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THE ROLE OF NEUROTROPHINS AND NEUROTROPHIN RECEPTORS IN THE PATHOGENESIS OF NEURODEGENERATION AND NEUROREGENERATION

Memòria presentada per
Paola Marco Salazar
per optar al grau de Doctor

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Departament de Medicina i Cirurgia Animals

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Tesi Doctoral dirigida per
Martí Pumarola i Batlle
Enric Vidal Barba

Universitat Autònoma de Barcelona

MARTÍ PUMAROLA I BATLLE, Catedràtic d´Universitat del Departament de Medicina I Cirurgía Animals de la Universitat Autòma de Barcelona.
I

ENRIC VIDAL BARBA, Investigador en Centre de Reserca en Sanitat Animal (CReSA), UAB-IRTA.

FAN CONSTAR

Que la memòria titulada “The role of neurotrophins and neurotrophin receptors in the pathogenesis of neurodegeneration and neuroregeneration” presentada per Paola Marco Salazar per a optar al Títol de Doctor, s´ha realitzat sota la nostra direcció, i, en considerar-la conclosa, autoritzem la seva presentación per ser jutjada pel Tribunal corresponent.

I per tal que consti als efectes que s´escaigui, signem la present a Bellaterra, el 30 de Setembre de 2014

Dr. Martì Pumarola i Batlle

Dr. Enric Vidal Barba

“No conozco la clave del éxito, pero sé que la clave del fracaso es tratar de complacer a todo el mundo”

Woody Allen

A mis aitas, Monly y Juan

A mis hermanos, Jontxu y Adri

A Jaime

A Sílvia

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FIN

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ABBREVIATIONS

BSA	Bovine serum albumin
BSE	Bovine Spongiform Encephalopathy
BDNF	Brain derived neurotrophic factor
CNS	Central nervous system
DRG	Dorsal root ganglia
GFAP	Glial fibrillary acidic protein
HD	Huntington´s disease
HIER	Heat induced epitope retrieval
IHC	Immunohistochemistry
IR	Immunoreactivity
ISH	In situ hybridisation
NTs	Neurotrophins
NT-3	Neurotrophin 3
NTRs	Neurotrophin receptors
NGF	Nerve growth factor
PBS	Phosphate buffered saline
PD	Parkinson´s disease
PNI	Peripheral nerve injury
PNS	Peripheral nervous system
PFA	Paraformaldehyde
Pro-NTs	Proneurotrophins
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RIP-I/hIFNβ	Rat insulin I promoter- human interferon β
SNI	Sciatic Nerve Injury
TSEs	Transmissible spongiform encephalopathies
TrK	Tyrosine kinase receptor
W.a.s	Weeks after surgery

WD	Wallerian degeneration
WT	Wild Type

CONTENTS

SUMMARY

RESUMEN

1. Summary

Neurotrophins (NTs) are a unique family of structurally related polypeptide growth factors that influence the development, maintenance, survival, repair and death of neuronal and non neuronal cells in the nervous system.

Members belonging to this group include NGF, BDNF, NT-3 and NT 4/5. They exert their intracellular roles by binding to two different transmembrane types of receptors; the tyrosine kinase receptors (Trk A, B and C) and the p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumor necrosis factor receptor (TNF) superfamily.

Neurotrophins are under current investigation for their involvement in physiological and pathological conditions. Previously published literature points them out as promising therapeutic agents.

In this thesis, an immunohistochemical assessment of all these neurotrophins (with the exception of NT4/5) and their receptors was performed in the nervous system of different adult transgenic murine models in two different scenarios: central nervous system neurodegeneration and peripheral nervous system neuroregeneration.

In order to determine the role of NTs/NTRs in the neurodegenerative mechanisms associated to prion diseases pathogenesis, the BoTg 110, a murine model of bovine spongiform encephalopathy (BSE), which overexpresses the bovine prion cellular protein, was subjected to an intracerebral inoculation with a BSE isolate. Neuropathological features where assessed and compared to NTs/NTRs immunolabelling. Furthermore, in this experiment, a wild type mouse line (Balb-C) was included as a control for a thorough -normal- mouse brain mapping of the NTs/NTRs immunolabelling. An increased expression of $p75^{NTR}$, particularly in glial cells, was observed to correlate well with TSE related lessions. This may suggest that, among all neurotrophic factors evaluated, this receptor is involved in end stage brain pathology in BSE.

Additionally, the study of the peripheral nervous system neuroregeneration was carried out following an experimental unilateral mechanic injury (crush) in the sciatic nerve of male transgenic RIP-I/hIFNβ mice. In this model, the involvement of NTs/NTRs in the neuroregenerative process was evaluated in the nerve, in the corresponding dorsal root ganglia and in the lumbar spinal cord segments at different time points after surgery. Our findings indicated changes in the immunoreactivity for all factors studied in these three structures. Particularities depending on the time point and studied NTs were observed related to the neuroregenerative processes

Our results indicate that neurotrophins, and particularly the $p75^{NTR}$ could be further studied as possible therapeutic targets for prion diseases. Likewise, combined neurotrophins could be useful to treat patients affected by peripheral nerve injuries and therefore contributing to the peripheral nerve regeneration.A better understand ing of the mechanisms underlying the neutrophin function involvement is a prerequisite for the development of more effective treatments for the disorders affecting the nervous system.

2. Resumen

Las neurotrofinas son una familia de factores de crecimiento polipeptídicos estructuralmente relacionados que influyen en el desarrollo, mantenimiento, supervivencia, reparación y muerte de las células neuronales y no neuronales en el sistema nervioso.

Los miembros pertenecientes a este grupo que incluyen NGF, BDNF, NT-3 y NT 4/5, ejercen sus funciones intracelulares mediante la unión a dos tipos de receptores transmembrana muy diferentes; los receptores de tirosin quinasa (Trk A, B y C) y el receptor de neurotrofina p75 ($p75^{NTR}$), un miembro perteneciente a la superfamilia del factor de necrosis tumoral (TNF).

Las neurotrofinas son objeto de estudio de la investigación actual debido a su participación tanto en condiciones fisiológicas como patológicas. Estudios previos publicados señalan las neurotrofinas como agentes terapéuticos prometedores.

En esta tesis, se llevó a cabo un estudio inmunohistoquímico de todas estas neurotrofinas (a excepción de NT4 / 5) y sus receptores en el sistema nervioso de diferentes modelos transgénicos murinos en dos escenarios diferentes: neurodegeneración del sistema nervioso central y neuroregeneración en el sistema nervioso periférico.

Con el fin de dilucidar los mecanismos neurodegenerativos asociados a la patogénesis de las enfermedades priónicas, un modelo murino (BoTg 110) de la encefalopatía espongiforme bovina (EEB), que sobreexpresa la proteína priónica celular bovina, fué sometido a una inoculación intracerebral con un inóculo de EEB. Se evaluaron los cambios neuropatológicos en el encéfalo y se compararon con el marcaje inmunohistoquímico de las NTs/NTRs. Además, en este experimento, se incluyó un modelo “wild type” (Balb-C) como control para realizar un completo mapeo del inmunomarcaje de las NTs/NTRs en el encéfalo –normal- de ratón. Se observó una correlación entre el incremento en el marcaje inmunohistoquímico del receptor p75, especialmente en células gliales con la distribución de lesiones asociadas a la EEB. Esto podría sugerir que, entre todos los factores neurotróficos evaluados, este receptor podría está implicado en la fase terminal de la patología de la EEB.

Además, el estudio del sistema nervioso periférico se llevó a cabo tras inducir experimentalmente un daño mecánico (aplastamiento o “crush”) en el nervio ciático en ratones transgénicos macho RIP-I / hIFNβ. En este modelo, el papel de las NTs/NTRs en los procesos de neuroregeneración se evaluó en el nervio, en los correspondientes ganglios de la raíz dorsal y en médula espinal a diferentes tiempos después de la cirugía. Se observaron cambios en la inmunorreactividad de todos los factores estudiados en estas tres estructuras. Observamos algunas particularidades en función del tiempo y la neurotrofina o receptor estudiado que se correspondían con la regeneración nerviosa.

Nuestros resultados indicaron que, las neurotrofinas, en particular el receptor p75, podrían ser estudiadas como posibles dianas terapéuticas para el tratamiento de las enfermedades priónicas. Del mismo modo, una combinación de neurotrofinas podría ser de utilidad para tratar pacientes afectados por lesiones de nervios periféricos, ayudando así al proceso regenerativo.

Profundizar en el conocimiento del papel que juegan las neurotrofinas en este contexto es indispensable para poder desarrollar tratamientos más eficaces de aquellos trastornos que afectan el sistema nervioso.

PROLOGUE

3. Prologue

This thesis started early in 2010 when I was awarded a research grant within the COTSA project. This project, entitled: Transfrontier cooperation on small ruminant origined food safety, was a collaboration between UAB, UNIZAR (University of Zaragoza, Spain) and ENVT (*École Nationale Vétérinaire* of Toulouse, France) with the objective to study the permeability of atypical scrapie strains to humans and other domestic species of interest. In this scenario, I received training on the study of prion diseases. Namely bioassays, laboratory animal management, mice intracerebral inoculation, clinical signs assessment, necropsy and neuropathological techniques, including immunohistochemistry.

I started working in Professor Martí Pumarola's Veterinary Neuropathology group, which had been working with animal prion diseases since 1996, amongst other subjects. At the time of my incorporation several experiments were ongoing and, since the incubation times of the inocula included in my project (mainly atypical scrapie) were quite long, we decided to include in this thesis dissertation the results of other research lines within the group in which I was involved.

Dr. Enric Vidal in Priocat laboratory, *Centre de Recerca en Sanitat Animal* (CReSA) had some ongoing titration experiments using an inoculum prepared from one of the Bovine Spongiform Encephalopathy (BSE) cases diagnosed in Catalunya. A transgenic mouse model expressing bovine cellular prion protein (BoTg 110 mice) was used. This model was kindly provided by Dr. Juan Maria Torres from *Centro de Investigación en Sanidad Animal* (CISA-INIA) in Valdeolmos, Madrid. It was considered of great interest to apply the procedures that had been set up for the neuroregeneration studies to the opposite scenario: neurodegeneration. The availability of high value brain samples from a well characterised neurodegeneration mouse model (BSE in BoTg110 mice) paved our way. Since little data was found in the scientific literature on immunohistochemistry of neurotrophins and their receptors in the adult mouse brain we included in our experiments brain samples of a wild type (WT) mouse inbred strain as a control in our study.

Besides prion diseases, one of the fields the group was focused on was diabetic neuropathy. Leaded by Dr. Anna Serafin, Dr. Jessica Molin was performing the experiments in which I was also involved. A mouse model of peripheral nerve injury was being set up at that moment to study the effects of diabetes on neuroregeneration. The expression of neurotrophins and their receptors in peripheral nervous system structures (nerve and dorsal root ganglia) after trauma, during nerve regeneration, was one of the subjects of interest in that context and different immunohistochemical procedures were set up for these studies.

INTRODUCTION

4. Introduction

4.1. Neurotrophin concept

The nervous system development is characterized by remarkable neuronal death phenomena and therefore an optimal trophic factor supply is required for a proper development and survival.

In the mammalian nervous system, **growth factors**, a variety of secreted proteins, perform an essential role regulating various cellular biological activities, including proliferation, differentiation, regeneration, survival and death, from embryonic state to adulthood (1). Some of these factors have pleiotropic activities, and therefore exert their effects in multiple tissues. Others are restricted on neural cells and are classified as **neurotrophic factors** (2).

Neurotrophic factors are classified into several families based on similarities in their structure, including the nerve growth factor (NGF) superfamily, glial cell derived neurotrophic factors (GDNF) superfamily and the neurokine or neuropoietin superfamily (3).

Among them, the most intensively studied are the members of the nerve growth factor (NGF) family, known as **neurotrophins (NTRs)**.

Neurotrophins are structurally, functionally related and naturally produced polypeptides, synthesized by both neuronal and non-neuronal cell types.

All neurotrophins are initially synthesized as precursors called **pre-pro-neurotrophins**, containing a signal peptide for protein secretion, which is translocated to the endoplasmic reticulum (ER) to produce the precursor protein or **pro-neurotrophin (pro-NT)** (30-32 KDa). The pro-sequence (amino terminal fragment) is then enzymatically cleaved in the Golgi apparatus, either within the trans-Golgi compartment by furins, or in secretory vesicles by other protein convertases (PCs) belonging to the convertases family. Eventually they are secreted as mature homodimeric active proteins into the extracellular space (Fig. 4.1). The mature proteins, which are of about 12KDa in size, form stable, non-covalent dimers, and are normally expressed at very low levels during development (4).

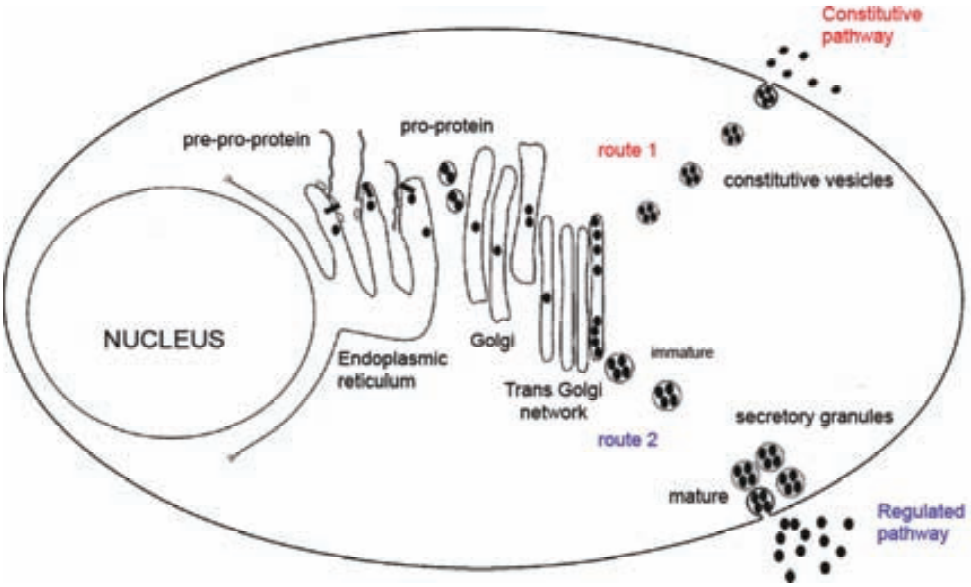

Figure 4.1: **The route of neurotrophin synthesis until secretion**: all known neurotrophin genes have in common that they encode for a precursor protein, named pre-pro-neurotrophin, which is further converted into the mature form. The unprocessed proteins can be cleaved intracellularly in the endoplasmic reticulum and proteolitically converted into the mature form (regulated pathway) or extracellularly transported to the plasma membrane, released in an unprocessed form and converted by proteases (constitutive pathway) *(Adapted from* (5)).

Neurotrophins are now known to be critical for the development and maturation during embryonic stage but also maintenance during adulthood of the nervous tissue and regeneration after injury (6).

In the Central Nervous System (CNS), they act on neurons and glia mediating physiological functions like synaptic plasticity, neural survival and death (7).

In the Peripheral Nervous System (PNS) both Schwann cells and macrophages act as a source for neurotrophic factors in myelin sheath maintenance, axonal regeneration and remyelination (8).

The first neurotrophic factor to be discovered was the **nerve growth factor** (NGF), while they were studying mouse sarcoma cultures *in vivo* (9). This discovery led to the neurotrophic factor hypothesis, which proposed that a given neuron would survive depending on the availability or absence of neurotrophic factors, suggesting that neurons must compete with each other for specific survival factors (10) . Since then, other members of the same family have been characterized in mammals**,** including **brain derived neurotrophic factor** (BDNF) (11) , **neurotrophin 3** (NT-3) (12) and **neurotrophin 4/5** (NT-4/5) (13) and, in other non-mammal species, NT-6 (14) and NT-7 (15).

Mature neurotrophins are structurally dimeric proteins composed by two subunits of 120 amino acid residues (16). They exert their function by the selective binding to two distinct classes of transmembrane receptors, the **tyrosine kinase family of receptors** (Trk A, Trk B and Trk C) and the **p75 neurotrophin receptor** (p75 ^{NTR}).

Thus, NGF binds to Trk A, BDNF and NT-4/5 to TrK B and NT-3 to TrkC (and Trk A, B with less affinity).

In contrast, the p75 ^{NTR} binds all four types of neurotrophins with relatively lower affinity, and also, appears to enhance the signalling of the tyrosine kinase receptors.

Binding to high affinity tyrosine receptors in the cell surface requires basic residues (17) whereas a set of other basic residues common to all neurotrophins are important for their binding to the low affinity p75 ^{NTR} (18). Although they have highly conserved domains that determine their basic structure, they also have variable domains that determine their neuronal specificity mediated by the differential affinity to bind receptors. These similarities and differences between the members of the neurotrophin family also influence their regional distribution, cellular localisation and developmental regulation (19).

Thus, proneurotrophins (Pro-NTs) themselves are active signalling molecules that have opposite effects on cell survival when compared to their mature forms. Differences depend on the cellular context, their processing step, which receptors their bind to, and the ratio of different receptors and cell types. Also, is probably due to differences in their molecular sizes. While mature neurotrophins are highly homologous, homology of the pro-region is low. Under physiological conditions, neurotrophins binding to Trk receptors show neuroprotective roles, whereas Pro-NTs do not activate Trk receptors directly (20). However, these unprocessed forms have been demonstrated to induce p75^{NTR} mediated neuronal death (21) .

The processing step to produce mature neurotrophins is essential for their functionality. Some researchers have highlighted the pro-domain (mainly the c-terminal part) as the principal responsible for the correct folding of the mature ligand as well as its targeting to the secretory pathway (22).

4.1.1.The neurotrophins

4.1.1.1. Nerve Growth Factor (NGF)

NGF was purified from the submandibular glands of male mice (9) and it is a small secreted protein, important for the growth, maintenance and survival of certain target neurons, like sympathetic and sensory neurons (23). In its absence, these neurons undergo protein synthesis-dependent apoptosis (24). NGF is required for their survival during development but not in the adulthood (25).

Its function is mediated through two specific receptors, Trk A and p75 ^{NTR}.

If NGF binds the Trk A, the receptor dimerizes and autophosporylates, leading to the activation of different prosurvival signalling pathways. In the event of p75^{NTR} binding it also mediates cytotoxic responses (26).

NGF levels usually vary widely in different brain regions. It is produced in the neocortex and hippocampus and retrogradely transported to the cholinergic neurons of the basal forebrain (27).

By blot hybridation assay, the highest concentration of NGF mRNA in the adult rat brain was found in the hippocampus, followed in order by piriform cortex and neocortex. The diencephalon, pons, medulla oblongata, had medium levels, but higher than those of the spinal cord and striatum. Midbrain, septal area and cerebellum had the lowest levels observed (28). Immunohistochemical studies support the presence of NGF in the adult rat brain, preferentially in fiber tracts (29).

In the normal adult spinal cord there is little effect on motor neurons and their neurite outgrowth (30) but levels are not as low as in the sciatic nerve, in which only trace levels are found. NGF supports and increases the survival in a subpopulation of small sized sensory neurons that mediate nociception in dorsal root ganglia, and sympathetic neurons, but not parasympathetic neurons (31).

4.1.1.2. Brain derived neurotrophic factor (BDNF)

BDNF was the second factor to be discovered and the first purified from pig brain based on its trophic (survival-growth promoting) effect on cultured embryonic chick dorsal root ganglion (11).

It is produced by target tissues and also by the neuron itself. Then, is transported anterogradely, stored in the nerve terminals and released in an activity dependent manner (32).

BDNF not only influences the survival, growth or maduration of subsets of neurons during embryonic development of the nervous system but also exerts continuing effects on neuronal function in adulthood (33).

Several lines of evidence indicate that it is essential in sustaining physiological processes of the normal, intact adult brain. It has a role in modulating dendritic branching and dendritic spine morphology (34), neuronal plasticity, long-term potentiation (LTP) (35) as well as regulating hypothalamic metabolic function (33).

Its precursor or pro-BDNF binds to $p75^{NTR}$, resulting in pro-apoptotic signalling through c-Jun N-terminal kinase cascade (JNK), or survival through the nuclear factor kβ cascade(NF-kβ) (36–38). In contrast, mature BDNF binds with greatest affinity to TrkB, mediating the transcription of functional genes essential for neuronal survival and differentiation (39).

BDNF is one of the most widely expressed neurotrophin in the nervous system, located in almost all cortical lobes and also in several subcortical regions in the brain (36).

In the adult unlesioned spinal cord, both astrocytic and non-astrocytic populations in the white matter highly express BDNF, as well as motoneurons in the ventral horn (40,41). Strong labelling has been found in the nerve terminals in the superficial layers of the dorsal horn too (42).

In the dorsal root ganglion (DRG), BDNF is found in the medium-sized primary sensory neurons (43) whereas in an intact nerve is mainly expressed by Schwann cells and fibroblasts (44).

4.1.1.3. Neurotrophin 3 (NT-3)

NT-3 is a protein growth factor unique in the number of neurons it can potentially stimulate, given its ability to activate two of the tyrosine kinase receptors Trk C and Trk B, and the p75 ^{NTR} (45–47).

It has activity, particularly during development, on neuronal survival and differentiation of both CNS and PNS, as well as encouraging the growth and differentiation of new neurons and synapses (48). NT-3 is not only distributed in the nervous system, but also in peripheral tissues, including pancreas, spleen, liver, adrenal gland, kidney, heart, thymus and diaphragm (49,50).

In the rat CNS, NT-3 immunopositive neurons are observed in the olfactory bulb, hippocampus (pyramidal layers and the dentate gyrus), cerebellum (granular neurons and Purkinje cells), and the septum; immunoreactive glia is also observed within the corpus callosum, the substantia nigra, the fimbria of the hippocampus, the subependymal layers of the ventricles and cerebellum (45,49,51–53).Few studies have also reported its distribution in the primate brain (54), with some particularities in the immunoreactive intensity, especially in the hippocampus, pons (locus coeruleus), medulla oblongata (hypoglossal nucleus) and cerebellum (Purkinje cells) (55).

In the rat spinal cord, strong labelling is found in the astrocytes as well as non-astrocyte populations of the white matter (idem to BDNF). In the grey matter, the staining is more weak (40).

Neurotrophin 3 supports large sensory proprioceptive neurons of DRG (56). NT-3 containing neurons are mainly localized in the trigeminal, cervical and lumbar spinal ganglia (57). Some authors reported that NT-3 levels are clearly detected in the intact sciatic nerve (58) whereas others do not (59). Likewise, Yamamoto *et al.* described weak NT-3 levels in the peripheral nerve tissues (sciatic nerve and DRG) and spinal cord, but more abundant in non-neural tissues (31).

4.1.1.4. Neurotrophin 4/5 (NT-4/5)

NT-4/5 is structurally the less conserved neurotrophin, sharing only half of the amino acid identity with the other neurotrophins (60). Based on similar structure it can be called either NT-4/5 or just NT-4 and NT-5 (61). It is expressed in embryonic as well as adult tissues and acts predominantly through the TrK B receptor. In the CNS, it is detected throughout the cortex, hippocampus, thalamus, septal area and cerebellum.

No literature has been found on its distribution in the spinal cord and PNS under normal conditions.

4.1.2.The neurotrophin receptors

As already mentioned before, the action of neurotrophins depends on two different transmembrane-receptor signalling systems:

- Three highly homologous members of the **tropomyosin receptor kinase** (Trk) family of receptor tyrosine kinases (Trk A, Trk B and Trk C)
- The **p75 neurotrophin receptor** (p75 ^{NTR}), member of the tumour necrosis factor receptor (TNFR) superfamily (4,60).

Different neurotrophins show binding specifity for particular receptors (Fig. 4.2). These receptor-ligand interactions were elucidated in cell lines overexpressing the receptors in recombinant forms and also in mice with targeted mutations in specific genes (62). They are confined to the caveolae-like domains of the cell membrane, a specialized region of signalling molecules concentration (63).

By electron microscopy examination of the m-RNAs, Trk A and C were localized only in the cell soma and Trk B and p75 ^{NTR} had a somatodendritic distribution (64). The ability of both receptors to present different binding sites and affinities to particular neurotrophins, determines both their responsiveness and specificity (4).

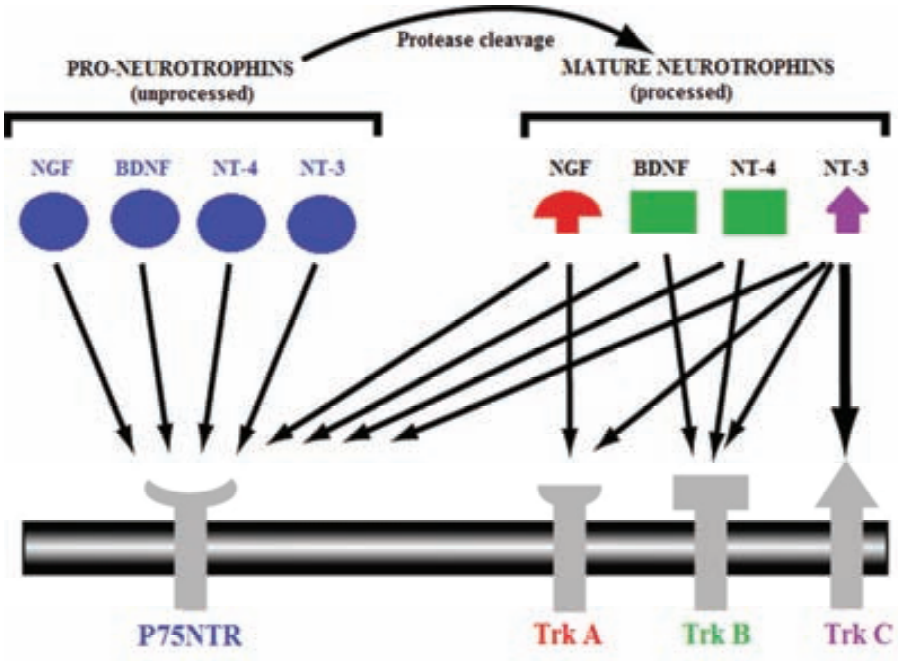

Figure 4.2: Neurotrophins and their receptors).The unprocessed forms of neurotrophins convert into mature neurotrophins by protease cleavage. Inmature neurotrophins (left) bind to the p75^{NTR} with higher affinity than mature neurotrophins do.The processed ligands bind preferentially to one or two TrK receptors (right) except for NT-3, which binds to Trk C but is able to activate all Trks. (*Adapted from* (65))

4.1.2.1. Trk receptors

Trk receptors are evolutionarily conserved type I transmembrane proteins and neurotrophins are their common ligands (2).

They all have three common structural regions (Fig. 4.3):

- An *extracellular ligand binding region* or extracellular domain (ECD), consisting of a signal peptide, a cysteine-rich cluster followed by three leucine-rich repeats, another cysteine-rich cluster and two immunoglobulin (Ig)-like domains. The second Ig-like domain is the major element for neurotrophin binding specificity. However, other extracellular domains also regulate Trk catalytic activity (60,66).
- A *transmembrane region*, that leads to the communication between the extracellular and intracellular regions.
- A *cytoplasmic region*, in which the tyrosine kinase catalytic domain is surrounded by several tyrosine residues. These serve as phosphorylation dependent docking sites for cytoplasmic adaptors and enzymes.

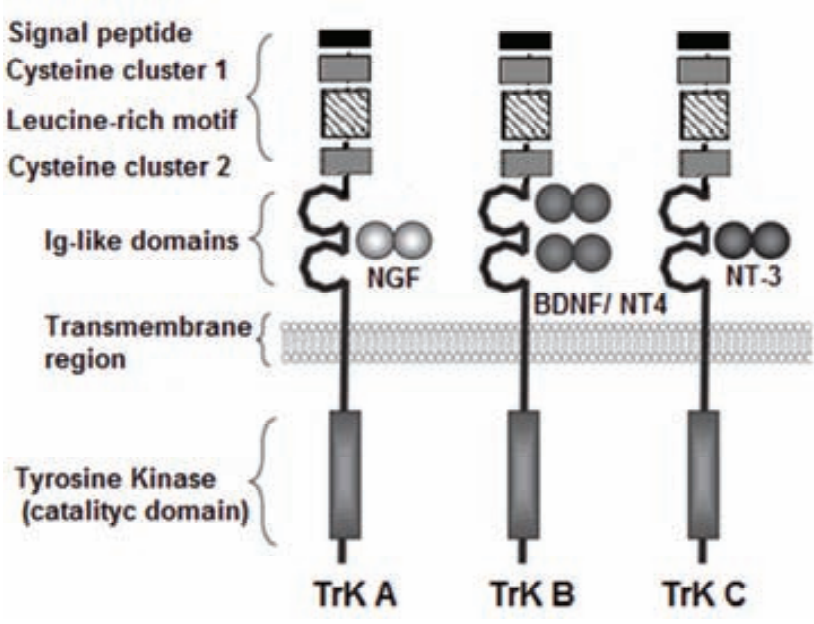

Figure 4.3: Structural organization of the Tyrosine kinase receptor superfamily. Each receptor consists of an N-terminal extracellular ligand-binding domain, a single transmembrane α helix, and a cytosolic C-terminal domain with protein-tyrosine kinase activity. Although these receptors share a common structural organization they have preference for different growth factors. Thus, Trk A has the highest affinity to NGF; Trk B to BDNF and NT 4/5 and Trk C to the neurotrophin NT-3 (*Adapted from* (67)).

The Trk receptors have selective binding properties (Fig 4.2): The Trk A is the catalytic receptor for the NGF; Trk B binds BDNF and NT-4/5; Trk C is the preferred ligand for NT-3, which is also the low affinity receptor for Trk A and B.

Although these interactions between the TrK receptors and their ligands have been considered to be of high affinity, the binding of NGF to Trk A, and of BDNF to Trk B is of low affinity, but it can be regulated by receptor dimerization, structural modifications or association with the p75 ^{NTR} (65).

Pro-NT are also active ligands but trigger functional effects opposite to those elicited when binding to mature neurotrophins (60). The binding of a neurotrophin to full-length Trk receptors results in receptor dimerization. Subsequently, the tyrosine kinase catalytic domain of the cytoplasmic region is activated through its auto trans-phosphorylation for adaptor proteins, thereby initiating downstream signalling pathways (68–70). The three major signalling cascades activated by the Trk receptors that mediate growth, survival responses and differentiation of many neuronal populations, are Phosphatidylinositol-3-kinase (PI3-K), phospholipase C (PLCγ), and RAS/mitogen-activated protein (MAP) kinase pathways (66), as illustrated in Figure 4.4.

There are many differential splicing variants of the full-length receptors. These isoforms lack most of the intracellular kinase domain and have truncations or insertions instead, this affects the specificity of neuronal responsiveness to neurotrophins binding (60,71). In the mammalian CNS, full-length Trk receptors are the major forms in early development, whereas the spliced variants become more prevalent with increasing age, even exceeding levels of full-length in adulthood (72,73).

The two **Trk A** types of biologically active isoforms are Trk A-I and Trk A-II; while Trk A-II has an extra insertion between the Ig-like domain and the transmembrane region, Trk A-I does not. The other known isoforms differ from the full-length not only in the structure but also that they are only expressed in the thymus (74). Trk A mRNA expression and immunoreactivity (IR) has been studied in the rat brain and coincided in specific neuronal populations in the forebrain and brainstem. Its ligand, NGF, responsiveness was also demonstrated for each of these populations (75). Thus, Trk A is expressed in the basal forebrain and neostriatum cholinergic neurons; also the diencephalon (paraventricular and reuniens thalamic nuclei) and midbrain (interpeduncular nucleus), pons and medulla oblongata (prepositus hypoglossal nucleus, area postrema, gigantocellular and paragigantocellular reticular nuclei) in noncholinergic neurons.

The **Trk B** isoforms contain a short C-terminal sequence (76), but not all of them are biologically active and do not bind its specific ligands either (77). It has been studied immunohistochemically in the rat brain (78), while all Trk B forms are coexpressed within neurons of the adult CNS, glial cells such as astrocytes, oligodendrocytes and Schwann cells, produce only truncated Trk B isoforms (79–81) and are also expressed in the choroid plexus and ependyma (72).

The **Trk C,** which is the earliest receptor expressed in the neural tube (82), has also intracelullar variations like inserts in the catalytic domain (60). They are

expressed in the CNS during fetal and adult life and present a restricted signalling capability in non neuronal cells (83) . The Trk C expression was examined by immunofluorescence and immunohistochemistry in the adult brain; truncated forms were found in the hippocampus and cerebellum but were remarkably evident in the neocortical layers , compared to other brainstem structures (84).

Individual Trk receptors are expressed in separate subsets of neurons.

Within the spinal cord, both truncated and full length Trk B and C are strongly present in a small subpopulation of motoneurons. In contrast, the Trk A mRNA level expression is very low (31,85).

In the peripheral nervous system, neurotrophins are released by the target tissue and binding occurs at the nerve terminals. Then they are internalized into vesicles and retrogradely transported along the axon to the soma to mediate survival. All three TrK receptors are found in discrete subpopulations of primary sensory neurons. In the DRG, Trk A is highly expressed in the small neurons, and Trk B and Trk C are moderately expressed in the mid-sized and large primary sensory neurons respectively (8).

In the sciatic nerve, while Trk B and C are present in the intact nerve in the Schwann cells membrane, Trk A is not (31).

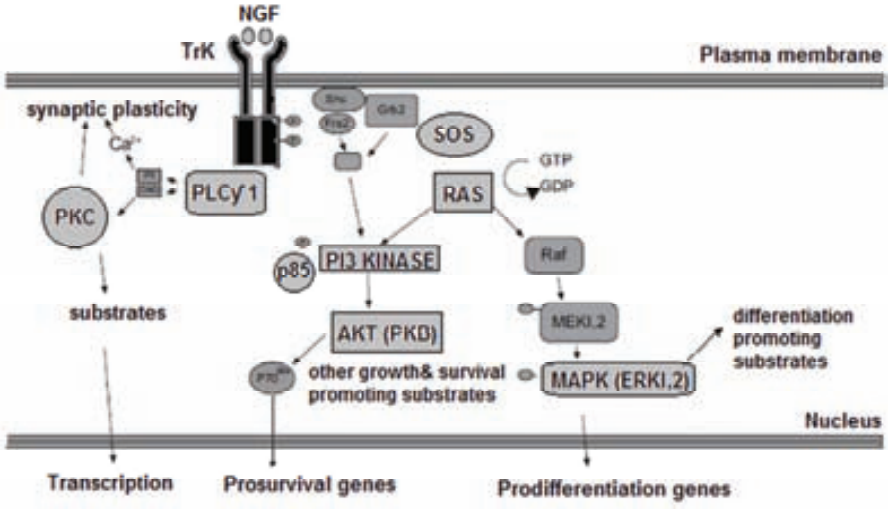

Figure 4.4: Neurotrophin major intracellular signalling pathways: interactions of neurotrophins (NGF is depicted) with TrK receptors lead to its dimerization and autophosphorylation.The pathway requires internalization of the TrK receptor into the endosomal compartment *(Adapted from* (66)).

4.1.2.2. The p75 ^{NTR}

The p75 ^{NTR} is a 75-kD (transmembrane) cell surface glycoprotein (86). It was first identified as a low-affinity receptor for NGF, but was subsequently shown to bind all neurotrophins with approximately similar affinity in most cells (87–89). Among

its multiple functions it is capable of inducing programmed cell death, axonal growth and degeneration, cell proliferation, myelination and synaptic plasticity. The multiplicity of cellular activities depends on the ligand bound to it, the cell type in which it is expressed and the absence or presence of other receptors (67).

The $p75^{NTR}$ also acts as a co-receptor and/or cooperates with the Trk signalling to increase the ligand selectivity and the binding sites that are thought to be responsible for mediating neurotrophin function (2,4); $P75^{NTR}$ initiates the autonomous pro-survival signal of some cells and apoptotic processes of others, as well as affects the cell cycle arrest, promotes axonal outgrowth and Schwann cell development (69). However, expression of $p75^{NTR}$ without Trk in both astrocytes and oligodendrocytes is not enough to elicit apoptosis, suggesting that the fate of cells depend not only upon TrK expression but possibly on other factors (90).

Several experiments have shown that, when $p75^{NTR}$ is present and Trk A is not, the $p75^{NTR}$ mediate neuronal cell death. However, if both receptors are present, $p75^{NTR}$ increases the NGF binding to Trk A, enhancing neurite outgrowth and neuronal survival (91).

In the absence of Trks, all Pro-NTs interact with high affinity to the $p75^{NTR}$.

The $p75^{NTR}$ structural organization it is well conserved in vertebrates from rodents to humans (71), and is composed of three main parts (Fig.4.5):

- *An extracellular domain*, as a distinguishing feature of the TNFR superfamily members, that includes four cysteine-rich domains (CRDs) responsible for interaction with neurotrophins, and several glycosylation sites.
- *A single transmembrane motif that links with the intracellular portion.*
- *An intracellular region that includes a death domain* (92).

