UNIVERSITAT DE BARCELONA

MAG, Nogo-A and NgR in Hippocampal Development and Regeneration

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Ana Mingorance Jiménez de la Espada

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DEPARTAMENTO DE BIOLOGÍA CELULAR
FACULTAD DE BIOLOGÍA
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Memoria presentada por la licenciada en Biología Ana Mingorance Jiménez de la Espada para optar al grado de Doctor en Biología.

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Barcelona, Octubre 2005

VºBº del Director de la Tesis | El Doctorando

Dr. José A. del Río Fernández | Ana Mingorance Jiménez de la Espada

Index i

Abbreviations iii

List of illustrations vi

List of tables viii

Prologue ix

Introduction 1

I. Axonal Regeneration........ 1

1.1 Axonal regeneration: the problem 1

1.2 Factors implicated in axonal regeneration failure 2

1.3 Strategies to promote axonal regeneration 7

1.4 Human trials: state-of-the-art 15

1.5 Lesion models 15

II. Inhibitory molecules for axonal regeneration........ 17

2.1 Axon guidance molecules 17

2.2 Proteoglycans 20

2.3 Myelin-Associated Inhibitors 21

III. Receptors and signal transduction........ 32

3.1 Membrane proteins interacting with the Myelin-Associated Inhibitors 32

3.2 Intracellular Pathways mediating inhibition of axonal regeneration 38

IV. The Hippocampus and Axonal Regeneration........ 45

4.1 Hippocampal basic anatomy 45

4.2 Intrinsic and Extrinsic hippocampal connections 47

4.3 Hippocampal development 48

4.4 The Perforant Pathway lesion as a model 49

4.5. In vivo vs. in vitro: entorhino-hippocampal organotypic cultures 54

Aims 59

Results 60

Chapter I. Regulation of Nogo and Nogo receptor during the development of the entorhino-hippocampal pathway and after adult hippocampal lesions 60

Chapter II. Overexpression of Myelin-Associated Glycoprotein after axotomy of the perforant pathway........ 76

Chapter III. Chondroitin sulfate and NgR-mediated inhibition prevent regeneration of the entorhino-hippocampal pathway 89

Summary of Results and Discussion 103

I. The role of the Myelin-Associated Inhibitors in axonal regeneration........ 103

1.1. The developmental expression of MAG and NgR, but not that of Nogo-A, correlates with the loss of axonal regeneration of the PP. 103

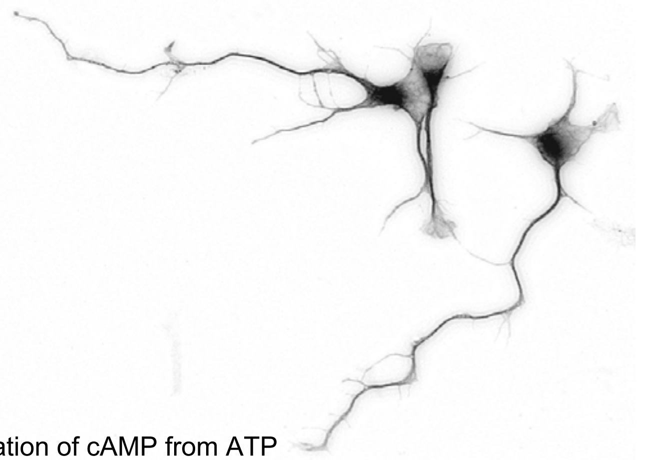
1.2. Neuronal Nogo-A and NgR expression is regulated by activity. 105
1.3. Nogo-A, MAG and NgR expression is differentially regulated following PP axotomy. 111
1.4 Blockade of Nogo-A and MAG promotes axonal regeneration after PP axotomy in vitro. 116
1.5 CSPG and Nogo-66 do not have synergistic effects on axonal regeneration. 118
II. Nogo-A during neuronal development…………………………………………………………….. 122
2.1. Nogo-A is expressed by neurons during development 122
2.2. Contact with Nogo-A induces fasciculation in vitro 123
2.3. IN-1 induces axonal growth independently of the presence of external Nogo-A 126
2.4. Nogo-A does not self-interact, but binds to Nogo-B. 127
III. Myelin contribution to axonal regeneration failure………………………………………………… 129
3.1 Myelin and Evolution. 129
3.2 Why inhibition? 130
IV. Many inhibitors but one single way to inhibit neurons: intracellular convergence………… 132
V. Development and Regeneration: protein recycling………………………………………………… 134

Conclusions 136

References 137

Summary in Spanish 159

Annex: Nogo-A Functions during the development of the CNS and in the adult. 181

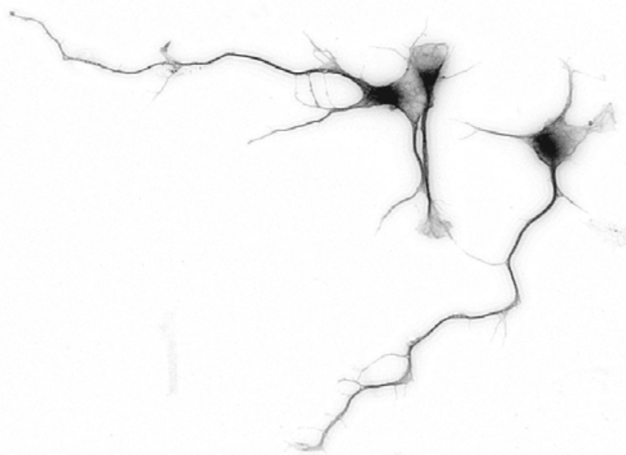


Abbreviations

293T	Human embryonic kidney cell line
AC	Adenylate cyclase, catalizes the formation of cAMP from ATP
AP	Alkaline-Phosphatase
BBB	Blod-Brain Barrier
BDNF	Brain Derived Neurotrophic Factor
CA3-CA1	Cornus Ammonis, hyppocampal pyramidal layer subdivisions
CALI	Chromophore-Assisted Laser Inactivation
CaMKII	Ca^{2+} -calmodulin-dependent protein kinase II
cAMP	Cyclic AMP
CaN	Ca2+ -calmodulin-dependent phosphatase, Calcineurin
CAP-23	Cytoskeleton-Associated Protein of 23 kDa
Caspr	Contactin-Associated PRotein
cGMP	Cyclic GMP
CGNs	Cerebellar Granule Neurons
ChABC	Chondroitinase ABC
CHO	Chinese hamster ovarian cell line
CNS	Central Nervous System
COS7	Transformed african green monkey kidney fibroblast cells
CREB	cAMP response element binding protein
CS, CSPG	Chondroitin Sulfate Proteoglycan
CST	CorticoSpinal Tract
DAG	Diacylglycerol
DAL	Days after lesion
db-cAMP	Dibutyril-cAMP, agonist of cAMP
DCC	Deleted in Colorectal Cancer, receptor for Netrin-1
DIV	Days *in vitro*
dMAG	Soluble derivative of MAG
dnNgR	Dominant negative form of NgR
DRG	Dorsal Root Ganglion
DS, DSPG	Dermatan Sulfate Proteoglycans
E12-E18	Embryonic stages, from E0 to E18 (or E0.5 to E18.5) in mice. E0 = day of plug.
ECL	Entorhinal Cortex Lesion
ECM	Extracellular Matrix Protein
EHP	Entorhino-Hippocampal Pathway
ER	Endoplasmic reticulum
Erk 1/2	Extracellularly Regulated Kinases, a MAP kinase (Mitogen Activated Protein)
Fab	Fragment Antigen Binding, portion of an antibody that includes the variable ends
FF	Fimbria-Fornix
GAG	GlycosAminGlycan
GAP-43	Growth-Associated Protein of 43 kDa
GD1a	Disialoganglioside 1a, Ganglioside, receptor for MAG
GDNF	Glial Derived Neurotrophic Factor
GDP	Guanosin di-phosphate
GFAP	Glial Fibrilar Acid Protein, marker of astroglya
GL	Granule Layer
GPI	Glycophosphatidylinositol, anchors some proteins to the cell membrane
Gsk3B	Glycogen Synthase Kinase-3

GT1b	Trisialoganglioside 1b
GTP	Guanosin tri-phosphate
GTPase	Enzyme that binds and Hydrolyzes GTP
H	Hilus, area of the dentate gyrus
HAL	Hours After Lesion
HS, HSPG	Heparan Sulfate Proteoglycans
ICC	Immunocytochemistry
Ig	Immunoglobulin
IN-1	Inhibition Neutralization, antibody against NI-250
IP3	Myo-inositol-1,4,5-trisphosphate
ISH	*In Situ* Hybridization
JNK	c-Jun amino-terminal kinase, a MAP kinase
KA	Kainic Acid
kDa	Kilo Daltons, unity of molecular weight
KS, KSPG	keratan sulfate proteoglycans
L1	Cell adhesion molecule from the immunoglobulin superfamily
LEA	Lateral Entorhinal Area
Lingo	LRR and Ig domain-containing, Nogo Receptor-interacting protein Also LERN1 (leucine-rich repeat neuronal protein 1)
LRR	Leucine-rich repeat. Domain characteristic of a family of proteins
MAG	Myelin-Associated Glycoprotein
MAI	Myelin-Associated Inhibitor
MBP	Myelin Basic Protein
MEA	Medial Entorhinal Area
mg/kg	Milligram per kilogram, unidy for doses
ML	Molecular Layer
NANase	Neuraminidase
NC	Neocortex
NCAM	Neural Cell Adhesion Molecule
NEP1-40	Nogo Extracellular Peptide, residues 1-40
NG2	CSPG4, expressed by OPCs
NgR	Nogo Receptor
NgR310	NgR extracellular fragment lacking the correceptor-interacting domain. Dominant negative form of NgR
NI-250, NI-35	For “Neurite growth Inhibitor” and the corresponding molecular weight
NiG	Nogo-A specific region
NIMP	Nogo Interacting Mitochondrial Protein
NP-1	NeuroPilin-1 receptor for semaphorins. Do not confound with Neuronal Pentraxin-1
NT3	Neurotrophin 3
OMgp	Oligodendrocyte Myelin Glycoprotein
OPCs	Oligodendrocyte precursor cells
P0-P21	Postnatal stages. In mice generally from P0 (birth day) to P21. Afterward refered as “adult”.
PDE4	Phosphodiesterase specific of neurons (in the CNS), enzyme that cleaves cAMP into AMP
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol-4,5-biphosphate
PKA	Protein Kinase A, activated by cAMP
PKC	Protein Kinase C, activated by calcium and DAG
PKG	Protein Kinase G, activated by cGPM
PLC, PI-PLC	Phospholipase-C, enzyme that cleaves GPI groups relearing GPI-anchored proteins from the plasma membrane
PNS	Peripheral Nervios System
PP	Perforant Pathway, also EHP (entorhino-hippocampal pathway)
PTX	Pertusis toxin
RFP	Red Fluorescent Protein, used as tag for recombinant proteins

RHD	Reticulon Homology Domain
Rho-GDI	GDP-dissociation inhibitor specific for the Rho family, keeps Rho-GTPases inhibited when bound to them
ROCK	Rho-Associated Coiled-Coil-Containing Protein Kinase, effector of Rho-A
RTN	Reticulon. Family of related proteins including Nogo (RTN4)
S	Subiculum
SCI	Spinal Cord Injury
SDS-PAGE	SDS- Polyacrylamide Gel Electrophoresis
Sema	Short form for Semaphorins, a family of guidance cues
SLM	Stratum Lacunosum Moleculare
SO	Stratum Oriens
SP	Stratum Piramidale
SR	Stratum Radiatum
TAG-1	Transiently Expressed Axonal Glycoprotein-1, a cell adhesion molecules
TBI	Traumatic Brain Injury
TNFR	Tumour Necrosis Factor Receptor, characterizes a superfamily of receptors
WGA	Wheat Germ Agglutinin, a lectin that binds to oligosaccharides abundantly found on the cell surface but also bound to intracellular organelles.



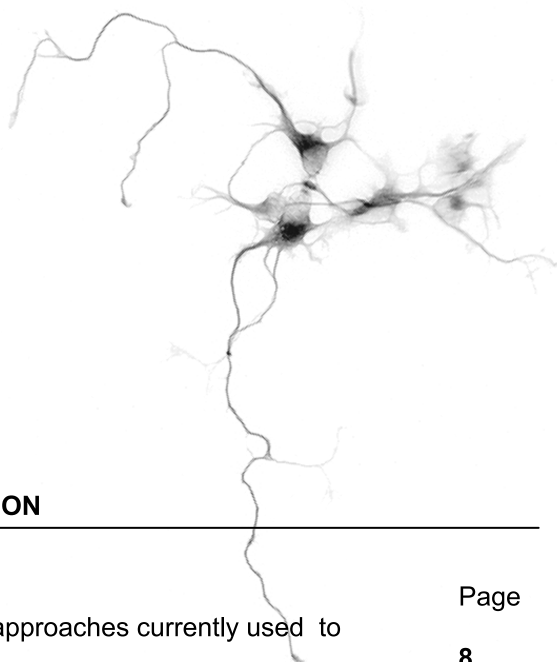
List of illustrations

INTRODUCTION

Number	Name	Page
1	Fig. 1.1. Scheme showing some of the changes occurring during CNS development	**2**
2	Fig. 1.2. Correlation between developmental changes in neurite outgrowth of DRG neurons on myelin and endogenous levels of cAMP	**3**
3	Fig. 1.3. Composition of the glial scar after three stereotypical CNS lesions	**5**
4	Fig. 1.4. Reactive astrocyte morphology during the development of the glial scar	**5**
5	Fig. 1.5. CNS myelin inhibits axonal extension in vitro	**6**
6	Fig. 1.6. Representation of the main approaches used to block the effects of myelin-associated inhibitors extracellularly.	**8**
7	Fig. 1.7. Scheme of the main elements involved in cAMP regulation of axonal regeneration	**12**
8	Fig. 1.8. Scheme of Rho-GTPases equilibrium in the regulation of axonal regeneration.	**13**
9	Fig. 1.9. Human trials	**15**
10	Fig 2.1. Scheme representing the main four families of axon guidance cues and their receptors	**17**
11	Fig. 2.2. Semiquantitative RT-PCR analysis of the expression of semaphorins by purified optic nerve glia	**19**
12	Fig. 2.3. Structure of a CSPG	**20**
13	Fig 2.4. Structure of some members of the Ig superfamily implicated in growth and guidance in the nervous system	**22**
14	Fig. 2.5. During development, MAG can act as both a receptor and a ligand	**23**
15	Fig. 2.6. Table and figure extracted from the original article by Caroni and Schwab where they first describe the existence of two myelin-associated proteins with inhibitiory properties	**25**
16	Fig. 2.7. Structure of Nogo-A, -B and –C, and their relationship to one another	**26**
17	Fig. 2.8. Taxonomic distribution of the RTN gene family	**26**
18	Fig. 2.9. Localization and topology of Nogo-A in different cells	**27**
19	Fig. 3.1. Scheme of the neuronal receptors that interact with the myelin-associated inhibitors	**32**
20	Fig. 3.2. Orthogonal stereoviews of the NgR structure	**33**
21	Fig. 3.3. MAI receptor complex formed by NgR, p75 and Lingo	**35**
22	Fig. 3.4. Structure of Lingo-1	**36**
23	Fig. 3.6. A cultured sensory neuron extending a growth cone with long thin filopodia	**35**
24	Fig. 3.7. The actin and microtubule cytoskeleton in the growth cone	**39**
25	Fig 3.8. MAI regulation of actin dynamics	**39**
26	Fig. 3.9. Calcium regulation of the bidirectional growth cone turning induced by MAG	**40**
27	Fig. 3.10. Regulation of Ca^{2+} signalling by cAMP /cGMP balance	**41**
28	Fig. 3.11. Generation of IP3 and activation of PKC following G_i and PLC activation	**42**
29	Fig. 3.12. Comaparison between the state of the three regulatory pathways described in this chapter in the embryonic and mature neurons	**44**
30	Fig. 4.1. Cytoarchitecture of the entorhino-hippocampal formation	**46**
31	Fig. 4.2. Dentate gyrus subdivisions	**47**
32	Fig. 4.3. Pattern of hippocampal innervation	**47**
33	Fig 4.4. Myelin in the entorhino-hippocampal formation	**49**
34	Fig 4.5. Scheme of the knife-lesioning used in our laboratory	**50**
35	Fig 4.6. (next page) Comparison between electrolytic lesion and axotomy of the PP	**51**
36	Fig 4.7. Representation of the interface culture method and the experimental design we have followed with the organotypic cultures	**55**

SUMMARY OF RESULTS AND DISCUSSION

Number	Name	Page
37	Fig 1.1. Developmental expression of Nogo-A, MAG and NgR	**104**
38	Fig. 1.2. Scheme of the three gradients of neuronal maturation observed in the hippocampus during development	**105**
39	Fig. 1.3. Kainic acid-induced regulation of Nogo-A expresión	**108**
40	Fig. 1.4. Regulation of Nogo-A and NgR by glutamate and BDNF in hippocampal cultures	**109**
41	Fig. 1.5. Model of the putative interactions between Nogo-A and NgR at the synapse	**110**
42	Figure 1.7 Scheme of Spinal Cord Injury (SCI) and Perfoant Patway (PP) axotomy comparing the reactivity of mature oligodendrocytes as assessed by regulation of MBP and MAG expression	**112**
43	Figure 1.8. Overexpression of Nogo-A in the entorhinal cortex after axotomy of the perforant pathway	**114**
44	Figure 1.9. Overexpression of Nogo-following fimbria-fornix lesioning	**115**
45	Fig. 1.10. Regulation of Sema 3a protein levels after axotomy in vitro	**116**
46	Figure 1.11. Analysis of maximal axon length and regeneration specificity obtained with the different treatments	**119**
47	Figure 2.1. Subcellular localization of Nogo-A in developing neurons	**122**
48	Fig. 2.2. Expression pattern of Nogo-A during embryonic development in mice	**124**
49	Figure 2.3. Effect of IN-1 on neurite length of P5 CGNs	**126**
50	Fig 2.4. Nogo-A intracellular interactions. A-B) Nogo-A does not interact with itself in the entoplasmic reticulum	**128**
51	Figure 3.1. Evolutionary tree illustrating the correlation of myelin inhibitors with the loss of axonal regeneration during mammalian evolution	**130**



List of tables

INTRODUCTION

Number	Name	Page
1	Table 1.1. Summary of the main therapeutic approaches currently used to promote axonal regeneration	**8**
2	Table 1.2. IN-1 in vivo treatments	**9**
3	Table 2.1. Nogo-deficient mice characteristics	**30**
4	Table 2.2. Some of the LRR-family proteins expressed in the CNS	**31**
5	Table 4.1. Summary of the main approaches that have been assayed to induce the regeneration of entorhinal axons following PP axotomy in organotypic cultures when we started this project	**57**

SUMMARY OF RESULTS AND DISCUSSION

Number	Name	Page
6	Table 1.1. Kainic acid-induced regulation of Nogo-A, NgR and related proteins.	**107**
7	Table 1.2. Lesion-induced Nogo-A regulation	**113**
8	Table 1.3. Regeneration of entorhinal axons following PP axotomy in organotypic cultures with different treatments	**117**

PROLOGUE

This thesis was begun in April 2002 in the Cellular Biology Department of the University of Barcelona. It arose from a research line initiated by Dr. Jose Antonio del Rio on the characterization of the factors that regulate the regeneration of the connection between the entorhinal cortex and the hippocampus. After several months of screening, we focused on the topic that seemed to have the greatest potential: the study of two proteins of myelin and their neuronal receptor, which had recently been cloned and had motivated the interest of the scientific community.

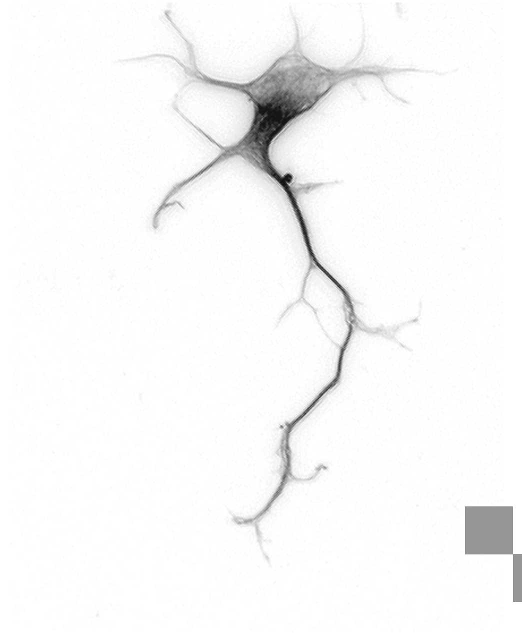
The question this thesis aims to address is, therefore, the *role of myelin-associated inhibitors in the regeneration of cortical connections*. The model we have used is the entorhino-hippocampal connection and the conceptual structure followed was i) characterizing the temporal expression of the proteins, ii) analysing their regulation after lesion, and iii) studying the effect of their blockade on axonal regeneration.

To place our work in context, the introduction provides the current general knowledge about CNS axonal regeneration. The articles that comprise the main body of this thesis are organized transversally to the conceptual structure described above. This responds to the sequence in which the results were obtained, and also to the greater facility of publishing articles that cover a range of aspects about the functional characterization of a protein. However, in order to preserve the thematic organization of this thesis and to facilitate its understanding, these studies will be discussed following the three thematic blocks (i.e. development, regulation and blockade).

Finally, I would like to remark that this thesis, like many others, is not the story of *what we wanted to do*, but that of *what worked out well*.

Ana Mingorance,

Barcelona, September 2005.



Introduction

Introduction I

Axonal Regeneration

1.1 Axonal regeneration: the problem

Every year, over 1000 Spaniards sustain traumatic spinal cord injury (SCI). It is estimated that only 0.9% of them will have a complete neurological recovery, while the rest will suffer sever motor, sensorial and cognitive sequelae. More than half have of these individuals are under 30 years of age[1].

Spinal cord injury is one of the main examples of central nervous system (CNS) lesion. Unlike the peripheral nervous system (PNS) or the embryonic CNS, axons from the adult CNS are unable to regenerate spontaneously after lesion (Horner and Gage, 2000; David and Lacroix, 2003). Advances in cellular biology during the last decade have provided us with a wide knowledge about the mechanisms that regulate neuronal development. However, this knowledge is still partial and an effective solution for CNS injuries remains to be found (for review Horner and Gage, 2000; David and Lacroix, 2003).

Pioneering work by Tello and Ramón y Cajal, during the first decades of the XX century, demonstrated that axonal regeneration failure in the CNS is not due to intrinsic properties of central neurons and reported, for the fist time, the presence of neurite outgrowth inhibitors in the CNS (Ramón y Cajal, 1928). Efforts by David and Aguallo (1981), and later by Caroni and Schwab (1988), ultimately demonstrated that adult CNS is non-permissive for axonal regeneration, and that part of this inhibition is due to the presence of inhibitory molecules in association with myelin (David and Aguayo, 1981; Caroni and Schwab, 1988). Currently, it is accepted that the reduced regeneration capacity of CNS axons results from a combination of factors, including the partial activation of axonal-growth programs after lesion and the presence of inhibitors in the adult CNS.

Given this multifactorial problem, the characterization of the distinct mechanisms that regulate axonal regeneration is essential for the design of strategies to repair CNS injury. In this regard, this thesis focuses on the characterization of several of the inhibitory molecules present in the CNS. We have analysed the role of myelin-associated inhibitors and their common receptor in the adult CNS under normal and pathological conditions, comparing their participation in axonal regeneration failure with that of other known inhibitors (such as proteoglycans) and explored the possible functions performed by these proteins during CNS development. In this introduction we aim to provide a general overview of the processes that occur after CNS traumatic lesion, and the mechanisms that prevent axonal regeneration, focusing on the role of myelin-associated inhibitors.

1.2 Factors involved in axonal regeneration failure

For axonal regeneration to succeed, a number of conditions must be fulfilled. First, neuronal soma must survive after lesion; afterwards, the lesioned axon must be able to form a new growth cone, reach its original target and make synapsis with it, and finally, the new connection must be myelinated. While the whole process is completed in the PNS after lesion, in the CNS it is limited to an abortive formation of a growth cone, after which there is no elongation, and regeneration fails. Another dichotomy occurs between embryonic and mature CNS. Embryonic CNS neurons of many vertebrates regenerate quickly after diverse traumatisms, as occurs after complete transection of spinal cord in chicken. In these cases, fully functional recovery is achieved when the lesion is performed before embryonic day 13 (of the 21-day developmental period; Hasan *et al*., 1993). As the CNS matures, there is a decrease in the capacity of neurons to regenerate after lesion, culminating in a complete lack of regeneration after early postnatal stages.

