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ANATOMICAL ORGANISATION AND FUNCTIONAL RECOVERY IN SPINAL INJURED NON-HUMAN PRIMATES TREATED WITH EITHER AN ANTIBODY AGAINST NOGO-A OR A COMBINATION OF THIS ANTIBODY WITH BRAIN-DERIVED NEUROTROPHIC FACTOR (BDNF)

THESIS

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Fribourg, le ……

Thesis supervisor: Dean:

…… ……

« La science restera toujours la satisfaction du plus haut désir de notre nature, la curiosité. »

Ernest Renan

« La réussite appartient à tout le monde mais c'est au travail d'équipe que revient le mérite. »

Franck Piccard

A mes 3 Hommes

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General introduction

The present work aimed at addressing several issues that were focused as much on neuroanatomy as on behaviour. All the investigations presented here concern nonhuman primates subjected to an incomplete cervical cord lesion located at cervical level C7/C8 and treated with one of three possible treatments: i) a control antibody, ii) an antibody neutralizing the growth-inhibitor protein Nogo-A and iii) a combination of the antibody directed against Nogo-A and the brain-derived neurotrophic factor (BDNF). In addition to these injured monkeys, corresponding anatomical data were also collected from intact animals. Looking at the anatomical changes related to the lesion and/or the treatment, at first purpose of the present work was to examine the fate of the positive SMI-32 corticospinal (CS) neurons in the layer V of the primary motor cortex (M1) in two groups of monkeys: intact and injured/treated animals. Using the same staining, a second investigation evaluated the presence of SMI-32 positive fibers into the scar tissue in the three groups of treated animals. Finally, the last investigation concerned both behavioural and anatomical assessments of injured monkeys related to the interactions between the three different types of treatments. In the latter study, the manual dexterity was evaluated using motor tasks before and after the cervical cord lesion and was correlated to the three types of injured/treated monkeys. Then, in order to associate the behavioural observations to anatomical data, the injured CS axons of these animals were counted rostrally to the lesion at cervical level C3 and their soma evaluated as in the first study.

However, before describing in more detail these three investigations, it is important to provide the background information required to understand the various research projects. Therefore, the first part of the introduction will present various basic physiological concepts about the motor system. It will begin at the brain level and follow down towards the spinal cord. Then, the structure of the spinal cord will be described as well as how the spinal cord is affected by a lesion. After that, the potential regenerating strategies employed to thwart the effects of a spinal cord injury (SCI) will be examined, with particular attention on the two molecules used as treatment in this work: an antibody directed against the neurite outgrowth inhibition Nogo-A and the neurotrophic factor BDNF. Finally, this general introduction will end with a small subsection focusing on the interest of using nonhuman primates in fundamental biomedical research.

The motor system organisation

Motor areas of the cerebral cortex in primates

Several regions of the cerebral cortex contribute to the control of voluntary movements[1]: a primary motor area called M1 but also referred to as F1 and three secondary motor areas or premotor areas subdivided on the basis of anatomical and functional criteria, the premotor cortex (PM), the supplementary motor area (SMA) and the cingulate motor area (CMA) (Fig. 1A). M1 and the premotor areas correspond respectively to the Brodmann's area 4 and 6 of the frontal lobe. At the time of the movement execution, information of the premotor areas, in addition to information from parietal cortex, converges to M1 (Fig. 1A right panel). In M1, neurons generate impulses that will account for the execution of the movement for instance via the activation corticospinal neurons. Most of the corticospinal (CS) fibers cross the midline of the body, then travel along the spinal cord, and make connections with neurons located in the spinal cord and associated with skeletal muscles (on the opposite site of the body). Thus, the right hemisphere of the brain mainly controls the left parts of the body and vice-versa. Several studies have demonstrated that electrical stimulation of a precise part of the motor cortex corresponds to a specific contraction of muscles located in a restricted part of the body (Fritsch and Hitzig 70;Penfield and Ramussen 52;Penfield and Welch 51). In other words, every part of the body is under control of a particular region of the motor cortex from which movements can be elicited. Stimulating adjacent regions in M1 elicited movements in adjacent body regions. There is thus a kind of body representation in M1 and this notion is referred to as a somatotopic arrangement (Fig. 1B). Furthermore, according to the species, the areas assigned to various body parts on the motor cortex are proportional not to their size, but rather to the complexity of the movements that they can perform.

[1] http://thebrain.mcgill.ca/flash/i/i_06/i_06_cr/i_06_cr_mou/i_06_cr_mou.html

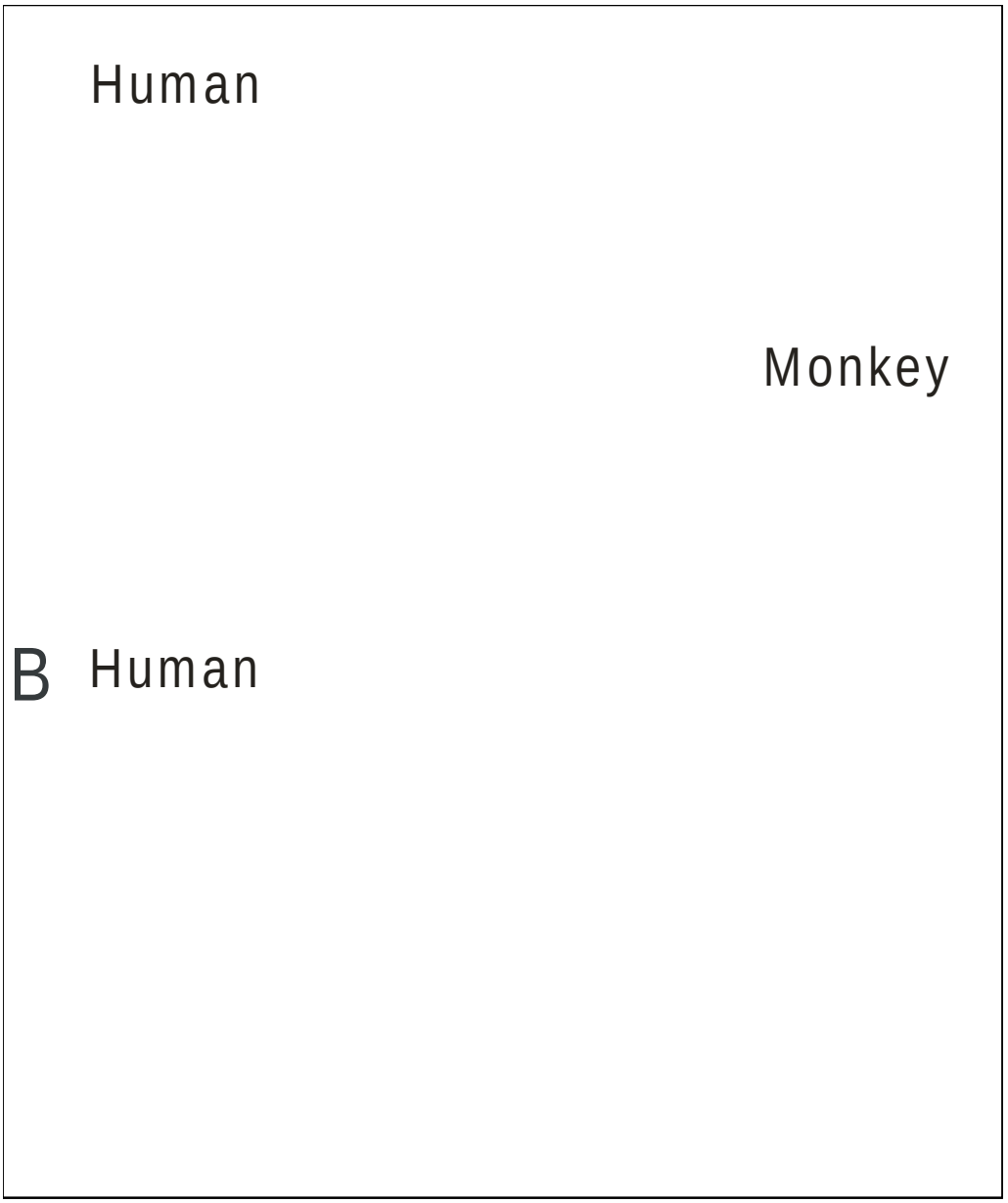


Figure 1: A) Main cortical domains of the motor system. The primary motor cortex (M1) is localized along the precentral gyrus and corresponds to the blue area. The secondary motor areas are localized in front of the central gyrus and behind M1 in frontal and parietal lobes and from which information converges towards M1 (right panel). Another part of the information originates from the secondary motor areas project towards the trunk and the spinal cord representing the parallel organisation of the motor system. (PMd=dorsal premotor area; PMv=ventral premotor area; S1=primary somatosensory cortex; SMA=supplementary motor area; CMA=cingulate motor area.). B) The motor homunculus in M1. (Right panel taken from Eric R. Kandel, James H. Schwartz, Thomas M. Jessell; Principles of neural science fourth edition; McGraw-Hill Health professions division New-York; page 760) (Left panels taken from http://brainconnection.positscience.com/topics/?main=anat/motor-anat).

The three premotor areas can be described as follows:

1. The premotor area (PM) also called post-arcuate area is involved in the control of movements in response to sensory stimuli as well as motor preparation (e.g. Godschalk et al 85;Weinrich and Wise 82;Wise and Mauritz 85). In macaque monkeys, PM corresponds to the lateral area 6 (Godschalk et al 84;Rizzolatti et al 83;Schell and Strick 84). Globally, PM is responsible to direct body movements by integrating sensory information and controls proximal muscles that are related to the body's main axis. PM neurons are often active until the movement is performed, corresponding to programming and preparation of the voluntary movements. PM is often subdivided into two subareas, the dorsal premotor area (PMd) and the ventral premotor area (PMv) because they play different roles (Fig. 1A right panel) (Kurata 91). PMd is in particular involved in coordinating hand and eye movements by computing their relative position (Pesaran et al 06), whereas PMv is involved in the transformation of the visual information about the position of an object into a motor reference frame for movement coordination. In other words, PMv is a part of the cortical circuit controlling visuomotor grasping with the hand (e.g. Shimazu et al 04). In addition, PMv, like other premotor areas, is characterized both by having specific corticospinal projections to the spinal cord and by reciprocal cortical connections with M1 (Dum and Strick 91;Dum and Strick 02) .
2. The supplementary motor area (SMA) performs somewhat comparable functions as PM. However, while PM is connected primarily with reticulospinal neurons, SMA sends axons directly to distal motor units. SMA is involved in behavioural planning and execution as well as in speech production (e.g. Alario et al 06). SMA is an important component in motor tasks demanding retrieval of motor memory. It appears also to be crucial in temporal organization of movements, particularly in sequential performance of multiple movements (Tanji 94). As PM, SMA is also subdivided into two subareas: a rostral zone (pre-SMA or area F6) and caudal zone (SMA-proper or F3). Pre-SMA is involved in higher level processes while SMA-proper seems to be more closely related to motor output. In summary, SMA neurons which are also often active few seconds before executing the movement are engaged in central organization of the voluntary movements.

3. The cingulate motor area (CMA) is the third premotor area contributing to voluntary movements (e.g. Kermadi et al 00). CMA participates to the generation and the control of voluntary movement either through M1 (Fig. 1A right panel) or through direct projections to the spinal cord (Darian-Smith and Darian-Smith 93;Darian-Smith et al 93;Dum and Strick 91;Dum and Strick 96;Dum and Strick 02;Galea and Darian-Smith 94;Rouiller et al 96). This premotor area can be subdivided on the basis of corticospinal projections into three subareas (Dum and Strick 91) : the caudal cingulate motor area on the dorsal bank (CMAd), the caudal area on the ventral bank (CMAv) and finally the rostral cingulate motor area (CMAr).

The cortical layers

The cerebral cortex of all vertebrates shares numerous common features. For example, the cell bodies of the cortical neurons of all vertebrates are always organized in a laminar construction arranged in a parallel direction to the surface of the brain (Fig. 2). In the nineteenth century, the neuroanatomists have only described this laminar organization of the cerebral cortex without having correlated it to physiological properties like it was the case later for the striate cortex of the macaque monkey for instance (Lund 87;Lund et al 88;Lund and Wu 97;Lund and Yoshioka 91). At that time, the precise number of cortical layers was subjected to debate. In his book "The Brain of Mammals" published in 1873, Theodore Meynert was the first scientist to consider the arrangement in different cortical layers (Meynert 73). This neuroanatomist claimed that the brain is composed of five separate layers and this view has been supported by other scientists (Betz 74;Mierzejewski 75). However, others have adopted a different approach of studying the cerebral cortex and have asserted that the brain is composed of six layers (Baillarger 40;Major 76). Nowadays, the neuroanatomists distinguish traditionally six cortical layers; where the *layer I* is the most superficial cell layer situated just under the pia matter and corresponds to a zone devoid of neurons. Then, descending form the pia matter towards the white matter the other layers follow each other: *layer II*, *layer III*, *layer IV*…etc. Thus, the cerebral cortex is built as a pastry with different horizontal layers. Nevertheless, nowadays the number of cortical layers appears still variable and can be subjected to some lumping or splitting according to the type of the cortical areas. For example, in the visual cortex, the *layer IV* can be subdivided into four sub-layers: the *layer IVa, IVb, IVcα* and *IVcβ* (Fig.2)

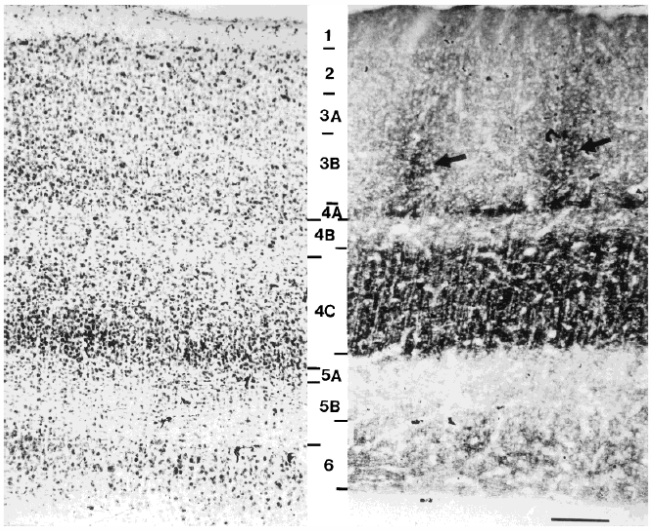


Figure 2: Laminar organization of the cortex (here macaque monkey) stained with cresyl violet (left) and cytochrome oxidase (CO) (right) showing the different types of layers and sub-layers. The sections are coming from the parafoveal region of V1 cortex. In the section stained with CO, the arrows represent the positions of two CO-rich blob regions. Scale bar = 200 µm and thickness of the section = 40 µm (Lund and Wu 97).

The neuroanatomists proposed a detailed description of the laminar structure of the cortex using different histological techniques. First, the cerebral tissue was fixed, using for instance formaldehyde, in order to cut thin slices. Then, the cortical sections were stained using dyes that color selectively different structure components of the cortex. In this way, the stainings allow the visualization of specific cortical structures like neuronal cell bodies and/or their associated intracortical axons. Finally, the stained sections were placed on a support (slides) allowing their visualization under the microscope. One of the oldest histological staining method which is still used in microscopy today is the Nissl staining. The Nissl staining is a nonspecific staining. This property is very useful in anatomical analyses because it allows, for example, the differentiation of the different types of neurons from the glia or the different cortical layers. On the other hand, there are other stainings that are more specific than the Nissl staining, like the Sternberger monoclonal incorporated antibody 32 (SMI-32) which allows the visualization of some specific anatomical features. In fact, the SMI-32 staining reacts with the epitope of nonphosphorylated neurofilaments M (150kDA) and H (200kDa) present only in a subpopulation of cortical neurons in the *layers III* and *V* (Fig. 3) (Lee et al 88;Sternberger and Sternberger 83).

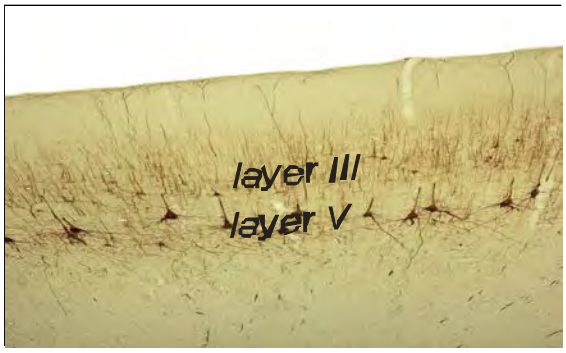


Figure 3: 50 µm thick section from macaque monkey M1 cortex to illustrate layers III and V neurons stained with SMI-32. In the layer V, the giant pyramidal cells of Betz are easily identifiable.

Neurons of M1: the pyramidal neurons

The cerebral cortex contains a great diversity of pyramidal and nonpyramidal neuronal types. The pyramidal neurons represent a specific type of neurons which can be stained for instance in the *layers III* and *V* using SMI-32 antibody (see above paragraph). Cortical projection neurons exhibit diverse morphological, physiological and molecular phenotypes. In addition, neurons projecting to specific regions are found in a specific laminar localization. For instance, the pyramidal neurons of the *layer III* make cortico-cortical projections, whereas the neurons located in the *layer V* send their axons directly to the brainstem and the spinal cord (Clasca et al 95). Thus, because the pyramidal neurons extend their axons into the different parts of the CNS they are consequently Golgi type I neurons (in contrast to Golgi type II neurons which have only short projections). Physiologically, some of the pyramidal neurons of the *layer V* in M1 have a direct access to the motoneurons, as they project directly towards the spinal cord. Thus, the stimulation of the pyramidal cells located in the layer V of M1 easily generate muscle contractions. Histologically, as indicated by their name, the pyramidal neurons are characterized by a triangular shaped cell body. In addition, they have a single axon as well as a thick large apical dendrite and multiple basal dendrites. In humans, the soma diameter of a pyramidal cell averages from 10 to 50 micrometers but some giant cells can have a soma diameter of up to 100 micrometers[2]. These giant cells are also called Betz cells in memory of Vladimir Alekseyvich Betz who discovered and described them for the first time (Betz 74). The Betz cells represent 10% of the total pyramidal neurons' population and these neurons appear to be especially vulnerable to the process of aging and senescence (Rivara et al 03;Scheibel et al 77). Historically, the physiological correlation of the Betz cells to a motor activity was first made by Lewis in 1878 (Lewis 78).

[2] http://en.wikipedia.org/wiki/Pyramidal_cell

Motor descending pathways

Take hold of a cup of coffee or tee is a gesture that almost everyone is doing every morning. However, this voluntary movement that appears simple at the first sight involves a lot of pathways including the motor descending pathways (Lemon 08). In the primate, the main motor descending pathway is the corticospinal tract (CST) also named the pyramidal tract because this tract travels along the pyramids in the brain. The capacity to perform a precise opposition of our thumb and forefingers as well as independent movements of our fingers is under the control of the corticospinal (CS) system. The other descending tracts that participate to the motor control or the posture of the body are the rubrospinal tract, the reticulospinal tact, the vestibulospinal tract and the tectospinal tract. However, this work will focus particularly on the CST which is a member of the lateral descending pathways.

The descending lateral corticospinal tract

The CST is responsible for the voluntary movements involving mainly the distal musculature of the forelimbs. The CS projections arise from spatially distinct and somatopically organized cortical regions. For example, the macaque monkey has at least six CS neural subpopulations located within the frontal, cingulate, parietal and insular cortices (Galea and Darian-Smith 94;Galea and Darian-Smith 95;Kuypers 81a). Inside these cortical motor areas, the CST originates from the *layer V* (Asanuma 81;Humphrey and Corrie 78;Porter 81). While the majority of CS axons (about 95%) decussates at the pyramidal level (medulla-spinal cord juncture) and travels laterally in the spinal cord along the dorsolateral funiculus, a minority of CS axons (about 5%) does not cross the midline and continues its course ispilaterally either along the dorsolateral funiculus or along the ventromedial funiculus (Fig.4) (Galea and Darian-Smith 97a;Kuypers 81a;Lacroix et al 04;Phillips 79;Rouiller et al 96).

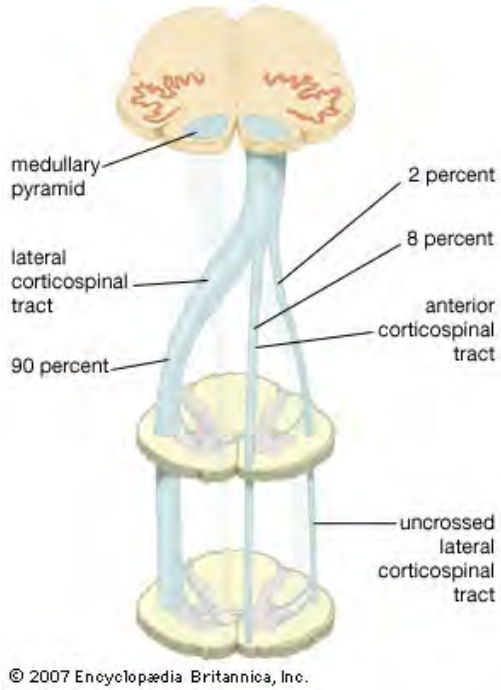


Figure 4: Drawing showing the trajectory of each fibers percentage of the CST along the rostro-caudal axis. About 90 % of the CS fibers decussate and continue their trajectory in the dorsolateral funiculus of the spinal cord whereas the rest of the fibers travel ipsilaterally in the dorsolateral funiculus (2%) or the ventromedial funiculus (8%) (Taken from: http://www.britannica.com).

In 2004, Lacroix and collaborators have shown this distribution by injecting an anterograde tracer named biotinylated dextran amine (BDA) in M1 in uninjured monkeys and have then observed on transverse section of the lumbar cord of these animals the distribution of BDA-labeled CS axons (Lacroix et al 04). In other words, they showed that the great majority of the CS fibers are located in the dorsolateral funiculus contralaterally to the BDA injections whereas a minority is situated on the ipsilateral side to the injections. Using similar methods and the same animal's model, Wannier and co-workers have found a largely comparable distribution of the CS projections at the cervical cord level (Fig. 5) (Wannier et al 05). These results were derived from a previous set of experiments conduct by Rouiller et al. (Rouiller et al 96). At their level of termination at all spinal segments, the axons of the CST leave the white matter and contact spinal neurons located in the different lamina of the grey matter, such as for instance in the Rexed lamina IX (ventral horn) which contain the α motoneurons (see Rexed lamina below). In macaque monkeys, the CST terminates most heavily in the medial part of the Rexed lamina VII and IX of the spinal grey matter where interneurons of various types are located (Fig. 6) (Armand et al 97;Dum and Strick 96;Kuypers 81b;Peschanski and Ralston 85).

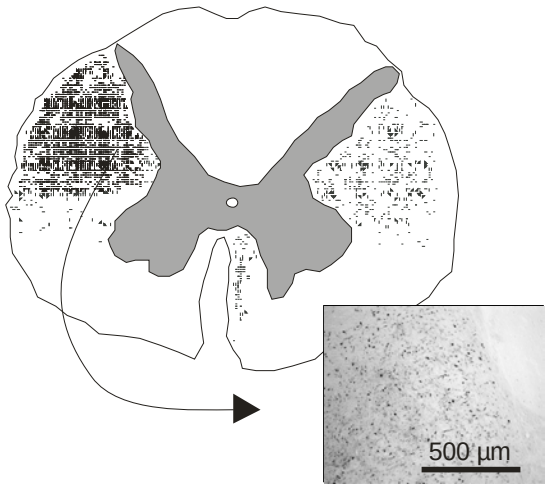


Figure 5: Transverse section of the cervical spinal cord showing the distribution of BDA-labeled CS axons as a result of BDA injection in the right M1 of an intact monkey. In the white matter, the position of each labeled CS axon is represented by a dot. The great majority of the CS fibers are visible in the dorsolateral funiculus located contralaterally to the injections. As indicated by the arrow, a small region of the section is illustrated by a photomicrograph where the cut BDA positive CS axons appear as black dots (Wannier et al 05).

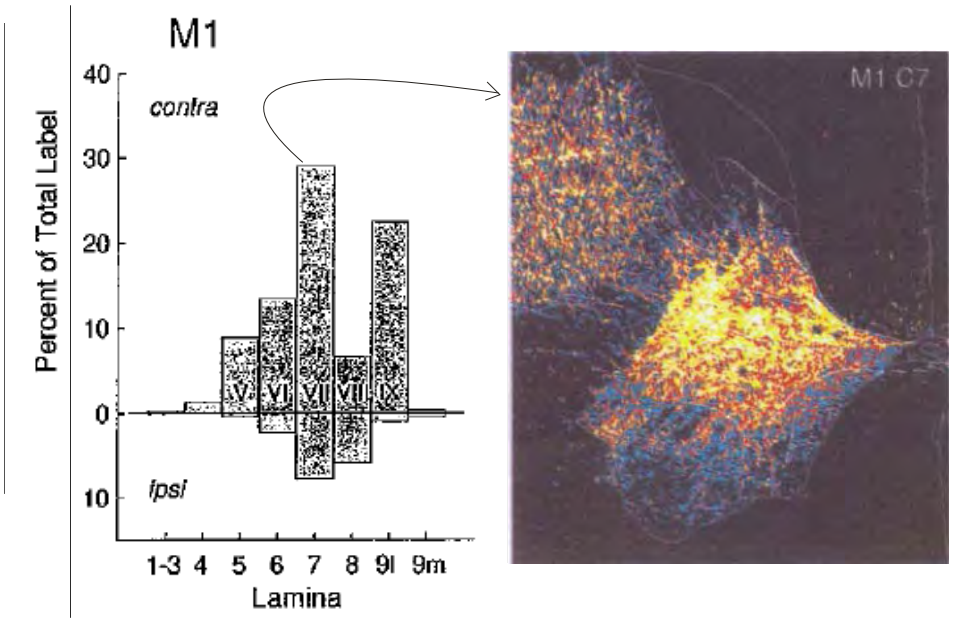


Figure 6: Left panel: histogram indicating the Quantitative analysis of CS terminations (percentage of total number of illuminated pixels that were found in each lamina). Most axons that originate from M1 terminate in the lamina VII and IX. Right panel: photomicrograph taken at cervical level C7 from macaque monkey illustrating the projections patterns of the CST from M1 after WGA-HRP injections. Most of the CS terminations contact the neurons located in the intermediate layers of the spinal grey matter. Compared to the premotor areas, the hand motor nuclei of M1 exhibit much more labelled axons (Dum and Strick 96).

Corticomotoneural (CM) system

The corticospinal tract provides the most direct pathway over which the cerebral cortex controls movement. In rodents and marsupials this influence is exerted largely upon interneurons in the dorsal horn of the spinal grey matter. However, ascending the phylogenetic scale through carnivores and primates, the number of corticospinal axons grows and corticospinal terminations shift progressively toward the interneurons of the intermediate zone and ventral horn, ultimately forming increasing numbers of synaptic terminations directly on the motoneurons themselves (Schieber 07). Thus, a unique feature of the primate motor system is the presence of the CM system with direct projections to motoneurons supplying hand and forearm muscles. However, it has been demonstrated that the dexterous finger movements in primate could also be realized via an indirect CM pathways (Sasaki et al 04). Nevertheless, abolition of CM influences causes a permanent deficit in the fractionation of use distal muscles and an inability to carry out independent movement of the fingers. Based on this phylogenetic trend, humans are believed to have more direct corticomotoneuronal synapses than any other species, consistent with observations that humans suffer more extensive loss of control of distal muscles from lesions of the corticospinal tract than do other mammals (Schieber 07). The CM fibers include large, rapidly conducting axons. They arise mostly from somatotopically arranged areas of precentral cortex and the largest concentration of CM cells is in the deep part of lamina V in area 4. The CM excitation has been demonstrated to be effective in natural functional states when the conscious animal is performing learned movement tasks (Porter 85). In monkey, the CM system contributes to the control of force in precision grip (Maier et al 93). In other words, it has been shown that CM neurons activity can covary with grip force.

The descending lateral rubrospinal tract

The rubrospinal bundle is another component of the lateral descending motor system. Both the CST and the rubrospinal tract belong to the dorsolateral system but compared to the CST, this pathway does not originate from the cortex but from the red nucleus in the rostral midbrain. The rubrospinal pathway is implicated in control of hand movements in parallel to the CST, in particular during coordinated whole limb movements. The axons coming from the red nucleus decussate at the pons level and join the CS fibres at the spinal cord level. Therefore, in the spinal cord, as the CST, the rubrospinal pathway sends direct crossed axons mainly within the dorsolateral funiculus. It is interesting to consider that during evolution in mammals, this

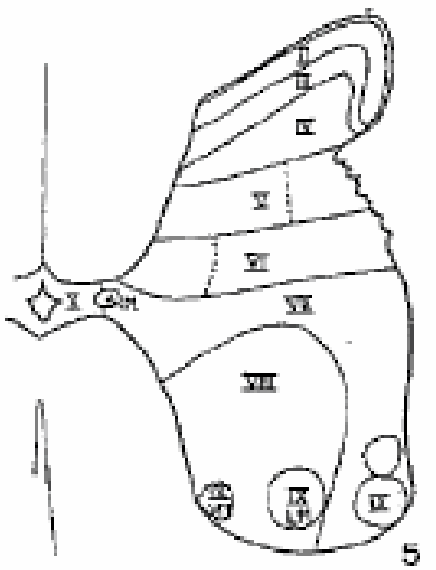
indirect pathway was largely replaced by the direct CS system in primates. In nonhuman primates, the rubrospinal projections are about 100 times less numerous than the CS projections (Holstege et al 88;Ralston et al 88). Thus, whereas the rubrospinal system contributes importantly to control the motor system of many species of vertebrates and some mammals, it appears to have a fairly minor function in primates and to have been largely supplanted by the corticospinal system.

Spinal cord organisation

The spinal cord and the brain are the two components of the central nervous system (CNS). The spinal cord is the hyphen between the brain and the body which transmits both the motor and the sensory information. To understand what happens after a spinal cord injury, it is first imperative to understand the basic anatomical structures of this part of the CNS. First, the spinal cord is composed of in its centre the grey matter forming a kind of butterfly surrounded by the white matter (Fig. 5). The grey matter is composed of neuronal cell bodies, dendrites and axons whereas the white matter is only composed of axons. In the white matter, the axons are grouped in different bundles and one distinguishes the descending tracts which carry the motor information from the ascending tracts which carry the sensory information.

As Brodmann described the structural organization of the brain, Rexed made the same for the spinal cord (Fig. 7) (Rexed 54). In fact, in 1954, Rexed has constructed an atlas of the cat's spinal cord in order the help the physiologist who wants localize their stimulating or recording electrodes. Using 100 μm thick sections stained with toluidine blue, Rexed made a cytoarchitetonic and topographic description of the spinal grey matter and subdivided it into ten different lamina numbered from dorsal to ventral.

Briefly, the lamina I is a thin layer covering the top of the dorsal column and bending the apex down on the lateral side. The lamina II forms a well-defined band across the dorsal cell column and around the apex. The lamina III and IV take up the rest of the head of the dorsal horn. The lamina V goes straight across the dorsal cell column with a medial and a lateral zone. The lamina VI occupies the base of the dorsal cell column. The lamina VII contains the cell of the zone intermedia but in the intumescences it dips far down into the ventral cell column. The lamina IX is built up by the medial and lateral nuclei of large, heavily-stained motoneurons. Finally the lamina X is the grey matter around the central canal.



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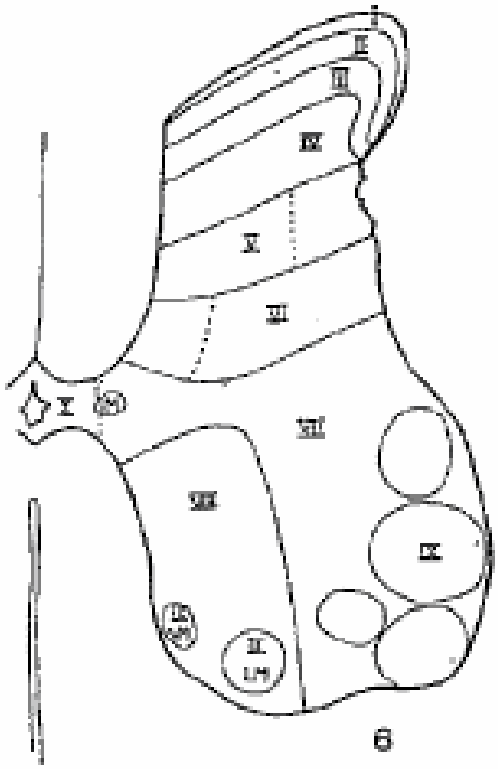

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Fig. 5 Schematic drawing of the 5th cervical segment. (For abbreviations see fig. 1.)
Fig. 6 Schematic drawing of the 6th cervical segment. (For abbreviations see fig. 1.)

Figure 7: Cytoarchitectural division of the cat's grey matter made by Rexed at cervical level 5 (Rexed 54).

Spinal cord injury (SCI)

SCI disrupts numerous pathways and leads to a partial or total loss of functions depending on the extent of the lesion. The cellular consequence of SCI can be a retraction of the proximal or cut end of the axon, neuronal atrophy or neuronal cell death of different types of cells including oligodendrocytes, astrocytes and precursor cells (Horky et al 06;Lieberman 71). Several aspects strongly influence the effects of SCI and its associated functional recovery: the type of the lesion (i.e. the mechanisms that have conduced to the injury), its location and its extent. SCI can occur for many causes including: trauma such as automobile crashes, falls, gunshots...etc, tumor, ischemia, developmental disorders, neurodegenerative disorders such as spina bifida, demyelinative diseases such as multiple sclerosis, transverse myelitis resulting from stroke, inflammation or other causes and vascular malformations[3].

SCI triggers a cascade of biological events that unfold within seconds and that proceed for months or even years. SCI affects three major bodily systems: the nervous system, the immune system and the vascular system. These systems respond directly and interact dynamically to the injury. SCI immediately affects or kills cells, but causes also delayed damage and death to cells that may survive the original trauma. The biological response to a SCI is triphasic. These phases follow a distinct but somewhat overlapping temporal sequence. It begins with an acute phase which occurs immediately after the injury, followed by a subacute phase corresponding to a local reaction of the injured cells minutes to weeks after the injury and finishes by a late-phase or chronic phase, lasting from months to years after the injury. The acute phase begins directly after the alteration of the spinal tissue and is marked by systemic and local events (Hulsebosch 02;Tator 98). In the present studies, the injury performed is a lateral transection of the spinal cord using a surgical blade. The direct trauma from injury causes cell death (necrosis) to the spinal neurons and the endothelial cells lining the blood vessels of the spinal cord. The principal systemic event of the acute phase, after a brief increase in blood pressure, is a prolonged decrease in blood pressure (hypotension) that sometimes coincides with a decrease in blood volume. This vascular perturbation results in ischemia that induces cells death. Perhaps because it is more vascularized, the spinal cord's grey matter that contains the neuronal cell bodies is far more necrotic after the injury than the white matter (which contains large tracts of myelinated nerve fibers) (WOLMAN 65). An additional cause of hypotension is the rapid bleeding into the normal fluid-filled spaces of the spinal cord, contributing also to the local oedema formation. The oedema reaches its

[3] http://en.wikipedia.org/wiki/Spinal_cord_injury

maximum the first days following the lesion and increases the local compression of the axons of the spinal cord. Moreover, in addition to these vascular perturbations, a significant local shift in ion levels in the neural environment, particularly of ion Ca^{2+}, occurs in the acute phase, modifying the excitability of the surviving cells and causing membrane' s rupture and release of the cellular content. Higher ion concentrations can also reach toxic levels sufficient to kill nearby neurons. The secondary phase of the injury sets in minutes after the lesion and lasts for weeks. During this phase, the area of the injury markedly expands. The secondary phase features a continuation of some events from the acute phase, i.e. electrolyte shifts, oedema and necrotic cell death, as well as novel perturbations including the formation of free radicals, delayed Ca^{2+} influx, immune system response (inflammation) and apoptotic cell death. Finally, some events occur month to years after the injury and belong therefore to the long-term phase. The long-term phase is accompanied with the loss of the myelin sheaths in the white matter that often terminates with the formation of a fluid-filled cyst. The major event that composes that late-phase is the scar formation that will separate the wound from the intact spinal cord tissue. In addition to the scar formation, the Wallerian degeneration takes place especially in axons with large diameter. This biological mechanism corresponds to a degeneration of the distal stump of the nerve fibers. This process occurs in both CNS and PNS (Schwab and Bartholdi 96). Two opposite forces which act simultaneously may account for the formation of retraction terminal bulbs: 1) the pressure of the axoplasmic flow towards the cut ends of the sectioned nerve fibers and 2) the interferences of structural changes within the axon and the myelin sheaths (Kao et al 77). Once a terminal retraction bulb formed, the axon becomes stabilized and stops to retract but fails to re-grow (Kalil and Schneider 75).

As already mentioned, the injured peripheral axons spontaneously recover whereas the injured axons of the CNS do not possess this natural ability. It has been suggested that the difference between these two systems consist in the capacity of "cleaning" the damages of the injury. In fact, in the CNS the axonal debris and the myelin persist for months in the areas of the injury due to the poor recruitment of the macrophages (Brown et al 91;Perry et al 93); whereas in the PNS the myelin is phagocytosed by the macrophage after 3-5 days (Bignami and Ralston, III 69;Cook and Wisniewski 73) . In addition, in PNS the properties of the Schwann cells also help to the regeneration. They also phagocyte the peripheral nerve debris but, in addition, they produce neurotrophic factors and secrete extracellular matrix molecules that support axonal regrowth. This explains why, for example, some studies have adopted the strategy to deliver Schwann cells in addition to neurotrophic factors into the sites of SCI for augmenting the regeneration (Jones et al 01).

Moreover, the effects of a spinal cord lesion may vary depending on the type, level, and severity of injury. SCIt can be classified into two general categories: the complete SCI and the incomplete SCI. In the complete injury, the "neurological" level is lost and no motor or sensory functions are available below the injured spinal level. On the other hand, in the incomplete injury, only a part of the "neurological" level is affected by the lesion explaining why some motor and/or sensory functions are preserved. In this case, the consequences of a SCI depend on its extent and its location along the spinal cord because these two factors will determine which motor and/or sensory functions will be affected by the injury. The location of the SCI is determined in the rostro-caudal axis (the segmental level) while the extent is looked at the medio-lateral axis establishing respectively which levels of the body parts could be affected by the lesion and which tracts and/or Rexed lamina are injured. Traditionally in the clinics, the SCI are classified according to these two different criteria and most injuries are located in both the cervical and the thoracolumbar regions (Ditunno, Jr. 92). Along the rostro-caudal axis, the principle is simple: the more the lesion is close from the head, more parts of the body will be affected (Fig.8). In adition to the paralysis and the loss of motor and/or sensory functions below the segental level of the SCI, some other complications may also derive from the injury such as: bowels, bladder and sexual disfunctions, neuropathic pain, muscle atrophy, spacitiy (increased reflexes and stiffness of the limbs), osteoporosis…etc.

