Insights into Fas Ligand reverse signalling in Schwann cells and astrocytes

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Abstract

Fas Ligand has been widely associated with Fas-mediated apoptotic cell death through binding to Fas, a member of the tumor necrosis factor receptor superfamily able to induce apoptosis. The Fas Ligand homotrimeric molecule belongs to the tumor necrosis factor superfamily of ligands and is expressed in the immune, nervous, and reproductive systems with cell surface and secreted isoforms. In the immune system, Fas Ligand can also act as a counter-receptor and signal in the Fas Ligand-expressing cell, a mechanism called reverse signalling. Apart from its known role as a trigger for apoptosis, Fas has recently been implicated in neuroprotective and neuroregenerative functions. Since Fas Ligand is expressed at the surface of glial cells, the cells surrounding and supporting neurons in the nervous system, we hypothesized that Fas Ligand could transduce signals in glial cells upon engagement by Fas and that the biological effect of reverse signalling might also be neuroprotective or neuroregenerative. We studied Fas Ligand reverse signalling in Schwann cell and astrocyte glia, the principal neurotrophin-secreting cells of the peripheral and central nervous system, respectively. We show that agonistic anti-Fas Ligand antibodies or Fas protein constructs are able to trigger Fas Ligand reverse signalling in mouse, rat, and human glia. Nerve growth factor protein secretion and mRNA levels were increased in glia treated with Fas Ligand agonists. Following Fas Ligand engagement, phosphorylation levels of the signalling kinases extracellular-signal regulated protein kinase 1/2 and the cytosolic tyrosine kinase Src were rapidly increased in Schwann cells. We also show that functional recovery after sciatic nerve crush injury is delayed in mice when FasL reverse signalling cannot be engaged. Microarray analysis of changes in gene expression following Fas Ligand engagement in mouse astrocytes revealed that Fas Ligand signalling only modestly affects transcription, at least at the time points studied. However, the genes regulated by Fas Ligand signalling have been implicated in cell growth and survival. Since FasL is upregulated in peripheral and central nervous system glia after injury, recruitment of growth / survival pathways and secretion of growth

factors downstream of Fas Ligand may contribute to regeneration after injury in the nervous system.

Résumé

Fas Ligand est principalement connu pour son rôle dans la mort cellulaire par apoptose en tant que ligand de Fas, un membre de la super-famille des récepteurs du facteur de nécrose tumorale capable de médier la mort par apoptose. La protéine homotrimérique Fas Ligand appartient à la famille du facteur de nécrose tumoral et est exprimée dans les systèmes immunitaire, nerveux et reproducteur sous deux isoformes : un isoforme membranaire et un isoforme sécrété. Dans le système immunitaire, Fas Ligand peut également agir comme récepteur réverse et produire des signaux dans la cellule qui l'exprime, un méchanisme appelé signalisation réverse. Fas n'a pas uniquement un rôle dans l'apoptose mais peut également avoir des effets neuroprotecteurs et neurorégénérateurs. Fas Ligand étant exprimé à la surface des cellules gliales, cellules qui entourent et soutiennent les neurones dans le système nerveux, nous avons émis l'hypothèse que Fas Ligand pourrait transmettre des signaux dans les cellules gliales suite à la fixation de Fas et que les effets biologiques de la signalisation réverse pourraient également être neuroprotecteurs ou neurorégénérateurs. Nous avons étudié la signalisation réverse de Fas Ligand dans les cellules Schwann et les astrocytes, les principales cellules gliales sécrétant des neurotrophines dans les systèmes nerveux périphérique et central, respectivement. Nous montrons que des anticorps anti-Fas Ligand ou une protéine Fas chimérique agonistes peuvent déclencher la signalisation réverse de Fas Ligand dans les cellules gliales humaines, de souris, et de rat. La sécrétion du facteur de croissance neurale et les niveaux d'ARN messagers de cette protéine se sont accrus dans les cellules gliales traitées avec les agonistes de Fas Ligand. Suite à l'activation de Fas Ligand, une rapide augmentation de la phosphorylation des protéines kinases Erk1/2 et Src a été observée dans les cellules Schwann. Nous montrons également que le rétablissement fonctionnel après lesion du nerf sciatique par écrasement est retardé chez des souris qui ne peuvent recruter la voie de signalisation de Fas Ligand. L'analyse des modifications dans l'expression génique après activation de Fas Ligand, avec les puces à ADN, a montré que la

signalisation réverse avait un effet modeste sur la transcription, aux temps étudiés. Cependant, les gènes régulés par Fas Ligand sont connus pour être impliqués dans la croissance et la survie cellulaire. Comme l'expression de Fas Ligand est accrue dans les cellules gliales des systèmes nerveux périphérique et central après une lésion, le recrutement des voies de signalisation de croissance et de survie par Fas Ligand et la sécrétion de facteurs de croissance par les cellules gliales pourraient contribuer à la régénération après lésion du système nerveux.

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Original contribution to knowledge

Chapter 3. Results

Section 3.1

FasL reverse signalling and its biological and functional consequences have not been studied previously in the nervous system. The role of FasL signalling in Schwann cells is novel and suggests a potential role for FasL in peripheral nerve regeneration. We have shown that:

- FasL engagement stimulates Schwann cells to produce soluble mediators that induce neurite outgrowth
- FasL induces NGF secretion from mouse Schwann cells
- FasL engagement upregulates NGF mRNA and in addition stimulates NGF release independently of RNA synthesis
- FasL reverse signalling activates the ERK1/2 and Src pathways in Schwann cells
- FasL reverse signalling induces release of NGF by rat and human Schwann cells
- FasL signalling accelerates *in vivo* functional recovery after sciatic nerve crush

Section 3.2

The role of FasL reverse signalling in astrocytes is also novel. The study of changes in gene expression induced by FasL reverse signalling had never been previously performed in astrocytes. The release of NGF as well as the induction of transcription of genes involved in cell survival and cell proliferation may also be indicative of a potential role of FasL reverse signalling in central nervous system repair and regeneration. We have shown that:

- FasL reverse signalling induces release of NGF by primary mouse, rat, and human fetal astrocytes
- FasL engagement upregulates NGF mRNA in mouse and human astrocytes
- Human fetal astrocytes do not express FcγR in vitro
- FasL reverse signalling induces mRNA levels of Ier3, Fosl2, Adrb1, and Egr2 and decreases mRNA levels of Txnip after a 1.5 h treatment of mouse astrocytes with a FasL agonist
- FasL reverse signalling did not have an effect on gene expression after a 12 h treatment of mouse astrocytes with a FasL agonist

Contribution of authors

Chapter 3. Results

Section 3.1

Fas Ligand acts as a counter-receptor in Schwann cells and induces the secretion of bioactive nerve growth factor

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A substantial portion of the data presented here is included in the manuscript.

MR performed the majority of the work (experimental design and optimization of conditions, cell isolation and culture, data analysis and manuscript writing) presented in this section under the supervision of Dr. Julie Desbarats, and subsequently Dr. Guillermina Almazan and Dr. John H. White.

Dr. Desbarats helped with the PC12 neurite outgrowth assay (Fig. 3.1). Rosy Siegrist-Johnstone and Chris Young performed the sciatic nerve crush injuries and collected the paw prints for Figure 3.6. They also provided technical support. Dr. Weinstein provided the human Schwann cells. Dr. Anne Baron-van Evercooren kindly gave us the MSC80 cell line and Shireen Hussain provided the primary rat Schwann cells.

Section 3.2

Effect of FasL reverse signalling on NGF release and on global gene expression in astrocytes

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In preparation

MR performed the majority of the work (experimental design and optimization of conditions, cell isolation and culture, data analysis and manuscript writing) presented in this section under the supervision of Dr. Julie Desbarats, and subsequently Dr. Guillermina Almazan and Dr. John H. White.

Dr. Jack P. Antel provided the human fetal astrocytes. Jeffery D. Haines provided the rat astrocytes, Ken McDonald provided help with the flow cytometer, and Tian-Tian Wang provided training for the real-time PCR. The McGill University and Génome Québec Innovation Centre, Montréal, Canada, performed the cRNA synthesis from total RNA, labelling and hybridization to the Illumina microarray chips.

List of abbreviations

ActD	actinomycin D
ADAM	A disintegrin and metalloprotease
Adrb1	adrenergic receptor b1
AICD	activation-induced cell death
ALS	amyotrophic lateral sclerosis
AP-1	activator protein 1
APAF-1	apoptotic protease-activating factor 1
ARE	AU-rich element
ASK1	apoptosis signal-regulating kinase 1
ATF2	activating transcription factor 2
Bak	Bcl-2 antagonist/killer
Bax	Bcl-2-associated X protein
Bcl-2	B cell lymphoma 2
Bcl2a1b	B-cell leukemia/lymphoma 2 related protein A1b
BDNF	brain-derived neurotrophic factor
Bid	BH3-interacting domain death agonist
BIM	Bcl2-interacting mediator of cell death
BM	basal medium
FRS	fibroblast growth factor receptor substrate
cAMP	3'-5'-cyclic adenosine monophosphate
CD	cluster of differentiation
CD95L	CD95 ligand
cFLIP	cellular Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein
CGN	cerebellar granule neuron
CHX	cycloheximide
CKI	casein kinase I
CM	conditioned medium
CNS	central nervous system
CNTF	ciliary neurotrophic factor
CRD	cysteine-rich domain
CREB	cAMP-responsive element binding protein
Cyr61	cysteine rich protein 61
Daxx	death-associated protein 6
DD	death domain
DISC	death inducing signalling complex
DN	dominant negative
DRG	dorsal root ganglion

Egr	early growth response
ERK	extracellular-signal regulated kinase
FADD	Fas-associated death domain
fALS	familial ALS
FasL	Fas ligand
FBP	formin binding protein
FC	fold change
FcγR	Fcγ receptor
FCS	fetal calf serum
FITC	fluorescein isothiocyanate
Fosl2	Fos-like antigen 2
Gbx2	gastrulation brain homeobox 2
GDNF	glial derived neurotrophic factor
GFAP	glial fibrillary acidic protein
gld	generalized lymphoproliferative disease
Grb2	growth factor receptor-bound protein 2
h	hour
ICER	inducible cAMP early repressor
Ier3	early response 3 gene
IEX	immediate early response gene X
IFN	interferon
IGF	insulin like growth factor
IL	interleukin
IP3	inositol 1,4,5-trisphosphate
IRF	interferon regulatory factor
JNK	c-Jun N-terminal kinase
KCl	potassium chloride
LNGFR	low-affinity NGF receptor
lpr	lymphoproliferation
MAPK	mitogen-activated protein kinase
MEK	MAP/ERK kinase
mFasL	membrane-associated FasL
MMP	matrix metalloproteinase
MORT1	mediator of receptor-induced toxicity-1
MPD	membrane proximal domain
MPP+	1-methyl-4-phenylpyridinium ion
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine
mRNA	messenger RNA
mSOD	mutant superoxide dismutase
ND	not detectable
NFAT	nuclear factor in activated T cells

NFκB	nuclear factor kappa-light-chain-enhancer of activated B cell
NGF	nerve growth factor
NIK	NFκB inducing kinase
NK	natural killer cell
nNOS	neuronal nitric oxide synthase
NO	nitric oxide
NSF	N-ethylmaleimide sensitive fusion protein
NT	neurotropphin
NTR	neurotropphin receptor
PD	Parkinson's disease
PDGF	platelet derived growth factor
PE	phycoerythrin
PI3K	phosphoinositide 3-kinase
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLC	phospholipase C
PLO	poly-L-ornithine
PMA	phorbol myristic acetate
PNS	peripheral nervous system
PRD	proline rich domain
PSTPIP	proline serine threonin phosphatase-interacting protein
RAIDD	RIP associated ICH-1 / Ced-3-homologous protein with a death domain
Rb	retinoblastoma gene product
Rhoe	Rho family GTPase 3 (Rnd3)
RIP	receptor-interacting protein
RT	reverse transcription
S100a11	S100 calcium binding protein A11
SA	self-assembly domain
SAM	significance analysis of microarrays
sFas	soluble Fas
sFasL	soluble Fas ligand
SH	src-homology
SEM	standard error of the mean
siRNA	small interfering RNA
SLE	systemic lupus erythematosus
SN	substantia nigra
SNAP	soluble NSF attachment protein
t-SNARE	target SNAP receptor
v-SNARE	vesicle SNAP receptor

SNX	sorting nexin
SOD	superoxide dismutase
SOS	son-of-sevenless
SPPL	signal peptide peptidase-like
tBid	truncated Bid
TCR	T cell receptor
THD	tumour necrosis factor homology domain
TMD	transmembrane domain
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TNFSF	tumour necrosis factor superfamily
TRAF	TNF receptor associated factor
TrkA	tyrosine kinase A receptor
TUNEL	terminal deoxynucleotidyl transferase dUTP nick end labeling
Txnip	thioredoxin interacting protein
UTR	3' untranslated region
UV	ultraviolet
VDUP	vitamin D3 up-regulated protein
VTA	ventral tegmental area
wt	wild type

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Chapter 1. Literature review

1.1. General introduction

Fas Ligand (FasL; CD95L; tumor necrosis factor superfamily 6, TNFSF6), a type II transmembrane protein of the tumor necrosis factor superfamily (TNF), was first defined as a ligand for Fas and was always studied as such (Nagata and Golstein 1995). Fas was discovered as a cell surface receptor able to induce apoptosis in lymphocytes (Trauth, Klas et al. 1989; Yonehara, Ishii et al. 1989; Itoh, Yonehara et al. 1991). It was shown early on that binding of FasL to Fas lead to apoptotic cell death in the Fas-expressing cell. The most apparent phenotype of Fas- or FasL-deficient mice was enlargement of lymph nodes. The Fas / FasL pair was thus essentially studied in the immune system (Nagata and Golstein 1995; Aggarwal 2003). However, Fas and FasL expression is not restricted to the immune system and notably, they are normally expressed in the nervous system on neuronal and glial cell membranes (Choi and Benveniste 2004).

Fas and FasL in the nervous system have been involved in the establishment of immune privilege, in programmed cell death during embryogenesis and development, and in neuronal death in neurodegenerative diseases and following an injury (Choi and Benveniste 2004). More recently, new non-apoptotic roles have been elucidated for Fas in the immune and the nervous systems (Guicciardi and Gores 2009). The ability of FasL to transduce signals through its cytoplasmic domain and act as a counter-receptor was also unveiled in the immune system (Sun and Fink 2007). The paradigm of the Fas / FasL death factors is now giving way to a more diverse role for these molecules, not restricted to the immune system or to apoptosis.

This thesis aims at the identification of FasL reverse signalling pathways in the main neurotrophin-secreting cells of the peripheral nervous system (PNS) and the central nervous system (CNS), the Schwann cells and the astrocytes, respectively. The basic characteristics of the Fas / FasL pair in the immune

system as well as their main apoptotic and non apoptotic roles identified in the nervous system are first reviewed. An overview of the pro-regenerative and pro-apoptotic effects of the prototypical neurotrophin nerve growth factor (NGF) in both the PNS and CNS is then presented. Finally, the cells used in this study, Schwann cells and astrocytes, as well as the issue of regeneration in the nervous system are addressed.

1.2. Fas and FasL in the nervous system – apoptotic roles

1.2.1 Traditional view of Fas as a prototypical death receptor in the immune system

Fas (CD95; tumor necrosis factor receptor 6, TNFR6) was discovered in 1989 using antibodies which triggered apoptosis in Fas-bearing lymphocytes (Trauth, Klas et al. 1989; Yonehara, Ishii et al. 1989; Itoh, Yonehara et al. 1991). The human Fas molecule is a type I protein containing 319 amino acids with a calculated molecular weight of 36 kDa and an apparent molecular weight of 43 kDa due to glycosylation at two sites (Itoh, Yonehara et al. 1991). Fas belongs to the tumor necrosis factor receptor (TNFR) family which is comprised of 29 members (Aggarwal 2003). The extracellular domain of Fas contains three cysteine-rich domains (CRD) (Itoh, Yonehara et al. 1991) required for interaction with FasL (Orlinick, Vaishnaw et al. 1997). The intracellular portion of Fas contains an 85 amino acid region, present in the "death receptor" subclass of TNFR members, known as the death domain (DD) and required to trigger cell death (Itoh and Nagata 1993; Tartaglia, Ayres et al. 1993; Guicciardi and Gores 2009). Fas can also be produced in a soluble form (sFas) by alternative splicing (Cheng, Zhou et al. 1994).

Binding of FasL to preassembled Fas trimers (Chan, Chun et al. 2000; Siegel, Frederiksen et al. 2000) induces a conformational change of the intracellular domain which allows binding of adaptor proteins (Lambert, Landau et al. 2003). In the classical apoptotic pathway, the Fas DD interacts with Fas-associated death domain (FADD; mediator of receptor-induced toxicity-1, MORT1), a cytosolic adaptor protein (Boldin, Varfolomeev et al. 1995; Chinnaiyan, O'Rourke et al. 1995), to form the death-inducing signalling complex (DISC). Caspase 8 is recruited to the DISC and can initiate two different pathways, the caspase cascade and the mitochondrial pathway, which both lead to cell death through activation of caspases (Fig. 1.1). Caspases 8 and 9 are considered initiator caspases which activate the effector caspases. Activation of effector caspases 3, 6, and 7 lead to DNA cleavage and cell death (Salvesen and Riedl 2008; Bouillet and O'Reilly 2009). Caspase 8 may activate capsases 3, 6, and 7 directly through cleavage of their pro-domain or recruit the mitochondrial pathway by cleaving the pro-apoptotic BH3-interacting-domain death agonist Bid. Release of B cell lymphoma 2 (Bcl-2)-associated X protein (Bax) and Bcl-2 antagonist / killer (Bak) lead to mitochondrial membrane permeabilization, cytochrome *c* release, and activation of initiator caspase 9 (Fig. 1.1). The mitochondrial pathway is not uniquely activated by Fas / FasL interactions, it can be triggered independently by external stress stimuli such as starvation or exposure to radiation (Bouillet and O'Reilly 2009).

In the immune system, Fas activation is involved in T cell and B cell homeostasis (Strasser, Jost et al. 2009). Fas activation leads to activation-induced cell death (AICD) in CD4+ T cells to terminate an immune response (Brunner, Mogil et al. 1995; Dhein, Walczak et al. 1995). Fas is also involved in clonal deletion of potentially autoreactive thymocytes (Dhein, Walczak et al. 1995) and is an effector in the cytolytic activity of CD8+ T cells and natural killer (NK) cells (the cytotoxic cells of the immune system). FasL on the surface of these cytotoxic cells binds to Fas on target cells and triggers Fas-induced apoptosis (Rouvier, Luciani et al. 1993; Barry and Bleackley 2002; Lettau, Paulsen et al.

2008). Furthemore, the Fas / FasL system contributes to immune privilege in the testis, the eye and the brain (Griffith, Brunner et al. 1995; French, Hahne et al. 1996; Saas, Walker et al. 1997; Lettau, Paulsen et al. 2008). FasL is expressed constitutively in sites of immune priviledge, and protects these tissues from inflammatory damage by leading to the Fas-induced death of activated lymphocytes as they infiltrate the site (Bellgrau and Duke 1999).

The availability of spontaneously arising mutant mouse strains facilitated the study of Fas / FasL in the immune system and is now an asset in the study of their roles in other systems. The *lpr* (lymphoproliferation) mouse does not express Fas (or expresses only very low levels) due to the insertion of a transposable element in intron 2 of Fas, which causes its premature termination and aberrant splicing (Adachi, Watanabe-Fukunaga et al. 1993). Another mutation in Fas, lpr^{cg}, is a point-mutation that does not affect levels of Fas cell surface expression, but results in an unfolded death domain that is non-functional (Matsuzawa, Moriyama et al. 1990; Watanabe-Fukunaga, Brannan et al. 1992). However, in lpr^{cg} mice, non-apoptotic signalling, independent from the death domain, can still be engaged from the intracellular portion of Fas (Desbarats, Birge et al. 2003). Finally, the *gld* (generalized lymphoproliferative disease) mouse expresses FasL with a point mutation in its extracellular domain, preventing binding to Fas (Takahashi, Tanaka et al. 1994). *lpr*, lpr^{cg}, and *gld* mice present a severe dysfunction of the immune system, which is dependent on the background strain, and is characterized by lymphadenopathy and to a various degree, autoantibody production (Nagata and Golstein 1995; Matiba, Mariani et al. 1997). Interestingly, mice carrying both lpr^{cg} and *gld* heterozygous mutations develop a weaker phenotype (Nagata and Golstein 1995). Finally, a mouse strain was created with an engineered mutation in the Fas gene. This Fas-null mouse results from the knockout of exon 9 which encodes the death domain (Adachi, Suematsu et al. 1995). However, even though Fas-null cannot engage apoptosis signalling, it is not a true Fas deletion since a truncated form of Fas is still expressed at the cell surface (Landau, Luk et al. 2005).

Programmed cell death induced by the Fas / FasL system has mostly been studied in the immune system as reviewed above, but has also been implicated in the nervous system (Haase, Pettmann et al. 2008). Fas is expressed in the CNS and PNS at the surface of both neurons (cortical, hippocampal, sensory, cerebellar granule, and motor neurons) and glia (astrocytes, microglia, oligodendroctyes, and Schwann cells) (Felderhoff-Mueser, Taylor et al. 2000; Lambert, Landau et al. 2003). The roles of the Fas / FasL system in apoptotic signalling in the nervous system will be reviewed next.

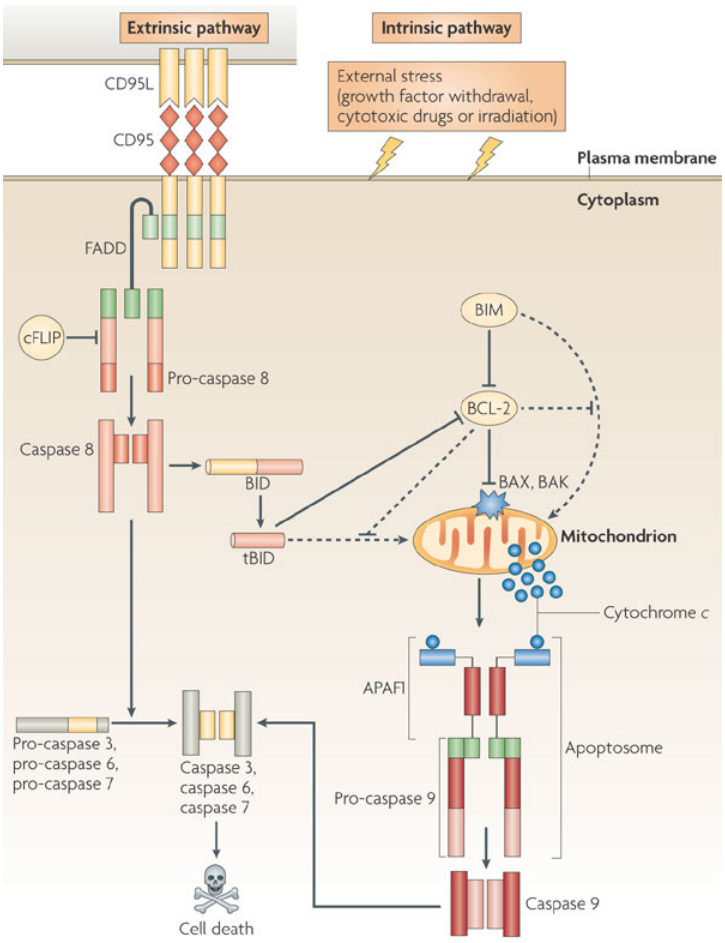

Figure 1.1 Caspase cascade and mitochondrial pathways leading to apoptosis following Fas engagement by FasL

Activation of caspase 8 by FADD can lead to cleavage and activation of caspases 3, 6, and 7 which in turn induce cell death. Activation of caspase 8 can be blocked by cellular FLICE-like inhibitory protein (cFLIP). Caspase 8 can also recruit the mitochondrial pathway by cleaving the pro-apoptotic Bid into truncated Bid (tBid). Activation of Bax and Bak lead to cytochrome *c* release and activation of caspase 9 in the apoptosome which then activates the effector caspases. APAF1, apoptotic protease-activating factor 1, is a component of the apoptosome. Following external stress stimuli, BIM (BCL-2-interacting mediator of cell death) can also initiate the mitochondrial pathway. Reproduced from (Bouillet and O'Reilly 2009)[1].

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1.2.2 Fas in cerebellar neuron cells

Primary cerebellar granule neurons (CGNs) are widely used as a model system to study neuronal death *in vitro*. The cerebellum constitutes a source of abundant and homogenous neuronal population since 90% of the neurons are granule neurons. In rodent cerebellum, neurogenesis occurs postnatally, and the cells are easy to isolate and grow *in vitro* (Contestabile 2002). Primary immature CGNs *in vitro* only survive under conditions of chronic membrane depolarization, obtained by culturing them in >20 mM potassium chloride (KCl). The cells differentiate to mature CGNs when they are maintained in culture in high KCl concentration for seven days. When the KCl concentration is decreased to 5 mM (physiological potassium concentration), both mature and immature cells die by apoptosis (Contestabile 2002).

Upon withdrawal of KCl (from 25 mM to 5 mM) from mature differentiated CGNs, cell death is evident after 16 h. The programmed cell death engaged displays the basic features of apoptosis such as morphological changes, DNA fragmentation into internucleosome-sized fragments, and the need for *de novo* RNA and protein synthesis (D'Mello, Galli et al. 1993). Fas is expressed constitutively in mature differentiated CGNs of mice *in vitro* (Le-Niculescu, Bonfoco et al. 1999; Hou, Xie et al. 2002) and in the murine developing cerebellum *in vivo* (Allais, Burel et al. 2009). Fas protein levels are rapidly increased following KCl withdrawal, with a peak at 2 h followed by a decrease to background levels at 10 h (Castiglione, Spinsanti et al. 2004). Overexpression of Fas in CGNs leads to apoptosis (Hou, Xie et al. 2002), and treatment of CGNs with agonistic anti-Fas antibodies also induces Fas upregulation and cell death (Hou, Xie et al. 2002). FasL is also induced after KCl withdrawal from CGN cultures (Le-Niculescu, Bonfoco et al. 1999; Ginham, Harrison et al. 2001; Castiglione, Spinsanti et al. 2004). Treatment of mature differentiated rat CGNs with cross-linked soluble FasL (sFasL) followed by a decrease in KCl from 25 mM to 5 mM results in increased apoptosis (Castiglione, Spinsanti et al. 2004). *In vivo*, injection of FasL in the subarachnoid space at the surface of the

cerebellum induces apoptosis of CGNs (Allais, Burel et al. 2009). On the other hand, sequestering of FasL by a Fas protein chimera (FasFc) or neutralizing anti-FasL antibodies leads to a decrease in apoptosis (Le-Niculescu, Bonfoco et al. 1999; Castiglione, Spinsanti et al. 2004). The use of mutant mice implicates further the Fas / FasL system in CGN apoptosis. In one study, CGNs from *gld* mice (FasL-deficient) were shown to be protected against apoptosis induced by KCl withdrawal (Le-Niculescu, Bonfoco et al. 1999). However, other studies have failed to show the involvement of Fas in potassium-deprivation induced apoptosis (Gerhardt, Kugler et al. 2001; Putcha, Harris et al. 2002; Beier, Wischhusen et al. 2005). CGNs from wild-type, *lpr* and *gld* mice have been found to be equally susceptible to cell death and treatment of the cells with anti-Fas antibodies or sFasL did not induce apoptosis (Gerhardt, Kugler et al. 2001; Putcha, Harris et al. 2002). In this context, caspase 9 was found to be the initiator caspase leading to activation of caspase 3 (Gerhardt, Kugler et al. 2001). It has been suggested that susceptibility to Fas-induced cell death was dependent on a balance in protein expression and activation. For example, inhibition of the phosphoinositide 3-kinase (PI3K) / Akt / protein kinase B (PKB) survival pathway could sensitize CGNs to Fas-induced cell death (Beier, Wischhusen et al. 2005).

Following removal of KCl from CGN culture medium, c-Jun N-terminal kinase (JNK) activation and c-Jun phosphorylation lead to FasL mRNA increase (Le-Niculescu, Bonfoco et al. 1999). Caspase 3 is also increased while unprocessed caspase 8 levels are stable and cleaved caspase 8 is undetectable. The timing for the activation / induction of these signalling proteins suggest that upon KCl removal, JNK / c-Jun induces FasL expression which is then available to bind Fas and induce a caspase 3-dependent apoptotic pathway (Le-Niculescu, Bonfoco et al. 1999; Ginham, Harrison et al. 2001). Caspase 3 activation was also detected following injection of FasL into the subarachnoid space of the cerebellum (Allais, Burel et al. 2009). The p38 mitogen-activated protein kinase (p38 MAPK), a member of the mitogen-activated protein kinases which is

activated by a number of stress stimuli including UV irradiation, heat shock, and pro-inflammatory cytokines, was also implicated in the apoptotic pathway downstream of Fas. Activating transcription factor 2 (ATF2), and the retinoblastoma gene product (Rb), two of p38 MAPK substrates, are phosphorylated when Fas is engaged with an anti-Fas antibody. Once phosphorylated, Rb releases the E2F1 transcription factor known to be an inducer of apoptosis (Hou, Xie et al. 2002). Following KCl withdrawal, the Bcl-2 family of proteins Bid and Bax are also involved in mature CGN apoptotic cell death. Bid translocates to the mitochondria where it binds Bax inducing a conformational change of Bax and the release of cytochrome *c* (Desagher, Osen-Sand et al. 1999).

From the studies of Fas-induced apoptosis in CGNs following KCl withdrawal, it appears that the Fas / FasL system plays a role in determining their survival but the signalling pathways engaged are not fully understood. Thus, several pathways may concomitantly be recruited to lead to apoptosis.

1.2.3 Fas in stroke

Stroke is a condition originating from multiple causes that can be separated into two different types. Ischemic stroke, constituting about 85% of the cases, happens when the supply of glucose and oxygen is decreased, generally due to occlusion of an artery or its branches. It can involve thrombosis or embolism of intracranial or extracranial arteries. Hemorrhagic stroke on the other hand is caused by blood spillage in the brain. An intracerebral hemorrhage is due to the rupture of a cerebral vessel and the formation of a hematoma, while a subarachnoid hemorrhage is due to the rupture of a vessel outside the brain and blood leakage in the cerebral spinal fluid of the subarachnoid space (Saenger and Christenson 2010).

Following an ischemic insult, neurons die by necrosis in the infarct zone, while cell death is mostly through apoptotic pathways in surrounding areas, called the penumbra (Ferrer and Planas 2003). The severity of the damage caused by an ischemic stroke depends on the duration of the hypoperfusion period and on the reperfusion event. Within minutes, there is bioenergetic failure, followed by excitotoxicity and within hours, oxidative stress, microvascular injury, post-ischemic inflammation and within days, blood-brain barrier dysfunction (Ferrer and Planas 2003; Saenger and Christenson 2010). In hemorrhagic stroke, damage is essentially due to the inflammatory response (Saenger and Christenson 2010).

There is evidence that the Fas / FasL system plays an important role in the pathophysiology of stroke. The expression of both Fas and FasL following stroke is a good indication of their involvement. It was shown by *in situ* hybridization that Fas mRNA, barely detectable in sham-operated mice, is expressed in neurons and glia in the brain starting 6 h following global cerebral ischemia. This induction of Fas is transient since the levels return to baseline after 24 h (Matsuyama, Hata et al. 1995). The Fas protein is also increased in mouse brain cortex at 3, 6 and 12 h following transient focal cerebral ischemia (Jia, Guan et al. 2009) and 24 and 48 h in rat brain following focal cerebral ischemia (Rosenbaum, Gupta et al. 2000). Fas, which is detectable in normal conditions in neurons and astrocytes by immunostaining, is increased 24 h following general ischemia in rat cortex (Liu, Kim et al. 2008). In rat hippocampal CA1 neurons, Fas mRNA and protein are also increased 24 and 72 h following global cerebral ischemia, while cell death is evident at 72 h (as shown by staining for DNA damage). However, Fas expression seems to be restricted to hippocampal CA1 neurons (Jin, Graham et al. 2001).

FasL is not detectable or hardly detectable under normal conditions in the cortex of rat brain (Martin-Villalba, Herr et al. 1999; Rosenbaum, Gupta et al. 2000). Following an ischemic insult, FasL mRNA and protein levels are increased in neurons (Martin-Villalba, Herr et al. 1999; Rosenbaum, Gupta et al. 2000) and microglia (Rosenbaum, Gupta et al. 2000). In rat hippocampal CA1

neurons, FasL mRNA and proteins are increased following global cerebral ischemia up to 72 h (Jin, Graham et al. 2001). However, one study found that FasL protein levels decrease following ischemia, a discrepancy which may be due to the differences in the ischemic injury protocol and / or in the area of the brain sampled (Liu, Kim et al. 2008). FasL protein can be detected by immunohistochemistry in the ischemic penumbra following focal cerebral ischemia in mouse but is undetectable on the contralateral side or in the brain of sham-operated mice (Martin-Villalba, Hahne et al. 2001). *Gld* mice, which lack FasL, display a 54% decrease in infarct volume compared to wild-type (Martin-Villalba, Hahne et al. 2001). Furthermore, primary cortical neurons *in vitro* are protected from cell death (55% decrease) when FasFc is added to the culture medium in a model of oxygen-glucose deprivation. The effect of sequestering FasL by FasFc on cell survival suggests that Fas is involved in cell death: when FasL is not available to bind Fas, cell death is reduced (Martin-Villalba, Hahne et al. 2001). Besides, addition of recombinant FasL induces neuronal apoptosis *in vitro* presumably by binding and engaging Fas (Martin-Villalba, Herr et al. 1999). Following focal cerebral ischemia, infarct volumes are decreased in Fas-deficient *lpr* mice compared to wild-type mice at 24 h (Martin-Villalba, Herr et al. 1999; Rosenbaum, Gupta et al. 2000). The increase in both Fas and FasL in the brain after an ischemic insult suggests that they could potentially signal apoptosis upon binding. This hypothesis is supported by the effect of FasFc and recombinant FasL on cell death.

