Summary of Results and Discussion
Results and Discussion I

The Role of Myelin-Associated Inhibitors in Axonal Regeneration

Nogo-A and MAG inhibit axonal growth \textit{in vitro} and have been proposed to be one of the main hindrances to axonal regeneration \textit{in vivo} (McKerracher \textit{et al.}, 1994; Mukhopadhyay \textit{et al.}, 1994; Chen \textit{et al.}, 2002; GrandPre \textit{et al.}, 2002; Prinjha \textit{et al.}, 2002; Wong \textit{et al.}, 2003). However, their regulation after axotomy was still unclear when this thesis was started. One of the main contributions of the present work is the characterisation of Nogo-A and MAG regulation after lesion, and the analysis of their contribution to axonal regeneration failure in the same model. This allows us to compare the results obtained to date and provides a more accurate view of what occurs after perforant pathway (PP) axotomy, thus providing more information than isolated reports on different models.

The present thesis demonstrates that both Nogo-A and MAG are key inhibitors of axonal regeneration following axotomy of adult CNS connections, since their regulation after \textit{in vivo} axotomy fits spatially and temporally with this putative role and their blockade strongly promotes regeneration of the perforant pathway after axotomy \textit{in vitro}.

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.

Axonal regeneration in the adult CNS is extremely limited, mainly owing to the presence of growth inhibitory proteins in association with CNS myelin (Schwab, 1998). This seems to be decisive for the PP, whose axons lose their capability to regenerate in a period matching the onset of myelination (Li D. \textit{et al.}, 1995; Savaskan \textit{et al.}, 1999; Prang \textit{et al.}, 2001). We have demonstrated that the developmental loss of regenerative capacity also correlates with the
appearance of MAG expression by oligodendrocytes and the expression of NgR by entorhinal axons, but not with Nogo-A expression (at least not completely, see below).

It has been proposed that the myelination of axons parallels the maturation of their connections. Long projecting cortical neurons are among the first to mature. Thus, the corpus callosum, formed by their axons, is the first site where MAG expression can be detected, around postnatal day 5 (P5) in mouse (Fig. 1.1). After this stage, transcription of MAG (indicative of myelin synthesis) increases and peaks between P15 and P21, together with synaptogenesis. Thereafter, MAG expression remains constant throughout adulthood. In the grey matter, however, the appearance of MAG expression is delayed (Fig. 1.1). It is first detected in the hippocampus at P10, mainly in areas enriched in oligodendrocytes such as the CA3 stratum radiatum, peaks around the third postnatal week and then decreases and remains steady. Its temporary regulation during development clearly matches myelination onset, indicating that during this period, MAG expression is probably linked to its function in myelin, and is regulated together with other myelin structural proteins, e.g. MBP (Li D. et al., 1995; Savaskan et al., 1999).

In contrast with MAG, which is not expressed when CNS axons can regenerate, the expression of Nogo-A is detected from the first developmental stage analyzed (E12) and is maximal around P0-P5 (figure 1.1 from E16). During these periods, it is restricted to neurons and to a lesser extent to radial glial cells, but is not detected in oligodendrocytes, which are not yet mature. However, as oligodendrocytes become myelinating, they begin to express Nogo-A (presumably exposing it at the myelin surface). This is consistent with data from Hunt et al., (2003), but not from Wang X. et al. (2002), who also failed to observe Nogo-A neuronal expression. Oligodendroglial expression of Nogo-A does indeed correlate with MAG expression and with the loss of axonal regeneration capability. Thus, Nogo-A expression by oligodendrocytes may be related to axonal regeneration inhibition, but not Nogo-A expression by neurons or radial glia.
Detection of Nogo-A by immunohistochemistry was highly conditioned by the technique used. In our hands, short fixation times revealed the greatest immunostaining, which was eminently neuronal but permitted the detection of Nogo-A in oligodendrocytes. This variability was not determined by our antibodies but by the protein, as other laboratories have reported Nogo-A expression almost exclusively in oligodendrocytes using long-term fixation (Wang X. et al., 2002) or by selectively modifying citrate buffer pH during antigen retrieval techniques (L. Dupuis, personal communication). In addition, Nogo-A mRNA was typically neuronal in our in situ hybridizations, as reported elsewhere. These technical limitations hindered the analysis of Nogo-A expression by oligodendrocytes, and Nogo-A expression regulation after lesion.

The functional role of a protein is determined by its receptor/s and the intracellular signalling it may induce in the target cell. The presence of the putative inhibitors in myelin by itself does not demonstrate these proteins are inhibiting axonal growth. To do this, neurons must express their corresponding receptors and the intracellular machinery necessary to induce growth arrest. Since intracellular molecules involved in transducing myelin-induced inhibition seem to be conserved among different processes (as we shall discuss bellow), the expression of the neuronal receptors to these ligands is the regulatory point that permit neurons become “sensitive” to MAI. Consistent with this, expression of NgR by entorhinal neurons correlates very precisely with the drop of axonal regeneration capability of the perforant pathway and is not detected until P5. Thus, NgR expression could directly determine the regenerative potential of entorhinal neurons once myelination has started. However, as we will discuss bellow, other molecules limit axonal regeneration of the perforant pathway.

1.2. Neuronal Nogo-A and NgR expression is regulated by activity.

NgR was reported at asymmetric synaptic contacts, with both pre- and postsynaptic localization (Wang X. et al., 2002). When Nogo-A neuronal localization was evident, it was also detected at the postsynaptic density in both symmetric and asymmetric synapses (Liu et al., 2003). These studies suggested that Nogo-A and NgR also regulate structural plasticity at synapses. This prompted us to analyze how Nogo-A and NgR expression is regulated by neuronal activity,
during the development of neuronal connections and in the adult, after kainic acid-induced seizures.

**During development**, hippocampal neurons maturate in a graded manner, following three defined patterns. First, pyramidal neurons maturate. This process starts around perinatal stages in the CA3 pyramidal neurons, which are older than CA1 counterparts, and then progress into the CA1 area (Fig. 1.2.1). Thereafter, as granule cells reach the prospective granule layer in the dentate gyrus, they begin to maturate following a gradient from the suprapyramidal granule layer to the infrapyramidal layer, in an outside-in pattern that coincides with the generation of granule neurons, oldest cells being the first to maturate (Fig 1.2.2 and 3).

Here, we demonstrate that NgR expression in the developing hippocampus closely resembles the gradient of maturation described above, both in pyramidal and granule cells (Fig. 1.2.1’ to 3’). One of the mechanisms that may modulate (and induce) NgR expression is synaptic activity. As hippocampal neurons begin to receive afferences and/or connect with postsynaptic targets, they may switch to a state in which expression of NgR allows them to stabilize synapses or to prevent additional sprouting once the right connections have been established through interaction with the MAI (or both). This is consistent with the finding that synaptic stabilization is accompanied during development by the removal (or pruning) of the branches that fail to establish those synapses and the inhibition of additional branching. Certain extracellular molecules, namely CAMs, neurotrophins, integrins and axon guidance molecules, regulate the three processes (Tessier-Lavigne, 1996; Seki and Rutishauser, 1998; Rohrbough et al., 2000; Bagri et al., 2003; Pascual et al., 2004), which indicates that these mechanisms are probably intracellularly related (Rico et al., 2004).

**In the mature brain**, the control of branch generation (sprouting) to create new synapses and synapse elimination are key mechanisms that ensure fine-tune networks (Rakic et al., 1986). As during development, these mechanisms can be regulated by activity. The hippocampus is one of the brain regions endowed with high plasticity, and hippocampal neurons express high levels of both Nogo-A and NgR. Since both proteins are localized at synapses, they may interact directly, and NgR expression by mature neurons suggests that this interaction leads to synapse stabilization. To test this hypothesis, one or both proteins should be downregulated during processes requiring synaptic plasticity.

In the present work, we confirm that at least some neurons can regulate both Nogo-A and NgR in an activity-dependent manner. The administration of the non-NMDA receptor agonist kainic acid (KA) is used as an animal model for human temporal lobe epilepsy as it induces an increase in neuronal activity (Ben-Ari, 1985). In situ hybridization in rats treated with KA at convulsive doses show that Nogo mRNA is strongly downregulated in the hippocampus, peaking at 24 hours after injection (that was the first time point analyzed). This downregulation was strikingly manifested in the granule layer, where neurons reduce Nogo-A expression to almost undetectable levels, contrasting with hilar interneurons that maintain high Nogo-A
expression. Similarly, NgR expression is reduced in the granule layer also reaching a minimum 24 hours after lesion, and NgR mRNA levels are partially recovered, but not completely, after 72 hours.