Both environmental changes in the CNS and endogenous changes in the neurons as they mature appear to contribute to this gradual loss of

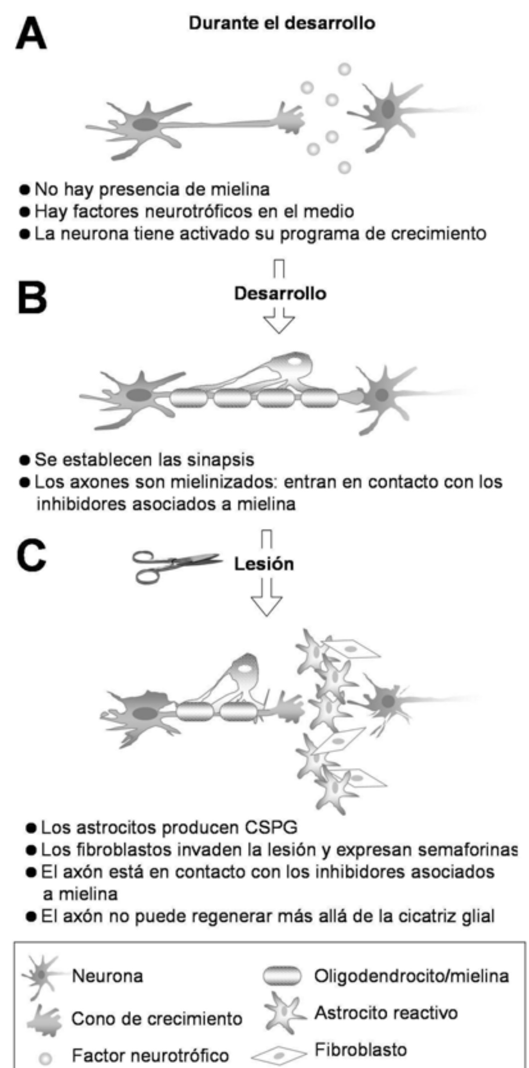


Fig. 1.1. **Scheme showing some of the changes occurring during CNS development** that ultimately contribute to preventing axonal regeneration, such as the appearance of myelin-associated inhibitors. (Taken from Mingorance *et al*., 2004b).

regenerative potential (Horner and Gage, 2000). Thus, factors determining regenerative potential loss can be classified as either **endogenous** or **exogenous**.

1.2.1 ENDOGENOUS FACTORS

Neurons in the adult CNS regenerate when provided with a permissive environment, as occurs with peripheral graft transplantation into the CNS (David and Aguayo, 1981). However, even in these particularly favourable conditions, the regeneration rate of adult neurons is slow, typically much slower than the elongation rate observed for the same neurons during embryonic stages (Condic, 2002). This decrease in axonal regeneration, parallel to neuronal maturation, also occurs in the PNS and is induced by various stimuli, including the activation of the endogenous program of the neuron; contact with factors released from the target tissue (so that regenerative potential loss is associated with the completion of a specific connection); or the arrival of synaptic afferents. The latter is the case of retinal ganglion cells, which, after contacting amacrine cells, switch from an “axon growth” state, in which axonal outgrowth is facilitated, to a new state in which dendritic growth is potentiated in detriment of axonal growth (Goldberg *et al.*, 2002).

Today, one of the main objectives of Neurobiology is to identify the intracellular changes responsible for the loss of intrinsic capacity to regenerate, and to manipulate them after lesion in order to achieve therapeutic benefits. Briefly, these changes can be summarized in three groups: a decrease in the expression levels of growth-promoting molecules (such as GAP-43 and CAP-23); the expression, after certain stages of development, of receptors for neurite outgrowth inhibitory molecules (such as NgR); and changes in cyclic nucleotide levels (Horner and Gage, 2000; Selzer, 2003). It is of interest to mention the role of cAMP in regulating axonal outgrowth, since this nucleotide has an important impact on the adult CNS after lesion (Spencer and Filbin, 2004). cAMP is expressed at high levels during neuronal development, when it promotes axonal outgrowth through various mechanisms, such as the inactivation of Rho GTPase. However, after a particular point of development, which depends on each neuronal population, there is a rapid drop in the concentration of this cyclic nucleotide. This decrease not only reduces the intrinsic capacity of the neuron to regenerate through the molecular mechanisms previously described, but also increases growth cone sensitivity to several axonal outgrowth inhibitory molecules, such as myelin-associated inhibitors (Fig 1.2; Cai *et al.*, 2001).

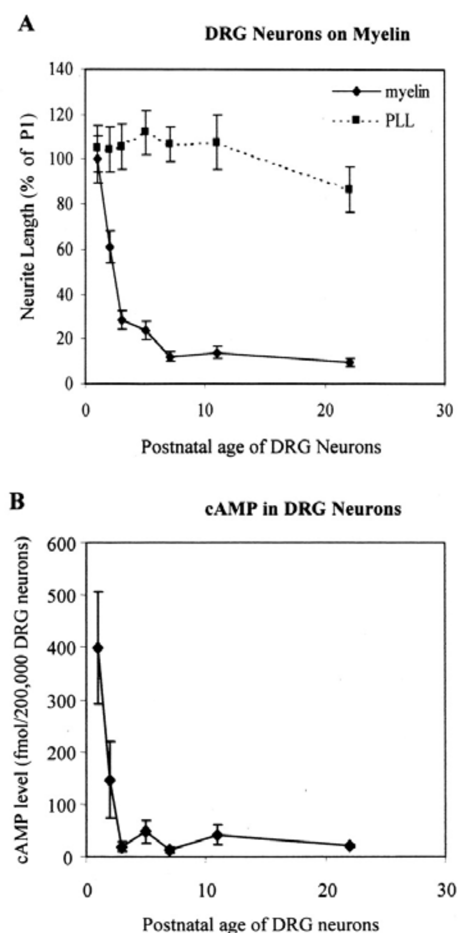


Fig. 1.2. Correlation between developmental changes in neurite outgrowth of DRG neurons on myelin and endogenous levels of cAMP (taken from Cai *et al.*, 2001)

1.2.2 EXOGENOUS FACTORS

When neuronal connections are established, the CNS switches from a permissive environment for axonal outgrowth to a much more restrictive milieu, in which synaptic stabilization is promoted through mechanisms that limit neuronal capacity to regenerate. In addition to this change, and linked to CNS maturation, several changes occur that are induced in this system only after lesion. These two groups of alterations constitute the exogenous factors that regulate axonal regeneration and seem to be determinant in preventing the outgrowth of lesioned axons. Here we will summarize them.

a) Lack of stimulus

One of the major differences between the CNS and PNS is the kind of glial cells. Schwann cells are the myelinating glial cells in the latter. Both central and peripheral axons grow easily on a substrate of Schwann cells, while axons in both nervous systems struggle on a substrate of oligodendrocytes or astrocytes (Schwab, 2002). Part of this difference is due to the expression of cell-adhesion molecules and neurotrophins by Schwann cells, which promotes axonal outgrowth, while levels of these proteins decrease in the CNS after postnatal stages (Rush, 1984). Compensation for this scarce production of neurotrophic factors, however, fails to promote axonal regeneration in the adult CNS, as commented later in this introduction.

b) Glial scar

One of the main obstacles to axonal regeneration is the glial scar (Silver and Miller, 2004). As a result of a CNS injury, the glial cells of the affected zone exhibit “reactive” behaviour, which includes cell proliferation, hypertrophy and changes in the expression of certain receptors and extracellular matrix proteins, such as the tenascins and proteoglycans (Fig. 1.3). When the integrity of the meninges is preserved, the main components of the scar are astrocytes. Reactive gliosis includes astrocyte proliferation in the so called “penumbra” zone (surrounding the lesion core) and is characterized by hypertrophy as a consequence of intermediate filament overexpression, such as Vimentin and GFAP (Fig. 1.4). However, when meninges are affected in more sever lesions, reactive gliosis is combined with cavitation, in which fibroblasts infiltrate into the neuronal tissue. The characteristics of the glial scar generated after three distinct models of lesion are summarized in Figure 1.3 (Silver and Miller, 2004).

The glial scar as a physical barrier. When the glial scar matures, processes from hypertrophic astrocytes group together to form a dense cellular barrier (Fig. 1.4). In these conditions, the arrangement of astrocytes mechanically prevents the growth of axons through the glial scar. The observation of this close net of reactive astrocytes surrounding collapsed growth cones leads to the notion that reactive glia cells cause axonal regeneration failure by generating a physical barrier (Ramón y Cajal., 1928). However, such a mature scar takes 7-10 days to develop, and axonal regeneration does not occur in the time window previous to scar

maturation, which indicates that other factors, presumably inhibitory molecules, limit axonal regeneration during this period, (Caroni and Schwab, 1988).

Fig. 1.3. **Composition of the glial scar after three stereotypical CNS lesions**. In all examples, macrophages invade the lesion, and overexpression of both CSPG (chondroitin sulphate proteoglycans) and KSPG (keratan sulphate proteoglycans) is present in all the examples. Differences between lesions are the result of the disruption of meninges and presence of cavitation.. ECM, extracellular matrix; TGF, transforming growth factor. (Taken from Silver and Miller, 2004)

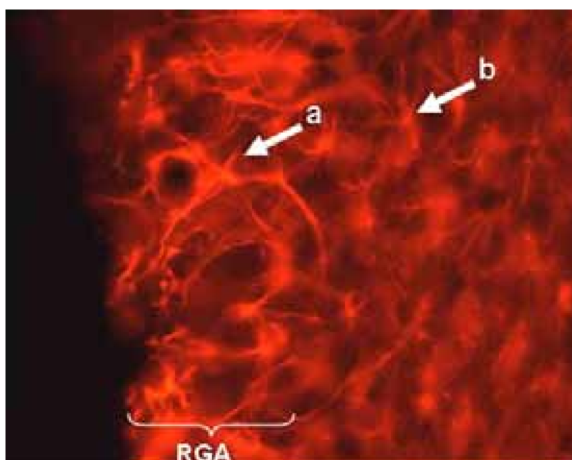


Fig. 1.4. Reactive astrocyte morphology during the development of the glial scar. The photograph shows immunocytochemistry for GFAP in the entorhinal cortex 3 days after axotomy. GFAP immunostaining demonstrates the different morphology of reactive astrocytes (a), near the lesion site (left side), and non-reactive astrocytes (b). Mature glial scar has not yet developed. RGA, reactive gliosis area.

The glial scar as a source of inhibitory molecules of axonal outgrowth. Constitutively, and at particularly high levels after lesion, astrocytes express a set of molecules named proteoglycans, some of which strongly inhibit axon outgrowth (Morgenstern *et al*., 2002). A class of proteoglycans, the chondroitin-sulphate proteoglycans (CSPG), is overexpressed in the glial scar after lesion for months (Carulli *et al*., 2005). Astrocytic reactivity and overexpression of CSPG occur only after a critical embryonic period, what contributes to the regenerative capacity of embryonic neurons (Carulli *et al*., 2005). The glycosaminglycan chains that characterize this family of proteoglycans are responsible for their inhibitory effect, and the enzymatic degradation of these chains is one of the current therapies used to promote axonal regeneration. Although CSPG are the main inhibitory molecules expressed in the glial scar, when the lesion includes the disruption of the blood-brain barrier, meningeal-derived cells infiltrate into neuronal tissue and overexpress several axon guidance molecules, such as Semaphorin 3A (Sema 3A), Slit, or Ephrin-B2, and the extracellular matrix proteins tenascins. All these molecules inhibit growth cone outgrowth in the adult CNS and contribute to the development of a barrier in the glial scar core. Indeed, some studies suggest that rather than the physical barrier that reactive astrocytes form in the glial scar, it is the astrocyte/meningeal cell limit that truly prevents axons from crossing the scar, at least *in vitro* (Shearer *et al*., 2003; Sandvig *et al*., 2004).

c) <u>Myelin-associated Inhibitors</u>

As previously described, the inhibition of axonal regeneration is due in part to the production of inhibitory molecules in the glial scar after lesion. However, it should be remembered that even in the absence of a lesion, adult CNS is particularly unfavourable for axon outgrowth. A large part of this inhibition is because of the presence of inhibitory molecules in association with myelin (Fig 1.1; Grados-Munro and Fournier, 2003). To date, three proteins have been classified in this category: Nogo-A, MAG and OMgp, for which a growing number of receptors and co-receptors is known (Sandvig *et al*., 2004). Given the current state of research on myelin-associated inhibitors, the future identification of new inhibitors (and receptors) is probable. Hence, we are just starting to characterize the intracellular pathways that transduce the inhibition of axonal growth from myelin to the neuronal cytoskeleton. Until every element involved in this inhibition is known, the contribution of each of the myelin-associated inhibitors to axonal regeneration failure will not be established. Since these topics, together with the non-pathological function of myelin-associated inhibitors, are the focus of this thesis, two later chapters are devoted to them. In this first chapter, to provide enough information to later

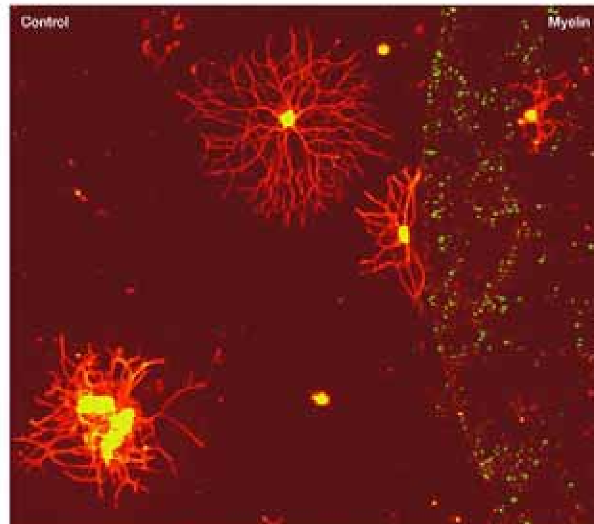


Fig. 1.5. CNS myelin inhibits axonal extension *in vitro*. Neonatal dorsal root ganglia cells growing on a coverslip coated with myelin drops (green dots). (Taken form Lee *et al*., 2003).

introduce some of the strategies used to promote axonal regeneration, we provide only a global view of the role of myelin in the inhibition of axonal growth.

As adult CNS neurons regrow on peripheral nerve tissue, it has been proposed that the lack of neurotrophic factors in the adult CNS could lead to axonal regeneration failure (David and Aguallo, 1981). The delivery of neurotrophic factors can indeed improve neuronal survival and permit the development of new axons from lesioned neurons, which have the capacity to grow a short distance into the lesioned CNS (e. g. Cheng *et al.*, 1996). However, these axons always avoid growing on white matter (illustrated in Fig. 1.5). At the end of the 80s, it was hypothesized for the first time that the inhibitory nature of the adult CNS was mainly due to one type of glia: the myelinating oligodendrocytes (Schwab, 1991). Pioneering work by Dr. Schwab provided the first evidence that the strong inhibitory properties of myelin depend on the presence of certain proteins on this molecule (Caroni and Schwab, 1988b). These researchers generated a monoclonal antibody, called IN-1, raised against an unknown myelin protein (which inhibited axonal growth) that blocked part of the myelin-induced inhibition in diverse regenerative paradigms (Caroni and Schwab, 1988b; the generation of the antibody and the experiments using it are described in depth in the following sections of this introduction). The cloning of the epitope recognized by IN-1 led to the identification of the first myelin-associated inhibitor: Nogo-A (Spillmann *et al.*, 1998). Soon after this, MAG (a protein previously known for its role in myelin formation) and OMgp (a previously unknown protein) were also identified as myelin-associated inhibitors that bind to the same receptor complex, although their molecular structures differ greatly (Wang *et al.*, 2002b). This receptor complex is integrated by the so-called “Nogo receptor” (NgR), which binds to the three ligands, the co-receptor Lingo-1, and one of the co-receptors p75 and Troy, both from the death receptor family (Wang *et al.*, 2002a; Mi *et al.*, 2004, Park *et al.*, 2005; Shao *et al.*, 2005). Together with their common receptor complex, these three ligands form the central skeleton of myelin-induced inhibition of axonal outgrowth.

Recent studies have demonstrated that NgR, Nogo-A and MAG are regulated after lesion (Hunt *et al.*, 2002; Meier *et al.*, 2003, this thesis). Since this regulation is rapid and not restricted to the glial scar, the myelin-associated inhibitors are one of the main families of molecules that prevent axonal regeneration out of the glial scar (mainly before the glial scar develops) and their blockade has been shown to promote axonal regeneration.

1.3 Strategies to promote axonal regeneration

As we have previously discussed, the regeneration of a cortical connection must be achieved following different stages (Horner and Gage, 2000; Selzer, 2003). Several strategies have been developed to promote each of these stages. Here we will focus on those strategies designed to

Table 1.1. (Next page) Summary of the main therapeutic approaches currently used to promote axonal regeneration. Because it is the focus of this thesis, we have detailed in the summary table those treatments addressed to reduce the effect of inhibitory molecules while other approaches, such as neurotrophic factor delivery or transplants of permissive substrates, are outlined only. Therefore, the space dedicated to the distinct treatments is not proportional to the research devoted to them.
Various[1]: cAMP analogs, flavonoids, forskolin, neutotrophins (see text for details). CSPG, chondroitin sulfate proteoglycans; MAI, myelin-associated inhibitors. Only one representative reference is given for each treatment, see the text for a more complete reference list.

THERAPEUTIC APPROACHES			
Treatment	**Agent**	**Target**	**References**
Blockade of inhibitory molecules			
A. **Extrinsic treatments**			
1. Myelin-associated inhibitors (MAI)			
Blocking antibodies	IN-1/α-MAG/ α -NgR	MAG/Nogo-A/NgR	Karim *et al*., 2001
Vaccination	Myelin/single MAI	Myelin	Sicotte *et al*., 2003
Antagonist peptides	NEP1-40	Nogo-A	Grandpre *et al*.,2002
Enzymatic degradation	Neuraminidase	MAG	Tang *et al*., 1997
Ectodomains	NgR310	MAI	Li *et al*., 2004
Dominant negative	dnNgR	MAI	Fischer *et al*., 2004
CALI	Laser	MAG	Wong *et al*., 2003
X-radiation removal of oligod. / myelin	X-rays	Myelin	Weibel *et al*., 1994
2. Chondroitin Sulfate Proteoglycans (CSPG)			
Enzymatic degradation	Condroitinase ABC	CSPG	Moon *et al*., 2001
3. Other inhibitory molecules			
Blocking antibodies	α- Neuropilin	Sema3A/3F	Giger *et al*., 1998
Dominant negative	dnNP	Sema	Renzi *et al*., 1999
Antagonist peptoides	SICHI	Sema3A	Montolio *et al*.,
B. **Intrinsic treatments**			
Cyclic nucleotides levels regulation	Various[1]	MAI	Cai *et al*., 1999
Rho and ROCK inactivation	C3, Y-27632	MAI/CSPG	Fournier *et al*., 2003
PKC inactivation	Go6976	MAI/CSPG	Sivasankaran *et al*., 2004
Delivery of neurotrophic factors	Neurotrophic factors		Xu *et al*., 1995
Transplant of permissive tissue			
Fetal tissue	Fetal tissue		Xu *et al*., 1995;1999
PNS	Peripheral nerve		Xu *et al*., 1995:1999
Ensheathing glia	Olfactory ensheathing cells		Ramon-Cueto *et al*., 2000
Prevention of Apoptosis			
Apoptotic / Anti-apoptotic gene regulation	Bcl-2		Goldberg *et al*.,2002
Cell replacement	Stem cells		Snyder *et al*., 1997

promote both the formation of a new growth cone and its following elongation. These strategies are mainly focused on increasing CNS permissiveness following lesion, either by directly enhancing its permissiveness, or by reducing its inhibition (or “non-permissiveness”). There is a large list of treatments that demonstrate both strategies are capable to induce some degree of functional regeneration after lesion. Due to space constrictions we will focus on those blocking inhibitory molecules effects while only outlining the therapies addressed to promote cell survival, or provide an alternative substrate for regenerating axons (see table 1.1).

1.3.1 BLOCKADE OF INHIBITORY MOLECULES

The knowledge that adult CNS neurons grow when provided with a permissive environment has made researchers focus on reversing the inhibitory properties of this system by blocking the effect of growth inhibitory molecules. This can be done through two approaches: by either blocking or eliminating the inhibitory molecules or their

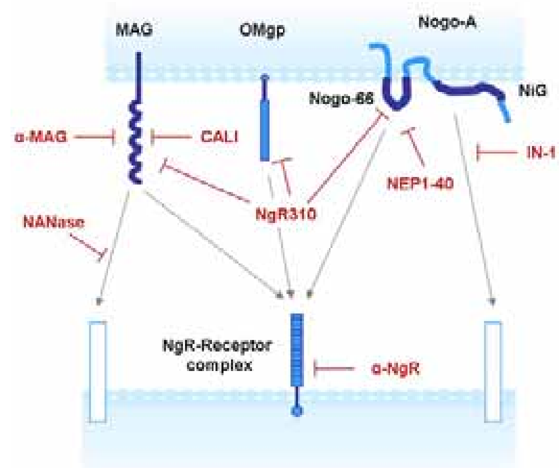


Fig. 1.6. Representation of the main approaches used to block the effects of myelin-associated inhibitors extracellularly. The agents used for the extrinsic treatments and the level where they act are labelled in red. For short, NgR drawing represents the whole NgR-receptor complex.

receptors (extrinsic treatments), or alternatively, by modifying neuronal responsiveness to these molecules (intrinsic treatments).

a). Extrinsic treatments.

a.1) Myelin-associated inhibitors

Blocking antibodies. The use of function-blocking antibodies is based on the binding of the antibody to a protein. When this binding prevents the epitope protein from interacting with its receptor or ligand, the antibody can be epitope protein from interacting with its receptor or ligand, the antibody can be used as a blocking tool and be delivered *in vivo*. For many years, the monoclonal antibody IN-1 was used to neutralize axonal growth inhibitors in myelin in many *in vitro* and *in vivo* studies performed before the molecular characterization of the Nogo-A antigen (Table 1.2 and references within). We now know that IN-1 recognizes the central domain of Nogo-A (Fiedler *et al*., 2002), a region with inhibitory effects on axonal growth that differs from the domain that interacts with NgR and whose receptor remains unknown (Fig. 1.6). The distinct articles reporting the use of IN-1 to promote axonal regeneration *in vivo* are summarized in Table 1.2. For a more detailed description of treatments with IN-1 see Mingorance *et al*., 2004c.

Reference	Type of lesion	Neuronal population studied	treatment	Anatomical Regeneration	functional regeneration
Schnell and Scwab, 1990; 1993; Schnell et al., 1994	Bilateral Pyramid.	CST	IN-1 secreting hybridoma intracerebral	Long-distance regeneration	Yes
Cadelli and Schwab, 1991	Fimbria/fornix	cholinergic Septohippocamp al tract	human amnion extracellular matrix material containing NGF and NI-1	Moderate	Non tested
Bregman et al., 1995; Thallmair et al, 1998 ; Z'Graggen et al., 1998	Unilateral pyramidotomy	CST	IN-1 secreting hybridoma cells into the hippocampal formation	Compensatory sprouting Long-distance regeneration	Yes
Kartje et al., 1999	Unilateral aspiration lesion	Corticostriatal tract	IN-1 secreting hybridoma cells into the lesion cavity	Compensatory sprouting	Yes
Brosamle et al., 2000	Unilateral pyramidotomy	CST	IN-1 Fab into the injury	Long-distance regeneration	Non tested
Oudega et al., 2000	Nerve graft paradigm	Sensory axons	IN-1 secreting hybridoma cells into the cerebral cortex or thoracic cord	None	Yes
Blochlinger et al., 2001	Unilateral pyramidotomy	Corticopontine tract	IN-1 secreting hybridoma cells into the contralateral cerebral cortex or hippocampus	Compensatory sprouting	Non tested
Merkler et al., 2001	Unilateral pyramidotomy	CST	IN-1 secreting hybridoma cells proxim to the ventricular system	Non tested	Yes
Raineteau et al., 1999; 2001	Bilateral Pyramid.	Rubrospinal tract and CST	IN-1 secreting hybridoma cells into the hippocampal formation	Compensatory sprouting	Yes
Bareyre et al., 2002	Unilat eral pyramidotomy	CST	IN-1 secreting hybridoma cells into the hippocampal formation	Compensatory sprouting	Non tested
Tatagiba et al., 2002	Section of audirory nerve	Cochlear nerve fibers	IN-1 Fab intrathecal	Long-distance regeneration	Yes
Papadopoulos et al., 2002	Ischemic lesion	Cortical neurons	IN-1 secreting hybridoma cells posterior to the lesion site	Compensatory sprouting	Yes
Weissner et al., 2003	Chronic injury CST	CST	Delayed IN-1 and NT3 treatment	moderate	Slight
Emerik et al., 2003	Unilateral SMC lesion	sensorimotor cortex	IN-1 secreting hybridoma cells into the hippocampal formation	functional reorganization of the intact motor cortex	Yes

Table 1.2. IN-1 *in vivo* treatments. References are given in chronological order. "yes" indicates functional recovery, "compensatory sprouting" means innervation form the unlesioned hemisphere (taken from Mingorance *et al*., 2004c).

In addition to IN-1, antibodies against MAG and a monoclonal antibody against NgR, which blocks its binding to Nogo-A, MAG and OMgp, also reduce myelin-induced inhibition *in vitro*,

effectively promoting neurite outgrowth on CNS myelin or MAG substrate (Mukhopadyay *et al*., 1994; Li *et al*., 2004). Although the therapeutic potential of these antibodies are apparent, they have been tested only in neurite outgrowth assays but not in alternative regeneration assays (*in vitro*) or after *in vivo* CNS injury.

An alternative approach that also uses antibodies to block protein function consists of **vaccination**. Vaccination with purified myelin permits the immunological neutralization of myelin-associated inhibitors, thereby avoiding the problems associated with antibody delivery. This approach has resulted in a strong regenerative growth of the cortical spinal tract (CST) axons (Huang *et al*., 1999), although it may lead to the development of autoimmune disease. To minimize this risk, since not all proteins are encephalitogenic, experiments have been performed using only recombinant proteins as immunogens (Nogo-66 and MAG). These proteins have been shown to promote axonal regeneration of the lesioned CST (Sicotte *et al*., 2003).

Antagonist peptides. Although antibodies have traditionally been used to specifically block ligand/receptor interaction, the capacity to generate efficient antibodies is lacking and advances in recombinant protein production have provided various alternatives. These alternatives include single chain antibodies, peptides, peptoids (synthetic peptides), and even the use of single-stranded nucleic acids (aptamers). One of these tools, an antagonist peptide that blocks Nogo-A interaction with NgR, has been successfully used to promote axonal regeneration *in vivo* after SCI either by intratectal delivery or systemic administration, even when delayed to seven days post-axotomy (Grandpre *et al*., 2002; Li *et al*, 2004). The peptide, named NEP1-40 (from Nogo Extracellular Peptide, residues 1-40), consists of the first 40 residues of the Nogo-66 loop of Nogo-A (the domain interacting with NgR) and acts as a competitive antagonist of NgR, preventing Nogo-66 binding but not that of MAG or OMgp (Grandpre *et al*., 2002; Li *et al*., 2004).