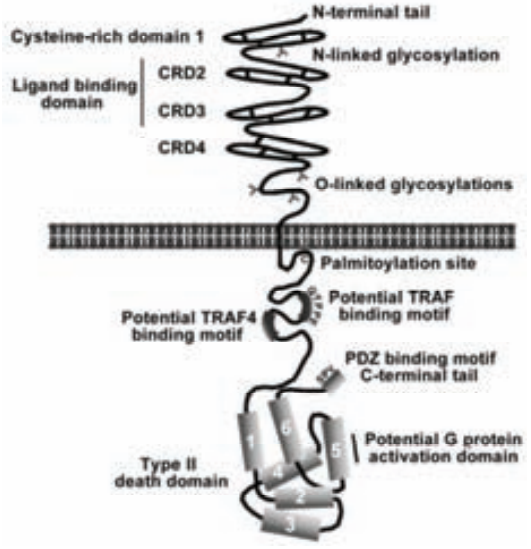

Figure 4.5: **Schematic representation of the structure of the p75 ^{NTR}**. It is a type I transmembrane receptor with an extracellular domain which contains four repeated modules (CRD) of six cysteines. The intracellular domain contains a palmytolation site at cysteine 279 and multiple binding motifs for protein-protein interactions. (*Adapted from* (71))

When binding to either Pro-NTs or mature neurotrophins, there is activation of different signalling pathways (Fig. 4.6).

Since $p75^{NTR}$ lacks intrinsic activity, intracellular cascades occur through the death domain in association with certain downstream signalling elements. These potential motifs interact with adaptor proteins that show preference for distinct regions of the cytoplasmic domain (71,93).

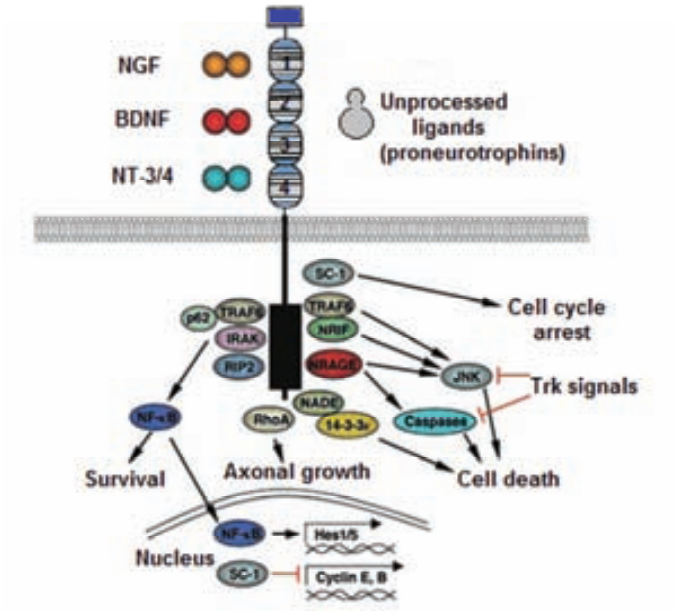

Figure 4.6: **$p75^{NTR}$ mediated signalling pathways**: p75 ^{NTR} interacts with different cytoplasmic proteins (TRAF6, RhoA, NRAGE, SC-1 and NRIF) and regulates gene expression, the cell cycle, apoptosis, survival, mitogenic responses and axonal growth. (*Adapted from* (67))

The death domain interacts with adaptor proteins to trigger caspases activation, but these interactions are not invariably associated with pro-apoptotic functions. Thus, the major proapoptotic signalling pathway mediated by $p75^{NTR}$ is the Jun N-terminal kinase pathway (JNK). The association with other adaptors, also promotes cell death via caspases, or affects the cell cycle through the Schwann cell 1 factor (SC-1) (26,69).

The $p75^{NTR}$ expression is controversial, probably due to its dual function in survival and apoptosis (31,58).

In response to mature neurotrophin binding, p75 ^{NTR} promotes nuclear factor *kappa beta* (NF-kß) activation, exerting effects on neuronal survival (94) and also modulates Rho family GTPase activity to stimulate neurite outgrowth (6) . In addition, all Pro-NTs interact with $p75^{NTR}$ or bind the co-receptor complex of $p75^{NTR}$ and sortilin (a transmembrane protein which is highly enriched in the

vertebrate CNS), triggering death (95) or survival (96).Thus, sortilin was found to be an essential cell-surface co-receptor with $p75^{NTR}$ for pro NGF-induced neuronal cell death (97).

$P75^{NTR}$ has a wide distribution, particularly in the developing nervous system and non-neuronal tissues. Numerous cell types, including meningeal cells, sensory and sympathetic neurons, spinal motoneurons and brainstem motor nuclei, neurons in the cerebral cortex, basal forebrain, caudate, putamen, hippocampus and Purkinje cells in the cerebellum, express $p75^{NTR}$ at some stage of their development (98–100). In subpopulations of central and peripheral glial cells (Schwann cells), $p75^{NTR}$ is also highly abundant (101).

The $p75^{NTR}$ expression is normally switched off in the adulthood (100). However it is maintained at lower levels in the basal forebrain cholinergic neurons, sensory neurons and motoneurons of the spinal cord (102–104).

4.1.2.3. Interaction between $p75^{NTR}$ and Trk receptors signalling pathways

Neurotrophic-mediated signalling through $p75^{NTR}$ and the Trk receptors can have opposing effects (Fig.4.7). The binding of neurotrophins to TrK receptors almost always leads to neuronal survival and differentiation. In contrast, the presence of $p75^{NTR}$ frequently promotes activation of pro-apoptotic cascades (6,95).

Thus, Trk receptors signalling pathways act at several steps to suppress some of them, such as the JNK-p53, while leaving others intact (105). However, Trk activation does not inhibit induction of NF-kß mediated signalling by $p75^{NTR}$, making a synergistic contribution to neural survival (26).

In any event, specificity in signalling via the two families of receptors appears to be regulated through proteolysis of the neurotrophins(6).

Studies performed in vivo and in vitro indicate that both receptor types collaborate together to control ligand discrimination, ligand binding, intracellular transport and signal transduction (106).

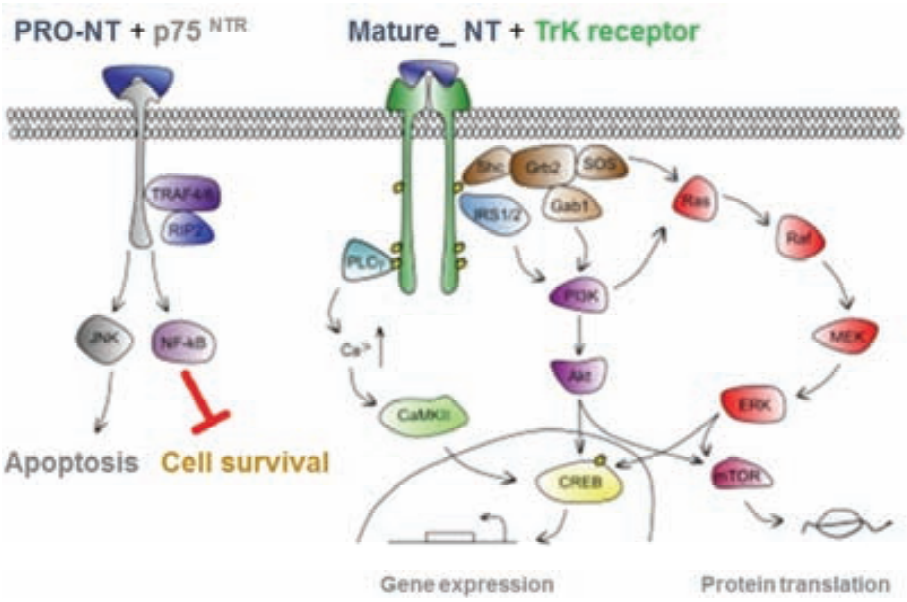

Figure 4.7: **Interaction of p75^{NTR} and Trk signalling pathways**: Pro-neurotrophin signalling via p75^{NTR} (left).Mature neurotrophin signalling via TrK activation (right). When both receptors are present p75^{NTR} mediated apoptotic signalling through the JNK (c-Jun N-terminal kinase) cascade suppresses the ability of neurotrophins to activate the three main signalling pathways (PLCγ (phospholipase C γ), PI3K (phosphatidylinositol 3-kinase) and ERK (extracelullar signal-regulated kinase)) responsible for the transcription of genes essential for the survival and differentiation of neurons *(Adapted from* (39)).

4.2. Prion diseases and other neurodegenerative disorders

The pathophysiology of most neurodegenerative disorders is vaguely understood, and suspected to be influenced by genetic and environmental factors. Altered brain development in the perinatal period can be a high risk factor. Different neuronal populations become atrophic or lost in the course of neurodegenerative diseases and it is well known that neurotrophin expression in these neurons is also altered (1).

Mechanisms by which neurotrophic factors might prevent age and disease-related neuronal degeneration include suppression of oxidative and metabolic stress, excitotoxicity and calcium overload, and damage to DNA and proteins (107).

Neurotrophins not only mediate protective functions in the nervous system during development and adulthood but have also been implicated in different age-related pathologies (67). Experiments in rats have described age related changes associated to decreased BDNF expression in the nervous system, particularly in the hippocampus, but the relationship between these factor and aging is controversial (108). In addition, data from patients and animal models compromise the role of neurotrophic factor signalling in age-related neurodegeneration (109).

4.2.1.Transmissible spongiform encephalopathies (TSEs)

Transmissible spongiform encephalopathies (TSE) constitute a group of fatal, neurodegenerative diseases, affecting both domestic animals and humans; currently all lack effective treatment (110).

Animal prion diseases encompass Scrapie in sheep and goats, Bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids and Transmissible mink encephalopathy (TME) in minks. Creutzfeldt-Jakob disease (CJD) is the most common human prion disease (111).

The main pathological hallmarks consist of spongiosis, reactive astrogliosis and activation of microglial cells, neuronal loss and neural tissue deposition of a misfolded protein which is believed to be the infectious agent and, apparently, it lacks nucleic acids (112).

The normal, cellular PrP (PrP^{C}) is converted into an abnormally misfolded isoform, named resistant prion protein (PrP^{Res}) or disease associated prion protein (PrP^{d}), through a posttranslational process during which it acquires high beta-sheet content and the ability to form amyloid fibrils. Both PrP^{C} and PrP^{Res} have the same primary amino acidic sequence but differ in the final structure (113,114).

PrP^{C} is a protein with two variably occupied Asparragine-glycosylation sites, attached at the outer membrane by a glycosylphosphatidyl-inositol (GPI) anchor; thus, PrP may exist as unglycosylated, monoglycosylated and di-glycosylated isoforms of different electrophoretic mobilities or glycoforms (115).

Moreover, its secondary structure is rich in alpha-helix and the protein is likely to be in a monomeric state in mild detergents (110).

When refolded into beta-sheet, PrP^{Res} leads to the formation of aggregates, sometimes of amyloid type, that can be differentiated from PrP^{C}, because of their partial resistance to protease digestion (most commonly proteinase K), and of their insolubility into non-denaturing detergents (116).

Prion diseases can be sporadic (CJD), inherited (fCJD, FFI, GSS) iatrogenic or infectious (vCJD, Scrapie, BSE, CWD...).

In ovine Scrapie, as an example of an infectious TSE, the alimentary tract is an entry portal of infectious agent (Fig. 4.8). The infectious agent first accumulates in Peyer's patches, gut associated lymphoid tissues (GALT) and ganglia of the enteric nervous system. Subsequently, infection most likely spreads to the CNS via the splanchnic or vagus nerve (117).

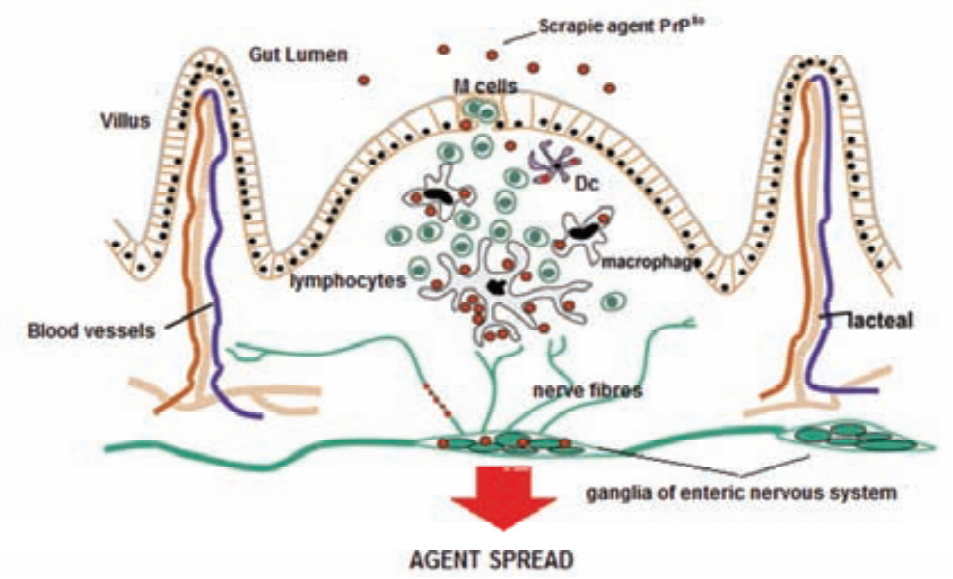

Figure 4.8: Possible spread of scrapie infectivity from the gut lumen to the nervous system following oral infection. Soon after ingestion, the abnormal protein, PrP^{res} is detected within Peyer's patches, macrophages, cells with morphology consistent with that of M cells and within ganglia of the enteric nervous system (*Adapted from* (117)).

In prion diseases, it has been demonstrated in a mouse neuroblastoma N2a cell model that, when PrP106-126, a synthetic peptide homologous to the human PrP region 106-126, binds to p75NTR induces translocation of the nuclear factor-kß (NF-kß), and consequently promotes apoptosis (86,112). An *in vivo* assay showed that NF-kβ activity was significantly increased in the brain of mice infected with Scrapie (118).

Although this suggests that neurotrophin receptors and particularly $p75^{NTR}$ binding to the prion protein might be involved in prion disease pathogenesis, it has not been possible to find further publications on the subject.

4.2.2. Other neurodegenerative disorders

Neurotrophic factors provide neuroprotection to specific neuronal populations against different types of brain insults. Thus, the interaction between neurotrophic factors and their receptors may be involved in the mechanisms that regulate the differential vulnerability observed in the neuronal populations affected in neurodegenerative diseases (53).

Alzheimer's disease (AD) is a neurodegenerative disorder characterized by the neuronal loss and dysfunction in brain regions, such as the neocortical association areas, hippocampus and olfactory bulb (37,102). The disease is associated with the presence of amyloid plaques and neurofibrillary tangles in the brain (106). The process is believed to involve mitochondrial alterations, membrane-associated oxidative stress, altered proteolytic processing of the β-amyloid precursor protein

(APP) and accumulation of both neurotoxic forms of the amyloid β-peptide (Aβ) and microtubule-associated protein, TAU (104).

Evidence is published indicating that NTs and NTRs have a certain role in AD pathogenesis, where amyloid beta (Aβ) protein fragment 1-42 is the predominant form found within the brain The NGF supply is decreased (119), supporting that responsiveness to NGF is impaired, possibly due to reduced expression of Trk A and p75 receptors (120). There is evidence that neurotrophic factors can protect neurons against toxicity but also promote the cell death (121). In vitro findings have shown that, not only the PrP 106-126 peptide but also the Aβ is a pro-apoptotic ligand for $p75^{NTR}$. Likewise, this complex induces the translocation of the NF-kβ depending on the intracellular domain of the $p75^{NTR}$ the bind to (122).

Therefore, trafficking of $p75^{NTR}$ possibly together with its death-promoting ligand β amyloid or its precursors appears to be involved in neuronal damage (123,124).

BDNF therapy has been examined in cultured entorhinal neurons and also in different animal models, suggesting that its functional effects on cell signalling, cell survival and cell death prevention could target the symptoms of memory loss associated to Alzheimer´s disease (33). Similarly, NT-3 was found to prevent the degeneration of noradrenergic neurons (53).

In patients with Parkinson´s disease (PD), BDNF is reduced in nigral dopaminergic neurons (125). Early studies demonstrated that this factor, NT-3 and their receptor Trk B exhibit therapeutic potential and can reverse neuronal injury, supporting and increasing the survival and preventing these neuron type loss (33,126).

Likewise, BDNF expression is reduced in affected brain regions of both Huntington´s disease patients and huntingtin mutant mice (127) pointing it as the most potent survival-promoting factor for striatal neurons (128).

4.3. Role of neurotrophic factors in the PNS neuroregeneration

Neurotrophins regulate the normal developing and adult peripheral neurons, including sensory, motor and autonomic neurons (129). Neurons in the PNS are able to regenerate their axons whereas the central nervous system does not repair itself (130) .In addition, the PNS is more susceptible to the ablation of neurotrophic factor genes than the CNS (13).

Repair of the peripheral nerve fibers is most likely to succeed if axons are simply crushed or have a very short interstump gap, up to 1 cm in length (131). Peripheral nerve injuries can be classified using different schemes, but there are two commonly used systems, the Seddon (132) and the Sunderland (133).

According to Seddon classification, nerve injuries are divided into 3 categories based upon the severity of tissue insult, the prognosis and time of recovery (Fig.4.9).

- *Neurapraxia* is the mildest injury, where there is no axon disruption or Wallerian degeneration but the nerve cannot transmit impulses. Recovery

after compression is always complete, ranging from hours to a few months.

- *Axonotmesis* is a more severe insult, including nerve crush or stretch injuries. The nerve sheaths are grossly intact, but the axon is disrupted and undergoes degeneration. The functional recovery is good, but reinnervation depends on the degree of internal organization and distance to the target organ.
- *Neurotmesis* is the most severe injury and can be seen following sharp injury, nerve transection or massive trauma. It comprises the disruption of the entire nerve trunk. In consequence, axons are often misdirected and reinnervate target organs incorrectly, leading to an antagonizing action and therefore a poor prognosis.

The Sunderland scheme is based on the degree of the tissue injury, leading to five degrees. Neurapraxia corresponds to first degree; axonotmesis is subdivided into three different degrees of nerve disruption (second, third and fourth); neurotmesis corresponds to fifth degree.

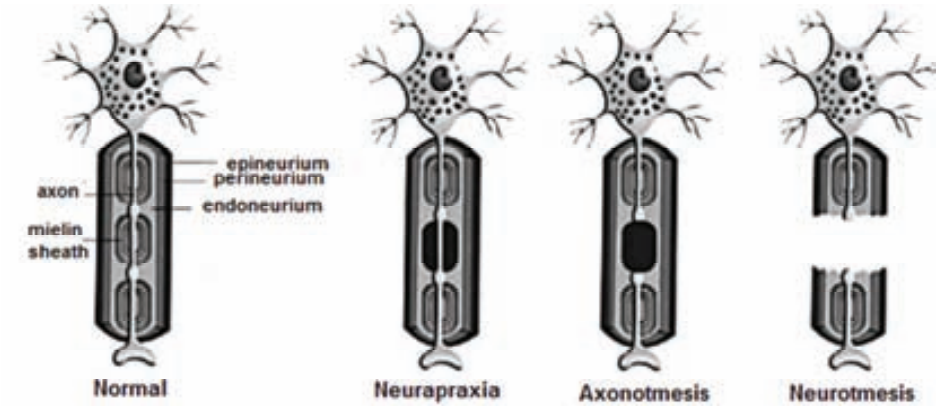

Figure 4.9: Schematic representation of an intact nerve fiber and the three types of nerve injury according to Seddon classification system**.** (*Adapted from* (134))

After nerve injury, the supply of retrogradely transported neurotrophic factors from the target organs is interrupted, leading to neuronal death. However, if the neuronal soma remains intact, the axon can regenerate back to reach their targets, indicating target derived neurotrophic factor dependence. It has been proposed that they are involved in axonal regeneration.

4.3.1. Cellular changes after Peripheral Nervous System injury (PNI)

In response to injury of the PNS, neurons change from a transmitting to a regenerative state in preparation for the regeneration process.

The process itself involves remarkable intracellular, structural and molecular changes (Fig. 4.10). There is downregulation of different cellular components and synthesis of new molecules that are not normally expressed in the adult neurons (8,135,136). Morphology and tissue organization in and around the area are affected, including neuronal cell body, the portion between the neuron and the injury site (proximal fragment), the injury site itself, the fragment between the injury and the organ (distal fragment) and the target organ (137).

After crush or axotomy, the connexion between the neuronal cell body and its target organ is disrupted leading to its denervation. The proximal and distal parts of the axon retract, the axoplasm leeks out and the membranes collapse.

The early changes begin with the enzymatic proteolysis and granular disintegration of axonal cytoskeleton. The granular disintegration is initiated by an increase in the calcium concentration in the axoplasm. Changes in the ion channel expression and neurotrophic signalling together with the impulse traffic are key factors in this regulation (135). Schwann cells stop making myelin proteins and discard their myelin sheaths.

In the neuronal soma, the metabolic activity is altered and morphological changes occur such as chromatolysis. The Nissl substance disappears; there is nuclear eccentricity, nuclear and nucleolar enlargement, cell swelling and retraction of dendrites (138).

In the proximal fragment there are retrograde signals from the damaged axons up to the first node of Ranvier, which give rise to multiple neuronal sprouts, to maximize the chances for each neuron to reach its target organ (138,139).

The distal nerve fragment undergoes Wallerian degeneration, which refers to the breakdown and demyelination of distal axonal fragments. Circulating macrophages quickly enter the endoneurium and, in association with the Schwann cells, they phagocytise myelin and axon debris. Both cells types are also going to be involved in the secretion of growth factors and mitogens that participate in axonal regeneration and remyelination (8,140).

Schwann cells are able to return to an immature state when lose axonal contact. In this proliferative phenotype, there is up-regulation or re-expression of certain neurotrophic factors, such as NGF, BDNF and NT-3, and other molecules like extracellular matrix glycoproteins (fribronectin, laminin, proteoglycans…) and also downregulation of the myelin proteins (136) . On its cell membrane there is expression of neurotrophin receptors, and neurotrophin release.

On a second stage they line the endoneurial tubes and form the bands of Büngner to create a favourable substrate for axonal regeneration and over which growth cones advance (141).

Neurotrophic factors along with other guidance molecules, determine the response of the axonal growth cone, which extends its protrusions depending on the chemoattractive or chemorepulsive gradients of the extracellular environment (136).

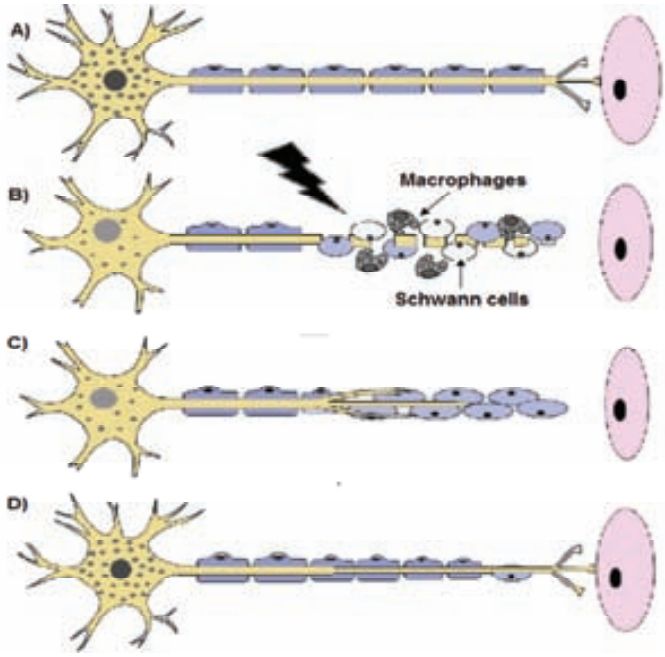

Figure 4.10: **Summary of the cellular and morphological events after injury of a peripheral nerve, between degeneration and the regeneration process**. **A)** Normal nerve fiber in contact with its target cell. **B)** Insult to the nerve fiber results in fragmentation of the axon and loose of the synaptic contact. In the neuronal soma, chromatolysis and dendritic retraction occur. Macrophages and Schwann cells phagocyte myelin debris **C)** Fine sprouts emerging from the proximal segment of the axon and proliferation of Schwann cells along the bands of Büngner in the distal segment **D)** Nerve fiber regeneration and reiinervation of the target cell *(Adapted from (136))*.

Axon regeneration does not always mean functional recovery. There must be a maturation process, which involves remyelination , axonal enlargement (neuroplasticity) and reestablishment of the connections with the target organ (neurorestoration) that eventually induce partial or complete functionality (4,138,142).

In summary, successful axon regeneration will depend, not only on the lesion type and severity, but also on the surrounding microenvironment (136).

4.3.2. Neurotrophin expression after PNI

Under physiological conditions, Schwann cells and multiple peripheral tissues such as skin and muscle, have a basal expression level of neurotrophins (143). In the event of a nerve injury, the expression changes in order to enhance the survival and growth of damaged axons and also to support the reinnervation of the target organ. Independently of the pattern, levels return to normal with regeneration, except the case of a chronic denervation, when the expression of neurotrophins continues for a few months (144).

4.3.2.1. Nerve growth factor (NGF)

When the nerve is injured and therefore disconnected from the periphery, the delivery of NGF from the target is decreased. Consequently, DRG sensory neurons decrease their immunoreactivity as well as the expression of both receptors, Trk A and $p75^{NTR}$ mRNA.

A crush to the sciatic nerve triggers upregulation of NGF expression in motor neurons: increased immunoreactivity is exclusively seen in injured motor neurons, but it is no longer detectable when they reinnervate their target (145) . In the distal segment of the nerve, the axon regeneration coincides both spatially and temporally with a reduction in Schwann cell NGF receptor density (146).

Recent unilateral and bilateral transection experiments of sciatic nerve in rats demonstrated that NGF is not only elevated when the contralateral site is intact but also further increase happens when is injured too (147).

However the effect that NGF has on nerve regeneration is controversial. While some authors reported that NGF improved nerve regeneration (148–150), others demonstrated that NGF stimulates collateral sprouting but not regeneration from uninjured neurons (151,152).

4.3.2.2. Brain derived neurotrophic factor (BDNF)

Peripheral damage changes both protein and m-RNA levels similarly during regeneration. Depending on the isoform, the lesion site and the injury type, neurotmesis or axonotmesis, the BDNF production increases gradually or less mildly respectively. However, for neurapraxia, since Wallerian degeneration process does not occur, there are no expression changes associated (41,153).

This upregulation has been described in the distal stump of axotomized nerves (154), axotomized DRG neurons, motoneurons of the ventral horn and denervated muscles (reviewed by (135)).

In the PNS, BDNF levels begin to decrease only after the myelination process starts, returning to normal in the later stage, with recovery (153).

Although the role in the regeneration process is controversial, the presence of its high affinity receptor Trk B seems to be determinant (reviewed by (136)).

4.3.2.3. Neurotrophin 3 (NT-3):

After damage, in the distal segment of the denervated sciatic nerve, NT-3 expression levels are lower when compared to the unlesioned. These changes occur earlier following neurotmesis (transection) than when axonotmesis (crush) is performed, but in both situations is more evident compared with that of neurapraxia. In contrast, the NT-3 protein is markedly increased, indicating that the NT-3 content may be regulated post-transcriptionally (153).

NT-3 is retrogradely transported within the crushed sciatic nerve to spinal cord motoneurons, although in the spinal cord, there is a fluctuation. Within the first hours post injury, there is an increase and levels are higher than the control, but

then slightly decrease to normal levels and even lower (58). There are no changes in the DRG when spinal ligation is done (reviewed by (135)).

Like other NT family members, binding to its high affinity receptor, Trk C, promotes nerve regeneration (155), and if there is success NT-3 levels return to normal (147).

4.3.2.4. Neurotrophin 4/5

NT4/5 is expressed by transformed Schwann cells in the proximal section of damaged nerves and has been proposed as a potent factor to improve nerve regeneration (156).

4.3.3. Neurotrophin receptors expression after PNI

In response to injury, neurotrophin receptors are differentially upregulated in the distal portion of the nerve, in the cell membrane of Schwann cells and growth cone of regenerating axons (136).

There is upregulation in the Schwann cell membrane of all receptors in the proximal and distal fragments, except for Trk A, which is not detected at all neither in the distal nor proximal nerve sites (31).

As the regenerative process proceeds, Trk B and Trk C gradually decrease to undetectable level, in contrast to $p75^{NTR}$, which remains abundant even a few weeks later.

4.4. Technical methods for studying neurotrophins and their receptors

Under physiological conditions or following an injury, there are different *in vivo and in vitro* techniques that allow the study of the expression pattern, distribution and function of NTs and their receptors in the nervous system.

They are studied in culture, using PC12 pheochromocytoma tumor cell lines (157); by electron microscopy (64) or using molecular techniques such as real time PCR (158), western blotting (84); or immunoassays like enzyme-linked immunosorbent assay (ELISA) (108) or antibody arrays (159,160). In addition, the expression pattern of genes encoding neurotrophins has been studied by in situ hybridisation (ISH) in the adult mouse brain (161).

The immunohistochemical analysis is not very frequent and is usually restricted to either the study of a unique neurotrophin factor or a specific area.

Until now, the majority of *in vivo* studies are performed in rodents, particularly rats (28,36,47,49). A few studies have also been reported in primate brains (55) and humans (53,162,163). However, there are not many experiments that use murine models to explore and map these molecules in normal nervous tissue, neurodegeneration and neuroregeneration.

4.5. The therapeutic use of neurotrophins

A wide range of neurodegenerative diseases are characterized by the aggregation and accumulation of misfolded proteins either intra or extracellularly.

To date, there is no an effective cure, but most efforts are focused on the use of neurotrophic factors as possible therapeutic agents to target the disease associated processes. The development of *in vitro* and *in vivo* systems, including animal models, has provided new insights into the disease pathogenesis.

In human medicine, the depletion of some NTs is linked to the pathology and symptomatology of certain CNS neurodegenerative disorders like Parkinson´s, Huntington's and Alzheimer´s diseases has been noticed (164). Thus, neurotrophin administration has been shown to make improvements in their functional recovery (165), indicating they could have a therapeutic role, either individually or in combination with other trophic factors.

Experiments performed in rodents and primates have proved the efficacy of neurotrophins in preventing neuronal death, in cell signalling improvement, restoration of cognitive functions and prevention of age related failures (166).

On the other hand, the treatment of PNI includes multiple and promising alternatives under continuing development, from the most conventional methods to combined therapy using conduits coated with stem cells and growth factors (167).

However, methods for achieving effective dosing and delivery in the CNS, due their large size and their inability to cross the blood-brain barrier, remain a substantial challenge (33,168,169). Neurotrophin based therapy is a good starting point for the treatment of peripheral nerve injuries and peripheral neuropathies as they have multiple properties. Therefore they are likely more feasible when compared to the CNS neurodegenerative disorders treatment (129).The current research is focused on the possible benefits of clinical treatment with these factors, but it has not been fully established yet.

Following our immunohistochemical results, and considering existing evidence that relates neurotrophin changes to nervous system alterations in either BSE infected mice or PNI induced model, it becomes necessary to design and fully characterize specific animal models, as an alternative to study neurotrophin-based potential treatment.

OBJECTIVES

5. Objectives

Our **main objective** has been to study the role and contribution of neurotrophins and their specific receptors in neurodegeneration and neuroregeneration. Hence, two genetically modified mouse models of neurological diseases have been studied with an immunhistochemical approach.

Our **working hypothesis** was that comparison of the NTs/NTRs immunolabelling of healthy *vs.* diseased animal and of injured v*s.* non-injured nerves at different time points after nerve surgery, would disclose differences that would yield information on the role of NTs/NTRs in these scenarios.

As little information was found regarding neurotrophins and mouse nervous tissue immunolabelling, a secondary objective was to typify the normal distribution of these molecules in the central and the peripheral nervous system elements in the chosen models.

The models selected included on one hand to evaluate the changes in the distribution of NTs/NTRs produced in the brain by BSE prions, a chronic neurodegenerative disease. And, on the other hand, studies performed on a diabetic neuropathy model suggested a role of some neurotrophins in the regeneration and healing of peripheral nerves after experimental nerve traumatism under diabetic condtions. Therefore, we decided to characterize these growth factors in peripheral and central nervous system elements after experimental nerve traumatism in non-diabetic contitions using the same transgenic model.

According to this, our specific objectives are detailed as follows:

- To study the possible role of neurotrophins and their receptors in the BSE pathogenesis and neurodegeneration.

- To map neurotrophins and their receptors immunolabelling in the brain of wild type (Balb-C) healthy mice and compare the distribution with a transgenic murine model (BoTg110) inoculated with “healthy” cow brain homogenate

- To evaluate the neurotrophins and their receptors immunolabelling changes in BSE inoculated BoTg110 mice compared to mock-inoculated boTg110 controls

- To map neurotrophins and their receptors in the peripheral nervous tissue (sciatic nerve and dorsal root ganglia) and related central nervous system elements (lumbar spinal cord) of an experimental nerve injury model in the transgenic mouse RIP-I/hIFNβ.

- To analyse immunohistochemically the spatio-temporal changes of these molecules following a sciatic nerve crush in the injured area, the proximal nerve segment dorsal root ganglia and spinal cord, along the regenerative process at three different time points after injury.

- To evaluate the efficacy of the murine transgenic models, BoTg110 and RIP-I/hIFNβ, to help the understanding of the neurodegenerative phenomena and neuroregenerative process of the nervous system.

STUDY 1:

Neurotrophins and CNS neurodegeneration: Mapping of neurotrophins and their receptors in the adult mouse brain and their role in the pathogenesis of a transgenic murine model of bovine spongiform encephalopathy.

6. Study 1

6.1. Introduction

Transmissible spongiform encephalopathies (TSEs or prion diseases) constitute a group of fatal, neurodegenerative diseases affecting both humans and a wide range of animal species and are characterized by a long incubation period (110). To date, there is no effective treatment for any TSE. In animals, prion diseases encompass scrapie in sheep and goats, bovine spongiform encephalopathy (BSE) in cattle, chronic wasting disease (CWD) in cervids, feline spongiform encephalopathy (FSE) in domestic cats and zoo-kept wild felines and transmissible mink encephalopathy (TME) (170) . Human TSEs include Creutzfeldt-Jakob disease (CJD), the most common human prion disease, fatal familial insomnia (FFI), Kuru and Gerstmann-Straussler-Scheinker disease (GSS) (171).

This group of diseases can be sporadic, genetic or acquired, but they are all transmissible and have a common feature, which is the accumulation in the brain of an abnormal conformer of the prion protein. Currently, the most accepted theory is that the infectious agent is a misfolded form of the host encoded cellular prion protein (PrP^C) which is infectious and lacks nucleic acids (172). This abnormal isoform, named disease associated prion protein (PrP^d or PrP^{res}), is the result of a post-translational process during which it acquires different properties. Among these changes, there is a higher beta-sheet content, the ability to aggregate and form amyloid fibrils and the capacity to resist protease digestion. Both PrP^C and PrP^d have the same primary amino acid sequence but differ in their respective final structures (113,171). Additional to PrP^d deposition in the brain, the main neuropathological features are spongiform change in the neuropil, vacuolation of neuronal bodies and astrocyte and microglial cell activation and neuronal loss (112).

Among the animal TSEs one of the best known is BSE, which was first reported in cattle in mid 1980's (173) and has had major public health implications as it is a food-borne zoonosis resulting in the invariably fatal variant CJD (vCJD) (174,175) .

Neurotrophins are a family of structurally and functionally closely related proteins consisting of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin 3 (NT-3) and NT-4/5. They are synthesized as precursors or proneurotrophins (Pro-NTs) by both neuronal and non-neuronal cell types prior to being either cleaved intracellularly by proconvertases or secreted in the unprocessed form. In the later case, conversion through proteolytic cleavage by plasmin or another extracellular protease to the mature form takes place (60). In the nervous system of vertebrates, neurotrophins control many aspects of embryonic development (cellular survival, differentiation, plasticity and regeneration) and adult function of most populations of neurons (66).

Their action depends on two different transmembrane-receptor signalling systems: (i) the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (Trk A, Trk B, Trk C) and (ii) the p75 neurotrophin receptor ($p75^{NTR}$), a member of the

tumour necrosis factor receptor (TNFR) superfamily (4,60). Different neurotrophins show binding specificity for particular receptors. NGF binds preferentially to Trk A; BDNF and NT-4 to Trk B and NT-3 to Trk C. These interactions are considered to be high affinity, but can be regulated by receptor dimerization, structural modifications or association with the $p75^{NTR}$. The $p75^{NTR}$ can bind to all NTs, and also acts as a co-receptor with Trk receptors. Proneurotrophins are also active ligands of Trk receptors, but their binding elicits functional effects opposite to those elicited by the binding of mature neurotrophins (4,176).

The interaction between neurotrophic factors and their receptors is involved in the mechanisms that regulate the differential vulnerability observed in the neuronal populations affected in neurodegenerative diseases (53). For example, BDNF expression is reduced in affected brain regions of both Huntington's disease (HD) patients and Huntington mutant mice (127). In addition, this neurotrophic factor plays a key role in HD as it has been shown to be the most potent survival-promoting factor for neurons in the striatum (128).

Likewise, BDNF has been observed to be reduced in neurons of the substantia nigra in Parkinson´s Disease (PD) (125). Several studies demonstrated that BDNF and its receptor Trk B exhibit therapeutic potential for PD, supporting the survival and preventing cell loss of dopaminergic neurons in the substantia nigra (33,126). It was proposed that neurotrophins could be used as therapeutic agents, either individually or in combination with other trophic factors, to treat several neurodegenerative disorders (165). However, methods for achieving effective dosing and delivery to the central nervous systems remain a substantial challenge (33).