Interestingly, the time at which Nogo-A and NgR are downregulated coincides with the beginning of kainic acid-induced sprouting and synaptic reorganization of granule cell axons (mossy fibers; Tauck and Nadler, 1985; Cronin and Dudek, 1988). This suggests that Nogo-A and NgR may regulate axonal plasticity, and so their downregulation may allow mossy fiber sprouting. This contrasts with p75 and Lingo regulation, which is overexpressed in the hippocampus following KA treatment (Table 1.1; Roux et al., 1999; Trifunovski et al., 2004), and supports the implication of these receptors in signalling pathways not involving NgR, whose function is negatively regulated by neurons after KA injection. While alternative neuronal functions are well known for p75 (Bandtlow and Dechant, 2004), this is only an assumption for Lingo (supported by its widespread neuronal expression during embryonic development; Carim-Todd et al., 2004), which negatively regulates myelination when expressed by oligodendrocytes (Mi et al., 2005).

Previous studies by Josephson and Meier reported no alterations of Nogo-A expression at 24 hours after KA injection, and no alterations or strong upregulation after 7-5 days (Table 1.1; Josephson et al., 2001; Meier et al., 2003). The doses used in the three studies were similar (10 mg/kg Josephson and Meier, 12-15mg/kg in our work), as well as the animal model (adult rats 200-250g), although our time course was the shortest (12-72 hours). In our study and that of Meier, only animals showing convulsive symptoms were analized, although this was not mentioned in Josephson’s manuscript. We have extensively confirmed in the laboratory that animals with the same genetic background and age respond differently to KA injection, depending, among other parameters, on the time the animal is permitted to convulse before sacrifice. Thus, although similar doses were used, Josephson’s lack of Nogo-A regulation may correspond to lower seizures in subconvulsive animals. However, mRNA form mice injected with KA at subconvulsive doses (which did not show seizure symptoms) was used for our Northern blot studies, and we observed a similar downregulation of Nogo-A expression (Fig. 1.3B), presuming that Nogo-A expression is regulated similarly in rats and mice. Artefacts due

| Regulation of Nogo-A, NgR and related proteins in the hippocampus after KA administration |
|--------------------------------------|---------------------------------|------------------------------------------|
| **Nogo-A (Nogo)**                    | **Normal levels**               | **Regulation**                           | **References**                           |
| High                                 | No changes                      | Increase. Peak 5DAL                      | Josephson et al., 2001                   |
|                                      |                                 | Decrease. Peak 24HAL                     | Meier et al., 2003                       |
|                                      |                                 |                                           | Mingorance et al., 2004                  |
| **NgR**                              | **High**                        | Decrease. Peak 4HAL                      | Josephson et al., 2003                   |
|                                      |                                 | Decrease. Peak 24HAL                     | Mingorance et al., 2004                  |
| **P75**                              | **Low**                         | Increase. Peak 3DAL                      | Roux et al., 1999                        |
| **Lingo**                            | **High**                        | Increase.                               | Trifunovski et al., 2004                 |
| **BDNF**                             | **Moderated**                    | Increase. Peak 3DAL                      | Josephson et al., 2003                   |

Table 1.1. Kainic acid-induced regulation of Nogo-A, NgR and related proteins. Our results are compared with reports from other laboratories and complemented with studies about p75, Lingo and BDNF regulation (the later is especially relevant, as it has been proposed to regulate NgR expression).
to differences in animal fixation, which can affect mRNA detection, can be ruled out from our study, since KA injection selectively affected Nogo-A expression in certain layers, like the granule cell layer, while it was not altered in the adjacent hilar interneurons (arrows in Fig. 1.3A).

The hippocampus of both sclerotic and non-sclerotic epileptic patients shows elevated levels of neuronal Nogo-A (Bandtlow et al., 2004). In these patients, the hippocampus has undergone extensive remodelling as a result of recurrent seizures and thus these data cannot be directly compared with those obtained after KA injection in experimental animals. However, the upregulation of Nogo-A reported by Meier at 5 days after KA injection is consistent with the hypothesis that Nogo-A has a biphasic regulation after kainic or epileptic seizure (Meier et al., 2003), and after the initial downregulation, Nogo-A levels may remain high for a long time (as observed in patients of chronic epilepsy; Bandtlow et al., 2004). These longer time points were not considered in our analysis.

We would like to highlight that after KA injection, NgR regulation has been shown to follow the same time course as BDNF regulation, but while NgR is strongly downregulated, BDNF is unperegulated (Wetmore et al., 1994; Josephson et al., 2003). In an elegant article, Josephson et al. show that this regulation (of both NgR and BDNF) also occurs during learning in rats exposed to running wheels, and proposed that NgR downregulation is essential during learning and memory, as KA injection may, to some extent, simulate neuronal activity during LTP, and NgR downregulation parallels learning and is recovered when the animal has adapted to the running wheel (Josephson et al., 2003).

The function of BDNF in synaptic transmission and plasticity in the hippocampus has been extensively studied. Acute application of exogenous BDNF rapidly enhances neuronal and synaptic activity and transmitter release in primary cultures of embryonic hippocampal neurons (Knipper et al. 1994; Levine et al. 1995). To assess the possible involvement of BDNF in NgR expression regulation, and confirm the in vitro glutamate effect on Nogo-A and NgR levels, we carried out a similar experiment in which long-term hippocampal primary cultures (15 DIV) were treated with glutamate at neurotoxic doses and with BDNF at non-neurotoxic doses (Fig. 1.4). Cultures had been kept in serum-free conditions and consisted primarily of neurons, which after
the first week *in vitro*, had established synaptic contacts and were synaptically active. PD98059, an inhibitor of MEK1 (a downstream activator of Erk1/2), was used to determine whether Erk1/2 blockade regulates Nogo-A and NgR expression, as Erk1/2 is known to mediate the effect of neurotrophins.

Although Josephson *et al.* did not implicate BDNF in NgR regulation, the same laboratory demonstrated one year later that NgR is downregulated 24 hours after BDNF delivery into rat hippocampus, thus linking both processes (Josephson *et al.*, 2003; Trifunovski *et al.*, 2004). Our *in vitro* assay also shows that NgR is downregulated in cells treated with BDNF at doses known to stimulate synaptic activity but not toxicity. This is particularly relevant, since overnight priming of neurons with neurotrophins has been shown to reduce MAI sensitivity (Cai *et al.*, 1999). Toxic doses of glutamate failed to regulate NgR expression at significant levels (Fig. 1.4B). Nogo-A expression, in turn, was strongly regulated by both toxic glutamate treatment and BDNF (when analyzed 12 hours after treatment initiation), further supporting the results obtained *in vivo* after KA injection.

How can Nogo-A and NgR lead to synapse stabilization? Or more precisely, why would neurons downregulate Nogo-A and NgR when plasticity is required? Granule cells express both Nogo-A and NgR, as do pyramidal cells from CA3. While NgR protein has been found both pre- and postsynaptically, Nogo-A has been detected mainly in the postsynaptic spine of the brain (Fig. 1.5C; Wang X. *et al.*, 2002; Liu *et al.*, 2003), but also in the presynaptic terminal of spinal motorneurons, where neuromuscular synaptogenesis is required (Dodd *et al.*, 2004; Esther Stoeckli, personal communication). Thus, NgR-Nogo-A interaction probably takes place in the mossy fiber-CA3 pyramidal neuron synapse. Based on the hypothesis that NgR and Nogo-A downregulation is advantageous for neurons when plasticity is required, we propose two models (illustrated in figure 1.5.)
Electronic microscopy data (Fig. 1.5C) suggest that NgR localises to the synaptic terminals of mossy fibers and Nogo-A is postsynaptic, in CA3 dendrites (and particularly in dendritic spines; Fig 1.5A-C). Since Nogo-A has at least two distinct subcellular localizations, two models (illustrated in fig. 1.5D1 and D2) are possible. First, Nogo-A and NgR may interact at the synapse. Cell adhesion molecules regulate various aspects of synaptogenesis, from initial contact formation to specific target recognition, and regulation of synaptic size and strength (for review Scheiffele, 2003; Washbourne et al., 2004). Hence, Nogo-A and NgR may function, locally at synapses, as adhesion molecules. Nogo-A is known to interact with Caspr, a protein structurally related with neurexins, at paranodes (Nie et al., 2003). Thus, it would be useful to determine whether Nogo-A, from its postsynaptic localization, also interacts with β-neurexins, a family of presynaptic proteins known to play a central role during synapse assembly (Scheiffele, 2003). As Nogo-A and NgR are negatively regulated during synaptic reorganization, their putative role may be synaptic stabilization. Alternatively, Nogo-A and NgR may be active in synapses even if they don’t interact directly (Fig. 1.5D2). While NgR may interact with other postsynaptic proteins, leading to synapse stabilization, Nogo-A may play this role from the endoplasmic reticulum (Fig. 1.5E), perhaps regulating calcium levels, as Nogo-A, -B and –C have been reported to form a cluster with channel-like characteristics and a putative calcium-binding domain was identified in the Nogo-A N-terminus domain (Oertle et al., 2003c; Dodd et al., 2005).