Fas engagement by anti-Fas antibody can lead to apoptosis in cortical and hippocampal primary neurons *in vitro*. Cell death is typical of apoptosis at the morphological level and it involves caspase 8 activation. Upon injection of anti-Fas antibody into the hippocampus of rats, neurons die at the injection site by apoptosis. Following ischemic insult, hippocampal neurons die by apoptosis and show increased expression of Fas suggesting that a Fas-dependent pathway triggers programmed cell death in the neurons (Felderhoff-Mueser, Taylor et al. 2000). Neuronal apoptosis (and some glial apoptosis) is evident in mouse brain

following focal ischemia as shown by DNA fragmentation and formation of apoptotic bodies (Li, Chopp et al. 1995). Morphological features as well as DNA fragmentation (revealed by terminal deoxynucleotidyl transferase dUTP nick end labelling, TUNEL, analysis) typical of apoptosis can be seen in cells of cortical and subcortial areas surrounding the infarct zone 24 h following focal cerebral ischemia in rat and mouse (Rosenbaum, Gupta et al. 2000). Interestingly, Fas, FasL, and TUNEL staining in ischemic rat brain are colocalized in the same cells, a majority of which are neurons (Rosenbaum, Gupta et al. 2000). Some molecules involved in Fas signaling are induced following stroke. Cerebral ischemia is followed by an increase in FADD (mRNA and protein), and an increase in caspase 8 and caspase 3 activity in mice cerebral cortex (Jia, Guan et al. 2009). In hippocampal CA1 neurons of rats, FADD is also induced with a peak at 24 h following global cerebral ischemia and colocalizes with Fas. Fas and FADD can co-immunoprecipitate in ischemic hippocampal extracts (Jin, Graham et al. 2001). However, in hippocampal CA1 neurons, cleaved caspase 8 is undetectable by Western blotting but capase 10, an initiator caspase with similar action as caspase 8, is detectable after 4 h and up to 72 h. There is also increased co-immunoprecipitation of caspase 10 with FADD from 4 h up to 72 h after ischemic insult, colocalization of caspase 10 and FADD after 72 h, and caspase 10 and caspase 3 after 24 h (Jin, Graham et al. 2001). This suggests that a pathway downstream of Fas is engaged by FasL binding following an ischemic injury which recruits FADD, caspase 10, and caspase 3 and leads to apoptosis following cerebral ischemia (Jin, Graham et al. 2001). Some treatments that are known to be neuroprotective after a stroke, such as estrogens or mild hypothermia, lead to a decrease in Fas and FADD expression, caspase 8 and 3 activity, apoptosis and in the size of the infarct (Liu, Kim et al. 2008; Jia, Guan et al. 2009).

Animal models of stroke showed the involvement of Fas / FasL in apoptotic cell death. This potential role of the Fas / FasL system was confirmed in human samples. In post-mortem tissue of human patients presenting fatal

ischemic stroke, apoptotic cells can be detected by morphological examination, TUNEL staining, and DNA ladder pattern in the peri-infarct area. Fas and FasL expression can also be detected in neurons by immunohistochemistry (Sairanen, Karjalainen-Lindsberg et al. 2006). In human patients, sFas decreases within 24 h following an intracerebral hemorrhage (Tarkowski, Rosengren et al. 1999; Delgado, Cuadrado et al. 2008) while FasL expression is increased in perihematomal areas (Delgado, Cuadrado et al. 2008). If sFas sequesters FasL thereby preventing Fas signalling, a decrease in sFas levels in parallel with an increase in FasL suggests that in stroke patients, Fas signalling is increased and possibly implicated in cell death by apoptosis (Delgado, Cuadrado et al. 2008). In fact, sFas levels and volume of infarct 3 months after acute stroke as well as sFas levels and neurological deficits 3 weeks after stroke are negatively correlated (Tarkowski, Rosengren et al. 1999). Histological sections of cerebral tissue from patients with intracerebral hemorrhage showed that apoptosis is involved following stroke based on morphological and TUNEL staining analysis (Qureshi, Suri et al. 2003).

1.2.4 Fas in motoneuron cell death

Amyotrophic lateral sclerosis (ALS), also called Lou Gehrig's disease, is a fatal neurodegenerative disease characterized by a progressive loss of both lower and upper motoneurons leading to paralysis and death by respiratory failure two to five years after onset (Shaw 2005). The symptoms include muscle weakness and muscle wasting due to loss of lower motoneurons in the spinal cord and the brain stem. Loss of upper motoneurons in the motor cortex leads to hyperreflexia and spasticity (tight and stiff muscles). The majority of cases are sporadic and only 5 to 10% are familial (fALS). In fALS, most mutations are inherited in a dominant autosomal pattern (Shaw 2005). Ten to 20% of the familial forms (1-2% of all cases) are due to mutations in the gene encoding cytosolic copper-zinc superoxide dismutase (CuZnSOD, SOD1) (Shaw 2005). Strong evidence points

to a gain of toxicity of mutated SOD1 being responsible for the disease, rather than a loss of function (Gurney, Pu et al. 1994; Shaw 2005). However, the etiology of most cases of ALS is still unknown and it is thought to be multifactorial. Evidence suggests that, apart from genetic causes, it can be induced by oxidative stress, excitotoxicity, inflammatory reaction, mitochondrial dysfunction or misfolded protein aggregation (Shaw 2005). In ALS, motoneurons die by a programmed cell death which presents the hallmarks of apoptosis. Motoneurons in spinal cord and motor cortex post-mortem samples from ALS patients present the morphological features (cell shrinkage, cytoplasmic and nuclear condensation), nuclear DNA fragmentation (as shown by TUNEL analysis) and internucleosomal pattern of DNA fragmentation (as demonstrated by agarose gel electrophoresis), increased caspase 3 activation, and modification in subcellular localization and expression levels of proapoptotic Bax and Bad proteins and anti-apoptotic Bcl-2 protein characteristic of apoptosis (Mu, He et al. 1996; Martin 1999).

Transgenic mice overexpressing human mutant SOD1 (mSOD1) develop a disease mimicking human ALS and are widely used as mouse models for the disease (Gurney, Pu et al. 1994; Guegan and Przedborski 2003; Kato 2008). In the first mSOD1 transgenic mouse created, the G93A-SOD1 mouse strain which bears a glycine to alanine substitution at position 93 in the SOD1 protein, the disease course is characterized by a presymptomatic phase, followed by an early symptomatic phase starting at 90 days of age, and an end stage around 140 days (Guegan and Przedborski 2003). Animal models of ALS develop diseases that are very similar but not identical to the human pathology. They are also based on gene mutations that only account for a very small proportion of human ALS since more than 90% of cases are sporadic. However, it is believed that understanding the causes and course of the disease in these models will give clues as to what is happening in sporadic ALS (Guegan and Przedborski 2003).

During CNS development, supernumerary neurons are eliminated by programmed cell death. One of the main causes of neuronal death is thought to be

trophic deprivation since neurons compete for a limited amount of neurotrophic factors released by their targets (Pettmann and Henderson 1998). In embryonic motoneurons, Fas and FasL are expressed at the time of programmed cell death (E12.5 in mice and E14 in rats). They can trigger cell death in trophic deprivation conditions since sequestering of FasL by addition of FasFc reduces cell death by 75% in a dose-dependent manner *in vitro* (Raoul, Henderson et al. 1999). Cell death occurs through a caspase 8 dependent pathway since cell death is reduced by 75% with pre-treatment with the caspase 8 inhibitor IETD-fmk (Raoul, Henderson et al. 1999). In the presence of trophic factors, activation of Fas with agonist anti-Fas antibody or cross-linked sFasL induces embryonic motoneuron death through a caspase 3 / caspase 8 dependent pathway. Interestingly, motoneurons in culture for 3 days in the presence of trophic factors become resistant to Fas-induced apoptosis. This is paralleled by a decrease in FasL expression and an increase in the cellular Fas-associated death domain-like interleukin-1β-converting enzyme inhibitory protein (cFLIP), an endogenous inhibitor of caspase 8 activation which inhibits binding of procaspase 8 to FADD (Raoul, Henderson et al. 1999). These elements provide indications that the Fas / FasL system plays a role in motoneuron biology and death during development.

Interestingly, activation of Fas in rodent embryonic motoneurons also engages another cell death pathway which is caspase 8 independent and seems to be unique to motoneurons (Fig. 1.2). This specific pathway is not found in other cells known to be susceptible to Fas-mediated cell death such as fibroblasts, thymocytes and cortical neurons (Raoul, Estevez et al. 2002). This pathway, possibly triggered by neighbouring cells like glia, induces the transcriptional upregulation of neuronal nitric oxide synthase (nNOS) downstream of p38 MAPK (Raoul, Estevez et al. 2002). Upon Fas engagement, p38 MAPK is phosphorylated and in turn enhances nNOS upregulation and nitric oxide (NO) formation (Raoul, Estevez et al. 2002). Transfection of embryonic motoneurons with Fas small interfering RNA (Fas siRNA) leads to a reduction in expression of nNOS at the RNA and protein level (Locatelli, Corti et al. 2007). nNOS

upregulation and Fas-induced cell death are inhibited by the p38 MAPK inhibitor SB203580 but addition of exogenous NO restores Fas-mediated death. Specific inhibitors of nNOS also block Fas-triggered death. Production of NO is therefore necessary for Fas-mediated cell death in motoneurons (Raoul, Estevez et al. 2002). Using a dominant-negative (DN) approach, Raoul *et al.* showed that death-associated protein 6 (Daxx), a death domain associated protein involved in apoptosis, and apoptosis signal-regulating kinase 1 (ASK1), a MAPK kinase kinase, are recruited downstream of Fas (Raoul, Estevez et al. 2002). Embryonic motoneurons from Daxx-DN transgenic mice are resistant to Fas-induced cell death but not to UV irradiation-induced death (Raoul, Barthelemy et al. 2005). The Fas / Daxx / ASK1 / p38 MAPK / nNOS pathway therefore represents a novel motoneuron-restricted death pathway engaged specifically by Fas activation. As previously discussed, the traditional Fas / FADD / caspase 8 is also present in motoneurons (Raoul, Henderson et al. 1999) and the two pathways act synergistically. Cross-talk between the two signalling pathways exists at the level of p38 MAPK which also regulates the caspase 8 dependent pathway (Raoul, Estevez et al. 2002).

This novel NO-dependent Fas-mediated death pathway was analyzed in cells from different mSOD1 and wild-type (wt) SOD1 transgenic mouse strains. Motoneurons from mSOD1 transgenic mice are more susceptible to cell death triggered by Fas and NO than control motoneurons (expressing no transgene) and motoneurons overexpressing wt human SOD1 (Raoul, Estevez et al. 2002). In mSOD1 motoneurons, the FasL increase following NO treatment leads to a Fas-dependent increase in p38 MAPK phosphorylation and further increase in NO synthesis which is not seen in control motoneurons (Raoul, Buhler et al. 2006). Other cells from mSOD1 mice (thymocytes, cortical neurons, cerebellar granule neurons, sensory neurons, and astrocytes) were analyzed and none displayed any increased sensitivity to Fas-activation and NO (Raoul, Estevez et al. 2002). This motoneuron-specific death pathway may be responsible for the selective loss of motoneurons in the ALS pathology. This pathway also establishes a Fas / NO

feedback loop that may start early, before occurrence of symptoms, and leads to the chronic death of motoneurons (Raoul, Buhler et al. 2006).

The existence of this novel pathway was confirmed *in vivo* in the lumbar region of spinal cord of G93A-SOD1 mice (Wengenack, Holasek et al. 2004) where an increase in phosphorylated forms of ASK1 and p38 MAPK, nNOS levels, and caspase 3 activation is detected (Tortarolo, Veglianese et al. 2003; Wengenack, Holasek et al. 2004). The same pattern is also found in cortical motoneurons of G93A-SOD1 mice (Holasek, Wengenack et al. 2005). In presymptomatic G93A-SOD1 mice and G85R-SOD1 mice, expression of FasL and Daxx is increased in motoneurons (Raoul, Buhler et al. 2006). In order to investigate the relevance of Fas signalling in motoneuron death, G93A-SOD1 mice were crossed with *gld* mice (Takahashi, Tanaka et al. 1994), which have a loss of function mutation in FasL (Petri, Kiaei et al. 2006). G93A-SOD1 mice homozygous for the $FasL^{gld}$ allele displayed a modest but significant increase in survival, a reduction in nNOS expression, and a decrease in spinal motoneuron loss (Petri, Kiaei et al. 2006). Intrathecal administration of Fas siRNA to mSOD1 mice at 90 days of age is effective at decreasing Fas expression in the spinal cord by 50% after 4 weeks. Silencing of Fas leads to reduction in nNOS, phosphorylation of p38 MAPK, and blocking of caspase 8 activation compared to spinal cord of mock-transfected or wild type mice. Spinal cord sections show an increased survival of motoneurons in Fas siRNA-treated mice. First motor deficits evaluated by the rotarod test, were delayed to 133 days in mice treated with Fas siRNA compared to 112 days in control and the life span increased from 140 days to 160 days (Locatelli, Corti et al. 2007). These results confirm a role for the Fas / FasL pathway in motoneuron death in the ALS murine model mSOD1.

The importance of Fas in ALS is also suggested by the detection of abnormal levels of anti-Fas antibodies in sera of more than 20% of ALS patients. About 25% of sera from sporadic ALS patients contain abnormal levels of anti-Fas antibodies and most of these autoantibodies are able to induce rat motoneuron

death by apoptosis (Yi, Lautrette et al. 2000). Sengun and Appel also found anti-Fas antibodies in 25% of serum from sporadic ALS patients and 22% from familial ALS patients. However, there is no correlation between the presence of Fas antibodies and the course of the disease; this is not specific to ALS since 50% of sera from Parkinson's disease patients also contain abnormal levels of anti-Fas antibodies (Sengun and Appel 2003). No anti-Fas antibodies are found in serum from Alzheimer's disease patients (Yi, Lautrette et al. 2000; Sengun and Appel 2003). Sections of lumbar spinal cord from ALS patients carrying a SOD1 mutation (I113T) as well as sporadic ALS patients display surface expression of FasL which is not seen in control tissues (Kiaei, Petri et al. 2006).

The Fas / FasL receptor system plays an important role in the pathophysiology of ALS in human and in murine models of the disease. It can trigger death of motoneurons via two distinct but interconnected pathways, the classical mitochondrial apoptotic pathway and the motoneuron-restricted cell death pathway through Fas / NO. These two pathways may function synergistically since caspase 8, which is activated by p38 MAPK and Fas, can in turn trigger the mitochondrial pathway.

The role of Fas / FasL in neuronal apoptosis has been extensively studied in stroke and in motoneuron disease. In addition, Fas has also been implicated in the death of neurons in other neurodegenerative diseases such as Parkinson's disease, Alzheimer's disease, multiple sclerosis, and prion diseases (reviewed in (Choi and Benveniste 2004)). More recently, the Fas / FasL system has also been shown to mediate non-apoptotic functions, including neural regeneration and neuroprotection.

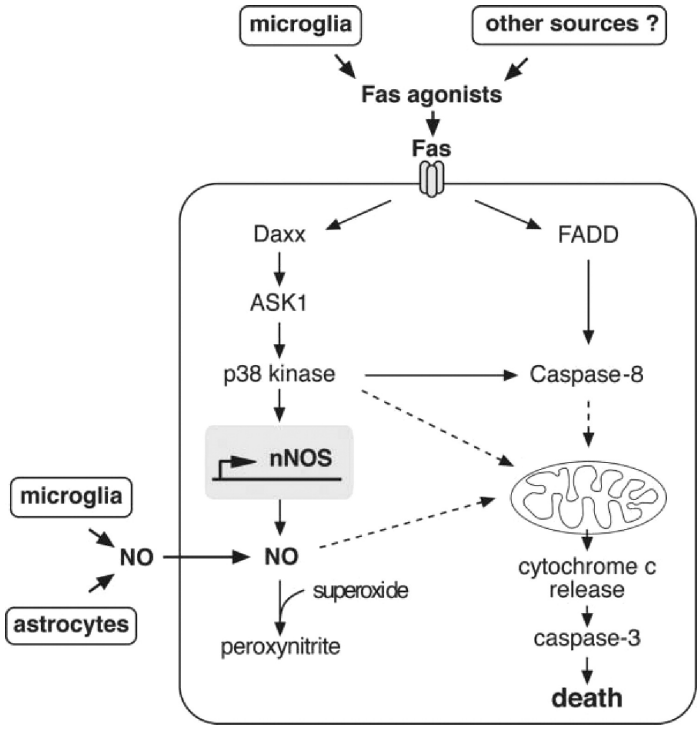

Figure 1.2 NO-dependent Fas-mediated death pathway unique to motoneurons

Fas engagement results in transcriptional upregulation of nNOS, through activation of Daxx / ASK / p38 MAPK, and motoneuron death. The traditional FADD / caspase 8 death pathway can also be engaged thanks to cross-talk at the level of p 38 MAPK. Reproduced from (Raoul, Estevez et al. 2002)[2].

[2] Reprinted from Neuron; Raoul, C., Estevez, A.G., Nishimune, H., Cleveland, D.W., deLapeyriere, O., Henderson, C.E., Haase, G., and B. Pettmann; Motoneuron death triggered by a specific pathway downstream of Fas: potentiation by ALS-linked SOD1 mutations, 35(6): 1067-83; Copyright 2002, with permission from Elsevier.

1.3. Fas and FasL in the nervous system – non-apoptotic roles

The Fas DD and a newly identified membrane proximal domain (MPD) can interact with different adapter proteins which determine the physiological outcome of Fas engagement (Lambert, Landau et al. 2003; Ruan, Lee et al. 2008). Upon binding of FasL to Fas, the intracellular domain conformational change induced allows binding of several adaptor proteins (Fig. 1.3) (Lambert, Landau et al. 2003). Along with FADD, Daxx and receptor-interacting protein (RIP) can be recruited to the Fas DD and become incorporated to the DISC (Lambert, Landau et al. 2003). While FADD recruits caspase 8 and the caspase cascade leading to apoptotic cell death, Daxx and RIP induce apoptosis through the JNK pathway (Fig. 1.3) (Lambert, Landau et al. 2003). However, these three adaptor proteins can also recruit non-apoptotic signalling pathways downstream of Fas which involve nuclear factor kappa-light-chain-enhancer of activated B cell (NF-κB) and MAPK kinase (MEK) / MAPK (extracellular-signal regulated kinase, ERK) (Lambert, Landau et al. 2003; Guicciardi and Gores 2009). For example, human fetal astrocytes, which express high levels of Fas, are resistant to Fas-induced apoptosis. The absence of caspase 8 expression and recruitement of cFLIP to the DISC can be accounted for this protection (Wosik, Becher et al. 2001). Through these non-apoptotic pathways, Fas can promote cell proliferation in T cell receptor (TCR)-activated T cells, thymocytes, and hepatocytes, liver and neuronal regeneration, cell survival or differentiation, and cytokine production (Interleukin-1 (IL-1), IL-6, IL-8). (Desbarats and Newell 2000; Desbarats, Birge et al. 2003; Guicciardi and Gores 2009).

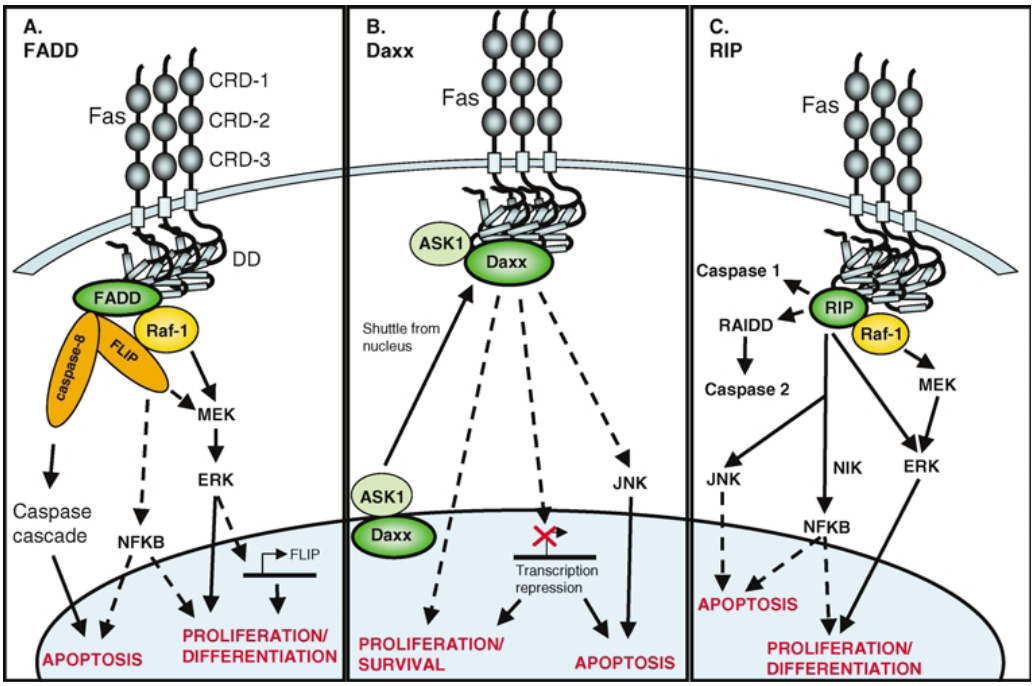

Figure 1.3 Binding of adapter protein FADD, Daxx, or RIP to Fas initiate different signalling pathways which determine the physiological outcome of Fas engagement

Recruitment of the caspase cascade by FADD (A), and the JNK pathway by Daxx (B) and RIP (C) following Fas engagement induce apoptosis. Proliferation, differentiation, and survival can also be induced by these adapter proteins through NF-κB and MEK / ERK signalling pathways. The pathways are shown separately but these events may happen simultaneously in the cells. RAIDD, RIP associated ICH-1 / Ced-3-homologous protein with a death domain; NIK, NFκB inducing kinase. Reproduced from (Lambert, Landau et al. 2003)[3].

[3] With kind permission from Springer Science+Business Media: Apoptosis, Fas–beyond death: a regenerative role for Fas in the nervous system, 2003, 8(6): 551-62, Lambert, C., Landau, A. M., Desbarats, J., Figure 2.

1.3.1 Neurodegeneration in Fas-deficient mice

A non-apoptotic role for Fas was suggested by the study of cognitive and neurological deficits of MRL-*Fas*$^{lpr/lpr}$ Fas-deficient mice. MRL mice are particularly prone to autoimmune disease and the MRL-*Fas*$^{lpr/lpr}$ strain is used as a model of systemic lupus erythematosus (SLE) disease (Brey, Sakic et al. 1997). MRL-*Fas*$^{lpr/lpr}$ mice develop an autoimmune disease which resembles the human SLE disease (Brey, Sakic et al. 1997). It was shown by Hess *et al*. that MRL-*Fas*$^{lpr/lpr}$ mice started to display spatial learning and memory (water maze test) deficits at 8 weeks of age and showed neurological deficits at 16 weeks of age (Hess, Taormina et al. 1993). The behavioural deficit exhibited by MRL-*Fas*$^{lpr/lpr}$ mice is called "autoimmunity-associated behavioral syndrome" (Brey, Sakic et al. 1997). The nigrostriatal dopamine system is affected in MRL-*Fas*$^{lpr/lpr}$ mice as evidenced by rotational behaviour, reduced locomotion when put in a new environment, reduced sucrose solution intake, and increased immobility time in forced swim tests in 17-week-old mice (Ballok, Earls et al. 2004). Dopaminergic cell loss is also evident in the midbrain, more specifically in the substantia nigra (SN) pars compacta and the ventral tegmental area (VTA) (Ballok, Earls et al. 2004).

Dorsal root ganglion (DRG) neurons from MRL-*Fas*$^{lpr/lpr}$ mice in culture present less neurite regeneration (measured as the percentage of neurons with extended neurites) than MRL-Fas$^{+/+}$ DRGs from mice older than 16 weeks but not younger than 6 weeks (Hikawa, Kiuchi et al. 1997). While brain weight at 5 weeks is equal between both strains, brains of MRL-*Fas*$^{lpr/lpr}$ mice older than 5 weeks were lighter than those of MRL-Fas$^{+/+}$ (Sakic, Szechtman et al. 1998; Ballok, Earls et al. 2004; Sled, Spring et al. 2009). Dendritic trees of pyramidal neurons in the parietal cortex and in the hippocampal CA1 area had fewer branches in MRL-*Fas*$^{lpr/lpr}$ than MRL-Fas$^{+/+}$ animals at both 5 and 14 weeks, and the difference was increased with time (Sakic, Szechtman et al. 1998). Pathological changes in the brain were evident in MRL-*Fas*$^{lpr/lpr}$ mice before onset of autoimmune pathology (Sled, Spring et al. 2009). There was a higher

number of TUNEL positive cells throughout the brain of Fas deficient MRL-*Fas*$^{lpr/lpr}$ mice than in MRL-Fas$^{+/+}$ mice (Sakic, Maric et al. 2000). The majority of dying cells were neurons and they died by a Fas-independent pathway (Ballok, Millward et al. 2003). In MRL mice treated with immunosuppressive drugs, the dendritic tree of pyramidal neurons from the hippocampus displayed more complexity than in untreated mice but the improvement was more pronounced in MRL-Fas$^{+/+}$ than in MRL-*Fas*$^{lpr/lpr}$ mice (Sakic, Kolb et al. 2000) suggesting that even though there may be a contribution of the immune dysregulation in the neurological deficits, it did not entirely account for the deficit, and Fas may play a role in maintenance of neuritic organization in the brain.

An effect of Fas on neuronal branching was also found in hippocampal explants *in vitro* (Zuliani, Kleber et al. 2006). Treatment of hippocampal explants with a FasL construct or an agonist anti-Fas antibody led to an increase in axonal and dendritic branching. In contrast, treatment with a neutralizing FasL antibody decreased branching. The Fas-induced signalling pathway leading to neuronal branching is independent of the caspase cascade and does not require transcription. Interestingly, dendritic trees in CA1 pyramidal neurons are smaller by about 25% in *lpr* and *gld* mice compared to wt neurons at P5 (Zuliani, Kleber et al. 2006).

In summary, the observation that the brain pathology in MRL-*Fas*$^{lpr/lpr}$ mouse starts prior to any autoimmune symptoms and that immunosuppressive drugs do not alleviate all the neurological deficits suggest that Fas may play a trophic or neuroprotective role in the brain, which is supported by the positive effect of Fas on neuronal branching *in vitro* and *in vivo*.

1.3.2 Fas as a neuroprotective factor in Parkinson's disease

Parkinson's disease (PD) is a progressive neurodegenerative disease characterized clinically by resting tremor, bradykinesia, rigidity, and postural

instability. It is the second most common neurodegenerative disease after Alzheimer's disease, with a lifetime risk of 2% (Dauer and Przedborski 2003; Fahn 2003). Neurodegeneration affects primarily the dopaminergic neurons of the substantia nigra (SN) and the loss of dopamine from the projections of the SN neurons into the striatum results in the characteristic symptoms of PD (Dauer and Przedborski 2003). Because the vast majority of PD cases are sporadic (95% of cases), PD has historically been considered a predominantly environmental disease (Dauer and Przedborski 2003). However, the study of familial forms of PD have implicated several types of genes: autosomal dominant (e.g. α-synuclein (Polymeropoulos, Lavedan et al. 1997)), autosomal recessive (e.g. parkin (Kitada, Asakawa et al. 1998)), and genetic risk factor (e.g. synphilin-1 (Marx, Holzmann et al. 2003)) (Healy, Abou-Sleiman et al. 2004). The multiplicity of genetic causes of PD, often resulting in disease clinically indistinguishable from sporadic PD, demonstrates that this disease is an etiologically heterogeneous disorder. Experimentally, exposure to the dopaminergic toxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) provides a well established model of PD in rodents and primates, and has been shown to cause PD in humans. MPTP crosses the blood-brain barrier where it is metabolized to its active form, the 1-methyl-4-phenylpyridinium ion (MPP^+), which is selectively concentrated in dopaminergic neurons by the dopamine transporter, resulting in dopaminergic neuronal death (Dauer and Przedborski 2003).

Fas-deficient *lpr* mice are more susceptible to Parkinson's-like disease induced by MPTP than wild-type mice (Landau, Luk et al. 2005). Subtoxic doses of MPTP led to a significant loss of dopaminergic neurons in the SN and VTA areas of the midbrain of *lpr* mice while the same areas in wild-type mouse brains were unaffected. In *gld* mice, in which Fas / FasL affinity is decreased and therefore Fas signalling is decreased, the dopaminergic neuronal loss was significant but less extensive than in *lpr* mice (Landau, Luk et al. 2005). The behavioural deficits of the mice correlated with the degree of dopaminergic neuronal loss. *Lpr* mice displayed reduced activity (hypokinesia) and displayed

tremors following exposure to MPTP, while wt mice were unaffected. Fas-deficient *lpr* mice also displayed significant deficits in coordination testing on the Rotarod device. *Gld* mice on the other hand did not display any gross motor impairment, in agreement with the reduced dopaminergic neuronal loss they presented compared to *lpr* mice (Landau, Luk et al. 2005). Furthermore, the DD was not involved in the neuroprotective signalling pathway engaged downstream of Fas, since Fas-null and lpr^{cg} mice, which have normal surface expression of Fas but do not express functional intracellular DDs, were protected from MPTP neurotoxicity to the same extent as wt mice. These mutant mice were in fact slightly more resistant to MPTP treatment than wt mice suggesting that Fas-mediated neuroprotection is even more effective when apoptosis through Fas cannot occur (Hayley, Crocker et al. 2004; Landau, Luk et al. 2005). *In vitro*, primary midbrain neurons and SH-SY5Y neuroblastoma cells treated with a FasL construct were protected against the neurotoxic agent MPP+, the active metabolite of MPTP. This protection was lost in SH-SY5Y cells that do not express Fas, and was not mediated by caspase 8 activation since inhibition of caspase 8 with IETD-fmk did not decrease Fas-mediated neuroprotection (Landau, Luk et al. 2005). A previous study also showed that SH-SY5Y cells could not be rescued from MPP+ toxicity by treatment with the caspase 8 inhibitor and that Fas and FasL expression was not upregulated by MPP,+ which indicates that the Fas / caspase 8 pathway may not be responsible for the cell death mechanism triggered by MPP+ (Gomez, Reiriz et al. 2001).

Interestingly, patients suffering from PD have the same expression level of Fas on peripheral blood leukocytes as controls, but upon treatment with mitogens, their peripheral blood T cells are not able to upregulate Fas expression to the same extent as control subjects without PD (Landau, Luk et al. 2005). In contrast, a recent study showed that a subset of CD4+ T cells from the blood of PD patients had higher levels of Fas expression than control subjects and that they were both able to upregulate Fas expression upon stimulation (Calopa, Bas et al. 2010). However, it is difficult to compare the two studies since the former examined Fas

expression on peripheral blood leukocytes while the latter analyzed Fas expression in different subsets of lymphocytes. In PD post-mortem tissue, sFas was increased in the midbrain compared to controls (Mogi, Harada et al. 1996). However, Fas and FasL expression on neurons in the substantia nigra of PD patients is decreased (Ferrer, Blanco et al. 2000). It is possible that the decrease in Fas expression in dopaminergic neurons seen in PD substantia nigra, and the further decrease in Fas signalling due to the decoy factor sFasL, decrease protective Fas signalling in dopaminergic neurons and thereby increase their susceptibility to degeneration (Landau, Luk et al. 2005).

These data suggest that Fas signalling in dopaminergic neurons is protective when the cells are exposed to a toxic insult, and absence or reduction of this signalling pathway renders neurons more susceptible to cell death.

1.3.3 Fas stimulates neurite growth in sensory neurons and cell lines

The study of cell lines and primary sensory neurons in culture provided evidence for the role of Fas signalling in neurite growth. Treatment of the SH-SY5Y neuroblastoma cell line with anti-Fas antibody or an agonist FasL protein chimera (a construct termed SuperFasL) do not induce apoptosis, although they induce cell death in Jurkat T cells (Desbarats, Birge et al. 2003). Upon engagement of Fas, caspase 8 is not cleaved but ERK1/2 is phosphorylated in a caspase 8-independent manner. Following Fas activation, ERK1/2 phosphorylation, which is maintained for up to 150 minutes, induces p35 expression, suggesting that the ERK1/2-p35 pathway, a known inducer of neurite outgrowth (Harada, Morooka et al. 2001), is involved. The effect of Fas engagement on neurite outgrowth was confirmed with dorsal root ganglion (DRG) explants and DRG dissociated sensory neurons in culture and involved an ERK-dependent pathway (Desbarats, Birge et al. 2003). Interestingly, DRG explants

from *lpr*^{cg} mice, which express Fas with a mutated DD that cannot signal apoptosis, also extend neurites in response to Fas engagement (Desbarats, Birge et al. 2003). Overexpression of a Fas construct in SH-SY5Y and COS-7 cells (which do not normally extend processes) induces process extension. These processes are even more numerous and contain more branches when the cells are treated with an agonist anti-Fas antibody (Ruan, Lee et al. 2008). The initiation of process growth by Fas is independent of ERK1/2 activation, but further process extension induced by Fas engagement depends on ERK1/2 activation. The intracellular DD is not involved in initiation of process growth, but another intracellular domain was found to be required for process extension induced by Fas. This domain was called the membrane proximal domain (MPD) and was shown to be an ezrin binding region (Ruan, Lee et al. 2008). Ezrin is known to link membrane proteins to the cytoskeleton (Niggli and Rossy 2008) and activate the Rho GTPase Rac1 (Bosco, Mulloy et al. 2009) which is involved in cytoskeletal rearrangement (Ruan, Lee et al. 2008). The same effect was reproduced in primary embryonic mouse cortical neurons where Fas induced an increase in number of neurites and branching points from the neurons (Ruan, Lee et al. 2008).