Enzymatic degradation. In order to be functional, some proteins require the presence of specific domains or residues, which are generally involved in ligand/receptor interaction. This is the case of MAG, which induces the inhibition of axonal outgrowth through binding with NgR, NgR2 and gangliosides. While binding to NgR appears to be sialic acid-independent, the fraction of MAG-induced inhibition caused by its interaction with NgR2 and gangliosides can be prevented with neuraminidase (NANase) treatment. NANase releases sialic acids from the neuronal surface and successfully reduces MAG inhibition *in vitro* (DeBellard *et al.*, 1996; Tang *et al.*, 1997; Tang *et al.*, 2001; Vinson *et al.*, 2001). However, enzymatic degradation of a widely used residue, such as sialic acid, is too unspecific to be considered a therapeutic tool.

Ectodomains. The previous reagents have the limitation of targeting only a single myelin-associated inhibitor. In contrast, the use of soluble receptor ectodomains is expected to induce a greater improvement in regeneration by binding to the different ligands and preventing them from interacting with their receptors. Recently, a soluble fragment of NgR, comprising the whole

ligand binding domain but lacking the capacity to bind co-receptors (NgR310), has been administered intrathectally to rats with SCI. This treatment induced the sprouting of CST fibers, leading to functional regeneration (Li *et al*., 2005).

Dominant negative. A similar construction to the soluble NgR ectodomain that lacks the co-receptor binding domain (C-terminal) but is attached to the membrane has been used *in vivo* as a dominant negative of NgR (dnNgR). A dominant negative is a modified protein that lacks several necessary domains but retains the capacity to interact with the partners of the original protein. When expressed in a cell, the dominant negative will compete with the endogenous protein for binding to partners (or ligands), thereby silencing it. When expressed in a neuronal cell line, dnNgR induces a loss of responsiveness to MAG (Domeniconi *et al*., 2002), and when overexpressed in retinal ganglion cells after transfection with adeno-associated viruses, it enhances optic nerve regeneration (Fischer *et al*., 2004), mainly when the expression of the NgR antagonist is combined with a stimulation of the neuronal growth state.

CALI. Chromophore-assisted laser inactivation of proteins is a highly specific approach to target single proteins in living cells (Jay and Keshishian., 1990). CALI inactivates a protein of interest by targeting laser energy using a specific antibody labeled with the dye Malachite Green. After laser irradiation with 620 nm light, the excited Malachite Green releases short-lived free radicals to inactivate proteins bound by the antibody. The wavelength of light used is absorbed by the dye but not significantly absorbed by cellular components. CALI has been used to selectively inactivate MAG after optic nerve crush in organotypic cultures by using an anti-MAG antibody conjugated with Malachite Green (Wong *et al*., 2003). CALI of MAG permits the regeneration of lesioned retinal axons, which provides one of the best lines of evidence in support of the contribution of MAG to axonal regeneration failure (Wong *et al*., 2003).

X-irradiation. Neonatal X-irradiation of CNS tissue kills the dividing oligodendrocyte precursor cells and markedly reduces the myelinating oligodendrocyte population (and hence myelin) after irradiation. This strategy has been used to locally irradiate optic nerves of newborn rats and promote the axonal regeneration of retinal axons when crushed at postnatal day 19 (Weibel *et al*., 1994).

a.2) Chondroitin Sulfate Proteoglycans

Enzymatic degradation. The characteristic that defines the class of CSPG is the presence of, chondroitin sulphate GAG chains wich also confer the inhibitory activity of many of them and can be degraded by the enzyme chondroitinase ABC. *In vivo*, chondroitinase treatment reduces axonal regrowth inhibition and permits the recovery of function after spinal cord lesion (Bradbury *et al*., 2002) and the regeneration of the nigrostriatal pathway (Moon *et al*., 2001).

a.3) Other inhibitors.

In addition to myelin-associated inhibitors and proteoglycans, the main inhibitory molecules expressed in the adult CNS, and whose function in preventing axonal regeneration has been

extensively studied, are semaphorins. The expression of Sema3A in the glial scar is thought to greatly contribute to axonal regeneration failure and several specific function-blocking tools have been designed (Pasterkamp and Verhaagen, 2001). The therapeutic approaches targeting Sema3A expression are similar to those described for the myelin-associated inhibitors, although they have not yet been tested *in vivo*. In particular, the use of **blocking antibodies** raised against Sema3A or Neuropilins (Semaphorin receptors) and the expression of **dominant negative receptor** constructs have been extensively used *in vitro* (see Pasterkamp and Verhaagen, 2001 for review). In addition, from a library screening, our laboratory obtained a **peptoid** that selectively prevents Sema3A binding to NP1. In addition to preventing Sema3A-induced growth cone collapse and repulsion *in vitro*, like the blocking antibodies and the dnNP constructions, the peptoid improves the regeneration of lesioned entorhinal axons (Montolio *et al.*, in press) in an *in vitro* model of axonal axotomy (perforant pathway axotomy in entorhino-hippocampal co-cultures).

b). <u>Intrinsic treatments</u>.

A second approach to reduce the effect of inhibitory molecules is by altering the intrinsic growth state of the adult neurons so that they no longer respond to these inhibitors. As we have previously mentioned (see Fig. 1.2), cAMP levels are elevated during development and after a particular point in development their concentration drops. It is not fully understood how **cAMP levels** control axonal regeneration but they are believed to regulate diverse processes (such as cytoskeleton dynamics and transcription of several proteins), which together increase the regenerative capacity of the neuron, and interestingly, block myelin-induced inhibition. The main skeleton of cAMP regulation of axonal regeneration is shown in Figure 1.7 and will be addressed in the third chapter of this introduction. Briefly, priming neurons with neurotrophins allows neurite outgrowth on myelin (Song *et al.*, 1998; Cai *et al.*, 1999). Subsequently, it was discovered that this priming effect was mediated by an increase in the intracellular levels of cAMP, which in turn led to PKA activation. Among various downstream effectors, PKA activation of CREB, and the consequent regulation of Arginase I (ArgI) expression appear to be essential to block the inhibitory effect of myelin and MAG (Neumann *et al.*, 2002; Cai *et al.*, 2002; Gao *et al.*, 2004). This pathway can be reinforced in many ways, all leading to the same blockade of myelin-induced inhibition. Including the elevation of cAMP with neurotrophins or Forskolin, delivery of the cAMP analogous dibutyryl-cAMP (db-cAMP), blockade of the phosphodiesterase 4 (PDE4; which

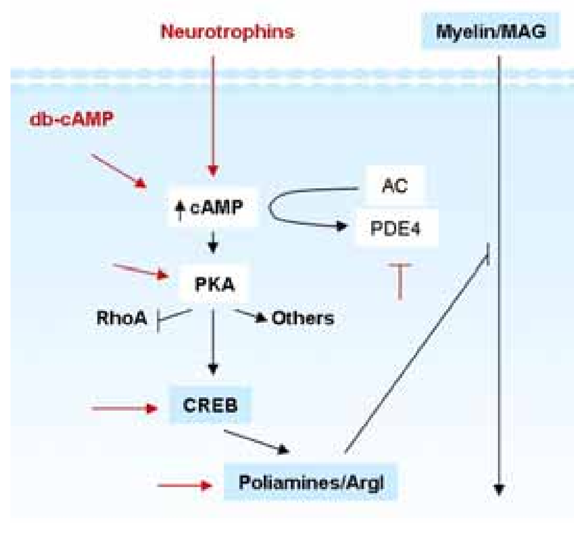
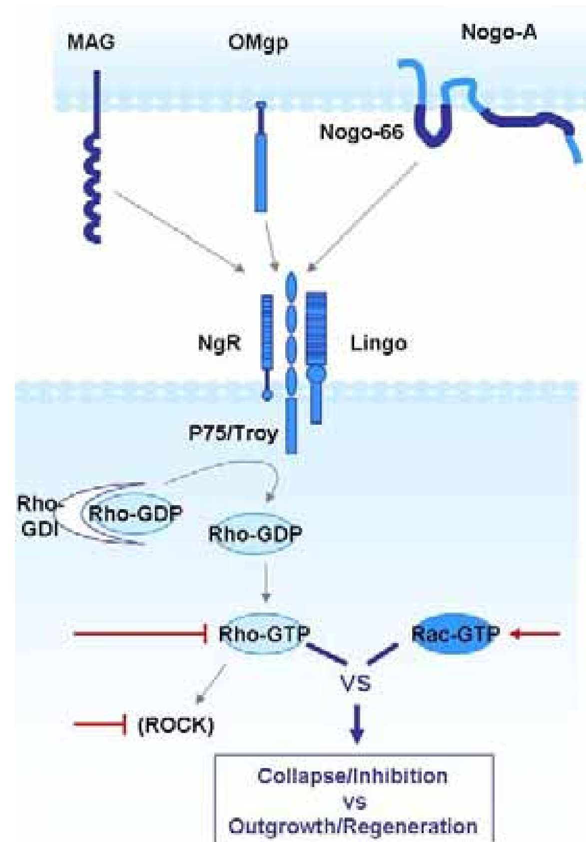


Fig. 1.7. Scheme of the main elements involved in cAMP regulation of axonal regeneration. cAMP effect on regeneration has been shown to need the activation of PKA, CREB, and an increase in ArgI and poliamines synthesis. Different manipulations have been performed *in vitro* and *in vivo* to strengthen this pathway and all of them lead to axon outgrowth on myelin and axonal regeneration. The points on which these manipulations act are shown in red.

Fig. 1.8. Scheme of Rho-GTPases equilibrium in the regulation of axonal regeneration. Rho is activated indirectly by p75 (and putatively by Troy), which dissociates the complex Rho-GDI. The balance of activated Rho and Rac determines the final effect on cytoskeleton dynamics of ligand binding to neuronal receptors. Both the reinforcement of Rac signalling and the inhibition of Rho and its downstream effectors block the pathway activated by the myelin-associated inhibitors and permit neurite outgrowth on inhibitory substrates and ultimately axonal regeneration after lesion (red lines).



degrades cAMP) with Rolipram, adenovirus-mediated overexpression of an activated form of CREB, and increasing polyamines or particularly ArgI levels.

Another key molecule in axonal regeneration control is **RhoA**. After injury, the neuron has to integrate a number of cues and transform them into a final response, which involves cytoskeletal remodelling. Processes associated with the reorganization of the actin cytoskeleton (such as neurite outgrowth and axon guidance) are closely associated with Rho family GTPases. Two of these proteins, RhoA and Rac, have antagonist effects, and generally molecules that inhibit axonal growth, such myelin-associated inhibitors and CSPG, activate Rho while they inactivate Rac, leading to growth cone collapse (Monnier *et al*., 2003; Yamashita and Tohyama, 2003). After the binding of myelin-associated inhibitors to NgR, p75 activates RhoA by releasing it from Rho-GDI (which allows Rho to be activated by other proteins) (Yamashita and Tohyama, 2003). Several lines of evidence indicate that manipulation of the activity state of Rho GTPases may modulate growth cone collapse and neurite outgrowth inhibition. First, the introduction of dominant-negative or constitutively active Rac *in vitro* activates Rac and blocks growth cone collapse in response to Sema 3A (Jin and Strittmatter, 1997) or myelin (Kuhn *et al*., 1999). Second, *in vivo* blockade of RhoA with a cell-permeable Rho antagonist promotes the regeneration of retinal ganglion cells after optic nerve axotomy (Bertrand *et al*., 2005), and treatment with C3 transferase or inactivation by dominant-negative Rho inactivate Rho and promote regeneration and functional recovery after SCI (Ellezam *et al*., 2002). Recently, the targeting of a downstream effector of Rho, named ROCK, has been shown to induce sprouting of CST fibers and improve locomotor recovery after spinal cord lesions in rats (Borisoff *et al*., 2003; Fournier *et al*., 2003).

Finally, both myelin inhibitors and CSPG mediate their effects by activating PKC. Blockade of PKC activity (either pharmacologically or genetically) attenuates the capacity of CNS myelin and CSPG to activate Rho and inhibit neurite outgrowth (Sivasankaran *et al*., 2004; Hasegawa *et al*., 2004), and intrathecal delivery of a PKC inhibitor after spinal cord lesion promotes the regeneration of dorsal column axons in rats *in vivo* (Sivasankaran *et al*., 2004).

1.3.2 DELIVERY OF NEUROTROPHIC FACTORS

Neurotrophic factors have both growth-promoting and cell survival effects on neurons. Experiments with neurotrophin delivery either intratectally or using engineered Schwann or fibroblast cells (secreting BDNF or NT3) have demonstrated the utility of neurotrophin delivery in promoting regeneration after SCI. However, the delivery of neurotrophic factors after injury to the CNS *in vivo* does not appear to be sufficient to promote axon growth in the inhibitory environment of white matter, and regenerating axons prefer to grow on the transplanted neurotrophin-secreting cells, which act as a permissive graft. Therefore, the best results are obtained when neutrophin delivery is combined with transplant of peripheral nerve or foetal CNS grafts (i.e. Xu *et al*., 1995; Bregman *et al*., 1997).

1.3.3 TRANSPLANT OF PERMISIVE TISSUE

When scarring is extensive or a part of the CNS is lost (for example after complete trasection of the spinal cord, when cut ends retract), a bridge is used to guide axons through these barriers and allow them to reach the intact parenchyma. Peripheral nerve and fetal tissue have been used as permissive substrates and bridge after injuries to the visual system and spinal cord. In these experiments, however, lesioned axons often invaded the graft to a limited extent, providing little functional recovery. In addition, new artificial substrates are being developed to be easily manipulated and tolerant for the immunitary system. These substrates, made of acrylonitrile and vinylclorhide, or rapidly absorbable substances, such as poly-α-hydroxyacids, can be seeded with cells or guidance molecules and used to promote cell survival and regeneration. Indeed, the use of artificial tubes seeded with Schwann cells has already been successfully applied to guide regenerating axons after trasection of the spinal cord (Xu *et al*., 1995; 1999).

1.3.4 PREVENTION OF APOPTOSIS

As a result of axotomy, many neurons undergo apoptosis. Delivery of neurotrophic factors can, to some extent, maintain neuronal survival after lesion. Other approaches include the manipulation of the intracellular cell-death program. Bcl-2 is an anti-apoptotic gene, and its overexpression can rescue neurons from axotomy-induced cell death. However, Bcl-2 (or survival promotion) alone is not sufficient to promote regeneration, as shown in several studies that indicate that neuronal survival is not necessarily linked to axonal regeneration (Bonfanti *et al*., 1996; Goldberg *et al*., 2002; Sole *et al*., 2002).

1.3.5 CELL REPLACEMENT

When neuronal loss is extensive, cell replacement is required to regenerate the CNS. The cell replacement source can be foetal tissue or stem cells. The latter are more homogeneous and easy to obtain than foetal grafts; in addition, since they are multipotent they can be propagated *in vitro*. However, when transplanted into non-neurogenic areas (such as the hippocampus), stem cells tend to adopt glial fates. Although this could have some advantages, as the differentiation of stem cells into oligodendrocytes could be used to treat some disorders of myelin, only a few articles report that stem cell grafts lead to neurogenesis (e. g. Snyder *et al*.,

1997), what seems to be dependent on the microenviroment. Although this approach is still promising, it requires further development before application as a therapy for traumatic injuries in humans.

1.4 Human trials: state-of-the-art

Recent therapeutic advances in experimentation animals point to the efficacy of combination therapies: combination of embryonic stem cell transplants with db-cAMP or ROCK inhibitors (Harper *et al*., 2004), Schwann cells with db-cAMP and rolipram (Pearse *et al*., 2004), Schwann cells with chondroitinase and GDNF (Xu *et al*., 2002; 2003), chondroitinase and lithium (an inhibitor of Gsk3β, an enzyme that participates in growth cone collapse, Yick *et al*., 2004), neuronal stem cells combined with the cell adhesion molecule L1 (Roonprapunt *et al*., 2003), and combination of Schwann cell bridges and olfactory-ensheathing glia grafts with chondroitinase (Fouad *et al*., 2005), this latter after complete transection of the spinal cord. These studies represent the forthcoming trials in humans.

- Olfactory ensheathing glia (OEG) transplants
  - Human fetal OEG (Beijing, Russia)
  - Human nasal mucosa (Lisbon)
  - Human nasal mucosa OEG autografts (Brisbane, Australia)
- IN-1 antibody to regenerate chronic SCI (Novartis, University of Zurich)
- Nogo receptor blockers (Biogen, Yale University)
- Inosine to stimulate sprouting in chronic spinal cord injury (BLSI, MGH)
- Schwann cell autografts (Yale & Miami Project)
- Stem cell transplants
  - Bone marrow stem cells (mesenchymal stromal cells)
  - Umbilical cord blood stem cell transplants
  - Genetically modified stem cell autografts (BDNF & NT-3)
- Chondroitinase (London, China)
- Rolipram & dibutyryl cAMP combined with cell transplants

Fig. 1.9. Human trials. Taken from W. M. Keck Center for Collaborative Neuroscience and the SCI Project. Rutgers University.

The only "effective" treatment currently used to treat SCI in humans is the neuroprotective glucocorticoid steroid methylprednisolone. The success of neuroprotective treatments lies in the fact that during the first minutes and hours after injury, a secondary degenerative process occurs, and when applied early, pharmacological treatments which interrupt this process can improve spinal cord tissue survival, and thus preserve the anatomic substrate required for functional recovery (Hall and Springer, 2004). To facilitate this recovery, clinical trials of first generation therapies in humans are underway, and second generation therapies should start soon. The regenerative therapies currently being tested in animals are listed above. Figure 1.9 summarizes several of the treatments in trial, or to be initiated soon.

1.5 Lesion models

CNS damage can occur in a number of ways. The most common include Neurodegenerative diseases (Huntington's disease, Parkinson's disease, Amyotrophic Lateral Sclerosis, and Alzheimer's disease), Multiple Sclerosis, Schizophrenia and Mood Disorders, Stroke, and finally

Neurotrauma. In this last category, two classes of injury are differentiated, depending on the CNS division affected: Spinal Cord Injury (SCI) and Traumatic Brain Injury (TBI).

Many human diseases and injuries that result in neurodegeneration can be mimicked using animal models. For example, Kainate, an excitotoxic agent, is a useful model of epileptogenesis and seizures (Zaczek *et al*., 1981), and the administration of MPTP selectively kills dopaminergic neurons, thereby providing a model of Parkinson's disease (Freyaldenhoven *et al*., 1997). In addition, ischemic stroke models are generated by occlusion of the middle cerebral artery (Tamura *et al*., 1980), and knockout and transgenic mice are commonly used to study chronic neurodegenerative diseases. Two main models are used to model CNS trauma: transection and contusion.

Transection, axotomy or blunt injury, all synonyms, uses surgical transection of a nerve tract. In the spinal cord, it can be either complete or partial (hemisection), while in the brain it would be referred to as unilateral or bilateral axotomy. After transection, the severe damage is restricted to the level of the injury. In contrast, following **contusion** (or crush injury), the injury leads to a characteristic egg-shaped zone of necrosis. The most extended animal model for SCI is the weight-drop injury, and for TBI, models such as the controlled cortical impact (Lighthall., 1988; 1990) and fluid percussion injury (Prins *et al*., 1996) are commonly used.

As in TBI, characterized by a blow to the head, most people with SCI do not have a completely severed spinal cord. Rather, they have a contused spinal cord, where the cord is stretched or compressed, causing tearing and bleeding. Thus, the contusion model most closely mimics a typical human SCI and TBI, and is therefore most likely to be predictive of efficacy in human beings. In contrast, the more selective transection model permits the differentiation of the various experimental parameters and is more suitable for basic science.

The model used in this thesis is the axotomy of the perforant pathway, a unilateral transection model of TBI. The perforant pathway is the main afferent connection to the hippocampus, and connects the entorhinal cortex with the granule cells in the dentate gyrus. This model has been chosen for reasons that will be discussed in the fourth chapter of this introduction. We will also discuss its advantages and disadvantages and address the utility of the organotypic culture system to model *in vivo* axotomy.

Introduction II

Inhibitory Molecules for Axonal Regeneration

2.1 Axon guidance molecules

During development, axons are guided to their targets by certain molecules that act as guidance cues. These cues can be growth-promoting (or attractive) or repulsive, and act either at short- or long-range (Tessier-Lavigne and Goodman, 1996). There are four families of well characterized chemorepellents: semaphorins, eprhins, netrins, and slits (Fig. 2.1). These guidance cues, however, continue to be expressed in the adult CNS, where they may play distinct roles. As discussed in the following chapter, the intracellular pathways mediating growth cone collapse, or growth inhibition, during development appear to be the same ones that regulate axonal extension in the mature CNS. The presence of inhibitory guidance cues in the adulthood, which is frequently regulated following lesion, may thus inhibit axonal outgrowth, contributing to the incapacity of injured axons to regenerate. Here we provide a concise summary of the role of these axon guidance cues in the adult CNS, followed by a brief description of their

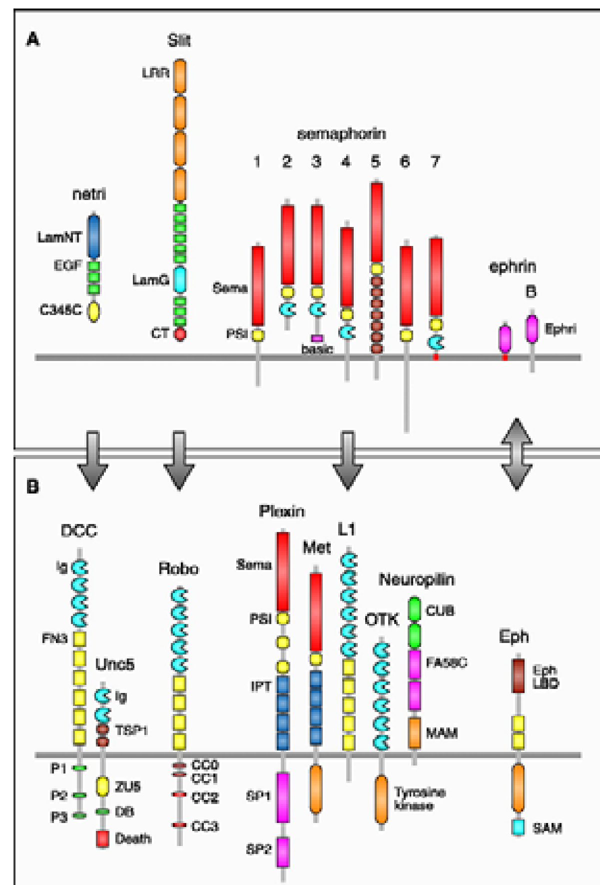


Fig 2.1. Scheme representing the main four families of axon guidance cues and their receptors. The expression of these proteins in the adult CNS may contribute to prevent axonal regeneration (taken from Dickson 2002).

involvement in CNS axonal regeneration failure after lesion.

2.1.1 NON-PATHOLOGICAL FUNCTION

Semaphorins. Semaphorins are a large family of secreted and transmembrane proteins which participate in tasks such as axon steering, fasciculation, branching, and synapse formation during development. In addition, semaphorins, together with eprhins, netrins, and slits, regulate neuronal migration. With some exceptions, semaphorins act as chemorepellents through binding to neuroplinins and/or plexins. In the healthy mature CNS, the expression of semaphorins is reduced in comparison with their widespread distribution in the developing CNS, and is limited to certain neuronal populations, predominantly in the motor system and olfactory-hippocampal pathway, and meningeal fibroblasts (de Wit and Verhaagen, 2003). The expression of semaphorins in these structures in the adult CNS could stabilize synaptic contacts, thereby inhibiting aberrant sprouting, a role that has also been proposed for other axonal growth inhibitors such as those present in myelin.

Ephrins. There are GPI-linked ephrins (ephrins-A) and transmembrane ephrin-B proteins, all of which interact with tyrosine-kinase receptors (Eph). During development, ephrins act as contact repellents, regulating axon fasciculation and topographic map formation. Most Eph receptors and ephrins are expressed at substantial levels in the adult CNS, predominantly in regions that undergo remodelling. Although they are typically expressed in neurons, some Eph and ephrins are also expressed in glial cells. The subcellular localization in pre- and post-synaptic areas indicates that, in the adult CNS, Eph-eprhin interactions participate in the regulation of synaptic architecture and plasticity, and their roles in the context of dendritic spine morphogenesis, neuroglial communication at the dendritic spines and regulation of plasticity and sprouting have been extensively studied (Yamaguchi and Pasquale, 2004

Netrins, which are involved in axon guidance processes during development, are secreted proteins that can both attract or repel the growth cone of distinct neurons through interaction with DCC and Unc-5H receptors. In the adult spinal cord, Netrins and their receptors are expressed by neurons and oligodendrocytes at similar levels to those found during development, but unlike development, in the adult CNS Netrin is expressed in association with membranes or extracellular matrix (ECM). This characteristic indicates that Netrin acts as a short-range cue in the adult CNS, probably regulating the interaction between neurons and oligodendrocytes (Manitt *et al*., 2001).

Slits are diffusible proteins that participate in multiple aspects of neural development, such as axon guidance (Brose *et al*., 1999; Nguyen Ba-Charvet *et al*., 1999, 2002; Plump *et al*., 2002) and neuronal migration (Zhu *et al*., 1999; Marin *et al*., 2003). In the adult, Slit family members and the receptor Robo-2 are expressed in regions such as the cerebral cortex, hippocampus and septum (Marillat *et al*., 2002; Nguyen-Ba-Charvet *et al*., 2004), where they may regulate the migration of subventricular zone neuroblasts (Nguyen-Ba-Charvet *et al*., 2004).