In prion diseases, it has been demonstrated that PrP 106-126, a synthetic peptide homologous to the human PrP region 106-126, induces apoptosis in a mouse neuroblastoma N2a cell model involving $p75^{NTR}$ and the nuclear factor-kß (NF-kß) signalling pathway (112,177). This suggests that NTRs, and particularly the $p75^{NTR}$, might be involved in prion disease pathogenesis. However it has not been possible to find further publications on the subject.

In this study we report the results of an immunohistochemical study to investigate the role of neurotrophins and their receptors in a transgenic murine model (BoTg110) of BSE. This transgenic mouse line is characterized by the over expression (up to eight times the expression of a normal cow brain) of the bovine prnp on a murine PRNP knockout background (178) and has been shown to be a good model to study BSE pathogenesis (179–183).

Little information was found regarding immunohistochemical investigations of NTs and NTRs in the mouse brain (60,78,184–186), thus the study was performed in parallel on a wild type mouse line (Balb-C) to ensure that the transgene did not have an influence on the studied molecules immunolabelled and to establish a baseline immunolabelling pattern in paraffin-wax embedded mice brain samples.

6.2. Material and methods

6.2.1. Animal models and inoculum

Female BoTg 110 transgenic mice, over expressing bovine PrP^c, under the murine prnp promoter in a murine PrP background, were used (178).

In this model the bovine PrP^c expression levels are up to eight times higher than the PrP^c levels found in bovine brain homogenates.

6.2.1.1. Titration study

A case of BSE was identified within the BSE active surveillance plan. Characterization of this case has been described elsewhere (BSE case 1; (187,188)). An inoculum was prepared from this case homogenizing different brain areas. A total of 17,81g were used; 31% mesencephalon, 20% thalamus, 17% spinal cord, 13% pons, 12% medulla oblongata and 8% cerebellar vermis.

Six groups of 10 BoTg110 transgenic female mice were included in the titration study and therefore inoculated with six dilutions (10^{-1} to 10^{-6}) of the inoculum. An end point was set at 531±5 days post inoculation (dpi).

Each 6-8 week old mouse received an intracerebral inoculation through the parietal bone of 20µL using a 50µL precision syringe, a 25 G gauge and a repeatability adaptor. During the inoculation procedure the mice were kept under deep gaseous anesthesia (isofluorane). A subcutaneous dose of buprenorfin was administered before awakening to reduce post inoculation pain.

The Log_{10} lethal dose 50 (LD_{50}) for the inoculum per 20µl was 4.9 (i.e. brain homogenized and diluted at $10^{-4.9}$) as determined by bioassay. It was calculated using the Spearman Karber formula; Origin 7.0 and Labwork 4.6 software were used for data analysis.

All procedures were approved by the Animal Experimentation Ethics Committee of the Autonomous University of Barcelona (procedure number 585-3487).

To evaluate transmissible spongiform encephalopathy (TSE) related clinical signs, mice were observed daily and their neurological status was assessed twice a week. The presence of three signs of neurological dysfunction (using ten different items) was necessary for a mouse to score positive for prion disease (189,190).

6.2.1.2. Neurotrophin study

From the mice included in the titration study, a total of 14 female BoTg 110 mice were selected for the neurotrophin study and divided into two groups. Those inoculated with BSE inoculum (n=8; at a 1 in 10 dilution) and the control group (n=6) inoculated with a healthy cow brain homogenate at 1 in 1000 dilution (from now on called BSE infected and control group).

Animals from an additional mouse model were also included in this study. These were Balb-C wild type (WT) mice and 10 healthy non-inoculated females, 367 days old, were used to compare the immunostaining pattern with the BoTg 110_control transgenic model.

In summary, three experimental groups were used:

- BoTg 110_BSE infected
- BoTg 110_control
- Balb-C WT

Mice were kept under controlled conditions at room temperature (RT) of 21-22ºC; 12 hour light-darkness cycle and 60% relative humidity. Cages were isolated in HEPA filtered (both air infusion and extraction) ventilated racks. Food and water was administered *ad libitum* with 19% protein extruded rodent diet containing: crude protein no less than 19%, crude fat no less than 9% and crude fiber no more than 5%) (Teklad Global, Harlan-Teklad).

6.2.2. Post mortem studies and sample processing

When scored positive for clinical BSE, mice were sacrificed with an intraperitoneal overdose of sodium pentobarbital followed by cervical dislocation, in accordance with the recommendations of the ethics committee.

At necropsy examination, brain tissue was collected and placed in 10% neutral buffered formalin solution. Transverse sections were taken at three different levels of the brain (optic chiasm, piriform cortex and cerebellum/medulla oblongata) and these were processed routinely prior to being embedded in paraffin wax for histopathological, histochemical and immunohistochemical studies.

6.2.3. Immunohistochemistry , affinity histochemistry and histopathology

Using a conventional microtome (Leica RM2135), four micrometer thick sections were cut, mounted on glass slides and subsequently dewaxed and rehydrated. To carry out the histopathological study to evaluate the spongiform change, sections were stained with haematoxylin and eosin (HE).

- Immunohistochemistry for PrP^d: this was performed as previously described in our group (188) to visualize the PrP deposits. Briefly, sections were immersed in formic acid. Endogenous peroxidase activity was blocked by incubation with 3% H_2O_2 in methanol for 40 min and then sections were boiled at pH 6.15 in a pressure cooker. Following treatment with proteinase K, sections were incubated overnight with mouse anti-PrP 6H4 monoclonal antibody (diluted 1 in 400, Prionics, Schlieren, Switzerland) and visualized using the anti-mouse Dako EnVison Plus™ system with DAB as chromogen substrate.
- Immunohistochemistry for *glial fibrillary acidic protein* (GFAP): a rabbit polyclonal antibody was used for the astrocytic cells labelling (diluted 1 in

400, Dako Z0334). The antigen retrieval was done with citrate buffer (pH 6.0,96-98ºC in a water bath for 20´)

- *Lycopersicum esculentum* agglutinin (LEA; 1 in 100 dilution; Sigma L-0651, St Louis, Missouri, USA) histochemistry was performed on brain tissue sections for microglial cell membrane staining.

Blockage of the endogenous peroxidase activity was carried out with H_2O_2 diluted in methanol (20´). Slides were washed twice with PBS (5´) and mounted vertically. An additional wash was performed with PBST (2x5´). The washing buffer was supplemented with $CaCl_2$, $MgCl_2$ and $MnCl_2$ 1nM. Sections were incubated with the lectin at RT (2h) and washed with PBS (3x5´). The binding was visualized with Bottle 2 of Universal LSAB^{TM}+KitHRP, Rabbit/ Mouse/Goat (Dako K0690) and DAB as the chromogen substrate with another wash with PBS in between (3x5´).

- *NTs* and *NTRs* immunohistochemistry

  Sections were mounted on silanized glass slides (Knittel Glass, Germany) that were manually treated with 3-(triethoxysilyl)-propylamine (Merck, Germany). After dewaxing, endogenous peroxidase activity was blocked in the darkness by incubation in 3% hydrogen peroxide (H_2O_2) in methanol for 40 minutes. Two different heat-induced epitope retrieval procedures in citrate buffer (pH 6.0) were used. Sections were then cooled at RT for 30 min. Non-specific antibody binding was blocked with a blocking solution made of 2% bovine serum albumin (BSA) in PBS.Sections were incubated overnight at 4°C with corresponding primary antibodies against either NTs or NTRs. To visualize binding of primary antibodies, an anti-rabbit EnVision Plus^{TM} System (Dako, Glostrup, Denmark) was used, incubating the slides with the secondary reagent at RT for 30 min. The peroxidase substrate was 3, 3´-diaminobenzidine (DAB) in 200µl PBS and 100µl H_2O_2. Development time was set at maximum of 10 minutes depending on the immunopositivity of each antibody. An optical microscope was used to control the signal intensity.
  Sections were washed with PBS for the last time and counterstained with haematoxylin (Merck) for three seconds, dehydrated and mounted automatically (Leica Autostainer XI, Leica CV5030).
  In every case, omission of the primary antibody was used as a nonspecific secondary antibody binding control. Details on the IHC protocol for each marker used are summarized in Table 6.1.

6.2.4.Assessment of sections:

Spongiform lesions and the different immunolabeling patterns were semi-quantitatively evaluated by eye under the microscope (Olympus BH2) in 14 selected areas of the mice brain (Fig. 6.1)

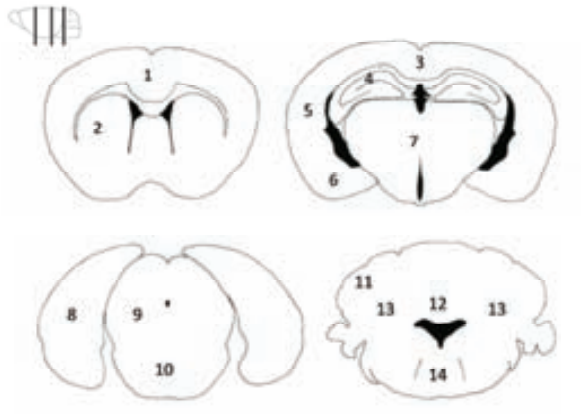

Figure 6.1: Schematic representation of the brain areas studied (transverse sections of the mouse brain): **1:** Frontal cortex; **2:** Striatum; **3:** Parietal cortex; **4:** Hippocampus; **5:** Temporal cortex; **6:** Piriform cortex **7**: Thalamus; **8:** Occipital cortex; **9:** Mesencephalon; **10:** Pons; **11:** Cerebellar hemispheres; **12:** Cerebellar vermis; **13:** Cerebellar nuclei; **14:** Medulla oblongata.

The scoring system established consisted of an scale between 0 and 4, where 0 represented the absence of lesion or labelling, 1 represented mild labelling; 2 moderate labelling; 3 when the immunolabelling was intense and 4 was attributed to the highest intensity of lesion or labelling (181).

Then, for each area, a mean value of all the animals in each group was obtained. These values corresponding to the 14 brain areas studied were represented graphically in order to obtain the final brain profile for each antibody used and compare the different mice groups (BSE vs. controls).

The U-Mann Whitney statistical test for non-parametrical data was applied (P<0,05 with a 95% confidence interval and P<0,01 with a 99% confidence interval). Photomicrographs were taken with a Leica DM 6000 B microscope, a Leica DFC 480 digital camera and Software Leica Application suite version 2.7.1.

Antibody	Target	Species	Dilution (Study 1/Study 2)	Supplier	Epitope retrieval
Human NGF	NGF protein	Rabbit (polyclonal)	1: 500 / 1:250	Sigma Chemicals, Saint Louis, Missouri USA (N665)	HIER citrate buffer (pressure cooker)
Anti BDNF	BDNF protein	Rabbit (polyclonal)	1:100	Abcam, Cambridge, UK (ab72439)	HIER citrate buffer (pressure cooker)
Anti NT-3	NT-3 protein	Rabbit (polyclonal)	1: 50	Abcam, Cambridge, UK (ab65804)	HIER citrate buffer (pressure cooker)
Anti Trk A	Intracellular domain of TrKA	Rabbit (monoclonal)	1: 50 / 1:100	Abcam, Cambridge, UK (ab76291)	HIER citrate buffer (pressure cooker)
Anti Trk B	Intracellular domain of TrKB	Rabbit (polyclonal)	1:200	Abcam, Cambridge, UK (ab51190)	HIER citrate buffer (pressure cooker)
Anti Trk C	Extracellular domain of TrKC	Rabbit (polyclonal)	1:100/ 1:250	Abcam, Cambridge, UK (ab75174)	HIER citrate buffer (pressure cooker)
Anti p75 neurotrophin receptor	Extracellular domain of $p75^{NTR}$	Rabbit (polyclonal)	1:500	Abcam, Cambridge, UK (ab8874)	HIER citrate buffer (water bath at 95º)

HIER: heat induced epitope retrieval

Table 6.1: Details of the immunolabelling procedures: data of the primary antibodies used for both studies 1 and 2.

6.3. Results

6.3.1. Experimental inoculation (disease induction) and inoculum titration

BSE was successfully transmitted to BoTg 110 mice. Animals inoculated with lower dilutions showed the shortest incubation periods and the highest attack rates in a dose dependent manner. Using the Spearmann Karber formula, the mice intracerebral Log10 Lethal Dose 50 (LD50) for the IPB inoculum per 20µL was 4.9 (i.e. brain homogenized and diluted at $10^{-4.9}$). If a weight correction factor was applied the mice intracerebral Log10 LD50 per 1g of brain tissue was 6.6.

The animals belonging to the group inoculated with the lowest dilution were chosen for the NTs/NTRs immunohistochemical study. Animals in this group were all TSE positive, had a mean incubation period of 350 dpi and all were considered to be at a terminal stage of the disease as they were all euthanized before the chosen endpoint (513 dpi) due to severe neurological dysfunction.

6.3.2. Spongiosis, PrP^d deposition and gliosis

The most characteristic TSE lesions (neuropil spongiosis, PrP^d deposition and gliosis) were evaluated in the BSE infected mice brains and compared with the group inoculated with healthy cow brain homogenate (negative control or healthy group) (Fig. 6.2).

In the BSE infected group, the haematoxylin-eosin staining revealed a very characteristic neuropil spongiosis lesion pattern consisting of multiple and different sized vacuoles mainly confined to the thalamus, mesencephalon, pons and medulla oblongata, but also, to some extent, in the hippocampus and cerebellar nuclei. The cerebral cortices were generally not as much affected with the exception of the occipital cortex (Fig.6.3 a). The control group showed no or slight spongiosis, the latter probably due to ageing and this was most notable in the cerebellar white matter.

Granular and plaque like rounded PrP^d deposits labelled positively in the mesencephalon, medulla oblongata, thalamus, striated body and hippocampus (more frequently in the *cornu ammonis* than in the dentate gyrus). The least affected areas were the temporal, frontal and parietal cerebral cortices and the piriform cortex. However, in the occipital cortex, the immunolabelling was slightly greater. None of the control animals showed any PrP^d immunolabelling (Fig.6.3b and 6.4).

Immunohistochemistry for Glial fibrillary acidic protein (GFAP; 1 in 400 dilution; Dako, Glostrup, Denmark) revealed the typical stellate cell immunolabelling pattern, which was particularly evident in the white matter (corpus callosum and cerebellar white matter) in the negative control group. In the BSE infected group cells with numerous prolongations, corresponding to hypertrophic astrocytes, were abundant and strongly positive for GFAP in the striatum, thalamus, mesencephalon (Fig.6.2) (particularly in the tegmentum), pons, medulla oblongata and cerebellar nuclei. Semiquantification revealed statistically significant

differences in the aforementioned regions. No significant differences were found in the degree of immunolabelling for GFAP in the neocortices of the BSE infected and negative control mice (Fig.6.3c).

When histochemistry to *Lycopersicum esculentum* agglutinin (LEA, 1 in 100 dilution, Sigma, St.Louis, Missouri, USA) was performed, ramified cells, corresponding to microglia, were stained throughout the brain, both in the white and grey matter. In the BSE infected mice brains these cells were appreciably increased in number, hypertrophic and, occasionally, some of them were binucleated. LEA also stained the vascular endothelia and some intraneuronal organelles.

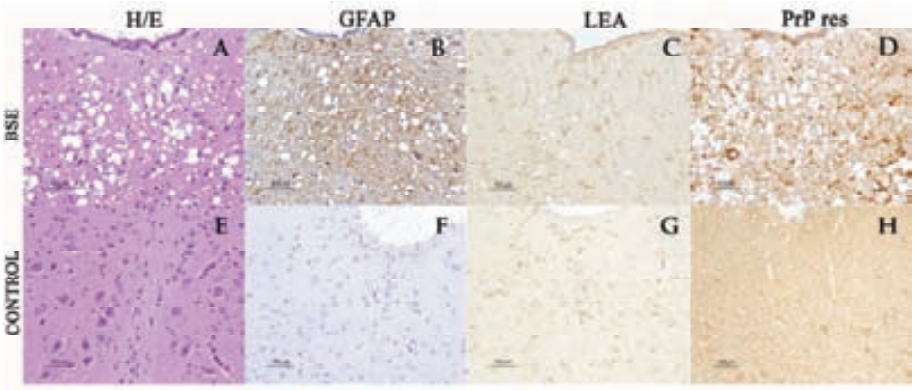

Figure 6.2: Neuropathological characterization of the mesencephalon of BoTg 110 BSE infected mice (top panel, A-D) **and BoTg 110 control mice** (bottom panel, E-F). Haematoxylin eosin staining (A, E), immunohistochemistry for GFAP (B, F), histochemistry for *Lycopersicum Esculentum* agglutinin (C, G) and immunohistochemistry for PrPd (D and H). Scale bars 50 µm.

When both groups were compared (BoTg 110 BSE infected *vs.* BoTg 110 control), statistically significant differences were found in thalamus (*P*=0.01917), medulla oblongata (*P*=0.00178) and cerebellar nuclei (*P*=0.01183), where the score was higher in the BSE infected group (Fig.6.3 d). (Table 1 in section 11 Annex)

6.3.3. Neurotrophins and their receptors immunolabelling

- **Nerve growth factor (NGF)**

Mild, diffuse positive immunolabelling for NGF was observed in the perikaryon of the majority of neurons throughout the brain. Very mild immunolabelling of the neuropil in the gray matter was also observed. White matter was generally devoid of immunolabelling with the exception of the cerebellum where mild diffuse immunolabelling in the white matter was present. The pontine nuclei showed slightly stronger immunolabelling. In the cerebellar cortex the neuropil of the molecular layer was mildly positive while Purkinje cell perikarya immunolabelling was variable. Sometimes intracytoplasmic labelling was observed while on occasions the cytoplasm was negative and extracellular peri-neuronal immunolabelling was present. In the hippocampus, the labelling was more intense

in the cornu ammonis than in the other areas. The parietal and temporal lobes of the cerebrum showed slightly stronger neuronal immunolabelling in layers II/III. The choroid plexus and ependymal cells also labelled positively (Fig.6.4a and 6.4b).

No significant differences were observed when WT (Balb-C) mice were compared with healthy BoTg110 mice (P> 0,05); the medulla oblongata was slightly more intensely labelled in WT mice but this was not significant upon semiquantification. The immunolabelling pattern remained unchanged in the BSE infected group compared with the healthy group. No statistically significant differences were obtained when comparing the semiquantitative evaluation of the pattern described above (Fig.6.3e).

- **Brain derived neurotrophic factor (BDNF)**

The anti-BDNF antibody revealed a diffuse intracytoplasmic and nuclear immunolabelling pattern in the neurons (Fig.6.4C and 6.4D). The neuropil also showed mild immunolabelling, which, at higher magnification, consisted of a fine, punctuate pattern; this was particularly intense in the perivascular compartment (probably depicting the external *glial limitans*) and was also seen associated with scattered glial cell prolongations. The white matter was devoid of immunolabelling. The choroid plexus and ependymal cells were positive in all mice studied.

Immunolabelling distribution was rather homogeneous throughout the brain with higher intensity in some of the examined areas, such as the hippocampus, thalamus, mesencephalon, pons, medulla oblongata and the cerebellum. In contrast, the striatum was less intensely immunolabelled than other areas (Fig.6.3f).

Of note, in the hippocampus, the cornu ammonis, and particularly the stratum lucidum layer, showed stronger immunolabelling than the dentate gyrus. In the thalamus, a fine, perineuronal and intense punctuate immunolabelling pattern was found mainly in the habenular nucleus (Fig.6.4D). In the mesencephalon, the positive immunolabelling was mainly found in the lateral, medial and ventral areas (Fig.6.4C). Both oculomotor and red nuclei were always BDNF positive, the pontine nuclei were also strongly positive and the cerebellar nuclei were intensely immunolabelled. Additionally, the three different cerebellar layers had different intensities of immunolabelling: in the molecular layer the immunolabelling was associated with the dendrites of Purkinje cells. The Purkinje cell perikarya were also immunoreactive. In the granular layer only the Golgi neurons were labelled. Neurons in the medulla oblongata were also positively immunolabelled for BDNF, particularly the ones in the superior vestibular nuclei (SuVe), ventral cochlear nuclei (VCoA), raphe magnus nucleus (RMg), raphe nuclei (RA), pallidus nucleus (Rpa) and facial nuclei (7N).

With respect to the immunolabelling pattern and distribution no significant differences were found between negative control and BSE infected groups (Fig.6.3f). In the mesencephalon the spongiform change was restricted to the areas positive for BDNF. No significant differences were present between control

and BSE infected groups, when comparing the semi-quantitative evaluation of the pattern described above.

Although no statistically significant differences were detected between the healthy BoTg 110 mice and the WT model (Balb-C mice) slightly stronger immunolabelling was seen in the Purkinje cell layer of the WT group.

- **Neurotrophin 3 (NT-3)**

Strong intraneuronal immunolabelling for NT-3 was observed, but less intensity was found in the neuropil and the nucleus of neurons was devoid of immunolabelling (Fig. 6.4E and 6.4F).

In the white matter only some glial cells, probably oligodendrocytes, and the choroid plexus labelled positively for NT-3 but the ependymal cells and other glial cells were devoid of immunolabelling.

In the cerebellum, strong labelling was detected in the perikarya of the Purkinje cells (Fig.6.4F), and also in the neurons of the cerebellar nuclei. Mild to moderate labelling of the molecular layer was observed and it was mild to absent in the granular layer where Golgi neurons labelled positively. In the medulla oblongata, NT-3 was distributed homogenously intra-cellular, but the labelling was stronger in the ventral area. The cerebrocortical areas were also homogenously and strongly labelled in the neuropil and intra-neuronal except for the superficial molecular layer, which was less intensely labelled. In the hippocampus the pyramidal cell layer of the CA3 and CA4 regions was more intensely labelled than the other layers and the dentate gyrus. The neuropil was mildly labelled. In the striated body the neuropil of the grey matter was intensely labelled as was the perikarya of neurons. In the mesencephalon an intraneuronal, homogenous immunolabelling pattern was observed and the neuropil was almost devoid of labelling. Similarly in the thalamus an intracytoplasmic labelling was present and the neuropil labelling very mild.

Statistical significance was not achieved when assessing differences between the control and BSE groups (Fig.6.3g) nor between healthy BoTg 110 and Balb-C mice. In the control BoTg 110 group no samples of the pons were available for study.

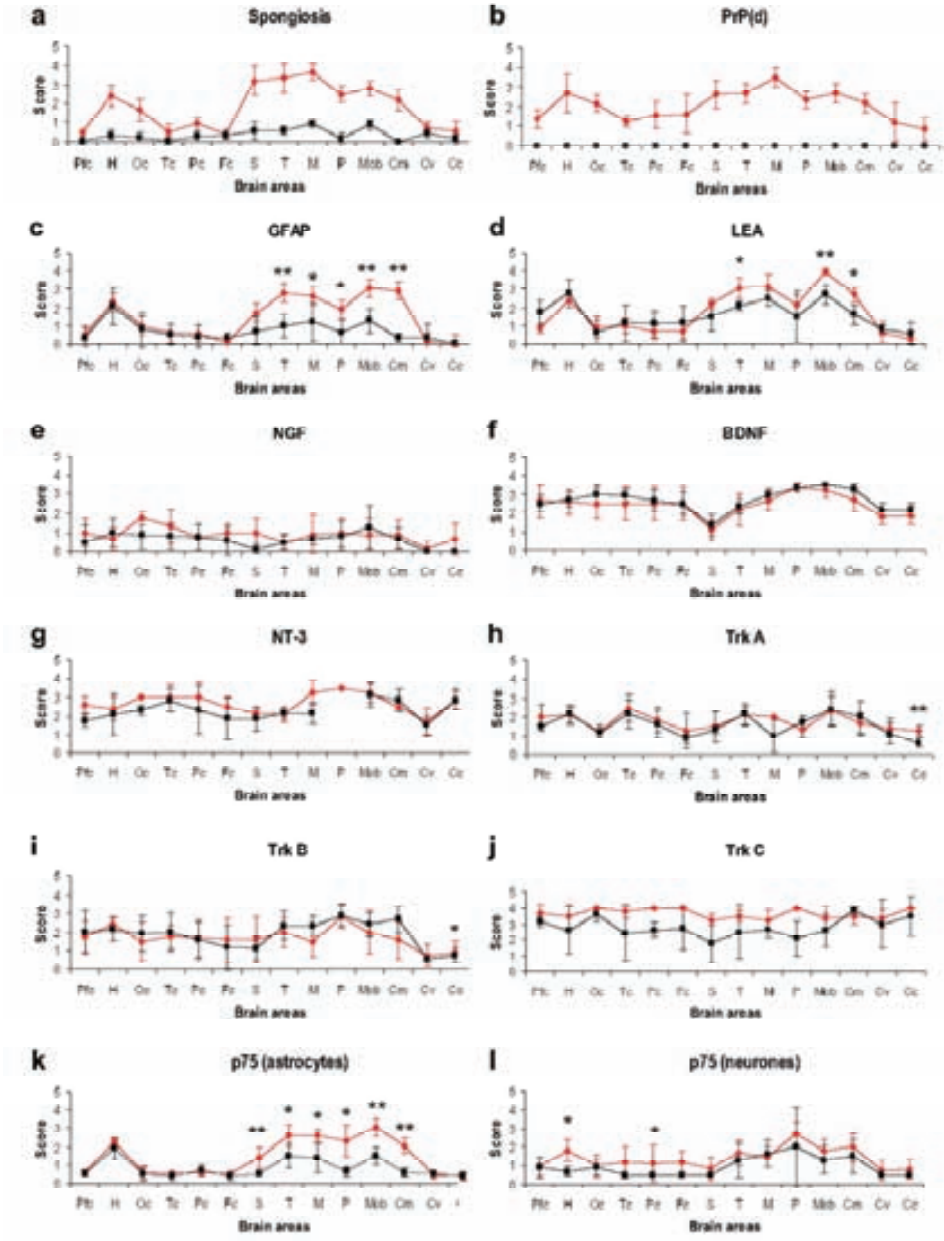

Figure 6.3: Graphic representation of the semiquantitative expression of different markers in the mouse brain. Comparison of the mean scores in Control (black lines -----) and BSE inoculated (red lines -----) mice groups. Scores for **(a)** spongiosis, **(b)** PrP^d deposition, **(c)** astrogliosis, **(d)** microglia, **(e-g)** neurotrophins and **(h-l)** neurotrophin receptors. Vertical bars indicate standard deviation. Mann Whitney U test (*****P* < 0, 05 with a 95% confidence interval and ****P*< 0,01 with a 90% confidence interval).Pfc:piriform cortex; H:hippocampus; Oc:occipital cortex; Tc:temporal cortex; Pc: parietal cortex; Fc:frontal cortex; S:striatum; T:thalamus; M:mesencephalon; P:pons; Mob:medulla oblongata; Cm:cerebellar nuclei; Cv:cerebellar vermis; Cc:cerebellar cortex.

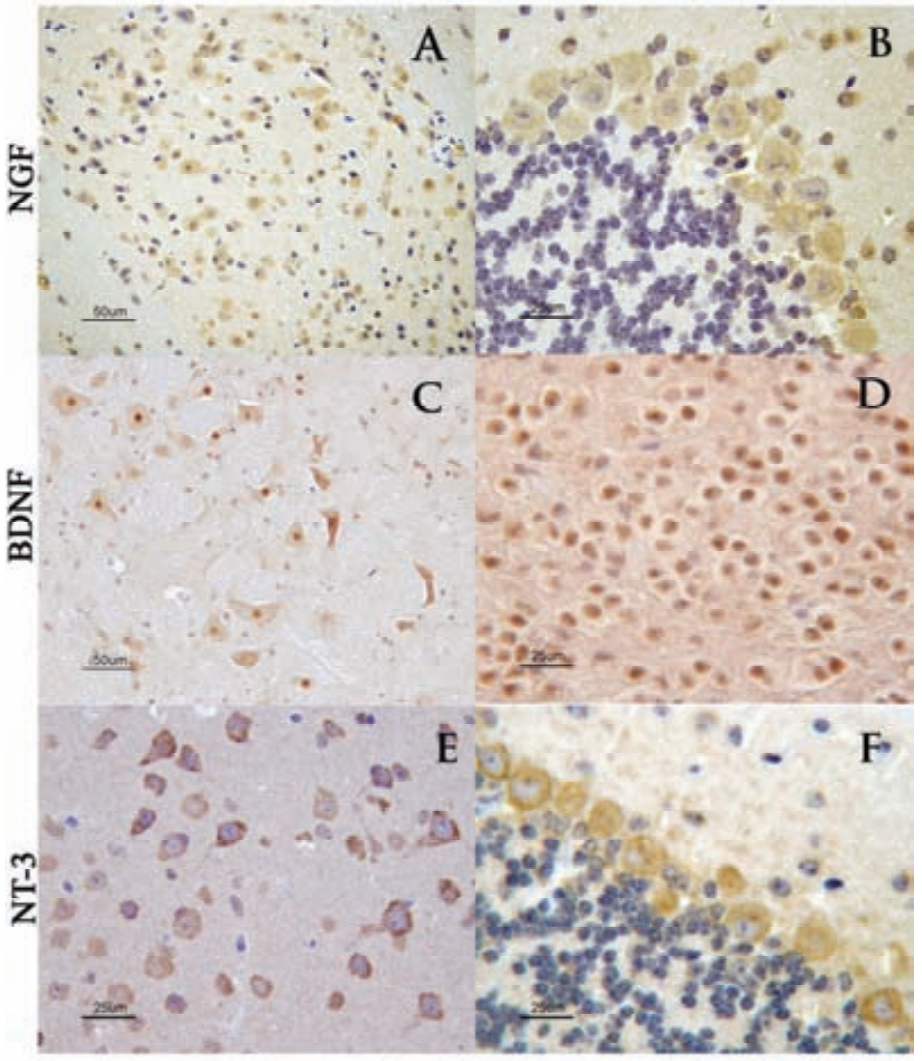

Figure 6.4: Neurotrophin immunolabelling patterns in the brain of the control BoTg 110 murine model. NGF: **(A)** pontine nucleus **(B)** cerebellar cortex; BDNF: **(C)** mesencephalon **(D)** habenular nuclei. NT-3: **(E)** neocortex; **(F)** cerebellar cortex. Scale bars a and c: 50 µm; b, d, e and f: 25 µm.

- **Trk A:**

Immunohistochemistry for the NGF receptor showed a diffuse, moderate to intense labelling of the neuropil, which was particularly intense in the peri-neuronal areas (Fig.6.5A). Additionally, intracytoplasmic labelling was detected in the following areas: the interpeduncular nuclei in the tegmentum of the mesencephalon, the pontine nuclei, the cerebellar nuclei (Fig.6.5B) and the medulla oblongata (facial, paragigantocelullar reticular and raphe magnus nuclei).

Both peri-neuronal and neuropil labelling for NFG were present within the inferior olive nuclei (the labelling was more evident in the medial nuclei of this area). The neocortical areas were strongly reactive to the anti-Trk A antibody. In the cerebellum, mild to moderate labelling was found in the neuropil of both the granular and molecular layers. In contrast, mild or no labelling was present in the cytoplasm of Purkinje cells. Within the hippocampus the labelling was uniform except for the *stratum lucidum* layer, which was less intensely labelled. Only some neurons in the *cornu ammonis* showed intracytoplasmic labelling. In the diencephalon, the following thalamic nuclei showed intense labelling: the ventral posteromedial (VPM), the ventral posterolateral (VPL) and ventrolateral (VL). Neither choroid plexus, ependymal cells nor the white matter were immunoreactive.

No statistically significant differences were detected between the WT model and BoTg110 control mice with the exception of the frontal cortex (P=0.008475) and striatum (P=0.006928) where the immunolabelling was slightly higher in the WT animals (Balb-C mice). When both, negative control and BSE infected group were compared, statistical significance was only achieved in the cerebellar cortex (Fig.6.3h), due to a more intense labelling in the granular layer of the infected animals. Additionally, in the hippocampus of the infected animals a granular labelling was detected associated to spongiform foci. The thalamic nuclei described above were markedly less labelled but only in those animals, which showed intense lesions in the region. However, since variation in lesion intensity existed between animals, the mean score was not significantly different from the control group.

- **Trk B**

The main immunolabelling pattern observed with the anti Trk B antibody was peri-neuronal although some areas showed either a diffuse labelling of the neuropil or intraneuronal labelling (Fig.6.5C and 6.5D). Choroid plexus and ependymal cells were also immunoreactive. A mild to absent labelling was present in the white matter.

In both temporal and parietal cerebral cortices a mild to moderate labelling for Trk B was present and restricted to the neuropil. In the piriform cortex this pattern was less intense. In the hippocampus the immunolabelling was more intense in the pyramidal cell layer of the CA3 and CA4 regions of the *cornu ammonis*. Surrounding both the aqueduct and ventricle walls, numerous, intensely labelled, amorphous structures were observed, some of them had an unstained central core (Fig.6.5D).The thalamus showed strong peri-neuronal and intraneuronal labelling, mainly in the VPM, VPL and VL nuclei. The thalamic neuropil was also intensely labelled. In the mesencephalon, the peri-neuronal labelling was evident in the nuclei of the tegmental region. Additionally, the pontine nuclei showed very strong intraneuronal immunolabelling.

In the cerebellum, immunoreactivity of the Purkinje cell layer was inconsistent, occasionally peri-neuronal and also intraneuronal immunolabelling was seen but also Purkinje cells were frequently devoid of labelling. The granular layer was also devoid of immunolabelling and the molecular layer showed a moderate to intense

immunolabelling of the neuropil. In the cerebellar nuclei both neuropil and peri-neuronal immunolabelling was intense. The peri-neuronal immunolabelling pattern was evident in the vestibular, cochlear and ventral nuclei of the *medulla oblongata* (Fig.6.5C). No significant differences were found when the WT animals (Balb-C) and the BoTg 110 control groups were compared, with the exception of the *medulla oblongata* (*P*=0.005614), which was slightly less intensely immunolabelled in the BoTg 110 (Fig.6.3i). This was also the case in the cerebellar cortex where the Purkinje cell perikarya were more intensely labelled in the wild type murine model.

No significant differences were observed between BSE inoculated and healthy BoTg 110 mice.

- **Trk C**

The immunolabelling pattern observed with the anti-Trk C antibody consisted of intense labelling of the neuronal perikaryon and neuronal prolongations. Moderate diffuse labelling of the neuropil was also present in some areas. Ependymal cells and the choroid plexus were occasionally labelled. The white matter was mildly labelled.

In the neocortex, intraneuronal labelling was prominent, particularly in pyramidal neuron layers (III and V) (Fig.6.5E). In the neuropil, labelling of neurites was very prominent, particularly in the temporal lobe. Neurons of the piriform cortex were also strongly labelled. Conversely, in the striatum positive labelling was very mild. In the hippocampus the intraneuronal labelling was also very mild and restricted to the pyramidal layer of the *cornu ammonis*. In the *stratum radiatum* the radial dendrites of the pyramidal layer neurons appeared intensely labelled and, with a more disorganized aspect, the neurites of the *lacunosum moleculare* layer. In the dentate gyrus, neurite labelling was evident in both granular and molecular layers. In the thalamus the immunoreactivity was mainly localized in the habenular nuclei (Fig.6.5F), the geniculate nuclei, the posterior thalamic nuclear group (Po), VPM and VPL, and also in the median eminence (ME) in the hypothalamic area. In the mesencephalon, the immunolabelling was stronger in the neurites of the tectum (mesencephalic colicles). Both red and oculomotor nuclei showed mild to moderate intraneuronal labelling and the pontine nuclei were also intensely labelled. In the cerebellum, the immunolabelling of the molecular layer was due to the dendrites of the Purkinje neurons, the perikaryon of which was intensely labelled also. The granular layer was devoid of labelling except for the Golgi neurons. In the cerebellar nuclei an intracytoplasmic labelling pattern was observed. In the medulla oblongata the neurites were positive, particularly in the facial and the ventral cochlear nuclei were an intense intraneuronal labelling was present.

When comparing the Trk C immunolabelling of BoTg 110 control group and the WT model (Balb C) no significant differences were found except in the cerebellar nuclei (Fig.6.3j), where the BoTg 110 mice showed slightly stronger immunolabelling (*P*=0.01421).

Again, BSE inoculated mice did not show any significant differences compared to negative control animals.