Neurite outgrowth was also induced in PC12 cells treated with Fas agonists (Waetzig, Loose et al. 2008). Interestingly, NGF treatment of PC12 cells, which induces neurite extension, also induced Fas and FasL expression. PC12 cells co-treated with nerve growth factor (NGF) and FasFc, which blocks Fas / FasL interactions, grow fewer neurites than cells treated with NGF alone, suggesting that both NGF / tyrosine receptor kinase A (TrkA) and Fas / FasL activate parallel signalling pathways that lead to neurite outgrowth. However, while NGF / TrkA recruits ERK1/2 and JNK, Fas signalling was shown to recruit only JNK and not ERK1/2, in contrast to what was shown in SH-SY5Y and primary sensory neurons (Waetzig, Loose et al. 2008). Fas signalling pathways inducing neurite outgrowth may recruit different proteins depending on the cell types involved and the level of expression of different signalling proteins. Fas-

induced neurite outgrowth pathways are not well characterized and further experiments are needed in order to better identify the proteins recruited and the events triggered downstream.

1.3.4 Fas / FasL in peripheral nerve regeneration

The role of Fas / FasL signalling in peripheral nerve regeneration was studied in a model of sciatic nerve crush injury in mice. *Lpr* mice displayed a delayed recovery after sciatic nerve crush injury when compared to wt mice. In contrast, recovery after sciatic nerve crush injury was not delayed in lpr^{cg} mice, which express normal cell surface levels of Fas, compared to wt mice. Injection of anti-Fas antibodies into the sciatic nerves of mice after a crush injury also accelerated functional recovery compared to isotype-control injected nerves. Furtermore, sciatic nerve sections 8 days after injury showed a better organization of myelinated fibers and Schwann cells as well as a decrease in inflammation in the anti-Fas antibody treated compared to isotype control treated sciatic nerves. These results suggests that Fas signalling accelerates functional recovery after injury in the PNS (Desbarats, Birge et al. 2003).

1.4. FasL reverse signalling

1.4.1 Characteristics of FasL

Until recently, FasL was mostly studied as a ligand for Fas receptor, and was identified for its ability to bind Fas and trigger apoptosis in Fas-expressing cells in the immune system (Trauth, Klas et al. 1989; Yonehara, Ishii et al. 1989; Suda, Takahashi et al. 1993). FasL, a member of the TNF superfamily, is a type II homotrimeric transmembrane protein with both cell surface and secreted isoforms (Suda, Takahashi et al. 1993; Takahashi, Tanaka et al. 1994; Takahashi, Tanaka et

al. 1994; Tanaka, Suda et al. 1995). Murine FasL is a 279 amino acid protein with a molecular weight of 31 kDa (40 kD after glycosylation) (Takahashi, Tanaka et al. 1994) while rat FasL contains 278 amino acids (Suda, Takahashi et al. 1993) and human FasL 281 amino acids (Takahashi, Tanaka et al. 1994) (Fig. 1.4). There is a 90.6% identity between mouse and rat nucleotide sequences and a 91.4% identity between amino acid sequences (Takahashi, Tanaka et al. 1994). Amino acid sequence identity between human and murine FasL is of 76.9% and between human and rat, 75.8% (Takahashi, Tanaka et al. 1994). The homology between human and mouse FasL is such that there is no functional species-specificity and either one can induce apoptosis in cells expressing human or mouse Fas (Takahashi, Tanaka et al. 1994). Interestingly, the homology is higher for FasL than for Fas since there is only 49.3% identity at the amino acid level between human and mouse Fas (Takahashi, Tanaka et al. 1994).

FasL structure is depicted in Figure 1.4. The intracellular part of FasL contains several features indicative of a signalling protein, among which a proline-rich domain (PRD) (Suda, Takahashi et al. 1993; Takahashi, Tanaka et al. 1994; Takahashi, Tanaka et al. 1994). Proline-rich motifs bind proteins containing src-homology regions (SH3) suggesting that the FasL intracellular domain may be involved in signal transduction pathways. There is also a casein kinase I (CKI) motif, –SXXS–, in the intracellular portion of FasL. The serine / threonine kinase CKI has been shown to phosphorylate the intracellular domain of TNF and may also phosphorylate FasL and be one of the first players in reverse signalling (Watts, Hunt et al. 1999). Phosphorylation at the PRD along with the mono-ubiquitination of lysine residues adjacent to the PRD are involved in the sorting of FasL into secretory lysosomes which are exocytosed at the immunological synapse (cleft formed between an antigen-presenting cell and a lymphocyte) to provide a source of active FasL when needed (Blott, Bossi et al. 2001; Zuccato, Blott et al. 2007). The extracellular domain contains several potential glycosylation sites (4 in mouse, 3 in human) and two cysteine residues which suggest the existence of a disulfide bond (Suda, Takahashi et al. 1993). The homology between FasL and other TNF family members lies essentially in

the extracellular domain (Suda, Takahashi et al. 1993). The receptor-binding domain (TNF homology domain, THD) and the self-assembly domain (SA) of FasL (Fig. 1.4) are separate domains in the extracellular portion (Orlinick, Elkon et al. 1997). The glycosylation state of FasL does not affect receptor binding (Orlinick, Elkon et al. 1997).

The FasL gene is located on mouse and human chromosome 1 (Lynch, Watson et al. 1994; Takahashi, Tanaka et al. 1994). FasL is expressed constitutively at high levels in the testis and at low levels in small intestine, lung, and kidney. FasL mRNA is weakly expressed in rat splenocytes and thymocytes but expression is increased when the cells are activated with a variety of stimuli, namely phorbol myristic acetate (PMA) and ionomycin, or with concanavalin A and IL-2 (Suda, Takahashi et al. 1993). FasL expression is tightly regulated at the transcriptional level with protein / DNA interactions at the FasL promoter. Inducers of FasL mRNA synthesis include nuclear factor in activated T cells (NFAT), NFκB, activator protein 1 (AP-1), early growth response (Egr) factors, c-myc, and interferon regulatory factor (IRF) family proteins. In addition, retinoic acid, nitric oxide, vitamin D3 and the transcriptional repressor inducible 3'-5'-cyclic adenosine monophosphate (cAMP) early repressor (ICER) are known transcriptional repressors of FasL expression (Kavurma and Khachigian 2003).

The *gld* mouse expresses a loss-of-function mutation in the FasL gene, a T to C change at nucleotide 847, resulting in the replacement of phenylalanine with leucine in the extracellular domain (Lynch, Watson et al. 1994; Takahashi, Tanaka et al. 1994). Mutated FasL mRNA is produced but the mutated protein is not able to induce apoptosis in Fas-expressing cells (Takahashi, Tanaka et al. 1994). As mentioned earlier, *gld* mice, like *lpr* mice, develop lymphadenopathy in an autosomal dominant manner (Cohen and Eisenberg 1991).

FasL expression at the cell surface is tightly regulated, possibly because it has the potential to induce death by binding to the Fas receptor. Upon synthesis in immune cells, FasL molecules are retained within the same secretory lysosomes as granzyme and perforin, the cytolytic effectors of cytotoxic T cells

and NK cells. FasL is then released by degranulation of the vesicles upon contact with a target cell through a TCR-dependent mechanism. This mechanism is also present in CD4+ T cells (Bossi and Griffiths 1999).

The extracellular part of FasL can be cleaved by matrix metalloproteinases (MMPs) to generate sFasL (Suda, Takahashi et al. 1993; Kayagaki, Kawasaki et al. 1995; Mariani, Matiba et al. 1995; Tanaka, Suda et al. 1995). Human FasL is cleaved between Ser126 and Leu127 and mouse FasL between Lys129 and Gln130 (Fig. 1.4) (Schneider, Holler et al. 1998). However, the pro-apoptotic activity of the trimeric sFasL is decreased 1000 fold compared with membrane-bound FasL (mFasL) (Schneider, Holler et al. 1998; Tanaka, Itai et al. 1998), but cross-linking of sFasL with antibodies re-establishes its activity (Schneider, Holler et al. 1998). It was shown in transgenic mice that mFasL was the only form responsible for cytotoxic activity through Fas-induced apoptosis and was necessary to maintain normal function of the immune system (O' Reilly, Tai et al. 2009). MMPs therefore regulate the availability of functionally active FasL at the surface of the cells (Lettau, Paulsen et al. 2008). Another type of protease has been shown to cleave FasL in human T cells, a member of the A-disintegrin and metalloprotease family, ADAM10 (Kirkin, Cahuzac et al. 2007; Schulte, Reiss et al. 2007). Inhibition of FasL shedding by an ADAM10 inhibitor increases the availability of membrane-bound FasL and leads to an increase in activation-induced cell death in T cells (Schulte, Reiss et al. 2007).

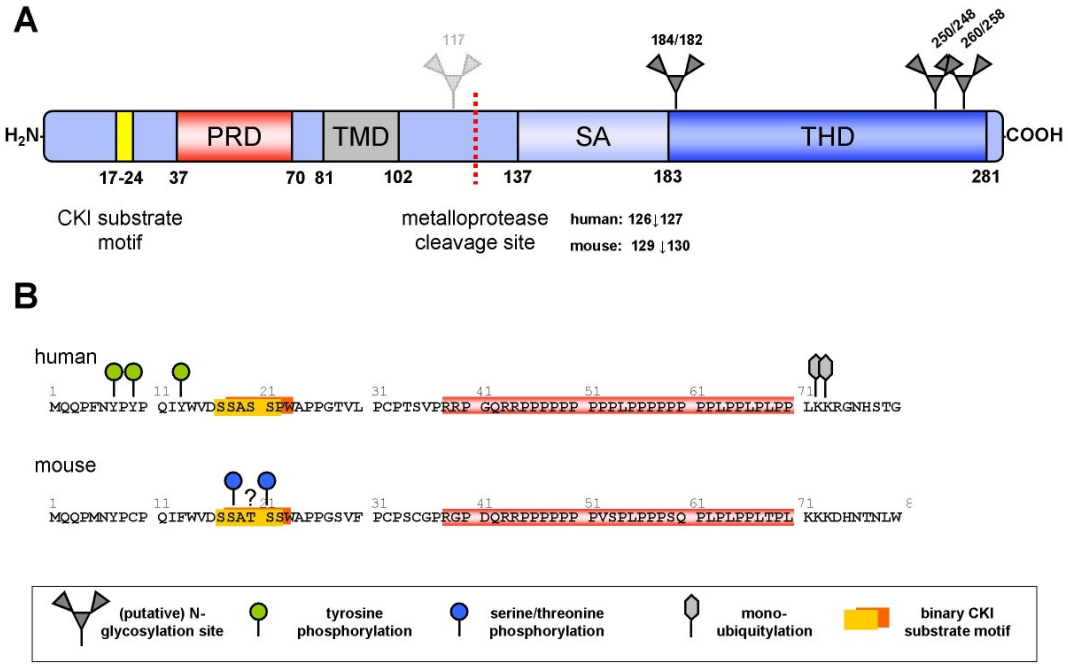

Figure 1.4 Structure and basic characteristics of the FasL protein

Schematic representation of FasL (A) showing the different domains of FasL. CKI substrate motif is displayed in yellow. TMD, transmembrane domain. Sites of posttranslational modifications of the human FasL and murine FasL cytosolic tails (B). Reproduced from (Voss, Lettau et al. 2008)[4].

[4] BioMed Central Open Access

1.4.2 FasL reverse signalling in the immune system: physiological outcomes

In recent years, Fas has been implicated in non-apoptotic functions in the immune system, including stimulation of T cell responses and enhancement of pro-inflammatory cytokine secretion (Peter, Budd et al. 2007). Although FasL is primarily studied as a ligand for the Fas receptor, FasL itself is able to transduce intracellular signals, thus acting as a counter-receptor. Ligands acting as counter-receptors is a feature common to many members of the TNF superfamily, which have been considered "bi-directional" receptors (reviewed in (Eissner, Kolch et al. 2004). The intracellular domain of FasL is highly conserved across species and presents several features of a signalling molecule (Fig. 1.4) (Wenzel, Sanzenbacher et al. 2001; Ghadimi, Sanzenbacher et al. 2002; Linkermann, Qian et al. 2003; Sun and Fink 2007). In T cells, FasL can transduce "reverse" or "counter" signals that regulate cell cycle progression and cytokine secretion (Desbarats, Duke et al. 1998; Suzuki and Fink 1998), (reviewed in (Newell and Desbarats 1999)), as well it can regulate its own expression and trafficking (Sun and Fink 2007).

In the immune system, FasL acts as a costimulatory signal, that is, a signal that amplifies T cell activation resulting from TCR signalling. The first demonstration that FasL was itself able to transduce a signal inside the FasL-expressing cell was made in 1998 by Suzuki and Fink (Suzuki and Fink 1998). They found that CD8+ T cells from *gld* mice did not proliferate as much as wt T cells upon antigenic stimulation, and this effect could be mimicked by treating wt CD8+ T cells with FasFc which blocks Fas / FasL interactions (Suzuki and Fink 1998). In the presence of the activating stimulus anti-CD3, CD4+ T cell proliferation was inhibited by co-treatment with the FasL agonist FasFc construct *in vitro*. Signalling through FasL via the FasFc construct led to an inhibition of IL-2 production, cell cycle arrest, and finally cell death induction. *In vivo*, CD4+ T cell expansion was also abolished upon treatment with FasFc in a model of superantigen driven CD4+ T cell proliferation (Desbarats, Duke et al. 1998). Co-

stimulation of CD4+ and CD8 + naïve T cells resulted in the proliferation of these cells and in the upregulation of FasL expression. Accumulation of FasL in the Fas-susceptible CD4+ T cells leads to a second phase of cell death due to Fas / FasL ligation which does not occur in CD8+ T cells, more resistant to Fas-induced cell death, perhaps because they downregulate FasL expression in this second phase following antigen-activation (Suzuki and Fink 2000). This role of FasL as a co-stimulatory receptor in CD8+ T cells was confirmed in mice where it is also required for maximal proliferation of naïve and antigen-experienced CD8+ T cells (Suzuki, Martin et al. 2000). FasL reverse signaling is also involved in T cell development and modulates the positive selection of T cells (Boursalian and Fink 2003). FasL co-stimulation in T cells requires the PRD of FasL (Sun, Ames et al. 2006).

1.4.3 Signal transduction mechanisms of FasL reverse signalling in T cells

In CD8+ T cells, co-stimulation through FasL induces phosphorylation of the FasL intracellular domain and binding to SH3-containing proteins such as growth factor receptor-bound protein 2 (Grb2), Fyn, and p85 subunit of PI3K (Fig. 1.5) (Sun, Ames et al. 2006). FasL crosslinking results in the association of FasL with the p85 subunit of PI3K, the phosphorylation of Akt and the activation of the Ras / MAPK pathway. It also leads to the nuclear translocation of NFAT and the activation of AP-1 as detected by c-Jun phosphorylation. These signalling events lead to interferon (IFN)-γ secretion and recruitment of FasL into lipid rafts (Sun, Ames et al. 2006). Activation of the ERK / MAPK pathway was also shown downstream of FasL in a mouse Sertoli cell line (Ulisse, Cinque et al. 2000). Deletions in the PRD domain of FasL block FasL reverse signalling and FasL-mediated costimulation in CD8+ T cells, but do not impair Fas-induced cell death (Sun, Lee et al. 2007). The CKI motif of FasL is phosphorylated upon co-stimulation and this regulates NFAT activation (Sun, Lee et al. 2007).

Interestingly, in human peripheral T cells, FasL co-stimulation inhibits activation of the T cells and decreases the level of ERK1/2 phosphorylation (Paulsen, Mathew et al. 2009), which is the opposite to what was shown in mice CD8+ T cells (Suzuki and Fink 1998) but similar to what was found in activated mouse CD4+ T cells *in vitro* and *in vivo* (Desbarats, Duke et al. 1998). These results confirm that FasL reverse signalling is involved in the regulation of T cell activation. However,it is not clear whether these contradictory results are due to species-specific outcomes following FasL reverse signalling or to differences in experimental setup (Paulsen, Mathew et al. 2009).

The PRD of FasL interacts with the SH3 domains of Fyn, Lyn, Src, Fgr, Lck, Nck, Grb2, PI3K p85 subunit, PACSIN1-3, and with WW domains of dystrophin, the nuclear adaptor protein FE65, and the formin binding proteins FBP11 and FBP17 (Hane, Lowin et al. 1995; Wenzel, Sanzenbacher et al. 2001; Ghadimi, Sanzenbacher et al. 2002; Lettau, Qian et al. 2006; Qian, Chen et al. 2006; Sun, Ames et al. 2006; Zuccato, Blott et al. 2007; Voss, Lettau et al. 2009). WW domains are stretches of about 20 amino acids flanked with a tryptophan (W) residue on each side (Macias, Wiesner et al. 2002). Recombinant CKI is able to phosphorylate membrane-bound TNF at the CKI consensus site, at serine / threonine residues, and it is believed that it can do so with FasL (Voss, Lettau et al. 2008). CKI phosphorylation is thought to be the first step of signalling events downstream of the TNF family members (Watts, Hunt et al. 1999).

Many of the FasL interacting partners are involved in vesicle trafficking and are thought to be important for the tight regulation of cell surface expression of FasL (Lettau, Qian et al. 2006; Thornhill, Cohn et al. 2008). For example, Nck colocalizes with FasL in secretory vesicles and at the immunological synapse in activated T cells. Nck activates actin filament formation upon T cell activation and may direct the translocation of FasL-containing lysosomes to the cell surface (Lettau, Qian et al. 2006). Phosphorylation of Fas by the Fgr protein tyrosine kinase is necessary for localization of FasL in lysosomes (Zuccato, Blott et al. 2007). The adaptor protein proline serine / threonine phosphatase-interacting protein (PSTPIP), another FasL-interacting protein, binds the PRD of FasL

through SH3 domains. PSTPIP is involved in the regulation of cell surface expression of FasL and may also contribute to the targeting of FasL to secretory lysosomes (Baum, Kirkin et al. 2005). PACSIN1, 2, and 3 are also involved in the regulation of vesicular trafficking (Modregger, Ritter et al. 2000; Wasiak, Quinn et al. 2001).

Grb2 is an adapter protein involved in growth factor receptor and TCR signalling. The FasL cytoplasmic domain, which interacts with Grb2, is sufficient to trigger ERK1/2 activation suggesting that Grb2 may recruit the Ras / MAPK pathway upon FasL engagement (Sun, Ames et al. 2006). In Sertoli cells of the testis, FasL reverse signalling activates the MAPK pathway leading to a rapid phosphorylation of ERK1/2 (Ulisse, Cinque et al. 2000). Interestingly, mFasL levels are increased and sFasL levels decreased when the MAPK pathway is inhibited by PD98059 (Ulisse, Cinque et al. 2000).

Finally, another form of FasL reverse signalling occurs with the release of the intracellular domain of FasL by proteolytic cleavage of the extracellular domain by ADAM10. This cleavage results in a 17 kDa intracellular protein. This fragment is further cleaved by an intramembrane protease of the signal peptide peptidase-like family (SPPL2a) to generate a 13 kDa fragment which translocates to the nucleus. The 13kDa protein was shown to act as a transcriptional repressor in a Gal4 reporter assay, suggesting that FasL reverse signalling may also occur through regulation of transcription by a portion of the intracellular domain of FasL itself (Kirkin, Cahuzac et al. 2007).

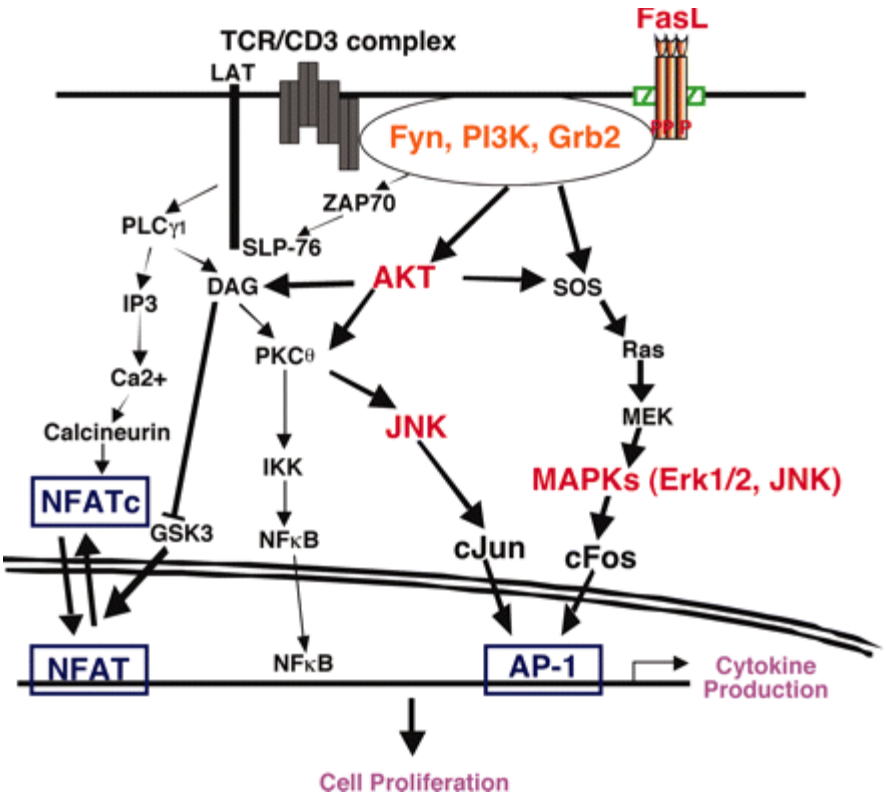

Figure 1.5 Summary of the signal transduction mechanisms of FasL reverse signalling in T cells

TCR cross-linking and FasL engagement initiate different signalling events which trigger nuclear translocation of transcription factors NFAT and AP-1. Proteins in orange were identified to interact with FasL and proteins in red can be phosphorylated upon FasL cross-linking. Reproduced from (Sun and Fink 2007)[5].

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1.4.4 FasL in Schwann cells and astrocytes

The expression of Fas and FasL is not restricted to the immune system, but are also co-expressed in the reproductive and nervous systems (Suda, Takahashi et al. 1993; French, Hahne et al. 1996; Moalem, Monsonego et al. 1999; Bonetti, Valdo et al. 2003). In the nervous system, Fas and FasL are constitutively expressed by neurons and glial cells *in vivo* and *in vitro*, and both molecules are upregulated after nervous system injury (Choi and Benveniste 2004). FasL engagement on Schwann cells leads to mobilization of a number of intracellular signalling pathways (Thornhill, Cohn et al. 2007), suggesting that FasL reverse signalling may occur in the nervous system, as well as in the immune system. Schwann cells form the myelin sheath around peripheral axons (Jessen and Mirsky 2005) and are the neurotrophin-secreting cells of the PNS (Reynolds and Woolf 1993; Bunge 1994). PNS regeneration following injury cannot occur without Schwann cells (Bunge 1994), but even in the presence of Schwann cells, recovery is never complete (Frostick, Yin et al. 1998).

In Schwann cells, FasL binds PACSIN2 and PACSIN3 as previously reported in T cells (Ghadimi, Sanzenbacher et al. 2002; Qian, Chen et al. 2006; Thornhill, Cohn et al. 2007). Two novel FasL interacting partners were identified: sorting nexin 18 (SNX18) and adaptin β (Thornhill, Cohn et al. 2007). While binding of FasL to adaptin β is not mediated by the PRD, the SH3 domain of SNX18 interacts with the PRD of FasL (Thornhill, Cohn et al. 2007). SNX18 may trigger the ERK / MAPK cascade in Schwann cells through binding to the Ras activating protein SOS (son of sevenless). SNX18 and β adaptin are known to be involved in protein trafficking and they may be involved in the control of FasL cell surface expression (Thornhill, Cohn et al. 2007). In Schwann cells, Grb2 also interacts with the PRD of FasL and it regulates FasL surface expression. Grb2 seems to be an adaptor protein between the FasL intracellular domain and β adaptin which is known to be involved in protein sorting (Traub 2005; Thornhill, Cohn et al. 2008).

In the CNS, Fas and FasL are constitutively expressed by neurons and glial cells *in vivo* and *in vitro* and they are upregulated following injury (Choi and Benveniste 2004). FasL expression can be constitutively detected in primary cultures of rat astrocytes, microglia, and oligodendrocytes (Moalem, Monsonego et al. 1999). In human and rat brain, FasL is expressed in neurons, astrocytes and microglia. Following brain lesion, FasL is upregulated at the site of injury in reactive astrocytes and in microglia (Bechmann, Mor et al. 1999). Human fetal and adult astrocytes express Fas and FasL and upregulate their expression upon treatment with IL-1, IL-6, TNFα, or IFNγ (Choi, Park et al. 1999). Interestingly, despite the upregulation of Fas and FasL in rat astrocytes following an injury, there is no evidence of astrocytic cell death by apoptosis (Bechmann, Lossau et al. 2000).

In cultured Schwann cells from neonatal rat sciatic nerve, FasL is either undetectable or detectable at very low levels, while Fas is constitutively expressed (Wohlleben, Ibrahim et al. 2000). Treatment with the proinflammatory cytokines TNFα and IFNγ upregulates FasL expression but downregulates expression of Fas (Wohlleben, Ibrahim et al. 2000). FasL is not detectable by immunocytochemistry in Schwann cells from intact sciatic nerves, but FasL staining is visible in Schwann cells from inflamed nerves in rats with experimental autoimmune neuritis. These results suggest that in the PNS, FasL is upregulated following injury (Wohlleben, Ibrahim et al. 2000). In Schwann cells cultured from normal human sciatic nerves, however, FasL is expressed constitutively and is upregulated upon treatment with TNFα or IFNγ (Bonetti, Valdo et al. 2003).

1.5. Roles of NGF in the nervous system

1.5.1 Overview of NGF and its receptors

1.5.1.1 NGF structure and expression

NGF is one of the key survival factors in nervous system development. Secreted by target cells, NGF is taken up by innervating neurons and retroactively transported. Only neurons able to establish contact with a target cell and obtain trophic signals can survive (Fig. 1.6). Others die through programmed cell death, an important element in morphogenesis during embryonic development (Pettmann and Henderson 1998; Nat, Voiculescu et al. 2001; Skaper 2008).

The first *in vitro* evidence that a diffusible factor produced by a tumor was able to induce neurite outgrowth from dorsal root and sympathetic chick ganglia was provided by Rita Levi-Montalcini and Viktor Hamburger in 1954 (Levi-Montalcini, Meyer et al. 1954). The nerve-growth promoting protein was purified by Stanley Cohen from mouse sarcoma (Cohen and Levi-Montalcini 1957), snake venom (Cohen 1959), and mouse salivary gland (Cohen 1960). Drs. Levi-Montalcini and Cohen received the Nobel Prize in Medicine in 1986 for their work on this protein, now known as NGF. NGF belongs to the neurotrophin (NT) family of growth factors, along with brain-derived neurotrophin factor (BDNF), NT-3, and NT-4 / 5 (Lu, Pang et al. 2005).

The characterization of NGF was not straightforward since the growth promoting factor originated from a 7S protein complex in the mouse salivary gland. This complex consists of the association of 3 types of subunits, alpha (α), beta (β), and gamma (γ) with the β subunit dimer being solely responsible for the biological activity. However, this complex is specific to the mouse salivary gland and is not found in the nervous system (Shooter 2001). A different isolation protocol led to the purification of a 2.5S NGF complex (Shooter 2001). This 2.5S NGF results from the non-covalent binding of two polypeptides of 118 amino acids (13 kDa) each (Varon, Normura et al. 1968; Angeletti and Bradshaw 1971;

Angeletti, Hermodson et al. 1973; Angeletti, Mercanti et al. 1973). βNGF / 2.5S NGF is commonly referred to as NGF (Shooter 2001).

NGF is synthesized as a prepro-NGF protein. After removal of the signal peptide in the endoplasmic reticulum, and N-glycosylation, proNGF has a molecular weight of 35 kDa (Darling, Petrides et al. 1983; Edwards, Selby et al. 1988). ProNGF is then processed to release the mature NGF protein. This enzymatic cleavage is performed mostly by furin but also by other protein convertases in the *trans*-golgi network (Seidah, Benjannet et al. 1996). In cells that have a regulated secretory pathway, NGF accumulates in secretory vesicles and is not released in the medium unless the cells are externally stimulated to release NGF (for example by a cAMP analog or by membrane depolarization) (Edwards, Selby et al. 1988; Seidah, Benjannet et al. 1996; Mowla, Pareek et al. 1999; Wu, Kruttgen et al. 2004). Cells with a constitutive secretory pathway do not accumulate NGF intracellularly but secrete it into the medium (Edwards, Selby et al. 1988; Seidah, Benjannet et al. 1996). Interestingly, cells with a constitutive secretory pathway generate mature NGF proteins of 13.5 and 16.5 kDa while cells with a regulated pathway secrete only a 13.5 kDa form (Seidah, Benjannet et al. 1996). More recently, it was shown that the pro-form of NGF could be secreted and could bind $p75^{NTR}$ with high affinity. Secreted proNGF can then be processed extracellularly by metalloproteinase 7 (MMP7) and plasmin (Lu, Pang et al. 2005). Since binding of NGF and proNGF to the two alternative receptors, the high affinity TrkA versus the low affinity $p75^{NTR}$ can have opposite biological consequences, namely survival or death, the regulation of proNGF cleavage appears to be a way to control the outcome of NGF function (Lu, Pang et al. 2005).

The βNGF gene is located on chromosome 1 in humans (Francke, de Martinville et al. 1983; Zabel, Eddy et al. 1985) and on chromosome 3 in mice (Zabel, Eddy et al. 1985). Four mRNAs are generated from the mouse NGF gene as a result of alternative splicing and transcription initiation from two promoters (Fig. 1.7). The largest exon, exon 4, is present in all 4 types of mRNAs produced. The difference in the 4 mRNAs transcripts lies in the arrangement of the other

small 5' exons, 1a, 1b, 2, 3a, and 3b. In mouse brain and peripheral organs, the predominant transcript is B which is 4 times more abundant than A; transcripts C and D are rare (Selby, Edwards et al. 1987). However, removal of the signal peptide in the endoplasmic reticulum generates an identical protein from transcripts A and B (Edwards, Selby et al. 1988). The murine NGF gene promoter is surrounded by an upstream suppressor and activator as wells as a downstream activator containing an AP-1 element, known to be a binding site for the transcription factor AP-1 (c-fos / c-jun heterodimer) (D'Mello and Heinrich 1991) while the human NGF gene has two activator regions, one upstream and one downstream of the promoter, both of which contain an AP-1 element (Cartwright, Martin et al. 1992). This suggests that there may be differences in the regulation of NGF transcription between mouse and human (Cartwright, Martin et al. 1992). The murine downstream AP-1 site is effectively bound by c-fos and c-jun. This AP-1 element is necessary for both the basal expression of the NGF mRNA as well as its induction (D'Melo and Heinrich 1991). A study of the murine NGF promoter in fibroblasts did not reveal any activity from the upstream regions but confirmed the role of the downstream AP-1 element in regulation of NGF expression (Cowie, Ivanco et al. 1994). The NGF mRNA contains a long 3'-untranslated region (UTR) with AU-rich elements (ARE), notably repetitive AUUUA stretches known to be involved in mRNA destabilization (Shaw and Kamen 1986; Tang, Wang et al. 1997). The half-life of the NGF mRNA in astrocytes, Schwann cells and fibroblasts is short, between 30 and 60 minutes, due to the destabilizing effect of the 3'-UTR. However, the AU-rich binding proteins involved in NGF mRNA destabilization are unknown (Tang, Wang et al. 1997; Bolognani and Perrone-Bizzozero 2008).

Schwann cells are thought to have only a constitutive secretory pathway (Mowla, Pareek et al. 1999) suggesting that any difference in NGF release from these cells upon stimulation is due to a modification in NGF synthesis at the translational and / or transcriptional level but not to a regulation of secretion. In the sciatic nerve of newborn rats, NGF is expressed by Schwann cells (Bandtlow, Heumann et al. 1987). Transcription of NGF is activated in Schwann cells by

cAMP through a protein kinase A (PKA) dependent pathway (Matsuoka, Meyer et al. 1991). NGF is upregulated in Schwann cells after injury (Krenz and Weaver 2000). Sciatic nerve lesions in mice induce an increase in NGF mRNA detectable after 2 h and with a peak at 12 h. This increase is preceded by an increase in c-fos and c-jun mRNAs starting at 30 minutes and peaking at 2 h. The increase in NGF mRNA is mediated by the downstream AP-1 binding site (Hengerer, Lindholm et al. 1990). Sciatic nerve lesions in rats are also followed by an increase in NGF protein and mRNA at the lesion site (Heumann, Korsching et al. 1987; Heumann, Lindholm et al. 1987).

In astrocytes, NGF expression is regulated by cytokines such as IL-1 (Pshenichkin, Szekely et al. 1994), steroids, vitamin D3, thyroid hormone, serum, depolarization, and β-adrenergic receptor activation (Rush, Mayo et al. 1995). β-adrenergic receptor activation results in an increase in cAMP followed by upregulation of c-fos expression and accumulation of c-fos in the nucleus where it can induce NGF gene transcription, possibly through binding to the AP-1 site of the NGF promoter (Mocchetti, De Bernardi et al. 1989). NGF is upregulated in astrocytes after an injury (Krenz and Weaver 2000). Cytokines (TNFα, IL-1β) induce an increase in NGF synthesis through a pathway involving protein kinase C (PKC) (Miklic, Juric et al. 2004; Kuno, Yoshida et al. 2006). NFκB is also involved in the regulation of NGF mRNA levels following exposure to IL-1 (Friedman, Thakur et al. 1996).