1.3. Nogo-A, MAG and NgR expression is differentially regulated following PP axotomy.
Inhibitors are present in the mature brain in either a constitutive manner or induced following lesion. Class-3 semaphorins are a classical example of the second case (Pasterkamp et al., 1999). MAI, in contrast, are the classic example of constitutive inhibitors. Our studies show that although Nogo-A and MAG are found in association with myelin since myelination begins, both are regulated by axotomy in a spatial and temporary pattern that indicates they can also be regarded as lesion-induced axonal regeneration inhibitors.

1.3.1. MAG OVEREXPRESSION AFTER AXOTOMY: EVIDENCES OF REACTIVE MATURE OLIGODENDROCYTES.

The myelin-associated proteins MAG and MBP have been classically used as mature oligodendrocyte markers. Analysis of MBP expression after electrolytic lesion or axotomy has revealed an increase in the number of mature oligodendrocytes in the hippocampus accompanied by overexpression of MBP (Jensen et al., 2000; Meier et al., 2003). In our study, we demonstrate that in the hippocampus, MAG is also overexpressed and the number of MAG-expressing cells is increased in the denervated SLM around 3 DAL. However, in the entorhinal cortex, the number of mature oligodendrocytes after PP lesion does not increase (or decrease), as assessed by MAG expression. On the contrary, mature oligodendrocytes survive to the axotomy and actively react displaying certain characteristics that lead us to refer to them as "reactive" oligodendrocytes.

Mature oligodendrocytes may have two reactions after lesion: extensive death of mature oligodendrocytes accompanied by downregulation of myelin related genes (Morin-Richaud et al., 1998; Wrathall et al., 1998; Zai and Wrathall, 2005), or, when mature oligodendrocytes survive to axotomy, active reaction and overexpression of these genes (Frei et al., 2000; Jensen et al., 2000; Meier et al., 2003; Li and Blakemore, 2004). The term reactive oligodendrocytes, referring to mature oligodendrocytes, was first used in spinal cord injury by Bartholdi and Schwab (see fig. 1.7), who showed transient overexpression of MBP by mature oligodendrocytes around the lesion during the first week following axotomy (Bartholdi and Schwab, 1998). This regulation resembles that observed with MAG hybridization in the entorhinal cortex (Fig. 1.7), indicating that MAG overexpression, as that of MBP, is not an isolated regulation but a sign of glial reactivity. Glia reactivity has been defined as a complex process involving hyperplasia, proliferation, migration and overexpression of certain genes, in response to brain injury (Ridet et al., 1997; Acarin et al., 2001). Only some of these events or all of them occur after lesion, which depends mainly on the type of lesion and the glial cell type. While oligodendrocyte progenitors and microglia proliferate rapidly after transection, proliferation of astrocytes is less frequent and restricted to the proximity of the lesion (Silver and Miller, 2004).

The fourth glial population (separating oligodendrocyte precursor cells from mature oligodendrocytes), formed by mature oligodendrocytes, is probably incapable of reacting after lesion, like the other populations. First, mature oligodendrocytes are postmitotic cells, and in order to proliferate, dedifferentiation would be necessary, which would be manifested as a
decrease in the number of mature oligodendrocytes rather than a proliferative reaction. Second, mature oligodendrocytes are myelinating cells and their processes, enwrapping axons, prevent them from migrating, as the other populations do. Thus, if mature oligodendrocytes undergo a reactive response to axotomy, this would be characterized by hyperplasia and overexpression of certain genes. This is exactly what we found in the entorhinal cortex after PP axotomy. As reactive astrocytes overexpress structural proteins, e.g. GFAP and Vimentin (Yang et al., 1994), mature oligodendrocytes overexpress myelin-related genes, e.g. MAG and MBP, and become hyperplastic during the first week after lesion.

The parallelism between the reactivity of mature oligodendrocytes after axotomy observed in the spinal cord (Fig. 1.7A, C) and in the entorhinal cortex (Fig. 1.7B, D) indicates that the cellular response is the same in both cases. A different process occurs in the hippocampus, where new mature oligodendrocytes are generated after lesion, probably following the differentiation of precursor cells (Fig. 1.7E). It would be useful to establish the factors determining the final outcome of the mature oligodendrocytes population (cell death or survival and reactivity). Although the picture if far from being complete, one of these factors seems to be neuronal survival, as oligodendrocyte death is associated with Wallerian degeneration (Abe et al., 1999). Conversely, as will be discussed below, myelin has a trophic effect on neurons and the survival of mature oligodendrocytes after PP axotomy may contribute to support neuronal survival (Windebank et al., 1985; Sanchez et al., 1996).

1.3.2 NOGO-A EXPRESSION AFTER AXOTOMY: RE-EXPRESSION BY ASTROCYTES.

Mature oligodendrocytes overexpress MAG and MBP after lesion, and therefore they could also overexpress Nogo-A. However, as commented in the introduction, the detection of
oligodendrocytic Nogo-A is not easy neither by western blotting nor by in situ hybridization (ISH), so descriptions have been made about neuronal Nogo-A regulation after lesion but are lacking about the myelin-associated Nogo-A.

During the preparation of this thesis, several articles reported Nogo-A regulation after spinal cord lesions. Most of them described the downregulation of Nogo-A in the lesion centre, probably following the death of Nogo-A expressing cells, but overexpression around the lesion site (table 1.2). Some studies localized the overexpression of Nogo-A in sectioned axons and probably oligodendrocyte processes bordering the lesion (Huber et al., 2002; Hunt et al., 2003).

Here, we report Nogo-A overexpression after PP axotomy around the lesion site by ISH. We have also confirmed this overexpression by immunohistochemistry (fig. 1.8). Nogo-A overexpression at the borders of the axotomy is transient, as it was detected by immunohistochemistry from 4 DAL to 7 DAL (fig. 1.8B-C) and by ISH at 3 DAL. The morphology and localization of these Nogo-A-overexpressing cells indicates they are probably glial cells. In contrast, entorhinal projecting neurons also display higher levels of Nogo-A, which accumulates mainly around the cell soma during longer post-axotomy times (fig. 1.8F-H), and is also observed in sectioned axons. This overexpression can be detected, to a lower extent, by ISH.

In the hippocampus, Nogo-A is slightly overexpressed by granule cells around 3 DAL, as also observed by Meier (2003). We also analyzed Nogo-A regulation after fimbria-fornix transection (Fig. 1.9). The fimbria-fornix (FF) constitutes a major afferent and efferent fiber tract connecting the hippocampus with the diencephalon, forebrain, striatum and prefrontal cortex (Cassel et al. 1997). Lesioning a fibre tract may facilitate the observation of mature oligodendrocytes, and thus allow us to assess whether they overexpress Nogo-A after lesion. A similar work, performed by David Hunt using optic nerve crush, revealed the upregulation of Nogo-A around

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<td>Spinal cord injury (transsection)</td>
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Table 1.2. Lesion-induced Nogo-A regulation. Nogo-A regulation by injury has been mainly studied by in situ hybridization and the data reported normally refer to neuronal Nogo-A. References are given in chronologic order.
the lesion border but failed to determine the identity of these Nogo-A-overexpressing cells (Hunt et al., 2003; Table 1.2). After FF transection, Nogo-A is also overexpressed around the lesion (Fig. 1.9). Closer examination shows that this overexpression is apparently localized in three cell types, presumably infiltrated fibroblasts (Fig. 1.9C, F), mature oligodendrocytes (Fig. 1.9E) and neurons. Transected axons, with collapsed terminals, were particularly immunoreactive to Nogo-A (Fig. 1.9D, G). Accumulation proximal to lesions is typical of molecules that undergo fast anterograde axonal transport (Tonra et al., 1998) and molecular motors are upregulated after nerve transection (Su et al., 1997). Thus, Nogo-A accumulation in the proximal endbulbs may be linked to an increase in the anterograde transport of vesicles (including Nogo-A associated with ER) to provide the membrane material needed for axonal regeneration (Su et al., 1997).

An interesting feature about the regulation of Nogo-A after axotomy is that it is re-expressed by astrocytes. We have shown that during development, Nogo-A is expressed by radial glia cells, which become astrocytes once their developmental role is completed (Culican et al., 1990). After axotomy, reactive astrocytes re-express proteins characteristic of radial glia such as Vimentin and Nestin (Clarke et al., 1994). Similarly, they express Nogo-A transiently after PP axotomy, especially in the deafferented molecular layer of the dentate gyrus. This expression was confirmed by analysis of the C6 cell line derived from a rat astrogliaoma, whose cells behave as reactive astrocytes (Grobben et al., 2002) and constitutively express Nogo-A. Nogo-A expression by reactive astrocytes is also observed around degenerating neurons in the spinal cord of patients with amyotrophic lateral sclerosis (Luc Dupuis personal communication). Although the expression of Nogo-A in the adult CNS by reactive astrocytes is probably associated with its role in radial glia cells and not intended to interact with neuronal NgR and...
inhibit axonal regeneration, its presence in the deafferented hippocampal layers may contribute to this effect.