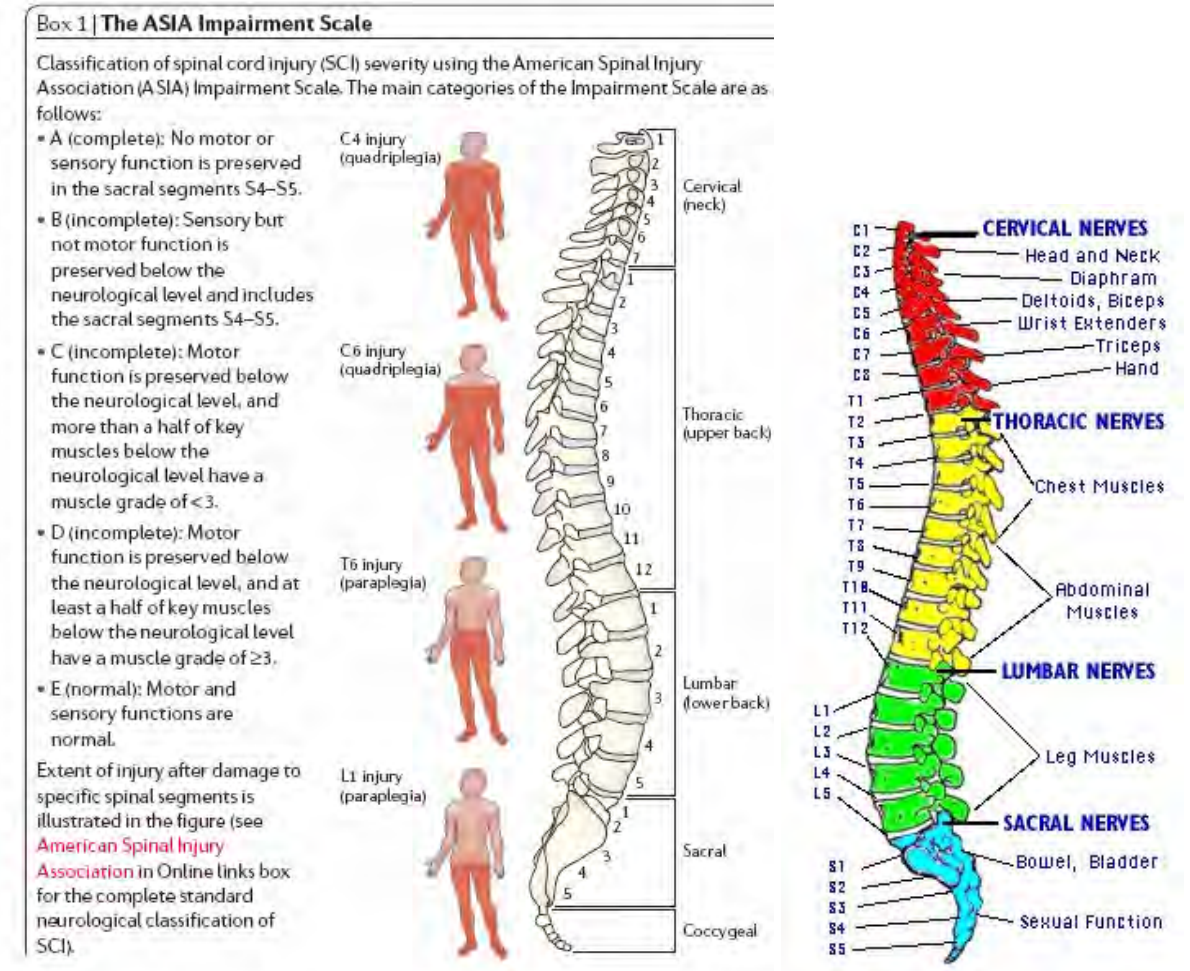


Figure 8: Left panel: classification of the "completeness" of the SCI in human according to the American Spinal Injury Association (ASIA) Impairment Scale (Thuret and Moon 06). Right panel: division of the spinal segments allowing to predict which parts of the body will be affected following a SCI (Taken from http://www.spinalinjury.net/html/_spinal_cord_101.html).

Because the distribution of the spinal motoneurons is also topographically organized it is possible to predict which parts of the body will be affected following SCI. In fact, in the grey matter of the spinal ventral horn, the motoneurons are spatially distributed scording to two anatomical rules: the proximal-distal rule and the flexor-extensor rule (Fig. 9). Regarding the proximo-distal principle, the motoneurons that are located most medially in the ventral horn innervate the axial muscles, whereas the distal muscles are innervated by the motoneurones that are found most laterally.

Thus, it appears that because these latter motoneurons are placed most closely to the lateral CST, they receive consequently the highest density of the projections of this tract (Holstege 91;Ralston and Ralston 85). Likewise, the motoneurons that are located most dorsally in the ventral horn innervate the flexor muscles, whereas the extensor muscles are innervated by the motoneurones that are found most ventrally.

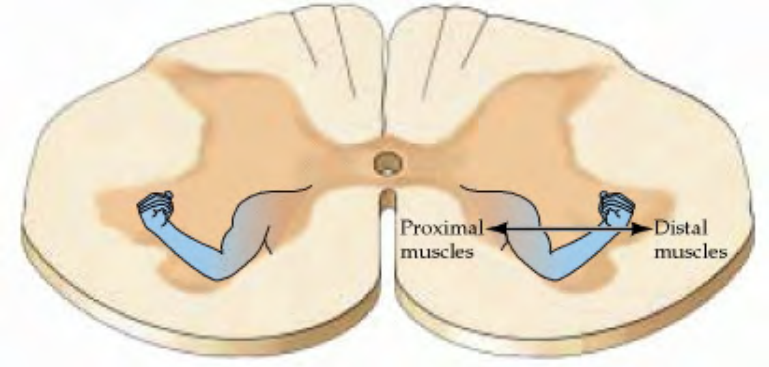


Figure 9: Spatial distribution of the motoneurons in the ventral horn cross section of the spinal cord at the cervical level. Motoneurons innervating axial musculature are located medially, whereas those innervating the distal musculature are located more laterally. Likewise, the motoneurons that control the flexor musculature are situated more dorsally as compared to the motoneurons that control the extensor muscles. Thus, it appears that the lower neurons of the spinal cord have a somatotopic organization. (Taken from http://www.ncbi.nlm.nih.gov/bookshelf/br.fcgi?book=neurosci&part=A1090&rendertype=figure&id=A1092).

In addition to have a somatotopic organization inside the ventral horn, the motoneurons have also a segmental organization. The technique of retrograde transport of horseradish peroxidase allows the analysis of the organization of the spinal cord motor columns in macaque monkeys' ventral horn. Using this method, Jenny and Inukai have demonstrated that the vast majority of hand (finger), wrist and arm muscles are located in the spinal segment C8-T1 (Fig. 10) (Jenny and Inukai 83). In the present study, the location of the hemi-section of the spinal cord on the rostro-caudal axis was determined at the border of the cervical segments C7-C8. This type of lesion is between the populations of motoneurons responsible for the biceps contraction (above the lesion) and the triceps contraction (below the lesion). Thus, after such lesion, the injured animal can move its arms (shoulder, elbow and wrist articulations) but its finger are completely paralysed by the lesion, making impossible to perform the precision grip.

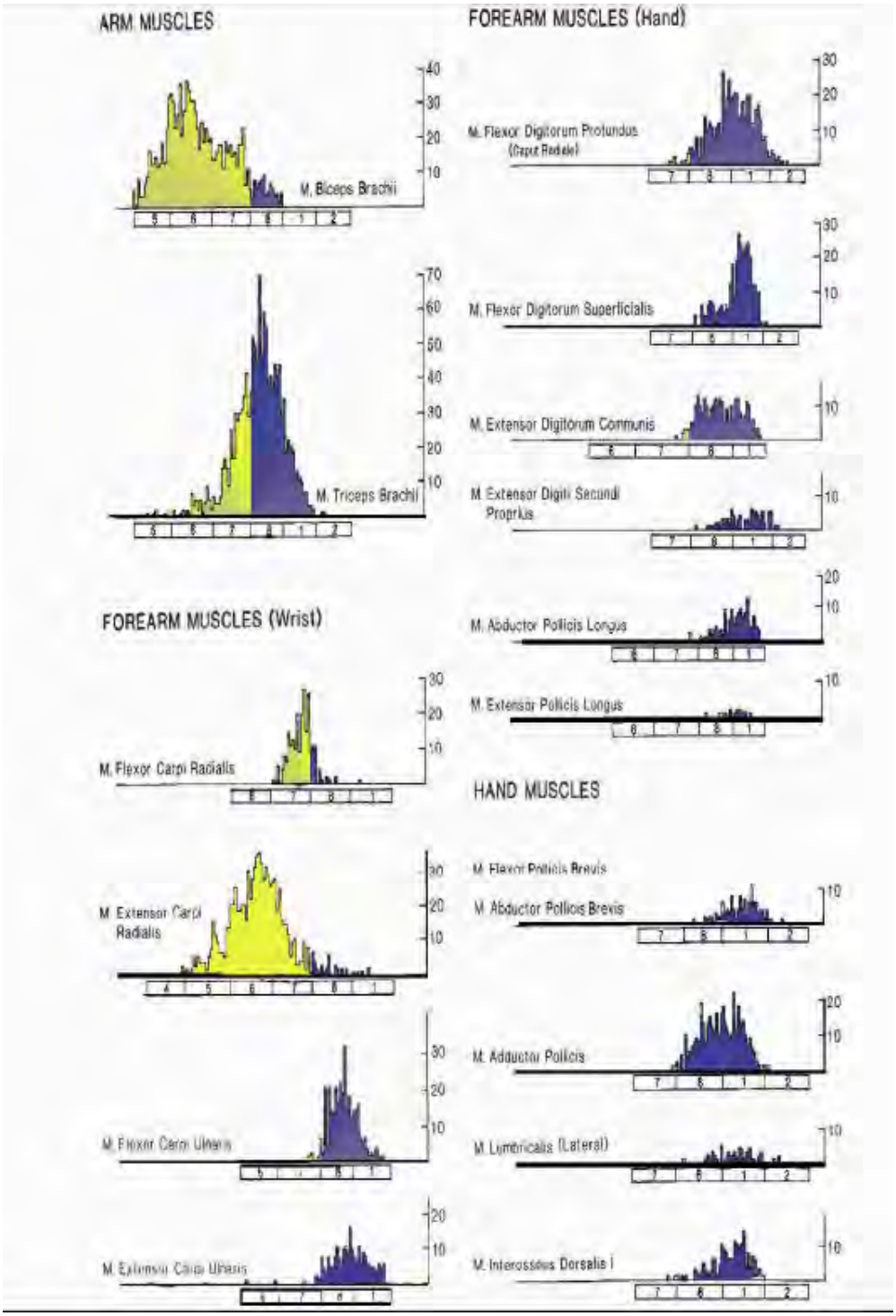


Figure 10: Segmental distribution of motoneurons innervating selected muscles of macaque monkey expressed as the number of HRP-positive motoneurons counted in the spinal cord after injection of HRP in forelimb muscles. In these graphs, the blue parts of the stippled bars represented the motoneurons affected by the lesion performed in this study at the cervical level C7/C8, whereas the yellow parts correspond to the not affected motoneurons. The stippled bars represent physiological data and the open bars represent anatomical data (Jenny and Inukai 83).

Spinal lesion of the corticospinal tract (CS) and functional recovery

Axonal regrowth capacity decreases as the age of the animal increases (Bregman and Goldberger 82). In fact, when the spinal cord of embryos or newborns higher vertebrates is injured, the regeneration occurs only during a regenerating-permissive period. In embryonic chicks and opossum the lesioned spinal cord exhibits axonal regeneration across the site of the injury but only during a certain time (Keirstead et al 95;Keirstead et al 97;Nicholls and Saunders 96). It has been suggested that the end of this regenerative capacity in newborn animals corresponds in the CNS to the beginning of the myelin formation (Schwab and Bartholdi 96). Indeed, the myelin of the CNS contains molecules that inhibit nerve fibers' regrowth after injury. Thus, lesion of mammals' tracts of the CNS, like the CST of cats, hamsters or rats are followed inexorably by a weak axonal regeneration (0.5 to 1 mm around the lesion) associated to a loss of the motor and/or sensory capacities (Bregman and Goldberger 83;Keifer and Kalil 91;Schnell and Schwab 90;Schnell and Schwab 93;Schwab and Bartholdi 96).

In nonhuman-primate studies, three levels of injuries were performed in order to affect the CST: 1) motor cortex lesions (Liu and Rouiller 99;Nudo 99;Nudo et al 96;Passingham et al 83;Rouiller et al 98;Sloper et al 83), 2) lesions of the pyramids (Beck and Chambers 70;Bucy 57;Bucy 66;Chapman and Wiesendanger 82;Hepp-Reymond et al 74;Hepp-Reymond and Wiesendanger 72;Hepp-Reymond 82;Kucera and Wiesendanger 82;Lawrence and Hopkins 76;Lawrence and Kuypers 68a;Lawrence and Kuypers 68b;Schwartzmann 78;Tower 40;Woolsey et al 72) and 3) lesions of the spinal cord (Aoki and Mori 79;Denny-Brown 66;Freund et al 06;Freund et al 09;Freund et al 07;Galea and Darian-Smith 97a;Galea and Darian-Smith 97b;Holmes and May 09;Pettersson et al 07;Sasaki et al 04;Schmidlin et al 05;Schmidlin et al 04;Wannier et al 05). Because this report concerns only injuries aimed at the spinal cord level, the next section will focus only on the anatomical and behavioural consequences of injured CST at spinal cord level.

Anatomically, lesions performed on the CST at spinal cord level affect both the transectioned nerve fibers and their associated cell body. At cortical level, the question whether the soma of the axotomized CS neurons die or not following SCI remains a matter of debate. In other words, there is still controversy about the fate of the CS neurons (whether their undergo retrograde cell death or not) after SCI. Focusing on injured CST (pyramidotomy or spinal cord lesion), some studies have concluded that most injured CS neurons survived to their axotomy, whereas studies claimed the opposite (Holmes and May 09;Levin and Bradford

38;Pernet and Hepp-Reymond 75;Tower 40;Wannier et al 05). Recently, Nielson et al. have proposed to resolve this question definitively in a novel way, by assessing CST axons in the medullary pyramid (Nielson et al 10). They have demonstrated in rats subjected to various types of SCI that the injury does not cause death of the CS cell bodies in the cortex. Thus, according to this study, the interventions to promote regeneration of the CST do not require separate manipulations to preserve the cells of origin in the cortex. However, following SCI the cut axons of the adult mammalian CSN fail to regenerate and a series of structural changes occur at the proximal and the distal level of the transection. Whereas the distal stump of the axon degenerates, corresponding to the so-called Wallerian degeneration, the proximal stump of the axon dies back (i.e. retracts) on a distance of several hundreds microns, as observed for instance in the CST of mice (Kerschensteiner et al 05).

In addition to anatomical changes, the transection of the CST provokes also behavioural modifications. In 1968, Donald Lawrence and Hans Kuypers investigated the function of the lateral system after lesions in monkey of both corticospinal and rubrospinal systems (Lawrence and Kuypers 68b). In these animals, the voluntary movements were slower and less precise. Moreover, the monkeys were unable to move independently their different arm parts, i.e. it was impossible for them to move separately the shoulder articulation, that of the elbow, the wrist or their fingers. But in spite of these lesions, they were always capable to held upright and to had a normal posture for instance while sitting. If only the CST of the monkeys was damaged, the motor deficits were comparable to those observed at the time of a global lesion of the lateral system. In monkeys numerous studies have attempted to damage the CST at various spinal cord levels such as at the third cervical segment (C3) (Galea and Darian-Smith 97a;Galea and Darian-Smith 97b), at C4 and C5 (Sasaki et al 04), as well as at C7 and C8 (Schmidlin et al 04). However, these lesions were not selective because they cut other fibre tracts in addition to the CS fibres, such as the rubrospinal or the reticulospinal tracts or ascending projections. This is the major difference between the lesion of the CST at the pyramids or at cervical cord level. Whereas both types of lesion lead to behavioural deficits in nonhuman primate, the pyramidotomy interrupts selectively the CST and a cervical cord lesion gives rise to a more complex injury situation. In fact, the animals that were subjected to a bilateral pyramidotomy loss their posture and limb movements but this situation returned to normal around 6-8 months after the injury (Hepp-Reymond and Wiesendanger 72). Animals with unilateral pyramidotomy show no impairment of the hand situated on the contralesional side, while the ipsilesional hand exhibited a better recovery than animals subjected to bilateral pyramidotomy (Lawrence and Kuypers 68b). These results led to the conclusion that the monosynaptic direct projections of the CST to the spinal cord are

responsible for the control of the voluntary movements. On the other hand, monkeys subjected to a unilateral lesion of the midcervical cord that interrupts the dorsolateral funiculus as well as the lateral and the ventral columns recover more rapidly their ability to use hand successfully during a prehension task which requires precision grip. This movement consists of the opposition of thumb and index finger typically used for grasping small objects. The precision grip originated as an adaptation in primates for grooming and finger-feeding. The higher primates could oppose the thumb pad to the side of the second digit to clean insects from fur, pluck berries from bushes as well as bring food to the mouth for example. Bortoff and his colleague Strick have related the extent of the CS terminations in the ventral horn of the primate to the capacity to perform the precision grip. In fact, using the anterograde tracer WGA-HRP, they observed marked differences between the CS stained fibers into the spinal cord of two different species of monkeys (*Cebus apella* and *Saimiri sciureus*), only one being able to perform the precision grip although both have the anatomical hand ability (Bortoff and Strick 93).

After such lesion, the degree of recovery of the manual dexterity is directly related to the size and the level of the lesion. In a study performed on new born and juvenile macaque monkeys lesioned unilaterally at the cervical level C3, a dramatic loss of function in manual dexterity task was reported, but only transient (Galea and Darian-Smith 97a;Galea and Darian-Smith 97b). In fact, 60 to 90 days following the lesion of the spinal cord, a remarkable recovery of the manual functions was observed in the hand affected by the injury. However, some behavioural deficits persisted nevertheless, like a loss of the pre-shaping prior grasping and as well as a weakening of the muscles contributing to precision grip. On the other hand, the hand that was not affected by the lesion remains totally normal. This noticeable recovery following SCI was attributed to several factors, such as a change of strategy to perform the manual task or because the unilateral hemisection of the spinal cord spared the undecussated descending CST axons, which constitute around 5-10% of the total amount of the fibers of this tract (see above) (Jankowska and Edgley 06;Rouiller et al 96). Another interpretation of this recovery is the indirect access of the cortical signals to spinal motor neurons via other descending systems like the rubrospinal tract.

In summary, in case of lesions cutting principally the CST, a more or less extensive recovery of the motor functions takes place. A number of factors could play a role in the marked recovery observed after unilateral hemisection of the spinal cord like the implication of the spared axons. The recovery happened gradually during the months following the lesion but this improvement was only partial and two deficits remained visible after the SCI: a weakness of the distal flexor as well as the incapacity of mobilizing the fingers independently from each others (Fig. 11). These two deficits cause a reduced capacity to make fine manipulative movements by the distal appendages, like those done during the precision grip.

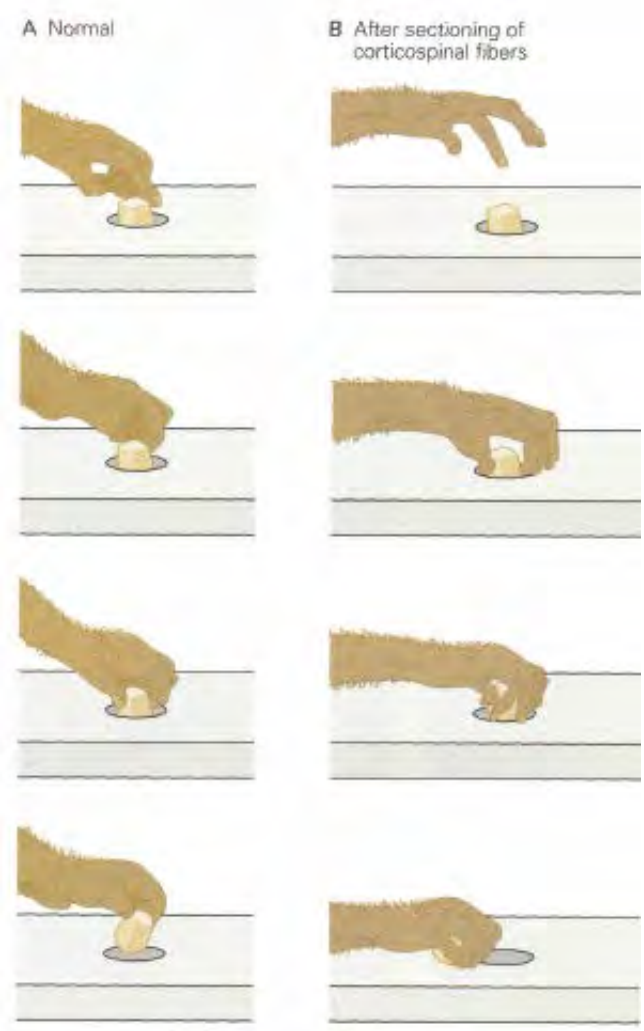


Figure 11: The CST is required for fine control of the digits when performing the precision grip. A. A normal monkey is able to pick up a food morsel from a small well using the index finger and thumb. B. After bilateral sectioning of the pyramidal tract the monkey can only tentatively remove food from the well by grabbing with the whole hand (Lawrence and Kuypers 68b).

The glial scar: consequence of a SCI

As in other parts of the body, the injury of the CNS is followed by the formation of a scar. The glial scar is considered as the third main component impeding the axon regeneration after SCI in the CNS following the damaged oligodendrocytes and the degenerating myelin. This tissue is predominately composed of reactive astrocytes and proteoglycans but additional cells contribute also to its formation, such as microglia cells, oligodendrocyte precursors, meningeal fibroblasts and inflammatory cells like macrophages, lymphocytes and neutrophil granulocytes (Schwab and Bartholdi 96) (Fig.12). The proteoglycans which enter in the composition of the extracellular matrix (ECM) are produced by reactive astrocytes and these glycoproteins have a protein core linked covalently to glycosaminogylcan (GAG) chain (s). Four classes of proteoglycans are known:

1) The heparan sulphate proteoglycan (HSPG)
2) The dermatan sulphate proteoglycan (DSPG)
3) The keratan sulphate proteoglycan (KSPG)
4) The chondroitin sulphate proteoglycan (CSPG) (Johnson-Green et al 91)

The CSPGs form a relatively large familiy, which inculdes aggrecan, brevican, neurocan, NG2, versican and phosphacan (sometimes classed as a KSPG) (Silver and Miller 04). The CSPGs are known to prevent axonal regeneration. One strategy applies to thwart the inhibition of the CSPGs is the enzymatic digestion of its GAG chains which show to stimulate the regeneration of the nerve fibers through the site of the spinal cord injury (Fawcett and Asher 99;McKeon et al 95;Smith-Thomas et al 95). In addition to the proteoglycans, other proteins present in the glial scar are also known to contribute to axonal regeneration impediment such as the semaphorin 3 (SEMA3) and its receptors neuropilin 1 or ephrin-B2 and its receptor EPHB2 (Bundesen et al 03).

Lesion scar components

Cellular	Extracellular
Reactive astrocytes	Collagen IV
Microglia/macrophages	Laminin
Endothelial cells	Fibronectin
Fibroblasts	Chondroitin sulfate proteoglycans
	Heparan sulfate proteoglycans (perlecan)
	Keratan sulfate proteoglycans
	Tenascin C
	Thrombospondin
	Basal membrane

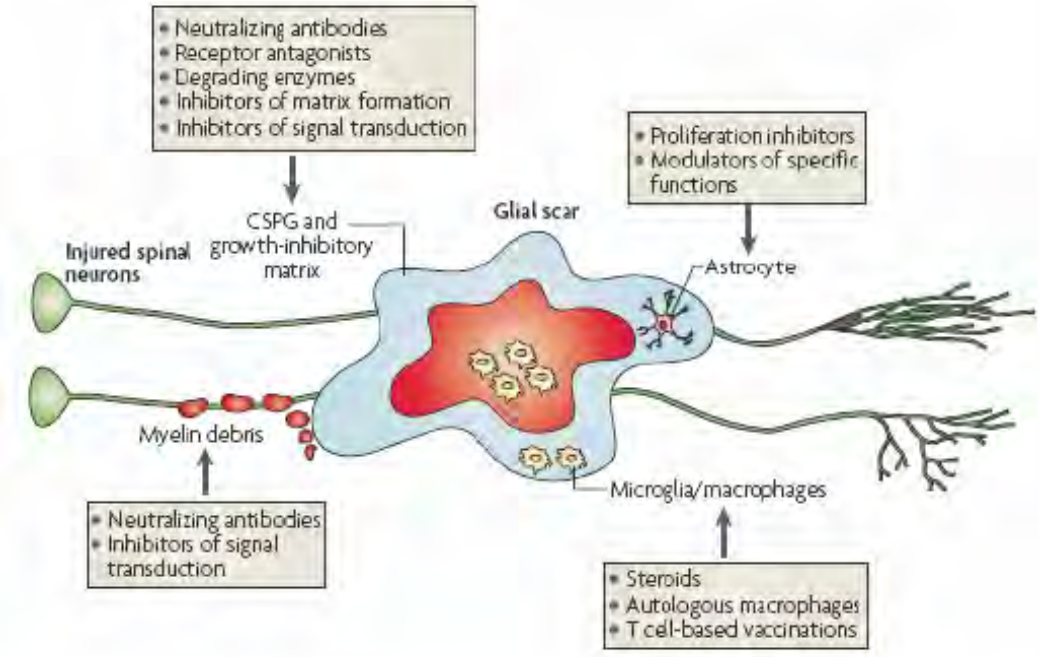


Figure 12: Up panel: summary of the different elements that compose the glial scar (Stichel and Muller 98). Down panel: the potential therapeutic interventions associated to the scar components (Rolls et al 09).

Frequently, the scar tissue is composed of two distinct regions: the glial scar and the fibrous scar (Fig. 13). The fibrous scar is located in the centre part of the lesion and is composed of dense extracellular matrix (ECM) network whereas the glial scars is rather located in its periphery and is composed of reactive astroglia cells (Brazda and Muller 09;Klapka N et al 02;Shearer and Fawcett 01;Silver and Miller 04). These two types of scars are separated by a basement membrane composed mostly of type IV collagen (Stichel and Muller 98).While it appears that the glial scar plays a major role in the failure of axonal regeneration in the CNS by expressing inhibitory molecules, functional recovery could be directly correlated with the amount of fibrous scarring (Reier et al 83;Seitz et al 02). In fact, fibrous scar-suppressing

treatment promotes not only extensive long-distance regeneration on injured CST axons but also neuroprotective effects on the axotomized cortical motoneurons (Klapka et al 05).

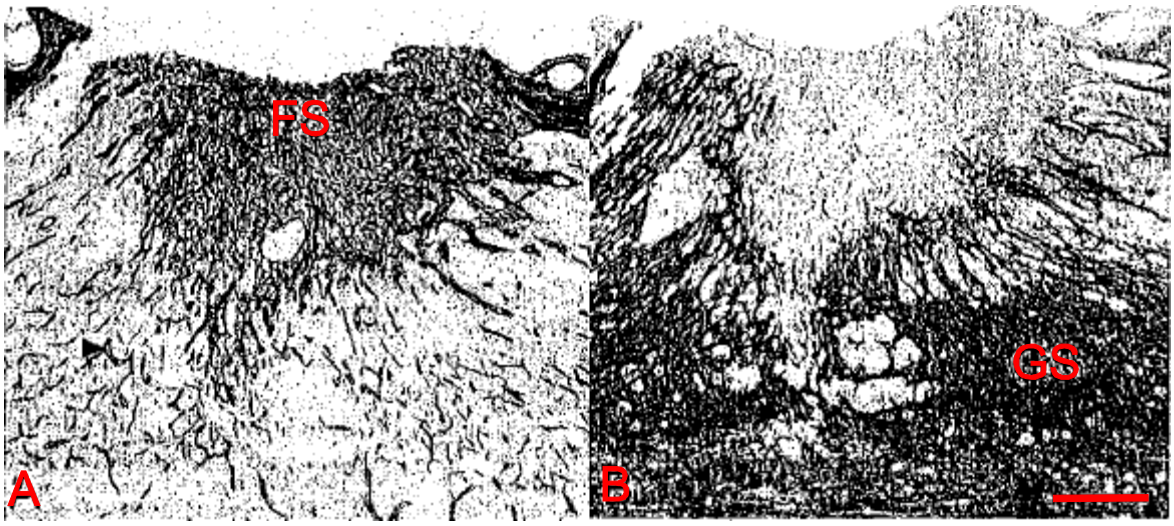


Figure 13: Transection of a thoracic rat spinal cord with immunohistological staining of the collagenous scar with antibodies directed against Coll IV (A) and the glial scar with antibodies against GFAP (B). The two different scar types are located complementary to each other: FS, fibrous scar; GS, glial scar. Magnification bar: 500 µm (Brazda and Muller 09).

In the context of neurodegeneration, the glial scar formation has also been shown to have beneficial effect. In fact, the glial scar allows to re-establish the physical and chemical integrity of the CNS by reestablishing the blood brain barrier. Thus, the CNS is again protected from infections and spreading of further damages. Moreover, the glial scar is also known to stimulate the revascularization in order to increase the nutritional, trophic and metabolic support of the nervous system (Stichel and Muller 98). Nevertheless, these beneficial effects contrast with its secreted and transmembrane molecular inhibitors which interrupted the progression of the regenerating projections (Fawcett 06;Silver and Miller 04).

Regeneration strategies

In addition to the suffering endured by lesioned people, SCI has long-term health, economic and social consequences, giving a strong motivation to the development of treatments, even if they were leading only to partial improvements.

Following SCI in the mature mammalian CNS, both intrinsic and extrinsic factors contribute to the failure of axonal regeneration and the limited associated spontaneous functional recovery such as:

- Cell death (Beattie et al 00)
- Glial scar formation (Fawcett and Asher 99)
- Inhibition related to the myelin (Huber and Schwab 00)
- Insufficiency of permissive substrates for axonal regeneration
- A lack of growth factors support

In order to change this non-permissive situation for regeneration, four main types of therapeutic strategies may be used to thwart the SCI effects:

1. Avoid scar formation
2. Rescue of injured neurons with prevention of neuronal death
3. Promotion of target-directed axonal regeneration
4. Neural replacement by endogenous or transplanted neural stem cells or other tissue transplantation

In this work, two strategies are used in order to promote axonal regeneration and functional recovery: first an intrathecal infusion of an antibody directed against the Nogo-A molecule in order to block the inhibition exerted by this myelin growth-inhibitory molecule, thus stimulating the axonal regrowth (Beaud et al 08;Freund et al 06;Freund et al 07); second, a combination of this therapeutic approach with the brain derived neurotrophic factor (BNDF) with the intention to strengthen the axonal regeneration obtained by the Nogo-A antibody. In other words, the first type of treatment (anti-Nogo-A antibody) focus on creating a permissive environment for axonal regrowth by thwarting the inhibition caused by the myelin, whereas the second type of treatment (additional infusion of BDNF into the spinal cord injury) attempts to create better regrowth environment by stimulating the cellular survival and axonal regeneration.

The infusion of an antibody directed against Nogo-A molecule as a strategy against the SCI consequences

Without treatments, injuries of adult mammalian axons of the CNS are always followed by a failure of nerve fibers regeneration and neuronal cell death or neuronal atrophy (Ramon y Cajal 28;Wannier et al 05). In contrast to the axons located in the CNS, the lesioned nerve fibers that belong to the peripheral nervous system (PSN) are not only able to regenerate their axons but their soma neurons survive to an injury. Understanding why these two systems react so differently is one of the most challenging and essential problems confronting the neurobiologists nowadays (Goldberg and Barres 00b). During many years, it was at first believed that the CNS did not possess any ability to regenerate. However, this point of view has changed during the 80s when peripheral nerve grafts were used as bridges aiming to bypass the lesion site. With this kind of bridges, the injured axons of rats were able to regrow over marked distances through the peripheral grafts but then stop when living this growth permissive tissue (David and Aguayo 81;Richardson et al 80). Thus, with these experiments it was demonstrated that as the PNS fibers, the CNS fibers possess also the intrinsic capacity to regrow after a lesion. These findings were then followed by the demonstration that the glia (astrocytes and oligodendrocytes) is a substrate contributing to the growth-inhibitory nature of the CNS (see above the chapter about the glial scar) (Caroni et al 88). Therefore, the origin of the axonal regeneration failure in the CNS is a non-permissive environment. In fact, a comparison between the myelin of both CNS and PNS has revealed that CNS white matter is selectively inhibitory for axon outgrowth (Schwab and Thoenen 85). It has been also demonstrated that the CNS of mammals is able to regenerate during the early periods of life but only as much as the fibers are still not myelinated (Schwab and Bartholdi 96). Moreover, in the rat and in the chicken species the deletion of the oligodendrocytes or the delay of the process of myelination in the early postnatal period of life facilitates the regeneration of injured axons in the spinal cord (Keirstead et al 92;Savio and Schwab 89). Among the glia, numerous growth inhibitor molecules associated with the myelin were found such as Nogo (Chen et al 00;GrandPré et al 00;Prinjha et al 00;Schwab and Bartholdi 96), myelin-associated glycoprotein (MAG) (McKerracher et al 94;Mukhopadhyay et al 94), oligodendrocytes myelin glycoprotein (OMgp) (Kottis et al 02;Wang et al 02), CSPG, Brevican, Ephrin B3, Sema 4D and others. Among these inhibitory molecules, the family of Nogo molecules, and Nogo-A in particular, appear to play a key role in the lack of axonal regeneration observed following SCI and will be investigated in more details in the next chapter.

Discovery of the Nogo molecule

Molecules capable of inhibiting axonal growth *in vitro* have been found both in the intact CNS (Caroni and Schwab 88b;Schwab 90) and at sites of injury (Fawcett and Asher 99;Pasterkamp et al 01). Among these molecules, Nogo has been identified as one of the major myelin components able of inhibiting the axonal regrowth in the lesioned CNS. This glycosylated transmembrane protein (molecular weight of around 220 kDa) is released in the CSN environment when the oligodendrocytes are injured (Fig 14).

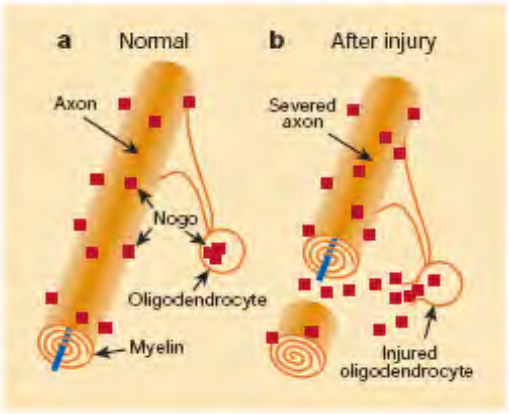


Figure 14: Inhibition of axon regeneration. A) The Myelin-associated molecules Nogo is found in normal condition in the inner and outer myelin sheet of the oligodendrocytes. B) However, following a spinal cord lesion, Nogo is released in the CSN environment which causes failure of axonal regeneration (Goldberg and Barres 00a).

The story of Nogo began in 1982 when it was postulated for the first time that the degeneration products of the CNS myelin were involved in the inhibition of axonal regeneration (Berry 82). Then, in 1988, it was demonstrated subsequently that CNS myelin inhibited neurite growth *in vitro* and that two proteins extracted fro the CNS myelin had most of the inhibitory activity (Caroni and Schwab 88a;Caroni and Schwab 88b). These studies promoted the generation of a function-blocking antibody, IN-1, directed against one of these myelin-related protein (later known as the Nogo-A isoform of the gene nogo) (Caroni and Schwab 88b). Following these investigations, Schwab and his colleagues have examined the mechanisms of axonal growth inhibition after a spinal cord lesion using the monoclonal antibody IN-1 (mAb IN-1 treatment). Surprisingly the antibody recognizes different membrane proteins with different molecular mass (Mr): NI-35 (a 35-kDa glycoprotein), NI-220 (a 220-kDa glycoprotein) and NI-250 (a 250-kDa glycoprotein). As assumed by Schwab and collaborators, IN-1 neutralizes Nogo inhibitory effect allowing axonal regeneration: in

rats, a massive sprouting, up to 7−11 mm, occurred caudally to the lesion site within 2−3 weeks following a spinal cord lesion (Schnell and Schwab 90). This capacity for CNS axons to regenerate and elongate after the neutralization of myelin-associated neurite growth inhibitors was observed both *in myelin culture* (Caroni et al 88;Rubin et al 94) and *in vivo* (Schnell and Schwab 90;Schnell and Schwab 93). In parallel to the axonal regeneration after spinal cord injury, rats exhibited functional improvements such as recovery of specific reflexes and locomotor functions (Bregman and others, 1995). In addition to have shown these promising results, in 1998 Schwab and his colleagues were the first group who described one bovine inhibitory molecule recognized by monoclonal antibody IN-1 (Spillmann et al 98). Then, in year 2000 the nogo gene was cloned. Indeed, during this year Chen and his colleagues used rat complementary DNA (cDNA) encoding NI-220/225 sequences and they identified the rat homologous antigen for IN-1 (Chen and others, 2000), while Prinjha and his collaborators, using the same method as Chen et al. found the corresponding human cDNA (Prinjha et al 00). Finally, at the same period GrandPré and colleagues established the full-length of this human cDNA that had previously been deposited in the Genbank sequence database (GrandPré et al 00). Thus, all of these three groups had identified a previously unknown gene coding for the protein named Nogo.

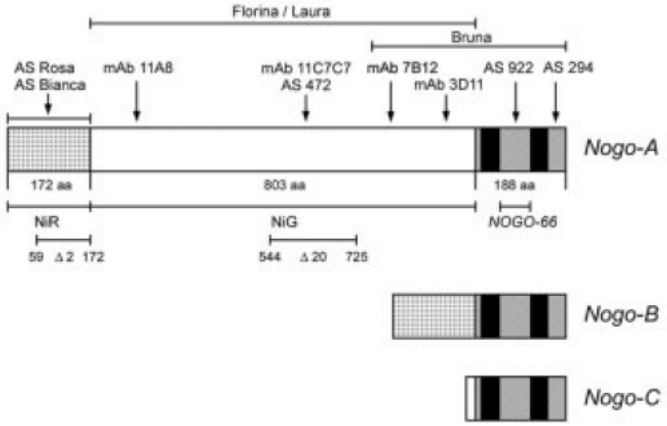
The three isoforms of Nogo protein

The reticulon (RTN) family

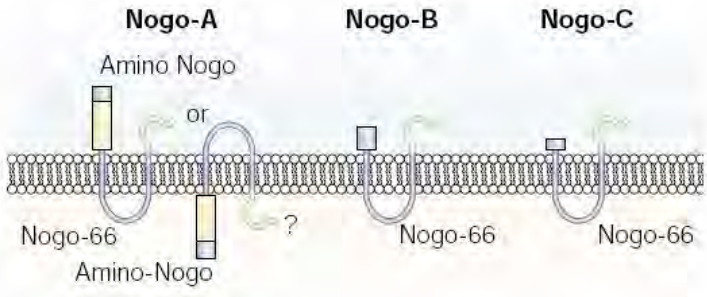
In the nervous system, via both alternative splicing and alternative promoter usage, the nogo gene gives rise to three different isoforms of the Nogo protein named respectively Nogo-A, Nogo-B and Nogo-C (Chen and others, 2000; Filbin, 2003; Goldberg and Barres, 2000a; Oertle and others, 2003a; Schweigreiter and Bandtlow, 2006) (Fig 15). A 66-residue extracellular domain sequence (named Nogo-66) is common to all the three isoforms. This particular sequence is known to be responsible for the inhibition of the axonal extension and induces growth cone collapse (GrandPré et al 02).