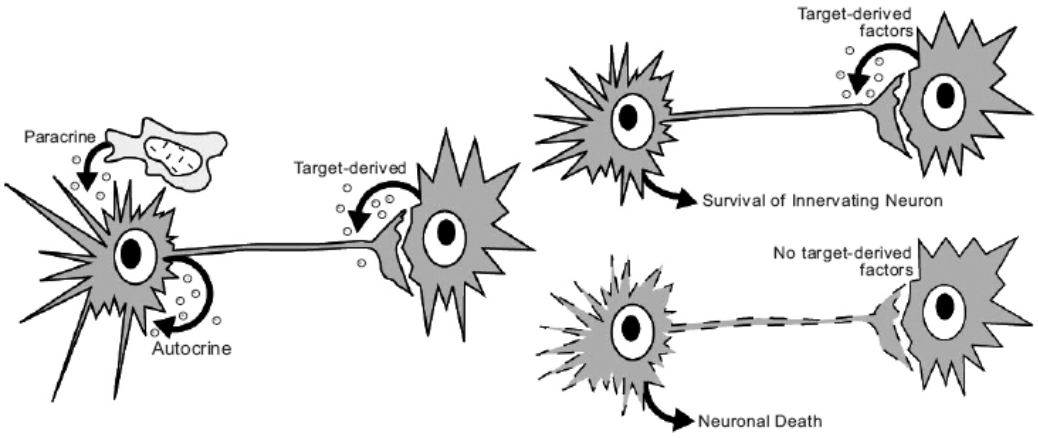

Figure 1.6 During development, neuronal survival is dependent on target-derived NGF secretion

Reproduced from (Skaper 2008)[6].

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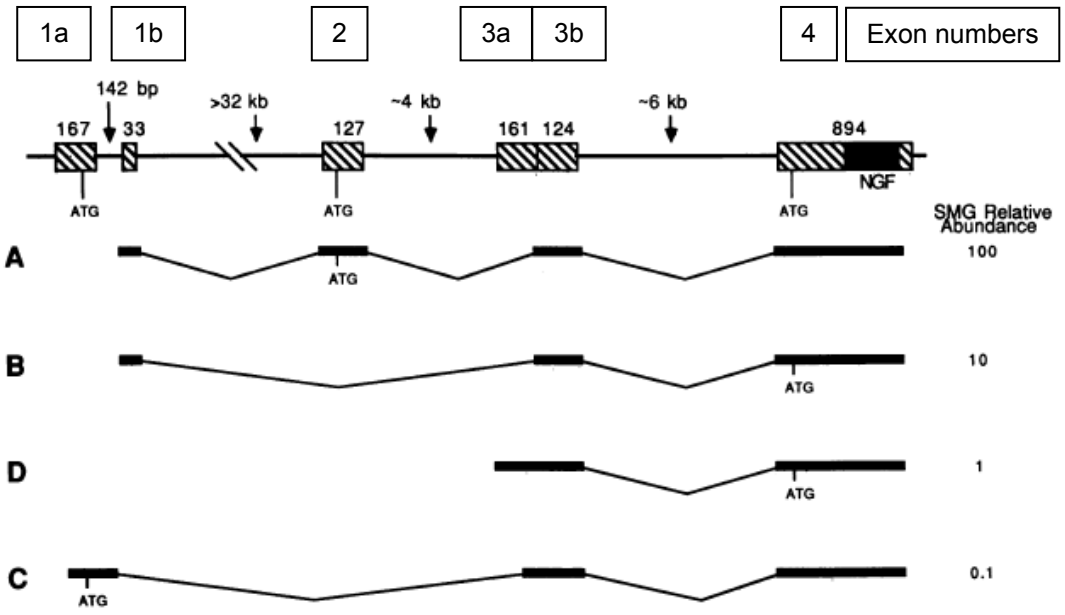

Figure 1.7 Diagram of the NGF gene and transcripts

Exons are represented as boxes and introns as lines and their size is indicated above. Exons numbers are indicated at the top. The relative abundance of each transcript (A, B, C, and D) in the submaxillary gland (SMG) is indicated on the right. Mature mRNAs are formed by splicing of the thin lines and joining of the thick lines. Adapted from (Selby, Edwards et al. 1987)[7].

[7] Amended with permission from American Society for Microbiology

1.5.1.2 NGF receptors, TrkA and p75NTR

NGF can bind two receptors, TrkA (Klein, Jing et al. 1991) and $p75^{NTR}$ (Johnson, Lanahan et al. 1986), also called low-affinity NGF receptor (LNGFR) (Fig. 1.8) (Radeke, Misko et al. 1987). $P75^{NTR}$ binds all neurotrophins with the same affinity while TrkA binds NGF specifically (Skaper 2008).

TrkA dimerizes upon binding of NGF and autophosphorylates, thereby activating downstream signalling molecules (Jing, Tapley et al. 1992) (Fig. 1.8). TrkA can recruit different signalling pathways, among them, the phospholipase C (PLC)-γ1 / inositol 1,4,5-trisphosphate (IP3) pathway which leads to Ca^{2+} release from intracellular stores, the Ras-MAPK / ERK signalling cascade, and the PI3K signalling pathway (Yano and Chao 2000; Huang and Reichardt 2003). On target neurons, the TrkA / NGF complex can be internalized from the cell membrane upon binding of NGF (Jing, Tapley et al. 1992) and transported to the nucleus by retrograde transport (Huang and Reichardt 2003). High-affinity binding of NGF requires the formation of a complex with both TrkA and $p75^{NTR}$ (Hempstead, Martin-Zanca et al. 1991; Battleman, Geller et al. 1993). Formation of this complex enhances auto-phosphorylation of TrkA and modulates the biological outcome (Verdi, Birren et al. 1994).

The $p75^{NTR}$ receptor belongs to the TNFR family, like Fas, and contains a cytoplasmic death domain (Itoh, Yonehara et al. 1991; Hofmann and Tschopp 1995; Yano and Chao 2000) (Fig. 1.8). Engagement of $p75^{NTR}$ can trigger the JNK and NFκB pathways (Yano and Chao 2000). ProNGF binds $p75^{NTR}$ with high affinity but does not bind TrkA efficiently and does not activate TrkA (Lee, Kermani et al. 2001).

NGF can signal from the cell surface, once bound to its receptor, or from endosomes after internalization of the NGF / TrkA complex. Endosomes containing NGF bound to phosphorylated TrkA are transported to cell bodies of neurons along microtubules of axons, a process called retrograde transport (Campenot and MacInnis 2004). When the endosomes reach the cell body, the

NGF / TrkA complex activates signals that modulate gene transcription in the nucleus and promote cell survival (Campenot and MacInnis 2004).

The interaction of mature NGF and proNGF with TrkA and $p75^{NTR}$ receptors can induce many different outcomes, from neuronal survival and synaptic plasticity to activation of pro-apoptotic pathways. These pro-regenerative and pro-apoptotic roles of NGF / proNGF will be reviewed next.

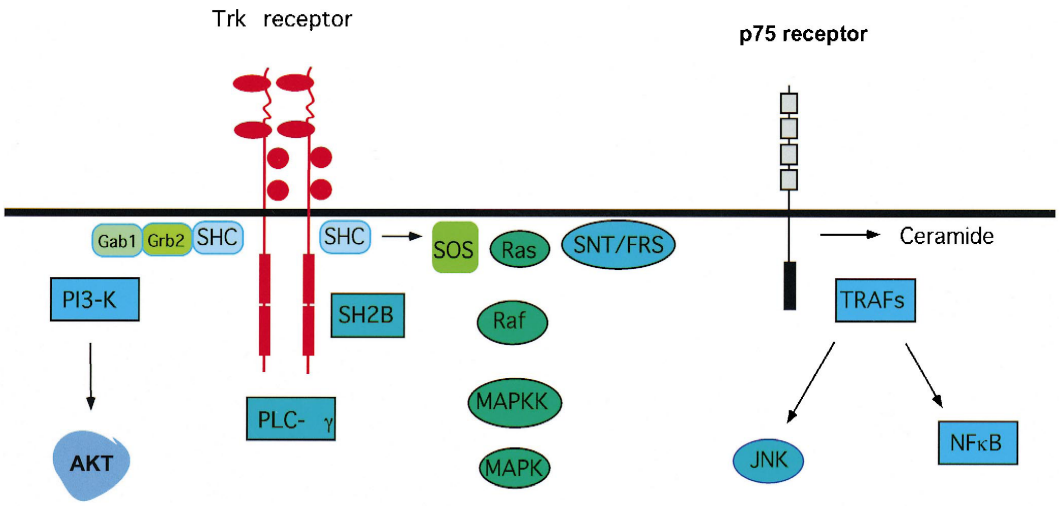

Figure 1.8 Diagram of NGF receptors TrkA and $p75^{NTR}$

The adaptor proteins and signalling pathways recruited by these two receptors are shown. FRS, fibroblast growth factor receptor substrate; SH2B, Src homology 2 B; TRAFs, TNF receptor associated factor family of proteins. Reproduced from (Yano and Chao 2000)[8].

[8] Reprinted from Pharmaceutica Acta Helvetiae, Yano, H. and Chao, M. V, Neurotrophin receptor structure and interactions, 74(2-3): 253-60, Copyright 2000, with permission from Elsevier

1.5.2 Pro-regenerative roles of NGF in the nervous system

In the first years after the discovery of NGF, it was shown that injection of an anti-serum to NGF in newborn mammals (mouse, rat, rabbit and cat) for 8 days led to the loss of 99% of sympathetic neurons (Cohen 1960; Levi-Montalcini and Booker 1960). Exposure of rat and guinea pig fetuses to anti-NGF antibodies *in utero* also resulted in the loss of 85% of DRG neurons and loss of most sympathetic neurons (Johnson, Gorin et al. 1980). The growth-promoting effect of NGF on sensory ganglia from chick embryos *in vitro* is abolished by NGF anti-serum. These results suggest that NGF is necessary for sensory and sympathetic neuron survival during development *in vivo* (Cohen 1960; Levi-Montalcini and Booker 1960). Sensory and sympathetic neurons in culture require NGF for survival and maintenance (Levi-Montalcini and Angeletti 1963). NGF deprivations results in neuronal death during development but most mature neurons are not dependent on NGF for survival. However, NGF-deprived mature neurons tend to die after several months (about 40% of cell death after 3 months for adult mouse sympathetic neurons) suggesting that NGF may still play a role in biology and function of adult neurons (Sofroniew, Howe et al. 2001).

The study of transgenic mice also confirmed the major role of NGF and NGF receptors in neuronal survival. NGF- and TrkA-deficient mice display significant sensory and sympathetic neuronal loss (Crowley, Spencer et al. 1994; Smeyne, Klein et al. 1994). In $p75^{NTR}$-deficient mice, sensory neurons, but not sympathetic neurons, are affected (Lee, Li et al. 1992). TrkA-deficient mice have a more extensive phenotype than $p75^{NTR}$-deficient mice suggesting that the main receptor for NGF during development is TrkA (Smeyne, Klein et al. 1994). Interestingly, in the CNS, NGF and NGF receptor deficiencies do not affect neuronal viability but affect neuronal projections (Crowley, Spencer et al. 1994; Smeyne, Klein et al. 1994). The effect of NGF on sympathetic neuron survival is mediated by the cAMP-responsive element binding (CREB) transcription factor, and induction of anti-apoptotic Bcl-2 protein expression (Riccio, Ahn et al. 1999).

NGF is also involved in myelination. NGF neutralization in sciatic nerve *in vivo* during development reduces the production of myelin proteins (Chan, Watkins et al. 2004). In co-cultures, NGF promotes myelination from Schwann cells but inhibits myelination from oligodendrocytes through a neuronal TrkA-dependent pathway (Chan, Watkins et al. 2004). Myelination in PNS and CNS may therefore be regulated by different signalling pathways downstream of the neurotrophin receptors (Xiao, Kilpatrick et al. 2009). Aside from neuronal TrkA, $p75^{NTR}$ on Schwann cells is also implicated in promotion of PNS myelination during development. However, the mechanism by which NGF controls myelination through neuronal TrkA and glial $p75^{NTR}$ is not completely understood (Xiao, Kilpatrick et al. 2009). A role for pro-NGF in myelination has been suggested *in vitro* but there is no clear evidence that this is relevant to *in vivo* myelination (Xiao, Kilpatrick et al. 2009).

Survival and differentiation signals from NGF are mainly due to binding of mature NGF to TrkA. On the other hand, the major biological outcome of proNGF binding to $p75^{NTR}$ is cell death (Lee, Kermani et al. 2001). In the context of TrkA-deficient cells, engagement of $p75^{NTR}$ by NGF induces apoptosis. However, in cells co-expressing both receptors, cell survival is promoted at low NGF concentration (Yoon, Casaccia-Bonnefil et al. 1998). Interestingly, proNGF is also able to trigger non apoptotic signalling through binding to TrkA. ProNGF / TrkA can engage the Ras-MAPK pathway and induce neurite outgrowth from murine superior cervical ganglion neurons. However, proNGF is four times less potent than mature NGF (Fahnestock, Yu et al. 2004). Even though proNGF is not as efficient as NGF at inducing TrkA phosphorylation and recruiting downstream effectors, the authors suggests that it may be the most relevant form of NGF in the brain (Fahnestock, Yu et al. 2004) since only proNGF, and not NGF, could be detected in brain tissue from mouse, rat, and human (Fahnestock, Michalski et al. 2001).

Apart for a role in survival, peripheral axonal branching, and dendritic arborisation, NGF is known to play a role in plasticity of NGF-reponsive neurons, and on the regulation of neuropeptide synthesis and release (Sofroniew, Howe et

al. 2001). NGF also induces hyperalgesia when injected into adult rodents by lowering the threshold of nociceptors (Levi-Montalcini, Dal Toso et al. 1995).

In vitro, Schwann cells express NGF and $p75^{NTR}$ but not TrkA (Yamamoto, Sobue et al. 1993). After an injury, expression of NGF and $p75^{NTR}$ is upregulated (Heumann, Korsching et al. 1987; Funakoshi, Frisen et al. 1993). Signalling through the $p75^{NTR}$ receptor on Schwann cells promotes myelination and functional recovery after PNS injury (Tomita, Kubo et al. 2007). Schwann cell migration on denervated sciatic nerves *in vitro* is increased by engagement of $p75^{NTR}$ following NGF treatment (Anton, Weskamp et al. 1994). In Schwann cells, binding of NGF to the $p75^{NTR}$ induces the activation of the transcription factor NFκB despite the absence of TrkA. Since NFκB is known to be involved in the secretion of extracellular matrix, this signalling pathway may explain the role of $p75^{NTR}$ stimulation on Schwann cell migration (Carter, Kaltschmidt et al. 1996).

Similarly, astrocytes in culture do not express TrkA but they express $p75^{NTR}$ (Rudge, Li et al. 1994). Astrocytes also express NGF and they upregulate NGF expression following injury or exposure to cytokines (Friedman, Thakur et al. 1996; Miklic, Juric et al. 2004). Like Schwann cells, expression of $p75^{NTR}$ is upregulated after injury in astrocytes (Hanbury, Charles et al. 2002; Cragnolini, Huang et al. 2009). $P75^{NTR}$ engagement by NGF reduces astrocyte proliferation but does not induce cell death (Cragnolini, Huang et al. 2009).

1.5.3 Pro-apoptotic roles of NGF in the nervous system

The biological outcome of NGF signalling depends on a balance between TrkA and $p75^{NTR}$ signalling pathways. While low doses of mature NGF (1-10 ng/mL) do not affect viability of hippocampal neurons expressing $p75^{NTR}$ but not TrkA, overnight exposure to high doses (>100 ng/mL) induces death of 30-40% of the cells through the JNK pathway (Friedman 2000). In oligodendrocytes expressing only $p75^{NTR}$, treatment with a high dose of NGF (100 ng/mL) also

induces cell death but expression of TrkA rescues them from apoptosis (Yoon, Casaccia-Bonnefil et al. 1998). Upon binding of mature NGF, $p75^{NTR}$ activates Rac GTPase and the JNK / c-Jun pathway, which is required for cell death induction (Yoon, Casaccia-Bonnefil et al. 1998; Harrington, Kim et al. 2002). However, upon activation of both $p75^{NTR}$ and TrkA, JNK activity is suppressed while in parallel, the MAPK / ERK pathway is activated. However, NFκB activation, which is recruited by $p75^{NTR}$ and is involved in survival, is unaffected by signalling through TrkA (Yoon, Casaccia-Bonnefil et al. 1998).

Following axotomy, Schwann cells upregulate expression of both NGF and $p75^{NTR}$ and die through a $p75^{NTR}$-dependent pathway (Syroid, Maycox et al. 2000). In Schwann cells, $p75^{NTR}$ can induce both cell death and non-apoptotic pathways depending on the context. RIP2, which is an adaptor protein containing a serine threonine kinase domain and a caspase recruitment domain, seems to be responsible for the regulation of the outcome of $p75^{NTR}$ engagement by NGF in Schwann cells. NGF enhances binding of $p75^{NTR}$ to RIP2 and activates downstream NFκB-dependent survival pathways. Cells deficient in RIP2 undergo apoptosis upon engagement of $p75^{NTR}$ by NGF (Khursigara, Bertin et al. 2001).

ProNGF does not bind TrkA efficiently, does not activate TrkA, and is less effective at inducing neurite outgrowth from PC12 cells or superior cervical ganglion neurons. On the other hand, proNGF binds $p75^{NTR}$ with much higher affinity than TrkA and is much more potent at inducing apoptosis through $p75^{NTR}$ than mature NGF (Lee, Kermani et al. 2001). For example, following spinal cord injury, proNGF induces death of oligodendrocytes through binding to $p75^{NTR}$ (Beattie, Harrington et al. 2002). Activation of $p75^{NTR}$ on photoreceptors by proNGF secreted by microglial cells also induces cell death (Srinivasan, Roque et al. 2004). In the cortex, $p75^{NTR}$ expression is induced following axotomy with a maximum corresponding to the peak in cell death. In parallel, proNGF is secreted in the cerebrospinal fluid and activates cortical neuron death by binding to $p75^{NTR}$ (Harrington, Leiner et al. 2004). It was recently found that the recruitment of a co-receptor, sortilin, to the proNGF / $p75^{NTR}$ complex was required for the activation of apoptotic pathways. However, sortilin is not involved in signalling

pathways engaged by mature NGF binding to $p75^{NTR}$ or TrkA. It is hypothesised that in the absence of sortilin, which binds the proNGF pro-domain, proNGF is accessible to metalloproteases. Mature NGF generated by cleavage can then bind TrkA and induce survival signals (Nykjaer, Lee et al. 2004). Under oxidative stress conditions, reactive astrocytes secrete proNGF which activates the $p75^{NTR}$ / sortilin receptor complex and induces motoneuron death (Domeniconi, Hempstead et al. 2007). Interestingly, when cells that co-express TrkA and $p75^{NTR}$ are treated with proNGF, they undergo cell death (Lee, Kermani et al. 2001).

1.6. Schwann cells and PNS regeneration

Schwann cells are the main glial cells of the PNS (Jessen and Mirsky 1998). There are two types of Schwann cells, the myelinating Schwann cells which wrap large axons and the non-myelinating Schwann cells that surround small diameter axons (Mirsky, Woodhoo et al. 2008). Schwann cells originate from the neural crest during embryogenesis. Migrating neural crest cells differentiate into Schwann cell precursors which become immature Schwann cells (Jessen and Mirsky 2005). Of the immature Schwann cells, some will wrap an axon and myelinate, and others will not myelinate but support neuronal survival (Fig. 1.9) (Jessen and Mirsky 2005). The transition from immature Schwann cells to mature Schwann cells (myelinating or non-myelinating) is marked by a stop in proliferation and an exit from the cell cycle. This cell cycle arrest is reversible and following injury, the Schwann cells are able to de-differentiate and re-enter the cell cycle (Jessen and Mirsky 2005). While Schwann cell precursors need to be in close proximity with axons for survival, immature and mature Schwann cells can survive independently from axons thanks to the establishment of an autocrine survival loop (Meier, Parmantier et al. 1999; Jessen and Mirsky 2005). Among the survival factors secreted by the Schwann cells are insulin-like growth factors (IGFs), neurotrophin 3 (NT-3), and platelet derived growth factor (PDGF)-BB which are also known to be important for neuronal survival and differentiation

(Meier, Parmantier et al. 1999). This feature may explain why Schwann cells can survive following an injury and sustain axonal regrowth (Jessen and Mirsky 2005). Schwann cell development is controlled by extracellular signals including neuregulin-1, endothelin, and Notch. The extracellular matrix also plays a role in Schwann cell proliferation and myelination. The myelination program is also controlled by the transcription factors Sox-10, Krox-20 (Egr-2), and Oct-6 (Mirsky, Woodhoo et al. 2008).

Schwann cells form a one-to-one relationship with axons and produce the myelin sheet around the axon (Jessen and Mirsky 2005). Myelin is required for axon survival and saltatory conduction of action potential in the nervous system of vertebrates (Xiao, Kilpatrick et al. 2009) They also secrete neurotrophic factors including NGF, brain-derived neurotrophic factor (BDNF), NT-3, ciliary neurotrophic factor (CNTF), and glial cell-derived neurotrophic factor (GDNF) (Reynolds and Woolf 1993; Bunge 1994; Watabe, Fukuda et al. 1995; Frostick, Yin et al. 1998), components of the extracellular matrix and cell adhesion molecules (Reynolds and Woolf 1993; Mirsky, Woodhoo et al. 2008). They can also phagocytose exogenous antigens and act as nonprofessional antigen-presenting cells (Stoll and Muller 1999; Meyer zu Horste, Hu et al. 2008). Schwann cells express MHC class I molecules constitutively and are able to induce expression of MHC class II molecules in the context of injury or disease (Meyer zu Horste, Hu et al. 2008). Schwann cells also have immunoregulatory properties since they can secrete proinflamatory cytokines such as IL-1, IL-6 and immunomudulators such as prostaglandin E_2 and thromboxane A_2 (Meyer zu Horste, Hu et al. 2008).

Following injury, Schwann cells de-differentiate during Wallerian degeneration and adopt a proliferating phenotype. They also upregulate the synthesis of growth factors, extracellular matrix proteins, and cell adhesion molecules (Reynolds and Woolf 1993; Mirsky, Woodhoo et al. 2008). Upon removal of myelin debris, proliferating Schwann cells form the bands of Büngner, an ensemble of cells lined up in the basal lamina tube, which will guide regenerating axons (Stoll and Muller 1999). Myelin debris are phagocytosed

mainly by invading macrophages but Schwann cells also participate in this process (Stoll and Muller 1999). Following an injury, Schwann cells at the proximal stump, and some at the distal stump, adopt a migrating phenotype. They migrate along with, or towards, the regenerating axons through the bands of Büngner. These migrating Schwann cells promote elongation of axons (Torigoe, Tanaka et al. 1996).

Regeneration in the PNS following injury is never complete and strategies to improve PNS regeneration are being studied. The use of exogenous, modified Schwann cells and supplementation with neurotrophins are avenues that have been examined (Frostick, Yin et al. 1998). The observation that NGF (Heumann, Korsching et al. 1987; Heumann, Lindholm et al. 1987) and $p75^{NTR}$ (Funakoshi, Frisen et al. 1993) are upregulated after an injury suggests a role for these molecules in regeneration. However, binding of NGF to $p75^{NTR}$ can promote survival or death depending on the adaptor protein and signalling pathways triggered (Khursigara, Bertin et al. 2001).

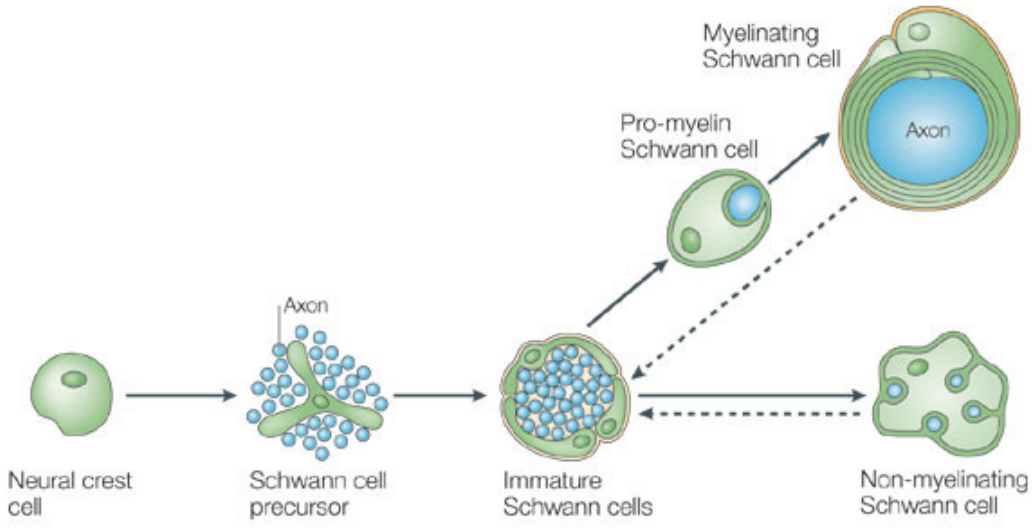

Figure 1.9 Schwann cell development from neural crest cells to mature myelinating and non-myelinating cells

Neural crest cells are migrating cells. Immature Schwann cells have all the potential to become mature myelinating Schwann cells but only cells enveloping a large diameter axon will myelinate. Dashed arrows indicate reversible steps. Reproduced from (Jessen and Mirsky 2005).[9]

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1.7. Astrocytes and CNS regeneration

Astrocytes are the most abundant cells in the CNS, and they are present in all CNS regions (Sofroniew and Vinters 2010). They do not form the myelin sheath in the CNS, a role performed by the oligodendrocytes, but like the Schwann cells in the PNS, they provide neurotrophic support to the axons (Seth and Koul 2008). Astrocytes have long been viewed as passive cells offering only structural support to neurons. However, in recent years, research has provided evidence that astrocytes perform many different functions in normal physiology as well as in disease (Sofroniew and Vinters 2010). Contrary to the Schwann cells, astrocytes are a heterogenous cell population with multiple cell lineage origins. Some progenitors originate from the ventricular and subventricular zones. Radial glia are one of the progenitors of astrocytes which also generate neurons and oligodendrocytes. $NG2^+$ precursors, known as oligodendrocyte precursors, and $Olig2^+$ cells, precursors of both neurons and oligodendrocytes, also generate cells of the astroglial lineage (Hewett 2009).

In vivo, two main types of astrocytes exist: protoplasmic astrocytes in the gray matter and fibrous astrocytes in the white matter. Processes from both types are intimately associated with neurons, the former surrounding synapses while the latter are in close proximity to nodes of Ranvier (Sofroniew and Vinters 2010). However, both types of astrocytes form a network of interconnected cells through gap junctions and participate in the blood-brain barrier (Sofroniew and Vinters 2010). Astrocytes do not only have a structural role in CNS organization, they are also important players in brain physiology. They are involved in the maintenance of homeostasis at the synapses by re-uptake of neurotransmitters released by neurons. For example, uptake of glutamate is crucial in preventing glutamate excitotoxicity. They are able to release neurotransmitters as well, called gliotransmitters (eg. glutamate and GABA). Astrocytes play a role in neuron metabolism since they are able to transfer nutrients from the blood to the neurons; they also store glycogen which can be provided to neurons in hypoglycaemia conditions (Sofroniew and Vinters 2010). Astrocytes also secrete neurotrophins

and express neurotrophin receptors (Rudge, Li et al. 1994; Seth and Koul 2008). Finally, astrocytes are also a type of excitable cell, even though they cannot generate action potentials like neurons. They can produce waves of calcium and propagate them to neighbouring cells through the gap junctions in a spontaneous way or in response to neuronal signals (neurotransmitter release). This glial excitation leads to the release of gliotransmitters that affects neurons and glia in proximity (Volterra and Meldolesi 2005). Astrocytes are therefore important players in brain function, and dysfunction of these cells has been linked to neurodegenerative diseases such as epilepsy, multiple sclerosis, and Alzheimer's disease (Seth and Koul 2008).

Following CNS injury and axonal degeneration, myelin is removed over a period of several months instead of days as in the PNS. Myelin proteins and growth inhibitors associated with myelin such as NOGO proteins act as inhibitors of axonal outgrowth. Due to the absence of blood-borne macrophages, when the blood-brain barrier is intact, myelin debris are inefficiently removed by microglia only. This explains in part why the CNS fails to regenerate (Stoll, Jander et al. 2002). Astrocytes also contribute to the establishment of this unpermissive environment for regeneration with the reactive gliosis reaction, the formation of the glial scar, and the secretion of inhibitory extracellular matrix components (Stoll, Jander et al. 2002). Reactive gliosis is characterized by astrocyte proliferation and hypertrophy, secretion of inhibitory extracellular matrix molecules such as chondroitin sulphate proteoglycans, and secretion of anti- and pro-inflammatory molecules. However, reactive astrocytes seem to have both beneficial and detrimental roles (Sofroniew and Vinters 2010).

Following CNS injury, recovery does not occur spontaneously as in the PNS and no treatment allowing functional recovery is available yet. Strategies to promote regeneration are being studied such as treatment by transplantation of peripheral nerves, Schwann cells, and stem and progenitor cells for the treatment of spinal cord injuries. Delivery of growth factors (BDNF, NGF, NT-3, and others) into the injured area is also a promising intervention strategy. However, local administration of these growth factors is necessary (systemic distribution has

side effects and does not allow a high enough dose to be effective) but difficult to accomplish. Despite promising results in animal models and early clinical trials (Thuret, Moon et al. 2006), none of these avenues have yet been approved for use in humans.

1.8. Thesis aim

FasL reverse signalling has been studied in the immune system but to our knowledge, it has never previously been studied in the nervous system. As reviewed in the introduction, FasL is expressed by glial cells and is upregulated following injury. On the other hand, neurons and glia also express Fas, the cognate ligand for FasL. Thus, we hypothesized that engagement of FasL in glial cells by Fas might trigger FasL reverse signalling. We started to explore the biological and functional consequences of FasL reverse signalling in Schwann cells, the glial cells of the PNS. We then verified whether FasL reverse signalling could also occur in astrocytes in the CNS and analysed the effect of FasL signalling on gene expression.

Chapter 2. Materials and methods

2.1. Mice and rats

C3H/HeJ (C3.wt) and C57BL/6 (wt) mice were purchased from Charles River Canada and C3.MRL-Fas^{lpr}/J (C3.lpr) and B6.MRL-Fas^{lpr} (*lpr*) mice were bred in our animal facility. Sprague-Dawley rats were purchased from Charles River Canada. All experiments followed Canadian Council on Animal Care ethical guidelines and were approved by the McGill University Animal Care Committee.

2.2. Schwann cell preparation and culture

Primary mouse Schwann cells were prepared from dorsal root ganglia of C3H newborn mice (post-natal day 0 to 4) or adult C57BL/6 (*wt*) and B6.MRL-Fas^{lpr} (*lpr*) mice. Schwann cells from newborn mice were isolated following a method modified from Weinstein and Wu (Weinstein and Wu 2001). In brief, nerves were digested in 0.25% collagenase (EC 3.4.24.34; Sigma), followed by 0.125% trypsin (EC 3.4.21.4; Worthington Biochemicals) and 4 U/mL DNase I (EC 3.1.21.1; Worthington Biochemicals). Cell suspensions were plated on 60-mm BD Primaria^{TM} cell culture dishes (BD Biosciences) in Dulbecco's Modified Eagle's Medium (DMEM; GIBCO® Invitrogen) plus 10% fetal calf serum (FCS; HyClone), 100 U/mL penicillin / 100 μg/mL streptomycin (GIBCO® Invitrogen), and 0.1 mM non-essential amino acids (GIBCO® Invitrogen). Cells were treated for 2 days with cytosine arabinosine (AraC; Sigma; 10 μM) and then grown in DMEM supplemented with 10% FCS, penicillin / streptomycin, non-essential amino acids, bovine pituitary extract (Sigma; 10 μg/mL) and forskolin (EMD Chemicals; 2 μM). At confluency, Schwann cells were purified by antibody-mediated cytolysis with anti-Thy 1.2 (AbD Serotec) and Low-Tox®-M rabbit complement (Cedarlane) to eliminate contaminating fibroblasts (Weinstein and Wu 2001). For preparation of conditioned medium (CM), cells were plated in

DMEM / 2% FCS and treated for 48 h with 5 μg/mL anti-FasL antibodies (clone MFL3), or isotype-matched control antibodies (BD Biosciences).

Schwann cells from adult C57BL/6 (*wt*) and B6.MRL-Fas^{lpr} (*lpr*) mice were isolated from sciatic nerves 7 days post crush injury following a method modified from Pannunzio *et al*. (Pannunzio, Jou et al. 2005). In short, sciatic nerves were dissected, epineurium was removed, and the nerves were digested in 0.05% collagenase (Sigma) / 0.1% hyaluronidase (EC 3.2.1.35; Sigma). The resulting cell suspension was plated on laminin-coated plates in DMEM supplemented with 10% FCS, 100 U/mL penicillin / 100 μg/mL streptomycin, and non-essential amino acids. Cells were kept in culture for 5 days after isolation, and medium was changed every 2 days. Before treating the cells for FasL engagement, the cells were serum-starved for 18 h in DMEM supplemented with F12 nutrient mixture (Sigma) and N2 supplement (GIBCO® Invitrogen). Cells were then treated with 5 μg/mL murine FasFc protein chimera (mFasFc; R&D Systems) or 5 μg/mL control antibody (BD Biosciences or Jackson Immunoresearch Laboratories, Inc).