2.1.2 PATHOLOGICAL FUNCTION

While the balance between attractant and repellent factors favors axonal outgrowth during development, in the mature CNS this relationship changes to favor the stability of synaptic contacts (although allowing certain degree of morphological plasticity). Following injury, the expression of growth inhibitory molecules, which normally prevent aberrant sprouting, may hinder axonal regeneration. This is the case of semaphorins. After lesion to the olfactory system, cerebral cortex and perforant pathway, several secreted and transmembrane repulsive semaphorins (significantly Sema 3A) are overexpressed by meningeal fibroblasts, which infiltrate into the neural tissue and secrete Sema 3A into the ECM in the lesion site (Pasterkamp *et al.*, 1999). This injury-induced overexpression of semaphorins in the glial scar by meningeal cells contributes to the formation of a chemical barrier for regenerating axons. In addition to meningeal fibroblasts, glial cells express several members of the semaphorin family (Fig. 2.2). For instance, the expression of Sema 5A by oligodendrocyte lineage cells has been shown to prevent axonal regeneration of retinal ganglion cells, and its blockade *in vitro* promotes axonal regeneration, although its expression is not up-regulated after axotomy (Goldberg *et al.*, 2004). Also, Sema 4D is expressed by mature oligodendrocytes, and although its blockade has not been tested, it is up-regulated after CNS injury (Moreau-Fauvarque *et al.*,2003). The expression of semaphorins by glial cells and their regulation by injury indicate that these molecules may act as constitutive inhibitors of neurite outgrowth in the adult CNS, and after injury, their negative consequences on axonal regeneration may be supplemented by fibroblast-secreted semaphorins and those overexpressed by glial cells.

Sema	OPC	Oligo	Astro
3A	XXX	-	-
3B	XX	X	XX
3E	XX	XX	X
4A	X	XX	XX
4B	XX	XX	XX
4C	XX	XXX	-
4D	XX	XX	-
5A	XX	XXX	-
5B	XX	XX	-
6A	X	XXX	XX
Actin	XXXX	XXXX	XXXX

Fig. 2.2. Semiquantitative RT-PCR analysis of the expression of semaphorins by purified optic nerve glia.. OPC, Oligodendrocyte precursor cells; Oligo, oligodendrocytes; Astro, astrocytes. (Taken form Goldberg *et al*., 2004)

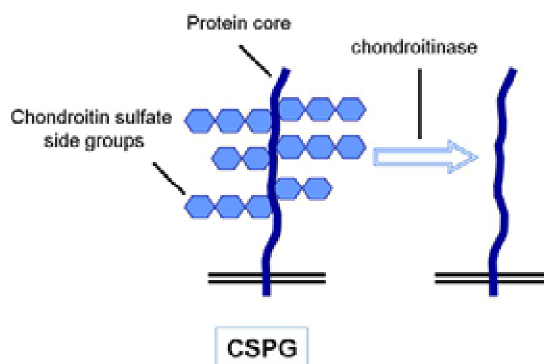
After injury, many ephrin- and Eph-mRNAs are up-regulated. Reactive astrocytes overexpress ephrin-B2 Eph-B2, while meningeal fibroblasts up-regulate Eph-B2. It is likely that this interaction generates a boundary in the glial scar, where astrocytes and fibroblasts create segregated bands that contribute to the development of the cellular structure of the meningo-glial scar. In addition, recent analyses of mice lacking Eph-A4 has revealed that in the absence of this receptor, the astrocytic gliosis and the glial scar fail to develop normally, thereby allowing functional recovery after spinal cord hemisection. Thus, after lesion, Ephs and Ephrins prevent axonal regeneration not only by mediating contact repulsion, but also by regulating the development of the glial scar. Similarly, Slit proteins, together with their glypican-1 receptors, are overexpressed by reactive astrocytes after cortical injury (Hagino *et al.*, 2003). This combined expression of Slits and glypican might enhance the binding of Slit to Robo (Hagino *et al.*, 2003), which is expressed by adult cortical neurons (Nguyen-Ba-Charvet *et al.*, 2004), and may be involved in regeneration failure. Finally, in the adult CNS, the expression of Unc-5H, the netrin receptor that mediates axon repulsion during development, dominates over DCC (Manitt

et al., 2004). This indicates that the main function of Netrin in the adult CNS could be the inhibition of process extension and collateral sprouting, and following injury, it could contribute to the inhibition of axonal regeneration (Manitt *et al*., 2004).

2.2 Proteoglycans

Proteoglycans are extracellular matrix proteins comprising a core protein linked to glycosaminglycan (GAG) sugar chains, which are made of a polymer of disaccharide units. These disaccharide units differ for each group of proteoglycans, which are classified into heparan sulfate proteoglycans (HSPG), chondroitin sulphate proteoglycans (CSPG), dermatan sulfate proteoglycans (DSPG), and keratan sulfate proteoglycans (KSPG), and define many of the functional properties of the proteoglycan (Bandtlow and Zimmermann, 2000). Here, we will focus on CSPG since they are strong inhibitors of axonal growth and their expression increases in the glial scar after lesion. CSPG are not only expressed by astrocytes, but have a widespread distribution in the organism and regulate diverse biological processes, such as cell migration and recognition. Indeed, during CNS development these molecules participate in the modulation of neuronal migration, pathfinding and neurite outgrowth (Bovolenta and Fernaud-Espinosa, 2000).

Fig. 2.3. Structure of a CSPG. After chondroitinase treatment, CSPG molecule is reduced to a core protein, which generally has no inhibitory effect on axon growth.



One characteristic of CSPG structure is the presence of chondoritin sulphate GAGs chains, which can be removed by Chondoritinase treatment (Fig. 2.3). In addition to the GAG chains, the core protein of CSPG can also interact with a wide range of molecules, thus conferring part of the protein properties. Some CSPG are brevican, neurocan, versican and aggregacan (all secreted molecules that participate in the formation of the ECM), NG2 and phosphacan, which inhibit neurite outgrowth either directly or indirectly via interaction with cell adhesion molecules such as N-CAM and L1 (Bovolenta and Fernaud-Espinosa, 2000). The monoclonal antibody CS-56, raised against chondroitin sulphate chains, allows the detection of the general expression of CSPG and has been used to characterize the regulation of these molecules after lesion (Davies and Silver., 1998).

2.2.1 NON- PATHOLOGICAL FUNCTION

In the CNS, proteoglycans regulate several aspects of neurite outgrowth, including the promotion and inhibition of neurite outgrowth and the regulation of neuronal polarity (Bovolenta and Fernaud-Espinosa, 2000). HSPG are promoters of neurite outgrowth during neuronal development *in vivo*; however, CSPG, like neurocan or phosphacan, have bi-functional activity,

and while they are outgrowth-permissive molecules during development, at high concentrations they strongly inhibit neurite growth (Asher *et al*., 2001). Indeed, all glia boundaries in the developing mammalian CNS are characterized by high expression of either CS, DS or KS proteoglycans, which may play a role in axon guidance (Bovolenta and Fernaud-Espinosa, 2000).

Like many inhibitory proteins, CSPG contribute to the regulation of CNS plasticity in the adult CNS, and chondroitinase treatment restores ocular dominance plasticity, which is lost after a critical period during development (Pizzorusso *et al*., 2002).

2.2.2 PATHOLOGICAL FUNCTION

CSPG and the inhibition of axonal regeneration. Astrocytes, oligodendrocyte lineage cells and meningeal cells are the cell types that produce CSPG (Asher *et al*., 2001). In response to a CNS lesion, CSPG expression increases in the glial scar, starting immediately after injury and peaking between 4 and 7 days post-injury (Pasterkamp *et al*., 1999; Properzi *et al*., 2003). In particular, reactive astrocytes and oligodendrocyte progenitor cells up-regulate neurocan, NG2, phosphacan and versican in the glial scar (Rhodes and Fawcett, 2004). Although chondoritinase abolishes the inhibitory effects of many CSPG, there are certain exceptions, such as NG2, which is expressed by oligodendrocyte progenitors and whose inhibition lies in the core protein (Dou and Levine., 1994). In addition to the direct inhibition of axonal growth, CSPG may prevent axonal regeneration by modifying axonal responsiveness to guidance cues, such as Sema 5A. While CSPG are required for Sema 5A-mediated inhibition, HSPG may convert Sema 5A into an attractive cue (Kantor *et al*., 2004). Although this regulation occurs during development, the participation of Sema 5A in preventing axonal regeneration in the adult has been demonstrated and CSPG could contribute to the induction of axonal inhibition in response to this bi-functional molecule. Finally, CSPG overexpression after injury could have beneficial effects and it has been proposed to limit cavitation, as part of the rapid reactivity of local glial cells (Rhodes *et al*., 2004).

2.3 Myelin-associated Inhibitors

This name designates a family of functionally related proteins that includes MAG, Nogo-A and OMgp. They are all expressed by oligodendrocytes and bind to NgR. The result of this interaction is a strong inhibition of neurite outgrowth. In addition to this role, these three proteins are involved in distinct tasks. Here we will comment on the molecular structure, cellular localization, distribution and roles (both in the healthy and in lesioned CNS) of MAG, Nogo-A and OMgp, and will address their receptors and the signaling pathways that participate in axonal inhibition in the following chapter.

2.3.1 MAG

MAG was the first protein in myelin to be characterized as an inhibitor of axon growth (Filbin *et al*., 1996). Although it is expressed by oligodendrocytes and Schwann cells in central and peripheral myelin respectively, MAG accounts for only 0.1% of myelin protein in the PNS, while it is one of the main proteins in CNS myelin (1% of total protein). Like NCAM, L1 and DCC, it is also a member of the Immunoglobulin Superfamily (Fig. 2.4). Immunoglobulin cell adhesion molecules (Ig CAMs) are widely expressed during development and play a role in the development of specific axonal projections (Walsh and Doherty, 1997). MAG contains five Ig domains, a transmembrane domain and a short intracellular domain that differs for two isoforms, called S-MAG and L-MAG, which are generated by alternative splicing (Tropak *et al*., 1988). In addition, MAG is a Sialoadhesin (Kelm *et al*., 1996), and binds to sialic acid residues on the surface of gangliosides and proteins (Tang *et al*., 1997a, b; Tropak *et al*., 1997; Vinson *et al*., 2001; Vyas *et al*., 2002). Arg118, within the first Ig domain, is a crucial residue for the binding of MAG to sialic acids (Tang *et al*., 1997).

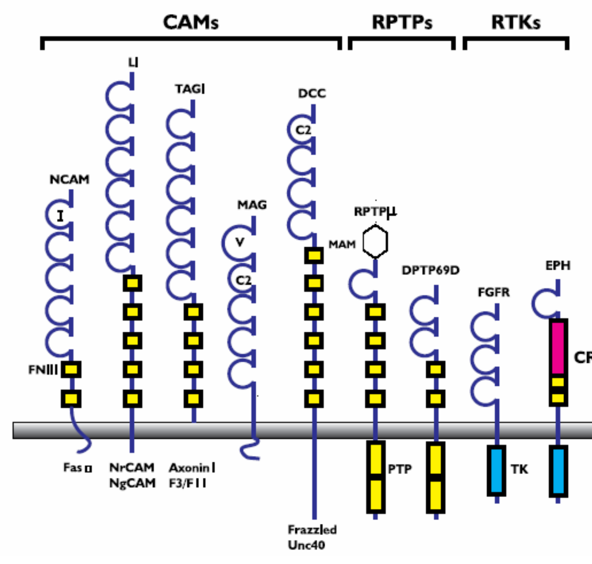


Fig 2.4. Structure of some members of the Ig superfamily implicated in growth and guidance in the nervous system. Shown are examples of three main subgroups, named CAMs, RPTPs, and RTKs.

The primary sequence of the two MAG intracellular domains share no significantly homology with other proteins, and few studies have addressed the differential functions of the two isoforms (Kursula *et al*., 2001, 2002). In this thesis, we have studied the roles of MAG and its regulation by injury in the context of axonal regeneration. Since the two isoforms share the extracellular domain, and putatively interact with the same neuronal receptors, treat them as a single protein.

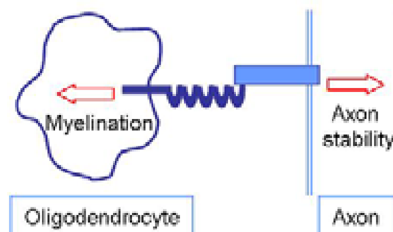
2.3.1.1 NON- PATHOLOGICAL FUNCTION

Myelination. It has been proposed that MAG plays a critical role in initial stages of myelination and contributes to the maintenance of the myelin sheath (Schachner and Bartsch, 2000) (Fig. 2.5 a and b). A significant delay in the formation of compact myelin has been observed in MAG-deficient mice (Montag *et al*., 1994), and distal oligodendrocyte processes degenerate in these animals (Lassmann *et al*., 1997). However, the subtle phenotype associated with MAG-deficiency indicates that other molecules compensate for the lack of MAG in these animals.

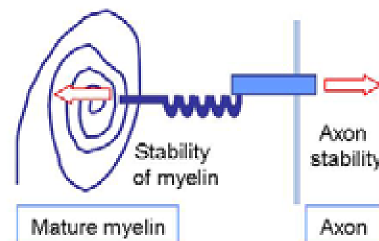
Intercellular adhesion. After formation of myelin, MAG is restricted to the periaxonal region and is especially enriched in the paranodal regions of myelin sheaths (e.g., Bartsch *et al*., 1989; Martini and Schachner, 1986). Because of its location, it has been proposed that MAG participates in the stabilization of contacts between axons and glial processes (for a review, see Bartsch, 1996). Also, axonal atrophy is observed in MAG-knockout mice, which indicates that MAG may participate in axonal integrity maintaince (Yin *et al*., 1998). The neuronal receptor (or receptors) involved in these processes are still unknown.

Promotion of neurite outgrowth. It is interesting that the effect of MAG on neurite outgrowth was first described to be attraction (Johnson *et al*., 1989; Mukhopadhyay *et al*., 1994). Depending on the type and age of the neuron, contact with MAG either promotes or inhibits neurite outgrowth. This is the case of dorsal root ganglia (DRG) neurons. While newborn or early postnatal neurons extend neurites twice as long on MAG-transfected than on control cells, axon outgrowth from adult DRG is inhibited by MAG (Mukhopadhyay *et al*., 1994). However, it is not clear whether this outgrowth-promoting effect has a physiological role, since myelination, and hence MAG expression, starts after neurons have switched their response to MAG from attraction (during development) to inhibition.

(a) At the initiation of myelination, MAG is both a receptor and a ligand



(b) In mature myelin, MAG is both a receptor and a ligand



(c) When affecting axonal regeneration, MAG is a ligand

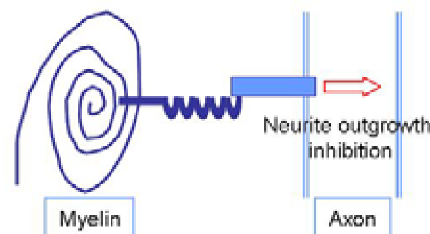


Fig. 2.5. During development, MAG can act as both a receptor and a ligand. When inducing myelination or contributing to the stability of myelin, MAG acts a receptor for the oligodendrocyte and as a ligand for unknown neuronal receptors, which activation promotes axon stability. In the adult, MAG interaction with NgR, NgR2 or gangliosides prevents axon outgrowth and hence, axonal regeneration. To simplify the scheme, soluble MAG has not been represented (adapted from Filbin 1995).

2.3.1.2 PATHOLOGICAL FUNCTION

Before the antigen of IN-1 antibody was known, MAG was described as a putative myelin inhibitor of regeneration (McKerracher *et al*., 1994; Mukhopadhyay *et al*., 1994). MAG prevents axonal growth of postnatal neurons *in vitro*, and its inactivation promotes axonal regeneration both *in vitro* and *in vivo* (McKerracher *et al*., 1994; Mukhopadhyay *et al*., 1994; Wong *et al*., 2003) (Fig. 2.5 c). Its binding to the neuronal surface has been found to be, at least in part, sialic-acid dependent and neuraminidase-sensitive (Kelm *et al*., 1996; DeBellard *et al*., 1996; Tang *et al*., 1997b; Vinson *et al*., 2001). The first putative neuronal receptors to be described for MAG were gangliosides GD1a and GT1b (Vinson *et al*., 2001; Vyas *et al*., 2002). However, the

implication of gangliosides in MAG-mediated neurite outgrowth inhibition is not clear, and two related proteins have been reported to be functional receptors for MAG: NgR and NgR2. While the binding to the first appears to be sialic acid-independent, at least in non-neuronal cells, (Domeniconi *et al*., 2002; Liu *et al*., 2002), NgR2 has been recently identified as a high-affinity and sialic acid-dependent neuronal receptor for MAG. Even before the discovery of NgR, it was found that p75 mediated MAG signalling (Yamashita *et al*., 2002). It is now known that gangliosides and NgR, but probably not NgR2, use p75 as a co-receptor, and neurons from p75-knockout mice are not inhibited by MAG. In addition, depending on the neuronal population and developmental stage, p75 is replaced by Troy in the receptor complex. A complete discussion about MAG receptors and the signalling pathways they use will be addressed in the following chapter.

One characteristic that differentiates MAG form the other myelin-associated inhibitors is the a proteolytic fragment, termed **dMAG**, which is released form myelin *in vivo* (Sato *et al*., 1984, Yim and Quarles, 1992, Tang *et al*., 9197b). This fragment, which consists of the entire extracellular domain of MAG, inhibits axonal regeneration *in vitro* (Tang *et al*., 1997b). Importantly, it has been shown that factors secreted from damaged white matter inhibit neurite outgrowth in a collagen assay, and that the use of antibodies against MAG in these assays neutralizes this inhibition (Tang *et al*., 2001). Therefore, dMAG appears to account for most of the inhibitory activity secreted from damaged white matter.

The regulation of MAG after axotomy appears to be specific to the cellular population expressing MAG (Schwann cells or oligodendrocytes) or the particular injury. Thus, there is not a defined regulation of MAG expression after axotomy, and while it is down-regulated after transection of rat sciatic nerve (PNS) (Tacke and Martini, 1990), it increases following axotomy of cortical connections (the present work). Although in the last case MAG expression excluded the glial scar, it may explain why the PNS is more permissive to axonal regeneration than its central counterpart while expressing some of the inhibitory molecules thought to contribute to axonal regeneration failure. It is important to highlight that the regenerative capacity of MAG-deficient mice is scarce, as is also that of the Nogo-A- and NgR-knockouts, as described later (Bartsch *et al*., 1995). This low regenerative capacity appears to be a common characteristic of many knockout mice, and will be discussed in the last part of this thesis. However, several *in vivo* blockades have demonstrated that MAG actively participates in the lack of axonal regeneration, and interference with MAG activity can be useful approach to enhance regeneration. This is the case of vaccination against MAG (Sicotte *et al*., 2003), the use of blocking antibodies (Tang *et al*., 2001), neutralization of MAG with CALI (Wong *et al*., 2004) and Neuraminidase treatment (the present work).

2.3.2 Nogo

The previous chapter mentioned the pioneering experiments made by Caroni and Schwab that led to the discovery of Nogo in 2000 (Caroni and Schwab 88a, b). This initial work showed that

a considerable part of the inhibitory activity present in oligodendrocytes, and particularly in CNS myelin, lies in two protein fractions of 35 and 250 kD, which strongly inhibit neurite outgrowth when used as substrate (Caroni and Schwab 1988b). These original myelin-associated inhibitors were named NI-250 and NI-35 (for "neurite growth inhibitor" and a number corresponding with their molecular weight) (Fig. 2.6). The monoclonal antibody IN-1 (for inhibition neutralization) was raised against NI-250 (Caroni and Schwab 1988b), and was shown to partially neutralize the growth inhibitory effect of CNS myelin and oligodendrocytes, and result in long distance fiber regeneration in the lesioned adult mammalian CNS *in vivo* (see Chapter I). Since IN-1 neutralized the inhibitory activity of NI-35, this protein was assumed to be a proteolytic product of NI-35 (Bandtlow and Schwab, 2000).

Table III. Nonpermissive Substrate Property of CNS Myelin Is Preserved upon Reconstitution into Artificial Lipid Vesicles

Reconstituted protein fraction	3T3 spreading	Dish-adsorbed lipids
	μm²	*cpm [³H]cholesterol*
No protein	1,638 ± 91	521 ± 65
CNS myelin	136 ± 30	650 ± 58
CNS myelin; resulting liposomes trypsinized	1,397 ± 152	630 ± 32
PNS myelin	1,570 ± 136	620 ± 41
liver membranes	1,445 ± 121	750 ± 47

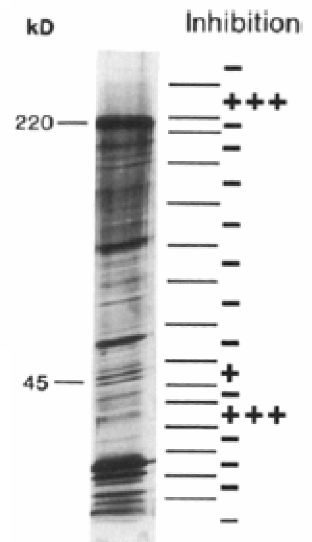


Fig. 2.6. Table and figure extracted from the original article by Caroni and Schwab where they first describe the existence of two myelin-associated proteins with inhibitiory properties. Different protein fractions (see table for details) were reconstituted as liposomes that were used to coat culture dishes. The main inhibitory fraction corresponded with CNS-myelin (compared PNS myelin or non-myelin fractions) and its effects could be reverted with trypsin treatment,, what indicates they are associated with proteins. Fractions from CNS myelin were tested and the main non-permissive liposomes corresponded to 250 and 35kD-containin protein fractions (see figure). (Taken from Caroni and Schwab 1988a).

The IN-1 epitope was purified and partially sequenced in 1998 by Spillmann *et al*. (Spillmann *et al*., 1998) and culminated in the cloning of *nogo*, the gene codifying the protein Nogo-A (Ni-250) (Chen *et al*., 2000, GrandPre *et al*., 2000; Prinjha *et al*., 2000).

The *nogo* gene. The *nogo* gene (also RTN4, see below) putatively gives rise to seven proteins by splice variation and alternative promoter usage, although only three are finally transcribed in the CNS, Nogo-A, Nogo-B and Nogo-C (Hunt *et al*., 2002). Human *nogo* gene consists of 14 exons and two promoters. While Nogo-A and –B are transcribed form the same promoter and differ only in the exons 3 and 4, which are lost in Nogo-B by splicing, Nogo-C is transcribed from different promoters and shares the segments encoded by exons 6-11 with Nogo-A and –B. *nogo* transcripts have a broad tissue expression pattern, and most transcripts are enriched in the CNS (Oertle *et al*., 2003b).

The Nogo proteins. Human Nogo-A, -B and –C proteins are 1192-aa, 373-aa and 199-aa in length respectively (Fig. 2.7), and appear in SDS-PAGE as 220kDa, 55kDa and 25kDa bands. They lack an amino terminal signal sequence, which would cause them to localize at the cell surface, whereas all Nogo forms exhibit a dilysine endoplasmic reticulum (ER) retention signal within the conserved C-terminal.

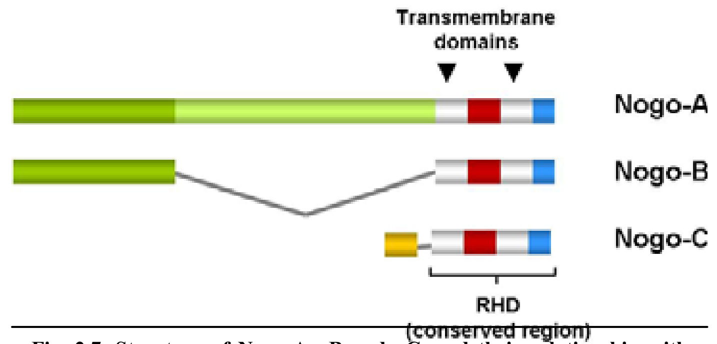


Fig. 2.7. Structure of Nogo-A, -B and –C, and their relationship with each other. RHD: Reticulon-Homology domain. Putative tramsmembrane domains are shown in white and Nogo-66 in red (see text for details).

The C-terminus of Nogo, present in the three forms, contains two large transmembrane domains, both of which could both span the membrane either once or twice (Fig. 2.7). These transmembrane domains are separated by a 66-aa fragment called **Nogo-66**, which is also conserved. Nogo-A and -B also share a 172 aa N-terminus domain (dark green, Fig. 2.8), followed in Nogo-A by a central region, named **NiG**, which is missing in Nogo-B and –C (light green). Both the Nogo-66 and the NiG regions are believed to participate in Nogo-A-induced neurite outgrowth inhibition. In addition, Nogo-C has a short 11 aa N-terminus domain which is not present in any other Nogo form. Neither Nogo-A, -B or -C contain any motifs of known cell-adhesion molecules, ECM proteins, axonal guidance molecules or growth cone repulsive molecules (Chen *et al.*, 2000) that could facilitate the analysis of the putative roles of Nogo-A.