A)

B)



C)



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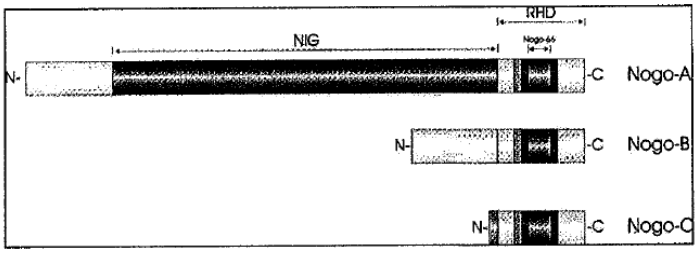

Figure 15: Four illustrations coming from four different publications showing the three possible isoforms of the Nogo protein. Among the three isoforms, Nogo-A appears to be the largest. Compared to the three other representations, panel C) shows clearly the common Nogo-66 sequence responsible for the axonal inhibition. A) Illustration taken from Goldberg and Barres, 2000a; B) illustration taken from Oertle and other, 2003; C) Filbin 2003 and D) illustration taken from Schweigreiter and Bandtlow, 2006.

In addition to Nogo-66 sequence, all the three isoforms of the Nogo protein have a common C-terminus of 188 amino acids (aa) named reticulon-homology domain (RHD). Therefore, this sequence characterizes the Nogo proteins as members of the reticulon (RTN) family. The RTN proteins, which are evolutionary very old proteins, are present in all eukaryote organisms including plants and fungi (Oertle and others, 2003a) (Fig. 16). One named *reticulons* (RTNs), proteins which belong to the vertebrate and *reticulon-like proteins* (RTNLs), proteins of the other eukaryotes. In the vertebrate, the RTNs are mostly localized to the endoplasmic, reticulum (ER) (van de Velde and others, 1994). The presence of RTNs in eukaryotic but not in prokaryotic organisms, in addition to their close association with the ER suggests that RTNs evolved along with the eukaryotic endomembrane system (Yang and Strittmatter, 2007). Therefore, it is not surprising that the RTNs influence the functions related to the ER such as the endoplasmic reticulum-Golgi trafficking, the vesicle formation and the membrane morphogenesis. Despite the fact that many aspects and functions of the RTNs remain incomplete, there are growing supports that the RTNs play a role in neurodegenerative diseases such as Alzheimer's disease, amyotrophic lateral sclerosis (ALS), multiple sclerosis and perhaps hereditary spastic paraplegia (Dupuis and others, 2002; He and others, 2004; Jokic and others, 2006; Jokic and others, 2005; Reindl and others, 2003).

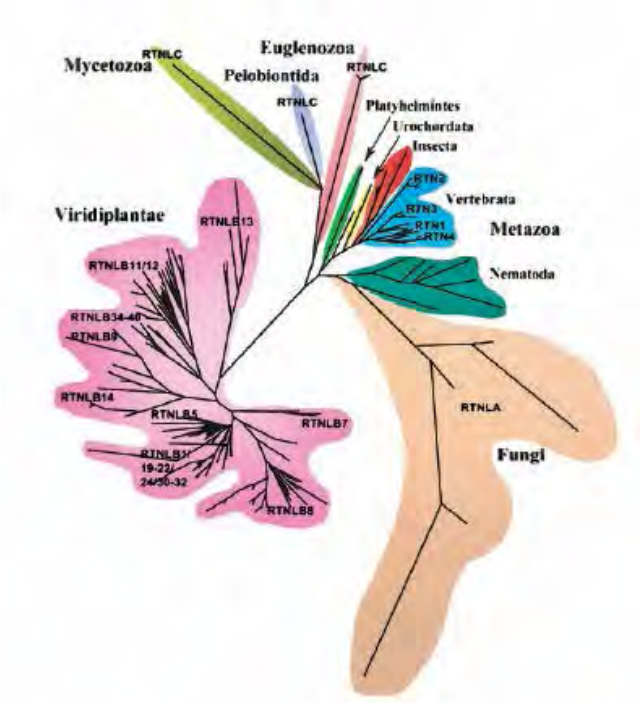

Figure 16: Tree diagram representing taxonomic distribution of the RTN gene family based on alignment of the reticulon homology domain. Reticulon-like (RTNL) genes are found is most eukaryotic kingdoms from protists to animals (Oertle and others, 2003a).

In the higher vertebrates RTN family, four paralogues have been identified and named: RTN1 (previously named neuroendocrine specific protein NSP1), RTN2, RTN3 and RTN4. Among these paralogues, RTN1 was found to be a specific marker for neuroendocrine cells (Roebroek and others, 1993) and RTN4 corresponds to the Nogo proteins explaining why the three isoforms of the nogo gene are also sometimes named in the literature RTN4-A, RTN4-B, RTN4-C.

Localization

The localization of the three isoforms of Nogo protein appears to be at variance in different body parts. Nogo-B and –C are found in certain neurons and a range of non-neuronal tissues. For example, Nogo-C is predominantly localized in skeletal muscle, whereas small concentrations of Nogo-B and –C were detected in kidney, cartilage, skin, lung and spleen (Chen and others, 2000). In human, the distribution of Nogo-A in the CNS corresponds globally to the distribution found in rodents except for the substantia nigra, the cerebellum, and the granule cells and for the cells located in the deep cerebellar nuclei where the concentrations were lower (Buss and others, 2005). It has also been demonstrated that Nogo-A is also expressed in neurons, especially during development. All Nogo proteins, and Nogo-A in particular, are expressed on the cell surface of the myelin of oligodendrocytes. On the other hand, Nogo-A is not expressed by Schwann cells. Focusing on the progression of the functions of Nogo-A, it was surprisingly shown that the evolution of the Nogo-A protein has undergone a profound change of direction at the transition from fish to tetrapods, shifting towards a novel role as a restrictor of axonal plasticity (Schweigreiter, 2008). In fact, whereas higher vertebrates are only poorly able to regenerate severed axons in their CNS, fishes possess a regrowth permissive CNS environment (Diekmann and others, 2005). Indeed, after injury, retinal growth cones of fishes can regenerate in spite of the presence of myelin in their CNS, while they collapse or grow around when contacting higher vertebrate oligodendrocytes (Bastmeyer and others, 1991). It has been shown that this particular situation is due to a differential recruitment of exons of the nogo gene which proceeded along at least two independent lines during the early vertebrate evolution (Bastmeyer and others, 1991; Diekmann and others, 2005; Schweigreiter and Bandtlow, 2006; Wanner and others, 1995). In other words, fishes do not possess the sequence called Δ20 or NiG Δ20, region of the higher vertebrate Nogo-A protein related to neurite growth inhibition. Thus, despite their difference of splicing, retinal axons of fishes are sensitive to neurite growth inhibiting molecules present in the higher vertebrate CNS myelin and the presence or the absence of Δ20 can change the regeneration capability of an organism.

Functions

Compared to Nogo-B and –C, whose exact functions remain unknown for the moment, and compared to the other myelin-derived molecules, Nogo-A is probably the best characterized inhibitor of nerve growth in CNS of adult mammalian. As already mentioned, Nogo-A has been identified as a strong inhibitor of neurite outgrowth and cell spreading in the injured CNS (Oertle and others, 2003b). Therefore, Nogo-A function-blocking antibodies have been applied in different lesion models, in particular in the adult rats and nonhuman primates spinal cord in order to improve the neuronal regeneration. Spinal cord injury (SCI) in both rats and monkeys followed by the application of antibodies directed against Nogo-A lead to an increased regenerative sprouting of the axotomized nerve fibers associated with an improvement of functional recovery (Fig. 17 and 18) (Buchli and Schwab 05;Freund et al 06;Freund et al 09;Liebscher et al 05). In addition, it has been demonstrated that following a cervical cord lesion, monkeys treated with the anti-Nogo-A antibody show enhanced presence of CS axons rostral and around (some fibers) the lesion as well as into caudal spinal segments (Freund et al 07). However, it has been established that both the effects on regeneration and on functional recovery were reduced if the treatment was delayed by more than one week after lesioning (Von Meyenburg et al 98). The most robust evidence of the efficiency of the IN-1 antibody on the CST comes from pyramidotomy experiments (which allowed to sever the entire CST) where axonal regeneration was observed 2 mm caudal to the lesion (Raineteau et al 99). In fact, after pyramidotomy, compensatory sprouting of contralateral CST fibers was stabilized by the anti-Nogo-A antibody (Bareyre et al 02). Thus, (mAb) IN-1 monoclonal antibody is the most frequently used antibody in SCI investigations. However, additional antibodies also directed against Nogo-A, but targeting other parts of this protein, have been developed in the context of SCI experiments (Fig. 19). For example, a recombinant of IN-1 antibody partially humanized (called mAb hNogo-A) was infused in rats subjected to a thoracic dorsal and dorsolateral spinal cord lesion and produced enhanced CST sprouting with labeled axons observed up to 9 mm beyond the lesion (Brösamle et al 00).

Likewise, monoclonal antibodies directed against the active sites of the Nogo-A-specific region (called mAb 7B12) were produced and infused intrathecally in rats and were shown to enhance the functional recovery suggesting that both, IN-1 antibody and other antibodies directed against Nogo-A peptides can stimulate the regeneration of the CST following injury (Liebscher et al 05). Together, these findings demonstrate that compensatory sprouting and neurite outgrowth can be enhanced by blocking Nogo-A with antibodies after CNS injury.

Though the concept of Nogo-A as a strong inhibitor of nerve regeneration is supported by significant amount of data, there remains still open questions. For example, discussions subsist about the variety of results obtained by three different lines of *nogo* knockout (KO) mice with regard to their regenerative ability. In 2003, Kim et al. and Simonen et al, both reported that the fibers of injured CST of *nogo* KO mice is able to regenerate respectively above and below the lesion but Zheng et al. published the same year opposite results (Kim et al 03;Simonen et al 03;Zheng et al 03). Among these studies, Simonen et al. obtained however moderate results because no significant difference was observed between the control and the KO animals (Simonen et al 03). These three papers published in the journal *Neuron* were then followed by impetuous discussions between the different laboratories. Some criticized the age of the animals used in the experiments, others disapproved the mutant allele and the lesion model, while others deplored artifactual labeling caused by the leakage of the staining into the cerebrospinal fluid (Cafferty et al 07;Steward et al 07;Woolf 03). Recently, the debate on the mutant mice started again by the publication of data related to triple-mutant mice lacking the three major myelin inhibitors, Nogo, MAG and Omgp (Lee et al 10). According to this study, deleting any one of these three inhibitors in mice enhanced sprouting of healthy and injured axons but this regeneration was no associated with behavioural improvement and no synergistic effect was observed in triple mutants. Moreover, triple-mutant mice showed no enhanced regeneration of axonal tracts after SCI. The authors concluded therefore that Nogo, MAG and OMgp do not have a role in axonal regeneration failure. This conclusion was challenged by different authors who criticized several aspects put aside by Lee et al. (Schwab and Tuszynski 10). First, despite the deletion of three major myelin-related inhibitors, the lack of regeneration observed in the triple-mutant mice could be attributed to compensatory upregulation of other inhibitors factors such as netrins, semaphorins, ephrins or proteoglycans. Second, knockdown of any molecules during the development may alter the development itself and may change consequently the manner an adult individual could respond. Thus, the conclusions of Lee et al. (2010) appear controversial and additional studies are required to define precisely the basis for the discrepancies in the mutant mice.

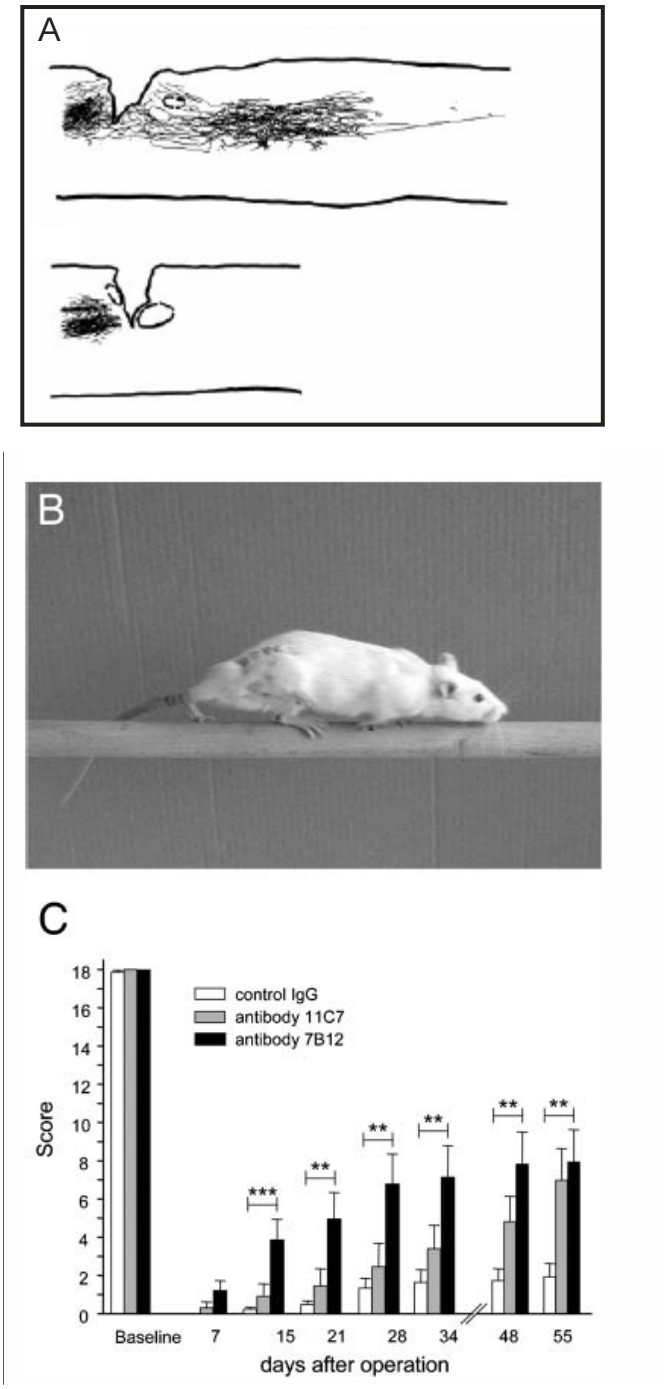

Figure 17: A) Camera Lucida reconstructions of parasagitall sections (50 µm thick) of injured spinal cord in rats. Upper panel: animal treated with an antibody directed against Nogo-A; lower panel: animal treated with a control antibody. The Nogo-A treated animal exhibits long-distance axonal regeneration into the grey matter areas, whereas the control animal shows no regenerative growth (Brösamle and others, 2000). B, and C: functional recovery in anti-Nogo-A antibody treated rats after SCI. B: rat balancing over a narrow beam. C: anti-Nogo-A antibody treated animals (here 11C7 and 7B12) show improved performance on the narrow beam as compared with the control IgG treated spinal cord injured rats (Buchli and Schwab 05).

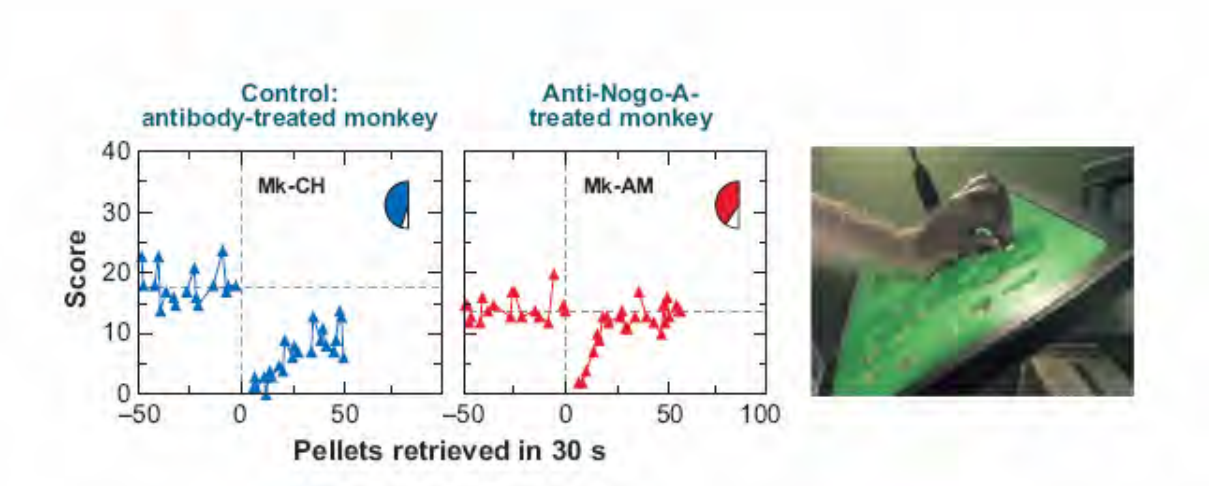

Figure 18: Effect of lesions to the CST at the cervical level on skilled independent finger movements in the macaque monkey, and partial recovery following treatment with the anti-Nogo-A antibody. Left panel: quantitative measurement of monkey manual dexterity (number of pellets retrieved within 30 seconds) using the modified Brinkman board pre- and post-lesion. The extent of the blue/red zones in the semicircles located on the right-up of each graphs represent the extent of the hemi-cord lesion performed in each animal. The day 0 indicates the time of the lesion. The untreated monkey (blue, Mk-CH) showed some limited recovery in its performance, whereas the monkey that received the anti-Nogo-A antibody (red, Mk-AM) rapidly returned to control levels of dexterity Right panel: in the modified Brinkman board task, the monkey performs the precision grip (opposition of thumb and index finger) to retrieve the pellet located in each well (Freund et al 06).

- **mAb IN – 1 (whole length IgM; rFab)** (Caroni and Schwab, 1988; Chen et al., 2000).
  Raised against a myelin fraction enriched in Nogo-A that is able to bind Nogo-A and neutralize its growth inhibitory properties.
- **mAb 7B12 (rFab)** (Oertle et al., 2003; Liebscher et al., 2005).
  Has an epitop towards the C-terminus of the Nogo-A specific region; Binds to the outer surface of living cultured oligodendrocytes.
- **mAb 11C7 (rFab)** (Oertle et al 2003).
  Directed against a 18 amino acid peptide (aa 623-640) close to the active site of the rat Nogo-A specific region. Binds to the outer surface of living cultured oligodendrocytes.
- **mAb hNogo – A (rFab)** is a humanized monoclonal antibody raised against the Nogo-A specific region of the human Nogo-A sequence. Recognizes human and primate Nogo-A specifically on Western blots.

Figure 19: Summary of the different subtypes of antibodies directed against Nogo-A used in the research (taken from the thesis of Alexander Wyss; chapter 1.9.12.2.).

Nogo-A signaling pathways

Nogo-A is the largest protein encoded by the nogo gene and is divided into three main domains (Fig 20):

1) The NiR domain lying at the N-terminus (aa 1 to 168)
2) The NiG domain corresponding to the large central domain (aa 169 to 974)
3) The RDH domain locating at the C-terminus (aa 975 to 1162)

Figure 20: The three different domains of the Nogo-A protein (Schweigreiter, 2008).

Although the function and the mechanism of action of the Nogo-B and –C proteins remain to be clarified, the mechanism by which Nogo-A inhibits neurite outgrowth and axon regeneration in the CNS is well characterized. First, it has been demonstrated that two different regions of the Nogo-A protein confer the inhibitory function of Nogo-A in the CNS by binding to neuronal receptors:

1) The Nogo66 domain, corresponding to the extracellular/endoplasmic reticulum (ER) luminal portion of Nogo-A (the 66-loop), which binds to the receptor NgR1 (Fig. 21 red part of the Nogo-A protein) (Fournier et al 01;GrandPré et al 00)
2) The Δ20 region (also called NiGΔ20 because it is localized in the NiG domain), which binds to integrins present on the neuronal surface (Fig 21 blue part of the Nogo-A protein) (Fournier and others, 2001; Oertle and others, 2003b). This part of Nogo-A mediates its action (fibroblast and growth-cone collapse) independently of the Nogo receptor suggesting the existence of another receptor unknown for the moment (Oertle et al 03).

Subsequently, a third inhibitory region, called Nogo-A-24, has also been identified in the Nogo-A protein (Fig. 21 green part of the Nogo-a protein). As Nogo66, this region can also bind directly to the receptor NogoR. It has been demonstrated that this region of Nogo-A protein, when fused with Nogo66, is responsible to enhance the binding affinity of Nogo66 to NogoR (Fig. 21 orange part) (Hu et al 05).

The receptor NgR1 is known to team up with a selection of transmembrane co-receptors or signaling transducers for the signaling transduction such as:

1. -The neurotrophin receptor p75
2. -The transmembrane protein LINGO-1

3. The orphan tumor necrosis factor family member TAJ/TROY (Fig. 21 yellow part and 22 down panel) (Yang and Strittmatter 07)

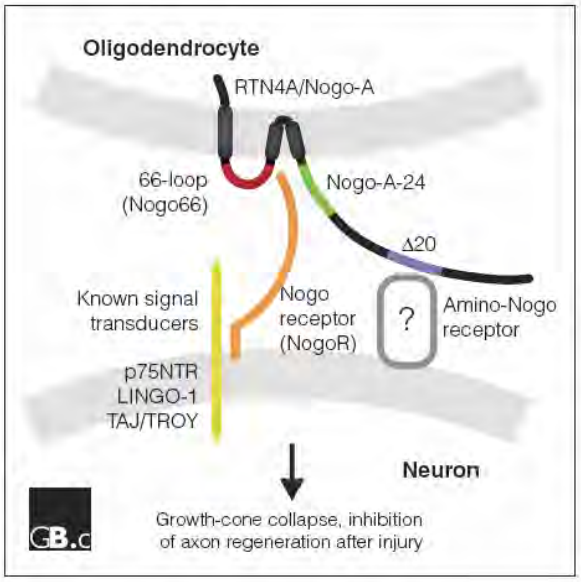

Figure 21: The three different inhibitory regions of Nogo-A: Nogo66 in red; Nogo-A-24 in green and Δ20 in blue. Nogo66 and Nogo-A-24 are known to interact with the Nogo receptor, whereas Δ20 interacts with a receptor not yet identified (Yang and Strittmatter 07).

Recently, it has also been shown that during the development, Nogo-A interacts with another receptor termed Caspr/F3 (contactin) complex at the CNS paranodes. It has been then proposed that this axonal interaction may play a role during the myelination process via the regulation of the potassium channel localization and also via the maintenance of the architecture of the axoglial apparatus (Girault and Peles 02;Nie et al 03). However, the physiological role of the Nogo-A binding to Caspr/F3 complex still remains an open question (Teng et al 04).

Although not all signaling pathways have been clarified yet, interestingly both inhibitory regions of the Nogo-A protein, Nogo66 and Δ20, transduce their signal downstream via the up-regulation of the GTPase RhoA (Fig. 22). In fact, RhoA inhibition promotes regeneration, neuroprotection and functional recovery *in vivo* after SCI (McKerracher and Higuchi 06). Following its activation, Rho-A down-regulates the protein Rac1 which produces in turn the activation of the kinase termed ROCK (Fig. 22). At last, ROCK stimulates the actinomyosin activity in the cytoskeleton leading to the inhibition of the neurite growth like via the collapse of the growth cone (Fournier et al 00;Liu et al 06). Finally, three other, structurally distinct inhibitor components, named MAG, OMgp and Versican V2, have also been found to interact with NgR1 meaning that the Nogo-66 loop shares its signaling complex (Fig. 22). Among

these components, MAG and Omgp are myelin-derived molecules. Whereas MAG is found in both the CNS and the PNS in periaxonal membrane, OMgp is a relatively minor element of the myelin and is believed to be localized largely in paranodal loops, next to the node of Ranvier (Filbin 03;Wang et al 02).

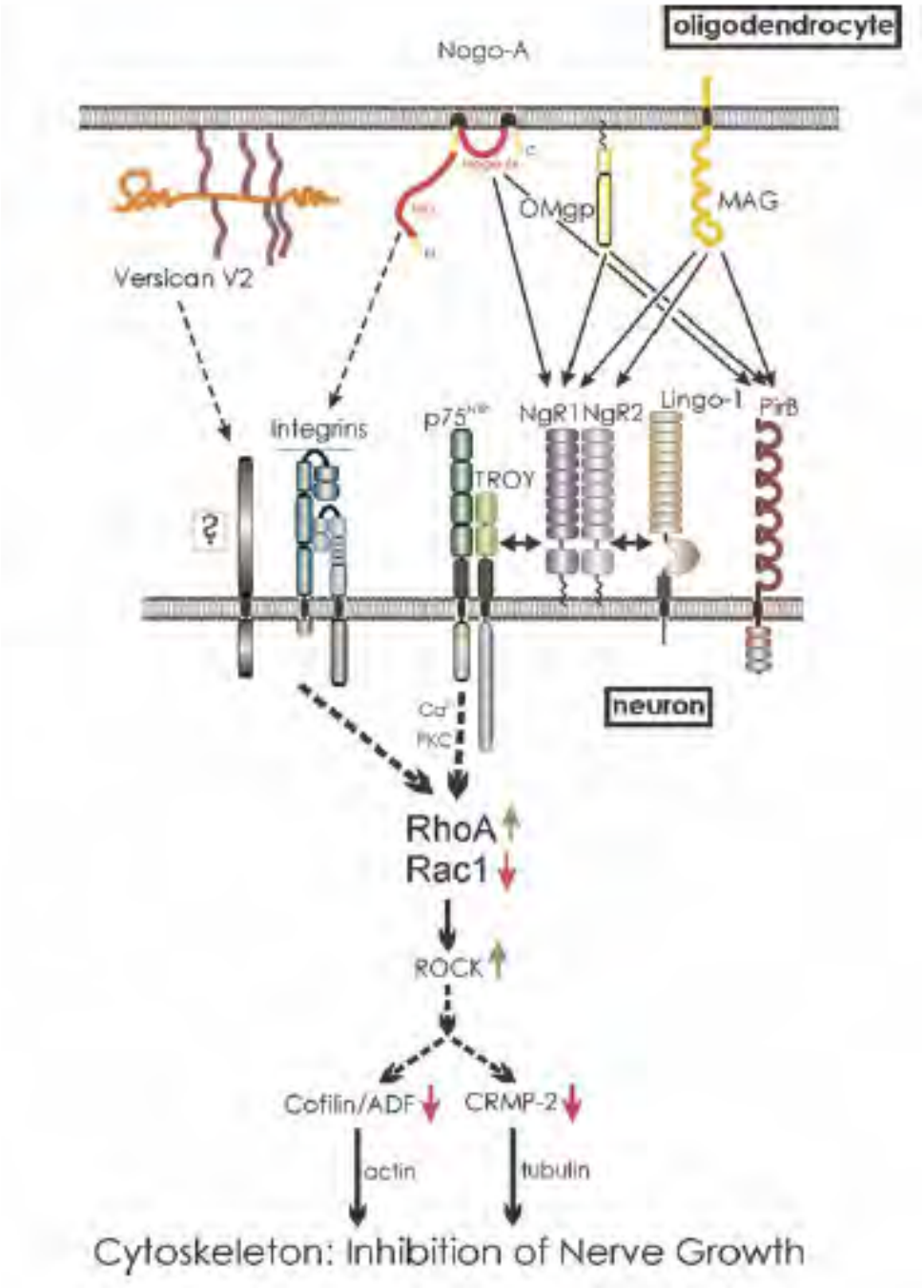

Figure 22: Signaling pathway of Nogo-A (Schweigreiter 08). The interaction mediated via Nogo-A and its receptor NogoR results in inhibition of axon regeneration after injury.

Is there a role for Nogo-A beyond limiting axonal plasticity?

Recently, some investigations have demonstrated that Nogo-A is a multifunctional protein not only involved in the neurite outgrowth inhibition. First, new debates arise these last years about other roles that could play Nogo-A in neuronal cells. In 1994, a study has shown that Nogo-A is expressed into the ER but it is only in 2006 that it was demonstrated that Nogo-A could possesses a function related to this organelle (van de Velde et al 94;Voeltz et al 06). However, in spite of its predominant localization in oligodendrocytes, Nogo-A is also expressed in neurons, suggesting that Nogo-A may play other roles different from its inhibiting function in oligodendroglial myelin sheath. In 2006, Voeltz et al. have identified via an in vitro system that Nogo-A and the interacting protein DP1/Yop1p are "morphogenic" proteins involved in the ER network formation and maintenance of the peripheral tubular ER shaping, but not for the peripheral sheets or the nuclear envelope (Voeltz et al 06). Nogo-A and the interacting protein DP1/Yop1p possess the particularity to be exclusively localized in the ER tubules and are excluded from flat membrane sheets such as the nuclear envelope. In addition, it appears that these proteins can form oligomers in vitro owing to their immobility and that oligomerization could be important for both their localization in the tubular domains of the ER and for their ability to form tubules (Shibata et al 08). Furthermore, the overexpression of Nogo-A abolishes peripheral ER sheets and leads to the ER morphology modifications (in COS cells) and significantly impaired the NE expansion at the end of the mitosis (Anderson and Hetzer 08;Voeltz et al 06). Finally, it has been demonstrated that this function ER-related of Nogo-A seems to be conserved from amphibians to mammals because the same cellular expression pattern was also found in Xenopus (Klinger et al 04).

Then, direct evidence support the idea that Nogo-A, as other reticulons, may play a role in nuclear envelope growth and assembly because it is also localized into high curvature membranes. In fact, in Xenopus, Nogo-A is also localised to junctions between membranes structures and at the edges of flattened membrane of oocytes and egg extracts associated with growing nuclear envelope (Kiseleva et al 07;Voeltz et al 06). In addition, highly curved membrane regions of forming or growing nuclear envelope preferentially contain Nogo-A and antibodies directed against Nogo-A perturbs the nuclear envelope growth (Kiseleva et al 07).

In summary, it appears that Nogo-A is a multifunctional protein. In oligodendrocytes, Nogo-A inhibits the neurite outgrowth; in the ER, Nogo-A is responsible of the network formation and maintenance of the peripheral tubular ER shaping and finally Nogo-A plays a role in nuclear envelope growth and assembly. It remains thus possible that other cellular functions could be related to Nogo-A in the future.

Neurotrophins infusions as a strategy against SCI consequences

The neurotrophic theory

Working with neurotrophic factors, also called neurotrophins, imply to know about the neurotrophic theory. The concept of this theory is based on the idea that supplies of neurotrophic factors delivered by their cellular environment are related to survival, proliferation, differentiation of the developing or injured neurons as well as promoting the axonal growth, synaptic plasticity and neurotransmission (Jones et al 01). The neurons might obtain trophic substances via different sources such as (Fig. 23):

1. From afferent neurons via an anterograde transport
2. From themselves via an autocrine loop
3. From their innervated neurons via a retrograde transport
4. From their ensheating glia cells

Figure 23: Drawing representing the possible sources for trophic support. The arrows correspond to the trophic influence. The neurotrophic factors are consequently secreted proteins (Korsching 93).

What are the neurotrophins?

The neurotrophins consist of a small family of five related molecules named respectively nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), neurotrophin-4 also known as neurotrophin-5 (NT-4/5) and neurotrophin-6 (NT-6) (Gotz et al 94). In addition to these classical molecules, others neurotrophic factors have been shown to have neurotrophic effects such as cytokine growth factors (CTNF) or the glial cell-line derived neurotrophic factor (GDNF).

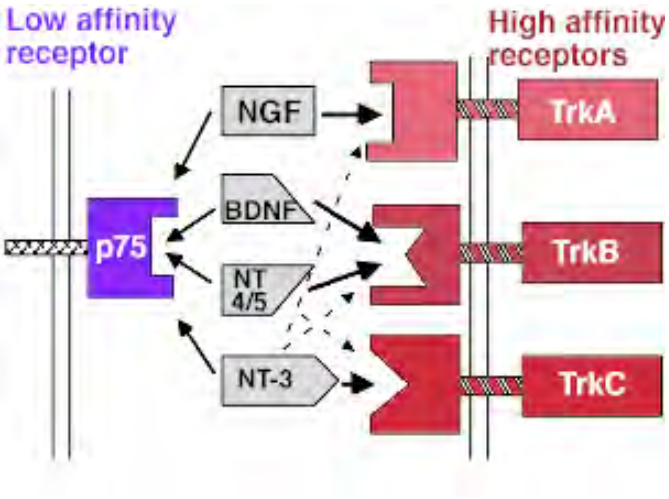

Figure 24: Neurotrophins and their corresponding receptors. The neurotrophins are representing in grey boxes and their high affinity trk receptors are symbolized by red boxes (TrkA, TrkB and TrkC). The bold arrows indicate strong interaction between the neurotrophins and its corresponding trk receptor, whereas dashed arrows indicate weak interactions of the neurotrophins and others trk receptors. All the neurotrophins bind to the low affinity receptor p75 (thin arrows) (Gilbert 06).

The neurotrophins exert their effects through a family of tyrosine kinase (trk) receptors. There are three kinds of trk receptors (Fig.24):

1) The receptor trkA which is selective for NGF
2) The receptor trkB which is selective for BDNF and NT-4/5
3) The receptor trkC which is the receptor for NT-3

In addition, NT-3 can also bind to trkA and trkB receptors but with lower affinity than the trkC receptor. In the same way, trkA can be also the receptor of NT-4/5 but with lower affinity than the trkB receptor (Fig. 24 dotted arrows). Moreover, the issue is made more complicated by the existence of isoforms of trkB and trkC receptors, which lack the cytoplasmic tyrosine kinase catalytic region (Barbacid, 1995; Barbacid, 2002; Gilbert, 2006). These receptors are found throughout the developing body as well, and it is not known if these noncatalytic forms of the receptors act as agonist or inhibitor.

Finally, in addition to the three trk receptors, there is also a common not cytoplasmic trk low affinity receptor to four neurotrophins called p75 (Fig. 24) (Chao and Hempstead, 1995; Greene and Kaplan, 1995; Segal and Greenberg, 1996). Even if this receptor does not have a trk catalytic domain, it is able nevertheless to mediate the neurotrophin signals. P75 was first identified to bind the human NGF receptor (Johnson et al 86). The roles of this receptor are controversial, as it may also be involved in promoting or downregulating the response to the neurotrophin.

P75 may function by increasing the affinity of the trk receptors for their respective neurotrophins, it may bind the neurotrophins and prevent them from binding to the high affinity receptors (Gilbert 06). An emerging concept concerning the neurotrophins receptor is that the trk receptors preferentially bind mature neurotrophins while p75 has more affinity to bind proneurotrophins (neurotrophin precursors) in order to elicit opposite biological responses such as long-term depression (LTD) versus long-term potentiation (LTP) (Fig. 25) (Lu et al 05).

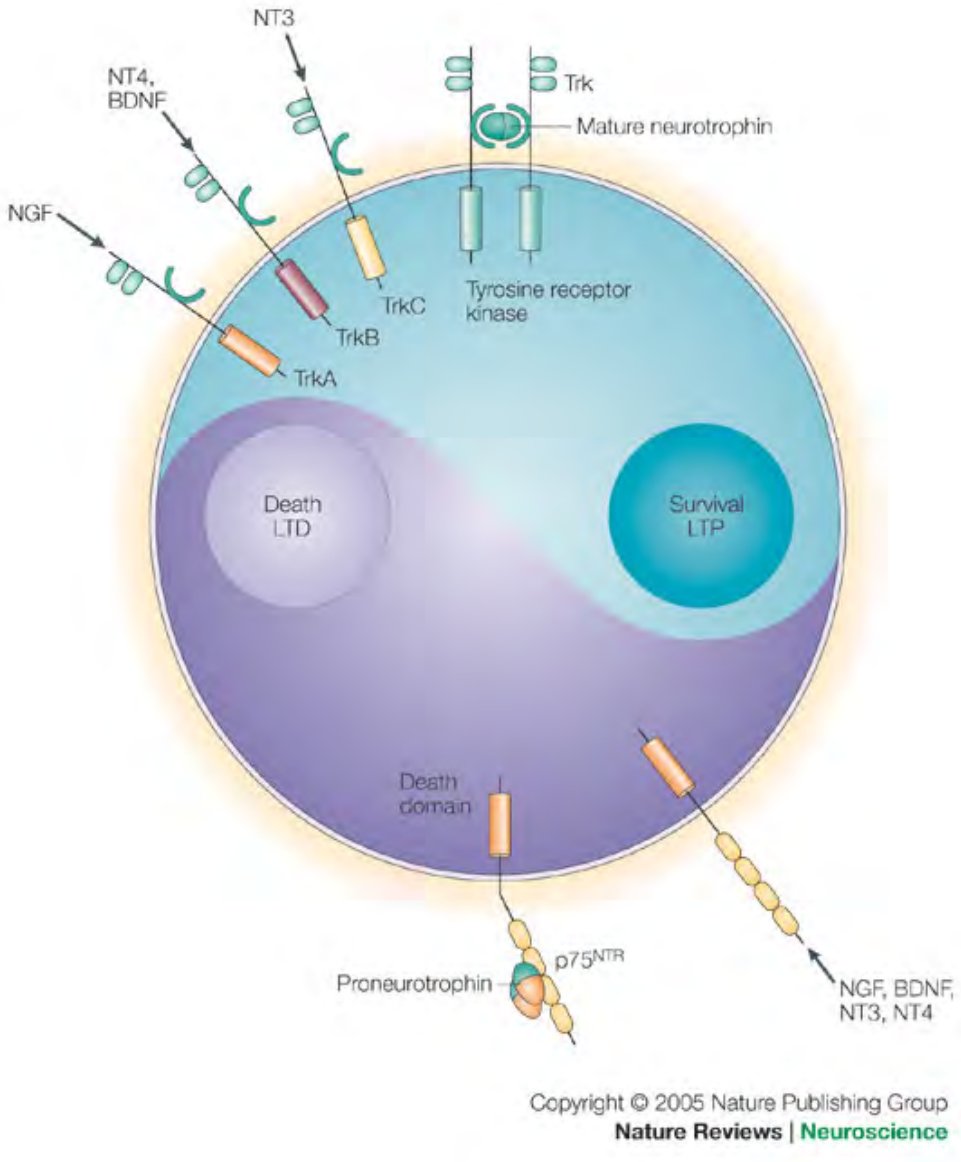

Figure 25: The different trk receptors preferentially bind to specific mature neurotrophins whereas the p75 receptor binds also to these proteins but to their precursor molecules too, resulting contradictory effects to those mediated by the trk receptors. The ligand-induced receptor dimerization results in trk activation (Lu et al 05).

Brain-derived neurotrophic factor (BDNF) as a strategy to improve regeneration after a SCI

NGF was the first member of the neurotrophic factors to be discovered (Levi-Montalcini and Hamburger 51). Thirty-one years later, a second member of this family purified from pig brain was identified and named BDNF (Barde et al 82). As NGF, BDNF is a homodimeric polypeptide implicated in neural cell growth and differentiation (Fig. 26B). In human, the bdnf gene is located on the chromosome 11p and the BDNF protein shares about 50% amino acid identity with the other member of the neurotrophic family (Binder and Scharfman 04). Similarly to the nogo gene, eight different BDNF mRNAs were identified in 1993 to rise from the bdnf gene via the alternative usage of four promoters and differential splicing control (Timmusk et al 93). However, in 2007 Aid and her co-workers proposed a novel nomenclature of the various exons previously named by Timmusk et al. because they showed the existence in rodent of three novel exons rising from the same gene (Aid et al 07). As a consequence, rodents have eleven BDNF exons (Fig 26A). The use of different promoters allows a tissue-specific expression of BNDFs' exons. For example, the exons I, II and III (Timmusk nomenclature) are predominantly expressed in the brain while the exon IV (Timmusk nomenclature) is found in heart and lung (Timmusk et al 93).