The transformed Schwann cell line, MSC80, was provided by Dr. Anne Baron-Van Evercooren (INSERM, Paris, France) (Boutry, Hauw et al. 1992). MSC80 cells were serum-starved for 18 h and then treated for the indicated times with 5 μg/mL murine FasFc protein chimera (mFasFc; R&D Systems) or 5 μg/mL control antibody (BD Biosciences or Jackson Immunoresearch Laboratories, Inc). When indicated, the cells were pre-treated for 30 min with the following inhibitors: PD98059 (30 μM, EMD Chemicals), PP2 (10 μM; EMD Chemicals), actinomycin D (ActD; 5 μg/mL; Sigma), or cycloheximide (CHX; 10 μg/mL; Sigma).

Human Schwann cells were obtained from embryonic sciatic nerves from late term abortuses as previously described (Weinstein and Wu 2001; Wu, Jurek et al. 2001). All procedures were reviewed and approved by the Institutional Review Boards at the Albert Einstein College of Medicine and the Bronx Municipal Medical Center. Human Schwann cells were grown in DMEM supplemented with 10% FCS, penicillin / streptomycin, non-essential amino

acids, bovine pituitary extract (Sigma; 10 μg/mL) and forskolin (EMD Chemicals; 2 μM). For experiments, cells were plated for 18 h in DMEM supplemented with 5% FCS and then treated with anti-FasL antibody clone NOK1 or clone MFL3 (5 μg/mL; BD Biosciences).

Rat Schwann cells were provided by Shireen Hossain (McGill University, Montreal, Canada). They were obtained from dorsal root ganglia of embryonic (E15) Sprague-Dawley rats as previously described (Fragoso, Robertson et al. 2003). Rat Schwann cells were serum-starved for 18 h before treatment with anti-FasL antibody clone MFL4 (BD Biosciences) or mFasFc at 5 μg/mL.

2.3. Astrocyte preparation and culture

Mouse astrocytes were isolated from the brain of newborn C3H/HeJ (C3.*wt*) or C3.MRL-*Fas*^{lpr}/J (C3.*lpr*) mice, as indicated, at postnatal day 2 or 3. Meninges were removed and brain was passaged through a sterile 350-nm nylon mesh and dissociated with DNaseI (12 units/mL) by trituration following a method modified from (Weinstein 1997). Cell suspensions were plated in DMEM / 10% FCS in 75-cm^2 flasks and cultures were expanded. Purity of each culture was verified by flow cytometry following staining with anti-GFAP antibodies. Cultures contained typically more than 90% GFAP$^+$ cells, an intracellular marker for astrocytes *in vitro*. Cells for the microarray analysis were plated on 15 μg/mL poly-L-ornithine (Sigma).

Rat astrocytes were provided by Jeffery Haines (McGill University, Montreal, Canada). They were obtained from the brain of Sprague-Dawley rats at postnatal day 2 or 3 as previously described (McCarthy and de Vellis 1980). Rat astrocytes were cultured in DMEM / 10% FCS.

Human fetal astrocytes were provided by Dr. Antel (Montreal Neurological Institute, Montreal, Canada) at passage 3 or 4. They were isolated from the brain of fetuses at gestational age 16-19 weeks as previously described

(Blain, Miron et al. 2010). Briefly, fetal CNS was dissociated in 0.05% trypsin / 50 mg/mL DNaseI followed by mechanical dissociation. Cells were plated on poly-L-lysine (10 mg/mL; Sigma) for the first 2 passages. Human astrocytes were grown in DMEM / 10% FCS. All procedures were reviewed and approved by the Research Ethics board of the Montreal Neurological Institute (MNI) and Hospital and the McGill University Health Centre (MUHC) and complied with the Canadian Institutes for Health Research (CIHR) guidelines

For experiments, mouse, rat and human astrocytes were serum-starved in DMEM overnight. Cultures were then supplemented with 5 μg/mL mFasFc protein chimera (Sigma), 5 μg/ml anti-FasL antibodies (clone MFL3, MFL4, or NOK-1; BD Biosciences), or isotype-matched control antibodies (BD Biosciences or Sigma), as indicated.

2.4. PC12 neurite outgrowth assay

PC12 cells were obtained from the American Type Culture Collection (ATCC) and grown in DMEM supplemented with 10% FCS and 5% horse serum (HyClone). For assays, PC12 cells were seeded in 96-well plates in CM from Schwann cells stimulated with anti-FasL antibody clone MFL3 (BD Biosciences), isotype matched control antibodies, or CM from untreated Schwann cell as a further control. PC12 cells were grown for 3 days in CM at 37°C / 5% CO_2. 5% horse serum was added to CM in each well. PC12 cells were then fixed in 4% paraformaldehyde, stained with 0.04% Coomassie Brilliant Blue Solution (0.04% Coomassie Brilliant Blue, 25% isopropanol, 10% acetic acid in H_2O), and photographed under an inverted microscope (DM IRE2; Leica, Wetzlar, Germany) at 10X magnification using MetaMorph software (Molecular Devices, Sunnyvale, CA). Quantification of neurite length and number was done with ImageJ software (NIH).

2.5. Quantification of nerve growth factor in conditioned medium and in cellular extracts

Released NGF protein in CM was quantified with a commercial NGF ELISA kit (Promega) according to manufacturer's instructions. Intracellular NGF content was measured from cellular extracts in ELISA lysis buffer (1% NP-40, 50 mM Tris-HCl pH8, 150 mM NaCl, 0.1 mM Na_3VO_4, 20 mM NaF) supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche Applied Science), and acid-treated.

2.6. Quantification of NGF mRNA levels by RT-PCR and RT-qPCR

Cells were homogenized in Ambion's RNAqueous®- Micro RNA Isolation kit lysis buffer and total RNA was extracted according to the manufacturer's instructions. For Schwann cells and human fetal astrocyte samples, reverse transcription (RT) was performed on 50 ng total RNA with random decamer primers (Ambion) using the Sensiscript® reverse transciption kit (Qiagen). For mouse astrocyte samples, reverse transcription (RT) was performed on 150 ng total RNA with random decamer primers (Ambion) using the Omniscript® reverse transciption kit (Qiagen). The HotStarTaq® PCR kit from Qiagen was used to amplify cDNAs of interest with the following primers:

18S rRNA	sense	5'-TGAGAAACGGCTACCACATCC-3'
	antisense	5'-TCGCTCTGGTCCGTCTTGC-3';
murine NGFβ	sense	5'-AAGGTTTTGCCAAGGACG-3'
	antisense	5'-GTGATGTTGCGGGTCTGC-3'.

A Touchgene Gradient Thermal Cycler (Techne, Inc.) was used with cycles programmed as follows: 18S rRNA, 15 min. at 95°C, cycled 17 x [60 s. at

94°C, 40 s. at 66.2°C, 30 s. at 72°C], then final elongation 10 min. at 72°C; NGFβ mRNA, 15 min. at 95°C, cycled 28 x [60 s. at 94°C, 60 s. at 61.3°C, 30s. at 72°C], then final elongation 10 min. at 72°C. PCR products were resolved on 1.5% agarose gels by ethidium bromide staining and bands were quantified by densitometry on an Alpha-Innotech Corporation imager with AlphaEase FC software.

For real-time PCR (qPCR), the MiniOpticon real-time PCR system with the iQ SYBR Green Supermix (Bio-Rad) was used using the same primers for murine NGFβ as described above and the following primers for human NGFβ:

human NGFβ	sense	5'-AACAGTTTTACCAAGGGAGCAGC-3'
	anti-sense	5'-CACCGACCTCGAAGTCCAGAT-3'

Results were normalized to GAPDH mRNA expression using the following primers:

mouse GAPDH	sense	5'-AGCCTCGTCCCGTAGACA-3'
	anti-sense	5'-CTCGCTCCTGGAAGATGG-3'.
human GAPDH	sense	5'-GAGTCAACGGATTTGGTCGT-3'
	anti-sense	5'-TTGATTTTGGAGGGATCTCG-3'

The program used was as follows: 3 min at 95°C, cycled 6 x [20 s at 95°C, 20 s at 60°C (increment temperature by -1°C per cycle), 20 s at 70°C, plate reading], cycled 41 x [20 s at 95°C, 20 s at 57.5°C, 20 s at 70°C, plate reading], followed by melting curve (65°C to 95°C, increment of 0.5°C for 5 s), plate reading, and end.

For validation of microarray data, reverse transcription (RT) was performed on 300 ng total RNA with random decamer primers (Ambion) using the Omniscript® reverse transciption kit (Qiagen). Real-time PCRs were carried out as detailed above using GAPDH as a reference gene. cDNAs for the genes of interest were amplified with the following primers:

Ier3	sense	5’-CTACCCTCGAGTGGTCCGGC-3’
	anti-sense	5’-CAGGTGTCACGGCGCTGGTA-3’.
Cyr61	sense	5’-GGGCGCATTTGATCCCGCTG-3’
	anti-sense	5’-CGCACTTGGGTGCCTCCAGA-3’.
Fosl2	sense	5’-GGCGGCCAGCAGAAGTTCC-3’
	anti-sense	5’-GCCCAGGGACTGAAGCCAGG-3’.
Adrb1	sense	5’-CGGCTGCAGACGCTCACCAA-3’
	anti-sense	5’-AGGAGCCGTACTCCCAGCGG-3’.
Gbx2	sense	5’-CGCTGCTCGCTTTCTCTGCG-3’
	anti-sense	5’-GGGGTCTCCTCCAGTGCGTG-3’.
Egr2	sense	5’-CGCCTCGTCGGTGACCATCT-3’
	anti-sense	5’-GCAGCTGGCACCAGGGTACT-3’.
Rhoe	sense	5’-AGCCTGTGGGACACTTCAGGTTC-3’
	anti-sense	5’-ACTTGCAGCCCACCAACAGC-3’.
Txnip	sense	5’-TCCTAGAAGAGCAGCCTACAGGTGA-3’
	anti-sense	5’-CTGGCTGGGGCGATCGAGAA-3’.
Bcl2a1b	sense	5’-ACTTCCGCAAGAGCAGATTGCC-3’
	anti-sense	5’-AGCATTTCCCAGAACTGTCCTGTC-3’.
CD72	sense	5’-CAGAGGCGCCCAGGGCTATG-3’
	anti-sense	5’-ACCCGACCCCTGCTTTGTCC-3’.
S100a11	sense	5’-CACTCCTGTCCCAGCCACCG-3’
	anti-sense	5’-ACTCTTGGAAATCTAGCTGCCCG-3’.
Fcgr3	sense	5’-ATGGAGCAGACCCGCCTCAG-3’
	anti-sense	5’-AGGCCCGTGTCCACTGCAAA-3’.

All primers were designed using National Center for Biotechnology Information (NCBI) Primer-BLAST and were supplied by Invitrogen.

2.7. DNA microarray analysis

Primary mouse astrocytes were treated in parallel with mFasFc (5 μg/mL; Sigma) or control antibody (5 μg/mL) in triplicate wells for 1.5 h and 12 h. Cells were homogenized in Ambion's RNAqueous®- Micro RNA Isolation kit lysis buffer and total RNA was extracted according to the manufacturer's instructions. Total RNA samples were sent to the McGill University and Génome Québec Innovation Centre, Montréal, Canada (http://www.gqinnovationcenter.com/). The centre performed the cRNA synthesis from total RNA, labelling and hybridization to Illumina Sentrix Genome Wide Expression Array mouse WG-6 v1.1 BeadChip® containing more than 45,000 probes.

2.8. Immunoblot analysis

Cells were harvested in immunoblotting lysis buffer (1% Triton X-100, 10% glycerol, 20 mM HEPES, 150 mM NaCl, 0.1 mM Na_3VO_4, 20 mM NaF) supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche Applied Science). Proteins were resolved in 12% SDS-polyacrylamide gels, transferred to nitrocellulose membranes and blotted with polyclonal phospho-specific ERK1/2 antibodies or phospho-Src antibodies (Cell Signalling Technologies), followed by anti-rabbit IgG-HRP antibodies (Amersham Biosciences). Bands were developed using Western Lightning™-ECL kit reagents (PerkinElmer). Membranes were stripped and re-blotted for β-actin (Chemicon International). Films were scanned with the AlphaImager® Gel Documentation System and AlphaEase software (Alpha Innotech Corporation). Densitometric values for ERK1/2 and Src activation are expressed as the ratio of phospho-protein to β-actin for each lane.

2.9. Flow cytometry

For staining of human fetal astrocytes with cell surface Fcγ receptors (FcγR), cell suspensions were washed in PBS / 1% FCS and then incubated 30 min at 4°C in PBS / 1% FCS with fluorescein isothiocyanate (FITC)-labelled antibodies against CD16 (FcγRIII), CD32 (FcγRII), and CD64 (FcγRI) (BD Biosciences). Following two washes in PBS / 1% FCS, cells were resuspended in PBS / 1% FCS for analysis with a FACScan flow cytometer (Becton Dickinson). Data was analysed with BD CellQuest™ software.

Staining of mouse and human fetal astrocytes with intracellular GFAP was performed using BD Cytofix / Cytoperm™ kit (BD Biosciences) according to manufacturer's instructions. Cells were incubated with primary anti-GFAP or isotype-matched control antibody followed by incubation with phycoerythrin (PE)-labelled secondary antibodies. Samples were then run through a FACScan flow cytometer (Becton Dickinson) and data was analysed with BD CellQuest™ software.

2.10. Sciatic nerve crush injury

Sciatic nerve crush injuries were performed on 4 to 5 month-old C57BL/6-Fas^{lpr} (*lpr*) and C57BL/6-Fas^{null} (Fas-null) mice (3 mice per strain). A 1-cm long incision was made through the skin in avertin-anesthetized mice, and the gluteal and hamstring muscles of the upper dorsal thigh were separated to reveal the sciatic nerve. The nerve was crushed twice, by the application of pressure for 30 seconds with Dumont #5 forceps, 5 mm distal to the sciatic notch. The muscles and skin were closed with absorbable Dexon sutures. After the first post-operative day, the mice stop dragging their operated limb and gradually resume weight-bearing.

2.11. Walking track analysis

Walking track analyses were performed by coating the hind paws of mice with India Ink, and allowing the mice to walk along an 18 inch track lined with paper. The toe-spread of each paw correlates with the weight-bearing capacity of the limb (de Medinaceli, Freed et al. 1982). The tracks were analyzed by measuring the toe-spread of each paw print every three or four days.

2.12. Statistical analyses

Statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA). All figures show mean ± SEM of representative experiment unless otherwise indicated. Statistical tests used are detailed in the figure legends.

Chapter 3. Results

3.1. FasL reverse signalling in Schwann cells

3.1.1 FasL engagement stimulates Schwann cells to produce soluble mediators that induce neurite outgrowth

FasL reverse signalling was examined in Schwann cell cultures derived from newborn C3H mouse dorsal root ganglia treated with stimulatory antibodies to FasL. Cultures were serum-starved for 18 h prior to stimulation for 48 h with antibodies to FasL, using a protocol previously shown to activate intracellular signalling pathways via FasL (Thornhill, Cohn et al. 2007). Cells appeared healthy by phase contrast microscopy and no difference in survival or proliferation between treatments could be observed (data not shown). Conditioned medium (CM) was collected and used to stimulate PC12 cells, a rat pheochromocytoma cell line known to differentiate and grow neurites in response to NGF (Greene and Tischler 1976). PC12 cells grown in CM from control, untreated Schwann cells (CM-untr) or Schwann cells treated with control isotype-matched IgG antibodies (CM-IgG), kept an undifferentiated phenotype and adhered poorly to the surface of the plates **(**Fig. 3.1**)**. In contrast, PC12 grown in CM from Schwann cells stimulated with anti-FasL antibody (CM-FasL Ab) had a more adherent phenotype and extended neurites **(**Fig. 3.1 a and b**)**. Average neurite length was increased by more than 2-fold with CM-FasL Ab compared to all other treatments ($p<0.05$). The frequency of neurites longer than 10 μm was very low in all controls (~2 / field), however in the CM-FasL Ab, there were more than 50 neurites / field **(**Fig. 3.1 b**)**. The number of neurites per cell clump was significantly increased with CM-FasL Ab compared to other groups ($p<0.001$). Addition of anti-FasL antibodies to basal PC12 growth medium (BM-FasL Ab) did not induce a differentiated phenotype in the PC12 cells, ruling out a possible carry-over effect of anti-FasL antibody on neurite outgrowth in the PC12 cells **(**Fig. 3.1 a and b**)**. These results showed that FasL engagement stimulates Schwann cells to secrete an active soluble mediator(s) that stimulates significant neurite outgrowth.

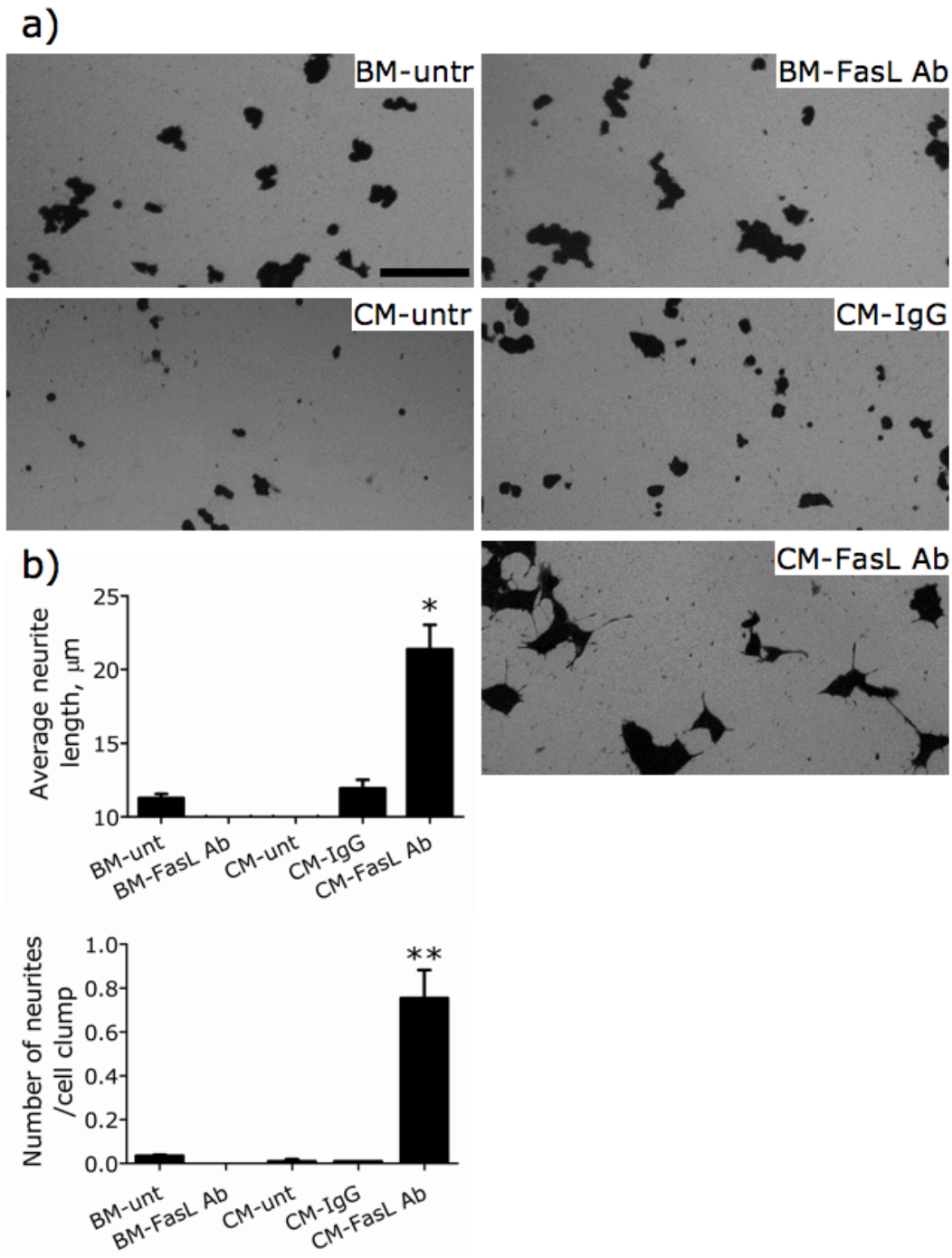

a)
BM-untr
BM-FasL Ab
CM-untr
CM-IgG
CM-FasL Ab
b)
Average neurite length, µm
25
20
15
10
*
BM-unt
BM-FasL Ab
CM-unt
CM-IgG
CM-FasL Ab
Number of neurites /cell clump
1.0
0.8
0.6
0.4
0.2
0.0
**
BM-unt
BM-FasL Ab
CM-unt
CM-IgG
CM-FasL Ab

Figure 3.1 FasL engagement stimulates Schwann cells to produce soluble mediators that induce neurite outgrowth

a) Representative photomicrographs of PC12 cells grown for 3 days in CM from primary mouse Schwann cells. As controls, PC12 cells were plated in basal PC12 culture medium (BM-untr) or basal medium supplemented with anti-FasL antibody (BM-FasL Ab). PC12 cells were plated in CM from untreated Schwann cells (CM-untr), or from Schwann cells treated with isotype control (CM-IgG) or anti-FasL antibody (CM-FasL Ab). Scale bar = 100 μm.

b) Quantification of neurite length and number of neurites per cell clump. All processes longer than 10 μm were considered as neurites. Means of triplicates + SEM are shown (except for BM-unt and CM-IgG, in duplicate). *, $p<0.05$, Student's t-test and one-sample t-test between CM-FasL Ab and all other groups; **, $p<0.01$, Student's t-test and one-sample t-test between CM-FasL Ab and all other groups.

3.1.2 FasL engagement induces NGF secretion from mouse Schwann cells

Since PC12 cells are well known to differentiate in response to NGF (Greene and Tischler 1976; Levi, Biocca et al. 1988), whether FasL engagement increased NGF secretion by Schwann cells was next assessed. The effect of FasL signalling on NGF secretion was analyzed in a mouse Schwann cell line, MSC80, obtained by spontaneous immortalization of neonatal cells grown in high concentrations of fetal calf serum (Boutry, Hauw et al. 1992) and a convenient source of unlimited number of cells. MSC80 cells were serum-starved and treated with the agonist murine Fas-Fc chimeric protein (mFasFc), consisting of Fas linked to the Fc portion of human IgG_1 antibody, for time periods ranging from 1 to 48 h. NGF released into the culture medium was then quantified by ELISA. NGF was detected as early as 1 h following stimulation of FasL signalling (1h vs. 0, $p<0.01$) **(**Fig. 3.2 a), and NGF levels were maintained for 24 h and further increased after 48 h of treatment (48 h vs. 24 h, $p < 0.01$) (Fig. 3.2 a**)**.

NGF release by FasL engagement observed in MSC80 cells was also verified in primary Schwann cells. Isolated Schwann cells from the sciatic nerve of adult C57/BL6 mice (wild-type, *wt*), seven days after a crush injury (Pannunzio, Jou et al. 2005), were serum-starved and treated with mFasFc or a control antibody for 48 h. NGF secretion was significantly increased by FasL stimulation by more than 50% in mFasFC-treated medium as compared to untreated or control groups ($p<0.05$) **(**Fig 3.2 b, *wt***)**. To rule out the possibility that FasL antibodies were blocking endogenous Fas / FasL interactions instead of stimulating FasL, experiments were designed using Schwann cells from C57/BL6 *lpr* mice, which express FasL but lack significant Fas expression (Adachi, Watanabe-Fukunaga et al. 1993). NGF secretion was significantly increased by FasL stimulation in *lpr* Schwann cells (~90% increase, $p<0.01$) **(**Fig 3.2 b, *lpr***)**. Thus, these results demonstrate that FasL antibodies induce NGF secretion from both the MSC80 cell line and from primary Schwann cells by engagement of FasL, independently of Fas expression.

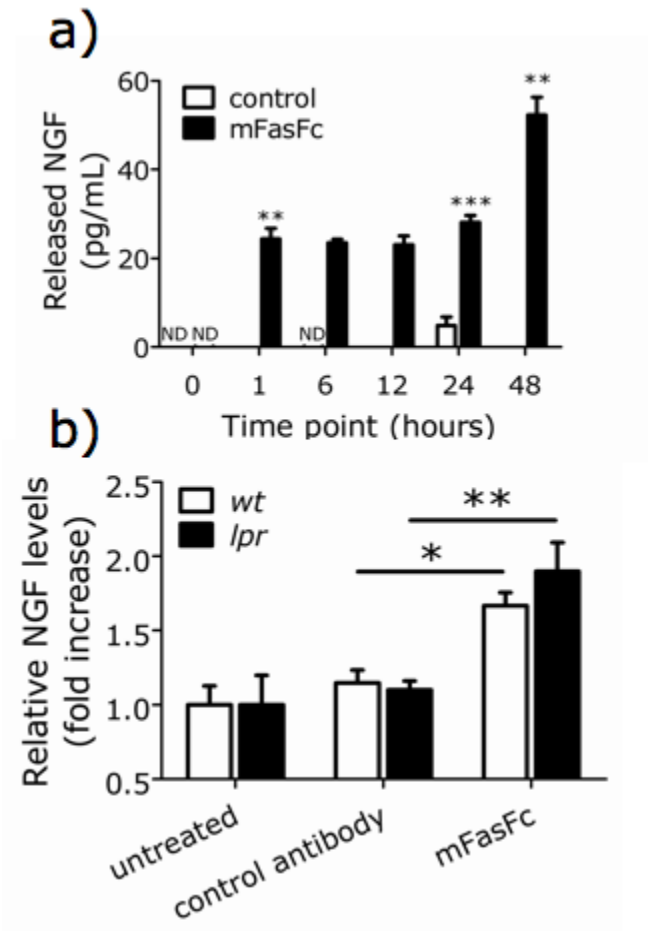


Figure 3.2 FasL induces NGF secretion from mouse Schwann cells

a) FasL induced NGF secretion from the mouse Schwann cell line MSC80. MSC80 cells were serum-starved overnight and then treated for the times indicated with mFasFc (black bars) or an isotype antibody control (white bars) at time 0, 6 h and 24 h. CM was collected and NGF level was quantified by ELISA. The results are expressed in pg/mL of medium. **, $p<0.01$, one-sample t-test between 1 h and 0 h, mFasFc-treated; ***; $p<0.001$, Student's t-test between mFasFc-treated and control at 24 h; **, $p<0.01$, Student's t-test between 48 h and 24 h, mFasFc-treated. ND, not detectable.

b) FasL induced NGF secretion from primary Schwann cells from wild-type and Fas-deficient adult mice. Schwann cells from C57BL/6 (*wt*, white bars) and B6.MRL-Fas^{lpr} (*lpr*, black bars) mice were serum-starved overnight and treated with control antibody or mFasFc for 48 h. NGF levels in CM were quantified by ELISA. NGF concentration in untreated groups was 80 pg/mL for *wt* and 103 pg/mL for *lpr*. *, $p<0.05$; **, $p<0.01$; two-way ANOVA followed by Bonferroni post-tests.

3.1.3 FasL engagement upregulates NGF mRNA, and in addition, stimulates NGF release independently of RNA synthesis

To examine whether FasL-induced NGF secretion required transcription, levels of NGF mRNA were next assessed by RT-PCR. FasL engagement significantly increased NGF mRNA levels in mouse primary Schwann cells (~50% increase compared to untreated or control groups; $p<0.05$) (Fig. 3.3 a**)**. In the MSC80 cell line, FasL engagement caused a time-dependent increase in NGF mRNA, with a maximal increase (3-fold above control) between 3 and 4 h following treatment ($p<0.001$) **(**Fig. 3.2 b**)**, suggesting that stimulation of FasL triggered new NGF synthesis. In order to determine whether the NGF released by the cells in response to FasL stimulation was due to *de novo* protein synthesis, MSC80 cells were serum-starved as previously described, pretreated with either actinomycin D (ActD, 5 μg/mL) or cycloheximide (CHX, 10 μg/mL) for 30 minutes to inhibit transcription or translation respectively, then treated for 6 h with mFasFc. There was a significant decrease (30%; $p<0.001$) in NGF release into the medium when transcription was inhibited with actinomycin D **(**Fig. 3.3 c**)**. Inhibiting translation with cycloheximide also produced a significant decrease in NGF release (13%; $p<0.05$) **(**Fig. 3.3 c**).** Intracellular NGF content was measured in parallel in cell lysates in order to assess whether intracellular NGF was also modified by the inhibitors of transcription and translation **(**Fig. 3.3 d**)**. There was no difference in intracellular NGF levels between untreated and mFasFc-treated groups. During the 6-hr treatment, the cells may be able to replenish the pool of NGF that was released in the first hour following FasL engagement. However, both cycloheximide and actinomycin D treatments induced a significant decrease in NGF levels in both untreated and FasFc-treated groups. Thus, there may be a constant replenishment (turn-over) of an NGF pool inside the cells which is reduced when *de novo* synthesis is inhibited at the level of transcription or translation. The data indicate that FasL reverse signalling primarily leads to release of pre-synthesized NGF from Schwann cells, with a

minor component involving *de novo* transcription and translation of NGF. The fact that the increase in NGF released in the culture medium is detectable within one hour of mFasFc treatment, long before the peak in NGF mRNA is achieved, supports the hypothesis that pre-made NGF is released in a first phase. This may be followed by a second wave of newly synthesized NGF protein. Our results suggest that FasL engagement increases NGF release as well as upregulates NGF mRNA synthesis and protein production.

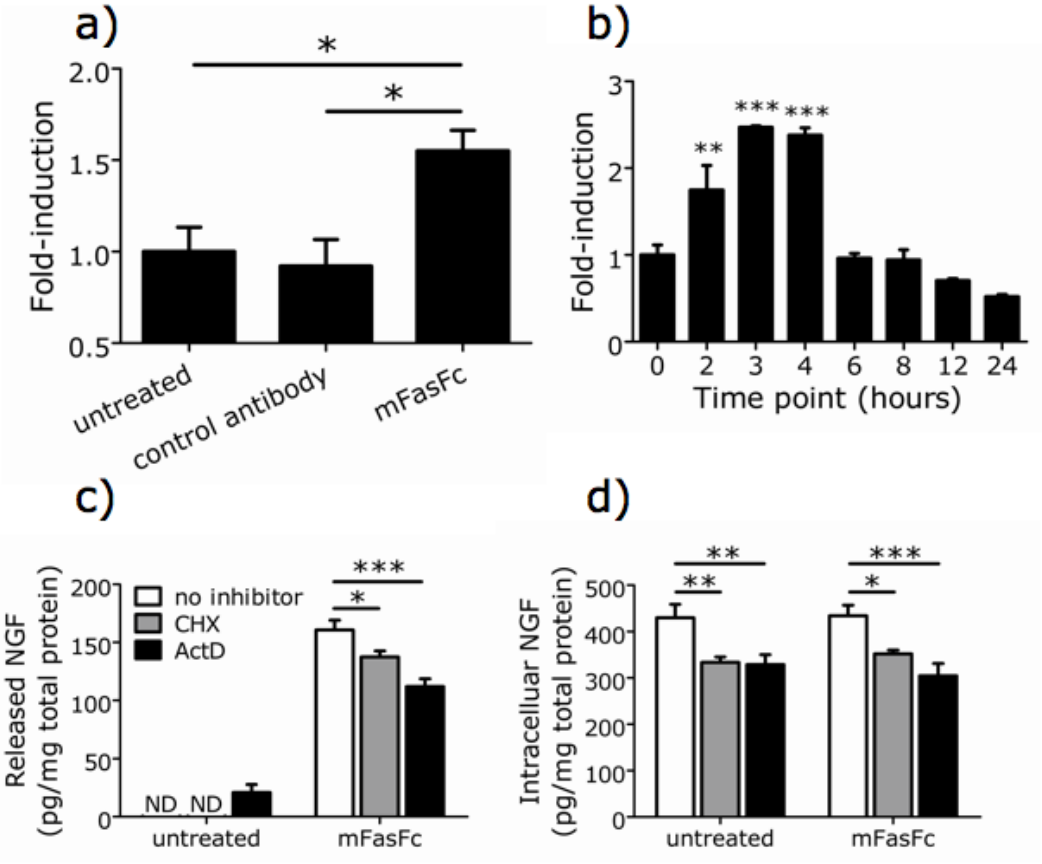

a)
Fold-induction
2.0
1.5
1.0
0.5
*
*
untreated
control antibody
mFasFc
b)
Fold-induction
3
2
1
0
**
***
***
0 2 3 4 6 8 12 24
Time point (hours)
c)
Released NGF (pg/mg total protein)
200
150
100
50
0
no inhibitor
CHX
ActD
***
*
ND ND
untreated
mFasFc
d)
Intracelluar NGF (pg/mg total protein)
500
400
300
200
100
0
**
**
***
*
untreated
mFasFc

Figure 3.3 FasL engagement upregulates NGF mRNA and in addition stimulates NGF release independently of RNA synthesis

FasL engagement upregulates NGF mRNA in **a)** primary mouse Schwann cells and **b)** in the mouse Schwann cell line MSC80.
a) C57BL/6 Schwann cells were serum-starved overnight and treated with control antibody or mFasFc for 48 h then harvested for RNA extraction and RT-PCR.
b) MSC80 cells were serum-starved overnight and treated with mFasFc for up to 24 h and harvested for RNA extraction and RT-qPCR at different time points. *, $p<0.05$; one-way ANOVA followed by Newman-Keuls multiple comparison test. **, $p<0.01$; ***, $p<0.001$; one-way ANOVA followed by Dunnett's multiple comparison test.

c) and **d)** Quantification of released NGF **(c)**, and intracellular NGF **(d)**, in MSC80 cells pre-treated for 30 min with CHX or ActD before the addition of mFasFc for a 6 h treatment. NGF levels were quantified in CM and in cell lysates by ELISA. The results are expressed in pg of NGF over mg of total cellular proteins to allow comparison of levels of NGF between CM and cell lysate. The amount of NGF in CM in mFasFc / no inhibitor corresponds to 29 pg/mL. Average + SEM of triplicates from two independent experiments are shown. Panel c), *, $p<0.05$; ***, $p<0.001$; Student's t-test. Panel d), *, $p<0.05$; **, $p<0.01$; ***, $p<0.001$; two-way ANOVA followed by Bonferroni post-tests. ND, not detectable.