The Reticulon Family. The C-terminal region conserved in all Nogo proteins has homology with another 3 mammalian genes. The four paraloges code for a family of proteins that share the characteristic of being largely localized in the ER, so it has been named the Reticulon Family (RTN). RTN proteins are ubiquitously expressed in vertebrates and have been described in most eukaryotic taxa but not in prokaryotes (Oertle *et al.*, 2003b, c). The four mammalial reticulon genes (*RTN1*, *RTN2*, *RTN3* and *Nogo*/*RTN4*) have a similar gene structure and give rise to distinct splicing forms (see Fig. 2.7 for *Nogo*/*RTN4*). In addition to the Reticulon-Homology Domain (RHD) in the C-terminus of all the RTN proteins, the N-terminus differs greatly, and N-terminus of Nogo-A appears in evolution during the transition from fish to land vertebrates (Oertle *et al.*, 2003b). The wide distribution of RTN proteins indicates that they exert very basic functions. They may act as pore or transporter complexes forming heteropolymers through the binding of the RHD (this association has been recently demonstrated for Nogo-A, -B and –C, Dodd *et al.*, 2005; see also Oertle *et al.*, 2003d); participate in the transport of proteins from the ER to other membrane compartments; stabilize ER network or play a role in cell division (Oertle *et al.*, 2003d).

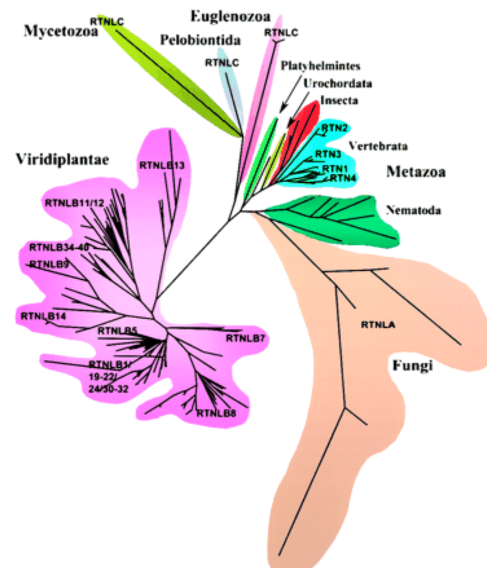


Fig. 2.8. Taxonomic distribution of the RTN gene family. (Taken from Oertle *et al*., 2003d)

Nogo-A corresponds to RTN4-A, and although it was first identified as a myelin component, it is also expressed by neurons, mainly in the cerebral cortex, the hippocampus and Purkinje cells, in the adult CNS (see Hunt *et al.*, 2003 for review). The relative abundance of Nogo-A in oligodendrocytes and neurons is not clear, as Nogo-A antigenicity seems to be highly sensitive to fixation techniques. However, there are many lines of evidence that Nogo-A is expressed by all myelinating oligodendrocytes (Chen *et al.*, 2000, GrandPre *et al.*, 2000, the present work) as well as by many neuronal populations, mainly during development (Josephson *et al.*, 2001; Hunt *et al.*, 2002; Huber *et al.*, 2002; Tozaki *et al.*, 2002, the present work). To inhibit axonal regeneration when expressed by oligodendrocytes, Nogo-A has to reach the cellular surface, and extracellularly expose those domains involved in neurite growth inhibition. The predicted topology of Nogo-A indicates that the two transmembrane domains may span the membrane once or twice and the 66 aminoacids between these segments are exposed at the ER lumen, or at the extracellular surface. These predictions also indicate that the N-terminus of Nogo-A is cytosolic when localized in the ER (Fig. 2.9 A). Like several myelin membrane proteins, such as PMP-22 and MAL, Nogo-A has an ER retention signal yet it can be found on the cell surface, where it adopts distinct topologies (Chen *et al.*, 2000; Oertle *et al.*, 2003d: Fig. 2.9 A).The mechanisms through which Nogo-A translocates to the plasma membrane are unknown and appear to be absent from cell lines such as CHO, COS7 or 293T, where Nogo-A is fully retained in the ER when transfected (Fig. 2.9 B).

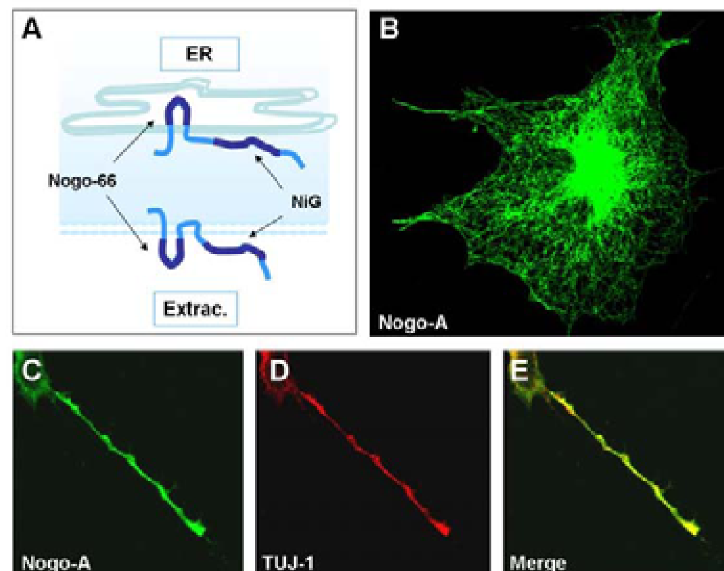


Fig. 2.9. Localization and topology of Nogo-A in different cell types. A) Putative topology of Nogo-A in neurons and oligodendrocytes. At the ER, Nogo-66 is luminal while the N-terminus is cytoplasmatic. However, at the cell surface, the second transmembrane domain of Nogo-A spans the membrane twice and both Nogo-66 and NiG are exposed extracellularly. B) When transfected in cell lines (COS7 cell in the figure), Nogo-A is retained in the ER as is not detectable without permeabilization. C) In neurons, Nogo-A is localized both in the ER and in the plasma membrane, where it can be found in the neuronal soma and neurites. The image shows Nogo-A expression in the axon and growth cone of a cultured cerebellar granule neuron compared with tubulin staining (TUJ-1).

The exact localization and topology of Nogo-A at the plasma surface has important biological significance (Oertle *et al.*, 2003d; Dodd *et al.*, 2005) and has not been clear until recent reports. Nogo-A on the surface of oligodendrocytes in the adult CNS inhibits neurite growth and regeneration through its N-terminus and the Nogo-66 domain. These domains are exposed

extracellularly (Fig. 2.9 A) and bind to cortical membranes when produced as fragments, which indicates the presence of specific receptor molecules for these domains (Oertle *et al*., 2003d). However, a large intracellular pool of Nogo-A remains associated with the ER and the Golgi complex (Oertle *et al*., 2003d). Figure 2.9 A shows the distinct topology of Nogo-A on each localization. In addition to being expressed by oligodendrocytes, Nogo-A is also expressed by neurons and fibroblasts, where it reaches the plasma membrane (Oertle *et al*., 2003d; Dodd *et al*., 2005). The N-terminus of Nogo-A is also exposed extracellularly in these cells (Oertle *et al*., 2003d; Dodd *et al*., 2005), which indicates that Nogo-A may exert multiple functions both when expressed by distinct cell populations and in its diverse subcellular localizations.

2.3.2.1 NON- PATHOLOGICAL FUNCTION

Nogo-A functions during development. During CNS development, Nogo-A is extensively expressed by neurons, including those that are proliferating and migrating, thus it is unlikely that Nogo-A is involved in preventing neurite outgrowth (Tozaki *et al*., 2002). The potential roles for neuronal Nogo-A during development, however, are totally unknown, and most research has focused on its role as an inhibitor in the adult (when expressed by oligodendorcytes), overlooking the first lines of evidences available (based on Nogo-A expression pattern and the first *in vitro* experiments) which indicate that neuronal Nogo-A is probably a cell-adhesion molecule. During recent years, we have assessed the possible roles of neuronal Nogo-A during development. The data available on these functions will be addressed in the discussion of this thesis, since they are mainly limited to our own work.

Nogo-A intracellular functions. From its localization in the ER, Nogo-A may play many diverse roles in oligodendrocytes and neurons. Although there is no evidence of intracellular roles for Nogo-A, it is reasonable to assume that the large pool of ER-retained Nogo-A has a function, probably related to the roles played by RTN proteins. However, there is evidence of the implication of Nogo-B in apoptosis (Oertle *et al*., 2003a; Watari and Yutsudo, 2003). Nogo-B modulates Bcl-2 proto-oncogen activity by changing it localization to the ER, and thus preventing its anti-apoptotic activity (Oertle *et al*., 2003a; Watari and Yutsudo, 2003). Since Nogo-A holds the full sequence of Nogo-B, it is reasonable to hypothesize that it also shares the pro-apoptotic role. Interestingly, Nogo-A interacts with a mitochondrial protein of unknown function called NIMP (from Nogo Interacting Mitochondrial Protein) (Hu *et al*., 2002). Given the central role that mitochondria has in apoptosis, the interaction with NIMP is a second line of evidence, in addition to homology with Nogo-B, that could relate Nogo-A with apoptosis control. Other proteins that interact with Nogo-A are tubulin and MBP, although their functional relevance is unknown. In addition, several members of the reticulon family regulate vesicular traffic and have been recently shown to interact with SNARE proteins (Steiner *et al*., 2004). Again, given the high homology of the RTN family members, a similar function for Nogo-A cannot be ruled out. Finally, RTN-1 members can form homo- and heteromers that could act as pore or transporter complexes (Oertle *et al*., 2003c). Nogo-A, -B and –C also form complexes (although Nogo-A does not interact with itself) when overexpressed and also when expressed

endogenously in oligodendrocytes (Dodd *et al*., 2005). These protein complexes may act as ion channels, pores or transporters, and given that Nogo-A and –B have a putative calcium binding site on their N-termini, the Nogo-A/B/C complex could be a channel or transporter that regulates calcium (Dodd *et al*., 2005).

Nogo-A function at the axo-glial junction. During the beginning of myelination, Nogo-A is preferentially localized in the paranodal region, in the myelin sheath. This region, flaking Ranvier nodes, allows the tightest contact between the oligodendrocyte and neuron. During this period, Nogo-66 is exposed at the paranodes and interacts with the complex Caspr/F3 at the neuronal surface (Nie *et al*., 2003). This binding contributes to the sorting of potassium channels from the nodes to the juxtaparanodes, which is required to ensure salutatory conduction (Nie *et al*., 2003). It is supposed that this role for Nogo-A is punctual, and after this period Nogo-A distribution at the myelin surface changes and is expected to interact with NgR.

2.3.2.2 PATHOLOGICAL FUNCTION

Certainly, the best characterized role of Nogo-A is that related to myelin-induced inhibition. Nogo-A, together with MAG and OMgp, localizes at the outermost myelin membrane, in close contact with the myelinated axon, which expresses a receptor complex that induces growth cone collapse in response to these ligands. The growth cone collapses when challenged with purified Nogo-A *in vitro*. The physiological relevance of this inhibition has been supported by *in vivo* neutralizing studies, in which axonal regeneration was substantially improved. To name a few, since they have already been described in the previous chapter, treatment with IN-1 (IgM and Fab fragments), raised against the N-terminus of Nogo-A, promotes the regeneration or sprouting of different tracts and increases functional recovery (Bregman *et al*., 1995; Merkler *et al*., 2001); Raineteau *et al*., 2001). Vaccination with Nogo-A-derived peptide also improves functional recovery (Hauben *et al*., 2001). Interference with Nogo-66 binding to NgR also enhances axonal regeneration, as observed *in vitro* by cleaving NgR from the axolemma (Fournier *et al*., 2001), *in vivo* treatment with the NgR antagonist peptide NEP1-40 (GrandPre *et al*., 2002; Li and Strittmatter, 2003), and *in vivo* delivery of a soluble truncated NgR (Li S *et al*., 2004). Indeed, axonal regeneration after sciatic nerve crush is impaired in animals expressing Nogo-A in Schwann cells (Pot *et al*., 2002). This observation demonstrates that Nogo-A is a potent inhibitor of axonal regeneration *in vivo*, and raises the expectations of promoting functional recovery after spinal cord lesions by neutralizing Nogo-A in humans.

The other face of the coin, however, is the report on the regenerative potential of the Nogo-A-deficient mouse. Three papers have reported Nogo deletions using different strategies, with several regeneration phenotypes. A comparison between the mouse lines is presented in Table 2.1. From the four lines of Nogo-deficient mice generated, one lacks Nogo-A expression (Simonen *et al*., 2003), two lack Nogo-A/-B expression (Kim *et al*., 2003; Zheng *et al*., 2003) and one lacks the expression of the three transcripts (Zheng *et al*., 2003). The phenotype, behavior and viability of all of them is normal; however, myelin from all of the Nogo-deficient

mice has reduced inhibitory properties, highlighting the contribution of Nogo- A to axonal growth inhibition in *in vitro* assays (Simonen *et al*., 2003; Kim *et al*., 2003; Zheng *et al*., 2003). Unexpectedly, while one of the mutant lines lacking Nogo-A and –B displayed enhanced axonal regeneration after CST lesion accompanied by functional recovery (Kim *et al*., 2003), at least in the youngest animals, the results for the simple mutant were much more modest (Simonen *et al*., 2003), and in the last two lines, there was no evidence of enhanced axonal regeneration (Zheng *et al*., 2003). Currently, we cannot provide an explanation for this divergence, and far form clarifying Nogo-A role on axonal regeneration failure, the reports on the knockout mice have evidenced the need of being cautious when considering therapeutic approaches for patients.

2.3.3 OMgp

	Simonen *et al*., 2003 *Nogo A*	**Kim *et al.* 2003** *Nogo A/B*	**Zheng *et al.* 2003** *Nogo A/B*	**Zheng *et al.* 2003** *Nogo A/B/C*
Changes in viability, fertility or behavior	N	N	N	N[a]
Changes in CNS anatomy	N	N	N	N
Changes in myelin structure	N	N	N	N
Nogo-B expression	upregulated	-	-	-
Nogo-C expression	unchanged	unchanged	not tested	no functional
Myelin inhibitory activity *in vitro*	N	N	N	N
Enhanced regeneration *in vivo*	modest	Y[b]	N	N

Table 2.1. Nogo-deficient mice characteristics. Summary of the main characteristic presented by the different Nogo-deficient mice. Groups that generated the different lines and mice genotypes are indicated. Y and N refer to the presence or absence of the characteristic indicated. [a] Nogo-A/B/C knockout line was originally lethal, except for one mouse, carrying a mutation that lead to the expression of a non functional Nogo-C.[b] only young mice. (Adapted from Mingorance *et al*., 2005)

Although first identified in 1988 (Mikol and Stefansson, 1988), OMgp is the least known of the three myelin-associated inhibitors. It was originally cloned from human white matter and named oligodendrocyte myelin glycoprotein because of its selective localization in CNS myelin (although it was later shown to be expressed by neurons). The mature OMgp consists of a 401-aa polypeptide (440 aa in the immature form) formed by four domains: one cystein-rich N-terminal domain, followed by a leucine-rich repeat (LRR) domain, a serine-threonine rich domain and a gycosyphosphatidylinositol (GPI)-link on its C-terminus (Mikol 90). The largest part of the OMgp protein is the LRR domain, which comprises eight tandem leucine-rich repeats (Mikol *et al*., 1990). This domain appears to be involved in many functions of OMgp and characterizes this molecule as a member of the LRR protein family (Kobe and Kajava, 2001). This extended protein family is involved in diverse biological processes, with some members functioning as membrane-associated adhesion molecules, some as transmembrane receptors, and still others as soluble binding proteins or ligands. Some of the LRR family members expressed in the CNS and their main functions are listed in the following table (Table 2.2).

In addition to oligodendrocyte myelin, OMgp is also expressed in neurons, mainly in projecting neurons such as the pyramidal layer of the hippocampus, the cortical layer V and Purkinje cells,

in the cerebellum (Habib *et al.*, 1998). The temporal expression pattern of OMgp has been characterized during postnatal CNS development, where it increases with age, but not in the embryonic brain (Vourc'h *et al.*, 2003).

2.3.3.1 NON- PATHOLOGICAL FUNCTION

LRR-family proteins expressed in the CNS		
Protein	**Function**	**References**
Alivin-1	Neuronal survival	Ono *et al.*, 2003
Amigo	Fasciculation, promotion of Neurite outgrowth	Kuja-Panula *et al.*, 2003
LERN/Lingo	Axon growth inhibition	Mi *et al.*, 2004
Neuregulin-1	Axon guidance and repulsion	Lin *et al.*, 2003
NgR	Axon growth inhibition	Fournier *et al.*, 2001
OMgp	Axon growth inhibition	Wang *et al.*, 2002
Slit	Axon guidance and repulsion	Nguyen-Ba-Charvet and Chedotal, 2002
Trk	Neurotrophin signaling	Huang and Reichardt, 2003

Table 2.2. Some of the LRR-family proteins expressed in the CNS. Most of the members of this family are implicated in protein-protein interaction and they frequently have homophilic interactions. Among their multiple functions, only a general role is specified. Proteins are listed in alphabetic order. For each protein, a reference describing its physiological role has been chosen.

The localization of OMgp near the nodes of Ranvier in myelin, and its expression profile, indicate that this protein could exert a function related to myelin (Vourc'h *et al.*, 2004). In addition, the only exon coding the OMgp gene is embedded within an intron of the NF1 gene, and mutations in this gene are responsible for neurobibromastosis type 1 disorder. Also, OMgp, like NF1, induces growth inhibition when expressed in cell lines. These observations indicate that OMgp could regulate oligodendrocyte proliferation and differentiation (Vourc'h *et al.*, 2004).

2.3.3.4 PATHOLOGICAL FUNCTION

OMgp was purified from a Phospholipase-C (PI-PLC) fraction obtained from the treatment of myelin with PI-PLC, an enzyme which hydrolyzes GPI-anchor. This fraction and subsequently the purified OMgp were tested in a growth cone collapse assay, where they were shown to induce neurite outgrowth inhibition and growth cone collapse (Wang *et al.*, 2002). A screening in an expression library led to the identification of the Nogo receptor as a high-affinity OMgp-binding protein, and the classification of OMgp as the third myelin-associated inhibitor (Wang *et al.*, 2002). However, unlike MAG and Nogo-A, there are no studies on the participation of OMgp in preventing axonal regeneration *in vivo*, and the *in vitro* observations require confirmation *in vivo* (Vourc'h *et al.*, 2004).

Introduction III

Receptors and Signal Transduction

The neuronal receptors for environmental cues and the signalling proteins downstream of them determine whether these cues will be interpreted as inhibitory or growth-promoting. Here we will focus on **the neuronal receptors** for MAG, Nogo-A and OMgp when expressed at the oligodendrocyte surface: that is to say, when acting as myelin-associated inhibitors (MAI). In addition, we will discuss **the main signalling pathways** that mediate the inhibition induced by MAI.

3.1 Membrane proteins interacting with Myelin-Associated Inhibitors

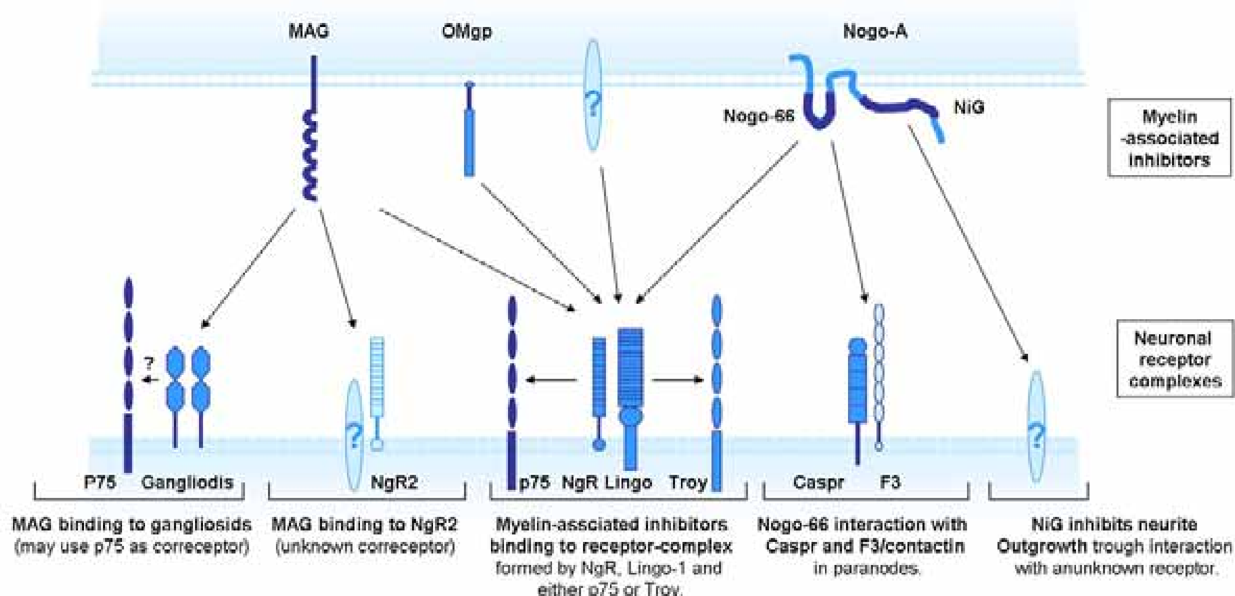


Fig. 3.1. Scheme of the neuronal receptors that interact with myelin-associated inhibitors. In addition to the central point of NgR/Lingo/p75/Troy complex, there are other receptors, some of which also participate in neurite outgrowth inhibition. Furthermore, some receptors are yet unknown, although believed to exist, and are represented as question marks, and the existence of additional MAI cannot be discarded

Since the discovery of the Nogo Receptor (NgR) in 2001, a growing number of neuronal receptors that participate in the transduction induced by MAI have been discovered, and the list is far from complete. Figure 3.1 provides an overview of the receptors and receptor complexes present at the neuronal surface that are known to interact with myelin-associated MAG, Nogo-A and OMgp.

3.1.1 RECEPTORS

a) The Nogo Receptor Family

NgR was identified in an alkaline-phosphatase (AP)-fusion protein expression screening as a receptor for the Nogo-66 domain of Nogo-A (Fournier *et al*., 2001). The *ngr* gene encodes a 473 aa protein anchored to the neuronal membrane by a GPI linkage that is concentrated in lipid rafts (Fournier *et al*., 2002; Liu *et al*., 2002). As previously mentioned, NgR belongs to the LRR family of proteins (see Table 2.2). It contains eight central LRRs flanked by a cysteine-rich C-terminal subdomain (LRR-CT) and by a smaller leucine-rich N-terminal subdomain (LRR-NT). This 311 aa NgR region (the LRR region) is necessary and sufficient for binding to Nogo-66, MAG and OMgp (Fournier *et al*., 2002; Domeniconi *et al*., 2002; Wang *et al*., 2002b). Most C-terminal 100 NgR residues are probably unstructured, but appear to participate in the interactions of Nogo with its co-receptors (Fournier *et al*., 2002; Wang *et al*., 2002b). In addition, as a soluble recombinant protein, NgR has affinity for cell-surface NgR, which indicates that it may form homodimers *in vivo*, although the physiological role of this interaction remains to be elucidated (Fournier *et al*., 2002; Liu *et al*., 2002).

Fig. 3.2. Orthogonal stereoviews of the NgR structure, the LRRNT subdomain is in blue, the central LRR subdomain in green and the LRRCT subdomain in red. The protein N- and C-termini are indicated (taken from Barton et al., 2003).

The crystal structure of NgR has been analysed, and the central part of the LRR has been reported to adopt a curved conformation with a concave exterior surface containing evolutionary conserved aa and a deep cleft at the C-terminal base of the LRR, where the association with the p75 co-receptor is expected to occur (Figure 3.2; Barton *et al*., 2003; He *et al*., 2003). This curved conformation, together with the high affinity of NgR homodimerization in solution (Fournier *et al*., 2001), has lead to the proposal that two molecules of NgR interact with a single ligand (concave sides pointing outward), and recruit two receptor molecules (Barton *et al*., 2003).

NgR protein expression is restricted to postnatal neurons and their axons, where it is also present at synaptic contacts (Wang X *et al*., 2002; the present study). During development, NgR protein is barely detectable in most brain regions in prenatal stages in mouse, and after P0, it starts to be widely expressed in the CNS, predominantly in the hippocampus and cerebral cortex (Josephson *et al*., 2002; this work). Interestingly, some areas,

such as the neostriatum, show little or no signal for NgR mRNA, while others, such as the spinal cord and the DRG, down-regulate it in the adult (Josephson *et al*., 2002; Wang X *et al*., 2002; see also Hunt *et al*., 2002 for review). These data indicated that NgR was probably not the only neuronal receptor mediating myelin-induced inhibition and prompted the search for homologs on databases. As a result of this search, two homologs of NgR (renamed NgR1, although for short we will refer to it as NgR) were found in mouse and human, four in zebrafish and five in fugu (Barton *et al*., 2003; Pignot *et al*., 2003; Klinger at al., 2003). The two mammalian homologs, NgR2 and NgR3, have a 55% LRR identity between them and share 45% homology with NgR (Pignot *et al*., 2003). The three homologs share, to a large extent, primary structures, biochemical properties and expression patterns (Pignot *et al*., 2003). However, NgR2 expression differs in the cerebellum and the thalamus, where it is expressed at high levels in comparison to NgR and NgR3.

Although the first reports indicated that none of the NgR homologs share the capacity to bind Nogo-66, MAG or OMgp (Barton *et al*., 2003), NgR2 has been recently identified as a high affinity receptor for MAG (Venkatesh *et al*., 2005). This interaction seems to be sialic acid-dependent, which sheds light on the observations that axon growth inhibition by MAG is reduced with neuraminidase treatment, although MAG binding to NgR has been reported to be sialic acid-independent (Domeniconi *et al*., 2002; Liu *et al*., 2002). However, there are discrepancies depending on the experimental model used (Venkatesh *et al*., 2005). Thus, MAG has at least two receptors at the neuronal surface, the first is NgR, which recruits p75 or Troy and Lingo-1 to the receptor complex (see below), and the alternative receptor is NgR2. These two receptors associate with gangliosides in lipid rafts, which support MAG binding in a sialic acid-dependent manner, to form a high affinity MAG-binding complex (Venkatesh *et al*., 2005). It is interesting to speculate that p75 could also be the co-receptor for NgR2, since NgR KO neurons, but not those from p75-deficient mice, are inhibited by MAG (and myelin in general) (Zheng *et al*., 2005). However, preliminary studies indicate that this may not be the case since NgR2 fails to bind to p75 in neurons (Venkatesh *et al*., 2005).