In adult mouse and rat brain, highest levels of BDNF mRNAs are found in neurons of the hippocampus (Conner et al 97;Ernfors et al 90;Hofer et al 90;Kawamoto et al 96;Yan et al 97). Therefore, it is not a surprise if one of the multiple functions of BDNF is its involvement in brain plasticity-related processes including learning and memory (Tyler et al 02;Yamada et al 02). BDNF is also involved in a variety of pathologies such as Alzheimer, Huntington and Parkinson's diseases but also in depression, drug addiction or epilepsy (Bibel and Barde 00;Binder and Scharfman 04;Castren 04;Cattaneo et al 05;Murer et al 01;Russo-Neustadt and Chen 05).

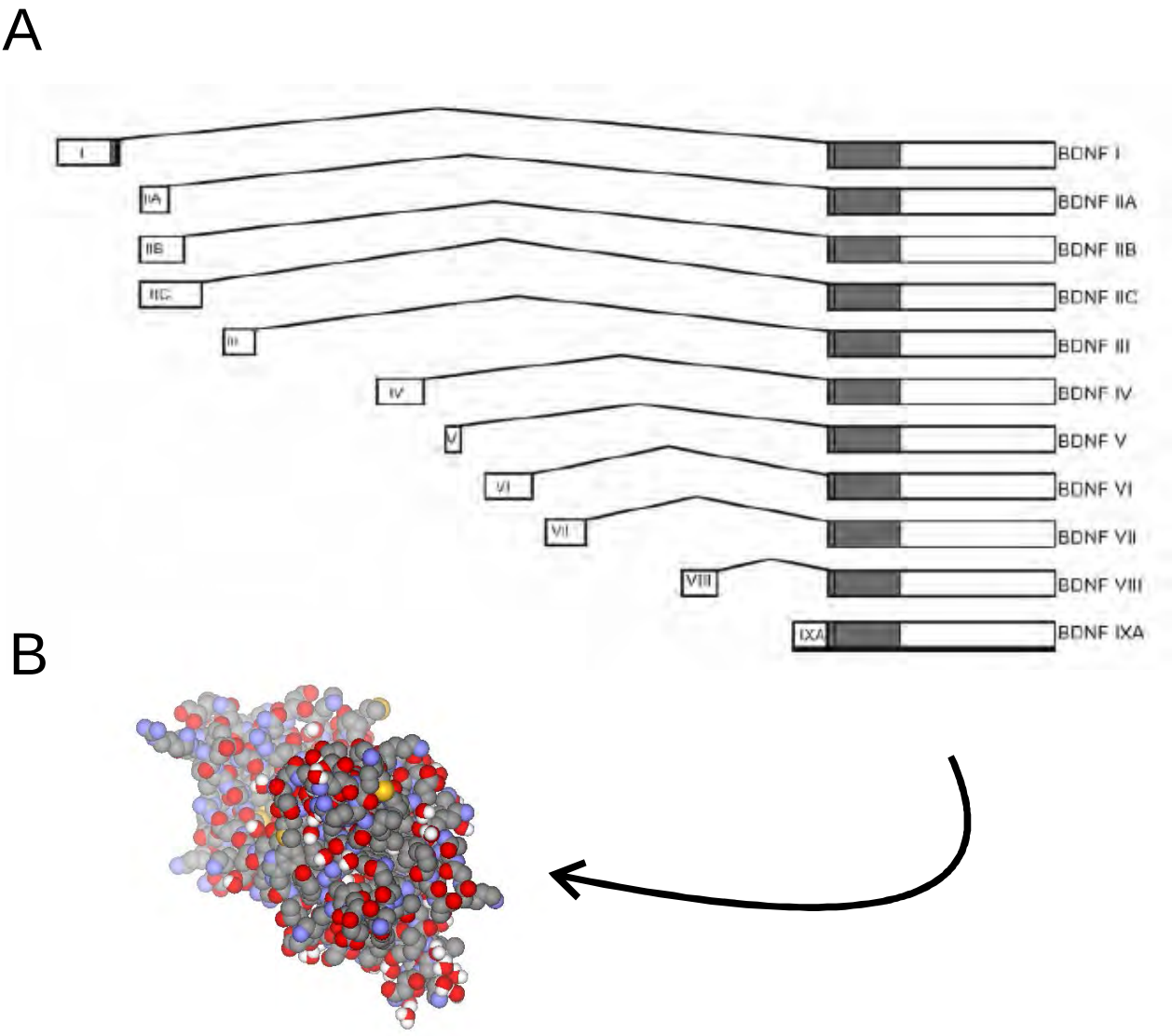

Figure 26: A) Rodents possess eleven BDNF exons (Aid et al 07). B) The homodimeric structure of the BDNF protein. (Taken from http://en.wikipedia.org/wiki/File:1BND.png)

In the spinal cord, BDNF interacts with various types of cells like sensory neurons, motoneurons, astrocytes, microglia/macrophages or oligodendrocytes. With sensory neurons, it has been shown that BDNF plays a role, as the other neurotrophins, in the development of these cells. Moreover, it has been shown that bdnf -/- animals lack slowly adapting mechano-receptors (Fig 27) (Carroll et al 98). In addition, BDNF appears to be related to dorsal root ganglion neurons because it has been demonstrated that it promotes their survival (Barde et al 82). With motoneurons, Sendtner et al. have shown that the administration of BDNF in newborn rats prevents the death of axotomized facial motoneuron (Sendtner et al 92). Finally, Dougherty et al. have shown that BDNF is expressed *in vivo* in normal adult spinal cord by different subsets of cells such astrocytes, microglia/macrophages and oligodendrocytes

(Dougherty et al 00). This study suggests that these cells are able to synthesize or take up BDNF themselves but it is not clear, for the moment, where BDNF comes from. Furthermore, this study has also investigated the expression of BDNF by these cells in injury conditions. The expression of BDNF was increased even six weeks after the injury in astrocytes and the microglia/macrophages of laminectomized rats only in the neighbouring areas of the lesion (0 to 10 mm). In contrast, this increase of BDNF was only visible during the first days after the injury in oligodendrocytes. Then, the expression of BDNF decreases and is largely deficient six weeks post-injury. Interestingly, this phenomenon was only visible at the injury-site, rising questions about the role of BDNF in SCI. Several lines of evidence suggest that increasing neurotrophin content following CNS injury may improve recovery. In fact, it has been demonstrated that following SCI, BDNF and NT-3 delivered either by injection (Schnell et al 94;Zhou and Shine 03), or gelfoam (Ye and Houle 97), or intrathecal infusion (Novikova et al 97), or modified fibroblasts (Kim et al 01;Liu et al 99;Nakahara et al 96) or Schwann cells (Menei et al 98), alone or combined with embryonic spinal cord (Bregman et al 98;Bregman et al 97) promote axonal regeneration and reduce atrophy of adult neurons projections in spinal cord especially on motoneurons and rubrospinal neurons cell-death (Diener and Bregman 94;Kobayashi et al 97;Novikova et al 97;Yan et al 92;Yan et al 93).

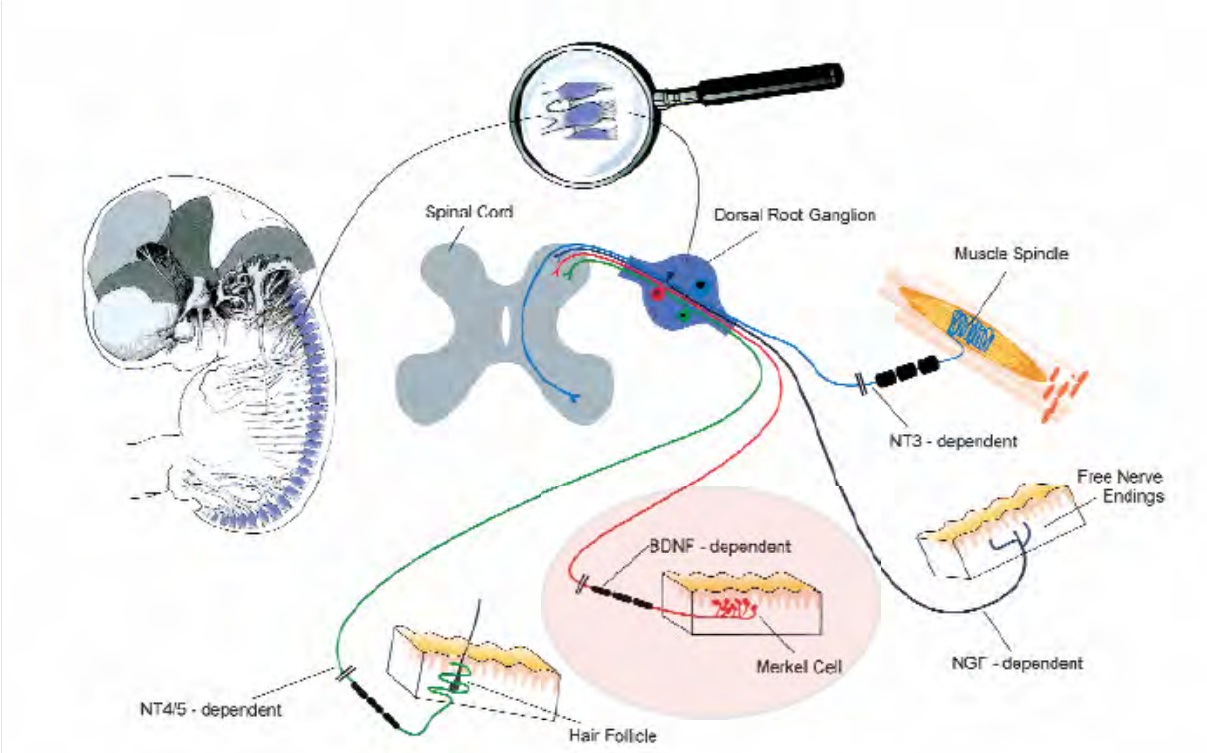

Figure 27: The development of the mechano-receptors terminating on Merkel cells is affected in BDNF KO mice (Carroll et al 98).

These studies rises the question of the real efficacy of BDNF delivered alone or combined with other substances or other neurotrophic factors as therapeutically strategy in SCI. Regarding the complexity and the disparity of the results published in the literature, the choice of using BDNF as treatment following SCI represents a real challenge. In a series of experiments, Novikova et al. have showed the complexity to use BDNF as a therapeutically strategy in rodents subjected to SCI. In 2000, this group shown that intrathecal delivery of BDNF in adult lesioned rats was ineffective on survival and atrophy of the ascending spinocerebellar neurons of the Clarke nucleus whereas the administration of NT-3 alone showed neuroprotective effect and the association of these two neurotrophic factors abolished the effect of NT-3 (Novikova et al 00). These results were surprising because the Clarke nucleus neurons expressed both types of receptors (*trkB* and *trkC*). Later, in another study, the same group has also observed that long-term infusion of BDNF and NT-3 in cervical SCI rats does not promote regeneration of the rubrospinal pathway or other descending tracts into implanted peripheral nerve graft in addition to significantly diminished sprouting of the injured rubrospinal axons rostral to the lesion site and decreased the regenerative response of intrinsic spinal cord neurons (Novikova et al 02). Similar results were obtained by Bradbury et al (Bradbury et al 98). An intrathecal infusion of BDNF was inefficient on the cell atrophy and the number of surviving cells on axotomized spinal cord neurons, while NT-3 infusion shows opposed results but both *trkB* and *trkC* receptors were reported to be expressed in the neurons of interest. It has been suggested that these results could be the consequence of an antagonistic regulation between these different neurotrophins (Giehl et al 01). It is however very surprising to observe that these studies are in complete contradiction with others, like the study conduced by Zhou and his coworkers (Zhou and Shine 03). In fact, this group has shown that overexpression of BDNF in rat sensorimotor cortex (near the CST soma) together with overexpression of NT-3 at the lumbar spinal level significantly amplifies the axonal sprouting of the injured CST compared to that induced by the NT-3 alone. Likewise, a recent study performed on primates has shown that implants of autologous BDNF/NT-3-secreting cell grafts placed within the sites of a C7 hemisection lesions promote regeneration of axons locally into the lesion and reduces the atrophy of the CS soma cells (Brock et al 10). Thus, it appears that use BDNF as a therapeutical treatment following SCI is a delicate purpose because it appears that this neurotrophin possesses various efficacy.

However, it seems that the CST reacts relatively favorably to this neurotrophic factor. Vavrek et al. have shown that following thoracic SCI, the application of BDNF to the cell bodies of the lesioned CST neurons in rats promotes collateral spouting of the axotomized CS axons rostrally to the lesion (Vavrek et al 06). Hibert and al. have shown that the infusion of BDNF on the motor cortex promotes sprouting of CS axons rostrally to the injury site but failed to produce regeneration into peripheral nerve grafts (Hiebert et al 02). Another study mentioned that injections of BDNF-hypersecreting human mesenchymal stem cells rostrally and caudally to a thoracic lesion in adult rat results in structural changes in brain and spinal cord associated with improved locomotor recovery and an increase of sprouting of the CST as well as serotonergic projections in addition to an increase of CS neurons cell survival in M1 cortex (Sasaki et al 09). In the same way, other studies have shown that the delivery of BDNF protects injured CST neurons and can improve functional recovery (Giehl and Tetzlaff 96;Hammond et al 99;Jakeman et al 98;Kim and Jahng 04;Namiki et al 00). Finally, an additional report has demonstrated that the exogenous application of different neurotrophins, including BDNF, increase the growth of raphespinal, coeruleospinal and CS axons (Bregman et al 97). Thus, it appears that the CST responds positively the BDNF given alone. However, very little is known on the effects of combining BDNF to the IN-1 antibody or other neurotrophins with this monoclonal antibody. An investigation conducted on neonatal rats had established that the regeneration of injured optic nerve fibers is improved after the association of IN-1 and BDNF (Weibel et al 94;Weibel et al 95). Contradictorily, regeneration of lesioned CS axons on rats subjected to SCI was improved by combining IN-1 antibody in conjunction with NT-3 but no effects were observed with the combination of IN-1 antibody with injections of BDNF under the same conditions (Schnell et al 94). On the other hand, Cui et al. have shown that the application of IN-1 antibody alone via peripheral nerve graft in adult rodents failed to enhance axonal regeneration in transected retinal ganglions axons while a combined application of both IN-1 and the ciliary neurotrophic factor (CTNF) showed a synergic effect (Cui et al 04). However, Satoh et al. have shown that the mRNA levels of Nogo-A, Nogo-B, Nogo receptor (NgR) and the neurite growth-associated protein GAP-43 were unchanged *in vitro* in the NTera2-derived differentiated human neurons following 48-h exposure of 100ng/ml of BDNF (Satoh and Kuroda 02). Thus, in the view of these different results, the issue of combining both BDNF and antibodies directed against anti-Nogo-A as a therapeutical treatment for animals subjected to SCI appears to be very complex.

Study regeneration and functional recovery: why choose nonhuman primates?

It was for a long time believed that spinal cord damages were incurable. However, the experimental studies conducted during the last years have shown the existence of potential and even promising treatments. All these promising treatments are first assessed on animal models. In fact, animals, rodents in particular, offer the opportunity to test potential treatments while decreasing the variability by being as reproducible as possible. Among animal models, nonhuman primates represent the ultimate animal model before testing it on human. In fact, the general organisation of the motor system of the nonhuman primates is more analogous to human compared to those of rats or mouse for instance. Although the comparison between the rodent and the primate motor system reveals many similarities, some evolutionary changes are evident when comparing the CST of these two species. For instance, through the spinal cord evolution, the CST undergoes a location change moving from the dorsal column (rodent) to the lateral column in monkeys (Rouiller et al 96). Additionally, this tract also increased its size and fibers number and direct connections to motoneurons have emerged leading changes like an enhancement of manual dexterity (Fig: 28) (Courtine et al 07). Even if rodents are able to control their digit in order to catch some small rewards, this ability is far less developed as compared to that of primates, who are the only ones to perform the precision grip (Lemon 04). This capacity of monkeys to perform fine digits movements makes of monkeys the most pertinent animal model when investigating behavioural, particularly manual, recovery. Another behavioural characteristic similar to human which represents an advantage of the nonhuman primate model used in science is the ability of monkeys to perform bipedal walking. Although the quadrupedal locomotion remains for this animal model the main way to walk, some monkeys like the Japanese *Macaca fuscata* can be trained to perform bipedal gait on a treadmill belt in order to compare the kinematics of multiple body segments (Nakajima et al 04). In addition, the evolutionary proximity between human and monkeys could also be relevant when looking at the possible side effects of a treatment given. For example, the sprouting resulting from the Nogo-A antibody therapy could cause aberrant connections and generate pathologies like allodynia. Finally, there are ethical and economical justifications to conduct experiments on nonhuman primates before initiating preclinical tests in humans. In fact, the financial cost associated with a single human clinical trial could support several primate studies and generate more rapidly significant results (Courtine et al 07).

In conclusion, the use of nonhuman primates in research is the source of ethical concern because monkeys are evolutionary very close to human. This explains why other animal models should be used first to develop the current treatment like rodent and feline. However, for some researches, no animal model alternatives are available, thus justifying the use of monkeys in experiments conducted on CNS for example. In addition, nonhuman primates offer the unique opportunity the test the finger-thumb precision grip as well as non-constrained bipedal locomotion. Finally, looking the cost generated by a treatment as well as its side effects apparition, its efficacy and its safety, monkeys remain the best solution before clinical trials in humans.

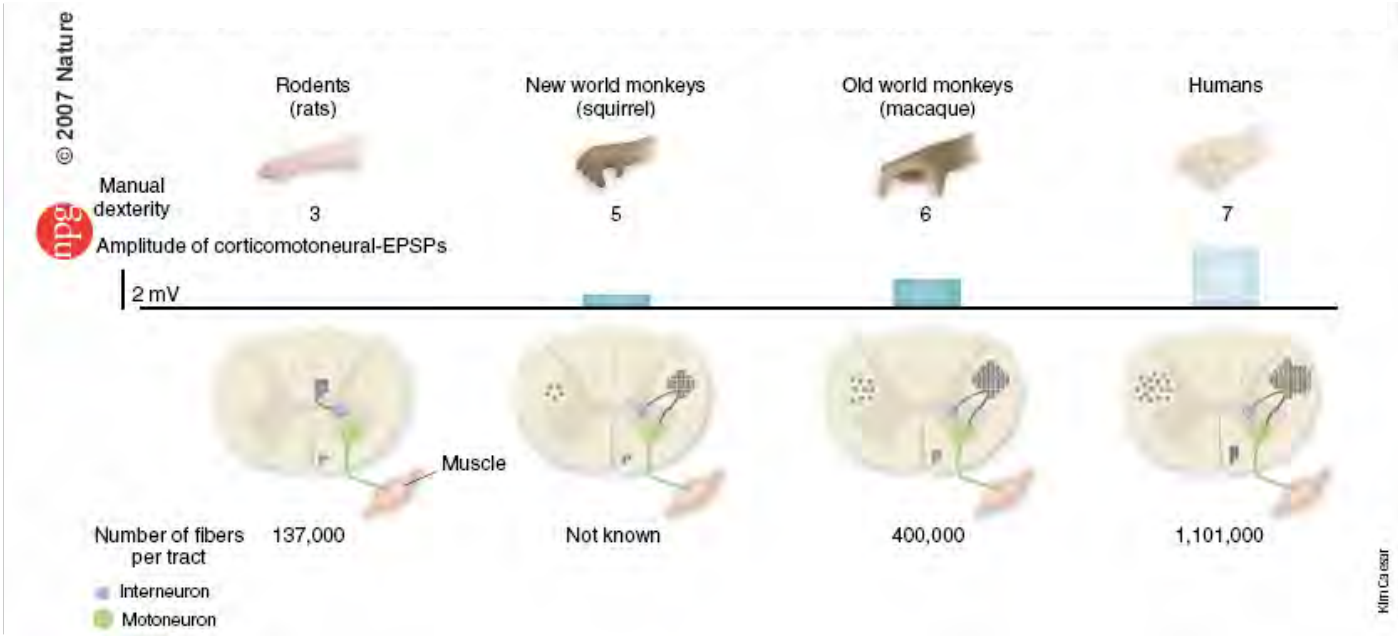

Figure 28: Relationship between the development of the corticospinal tract and the emergence of fine motor control abilities. In rodents, there are no direct connections between corticospinal neurons and the cervical motoneurons that innerve forelimb muscles. Thus, interneurons relay cortical input to motoneurons. In the evolution of the corticospinal tract in nonhuman primates and human, direct corticospinal connections with motoneurons have emerged, together with and increased in size and number of the corticospinal fibers. Accordingly, the size of the excitatory postsynaptic potential (EPSP) elicited by cortical neurons on motoneurons has increased during primate evolution. Furthermore, most of the CST fibers in rodents travel in the dorsal column. In contrast, the primate CST is mostly located in the lateral column, and a significant proportion of the corticospinal fibers (10-20%) descend ipsilaterally. Development of the CST correlates with the improvement in the index of dexterity, particularly in the ability to perform finger-thumb precision grip (legend cited from (Courtine et al 07).

Aims of the thesis

This work addresses several questions related to the effects of two types of potential regenerative treatments to adult macaques that have been subjected to an incomplete section of the cervical cord. The first treatment consisted in the use of an anti-Nogo-A antibody alone and the second treatment in the use of a combination of an anti-Nogo-A antibody with the brain-derived neurotrophic factor (BDNF). In the next section, the questions investigated are presented in relation to one of three manuscripts, which form altogether the results section of the present thesis.

Question 1: Does an anti-Nogo-A antibody treatment prevent or reduce cell body shrinkage in the motor cortex of adult macaque monkeys subjected to unilateral cervical cord lesion?

Following spinal cord injury (SCI), it has been demonstrated in adult nonhuman primates that a unilateral lesion performed at the cervical cord level C7/C8 and interrupting most of the corticospinal (CS) axons does not lead to measurable SMI-32 positive cell loss in layer V in the primary motor cortex (M1), meaning that most of the pyramidal CS neurons located in the layer V in M1 survived to their axotomy (Wannier et al 05). However, in the same study, the analysis of the cross-sectional somatic area of the same neurons has revealed that lesioned animals that have been treated with a control antibody exhibit a significant difference of soma area across their hemispheres: the cells were smaller in the contralesional hemisphere as compared to the ipsilesional hemisphere, whereas in intact animals (i.e. uninjured and untreated) no significant statistical difference was observed. In other words, the soma area of the layer V neurons in M1 of monkeys that were subjected to the unilateral cervical cord lesion shrinks in the hemisphere affected by the lesion. Afterwards, it has been shown in a serie of experiments that monkeys subjected to SCI but treated this time with a monoclonal antibody directed against Nogo-A exhibited sprouting and regeneration of CS axons as well as behavioural recovery (Fouad et al 04;Freund et al 06;Freund et al 09;Freund et al 07). As an increased arborisation of CS fibers could improve their access to neurotrophic factors, it is possible that the anti-Nogo-A antibody treatment could also prevent or reduce the soma shrinkage observed in the contralesional hemisphere in M1 of injured monkeys.

Question 2: In adult macaque monkeys subjected to unilateral cervical cord lesion, does anti-Nogo-A antibody alone or combined with BDNF improve the number and/or the cumulated length of SMI-32 positive fibers observed into the lesion territory?

In the adult mammals, after a SCI, axotomized nerve fibers fail to regenerate. The scar tissue formed after such a lesion is known to contribute to this failure because it contains and/or produces various growth inhibiting factors (Fawcett and Asher 99). Focusing on monkey's CS axonal regeneration, it was shown that an anti-Nogo-A antibody treatment promote regenerative sprouting rostrally and caudally to the spinal lesion (Fouad et al 04;Freund et al 06;Freund et al 09), but that only rare CS fibers can be found inside the scar tissue (Freund et al 07). Despite the fact that the scar tissue appears impermeable to the CS nerve fibers even in presence of the anti-Nogo-A antibodies, it remains however possible that neurons distinct from CS neurons possess the ability to regenerate and to grow into or even through the scar tissue. Moreover, it is possible that the regenerating capacity of these "non-CS fibers" to colonize the scar tissue can be strengthen by an anti-Nogo-A antibody treatment alone or combined with BDNF.

Question 3: In adult macaque monkeys subjected to unilateral cervical cord lesion, does BDNF interact with the anti-Nogo-A antibody treatment on the regenerative capacity of CS axons and on the functional recovery?

In rodents and monkeys with SCI, neutralizing the neurite growth inhibiting molecule Nogo-A with a specific antibody promotes both the regeneration of the CS nerve fibers and functional recovery (Bergman et al 95;Brösamle et al 00;Fouad et al 04;Freund et al 06;Freund et al 09;Liebscher et al 05;Schnell and Schwab 90). However, the rostro-caudal extent of the spinal cord covered by the regenerating CS fibers remains limited and the treatment does not prevent most axotomized CS neurons from shrinkage (Beaud et al 08). A different line of studies has shown that neurotrophic factors such as BDNF may induce neurite growth and protect axotomized neurons (Brock et al 10;Giehl and Tetzlaff 96;Hammond et al 99;Hiebert et al 02;Lu et al 01;Vavrek et al 06;Yan et al 92). This raises the question as to whether the effects obtained by neutralizing Nogo-A can be strengthened by adding BDNF to

the antibody treatment. To investigate whether the effect obtained by neutralizing Nogo-A can be reinforced by BDNF, the functional recovery of manual dexterity of lesioned monkeys (reflecting the integrity of the CS system) was investigated as well as the amount of sprouting of the BDA positive CS axons located rostral to the lesion, at the C3 level.

Material and methods

Overview of the experimental protocol

Detailed information concerning each experimental procedure is provided in the Material and Methods section of each following chapter.

Behavioural testing and pre-lesion procedures

The experiments were carried out on two species of monkeys: *Macaca mulatta* (rhesus monkeys) and *Macaca fascicularis* (cynomolgus monkeys). The monkeys were obtained from our own colony in our animal facility or were purchased from two certified suppliers (BioPrim; 31450 Baziège; France and Harlan, Buckshire USA), with the authorization to import the animals delivered by the Swiss Federal Veterinary Office (BVET, Bern, Switzerland). In total, 21 young adult macaque monkeys (3.5 to 6.9 years at sacrifice) of either sex were subjected to behavioural testing. Among these animals, all were subjected to spinal cord lesion, eight received a control antibody, eight received an anti-Nogo-A antibody and five received a combined treatment of the anti-Nogo-A antibody and the brain-derived neurotrophic factor (BDNF). To reduce inter-animal variability, "pairs" of monkeys were formed, placed under the responsibility of the same experimenter, and were subjected simultaneously to the same experimental protocol. During all the behavioural procedure and during a part of the anatomical analyses, the experimenter was blind to the treatment given to the animals, meaning that the type of treatment administered to each animal remained unknown during this period. During the whole study, the general health condition of the monkeys was assessed quantitatively via their body weight, which was measured regularly (generally before each daily behavioral session). A loss of 10 % body weight was set as a criterion for interrupting the experiment (mainly the behavioural session). This criterion was never encountered in the present study. Indeed, an increase of body weight was the normal observation. In addition, the monkeys did not show any reduction of co-operativity and motivation to perform the behavioral tasks. No signs of epilepsy, aggression or excessive alarm with respect to the experimenter and animal keeper were observed.

The monkeys' identification codes refer to individual monkeys and contain for sake of clarity, a "**C**" or an "**A**" or "**AB**" at fourth digit position, indicating whether the monkey was respectively **c**ontrol antibody-treated or **a**nti-Nogo-A antibody-treated or treated with a combinatory treatment composed of **a**nti-Nogo-A antibody associated with the neurotrophic factor **B**DNF. In addition, some anatomical investigations were also performed on intact animals. In these cases, "**I**" was placed at fourth digit position of their identification codes indicating that they are **i**ntact (i.e. uninjured and untreated). However, during the course of the experiments, the animals had different names from which the experimenter could not deduce which antibody was infused, explaining why the initials of these other names appear in the identification codes at a digit position after the treatment information.

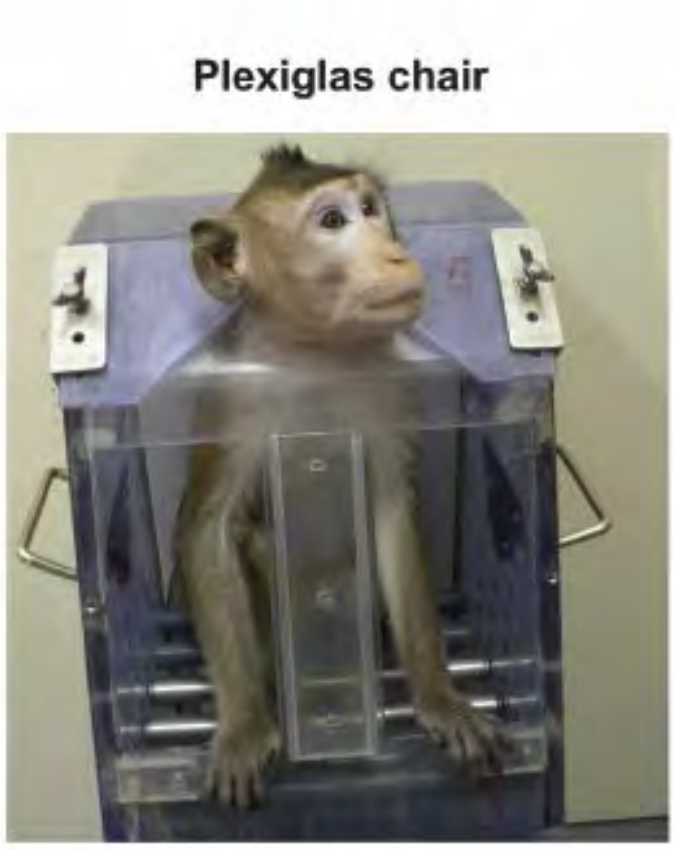

Figure 29: Plexiglas primate chair used during the behavioural tests. The monkey performs the tasks using one hand only or, in rare cases, both hands.

Retention and experimental procedures were in accordance with the Swiss federal law for animal protection and controlled by the local veterinary authorities. Monkeys were housed in our animal facilities in rooms of 12 m^3, each containing usually 2-4 animals free to move in the room and to interact with each others. Usually, animals of the same sex were housed together. The retention room contained two smaller temporary cages into which the monkeys were transferred by the animal caretakers at 7 am during weekdays. During the first couple of weeks, the monkeys were habituated to the animal housekeeping environment and their room-mates. Once habituated to their new environment, the animals were trained to freely transfer from the smaller temporary cage into a Plexiglas primate chair (Fig. 29). This step usually takes three to four weeks to learn. Then, once the monkey feels comfortable in the Plexiglas primate chair, one can start the training to the different behavioural tasks into the laboratories. At the beginning, all animals were trained to perform alternatively with each of the two hands a unimanual dexterity task called "modified Brinkman board task" (Fig. 30). This test is built as followed: a Perspex board (10 cm x 20 cm) was pierced of 50 randomly distributed wells, each filled with a food pellet at the beginning of the test. Twenty-five wells were oriented horizontally and twenty-five vertically. The dimension of the wells was 15 mm long, 8 mm wide and 6 mm deep. Retrieval of the food pellets required fractionated finger movements, consisting normally in a dexterous opposition of the index finger and the thumb, corresponding to the so called "precision grip". This manual prehension dexterity task was executed daily, alternatively with one and then with the other hand, 4 to 5 times per week for several months before and after the unilateral cervical cord lesion. Scoring the animal's performances started after the monkeys were habituated to the test. At least 40 sessions were taken to establish the level of the pre-lesion baseline. The performance of each hand was videotaped and the behavioural scores were established by counting the number of wells from which the food pellets were successfully retrieved and brought to the mouth during 30 seconds (Fig. 31). Then, once the manual skill of the modified Brinkman board task was acquired, the monkeys were trained to perform other manual tasks like the Rotative Brinkman board test or the hidden Brinkman board test (Fig. 30). These other manual tests assess additional aspects of the manual dexterity. However, in the present work, only the behavioural data related to the modified Brinkman board task are presented. The monkeys had free access to water and were not food deprived but had a unique daily meal. The rewards obtained during the behavioural tests represented the first daily access to food. After the tests, the monkeys received additional food (fruits, cereals).

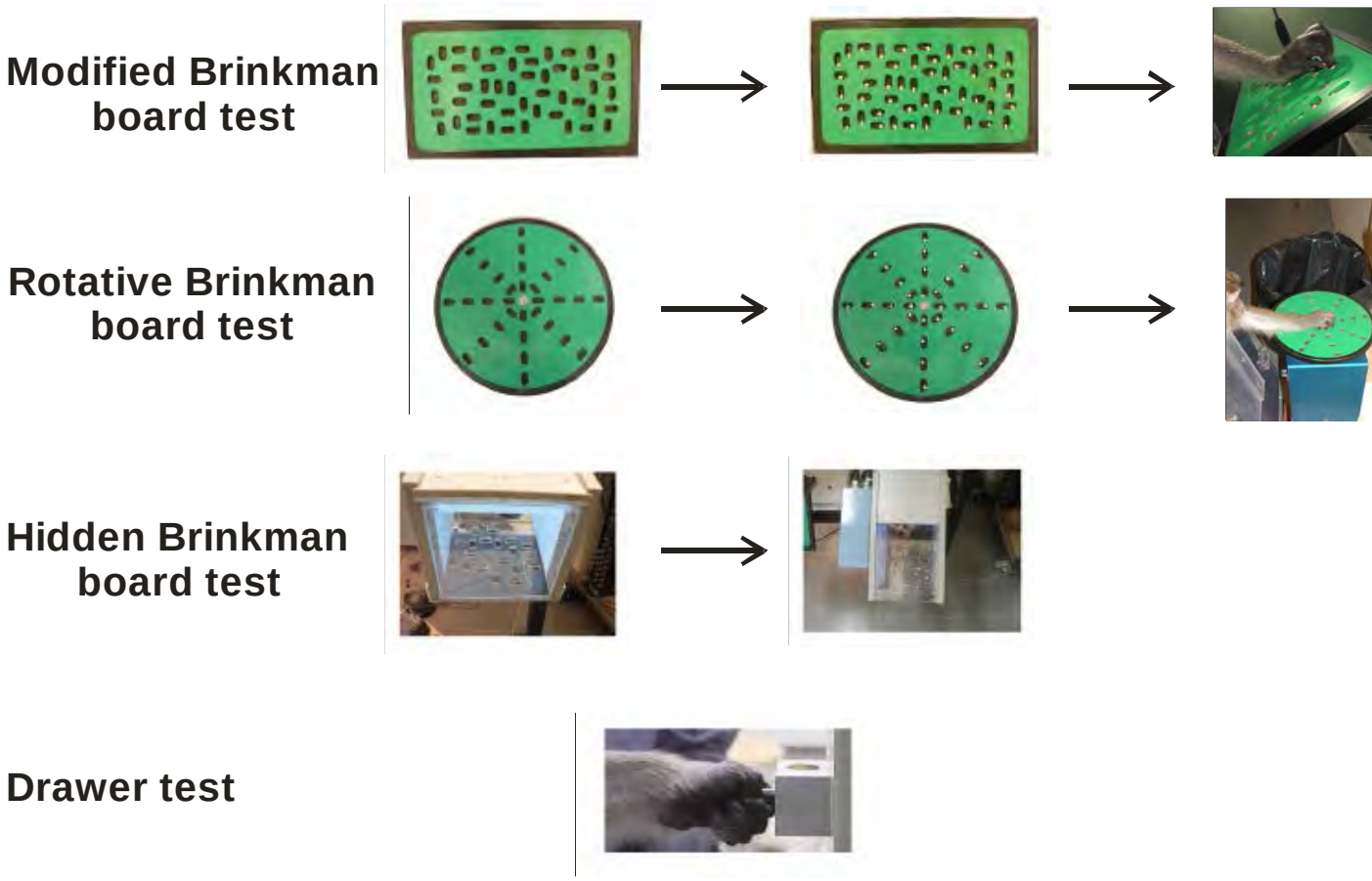

Figure 30: The monkeys were trained to perform different manual tasks[4].

Surgical procedures, treatments administration and post-lesion procedures

After the monkeys reached a stable level of performance, as indicated by a plateau of behavioural performance (Fig.31), the animal was subjected to an unilateral cervical cord lesion. Therefore, the behavioural scores that precede the lesion are considered to represent the pre-lesion behavioural performance, whereas the behavioural scores that follow the lesion are considered as the post-lesion behavioural performance. The lesion was performed at the cervical level at the C7/C8 spinal cord segments level. The injury affects most of the corticospinal tract (CST) axons located on the ipsilesional side of the animal. Immediately after the transection, an osmotic pump containing one of the three possible treatments was placed under the skin and the catheter of the osmotic pump was positioned few millimetres above the lesion. Independently of the treatment, the catheter of the osmotic pump remained four weeks in the animal's lesion site. Few days after the lesion, the animal began to recover even if it was control antibody treated. During this period the manual dexterity score re-increased progressively. Generally, the recovery period extends over 60 to 80 days. After that,

[4] http://www.unifr.ch/neuro/rouiller/research/motorcontcadre.php

the manual dexterity scores remained again stable (post-lesion plateau) and the animal was sacrificed. However, before the sacrifice, when the acquisition of behavioural data was finished, the animal received injections of anterograde tracers in M1 and remained alive during a certain period in order to allow the transport of the tracers. Under aseptic conditions in fully anesthetized animals, biotinylated dextran amine (BDA) was injected in the contralesional motor cortex, whereas dextran fluorescein was injected into the ipsilateral motor cortex. After the injections, a survival period of approximately 70 days was used to allow the different tracers to diffuse into the spinal cord tissue and obtain an optimal labelling of the descending CST. At the end of this survival period, the animals were sacrificed by lethal injection of pentobarbital and perfused transcardiacally, followed by a 4% paraformaldehyde solution in phosphate buffer perfusion and then continued with solutions of the same fixative containing increasing concentrations of sucrose. The brain and the spinal cord were dissected and placed in a 30% solution of sucrose of cryoprotection for few days. Frozen sections (50 µm thick) of cervical cord (approximately the segments C6-Th2) were cut in the parasagittal longitudinal plane and collected in three series for later histological processing. Additional material originating from segments located rostrally and caudally to the spinal cord lesion were cut in the frontal plane at 50 µm thick and collected in three series. BDA and Dextran-Fluorescein staining were revealed in two series of spinal cord sections, whereas the third serie of spinal cord section was immunohistochemically stained with the SMI-32 antibody (Sternberger Monoclonals, Baltimore, MD). The SMI-32 staining was chosen to reconstruct the extent of the lesion because the lesion contour was better defined as compare to the BDA staining. Similarly, a series of brain sections was also stained with the SMI-32 staining in order to visualize the layer III and V of pyramidal neurons. In fact, the epitope recognized by the SMI-32 antibody lies on non-phosphorylated regions of neurofilament protein and is only expressed by specific categories of neurons (Campbell and Morrison 89;Tang et al 06).

All the anatomical analyses were conducted using Neurolucida® software. BDA labeled sections rostral to the cervical cord lesion were analyzed to assess the regenerative sprouting of the severed CS axons, whereas the soma surface of neurons of the primary cortex (M1) as well as their number and the fibers visible into the lesion site were analyzed using SMI-32 labeled brain and spinal cord tissue.