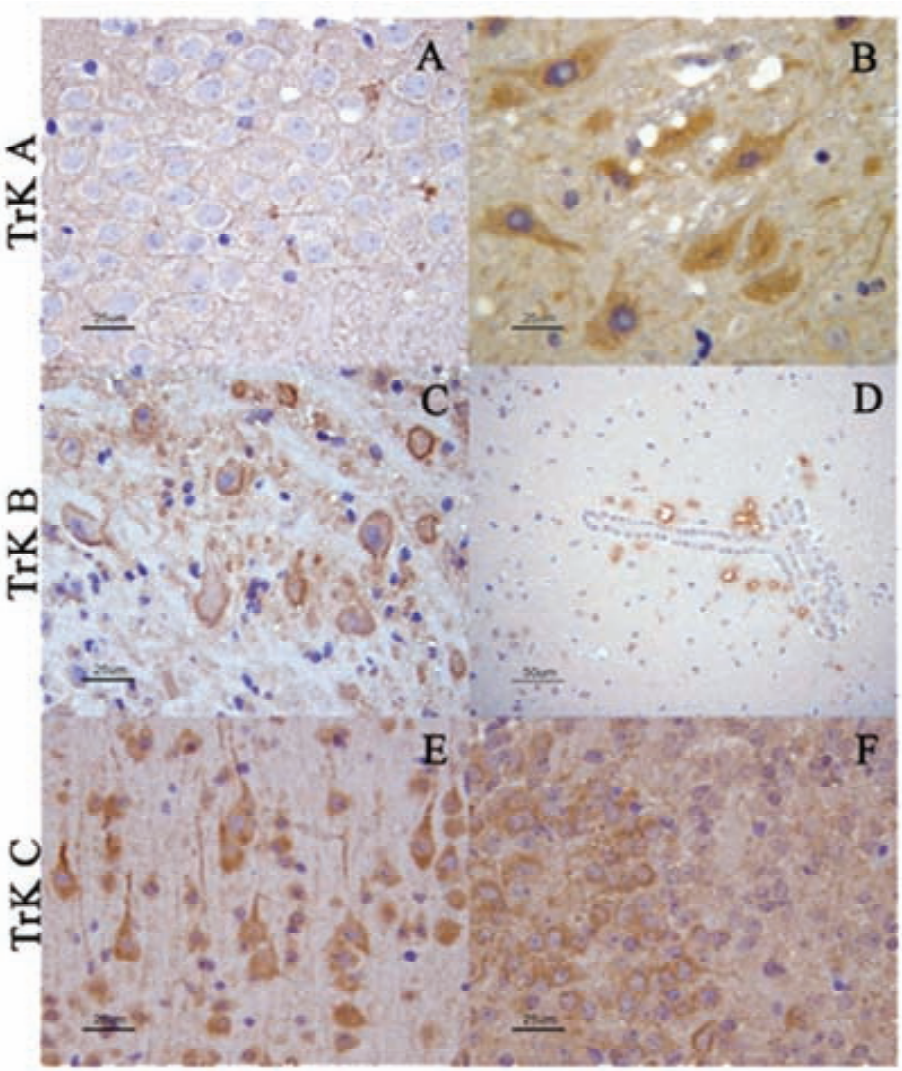

Figure 6.5: **Neurotrophin receptors immunolabelling patterns in the brain of the control BoTg 110 murine model**. Trk A: **(A)** hippocampus **(B)** medulla oblongata; Trk B: **(C)** medulla oblongata **(D)** amorphous structures in the aqueduct. Trk C: **(E)** frontal cortex **(F)** habenular nucleus. Scale bars A,B,C,E,F: 25 µm ; D: 50 µm.

- **p75 ^{NTR}**

An intraneuronal, occasionally finely granular, immunolabelling pattern for p75^{NTR} of mild to moderate intensity depending on the area studied was present. Additionally, a variable number of stellate shaped, glial cells were also positive and particularly numerous in the subependymal region and perivascularly. Its morphology and anatomical distribution strongly suggests that these cells are astrocytes. However it cannot be ruled out that some microglial cells are also stained. In all the mice the neuropil was mildly labelled. Ependymal cells were devoid of labelling, however, the apical membrane of the choroid plexus cells showed intense immunoreactivity (Fig.6.6F).

Neuronal and glial cells immunolabelling intensities were scored separately. The glial cell distribution was as follows: in the white matter, particularly that of the *corpus callosum* and cerebellum, many abundantly ramified cells, probably fibrillary astrocytes, labelled intensely for the p75^{NTR}. With respect to the grey matter, in the cerebral cortical areas a low number of strongly labelling ramified cells was present, particularly in the deeper layers. In the hippocampus, a high number of positively labelled glial cells was found, mainly in the *oriens, radiatum, lacunosum moleculare* layers of the *cornu ammonis* and molecular layer of the dentate gyrus. In comparison, in the thalamus, these cells were fewer but present in the habenular nuclei. The presence of positively labelled glial cells in the mesencephalon was quite similar, in number, to the thalamus, particularly in the tegmentum. The same applied to the pontine nuclei.

A great number of positively labelled glial cells was detected in the medulla oblongata and slightly more in the facial nuclei. In the cerebellar cortex, few cells were labelled in the grey matter.

As described previously with the GFAP antibody, in the BSE infected mice increased immunolabelling was present compared to the control group (Fig.6.6A and 6.6B) and this was due to the presence of a higher number of hypertrophic glial cells. Positively labelled amoeboid shaped glial cells were also detected in the thalamus, mesencephalon, cerebellar nuclei and medulla oblongata. These differences were statistically significant when the scoring of the striatum (P=0.00811), thalamus (P=0.02811), mesencephalon (P=0.01917), pons (P=0.01902), medulla oblongata (P=0.006766) and cerebellar nuclei (P= 0.00431) were compared (Fig.6.3k).

In the neocortex the intraneuronal immunolabelling was restricted to both internal and external pyramidal layers. The parietal cortex was the most intensely labelled region (Fig.6.6C). In contrast, in the striatum the labelling for p75^{NTR} was very mild. In the hippocampus the pyramidal layer of the *cornu ammonis* was much more intensely labelled than the dentate gyrus. Mild labelling was observed in the hypothalamus and mild to moderate in the thalamus and the medulla oblongata was moderately labelled (Fig.6.6E). In the cerebellum the labelling was confined to the neuropil, except for the cerebellar nuclei in which a moderate intraneuronal signalling was seen.

With respect to the neuronal pattern, a statistically significant increase in immunolabelling intensity was found only in the hippocampus (*P*=0.01041) and frontal cortex (*P*=0.03379) of the BSE inoculated group compared to the negative control group (Fig.6.3l).

No differences were observed when WT animals were compared with healthy (control) BoTg 110 mice.

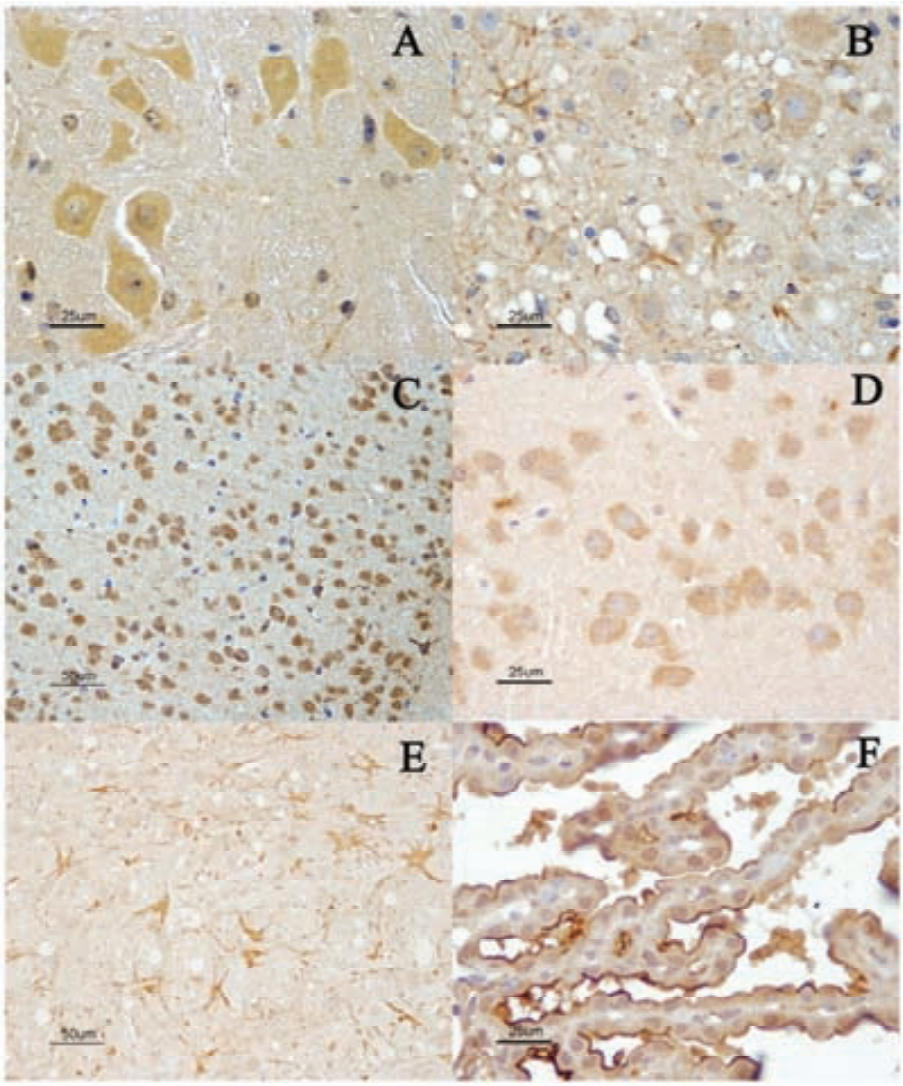

Figure 6.6: p75^{NTR} immunolabelling in the mouse brain. (A) Mesencephalon of BoTg110 control mice**. (B)** Mesencephalon of BoTg110 BSE infected mice. **(C**) Parietal cortex and **(D)** occipital cortex of BoTg110 control mice. **(E)** Hypertrophic astrocytes in the medulla oblongata of BSE infected mice. **(F)** Choroid plexus of BalbC WT control mice. Scale bars A,B,D and F: 25 µm; C and e: 50 µm

6.4. Discussion:

In the present study, an immunohistochemical assessment of the role of neurotrophins NGF, BDNF, NT-3 and their receptors (TrK A, TrK B, TrK C, $p75^{NTR}$) in the pathogenesis of BSE has been performed in adult mice brains. For this purpose, a BSE infected group of mice and a healthy cow brain inoculated group belonging to a transgenic line (BoTg 110) were compared. The immunolabelling was semiquantitatively assessed and the results analysed statistically in order to establish any significant differences between both groups. Additionally, a wild type mouse model (BalB-C) was also included in this study to ensure that the transgene had no independent influence on the results. This group and the BoTg 110 control group were used for the NTs+NTRs brain mapping.

To the authors' knowledge, little literature has been published regarding the distribution of neurotrophins in the healthy mouse brain as detected by immunohistochemistry. However, the expression patterns of approximately 20,000 genes (including the ones encoding NT) have been fully studied by ISH (161).

To date, in rodents, the majority of studies that have explored the expression, distribution and function of neurotrophin proteins in the CNS are limited to rats (28,36,47,49) although some studies have been reported in primates (55,191) and human brains (53,162,163).

The immunohistochemical approach in adult mice brains, allowed us to thoroughly study the distribution of these proteins throughout the organ and in which cell populations the protein accumulate rather than just where they are expressed as shown by ISH studies. The main advantage of using IHC is that it is an ideal method to determine whether any correlation existed between the studied molecules and BSE associated neuropathological changes (spongiform lesions, glial proliferation and PrP^{d} deposition).

In both the BoTg110 control group and the Balb-C (wild type) mouse model, all the NTs+NTRs were shown to be widely distributed throughout the brain. Comparison between these two groups did not show any significant differences regarding the NTs+NTRs cellular and neuroanatomical distribution. When the studied brain areas were semi-quantitatively scored, minor differences were detected in the NTRs immunolabelling of only a few areas (Table 2 section 11, Annex). Therefore our results suggested that when compared to WT mice, the BoTg110 model transgene did not significantly alter the NTs+NTRs expression.

NGF quantification in the rat CNS by immunolabelling showed widespread NGF-like immunoreactivity in the cell bodies and also in the dendrites and axons of neurons (192). In the present study we showed intraneuronal immunolabelling and mild immunolabelling of the neuropil, which was less widespread. However, since the technique used in the former study was performed on frozen tissue sections rather than formalin fixed paraffin wax embedded samples, this may explain the variations in the labelling pattern and intensity described, though a species specific distribution cannot be ruled out.

The NGF content of the cerebral cortex was reported to be more dense in layers II/III and V-VI in rats, while in the mice of this study this enhanced signalling was

only seen in layers II and III. The differences observed in staining intensity between areas seem to be subtler in our case, although, as previously described in the rat (192), a more intense immunolabelling was observed in the hippocampus (*cornu ammonis* pyramidal layer) and pontine nuclei (*Raphe pontis nucleus*) of our mice**.** Using *in situ* hybridization NGF gene expression was mild and widespread through the adult mouse brain (161) and had a similar distribution to the protein as denoted by our immunohistochemical results. In the rat brain the highest levels of NGF m-RNA expression were found in the olfactory bulb, neocortex and the hippocampus, and lower in other brain regions (193).

In a study which focused solely on the hippocampus of the primate brain, immunoreactivity was found not only in the *cornu ammonis* (CA2 and CA3) but also in the dentate gyrus (191). Other studies have examined the CNS expression of NGF in human brain, where NGF distribution was reported to be widespread but particularly high in the hippocampus and neocortex (53).

BDNF was homogenously found in all the brain areas studied, the striatum, however, appeared less intensely labelled than the other brain areas. Although the immunolabelling was mostly confined to neurons scattered glial cell prolongations were also labelled; this was in agreement with previous publications which state that not only neurons but also astrocytes are capable of producing NTs in the normal CNS (47).

Immunohistochemical studies in frozen brain sections from rat brains (36,194) are mostly in agreement with the distribution seen in the mouse brain in this study, i.e. stronger labelling of the hippocampus, thalamus, mesencephalon, pons and medulla oblongata. However in the cerebellum only scattered Purkinje cells were labelled in the rat, also labelling of the striatum seemed to be more intense in the rat than what we observed in the mouse. Other differences were detected such as in the hippocampus, while in pigs and rats immunoreactivity was detected in the dentate gyrus and *cornu ammonis* (195–197), in our mice only the *cornu ammonis* was labelled.

BDNF nuclear labelling was present only in neurons. While some authors report BDNF immunoreactivity only in the neuronal perikaryon (186,194) other studies also describe immunolabelling in the nucleus, such as occurred in this study, and discuss its specificity (198).

NT-3 was detected in the neuronal perikarya (but not in the nuclei) and, occasionally, a few glial cells appeared immunoreactive in the white matter. Previous description established that, in the rat central nervous system, both neuronal and glial populations are NT-3 immunopositive (49). Also, in humans, rats and monkeys brains similar results were reported with the exception of the NT-3 immunoreactivity within the glial cells, which was present in many more brain areas such as the substantia nigra, the fimbria of the hippocampus, and the cerebellum (47,53,55).

Our results showed that **Trk A** was primarily present perineuronally. Neuropil and intracytoplasmic labelling were also positive but with less intensity. The

immunolabelling was particularly prevalent in the hippocampus, neocortex and the brainstem.

By ISH and IHC, the Trk A expression has been reported in cholinergic neurons (basal forebrain and neostriatum) and non-cholinergic neurons (diencephalon and brainstem) of the rat brain (75). In the brainstem, specific nuclei were stained with Trk A antibody in rats and this was also the case for our mice: interpeduncular, pontine, inferior olive and paragigantocellular nuclei. Other nuclei/areas were labelled in the rat but failed to do so in our mice: gigantocellular, hypoglossal nuclei and the area postrema (75).

No evidence of Trk A expression was found in the cerebellum of the rat, in contrast, in the mouse brain an intracytoplasmic labelling was found in a small number of scattered Purkinje cells and some in the cerebellar nuclei. This difference might be explained by the different antibody used in each study; while an antibody against an intracellular domain was used in our study, Holtzman *et al*. used an antibody against the extracellular domain of Trk A. ISH studies in the mouse brain show an intense expression of Trk A mRNA particularly in the striatum, and moderate levels elsewhere (161); immunolabelling, however, revealed only small differences between areas.

The labelling of **Trk B** was perineuronal and occasionally in neuronal perikarya. Choroid plexus and ependymal cells labelled mildly and the glia failed to label. The positively labelled amorphous structures described in the results were GFAP and LEA negative and thus were presumably not astroglial or microglial in origin. Neither were they positive by any of the other markers used.

This receptor is expressed in several isoforms, including both the “full length” kinase-containing form and truncated isoforms, which lack the kinase domain (78). High levels of expression of the truncated receptors have been found in the adult rat CNS (80) where expression is also observed in glial cells such as astrocytes, oligodendrocytes and Schwann cells (81) and in the choroid plexus and ependymal (72). Positive labelling of glial cells was not observed in our mice. The use of an antibody against the ‘‘full length’’ Trk B might explain the differences if the truncated forms did not have the recognised epitope.

Immunolabelling for **Trk C** was strongly positive in neuronal perikarya and the prolongations throughout the brain. Similar results were found in the rat central nervous system by *in situ* hybridization in other studies: Trk C receptor is widely distributed in the brain including the neocortex, hippocampus, brainstem nuclei, and cerebellum (199).

An antibody against the extracellular domain of the **p75 ^{NTR}** was used in this study and immunohistochemical analysis showed the presence of this receptor in all brain areas examined. Both glial and neuronal populations were immunoreactive for p75 ^{NTR}. As two immunolabelling patterns were clearly identifiable for p75 ^{NTR}, the neuronal and glial immunolabelling was scored separately. Although this receptor was found initially in neurons, it has now been shown to have a crucial role also in glial biology (101,200,201).

In the healthy brain, astrocytes and neurons immunopositive for p75 ^{NTR} were observed throughout the brain but predominantly in the brainstem (thalamus, mesencephalon, pons and medulla oblongata). Additionally, the astrocytic labelling was more evident in the hippocampal area.

By ISH, p75^{NTR} was also observed throughout the mouse brain but showing different levels of expression. The highest expression was found in the pallidum followed by the hypothalamus, cerebellum, mesencephalon, pons, medulla oblongata and neocortex (188). Regarding the general brain distribution of p75 ^{NTR} in other species, no reports have been found except for a few focused only in specific areas (185,202–204).

Based on immunohistochemical results, no evidence was found between BSE infected and control animals to indicate that NTs and NTRs are involved in the pathogenesis of BSE, with the exception of p75 ^{NTR}.

In the BSE infected mice, a statistical significant increase of the p75 ^{NTR} glial-type labelling was observed in the striatum, thalamus, mesencephalon, medulla oblongata and cerebellar nuclei. This increase was parallel to the increase in astrocytes in the BSE infected group reflecting the glial activation seen in BSE, which is apparently accompanied by an increased expression of the p75 ^{NTR}.

Interestingly, when analysing the levels of immunolabelling in uninfected control animals, particularly that of the glial cells, that of p75^{NTR} is significantly enhanced in the thalamus, hippocampus, mesencephalon and medulla oblongata, when compared to other areas in both WT and BoTg 110 control animals (Table 3 in section 11, Annex). In these areas, an increase in the p75^{NTR} signalling is observed in BSE inoculated animals, with the exception of the hippocampus, where the p75^{NTR} levels remain unchanged. Additionally in these areas, a notable PrP^{d} deposition and spongiform lesion were observed. This suggests a relationship might exist between the regional expression of the p75^{NTR} in the normal brain and the BSE associated brain lesion distribution.

Whether the topographical distribution of the p75^{NTR} in the brain governs, at least in part, the distribution of BSE related pathology is unknown. However, Della-Bianca *et al*. (2001) showed that the binding of the synthetic PrP 106-126 peptide to p75^{NTR} triggered cell death mechanisms in cultured neural cells by caspase 8 and NDAPH oxidase dependant mechanisms (112). Therefore it is plausible to hypothesize that the extracellular deposits of BSE associated PrP^{d} might trigger a similar mechanism *in vivo*.

The brain lesion and PrP^{d} distribution are known to be features which vary according to the strain of abnormal prion protein, thus in an identical mouse model different strains yield different PrP^{d} profiles (205). The distribution of lesions and PrP^{d} observed in the present model largely coincides with that observed in BSE infected cattle (188) and with studies performed in the same model with different BSE inocula (178,182,187,188). Should the above hypothesis be confirmed, it would be dependent on a strain specific interaction between p75^{NTR} and BSE-misfolded PrP^{d}.

The $p75^{NTR}$ is widely expressed in developmental stages and decreases dramatically in adulthood. However it can increase in pathological states associated with neural cell death or neurodegeneration (4,206). An immunohistochemical study performed on normal human brain samples, non-human primate tissue and adult human tissue affected by different neurodegenerative disorders revealed $p75^{NTR}$ re-expression in the cortical neurons, mainly in patients affected by Alzheimer´s Disease (AD) (207). It is well established that, depending on the cellular context, $p75^{NTR}$ has a dual function; it promotes neuronal survival by its interaction with neurotrophins and Trk receptors but can also trigger cell death when other neurodegenerative molecules bind directly to its extracellular domain (93,101,124,208,209). Not only the neurotoxic fragment of the prion protein (PrP 106-126), as discussed above, but also the peptide of the amyloid precursor protein (APP) has been described as a neurotoxic ligand binding to the extracellular domain of the $p75^{NTR}$ (124,206,210,211). This evidence, taken together with our results suggest that BSE might share with other neurodegenerative diseases, such as AD, cell death mechanisms mediated by non-neurotrophin ligands binding to $p75^{NTR}$.

Links between other members of the neurotrophin family and neurodegeneration also exist in other similar disorders. In the parkinsonian brain, a recent study performed both *in vivo* and *in vitro* discusses the neuroprotective effect and the possible clinical benefits that NTs and NTRs have on dopaminergic neurons (212). Using a specific antibody directed against human recombinant BNDF, it was shown that levels of this neurotrophin were diminished at the cellular level in the substantia nigra of PD patients (125). By ELISA the same result was also achieved not only with BDNF but also with NGF (213). This was in contrast to our results since no statistically significant differences were found between infected and control group regarding any of the markers used (except for $p75^{NTR}$), suggesting that these neurotrophic factors are not involved in the prion protein mediated neurodegeneration. In a similar manner, in Huntington's disease patients, both BDNF immunoreactivity and protein expression were examined in brain samples and a significant reduction was shown (127). BDNF levels have been shown to decline also in the cerebral cortex of these patients (214). In our mouse model of BSE, a similar BDNF profile was found in infected and control groups, thus it is plausible to conclude that BSE infection does not greatly affect BDNF expression.

With the exception of the $p75^{NTR}$, our results suggest that the NTs and NTRs do not have a critical role in terminal stage brain pathology in BSE. Several research groups have proposed the use of other neurotrophic factors as a therapeutic method for limiting the severity of nervous system injury in disease (33,100,215–217). In the case of BSE, targeting PrP^{d} binding to $p75^{NTR}$ may have a therapeutic effect in prion diseases. However, additional studies are required to further understand the neuronal damage elicited by the interaction between $p75^{NTR}$ and BSE associated PrP^{d}.

STUDY 2:

Neurotrophins in the degeneration and regeneration of the PNS:

Immunohistochemical assessment of neurotrophins and their receptors in a transgenic mouse nerve injury model: sciatic nerve crush.

7. Study 2

7.1. Introduction

7.1.1. Anatomic compartments of the Peripheral Nervous System:

The PNS encompasses all nervous system elements outside the central nervous system (CNS). The PNS itself is divided into the somatic nervous system, the autonomic nervous system (ANS) and the enteric nervous system (ENS). The former is composed of **neuronal cell bodies** lying within sensory somatic ganglia, and **peripheral nerves** including cranial and spinal nerves. The autonomic ganglia and enteric plexi enclose neurons and nerve fibers.

The somatic nervous system consists of those neurons within the dorsal root ganglia (DRG), and nerve fibers that send sensory ascending information to the CNS and descending motor nerve fibers from the CNS that project to the skeletal striated muscle. Instead, the ANS controls smooth muscle of the viscera and glands. It is subdivided into the sympathetic nervous system and the parasympathetic nervous system.

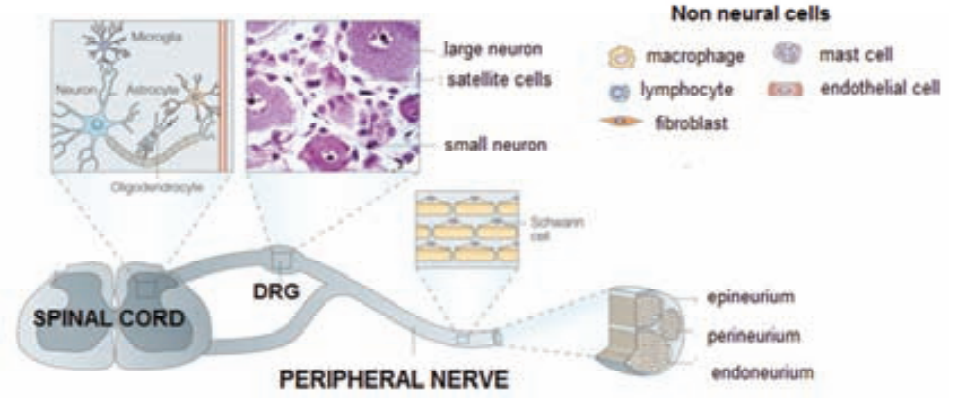

Figure 7.1: Schematic diagram of the spinal cord (CNS) and the PNS. Main neural and non neural cell types found in the spinal cord, DRG and peripheral nerve. (*Adapted from* (218).

7.1.2. Histology of the PNS

In contrast to the CNS, where neuronal somas are grouped in specific nuclei and collection of axons are called tracts, in the PNS they are grouped in ganglia and their axons are organized in nerve trunks. Each neuronal soma is surrounded by satellite cells (Figures 7.1, 7.3). Each axon is individually ensheathed by Schwann cells, which provide structural and metabolic support and can be myelinated (if

one Schwann cell wraps around the axon) or unmyelinated (when a single Schwann cell envelops various axons) (219).

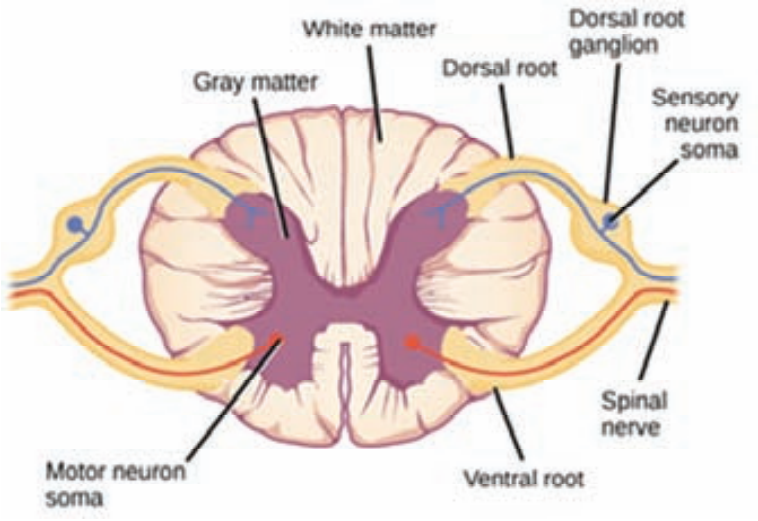

Figure 7.2: **Transversal section of a spinal cord.** The distal part of each DRG fuses with its corresponding spinal ventral root at the same level of each vertebra to form a spinal nerve, which intermingles distally with other spinal nerves to form the peripheral nerves (220).

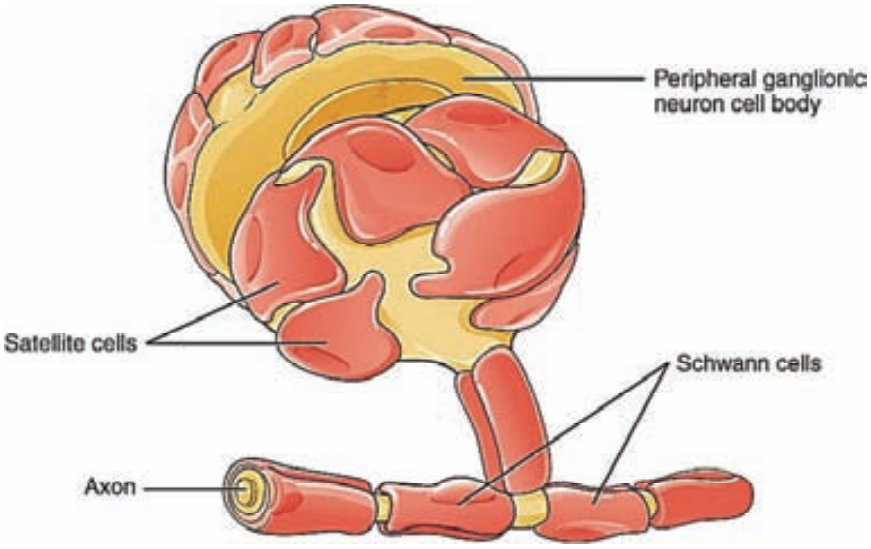

Figure 7.3: Dorsal root ganglion as part of the Peripheral Nervous System (PNS): Schematic representation of a dorsal root ganglion and encapsulating satellite glial cells, which provide nutrients and structural support to neurons. Schwann cells, form the myelin sheath (220).

Structurally, peripheral nerves are additionally supported by three layers of connective tissue: the epineurium, the perineurium and the endoneurium. The **epineurium** covers the entire nerve and is composed by type I collagen and fibroblasts. Within the nerve, the **perineurium** segregates nerve fibers into fascicles. Multiple concentric layers of collagen fibers, fibroblasts and perineurial cells form the perineurium. These cells are joined by tight junctions to form the blood-nerve barrier. The **endoneurium** is the inner layer surrounding each nerve fiber individually. Is formed by type III collagen fibrils and scattered fibroblasts between individual nerve fibers (Fig. 7.4).

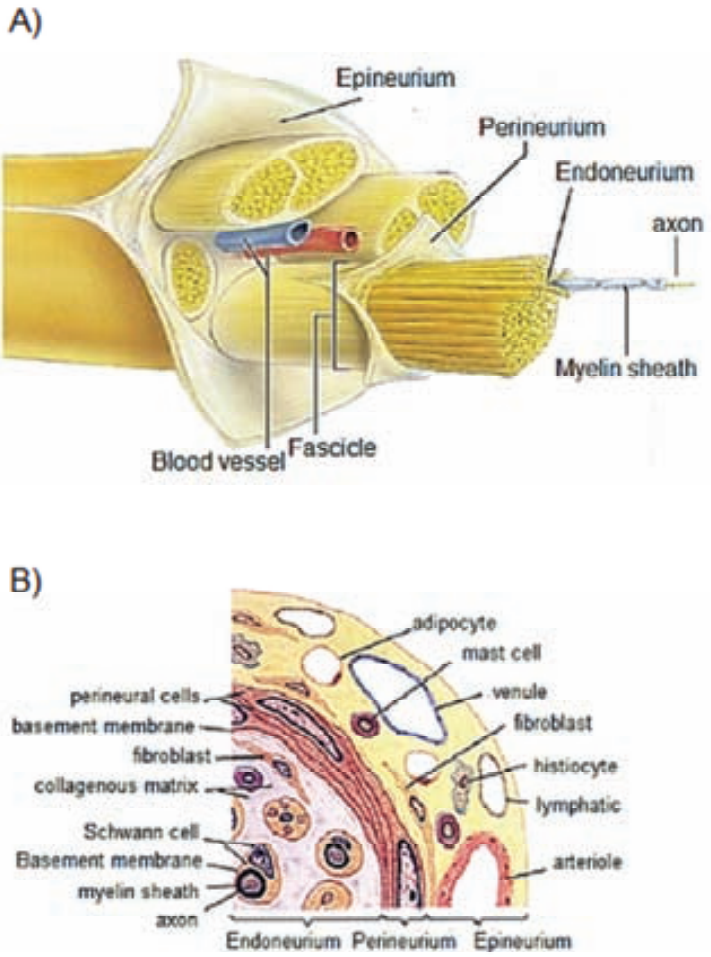

Figure 7.4: (A) **Architecture of the peripheral nerve** and (B) **Internal organization of the cellular elements**. The PNS is formed by the parenchyma (nerve fibers: axons and surrounding Schwann cells) and the stroma (the scaffold made of multiple connective elements such blood vessels and mesenchymal elements (fibroblasts, collagen fibers, adipocytes, etc.) *Adapted from* (221).

7.1.3.Wallerian-like degeneration and nerve regeneration

A peripheral neuropathy refers to a condition where damage resulting from mechanical or pathological mechanisms affects nerves. Traumatic injury is one of the most common causes resulting in partial or complete crush, compression or stretching of the nerves.

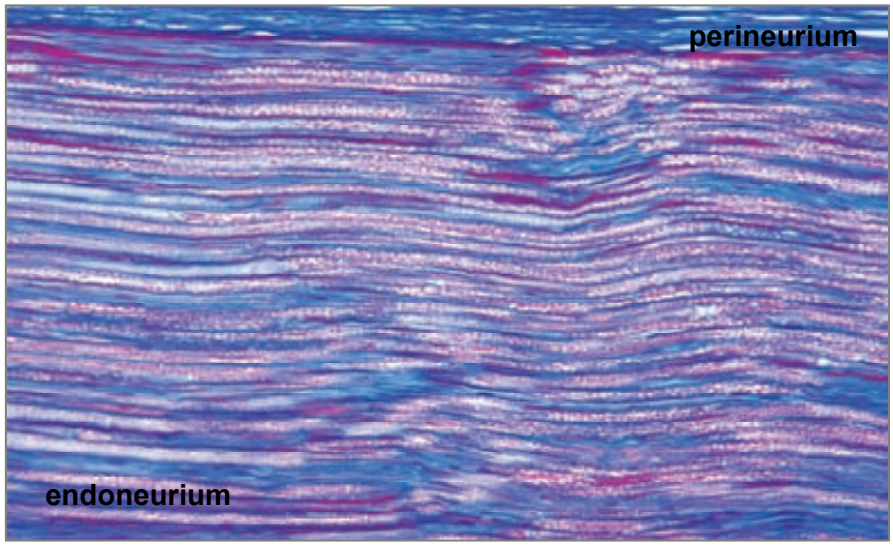

Figure 7.5: Longitudinal section of a normal sciatic nerve. Masson's Trichrome highlights the glycoprotein component of the myelin sheath in bright pink and in blue the fibrous endoneurium dissecting between the nerve fibers.
The central axon within each nerve fiber is no apparent with this particular staining.

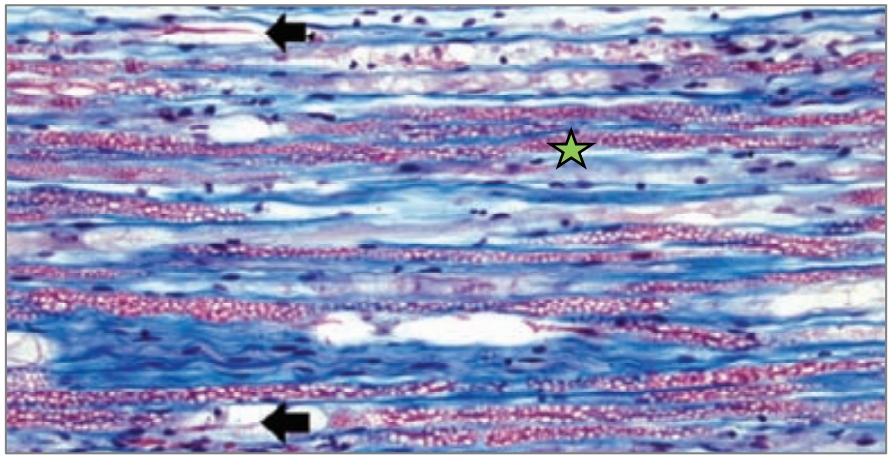

Figure 7.6: Longitudinal section of an injured nerve. Masson´s Trichrome stain.Black arrows highlight degenerative axons, which are replaced by macrophages containing myelin debris (green star) (222).

Peripheral nerve injury, including axotomy or nerve crush, disrupts the normal functionality of sensory and motor neurons components, including Schwann cells. Consequently, the distal segments undergo distinct morphological and molecular changes known as Wallerian-like degeneration (WD), the alteration of the retrograde transport and even compromising neuronal survival; In addition, the reaction within the perikaryon begins (Fig 7.8).

Proximal to the site of injury, entailing neuronal soma, morphologic changes are observed within the first few hours that are collectively referred as chromatolysis. These include the breakup and dispersion of the rough endoplasmic reticulum (Nissl substance) as well as nuclear *eccentricity* and nucleolar swelling (223). In association with these alterations are the upregulation of regeneration associated genes or “RAGs” (140) together with genes that transcribe cytoskeletal proteins, such as actin and tubulin (224).

The term of WD refers to a coordinated series of degenerative events in the distal axonal segment of an injured nerve that take place in order to provide a suitable environment for regeneration.

Beside this, disintegration and degeneration of the axolemma (cell membrane surrounding the axon) and axoplasm (cytoplasm of the axon) occur, affecting the structure of the myelin sheath and leaving the target organ denervated. The axonal degeneration is mediated by calcium influx via ion specific channels which activates axonal proteases (225).

During WD, Schwann cells dedifferentiate and phagocytize the myelin and axonal debris independently, and by recruiting a large number of macrophages, which infiltrate in response to release chemoattractive factors (226).

Macrophages permeate the entire area and remain at least a month clearing, supported by Schwann cells which form small ovoids from their own debris and proliferate (227,228).

Proliferating Schwann cells, which undergo phenotypical changes, are confined to their basal lamina tubes where they align to form Büngner bands to guide and provide a supportive substrate and growth factors for regenerating axons (axonal sprouting).

If axonal regeneration succeeds, former connection is reestablished, involving remyelination and reinnervation of the target organ.