Currently, the function of NgR3 is not known and it has not yet been established whether NgR and NgR2 play additional roles to axon growth inhibition. However, the presence of these proteins in fish, where axonal regeneration exists and oligodendrocytes lack growth-inhibiting molecules (Klinger *et al*., 2003), indicate that NgR-family members may had a distinct role, probably not related with axonal regeneration, before the appearance of MAI.

b) p75 and Troy

Gangliosides and NgR family proteins, the only molecules known to act as receptors for MAI (when inhibiting axonal growth), lack intracellular domains and thus cannot initiate signal transduction. At present, two proteins are known to interact with NgR (one of which also interacts with the gangliosides that bind MAG) and regulate neurite outgrowth inhibition in response to the MAG: p75 and Troy.

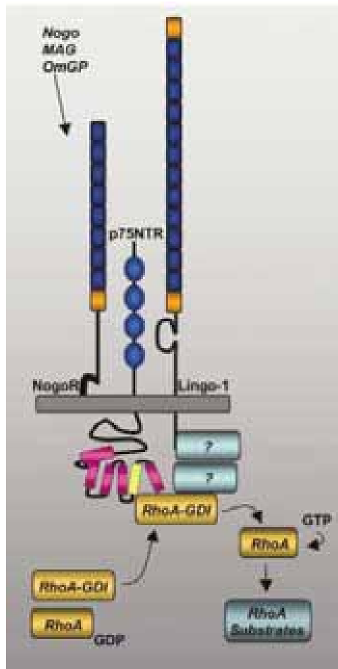


Fig. 3.3. MAI receptor complex formed by NgR, p75 and Lingo. After ligand binding to NgR, p75 interaction with Rho-GDI releases RhoA, resulting in axon growth inhibition. (Taken from Baker *et al*., 2004)

p75 and Troy are members of the tumour necrosis factor receptor (TNFR) superfamily. Members of this superfamily play an important role in regulating diverse biological activities, such as cell proliferation, differentiation, and programmed cell death or apoptosis. Most TNFR family members are type I membrane proteins that share significant sequence homology in their extracellular domains. The cytoplasmic domains of TNFRs, however, are divergent in length and sequence. Some apoptosis-inducing members of this superfamily have an 80 aa intracellular death domain (such as FAS and p75), while others induce apoptosis without a death domain (such as CD30 or Troy).

P75. Initially identified as a low-affinity receptor for neurotrophins, p75 is highly expressed in the developing nervous system during periods of axon outgrowth and dendritic arborization, but decreases over the postnatal period and adulthood (Chao, 2003). In fact, only subpopulations of a few types of mature neurons, such as subsets of dorsal root ganglion (DRG) neurons, have been shown to express p75 in adults (McMahon *et al*., 1994). Nevertheless, soon after, a study proposed that p75 expression is required for MAG inhibitory activity, although these two molecules do not interact directly (Yamashita *et al*., 2002), p75 was identified as a NgR co-receptor for the three myelin-associated inhibitors (Fig. 3.3; Wang *et al*., 2002b; Wong *et al*., 2002). Indeed, myelin-dependent inhibition of neurite outgrowth is reduced in cultured DRG or cerebellar neurons from p75- knockout mice (Yamashita *et al*., 2002; Zheng *et al*., 2005) or when expression of a dominant negative-p75 is induced on cerebellar neurons (Wang *et al*., 2002b). These observations indicate that a major part of myelin-induced inhibitory signalling is mediated by p75. In contrast to this evidence of p75 relevance in axonal regeneration failure, the suppression of p75 (in p75-knockout mice) does not sustain regeneration after spinal cord lesion (Song *et al*., 2004), although mentioned in the previous chapter, this seems to be a common feature of mice deficient in a single protein. This observation, together with the scarce expression of p75 in the adult CNS (being absent in some MAI-responsive neurons), prompted the search for a second co-receptor for NgR.

Troy, also referred to as TAJ or TNFRSF19, is an orphan receptor member of the TNFR family (Eby *et al*., 2000; Hisaoka *et al*., 2003). Among these orphan receptors, Troy is the one that most closely resembles p75 (Eby *et al*., 2000), which makes it a good candidate to functionally replace p75. Indeed, Troy forms a receptor complex with NgR and Lingo and mediates outgrowth inhibition and signalling pathways triggered by myelin inhibitors (as RhoA activation; Park *et al*., 2005; Shao *et al*., 2005). Neurons from Troy-deficient mice have a reduced sensitivity to myelin (Shao *et al*., 2005) and dominant-negative expression of Troy enhances

neurite outgrowth of DRG cells on myelin (Park *et al*., 2005). Interestingly, Troy is more widely expressed in the postnatal and adult brain than p75 and can substitute p75 in the receptor complex formed by NgR-Lingo-p75 in those cells lacking expression of the neurotrophin receptor (Park *et al*., 2005; Shao *et al*., 2005). This observation indicates that Troy may play a more important role than p75 in CNS axon regeneration in adulthood.

Like p75, Troy may have many additional roles to its participation in myelin-induced inhibition. In fact, p75 participates in three receptor complexes (with Trk receptors, NgR/Lingo, and sortilin) that bind distinct ligands (neurotrophins, myelin inhibitors, and proNGF). Therefore, under different conditions, p75 facilitates cell survival, growth inhibition, or cell death, respectively (Barker, 2004). Troy, in contrast, does not interact with neurotrophins (Shao *et al*., 2005), and when expressed in cell lines, it induces apoptosis independently of caspase activation (Eby *et al*., 2000). Before the finding of Troy-induced activation of RhoA, as a result of binding with NgR and Lingo, Troy was known to activate JNK and cell death pathways and it was proposed to regulate death during embryonic development (Eby *et al*., 2000). Nevertheless, Troy functions in the adult, in addition to participation in the NgR receptor complex, remain to be clarified.

c) Lingo-1/LERN1

Although p75 and Troy activate signalling pathways in the presence of MAI, when only NgR and p75 (and presumably Troy) are co-transfected in non-neuronal cells the ligands are unable to activate RhoA, indicating that an element of the receptor complex, expressed in neuronal cells, is missing (Mi *et al*., 2004). The search for additional proteins with NgR binding capacity led to the discovery of Lingo-1 (Mi *et al*., 2004).

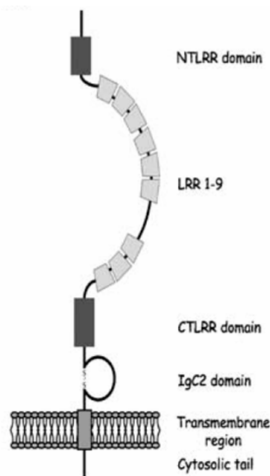


Fig. 3.4. Structure of Lingo-1. Taken from Carim-Todd *et al*., 2004.

Lingo-1 is a CNS-specific protein that had been simultaneously cloned in a screening for candidate gene transcripts involved in panic/anxious phenotype mapped in the chromosome 15q24, and named LRRN-A (leucine-rich repeat neuronal 6A) and LERN1 (leucine-rich repeat neuronal protein 1) (Carim-Todd *et al*., 2004). Lingo-1 has three human paralogs (Lingo2-4) and contains 12 LRR motifs, like NgR and OMgp, flanked by N- and C-terminal capping domains, one immunoglobulin (Ig) domain, a transmembrane domain and a short cytoplasmic tail, which contains a canonical epidermal growth factor receptor-like tyrosine phosphorylation site (Fig. 3.4; Carim-Todd *et al*., 2004; Mi *et al*., 2004). Lingo-1 interacts with NgR, and p75 or Troy, and forms a trimeric receptor complex (Fig. 3.3; Mi *et al*., 2004; Park *et al*., 2005; Shao *et al*., 2005), in which the presence of Lingo-1 is required for RhoA activation, as assessed in COS cell co-transfection experiments (Mi *et al*., 2004). Similarly, when expressed in neurons, dnLingo-1 inhibits myelin-induced inhibition (Mi *et*

al., 2004), indicating that Lingo-1 may play an important role in the assembly or signalling of the receptor complex, although its exact role in the receptor complex is yet unknown.

In addition to its involvement in axon outgrowth inhibition, Lingo-1 may play additional roles, mainly during CNS development. First, Lingo-1 expression is highly specific to the CNS and regulated during development, indicating that it may play exert some function during development, before MAI are expressed at the oligodendrocyte surface (Carim-Todd *et al*., 2004; Mi *et al*., 2004). In addition, Lingo-1 was discovered in a sequence database search for human Slit homologs that were selectively expressed in brain (Mi *et al*., 2004). The Slits are receptors for Robo and play a pivotal role during development by regulating axon guidance and cell migration (Wong *et al*., 2002). Another protein with structural similarity to Lingo-1 is NGL-1, the extracellular domain of which consists of nine LRRs plus the flanking LRR N-terminal and LRR C-terminal domains, followed by an Ig domain, and whose cytoplasmic region lacks any evident structural consensus sequence (Lin *et al*., 2003). NGL-1 is a specific binding partner of Netrin-G1 (a GPI-anchored form of Netrin), which plays a critical role in the development of the thalamic-cortical connection, stimulating neurite outgrowth of thalamic axons (Lin *et al*., 2003). Thus, the expression pattern of Lingo-1 and its structural similarity with guidance cue receptors strongly suggests that this protein is a candidate to interact with others that regulate axon guidance and also neuronal and glial migration during development.

d) Other possible receptors

Given the numerous inhibitors in the CNS (previous chapter), many receptors may mediate axon-growth inhibition. However, regarding the three MAI, they are additional receptors to those mentioned above. The first receptors have been previously mentioned in diverse occasions before, and are some gangliosides. In particular, GD1a and GT1b, two gangliosides (sialylated glycans), act as MAG receptors (Fig. 3.1), and their blockade (with soluble gangliosides or blocking antibody) reduces MAG-induced inhibition (Vinson *et al*., 2001; Vyas *et al*., 2001; 2002). In the absence of an intracellular signalling domain, gangliosides signal growth cone collapse by clustering with many proteins, both extra- and intracellularly, in membrane rafts. These proteins may include RhoA, which is present in the cytoplasmic face of lipid rafts. In addition, GT1b specifically binds p75 (which could trigger RhoA activation and is also concentrated in rafts) and ganglioside clustering is sufficient to induce axon outgrowth inhibition (Vinson 2001). Whether gangliosides mediate the activity of other MAI is unknown, but the conservation of p75 as a transducer element in the distinct receptor complexes described for MAG is surprising, and indicates that they may be only part of a larger receptor complex in membrane rafts integrated by NgR1 and 2, p75 and/or Troy, Lingo-1, and gangliosides.

Finally, Nogo-A specific central domain (NiG) inhibits axon outgrowth and fibroblast spreading (Fig. 3.1) and binds specifically to fibroblast surfaces and rat brain cortical membranes. These observations indicate the existence of a so far unidentified receptor (Oertle *et al*., 2003c). The

receptor for NiG is a protein, since this binding is protease-sensitive, and its inhibitory effects occur in the absence of NgR and p75 (Oertle *et al*., 2003c; Schweigreiter *et al*., 2004).

3.1.2 OTHER PARTNERS

The only extracellular binding partner of a MAI (when not acting as an axon growth inhibitor) is Caspr, a neuronal receptor for Nogo-A. Caspr is a homolog of Neurexin IV, whose expression is restricted to neurons (Einheber *et al*., 1997; Bellen *et al*., 1998). Caspr is concentrated in the paranodal regions of axons where it associates with F3 (also named Contactin). As a result of their binding with proteins on the paranodal region of the myelin, potassium channels are sorted from the node to the juxtaparanode. This sorting, which is required to ensure that action potential is not lost in the Rainvier nodes, occurs at the beginning of myelination. One of the proteins present on the oligodendorcyte surface that interacts with Caspr/F3 at the neuronal membrane is Neurofascin. Another protein is Nogo-A (Nie *et al*., 2003). During this period of development, when myelination occurs, Nogo-A localization on oligodendrocytes is restricted to the paranodes., Subsequently, as the sorting of channels is completed and the assembly of neuronal and oligodendroglial proteins at the paranodal region is completed, Nogo-A distribution becomes wider, and it is thought to interact preferentially with the neuronal NgR (Nie *et al*., 2003). The interaction of Nogo with Caspr is mediated by the Nogo-66 loop and occurs in the absence of F3 (Nie *et al*., 2003). It is interesting to highlight that Caspr shares homology with the Neurexin domain (Bellen *et al*., 1998), and Neurexin family members play crucial roles in synaptogenesis (Yamagata *et al*., 2003). Whether Nogo-A interacts with Neurexins has not yet been established. However, this notion is feasible because of the localization of Nogo-A at synaptic contacts (Wang X *et al*., 2002; Liu *et al*., 2003) and its capacity to bind the Neurexin homolog Caspr.

3.2 Intracellular Pathways mediating the inhibition of axonal regeneration

Extracellular factors are classified as either permissive/attractive or inhibitory/repulsive depending on their capacity to induce growth cone extension or retraction. The control of axon pathfinding during development shares molecular mechanisms with axonal regeneration in the mature CNS. Both processes result from local modulation of cytoskeleton dynamics within the growth cone. The molecular mechanisms that link these factors (from the cell surface) with the cytoskeleton are only partially described, although there are several main molecules and pathways involved in this

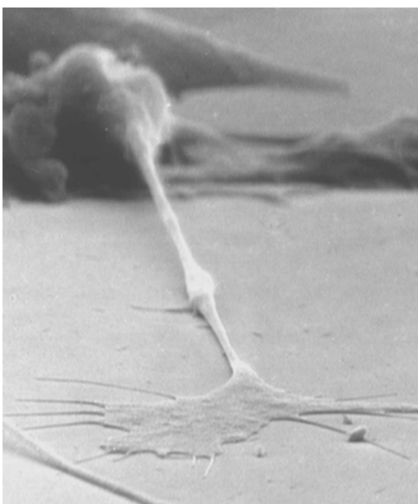


Fig. 3.6. A cultured sensory neuron extending a growth cone with long thin filopodia. Taken from Tosney lab webpage, University of Michigan.

signalling. Here we provide an overview of the main molecules and pathways implicated in the regulation of axon growth/regeneration, as the information available is too extensive to cover in this introductory chapter, and the picture is yet far from complete. Thus, we will focus on summarizing the key points.

3.1 Rho GTPases

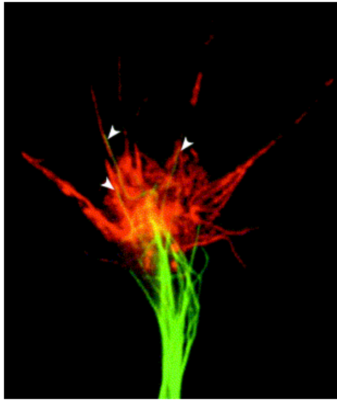


Fig. 3.7. The actin and microtubule cytoskeleton in the growth cone. Fluorescence image shows the distribution of F-actin (red) and microtubules (green) in the growth cone of a *Xenopus* spinal neuron *in vitro*. Taken from Henley and Poo, 2004.

The p21 Rho family of small guanosine triphosphatases (GTPases) is a key regulator of actin filaments in response to extracellular signals (Luo *et al*., 1997). Three of its members, Cdc42, Rac1 and RhoA, are regulated by the neuronal receptors involved in processes that require modulation of the cytoskeleton and growth cone dynamics, such as those responding to axon guidance cues or myelin-associated inhibitors (among many others). The growth cone is a motile terminal structure at the growing axon (see Fig. 3.6) whose peripheral domain includes filopodia and lamellipodia; the latter being flattened veils between filopodia. These projections extend and retract thereby sampling the environment and are retained in response to extracellular cues (O'Connor and Bentley, 1993; Gallo and Letourneau, 2004). Lamellipodia and filopodia are dependent on the polymerization and organization of actin filaments (F-actin, Fig. 3.7), a process that is modulated by Rho GTPases in response to extracellular factors.

Axonal repellents or inhibitors exert their effects through a common signalling event that is activation of RhoA. While activation of Rac1 or Cdc42 leads to axonal extension, as is the case of Netrin attractive effects, activation of RhoA by Semaphorins, Ephrins or MAI (concurrent with decreased Cdc42 and Rac1 activity) leads to growth cone collapse and inhibition of potential axonal regeneration (Jalink *et al*., 1994; He and Koprivica, 2004).

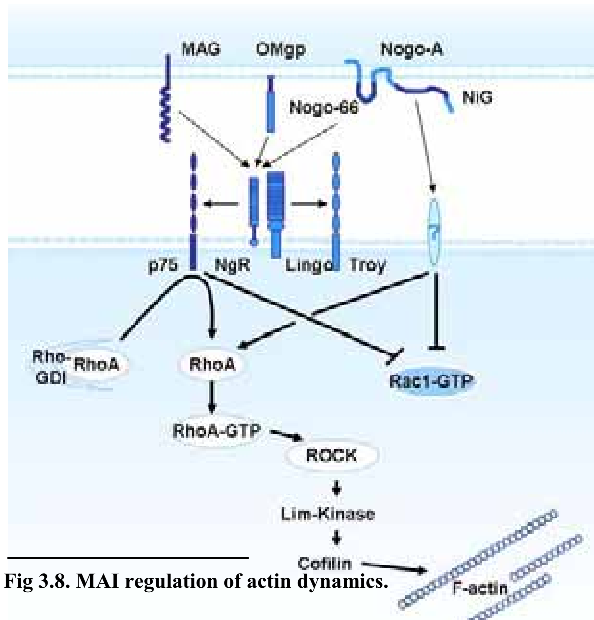


Fig 3.8. MAI regulation of actin dynamics.

Myelin components activate RhoA by recruiting Rho-GDI, which binds and retains Rho in its inactive state (Rho-GDP) to the cell surface via p75 (and putatively via Troy), thus allowing the release of Rho-GDP and permitting its activation (Fig. 3.8) (Yamashita and Tohyama, 2003). Simultaneously to RhoA activation, Ephrins, Semaphorins, NgR-ligands, NiG and some CSPGs inhibit Rac (Fig. 3.8; Giniger, 2002; Niederost *et al*., 2002; Schweigreiter *et al*., 2004). Downstream of RhoA, ROCK (RhoA effector) activates Lim-kinase, which in turns

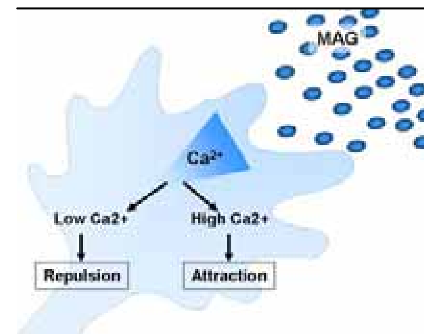
inhibits cofilin factor which stabilizes F-actin and induces growth collapse. This collapse is particularly important when following injury, axons form a new growth cone, which in contact with the inhibitors present in the mature CNS collapses and retract, preventing regeneration of the lesioned axon.

The second prominent cytoskeletal components in the growth cone are microtubules (Fig. 3.7), although the pathways controlling microtubule dynamics are less characterized than those leading to F-actin-regulation and the effect of MAI on microtubule dynamics have not been addressed.

Extracellular cue receptors initiate signalling cascades that integrate the variety of cues, either permissive/attractive or inhibitory/repulsive, present in a spatially and temporally regulated pattern, and instruct the growth cone to turn toward or away from a source of ligand, change speed, expand or collapse. Among these signalling cascades, three key points regulate axon growth inhibition: the intracellular levels of calcium, the levels of cyclic nucleotides, and the balance between PKC and IP3.

3.2 Calcium (Ca^{2+})

Fig. 3.9. Calcium regulation of the bidirectional growth cone turning induced by MAG..



One of the second messengers that translates extracellular information to directional motility is Ca^{2+} (Zheng, 2000). Extracellular gradients of molecules such as Netrin or dMAG (the soluble form of MAG released from myelin) generate an intracellular gradient of Ca^{2+}, and both proteins induce distinct turning responses in the growth cone depending on Ca^{2+} signals (Song *et al*., 1997; 1998). Activation of the NgR-receptor complex by MAG, the only MAI with a soluble form, increases cytoplasmic Ca^{2+} (Wong *et al*., 2002), in addition to RhoA activation, which is higher on the side facing the MAG source (Fig. 3.9; Henley *et al*., 2004). This Ca^{2+} signal is caused by Ca^{2+} influx from intracellular stores and, depending on the basal growth cone levels of intracellular Ca^{2+} , is traduced in a repulsive (low basal levels) or attractive (high levels) response (Henley *et al*., 2004). Thus, intracellular Ca^{2+} elevations with the same polarity but distinct magnitudes elicit opposite responses in the growth cone (Fig. 3.9).

How does Ca^{2+} regulate axon outgrowth or inhibition? Two Ca^{2+} -dependent proteins have been implicated in the directional motility of the growth cone: Ca^{2+} -calmodulin-dependent protein kinase II (CaMKII) and the Ca^{2+} -calmodulin-dependent phosphatase Calcineurin (CaN). Using growth cone turning assays, Wen *et al*. demonstrated that these two proteins act in the cone as a bimodal directional switch, that is to say, a greater activity of CaMKII over Calcineurin mediates attractive turning, while higher activity of Calcineurin (than that of CaMKII) induces repulsive turning. Calcineurin, which induces repulsion, has higher Ca^{2+} affinity than CaMKII,

thus, when cytoplasmic Ca^{2+} levels are low, Calcineurin activation prevails over CaMKII, and the growth cone is repelled. Conversely, when the basal levels of Ca^{2+} are higher, CaMKII activation is strong enough to determine an attractive turning (Wen *et al*., 2004).

Interestingly, during CNS development, intracellular Ca^{2+} levels are high, and as we have described (and will comment in detail in the following section) for cAMP levels, after a certain point during development these levels drop in the neurons. Moreover, stimulus able to elevate intracellular Ca^{2+} in the mature neuron, such as depolarization or elevation of cAMP, transform MAG-repulsive effects into attraction, since the MAG gradient provides the positional cue but the final response, attractive or repulsive, depends on Ca^{2+} levels (Henley *et al*., 2004). If intracellular Ca^{2+} levels participate in mediating the effects of more inhibitory molecules, manipulations of basal Ca^{2+} levels could have interesting therapeutic benefits, and are probably partially responsible of the promotion in axonal regeneration observed *in vivo* when increasing cAMP levels (Neumann *et al*., 2002; Qiu *et al*., 2002).

3.3 Cyclic nucleotides

As occurs with calcium, cyclic nucleotides are second messengers that regulate attraction and repulsion of the growth cone by extracellular factors, and in particular cAMP has gained much attention in studies of axonal regeneration. There is some clear evidence that relates high levels of cAMP in neurons with axonal regeneration: the priming with neurotrophins (treating neurons overnight before exposure to MAI) induces an increase of cAMP and is dependent on PKA activation; second, the same is observed after conditioning-lesion (lesioning the peripheral axon branch or DRG cells and one week later lesioning the central branch, which regenerates extensively; Richardson and Issa, 1984); and finally, the dramatic decrease in endogenous cAMP levels appears to be a determining factor in the spontaneous regeneration of CNS embryonic neurons. In fact, elevation of cAMP alone, even in the absence of other cues, mimics these effects and promotes axonal regeneration (for review Spencer and Filbin, 2004).

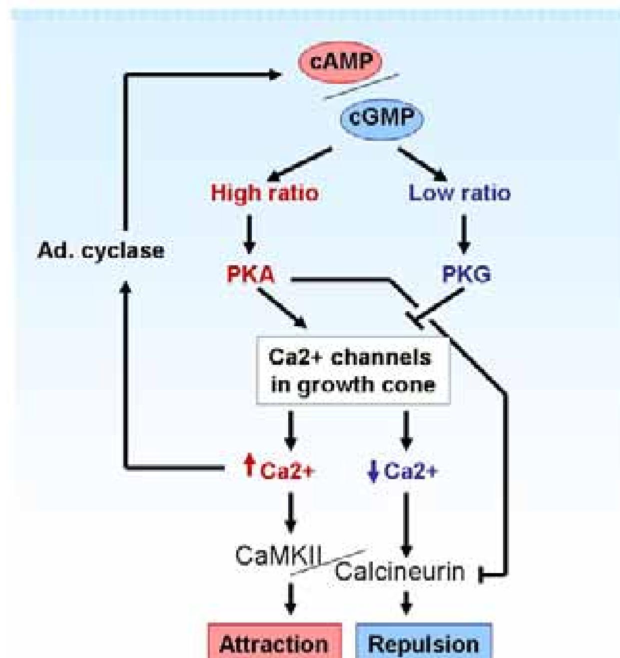


Fig. 3.10. Regulation of Ca^{2+} signalling by cAMP /cGMP balance. In the presence of high levels of cAMP (compared with those of cGMP), PKA modulates Ca^{2+} channels leading to an increase in the basal Ca^{2+} levels, which favours CaMKII-mediated growth cone attraction. Conversely, cGMP activation of PKG leads to a decrease in Ca^{2+} levels and the activation of Calcineurin over CamKII.