**1.3.3 NgR Biphasic Regulation After Axotomy.**

In our study, we show that NgR mRNA is upregulated by granule cell neurons after PP axotomy in the ipsilateral dentate gyrus soon after lesion, and this increase is followed by a decrease in NgR expression below control levels around two weeks after axotomy. This was also observed by Western blotting from total hippocampus. A similar regulation was described by Meier *et al.* (2003), who proposed that long-term NgR downregulation may permit sprouting of granule cell axons, which are known to sprout into the denervated molecular layer in a period matching the downregulation of NgR. This hypothesis is fairly possible, as supported by the downregulation of NgR after KA injection or learning (which also involve sprouting), and the fact that septal neurons, which are the first neuronal population to invade the molecular layer, do not express detectable levels of NgR. In contrast, we failed to detect a clear regulation of NgR mRNA in entorhinal neurons after transection, mainly because NgR expression levels in the adult entorhinal cortex are very low in our ISH. The putative expression of NgR by reactive astrocytes around the lesion site, so far observed by only one group in humans (Satoh *et al.*, 2005), was neither detected in our ISH.

We would like to highlight that although NgR is overexpressed soon after PP axotomy, it has been shown to be subsequently downregulated and in chronic diseases, like amyotrophic lateral sclerosis, neurons even stop expressing NgR (Luc Dupuis personal communication). This may represent a survival response of neurons that try to release themselves from CNS inhibitors in an attempt to overcome their critical situation.

**Figure 1.9. Overexpression of Nogo-following fimbria-fornix lesioning.** A) In uninjured fimbria, Nogo-A expression follows a fascicular pattern and also labels scattered oligodendrocytes. B-G) 4 and 7 DAL, Nogo-A is expressed at the lesion site by putative infiltrated fibroblasts (C, F), sectioned axons (D, G; arrowheads) and mature oligodendrocytes (insert in E).
1.3.4 The expression of other inhibitors is also regulated by axotomy.

Although this thesis focuses on MAI, we have also addressed the regulation of other known inhibitory proteins. CSPG are overexpressed by reactive glia after entorhinal cortex lesion, preferentially around the lesion site and along the denervated hippocampal areas. This overexpression is delayed compared with that of MAG and Nogo-A and lasts for months, suggesting that CSPG are the main inhibitors once the overexpression of MAI is over. Another inhibitor whose expression is regulated after entorhinal cortex lesion is Sema 3a (Fig. 1.10). In lesioned organotypic cultures, its protein levels are high soon after lesion and this increase lasts at least 10 days (Fig. 1.10). Although there are no meninges around organotypic cultures, and therefore no infiltration of Sema 3a expressing fibroblasts, Sema 3a is overexpressed by neurons after injury (Pasterkamp and Verhaagen, 2001). In addition, blockade of Sema 3a binding to its receptor, NP-1, promotes axonal regeneration in the same in vitro model, pointing to the inhibitory role of Sema 3a after lesion (Montolio et al., in preparation). Our results on the blockade of CSPG and Nogo-66/NgR binding are described in the following sections. They are the first direct evidence of the participation of CSPG and Nogo-A in the prevention of PP regeneration.

1.4 Blockade of Nogo-A and MAG promotes axonal regeneration after PP axotomy in vitro.

The inhibitory role of Nogo-A after SCI has been demonstrated by several in vivo studies (see the introduction of this thesis and references within). The most extended Nogo-A blocking reagents have been the antibody IN-1 (which binds to Nogo-A NIG domain) and the NEP1-40 peptide (directed against Nogo-66 domain binding to NgR) (GrandPre et al., 2002; Schwab et al., 2003). In contrast, in vivo studies are lacking for MAG, although sialic acid removal by neuraminidase was shown to interfere with MAG binding to its receptors and its capability to inhibit neurite outgrowth in vitro (Tang et al., 1997; Vinson et al., 2001; Venkatesh et al., 2005). In this work we have used these tools to assess the contribution of Nogo-A and MAG inhibition to perforant pathway regeneration in organotypic cultures.

Organotypic cultures have been considered as an ex vivo technique, as they conserve the cytoarchitecture of the tissue of origin. In addition, we have reported that proliferation, glial activation and regulation of inhibitors finely reproduce what has been described in vivo. Thus, the blockade of MAG and Nogo-A in organotypic cultures should closely resemble the in vivo blockade, having the advantage of easy access to the lesion site and drug delivery.
summarizes the results obtained from the various blockades assayed in organotypic cultures and complements the blockades listed in table 4.1 of the introduction.

<table>
<thead>
<tr>
<th>Target/Loop-66</th>
<th>Control</th>
<th>Acute Treatment</th>
<th>Delayed Treatment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neuraminidase</td>
<td>MAG(^1)</td>
<td>5.7 ± 1.4</td>
<td>23.8 ± 6.7</td>
</tr>
<tr>
<td>IN-1 IgM</td>
<td>NiG (Nogo-A)</td>
<td>4.5 ± 0.9</td>
<td>6.7 ± 2.4</td>
</tr>
<tr>
<td>IN-1 Fab</td>
<td>NiG (Nogo-A)</td>
<td>4.0 ± 0.6</td>
<td>5.7 ± 2.1</td>
</tr>
<tr>
<td>NEP1-40</td>
<td>Loop-66 (Nogo-A)</td>
<td>3.4 ± 1.2</td>
<td>19.7 ± 4.2</td>
</tr>
<tr>
<td>ChABC</td>
<td>CSPG</td>
<td>3.7 ± 1.3</td>
<td>37.2 ± 8.4</td>
</tr>
<tr>
<td>NEP1-40 + ChABC</td>
<td>Loop-66 + CSPG</td>
<td>3.8 ± 1.0</td>
<td>43.3 ± 6.9</td>
</tr>
</tbody>
</table>

The perforant pathway loses its capability to regenerate in organotypic cultures after 15 days in vitro (DIV). Neuraminidase treatment of cultures for seven days after axotomy (performed at 15 DAL and starting drug delivery the day after) increases the number of regenerating axons that enter the hippocampus. This increase is at the limit of significance by statistical analysis (p=0.051; Student’s t-test at 95% confidence level) but clearly important in biological terms, as in control cultures, a mean of 5.7 axons entered the hippocampus (stopping short after) and neuraminidase treatment allowed a mean of 23.8 axons to innervate again the hippocampus (four-fold increase), growing long distances and ending often in the prospective target layers. This result clearly indicates that sialic acid-dependent inhibitors, such as MAG, prevent the axonal regeneration of entorhinal axons in vitro. Moreover, neuraminidase did not elicit any major adverse effect on growing axons or interfered with PP termination specificity. A recent article has identified NgR2 as a neuraminic acid-dependent MAG receptor and shown that MAG binding to NgR also depends on neuraminic acids (Venkatesh et al., 2005). This indicates that MAG-induced inhibition totally depends on the presence of neuraminic acids and neuraminidase treatment should fully prevent it. The only published article that had previously reported the effects of neuraminidase in axonal regeneration also used, curiously, hippocampal organotypic cultures (Muller et al., 1994). This article describes the effect of neuraminic acid removal on the regeneration of the CA3-CA1 connection, whose sprouting seems to be dependent on PSA-NCAM. Removal of polysialic acids from PSA-NCAM significantly delayed the sprouting of CA3 axons but did not affect the total degree of regeneration achieved (Muller et al., 1994). At least a subset of entorhinal neurons also express PSA-NCAM, therefore the regeneration accomplished by MAG-specific blockade may be greater than the one observed, as neuraminidase may negatively affect certain growth promoting proteins.

However, IN-1 delivery failed to clearly increase axonal regeneration, although it did enhance the neurite length of entorhinal axons growing into the hippocampus (Table 1.3). We would like to highlight that these results should not necessarily be understood to reflect the role of the nogo-a specific domain in axonal regeneration, and may respond to differences in tissue penetration, as discussed below.
Targeting of the second inhibitory domain in Nogo, the loop-66, with the antagonistic peptide **NEP1-40**, promotes axonal regeneration of corticospinal axons *in vivo* (Grandpre et al., 2002; Li and Strittmatter, 2003). NEP1-40 seems to be highly specific for Nogo-66-NgR binding, as it does not interfere with MAG or OMgp binding, and has been shown to be extremely innocuous after systemic delivery. Thus, NEP1-40 is so far the most promising therapeutic tool that has been designed to target the MAI (Lee et al., 2003). In our model, acute treatment with NEP1-40 strongly promoted axonal regeneration at a similar rate to neuraminidase, supporting that Nogo-A binding to NgR contributes to prevent axonal regeneration of the perforant pathway (Table 1.3).