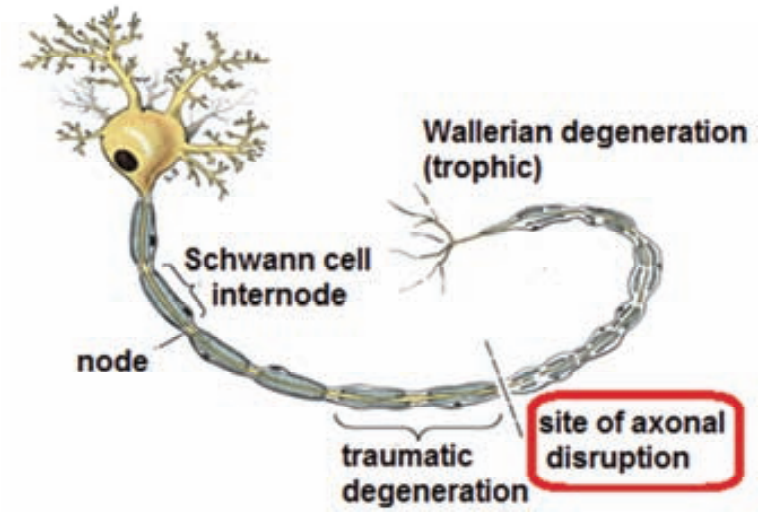

Figure 7.7: Schematic representation of an axonal disruption.

The temporal changes occurring in a damaged nerve as part of WD and regeneration can be summarized in different steps (as shown in Fig 7.8):

- Proximal to the site of injury: neuronal body chromatolysis as initial reaction to the injury.
- Distal to the site of injury: fragmentation of the axon and myelin degeneration.
- WD: phagocytosis of cellular debris by recruited macrophages, helped by Schwann cells, which dedifferentiate.
- Injured axon begins to regrow guided by Schwann cells, which proliferate and align to form Büngner bands, therefore connecting proximal segment with its initial target.
- Neuronal reaction forming new axonal elements with axonal sprouting.
- Axonal regeneration, target reinnervation and remyelination.

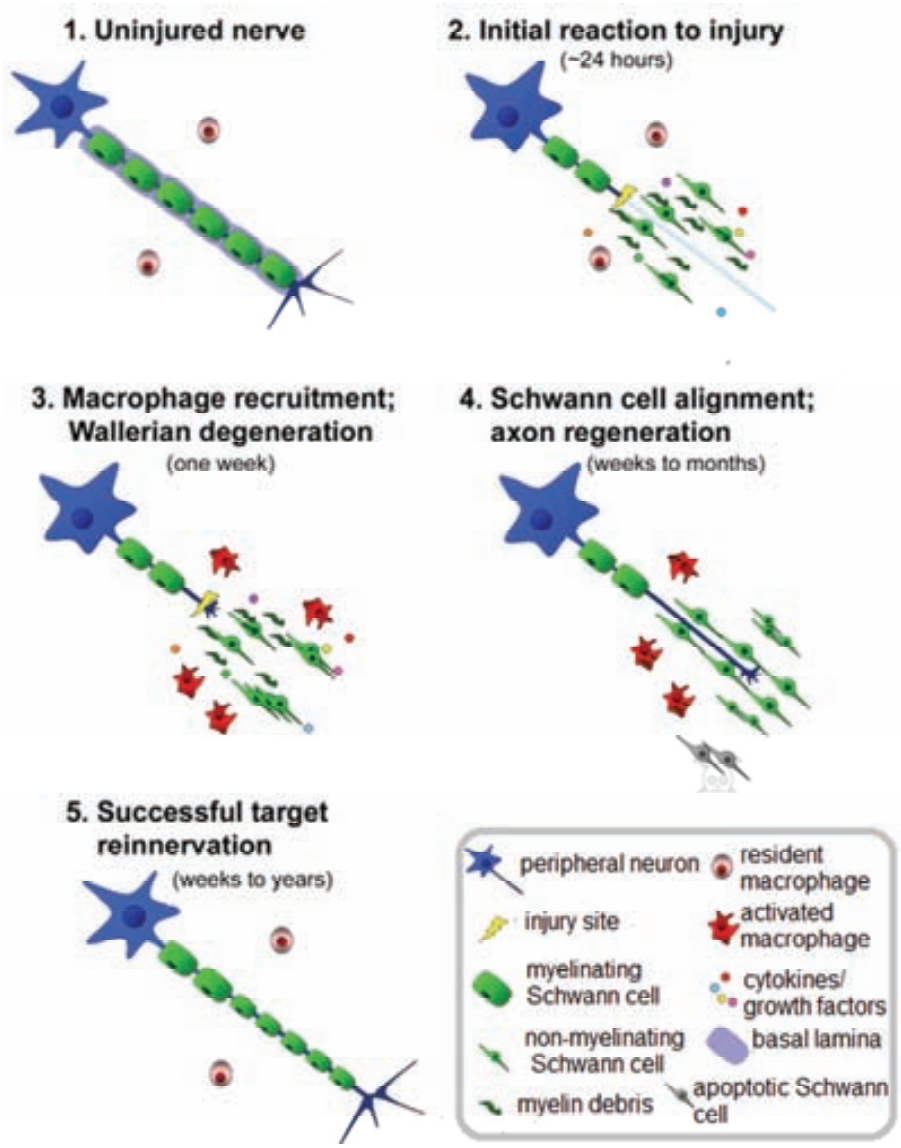

Figure 7.8: Time course of Wallerian degeneration and axonal regeneration following nerve injury: Morphological and molecular changes take place in the perikaryon and in the distal fragment of the injured axon in order to set the stage for the nerve regeneration Adapted from (229).

PNS displays a considerable regrowth capacity upon damage, but rarely returns to pre-injury levels (230). Thus, in humans is often incomplete; the reason is that the regenerative response of the injured neurons and of the cells surrounding the

injured neuron´s axon are not able to maintain an effective growth promoting response for long periods (229).

This study will only focus on nerve crush as an injury model (axonotmesis), which allow continuity between the local neuroma and cell bodies of the injured neurons as well as with the peripheral part of the nerve and peripheral target tissue (230).

7.1.4. The involvement of neurotrophins in nerve degeneration and regeneration:

In addition to morphological changes described in WD (see section 7.1.3), remarkable molecular changes occur in the distal nerve after injury to guide and support regeneration. This results in a rapid increase in the neurotrophin synthesis in all cellular components of the PNS (neurons, satellite glial cell and Schwann cells) as well as in the CNS (motor neurons and microglia) (230). More in detail, disruption in neurotrophin signalling is believed to be crucial for numerous of the biological responses to peripheral nerve injury (PNI).

PNI induced changes affect NTs and NTRs expression in the major structural areas of the peripheral and central nervous system. In particular, upon transection or crush injury, changes can be observed in the DRG neuronal soma, the motor neurons in the ventral horn and also in the distal part of the damaged nerve nerve (230,231).

Neurotrophins are key elements in the regenerative mechanisms. As part of the process, there is up regulation of regeneration associated genes, which include NTs and NTRs. Its expression is controlled by complex cell interactions that determine NT gene expression levels. While most of the transcription factors involved in NT expression are identified, specific interactions are not well described yet (176). When produced in the distal part, NTs are considered major chemoattractants for the regenerating cone, allowing outgrowing axons to reconnect and subsequently reinnervate of the target organs. Apparently, after PNI, neurotrophins act together to promote effective axonal regeneration (147).

In different *in vivo* (animal models) and *in vitro* (cell culture) experiments neurotrophins have long been proved to enhance peripheral nerve regeneration (232).

The present study aims to investigate the histological and immunohistochemical alterations of neurotrophins and their receptors associated with sciatic nerve degeneration and regeneration after experimental injury. This study has been focused on the injured nerve area, corresponding DRG and nerve roots and lumbar segments of the spinal cord, using non diabetic transgenic RIP-I/hIFNβ mice. As an uninjured control the contralateral structures were also studied.

Previous experiments carried out by our group using this transgenic murine model demonstrated that, when subjected to sciatic nerve crush, these mice developed a distal sensory and motor degeneration of multiple nerves with delayed peripheral nerve regeneration (233). Therefore, this model was suitable to study nerve degeneration and regeneration in mice with low dose streptozotocin-induced diabetes to mimic diabetic neuropathy. Additional functional, morphological and immunohistochemical experiments also performed in our group, confirmed dysfunctionality with degeneration of the nerves at 2 weeks post injury and regeneration at 8 weeks (234).

In the current study we aim to characterize the spatiotemporal changes of NT and their receptors in control, non-diabetic mice of the above described model, and highlight selective members of NTs that are involved primarily in the degenerative process, in regenerative process or in both; therefore, contributing to the understanding of the pathogenesis of WD and nerve fiber regeneration.

7.2. Material and methods

7.2.1. Animal model:

A total of 15 male transgenic RIP-I/hIFNβ mice on an imprinting control region (ICR) background were obtained from the *Centre de Biotecnologia Animal i de Terapia Gènica* (CBATEG) at the *Universitat Autónoma de Barcelona* (Spain).

This murine model is known for expressing human IFNβ in pancreatic β cells under the control of the rat insulin I promoter. Previous experiments carried out by our group demonstrated that 3.5 months following intraperitoneal administration of streptozotocin at multiple low doses (20-30 mg/kg), this model developed signs of diabetic autonomic polyneuropathy (235).

Control non-diabetic mice were used for the NTs/NTRs immunohistochemical characterization of the neuroregenerative events described in this study. During their growth mice gained weight and maintained normal blood glucose levels.

In the CBATEG facilities, animals were housed under specific pathogen-free conditions, at controlled temperature (22º±2ºC) and humidity (55±10%) conditions with a 12 hour light/12hour dark period cycle, standard diet (Teklad 2018S Harlan Teklad, Blackthorn, UK) and water *ad libitum*.

The Ethics Committee for human and animal research of the Universitat Autònoma de Barcelona (UAB) approved all the experimental procedures.

7.2.2. Surgical approach:

Technical procedure was previously described by our group (233,234) and it is described in detail as follows.

At the age of 3 months sciatic nerve damage (crush or axonotmesis) of the mouse left hind limb was performed.

The surgical procedure was carried out under anaesthesia with intraperitoneal ketamine-xylazine injection (100mg/kg- 10mg/kg), followed by a skin incision in the left upper thigh. Using a silk suture, the sciatic nerve was gently isolated from the surrounding connective tissue and subsequently crushed by the same person at the sciatic notch with non-serrated forceps (Dimeda Instrumente, Tuttlingen, Germany) (Fig. 7.9).

This procedure was repeated three times successively for 30 seconds, while rotating the forceps 180º each time. The nerve persisted translucent but continuity existed.

When the surgery was completed, the skin was closed with surgical clips (Dimeda Instrumente, Tuttlingen, Germany).

The unharmed contralateral sciatic nerve (right) served as an internal control.

In order to evaluate peripheral nerve regeneration at different stages following the nerve crush, three time points were established and animals were subsequently sacrificed 2, 4 and 8 weeks after the lesion was induced.

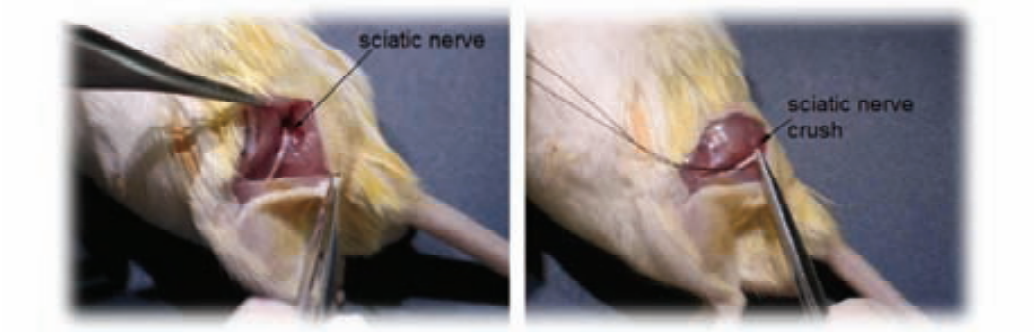

Figure 7.9: Surgical procedure: following skin removal and disinfection of the area, the sciatic nerve was exposed by skin incision and dissection of the musculature (left panel). Once the nerve was isolated it was crushed with microforceps (right panel).

7.2.3.Post mortem studies:

7.2.3.1. Tissue collection and preparation

Mice were divided in 3 groups of 5 animals each and sampled at three different time points after sciatic nerve damage (2, 4 and 8 weeks after crush).

At each time point, mice were deeply anesthetized with CO_2 and intracardially perfused with 40 ml of 4% PFA in PBS (pH 7.4).

After gross examination of both external appearance and the viscera, the following samples were collected and processed: the bilateral L3-L5 DRG, the associated lumbar spinal cord segments and two fragments of the sciatic nerve, one distal to the site of injury up at the femur major trochanter (left side) and the other one matching the intact region (right side).

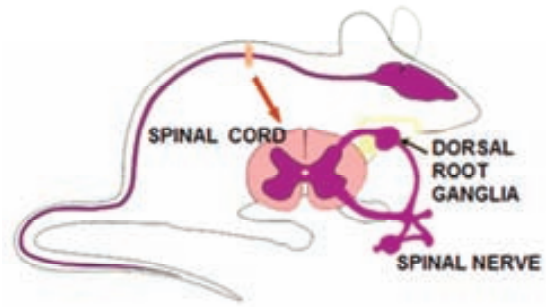

Figure 7.10: Nervous system in mouse. Main nervous tissue samples included in the study.

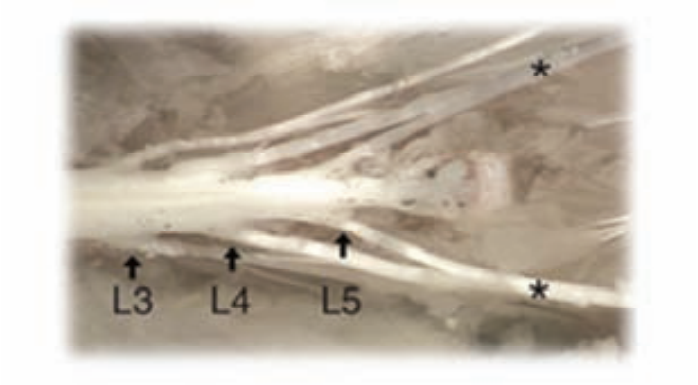

Figure 7.11: Lumbar segments exposed in the spinal cord of the mouse (L3, L4, and L5). Sciatic nerves are highlighted with asterisks (236).

Samples were trimmed under a dissection microscope (Leica S4E), fixed overnight in a 4% PFA solution in 0,1M PBS (pH 7.4) and automatically processed in a tissue processor robot (Leica TP1020).

The following day, tissues were included in cassettes and paraffin embedded in the paraffin embedding station (Leica EG1150H) for histopathological analysis and immunohistochemical studies.

7.2.3.2. Histological and immunohistochemical studies in DRG, spinal cord and peripheral nerve samples: experimental protocol.

Using a conventional microtome (Leica RM2135), three micrometer (μm) thick transverse sections were obtained from paraffin embedded sciatic nerve distal to the crushed site (or equivalent in the uninjured nerve), L3-L5 DRG and lumbar spinal cord samples.

Sections were then mounted on silanized glass slides, depparafined and prepared for histopathological and immunohistochemical examination according to the previously mentioned protocol (see section 6.2.3, study 1).

Slides were incubated overnight at 4ºC with the corresponding primary antibodies targeting NTs and NTRs. In the case of the tyrosine kinase receptors Trk A and Trk B, these antibodies targeted the intracellular domain respectively, whereas for $p75^{NTR}$ and Trk C the targeted portion was the extracellular domain. When compared to the study 1, some of them, including NGF, Trk A and Trk C were used at different concentrations (see Table 6.1, study 1).

Incubation with primary antibody was followed by five minute washes (x3) with PBS. A biotinylated anti-rabbit Ig G (raised in goat 1:200; Dakocytomation, Glostrup, Denmark) was used as secondary antibody for 1 hour at RT.

Slides were then washed (PBS, 3x 5min), incubated (1 hour at RT) with an avidin-biotin-peroxidase complex (ABC) (Pierce, Rockford, Illinois) and PBS washes were repeated (3x 5min).

For the final step, incubation was performed with a chromogen substrate consisting of 0, 05% 3-3`-diaminobenzidine (DAB, Sigma) and 0, 01% H_2O_2 in PBS, for a maximum of 10 minutes, depending on the antibody and the expected signal.

Slides were subsequently counterstained with Mayer´s haematoxylin for a maximum of three seconds (Merck, Darmstadt, Germany), dehydrated and mounted automatically (Leica Autostainer XI, Leica CV5030).

7.2.3.3. Examination of the slides:

The histopathological characterization of crushed nerves was performed on HE stained sections of sciatic nerve transversally trimmed with the aim of classifying animals into groups according to findings denoting features of degeneration and/or regeneration in the left limb.

Once the group of each mouse was confirmed by histology, animals were further processed for the IHC studies. For each marker, including NTs and NTRs, immunohistochemical changes in the sciatic nerve, lumbar spinal cord and DRG from both the intact and the crushed side of all animals were separately analysed, resulting in two conditions being assessed (non injured *vs*. injured)

Nervous tissue samples were evaluated and semiquantification was performed using a scoring system based on the signal intensity of the immunoreactivity and the number of positive cells. Thus 0 was considered absence of signal, 1 for a mild signal, 2 for a clear but scarce signal and 3 for strong intensity.

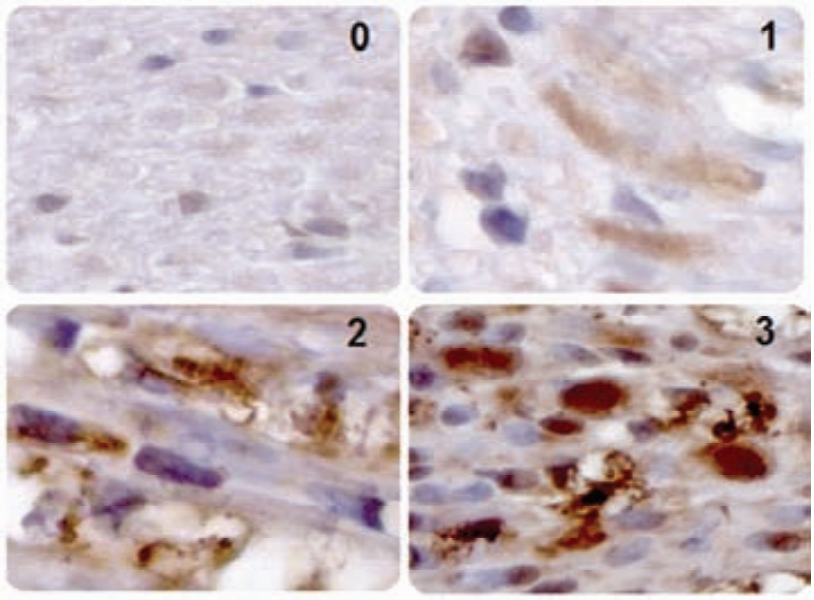

Figure 7.12: Scoring system used for the semiquantitative assessment of the samples (0: absence of signal, 1: mild signal, 2: clear but scarce signal and 3: strong intensity).

Data were graphically represented as mean ± standard deviation (SD) and "n" was the animal number assessed.

For each antibody, inter and intra group comparisons were evaluated between injured (I) and non injured sites (NI), in the sciatic nerves, ipsilateral DRG, and in the spinal cord, at each time point (2, 4 and 8 weeks).

To test the normal distribution of the data, Kolmogorov Smirnov test was done.

According to normality and the fact that there were more than 2 groups in the study, parametric methods should have been applied, particularly a way analysis of variance (ANOVA).

However, due to the reduced number of samples included in the study (less than 30), and also because they are considered more robust and less sensitive to extreme data, non-parametric tests were applied.

In this experiment, more than two categories were to be compared (this is three different time points: 2, 4 and 8 weeks).Therefore Kruskal Wallis test was applied to check global differences between data.

After this, the U-Mann Whitney test was done to evaluate intragroup differences (NI group or I group respectively) between time categories (2 weeks vs 4 weeks; 4 weeks vs 8 weeks; and 2 weeks vs 8 weeks).

Wilcoxon test was done to compare related samples, i.e within the same animal. This was the case of the BDNF labelling in the white and grey matter of the spinal

cord, the cell and axon labelling in the sciatic nerve, and the DRG labelling in NI and I sites.

The statistical significance level was set at p <0, 05 (with a 95% confidence interval) for all tests. All calculations were carried out using the SPSS software V.2.0 (IBM, Chicago Il, USA).

7.3. Results

7.3.1. Histological studies

The histological evaluation was performed at 2, 4 and 8 weeks following sciatic crush injury (weeks after surgery, abbreviated **"w.a.s"** herein). For this purpose, HE stained sections of all the sciatic nerves, both injured and the contralateral/intact nerve sites were examined under optic light microscopy.

In the 2 w.a.s. nerves, a consistent alteration of the endoneurium was observed with clear evidence of WD features. The multiple and extensive degenerative changes, included loss of nerve fibers, mostly myelinated axons, and round cell infiltrates with macrophage activation.

Nerve fiber regeneration was subsequently detected only in those mice sacrificed at 4 and 8 w.a.s, accompanied by a decrease in the endoneurial cellularity.

Therefore, the following three groups were established for the IHC study of neurotrophins and their receptors:

- Group 1: degeneration (2 w.a.s)
- Group 2: degeneration and regeneration (4 w.a.s)
- Group 3: regeneration (8 w.a.s).

7.3.2. Immunohistochemical studies

For each marker, including NTs and NTRs, immunohistochemical changes were individually analysed in the sciatic nerve, DRG and lumbar spinal cord from both the intact and the crushed site of all animals. This resulted in two conditions being assessed, non injured (NI) *vs.* injured (I).

7.3.2.1. Sciatic Nerve

7.3.2.1.1. Immunohistochemical changes in NTs and NTRs

Immunolabelling was individually scored in both Schwann cells (see discontinuous lines in Fig.7.13) and nerve fibers (see continuous lines in Fig.7.13) in the injured and the contralateral (non injured) sides (Fig.7.13).

At 2 w.a.s, since nerve fibers were not preserved due to the crushed lesion, no immunopositivity was observed in the **injured axon** with neither of the neurotrophin markers. A mild immunolabelling was detected at 4 w.a.s. of NGF, NT-3, Trk A and Trk B while strong intensity was noted with BDNF, Trk C and $p75^{NTR}$. At 8 w.a.s., NGF, NT-3, Trk A and Trk B maintained nearly the same levels as detected at 4 w.a.s., whereas this signal decreased in BDNF, Trk C and $p75^{NTR}$ stained nerve fibers.

In relation to the immunolabelling in **Schwann cells of injured nerves**, at 2 w.a.s. strong NGF, NT-3, Trk B and $p75^{NTR}$ immunolabelling was observed while clear but scarce immunoreactivity was detected with BDNF, Trk A and Trk C. From this

point to the end of the study, cellular staining progressively decreased to mild levels, except for Trk B, which was dramatically lower at 4 w.a.s. and BDNF immunoreactivity, which was augmented at this time point.

The immunoreactive profiles in both **Schwann cells** and **axons** of the **non-injured** site were variable depending on the assessed neurotrophin.

Relatively similar for Trk A and Trk B, a decrease was noticed from 2 to 4 w.a.s, and a slight increase was observed between 4 and 8 weeks.

Similar pattern was described for BDNF, with one exception detected at 8 w.a.s, when immunoreactivity was stronger in the axon.

For NGF, the immunoresponse was similar at 4 w.a.s and higher in the axon than in the cell at 2 and 8 w.a.s, but significance was only detectable at the end of the study. In contrast, for NT-3, Trk C and $p75^{NTR}$, this response was contrary between the axons and the Schwann cells, particularly from the 4^{th} week. While the immunoreactivity tended to decreased in the Schwann cells, the signal increased in the axons.

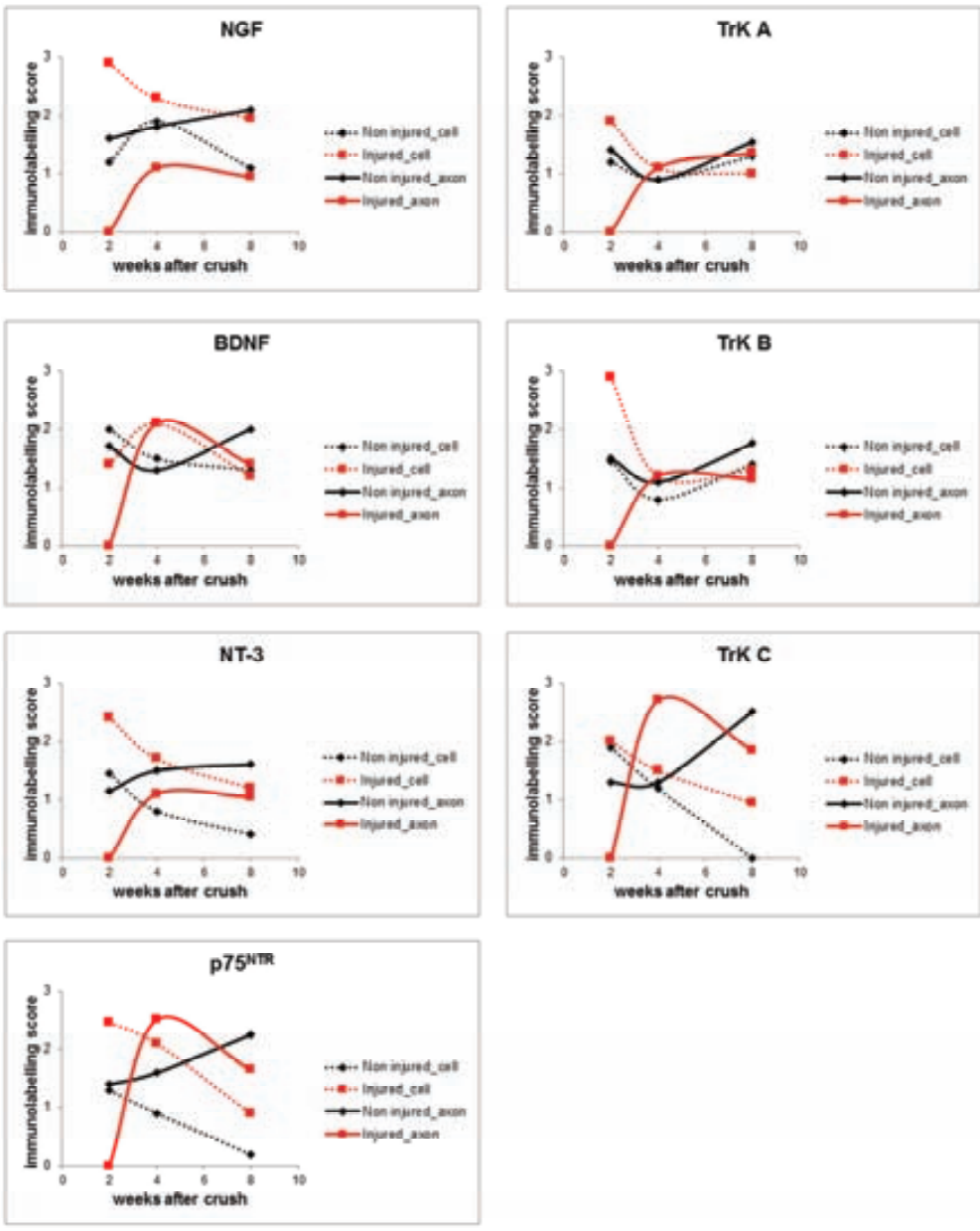

Figure 7.13: **Graphic representation of the semiquantitative immunolabelling score of NTs** (NGF, BDNF, NT-3) **and NTRs** (TrkA, TrkB, TrkC and $p75^{NTR}$) in the sciatic nerve. Comparisons of the mean scores in Schwann cells (discontinuous line) and the axon (continuous line) of the injured site **(red lines)** and the intact contralateral site **(black lines)**.

7.3.2.1.2. Intra/Intergroup differences in the NTs and NTRs expression between injured and non injured sides.

Statistical analysis was performed considering differences between injured and non-injured tibial nerves at each time point, and Schwann cells and nerve fibers along the time (Table 4 in section 11, Annex). Multiple and variable data were obtained. However, the most remarkable results, affecting all neurotrophins were observed in the **injured nerve**, when comparing 2 *vs*. 4 w.a.s and 2 *vs.* 8 w.a.s. Moreover, significance was achieved with intracellular labelling scores in all markers but BDNF between 2 and 8 w.a.s.

In addition, statistically significance was also achieved between the lesioned and the intact sides, particularly in Schwann cells.

7.3.2.1.3. Immunolabelling distribution in the sciatic nerve:

Regarding the signal distribution, the cellular staining was mainly confined in the nucleus and less frequently in the cytoplasm of Schwann cells.

In Figs. 7.14 and 7.15 the NTs and NTRs immunolabelling patterns observed in the sciatic nerve are respectively shown

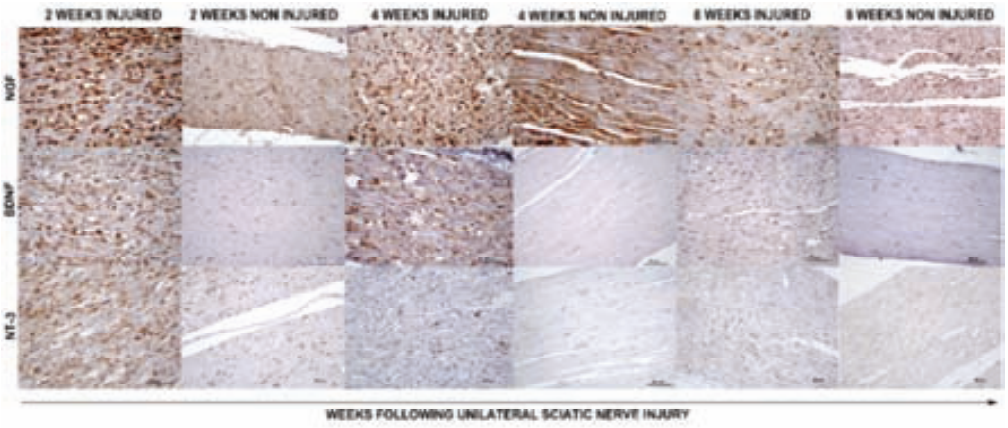

Figure 7.14: Neurotrophins immunoreactivity (NGF, BDNF and NT-3) in the sciatic nerve of male transgenic RIP-I/hIFNβ mice, at the three time points established following sciatic nerve injury, in injured and non injured sites. Scale bar: 50 mμ

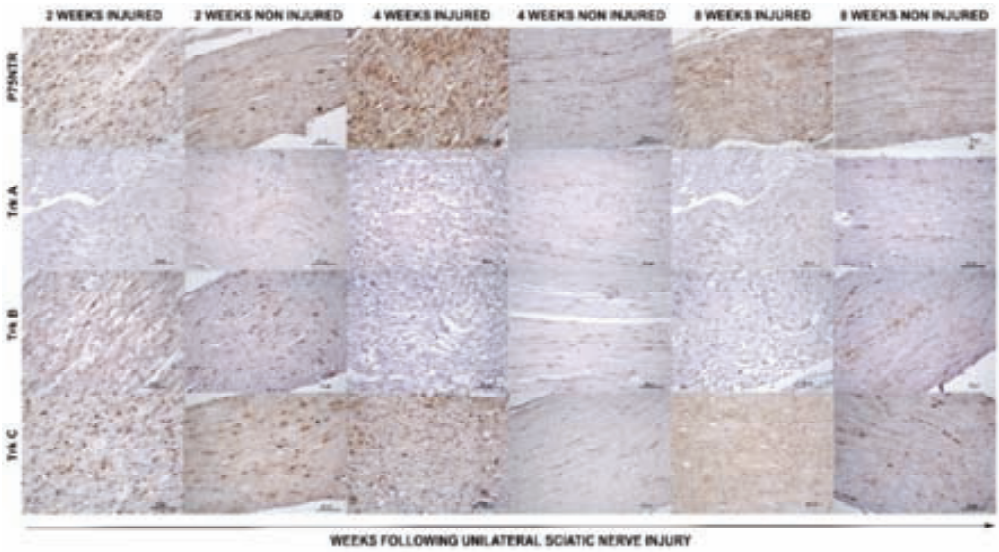

Figure 7.15: Neurotrophin receptors immunoreactivity (Trk receptors and $p75^{NTR}$) in the sciatic nerve of male transgenic RIP-I/hIFNβ mice, at the three time points established following sciatic nerve injury, in injured and non injured sites. Scale bar: 50 mμ.

7.3.2.2. Dorsal root ganglia (DRG)

NTs and NTRs immunoreactivity was detected in all DRG sections studied in both injured and non-injured sides of all animals.

7.3.2.2.1. Immunohistochemical changes in NTs and NTRs

In both I and NI sides, from 2 to 4 w.a.s, NGF, Trk A and BDNF signalling progressively decreased bilaterally, but was still detectable 8 weeks post injury.

For Trk B, NT-3, Trk C and $p75^{NTR}$, the immunolabelling increased from 2 to 4 w.a.s but decreased at 8 w.a.s, reaching initial levels or even lower (Fig. 7.16).

At 8 w.a.s, the injured site displayed higher NGF and $p75^{NTR}$ signal intensity than the contralateral site, but without statistically significant differences (Table 4 in section 11, Annex).

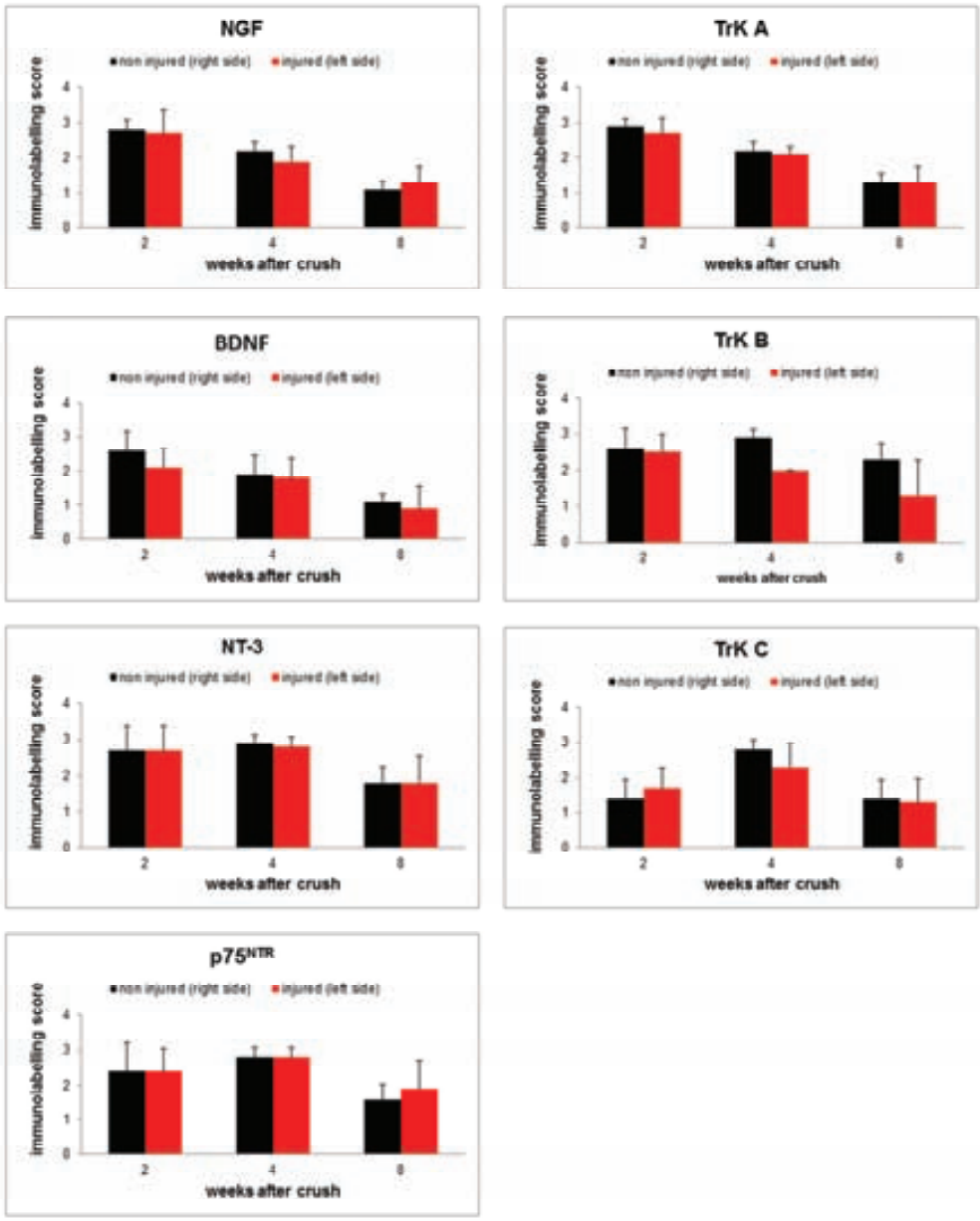

Figure 7.16: Graphic representation of the semiquantitative immunolabelling scoring of different NTs (NGF, BDNF, NT-3) and NTRs (Trk A, Trk B, Trk C and $p75^{NTR}$) in the DRG. Comparison of the mean scores in the injured site **(red bars)** and the intact contralateral site **(black bars)** at the three different time phases established after sciatic nerve crush (2, 4 and 8 weeks)

7.3.2.2.2. Intra/Intergroup differences in the NTs and NTRs expression between injured and non injured sides

Statistical analysis was carried out and data are summarized in Table 5 (see section 11, Annex). However, in the following result section, only significant results will be mentioned.