3.1.4 Molecular mechanism of FasL-induced NGF production – activation of the Src and ERK1/2 pathways

The intracellular domain of FasL contains a proline rich region which interacts with multiple Src homology 3 (SH3) domain containing proteins in T cells, including several members of the Src tyrosine kinase family (Hane, Lowin et al. 1995; Wenzel, Sanzenbacher et al. 2001; Voss, Lettau et al. 2009). Activation of FasL signalling is followed by binding of the Src proteins to the FasL intracellular domain and activation of the ERK / MAPK pathway in T cells (Sun, Ames et al. 2006; Sun, Lee et al. 2007) and Sertoli cells (Ulisse, Cinque et al. 2000). In order to better understand the signalling pathways recruited downstream of FasL in Schwann cells, the phosphorylation levels of the cytoplasmic tyrosine kinase Src and the serine / threonine kinase ERK1/2 were determined following treatment with mFasFc in the mouse Schwann cell line MSC80. Both ERK1/2 and Src were activated by FasL engagement in Schwann cells, although with different kinetics **(**Fig. 3.4 a**)**. mFasFc caused a rapid ERK1/2 phosphorylation with a peak around 10 min, followed by a progressive decline and returned to baseline after 120 min. On the other hand, Src was gradually phosphorylated up to 120 min following addition of mFasFc and did not return to control level within the time frame studied. To determine whether these pathways were involved in FasL-induced NGF release, PD98059 and PP2, selective inhibitors of the ERK / MAPK and Src pathways respectively, were used. In serum-starved MSC80 cells, pre-treatment for 30 min with PD98059 (30 μM) prior to a 6-hr treatment with mFasFc resulted in a 60% increase in NGF in the culture medium ($p<0.001$) **(**Fig. 3.4 b**)** but did not cause any significant change in intracellular NGF levels in response to FasL engagement **(**Fig. 3.4 c). This counter-intuitive result showed that blocking ERK1/2 activation increased NGF release, suggesting that ERK1/2 activation upon FasL engagement may inhibit the release of NGF. In contrast, inhibition of Src activation with PP2 (10 μM) led to a small (15%) but significant decrease in both intracellular and released NGF ($p<0.05$ and $p<0.05$, respectively) **(**Fig. 3.4 b and c**)**. These data

suggest that the signalling pathways involved in NGF production and NGF release in Schwann cells upon FasL engagement are at least partially different. FasL-mediated Src activation may induce an increase in NGF synthesis, while the MAPK / ERK pathway seems to inhibit the release of NGF from the Schwann cells.

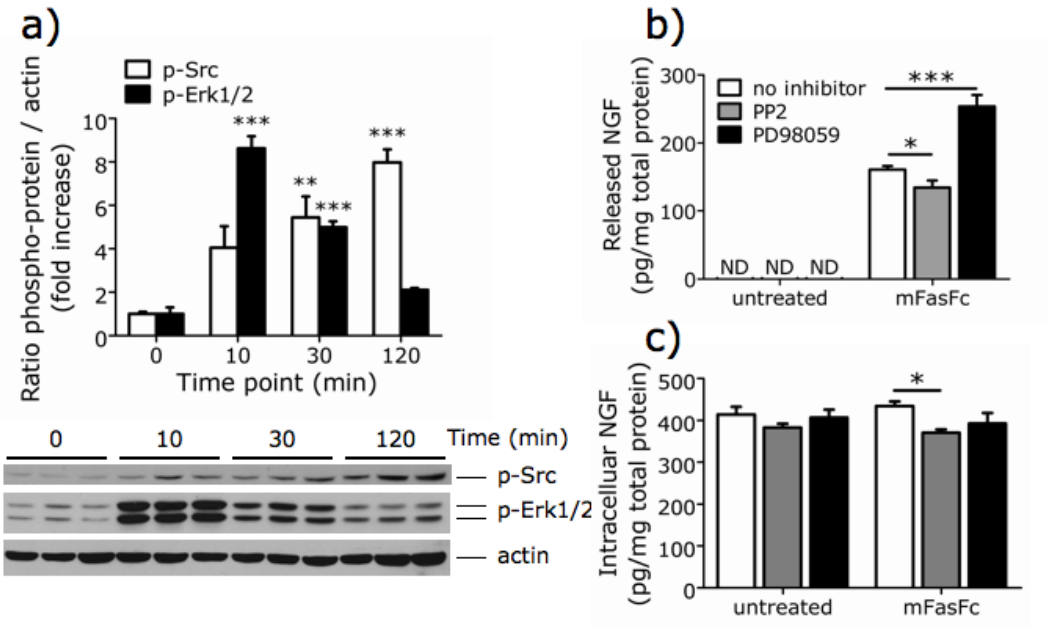


Figure 3.4 Molecular mechanism of FasL-induced NGF production

a) ERK1/2 and Src are activated by FasL reverse signalling in MSC80 cells. MSC80 cells were treated with mFasFc for the indicated times. Phosphorylation of ERK1/2 and Src were assessed by immunoblotting. Top panel, quantification of bands shown below. **, $p<0.01$; ***, $p<0.001$; one-way ANOVA followed by Dunnett's multiple comparison test of different time points versus time 0.

b) and **c)** Inhibition of Src and ERK1/2 activation modulates NGF release and intracellular NGF content in MSC80 cells. MSC80 cells were serum-starved and pre-treated for 30 min with PD98059 (30 μM) or PP2 (10 μM) before the addition of mFasFc for 6 h. NGF levels were quantified in CM (**b**) and in cell lysates (**c**) by ELISA. The results are expressed in pg of NGF over mg of total cellular proteins to allow comparison of levels of NGF between CM and cell lysate. The amount of NGF in CM in mFasFc / no inhibitor corresponds to 29 pg/mL. Average + SEM of triplicates from three and two independent experiments for

PD98059 and PP2 treatment, respectively, are shown. *, $p<0.05$; ***, $p<0.001$; Student's t-test.

3.1.5 Antibodies to FasL stimulate rat and human primary Schwann cells to produce NGF

Whether FasL engagement in Schwann cells from rat and human would also result in NGF secretion was next examined. Primary Schwann cells were serum-starved and treated for 48 h with species-specific agonistic antibodies, anti-FasL clone MFL4 for rat cells and clone NOK1 for human cells. NGF release in CM from rat Schwann cells increased by almost 6-fold compared to untreated cells ($p<0.01$) **(**Fig. 3.5 a**)**. Treatment of human Schwann cells with NOK1 antibody led to more than 2-fold increase in NGF release compared to untreated cells ($p<0.001$) **(**Fig. 3.5 b**)**. Interestingly, mFasFc and anti-FasL clone MFL3, both specific to mouse FasL, did not significantly increase NGF release in rat and human Schwann cells, respectively, suggesting that murine Fas does not bind rat and human FasL appropriately for stimulation of FasL reverse signalling **(**Fig. 3.5 a and b**)**. The effect of FasL reverse signalling on NGF secretion is therefore conserved across species. Biological relevance of FasL reverse signalling in the human peripheral nervous system is suggested.

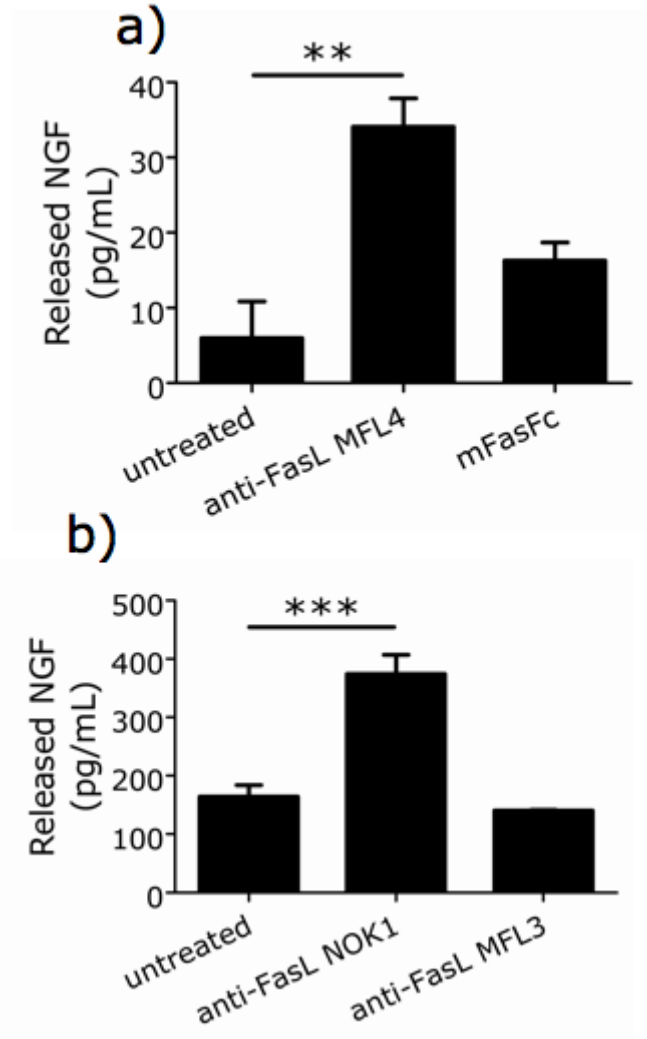


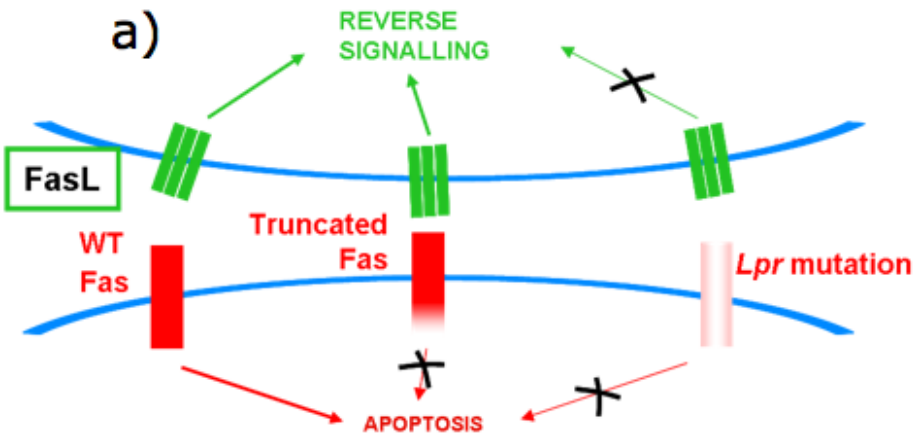
Figure 3.5 FasL reverse signalling induces release of NGF by rat and human Schwann cells

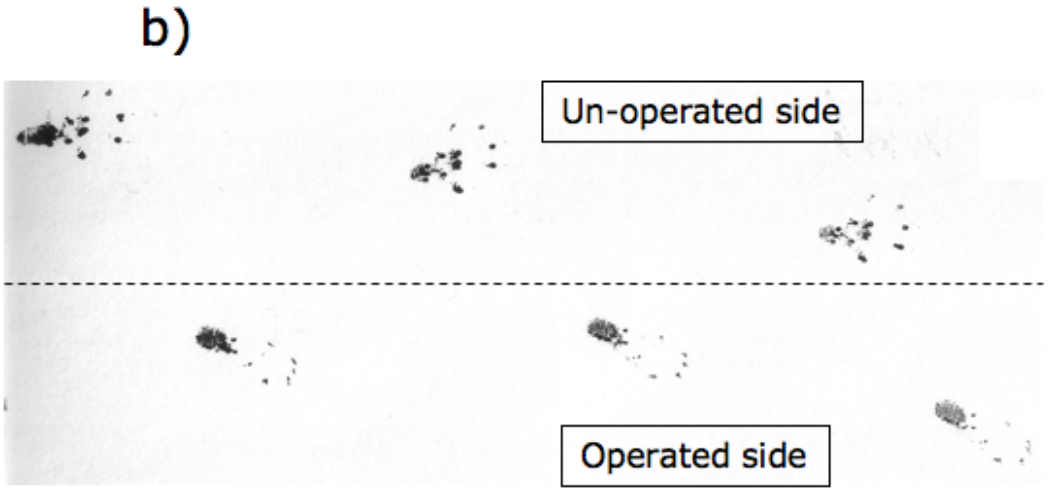
a) Rat Schwann cells were serum-starved and treated with anti-FasL antibody clone MFL4 or mFasFc for 48 h.

b) Human Schwann cells were serum starved and treated with anti-FasL antibody clone NOK1 or clone MFL3 for 48 h. NGF levels in CM were quantified by ELISA. **, $p<0.01$; ***, $p<0.001$; one-way ANOVA followed by Dunnett's multiple comparison test.

3.1.6 FasL signalling accelerates *in vivo* functional recovery after sciatic nerve crush

FasL engagement induces release of NGF by Schwann cells, suggestive of a potential role of FasL reverse signalling in regeneration. In order to study the effect of FasL on regeneration *in vivo*, functional recovery after sciatic nerve crush injury was examined. The rate of functional recovery in the *lpr* and Fas-null strains of mice was compared. As pictured in Fig. 3.6 a, *lpr* mice do not express Fas and there is no ligand available to bind FasL and engage reverse signalling. On the other hand, Fas-null mice express a truncated form of Fas with no intracellular death domains. In Fas-null mice, the extracellular portion of mutated Fas can bind FasL and engage signalling without the confounding factor of apoptosis. Recovery of motor function was assessed by comparing the ability of mice to bear weight on the injured limb using walking track analysis (measurement of toe spread on hindlimb paw prints, Fig. 3.6 b). At day 17 and 21, there was a significant difference in toe spread between *lpr* and Fas-null mice (Fig. 3.6, c; $p<0.05$ and $p<0.001$). At day 21 post-crush, Fas-null mice had fully recovered to uninjured values. Recovery was significantly delayed in *lpr* mice compared to Fas-null suggesting that FasL-induced growth signals contribute to recovery in Fas-null mice.


a)
REVERSE
SIGNALLING
FasL
WT
Fas
Truncated
Fas
Lpr mutation
APOPTOSIS


b)
Un-operated side
Operated side

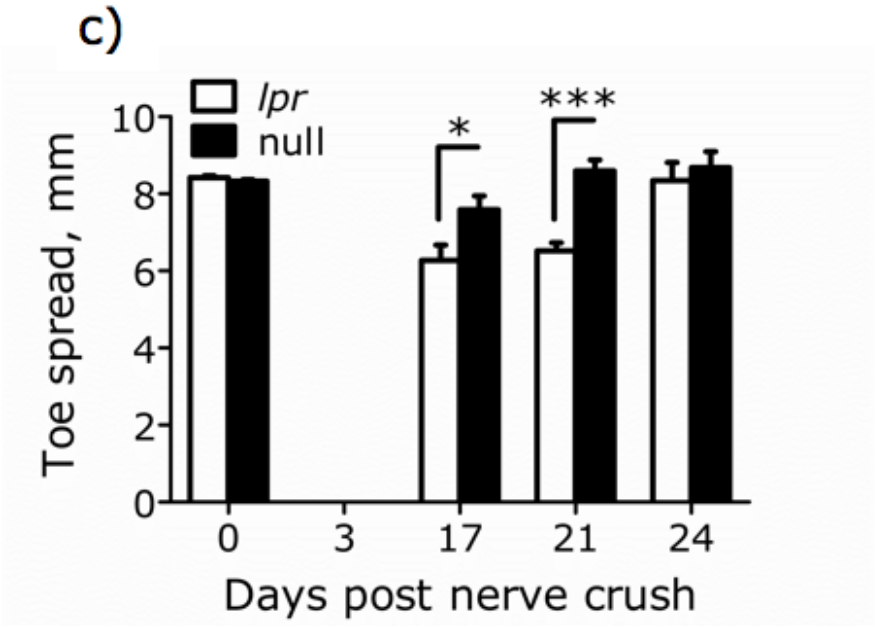

c)
lpr
null
*
***
Toe spread, mm
10
8
6
4
2
0
0
3
17
21
24
Days post nerve crush

Figure 3.6 FasL signalling accelerates *in vivo* functional recovery after sciatic nerve crush

a) Schematic representation of the signalling events that can be triggered by Fas and FasL in wt, *lpr*, and Fas-null mice.

b) Example of a walking track showing hindlimb paw prints of a mouse operated on the right side sciatic nerve (*lpr* mouse, 7 days post-crush injury).

c) Motor functional recovery was assessed by measure of toe spread on the injured limb after injury. *, $p<0.05$; ***, $p<0.001$; two-way ANOVA followed by Bonferroni's post-test (n=3 for each strain).

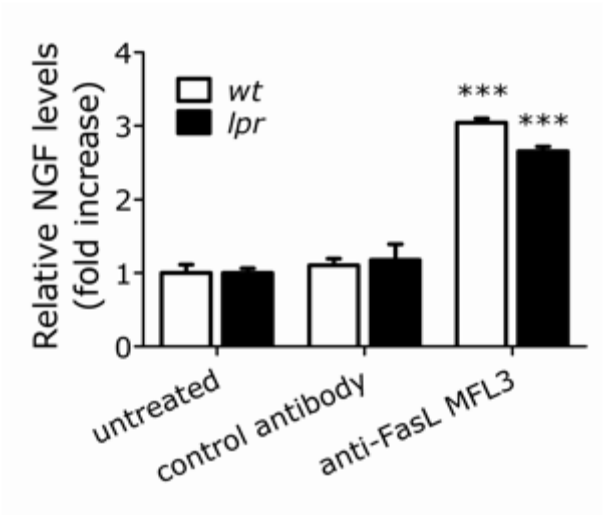
3.2. Fas Ligand reverse signalling in astrocytes

In the first part of this chapter, it was shown that FasL can transduce reverse signals in the main glial cells of the PNS, the Schwann cells, and induce the secretion of bioactive NGF. Since FasL is also expressed in glial cells in the CNS, the study of FasL reverse signalling in astrocytes was undertaken.

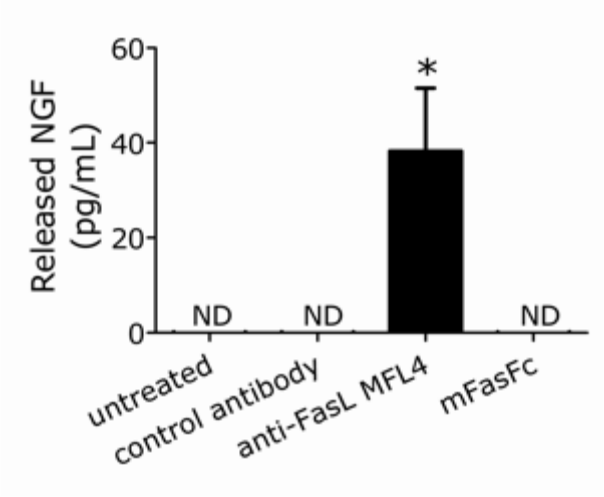
3.2.1 FasL engagement induces NGF secretion from mouse, rat, and human astrocytes

Astrocytes play supporting roles in the central nervous system by releasing growth factors and neurotrophins (Seth and Koul 2008). Therefore, these abundant glial cells could in principle respond to FasL engagement by increasing NGF secretion. We determined NGF levels in supernatants from primary murine astrocytes following treatment with anti-FasL agonistic antibodies and found that, like Schwann cells in the peripheral nervous system, astrocytes increase NGF secretion ($p<0.001$) (Fig. 3.7 a, C3.*wt*). NGF secretion was also increased by FasL engagement in astrocytes from Fas-deficient C3.*lpr* mice ($p<0.001$) (Fig 3.7 a, C3.*lpr*). The release of NGF by astrocytes upon treatment with anti-FasL antibodies therefore does not require Fas expression, and consequently is not due to blocking of Fas / FasL interactions, but to the engagement of FasL reverse signalling. Interestingly, anti-FasL antibody treatment also leads to increased NGF secretion in primary rat and human fetal astrocytes (Fig 3.7 b and c), suggesting that FasL reverse signalling in human and rodent cells may produce similar effects in the nervous system. Similarly, as in Schwann cells, mFasFc, specific to mouse FasL, did not induce NGF release in rat astrocytes (Fig. 3.7 b), reinforcing the hypothesis that murine Fas does not bind FasL appropriately to induce FasL reverse signalling.

a)


Relative NGF levels
(fold increase)
4
3
2
1
0
wt
lpr
***
***
untreated
control antibody
anti-FasL MFL3

b)


Released NGF
(pg/mL)
60
40
20
0
*
ND
ND
ND
untreated
control antibody
anti-FasL MFL4
mFasFc

c)

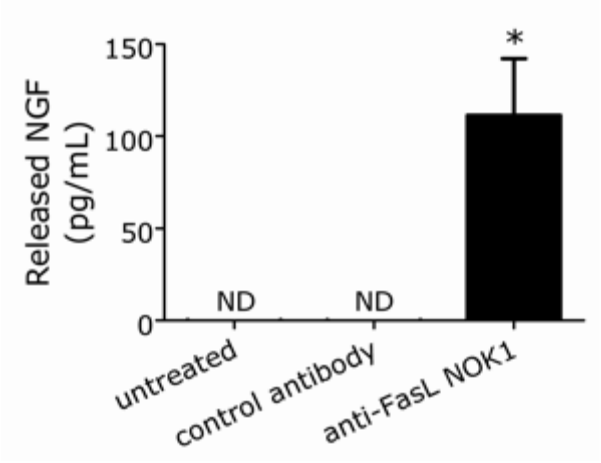

Released NGF
(pg/mL)
150
100
50
0
*
ND
ND
untreated
control antibody
anti-FasL NOK1

Figure 3.7 FasL reverse signalling induces release of NGF by primary mouse, rat, and human fetal astrocytes

a) FasL-induced NGF secretion from wild-type and Fas-deficient mice primary astrocytes. Astrocytes from C3.*wt* (*wt*, white bars) and C3.*lpr* (*lpr*, black bars) mice were serum-starved overnight and treated with control antibody or anti-FasL clone MFL3 for 48 h. NGF levels in CM were quantified by ELISA. NGF concentration in untreated groups was 203 pg/mL for *wt* and 215 pg/mL for *lpr*. ***, $p<0.001$; two-way ANOVA followed by Bonferroni post-tests comparing mFasFc-treated to untreated and control-antibody treated.

b) FasL-induced NGF secretion from primary rat astrocytes. Rat astrocytes were serum-starved overnight and treated with control antibody, anti-FasL antibody, clone MFL4, or mouse-specific mFasFc for 48 h. NGF levels in CM were quantified by ELISA. *, $p<0.05$; Kruskal-Wallis test followed by Dunn's comparison test.

c) FasL-induced NGF secretion from human fetal astrocytes. Human astrocytes were serum-starved overnight and treated with control antibody or anti-FasL antibody, clone NOK1, for 48 h. NGF levels in CM were quantified by ELISA. *, $p<0.05$; Kruskal-Wallis test followed by Dunn's comparison test.

3.2.2 FasL engagement upregulates NGF mRNA in mouse and human astrocytes

In Schwann cells, FasL engagement induces an increase in NGF mRNA in a time-dependent fashion. The effect of FasL signalling on NGF mRNA levels was examined in mouse and human astrocytes. NGF mRNA levels were measured in mouse and human fetal astrocytes at different time points following the addition of mouse-specific or human specific anti-FasL antibodies (clone MFL3 or NOK1, respectively). In mouse astrocytes, NGF mRNA levels decreased slightly at 3 h, but the difference was not statistically significant ($p>0.05$), and then increased steadily to achieve a ~50% increase at 24 h and 48 h compared to time 0 ($p<0.01$) (Fig. 3.8 a). On the other hand, NGF mRNA levels in human fetal astrocytes increased quickly 5.5 fold at 2 h compared to time 0 ($p<0.001$) and then progressively returned to baseline levels at 12 h (Fig. 3.8 b). The time-course of induction of NGF mRNA in mouse astrocytes and human fetal astrocytes was different. The difference in developmental stage (postnatal for mouse astrocytes and fetal for human astrocytes) and species differences (such as species-specific regulatory factors or receptor density) may explain this difference in NGF mRNA expression pattern following FasL engagement.

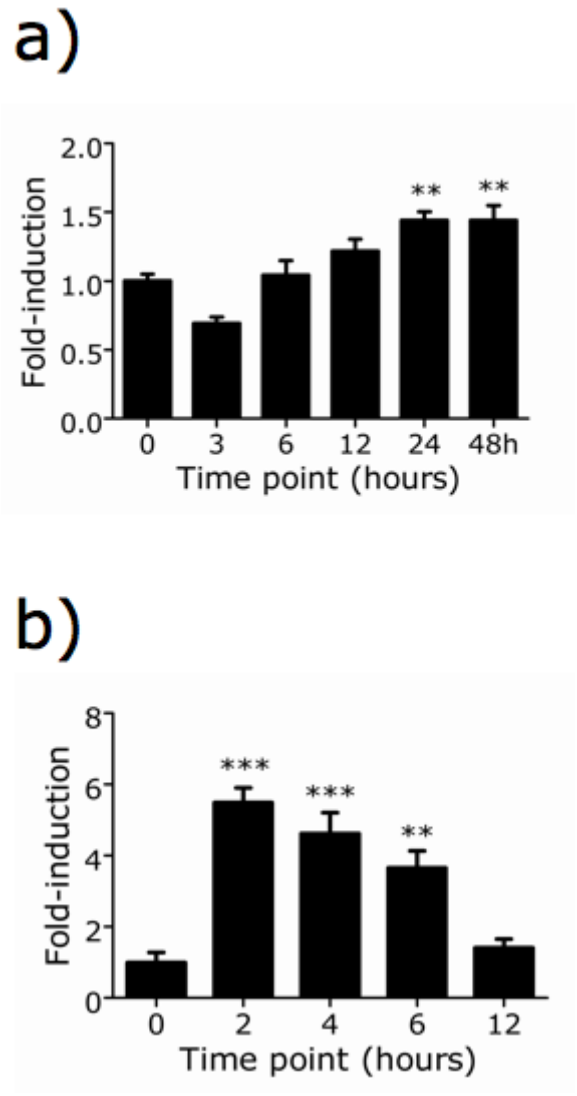


Figure 3.8 FasL engagement upregulates NGF mRNA in mouse and human astrocytes

a) Mouse astrocytes were serum-starved overnight and treated with anti-FasL antibody, clone MFL3, for different time points up to 48 h. Cells were harvested for RNA extraction and RT-PCR at the time points shown. **, $p<0.01$; one-way ANOVA followed by Dunnett's multiple comparison test.

b) Human fetal astrocytes were serum-starved overnight and treated with anti-FasL antibody, clone NOK1, for different time points up to 12 h. Cells were harvested for RNA extraction and RT-qPCR at the time points shown. ***, $p<0.001$; **, $p<0.01$; one-way ANOVA followed by Dunnett's multiple comparison test.

3.2.3 Human fetal astrocytes do not express Fcγ receptors

In our study, cells are treated with antibodies or a protein chimera containing the Fc portion of human IgG. There is a possibility that upon treatment, the Fc portion of the antibodies could bind Fcγ receptors (FcγRs) and trigger signalling events in human fetal astrocytes used in this study. From the literature, it is not clear whether the astrocytes express FcγRs in culture (Nitta, Yagita et al. 1992; Vedeler, Ulvestad et al. 1994; Li, Qin et al. 2008). In order to assess whether human fetal astrocytes expressed FcγRs, cells were labelled with anti-FcγR antibodies and analysed by flow cytometry. There are three classes of FcγRs, FcγRI (CD64), FcγRII (CD32), and FcγRIII (CD16) (Schmidt and Gessner 2005), therefore human fetal astrocytes were immunolabelled with anti-CD16, CD32, and CD64-FITC labelled and isotype-matched control-FITC antibodies. The cells were analysed by flow cytometry, and analysis gates were set according to isotype control staining. No significant surface expression of CD16, CD32, and CD64 was detected by flow cytometry (Fig. 3.9). Therefore, FcγRs are not a confounding factor when astrocytes are treated with anti-FasL antibodies or mFasFc protein chimera containing the Fc portion of human IgG_1.

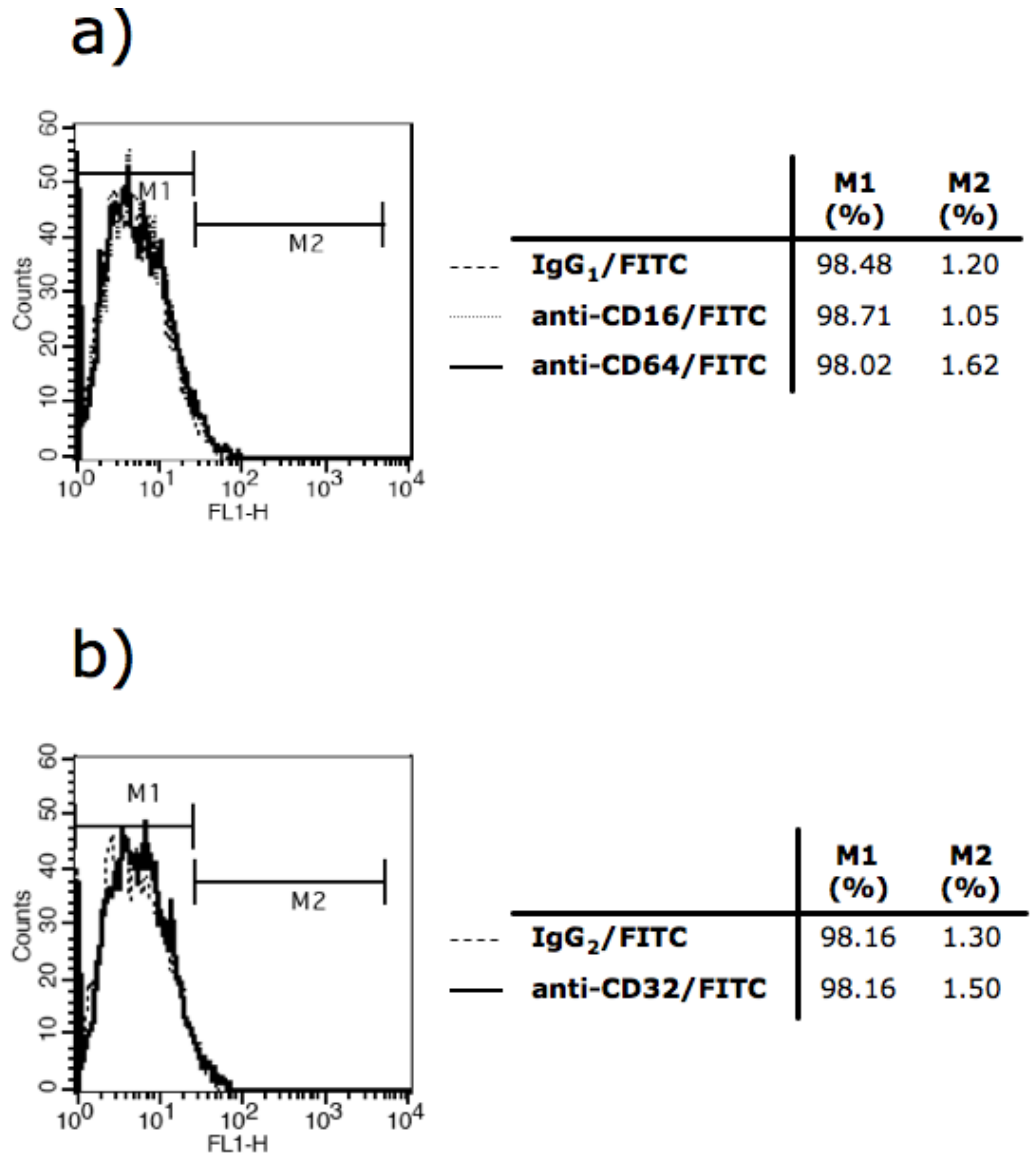


Figure 3.9 Human fetal astrocytes do not express FcγR *in vitro*

Flow cytometric profiles of human fetal astrocytes stained with (a) anti-CD16/FITC and anti-CD64/FITC antibodies or isotype-matched control antibody (IgG_1/FITC) and with anti-CD32/FITC antibody or isotype-matched control antibody (IgG_2/FITC) (**b**). Tables represent the percentage of cells located in M1 or M2 areas of fluorescence intensity.

3.2.4 Analysis of the effects of FasL reverse signalling on gene expression in mouse astrocytes

FasL reverse signalling has been shown to recruit several signalling pathways in T cells (Sun and Fink 2007) and in Schwann cells (section 3.1. of this thesis). In T cells, FasL signalling pathways activate transcription factors AP-1 (c-Jun / c-Fos), NFAT, and NFκB, and may therefore induce alterations of gene expression (Sun and Fink 2007). In order to better understand the biological significance of FasL reverse signalling in glial cells, changes in gene expression induced downstream of FasL reverse signalling were analyzed in mouse astrocytes using DNA microarray technology.

Primary mouse astrocytes were serum-starved overnight and treated with control antibody or mFasFc for either 1.5 h or 12 h in order to target early and late changes in gene expression induced by FasL reverse signalling (Fig. 3.10). Cells were treated in triplicate wells for each condition and processed in parallel.