1.5 CSPG and Nogo-66 do not have synergistic effects on axonal regeneration.

In addition to their role as myelin-associated inhibitors, CSPG are thought to be the main chemical hindrance to axonal regeneration after lesion. Although HSPG are involved in layer-specific termination of entorhinal axons in the hippocampus (Forster et al., 2001), *in vivo* blockade of CSPG, but not blockade of HSPG, promotes extensive axonal regeneration after various lesion models and enhances functional recovery (Moon et al., 2001; Bradbury et al., 2002; Zuo et al., 2002). Blockade of CSPG is achieved by degradation of chondroitin-sulfate groups with chondroitinase ABC (ChABC), which is extensively used in combinatorial therapies in animal models (e.g. Chau et al., 2004; Fouad et al., 2005).

Acute treatment of axotomized organotypic cultures with ChABC promotes a ten-fold increase in the number of regenerating axons and leads to the reinnervation of prospective targets (Table 1.3). In our hands, ChABC induces a degree of regeneration greater than NEP1-40 and more importantly, much more specific. As both drugs have been shown to have very good penetration on tissue (Bradbury et al., 2002; GrandPre et al., 2002) and the concentration used was optimized in preliminary experiments, this difference of efficiency may be due to several reasons. First, NEP1-40 only prevents Nogo-A binding to NgR but does not affect the binding of other MAI, and so its effect in preventing MAI inhibition is partial. Li et al. (2004) compared the efficiency of NEP1-40 with that of the NgR ectodomain, which binds to the different NgR ligands and is expected to abolish NgR-mediated inhibition. They reported no increase in functional recovery when using NgR ectodomain compared with NEP1-40 induced recovery, although axonal sprouting was more highly branched with the former (Li et al., 2004). The authors concluded that comparison between the two studies can be used to assess the relative roles of Nogo-66 versus MAG and OMgp, and thus Nogo-A binding to NgR is responsible for the overall myelin inhibition (Li et al., 2004). If this proves true, our NEP1-40/ChABC comparison would show that CSPG play a more determinant role in axonal regeneration failure than myelin. However, we believe that NgR ectodomain treatment underestimates the role of MAG and OMgp, as it is unlikely that a large Fc-NgR ectodomain recombinant protein has the same efficiency as a small 40-aa peptide, which would be more stable and soluble.
Interestingly, if both Nogo-66 and CSPG contribute to preventing axonal regeneration of entorhinal axons and represent the major inhibitors, **combination of NEP1-40 and ChABC** should achieve a much better regenerative degree than any of the blockades alone. Surprisingly, this is not true. As observed in table 1.3, the number of regenerating axons in cultures treated with the best performing concentration of NEP1-40 and ChABC in combination is not significantly greater than that obtained with ChABC alone. We have confirmed that ChABC does not affect MAI regulation and that NEP1-40 does not alter CSPG overexpression in the deafferented hippocampus. We also analyzed the putative effect of both drugs on microglial activation and glial scar development. From these experiments, we ruled out that ChABC or NEP1-40 affects glial reactivity.

Closer analysis reveals that the delivery of both drugs in combination does not prevent the effect of any of them, as the regeneration pattern achieved by the combined treatment (which will be referred to as “cocktail”) is actually a mix of both drugs when assessed by parameters other than the mean number of axons (Fig. 1.11). Axonal length in ChABC treated cultures reaches the normal length of PP axons, around 600µm (see drawing in fig. 1.11A). However, NEP1-40-treated cultures never displayed axons of such length and prevented them from doing so when added to ChABC in cocktail-treated cultures. Similarly, the high specificity of axonal regeneration observed in ChABC treated cultures was slightly reduced in cocktail-treated ones by the presence of NEP1-40 (Fig. 1.11B). Thus, although the number of regenerating axons was not additive, and resembled that obtained with ChABC alone, this is not due to the inefficiency of NEP1-40 when delivery in combination as the regeneration pattern of ChABC/NEP1-40-treated cultures is intermediate between both treatments.

We would like to underline that IN-1 also induced overall low-specific regeneration. One possible explanation is that MAI prevent axonal regeneration but do not participate in layer specificity. In fact, we observed that the amount of CSPG in the SLM and ML is particularly
lower than that of neighbouring areas, thus creating a permissive corridor for entorhinal axons. After axotomy in vitro, this corridor is filled following upregulation of CSPG, and is no longer appreciated. Similarly, regenerating axons (induced by release from MAI) would not grow preferentially along the SLM and ML if these layers are as inhibitory as the rest. On the other hand, ChABC treatment may permit axonal regeneration by removal of inhibitory groups (as NEP1-40 does) and reduce the amount of inhibitors located at the prospective layers, allowing regenerating axons to respond to the putative positive cues located in this layers.

However, why is axonal regeneration not increased, compared with ChABC-induced regeneration, when both Nogo-66/NgR and CSPG are blocked? Two possibilities may account for the lack of synergistic effect. First, inhibitors may only account for one part of axonal regeneration failure and in order to achieve greater regeneration degrees, physical barriers (like glial scar) should be removed or neurons treated to overcome endogenous limitations (e.g. by the activation of growth promoting genes). In this case, the higher effect of ChABC may be sufficient to overcome the “percentage" of axonal regeneration that corresponds to inhibitors, so the blockade of more inhibitors cannot increase axonal regeneration. This has been shown to be at least partially true, as the combination of treatments covering several “obstacles” to regeneration seems to have synergistic effects, e.g. the combination of ChABC with LiCl (a GSK-3β inhibitor; Yick et al., 2004), ChABC and nerve grafts (Krekoski et al., 2001; Fouad et al., 2005), and a dominant negative form of NgR with macrophage-derived factors (Fischer et al., 2004). In contrast, NEP1-40 and ChABC do not have synergistic effects on axonal regeneration because both MAI and CSPG follow the same signalling pathways. In this regard, both CSPG and myelin have been recently shown to activate RhoA via PKC, which is necessary for both their inhibitory effects, indicating that PKC (and downstream cascades) is a common point of signal convergence of the diverse inhibitors (Monnier et al., 2003; Fournier et al., 2003; Schweigreiter et al., 2004; Hasegawa et al., 2004; Sivasankaran et al., 2004). In this case, ChABC reduces PKC activation and reaches an activation threshold, after which the signalling pathway is no longer activated, and ChABC alone suffices to “inhibit" the downstream signalling leading to regeneration inhibition. However, NEP1-40 may not suffice to reach this threshold, as CSPG, MAG and OMgp are still functional. Note that the second hypothesis is not independent of the first, and can be considered a particular case.

Many treatments that are effective in vitro fail to promote axonal regeneration in vivo because they need to be started before injury or immediately after, which is normally not feasible in human patients. Therefore, in a last experimental set, we aimed to assess whether entorhinal neurons retain their capability to regenerate for some days, and whether there is a temporary window of effectiveness of ChABC and NEP1-40 beyond the initial hours. To this end, we delayed the beginning of the treatments up to five days after axotomy, and then proceeded as for acute treatments (same doses and every-other-day delivery for a week). The most efficient drug for acute treatments (ChABC) greatly lost its efficiency when treatment was delayed for five days. Although it still promoted slight regeneration when compared to controls, ChABC efficiency fell under the regeneration levels promoted by acute NEP1-40. Moreover, the
efficiency of the combined treatment decreased to similar levels, while NEP1-40 induced the same degree of regeneration in acute and delayed treatments.