Firstly, for each marker, differences were assessed at each time point (see the 3 groups previously established in section 7.3.1) comparing the intact (NI) and crushed sides (I).

Significance was only achieved with TrK B at 8 w.a.s (*P*= 0.041), which was lower in the I side.

In contrast, statistically significant differences were more evident between groups, i.e when comparing the three time points between each other.

Criteria were established as follows (2 *vs.* 4; 4 *vs.* 8 and 2 *vs.* 8 w.a.s).

Taking this criteria into account, in the **non-injured site** (Fig 7.16, black bars) this was the case for NGF (*P*=0.032; *P*=0.008; *P*=0.008 respectively) and its receptor Trk A (*P*=0.016; *P*=0.008; *P*=0.008 respectively). With BDNF, differences were only observed when comparing 2 *vs.* 8 w.a.s (*P*= 0.008) and with NT-3, only 4 *vs.* 8 w.a.s (*P*=0.008).

In the **injured site** (Fig 7.16, red bars), differences were observed when comparing 2 w.a.s *vs.* 8 w.a.s with NGF (*P*=0.01) BDNF (*P*=0.032) and for Trk A also when compared 4 and 8 w.a.s (*P*=0.032*)* and between 2 and 8 w.a.s too (*P*=0.008).

7.3.2.2.3. Immunolabelling distribution in the DRG:

Immunolabelling was observed in a variety of sensory neuronal sizes; small and medium size neurons were positive to NGF and Trk A whereas medium to large neurons were immunoreactive to BDNF, NT-3 and Trk B and C receptors.

Positive staining to $p75^{NTR}$ was found, in all neuronal sizes but displaying a high variability within each group.

Regarding the signal localization, intracytoplasmic staining was clearly observed with all markers used except for NGF, which also appeared in the cytoplasm but as a diffused reaction.

Nuclei of all sensory neurons were intensely positive to NGF, when compared to other NTs. Regarding the reminaing markers ,only scattered nuclei were stained to BDNF, NT-3, Trk B, Trk C and $p75^{NTR}$. However nuclear staining was negative to Trk A in all animals.

In addition, in some animals and only in the crushed site, Schwann cells were immunolabelled by all NT markers (NGF, BDNF, NT-3) and $p75^{NTR}$.

Surrounding sensory neuronal bodies, a large number of satellite cells were immunolabelled by NT-3 and $p75^{NTR}$, less frequently by BDNF, Trk B and Trk C. No immunoreactivity was noted with NGF and Trk A markers.

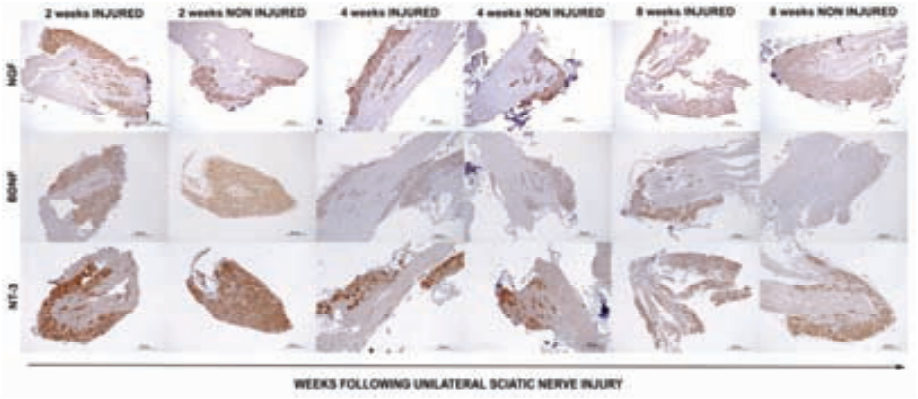

Figure 7.17: Neurotrophins immunoreactivity (NGF, BDNF and NT-3) in the DRG of male transgenic RIP-I/hIFNβ mice, at the three time points established following sciatic nerve injury, in injured and non injured sides. Scale bar: 200 µm.

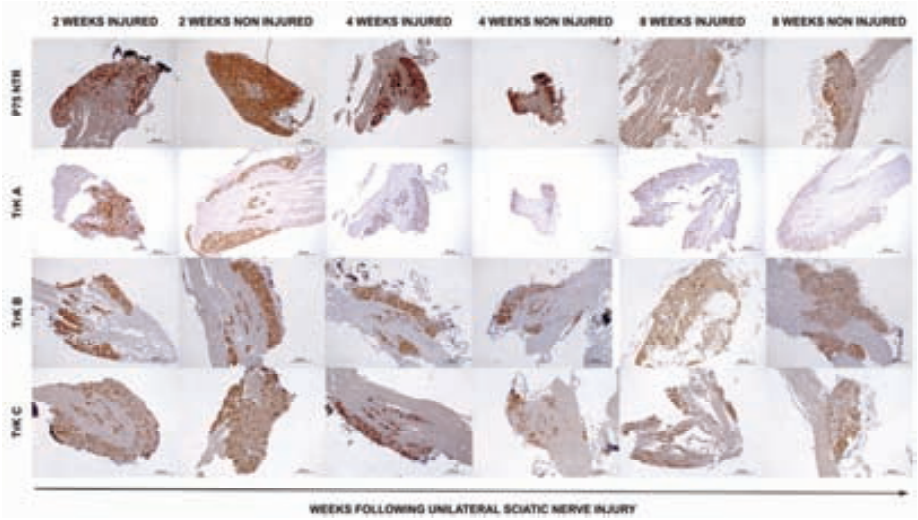

Figure 7.18: Neurotrophin receptors immunoreactivity ($p75^{NTRv}$and Trk receptors A, B and C) in the DRG of male transgenic RIP-I/hIFNβ mice, at the three time points established following sciatic nerve injury, in injured and non injured sides. Scale bar: 200 µm

7.3.2.3. Lumbar Spinal Cord

There was no evidence of stronger response on the ipsilateral site to nerve injury when compared to the contralateral in any of the markers evaluated.

7.3.2.3.1. Immunohistochemical changes in NTs and NTRs

NTs and NTRs immunoreactivity was detected in all lumbar levels studied (L3-L5). However, the immunolabeling was quite variable depending on the neurotrophin marker studied (Fig.7.19).

Two weeks after the crush, NGF and its receptor TrkA, showed a strong immunostaining, which decreased between 4 and 8 w.a.s.

In the case of BDNF grey and white matter was scored independently to avoid confusing interpretations since, while the white matter labelling increased progressively from 2 to 8 w.a.s., the grey matter showed the opposite response.

With NT-3 and Trk B, the immunoreactivity was rather similar at 2 and 4 w.a.s., but lower on the 8th week. The Trk C immunoreactivity was slightly higher at 4 w.a.s, but lately decreased.

Regarding $p75^{NTR}$, there was a marked immunolabelling increase between 2 and 4 w.a.s., which remained unchanged on the 8th week

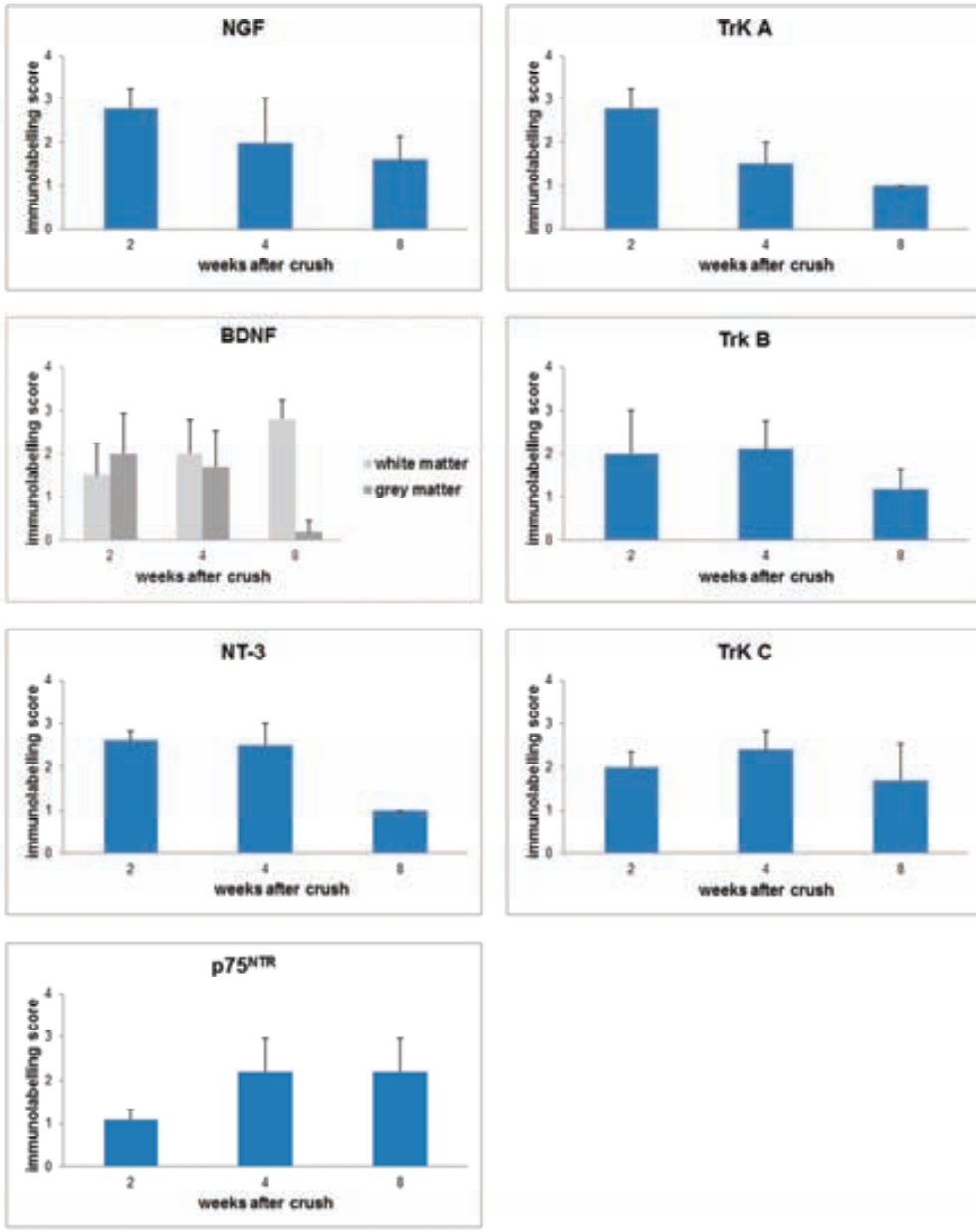

Figure 7.19: Graphic representation of the se miquantitative immunolabelling scoring of different NTs (NGF, BDNF, NT-3) **and NTRs** (Trk A, Trk B, Trk C and $p75^{NTR}$) in the spinal cord. Each bar represents the immunolabelling mean score observed at the three different time phases established after sciatic nerve crush (2, 4 and 8 weeks).

7.3.2.3.2. Intra/Intergroup differences in the NTs and NTRs expression at each time point after surgery.

Regarding the statistical analysis, for each NT and NTR, the three time points were compared (Table 6 in section 11, Annex).

Since differences between grey and white substances were evident with BDNF, intragroup comparisons were also statistically evaluated (grey matter *vs.* white matter). However differences were only significant at 8 w.a.s (*P*= 0.041).

In contrast, intergroup significant variability was more commonly found.

While significance was only seen with Trk A (*P*=0.016), between 2 and 4 weeks differences were established with NGF (*P*=0.016*)*, BDNF (*P*=0.016_white*; P*=0.008_grey*)*, NT-3 (*P*=0.008*)* and the receptors Trk A (*P*=0.008*)* and $p75^{NTR}$ (*P*=0.016) between 2 and 8 w.a.s.

7.3.2.3.3. Immunolabelling distribution in the spinal cord

The immunolabelling was distributed as shown in Fig.7.20; the grey matter was more intensely stained in the dorsal horns when compared to the ventral horns (see fig. 7.2 for anatomical location). This was particularly evident with NGF, Trk A and $p75^{NTR}$.

In the dorsal horns, both *laminae* I (posteromarginal zone) and II (gelatinous substance) were strongly reactive. In the ventral grey matter, motoneurons were intracellularly stained with less intensity. The neuropil and the cell membrane were particularly positive to Trk A and Trk B.

NT-3 appeared strongly but diffuse in the grey matter.

With the other two receptors Trk C and $p75^{NTR}$, an intracytoplasmic and occasionally intranuclear signal was appreciated throughtout the grey matter.

Positive glial cells were observed, particularly at 2 w.a.s.,in all lumbar spinal cord sections. A few ependymal cells were immunoreactive to NGF, Trk B, C and $p75^{NTR}$. In contrast, the majority were positive to NT-3. However, there was no ependymal BDNF and Trk A immunolabelling.

In the white matter, there was a mild immunolabelling to all neurotrophins when compared to the stronger grey matter immunolabelling, but in the case of BDNF the immunolabelling in the white matter was stronger than that of the grey matter, which was less intense than the remaining markers

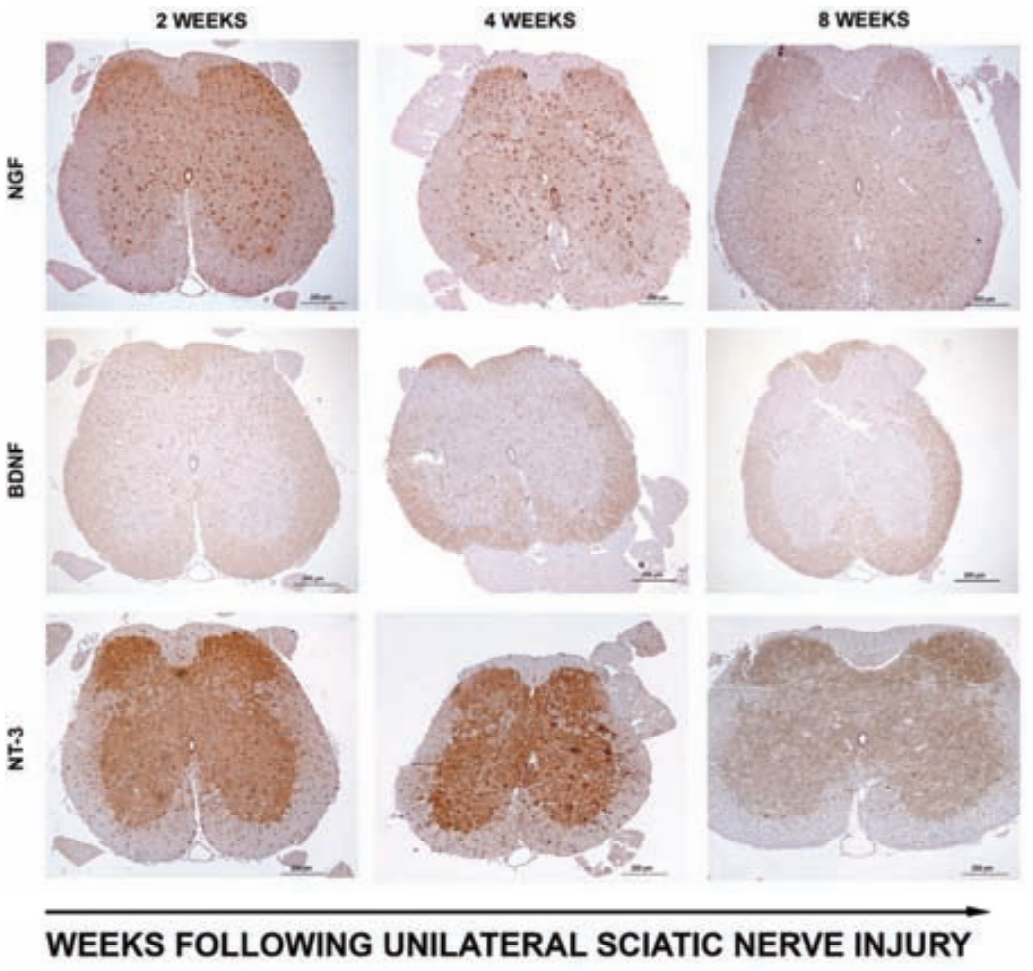

Figure 7.20: Neurotrophins immunoreactivity (NGF, BDNF and NT-3) in the lumbar spinal cord segment of male transgenic RIP-I/hIFNβ mice, at the three time points established following sciatic nerve injury. Scale bar: 200 mμ.

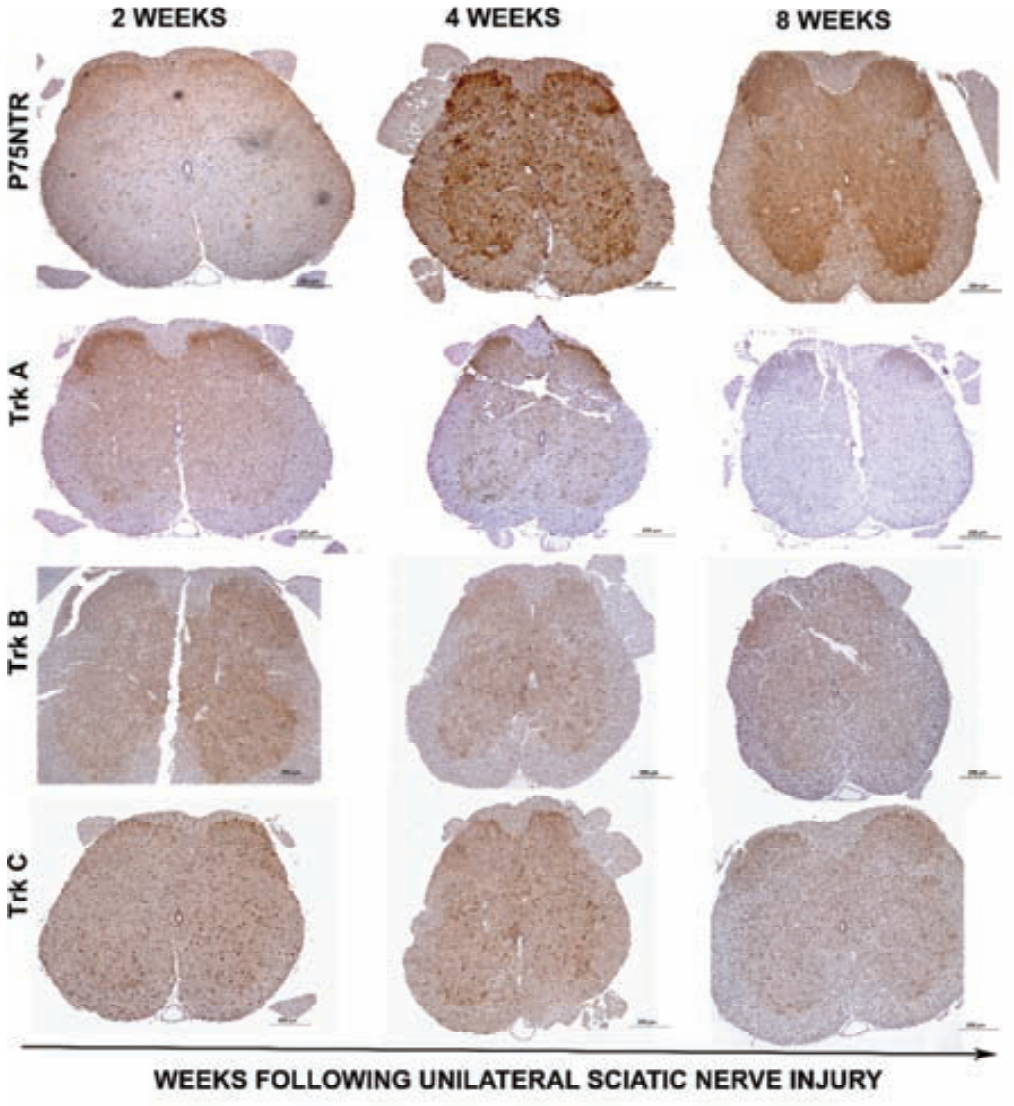

Figure 7.21: Neurotrophin receptors immunoreactivity (Trk receptors and $p75^{NTR}$) in the lumbar spinal cord segments of male transgenic RIP-I/hIFNβ mice, at the three time points established following sciatic nerve injury. Scale bar: 200 mμ

7.4. Discussion

Peripheral nerve injuries are a common event affecting the nerves (237) that induce multiple changes in the nerve fibers, DRG and spinal cord (230). Axonal degeneration results from the primary destruction of the axon and is followed by degeneration of the neuronal cell body and demyelination.

Since Waller first morphological characterization of changes in sectioned frog nerves in 1850, the understanding of the degenerative and regenerative responses of injured neurons has improved substantially (238).

The spatiotemporal evolution of WD is influenced by numerous experimental factors such as the neuroanatomical location of the injury, the injury type (crush, axotomy, intoxication, etc.), the distance from lesion site to the soma, the animal model, the age of the animals, the criteria and the study techniques used among many others (239).

In nerve regeneration research, the sciatic nerve crush (also called axonotmesis) in rodents is a widely used experimental model to study the injuries to the PNS (240). This is related to the numerous techniques currently available to study its outcome such as functional, morphological or behavioural tests (241,242). The majority of experiments that are focused on this field are performed in rat nerve. Although mouse nerves are smaller in size in comparison, both share similar qualities, making it a good choice too. Mouse models are increasingly valuable because of the wide availability of transgenic lines that allow a detailed dissection of the individual molecules critical for nerve regeneration (243). Beside this, other advantages are the robustness of the response, and that for manipulation and sampling there is no requirement of experienced microsurgical skills (241). Previous studies developed by our group using different transgenic mouse models have focused our attention in axonal regeneration under diabetic conditions (233–235) and have confirmed the utility of these murine models.

Axon regeneration in the peripheral nerve is a complex process. There are multiple molecules involved, from WD in the distal stump to upregulation of growth related genes at the cell body or synthesis of molecules required in the regenerating tip (223).

In order to initiate an appropriate response to traumatic injury, neurons switch to a regrowth mode, and synthesize different components to favour cytoskeletal and axonal reconstruction as well as produce several growth related proteins like neurotrophic factors (230). Although multiple families of neurotrophic factors are being studied, NTs and their NTRs are probably those members receiving most attention (230).

Thus, changes in neurotrophin signalling are believed to be an essential process for the multiple complex mechanisms underlying peripheral nerve regeneration (244).Up regulation of NT synthesis is an important mechanism of peripheral nerve regeneration after injury. NT expression is regulated by a complex series of events including cell interactions and multiple molecular stimuli

(147). Interestingly, each NT shows a different time course of expression following nerve injury (144,245).

In addition, experiments on neurotrophins in the PNS that analyze the time course of regeneration are restricted to fewer weeks than ours (147,242,246,247).

The results presented in this study described the immunohistochemical and histochemical changes of NTs and NTRs after PNI. Evaluation was done in the peripheral (sciatic nerve and the corresponding DRG) and central nervous system (lumbar spinal cord) of a transgenic murine model (RIP-I/hIFNβ), at three different stages (2, 4 and 8 weeks), following unilateral (left) sciatic nerve crush injury.

Neurotrophic factors are often evaluated at a molecular level: by western blot, to characterize the antibody specifity (247), by RT-PCR (248) or by ISH to study the gene expression, or in cell culture (249). Moreover, ultrastructural analysis are also used (250).

We found interesting to assess the immunolabelling to NTs and NTRs antibodies because, over other procedures, allowed us to study the spatial and temporal changes at each time point along the regenerative process. This involved precise anatomic localization, distribution and assessment of the immunolabelling intensity.

The murine model used will now be discussed followed by a discussion of the most relevant results obtained in the studied locations: sciatic nerve and corresponding DRG and lumbar spinal cord.

To end this study, we will conclude with a global discussion of the neuronal circuit and those changes detected in the neurotrophic factors and their receptors.

Discussion of the animal model: unilateral vs. bilateral changes

The contralateral unoperated side is commonly used as a control in multiple peripheral nerve injury models. It is considered equivalent to the sham operated animals, where the surgical technique is imitated but avoiding the experimental procedure (241,251). Taken this into account, we left the unharmed right sciatic nerve as a negative control.

Preliminary studies focused on these proteins after unilateral nerve injury described NTs and NTRs changes only in the spinal cord and DRG ipsilateral to the injured side (252).

Surprisingly, bilateral responses were achieved in all samples (sciatic nerve, corresponding DRG and lumbar spinal cord), except for sciatic nerves at early stages (2 w.a.s). However they reached what we think could be initial levels at the end of the study.

Our findings affecting bilateral structures are in agreement with those previously obtained by our group (234). In addition, these two sided changes could be attributed to the presence of inflammatory mediators released by certain cells like satellite cells in DRG or glial cells in the spinal cord, as reported by other authors (253).

Discussion of our findings in relation to the anatomical areas studied

7.4.1.Sciatic nerve:

Neurotrophin administration to injured peripheral nerves enhances axon regeneration and remyelination and improves functional recovery. In contrast, when blocking of these factors occurs, the regenerative process is compromised, causing deficits in recovery (254).

We observed an increased endoneurial cellularity on HE stained crushed sciatic nerve sections, mostly represented by active macrophages and proliferating Schwann cells. This endoneurial cellular density was detected predominantly 2 w.a.s and was less evident as regenerative profiles appeared. It has been reported that, after nerve injury, neurotrophic factors are synthesized by the target organ and non-neuronal cells, primarily Schwann cells thereby supporting the outgrowth of axons (228,255,256).

Besides, macrophages are the major immune cell population that respond to PNI and produce NTs and NTRs in transected peripheral nerve (257) and therefore contribute to sciatic nerve regeneration (248). In addition, fibroblasts, mast cells and epithelial cells have been reported to be a source of neurotrophin production supporting nerve regeneration (176).

In our study the term “cell compartment” from now on will encompass not only Schwann cells but also all these inflammatory and vascular elements.

From early to late stages after crush, we observed a decrease in the immunolabelling intensity in the nervous tissue samples included in the study (sciatic nerve, DRG and spinal cord) with all NTs and NTRs but $p75^{NTR}$.

NGF and its high affinity receptor Trk A labelling was absent in axons 2 w.a.s, but moderately increased later, at 4 and 8 weeks.

On the other hand, other cell types like Schwann cells showed the highest expression of both markers at 2 w.a.s., decreasing their expression at 4 and 8 w.a.s. but maintaining stronger immunolabelling than that found in the contralateral nerve samples

Our results are in agreement with previously published experiments, where Schwann cells are known to be the major source of NGF (176) , and also partially with others. Thus, Funakoshi and collaborators reported a variable Trk receptors response and an absence of Trk A expression in the injured site of the sciatic nerve, neither the proximal nor the distal segments (58).

Regarding **BDNF and its main receptor Trk B** findings, at 2 w.a.s, BDNF expression was absent in axons too, and mildly expressed in the cell compartment in crushed nerves, compared with contralateral expression.

The highest levels were observed at 4 weeks, and they were equivalent to contralateral samples at 8 weeks. Consistent with other studies, BDNF synthesis is also altered following damage, but when compared to NGF, a slower increase is

observed (232), showing a different spatial temporal pattern and distinct regulatory mechanisms for their expression (147) . In our animals, the Trk B expression was at its highest level only in the cell compartment 2 w.a.s, reaching similar levels than controls in both axons and cell compartment, at 4 and 8 weeks.

These findings are in line with previous studies reporting a Trk B increase in Schwann cell proximal to the damaged area up to 3 weeks (58).

Similar to NGF and BDNF, no **NT-3** expression was detected in axons 2 w.a.s. However, an augmented expression was progressively noted at 4 and 8 weeks, without reaching the contralateral levels.

Within the cellular compartment, the highest expression for this marker was observed at 2 weeks, declining progressively at 4 and 8 weeks, but maintaining always higher levels than in the contralateral uninjured nerve

Our results are somewhat in contradiction with a previously published study which reports that, within the first 12 hours after injury NT-3 expression decreases, but gradually returns to control levels within a month (258).

Again, in axons, no **Trk C** labelling was detected 2 w.a.s. This receptor reached its maximum expression at 4 weeks, and returned to initial levels at 8 weeks. No changes were observed in the cellular compartment in the meantime.

Early experiments reported a Trk C increase in Schwann cells proximal to the damaged area up to 3 weeks (58). Other studies on combined surgical approaches proved that rats subjected to unilateral PNI, showed elevated NT-3 levels in the unharmed nerve of the contralateral side (147). These divergences might be atributtable to the different animal model used in every study and to differences in surgical techniques.

In the injured nerve, **$p75^{NTR}$** appeared to be highly expressed 2 w.a.s only in the cellular compartment, being absent in axons. We cannot discard that inflammatory cells (lymphocytes and macrophages) present participated in its overexpression. At 4 weeks, expression was moderate and maintained at same levels until 8 w.a.s. In relation with this fact, it has long been shown that $p75^{NTR}$ is expressed on Schwann cells surface, and that this expression is upregulated following nerve injury (259). In a previous study $p75^{NTR}$ was undetectable in the intact sciatic nerve, but, upon damage, increased remarkably in the injury sites up to 3 months and then declined (260).

7.4.2.Dorsal root ganglia (DRG):

Primary sensory neurons whose neuronal soma is located in the DRG are responsible for transmitting sensory information. Three main classes of ganglion cell bodies exist in the mouse DRG: type A (large 30-50 microns), type B (medium, 20-35 microns) and type C, which are the smallest in size (small <20 microns) (261). They express Trk receptors, whose identity determines which neurotrophin will influence their function (262). The NTs and NTRs expression is only restricted to certain neuronal population. Thus, NGF and Trk A are mostly expressed in type C neurons, BDNF and Trk B in type B neurons, and NT-3 and

Trk C in the type A neurons (57). Trk A and C expressing neurons remain largely separate, whereas Trk B is coexpressed in overlapping populations of Trk A and Trk C positive cells (252,263). The $p75^{NTR}$ is normally coexpressed with Trk A and B receptors in all sizes of neurons as well as satellite cells (230,264). In our DRG study we have considered all types of neurons and satellite cells as a unique cellular compartment without differentiating them.

NGF and Trk A expression gradually decreased from 2 to 8 w.a.s. At each time point no statistical differences were noted between injured and non injured sides. However, with NGF this decrease was a little bit more evident in the injured site. Our results are not in agreement with similar experiments on unilateral crushing in the rat sciatic nerve, where NGF increase was firstly observed in the unharmed contralateral side, and lately in the lesioned side (253,265) .Throughout our study a mild decrease in the expression of both **BDNF and Trk B** was noted in the injured side ganglia compared to the contralateral side. For **NT-3 and Trk C**, no significant expression changes were observed. There is evidence of published data evaluating these neurotrophic factors but only within a 10 day period, which is not comparable with our time points (252).

The expression of **$p75^{NTR}$** was high at 2 w.a.s, and slightly increased until the 4^{th} week, then descending significantly at 8 weeks. Our results are in line with a previous study were immunolabelling to this receptor was detected in a variety of neuronal sizes and satellite glial cells (260). The $p75^{NTR}$ is also known for its role in the apoptotic pathway, so it could be related to axonal damage. Immunohistochemical studies revealed apoptotic transcript activation (primarily JNK) in DRG small B-cells following injury and particularly $p75^{NTR}$ dependent in early stages (266). During the course of our study, both sides DRG showed similar expression of this receptor. Our results are in agreement with the work of L.-T. Kuo *et al*. (2007) in a sciatic transected rat model indicating similar responses in small to medium neurons on the contralateral ganglia (267). But these results were discussed by others, who described no changes (268) or reduction in the number of immunoreactive neurons (269).

7.4.3.Lumbar spinal cord

To date, few neurotrophic factors studies on peripheral nerve injuries have included the spinal cord. Recently, a complete work has described that NTs are present in the spinal cord in normal conditions and have been shown to affect spinal circuit behavior (262).

Axonal damage leads to denervation of spinal cord motoneurons and related interneurons below the injury site (246). In our experiment, we have tried to analyze NTs and NTRs expression differences between motoneurons from injured and contralateral ventral roots. Nevertheless a diffuse expression of all markers has been observed in both sides of the lumbar spinal cord, not only in neurons but in glial cells as well. These results agree with those obtained by our group in a similar study (234). This could be due to the presence of inflammatory mediators released by glial cells in the spinal cord (253).

Diffuse overexpression of **NGF and its receptor Trk A** was evident 2 w.a.s in the gray matter. It decreased at 4 and particularly at 8 weeks. Interestingly, the dorsal horn containing sensory and interneurons, displayed the highest expression for Trk A. This outcome is in line with a previous study analyzing the spinal cord dorsal horns (270) and with the sensory rather than motor role of these proteins (144).

The expression of **BDNF and Trk B** was different when evaluating both gray and white matter staining. In the gray matter, both presented highest levels 2 w.a.s, decreasing at 4 weeks and reaching the lowest levels at 8 weeks. In contrast, the white matter showed the lowest expression for both markers at 2 w.a.s, increasing evidently at 4, and reaching their maximal expression at 8 weeks, predominantly BDNF. There is one existing study describing decreased Trk B expression in the spinal cord 1-7 days after injury (271), while other authors observed little or no changes not only for Trk B but also for Trk C (272). This variety of results compared with those obtained in our study indicates that time course expression of NTs and NTRs differs between them.

NT-3 and Trk C immunolabelling was diffusely spread and no changes were perceived until the eight week following surgery, when an evident diminution in their expression was observed, particularly in the white matter.

Our results are partly in agreement with the previously mentioned work of Curtis *et al*. (1998) (271).

A diffuse and strong expression of **p75^{NTR}** was observed 2 w.a.s, which increased at 4 weeks and was maintained until the end of the study. Our findings are in agreement with others describing motor neurons maximal expression of p75^{NTR} from the first week after injury (271).

The strongest immunoreactivity detected in our spinal cord study was at 2 weeks after crush for all NTs and corresponding NTRs, this is in line with other published data, indicating a protective role during the WD stages (247).

Differences shown between markers could indicate that they play different roles at different time points after injury (247).

Positive glial cells were observed in our samples throughout all lumbar spinal cord sections, predominantly at first stages. Our results are similar to those authors describing the increased expression in glial cells, especially around the cell bodies of those injured motoneurons (270). Additionally, activated microglia has been shown to release some of the NTs (273). We attribute part of our findings to glial expression of different NT, mainly in the white matter. For this reason, specific markers for glial cells should be used in order to clearly identify them.

In some animals, we found positive response in the ependymal cells and neighboring area to almost all NTs and NTRs, but BDNF and Trk A. Previous reports describe increased ependymal reactivity in front of some of these markers in all types of spinal injuries (274).

Global discussion of the neuronal circuit and the changes detected in NTs and NTRs.

When a peripheral nerve is severed, Schwann cells in that region express and release NTs, reducing the retrograde axonal transport (275). The retrograde transport of injury signals from the periphery side back to the perikaryon of sensory and motor neuronal bodies is one of the key mechanisms that take place, leading to regeneration (276). Signals are carried to the neuronal soma in vesicles called signalling endosomes containing NTs-Trks complexes that had been internalized at undamaged terminals (277). A single signalling pathway is unlikely to fully mediate nerve regeneration. Thus, coordination between different injury signals is essential to regulate the appropriate gene expression to promote neuronal survival and improve the intrinsic growth state of injured neurons (276).

If we consider the results obtained at three different levels (sciatic nerve, corresponding DRG and lumbar spinal cord) and for all the studied NT and NTRs, we can conclude that the highest expression for the majority of neurotrophic factors and matching receptors (NGF/Trk A, BDNF/Trk B and NT-3/Trk C) has been observed in Schwann cells, sensory and motor neuronal bodies from the beginning of the study. The exception was the proximal end of the injured axon, that began to show maximum expression of these markers from the 4^{th} week following injury. Our results correlate with previous studies performed using shorter post injury evaluation periods, and reporting highest accumulation in DRG only one day after injury, whereas in motor neurons, it was detected 3 days after injury (271). Likewise, Trk A and B expression decreased in the DRG one day after sciatic nerve crush up to 7 days, matching with maximal neurotrophin retrograde transport (268). The fact that neurotrophins transport increase within the injured neurons without corresponding changes in the receptors may be attributed to the redistribution of those preexisting receptors that facilitate internalization of greater amounts of neurotrophins (271).

Among all the studied factors, $p75^{NTR}$ was the exception, which started to be over expressed in both axon terminals and neuronal bodies from the 4^{th} week, even if Schwan cells started to overexpress it at 2 weeks. Preliminary data confirmed differences in response between Schwann cells, DRG and spinal cord neurons for this receptor. While sensory neurons display a rapid and transient response, peaking level at day 1 and returning to basal levels by 3-7 days, motoneurons maximal transport was shown at day three and was still high by the end of the first week (271). We attribute these differences to the animal species used in our experiment, the technical procedures and the delayed start of our study compared with the above mentioned ones. Some of the other functions attributed to $p75^{NTR}$ might be to enhance the binding affinity of Trk receptors (278) and facilitate the retrograde transport of certain neurotrophins (279).

In our model this late $p75^{NTR}$ overexpression correlates with the maintenance or increasing levels of other neurotrophic factors showed by neuronal components in all samples.

Injuries to a peripheral nerve disrupt the functionality of both sensory and motor neurons by damaging the integrity of axon and Schwann cells (230). Coordination

between different injury signals is required for the appropriate gene regulation that promotes neuronal survival and increases the intrinsic growth capacity of the injured neurons (276). In fact, the coordinated expression of neurotrophic factors in an injured nerve together with their increased retrograde transport is a common response, affecting both DRG sensory neurons and spinal motor neurons following sciatic nerve injury (271).