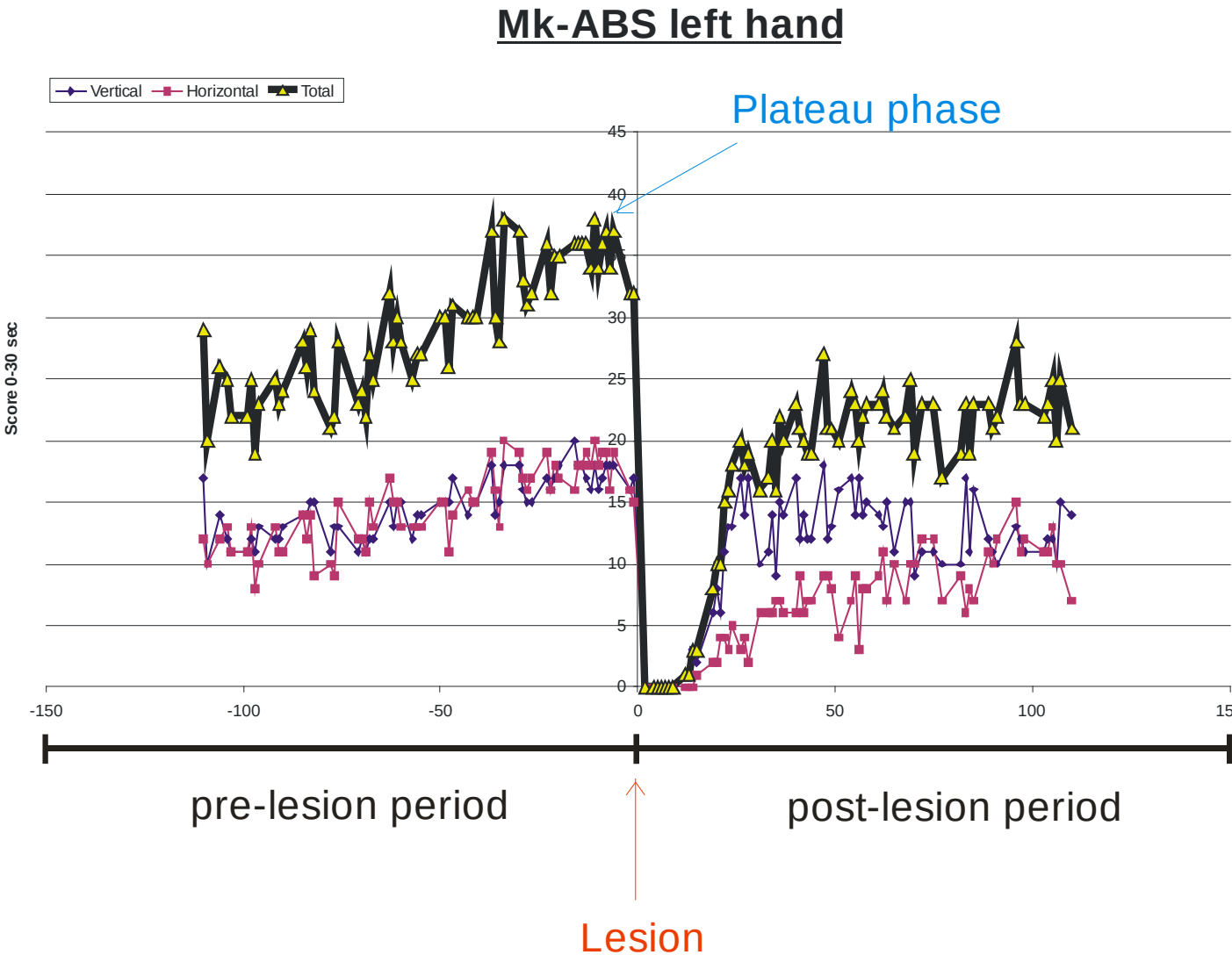

Figure 31: Graph representing the retrieval scores obtained for the monkey Mk-ABS for the left hand. In a pre-lesion phase, the animal learned the behavioural task (modified Brinkman board test) until it reached a plateau phase. At that time, a unilateral cervical cord lesion was performed affecting most of the corticospinal axons on the left side in this example. Consequently, the manual dexterity score fell dramatically and the scores remained very low several days following the lesion. Then, the animal recovered progressively during a post-lesion phase and the score progressively increased until a certain time where the animal stopped to recover further. At this time, the animal was sacrificed and the total functional recovery was calculated and plotted as a function of the size of the lesion.

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Results

Manuscript 1:

Anti-Nogo-A antibody treatment does not prevent cell body shrinkage in the motor cortex in adult monkeys subjected to unilateral cervical cord lesion.

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Anti-Nogo-A antibody treatment does not prevent cell body shrinkage in the motor cortex in adult monkeys subjected to unilateral cervical cord lesion

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Abstract

Background: After unilateral cervical cord lesion at the C7/C8 border interrupting the dorsolateral funiculus in adult monkeys, neutralization of Nogo-A using a specific monoclonal antibody promoted sprouting of corticospinal (CS) axons rostral and caudal to the lesion and, in parallel, improved functional recovery. In monkeys lesioned but not treated with the anti-Nogo-A antibody, the CS neurons in the contralesional primary motor cortex (M1) survived to the axotomy, but their soma shrank. Because the anti-Nogo-A treatment induces regeneration and/or sprouting of CS axons, it may improve access to neurotrophic factors. The question therefore arises as to whether anti-Nogo-A treatment prevents the soma shrinkage observed in the contralesional M1?

Results: Using the marker SMI-32, a quantitative and qualitative anatomical assessment of the pyramidal neurons in the layer V (thus including the CS cells) in M1 was performed and compared across three groups of animals: intact monkeys (n = 5); monkeys subjected to the cervical cord lesion and treated with a control antibody (n = 4); monkeys with the cervical lesion and treated with anti-Nogo-A antibody (n = 5). SMI-32 positive neurons on the side contralateral to the lesion were generally less well stained than those on the ipsilesional hemisphere, suggesting that they expressed less neurofilaments. Nevertheless, in all three groups of monkeys, the amount of SMI-32 positive neurons in both hemispheres was generally comparable, confirming the notion that most axotomized CS neurons survived. However, shrinkage of CS cell body area was observed in the contralesional hemisphere in the two groups of lesioned monkeys. The cell surface shrinkage was found to be of the same magnitude in the monkeys treated with the anti-Nogo-A antibody as in the control antibody treated monkeys.

Conclusion: The anti-Nogo-A antibody treatment did not preserve the axotomized CS cells from soma shrinkage, indicating that the anti-Nogo-A antibody treatment affects morphologically the axotomized CS neurons mainly at distal levels, especially the axon collateralization in the cervical cord, and little or not at all at the level of their soma.

Background

The motor deficits associated with interruption of the CS tract at a segmental level in monkeys were assessed in several studies [1-10]. More precisely, a surprisingly good and rapid recovery of dexterous finger movements of the ipsilateral hand took place after hemi-section at C3 level in either newborn and juvenile monkeys [4,5], or in adult monkeys after hemi-section at C4/C5 [8] or C7/C8 level [9,10]. Immediately after the cervical hemi-section and later on during the recovery, there was a dramatic reduction of the CS projection to the hemi-cord caudal to the lesion [4], indicating that the spontaneous recovery of manual dexterity was not due to a substantial reconstruction of the lesioned projection but rather to enhancement of the transmission of information from cortex to spinal cord in a reduced number of CS and/or corticobulbospinal projections together with a contribution of a more effective use of spinal circuits.

As far as the fate of the axotomized CS neurons is concerned, some controversy can be found in the literature. Some earlier anatomical studies suggested that pyramidotomy [11,12] or cervical cord lesion [6,13] induced the death of a substantial part of the large CS neurons in the contralateral primary motor cortex (M1), amounting up to 70% loss [11]. In sharp contrast, other authors concluded that there was no retrograde degeneration with breakdown and loss of neurons after section of the CS tract [14-16]. In a recent study [17], the issue of the fate of axotomized CS neurons was re-examined in two monkeys using SMI-32 as a specific marker for pyramidal neurons. We found that, after unilateral lesion of the dorsolateral funiculus at cervical level (C7-C8), the CS neurons in the contralesional primary motor cortex (M1) survived the axotomy, but their soma shrank [17].

In a recent report, evidence was provided in monkeys that the functional recovery from unilateral cervical cord lesion and CS axonal sprouting can be enhanced by an antibody treatment neutralizing the neurite growth inhibitor Nogo-A [10], extending to the primates previous results obtained in the rat [18-21]. Indeed, several functional readouts of manual dexterity showed a faster and more complete recovery of manual dexterity in a group of six anti-Nogo-A antibody treated monkeys subjected to cervical hemi-section than in a group of six monkeys subjected to a comparable lesion but treated with a control antibody [10]. Such enhancement of manual dexterity promoted by anti-Nogo-A antibody treatment was associated with an axonal sprouting of CS axons in the cervical cord rostral and caudal to the lesion [10,22]. These new fibers could provide the axotomized CS neurons an augmented access to neurotrophic factors compared to that available to the axotomized CS neurons in control antibody treated animals. The goal of the present study was thus to investigate whether the anti-Nogo-A antibody treatment also exerts an effect on the CS neurons at the level of their soma in the contralesional motor cortex, for instance by preventing the cell soma shrinkage occurring in the monkeys subjected to cervical lesion and treated with a control antibody [17]. This hypothesis is plausible because the treatment induces regeneration and/or sprouting of CS axons and may thus increase the access to neurotrophic substances. Moreover, although the anti-Nogo-A antibody was delivered intrathecally in the cervical cord close to the lesion, the antibody was shown to reach the entire brain through the CSF circulation [23] and, therefore, it may have affected the CS cell bodies in the cerebral cortex as well.

Results

Cervical cord lesion

For the animals subjected to the unilateral spinal cord section, the extent of the lesion was assessed by reconstructing the incision site from histological sections (Fig. 1A). In most monkeys, the lesion completely transected the dorsolateral funiculus, thus corresponding to a complete transection of the CS axons originating from the contralesional hemisphere (as checked with BDA labeling of CS axons in most monkeys [10]). In one monkey (asterisk in Fig. 1A), the dorsolateral funiculus was not completely transected and therefore a few CS axons as well as RS axons were spared by the lesion. The other seven monkeys had a complete transection of the dorsolateral funiculus on the lesioned hemi-cord (Table 1), thus corresponding to a complete interruption of the main CS tract originating from the opposite hemisphere (as discussed in detail earlier in [10]). The monkeys Mk-AF and Mk-CC clearly had an incomplete lesion of the RS tract, whereas monkeys Mk-CS and Mk-CB possibly had an incomplete lesion of the RS tract (though transected at 95% at least). The RS tract transection was complete in the other four monkeys with complete lesion of the dorsolateral funiculus (Mk-AG, Mk-AC, Mk-AM and Mk-CH).

Do some axotomized CS neurons degenerate?

In a recent paper, we reported that the number of SMI-32 positive pyramidal neurons in layer V in M1 was comparable in the contralesional and in the ipsilesional hemispheres in two monkeys subjected to an unilateral cervical lesion (treated with a control antibody), and amounted to a comparable figure as in two intact animals [17]. In the present study, these data were extended to a total of four monkeys subjected to the cervical lesion (treated with a control antibody) and five intact monkeys (Fig. 1C; blue and green bars, respectively). In the group of intact monkeys, as expected the majority (four out of five) of animals did not show a statistically significant difference of SMI-32 positive neurons between the two hemispheres ($p > 0.05$, n.s. in Fig. 1C). However, in one intact monkey (Mk-

Table 1: List of cervical cord lesioned and intact monkeys included in the present study with identification code (same as in [10]).

	Mk-AG	Mk-AI	Mk-AE	Mk-AC	Mk-AM	Mk-CS	Mk-CB	Mk-CC	Mk-CH	Mk-JR	Mk-JE	Mk-JZ	Mk-JRh	Mk-JG
species	fasc.	fasc.	mul.	fasc.	fasc.	mul.	fasc.	fasc.	fasc.	mul.	mul.	fasc.	mul.	mul
Antibody Treatment	**Anti-Nogo-A (hNogo)**	**Anti-NogoA (hNogo)**	**Anti-Nogo-A (11 C7)**	**Anti-Nogo-A (hNogo)**	**Anti-Nogo-A (hNogo)**	**Contr.**	**Contr.**	**Contr.**	**Contr.**	-	-	-	-	-
Hemi-section total extent (%)	78	*	56	85	80	63	75	38	90	-	-	-	-	-
Completeness of dorsolateral funiculus section	Yes	*	Yes	Yes	Yes	Yes	Yes	No	Yes	-	-	-	-	-
RS and CS lesion extent (%)	100	*	73	100	100	87	93	61	100	-	-	-	-	-
Functional Recovery (%)	100	(100)*	57	100	96	22	78	83	53	-	-	-	-	-
Completeness of CS/RS section	Yes	*	No	Yes	Yes	Yes	Yes	No	Yes	-	-	-	-	-
Survival time after lesion (in days)	112	97	144	135	138	198	225	105	138	-	-	-	-	-
ICMS	no	no	yes	no	no	yes	no	yes	no	no	no	no	no	no

At the time of the experiment, the lesioned monkeys had different names, not indicating whether the animal was infused with the control or the anti-Nogo-A antibody, corresponding to a double blind procedure (except Mk-AF, Mk-CS and Mk-CC). New names were assigned to the monkeys during the writing of the manuscript to improve its readability, in consistency with [10].

Under species, "mul." is for macaca mulatta while "fasc." is for macaca fascicularis.

The five anti-Nogo-A antibody treated monkeys are in the five leftmost columns ("Anti-Nogo-A") with indication of which of the two antibodies was used (mAB I C7 or mAB hNogo-A), whereas the four control antibody treated monkeys ("Contr.") are in the next four columns. The five intact monkeys are in the five rightmost columns.

Total hemi-section extent was calculated as the percentage of corresponding hemi-cord affected by the lesion on the frontal reconstruction of the cervical cord.

To tentatively determine more precisely the extent of the RS and CS lesions a quadrant of the lateral funiculus in the white matter was outlined going from the dorsal to the ventral rootlet zone. This was indicated as RS and CS lesion extent.

Functional recovery was assessed here for manual dexterity based on the "modified Brinkman board" task (see [10]), by giving in percent the ratio of the post-lesion score to the pre-lesion score.

In the row "completeness of RS section", "No" and "Yes" indicates whether the RS transection was considered as partial or as complete, respectively (for further explanation see text).

Survival time: number of days separating the cord hemi-section from the sacrifice of the animal.

* In this animal the total hemi-section extent and consequently also the RS and CS extent could not be calculated for technical reasons (poor quality of histology). In addition in this same animal the lesion was too caudal and thus the 100% functional recovery cannot be interpreted.

ICMS is for intracortical microstimulation. "Yes" means that the corresponding monkeys were subjected to extensive ICMS both pre- and post-lesion in order to study the change of motor maps (somatotopy) in relation to the lesion (see [9, 28]).

IU), the number of SMI-32 positive neurons per unit length was significantly higher ($p < 0.05$) in the right than in the left hemisphere (Fig. 1C), indicating that even in intact monkeys there may be cases in which the density of SMI-32 positive neurons in layer V of M1 differs between the two hemispheres. Accordingly, there was also one monkey (Mk-CS) in the group of lesioned monkeys treated with the control antibody (blue bars in Fig. 1C) exhibiting an inter-hemispheric difference of the number of SMI-32 positive neurons per unit length ($p < 0.05$), whereas in the other three monkeys the difference was not statistically significant. However, still in the group of lesioned monkeys treated with the control antibody (blue bars in Fig. 1C), there was no systematic trend with respect to the side of the lesion. These data thus confirm the notion that axotomy of the crossed CS tract at cervical level in macaque monkeys treated with a control antibody does not lead to significant neuronal loss. As anticipated, the same conclusion applies to the group of five monkeys subjected to cervical cord lesion and treated with anti-Nogo-A antibody (Fig. 1C; red bars): in two monkeys there were slightly more SMI-32 positive neurons in the contralesional hemisphere whereas in the other three monkeys the ipsilesional hemisphere had more SMI-32 positive neurons (in two of them, the difference was statistically significant, but to a similar extent as in the case observed in each of the other two groups of monkeys). Overall, the unsystematic inter-hemispheric differences in the two groups of lesioned monkeys were generally in the same order of magnitude as those observed in the group of intact monkeys.

Does anti-Nogo-A treatment prevent shrinkage of CS neurons after axotomy?

In our recent study [17], the two monkeys subjected to the cervical lesion and treated with the control antibody exhibited a shrinkage of their soma as a result of the CS axotomy at cervical level. This observation is confirmed here on a larger pool of four control-antibody treated monkeys (Fig. 2A; blue bars): in three of the four monkeys, the SMI-32 positive neurons of layer V were significantly smaller in the contralesional hemisphere than in the ipsilesional one. In the fourth monkey (Mk-CH), no somatic size difference was found, but in this particular animal the quality of the SMI-32 immunostaining was poor, which may in part explain this result. In contrast to the majority of control antibody treated monkeys, the intact monkeys did not show a statistically significant difference in the distribution of somatic area of layer V pyramidal neurons between the two hemispheres (Fig. 2A; green bars). It can be concluded that CS axotomy induced shrinkage of somatic area of the corresponding CS neurons in the lesioned monkeys treated with the control antibody.

To address the issue whether anti-Nogo-A antibody treatment, in addition to enhanced sprouting in the cervical cord near the lesion [10,22], also prevents shrinkage of the soma of the CS axotomized neurons, the same analysis was conducted here in the group of five anti-Nogo-A antibody treated monkeys. In all five monkeys (Fig. 2A; red bars), the soma cross-sectional areas of SMI-32 positive layer V pyramidal neurons were significantly reduced in the contralesional hemisphere as compared to the ipsilesional one, indicating that the CS axotomized neurons shrank. Moreover, the extent of shrinkage is generally comparable in the anti-Nogo-A antibody treated and control antibody treated monkeys (Fig. 2C). In other words, the anti-Nogo-A antibody treatment did not prevent or reduce shrinkage of the soma of axotomized CS neurons.

The data of the present study are summarized in Figures 2B and 2C, where the inter-hemispheric difference (in percent) of the number of SMI-32 positive neurons per unit length in the layer V of M1 and of the median of their cross-sectional soma area were plotted as a function of the extent of the lesion, estimated in percent of the lateral spinal cord. For the three groups of monkeys, there was no systematic inter-hemispheric difference for the number of SMI-32 positive neurons per unit length in layer V of M1 (Fig. 2B), indicating that the unilateral axotomy of the CS tract did not produce a substantial cell loss in the contralesional hemisphere. Indeed, in the two groups of lesioned monkeys, the inter-hemispheric cell number difference was in the same range as in intact monkeys. On the other hand, as compared to intact monkeys, the cervical hemisection led to a significant shrinkage of SMI-32 positive cells in layer V of the contralesional M1, irrespective of whether the monkey was treated with a control antibody or an anti-Nogo-A antibody (Fig. 2C).

Discussion

Based on a substantially increased number of animals, the present study confirms preliminary data [17] indicating that unilateral cervical lesion is not followed by a significant elimination of axotomized CS neurons. This conclusion thus adds a new piece of evidence to the long debated issue of whether CS neurons die after axotomy in monkeys. The present data are in line with previous reports claiming that there is no extensive CS cell loss after spinal cord lesion in monkeys [14-16]. As compared to the studies which previously addressed this question, the present investigation is based on a specific staining of pyramidal neurons in layer V (SMI-32), allowing better focus on the neurons of interest (CS neurons) than for instance in Nissl staining, as used in most cases previously. Although SMI-32 stains pyramidal cells in layer V in general and not only the CS neurons, the measurements conducted in the present study are thus somewhat more directed towards the subpopulation of CS neurons than measurements

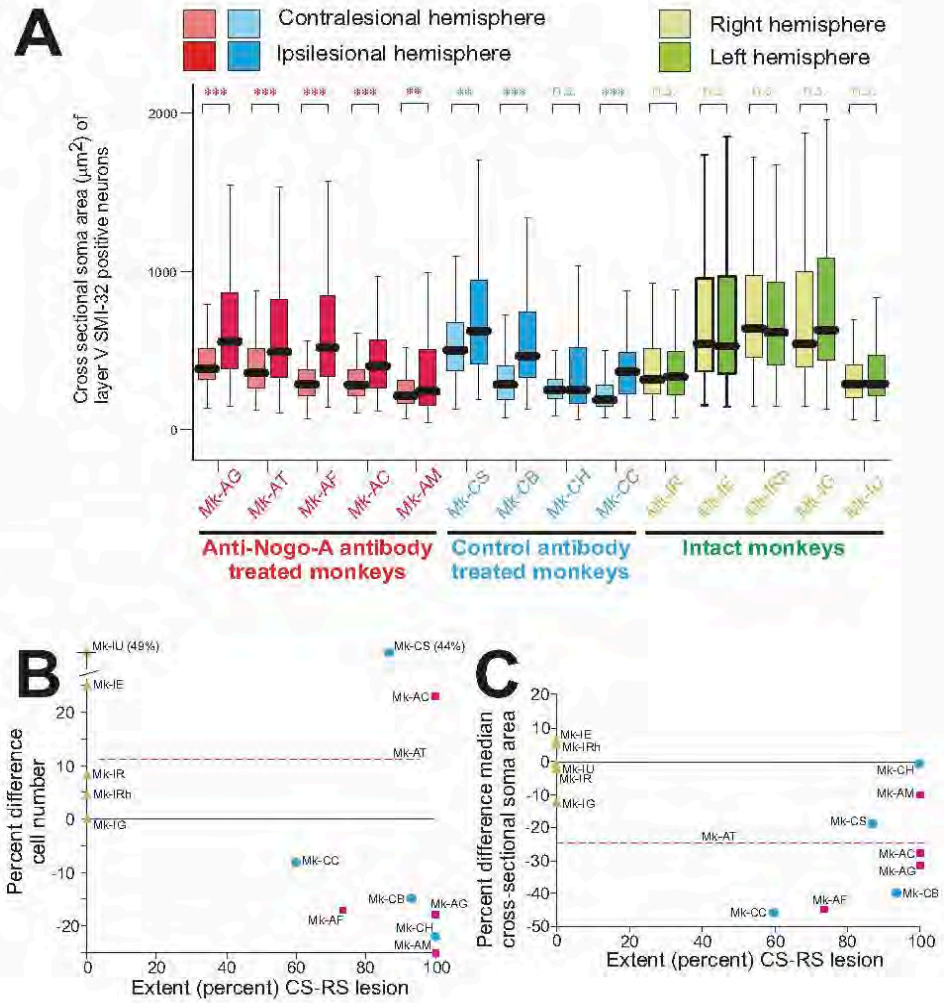

Figure 2

Somatic size of pyramidal neurons in layer V of the motor cortex and inter-hemispheric differences. A: Box and whisker plots showing the distribution of somatic cross-sectional areas of SMI-32 positive neurons in layer V in M1 for the three groups of monkeys. In the box and whisker plots, the thick horizontal line in the box corresponds to the median value, whereas the top and bottom of the box are for the 75 and 25 percentile values respectively. The top and bottom extremities of the vertical lines on each side of the box are for the 90 and 10 percentile values, respectively. All but one lesioned monkey (red and blue bars) exhibited a significant inter-hemispheric difference of cross-sectional soma area (* = $p < 0.05$; ** = $p < 0.001$; *** = $p < 0.0001$; Mann and Whitney test), whereas in the intact monkeys the difference was not statistically significant (n.s. = $p > 0.05$; Mann and Whitney test). B: Inter-hemispheric percent difference in the number of SMI-32 positive neurons per unit length in layer V in M1 for the same three groups of monkeys as in Figure 1C (same color code), plotted as a function of the lesion extent (percent of the territory corresponding to the CS and RS tracts affected by the lesion). Note that the differences are not systematic with respect to the side of the lesion in the two groups of lesioned animals (red squares and blue circles for the anti-Nogo-A and control antibody treated monkeys, respectively). In the percent comparison, 100% is for the number of SMI-32 positive neurons per unit length on the ipsilesional side (on the left side for the intact monkeys). The green triangles are for the intact monkeys. As the lesion extent could not be determined for Mk-AT (see Table 1), its corresponding percent value was represented by an horizontal dashed line. C: Inter-hemispheric percent difference of the median value of the cross-sectional soma area of SMI-32 positive neurons in layer V in M1 for the same three groups of monkeys as in panel A, plotted as a function of the lesion extent (percent of the territory corresponding to the CS and RS tracts affected by the lesion). Note that the difference is small for the intact monkeys (green symbols), but it is more prominent and systematic with respect to the side of the lesion for most lesioned monkeys, with considerable overlap between the two groups of lesioned monkeys. Same conventions as in panel B.

conducted for instance in Nissl material, where a much larger population of neurons would be involved. As a consequence, a difference between the two hemispheres affecting the CS neurons after a unilateral cervical lesion is detected with more sensitivity in SMI-32 than in Nissl material. Our measurements thus include the large pyramidal neurons of layer V in the motor cortex, which in the rat primary somatosensory (barrel) cortex express intraneuronal Nogo-A [24]. Whether this is also true in primates and relevant in the present context remains an open question.

To investigate whether cell death occurred in layer V in the contralesional hemisphere, we counted the number of SMI-32 positive neurons in layer V in M1 territories of roughly comparable size delineated on the same section in the two hemispheres. Moreover, the data were normalized with respect to the length of the layer V territory in which the measurements were conducted. Our goal here was not to establish the absolute number of SMI-32 positive neurons in a given cortical area but rather to compare between the two hemispheres their relative numbers. Furthermore, because the number of SMI-32 stained neurons in the delineated territory of interest was relatively low, we did not use a stereological probe to estimate their number, rather we counted all of them exhibiting their nucleus. This approach is considered as adequate for studies dealing with few neurons [25-27]. For example, in a recent morphometrical study in the hippocampus [25], the authors used a true stereological approach to estimate the volume of the structure of interest as well as the total absolute number of neurons. In contrast, to estimate the number of BrdU immunoreactive neurons, the authors did not use a stereological probe because "of the low number of BrdU-labelled cells [25]." In the present case, the number of SMI-32 positive neurons with a visible nucleus in the restricted zone of interest of M1 (layer V) was also very low, thus preventing the use of stereological probe. Furthermore, the data and their reproducibility obtained in the five intact monkeys strongly support the validity of the method used here to establish the number of SMI-32 positive neurons and their cross-sectional somatic area. Indeed, in all five intact monkeys, there was no statistically significant inter-hemispheric difference for their soma area. Furthermore, the number of SMI-32 positive cells did not show substantial inter-hemispheric difference in four out of five intact monkeys. In contrast, the somatic area difference observed in the two groups of lesioned monkeys was considerable and systematic with respect to the side of the lesion (except in one animal, Mk-CH). The morphometrical measurements were conducted in the hindlimb area of M1 and not in the hand area because the latter zone was subjected, in some animals, to extensive intracortical microstimulation [9,28] (see also Table 1) and, in all animals, to the injection of an anterograde tracer to label the CS tract [10,22]. The hand area in M1 was thus not suitable for an optimal detection of SMI-32 positive neurons as they may be obscured by the presence of the reaction product corresponding to the injection site. The hindlimb area, also affected by the cervical lesion, was thus more appropriate to conduct the anatomical analysis and we believe that the present data derived from the CS neurons of the hindlimb area is very likely to apply also to the CS neurons of the hand representation.

As shown in Figure 1C, there was no systematic inter-hemispheric difference in the number of SMI-32 positive neurons in the three groups of monkeys, thus supporting the conclusion that the cervical cord hemi-section did not provoke substantial loss of axotomized CS neurons. Considering the results in Figure 1C for the two groups of monkeys subjected to the cervical cord hemi-section (red and blue bar graphs), surprisingly three monkeys (Mk-AT, Mk-AC and Mk-CS) exhibited a higher number of SMI-32 labeled neurons on the contralesional hemisphere. In the first two monkeys, this difference was however not statistically significant, reflecting in part the variability of the method. For the third monkey (Mk-CS), the significant difference may reflect an episodic interhemispheric difference in a minority of monkeys, as actually observed in one intact monkey (Mk-IU in Fig. 1C) out of five intact animals. Nevertheless, the presence of some lesioned monkeys with more SMI-32 positive neurons on the contralesional hemisphere reinforces the conclusion of an absence of elimination of CS axotomized neurons after cervical cord hemi-section. Overall, as shown in Figure 1C, the number of SMI-32 positive neurons varied across animals, most likely reflecting individual variability both in the number of large layer V pyramidal neurons and in the intensity of SMI-32 staining.

The observation that there was no substantial loss of axotomized CS neuron after cervical hemi-section represents a quite favorable outcome for functional recovery. Indeed, the axons that have been severed, although they retract some distance from the lesion [22], are still present and potentially in position to regenerate or give rise to collateral sprouting. In other words, the functional recovery does not depend only on CS neurons spared by the lesion or on other preserved descending tracts. In recent reports, we demonstrated that the severed CS axons indeed can give rise to spontaneous axonal sprouting in absence of treatment, but only to a very limited extent [10,22]. In contrast, an anti-Nogo-A antibody treatment substantially enhanced CS axonal sprouting from the severed axons, as manifested by an increase of axonal arbors rostral to the lesion as well as axonal arbors and swellings caudal to the lesion [10,22].

As a result of cervical cord hemi-section, the axotomized CS neurons in the contralesional hemisphere exhibit shrinkage of their soma (Fig. 2A, blue bar graphs). Neuron atrophy in response to axotomy is very well known and widely observed, including cortical neurons. Neuronal metabolism depends on continuous retrograde trophic signals from the targets, which are absent or reduced in case of axotomy. As the normal intact axonal arborization may be much larger than the "spontaneously" newly formed arbor of the few sprouting neurons in the control antibody treated monkeys, the decrease of retrogradely transported trophic signals would thus contribute to the soma shrinkage. In the anti-Nogo-A antibody treated monkeys, although sprouting was enhanced [22], the quality and amount of trophic signals may be still insufficient to completely reverse the atrophy. Furthermore, the number of CS axons that successfully sprout and re-establish an axonal arbor with contacts is not precisely known. It is likely that only a relatively limited proportion of axotomized CS neurons managed to re-establish a large axonal arbor and to obtain enough trophic signals to prevent or reverse the atrophy. Increase of the somatic size of only of few neurons would remain undetected in the soma size distribution (Fig. 2A). Finally, the time course has to be taken into consideration. Shortly after the lesion, sprouting neurons are not in an atrophic state and this is true for the initial few weeks after the injury when sprouting takes place. The present morphometric measurements were conducted in our monkeys many months post-lesion at a time point when sprouting was completed. However, as the newly formed axonal arbors may not be as large as intact ones, the total amount of trophic support they receive may still not be enough to maintain their full soma size.

The present study provides evidence that the anti-Nogo-A antibody treatment limits its action on the distal part of the axotomized CS neurons, namely by enhancing axonal sprouting close to the lesion itself, but does not prevent changes taking place at the level of the CS cell body, at least as far as soma size shrinkage is concerned. This conclusion applies to the present experimental conditions and it cannot be excluded that soma shrinkage of axotomized CS neurons could have been better prevented, at least to some extent, if the dose of the anti-Nogo-A antibody would have been higher or if it would have been delivered not only near the cervical lesion but also at cortical level. Indeed, although the anti-Nogo-A antibody delivered intrathecally near the lesion site penetrates into the CNS, the penetration was less complete in the brain than in the spinal cord [23].

To our knowledge, the issue of CS neurons elimination after spinal cord lesion and treatment with anti-Nogo-A antibody has not been investigated before, even in rodents and therefore the present observation of an absence of anti-Nogo-A antibody treatment effect on the shrinkage of axotomized CS neurons is original. The issue of whether the anti-Nogo-A antibody treatment can contribute to rescue cells from elimination after axotomy could not be addressed here as there was no CS neuron loss, even in the control antibody treated monkeys. The absence of CS cell loss after cervical lesion contrasts with the number of RS neurons detected in the contralesional magnocellular red nucleus (RNm), which was up to 30% lower than in the ipsilesional RNm [29]. However, in the latter study conducted on the same groups of lesioned monkeys, it was observed that the anti-Nogo-A antibody treatment did not impact on the RS neurons, as the number of detected RS neurons was comparable in the anti-Nogo-A antibody and in the control antibody treated monkeys.

Conclusion

In adult macaque monkeys, following cervical cord hemi-section, neutralization of Nogo-A with a specific monoclonal antibody enhanced functional recovery [10] and promoted sprouting of the corticospinal tract both rostral [22] and caudal to the lesion [10,22], as compared to monkeys treated with a control antibody. In the control antibody treated monkeys, the corticospinal neurons in the contralesional primary motor cortex (M1) survived to the axotomy, but their soma shrank. The present study tested the hypothesis that the anti-Nogo-A treatment may prevent such soma shrinkage. We found that the anti-Nogo-A antibody treatment did not preserve the axotomized corticospinal cells from soma shrinkage, indicating that the anti-Nogo-A antibody treatment affects morphologically the axotomized corticospinal neurons mainly at distal levels, especially the axon collateralization in the cervical cord, and little or not at all at the level of their soma.

Methods

The present data have been derived from a long-term protocol, described earlier in detail [9,10,17,22,28], conducted on monkeys subjected to unilateral cervical cord lesion at the C7/C8 border, in accordance with the Guide for the Care and Use of Laboratory Animals (ISBN 0-309-05377-3; 1996) and approved by local (Swiss) veterinary authorities. The present study aimed at comparing three groups of monkeys (Table 1): (i) Intact monkeys (n = 5); (ii) Monkeys subjected to the cervical hemi-section and treated with anti-Nogo-A antibody (n = 5); (iii) Monkeys subjected to the cervical hemi-section and treated with a control antibody (n = 4).

Two monoclonal antibodies (mAbs) against different sites of the neurite inhibitor protein Nogo-A were employed (Table 1; see also [22] for more detail on the antibodies)

in the group of anti-Nogo-A antibody treated monkeys: the mouse mAB 11C7 was raised against a 18 amino acid sequence of rat Nogo-A (aa623 – 640) close to the most inhibitory region of the Nogo-A protein [30]. The second antibody used, mAb hNogo-A recognized the Nogo-A specific region of the human Nogo-A sequence. Both antibodies identify primate Nogo-A monospecifically on Western blots [23,30]. The antibodies were purified as IgGs and concentrated to 3.7–10 mg/ml in PBS. In the control antibody treated monkeys, purified IgG of a mouse mAb directed against wheat auxin (AMS Biotechnology, Oxon/UK) was used as control antibody (concentration: 3.7–10 mg/ml).

Briefly, the main steps of the protocol conducted on the lesioned monkeys were the following. First, an intensive pre-lesion training was initially performed in order to establish a stable behavioral score in manual dexterity tasks involving both hands [10]. A unilateral lesion was performed at the C7/C8 border [10,17] and the antibody (either control or anti-Nogo-A) was administered intrathecally a couple of mm rostral to the lesion. The manual dexterity tests were conducted at regular intervals for several months post-lesion, until the animals reached a plateau reflecting a stable level of functional recovery. Anterograde tracers were injected mainly in the hand representation of the primary motor cortex (M1) bilaterally to label the CS tract [10,22]. The monkeys were sacrificed under deep anesthesia and perfused transcardially with fixatives [17]. Frozen sections comprising each of the two hemispheres were cut in the coronal plane at a thickness of 50 μm. The spinal cord was cut in the paralongitudinal plane at the site of the lesion and transversally at the level of the first cervical segments as well as of thoracic segments caudal to the lesion. The lesion was reconstructed using SMI-32 stained material in all monkeys as previously reported [10,17] and its extent was expressed quantitatively by the percent of the territory delimited by two lines starting from the central channel and extending to the dorsal and ventral rootlets, thus covering completely the lateral funiculus and hence the zone occupied by the decussated CS and rubrospinal (RS) tracts. A series of brain and spinal cord sections was treated immunocytochemically with SMI-32 antibody in order to visualize in the cerebral cortex the layers III and V pyramidal neurons (Fig. 1B), as recently described in detail [31]. The epitope recognised by the SMI-32 antibody lies on non-phosphorylated regions of neurofilament protein and is only expressed by specific categories of neurons [32,33]. SMI-32 positive neurons (only those exhibiting the nucleus) in the primary motor cortex (M1) on both hemispheres were counted under the microscope at high magnification (400×), in a territory including layer V at the same dorsoventral location and defined of roughly equal length on both hemispheres (zone delineated with a dashed line in Fig. 1B). The typical appearance of SMI-32 positive neurons in layer V of M1 was illustrated in a recent report (see Figure 3 in [17]). At low magnification, as staining was stronger on the ipsilesional hemisphere, the number of SMI-32 positive neurons seems to be lower on the contralesional side (Fig. 1B). This is however a wrong impression as, at higher magnification (Fig. 1D), several SMI-32 positive neurons appear on the contralesional hemisphere (Fig. 1D): at high magnification, even lightly stained SMI-32 positive neurons can reliably be detected. On each section and separately for each hemisphere, the total number of SMI-32 positive neurons was divided by the length (in mm) of the territory in which the analysis was conducted (line segment in Fig. 1B). Moreover, the somatic cross-sectional silhouette area of these SMI-32 positive neurons in layer V was determined, as described in detail earlier [17]. In each monkey, four to six coronal histological sections were taken along the rostrocaudal extent of M1 (separated by 800 μm each) in which the analysis of SMI-32 positive neurons in layer V was conducted.

The number of SMI-32 positive neurons per unit length (mm) obtained in a given section was averaged across the sections analyzed in each monkey, separately for the two hemispheres, and a standard deviation was obtained reflecting the variability from one section to the next (Fig. 1C). This measure is proportional to the number of SMI-32 positive neurons per volume unit. The statistical comparison of the number of SMI-32 positive neurons per unit length between the two hemispheres was based on a paired t-test for small samples applied on the four to six sections analyzed in each monkey. A statistically significant difference of the number of SMI-32 positive neurons in layer V between the two hemispheres was obtained for a t value corresponding to $p <= 0.05$ ($df = 3$ to 5 for 4 to 6 sections analyzed, respectively). The soma areas did not follow a normal distribution (wider dispersion for large somatic areas than small ones) and therefore they were graphically represented in the form of box and whisker plots (putting emphasis on the median value rather than the mean value; Fig. 2A). Accordingly, the statistical comparison of soma areas of SMI-32 positive neurons between the two hemispheres was conducted for each animal using the non-parametric unpaired Mann and Whitney test (Fig. 2A), with a significance level of $p <= 0.05$.

List of abbreviations

CS = corticospinal

M1 = primary motor cortex

Mk = monkey

RNm = magnocellular red nucleus

RS = rubrospinal

SCI = spinal cord injury

Authors' contributions

EMR designed the study, contributed to the experiments and analysis of the data, and drafted the manuscript. MLB conducted the morphological measurements in most monkeys, contributed to the analysis and wrote a preliminary version of the manuscript in the context of her Master thesis. ES and TW designed the study, carried out the experiments, the morphological measurements on some monkeys and analyzed the corresponding data. PF contributed to the experiments and the analysis of the data. JB designed the study and performed the cervical lesions. AM contributed to the study design and antibody protocol. MES contributed to the study design and general concept, antibody protocol and data analysis. All authors read, commented and approved the final manuscript.

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Manuscript 2:

After spinal cord injury, adult macaque monkeys exhibit numerous SMI-32 positive fibers into the scar tissue insensitive to the application of an anti-Nogo-A antibody alone or co-applied with BDNF.

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Abstract

In adult macaque monkeys subjected to a partial spinal cord injury (SCI), corticospinal (CS) fibers are rarely observed into the scar tissue. This situation is little affected by the application of an anti-Nogo-A antibody which otherwise fosters the growth of CS fibers rostrally and caudally to the lesion. However, as little is known about the behavior of other groups of neurons, it is conceivable that some other fiber types colonize the scar tissue. To test this hypothesis, the presence of fibers containing non-phosphorylated neurofilaments inside the scar tissue was assessed using the SMI-32 staining. The investigation was conducted using anatomical material from 18 adult macaque monkeys: eight animals received a control antibody, five animals an anti-Nogo-A antibody, and five animals received an anti-Nogo-A antibody together with the brain-derived neurotrophic factor (BDNF). Numerous SMI-32 positive (+) fibers were observed into the scar tissue. These fibers occupied the whole dorso-ventral axis with a tendency to accumulate on the ventral side, and their trajectory was erratic. Most of these fibers (ca 87%) were larger than 1.3 μm and densely SMI-32 (+) stained. In the undamaged spinal tissue, motoneurons form the only large population of SMI-32 (+) neurons which are densely stained and which axons tend to be large. The data suggests that a sizeable proportion of the fibers seen into the scar tissue originate from motoneurons, but that fibers of other origins are also contributing. Neither the presence of the antibody neutralizing Nogo-A alone, nor the presence of the antibody neutralizing Nogo-A combined with BDNF influenced the number or the length of the SMI-32 (+) fibers inside the scar tissue. In summary, our data show that after a spinal cord lesion in adult monkeys, the scar tissue is colonized by fibers, a large portion of which presumably originate from motoneurons.