The most feasible explanation for the reduced effect of ChABC on axonal regeneration is that the doses used may be insufficient to degrade the growing accumulation of CSPG. When delivered right after lesion, 50 ng/ml of ChABC can degrade the initial amount of CSPG, and this may be enough to permit axonal regeneration during the initial, most critical period for lesioned neurons. After five days, the amount of CSPG accumulated may require higher doses of ChABC to be sufficiently degraded. Unfortunately, we assayed several dilutions of the enzyme (described to be efficient in the literature) and found that doses higher than 100 ng/ml reduced culture viability, as observed by a retraction of the section site that physically prevented axon crossing and culture death with the highest dose (600ng/ml). NEP1-40 showed no apparent toxicity for the cultures, but also lost efficiency at high doses. However, the functional dose used for acute treatment was enough to reduce the binding of Nogo-A to NgR to the same extent when used for delayed treatment. Altogether, we highlight the need to thoroughly test the doses and temporal delivery that optimize the efficiency of each drug and, in addition, the need of selecting treatments that use various mechanisms for combination. To this end, we should have detailed understanding of how these treatments work, as ChABC and NEP1-40 may appear as complimentary treatments (since they target distinct inhibitors), while the intracellular convergence of this inhibitors makes them ultimately redundant. The present thesis contributes to this critical knowledge, and reports the analysis of two of the most promising drugs available.
Results and Discussion II

Nogo-A During Neuronal Development

2.1. Nogo-A is expressed by neurons during development

One aspect that remains to be clarified is the neuronal role of Nogo-A. During development, Nogo-A is expressed by most, but not all, of neuronal populations. In fact, it is expressed at higher levels than in postnatal and adult stages. However, there are no reports on neuronal

*Figure 2.1. Subcellular localization of Nogo-A in developing neurons.* Dissociated hippocampal neurons from E13 mice were maintained in serum-containing medium for 15 hours and then fixed to analyze Nogo-A localization. A) Nogo-A (white) is localized to the neuronal soma, the major neurite (including growth cone) and minor neurites, but is not detected at the filopodia or lamellipodia, as observed by phalloidin binding to actin (red). B) This pattern coincides with endoplasmic reticulum localization, as observed by Calnexin immunostaining (green) in permeabilized cultures. C-D) Nogo-A is also present at the neuronal surface. WGA, a lectin that binds to glucids at the cell surface and at the Golgi apparatus, was used to assess plasma membrane localization. ICC was performed without permeabilizing agents and membrane integrity was determined by the absence of WGA-Golgi immunostaining and tubulin labelling. Cells II and III in C were permeabilized, but not cell I. All of them express Nogo-A (arrowhead points to non-permeabilized neuron with Nogo-A staining). Not every neuron, however, exposes Nogo-A at the cell surface, like neurons I and II in D (asterisks).
Nogo-A functions. We observed that during development, Nogo-A is expressed by neurons and radial glia cells (Fig. 2.2 A-K). In cultured embryonic neurons, Nogo-A is located at the plasma membrane (Fig. 2.1C-D), but also in association with the endoplasmic reticulum (ER; Fig. 2.1B). Association with the endoplasmic reticulum is evident after Nocodazole treatment, which depolymerizes microtubules and causes a retraction of the ER (together with Nogo-A) from neurites to the cell soma (not shown). This association is more manifest in neurites, particularly in the prospective axon and the central domain of the growth cone (Fig. 2.1A), and Nogo-A accumulation in the proximal branch of sectioned axons suggests that this reticular association remains until adulthood, and Nogo-A is transported anterogradely.

2.2. Contact with Nogo-A induces fasciculation in vitro

Nogo-A presence at the plasma membrane suggests it interacts with extracellular receptors. In fact, embryonic hippocampal neurons are sensitive to Nogo-A presence on the substrate, although they fail to express detectable levels of NgR. Hippocampal explants grow radially on ornithine-laminin substrate, and when membrane extract from wild type COS cells is dropped between the two coating agents. However, membrane extract form Nogo-A transfected cells induces a clear fasciculation of embryonic hippocampal explants (Fig. 2.2 L). This may be due to two reasons. First, fasciculation may respond to an inhibitory effect of Nogo-A on the substrate, favoring interaxonal adhesion over adhesion to substrate. However, axonal length was not reduced in fasciculated explants (only a small decrease, probably due to increased fasciculation) or in dissociated hippocampal cells, while it was clearly lower in postnatal cerebellar explants and dissociated cerebellar granule cell neurons (CGNs, which express NgR). Second, Nogo-A may directly induce fasciculation. In this regard, Nogo-A is preferentially localized to axon tracts during development (Fig. 2.2 G-K) and when these cells are dissociated, Nogo-A can be detected at the plasma membrane (Fig. 2.1).
Summary of Results and Discussión II

Fig. 2.2. Expression pattern of Nogo-A during embryonic development in mice. A-I) Pattern of Nogo-A expression during development. A-B) Nogo-A is expressed by pioneering layer-I neurons at E12 and also by tangential migrating neurons at E14-16. C) Nogo-A is not only expressed by neurons (demonstrated by colocalization with the postmitotic neuronal marker TUJ-1), but also by non-neuronal cells spanning the cortical width (arrowheads in C). D) These cells correspond to radial glia cells as shown by colocalization of Nogo-A with Nestin (a neuronal marker). E-F) Radial migrating neurons, in contrast, do not seem to express Nogo-A but to migrate on Nogo-A-positive glial cells. G-K) After neurons arrive to their prospective layer, they stop migrating and instead extend their axons, which occasionally form thick axon bundles or tracts. Most of the main axonal tracts are enriched in Nogo-A (G, H, J, K). These tracts are characteristically labelled with antibodies against some cell adhesion molecules such as TAG-1 (G-H), L1 (I) and DCC (not shown). (Continue)
The presence of Nogo-A at the surface of fasciculated axons is inconsistent with an inhibitory or repulsive role \textit{in vivo}. However, this does not explain how the presence of Nogo-A in the substrate induces fasciculation \textit{in vitro}, which fits more with an inhibiting role and sets a dichotomy between the \textit{in vivo} and \textit{in vitro} observations. It could be argued that laminin substrate may switch Nogo-A from adhesive (\textit{in vivo}) to antiadhesive (\textit{in vitro}), as it does with Netrin-1 attraction, probably by changing the intracellular levels of calcium (Song and Poo, 1999; 2001). However, we first observed Nogo-A-induced fasciculation in explants growing on collagen-coated transwells that were dropped with mock and Nogo-A membrane extracts, and the experiment was also reproduced in matrigel transwells. Another possibility is that the effect of Nogo-A on neurons depends on the context, i.e. on the proteins with which it is co-expressed. This occurs with many receptors, as suggested in figure 2.2 5-7. As for netrin, two receptors are involved: DCC and Unc-5H (Kennedy, 2000). While binding of Netrin to DCC induces attraction and binding to Unc-5H repulsion, DCC can also transduce repulsion when coexpressed with Unc-5H (Shu \textit{et al.}, 2000). Although this example involves receptors, a similar mechanism may occur with Nogo-A as a ligand. Neurons that express high levels of Nogo-A seem to adhere to each other forming fascicles (Fig. 2.2 G-K). However, neurons migrating on radial glia, which express Nogo-A (Fig. 2.2 C-D), do not express it until they stop migrating and detach from the glial cell (Fig. 2.2 F). Neurons may adhere to neuronal Nogo-A, but not to the one expressed by radial glia or COS cells because it needs to be expressed together with other neuronal proteins to induce neurite adhesion. If this proved true, as Nogo-A is expressed by non-neuronal cells in our \textit{in vitro} assay, it would be presented to the neurites \textit{individually} and induce deadhesion from substrate, while it would induce adhesion when expressed by neurons in combination with some/s neuronal protein/s (Fig. 2.2 L). Some candidates for this \textit{partner role} may be TAG-1, L1 and DCC, as they colocalize with Nogo-A in all the tracts where it is expressed, such as the corticothalamic tract (Fig. 2.2 I-J) and the commissural fibres in the embryonic spinal cord (Fig. 2.2 G).

\textbf{(From page 123)}

1-4) Schematic representation of cortex development illustrating the expression of Nogo-A (red cells) by certain cell populations, contrasting with cells that do not express Nogo-A (green cells). Initially, radial glia cells span the cortical width and express Nogo-A (1). In contrast, radial migrating neurons, which generate from the subventricular zone (and some of which are generated from radial glia cells; Fishell and Kriegstein, 2003), do not express Nogo-A (see also F). When radial migrating neurons stop migrating and detach from glial cells, they start expressing Nogo-A (2 and A) and some of them extend long projections that are particularly enriched in Nogo-A (G-K, 3). Last, the population of tangential migrating neurons also express high levels of Nogo-A (4). These cells generate in the ganglionic eminences and migrate toward the cortex following paths that at some stages involve migration over Nogo-A/Tag-1-positive tracts.

5-7) Hypothesis of how Nogo-A may participate in cell adhesion. Cells can be separated in non-neuronal (blue) and neuronal cells (brown). When neurons that do not express Nogo-A (i.e. radial migrating neurons) contact with Nogo-A expressing glial cells, the presence of Nogo-A does not seem to prevent cell adhesion and migration. (5). Thus, Nogo-A may be considered permissive when expressed unilaterally. However, no neuron expressing Nogo-A was observed in apposition with radial glia cells. We can propose that this bilateral expression of Nogo-A may result in the deadhesion of the neuron from the Nogo-A source (6). Last, Nogo-A is widely expressed in axonal tracts. As these axons are packed together, the bilateral expression of Nogo-A cannot be repulsive (or induce deadhesion), but instead be permissive and even facilitate cell adhesion (7). The difference between this last situation and the previous one (6) may be the expression, together with Nogo-A, of other cell adhesion molecules by neurons.