Figure 1.7 shows the main skeleton of cAMP regulation of axonal regeneration, via PKA activation of CREB and the transcription of specific proteins, which

is thought to interfere with MAI signalling (Spencer and Filbin, 2004). However, to prevent MAI inhibition of axonal regeneration, cAMP may also regulate cytosketal elements. This may be performed not as a direct effector but as a regulator of other crucial pathways involved in growth cone dynamics, such as Ca^{2+} signalling (Fig. 3.10). A recent study indicated that cAMP may regulate Ca^{2+} signalling and that the balance, or ratio, between cAMP and cGMP (but not the total concentration of cAMP) could determine the direction of Netrin-induced growth cone turning (Nishiyama *et al*., 2003). While high ratios of cAMP/cGMP favour attraction in response to Netrin (and presumably to MAG), low ratios favour repulsion (Nishiyama *et al*., 2003). This effect is mediated by PKA- and PKC-positive and negative (respectively) modulation of Ca^{2+} channels in the plasma membrane and ER. Thus, an elevation of cAMP activity creates a high gradient of intracellular Ca^{2+} concentration, favouring growth cone attraction for the mechanisms described above. This relationship is consistent with experiments demonstrating that the effects of Netrin and MAG, which trigger an increase in intracellular Ca^{2+} levels, are modulated by cAMP manipulation, but not those of Sema3A, which is not involved in Ca^{2+} -dependent guidance (Ming *et al*., 1997; Song *et al*., 1998; Cai e al., 2001).

In addition, two regulatory points have been described that reinforce the pathway initiated by high cAMP levels so that once this pathway has been activated, the final attractive output is ensured (Fig. 3.10; Wen *et al*., 2004). First, PKA negatively regulates Calcineurin signalling to inhibit repulsion, and second, elevation of Ca^{2+} levels further activates adenylate cyclase, leading to a sustained high cAMP/cGMP ratio (Wen *et al*., 2004). Thus, if a cue induces the development of a gradient of Ca^{2+} levels, as occurs with Nogo-A and MAG (Bandtlow *et al*., 1993; Wong *et al*., 2002; Henley *et al*., 2004), the basal levels of Ca^{2+} will determine the growth cone response, and any factor that regulates these levels, such as cAMP, will interfere with this response.

3.4 PKC/IP3 balance

Another key point regulating axon outgrowth inhibition is the balance between PKC and IP3. Interestingly, PKC is a key component of the signalling pathways that mediate the inhibition induced by MAI and CSPG (Hasegawa *et al*., 2004; Sivasankaran *et al*., 2004). In fact, these two families of proteins trigger the activation of PKC and its pharmacological blockade leads to axonal regeneration *in vitro* and *in vivo* (Sivasankaran *et al*., 2004). The characterization of the role of PKC in axonal regeneration has uncovered an additional bidirectional regulation pathway, such as those described for Calcineurin/CamKII and cAMP/cGMP: the PKC/IP3 balance.

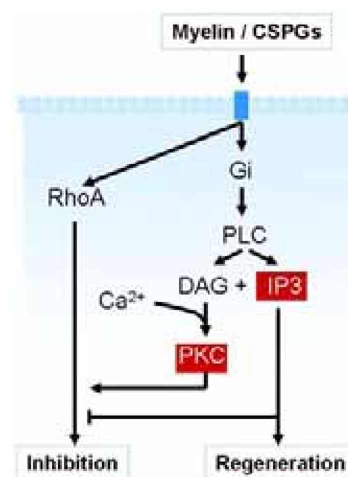


Fig. 3.11. Generation of IP3 and activation of PKC following G_i and PLC activation. While PKC activity inhibits axonal regeneration, IP3 promotes it. How these molecules affect cytoskeleton dynamics is unclear, but they seem to interfere with RhoA downstream signalling.

One of the first indications of PKC relationship with axon outgrowth inhibition was the regeneration of entorhinal axons

in vitro following treatment with the PTX toxin (using the same *in vitro* model we have used in this thesis; Prang *et al*., 2001). PTX is an inhibitor of the G_i and G_o proteins, which are upstream of phospholipase C (PLC), which in turns mediates the hydrolysis of PIP2 and produces two second messengers: diacylglycerdol (DAG) and IP3. PKC activation results from the production of DAG together with the elevation of Ca^{2+} levels (see Fig 3.11). While IP3 activation over PKC leads to axon growth promotion, when PKC activity is higher, axon growth in inhibited and collapse is induced (Fig 3.11; Hasegawa *et al*., 2004).These functions, and the implication of these proteins in mediated-MAI signalling, is clearly manifested in blocking experiments. MAG acts as an attractive molecule for growing embryonic axons *in vitro*. The blockade of IP3 prevents this attractive effect, while blockade of PKC in adult neurons turns the inhibitory effects of Nogo-A and MAG into attraction (Hasegawa *et al*., 2004; Sivasankaten *et al*., 2004). It is interesting that under these conditions, MAG and Nogo-A act as trophic factors for axotomized neurons, since PKC blockade not only prevents PKC activity, but also displaces the balance between PKC and IP3 toward the growth-promoting pathways initiated by IP3. Blockade of G_i or PLC fails to modulate neuronal responsiveness to MAI, reinforcing the notion that it is the balance between IP3 and PKC that determines regeneration versus inhibition.

The elements regulating growth cone outgrowth or inhibition that act downstream of PKC or IP3 are not clear, neither are the factors that determine the final balance between them. However, they are believed to regulate Rho GTPase activity. However, RhoA activation can be observed in the presence of MAG or Nogo-A despite the blockade of PKC (and the prevention of inhibition) (Hasegawa *et al*., 2004). Thus, RhoA activation by MAI may not be downstream of PKC, but rather PKC may modulate several downstream effectors of RhoA (Fig 3.11).

CONCLUSION

There are many proteins in the adult brain that act as inhibitory cues, and a growing number of receptors is being described for them. However, all this diversity converges beyond the plasma membrane, where growth inhibition or promotion relies on the activation or inhibition of certain cytoskeleton-associated proteins, which in turn are regulated by a small number of pathways. Basically, the modulation of Rho GTPases by Ca^{2+}, cyclic nucleotides and G_i-PLC divergent signals, as well as the interactions between them, are enough to account for the final output, and the capacity of a receptor to activate one or more of these pathways in a given context determines the behaviour of its ligand as an attractive/growth promoting cue or as an inhibitor.

Curiously, throughout evolution, these regulatory pathways have developed in a way such that, during development, axonal growth promotion is potentiated over inhibition, while in the mature CNS, the same stimulus is conduced preferentially toward the inhibition of axon growth and regeneration is prevented (see Fig. 3.12 as a summary).

Embryonic neuron	Mature neuron
↑ cAMP	↓ cAMP
↑ Ca2+	↓ Ca2+
IP3 dominates following Gi-PLC activation	PKC dominates following Gi-PLC activation

Fig. 3.12. Comparison of the state of the three regulatory pathways described in this chapter in embryonic and mature neurons.

Introduction IV

The Hippocampus and Axonal Regeneration

In this thesis, to analyze the regulation and the possible roles of MAI during development and after lesion, we have focused on the hippocampus. The hippocampus, in addition to being the first target of Alzheimer disease and common focus site in epilepsy, which makes it an interesting object of research in itself, provides a good model to analyze cortical connection development and regeneration. An important characteristic of the hippocampus is that connections form in a highly stereotyped fashion, following a laminar pattern, which allows observation of the specificity of the connection, both during normal development and after manipulation (such as after trasection). In addition, this patterning has been extensively studied and diverse *in vitro* techniques have been developed. Therefore, the hippocampus was chosen as a model for our studies. In the following section, we will summarize the main characteristics of hippocampal development and its connectivity, and will later focus on the changes that occur after axotomy of one of the main hippocampal connections: the perforant pathway, which we have used extensively to test the participation of Nogo-A, NgR and MAG in axonal regeneration. Finally, we will discuss the convenience of using entorhino-hippocampal organotypic cultures as an *in vitro* approach in our studies.

4.1 Hippocampal basic anatomy

Although we normally refer to the area on which we have focused as the hippocampus, it actually corresponds to the hippocampal formation. Under this name we distinguish three main subdivisions: the entorhinal cortex, the subicular complex and the hippocampus, each one including subsequent subdivisions (see Fig. 4.1).

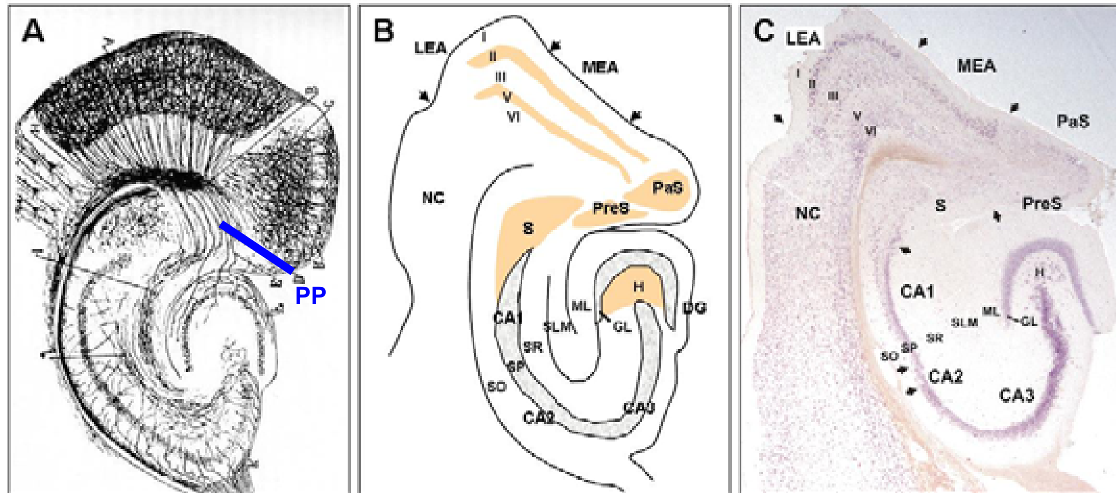


Fig. 4.1. Cytoarchitecture of the entorhino-hippocampal formation. A) Entorhino-hippocampal formation observed in an horizontal section of a 15-day-old mouse drawn by Ramón y Cajal. The perforant pathway is highlighted. B) Scheme of the mail cellular layers in entorhinal cortex and hippocampus of the adult mice. C) *In situ* hybridization of Nogo-A/B-C (pan-Nogo probe) in an equivalent section of that represented in B. The different layers shown in B are clearly observed by the *in situ* labelling. Abbreviations: I-VI, cortical cell layers; CA1-CA3, hippocampal fields; GL, granule layer; H, hilus; LEA, lateral entorhinal area, MEA, medial entorhinal area; ML, molecular layer, NC, neocortex; S, subiculum; SLM, stratum lacunosum moleculare; SO, stratum oriens; SP, stratum piramidale; SR, stratum radiatum; PaS, parasubiculum; PreS, presubiculum; PP, perforant pathway. (Taken from Ramón y Cajal, 1901, and Mingorance *et al*., 2004a).

The entorhinal cortex (EC) is the target of cortical afferents and gives rise to the perforant pathway (PP), the major cortical source of input to the hippocampus (Fig. 4.1A). As a cortex, the EC is divided in clearly distinguished layers from layer I (which, together with layer IV, lacks cells) to layer VI. The cells that give rise to the perforant pathway are predominantly present in layers II (pyramidal and stellate cells) and III (small pyramidal neurons), and project to the dentate gyrus, the stratum lacunosum-moleculare of the hippocampus proper, and the subiculum. Neurons from layers V and VI do not project to the hippocampus, but to the cortex and thalamus (Ramón y Cajal, 1901; Amaral and Witter, 1995). **The subiculum** (S) is situated in the transition area between the entorhinal cortex and the hippocampus. It is subdivided in parasubiculum (PaS), presubiculum (PreS) and subiculum (S), which are the output region from the hippocampus (Fig. 4.1B-C; Amaral and Witter, 1995).

Here we will use the term hippocampus to refer to the dentate gyrus together with fields CA3, CA2 and CA1 (from *cornus ammonis*). **The hippocampus proper**, formed by the CA1-3 areas, has a laminar organization that is conserved in its different regions (Fig. 4.1). Bordering the hippocampus, in contact with the ventricles, there is a thin layer of fibres called the alveus (the white matter of the hippocampus). Immediately over this layer is the stratum oriens (SO), which contains the basal dendrites of pyramidal neurons, whose somas constitute the stratum piramidale (SP). Following the SP, in all the hippocampal fields, we distinguish two layers containing the apical dendrites of pyramidal neurons, the stratum radiatum (SR) and the more superficial stratum lacunosum-moleculare (SLM). Finally, only in the CA3 area, there is an acellular stratum formed by the axons of mossy fibers (the axons from the dentate gyrus granule cells) that runs right over the SP and finishes in the border CA3-CA2, called the stratum lucidum (SL; arrows in Fig. 4.2)

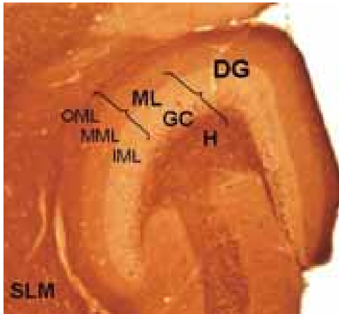


Fig. 4.2. Dentate gyrus subdivisions evidenced with anti-NIMP antibody labelling.

The dentate gyrus (DG) is also subdivided in layers. The outermost is called the stratum moleculare (SM) and includes the apical dendrites of granule cells. The SM is also divided in three layers, called the inner, middle and outer molecular layers, which are innervated by afferences from several sources, as mentioned previously (Fig. 4.2). The main cell type of the DG, granule cells, define the stratum granulare (SG), and project their axons or mossy fibers through the hilus (H) to form the SL (Fig. 4.2). There are many different cell types in the hilar region, the most renowned being mossy cells (interneurons), and between this region and the granule cells a thin layer can be differentiated in which neuronal precursor proliferation persists during adulthood of the animal: the subgranular zone (Amaral and Witter, 1995).

4.2 Intrinsic and Extrinsic hippocampal connections

During this thesis, we have used the PP as a model of cortical connection to study the role of MAI. However, there are many other connections between areas that form the hippocampal formation, or between these and external regions. In this introduction we will focus on the PP and the pathways that could be relevant to understand the processes that occur after PP axotomy, while only mentioning the others.

4.2.1 INTRINSIC CONNECTIONS

a) Entorhino-hippocampal connection: the perforant pathway.

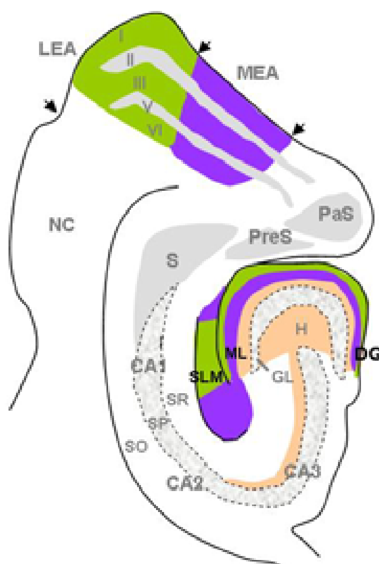


Fig. 4.3. Pattern of hippocampal innervation by entorhinal axons (green and blue) and mossy fibers (orange).

Neurons form the entorhinal layers II and III originate a connection that perforates the subiculum (hence its name) and establish synapses with the principal cells of the target tissue: the granule cells of the dentate gyrus and the pyramidal neurons of the CA1-CA3 areas. An important characteristic of this connection is that axons from the lateral entorhinal area (LEA) terminate in a very specific fashion in the outer third of the dentate gyrus molecular layer (outer molecular layer, OML), while those originating in the medial entorhinal area (MEA) are found in the middle molecular layer (MML) (Fig 4.3; Amaral and Witter, 1995).

b) Associational and commissural connections.

Associational connections link homolog areas in the same hemisphere, while commissural ones connect those with the contralateral hemisphere. These axons cross the brain by the dorsal and ventral commisures. In the hippocampus,

CA3 pyramidal cell axons innervate both the ipsi- and the contralateral hippocampus giving rise to commissural and associational fibres that run through the SR and SO. The connection from CA3 to CA1, named the Schaffer collaterals, plays a fundamental role in hippocampal connectivity, and together with the PP and mossy fibres, forms part of the so-called trisynaptic circuit. In the dentate gyrus, granule cells axons, known as the mossy fibres, project to the CA3 area and stop at the limit between CA3 and CA2. Mainly, these axons innervate CA3 pyramidal dendrites in the suprapyramidal side, the stratum lucidum (orange in Fig. 4.3) and display characteristic presynaptic varicosities. From the rest of intrinsic connections, we mention only that originated from mossy cells (hilar interneurons) that innervate the ipsi-and contralateral inner molecular layer (IML, orange in Fig. 4.3) which in response to PP axotomy sprout (form new collaterals that will establish new synaptic contacts) into the IML increasing its size with respect to the denervated MMl and OML.

4.2.2 EXTRINSIC CONNECTIONS

One of the main efferences from the hippocampus is the connection with the septum. This connection is reciprocal, as septal axons enter the hippocampus through the fimbria and establish synaptic contacts with interneurons in the CA3-CA1 areas and the DG, particularly in the ML. This last connection is relevant after PP axotomy as it sprouts into the ML when it is denervated. There are additional connections, such as that between the CA1 area with the subiculum and entorhinal cortex layer V, or the input itreceives from hypothalamic nuclei, which is not discussed in this introduction.

4.3 Hippocampal development

The development of the hippocampus is a highly ordered process with a number of important steps. Cells must be directed to the proper fate in the ventricular zone, must migrate to the appropriate laminar location once born and must establish connectivity with their targets. When the neuronal tube develops from the ectoderm, the anterior part experiments a differential growth in vesicles (diencephalon, mesencephalon and rombencephalon or hindbrain) while the rest will develop the spinal cord.

Initially, the ventricular side of the hindbrain is formed by a germinative zone, called the ventricular zone, which contains progenitor cells. These cells will give rise to glial cells and neurons which will start becoming postmitotic between embryonic days 10 and 14 (E10-E14). The radial migration of these initial postmitotic neurons forms the preplate, which, at E14, is composed by GABAergic neurons and Calretinin-possitive cells, which are situated in the most superficial layer of the hippocampus (Cajal-Retzius cells) (Soriano *et al.*, 1994; del Río *et al.*, 1995). After E15, the preplate splits, with the income of new neuroblasts, into subplate and marginal zone. This marginal zone is populated by GABAergic neurons and Cajal-Retzius cells. In parallel, the dentate gyrus starts to develop as a fold in the extreme of the hippocampus. At E15, granule cell precursors are generated in the subventricular zone, from which they migrate

away from the fimbria. Afterward, between E15 and E16, granule cells form the granule layer. In this layer, younger neurons will be localized deeper than the first ones generated, in a gradient called "outside-in", and after E16 the different subdivisions are distinguishable: marginal zone (corresponding to the molecular layer and containing Cajal-Retzius cells and GABAergic neurons), granule cell layer (containing only suprapiramidal granule cells), and the hilus (mainly populated by GABAergic interneurons). In contrast to the dentate gyrus, as pyramidal neurons generate in the hippocampus proper they follow an inside-out gradient, in which initial pyramids are crossed by the newly generated ones, which are located in more superficial positions. The neurogenesis of these pyramidal cells occurs earlier in CA3 with respect to the CA1 area, and lasts until E17. Around perinatal stages, the layer disposition in the hippocampus proper is that described at the beginning of this chapter for the mature hippocampus.

Around E15, first entorhinal axons reach hippocampal white matter, and at E16 they reach the marginal zone (corresponding with the future) SLM in the CA1-CA2 area (E17-E19), and a little later they extend in the CA3 region (Supèr and Soriano, 1994). The dentate gyrus will be innervated in perinatal stages, when granule cell dendrites are still immature as were those of pyramidal cells in the moment that PP arrived (Supèr *et al*., 1998). In this situation, entorhinal axons establish asymmetric synaptic contacts with the transient population of Cajal-Retzius cells (Supèr *et al*., 1998). After P5, the number of synapses between PP axons and Cajal-Retzius cells is progressively reduced as synaptogenesis with granule cell dendrites increases.

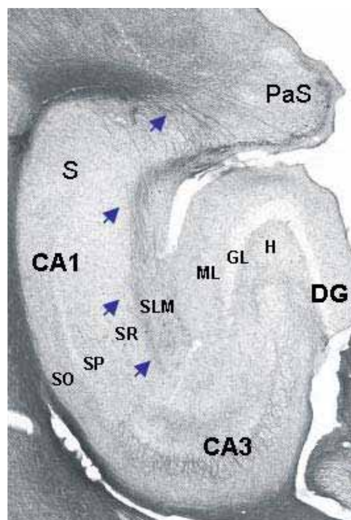


Fig 4.4. Myelin in the entorhino-hippocampal formation observed with myelin-basic protein (MBP) immunostaining. The course of the perforant pathway in the hippocampus is highlighted with blue arrows.

4.4 The Perforant Pathway lesion as a model

One of the characteristics of the hippocampal formation is its laminated organization, in which different afferents innervate different layers in a highly organized fashion. Therefore, transection of PP axons, originating on layers II and III of the lateral and medial entorhinal cortex, leads to localized laminar anterograde axonal and terminal degeneration of presynaptic elements of the outer two-thirds of the dentate molecular layer as well as the SLM (which includes the dendrites of the CA3-CA1 areas). Concomitant with this degeneration, microglia and astroglial cells are rapidly activated (a process described below), oligodendrocytes alter myelin genes expression and the blood-brain barrier (BBB) is distorted. In addition, PP axotomy leads to a so-called reactive sprouting and synaptogenesis by intact axonal afferents within the denervated areas.

These processes have been widely characterized, making the entorhinal cortex lesion (ECL), a well described model for the study of anterograde axonal degeneration, sprouting and reactive

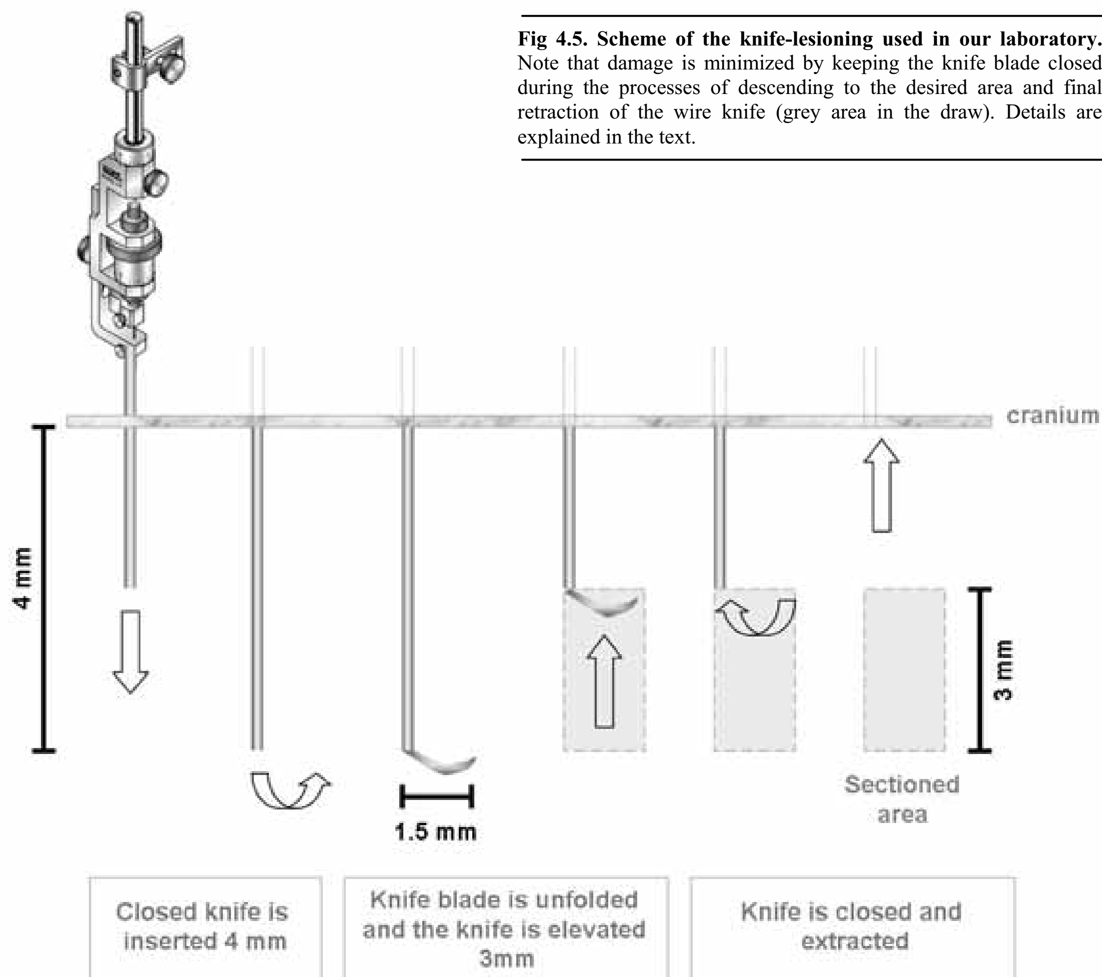


Fig 4.5. Scheme of the knife-lesioning used in our laboratory. Note that damage is minimized by keeping the knife blade closed during the processes of descending to the desired area and final retraction of the wire knife (grey area in the draw). Details are explained in the text.