Introduction

In the adult central nervous system (CNS) of mammals, axotomized nerve fibers fail to regenerate over long distances. Among the factors contributing to this failure, the development of a scar tissue at the lesion site leads to the formation of an environment hostile to nerve fibers' regrowth and acting as a barrier against axonal regeneration. Indeed, the scar tissue contains various neurite growth inhibiting factors produced by cells such as microglia cells, meningeal cells, astrocytes or oligodendrocytes (David and Lacroix 03;Reier et al 83;Schwab 02;Schwab and Bartholdi 96). These last years, several growth inhibiting molecules expressed in the scar tissue were identified such as the chondroitin sulfate proteoglycans (CSPGs) or the myelin-associated neurite outgrowth inhibitors (Davies SJ 97;Davies et al 99;Fawcett and Asher 99;McKeon et al 91;Rudge and Silver 90). Among these molecules, it has been shown that Nogo-A produced by reactive oligodendrocytes inhibits the regeneration of spinal lesioned axons (Caroni et al 88;Caroni and Schwab 88). In rodents, the application at the level of a spinal lesion of an antibody neutralizing Nogo-A promotes both corticospinal (CS) fibers regeneration and functional recovery (Bergman et al 95;Brösamle et al 00;Liebscher et al 05;Schnell and Schwab 90;Schwab 04;Thallmair et al 98). Experiments conducted on primates lead to similar results (Freund et al 06;Freund et al 09;Freund et al 07). In particular, these experiments have shown that in anti-Nogo-A antibody treated monkeys, CS fibers sprout caudally and rostrally to the spinal lesion (Fouad et al 04;Freund et al 06;Freund et al 07). However, in these animals too, CS fibers were only exceptionally observed inside the scar tissue. Nevertheless, it remains possible that following SCI, neurons distinct from CS neurons possess yet the ability to regenerate and to grow into or even through the scar tissue. The goal of the present study was to investigate, using the SMI-32 staining, whether neurites can colonize the scar tissue and whether the anti-Nogo-A antibody treatment alone or combined with BDNF can influence such regenerating nerve fibers.

Materials and Methods

Animals

Eighteen young adult macaques (3.5-6.9 years old; weight ranging from 3.0 to 5.0 kg) were subjected to an incomplete spinal cord hemisection performed at cervical level C7/C8. These animals are part of a large study on the consequences of an anti-Nogo-A antibody treatment on the regeneration of CS axons as well as on behavioural recovery after cervical cord injury. Separate data from 10 of these animals have appeared in previous reports (Beaud et al 08;Freund et al 06;Freund et al 09;Freund et al 07;Wannier-Morino et al 08). Surgery and care of the animals was done in conformity to the Guide for the Care and Use of Laboratory Animals (ISBN 0-309-05377-3; 1996) and authorized by local Swiss veterinary authorities. The housing conditions as well as the surgical procedures were reported in detail in earlier publications (Freund et al 06;Freund et al 09;Freund et al 07;Schmidlin et al 05;Schmidlin et al 04). The experimental protocol can be summarized as follow. In a first stage, the animals were trained to perform behavioral (manual dexterity) tasks until they reached a stable behavioral score (pre-lesion plateau). In the second stage, an incomplete unilateral cervical cord hemisection was performed and the tip of a catheter attached to an osmotic pump was fixed at the lesion site. A group of eight animals received a control antibody (14-80 mg/animal), a second group of five animals received an anti-Nogo-A antibody (total: 14.6-36 mg/animal), and a last group of five animals received the anti-Nogo-A antibody (36mg/animal) as well as BDNF (1.4mg/animal). The treatment was initiated immediately after the lesion and lasted one month.

In a third stage, starting immediately after the lesion, each animal was assessed behaviourally until it reached a stable score (post-lesion plateau). In the fourth stage, the anterograde tracer BDA was injected into the contralesional motor cortex. In some animals, a dextran-fluorescein tracer was injected in the ipsilesional motor cortex. After about two months, delay to allow anterograde transport of the tracers, the animals were sacrificed. In this report, the code identifying each animal is built in two parts: the first letters of the code indicate the treatment given (**Mk-C** for the control antibody treatment, **Mk-A** for the anti-Nogo-A antibody treatment, **Mk-AB** for the anti-Nogo-A antibody/BDNF treatment) and the additional letter(s) individuate the corresponding animal. The experimenter was blind as to the treatment of the animals and a different identification code was used during the whole duration of the experimental period. The final identification code was set after the sacrifice of the animals when the lesion was reconstructed and some of the behavioral and anatomical data analyzed.

Histology (BDA/ SMI-32/Dextran-Fluorescein stainings)

When the animals had reached a stable behavioural score post-lesion, approximately 100 days following the lesion, the BDA tracer was injected unilaterally in the contralesional motor cortex (hand territory) using Hamilton syringes (Table 1). After transport time (Table 1), the animals were sacrificed as follows. First, the monkeys were sedated with ketamine. Then, they received a lethal injection of sodium pentobarbital (90 mg/kg) intraperitoneally. They were then perfused transcardially with 0.4 liter of saline (0.9%), followed by 3 liters of a fixative solution (4% of paraformaldehyde in 0.1 M of phosphate buffer, pH=7.6). The perfusion continued subsequently with 2 solutions of similar fixative containing sucrose of increasing concentrations (10%, 20%) and ended with a 30 % sucrose solution in phosphate buffer (pH=7.6). At the end of the perfusion, the entire CNS was isolated and placed during few days in a 30% solution of sucrose in phosphate buffer (pH=7.6). The spinal portion around the lesion was cut parasagitally in either three or five series of respectively 50 or 30 µm sections and processed to visualize either BDA or SMI-32 staining. The segments just rostral and caudal to this portion were cut in 50 µm thick coronal sections and also collected in series. The SMI-32 staining was carried out according to the following protocol: first, to remove the endogenous peroxidase activity, the free-floating sections were preincubated during 10 minutes in 1.5% H_2O_2 in phosphate-buffered saline (PBS; pH 7.2) and incubated overnight at 4 ^{o}C in SMI-32 monoclonal antibody (dilution 1:3000) in addition to 2% normal horse serum and 0.2% Triton X-100; then the sections were rinsed several times In PBS. After that, the sections were rinsed several times again and incubated in a biotinylated secondary antibody (1:200, Vector Burlingame, CA) during 30-60 minutes at room temperature. Finally, the sections were stained with the avidin-biotin complex (ABC) immunoperoxidase method (Vectastain Elite kits, Vector). The reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB 0.05%) as the chromogen, diluted in Tris-saline with 0.001% H_2O_2. The sections were then washed, mounted on gelatin-coated slides, dehydrated, and coverlipped. In the present investigation, the number and the cumulative length of the SMI-32 (+) fibers visible into the lesion site were assessed at 400x magnification using a light microscope (Olympus) and the software Neurolucida. The sections analyzed in the present study were used previously in some of the monkeys to reconstruct the location and the extent of the cervical lesion in previous reports (Freund et al 09;Schmidlin et al 04;Wannier et al 05). Finally, for nine animals (five control treated; one anti-Nogo-A antibody treated and three anti-Nogo-A antibody/BDNF treated) the fibers thickness was also measured and their distribution established.

Treatments

Three different purified IgG antibodies (concentrated to 3.7-10 mg/ml in PBS) were used in the present study. The antibodies were delivered intrathecally at the site of the lesion via a catheter attached to an osmotic pump (Alzet 2M2 or 2M4). The pumps were placed in the back of the animal and the treatment started within minutes following the spinal cord lesion. After about 30 days, the pumps were removed. The control antibody corresponds to a mouse monoclonal antibody directed against wheat auxin (AMS Biotechnology, Oxon/UK) and it was administrated up to a total amount of 14 to 36 mg in 8 animals. The anti-Nogo-A antibody (14 to 36 mg) delivered during four weeks to four animals (Table 1) corresponded to a mAB hNogo-A monoclonal antibody which recognizes a defined Nogo-A region of the human Nogo-A sequence. The monkey Mk-AF (Table1) received a monoclonal antibody (11C7; 14 mg in four weeks) directed against a rat Nogo-A sequence of 18 amino acids (aa623-640) which builds a strong inhibitory region of the Nogo-A protein . On Western blots, both anti-Nogo-A antibodies can identify in a monospecifical manner the primate-Nogo-A protein (Oertle et al 03;Weinmann et al 06). The question of whether the two types of anti-Nogo-A antibodies present a difference in efficacy is not address in the present study because only one animal was treated with the 11C7 type of anti-Nogo-A antibody. Five animals (Mk-ABBo, Mk-ABMa, Mk-ABMx, Mk-ABP and Mk-ABS) have received the anti-Nogo-A antibody treatment (36 mg in 4 weeks), as well as 1.4 mg of the neurotrophic factor BDNF (Peprotech, UK) diluted in artificial CSF (150 mM Na, 3 mM K, 1.4 mM Ca, 0.8 mM Mg, 1 mM P, 155 mM Cl) and delivered intrathecally over the lesion during the same period of 4 weeks (Table1).

Table 1. Results from cervical cord-lesioned monkeys included in the present study with identification code

name	Mk-CB	Mk-CBo	Mk-CC	Mk-CGa	Mk-CG	Mk-CH	Mk-CP	MK-CS
	1,2,3,4	-	1,3	-	1,2,3,4	1,2,3,4	1,2,3	1,3,4
species	Fasc.	Fasc.	Mul.	Fasc.	Fasc.	Fasc.	Fasc.	Mul.
Sex	?	?	?	?	?	?	?	?
mAB received	control	control	control	control	control	control	control	control
amount mAB (mg)	80	14	14.8	14	36	36	14.8	14.8
BDNF treatment	No	No	No	No	No	No	No	No
mg	-	-	-	-	-	-	-	-
BDA transport time (days)	78	99	21	87	70	62	78	35
Lesion to BDA injection delay (days)	147	93	86	69	70	76	81	167
Hemisection extent (%)	75	93	38	73	51	90	45	63
Functional recovery (%)	78	99	83	100	90	53	83	22
Age of the animal at the sacrifice (years)	5	~4	~4	~4	~4	~4	6.9	4.5
N fibers with Ø < 1.3 µm	**266**	**190**	**64**	**83**	**34**	**232**	**143**	**24**
N fibers with Ø > 1.3 µm	**780**	**884**	**305**	**989**	**404**	**2307**	**657**	**158**
N fibers in the lesion	**1046**	**1074**	**369**	**1072**	**438**	**2539**	**800**	**182**
Cumulative lenght for fibers with Ø < 1.3 µm	**13.6**	**21.6**	**4.5**	**6.0**	**3.1**	**21.9**	**20.5**	**2.7**
Cumulative lenght for fibers with Ø > 1.3 µm	**63.0**	**94.0**	**27.6**	**72.6**	**31.2**	**178.9**	**86.5**	**18.9**
Cumulative lenght for all fibers in the lesion	**76.5**	**115.6**	**32.0**	**78.6**	**34.3**	**200.8**	**107.0**	**21.6**
Volume of BDA injection (in µl)	20			34.5	24	20	24	
No. of BDA injection sites	10			21	12	10	12	

name	Mk-AC	Mk-AF	Mk-AG	Mk-AK	Mk-AM	Mk-ABB	Mk-ABMa	Mk-ABMx	Mk-ABP	Mk-ABS
	1,2,3,4	1,3,4	2,4	3	1,2,3,4	-	-	-	-	-
species	Fasc.	Mul.	Fasc.	Fasc.	Fasc.	Fasc.	Fasc.	Fasc.	Fasc.	Fasc.
Sex	?	?	?	?	?	?	?	?	?	?
mAB received	hNogoA	11C7	hNogoA	hNogoA	hNogoA	hNogoA	hNogoA	hNogoA	hNogoA	hNogoA
amount mAB (mg)	36	14.8	14.6	36	36	36	36	36	36	36
BDNF treatment	No	No	No	No	No	Yes	Yes	Yes	Yes	Yes
mg	-	-	-	-	-	1.4	1.4	1.4	1.4	1.4
BDA transport time (days)	64	71	70	62	69	76	65	79	69	62
Lesion to BDA injection delay (days)	71	187	42	90	69	91	84	179	111	111
% Extent of the lesion	85	56	78	86	80	83	94	95	77	93
Functional recovery	100	57	100	100	96	93	68	78	70	66
Age of the animal at the sacrifice (years)	~4	6.25	3.5	3.5	~4	5	~4	5	4.5	4.5
N fibers with Ø < 1.3 µm	**73**	**47**	**46**	**301**	**145**	**32**	**49**	**124**	**705**	**328**
N fibers with Ø > 1.3 µm	**821**	**82**	**94**	**505**	**1607**	**526**	**918**	**1017**	**1199**	**953**
N fibers in the lesion	**894**	**129**	**140**	**806**	**1752**	**558**	**967**	**1141**	**1904**	**1281**
Cumulative lenght for fibers with Ø < 1.3 µm	**6.6**	**4.7**	**3.2**	**26.2**	**17.5**	**4.6**	**3.1**	**12.3**	**41.2**	**23.6**
Cumulative lenght for fibers with Ø > 1.3 µm	**59.9**	**4.3**	**8.3**	**75.9**	**131.3**	**56.3**	**77.2**	**93.4**	**88.0**	**67.9**
Cumulative lenght for all fibers in the lesion	**66.5**	**9.0**	**11.5**	**102.1**	**148.8**	**60.9**	**80.4**	**105.7**	**129.2**	**91.5**
Volume of BDA injection (in µl)	20		28		19	26	28.5	34.5		
No. Of injection tracks	10		15		10	12	19	27		

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3 EJN 29 (2009) 983-996
4 Brain Res 1217 (2008) 96-109

The functional recovery was established by calculating the ration in percent of the retrieval score at post-lesion plateau to that at pre-lesion plateau.

Results

Cervical cord lesion

All animals presented were subjected to an incomplete spinal cord hemisection at the level of the transition between the segments C7 and C8. For 16 animals, the lesions sites were reconstructed from the histological sections and their relative size were determined as a proportion (in percent) of the extent of the hemi-cord surface (Table 1). The lesion extent ranged between 38 % and 95 % of the hemicord surface (Fig. 1). In nearly all monkeys, the lesion interrupted completely the dorsolateral funiculus, spared large portions of the dorsal columns and spreaded into the ventral quadrant.

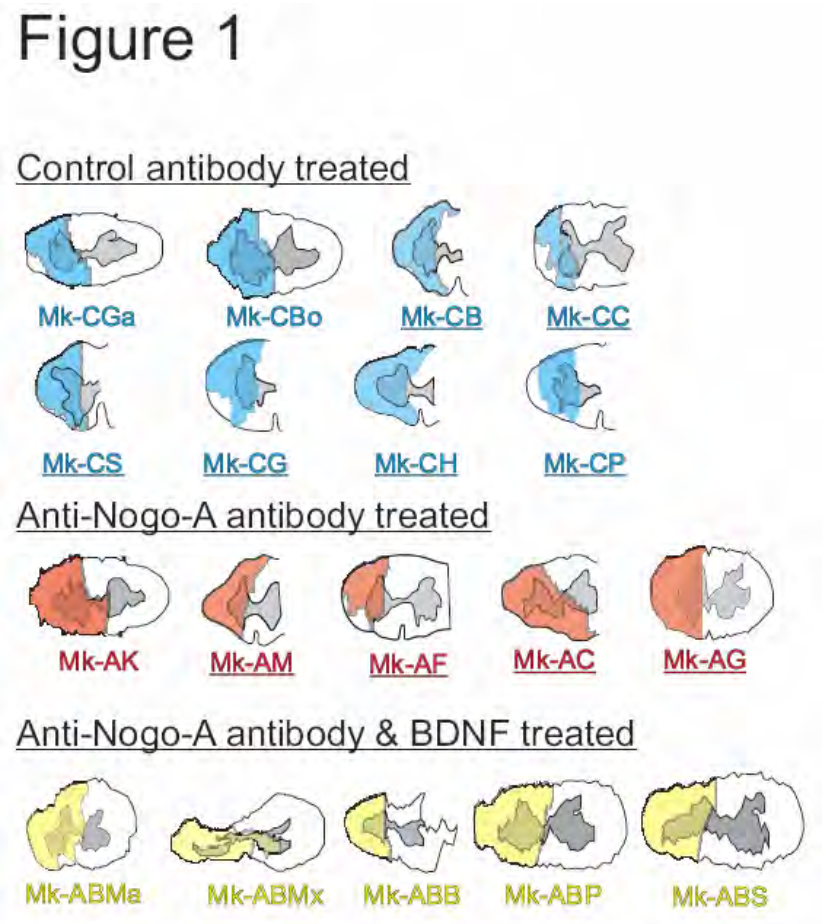

Figure 1: Reconstruction of the spinal cord lesion sites. On a frontal section of cervical cord, the lesion area and the position are indicated in blue for the control antibody treated monkeys, in red for the anti-Nogo-A antibody treated monkeys and in yellow for the anti-Nogo-A antibody/BDNF treated monkeys. The gray zone in the center corresponds to the gray matter. In two animals (Mk-ABMx and Mk-ABBo), the catheter attached to the osmotic pump has pressed on the spinal cord and led to deformations of the tissue. The reconstructions of the lesions of the animals whose name is underlined have already been reported previously (Freund et al 06;Freund et al 09;Freund et al 07).

Which spinal elements are labeled by SMI-32 staining?

The SMI-32 antibody recognizes non-phosphorylated neurofilaments, which are only expressed by specific categories of neurons (Campbell and Morrison 89;Sternberger and Sternberger 83). Coronal sections from segments located rostrally to the spinal cord lesion and stained for SMI-32 revealed that among spinal neurons, motoneurons were the most heavily stained (Fig. 2A and 2F white arrowheads). Other SMI-32 stained neurons are scattered inside the entire gray matter but only few of them were as heavily stained as motoneurons (Fig 2E, white arrowhead). In the white matter, lightly stained axons were often visible, as for instance in the dorsal funiculi (Fig 2B) or in the dorsolateral funiculi (Fig 2D), whereas heavily stained axons were rare, with the exception of axons present in the spino-cervical tract (Fig 2C). Motoneuronal axons in the white matter were also heavily stained (Fig 2A, 2F, white arrows). Figure 3 depicts two SMI-32 stained longitudinal sections of the spinal cord at the lesion site. In the first section, the lesion extends through the entire dorso-ventral axis (Fig 3A). Numerous fibers and many neurons can be seen in the entire gray matter (Fig 3A). Heavily stained neurons with a large cell body are concentrated in the ventral grey matter, most of them most likely corresponding to motoneurons (Fig 3A, arrowheads). Numerous SMI-32 (+) fibers are present inside the scar tissue (Fig 3B, black arrowheads), some of which can unequivocally be recognized as motoneurons axons, as they are prolongations of motoneurons' axon bundles present in the intact white matter (Fig 3B, arrows). The lower panels (Fig 3C and D) depict a section from another animal and located at a more medial position than in the upper panels of Figure 3. On this section, the lesion occupies only the ventral part of the spinal cord (Fig 3C). Here too, numerous SMI-32 (+) stained fibers were found inside the scar tissue. In all animals, the fiber growth did not following a specific orientation (Fig 3B, 3D) and only few exceptions were observed to bridge the rostro-caudal extent of the lesion (Fig. 3D, black arrowhead).

Figure 2

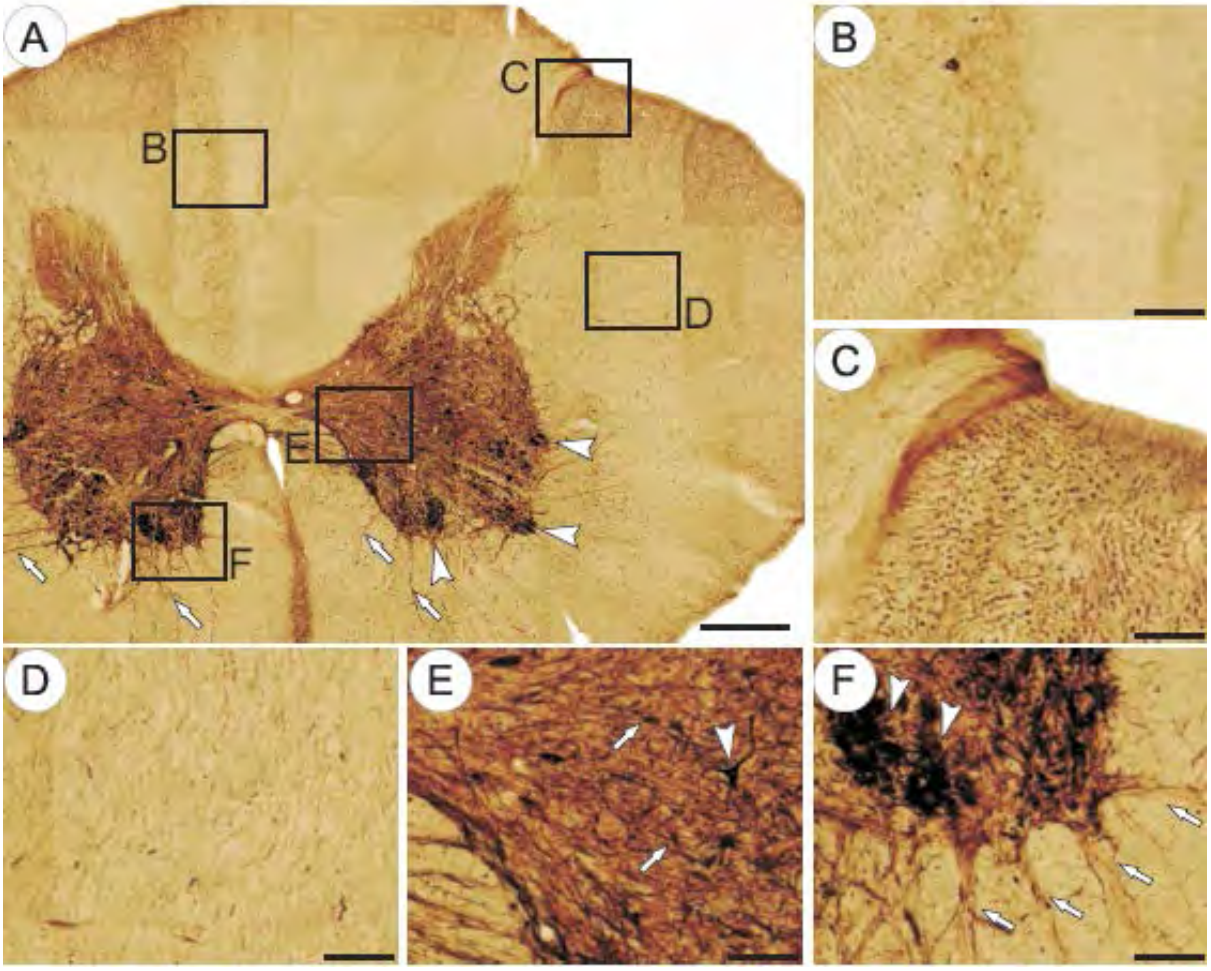

Figure 2: Distribution of SMI-32 (+) elements inside the spinal cord. A: SMI-32 stained coronal section from a level rostral to the lesion. The grey matter is easily distinguishable from the white matter and presents numerous sites with darkly stained elements. In particular, in the ventral grey matter, strongly stained pools of motoneurons are well recognizable (white arrowheads; F). In the white matter, apart from bundles of fibres spreading in the plane of the section (white arrows), no conspicuous elements are seen. B: At higher magnification, lightly stained SMI-32 (+) fibers can be seen in the left portion of this microphotograph of the dorsal columns. This portion is on the region containing ascending axons which have not been sectioned by the lesion. On the right of the microphotograph, which corresponds to a region where axons have degenerated after the lesion, no such staining is found. C: numerous well stained SMI-32 (+) fibers are present in the posterior spinocerebellar tract. D: moderately stained SMI-32 (+) fibers are present in the dorsolateral funiculus. E: The grey matter is filled with a fine mesh of SMI-32 (+) neuropile components in which few large (white arrowhead) and frequent small (white arrows) positively stained cell bodies are disseminated. F: the motoneurons pools (white arrowheads) build the only sites where numerous heavily SMI-32 (+) neurons are grouped. Scale bars: A: 500 µm; B-F: 100 µm.

Figure 3

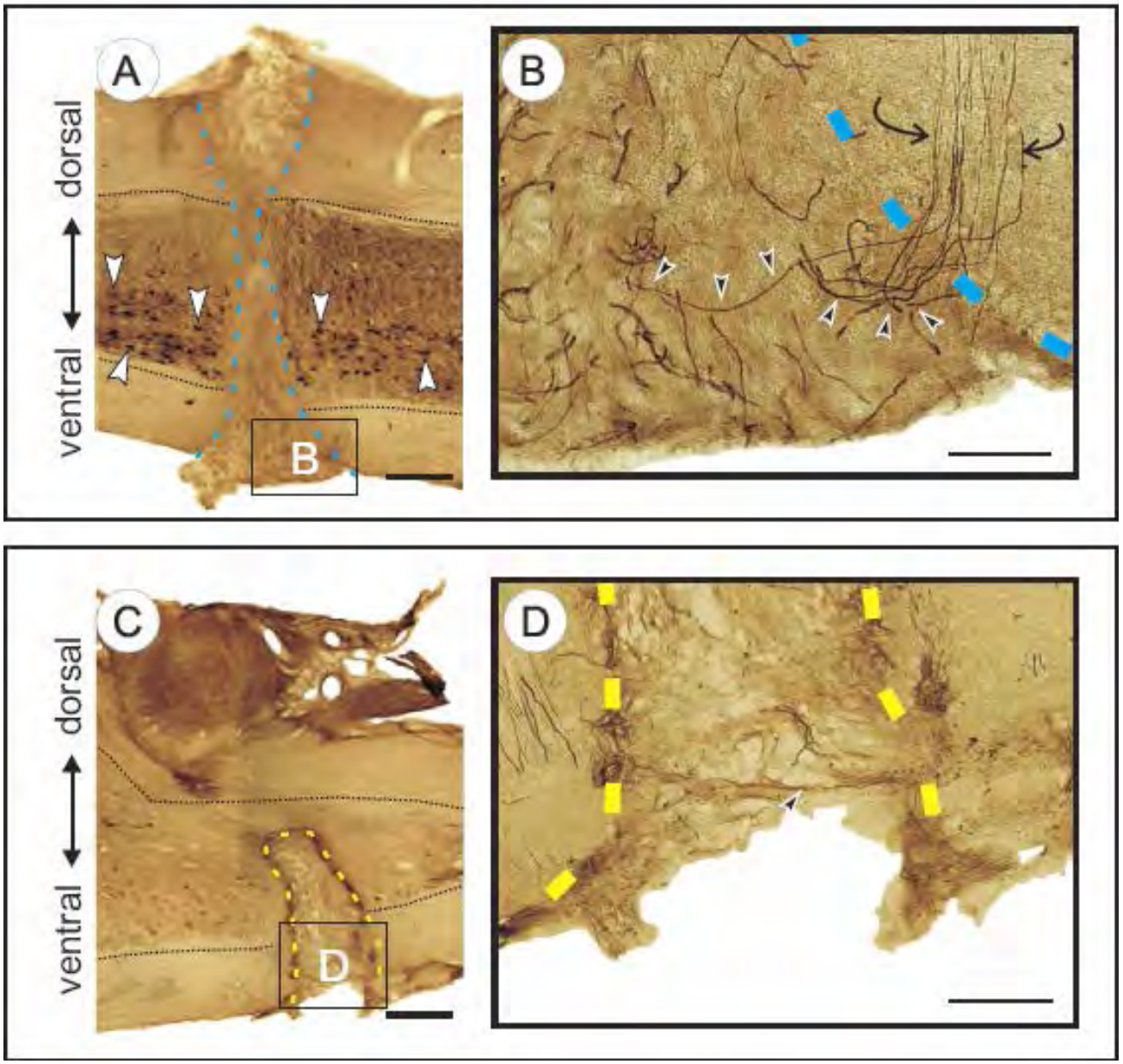

Figure 3: SMI-32 (+) elements inside the scar tissue. A: SMI-32 stained longitudinal section of the spinal cord at the level of the lesion. The material originates from a monkey that received the control antibody (Mk-CH). The limits of the lesion are indicated by the blue dashed line and the limits of the gray matter are shown with black dotted lines. The lesion extends throughout the entire dorso-ventral axis. The large darkly stained SMI-32 (+) neurons that accumulate in the ventral part of the grey matter are mostly motoneurons (white arrowheads). B: enlargement of the zone marked by a rectangle in A. Numerous SMI-32 (+) fibers are visible into the scar tissue (arrowheads) and in the nearby intact white matter (curved arrows). These last fibers form a bundle of darkly stained fibers running in a plane orthogonal to the axis of the spinal cord. Thus bundles are typical of motoneurons axons crossing the white matter to exit the spinal cord and

form ventral roots. Note that these fibers do not change noticeably their diameter as they cross the border between the intact tissue and the scar tissue. C: SMI-32 stained longitudinal section of the spinal cord at the level of the lesion. The material originates from a monkey that received the combination of the anti-Nogo-A antibody and BDNF (Mk-ABBo). The limits of the lesion are indicated by the yellow dashed line and the limits of the gray matter are shown with black dotted lines. At this level, the lesion extends only throughout the ventral portion of the spinal cord. D: enlargement of the zone marked by a rectangle in C. Numerous SMI-32 (+) fibers are visible into the scar tissue. These fibers normally grow erratically inside the scar tissue, but single fibers may keep a fixed orientation across the rostro-caudal extent of the scar (black arrowhead).

Do CS fibers contribute to a part of the SMI-32 (+) fibers present inside the scar tissue?

The histological procedure used to reveal SMI-32 (+) elements also stained CS axons filled with BDA injected in M1. To clarify whether CS fibers are present and common among the SMI-32 (+) fibers detected into the lesion, SMI-32 stained sections were compared to adjacent sections processed only for BDA. As shown in Figure 4, two regions of a BDA stained section, one ventral and one dorsal (Fig. 4D and 4F), were compared to corresponding regions of one adjacent SMI-32 stained section (Fig. 4C and 4E). As already described (Figs 2 & 3), the SMI-32 staining reveals positive stained fibers and neurons into the spinal grey matter as well as into the white matter. Numerous SMI-32 (+) stained fibers were seen into the scar tissue, particularly in its ventral portion (Figs 4E). In contrast, BDA (+) stained fibers were not found in the corresponding region of an adjacent BDA processed section (Fig. 4D and 4F). As expected, there was no BDA-labelled cell body into the spinal gray matter but BDA (+) stained fibers were common in the dorsolateral funiculus rostrally to the lesion site (Fig 4B). These data thus indicate that the overwhelming majority of the SMI-32 (+) fibers observed into the scar tissue do not comprise CS axons.

Figure 4

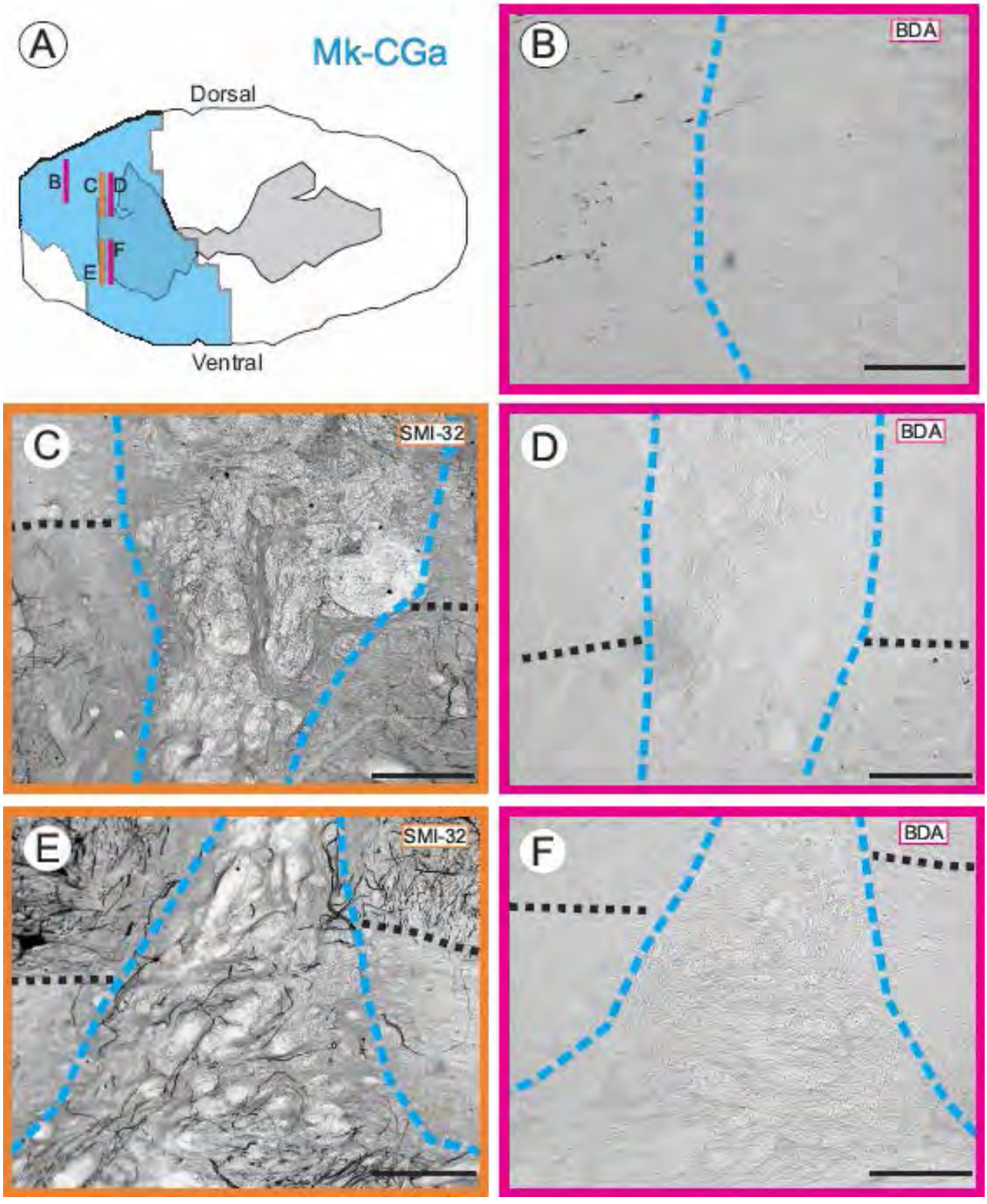

Figure 4: The SMI-32 (+) elements inside the scar tissue are not CS axons. A: Reconstruction of the spinal lesion in monkey Mk-CGa illustrating the localization inside the spinal cord of the photomicrographs illustrated in B to F. B: Section stained for BDA at the level of the dorsolateral funiculus. CS axons normally barely penetrate inside the scar tissue. C: In the scar tissue at the level of the dorsal horn, few SMI-32 (+) fibers can be seen. D: section adjacent to that depicted in C and stained for BDA. Note the absence of BDA (+) fibers in and outside of the scar tissue. E: In the scar tissue at the level of the ventral horn, numerous SMI-32 (+) fibers can be seen. F: section adjacent to that depicted in E and stained for BDA. Note here also the absence of BDA (+) fibers in and outside of the scar tissue. In all photomicrographs, the limits between the intact and the scar tissue are indicated by blue dashed lines and the limits between the white and the gray matter are shown with black dotted lines. Scale bars: 250 µm.

What is the origin of the SMI-32 (+) fibers visible into the lesion site?

As seen above, SMI-32 (+) motoneuronal axons are sometimes clearly seen to penetrate into the scar tissue (Fig. 3B) but the question remains as to whether other fiber populations also significantly contribute. To clarify this issue, we measured the diameter of the SMI-32 (+) axons inside the scar tissue, and compared it to that of the SMI-32 (+) fiber bundles formed of motoneuronal axons in the intact white matter. We hypothesized that if most SMI-32 (+) fibers inside the scar tissue originate from motoneurons, then the diameter distributions of the SMI-32 (+) fibers inside the motoneuronal axons bundles crossing the ventral white matter to form the ventral roots and of the SMI-32 (+) fibers inside the scar tissue should match. This hypothesis assumes that the diameter of motoneuronal axons does not change after they crossed the limit between the intact tissue and the scar tissue. This seems to be the case, as individual fibers whose diameter could be measured both inside the intact white matter and inside the tissue scar (Fig 3B) exhibited a comparable diameter independently of the region where the measure was performed. Into the scar tissue, both thick and thin SMI-32 (+) fibers were found (Fig 5A). The thick fibers were usually densely stained (Fig 5A, white arrowhead) and looked similar to motoneuronal axons in the intact white matter. Others fibers were rather thin and less densely stained (Fig 5A, black arrowhead), features that are exceptional for motoneurons axons inside the intact white matter. For Mk-ABS, the diameters' distribution of the SMI-32 (+) fibers present into the scar tissue was compared to that of the motoneuronal fibers. Both distributions overlapped, but the proportion of small diameter fibers was higher inside the scar that for motoneuronal axons (Fig 5B). This difference in fiber distribution has been consistently observed in all animals for which diameter measurements have been

obtained (Fig. 5C; N=9) and was statistically significant in all cases (Student's t-test, $p<0.01$). The data thus suggest that motoneurons axons are not the only fibers having the capacity to invade the scar tissue.