L) Nogo-A induces fasciculation \textit{in vitro}. Hippocampal explants express Nogo-A but not NgR. While they display radial growth when cultured on permissive substrates (laminin, collagen, or matrigel), they fasciculate when growing in the presence of Nogo-A. This may correspond to the mechanism explained in (6).
2.3. IN-1 supernatant induces axonal growth regardless of external Nogo-A

Another cue about Nogo-A neuronal role comes from observations about IN-1 effect on neurons. Postnatal CGNs express NgR and are inhibited by the presence of Nogo-A on the substrate, but they also express high levels of Nogo-A. Adding IN-1 supernatant to these substrates enhances axonal length, restoring control length, which may be attributed to a blockade of the inhibitory Nogo-A present in the coating (Fig. 2.3, third column). A similar experiment using DRG neurons was performed by Chen et al. in an article describing the cloning of Nogo-A (Fig. 3 in Chen et al., 2000). However, axonal length also increases when IN-1 is included in cultures not exposed to Nogo-A (Fig. 2.3 first two columns), indicating that IN-1-induced axonal growth is a direct effect of the antibody on neurons. This control was omitted in Chen et al. (2000). Although the increase observed in neurons growing on Nogo-A is higher (around 40%), it is significant and very consistent in cells growing on non-inhibitory substrates (around 20%), so that the blockade of Nogo-A in the substrate is combined with the enhancement of neuronal growth through direct binding to neurons.

The growth-promoting effect of IN-1 in non-lesioned neurons had been reported elsewhere, but has always been attributed to a release from the constitutive inhibition exerted by (non-neuronal) Nogo-A (Z’Graggen et al., 1998; Thallmair et al., 1998; Buffo et al., 2000). However, our in vitro assay allowed us to assess the effect of IN-1 in isolated neurons. Although the specificity of IN-1 for Nogo-A is somehow uncertain, the growth promoting effect seems to be specific, as monoclonal antibodies other than IN-1 raised against Nogo-A induce axonal growth and sprouting in unlesioned Purkinje cells (Buffo et al., 2000). Moreover, Purkinje cells express high levels of Nogo-A and at least part of the IN-1 effect may be due to binding to neuronal Nogo-A (as in Fig. 2.3).

How can the binding of IN-1 to neurons promote axon growth? One possibility is that IN-1 binding to neuronal Nogo-A activates it, and initiates signaling cascades leading to neurite extension. This effect is known for cell adhesion molecules or receptors that need to dimerize in order to start intracellular signaling (for example L1 and Fas/CD95, Dickson et al., 2002; Suda et al., 1997). Antibody binding to these proteins induces clustering, as the endogenous ligand would do, and mimics ligand-induced signaling. Likewise, IN-1 binding to Nogo-A at the plasma membrane may induce clustering of Nogo-A. The IN-1 epitope is located in the NiG domain of Nogo-A, which has inhibitory effects on postnatal neurons and fibroblast spreading but whose receptor, like the binding partners for Nogo-A during development, is yet unknown. Although in
the spinal cord, IN-1-induced sprouting of unlesioned corticospinal tract neurons is accompanied by the upregulation of growth promoting genes such as GAP-43 (Bareyre et al., 2002), GAP-43 was not upregulated in our cultures (not shown).

2.4. Nogo-A does not self-interact, but binds to Nogo-B.

We have suggested that Nogo-A self-interacts at the neuronal surface and IN-1 binding may facilitate this interaction. To test whether this interaction is possible, we cotransfected COS-cells (which do not express Nogo-A) with two Nogo-A constructions carrying different tags: haemaglutinin (HA) and the red fluorescent protein (RFP). Although both constructions were visualized by Western blot as two single bands (RFP molecular weight is about 27 kDa), when immunoprecipitation was performed using anti-HA antibody, the HA tagged Nogo-A was recovered, but not the heavier Nogo-A-RFP (Fig 2.4B). This indicates that Nogo-A does not form homophilic complexes, at least in the ER, as it is hardly detectable at the cell surface in COS cells (GrandPre et al., 2000; personal observations) and in the absence of its putative ligand (that is not necessarily expressed in COS cells). However, it does bind to Nogo-B (Fig 2.4C; also reported by Dodd et al., 2005). This interaction, which probably takes place in the ER (owing to the patterns of both proteins when transfected in COS cells), prompted us to further study the possible Nogo-A intracellular roles by analysing its intracellular partners. To this end, we contacted John R. Bethea (from the Miami Project to Cure Paralysis), who had recently cloned a Nogo-A interacting protein following a yeast two-hybrid assay (Hu et al., 2002). This protein, called NIMP (from Nogo-Interacting Mitochondrial Protein), is a novel mitochondrial protein with unknown function (Hu et al., 2002). After performing immunohistochemistry with anti-NIMP antibody in several tissues (covering the complete mouse CNS development, as well as perforant pathway lesions and fimbria-fornix axotomy) and obtaining a complex pattern, which suggested that more than one protein were recognized by the antibody, we tried to reproduce the experiments described in Hu et al. to confirm NIMP interaction with Nogo-A and the specificity of the anti-NIMP antibody (Fig. 2.4D-F). Although NIMP antibody recognized a band of the predicted NIMP-RFP molecular weight in lysates from transfected cells, it failed to detect this band following Nogo-A immunoprecipitation (Fig 2.4D). Moreover, the distribution of Nogo-A (by ICC) and NIMP (by RFP fluorescence) in cotransfected COS cells is not overlapped (they are expressed in distinct subcellular compartments), which renders the putative interaction between both proteins, if any, punctual. Unfortunately, NIMP antibody labelling of transfected COS-cells differed greatly from recombinant NIMP localization, as assessed by the fluorescent tag, and not only labelled most of the cell (which may be attributed to the high expression of endogenous NIMP in kidney; Hu et al., 2002), but also failed to detect NIMP-RFP enriched mitochondria, thus rendering the immunohistochemical analysis of NIMP pattern in brain tissue arguable. Therefore, although Nogo-A may behave as a cell adhesion molecule by interacting with proteins from this family when exposed at the plasma membrane, it may also associate with proteins in the ER and be involved in ER functioning (e.g. vesicle formation).
Fig 2.4. Nogo-A intracellular interactions. A-B) Nogo-A does not interact with itself in the endoplasmic reticulum (ER). Two tagged Nogo-A constructions (with HA epitope and RFP) were transfected in COS cells (A). B) Immunoprecipitation against HA epitope pulls down Nogo-A-HA but fails to coprecipitate Nogo-A-RFP. C) Nogo-A immunoprecipitates with Nogo-B when cotransfected in COS-cells. Again, HA epitope was used to immunoprecipitate Nogo-A. N-18 antibody recognizes both Nogo-A and –B. D-F) Nogo-A does not interact with NIMP in COS cells. Antibody against NIMP was tested in Western blotting and efficiently detected a 70kDa band corresponding with transfected NIMP-RFP. However, immunoprecipitation with HA antibody failed to detect NIMP (compared with cells not transfected with NIMP). E) Nogo-A distribution in the ER contrasts with NIMP mitochondrial pattern, as observed by NIMP-fluorescent tag. F) NIMP antibody failed to specifically detect NIMP in transfected cells (as assessed by RFP pattern).
Results and Discussion III

Myelin Contribution to Axonal Regeneration Failure

3.1 Myelin and Evolution.

One interesting analysis that may shed light on the contribution of MAI to axonal regeneration failure is the extent to which the appearance of MAI during evolution correlates with the loss of axonal regeneration capability. Figure 3.1 illustrates the appearance of the main guidance cue families (blue), and the myelin-associated inhibitors and other myelin proteins (red), during evolution.

Axonal regeneration occurs spontaneously in amphibians and fish but not in mammals. The loss of regenerative capacity during the transition from fish to land vertebrates may be due to the presence of inhibitory molecules in mammalian CNS that are absent in lower vertebrates. However, only Nogo-A specific sequences appear in the evolution approximately when regenerative capacity decreases (Diekmann et al., 2005). CSPGs, tenascins and the rest of MAI, in contrast, are already present in fish myelin (Battisti et al., 1995; Becker et al., 1995; Vourch and Andres, 2004; Diekmann et al., 2005). Goldfish oligodendrocytes express rtn4 (including the loop-66) and fish express the corresponding NgR (Klinger et al., 2004b), and so both proteins probably interact. As fish neurons can grow over purified fish oligodendrocytes and regenerate after lesion in vivo (Stuermer et al., 1992), this interaction does not lead to axonal inhibition as it does in mammals (probably because fish neurons follow different signalling pathways). Another possibility is that the regulation of the protein after lesion, but not the presence of the protein itself, determines the final capability to regenerate. Evidence points to the latter hypothesis. Oligodendrocytes in the salamander, for instance, downregulate tenascin-R and MAG following optic nerve crush in the same time window as retinal ganglion regeneration (Becker et al., 1999). Moreover, in the peripheral nervous system of lower
vertebrates, inhibitory molecules are rapidly removed after injury. Particularly, myelin debris are rapidly removed from the optic nerve in amphibians (Wilson et al., 1992; Sivron and Schwartz, 1995), while in mammals, inhibitors remain for weeks or are even upregulated. Thus, the complex reaction following lesion in mammals, but not the presence of inhibitory molecules, may determine the failure of axonal regeneration.