It becomes clear that a fundamental understanding of the cellular events is essential for improving treatment and optimizing repair (280).

Neurotrophins are considered to represent one of the most promising research areas that focus on the finding of new effective methods for peripheral neuropathies treatment promoting axonal regeneration (232,281–283). Despite the accumulating evidence pointing out neurotrophins as key molecules for nerve regeneration and repair, intracellular mechanisms controlling the synthesis have yet to be investigated (249).

Our histological and immunohistochemical findings along with additional, morphological and morphometrical experiments performed in our group (234) confirm a key role of different NTs and NTRs in promoting axonal regeneration after peripheral nerve injury.

This study has been primarily useful to understand, from a broad point of view, how these proteins behave along the regenerative process during long periods following injury.

However, further studies need to be done with other markers, possibly including double labelling experiments, to deeply explore the exact roles of these molecules through which they may exert a positive influence the regenerative process.

DISCUSSION

8. Discussion

It is well established that neurodegenerative diseases, among other brain disorders, constitute 40% of the incidences that result in human patients' disability and death (284). The clinical manifestation is often the appearance of neurological signs, which progress and worsen until a fatal end. Currently, an effective treatment for the majority of these diseases is not available

Likewise, peripheral nerve injuries are also common. Depending on the etiology of the injury and which structures are affected, the neurological signs are restricted to specific areas. Despite the breakdown of the affected structures, the PNS display a great regenerative capacity. Studies on nerve repair and regeneration have long been known to be of great importance (285). The strong regenerative response generated by neurons could be useful to understand the limited CNS regeneration capacity. In this respect, PNS repair is considered an easier target when compared to the repair of the CNS, but still multiple mechanisms underlying the regenerative process are not fully understood (136).

In line with this, some of the current clinical treatments of nervous system injuries are focused on the use of neurotrophic factors (286–289).

The protein family of neurotrophins is of great importance due to their widespread expression in the majority of neuronal populations in the CNS and PNS, together with their well characterized physiological functions (5).

Numerous studies examining the role of neurotrophins in the nervous system have highlighted their role from development to adulthood, in both physiological and pathological conditions (176,290–292). Several groups have already started to study their involvement in neurodegenerative diseases, such as Alzheimer´s disease (293), Parkinson´s disease (294), Huntington´s disease (295) among others. Only a limited number of references suggest that NTs NTRs could be involved in prion diseases(112).

Regarding peripheral nerve regeneration, the role of neurotrophic factors in the maintenance and survival of peripheral neuronal cells has been the subject of numerous studies (153,296–298).

Multiple therapies involving neurotrophins are ongoing intense research, and one of the main disadvantages is their short half-life. However, these studies rarely cover all NTs and their NTRs at once but some of them, or even other neurotrophic family members (299–302).

In the present thesis, two experiments were designed to immunohistochemically examine NTs and NTRs changes in nervous tissue samples in two very different scenarios. To a limited extend both shared certain characteristics; our goal was not to make comparisons between them but evaluate how neurotrophic factors behaved upon two very different types of nervosu tissue injury.

One study was performed in the CNS by an intracerebral prion inoculation with a BSE homogenate. The other study was made on the PNS by inducing focal and mechanic nerve damage that lead to neuroregeneration events.

Exploration of the nervous system function and dysfunction is infeasible in human beings. Therefore, the availability of in vivo models, which try to mimic how neural cells act and interact, together with the election of an accurate diagnostic method are important in neurobiological research. The use of mice is becoming increasingly valuable due to similarity to humans in anatomy, physiology and genetics. Indeed, they share 95% of the genome, but with the advantage of a wide availability of transgenic lines targeting any imaginable gene (303,304).

To achieve our experiments, two unrelated but transgenic murine lines were included. The BoTg 110 model belonging to the prion infectivity study, which had the PRNP gene modified and consequently overexpressed the bovine PrP (178); and the RIP-I/hIFNβ model, which expressed a rat insulin promoter/ human interferon beta chimeric gene (305) and developed signs of hyperglycemia when exposed to low doses of streptozotocin, useful for the investigation of those mechanisms contributing to impaired nerve regeneration in patients suffering from diabetes (234). This thesis was focused on the characterization of neuroregeneration in non diabetic control animals after PNI.

In neither of the experiments, changes in the NT/NTR immunolabelling were attributed to the transgene, but to very different reponses chronologically speaking: CNS neuronal damage due to prion diseases initiates slow but chronic molecular events (306) while axonal damage due to peripheral nerve crush triggers a rather acute neuroregenerative response in affected structures (138).

To date, we have not found any reference on the literature that immunohistochemically examines all neurotrophic factors (NGF, BDNF and NT-3) and their receptors (Trk receptors and p75 ^{NTR}) at the same time in mice in these contexts. In addition, the vast majority of these studies focus on the rat as the main model for experimental investigations and more specifically only in certain nervous system areas (47,78,177,194,307).

On one hand, this is advantage for our investigation because we may contribute to the NTs and NTRs research field somehow. But, on the other hand, it makes the results interpretation and their discussion more difficult.

The immunohistochemical approach, presents a series of limitations, as it is a static assay being applied to a dynamic biological system. Firstly, it is unable to interpreting information pertaining to the rate of protein production in a given tissue Strong labelling for a protein in a given location does not necessarily show that protein production in that tissue occurs at a high level (i.e. is not necessarily synonimus to changes in gene expression levels), but it might indicate that its storage at that location is relatively abundant. Another limitation is linked to the detection limit, which is the ability of the assay and the person who interprets the results to distinguish between signal and background in the tissue (308).

Despite these limitations, we think that the immunohistochemical approach is a good and suitable method to map and evaluate the labelling in different stages in the nervous system considering that our results are mostly in agreement with those previously published (100,112,147,230) .Nevertheless, complementary studies should be undertaken, involving other molecular techniques or the use of additional markers to confirm or not the results obtained in our studies.

For instance, the study of the retrograde signalling using alternative methodology may be also desirable, as it has been reported to be highly relevant to find treatments for the major neurodegenerative diseases, peripheral neuropathies and neurotraumatic injuries (277).

Since our two experiments have been discussed in correspondent previous sections (see sections 6.4, 7.4), merely a global overview of the most relevant contribution of NTs and NTRs to the neurodegenerative process triggered upon prion inoculation and neuroregenerative events after sciatic nerve crush, will be given.

The immunohistochemical analyses evidenced NTs and NTRs upregulation in the nervous system following each type of damage. These findings agree with a previous report pointing that each member of the NT family regulate processes in distinct but overlapping population of neurons in the nervous system under normal and pathological conditions (277); participating in both brain repair (309) and PNS regeneration (230).

In the CNS study (section 6), $p75^{NTR}$ was the only factor with apparently a critical involvement in the neurodegenerative events associated to BSE pathogenesis. Our findings can be linked, in part, to the only studies reported in the literature about prion diseases and NTs/NTRs (112).

In contrast, in the PNS study (section 7), differences were analyzed from a much more wider point of view and considering the time variable. Here, all the studied factors but not any in particular seemed to contribute to the regenerative process. Although the relation between neurotrophins and regeneration dependent mechanisms has been more widely studied (147,153,234), we consider that our investigations contribute a basic knowledge of how these NTs and NTRs act in the peripheral nerve regeneration, particularly in this transgenic model, and would be useful for future studies evaluating these factors in different diseases or abnormal conditions.

Taken together the results presented on this thesis, suggest that the role of neurotrophins and its receptors needs to be considered both in neurodegenerative diseases pathogenesis and, in those processes involving PNI, concerning their potential therapeutic use to improve neuroregeneration. Therefore, we consider that NT/NTR research in murine models is a valuable tool which can be pursued and improved as a step towards the study of human neurodegenerative disorders, and prion diseases in particular, as well as injuries affecting peripheral nerves.

CONCLUSIONS

9. Conclusions

1. The immunohistochemical approach is a good method to detect the presence and distribution of the neurotrophins, Nerve growth factor (NGF), Brain derived neurotrophic factor (BDNF) and Neurotrophin 3 (NT-3) and their receptors: the tyrosine kinase receptors (TRK-A, TRK-B and TRK-C) and the p75 neurotrophin receptor ($p75^{NTR}$) on formalin-fixed and paraffin embedded samples of central (brain, spinal cord) and peripheral nervous system (dorsal root ganglia and peripheral nerve) of wild type and transgenically modified mice.

2. A thourough brain mapping in control animals (healthy BoTg 110 and wild type Balb-C) of the immunolabelling of all the neurotrophins and their receptors was shown to be widely distributed throughout the brain without showing significant differences betwee the two models. Therefore, the transgene did not significantly alter their expression

3. The expression of neurotrophins and their receptors in the brain of control animals (healthy BoTg 110 and wild type Balb-C) was mainly neuronal, while the p75NTR was detected in both neuronal and glial cell populations.

4. Based on immunohistochemical results, no evidence was found that suggested the involvement of any of the studied neurotrophins and their receptors in the pathogenesis of BSE with the exeption of $p75^{NTR}$. A relationship might exist between the regional expression of $p75^{NTR}$ and the distribution of BSE associated brain lesions.

5. Our results suggest that BSE might share with other neurodegenerative diseases such as Alzheimer´s disease, cell death mechanisms mediated by non-neurotrophin ligands (PrPd in this case) binding to $p75^{NTR}$. The study of this receptor as a possible therapeutic target could be of interest in prion diseases.

6. Immunoreactivity against neurotrophins and their receptors was detected in all studied lumbar spinal cord segments (L3-L5), dorsal root ganglia and sciatic nerves in RIP/hIFNB non-diabetic mice, with a variable distribution depending on the neurotrophin marker and the location studied. In the spinal cord, the majority of markers displayed a gray matter tropism, involving both neurons and glial cells

7. Evidence was found of neurotrophins and neurotrophin receptors immunolabelling changes as a consequence of the peripheral nervous system injury in the spinal cord, dorsal root ganglia and nerves. Following a unilateral

sciatic nerve injury, contralateral structures also displayed differential immunolabelling. These two sided changes might be attributed to the presence of inflammatory mediators released by affected structures (satellite cells in dorsal root ganglia or glial cells in the spinal cord).

8. Our histological and immunohistochemical findings confirm a role of the different neurotrophins and their receptors in axonal regeneration events after a peripheral nerve injury. Immunoreactivity of these markers was detected in Schwann cells, nerve fibers, sensory and motor neuron perycaria and glial cells. Immunolabelling varied througout the regenerative process depending on the time point and location studied.

9. The transgenic murine models BoTg 110 and RIP/hIFNβ could be of possible interest towards future studies related to neurodegeneration and neuroregeneration.

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ANNEX

11. Annex

- **Syudy 1: Neurotrophins and CNS degeneration**

Mann-Whitney test : p-value
(BoTg 110 BSE vs. Control)

	Pfc	H	Oc	Tc	Pc	Fc	S	T	M	P	Mob	Cm	Cv	Cc
BDNF	NO STATISTICALLY SIGNIFICANT DIFFERENCES													
GFAP							0,0758							
LEA					0,40140	0,48750	0,17290							
NGF											0,3067			0,3472
P75e														
P75n				0,2027	0,3058		0,4555			0,8636				
TrkA	0,4875								0,1797	0,4875				
TrkC										0,0927			0,5203	
TrkB			0,4062					0,3934	0,0962					
NT-3	0,1699								0,1116					

P<0.05 95%Confidence*
P<0.01 90%Confidence**

Table 1: Statistical analysis (p-values) in the different brain areas studied (top horizontal first row) for each of the markers included in the study 1 (first column on the left). In grey all the non significant data. Statistically significant data are colored in blue and green.

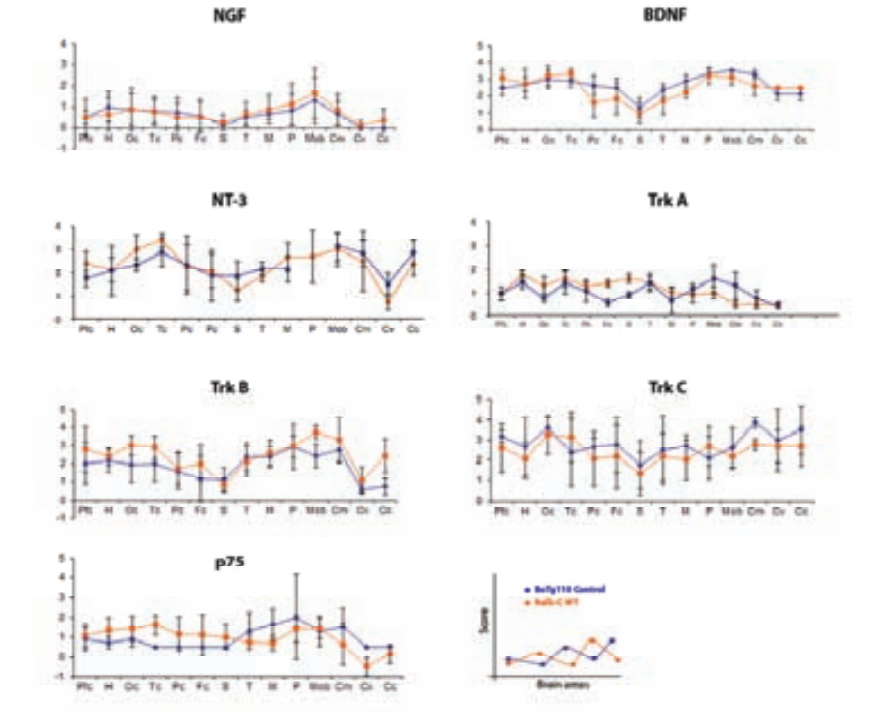
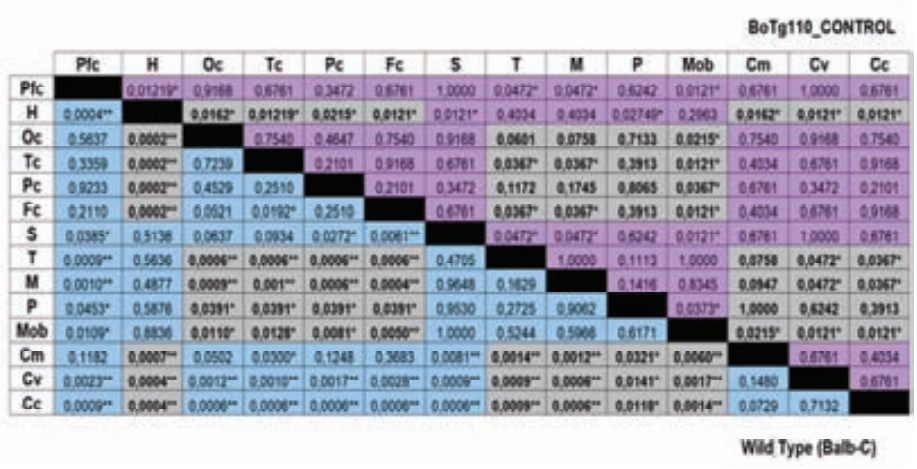


Table 2: Semiquantification of different NT and NTRs immunolabelling in different brain areas of BoTg 110 (blue) and Balb C (orange) murine models.



BoTg110_CONTROL

	Pfc	H	Oc	Tc	Pc	Fc	S	T	M	P	Mob	Cm	Cv	Cc
Pfc		0,01219*	0,9168	0,6761	0,3472	0,6761	1,0000	0,0472*	0,0472*	0,6242	0,0121*	0,6761	1,0000	0,6761
H	0,0004**		0,0162*	0,01219*	0,0215*	0,0121*	0,0121*	0,4034	0,4034	0,02749*	0,2963	0,0162*	0,0121*	0,0121*
Oc	0,5637	0,0002**		0,7540	0,4647	0,7540	0,9168	0,0601	0,0758	0,7133	0,0215*	0,7540	0,9168	0,7540
Tc	0,3359	0,0002**	0,7239		0,2101	0,9168	0,6761	0,0367*	0,0367*	0,3913	0,0121*	0,4034	0,6761	0,9168
Pc	0,9233	0,0002**	0,4529	0,2510		0,2101	0,3472	0,1172	0,1745	0,8065	0,0367*	0,6761	0,3472	0,2101
Fc	0,2110	0,0002**	0,6521	0,0192*	0,2510		0,6761	0,0367*	0,0367*	0,3913	0,0121*	0,4034	0,6761	0,9168
S	0,0385*	0,5136	0,0637	0,0934	0,0272*	0,0081**		0,0472*	0,0472*	0,6242	0,0121*	0,6761	1,0000	0,6761
T	0,0009**	0,5636	0,0006**	0,0006**	0,0006**	0,0006**	0,4705		1,0000	0,1113	1,0000	0,0758	0,0472*	0,0367*
M	0,0010**	0,4877	0,0009**	0,001**	0,0006**	0,0004**	0,9648	0,1629		0,1416	0,8345	0,0947	0,0472*	0,0367*
P	0,0453*	0,5876	0,0391*	0,0391*	0,0391*	0,0391*	0,9530	0,2725	0,9062		0,0373*	1,0000	0,6242	0,3913
Mob	0,0109*	0,8836	0,0110*	0,0128*	0,0081*	0,0050**	1,0000	0,5244	0,5966	0,6171		0,0215*	0,0121*	0,0121*
Cm	0,1182	0,0007**	0,0502	0,0300*	0,1248	0,3683	0,0081**	0,0014**	0,0012**	0,0321*	0,0060**		0,6761	0,4034
Cv	0,0023**	0,0004**	0,0012**	0,0010**	0,0017**	0,0028**	0,0009**	0,0009**	0,0006**	0,0141*	0,0017**	0,1480		0,6761
Cc	0,0009**	0,0004**	0,0006**	0,0006**	0,0006**	0,0006**	0,0006**	0,0009**	0,0006**	0,0118*	0,0014**	0,0729	0,7132	

Wild Type (Balb-C)

Table 3: Statistical analysis to compare differences between brain areas (white rows) in the BoTg 110 control model and the Balb-C wild type model. Mann Whitney test was applied and the statistically significant p-values are highlighted with a single * (p< 0,05) or double ** (p<0,01)

- **Study 2: Neurotrophins in the degeneration and regeneration of the PNS**.

Statistical test	Groups compared	NGF	BDNF	NT-3	Trk A	Trk B	Trk C	$p75^{NTR}$
U_Mann Whitney test								
Cell-I	2 weeks I vs 4 weeks I	0.016	0.056	0.056	0.056	0.008	0.310	0.151
	4 weeks I vs 8 weeks I	0.095	0.032	0.151	0.690	0.421	0.151	0.008
	2 weeks I vs 8 weeks I	0.008	0.421	0.008	0.032	0.008	0.032	0.008
Cell_NI	2 weeks NI vs 4 weeks NI	0.016	0.151	0.056	0.222	0.016	0.016	0.095
	4 weeks NI vs 8 weeks NI	0.008	0.69	0.222	0.222	0.095	0.008	0.056
	2 weeks NI vs 8wksNI	0.690	0.056	0.008	0.690	0.548	0.008	0.016
Axon-I	2 weeks I vs 4 weeks I	0.008	0.008	0.008	0.008	0.008	0.008	0.008
	4 weeks I vs 8 weeks I	0.421	0.056	0.841	0.548	0.841	0.016	0.008
	2 weeks I vs 8 weeks I	0.008	0.008	0.008	0.008	0.008	0.008	0.008
Axon-NI	2 weeks NI vs 4 weeks NI	0.31	0.151	0.31	0.095	0.095	1	0.548
	4 weeks NI vs 8 weeks NI	0.31	0.016	0.548	0.056	0.016	0.008	0.095
	2 weeks NI vs 8 weeks NI	0.095	0.421	0.151	0.548	0.310	0.008	0.016
Wilcoxon test								
Cell_I-Cell_NI	2 weeks I vs 2 weeks NI	0.038	0.063	0.043	0.059	0.039	0.683	0.042
	4 weeks I vs 4 weeks NI	0.046	0.034	0.038	0.314	0.102	0.276	0.034
	8 weeks I vs 8 weeks NI	0.039	0.705	0.039	0.414	0.655	0.042	0.066
Axon_I-Axon_NI	2 weeks I vs 2 weeks NI	0.042	0.039	0.042	0.041	0.039	0.038	0.041
	4 weeks I vs 4 weeks NI	0.038	0.038	0.102	0.414	0.564	0.034	0.041
	8weeks I vs 8weeks NI	0.043	0.063	0.068	0.492	0.042	0.041	0.066
Cell_I - Axon _I	2 weeks Injured	0.034	0.041	0.042	0.039	0.038	0.043	0.042
	4 weeks Injured	0.039	1	0.059	1	1	0.041	0.102
	8 weeks Injured	0.043	0.577	0.414	0.216	0.357	0.042	0.039
Cell_NI - Axon_NI	2weeks Non Injured	0.066	0.414	0.109	0.276	0.705	0.063	0.157
	4weeks Non Injured	0.564	0.317	0.102	1	0.083	0.317	0.066
	8weeks Non injured	0.042	0.102	0.043	0.465	0.059	0.043	0.043

Table 4: Statistical analysis (p values) in the sciatic nerve (cellular and axonal immunolabelling) of RIP-I /hIFNβ mice at three different time points (2, 4 and 8 weeks) in the injured and non injured sides.Two different tests were performed (U-mann Whitney test and Wilcoxon test). Statistically significant data are coloured in red.

		P values						
Statistical test	**Groups compared**	**NGF**	**BDNF**	**NT-3**	**Trk A**	**Trk B**	**Trk C**	**$p75^{NTR}$**
Wilcoxon test Non injured vs Injured	2 weeks_NI vs 2weeks_I	1	0.102	1	0.317	0.655	0.414	1
	4 weeks _NI vs 4 weeks _I	0.257	0.705	0.564	0.317	0.034	0.197	1
	8 weeks _NI vs 8 weeks _I	0.157	0.480	0.890	1	0.041	0.655	0.334
U-Mann Whitney Non injured	2 weeks _NI vs 4 weeks _NI	0.032	0.151	1	0.016	0.054	0.008	0.690
	4 weeks _NI vs 8 weeks _NI	0.008	0.056	0.008	0.008	0.056	0.008	0.008
	2 weeks _NI vs 8 weeks _NI	0.008	0.008	0.095	0.008	0.421	1	0.222
U-Mann Whitney Injured	2 weeks _ I vs 4 weeks _I	0.095	0.548	0.841	0.056	0.151	0.222	0.421
	4 weeks _I vs 8 weeks _I	0.095	0.056	0.056	0.032	0.31	0.056	0.095
	2 weeks _ I vs 8 weeks _I	0.01	0.032	0.151	0.008	0.056	0.222	0.31

Table 5: **Statistical analysis (p values) in the DRG of RIP-I /hIFNβ mice**,in the injured and non injured sides at three different time points (2, 4 and 8 weeks). Two different tests were applied (Wilcoxon and U-Mann Whitney) depending on the group(s) compared (see second column). Statistically significant data are coloured in red.

Statistical test	Groups compared	*P* values							
		NGF	BDNF_WM	BDNF_GM	NT-3	Trk A	Trk B	Trk C	$p75^{NTR}$
U-Mann Whitney	2 weeks *vs* 4 weeks	0.222	0.421	0.69	0.841	0.016	0.841	0.222	0.056
	4 weeks *vs* 8 weeks	0.548	0.095	0.008	0.008	0.151	0.032	0.151	1
	2 weeks *vs* 8 weeks	0.016	0.016	0.008	0.008	0.008	0.222	0.421	0.016
Wilcoxon test	2wks_white *vs* 2 wks_grey		0.129						
	4wks_white *vs* 4 wks_grey		0.257						
	8wks_white *vs* 8 wks_grey		0.041						

Table 6: **Statistical analysis (p values) in the spinal cord of RIP-I /hIFNβ mice.** Two different tests were applied (U-Mann Whitney and Wilcoxon) depending on the group(s) compared (see second column). Statistically significant data are coloured in red.

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EXPERIMENTALLY INDUCED DISEASE

Mapping of Neurotrophins and their Receptors in the Adult Mouse Brain and their Role in the Pathogenesis of a Transgenic Murine Model of Bovine Spongiform Encephalopathy

P. Marco-Salazar*, M. Márquez*, D. Fondevila*, R. M. Rabanal*, J. M. Torres , M. Pumarola* and E. Vidal

** Department of Animal Medicine and Surgery, Veterinary Faculty, Universitat Autònoma de Barcelona (UAB), 08193 Bellaterra (Cerdanyola del Vallès), Barcelona, CISA–INIA, 28130 Valdeolmos, Madrid and Centre de Recerca en Sanitat Animal (CReSA), UAB-IRTA, Campus de la Universitat Autònoma de Barcelona, 08193 Bellaterra, Barcelona, Spain*

Summary

Neurotrophins are a family of growth factors that act on neuronal cells. The neurotrophins include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin (NT)-3, -4 and -5. The action of neurotrophins depends on two transmembrane-receptor signalling systems: (1) the tropomyosin-related kinase (Trk) family of tyrosine kinase receptors (Trk A, Trk B and Trk C) and (2) the p75 neurotrophin receptor ($p75^{NTR}$). The interaction between neurotrophic factors and their receptors may be involved in the mechanisms that regulate the differential susceptibility of neuronal populations in neurodegenerative diseases. The aim of the present study was to evaluate the role of neurotrophins in the pathogenesis of bovine spongiform encephalopathy (BSE) using a transgenic mouse overexpressing bovine *prnp* (BoTg 110). Histochemistry for *Lycopersicum esculentum* agglutinin, haematoxylin and eosin staining and immunohistochemistry for the abnormal isoform of the prion protein (PrP^{d}), glial fibrillary acidic protein (GFAP), NGF, BDNF, NT-3 and the receptors Trk A, Trk B, Trk C and $p75^{NTR}$ was performed. The lesions and the immunolabelling patterns were assessed semiquantitatively in different areas of the brain. No significant differences in the immunolabelling of neurotrophins and their receptors were observed between BSE-inoculated and control animals, except for $p75^{NTR}$, which showed increased expression correlating with the distribution of lesions, PrP^{d} deposition and gliosis in the BSE-inoculated mice.

Keywords: brain; BSE prion; immunohistochemistry; neurotrophins

Introduction

Transmissible spongiform encephalopathies (TSEs or prion diseases) are a group of fatal neurodegenerative diseases affecting both man and animals and are characterized by having a long incubation period (Beringue *et al*., 2008). This group of diseases can be sporadic, genetic or acquired, but they are all transmissible and have a common feature, which is the accumulation in the brain of an abnormal form of the host-encoded cellular prion protein (PrP^{C}). Additional to the deposition of disease-associated prion protein (PrP^{d}) in the brain, the main neuropathological features are spongiform change in the neuropil, vacuolation of neuronal bodies and astrocyte and microglial cell activation and neuronal loss (Della-Bianca *et al*., 2001).

Among the animal TSEs, one of the best known is bovine spongiform encephalopathy (BSE), which was first reported in cattle in the mid 1980s (Wells and Wilesmith, 1995) and has had major

Correspondence to: E. Vidal (e-mail: enric.vidal@cresa.uab.cat).

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public health implications as it is a food-borne zoonosis resulting in the invariably fatal variant Creutzfeldt–Jacob disease (vCJD) (Wilesmith *et al.*, 1988; Bruce *et al.*, 1997).

Neurotrophins are a family of structurally and functionally related proteins consisting of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophin (NT)-3, -4 and -5. They are synthesized as precursors (proneurotrophins) by both neuronal and non-neuronal cells prior to being either cleaved intracellularly by proconvertases or secreted in the unprocessed form. In the latter case, there is conversion through proteolytic cleavage by plasmin or other extracellular proteases to the mature form (Bartkowska *et al.*, 2010). In the nervous system of vertebrates, neurotrophins control many aspects of embryonic development (e.g. cellular survival, differentiation, plasticity and regeneration) and the functions of most adult neurons (Skaper, 2008).

The action of neurotrophins depends on two transmembrane-receptor signalling systems: (1) the tropomyosin-related kinase (Trk) family of tyrosine kinase receptors (Trk A, Trk B and Trk C) and (2) the p75 neurotrophin receptor ($p75^{NTR}$), a member of the tumour necrosis factor receptor superfamily (Chao, 2003; Bartkowska *et al.*, 2010). Different neurotrophins show binding specificity for particular receptors. NGF binds preferentially to Trk A, BDNF and NT-4 to Trk B and NT-3 to Trk C. These interactions are considered to be of high affinity, but can be regulated by receptor dimerization, structural modifications or association with $p75^{NTR}$. The $p75^{NTR}$ can bind to all neurotrophins and also acts as a co-receptor with Trk receptors. Proneurotrophins are also active ligands of Trk receptors, but their binding elicits functional effects opposite to those elicited by the binding of mature neurotrophins (Chao, 2003; Reichardt, 2006).

The interaction between neurotrophic factors and their receptors is involved in the mechanisms that regulate the differential susceptibility of neuronal populations in neurodegenerative diseases (Connor and Dragunow, 1998). In prion diseases, PrP 106–126, a synthetic peptide homologous to the human PrP region 106–126, induces apoptosis in mouse neuroblastoma N2a cells, involving $p75^{NTR}$ and the nuclear factor-κB (NF-κB) signalling pathway (Della-Bianca *et al.*, 2001; Bai *et al.*, 2008). This suggests that neurotrophin receptors, and particularly $p75^{NTR}$, might be involved in prion disease pathogenesis. However, it has not been possible to find further publications on the subject.

The aim of the present study was to evaluate the role of neurotrophins and their receptors in a transgenic murine model (BoTg 110) of BSE. This transgenic mouse line is characterized by the overexpression (up to eight times the expression of a normal cow brain) of bovine *prnp* on a murine PRNP-knockout background (Castilla *et al.*, 2003) and has been shown to be a good model for study of the pathogenesis of BSE (Costa *et al.*, 2007, 2009; Espinosa *et al.*, 2007; Tortosa *et al.*, 2008, 2011). Little information was found regarding immunohistochemical investigations of NTs and NTRs in the mouse brain (Yan *et al.*, 1997a; Zermeno *et al.*, 2009; Bartkowska *et al.*, 2010; Parkhurst *et al.*, 2010), thus the study was performed in parallel with a wild type mouse line (Balb-C) to ensure that the transgene did not have an in uence on the studied molecules and to establish a baseline immunolabelling pattern in paraffin wax-embedded samples of mouse brain.

Materials and Methods

Animals and Inoculum

A case of BSE was identified within the BSE active surveillance plan and characterization of this case has been described elsewhere (BSE case 1; Vidal *et al.*, 2005, 2006). An inoculum was prepared from this case. The Log_{10} lethal dose 50 (LD_{50}) for the inoculum per 20 µl was 4.9 (i.e. brain homogenized and diluted at $10^{-4.9}$) as determined by bioassay in BoTg 110 mice. All procedures were approved by the Animal Experimentation Ethics Committee of the Autonomous University of Barcelona (procedure number 585-3487).

A total of 14 female BoTg 110 mice were used for the neurotrophin study and were divided into two groups: those inoculated with BSE inoculum ($n = 8$; at a 1 in 10 dilution) and the control group ($n = 6$) inoculated with a healthy cow brain homogenate at 1 in 1,000 dilution. Each 6–8-week-old mouse received a 20 µl intracerebral inoculation.

Animals from an additional mouse model were used to perform the neurotrophin study. These were Balb-C wild type (WT) mice and 10 healthy non-inoculated females, 367 days old, were included.

Sample Processing

When scored positive for clinical BSE, mice were killed in accordance with the recommendations of the ethics committee. At necropsy examination, brain tissue was collected and placed in 10% neutral buffered formalin. Transverse sections were taken at three different levels of the brain (optic chiasm, piriform cortex and medulla oblongata) and these were processed routinely prior to being embedded in paraffin

wax for histopathological, histochemical and immunohistochemical studies.

Histopathology, Histochemistry and Immunohistochemistry

Sections (4 μm) were dewaxed and rehydrated. For microscopical examination, sections were stained with haematoxylin and eosin (HE).

For NT and NTR immunohistochemistry (IHC), sections were mounted on silanized slides (Knittel Glass, Bielefeld, Germany) that were treated with 3-(triethoxysilyl)-propylamine (Merck, Darmstadt, Germany). After dewaxing, endogenous peroxidase activity was blocked by incubation in H_2O_2 3% in methanol for 40 min. Two different heat-induced epitope retrieval procedures in citrate buffer (pH 6.0) were used (Table 1). Sections were then cooled at room temperature for 30 min. Non-specific antibody binding was blocked with 2% bovine serum albumin in phosphate buffered saline (PBS) (blocking solution). Sections were incubated overnight at 4°C with primary antibodies against either NTs or NTRs. To visualize binding of primary antibodies, an anti-rabbit EnVision Plus™ System (Dako, Glostrup, Denmark) was used, incubating the slides with the secondary reagent at room temperature for 30 min. The peroxidase substrate was 3,3′-diaminobenzidine (DAB) in 200 μl PBS and 100 μl H_2O_2. Sections were counterstained with haematoxylin. Omission of the primary antibody was used as a negative control.

Immunohistochemistry for PrP^d was performed as described previously (Vidal *et al*., 2006). Brie y, sections were immersed in formic acid, endogenous peroxidase activity was blocked by incubation with H_2O_2 3% in methanol for 40 min and then the sections were boiled at pH 6.15 in a pressure cooker. After treatment with proteinase K, the sections were incubated overnight with mouse anti-PrP mAb 6H4 (diluted 1 in 400; Prionics, Schlieren, Switzerland) and the reaction was visualized using the anti-mouse Dako EnVision Plus™ system with DAB as chromogen (Table 1).

Lycopersicum esculentum agglutinin (LEA; 1 in 100 dilution; Sigma, St Louis, Missouri, USA) histochemistry was performed in order to stain microglial cells. The washing buffer was supplemented with $CaCl_2$, $MgCl_2$ and $MnCl_2$ 1 nM. The binding was visualized with bottle 2 of a Universal LSAB™ + Kit HRP, rabbit/mouse/goat (Dako; catalogue number K0690) and DAB was used as chromogen.

Assessment of Sections

Spongiform lesions and the immunolabelling patterns were evaluated semiquantitatively in 14 selected areas of the brain. The scoring system was: 0, no lesions or labelling; 1, mild labelling; 2, moderate labelling; 3, intense immunolabelling; and 4, the highest intensity of lesion or labelling (Tortosa *et al*., 2008).

For each area, a mean value from assessment of all the animals in each group was obtained. These values

Table 1
Details of the immunolabelling procedures

Antibody	*Target protein*	*Species*	*Dilution*	*Supplier*	*Epitope retrieval*
6H4	Prion protein	Mouse (monoclonal)	1 in 400	Prionics 01-010	Formic acid, pressure cooker, proteinase K
Glial fibrillary acidic protein	Astrocytes	Rabbit (polyclonal)	1 in 400	Dako (Z0334)	HIER citrate buffer (pressure cooker)
Human nerve growth factor	NGF protein	Rabbit (polyclonal)	1 in 500	Sigma (N665)	HIER citrate buffer (pressure cooker)
Brain-derived neurotrophic factor	BDNF protein	Rabbit (polyclonal)	1 in 100	Abcam (ab72439)	HIER citrate buffer (pressure cooker)
Neurotrophin 3	NT-3 protein	Rabbit (polyclonal)	1 in 50	Abcam (ab65804)	HIER citrate buffer (pressure cooker)
Tyrosine kinase A	intracellular domain of Trk A	Rabbit (monoclonal)	1 in 50	Abcam (ab76291)	HIER citrate buffer (pressure cooker)
Tyrosine kinase B	Intracellular domain of Trk B	Rabbit (polyclonal)	1 in 200	Abcam (ab51190)	HIER citrate buffer (pressure cooker)
Tyrosine kinase C	Extracellular domain of Trk C	Rabbit (polyclonal)	1 in 100	Abcam (ab75174)	HIER citrate buffer (pressure cooker)
P75 neurotrophin receptor	Extracellular domain of P75 neurotrophin receptor	Rabbit (polyclonal)	1 in 500	Abcam (ab8874)	HIER citrate buffer (water bath at 95°)
Lycopersicum esculentum agglutinin	Microglial cell membrane	Lectin	1 in 100	Sigma (L-0651)	Not applicable

HIER, heat-induced epitope retrieval.

corresponding to the 14 areas of brain studied were represented graphically in order to obtain the final brain profile for each antibody used and to compare the BSE-infected versus control mice. The Mann–Whitney *U* test for non-parametric data was applied ($P < 0.05$ with a 95% confidence interval and $P < 0.01$ with a 90% confidence interval; Microsoft Excel). Photomicrographs were taken with a Leica DM 6000 B microscope, a Leica DFC 480 digital camera and the Leica Application suite version 2.7.1 software.

Results

Disease Induction

BSE was transmitted successfully to BoTg 110 mice with a mean incubation period of 350 days. All mice progressed to the terminal stage of the disease with severe neurological dysfunction (i.e. abnormal gait, ataxia, kyphosis and altered, usually depressed, mental state).