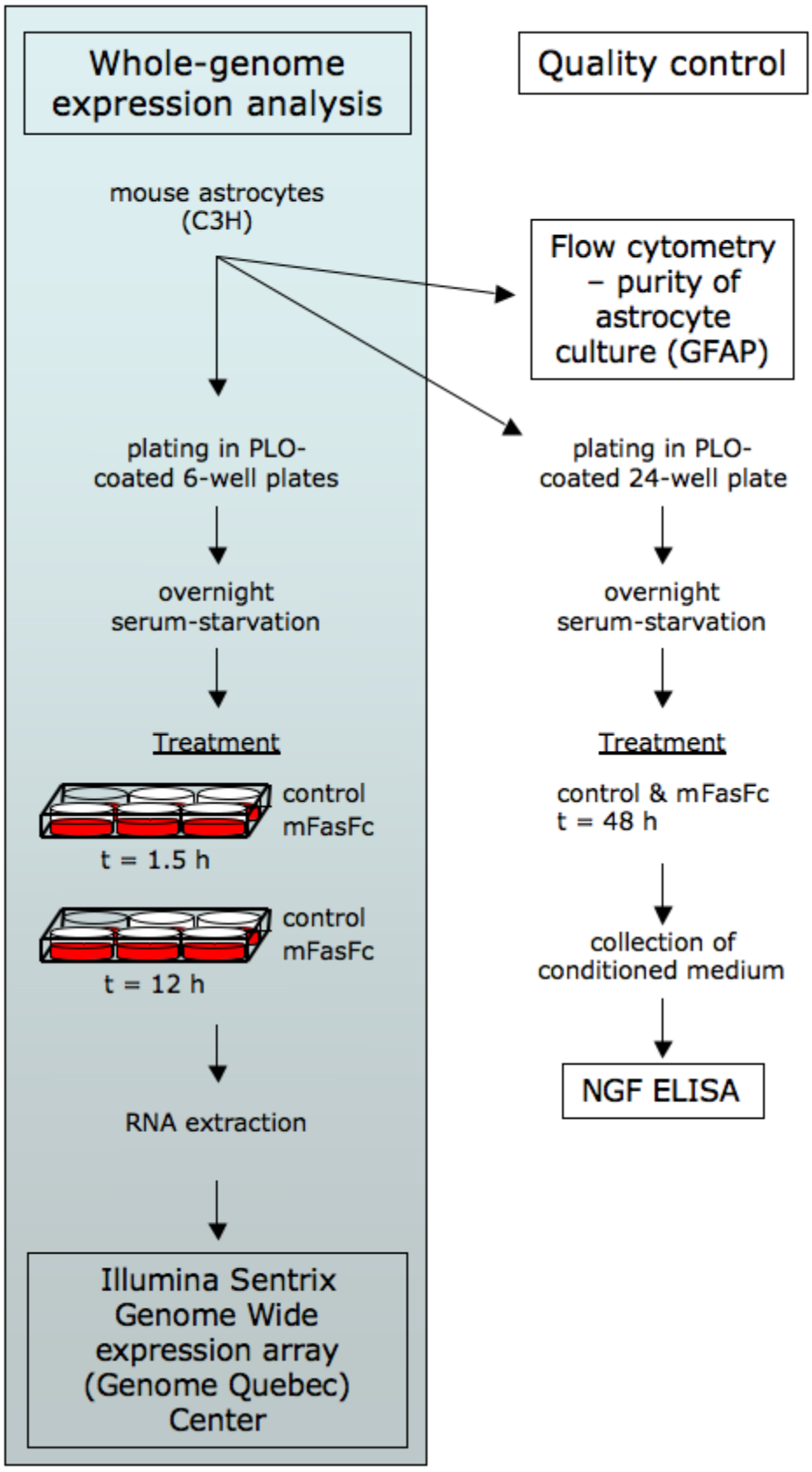

Whole-genome expression analysis
Quality control
mouse astrocytes (C3H)
Flow cytometry – purity of astrocyte culture (GFAP)
plating in PLO-coated 6-well plates
plating in PLO-coated 24-well plate
overnight serum-starvation
overnight serum-starvation
Treatment
Treatment
control
mFasFc
t = 1.5 h
control
mFasFc
t = 12 h
control & mFasFc
t = 48 h
collection of conditioned medium
NGF ELISA
RNA extraction
Illumina Sentrix Genome Wide expression array (Genome Quebec) Center

Figure 3.10 Experimental design of sample preparation and quality control for changes in gene expression triggered by FasL reverse signalling in mouse astrocytes

Astrocytes were plated on 6-well culture dishes for microarray analysis and in a 24-well plate to control for efficacy of the mFasFc treatment by NGF ELISA before performing the DNA microarray experiment. PLO, poly-L-ornithine.

3.2.4.1 Quality control

The purity of the astrocyte culture was assessed by flow cytometry following immunolabelling with an anti-glial fibrillary acidic protein (GFAP) antibody. GFAP is an intermediate filament protein expressed abundantly in astrocytes, which serves as an intracellular marker for astrocytes (Eng, Ghirnikar et al. 2000). About 95% of the cells in the culture were $GFAP^+$ (Fig. 3.11 a). As shown in the diagram of the experimental setup (Fig. 3.10), in parallel to the treatment for the microarray analysis, astrocytes were treated for 48 h with mFasFc and control antibody. NGF ELISA was performed on the conditioned medium collected and confirmed that the mFasFc treatment induced an increase in NGF release (1.7 fold increase, $p<0.01$) (Fig. 3.11 b). Subsequently, RNA was extracted from the cells and the analysis of gene expression was performed at the McGill University and Génome Québec Innovation Centre, Montréal, Canada.

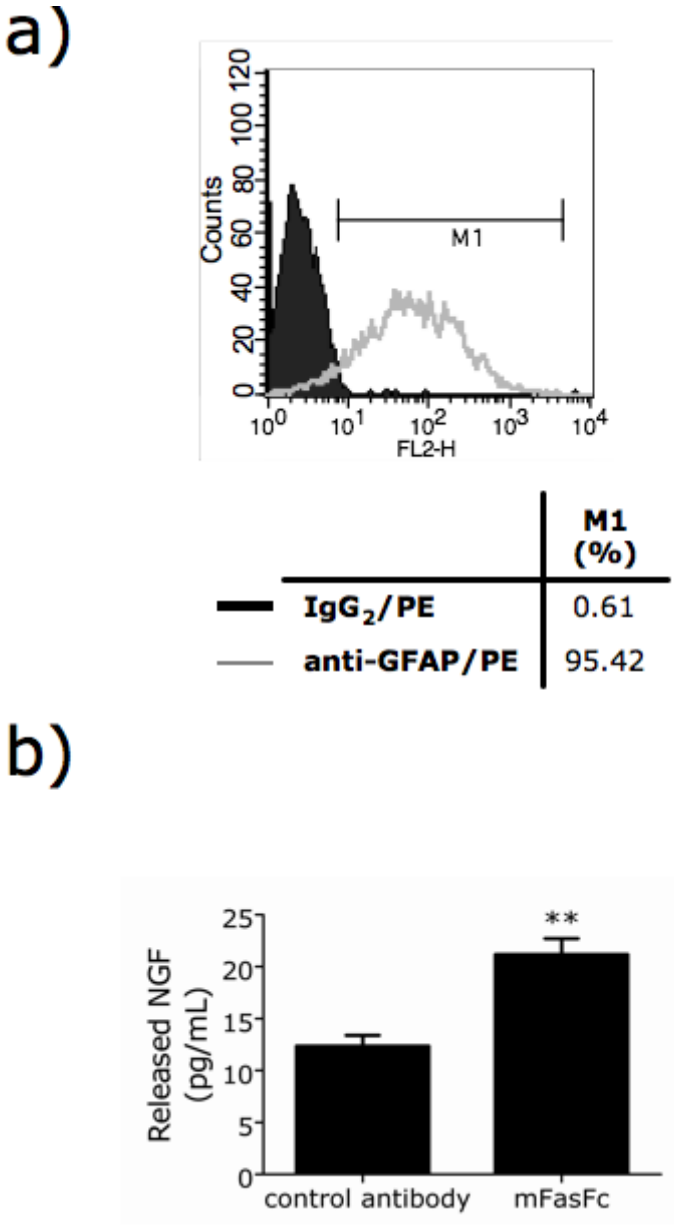


Figure 3.11 Quality control of the microarray experiment

a) Flow cytometric profile of mouse astrocytes stained for intracellular GFAP (anti-GFAP/PE) or isotype-matched control antibody (IgG_2/PE). The table shows the percentage of cells positive for GFAP.

b) FasL engagement induces NGF release in mouse astrocytes treated with mFasFc. Cells were serum-starved overnight and treated for 48 h with mFasFc or control antibody. NGF levels in CM were quantified by ELISA. **, $p<0.01$; Student's t-test.

3.2.4.2 DNA microarray analysis

Data from the DNA microarrays were analysed with FlexArray (Blazejczyk, Miron et al. 2007). In brief, raw data were normalized using the Lumi algorithm designed for the normalization of Illumina BeadArray data. Normalized data was then submitted to the significance analysis of microarrays (SAM) algorithm.

Unexpectedly, the scatter plot obtained for both time points, 1.5 h and 12 h, suggested that the FasFc treatment did not have a broad effect on gene expression (Fig. 3.12 a and 3.13 a). Each dot represents a pair of observations for one gene (x-coordinate representing the measurement in control and y-coordinate the measurement in treated samples for one gene). Since the cloud of dots is tightly placed around the identity line, the majority of gene expression is not altered by FasL engagement with mFasFc.

The Volcano plot obtained after analysis of the normalized data with the SAM algorithm at the 1.5 h time point confirmed that there was no change in expression of the majority of genes between mFasFc-treated and control groups (Fig. 3.12 b). No gene was upregulated or downregulated more than 2 fold and only three genes were induced and one downregulated more than 1.5 fold (with a p value threshold set at 0.04). With a threshold of 1.3 fold change in expression and p values <0.04, only 16 genes displayed a significant increase and one gene a decrease in expression. Out of these genes, five have no known function (Supplementary Tables 3.1 and 3.2). At 12 h, the Volcano plot also showed that the majority of genes were not affected by the mFasFc treatment compared to control (Fig. 3.13 b). With a 1.3 fold threshold and p values <0.04, 21 genes displayed a significant increase in gene expression and no gene was downregulated (Supplementary Table 3.3). At 12 h, no gene was affected more than 1.5 fold by FasL engagement.

The microarray analysis showed that, at least at 1.5 h and 12 h following addition of mFasFc to mouse astrocytes, FasL engagement does not have a major impact on regulation of gene expression.

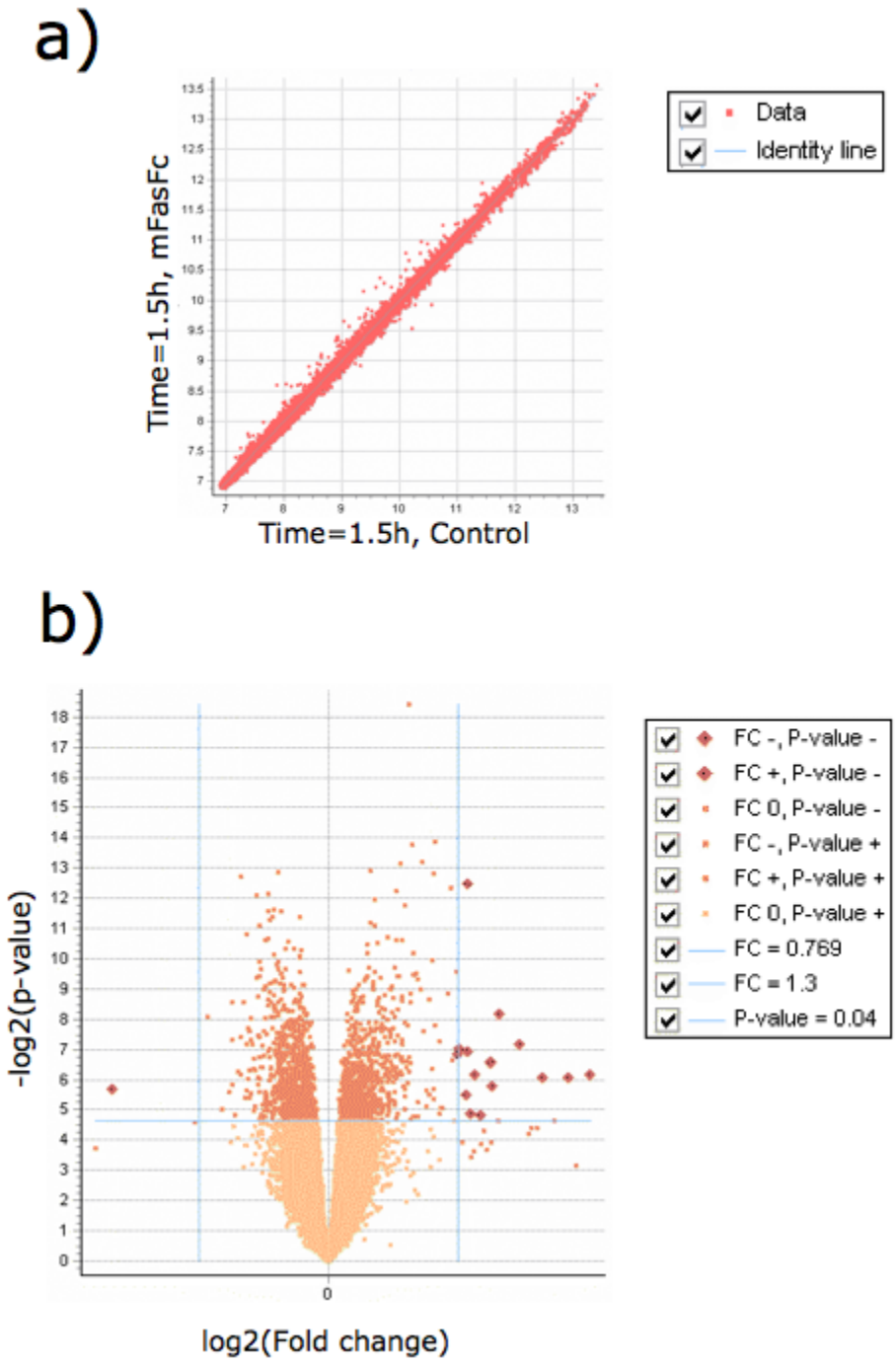


Figure 3.12 Graphical overview of the effects of FasL reverse signalling on gene expression after a 1.5 h treatment with mFasFc compared to control antibodies

a) Scatter plot of data following Lumi normalization of raw data.

b) Volcano plot representing each data point as a function of p-value and fold change following SAM analysis of the normalized data. The thresholds were set at $p<0.04$ and 1.3 fold change in expression. FC, fold change.

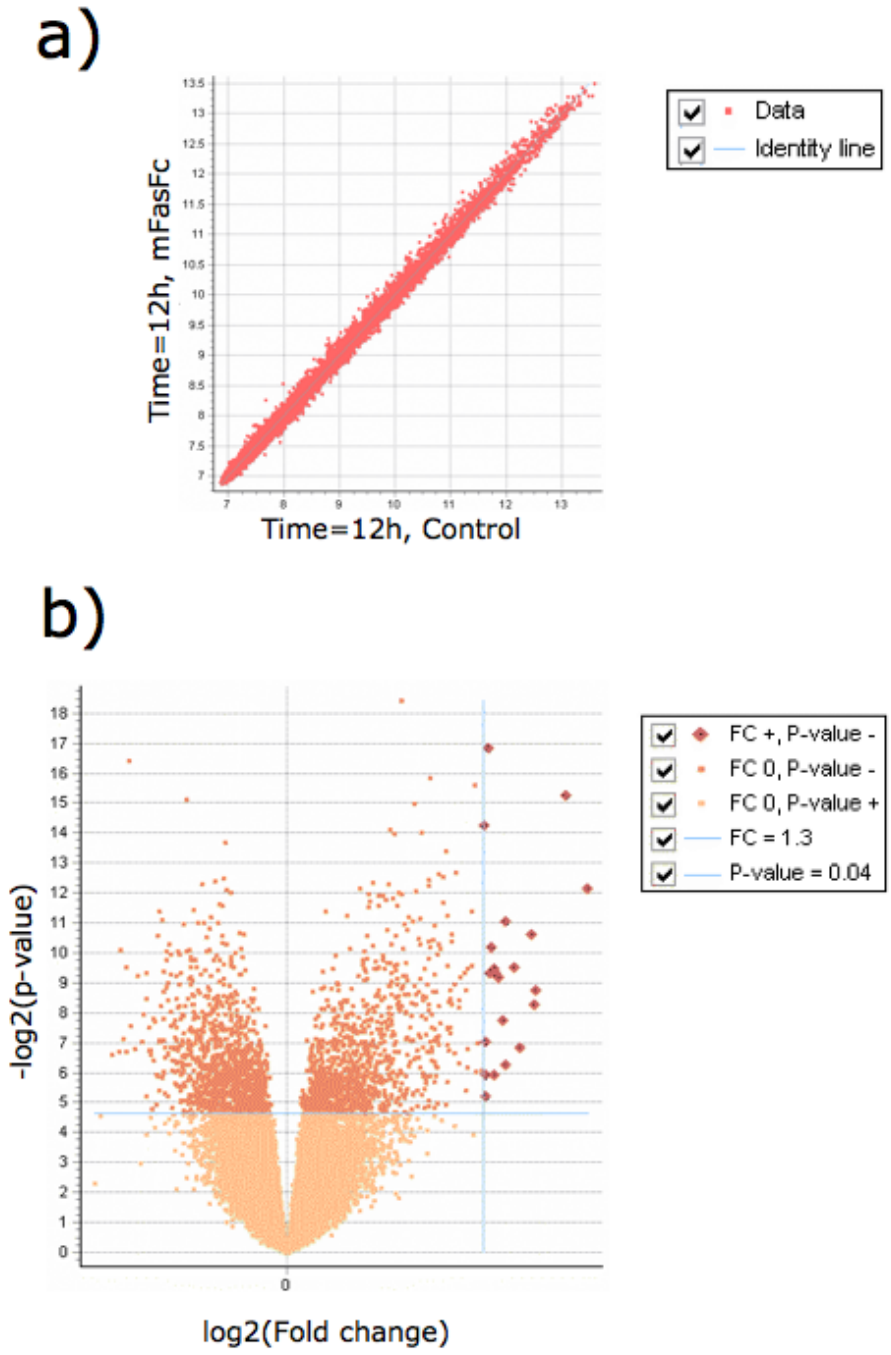


Figure 3.13 Graphical overview of the effects of FasL reverse signalling on gene expression after a 12 h treatment with mFasFc compared to control antibodies

a) Scatter plot of data following Lumi normalization of raw data.

b) Volcano plot representing each data point as a function of p-value and fold change following SAM analysis of the normalized data. The thresholds were set at $p<0.04$ and 1.3 fold change in expression. FC, fold change.

3.2.4.3 Validation

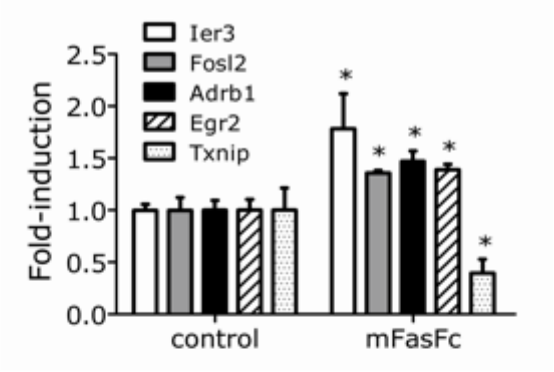
From the lists of genes that displayed a significant change in expression with mFasFc compared to control, a set of relevant genes (genes already known to play a role in either the immune system or the nervous system) were selected for validation of microarray data by RT-qPCR. These genes are presented in Tables 3.1 and 3.2 for the 1.5 h time point and Table 3.3 for the 12 h time point.

Upregulation of the immediate early response 3 gene (Ier3), involved in the regulation of cell growth and apoptosis, as well as differentiation of astrocytes (Wu 2003; You, Osawa et al. 2007), was confirmed at 1.5 h but not at 12 h (Fig. 3.14 a and c). Upregulation of Fos-like antigen 2 (Fosl2), coding for an AP-1 transcription factor (Milde-Langosch 2005), adrenergic receptor β1 (Adrb1), a G-protein coupled receptor (Hein 2006), and the transcription factor early growth response 2 (Egr2 / Krox20) (Svaren and Meijer 2008) was also confirmed at 1.5 h ($p<0.05$; Fig. 3.14 a). The only gene downregulated by mFasFc treatment, thioredoxin interacting protein (Txnip), as detected in the microarray data was also validated ($p<0.05$; Fig. 3.14 a). In contrast, the upregulation of Cyr61, Gbx2, and Rhoe was not validated ($p>0.05$; Fig. 3.14 b). Furthermore, none of the genes displaying an upregulation in the microarray data at 12 h and selected for validation confirmed the difference in expression levels between mFasFc and control treatment (Fig. 3.14 c).

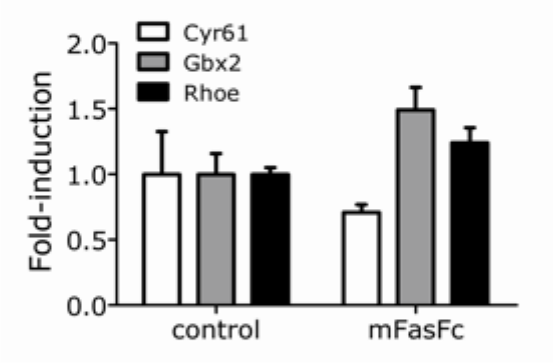
The DNA microarray experiment and the subsequent validation by RT-qPCR reveals that the effect of FasL reverse signalling on gene expression is not major and probably occurs early following engagement of FasL since none of the genes verified showed an increase in expression after 12 h of treatment. Even at 1.5 h, the change in expression levels was only confirmed for five genes out of nine tested. The NGF gene did not appear in the list of genes that displayed a significant change in expression at 1.5 h or 12 h. This is not surprising since the time course of NGF mRNA in mouse astrocytes showed that there was a decrease in NGF mRNA levels in the first 3 h following engagement of FasL (Fig. 3.8 a).

At 12 h, the levels of NGF mRNA were starting to increase but they were not significantly different from the levels at time 0.

a)


Ier3
Fosl2
Adrb1
Egr2
Txnip
Fold-induction
2.5
2.0
1.5
1.0
0.5
0.0
control
mFasFc
*
*
*
*
*

b)


Cyr61
Gbx2
Rhoe
Fold-induction
2.0
1.5
1.0
0.5
0.0
control
mFasFc

c)

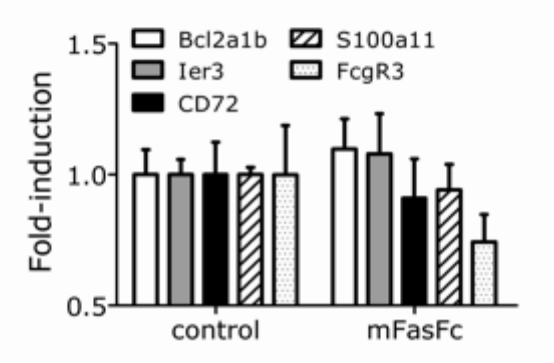

Bcl2a1b
Ier3
CD72
S100a11
FcgR3
Fold-induction
1.5
1.0
0.5
control
mFasFc

Figure 3.14 Validation of microarray data

a) Validation of the increase of Ier3, Fosl2, Adrb1, and Egr2 levels and decrease of Txnip levels after a 1.5 h treatment of mouse astrocytes with mFasFc. Validation was done by RT-qPCR. *, $p<0.05$; Student's t-test.

b) No significant increase in Cyr61, Gbx2, and Rhoe levels after a 1.5 h treatment of mouse astrocytes with mFasFc was detected by RT-qPCR ($p>0.05$; Student's t-test).

c) No significant increase in Bcl2a1b, Ier3, CD72, S100a11, and Fcgr3 levels after a 12 h treatment of mouse astrocytes with mFasFc was detected by RT-qPCR ($p>0.05$; Student's t-test).

Table 3.1 Genes induced by more than 1.3 fold (at 1.5 h) ($p<0.04$) according to microarray data and selected for validation by RT-qPCR

Gene	Definition	Known function	Fold change	P-value
Ier3	immediate early response 3 (Ier3)	regulation of cellular growth and apoptosis; astrocytic differentiation (Wu 2003; You, Osawa et al. 2007)	**1.55**	**0.0150**
Cyr61	cysteine rich protein 61 (Cyr61)	growth factor-inducible immediate early gene; regulator of apoptosis; cell adhesion, migration and differentiation; proangiogenic factor (Chen and Du 2007)	**1.47**	**0.0068**
Fosl2	fos-like antigen 2 (Fosl2)	AP-1 transcription factor; tumor progression; thymocyte development (Milde-Langosch 2005; Lawson, Maurice et al. 2009)	**1.40**	**0.0184**
Adrb1	adrenergic receptor, beta 1 (Adrb1)	G-protein coupled receptor, sympathetic response (Hein 2006)	**1.30**	**0.0076**
Gbx2	gastrulation brain homeobox 2 (Gbx2)	transcription factor; neural tube and hindbrain regions development (Joyner, Liu et al. 2000)	**1.39**	**0.0107**
Egr2	early growth response 2 (Egr2; Krox20)	transcription factor; cell cycle arrest & differentiation; peripheral nerve myelination (Mirsky, Woodhoo et al. 2008; Svaren and Meijer 2008)	**1.34**	**0.0343**
Rhoe	Rho family GTPase 3 (Rnd3)	regulation of cell cycle progression; cell migration, axon pathfinding (Chardin 2006)	**1.35**	**0.0139**

Table 3.2 Gene downregulated by more than 1.3 fold (at 1.5 h) ($p<0.04$) according to microarray data and selected for validation by RT-qPCR

Gene	Definition	Known function	Fold change	P-value
Txnip	thioredoxin interacting protein (Txnip), transcript variant 2	stress-response molecule, tumor suppressor, development and function of NK cells (Kim, Suh et al. 2007)	**0.65**	**0.0192**

Table 3.3 Genes induced by more than 1.3 fold (at 12 h) ($p<0.04$) according to microarray data and selected for validation by RT-qPCR

Gene	Definition	Known function	Fold change	P-value
Bcl2a1b	B-cell leukemia/lymphoma 2 related protein A1b (Bcl2a1b)	regulator of thymocyte survival (Mandal, Borowski et al. 2005)	**1.45**	**0.00003**
Ier3	immediate early response 3 (Ier3)	regulation of cellular growth and apoptosis; astrocytic differentiation (Wu 2003; You, Osawa et al. 2007)	**1.33**	**0.00172**
CD72	CD72 antigen (Cd72),	B-cell co-receptor; B-cell growth and differentiation (Wu and Bondada 2002)	**1.31**	**0.00086**
S100a11	S100 calcium binding protein A11 (S100a11)	Cell growth regulation (He, Li et al. 2009)	**1.39**	**0.00064**
Fcgr3	Fc receptor, IgG, low affinity III (Fcgr3)	regulation of acquired immunity and of innate immune responses (Nimmerjahn and Ravetch 2008)	**1.31**	**0.00001**

Chapter 4. Discussion

4.1. FasL acts as a counter-receptor in Schwann cells and induces the secretion of bioactive nerve growth factor

In this work, we have shown that engagement of FasL can mediate the synthesis and release of NGF in Schwann cells of mouse, rat, and human origin. We have established that FasL reverse signalling is not restricted to cells of the immune system, but also occurs in nervous system glial cells. FasL-induced NGF secretion is independent of Fas expression, since cells from Fas-deficient *lpr* mice release NGF upon FasL engagement to similar extent as Schwann cells from wild-type mice. We have demonstrated that FasL engagement increases NGF mRNA levels, but that *de novo* NGF mRNA synthesis only accounts for a small component of FasL-induced NGF secretion into the culture medium. FasL engagement triggers Src and ERK1/2 activation in Schwann cells, and FasL-induced Src activation is a mediator of downstream NGF synthesis. Finally, FasL reverse signalling may accelerate recovery after a crush injury in the PNS.

To asses whether the increase in NGF release required both *de novo* mRNA and protein synthesis, cultures were treated with actinomycin D and cycloheximide, two well-known inhibitors of transcription and translation, respectively. The less pronounced effect of cycloheximide on NGF protein levels as compared to effect of actinomycin D was surprising. However, cycloheximide is known to affect mRNA stabilization (Shaw and Kamen 1986; Matsuoka, Meyer et al. 1991; Friedman, Thakur et al. 1996) and in particular, NGF mRNA through an AU-rich Element (ARE) in its 3'-UTR (Tang, Wang et al. 1997). Cycloheximide inhibits the synthesis of a putative trans-acting AU-rich binding protein, thereby increasing the stability of the NGF mRNA (Matsuoka, Meyer et al. 1991; Friedman, Thakur et al. 1996). A leakage in translation (at sub-optimal concentration of cycloheximide) with increased mRNA stability may account for the differences observed at the translational and transcriptional levels on NGF production.

The signalling pathways engaged downstream of FasL include the Src tyrosine kinase family and the MAPK / ERK pathway. Activation of FasL signalling is followed by binding of the Src proteins to the FasL intracellular domain and activation of the MAPK / ERK pathway in T cells (Sun, Ames et al. 2006; Sun and Fink 2007; Sun, Lee et al. 2007) and Sertoli cells (Ulisse, Cinque et al. 2000). Here we showed that FasL engagement in Schwann cells recruits at least two different pathways, one through MAPK / ERK that affects NGF secretion, and one via Src which modulates NGF protein expression. It is intriguing that in Schwann cells, these two pathways, both recruited by FasL, appear to have opposite biological outcomes. On one hand, ERK1/2 activation reduces NGF secretion; while on the other hand, stimulation of Src increases NGF production. It is possible that these two effects counterbalance each other to fine-tune NGF secretion or to prevent over-secretion of NGF. In addition, other stimuli that independently activate or inhibit MAPK / ERK may also modulate the final output of NGF following FasL engagement *in vivo*. However, inhibition of Erk1/2 and Src phosphorylation only has modest effect on the synthesis and release of NGF suggesting that other signalling pathways may concurrently be recruited by FasL engagement. In T-cells, FasL cross-linking has been shown to result in the phosphorylation of AKT and JNK and the translocation of NFAT (Sun and Fink 2007) and these pathways may also play a role in Schwann cells.

Human or mouse FasL can engage apoptotic signalling by binding to either human or mouse Fas, suggesting cross-reactivity between species (Takahashi, Tanaka et al. 1994). However, our data show that mouse Fas is unable to trigger FasL reverse signalling in rat and human cells, suggesting that FasL reverse signalling requires higher affinity interactions, or binding of different motifs, than the interactions necessary for induction of apotosis by Fas. The amino-acid sequence identity between human and mouse FasL is 76.9%, and 75.8% between mouse and rat (Takahashi, Tanaka et al. 1994).

Schwann cells are known to upregulate NGF mRNA levels and subsequent NGF protein release into the medium in response to different types of

stimuli, including those that result in PKA activation (the adenylate cyclase activator forskolin or cAMP analogs) or increased intracellular calcium (ionomycin) (Matsuoka, Meyer et al. 1991), sciatic nerve injury (Hengerer, Lindholm et al. 1990), and electrical stimulation (Huang, Ye et al. 2010). It is generally believed that NGF is secreted from Schwann cells through a constitutive pathway via the trans-Golgi network, and that these glial cells do not have a regulated secretory pathway (Mowla, Pareek et al. 1999). On the other hand, a regulated secretory pathway has been described in neurons releasing BDNF, and characterized by the fact that there is more BDNF inside the cells than in the culture medium (Mowla, Pareek et al. 1999). Here, we find that total intracellular NGF levels are higher than total extracellular levels, suggesting that Schwann cells, at least *in vitro,* can release NGF through a regulated secretory pathway. Several studies have shown that NGF release from Schwann cells can be induced through calcium-dependent mechanisms, including ionomycin treatment (Matsuoka, Meyer et al. 1991) and electrical stimulation (Huang, Ye et al. 2010). Furthermore, it was recently reported that vestibular Schwann cells express target soluble NSF attachment protein (SNAP) receptor t-SNAREs (SNAP23 and syntaxin I), vesicle SNAREs (v-SNAREs: synaptobrevin / VAMP2), and cellubrevin, proteins involved in regulated secretory pathways, and secrete BDNF through a regulated secretory pathway (Verderio, Bianco et al. 2006). Together with our data, these results suggest that Schwann cells may also possess a regulated secretory pathway, and that FasL signalling may enhance activity of this secretory pathway.

The analysis of recovery of motor functions following sciatic nerve crush injury in mice suggested that FasL reverse signalling is involved in regeneration since Fas-deficient *lpr* mice, in which FasL signalling cannot be engaged, display a delayed recovery compared to Fas-null mice which cannot induce apoptosis but can still recruit FasL signalling. Recovery from sciatic nerve crush injury in mice occurs spontaneously and is normally completed in 17 to 20 days (Taylor, Vancura et al. 2001; Desbarats, Birge et al. 2003; Narciso, Mietto Bde et al.

2009). Our results are in agreement with a previous study where recovery in *lpr* mice was delayed compared to wild-type (over 20 days vs 15 days), which was attributed to the regenerative role of Fas (Desbarats, Birge et al. 2003). Here, we find that Fas-null mice fully recovered by day 21 which seems to be intermediate between recovery times for *lpr* and wild type mice suggesting a role for the Fas /FasL pair in peripheral regeneration. The study of functional recovery after sciatic nerve crush injury in FasL-deficient *gld* mice would be important to confirm the role of FasL signalling in regeneration. Observation of sciatic nerve cross-sections to determine the myelinating profile of nerves from the different mutant strains compared to wild-type after crush injury would also provide a better insight into the physiological role of FasL signalling in regeneration.

We have shown previously that Fas engagement on sensory neurons can induce axon regeneration (Desbarats, Birge et al. 2003). Here we have found that the cognate ligand for Fas, FasL, can act as a counter-receptor in Schwann cells, induce the release of NGF from these cells, and accelerate functional recovery in an injured peripheral nerve. While FasL is upregulated on Schwann cells *in vivo* during inflammation (Wohlleben, Ibrahim et al. 2000), Schwann cell Fas expression is downregulated (Wohlleben, Ibrahim et al. 2000). In this context, Fas expressed on the surface of neurons is available to bind FasL on adjacent Schwann cells. Following Wallerian degeneration, Schwann cells de-differentiate and switch to a proliferative phenotype (Stoll, Jander et al. 2002). During peripheral nerve injury, engagement of FasL on Schwann cells by Fas expressed on neurons could trigger secretion of NGF and possibly other trophic factors from Schwann cells, while simultaneously stimulating axon regrowth via Fas signalling on neurons (Desbarats, Birge et al. 2003). NGF plays a critical role during PNS development by promoting the maturation and survival of sensory and sympathetic neurons, and is also involved in maintenance and repair after injury (Terenghi 1999; Sofroniew, Howe et al. 2001). NGF promotes migration of Schwann cells in denervated sciatic nerve (Anton, Weskamp et al. 1994) and its synthesis is upregulated by Schwann cells after injury (Heumann, Korsching et al.