3.2 Why inhibition?

One of the first things that one wonders when learning about axonal regeneration is “why”. Why does axonal regeneration not occur spontaneously? And what are the advantages of this? Most probably, spontaneous axonal regeneration was lost during evolution because natural selection favoured it. More complex nervous systems are less capable of regenerating. This occurred during evolution (especially in mammals), but also during normal development and in the same animal when comparing central and PNS. Therefore, CNS complexity may be somehow compromised with regenerative plasticity.

Reactive astrocytes and mature oligodendrocytes probably became the two strongest inhibitors of the CNS by different pathways. We have already mentioned that while the presence of inhibitory molecules in myelin may represent constitutive inhibition of neuronal plasticity, the lesion-induced glial reactivity involving glial scar development and proteoglycan overexpression may be linked to the prevention of secondary cellular degeneration (Faulkner et
Therefore, the primary function of MAI would be to stabilize axonal connections and restrict plasticity in normal CNS (McGee et al., 2005), while reactive astrocytes may prevent cavitation after lesion. However, despite these key roles, both oligodendrocytes and astrocytes may restrict axonal regeneration, as a secondary, undesired effect. In fact, this hypothesis may explain why evolution favoured the presence of these restrictive mechanisms in the adult mammal CNS. Mutations associated with the inhibition of neurons by myelin may have led to synaptic disorganization and functional defects that were negatively selected during evolution. Analogously, mutations in astroglial reactivity after lesion may compromise animal survival, as they protect CNS from cavitation, and thus are also negatively selected.

Then, why is MAI expression not downregulated after lesion and restored once axons have regenerated? Releasing axons from constitutive inhibition following lesion may permit axonal regeneration. Actually, this is what we observe when interfering with MAI, their receptors and the intracellular pathways they activate after lesion in diverse models. In this regard, MAG expression is not only maintained after lesion but also strongly increased. This indicates that at least for MAG, there must be a role other than constitutive inhibition that explains this regulation. One possibility is that MAG overexpression, and more generally the reactivity of mature oligodendrocytes after PP axotomy, contributes to maintaining neuronal survival. In this regard, it is known that in many other axotomy models, such as corticospinal tract transection, over 90% of oligodendrocytes die during the first week after lesion (although the population can be recovered following progenitor differentiation in subsequent weeks), which is accompanied by extensive neuronal death (Abe et al., 1999). In contrast, the loss of mature oligodendrocytes is not significant in the entorhinal cortex after PP lesion, and we characterized the reactive response of these cells to axotomy. Entorhinal neurons are particularly resistant to axotomy, as only 20% die during the first weeks after lesion (Peterson et al., 1996). Oligodendrocyte survival, or the overexpression of certain proteins, may be related to the increased survival of entorhinal neurons. In fact, animals deficient in MAG show axonal atrophy in the adulthood, suggesting that MAG has trophic effects on axons/neurons (Windebank et al., 1985; Sanchez et al., 1996).
Results and Discussion IV

Many inhibitors but one single way to inhibit neurons: intracellular convergence

As the molecular mechanisms that regulate axon growth and axon inhibition began to be revealed, a signalling convergence emerged. The cytoskeleton is the ultimate target of every extracellular molecule regulating axon dynamics. Rho GTPases have a central role in regulating cytoskeletal dynamics, and so their regulation is necessary to modulate it. Thus, the modulation of Rho GTPases activity has been found in the signalling cascades initiated by the majority of guidance cues and inhibitors. This regulation may occur at the beginning of the signalling pathway, as described for Plexins or p75, which bind directly to Rho GTPases, or more downstream, as described for Eph and Robo receptors (Yamashita and Tohyama, 2002; Dickson et al., 2002). Key regulatory steps that control Rho GTPases activity are limited (mainly calcium levels, cyclic nucleotide levels and PKC activation), as are the possible ways in which any extracellular molecule modulates axon dynamics. Thus, the finding that inhibitors seem to activate the same pathways is not so surprising; they are inhibitors because they do so.

Interestingly, at least two pathways, regulated by distinct key molecules, seem to ultimately converge in the cytoskeleton and regulate growth cone attraction, repulsion and axonal inhibition (Song and Poo, 1999). MAI, as well as BDNF and Netrin, increase calcium intracellular levels and are sensitive to cAMP manipulation (Song et al., 1998). On the other hand, Sema 3A and NT3 do not modify calcium levels and are sensitive to cGMP manipulation (Song et al., 1998). These manipulations allow us to regulate both growth cone behaviour (e.g. orientation of turning or collapse) and axonal inhibition after lesion in the adult, which indicates not only that the signalling pathways regulating cytoskeleton dynamics converge, but also that these pathways are the same for these developmentally separated processes. The results obtained with ChABC and NEP1-40 combined treatment point to the relevance of this convergence. The targeting of two or more inhibitors is probably be less effective than targeting one inhibitor and provide a permissive substrate (as one of many possible combinations), as many inhibitors may be considered the same thing with a different face.
Results and Discussion IV

Development and Regeneration: protein recycling

Although MAI consist of only three proteins, many other inhibitory molecules are present in myelin in a constitutive manner. These include members of the CSPG family, like versican and brevican, and guidance cues like Sema 4D/CD100, Netrin 1 and Ephrin B3. Some of them, like Ephrin B3, are exclusively expressed by mature oligodendrocytes, and others, like Netrin 1, are also expressed by neurons. The presence of these guidance cues in the adult is thought to restrict axonal sprouting, leading to inhibition after lesion, probably through the same mechanisms that control axonal chemorepulsion during development. It is quite attractive to propose that the contrary is also true, and molecules restricting plasticity in the mature CNS are probably involved in the control of attraction, repulsion and branching, if expressed during development.

Our results suggest that neuronal Nogo-A is involved in cell adhesion during development. We find an interesting parallelism between Nogo-A and TAG-1, a cell adhesion molecule that is transiently expressed by neurons and regulates fasciculation (and interneuron migration; Denaxa et al., 2001; Kyriakopoulou et al., 2002; Rougon and Hobert, 2003; McManus et al., 2004) during development. As myelination begins, Nogo-A is expressed by myelinating oligodendrocytes and restricted to paranodes, where it regulates neuronal potassium channel sorting (Nie et al., 2003). TAG-1, when expressed by myelinating oligodendrocytes, regulates potassium channel sorting in the juxtaparanodes, interacting with a homolog of the binding partner of Nogo-A at the paranodes (Nie et al., 2003; Traka et al., 2003). Although TAG-1 and Nogo-A have been discovered in different contexts, these proteins seem to share a degree of functional parallelism during development. If this parallelism holds true, and since TAG-1 has been recently found to be expressed in the adult (Wolfer et al., 1998; Soares et al., 2005), TAG-1 and Nogo-A may also have homologous functions in the mature CNS, either in myelin or when expressed by neurons. Analogously, it is only a matter of time before many proteins that have been discovered in the context of axon guidance or adhesion are found to limit axonal regeneration in the mature CNS.
Conclusions
1. Nogo-A is expressed by hippocampal neurons during development and by myelinating oligodendrocytes, principal neurons and interneurons in the mature CNS. NgR expression is exclusively neuronal and starts during postnatal stages. In vitro, Nogo-A induces fasciculation of embryonic explants, but does not inhibit axon outgrowth. This suggests alternative functions for Nogo-A during development.

2. MAG expression by mature oligodendrocytes follows the onset of myelination and parallels the developmental loss of regeneration capability of the perforant pathway.

3. Neuronal expression of both Nogo-A and NgR is regulated by neuronal activity. Kainic acid, glutamate (for Nogo-A) and BDNF administration, downregulate Nogo-A and NgR expression. NgR is specifically downregulated by neurons when synaptic remodelling occurs. This indicates that both Nogo-A and NgR may regulate synaptic stabilization and plasticity.

4. Nogo-A and MAG are transiently overexpressed by reactive astrocytes and mature oligodendrocytes respectively in response to perforant pathway axotomy. NgR expression undergoes biphasic regulation after lesion in the deafferented granule neurons, and its subsequent downregulation coincides with the reactive sprouting of granule neurons. This agrees with a possible role of MAG, Nogo-A and NgR in preventing axonal regeneration.

5. The blockade of MAG and both Nogo-A inhibitory domains successfully promotes axonal regeneration of the perforant pathway in vitro.

6. Chondrotin sulphate proteoglycans are overexpressed after axotomy of the perforant pathway and their blockade permits axonal regeneration of the transected connection.

7. The combined blockade of Nogo-A binding to NgR and chondrotin sulphate proteoglycans activity does not have synergistic effects on perforant pathway regeneration.


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