Figure 5

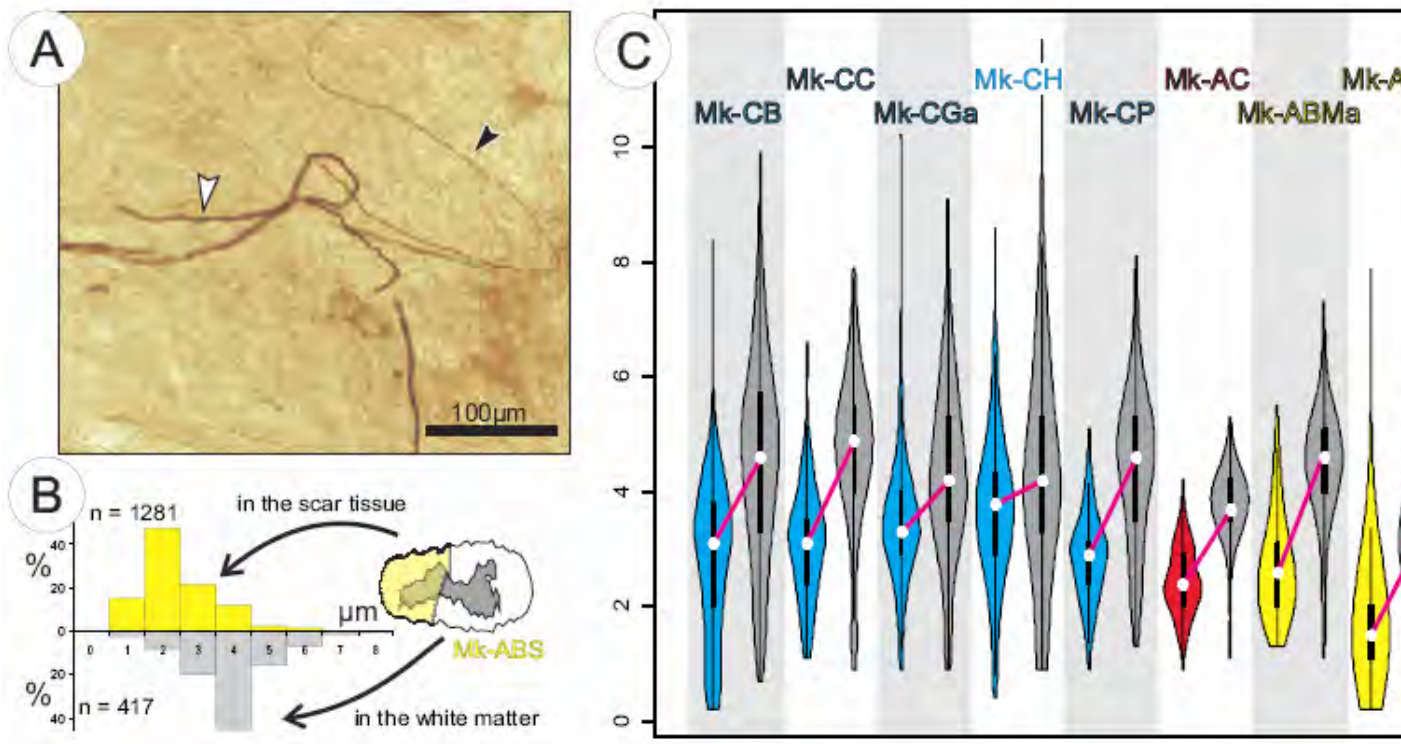

Figure 5: Distribution of the SMI-32 (+) fibers thickness in the scar and in the intact white matter. A: In the scar tissue, the thickness of SMI-32 (+) fibers differs: some fibers are rather thick and densely stained (white arrowhead) and some fibers are rather thin and less densely stained (black arrowhead). B: Diameter distribution of the SMI-32 (+) fibers observed inside the scar tissue (yellow) and into the motoneurons axons bundles inside the intact white matter (grey) of one animal (Mk-ABS). Note that though largely overlapping, the comparison of both distributions show that motoneuronal axons are generally large and cannot account for all SMI-32 (+) fibers found inside the scar tissue. C: Comparisons of the SMI-32 (+) fibers diameter distributions for nine animals. The violin plots indicate the median (white point), the first and third quartiles (thicker line segment), the minimum and maximum of the data as well as the distribution of the observations (envelope). A red segment line connects the median of the data obtained from one animal. Note that for all animals, the median diameter of fibers inside the scar

tissue is always lower than the median diameter of motoneurons axons inside the intact white matter. A statistical comparison between both fibers populations conducted for each animal using the student t-test indicated that the difference was statistically significant for all animals ($p<0.001$).

Do SMI-32 (+) fibers present into the scar respond to the growth promoting treatments?

To clarify whether the growth of SMI-32 (+) fibers into the lesion territory was affected by the presence of the anti-Nogo-A antibody, combined or not with BDNF, we counted the number of SMI-32 (+) fibers and measured their length inside the tissue scar. The number of SMI-32 (+) fibers counted into the scar tissue varied considerably across animas, but this value did not vary systematically in relation to the treatment (Figs. 6A and 6B). As it is conceivable that the SMI-32 (+) fibers increased in length, while their number remained constant, the length of all fiber segments found into the scar tissue was measured and added into a single cumulated value for each animal, plotted against the extent of the lesion. The percentage of SMI-32 (+) fibers having a diameter of less than 1.3 µm was determined (Fig. 6C). As for the number of SMI-32 (+) fibers, a large variability was observed across animals, and this variability was independent of the corresponding treatment or of the diameter of the fibers. Plotting the number of SMI-32 (+) fibers found in each animal in relation to their cumulated length revealed a clear linear relationship, unrelated to the treatments (Fig. 6D). Again, this tendency was independent from the diameter of the fibers. Thus, it appears that neither the number nor the cumulated length of the SMI-32 (+) fibers observed into the scar tissue are influenced by the anti-Nogo-A antibody alone or to its combination with the neurotrophic factor BDNF. SMI-32 (+) fibers into the scar belong mainly to motoneurons but also to other neuronal types. Since the thinnest fibers inside the scar did not have counterparts inside the motoneuronal axons bundles inside the intact white matter, these fibers were probably not belonging to motoneurons. By investigating separately the effects of the treatments on the largest and on the thinnest fibers, we can investigate whether the treatments act differently on different populations of neurons. Considering the distribution of the fibers thickness (Fig. 5B and 5C), we arbitrarily chose the limit of 1.3 µm to separate the population in thin fibers that, presumably, were predominantly not motoneuronal axons and large fibers where the proportion of motoneuronal axons is large. The number of SMI-32 (+) fibers inside the scar tissue was very different depending on the animal investigated (Fig 6A), the animal presenting the largest number of fibers having nearly as much as 20 times more SMI-32 (+) fibers into the scar tissue than the animal presenting the lowest number of fibers. This variability is not due to an effect of one of the treatment, as the animals with a large number

of fibers and those with a low number of fibers were found in all three groups of animals (Fig 6A). The same variability was also observed if thin fibers were investigated separately from the larger ones (Fig 6A). To clarify whether this variability could be accounted for by the dimension of the lesion, the number of fibers was plotted as a function of the lesion extent (Fig 6B). The data indicate that the number of SMI-32 (+) fibers tend to be greater in the larger lesions, but this tendency only accounts for a limited amount of the variability as shown by the large differences still observed in the animals with the largest lesions (Fig 6B). This variability was also observed for the thinnest fibers (Fig 6C). The treatments have thus no detectable effect on the number of SMI-32 (+) fibers present inside the scar tissue. This does however not exclude that the treatments may act on the length of the fibers present in the scar tissue. To evaluate this possibility, the number of SMI-32 (+) fibers inside the scar was plotted as a function of their cumulated length (Fig 6D). All the observations align along a single line, indicating that the mean length of a SMI-32 (+) fiber inside the scar tissue is not affected by the presence of the anti-Nogo-A antibody or of its combination with BDNF.

Figure 6

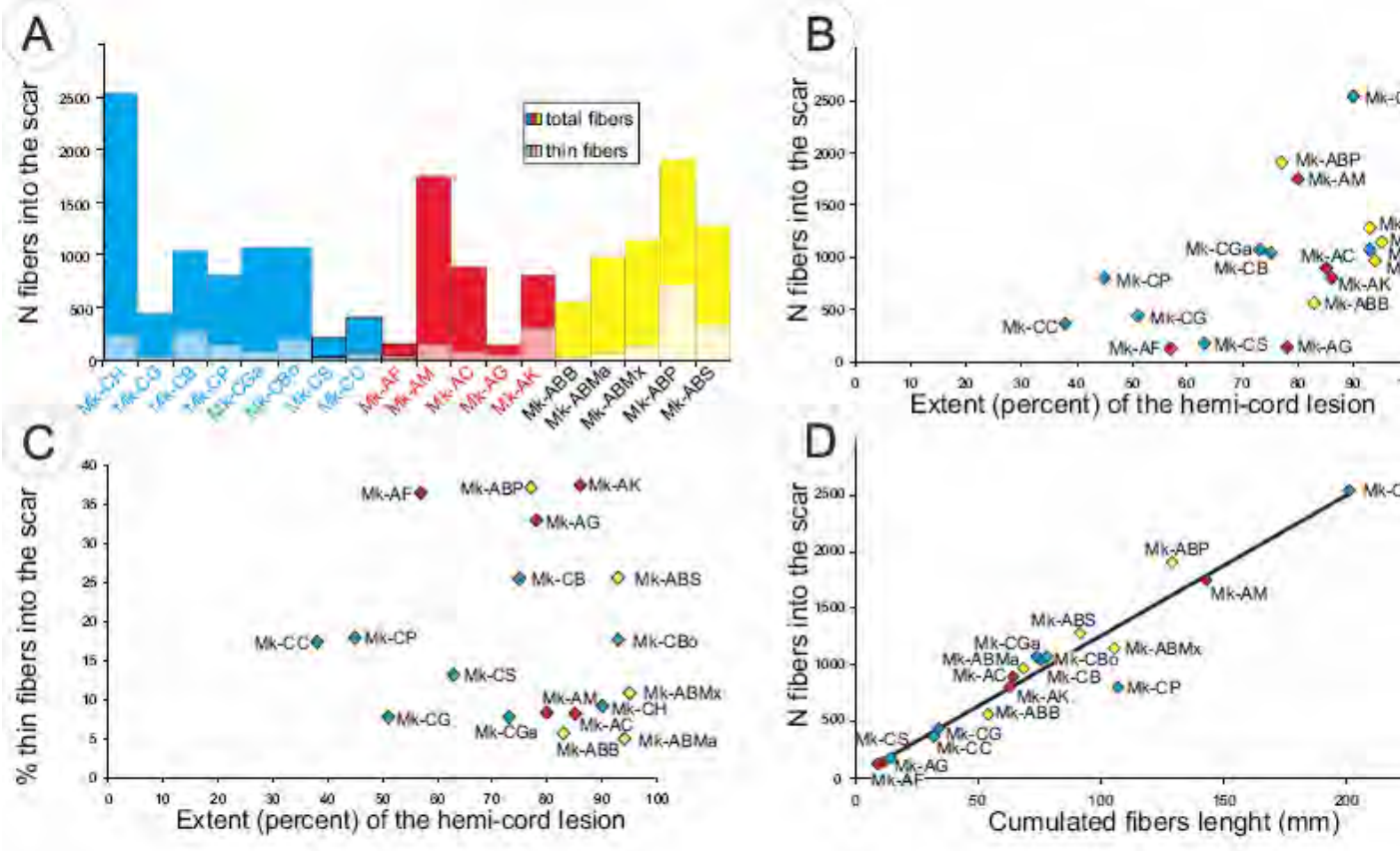

Figure 6: the number or length of SMI-32 (+) fibers inside the scar tissue is influenced neither by the anti-Nogo-A antibody nor applied together with BDNF. A: Bargraph representing the total number of SMI-32 (+) fibers counted into the scar tissue for each animal and each treatment. For all animals, the bar is divided in a lower region filled with a light color and corresponding to the fibers with a diameter lower than 1.3 µm, and a upper region filled with a darker shade of the color and corresponding to the larger fibers (see text). Note that the large individual variability is present in all three groups of animals and that the variability is equally important for the thinner and thicker fibers. No difference between treatment groups can be detected. B: Number of fibers counted into the scar plotted in function of the extent of the lesion and showing that this not the sole result of the lesion extent. No difference between treatment groups was detected. C: Percentage of fibers thinner than 1.3 µm counted into the scar plotted in function of the extent of the lesion and showing that for these fibers too, the variability is not accounted for the extent of the lesion and that no difference between treatment groups was detected. D: Number of fibers counted into the scar plotted in function of their cumulated length and showing that the growth of fibers is not affected by the treatments. Blue: control antibody treated animals; red: anti-Nogo-A treated animals; yellow: anti-Nogo-A / BDNF treated animals.

Discussion

The present study is a follow-up of investigations on the effects of a treatment neutralizing the neurite growth inhibitor Nogo-A in adult macaque monkeys subjected to incomplete spinal cord section. These investigations have indicated that animals treated with an anti-Nogo-A antibody recover better than control animals and that they present more CS fibers immediately above and under the lesion site (Freund et al 06;Freund et al 09;Freund et al 07). In these animals, CS fibers were only exceptionally found to penetrate inside the scar tissue (Freund et al 07) . The present data provide evidence that a sizable amount of neurites do however grow into the scar tissue. The bulk of these neurites presumably originate from motoneuronal axons, but it is likely that axons of other origins, though not of corticospinal neurons, also contribute to some extent. The growth of these fibers inside the scar tissue seems to be affected neither by the presence of the anti-Nogo-A antibody, nor by the presence of a mixture of this antibody with BDNF.

SMI-32 stained elements inside the spinal cord

The scar tissue formed after a spinal cord lesion is known to hinder the growth of nerve fibers (Brown and MacCouch 47;Clemente 55;Fawcett 06;Fawcett and Asher 99;Ramon y Cajal 28;Reier et al 83;Shearer and Fawcett 01;Silver and Miller 04;Stichel and Muller 98). In the present study, a sizable amount of SMI-32 (+) fibers was found within the scar tissue of all

animals. Indirect evidence suggests that a large proportion of these fibers are motoneuronal axons. First, in spinal cord sections, strongly stained SMI-32 (+) neurons were, for a majority, motoneurons. Moreover, none of the SMI-32 (+) descending or ascending axons present in the white matter do show such a darkly stained appearance. Second, motoneuronal axons are darkly stained and often present a large diameter, characteristics observed in the largest of the fibers present inside the scar tissue. Third, motoneuron axons crossing the white matter on their way to build the ventral roots form characteristic bundles. Axons were seen to leave such bundles located closely to the lesion and to penetrate the scar tissue. Although we consider it likely that most of the SMI-32 (+) fibers invading the scar tissue were motoneuronal axons, some were not, in particular those with axons of the thinnest diameter which were lacking from the bundles of motoneurons axons crossing the white matter.

Neurofilaments constitute the main structural element of the neuronal cytoskeleton and the presence in neurons of non-phosphorylated forms of neurofilaments can be shown using the Sternberger monoclonal incorporated antibody 32 (SMI-32) (Lee et al 88;Sternberger and Sternberger 83). Inside the spinal cord, motoneurons present a strong SMI-32 immunoreactivity (Tsang et al 00). Comparisons between the SMI-32 (+) neurons and those expressing choline acetyltransferase revealed that 82 to 100 % of the positive neurons located in the ventral horn of rats and primates (marmoset and rhesus monkeys) were double-stained. However, the SMI-32 staining is not specific for motoneurons. It also stains, though often less densely, neurons outside the ventral horn, such as for instance neurons in the Clark's columns or in the intermediate cell column (Carriedo et al 96;Tsang et al 00). Our observations are in accordance to those reports. As shown in Figures 2 and 3, the majority of the darkly SMI-32 stained neurons was located in the motoneuronal pools of the ventral horn. Other strongly SMI-32 stained neurons were detected in positions corresponding to Rexed laminae V to VIII, but they were infrequent.

In the cat, regeneration of motoneuron axons was observed after both peripheral and central injury (Havton and Kellerth 87;Linda et al 92;Linda et al 85). In this species, regeneration included the production of axons-like processes bearing structural characteristics of dendrites and axons, called "dendraxons" (Havton and Kellerth 87;Linda et al 92;Linda et al 85). These axons are myelinated, oriented towards the ventral root fascicles and kept a constant diameter. Although the existence of dendraxons has, at least to the best of our knowledge, never been reported in the primate, we cannot exclude that some of the SMI-32 (+) fibers observed here into the scar are "dendraxons".

CS fibers fail to grow inside scar tissue.

In a previous report on macaques that had received either a control antibody or the anti-Nogo-A antibody, CS fibers were only exceptionally found to penetrate the scar tissue (Freund et al 07). This observation is confirmed here on four additional animals receiving the control antibody and on two additional animals treated with the anti-Nogo-A antibody. It is now furthermore extended to five animals that received the combination of the anti-Nogo-A antibody together with BDNF. The failure of axotomized CS to grow inside the scar tissue has repeatedly been observed in rodents (Bregman et al 95;Inman and Steward 03;Schnell and Schwab 90) and primates (Fouad et al 04;Freund et al 06). In these species, while a treatment neutralizing Nogo-A often leads to regeneration of axotomized CS fibers, the fibers grow around the lesion instead of through the lesion and thus are not found inside the scar tissue. Interestingly, spontaneous invasion of the lesion site occurs from ascending tracts. In mice, ascending sensory fibers like those containing the calcitonin gene-related peptide (CGRP) or serotonin were reported to cross the inhibitory boundary formed by reactive astrocytes and to penetrate deeply into the scar (de, Jr. et al 05). In addition, neurites reactive to substance-P, to glycin and to GABA were also found within the scar (Brook et al 98). Recently, it has been shown that the axons of adult cat's spinal commissural interneurons can spontaneously grow through a local midline spinal section in spite of the presence of CSPGs in the lesion site and that these axons formed functional synaptic connections with appropriate neural targets (Fenrich and Rose 09). It thus appears that the mammalian spinal scar tissue does not constitute an environment hostile to regeneration for all fiber types. While this is firmly established for rodents and cats (Brook et al 98;Fenrich and Rose 09;Havton and Kellerth 87;Linda et al 92;Linda et al 85;Matthews et al 79;Wallace et al 87;Wang et al 96), it has, to our knowledge, never been reported for adult macaque monkeys. Our data show that some SMI-32 (+) fibers may even be attracted by the scar tissue.

Anti-Nogo-A antibody treatment alone or combined with BDNF do not induce detectable changes on the number or growth of the SMI-32 (+) fibers present within the scar tissue.

The SMI-32 (+) fibers encountered into the scar tissue in the present investigation demonstrates that this tissue is normally permissive to some types of neuronal fibers. Nogo-A is not expressed inside the scar tissue of rodents, but it was of interest to ascertain whether or not an antibody treatment neutralizing Nogo-A or this treatment

with the additional delivery of BDNF would influence the growth of SMI-32 (+) fibers inside the scar tissue. However, we did not detect any growth promoting effect of the anti-Nogo-A antibody alone or with BDNF on the SMI-32 (+) fibers of the scar. Nevertheless it remains possible that the infusion of the BDNF had an effect but that this effect could not been observed because it may not be permanent. In fact, it has been reported that in adult rats subjected to a SCI, BDNF increases the number of surviving motoneurons but only for three days postoperatively (Qin et al 06). After that, the number of cells decreased progressively to finally reach four days later the number of motoneurons in sham operated animals. Other studies have demonstrated that following both spinal cord compression and lumbar ventral root transection the level of BDNF mRNAs increases in motoneurons respectively 24 hours and 14 after the lesion (Ikeda et al 01;Li et al 06). Though opposite results, i.e. a down-regulation of the BDNF expression, were observed on injured lumbar motoneurons 30 minutes, 4 and 12 hours following a spinal cord hemisection (Gulino et al 04), all these studies also mention that the effect of BDNF was only transient, lasting only few days after the lesion. Thus, according to these results, it may well be that, in our conditions, BDNF had an effect on our treated animals but that, because the monkeys were not sacrificed rapidly after the lesion, these effects faded away with time and that they were undetectable at the time of the anatomical analysis. Finally, independently of the treatments and the thickness of the fibers, a great variability was observed across all animals. In spite of the fact that our study cannot explain this variability, the largely variable number and cumulative length of the SMI-32 (+) fibers presumably reflects the intrinsic variability inherent to each animal whether other types of treatment may reinforce the innate potential of the SMI-32 (+) fibers to invade the scar remains an open question. The presence of SMI-32 (+) fibers into the scar tissue reported into the present report demonstrates that some fibers can grow into this environment. As fibers such as the CS axons lack this capacity, the question arises as to the origin of this particularity. Understanding this origin could contribute to help transfer this characteristic to other fibers types, as thereby to help regenerating fibers to grow across a scar.

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Manuscript 3:

In adult macaque monkeys subjected to incomplete cervical cord lesion, infusions of BDNF combined with an anti-Nogo-A antibody treatment reduce the improvements observed with an anti-Nogo-A immunotherapy.

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Abstract

In adult mammals with a spinal cord injury, neutralizing the neurite growth inhibitor Nogo-A with antibodies promotes regeneration of corticospinal fibers and functional recovery. However, this treatment leads to regeneration over a limited length and does not impede most of the axotomized corticospinal neurons to shrink. Neurotrophic factors such as BDNF are known to stimulate neurite outgrowth and protect axotomized neurons. This raises the question of whether the effects obtained by neutralizing Nogo-A can be strengthened by adding BDNF during the antibody treatment.

In this study, a unilateral incomplete hemicord lesion was performed at C7/C8 level on three groups of monkeys: controls (using a control antibody, N=8), anti-Nogo-A (using an antibody neutralizing Nogo-A, N=5) and anti-Nogo-A/BDNF (using an antibody neutralizing Nogo-A and BDNF, N=5). Functional recovery was investigated by quantitatively assessing manual dexterity. Whereas the anti-Nogo-A animals returned to levels of performances similar to those observed before the lesion, both the control and the anti-Nogo-A/BDNF animals presented only a limited recovery. After completion of the behavioural investigations, both the presence of CS fibres at C3 and the size of motor cortex neurons were measured. The growth of CS fibers was promoted in the anti-Nogo-A animals but in the anti-Nogo-A/BDNF animals the presence of CS fibers remained comparable to that observed in control animals. In addition, examination of the size of CS neurons in the contralesional hemisphere did not indicate any treatment induced differences. In summary, our results suggest that adding BDNF to the treatment reduces the positive impact of the antibody neutralizing Nogo-A alone.

Introduction

In adult mammals, following spinal cord injury (SCI), transected axons do not regenerate and lost neurons are not replaced, resulting in persistent and often severe motor and sensory deficits. The absence of regeneration is attributed to various factors, in particular to the presence of myelin-associated neurite growth inhibitors such as Nogo-A in the central nervous system (CNS) environment and/or to the presence of insufficient levels of neurotrophic factors, such as BDNF, at the site of injury. ln recent years, several treatment strategies have emerged, promoting regeneration in the CNS, some with promising results. In particular, neutralizing Nogo-A with an antibody has lead to an improved functional recovery from SCI, as well as to regeneration and compensatory sprouting of corticospinal (CS) fibers in both rodents and monkeys (Bregman et al 95;Brösamle et al 00;Fouad et al 04;Freund et al 06;Freund et al 09;Freund et al 07;Gonzenbach and Schwab 08;Liebscher et al 05;Schnell and Schwab 90;Schwab 04;Thallmair et al 98). However, inhibiting Nogo-A did not prevent CS neurons to shrink and the length of the CS fibers regrowth remains limited (Beaud et al 08;Brösamle et al 00;Freund et al 07).

Neurotrophic factors like BDNF have also been proposed as therapeutic agents to promote regeneration inside the CNS, but the effects of such treatments remain controversial. Indeed, whereas several studies concluded that BNDF delivery protects axotomized CNS neurons, promotes sprouting of injured axons and improves functional outcome (Bregman et al 97;Giehl et al 98;Giehl and Tetzlaff 96;Hammond et al 99;Hiebert et al 02;Kamei et al 07;Namiki et al 00;Vavrek et al 06;Zhou and Shine 03), others reported conflicting results (Iarikov et al 07;Lu et al 01;Nakahara et al 96;Schnell et al 94;Schnell and Schwab 93). In addition, in adult rats subjected to SCI a combined treatment with NT-3 and anti-Nogo-A antibody increased regenerative sprouting of CS fibres, whereas treating with a combination of BDNF and anti-Nogo-A antibody had no effect (Schnell et al 94). However, no behavioural data were associated with the latter study and non-human primates were not investigated. Therefore, the aim of the present study was to assess the potential of functional recovery from SCI in macaque monkeys by combining BDNF and anti-Nogo-A antibody treatment, as well as the effect on the CS injured neurons and their axons. We compared three groups of SCI animals: i) monkeys treated with a control antibody, ii) monkeys treated with monoclonal anti-Nogo-A antibody alone, iii) monkeys treated with BDNF combined with anti-Nogo-A antibody.

Materials and Methods

Animals already published

In this report, behavioural assessments and anatomical evaluation of the size the SMI-32 positive neurons in the cortical layer V were performed on ten monkeys (see Table 1). However, in order to compare larger groups of animals, cases previously reported were also included in these functional and anatomical assessments (Beaud et al 08;Freund et al 06;Freund et al 09). These data are derived from fifteen lesioned monkeys that were either control antibody-treated (N=6) or anti-Nogo-A antibody-treated (N=9). All these 15 monkeys were handled similarly as the ten original monkeys. In consequence, most of the different methods presented below were also available for the animals already published. However, the BDNF treated and the anatomical analysis conducted at the C3 level on BDA stained CS axons in the spinal gray matter are new procedures.

Animals origin and identification

The experiments were carried out on ten (3.5-6.3 years old) cynomolgus (Macaca fascicularis) monkeys (male, 3.0 to 5.4 Kg; see Table 1), in accordance to the Guide for Care and Use of Laboratory Animals (ISBN 0-309-05377-3; 1996) and approved by local veterinary authorities, including the ethical assessment by the local (cantonal) Survey Committee on Animal Experimentation and a final acceptance delivered by the Federal Veterinary Office (BVET, Bern, Switzerland). The monkeys were either obtained from our own colony in our animal facility or were purchased from two certified suppliers (BioPrim, 31450 Baziège; France and Harlan Buckshire USA). During the course of the study, the general health condition of the monkeys was assessed quantitatively by measuring their body weight regularly (generally before each daily behavioural session). During the span of the experimental period, the animals tended progressively to increase their body weight. Moreover, after SCI, the monkeys did not turn uncooperative and did not show less motivation to perform the behavioural tasks. No signs of epilepsy, aggression or excessive fear with respect to the experimenter and animal care taker were observed.

In the present report, each monkeys' identification code contains for sake of clarity, a "C" or an "A" or "AB" at fourth digit position, indicating whether the monkey was respectively **c**ontrol antibody-treated, or **a**nti-Nogo-A antibody-treated, or treated with an combinatory treatment composed of **a**nti-Nogo-A antibody associated with the neurotrophic factor **B**DNF. For the examination of the fate of CS neurons, anatomical data from two additional intact monkeys were obtained. For these two animals, an "I" was placed at fourth digit position of

their identification code indicating that they are **i**ntact (i.e. uninjured and untreated). However, during the course of the experiments, the animals had different names from which the experimenters could not infer in which groups they belong.

Behavioural assessment

The monkeys were housed in our animal facility, in rooms of 12 m^3, each typically containing 2-4 monkeys free to move in the room and to interact with each other[5]. In the morning, before behavioural testing, the animal keeper placed the monkeys in cages used to allow subsequent transfer to the primate chair. The monkeys had free access to water and were not food deprived. The rewards obtained during the behavioural tests represented the first daily access to food. After the tests, the monkeys received additional food (fruits, cereals).

The dexterity of each hand was assessed separately in all lesioned monkeys with a finger prehension task, specifically our modified Brinkman board quantitative test (Rouiller et al., 1998; Liu & Rouiller, 1999; Schmidlin et al., 2004). The tests were conducted using a Perspex board (10 cm x 20 cm) containing 50 randomly distributed slots, each filled with a food pellet at the beginning of the test (home made behavioural apparatus) (Fig. 2B, inset). Twenty-five slots were oriented horizontally and twenty-five vertically. The dimension of the slots was 15 mm long, 8 mm wide and 6 mm deep. Retrieval of the food pellets was normally performed using the precision grip (opposition of thumb and index finger). This manual prehension dexterity task was executed daily, alternatively with one and the other hand, 4 to 5 times per week for several months before and after the unilateral cervical cord lesion. A daily behavioural session typically lasted 60 minutes. The performance of each hand was videotaped. In the present study, two parameters were assessed: (i) the retrieval score, i.e. the number of wells from which the food pellets were successfully retrieved and brought ton the mouth during 30 seconds, separately for vertical wells (Fig. 2A curves with blue diamantes), horizontal wells (Fig. 2A curves with pink squares) or both together (Fig. 2A curves with yellow triangles); (ii) the contact time, defined as the time of contact (in seconds) between the fingers and the pellets, calculated for the first vertical well and the first horizontal well targeted by the monkey in a given daily session. The contact time is comparable to the prehension time as introduced by Nishimura et al. (2007) for a different grasping task (Nishimura et al 07). T parameter reflects the capacity of performing the precision grip using the index finger and thumb. It is defined as the time separating the first contact of the index finger with the food pellet and the final successful grasp of the pellet utilising pad-to-pad

[5] A new Swiss regulation has been introduced in September 2010 requesting now a volume of 45m^3 at least to be given to a group of up to 5 macaque monkeys.

opposition of the index finger and thumb (i.e. when the pellet leaves the slots), for, respectively, the fist vertical and the first horizontal slots targeted by the monkey's hand.

After 30-60 days of initial training, the monkeys reached and maintained a stable level of performance (pre-lesion plateau) (Fig. 2A; red lines). Once reaching this level, they were subjected to the unilateral cervical cord lesion. After lesion, there was a progressive recovery of a various extent, lasting generally 30-40 days. Then, a stable level of recovered performance was reached, corresponding to a post-lesion plateau (Fig. 2A; green lines). The functional recovery was established by calculating the ratio in percent of the retrieval score at post-lesion plateau to that observed at pre-lesion plateau. In addition to the average score value, the contact time of the first vertical and first horizontal slots targeted by monkeys was measured, in order to minimize the impact of outliers the pre-lesion and post-lesion contact time was assessed as the median value (Fig. 3C and D). Considering that a good performance is reflected by a short contact time (in the pre-lesion), post-lesion performance (recovery) was expressed quantitatively as the ration (expressed as a percentage) of the pre-lesion median contact time to the post-lesion median contact time. For measures of both recovery of score and contact time, if the calculated values exceeded 100% (i.e. post-lesion performance was better than pre-lesion performance), the recovery was considered to be complete and therefore expressed quantitatively as 100%.

Surgical procedures

The anesthesia was introduced by intramuscular (i.m.) injection of ketamine (Ketalar®; Parke-Davis, 5 mg/kg, i.m.) and atropine was injected to reduce the production of bronchial secretions (0.05 mg/kg, i.m.). Before surgery, the animal was treated with the analgesic Carprofen (Rymadil®, 4 mg/kg, s.c.). Then, a continuous perfusion of a mixture of propofol 1% (Fresenius®) and a 4% glucose solution (1 volume of Propofol and 2 volumes of glucose solution) was delivered through an intravenous catheter placed in the femoral vein in order to maintain a deep anesthesia. Usually, the level of anesthesia remained deep and stable at a rate of venous perfusion of Propofol/glucose mixture of 0.1 ml/min/kg. When the animal was deeply anaesthetized, it was placed in a stereotaxic headholder, using ear bars covered at their tip with local anaesthetic. Surgery was carried out under aseptic conditions. During the surgery, different parameters were monitored: heart rate, respiration rate, expired CO_2, arterial O_2 saturation and body temperature. An extra intravenous bolus of 0.5 mg of ketamine diluted in saline (0.9%) was added at potentially more painful steps of the surgical procedure, such as laminectomy. In later experiments, ketamine was added to the perfusion solution and delivered throughout surgery (0.0625 mg/min/kg).

Placed in a ventral decubitus position, the animal had a pillow under the chest, and its head was kept in a flexed position ventrally to expose the spinal processes from C2 to Th1. After a vertical midline skin incision, the paravertebral muscles were retracted and the laminae of segments C6, C7 and Th1 were dissected. A complete C6 laminectomy and an upper C7 hemilaminectomy were then performed. The ligamentum flavum was removed in order to expose the dura mater, which was incised longitudinally. Observations on available anatomical material indicated that the transition level between the cervical spinal segments 7 and 8 was covered by the 6th cervical vertebra. The dorsal root entry zone at the C7/C8 border was then identified, providing a anatomical landmark for placing a surgical blade (No. 11; Paragon®), which was used to perform an incomplete hemisection of the cervical cord which completely cut the dorsolateral funiculus. The surgical blade was inserted 4 mm in depth perpendicularly to the spinal cord, and the section was prolonged laterally to completely cut the dorsolateral funiculus. In most cases, such a section completely interrupted the CS tract unilaterally. The lesion is located at C7/C8 level, caudal with respect to the main pool of biceps motoneurons but rostral to the pools of triceps, forearm, and hand muscle motoneurons (Jenny and Inukai 83). The muscles and the skin were sutured and the animal recovered from anesthesia usually 15-30 min after cessation of the venous perfusion with propofol. The animal was treated post-operatively with an antibiotic (Ampicilin 10%, 30mg/kg, s.c.). During the week following the surgery, Carprofen (4 my/kg) was given daily. After the spinal lesion, the animal was kept alone in a separate cage for a few days in order to get a perform a careful watch of its condition and allow better conditions for recovery than the usual housing in groups with other monkeys. 2-5 days after the surgery, the animal will be replaced in its groups.

Treatments

At the time of the lesion, the tip of a catheter was attached intrathecally in the vicinity of the lesion site. The other side of the catheter was attached to an osmotic pump which could deliver a volume of 2 ml. Two animals received a control antibody (Mk-CBo and MK-CGa), corresponding to a purified IgG of a mouse mAb directed against wheat auxin (AMS Biotechnology, Oxon, United Kingdom). (14.8 mg in 4 weeks). One animal (Mk-AG) received a monoclonal anti-Nogo-A antibody which was raised by immunization with the whole Nogo-A-specific region of the human Nogo-A sequence (14.8 mg in 4 weeks). Five animals (Mk-ABB, Mk-ABMx, Mk-ABMa, Mk-ABP and Mk-ABS) were implanted with one pump delivering this same anti-Nogo-A antibody and with a second pump delivering the neurotrophic factor BDNF (1.4 mg of BDNF in 4 weeks). The pumps were removed after 4

weeks. The various groups of monkeys with the different types of treatment are described in detail in Table 1. The characterization of the anti-Nogo-A antibody was performed as follows (Freund et al 07). Cynomolgus monkey brain tissue (cerebral cortex) was homogenized in T-PER lysis buffer (pierce, Rockford, IL) by using a rotor stator. For Western blots, aliquots corresponding to 10 µm total protein were separated on a 4-12% NuPAGE gel (Invitrogen, Carlsbad, CA). The protein bands were transferred to nitrocellulose membrane. The membrane was blocked for 1 hour at room temperature in blocking buffer [2% blocking reagent (Amersham, Arlington Heights, IL) in TBS-T], then incubated with 0.1 nM hNogo-A mAB in blocking buffer for 2 hours, followed by 1 hour of incubation with anti-human peroxidase-coupled secondary antibodies (1:500,000 dilution in blocking buffer). Signals were detected with ECL-Advance Western Blot detection reagents (Amersham) and exposure to film for 1 minute. The antibody recognizes primate Nogo-A monospecifically on Western blots (Oertle et al 03). The antibody was purified as IgGs and concentrated to 3-10 mg/ml in phosphate-buffered saline (PBS). The antibody hNogo-A mAB was found to be completely stable and shown to be distributed with the flow of cerebrospinal fluid over most of the spinal cord and brain within 7 days of infusion and penetrate deeply into the parenchyma (Weinmann et al 06). The antibody hNogo-A mAB is internalized together with endogenous Nogo-A protein into endosomal and lysosomal structures, leading to down-regulation of Nogo-A (Weinmann et al 06). The concentrations chosen for the anti-Nogo-A antibody treatment (3-10 mg/ml) are high in relation to the high-subnanomolar affinities of the antibody for Nogo-A. Therefore, differences in efficacy are not expected at these concentrations.

Histology

After removal of the pumps, the animals continued to be trained until they reached a stable post-lesion behavioural score. At that point, approximately three months before sacrifice of the animal, the anterograde tracer Biotinylated Dextran Amine (BDA; Molecular Probe®, Eugene, OR, USA) was injected in the primary motor cortex hand area on the contralesional hemisphere using Hamilton syringes in order to stain the CS tracts. Similarly, BDA was injected unilaterally into the dorsal part of the premotor cortex (PMd) of two intact monkeys included in a different study but in which dimensions of SMI-32 positive neurons in layer V of MI were examined in this study. The injections were performed under propofol anesthesia (see above). A craniotomy was carried out to expose the central and arcuate sulci. Injections of BDA were performed in the primary motor cortex (M1), i.e., in the rostral bank of the central sulcus, in a territory corresponding mainly to the hand representation. Based on

previously available monkeys, in which the hand representation was determined by intracortical microstimulation (Rouiller et al 96;Rouiller et al 98;Schmidlin et al 05;Schmidlin et al 04), the hand area was estimated to be situated immediately rostral to the central sulcus, extending mediolaterally between 10 and 15 mm from the midline, with its most lateral extent corresponding approximately to the genu of the arcuate sulcus. In this territory of about 5-6 mm along the mediolateral axis, three to four syringe penetrations were aimed perpendicularly to the cortical surface, at 1.5-2 mm distance from each other. To cover most of the rostral bank of the central sulcus, BDA was typically deposited along each syringe penetration at two (rarely three) depths, usually at sites located 3 and 7 mm below the pial surface. Usually one to three additional syringe penetrations were performed more medially, still along the central sulcus, to cover the representation of more proximal territories (writs, elbow, shoulder and trunk).

At the end of the survival period, the animals were sacrificed under deep (lethal) anesthesia (90 mg sodium pentobarbital/kg body weight) by transcardiac perfusion with 0.9% saline (400 ml). The perfusion was continued with fixative (3 litres of 4% phosphate-buffered paraformaldehyde in 0.1M phosphate buffer, pH 7.6) and solutions (2 litres each) of the same fixative containing increasing concentrations of sucrose (10%, 20% and 30%). The brain and the spinal cord were dissected and placed in a 30% solution of sucrose (in phosphate buffer) for cryoprotection for 7 days. Frozen sections (50 µm thick) of the brain were cut in the frontal plane, whereas frozen sections (50 µm thick) of cervical cord (approximately segments C6-T3) were cut in the parasagittal longitudinal plane and collected in three series for later histological processing. Upper cervical segments and lower thoracic spinal segments were cut in the frontal plane at 50 µm thick, and sections were also collected in three series. BDA staining was revealed in one series of the spinal cord sections. The second series of spinal cord sections was immunohistochemically processed to visualize corticospinal axons in the spinal cord using the marker SMI-32 (Covance, Berkeley, CA, USA). These series of spinal cord sections were used to reconstruct the location and the extent of the cervical cord lesion as described in detail in another report (Wannier et al 05) (Fig. 1). In addition to this parameter and as already introduced in Freund et al. (2009), the volume of each lesion was measured into the scar of SMI-32-stained sections in order to consider the extent of the lesion in 3-D rostro-caudal axis (Table 1 and Fig. 3) (Freund et al 09).The SMI-32 staining was chosen to reconstruct the extent of the lesion because the lesion contour was better defined, as compared to BDA staining. Similarly, a series of brain sections was also stained with the SMI-32 staining in order to visualize the layer III and V of MI. The epitope recognized by the SMI-32 antibody lies on non-phosphorylated regions of the neurofilament protein and is only

expressed by specific categories of neurons (Beaud et al 08;Campbell and Morrison 89;Tang et al 06). For all the anatomical analyses, a light microscope (Olympus) and Neurolucida software (MicroBrightField, Inc., Colchester, VT, USA) were used.

The BDA visualization procedure was initiated by rinsing the sections four times 5 min in a phosphate buffer (0.1 M, pH7.4). Sections were then incubated for 30 min at room temperature with ABC (avidin-biotinylated horseradish peroxidase complex) reactives (Vector Laboratories; dilution 1:500 in phosphate buffer with Triton). Sections were rinsed twice in phosphate buffer (0.1 M, pH 7.4), once in distilled water, and five times in cacodylate buffer 0.05 M (pH 7.2) before a 10-min preincubation in DAB solution (12.5 mg DAB, 250mg nickel ammonium sulfate, and 350 mg imidazole in 100 ml cacodylate buffer 0.05 M; pH 7.2). Sections were then incubated for 5-15 min in the same DAB solution in which 0.02% H_2O_2 was added. Finally, sections were rinsed three times in cacodylate buffer (0.05 M) and once each in distilled water and phosphate buffer. Because the final step in the immunocytochemical procedure was an ABC reaction (Wan et al 92), the BDA staining appeared as a brown reaction product (no use of nickel intensification).