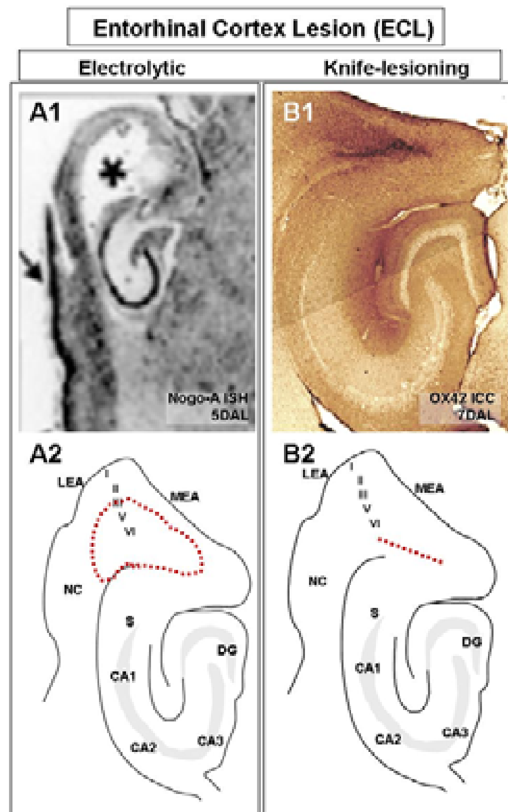
synaptogenesis in the hippocampus (see the following sections of this chapter for references). To study the role of MAI in axonal regeneration, we chose the ECL. In addition to the layer specificity of the PP and the extensive characterization of the processes that occur after axotomy, which alone substantiate the use of the entorhino-hippocampal connection as a model, the entorhinal cortex and the hippocampus can be easily cultured as a single piece slice. Furthermore, these slices can be maintained *in vitro* for long periods during which the system matures as *in vivo*, thereby permitting easy manipulation of the PP, such as axotomy or drug delivery. Lastly, and very importantly for our particular focus on MAI, the PP is a strongly myelinated connection (Fig. 4.4), and myelination parallels the loss of axon regeneration capacity of the PP. For all of these reasons, both *in vitro* and *in vivo*, the entorhino-hippocampal connection was used as the model in this thesis.

Although we also refer to the ECL (or PP lesion) as PP axotomy (and use both terms indistinctly), they are not synonyms. In fact, axotomy of the PP is only one of the ways in which the entorhino-hippocampal connection can be damaged (Fig. 4.5), while another way, used in most of the bibliography, is the electrolytic lesion (Fig. 4.6). In both techniques, the animal is deeply anesthetized and then placed in a stereotaxic holder. For electrolytic lesion surgery, an electrocoagulator is used to perform unilateral incisions (with a variable number of single pulses) in the entorhinal cortex. To perform PP axotomy, we and others (Jensen *et al*., 1997)

specifically section the PP, preserving entorhinal cortex structure. Briefly (see Fig. 4.5), animals are placed in a stereotaxic holder after pentobarbital anesthesia (50 mg/kg, b.w.) and a hole is drilled 1 mm posterior and 3 mm lateral to lambda. A closed wire knife is then inserted at an angle of 15º anterior and 15º lateral 4 mm ventral to dura. In this position, the wire is unfolded (extracting a 1.5 mm knife blade) and the perforant pathway is sectioned by taking back the knife 3 mm. The knife is then closed again and extracted, ensuring that, in addition to the 3x1.5mm section performed when the knife was extracted, the damage to the remaining brain is minimum.

The result of electrolytic and knife-lesioning (or axotomy) techniques is illustrated in Figure 4.6. The electrolytic lesion shown on the left of the panel corresponds to an entorhinal cortex five days after lesion, published in Meier *et al*., 2003. Knife lesioning of the PP performed in our laboratory is shown in B1 seven days after axotomy. While electrolytic lesioning leads to extensive entorhinal cortex damage (A2 in red), knife lesioning respects the integrity of this cortex (B2). While these two techniques permit the study of hippocampal deafferentation in terms of glial reactivity, the mayor advantage of knife lesioning is that axonal sprouting or gene expression regulation, both processes occurring in the entorhinal cortex, can only be examined in this model. At this point, we wish to highlight the importance of analyzing changes that occur in the entorhinal cortex, the tissue originating the PP, after lesion, and not restricting the study to the target tissue. In fact, after lesioning, the regulation of changes in glia, gene expression and, importantly, neuronal sprouting or death differs in the two areas. Thus, we used knife lesioning to section the PP.

Fig 4.6. (next page) Comparison between electrolytic lesion and axotomy of the PP. A1-2 illustrates the characteristics of the lesion performed with electrolysis. It corresponds with an in situ hybridization with Nogo-A probe 5 days after lesion. The tissue damaged is shown in A2 in red. Similarly a photograph of a knife lesion is shown in B1 and the extent of its damage illustrated in B2. B1 corresponds to immunohistochemical detection of ox-42, a marker of microglia, at 7 days after lesion (A1 has been taken from Meier *et al*., 2003).



To better understand the rationale followed in this study, in the following sections we will summarize the main changes occurring after PP axotomy that may contribute to preventing axonal regeneration. These changes include glial reactivity, neuronal changes and, importantly, overexpression of inhibitory molecules. Unfortunately, most studies have focused on hippocampal changes, ignoring those that occur in the entorhinal cortex, therefore data will be partial.

4.4.1 GLIAL CELLS REACTION FOLLOWING PERFORANT PATHWAY AXOTOMY

Astrocytes and microglia play a mayor role in regulating axonal regeneration after lesion. Typically, in response to injury that involves disruption of the BBB, astrocytes proliferate and become hypertrophic, extending and joining their processes and forming a glial scar (Silver and Miller, 2004). These changes define the characteristics of reactive astrocytes. Following CNS injury, microglia change from a highly ramificated cell to a cell with an enlarged body, loss of ramification and development of cytoplasmic phagosomes. These activated microglial cells are termed amoeboid microglia (Silver *et al*., 2004).

Following ECL, activation of microglial cells occurs rapidly in the deafferented dentate gyrus, typically within the first 24 hours, and reaches a maximum at 3DAL (days after lesion) (Jensen *et al*., 1994). At this time point there is a massive increase in the number of microglial cells in the molecular layer, as a combined result of proliferation and migration from adjacent areas (Hailer *et al*., 1999). Seven to nine days after ECL, microglia return to control situation (normal morphology and distribution). The reaction of astrocytes to ECL is delayed compared with that of microglia. Starting at 2DAL, astrocyte reaction peaks at 6-7DAL and remains detectable (on the basis of morphologic signs such as GFAP overexpression) for months (Gall *et al*., 1979; Steward *et al*., 1990; Fagan and Gage., 1994; Jensen *et al*., 1994).

Another important feature of activated microglia and astrocytes is their capacity to phagocytize PP axons that undergo anterograde degeneration after ECL and to degenerate granule cell dendrites (Benchmann and Nistch, 1997; Klinge *et al*., 1998).The peak of microglial phagocytosis is reached 3DAL while astrocytes engage in phagocytosis later, at day 6 post-ECL (Benchmann and Nistch, 1997). It has been suggested that microglial cells are first activated by complement factors derived from the disruption of the BBB, and in turn, cytokine production by activated microglia induce astroglial activation (Turner *et al*., 1998). This would explain the delay observed in astrocyte reactivity with respect to microglia activation.

Although microglia reactivity is transient, that of astroglia is more persistent, and includes the formation of the glial scar and the overexpression of inhibitory molecules, such as tenascin and CSPG, in the glial scar and the deafferented hippocampal areas (DG and SLM).

Regarding changes in oligodendrocytes after lesion, two populations should be differentiated: oligodendrocyte precursor cells (OPCs or oligodendrocyte progenitors), and mature oligodendrocytes. OPCs, as demonstrated by NG2 expression, proliferate near the lesion site and in the deafferented hippocampal areas between 4 and 7DAL (Fontana *et al*., 2005). After this period, they differentiate and contribute to myelinate the new connections formed after axotomy. The only data available on the response of mature oligodendrocytes to ECL when this thesis was started referred to an increase in mature oligodendrocyte numbers and MBP mRNA in the DG, SLM and CA3 area, peaking at 5 days post-lesion, which the authors attributed to response to axonal sprouting (Jensen *et al*., 2000b; Drojdahl *et al*., 2002, 2004).

4.4.2 NEURONAL REACTION FOLLOWING AXOTOMY

After ECL, approximately 85% of presynaptic elements to the outer two-thirds of the dentate molecular layer degenerate, while no denervation occurs in the IML. This degeneration is rapid, and is completed between 4 and 9DAL (Matthews *et al*., 1976a,b). Consequently, microglia, astrocytes and oligodendrocytes are activated (as described above) and degenerating dentate granule neurons are detected in the denervated hippocampus between 24 and 36 hours post-lesion, but are no longer detected after 48 hours (Kovac *et al*., 2004). In addition to target neuron degeneration, approximately 30% of the axotomized neurons in the lesioned entorhinal cortex undergo cell death following retrograde degeneration within the first 2 weeks after injury (Peterson *et al*., 1994).

Approximately one week after lesion, intact fibers engage in reactive sprouting and synaptogenesis, replacing 60-80% of the lost entorhinal input during the first 4 weeks after lesion (Matthews *et al*., 1976b; Steward and Vinsant, 1978). These fibres arise from three major surviving afferent fibre systems which normally project to the dentate gyrus: crossed entorhino-dentate fibres (from the contralateral entorhinal cortex) (Steward and Cotman, 1974, 1976; Steward, 1976; Deller *et al*., 1996a,b; Del Turco*et al*., 2003), the cholinergic septo-hippocampal fibres (Lynch *et al*., 1977, Nadler *et al*., 1977a, b; Zimmer *et al*., 1986; Deller *et al*., 1995) and the commissural/associational pathway (Deller *et al*., 1995a, b). This latter pathway, which normally terminates in the IML, sprouts in the same layer, expanding this termination zone with no translaminar sprouting (Deller *et al*., 1996).

4.4.3 INHIBITORY MOLECULES REGULATION FOLLOWING AXOTOMY

a) Chondroitin Sulfate Proteoglycans

Although proteoglycans and tenascins are regulated by axotomy, and are overexpressed at the glial scar, few data are available on their regulation after entorhinal cortex axotomy. The same occurs with axon guidance molecules, although Semaphorins are expected to be overexpressed in the glial scar that forms after ECL following the infiltration of Semaphorin-expressing fibroblasts, since this process occurs in other lesions involving BBB disruption.

Neurocan, which is expressed mainly by neurons during development but is developmentally downregulated, is reexpressed by reactive astrocytes in the deafferented molecular layer between 1 and 4 days post- ECL (Haas *et al*., 1999). Although there are no references of Neurocan expression at the lesion site, it has been reported that brevican is upregulated both in the deafferented areas and at the lesion site, where reactive glyosis takes place, starting 4DAL, peaking 4 weeks later and lasting for months (Thon *et al*., 2000). Also, the extracellular matrix protein tenascin-C is overexpressed by reactive astrocytes in the deafferented molecular layer between 2 and 10 days after lesion (Deller *et al*., 1997). Finally, the increase in OPCs after ECL (Fontanta *et al*., 2005) is also accompanied by an overexpression of the proteoglycan NG2 (Levine 1994), contributing, together with the inhibitory proteins expressed by reactive astrocytes after lesion, to a non-permissive corridor for axonal growth along the lesion site, the

dentate molecular layer and the SLM, during the time window (4-7 days after ECL) in which these glial populations experiment maximal reactivity.

b) Myelin-Associated Inhibitors (MAI) and their receptors.

When this project was started, nothing was known about the regulation of MAI after ECL. In fact, there were few data about their regulation after injury. However, in parallel with the first article of this thesis, where we describe Nogo-A and NgR regulation following ECL, a similar study was published by another group, reporting a 20-fold increase in Nogo-A expression levels in both the ipsilateral and contralateral cortices 1 day after electrolytic lesion of the PP, accompanied by a similar increase in the deafferented hippocampus. In contrast , NgR expression increased at 1 day after lesion and subsequently decreased, falling below normal levels by 15 days after lesion (Meier *et al*., 2003).

4.5. *In vivo* vs. *in vitro*: entorhino-hippocampal organotypic cultures

In this thesis we used enotorhino-hippocampal slice cultures as a model system to analyze the contribution of MAI and CSPG to axonal regeneration failure from a functional approach, by blocking these inhibitors after lesion. Analysis of regeneration in living animals is complex (including the surgery, the delivery of drugs after lesion and the subsequent histological analysis). However, dissociated cell cultures are not suitable to study the complex processes that occur in brain following lesion, although they may be useful to examine the capacity of certain molecules to induce, or prevent, axonal growth (when the complex environment cannot hide these effects). Organotypic cultures combine the easy obtaining and manipulation of the tissue, characteristics of *in vitro* assays, with the preservation of the cytoarchitecture of the tissue of origin, thereby making them highly suitable for questions requiring a three-dimensional structure. In the following sections, we will first address a number of methodological considerations, and then summarize the main data on axonal regeneration of the PP obtained using entorhino-hippocampal organotypic cultures when we initiated this project.

4.5.1 METODOLOGICAL CONSIDERATIONS: VARIABLES AND ADVANTAGES OF THE TECHNIQUE

The hippocampus has probably been the brain area most widely used in slice cultures and several techniques have been developed and optimized for its culture (see Gahwiler *et al*., 1997 for review). The various culture methods share some basic requirements: the tissue must be prepared from young animals, must be provided with a stable substratum, culture medium, and oxygenation, and must be maintained at 37º C. When these conditions are fulfilled, the tissue can be maintained in culture for many weeks and can develop and mature essentially as *in situ.* After an initial common stage of obtaining hippocampal slices, the several methods differ mainly in the way the slices are attached to a substrate to be cultured. In **roller-tube cultures**, slices are secured to a coverslip and then placed in plastic culture tubes that contain the culture medium. They then undergo slow rotation, thus alternating feeding and aeration (Gahwiler *et al*.,

1987). In these conditions they can be cultured for several weeks but after some time their thickness is reduced from the original 400µm to about 50µm. The main advantage of this technique is that it provides optimal optical conditions, therefore making it widely used for live tissue imaging. Slices can be also grown on culture dishes, placing the tissue directly on coated Petri dishes or embedded previously in collagen gels (Bahr *et al*., 1988). These slices, however, must be used after a few days since no special measure is taken to facilitate correct oxygenation. The most used organotypic culturing technique is the interface culture (also called filter culture or membrane culture; Stoppini *et al*., 1991), and has been the method used in this thesis.

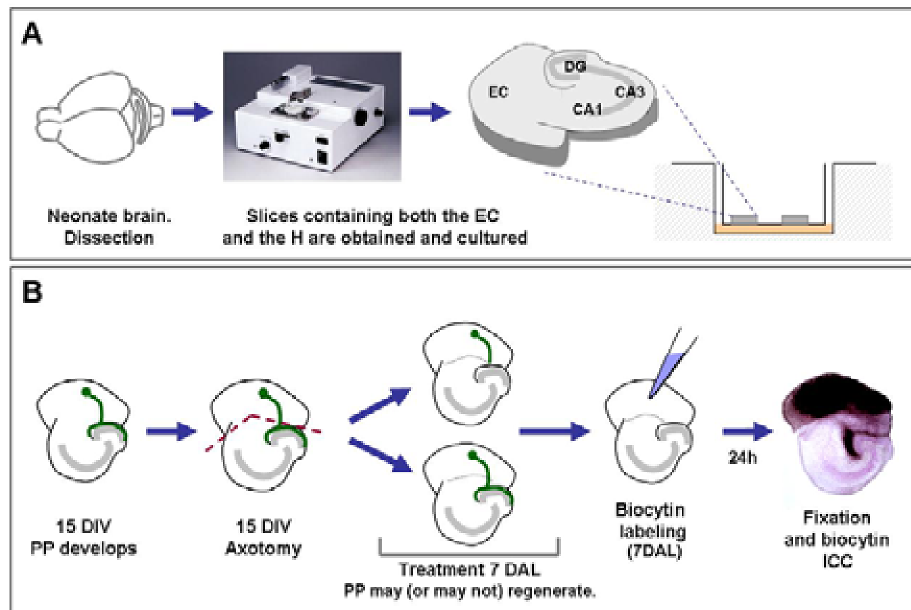


Fig 4.7. Representation of the interface culture method and the experimental design we have followed with the organotypic cultures. A) Early postnatal brain is dissected, chopped and slices containing both the entorhinal cortex and the hippocampus connected are cultured as a single piece on a semi-porous membrane. B) When axotomy is performed after 15DIV the PP cannot regenerate. This paradigm has been used to test the blockade of various inhibitors for approximately one week after axotomy and quantify the number of regenerating axons after biocytin tracing (this assay is described in more detail in the text)

The interface culture receives its name because once the slices have been obtained they are plated at the air-medium interface on a semi-porous membrane. The process that we perform in the laboratory starts by aseptically removing the dorsocaudal portion of the telencephalon, which contains the hippocampus, entorhinal cortex and annexes cortices (Fig. 4.7A). The source must be from early postnatal periods (P0-P1) since older tissue compromises the viability of the culture, especially for the entorhinal cortex. This portion is sliced into sections of 300µm thickness using a tissue chopper and slices containing both the entorhinal cortex and the hippocampus as a single piece are selected for culture (since although the entorhinal cortex innervates all the dorsoventral levels of the hippocampus *in situ,* it is localized ventrally and only the sections containing it can regenerate the PP *in vitro*). This selection is performed in a balanced salt solution to eliminate debris from the slices. Subsequently, slices are placed on a semi-porous membrane attached to a transwell, as observed in Figure 4.7. These transwells can be transferred to 6-well plates and cultures are fed with one millilitre of culture medium, which is replaced every other day, placed behind the transwell membrane.

In entorhino-hippocampal cultures, both neurons and glial cells survive the initial axotomy (the slicing procedure) that is required to obtain slices (Gahwiler *et al*., 1984). The anatomical organization and the phenotypic morphology of neuronal types are very similar to that observed *in situ*, and synaptogenesis occurs essentially as *in situ*, paralleling the temporal pattern (see Gahwiler *et al*., 1997 for review). The same occurs with axonal regeneration that is lost after the second postnatal week *in vivo* and the corresponding time *in vitro* (Woodhamns *et al*., 1993). This observation supports the notion that the same processes contributing to the maturation of the brain, which collaterally may prevent axonal regeneration after axotomy, also occur in organotypic cultures, as is the case of myelintation, which occurs during the first two weeks *in vitro*, coinciding with the loss of regeneration (Berger and Frotscher, 1994). This paralleling permitted us to follow a simple experimental design to assess the role of MAI and CSGP in axonal regeneration (Fig. 4.7). Our assay consisted of PP axotomy *in vitro* (sectioning the interface between the entorhinal cortex and the hippocampus with a knife blade) after 15 days *in vitro* (DIV). Under these conditions, the cultures receive no treatment and the PP does not regenerate spontaneously. Organotypics can be cultured for additional weeks and retain viability, therefore they allow the delivery of several drugs (peptides, neurotrophins, antibodies…), applying them directly to the cultures and analyzing axonal regeneration after 7-8 days. For this purpose, we have used the anterograde tracer biocytin, a biotin-polymer that is injected into the entorhinal cortex (crystals can be taken with a glass micropipette, placed on the entorhinal cortex and then facilitate the entrance of the biocytin to the cortex by gently pressing the cultures against the slice). After permitting the tracer to be transported along entorhinal fibres for 24 hours, tissue is fixed and after cutting, immunohistochemistry to detect biocytin clearly labels the growing axons that penetrate the hippocampus, which is devoid of labelling.

4.5.2 AXONAL REGENERATION IN ORGANOTYPIC CULTURES

Axonal regeneration of the PP in organotypic cultures has been attempted using various approaches, none have included the selective blockade of inhibitory molecules (Table 4.1). This is probably because most of these studies were performed before 2001-2002, when the participation of myelin in axonal regeneration failure gained importance for the research community after the cloning of NgR and the appearance of the “three ligands to one receptor” notion.

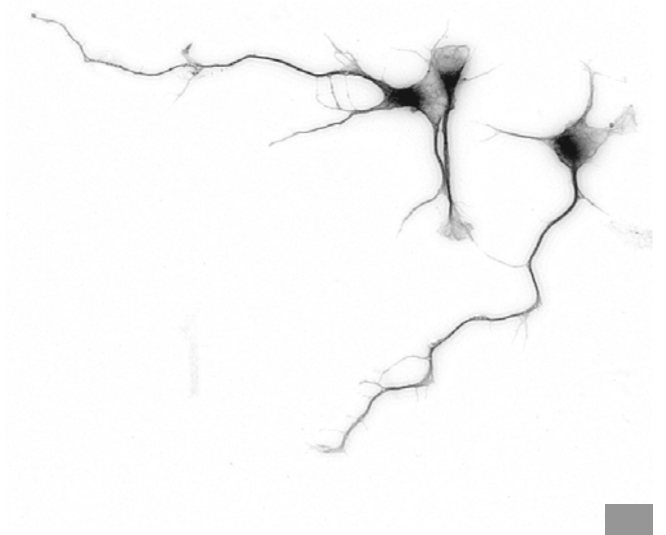
Early studies demonstrated that the capacity of entorhinal axons to reinnervate the hippocampus in organotypic cultures declined in parallel with the length of culture as a consequence of tissue maturation (Woodhams *et al*., 1993; Li *et al*., 1995). Thus, the first experiments used to regenerate the PP consisted of substituting the mature entorhinal cortex for young tissue (Li *et al*., 2005). In these conditions, the new entorhinal cortex projected to the hippocampus; however, this approach is far from being therapeutic and new approaches were used to improve neuronal survival. Neurotrophins can both increase survival and induce axonal growth. A wide spectrum of neurotrophins and growth factors, either as purified proteins or present in conditioned medium (obtained after culturing Schwann cells), were tested in

experimental assays very similar to that we have followed. In many cases the neurotrophins/growth factors failed to promote axonal regeneration and those that did achieved only a moderated degree of regeneration (Woodhams and Atkinson, 1996; Prang *et al*., 2001; unpublished results from our lab). The discrepancy effect of the numerous neurotrophins may respond to the selective neurotrophic requirements of entorhinal neurons. In addition, although entorhinal neurons may require certain neurotrophins to survive, it has been demonstrated that while neuronal death is extensive in other experimental paradigms, as is the case of retinal ganglion neurons, entorhinal neurons do not experiment massive death after PP axotomy. Thus, protection against cell death does not appear to contribute significantly to axonal regeneration of entorhinal axons, as demonstrated by Solé *et al*. (2004).

Procedure	Treatment category	Degree of regeneration	References
Bcl-2 transgenic mice	Prevention of apoptosis	None	*Solé et al., 2004*
Marginal zone graft	Transplant of permissive/attractive tissue	Robust	*del Río et al., 2002*
NGF, FGF-2, IGF-2, TGF-β, HGF NTN	Delivery of neurotrophic and growth factors	None	*Prang et al., 2001*
GDNF, NT4	Delivery of neurotrophic factors	Moderated	*Prang et al., 2001*
Pertusis Toxin (PTX)	Intrinsic treatment	Moderated - robust	*Prang et al., 2001*
PKC Inhibitor	Intrinsic treatment	Moderated - robust	*Prang et al., 2001*
Schwann cell conditioned medium	Delivery of neurotrophic and growth factors	Modest	*Woodhams and Atkinson, 1996*
aFGF	Delivery of neurotrophic factors	Modest	*Woodhams and Atkinson, 1996*
Young EC transplant	Cell replacement	Robust	*Li et al., 1995*

Table 4.1. Summary of the main approaches that have been assayed to induce the regeneration of entorhinal axons following PP axotomy in organotypic cultures when we started this project. As can be observed when comparing this table 1.1, none of the diverse extrinsic treatments aiming to reduce the effect of inhibitory molecules had been tried in the entorhino-hippocampal connection (neither *in vivo*).

The best results that had been achieved with this assay were the blockade of G-protein (with PTX) and PKC by using selective drugs and the transplant of young marginal zone to the hippocampus (Prang *et al*., 2001; del Río *et al*., 2002). In the first case, the two proteins targeted could correspond to the same signalling pathway, as we described for MAI and CSPG. Both classes of inhibitors stimulate PKC activity in a G-protein dependent mechanism, and this pathway is required for axonal growth inhibition mediated by these inhibitors (Hasegawa *et al*., 2004; Sivasankaran *et al*., 2004). Thus, the high degree of regeneration obtained following PKC and G-protein blockade may reduce the sensitivity of entorhinal axons to MAI and CSPG. In the article reporting the highest degree of regeneration, the main experiment consisted of providing the mature entorhinal cortex with a permissive/attractive graft obtained from young marginal zone (dentate molecular layer + SLM) (del Río *et al*., 2002). These young grafts are especially enriched in Cajal-Retzius cells, and their depletion from young hippocampal sections prevented mature entorhinal cortex innervation of these slices, thus indicating that Cajal-Retzius cells could be sufficient to induce or permit axonal regeneration in this system. As we will argue in the discussion, this table can be amplified with the various blockades of inhibitory molecules we have performed in this thesis.



Aims

This Doctoral Thesis was begun in April 2002. At that time, Nogo-A had been identified as the epitope of IN-1, and the first member of the receptor complex, NgR, had been cloned. The expression pattern of both proteins was beginning to be analyzed and the blockade of their interaction seemed to be a promising potential therapy to promote axonal regeneration after lesion (Fournier and Strittmatter, 2002). However, nothing was known about the presence of additional receptors, the signalling pathways they activate, or, importantly, about the convergence of the three myelin-associated inhibitors in the same receptor complex.

With this in mind, the aim of this thesis was to clarify the role of MAG, Nogo-A and NgR in axonal regeneration, and to explore their potential role in non-pathological conditions.

The specific objectives can be summarized as follows:

1. To characterize MAG, Nogo-A and NgR expression during the development of the hippocampal formation and in the adult.

2. To analyse the regulation of MAG, Nogo-A and NgR expression by axotomy of the perforant pathway and their contribution to the prevention of axonal regeneration.

3. To analyse regulation of chondroitin sulphate proteoglycan expression by axotomy, and to assess the involvement of chondroitin sulphate proteoglycans in the regeneration of the perforant pathway *in vitro*.

4. To examine the effects of combining chondroitin sulphate proteoglycan degradation with Nogo-A/NgR blockade on axonal regeneration of the perforant pathway