Histopathology and PrP^d Deposition

The most characteristic TSE lesions (i.e. neuropil spongiosis, PrP^d deposition and gliosis) were evaluated in the BSE-infected mice and compared with the control group (Fig. 2). In the BSE-infected mice, HE staining revealed characteristic neuropil spongiosis with multiple and variably sized vacuoles mainly confined to the thalamus, mesencephalon, pons and medulla oblongata, but also, to some extent, involving the hippocampus and cerebellar nuclei. The cerebral cortices were generally less affected, with the exception of the occipital cortex (Fig. 1a). The control group showed no or slight spongiosis, the latter being attributed to ageing. This change was most notable in the cerebellar white matter.

Granular and plaque-like rounded PrP^d deposits labelled positively in the mesencephalon, medulla oblongata, thalamus, striated body and hippocampus (more frequently in the cornu ammonis than in the dentate gyrus). The least affected areas were the temporal, frontal and parietal cerebral cortex and the piriform cortex. However, in the occipital cortex, the immunolabelling was slightly greater. None of the control animals showed any PrP^d immunolabelling (Figs. 1b and 3).

IHC for GFAP labelling revealed the typical stellate cell pattern, which was particularly evident in the white matter (corpus callosum and cerebellar white matter) in the negative control group. In the BSE-infected group, cells with numerous cytoplasmic extensions, corresponding to hypertrophic astrocytes, were abundant and strongly positive for GFAP in the striatum, thalamus, mesencephalon (Fig. 2) (particularly in the tegmentum), pons, medulla oblongata and cerebellar nuclei. Scoring of this expression showed significant differences in the aforementioned regions. No significant differences were found in the degree of immunolabelling for GFAP in the neocortices of the BSE-infected and negative control mice (Fig. 1c).

Histochemistry with LEA highlighted ramified cells, corresponding to microglia, throughout the white and grey matter of the brain. In the BSE-infected mice, these cells were appreciably increased in number, were hypertrophic and, occasionally, some of them were binucleate. LEA also stained the vascular endothelia and some intraneuronal organelles.

When both groups were compared (BSE-infected versus control), significant differences were found in the thalamus ($P = 0.01917$), medulla oblongata ($P = 0.00178$) and cerebellar nuclei ($P = 0.01183$), where the score was higher in the BSE-infected group (Fig. 1d).

Neurotrophin and Receptors Expression

Nerve Growth Factor. Mild, diffuse immunolabelling for NGF was observed in the perikaryon of the majority of neurons throughout the brain. Very mild labelling of the neuropil in the grey matter was also observed. The white matter was generally devoid of labelling with the exception of the cerebellum where mild diffuse labelling was present in the white matter. The pontine nuclei showed slightly stronger labelling. In the cerebellar cortex, the neuropil of the molecular layer was mildly positive, while the labelling of Purkinje cell perikarya was variable. Occasional intracytoplasmic labelling was observed, while on occasions the cytoplasm was negative and extracellular perineuronal labelling was present. In the hippocampus, the labelling was more intense in the cornu ammonis than in the other areas. The parietal and temporal lobes of the cerebrum showed slightly stronger neuronal labelling in layers II/III. The choroid plexus and ependymal cells also labelled positively (Fig. 3a and b).

No significant differences were observed when WT mice were compared with healthy BoTg 110 mice ($P > 0.05$); the medulla oblongata was slightly more intensely labelled in WT mice, but this was not significant when scored. The immunolabelling pattern remained unchanged in the BSE-infected group compared with the control group. No significant differences were obtained when comparing the scoring of the labelling (Fig. 1e).

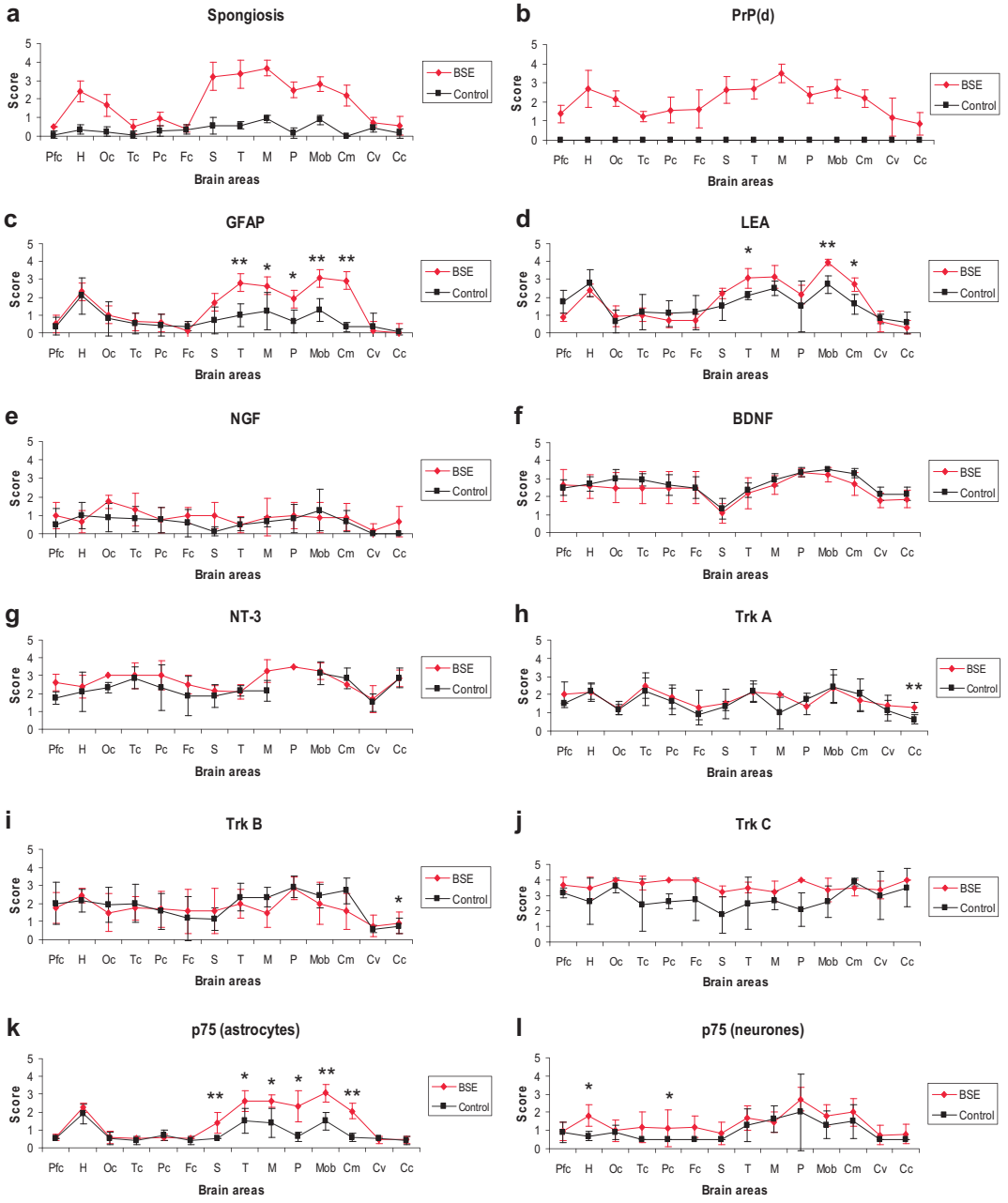

Fig. 1. Graphic representation of the scoring for expression of markers. Comparison of the mean scores in control (black lines) and BSE-inoculated (red lines) groups. Scores for (a) spongiosis, (b) PrP^{d} deposition, (c) astrocytosis, (d) microglia, (e–g) neurotrophins and (h–l) neurotrophin receptors. Bars indicate standard deviation. Mann–Whitney *U* test (*$P < 0.05$ with a 95% confidence interval and **$P < 0.01$ with a 90% confidence interval). Pfc, piriform cortex; H, hippocampus; Oc, occipital cortex; Tc, temporal cortex; Pc, parietal cortex; Fc, frontal cortex; S, striatum; T, thalamus; M, mesencephalon; P, pons; MObl, medulla oblongata; Cm, cerebellar nuclei; Cv, cerebellar vermis; Cc, cerebellar cortex.

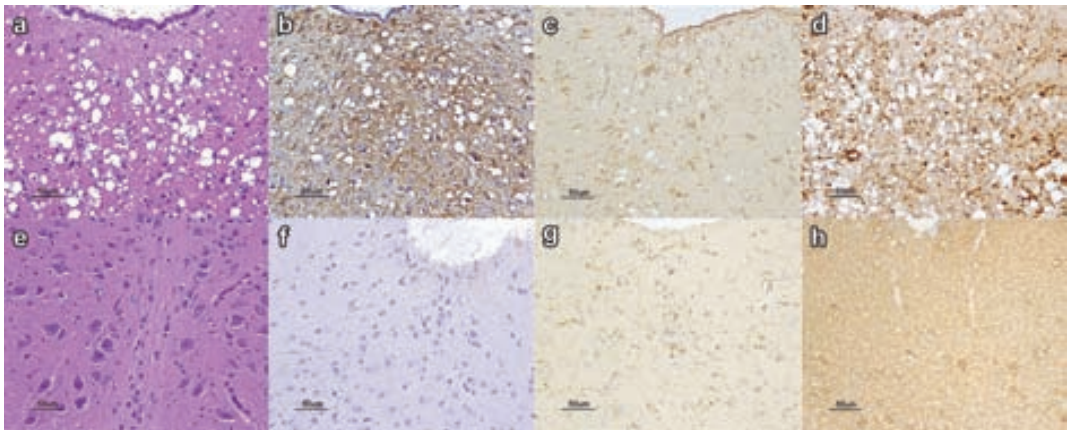

Fig. 2. Neuropathological characterization of the mesencephalon of BSE-infected mice (a, b, c and d) and control BoTg 110 mice (e, f, g and h). From left to right: HE staining (a and e), IHC for GFAP (b and f), histochemistry for LEA (c and g) and IHC for PrP^{d} (d and h). Bars, 50 μm.

Brain-Derived Neurotrophic Factor. The BDNF antibody revealed a diffuse intracytoplasmic and nuclear immunolabelling pattern in the neurons (Figs. 3c and d). The neuropil also showed mild labelling, which at higher magnification consisted of a fine punctuate pattern. This was particularly intense in the perivascular compartment (probably depicting the external glial limitans) and was also seen associated with scattered glial cell cytoplasmic extensions. The white matter was devoid of labelling. The choroid plexus and ependymal cells were positively labelled in all mice.

Immunolabelling was homogeneous throughout the brain with higher intensity in some of the examined areas, such as the hippocampus, thalamus, mesencephalon, pons, medulla oblongata and the cerebellum. In contrast, the striatum was less intensely labelled than other areas (Fig. 1f).

Of note, in the hippocampus, the cornu ammonis and particularly the stratum lucidum layer, showed stronger immunolabelling than the dentate gyrus. In the thalamus, a fine, perineuronal and intense punctuate labelling pattern was found, mainly in the habenular nucleus (Fig. 3d). In the mesencephalon, positive labelling was mainly found in the lateral, medial and ventral areas (Fig. 3c). Both oculomotor and red nuclei were always BDNF positive, the pontine nuclei were also strongly positive and the cerebellar nuclei were intensely labelled. Additionally, the three different cerebellar layers had different intensities of labelling. In the molecular layer the labelling was associated with the dendrites of Purkinje cells and the Purkinje cell perikarya were also immunoreactive. In the granular layer only the Golgi neurons were labelled. Neurons in the medulla oblongata were also positively labelled for BDNF, particularly those in the superior vestibular nuclei, ventral cochlear nuclei, raphe magnus nucleus, raphe nuclei, pallidus nucleus and facial nuclei.

With respect to the immunolabelling pattern and distribution, no significant differences were found between control and BSE-infected groups (Fig. 1f). In the mesencephalon the spongiform change was restricted to the areas positive for BDNF. No significant differences were present between control and BSE-infected groups when comparing the scoring of labelling; however, slightly stronger labelling was seen in the Purkinje cell layer of the WT mice.

Neurotrophin 3. Strong intraneuronal immunolabelling for NT-3 was observed, but less intensity was found in the neuropil. The nucleus of neurons was devoid of immunolabelling (Figs. 3e and f). In the white matter, only some glial cells, probably oligodendrocytes, and the choroid plexus labelled positively for NT-3, but the ependymal cells and other glial cells were devoid of labelling.

In the cerebellum, strong labelling was detected in the perikarya of the Purkinje cells (Fig. 3f) and also in the neurons of the cerebellar nuclei. Mild to moderate labelling of the molecular layer was observed, but labelling was mild to absent in the granular layer, where Golgi neurons labelled positively. In the medulla oblongata, NT-3 was distributed homogenously with an intracellular pattern, but the labelling was stronger in the ventral area. The cerebrocortical areas were also homogenously and strongly labelled in the neuropil and intraneuronally, except for the

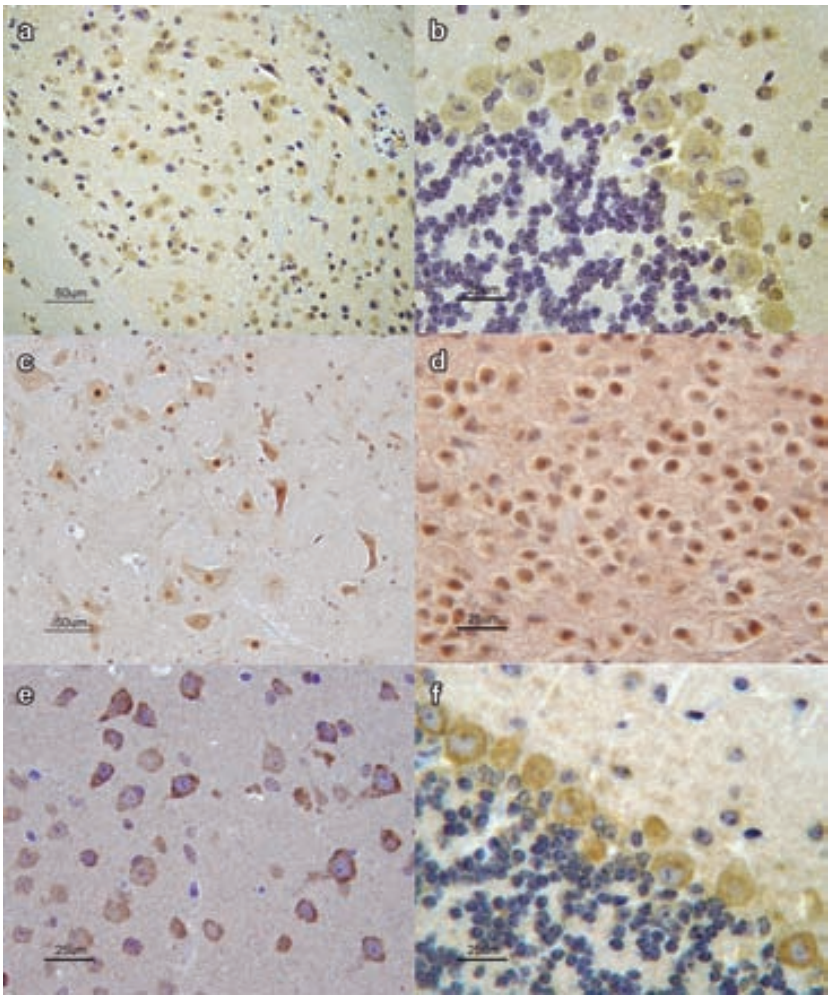

Fig. 3. Neurotrophin IHC in the brain of BoTg 110 control mice. NGF expression in (a) the pontine nucleus and (b) the cerebellar cortex. BDNF expression in (c) the mesencephalon and (d) the habenular nucleus. NT-3 expression in (e) the neocortex and (f) the cerebellar cortex. (a and c) Bars, 50 μm. (b, d, e and f) Bars, 25 μm.

superficial molecular layer, which was less intensely labelled. In the hippocampus, the pyramidal cell layer of the CA3 and CA4 regions was more intensely labelled than the other layers and the dentate gyrus. The neuropil was mildly labelled. In the striated body, the neuropil of the grey matter was intensely labelled as was the perikarya of neurons. In the mesencephalon an intraneuronal, homogeneous immunolabelling pattern was observed and the neuropil was almost devoid of labelling. Similarly, in the thalamus intracytoplasmic labelling was present and the neuropil labelling was very mild.

Significance was not achieved when assessing differences between the control and BSE-infected groups (Fig. 1g) nor between healthy BoTg 110 and Balb-C mice. In the control BoTg 110 group, no samples of the pons were available for study.

Nerve Growth Factor. IHC for NGF revealed diffuse, moderate to intense labelling of the neuropil, which was particularly intense in the perineuronal areas (Fig. 4a). Additionally, intracytoplasmic labelling was detected in the interpeduncular nucleus in the tegmentum of the mesencephalon, the pontine nucleus, the cerebellar nuclei (Fig. 4b) and the medulla

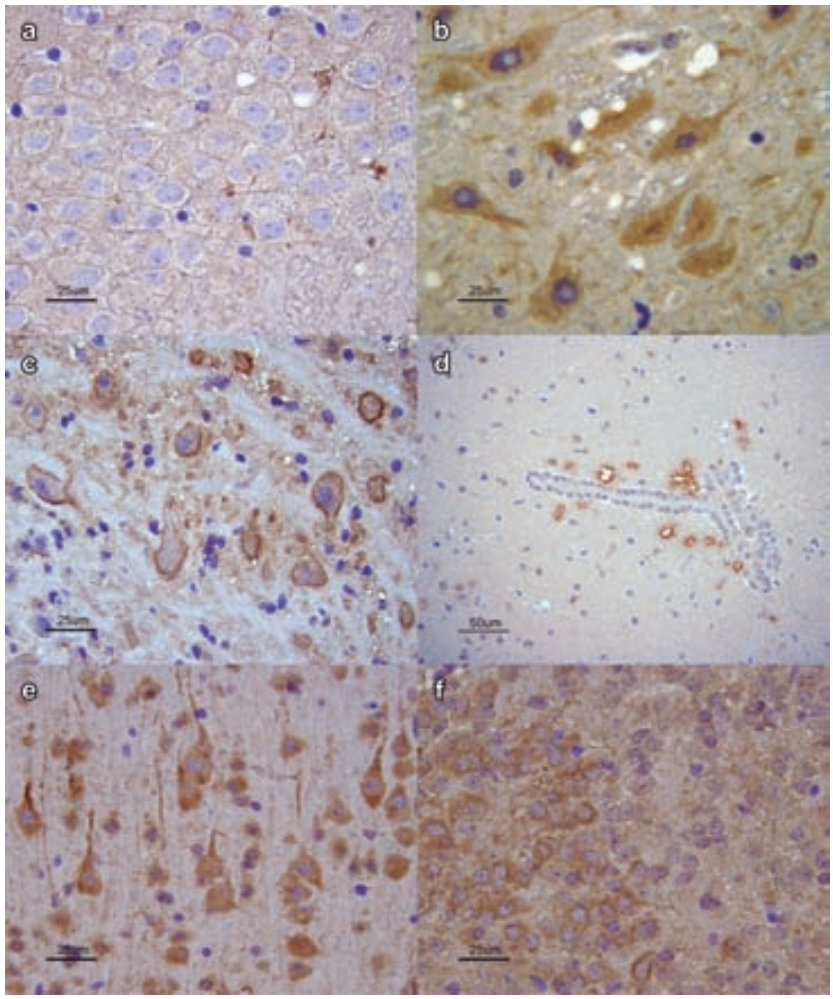

Fig. 4. Neurotrophin receptor IHC in the brain of BoTg 110 control mice. Expression of Trk A in (a) the hippocampus and (b) the medulla oblongata. Expression of Trk B in (c) the medulla oblongata and (d) the periaqueductal amorphous structures. Expression of Trk C in (e) the frontal cortex and (f) the habenular nucleus. (a, b, c, e and f) Bars, 25 μm. (d) Bar, 50 μm.

oblongata (i.e. facial nucleus, paragigantocellular reticular nucleus and raphe magnus nucleus). Both perineuronal and neuropil labelling for NFG were present within the inferior olive nuclei (the labelling was more evident in the medial nuclei of this area).

Tropomyosin-related Kinase A. The neocortical areas were strongly reactive with anti-Trk A antibody. In the cerebellum, mild to moderate labelling was found in the neuropil of both the granular and molecular layers. In contrast, mild or no labelling was present in the cytoplasm of Purkinje cells. Within the hippocampus the labelling was uniform, except for the stratum lucidum layer, which was less intensely labelled. Only some neurons in the cornu ammonis showed intracytoplasmic labelling. In the diencephalon, there was intense labelling of the ventral posteromedial (VPM), the ventral posterolateral (VPL) and the ventrolateral (VL) thalamic nuclei. Choroid plexus, ependymal cells and white matter were unlabelled.

No significant differences were detected between the WT mice and BoTg 110 control mice with the exception of the frontal cortex ($P = 0.008475$) and striatum ($P = 0.006928$), where the immunolabelling was slightly higher in the WT animals. When the negative control and BSE-infected group were compared, significance was only achieved in the

cerebellar cortex (Fig. 1h), due to a more intense labelling in the granular layer of the infected animals. Additionally, in the hippocampus of the infected animals granular labelling was associated with spongiform foci. The thalamic nuclei described above were markedly less labelled, but only in those animals with intense lesions in the region. However, since variation in lesion intensity existed between animals, the mean score was not significantly different from that of the control group.

Tropomyosin-related Kinase B. The main immunolabelling pattern for Trk B was perineuronal, although some areas showed either diffuse labelling of the neuropil or intraneuronal labelling (Figs. 4c and d). Choroid plexus and ependymal cells were also immunoreactive. Mild to absent labelling was present in the white matter.

In the temporal and parietal cortex there was mild to moderate labelling for Trk B and this was restricted to the neuropil. In the piriform cortex this pattern was less intense. In the hippocampus the immunolabelling was more intense in the pyramidal cell layer of the CA3 and CA4 regions of the cornu ammonis. Surrounding both the aqueduct and ventricle walls, there were numerous, intensely labelled, amorphous structures, some of which had an unstained central core (Fig. 4d). The thalamus showed strong perineuronal and intraneuronal labelling, mainly in the VPM, VPL and VL nuclei. The thalamic neuropil was also intensely labelled. In the mesencephalon, the perineuronal labelling was evident in the nuclei of the tegmental region. Additionally, the pontine nucleus showed very strong intraneuronal labelling.

In the cerebellum, immunoreactivity of the Purkinje cell layer was inconsistent. Occasional perineuronal and intraneuronal immunolabelling was seen, but Purkinje cells were also often devoid of labelling. The granular layer was also unlabelled, but the molecular layer showed moderate to intense labelling of the neuropil. In the cerebellar nuclei there was intense neuropil and perineuronal labelling. The perineuronal labelling pattern was evident in the vestibular, cochlear and ventral nuclei of the medulla oblongata (Fig. 4c). No significant differences were found when the WT animals and the BoTg 110 control groups were compared, with the exception of the medulla oblongata ($P = 0.005614$), which was slightly less intensely labelled in the BoTg 110 mice (Fig. 1i). This was also the case in the cerebellar cortex, where the Purkinje cell perikarya were more intensely labelled in the WT mice. No significant differences were observed between BSE-inoculated and healthy BoTg 110 mice.

Tropomyosin-related Kinase C. Trk C was expressed intensely by the neuronal perikaryon and neuronal cytoplasmic extensions. Moderate diffuse labelling of the neuropil was also present in some areas. Ependymal cells and the choroid plexus were occasionally labelled. The white matter was mildly labelled.

In the neocortex, intraneuronal labelling was prominent, particularly in the pyramidal neuron layers (III and V) (Fig. 4e). In the neuropil, labelling of neurites was very prominent, particularly in the temporal lobe. Neurons of the piriform cortex were also strongly labelled. Conversely, in the striatum body, positive labelling was mild. In the hippocampus the intraneuronal labelling was also mild and restricted to the pyramidal layer of the cornu ammonis. In the stratum radiatum, the radial dendrites of the pyramidal layer neurons were intensely labelled and, with a more disorganized aspect, the neurites of the lacunosum molecular layer were similarly labelled. In the dentate gyrus, neurite labelling was evident in both granular and molecular layers. In the thalamus the immunoreactivity was mainly localized to the habenular nucleus (Fig. 4f), the geniculate nuclei, the posterior thalamic nuclear group (Po), VPM and VPL and also in the median eminence (ME) in the hypothalamic area. In the mesencephalon, the immunolabelling was stronger in the neurites of the tectum (mesencephalic collicles). Both red and oculomotor nuclei showed mild to moderate intraneuronal labelling and the pontine nuclei were also intensely labelled. In the cerebellum, the labelling of the molecular layer was due to the dendrites of the Purkinje neurons, the perikaryon of which was intensely labelled. The granular layer was devoid of labelling except for the Golgi neurons. In the cerebellar nuclei an intracytoplasmic labelling pattern was observed. In the medulla oblongata the neurites were positively labelled, particularly in the facial and the ventral cochlear nuclei, where intense intraneuronal labelling was present.

When comparing the Trk C immunolabelling of the BoTg 110 control group and the WT mice, no significant differences were found except in the cerebellar nuclei (Fig. 1j), where the BoTg mice showed slightly stronger immunolabelling ($P = 0.01421$). BSE-inoculated mice did not show any significant differences compared with negative control animals.

Expression of $p75^{NTR}$. There was an intraneuronal, occasionally finely granular, immunolabelling pattern for $p75^{NTR}$ that was of mild to moderate intensity depending on the area studied. Additionally, a variable number of stellate glial cells were also positively labelled and were particularly numerous in the subependymal region and perivascularly. The morphology and anatomical distribution of these cells was consistent with astrocytes; however, the possibility that some microglial cells were also labelled could

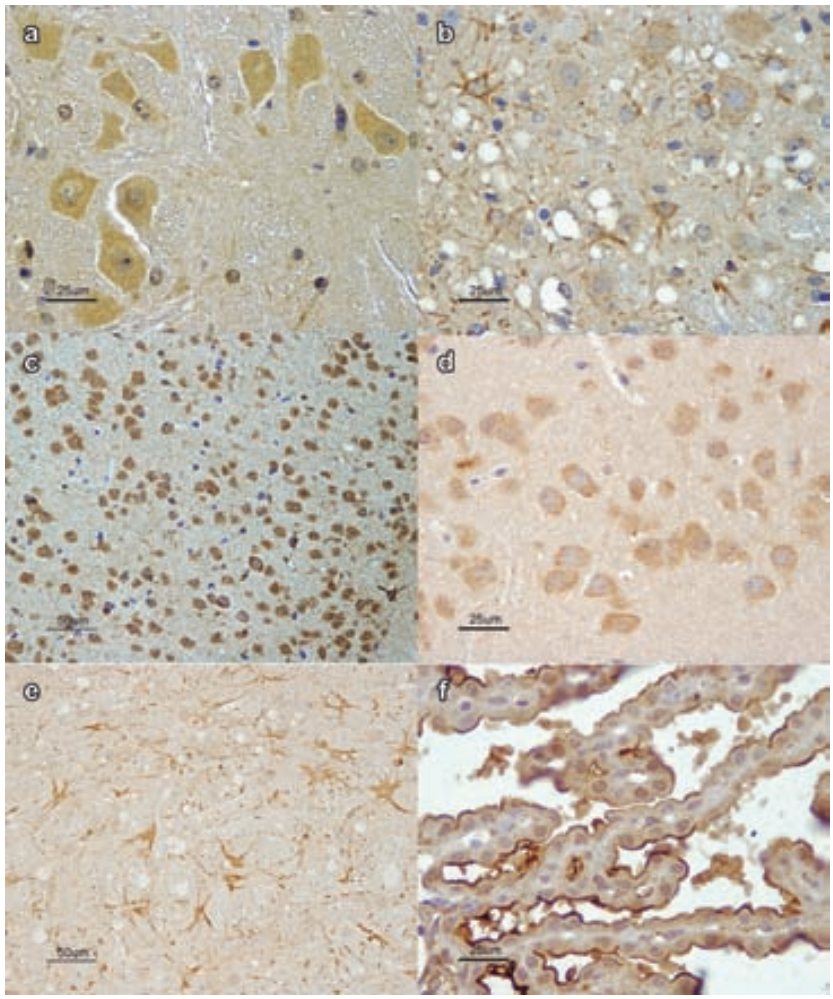

Fig. 5. $P75^{NTR}$ IHC in BoTg 110 mouse brain. (a) Mesencephalon of BoTg 110 control mice. (b) Mesencephalon of BoTg 110 BSE-infected mice. (c) Parietal cortex and (d) occipital cortex of BoTg 110 control mice. (e) Hypertrophic glial cells in the medulla oblongata of BSE-infected mice. (f) Choroid plexus of WT control mice. (a, b, d and f) Bars, 25 μm. (c and e) Bars, 50 μm.

not be excluded. The neuropil of all mice was mildly labelled. Ependymal cells were devoid of labelling; however, the apical membrane of the choroid plexus cells showed intense immunoreactivity (Fig. 5f).

Neuronal and glial cell immunolabelling intensity was scored separately. There was intense labelling of abundant ramified cells in white matter, particularly that of the corpus callosum and cerebellum. These cells, which labelled intensely for $p75^{NTR}$, were probably fibrillary astrocytes. In the cortical grey matter, there was a low number of strongly labelled ramified cells, particularly in the deeper layers. In the hippocampus, a large number of positively-labelled glial cells were found, mainly in the oriens, radiatum, lacunosum moleculare layers of the cornu ammonis and the molecular layer of the dentate gyrus. In comparison, in the thalamus, these cells were fewer in number and were present in the habenular nucleus. There were a similar number of positively-labelled glial cells in the mesencephalon and the thalamus, particularly in the tegmentum. The same applied to the pontine nucleus.

A large number of positively-labelled glial cells were detected in the medulla oblongata and there were slightly more in the facial nucleus. In the cerebellar cortex, a low number of cells were labelled in the grey matter.

As described previously with the GFAP antibody, in the BSE-infected mice increased immunolabelling was present compared with the control group (Figs. 5a and b) and this was due to the presence of a higher number of hypertrophic glial cells. Positively-labelled amoeboid glial cells were also detected in the thalamus, mesencephalon, cerebellar nuclei and medulla oblongata. These differences were significant when the scoring of the striatum ($P = 0.00811$), thalamus ($P = 0.02811$), mesencephalon ($P = 0.01917$), pons ($P = 0.01902$), medulla oblongata ($P = 0.006766$) and cerebellar nuclei ($P = 0.00431$) were compared (Fig. 1k).

The intraneuronal immunolabelling in the neocortex was restricted to the internal and external pyramidal layers. The parietal cortex was the most intensely labelled region (Fig. 5c). In contrast, in the striatum the labelling for $p75^{NTR}$ was mild. In the hippocampus the pyramidal layer of the cornu ammonis was much more intensely labelled than the dentate gyrus. Mild labelling was observed in the hypothalamus and mild to moderate labelling in the thalamus. The medulla oblongata was moderately labelled (Fig. 5e). In the cerebellum the labelling was confined to the neuropil, except for the cerebellar nuclei, in which moderate intraneuronal signalling was seen.

With respect to the neuronal pattern, a significant increase in labelling intensity was found only in the hippocampus ($P = 0.01041$) and frontal cortex ($P = 0.03379$) of the BSE-inoculated group compared with the negative control group (Fig. 1l). No differences were observed when WT animals were compared with healthy BoTg 110 mice.

Discussion

In the present study, an immunohistochemical assessment of the role of the neurotrophins NGF, BDNF, NT-3 and their receptors (Trk A, Trk B, Trk C and $p75^{NTR}$) in the pathogenesis of BSE was performed using a mouse model of the disease. For this purpose, a BSE-infected group of mice and a group of transgenic BoTg 110 mice inoculated with healthy cow brain were compared. The immunolabelling was scored semiquantitatively and the results analysed statistically in order to define differences between the groups. Additionally, a WT mouse strain (Balb-C) was included in the study to ensure that the transgene had no independent in uence on the results. This group and the BoTg 110 control group were used for mapping the distribution of neutrophins and their receptors in the brain.

There are few immunohistochemical studies of the distribution of neurotrophins in the mouse brain; however, the expression patterns of approximately 20,000 genes (including those encoding neurotrophins) have been studied by in-situ hybridization (Lein *et al*., 2007).

The majority of studies that have explored the expression, distribution and function of neurotrophins in the central nervous system have been performed with rats (Shelton and Reichardt, 1986; Zhou and Rush, 1994; Yan *et al*., 1997b; Friedman *et al*., 1998), although some studies have been reported in primates (Mufson *et al*., 1994; Zhang *et al*., 2007) and man (Connor and Dragunow, 1998; Murer *et al*., 1999; Tang *et al*., 2010).

The immunohistochemical approach permitted characterization of the distribution of these proteins throughout the brain and identification of the cell populations that express them. IHC is an ideal method for determining whether correlation existed between the studied molecules and BSE-associated neuropathological changes (e.g. spongiform lesions, glial proliferation and PrP^{d} deposition).

In the BoTg 110 control group and the WT mice, all of the neurotrophins and their receptors were distributed widely throughout the brain. Comparison between these two groups did not show any significant differences in the cellular and neuroanatomical distribution of the molecules. When the labelling was scored semiquantitatively, minor differences were detected in neurotrophin receptor expression in a few areas (data not shown). Therefore, the results suggest that the BoTg 110 transgene does not significantly alter the expression of these molecules.

No evidence was found, based on immunohistochemical differences between BSE-infected and control animals, to indicate that neurotrophins or their receptors are involved in the pathogenesis of BSE, with the exception of $p75^{NTR}$. In the BSE-infected mice, a significant increase in glial $p75^{NTR}$ labelling was observed in the striatum, thalamus, mesencephalon, brainstem and cerebellar nuclei. This increase was parallel to the increase in astrocytes in the BSE-infected mice, re ecting the glial activation seen in BSE, which was apparently accompanied by an increased expression of $p75^{NTR}$.

Interestingly, $p75^{NTR}$ expression in uninfected control mice was significantly enhanced in the thalamus, hippocampus, mesencephalon and medulla oblongata, when compared with other areas in WT and BoTg 110 control animals (data not shown). An increase in $p75^{NTR}$ signalling was also observed in these areas in BSE-inoculated animals, with the exception of the hippocampus where the $p75^{NTR}$ levels remained unchanged. Additionally, notable PrP^{d} deposition and spongiform lesions were observed in these areas. This suggests a relationship might exist

between the regional expression of $p75^{NTR}$ in the normal brain and the distribution of BSE-associated brain lesions.

Whether the topographical distribution of $p75^{NTR}$ in the brain governs, at least in part, the distribution of BSE-related pathology is unknown. However, Della-Bianca *et al.* (2001) showed that the binding of synthetic PrP 106–126 peptide to $p75^{NTR}$ triggered cell death mechanisms in cultured neural cells, mediated by caspase 8 and NADPH oxidase-dependent mechanisms. Therefore it is possible that extracellular deposits of BSE-associated PrP^{d} might trigger a similar mechanism *in vivo*.

Brain lesions and PrP^{d} distribution are features that vary according to the prion strain, so in an identical mouse model, different strains yield different PrP^{d} profiles (Bruce, 2003). The distribution of lesions and PrP^{d} in the present model largely coincides with that observed in BSE-infected cattle (Vidal *et al.*, 2005) and with studies performed in the same model with different BSE inocula (Castilla *et al.*, 2003; Vidal *et al.*, 2005, 2006; Tortosa *et al.*, 2011). Should the above hypothesis be confirmed, it would be dependent on a strain-specific interaction between $p75^{NTR}$ and BSE-misfolded PrP^{d}.

The $p75^{NTR}$ is widely expressed in developmental stages and decreases markedly in adulthood. However, its expression can increase in pathological states associated with neural cell death or neurodegeneration (Dechant and Barde, 2002; Chao, 2003). An immunohistochemical study performed on normal human brain, non-human primate tissue and adult human tissue affected by different neurodegenerative disorders revealed $p75^{NTR}$ re-expression in the cortical neurons, mainly in patients affected by Alzheimer s disease (Mufson and Kordower, 1992). It is well established that, depending on the cellular context, $p75^{NTR}$ has a dual function; it promotes neuronal survival by its interaction with neurotrophins and Trk receptors, but can also trigger cell death when other neurodegenerative molecules bind directly to its extracellular domain (Hempstead, 2002; Butowt and von Bartheld, 2003; Chen *et al.*, 2009; Diarra *et al.*, 2009; Zeng *et al.*, 2011). The neurotoxic fragment of the prion protein (PrP 101–126), as discussed above, and also the peptide of the amyloid precursor protein (APP) have been described as neurotoxic ligands binding to the extracellular domain of $p75^{NTR}$ (Yaar *et al.*, 1997; Dechant and Barde, 2002; Perini *et al.*, 2002; Butowt and von Bartheld, 2003). This evidence, together with our results, suggests that BSE might share with other neurodegenerative diseases, such as Alzheimer s disease, cell death mechanisms mediated by non-neurotrophin ligands binding to $p75^{NTR}$.

With the exception of $p75^{NTR}$, the present results suggest that the neurotrophins and their receptors do not have a critical role in terminal stage brain pathology in BSE. The use of other neurotrophic factors has been proposed by several research groups as a therapeutic method for limiting the severity of nervous system injury in disease (Knowles *et al.*, 2009; Nagahara and Tuszynski, 2011; Sari, 2011; Ibanez and Simi, 2012; Rosner *et al.*, 2012). In the case of BSE, targeting PrP^{d} binding to $P75^{NTR}$ may have a therapeutic effect in prion diseases. However, additional studies are required to further understand the neuronal damage elicited by the interaction between $p75^{NTR}$ and BSE-associated PrP^{d}.

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