1987; Anand, Terenghi et al. 1997). The local release of NGF by Schwann cells following FasL engagement in the context of injury may therefore promote peripheral nerve regeneration.

We have demonstrated a new physiological role for FasL in the nervous system, as a mediator of NGF release in Schwann cells via FasL reverse signalling and activation of the Src kinase pathway. Further work is needed to fully elucidate the molecular pathways of FasL signalling in Schwann cells and to define the physiological role of FasL in the peripheral nervous system in health and disease.

4.2. Effect of FasL reverse signalling on NGF release and on global gene expression in astrocytes

As a follow up to the work done on FasL reverse signalling in Schwann cells, the effect of FasL engagement in astrocytes was studied. Mouse, rat, and human astrocytes also responded to FasL binding by FasL agonists by releasing NGF into the culture medium. As in Schwann cells, this effect is independent of Fas expression since both C3.*wt* and C3.*lpr* astrocytes released NGF upon FasL engagement to the same extent. FasL can therefore trigger reverse signals in glial cells of the PNS and CNS of both rodent and human origin.

As in Schwann cells, NGF mRNA levels were affected by FasL engagement in astrocytes. However, the pattern of induction of NGF mRNA was different in mouse and human astrocytes. While the induction was rapid in human astrocytes, within 2 h, and the levels returned to baseline at 12 h, NGF mRNA levels in mouse astrocytes increased progressively to reach a plateau at 24 h. The extent of the induction was also different, with a maximum of more than five-fold increase in human astrocytes compared to about 1.5-fold in mouse astrocytes. The difference observed in NGF mRNA expression patterns may be

attributed to the species difference and / or to the different developmental stage of the cells, mouse cells being isolated from postnatal mice while human cells are isolated from fetal brain tissue.

It would be interesting to compare the time-courses of NGF mRNA expression versus NGF release into the medium in astrocytes. The regulation of NGF mRNA synthesis and NGF release may be different in astrocytes than in Schwann cells. Astrocytes are known to have a regulated secretory pathway (Calegari, Coco et al. 1999) and NGF may be secreted through this, rather than in a constitutive fashion. The quantification of intracellular NGF content would also give an indication of the type of release of NGF in astrocytes. Upon FasL engagement in Schwann cells, Src and ERK1/2 are activated. Whether this also occurs in astrocytes will be worthwhile examining. It has already been shown that the induction of NGF secretion by astrocytes following treatment with erythropoietin (Park, Lee et al. 2006) or phospholipids (Furukawa, Kita et al. 2007) was mediated by ERK1/2 activation. In these two studies, pre-treatment of the astrocytes with PD98059, a pharmacological inhibitor of the upstream MAPK activator kinase (MEK1/2), abolished the increase in NGF release into the medium following the treatment with erythropoietin or phospholipids. In contrast, another study concluded that the increase in NGF release from astrocytes triggered by TNFα was independent of the MAPK pathway (Galve-Roperh, Sanchez et al. 1998). In fact, the data showed an increase in NGF release when the cells were treated with PD98059, which is similar to what we observed with Schwann cells (section 3.1 of this work). We observed ERK1/2 phosphorylation was observed upon FasL engagement, suggesting that the ERK / MAPK pathway may exert a negative effect on NGF release, which was abolished by the inhibitor. It is therefore possible that in astrocytes, FasL induced NGF release is mediated by the ERK / MAPK pathway as it is in Schwann cells. The use of ERK1/2 and Src siRNAs would provide another mean to verify the involvement of these kinases in NGF mRNA synthesis and protein release.

The global effects of FasL engagement on gene expression were analysed using DNA microarrays. FasL reverse signalling may regulate gene expression in T cells by activating the transcription factors NFAT and AP-1 (Sun and Fink 2007) and was shown here to alter the levels of NGF mRNA in mouse Schwann cells and astrocytes, as well as in human astrocytes. DNA microrarray analysis was the experiment of choice in order to study changes in expression of genes not previously known to be regulated by FasL reverse signalling. Mouse astrocytes were chosen because they are easy to isolate and maintain and generate homogenous cultures (>95% purity). From the microarray data, it appears that in postnatal astrocytes at least, FasL reverse signalling does not induce a broad change in gene expression. However, this may not be the case in fetal astrocytes since NGF mRNA levels are quickly induced following engagement of FasL in human fetal astrocytes or in Schwann cells, where the peak of NGF mRNA occurs at around 3 and 4 h following engagement of FasL, respectively. It is also important to note that the effect of mFasFc treatment on NGF release in the astrocyte preparation used for the microarray experiment was not as high as usual (1.7 fold compared to 3 or more in standard experiments). This low level of response to FasL engagement may account for the weak effect of mFasFc treatment on global gene expression. It might have been better to perform the microarray experiment on a new, more representative preparation of astrocytes. However, the fact that an effect on NGF release was measured led us to believe that the potential effects of FasL engagement on gene expression would still be detectable by microarray analysis. The choice of time points, 1.5 h and 12 h, may also have been suboptimal. The 1.5 h time point was chosen because many immediate early genes reach peak levels around 1 h to 2 h following induction (Fambrough, McClure et al. 1999). However, some immediate early genes, like c-Fos, cJun, Cyr61, and Krox20, may have a peak of expression as early as 15 to 20 min after induction (Fambrough, McClure et al. 1999; Iyer, Eisen et al. 1999). It is possible that this set of genes is the most affected by FasL reverse signalling and was missed because they returned to baseline levels before 1.5 h. Following addition of a stimulus, genes whose expression is changed can be grouped in

clusters depending on the pattern of expression as a function of time (Iyer, Eisen et al. 1999). A 12-h time point was chosen as a time which would allow detection of genes from different clusters which would either be increasing toward a peak at a later time point or decreasing from a peak at an earlier time point (Iyer, Eisen et al. 1999). The observation that none of the genes that seemed induced from the microarray data at 12 h were validated by RT-qPCR suggests that the effects of FasL intracellular signals may occur in a short and transient period of time and that the 12 h time point may be too late to detect any of the changes. This rapid return to baseline may allow for a tighter control of the effects of FasL reverse signalling. However, NGF mRNA levels in mouse astrocytes only started to increase at 24 h and 48 h (it is interesting to note that NGF mRNA levels were not changed at the 1.5-h and 12-h time points, which is consistent with the time course of NGF mRNA levels obtained with mouse astrocytes). In post-natal astrocytes, there may be a delayed effect of FasL signalling via modulation of other pathways.

From the microarray data, it appears that even if FasL does not induce changes in expression of many genes, it does change a small number of them at an early time point. Only one gene, Txnip, was dowregulated by FasL signalling at 1.5 h. The genes validated as induced by FasL at 1.5 h include Ier3, Fosl2, Adrb1, and Egr2. The Ier3 gene is a stress-inducible gene which codes for a protein better known as immediate early response gene X-1 (IEX-1) (Wu 2003). This gene is upregulated by growth factors in fibroblasts and the half-life of Ier3 mRNA is very short (15-20 min) (Charles, Yoon et al. 1993). IEX-1 has been implicated in the regulation of apoptosis and cell survival (Wu 2003). Interestingly, in T cells from transgenic mice overexpressing IEX-1, activated T cell apoptosis is reduced and the mice develop an autoimmune disease similar to the pathology seen in *lpr* and *gld* mice (Zhang, Schlossman et al. 2002). Recently, a role for IEX-1 was found in the induction of differentiation of glioma cells towards an astrocytic phenotype (You, Osawa et al. 2007). These data suggest that Ier3 upregulation by FasL reverse signalling may protect cells from

apoptosis and / or contribute to glial cell differentiation. Further studies of FasL reverse signalling and expression of Ier3 in astrocytes will be necessary to better understand the biological effects of FasL in glial cells. Fosl2, also known as Fos-related antigen 2 (Fra-2), is a member of the Fos family of transcription factors that heterodimerizes with Jun proteins to form AP-1 transcription factors (Milde-Langosch 2005). Fosl2 plays a role in tumor progression and invasion in several types of cancer (Milde-Langosch 2005). It has also been implicated in the development and function of a thymocyte subpopulation (Lawson, Maurice et al. 2009). Adrb1, which codes for the β_1-adrenergic receptor, is important for cardiac function but also plays a role in the brain. It is expressed in the cerebral cortex, thalamus, and sympathetic ganglia (Hein 2006). β_1-adrenergic receptor functions include the regulation of sympathetic tone, modulation of synaptic activity, and retrieval of spatial memory (Hein 2006). β_1-adrenergic receptor activation in astrocytes promotes astrocyte activation and neuroprotection from glutamate toxicity (Junker, Becker et al. 2002), and BDNF synthesis (Juric, Loncar et al. 2008). Finally, the finding that the transcription factor Egr2 (Krox20) is induced in astrocytes by FasL engagement was surprising since Egr2 is mostly known for its role in myelination by Schwann cells in the PNS (Mirsky, Woodhoo et al. 2008; Svaren and Meijer 2008). However, a previous DNA microarray study found that Egr2 was induced in astrocytes in response to hydrostatic pressure (Yang, Agapova et al. 2004). Egr2 may therefore serve some neuroprotective functions in the central nervous system that remain to be elucidated. Furthermore, Egr2 function is not restricted to the nervous system and promotes T cell survival during positive selection (Lawson, Weston et al. 2010). Egr2 also downregulates the proliferation of activated T cells and protects against the development of autoimmune disease (Zhu, Symonds et al. 2008). In T cells, Egr2 expression is upregulated following activation and acts as an inducer of FasL transcription through binding to a FasL regulatory element (FLRE) in the promoter region of FasL (Mittelstadt and Ashwell 1999). Egr2 expression is regulated by NFAT (Rengarajan, Mittelstadt et al. 2000) which is known to be activated downstream of FasL reverse signalling (Sun and Fink 2007). Therefore,

there may be a positive feedback loop regulating FasL expression through NFAT and Egr2 upon engagement of FasL.

The only downregulated gene in this study, Txnip, also known as vitamin D3 up-regulated protein 1 (VDUP1), is a stress-response molecule (Kim, Suh et al. 2007). Upon stress stimuli, Txnip is up-regulated and inhibits cell proliferation. Depending on the cell type, it can also induce apoptosis. From the literature available, upregulation of Ier3, Fosl2, Adrb1, Egr2 and down-regulation of Txnip detected following FasL engagement seem to suggest a role for FasL reverse signalling in differentiation and cell survival. However, the role of these proteins on differentiation and survival has not been extensively studied in the nervous system. The effect of FasL engagement on astrocyte survival and proliferation should be verified at different time points following addition of FasL agonists by MTT cell proliferation assay or [3H]-thymidine incorporation assay.

In T-cells, FasL engagement leads to activation of the AP-1 transcription factor following recruitment of the MAPK pathway, thereby providing a means to modulate gene transcription (Sun and Fink 2007). From the literature (PubMed search), no evidence could be found that Ier3, Fosl3, and Egr2 contained any functional AP-1 binding site in their promoter region. Ovine Adrb1 contains at least one AP-1 binding site in its 5' untranslated region (Tseng, Waschek et al. 1995) but there is no evidence that this site is functional in regulating Adrb1 transcription. On the other hand, human Txnip contains a functional AP-1 binding site in its 5' untranslated region (Billiet, Furman et al. 2008). A search for putative AP-1 binding sites in the 5' region (-2000 bp to +1 bp) of these 5 genes using an internet-based program (Possum, http://zlab.bu.edu/~mfrith/possum/) revealed that all 5 genes contain at least 2 (Adrb1) and up to 10 (Ier3) AP-1 recognition sequences. A detailed study of the promoter region of these genes and of each AP-1 putative binding site would be necessary in order to assess whether AP-1 transcription factors are involved in regulation of the transcription of these genes downstream of FasL reverse signalling.

In summary, this DNA microarray study showed that FasL reverse signalling only modestly affected gene transcription in astrocytes, at least at the time points studied. However, the five genes regulated by FasL engagement have all been implicated in cell growth and survival, and in development in both the nervous and the immune systems. FasL reverse signalling in astrocytes may therefore engage survival pathways along with the secretion of NGF. It may also lead to the secretion of other factors that may affect the survival of neighbouring cells such as neurons. Interestingly, following traumatic brain injury, astrocytes upregulate NGF synthesis and are the primary source of NGF in the injured brain (Goss, O'Malley et al. 1998). Since FasL is upregulated in neurons and glia after injury (Choi and Benveniste 2004), engagement of FasL by Fas may provide a neuroprotective environment in the injured nervous system. The downstream targets of the genes induced by FasL reverse signalling remain to be elucidated to better understand the role of FasL engagement in glial cells.

4.3. Fas / FasL in the nervous system: a receptor / ligand circuit that could contribute to regeneration or apoptosis

Fas and FasL were identified and characterized in the immune system for their role in apoptotic cell death (Nagata and Golstein 1995; Aggarwal 2003). Most of the research on these molecules was therefore concentrated on the immune system and on their roles in T cell and B cell development, in cytolytic activity of CD8+ T cells and NK cells, and in the termination of the immune response by activation-induced cell death in CD4+ T cells (Brunner, Mogil et al. 1995; Dhein, Walczak et al. 1995; Barry and Bleackley 2002). The severe dysfunction of the immune system in mice with spontaneously arising Fas or FasL mutantations, characterized by lymphadenopathy and autoimmunity, was the most evident phenotype of these mice (Nagata and Golstein 1995; Nagata and Suda 1995). This observation contributed to the idea that the main functions of Fas and

FasL were restricted to the immune system. However, the observation that Fas and FasL were expressed in the nervous system (Choi and Benveniste 2004), along with the evidence of apoptotic cell death during development and following injury or in the course of neurodegenerative diseases (Lambert, Landau et al. 2003), opened a new field of study of the Fas / FasL pair in the nervous system. It appeared that signalling from Fas (following engagement by FasL) had many implications for the normal physiology of the nervous system as well as in the context of injury. In their classical role, Fas / FasL can induce apoptosis in supernumerary neurons that do not establish contact with a target cell during development of the nervous system (Contestabile 2002). Fas and FasL also contribute to cell death after stroke and other injuries and in the context of neurodegenerative disorders, exemplified in the literature review of this thesis by motoneuron death. FasL expression in the brain also contributes to the establishement of immune privilege in the nervous system (Lettau, Paulsen et al. 2008). However, the Fas / FasL system can also perform non-apoptotic functions in the CNS. Evidence of cognitive and neurological deficits in Fas-deficient mice demonstrated the importance of non-apoptotic Fas functions in the nervous system. The involvement of Fas function in neurite outgrowth, neuronal branching, and neuroprotection from toxic insults results from the activation of distinct signalling pathways downstream of Fas, through binding of adapter proteins to the Fas intracellular domain, via the DD or other domains of the protein tail (Desbarats, Birge et al. 2003; Lambert, Landau et al. 2003; Zuliani, Kleber et al. 2006; Ruan, Lee et al. 2008).

FasL is now recognized as a counter-receptor, able to trigger reverse signalling in the FasL expressing cells upon engagement by Fas. This new role was also identified and mainly studied in the immune system where it modulates T cell development and positive selection, and stimulates T cell proliferation (Sun and Fink 2007; Lettau, Paulsen et al. 2008). In this context, we explored the biological and functional consequences of FasL reverse signalling in glial cells. We found that FasL engagement resulted in the release of NGF from both

Schwann cells and astrocytes, glial cells of the PNS and CNS respectively. In Schwann cells, FasL-induced release of NGF seems to result from two different processes. A first pool of NGF is released within the first hour of stimulation with an agonistic FasFc protein, followed by release of newly formed NGF resulting from *de novo* transcription / translation of the NGF gene. It is generally believed that Schwann cells do not possess a regulated secretory pathway (Mowla, Pareek et al. 1999) which implies that they can only increase the release of NGF by increasing its synthesis. However, we observe an increase in the release of NGF by one hour while the peak in NGF mRNA levels only occurs around 3 to 4 h. We hypothesize that Schwann cells can store NGF, and potentially other proteins, and that upon stimulation, they can release the content of these vesicles via a regulated secretory pathway. Our hypothesis is supported by a recent study that identified SNARE proteins, known to be involved in regulated secretory pathways, in Schwann cells and showed their role in the regulated secretion of BDNF (Verderio, Bianco et al. 2006). Since the FasL intracellular domain can recruit proteins involved in vesicular trafficking in Schwann cells (Thornhill, Cohn et al. 2007; Thornhill, Cohn et al. 2008), it is possible that it mediates the transport and exocytosis of NGF upon stimulation. ERK1/2 is activated downstream of FasL engagement in Schwann cells, as in T cells (Sun and Fink 2007), but its effect appears to be on the release of NGF rather than on its synthesis. Unexpectedly, ERK1/2 inhibition results in an increase in NGF release suggesting that ERK1/2 activation inhibits release of NGF. SNARE protein phosphorylation is a means of control of exocytosis in neurons. However, ERK1/2 does not seem to be implicated in this control (Snyder, Kelly et al. 2006). SNARE-dependent release of BDNF has also been demonstrated in microglia and in this case as well, ERK1/2 phosphorylation does not seem to play a role in the release of neurotrophin (Trang, Beggs et al. 2009). It is possible that the regulation of secretion in Schwann cells is mediated by different kinases / signalling pathways. Inhibition of SNARE-mediated exocytosis with specific inhibitors such as TAT-NSF700, a peptide inhibiting the ATPase activity of N-ethylmaleimide sensitive fusion protein (NSF), would be

useful in order to confirm the involvement of the SNARE proteins in a potential regulated secretory pathway (Trang, Beggs et al. 2009) in Schwann cells, and to evaluate the role of ERK1/2 activation. Since astrocytes are known to have a regulated secretory pathway (Calegari, Coco et al. 1999), it would also be interesting to determine whether NGF can be stored inside the cells and exocytosed upon FasL engagement and whether ERK1/2 is involved. Immunocytochemistry for NGF in Schwann cells and astrocytes would also give indications on the intracellular localization of NGF, either diffuse in the cytoplasm if it is secreted in a constitutive way or as punctuate in vesicular structures in the case of regulated secretion (Mowla, Pareek et al. 1999). The second phase of the release of NGF is delayed and follows the increase in NGF mRNA levels. This second phase was partially dependent on transcription and translation, and involved activation of Src in Schwann cells. This second phase of NGF release is probably also present in astrocytes since NGF mRNA levels increased with time following engagement of FasL.

The PRD domain present in the intracellular portion of FasL was shown to be crucial for FasL reverse signalling, through binding of various adapter proteins. The intracellular domain of FasL recruits proteins involved in vesicular trafficking and can therefore regulate its own cell surface expression. In addition, signalling molecules are also recruited following FasL engagement and lead to activation and nuclear transport of transcription factors, suggesting that FasL reverse signalling can also regulate gene expression (Lettau, Paulsen et al. 2008). The study of FasL interacting partners in Schwann cells confirmed the involvement of adapter proteins involved in T cells, PACSIN 2 and 3, and identified novel interacting partners, SNX18 and adaptin β (Thornhill, Cohn et al. 2007). SNX18 and adaptin β are involved in endosomal vesicle trafficking in the context of clathrin-mediated endocytosis (Kirchhausen 2002; Park, Kim et al. 2010) and may therefore be involved in reducing the availability of FasL at the plasma membrane (Thornhill, Cohn et al. 2007). The adaptor protein Grb2 also interacts with FasL SH3 domains in T cells and Schwann cells (Sun and Fink

2007; Thornhill, Cohn et al. 2008). Upon receptor activation, Grb2 induces Ras and the MAPK / ERK signalling pathway (Tari and Lopez-Berestein 2001). Grb2 may therefore be involved in the signal transduction from FasL to downstream targets via activation of the MAPK cascade. Knocking down Grb2 with siRNA would be a way to verify whether Grb2 is involved in FasL reverse signalling. Binding of Src family kinases to FasL intracellular domain has been shown in T cells (Sun, Ames et al. 2006; Voss, Lettau et al. 2009) but has not been confirmed in Schwann cells. However, Src phosphorylation following FasL engagement in Schwann cells indicates a role for the Src family kinases which would need to be investigated further.

We showed that NGF secreted by Schwann cells upon FasL engagement was bioactive and induced significant neurite outgrowth from PC12 cells. PC12 cells express both TrkA and $p75^{NTR}$ and they respond to NGF stimulation by undergoing differentiation and by sprouting neurites (Meakin and Shooter 1992). The ELISA kit used to quantify released NGF measured both proNGF and mature NGF. In order to get a better insight into the physiological significance of NGF release by glial cell, it will be necessary to identify the type of NGF released. As discussed in the introduction, the outcome of mature NGF versus proNGF binding to NGF receptors is different and is also dependent on the type of receptor bound. NGF binding to TrkA or TrkA+$p75^{NTR}$ results in the engagement of survival pathways while NGF binding to $p75^{NTR}$ results in cell death (Yoon, Casaccia-Bonnefil et al. 1998; Lee, Kermani et al. 2001). On the other hand, proNGF interaction with $p75^{NTR}$ or $p75^{NTR}$+TrkA induces cell death (Lee, Kermani et al. 2001) and proNGF interaction with TrkA activates survival pathways (Fahnestock, Yu et al. 2004). In the context of the nervous system, multiple interactions between glial cells and neurons may determine the biological outcome of FasL reverse signalling. In the case of injury to the nervous system, Fas and FasL are upregulated in neurons and glia and can interact. Engagement of Fas can result in either apoptosis or death, depending on the intracellular pathways recruited in the cell. Fas engagement in sensory neurons resulted in

neurite outgrowth and accelerated recovery following sciatic nerve injury (Desbarats, Birge et al. 2003). We have shown here that FasL reverse signalling could lead to the release of bioactive NGF. Released NGF can then bind receptors in an autocrine or paracrine mode. Released NGF can bind $p75^{NTR}$ expressed and upregulated after injury on the glial cell surface (Syroid, Maycox et al. 2000), and promote cell migration or myelination in Schwann cells, or decrease the proliferation of astrocytes. NGF can also bind TrkA and / or $p75^{NTR}$ of neurons and result in different outcomes. The biological consequences of NGF release by glial cells depend on numerous factors. The availability of TrkA and / or $p75^{NTR}$ as well as the availability of intracellular signalling proteins is a first element. The second factor is the form of NGF secreted following transport through the *trans*-golgi network and the potential modification of proNGF to mature NGF in the extracellular space by cleavage of the prodomain by MMP7 or plasmin. The third level of regulation is the level of membrane-bound Fas and FasL available to transduce signals versus the level of sFas and sFasL. Cleavage of sFasL also depends on the enzymatic activity of MMPs while the production of sFas results from alternative splicing.

The outcome of Fas and FasL signalling in the nervous system is determined by a complex interplay of many different factors. In normal condition, the Fas / FasL pair probably does not play a significant role in nervous system homeostasis and function in the adult since FasL is expressed at very low levels or not at all (Wohlleben, Ibrahim et al. 2000; Choi and Benveniste 2004). In the context of injury or degeneration, upregulation of Fas and FasL suggests a role for these two molecules (Wohlleben, Ibrahim et al. 2000; Choi and Benveniste 2004). NGF release by glial cell could induce either survival and regeneration or death and further damages depending on the balance between all the different players. We showed that FasL reverse signalling was involved in functional recovery following sciatic nerve crush injury suggesting that NGF and other potential growth factors released following FasL activation are indeed involved in survival and regeneration.

FasL engagement induces NGF release and synthesis but also has the potential to alter global gene expression via the activation and nuclear translocation of transcription factors such as AP-1 and NFκB (Sun and Fink 2007). Interestingly, NFκB is involved in the regulation of NGF mRNA levels following exposure to IL-1 in astrocytes (Friedman, Thakur et al. 1996). NGF gene transcription in astrocytes has also been shown to be upregulated by β-adrenergic receptor activation via upregulation and nuclear translocation of c-fos and possibly through binding of c-fos to the AP-1 site of the NGF promoter (Mocchetti, De Bernardi et al. 1989). However, the analysis of changes in gene expression induced by FasL engagement in astrocytes did not uncover a major effect of FasL reverse signalling on mRNA transcription. Nonetheless, the alterations of gene expression identified early after FasL engagement (increase in IEX-1, Fosl2, β_1-adrenergic receptor, and Egr2; decrease in Txnip), all point to an effect of FasL signalling towards cell survival.

Fas / FasL signalling in PNS may contribute to regeneration by secretion of NGF and possibly other growth factors. In CNS however, the increase in NGF may not be sufficient to overcome the unpermissive environement. Myelin debris are a source of inhibitory molecules, including myelin-associated glycoprotein (MAG), Nogo-A, and oligodendrocyte-myelin glycoprotein (OMgp), which inhibit neurite outgrowth (Profyris, Cheema et al. 2004). However, there are indications that reactive astrocytes may serve neuroprotection roles (Sofroniew and Vinters 2010). Removal of reactive astrocytes from the lesion site after stab injury or moderate crush injury is detrimental for regeneration (Faulkner, Herrmann et al. 2004). Astrocytes appear to protect oligodendrocyte from cell death and demyelination and to be involved in repair after injury (Faulkner, Herrmann et al. 2004). In this context, Fas / FasL signalling may help astrocytes subserve their function. Systemic delivery of NGF as a therapeutic approach failed to induce significant regeneration in the CNS and promoted hyperalgesia (Thuret, Moon et al. 2006). Thus, induction of local secretion of NGF by resident cells may be a better way to help recovery without global side effects. In this

context, specifically engaging the Fas / FasL neuroregenerative pathways might be a targeted way to promote regeneration. However, favoring cell survival, migration, and neurite outgrowth over cell death might be difficult to achieve.

Summary and conclusion

The work presented in this thesis demonstrates that FasL reverse signalling is not restricted to the immune system and can be engaged on glial cells from both the PNS and the CNS. Engagement of FasL results in both NGF secretion and upregulation of NGF synthesis as well as a time-dependent phosphorylation of ERK1/2 and Src. Activation of FasL signalling also leads to the upregulation of a small subset of genes involved in cell proliferation and survival. Only one gene, a stress-response protein, was found to be repressed. FasL signalling did not have a global effect on gene transcription at the time points studied but this does not preclude a more significant effect at different time points. The Fas / FasL proteins are involved in both apoptotic and cell survival / neuroprotection in the nervous system and the regulation of this system is complex. However, the finding that FasL reverse signalling played a role in recovery after sciatic nerve crush injury suggests a role in regeneration for FasL reverse signalling in the nervous system.

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Appendix

Supplementary Table 3.1

List of genes induced by more than 1.3 fold with a 1.5 h mFacFc treatment compared to isotype control ($p<0.04$) (results from DNA microarray analysis)

Gene	Definition	Known function	ACCESSION	Fold change	P-value
Ier3	immediate early response 3 (Ier3)	regulation of cellular growth and apoptosis; role in astrocytic differentiation	NM_133662.1	**1.55**	**0.0150**
Cyr61	cysteine rich protein 61 (Cyr61), mRNA.	growth factor-inducible immediate early gene; regulator of apoptosis; cell adhesion, cell migration, and differentiation; proangiogenic factor	NM_010516.1	**1.47**	**0.0068**
Fosl2	fos-like antigen 2 (Fosl2), mRNA.	AP-1 transcription factor; tumor progression; thymocyte development	NM_008037.3	**1.40**	**0.0184**
Adrb1	adrenergic receptor, beta 1 (Adrb1), mRNA	G-protein coupled receptor, sympathetic response	NM_007419	**1.30**	**0.0076**
Gbx2	gastrulation brain homeobox 2 (Gbx2), mRNA.	transcription factor; neural tube and hindbrain regions development	NM_010262.2	**1.39**	**0.0107**
Egr2	early growth response 2 (Egr2), mRNA	transcription factor; cell cycle arrest & differentiation; peripheral nerve myelination	NM_010118.1	**1.34**	**0.0343**
Rhoe	Rho family GTPase 3 (Rnd3), mRNA	regulation of cell cycle progression; cell migration, axon pathfinding	NM_028810.1	**1.35**	**0.0139**
Sgk	serum/glucocorticoid regulated kinase 1 (Sgk1), transcript variant 6, mRNA	cell survival; cell-cycle progression	NM_011361	**1.32**	**0.0224**

Ldlr	low density lipoprotein receptor (Ldlr), mRNA	cholesterol homeostasis	NM_010700	**1.30**	**0.0088**
Bsf3	cardiotrophin-like cytokine factor 1 (Clcf1), mRNA.	gp130 cytokine family; B-cell stimulatory effects; neurotrophic factor	NM_019952.1	**1.33**	**0.0082**
Ccrn4l	CCR4 carbon catabolite repression 4-like, mRNA.	circadian deadenylase; circadian control of metabolism	NM_009834.1	**1.63**	**0.0149**
Ier5l	immediate early response 5-like (Ier5l), mRNA		NM_030244	**1.39**	**0.0103**
LOC240672	similar to MAP-kinase phosphatase (cpg21), mRNA		XM_140740.3	**1.70**	**0.0141**
2310004N11 Rik	serine/threonine kinase 40 (Stk40), transcript variant 2, mRNA		NM_028800	**1.41**	**0.0035**
4931402H11 Rik				**1.33**	**0.0002**
LOC381140			XM_355056.1	**1.36**	**0.0346**

Shaded boxes indicate genes for which change in expression was verified by RT-qPCR

Supplementary Table 3.2

List of genes downregulated by more than 1.3 fold with a 1.5 h mFacFc treatment compared to isotype control ($p<0.04$) (results from DNA microarray analysis)

Gene	Definition	Known function	ACCESSION	Fold change	P-value
Txnip	thioredoxin interacting protein (Txnip), transcript variant 2, mRNA	stress-response molecule, tumor suppressor, development and function of NK cells	NM_023719	**0.65**	**0.0192**

Shaded boxe indicate gene for which change in expression was verified by RT-qPCR

Supplementary Table 3.3

List of genes induced by more than 1.3 fold with a 12 h mFacFc treatment compared to control ($p<0.04$) (results from DNA microarray analysis)

Gene	Definition	Known function	ACCESSION	Fold change	P-value
Bcl2a1d	B-cell leukemia/lymphoma 2 related protein A1d (Bcl2a1d), mRNA	T-cell differentiation	NM_007536	**1.49**	**0.00022**
Bcl2a1b	B-cell leukemia/lymphoma 2 related protein A1b (Bcl2a1b), mRNA	regulator of thymocyte survival	NM_007534	**1.45**	**0.00003**
Ier3	immediate early response 3 (Ier3) mRNA	regulation of cellular growth and apoptosis; inhibited by 1, 25 D3; role in astrocytic differentiation	NM_133662.1	**1.33**	**0.00172**
Cd72	CD72 antigen (Cd72), mRNA	B-cell co-receptor; B-cell growth and differentiation	NM_007654.1	**1.31**	**0.00086**
S100a11	S100 calcium binding protein A11 (S100a11), mRNA	Cell growth regulation	NM_016740	**1.39**	**0.00064**
Fdps	farnesyl diphosphate synthase (Fdps), mRNA	key enzyme in isoprenoid biosynthesis	NM_134469	**1.34**	**0.00047**
Hmga1	high mobility group AT-hook 1 (Hmga1), transcript variant 1, mRNA	architectural transcription factor	NM_016660.1	**1.32**	**0.01663**
Fcgr3	Fc receptor, IgG, low affinity III (Fcgr3), mRNA	regulation of acquired immunity and of innate immune responses	NM_010188.2	**1.31**	**0.00001**
Ndufa3	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 3 (Ndufa3), mRNA		NM_025348	**1.30**	**0.00005**

D530030D03Rik	Cornichon homolog 4 (Drosophila) (Cnih4), mRNA	selective transport and maturation of TGFalpha family proteins		**1.35**	**0.00135**
LOC381649	Mus musculus similar to Peptidyl-prolyl cis-trans isomerase A (PPIase) (Rotamase) (Cyclophilin A) (Cyclosporin A-binding protein)		XM_355612.1	**1.34**	**0.01294**
Uqcrh	ubiquinol-cytochrome c reductase hinge protein (Uqcrh), mRNA	electron transport chain	NM_025641.2	**1.33**	**0.00458**
Tomm7	translocase of outer mitochondrial membrane 7 homolog (yeast) (Tomm7), mRNA	mitochondrial import machinery for preproteins	NM_025394	**1.32**	**0.00154**
A530032J19Rik			AK079976	**1.39**	**0.00228**
LOC384146			XM_357448.1	**1.39**	**0.00322**
1810022K09Rik			XM_485210	**1.37**	**0.00876**
LOC381860	similar to A430096B05Rik protein mRNA		XM_355873.1	**1.31**	**0.00153**
LOC381439	similar to ribosomal protein L31		XM_355397.1	**1.32**	**0.00140**
LOC382215	similar to farnesyl pyrophosphate synthase (LOC382215), mRNA		XM_356328.1	**1.31**	**0.01664**
LOC241737	similar to 60S ribosomal protein L9 (LOC241737), mRNA		XM_141567.3	**1.30**	**0.02680**
2610024B07				**1.30**	**0.00773**

Rik					

Shaded boxes indicate genes for which change in expression was verified by RT-qPCR

Other contribution to knowledge

Fas Ligand is a mediator of cachexia and muscle autophagy *in vivo*

Wenjing Ruan, Manuelle Mimouni-Rongy, Rosmarie Siegrist-Johnstone, Yoon Kow Young, and Julie Desbarats
Submitted