For SMI-32 visualization, the free-floating sections were preincubated during 10 minutes in 1.5% H_2O_2 in phosphate-buffered saline (PBS; pH 7.2) and incubated overnight at 4 ^{o}C in SMI-32 monoclonal antibody (dilution 1:3000) in addition to 2% normal horse serum and 0.2% Triton X-100. The sections were then rinsed several times in PBS and incubated in a biotinylated secondary antibody (1:200, Vector Burlingame, CA) during 30-60 minutes at room temperature. Finally, the sections were stained using the avidin-biotin complex (ABC) immunoperoxidase method (Vectastain Elite kits, Vector). The reaction was visualized with 3,3'-diaminobenzidine tetrahydrochloride (DAB, 0.05%) as the chromogen, diluted in Tris-saline with 0.001% H_2O_2. The sections were then washed, mounted on gelatine-coated slides, dehydrated, and coverlipped.

Measurement of the CS sprouting at C3

The presence of CS fibers into the gray matter placed rostrally to the lesion was assessed in twelve injured monkeys (control antibody-treated: n=3; anti-Nogo-A antibody-treated: n=4; anti-Nogo-A antibody/BDNF treated: n=5). For this analysis, ten BDA-labeled frontal sections from the C3 level of each animal were studied. The extend of the sprouting of the BDA-labeled CS axons was estimated by counting the number of time that stained axons crossed one of three vertically oriented lines traced over the gray matter of the ipsilesional hemicord of each frontal section. This quantification method is similar to that used by Maier et al (Maier et al 08) to quantify CS fibers stained with BDA in rats. According to their method, four vertical (M, D1, D2 and L) and one horizontally oriented lines (H1) were superimposed on the gray matter on the ipsilesional side of the sections at a magnification of 15x (Olympus® DP 10® and Neurolucida® 7.0; MicroBrightField). The first vertical line M was drawn through the central canal. Then, the horizontal line H1 was drawn perpendicular to M and also crossing the central canal. After that, L was drawn parallel to M and crossed H1 at the lateral border of the gray matter. Finally, D1 and D2 were drawn parallel to M at one third and two-thirds of the distance between M and L, respectively (Fig. 3B). In order to have a quantitative assessment of the CS fibers' growth and sprouting, the axons crossing these lines except the line L and M were counted at a total magnification of 400x. To prevent multiple counting of single collaterals because these fibers have an irregular course, the fibers crossing several times the same line were counted once. Then, the cumulative number derived from ten frontal sections was normalized by dividing it by the total number of CS axons present at C3 level in the whole white matter (Fig. 3A). This number was obtained by counting and averaging the total number of BDA labelled CS axons seen in the entire white matter on three frontal sections. Thus, results are expressed as a mean value of counted fibers at C3 level crossing D1, D2 and H1 in the ipsilesional part of the gray matter in ten sections divided by the mean value of CS fibers visible in the whole white matter at the same level in three sections.

Using a computerized tracer system (Neurolucida®), three representative reconstructions of coronal sections of cervical spinal cord (C3) were drawn for three differently treated animals in order to visualize the extent and specificity of the CST axons growth in response to injury and the three different treatments (Fig. 3C; dotted box).

Measurement of the soma size of the CS neurons in the layer V

In a recent report (Beaud et al 08), we have shown that, consequently to cervical cord lesion, a CS soma cell shrinkage occurred that could be not prevented by administrating an anti-Nogo-A antibody (Fig. 4C; underlined identification codes). Here, using the same methods, we examine the fate of the CS soma cells when a combination treatment of an anti-Nogo-A antibody and of BDNF was applied. For this analysis, two intact (Mk-IR13 and Mk-IC) and eight SCI monkeys were examined (control antibody-treated n=2; anti-Nogo-A antibody-treated n=1 and anti-Nogo-A antibody/BDNF treated n=5) (Fig 4C; identification codes not underlined). Because it has already been demonstrated that following cervical cord injury, and independently of the treatments administrated, the number of CS neurons on the contralesional hemisphere remains comparable to that on the ipsilesional hemisphere, the number of CS neurons was not assessed in the present report (Beaud et al 08;Wannier et al 05).

Briefly, the soma of CS neurons was evaluated on coronal SMI-32-labeled sections of motor cortical areas located in the medial wall of the gyri praecentralis in the primary motor cortex (M1). On SMI-32-stained sections, the well-stained pyramidal cells were easily identifiable even at low magnification (40x), especially in the ipsilesional hemisphere in the injured animals (Fig. 4Ba). First, a territory of approximately equal size was delineated on both hemispheres that included layer V at the same dorsoventral location in the same histological brain section at low magnification (40x) (Fig. 4B). Thus, the measurements were performed on sections of the hemispheres of comparable thickness for each section analyzed. Then, in these territories, the somatic cross-sectional silhouette area of the SMI-32 positive neurons on both hemispheres was determined at high magnification (400x) (Olympus® DP 10® and Neurolucida® 7.0; MicroBrightField). Only SMI-32 (+) neurons with a visible nucleus and positioned in M1 were taken into account for the measure. The typical appearance of SMI-32 positive neurons visible at low and high magnifications in layer V of M1 is illustrated in Figure 4A. The transition between of the soma and the apical dendrite was arbitrarily set at a distance of 20 µm from the nucleus (Fig. 4A black dotted line). In each monkeys, the analysis was conducted on eight coronal sections separated by 800 µm and covering much of the rostrocaudal extent of M1. Comparisons between hemispheres were performed using the Mann-Whitney two tailed test. A *p* value lower or equal to 0.05 was considered as indicating a significant difference between the neuronal populations investigated.

Table 1: List of the new monkeys included in the present study with identification code.

ID code	Mk-CGa	Mk-CBo	Mk-AG	Mk-ABMx	Mk-ABMa	Mk-ABB	Mk-ABS	Mk-ABP	Mk-IC	Mk-IR13
species	fasc.	fasc.	fasc.	fasc.	fasc.	fasc.	fasc.	fasc.	fasc.	fasc.
Treatment	Control antibody	Control antibody	hNogo	BNDF & hN	BNDF & hN	BNDF & hN	BNDF & hN	BNDF & hN	-	-
Weight	3	3.5	3.7	5.4	3.5	5.3	4.2	4.6	6.3	3.8
"Experimenter Blind" proce	Yes	Yes	Yes	Yes	Yes	Yes	Yes	Yes	No	No
Hemisection extent (%)	**73**	**93**	**78**	**95**	**94**	**83**	**93**	**77**	-	-
Volume of lesion (mm3)	**3.76**	**4.11**		**9.55**	**9.81**	**2.36**	**10.37**	**6.2**		
Functional recovery (%)										
Score (vert)	**100**	**100**	**100**	**78**	**89**	**84**	**71**	**72**	-	-
Score (horiz)	**100**	**97**	**100**	**71**	**43**	**100**	**59**	**65**	-	-
Contact time (vert)	**75**	**94**		**72**	**50**	**57**	**53**	**50**	-	-
Contact time (horiz)	**100**	**100**		**53**	**59**	**72**	**55**	**41**	-	-
Completeness of dlf sectior	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	**Yes**	-	-
									-	-
Stock farming	**BioPrim**	**BioPrim**	**BioPrim**	**BioPrim**	**BioPrim**	**BioPrim**	**Buckshire US**	**Buckshire US**	**BioPrim**	**Own colony**

At the time of the experiment, monkeys were assigned codes that did not allow experimenters to determine whether the animal was infused with the control or the anti-Nogo-A antibody or the anti-Nogo-A antibody combined with BDNF. New names were assigned to the monkeys during the writing of the manuscript to improve its readability.

Under species, "fasc." is for *Macaca fascicularis*.

The two new control antibody-treated monkeys are in the two leftmost columns, whereas the five anti-Nogo-A/BDNF treated monkeys are in the five middlemost columns ("BDNF/Anti-Nogo-A") with indication of which antibody was used (mAB hNogo-A). Between these two groups of animals there is the new animal treated with the anti-Nogo-A antibody alone.

Functional recovery (expressed in % for the behavioural parameter "score") was assessed here based on the modified Brinkman board task, by comparing the performance pre- and post-lesion, as explained in detail in the Materials and Methods section.

Abbreviations: hNogo: a monoclonal anti-Nogo-A antibody which was raised by immunization with the whole Nogo-A-specific region of the human Nogo-A sequence; fasc.: Macaca fascicularis; vert: vertical; horiz: horizontal; dlf: dorsolateral funiculus.

Results

Cervical cord lesion

The location and extent of the lesion was assessed by reconstructing the lesion site from histological sections. In four animals, few intact BDA stained fibers were observed in the white matter at the level of the lesion, indicating that in these animals the CS tract was not completely transected (Fig. 1; asterisks). The extent of the lesion was expressed as a percentage of the corresponding hemisection surface for each monkey (Fig.1 and Table 1). In one monkey (Mk-AT), the spinal cord was damaged during its extraction from the vertebrae and it was not possible to use the histological material to reconstruct the lesion.

While the position and the size of the cervical lesions were variable across the monkeys, the dorsolateral funiculus of all monkeys except four was unilaterally completely transected by the lesion (Fig. 1). Thus, in most animals, the lesion completely transected the majority of the CS axons resulting in a substantial manual deficit of the ipsilesional hand (Fig. 2B). In fact, as shown in Figure 4A by injecting BDA in the hand region of the contralesional motor cortex, the majority of the CS fibers (90-95%) that originated from the opposite hemisphere travel in the dorsolateral funiculus ipsilesionally. The cervical lesion affected also other descending tracts such as the rubrospinal tract, the reticulospinal tract, the tectospinal tract and the vestibulospinal tract, and also ascending tracts like the spinothalamic tract in the dorsal columns. However, in the present investigation, only the CS projections were labeled and analyzed.

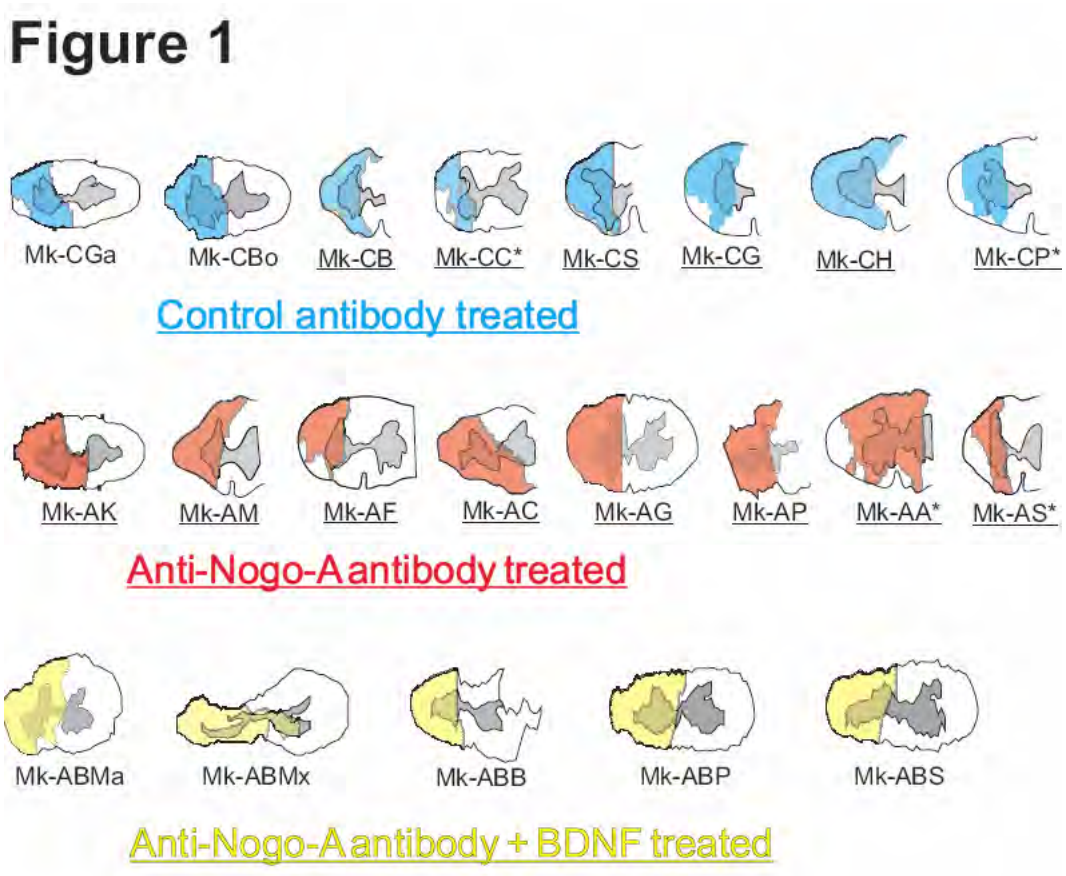

Figure 1: Reconstruction in the frontal plane, from sagittal sections, of lesion extent at cervical level C7/C8 in all monkeys. The grey area represents the grey matter of the cervical cord. Note that, due to the lesion, the hemi-cord on the side of the lesion has been distorted and that the lesions of thirteen monkeys have been previously publishes in recent reports (Beaud et al., 2008; Freund et al., 2006a, 2007) (underlined names). In addition, in four monkeys (asterisk), the dorsolateral funiculus was not completely transected (see Table 1; Freund et al., 2006a). The reconstruction of the lesion of the monkey Mk-AT was not presented here because the tissue was damaged in the area of the injury and it was therefore not possible to reconstruct the extent of the injury for this animal. For all the monkeys, a code colors was applied in relation to what treatment have received the animals: the blue color corresponds to the injured monkeys that have received that control antibody, the red color corresponds to the lesioned monkeys that have received that anti-Nogo-A antibody alone, the yellow color corresponds to the injured monkeys that have received that anti-Nogo-A antibody associated with the neurotrophic factor BDNF and the green color corresponds to the intact animal that have no spinal cord lesion and have received any types of treatments. Likewise, as explained in the Material and Methods section, another identification code placed at fourth digit position of the monkey's names was used for more clarity in order to refer also whether the monkeys were either injured and/or treated with the control antibody (Mk-CX), the anti-Nogo-A antibody alone (Mk-AX), the combination of both the anti-Nogo-A antibody and the neurotrophic factor BDNF (Mk-ABX) and finally the intact monkeys that were uninjured and untreated (Mk-IX).

Post-lesion recovery of manual dexterity

In all monkeys, the deficit in manual dexterity generated by the cervical cord lesion was followed by a progressive recovery (Fig. 2A). The manual dexterity evaluation was evaluated pre- and post-lesion using the modified Brinkman board task. This "reach and grasp" task allows to measure the ability of the monkey to use its index finger and thumb in the precision grip to retrieve rewards placed in vertical and horizontal oriented slots (Fig. 2B). On each daily session, the manual performance of the monkey was quantified for each hand by generating a score, given by the number of rewards retrieved in 30 seconds. This score varies in a characteristic way during the time course of the experiment (Fig.2A). First, the score increases progressively during the period where the monkey is learning the behavioural task. Then, the score is stabilizing into a pre-lesion plateau where the monkey has acquired the optimal dexterity to perform the behavioural task (Fig. 2A; red lines). Following this phase, a unilateral cervical cord lesion is performed in order to affect the hand with which the monkey reaches the highest score. After the lesion, the score is falling down drastically during several days to re-increase progressively. This period corresponds to the recovery phase. Finally, the score stabilizes again into a post-lesion plateau reflecting the moment when the monkey has recovered most of its manual capacity for this task (Fig. 2A; green lines). To assess how functional recovery is related to the size of the lesion, the extent of functional recovery (ration of post-lesion versus pre-lesion plateau in percent) was plotted as a function of the extent of the hemisection (Fig. 2B; see also Table 1 for individual values).

The Figure 2 shows the relationship between lesion extent and recovery of manual dexterity among the groups of injured monkeys that were either treated with the control antibody (blue diamonds) or with the anti-Nogo-A antibody (red squares) or with the associated treatment anti-Nogo-A antibody/BDNF (yellow triangles). For the animals that have received the control antibody, the recovery was variable but somewhat lower as compared to the anti-Nogo-A antibody-treated monkeys. In fact, the animals treated with the anti-Nogo-A antibody even those having large lesions tended to recovered significantly better compared to the control antibody-treated monkeys (Mann-Whitney one tailed test, p=0.08). In contrast, the monkeys treated with the combinatory treatment anti-Nogo-A antibody/BDNF exhibited a significantly lower functional recovery as compared to the animals that have received that anti-Nogo-A antibody alone (Mann-Whitney one tailed test p<0.05). In fact, anti-Nogo-A antibody/BDNF-treated monkeys showed a functional recovery comparable to the control antibody-treated monkeys even with smaller lesions. The movement for picking food pellets out of the horizontal and vertical slots differ, the positioning of the finger inside the horizontal

slots requiring a rotation of the wrist. The impact of a lesion on the capacity to empty horizontal slots reflects this difference and is often more pronounced than that regarding vertical slots. We hence verified whether any treatment would reveal a particular efficacy on the recovery of each type of movement. For this matter, the behavioural scores for the vertical and for the horizontal slots were established separately, and the effects of the treatments tested as described here above for the total score. The analysis presented the same pattern as that observed for the total score: for both vertical and horizontal slots, the recovery of the anti-Nogo-A antibody treated animals was improved with regard to the control antibody treated animals, but the recovery of the animals that received BDNF in addition to the anti-Nogo-A antibody kept in the range of what had been observed with the animals that had received the control antibody (data not shown).

The measure of the extent of the lesion only gives information about the degree of the interruption of pathways running along the spinal cord. To also take in account the magnitude of tissue damage, the recovery of score (number of pellets retrieved in 30 seconds) was plotted as a function of the estimated volume of the lesion, for both the vertical and the horizontal slots (Fig. 3A and B). Similarly, recovery of contact time was plotted as a function of the estimated volume of the lesion in Fig. 3C and D, for the vertical and horizontal slots, respectively. Functional recovery (both for the score and the contact time) was more prominent for anti-Nogo-A antibody-treated monkeys (largely irrespective of the estimated volume of the lesion) than for control antibody-treated monkeys (Fig. 3). In the latter group, the functional recovery for the score was not correlated with the estimated volume of the lesion for both the vertical slots ($r= 0.18$; $P< 0.006$) and the horizontal slots ($r= 0.159$: $P< 0.024$). Still in the group of control antibody-treated monkeys, the functional recovery for the contact time was uncorrelated with the estimated volume of the lesion for the horizontal slots ($r= 0.0233$; $P=0.956$) and for the vertical slots ($r= 0.00942$; $P= 0.982$). In the anti-Nogo-A antibody-treated monkeys (Fig. 3), recovery was not correlated or poorly correlated (in the case of the CT for horizontal slots) with the estimated volume of the lesion, as indicated by the coefficients of correlation of $r= 0.026$, $r= 0.097$, $r= 0.283$ and $r= 0.83$ in panels A, B, C and D, respectively, of Fig. 3; that were not statistically significant ($P>0.05$). Similarly, the anti-Nogo-A antibody/BDNF treated animals, recovery was not or only poorly correlated (in the case of the score for horizontal slots) with the volume of the lesion, as indicated here too ba the coefficients of correlation ($r=0.176$, $r=0.84$, $r=0.102$, $r=0.427$) that were not statistically significant ($P>0.05$).

It thus appears that following a unilateral cervical cord lesion the association of an antibody directed against the neurite growth inhibitor Nogo-A with the neurotrophic factor BDNF does not stimulate any improvement of dexterity, while the animals that received the anti-Nogo-A antibody alone, recovered better.

Figure 2

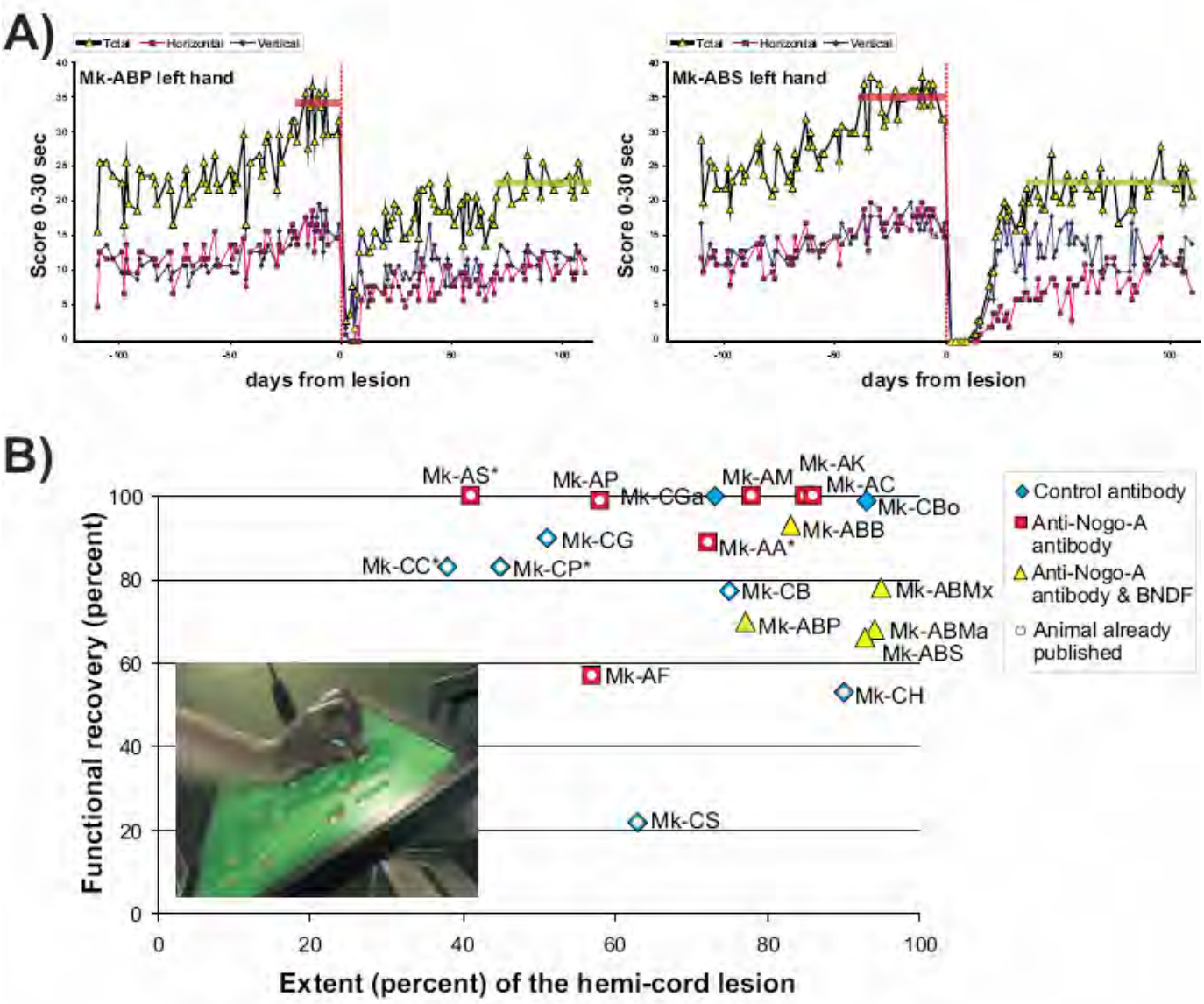

Figure 2: Quantitative assessment of manual dexterity before and after lesion. <u>Panel A:</u> Two examples of behavioural curves showing the retrieval scores of the hand on the side ipsilateral to the lesion as a function of the days from lesion (before and after lesion). These data were derived from the modified Brinkman board task (number of pellets retrieved in 30 seconds), for two lesioned animals treated with anti-Nogo-A antibody/BDNF combination. In these graphs, day 0 corresponds to the day when the lesion was performed and is represented by a red vertical

dashed line. Under each total score of each animal (yellow triangle), the scores for the vertical (blue diamantes) and horizontal (pink squares) slots were plotted separately. The two horizontal bars placed on each total score represent respectively, the pre-lesion plateau (red) and the post-lesion plateau (green). Panel B: Relationship between the extent of hemi-cord lesion in percent and degree of functional recovery of score in % for the modified Brinkman board test for vertically and horizontally oriented slots (blue diamonds for control antibody-treated monkeys, red squares for anti-Nogo-A antibody-treated monkeys and yellow triangles for anti-Nogo-A antibody/BDNF-treated monkeys). The monkeys that were already published were illustrated with an additional white circle inside their geometrical form. In this graph, on can see clearly the tendency that the anti-Nogo-A antibody treated animals recovery better compare to the other treated animals and thus, independently of the size of the performed lesion. On the other hand, the animals that have received either the control antibody or the associated treatment anti-Nogo-A antibody/BDNF recover less even when there lesion was smaller.

A photograph illustrating the modified Brinkman Board task used to assess manual dexterity pre- and post-lesion was inserted inside the graph for a better comprehension. In this image, one can see the board comprising 50 slots containing a food pellet, 25 oriented vertically and 25 horizontally. The monkey grasps the pellet in horizontal slot by performing the precision grip (opposition of index finger and thumb).

Figure 3

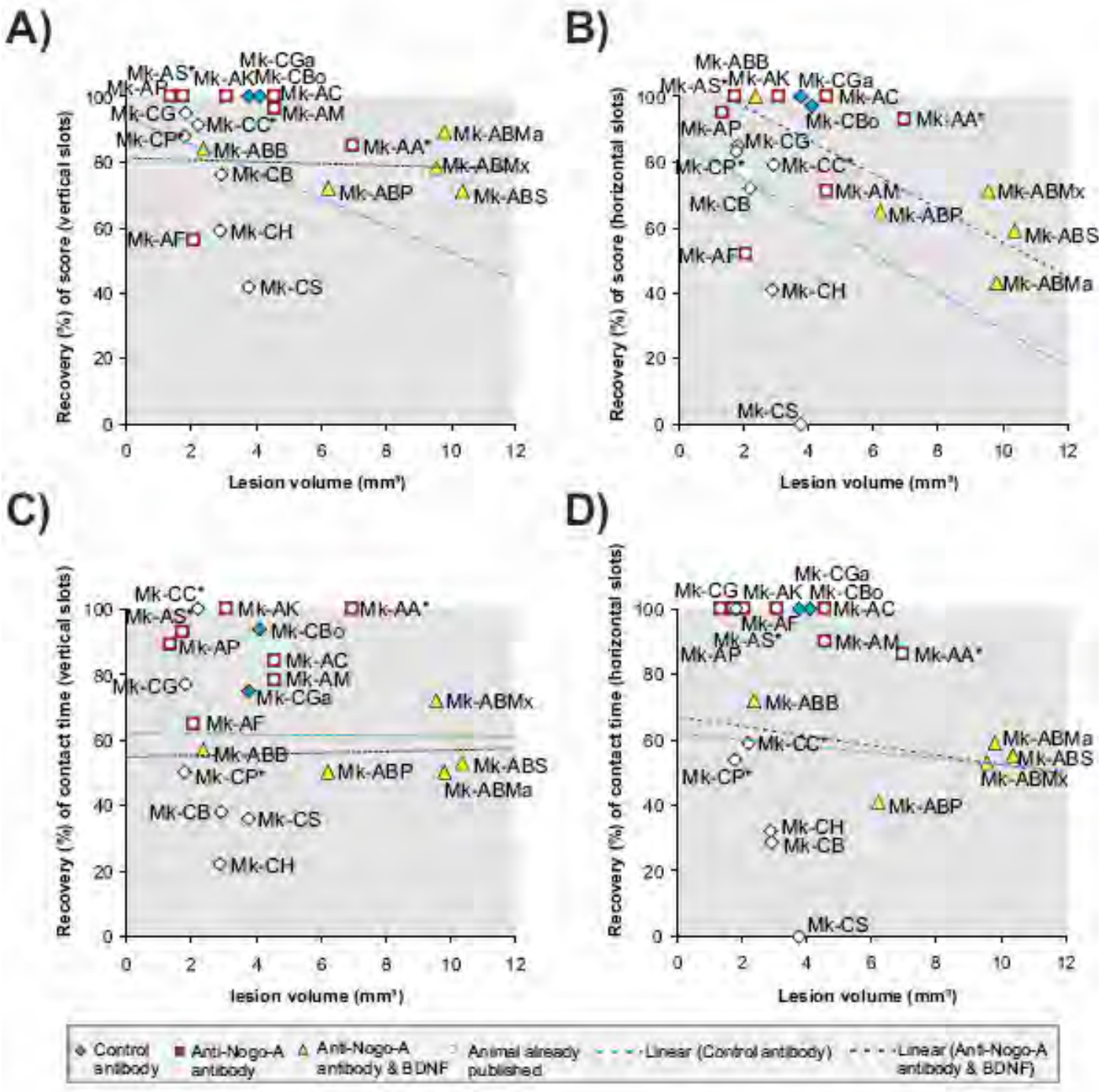

Figure 3: Relationship between the behavioural parameters score and contact time as function of the estimated volume of the cervical lesion. <u>Panels A and B:</u> Relationship between the degree of functional recovery of score (as a percentage) for the modified Brinkman board test and the estimated volume of the cervical lesion for (A) vertically and (B) horizontally oriented slots (blue diamantes for control antibody-treated monkeys, red squares for anti-Nogo-A antibody-treated monkeys and yellow triangles for anti-Nogo-A antibody/BDNF-treated monkeys). <u>Panels C and D:</u> Relationship between the degree of functional recovery of contact time needed for the first successful retrieval and the estimated volume of the cervical lesion, for (C) vertically and (D)

horizontally oriented slots (blue diamantes for control antibody-treated monkeys, red squares for anti-Nogo-A antibody-treated monkeys and yellow triangles for anti-Nogo-A antibody/BDNF-treated monkeys). In each panel, the dotted blue line and the black dotted line represent the linear regression line calculated for the score or contact time and the estimated volume of the lesion for the group of control antibody-treated monkeys (blue diamonds) and anti-Nogo-A antibody/BDNF-treated monkeys (yellow triangles), respectively. In four monkeys (*), the dorsolateral funiculus was not completely transected (see Fig. 1) and the monkey Mk-AK differ from the others because post-lesion treatment was delayed by 1 week.

Evaluation of the CS sprouting in the gray matter rostral to the lesion at C3 level

In view of these unexpected behavioural results (regarding the deleterious effect of BNDF), it is essential to confront these observations to anatomical data in order to evaluate how the CS axons respond to the combination of the anti-Nogo-A antibody and BDNF. For this assessment, BDA-labeled CS axon arbors leaving the cervical white matter and entering the gray matter on the ipsilesional side of the cord were analyzed rostrally to the lesion in frontal sections taken at C3 level for twelve monkeys that showed good quality of their histological sections. As result of multiple injections of BDA into the contralesional primary motor cortex (M1) mainly in the hand representation, the large majority of stained CS axons (90-95%) was found at the cervical cord level C3 in the ipsilesional dorsolateral funiculus, but a minority of uncrossed CS projections (5-10% of the CS axons) run in the contralesional dorsolateral and ventral funiculus (Fig. 3A). Such distribution in the macaque is in line with other recent reports (Lacroix et al 04;Rouiller et al 96;Wannier et al 05;Yoshino-Saito et al 10). In order to measure the strength of CS fibers projections into the grey matter, the number of BDA stained fibers crossing virtual lines placed over the gray matter were counted in the ipsilesional side (Fig 4B; see methods). This number was then normalized with respect to the mean total number of BDA-labeled CS axons counted into the whole white matter in three consecutive frontal sections (Fig. 4A) and plotted as a function of the extent of the lesion or as a function of the estimated volume of the lesion (Fig. 4C and D).

In the inset of figure 4C, three examples of reconstructions of frontal sections, one for each group of treated animals, show the pattern of the axonal arborization observed in the cervical gray matter (Fig. 4C; dotted box). The apparent density of CS neurites growing into the cervical gray matter was lower in the control antibody-treated group as compared to the two other groups of animals. In addition, the extent of sprouting of the CS fibers in the ventro-lateral direction appears to be enhanced by the anti-Nogo-A antibody alone or when combined with BNDF. However, after normalization (Fig. 4C), the group of animals treated with the

anti-Nogo-A antibody alone tended to have generally more neurites at C3 level than those treated with a control antibody (Mann-Whitney one tailed test, *p=0.07*). In addition, adding BDNF to the anti-Nogo-A antibody therapy decreases notably the amount of the CS fibers observed in the C3 spinal grey matter and this group of monkeys does not differ significantly from the one that has received the control antibody treatment only (Mann-Whitney one tailed test, *p=0.44*) (Fig 4C and D). Thus, in line with the functional recovery, the addition of BNDF to the anti-Nogo-A antibody therapy reduced or even suppressed the sprouting observed when using the anti-Nogo-A antibody alone as treatment. Indeed, the BDNF group of monkeys exhibits comparable normalized axonal sprouting as observed in control antibody-treated monkeys. In other words, the combination with BDNF appears to thwart the beneficial effect on axonal sprouting obtained by the administration of the anti-Nogo-A antibody alone.

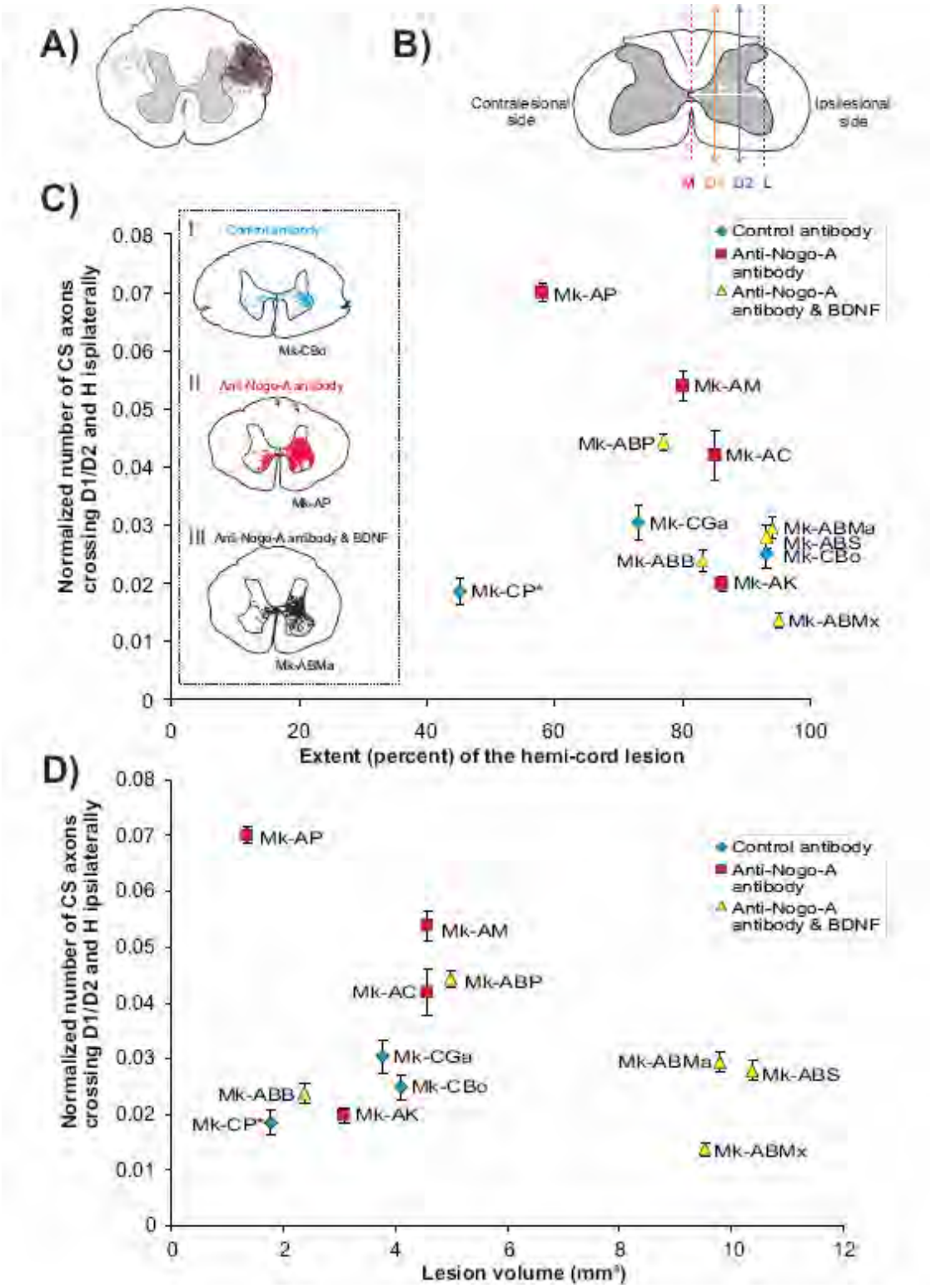

Figure 4: Relationship between the axonal arborization and hemi-cord lesion extent rostrally to the spinal cord injury at C3 level. <u>Panels A</u>: Transverse section of the cervical spinal cord at the C3 level cervical enlargement of the monkey Mk-ABMa showing the distribution of the BDA-labeled CS axons as a result of BDA injections in the left M1 principally in the hand representation. In this reconstruction, each BDA-positive CS axons is represented by a dot and the gray area corresponds to the gray matter. The small circle located in the middle part of the gray matter corresponds to the

central canal. The majority of the CS nerve fibers (90-95%) has decussated and travels in the opposite dorsolateral funiculus in the contralateral side of the spinal cord. The rest of the CS axons (5-10%) are uncrossed projections running ispilaterally and found in the homolateral dorsolateral and ventral funiculus. Panels B: BDA-positive CS axons were quantified in ten adjacent cross sections at C3 level by counting all intersections with lines H1, D1 and D2 in relation to the method published by Maier et al. . While H1, D1 and D2 were drawn for the quantitative analysis of the axonal arbors leaving the cervical white matter and entering the gray matter in the ipsilesional side of the cord (full lines), the lines M and L were drawn as reference for placing correctly the other lines (dotted lined). M was placed vertically through the midline. H1 was positioned in the ipsilesional side of the cord perpendicularly to M and touching the central canal and the lateral edge of the gray matter. L was located at the lateral rim of the gray matter perpendicularly to H1 and parallel to M. D1 and D2 were drawn parallel to M at one-third and two-thirds of the distance between the central canal and the lateral gray matter border. Panels C: Relationship between the extent of hemi-cord lesion in percent and the average of the total normalized number of CS nerve fibers crossing at C3 level the lines H1, D1 and D2 ipsilaterally. The normalization was performed by counting and averaging in three consecutive cross sections the number of CS axons running the whole white matter at C3 level. In the left lateral doted box, three reconstructions of cross sections were drawn in order to have an impression of the axonal arbors visible in the gray matter of three different treated monkeys (I is a control-treated animal, II is a anti-Nogo-A-antibody-treated animal and III is a anti-Nogo-A-antibody/BDNF-treated animal). In these reconstructions, it appears that the axonal arbors are reduced in the control-treated and the anti-Nogo-A-antibody/BDNF-treated monkeys compare to the monkey that received the anti-Nogo-A-antibody alone. This relation between the extend of the axonal arborization in the gray matter and the type of treatment given is confirm when the quantity of the axons counted in the ipsilesional gray matter is normalized by the total number of CS nerve fibers counted in the whole white matter. Compare to the other animals, the anti-Nogo-A treated monkeys have the tendency to have more axonal arborization in their gray matter. Thus, it seems that the association of the anti-Nogo-A-antibody and BDNF diminishes the nerve fibers arborescence in the cord gray matter rostrally to the lesion at C3 level. Panels D: Relationship between the estimated volume of the lesion and the average of the total normalized number of CS nerve fibers crossing at C3 level the lines H1, D1 and D2 ipsilaterally.

Does the administration of BDNF influence the shrinkage of the CS neurons?

In recent reports, we have shown in control antibody or anti-Nogo-A antibody treated SCI monkeys, that the number of layer V pyramidal neurons in the contralesional M1 was comparable to that observed in the ipsilesional M1, but their mean cell surface diminished (Beaud et al 08;Wannier et al 05). It was concluded that axotomized CS neurons did not die but shrank. Moreover, following SCI, the intrathecal infusion of the anti-Nogo-A antibody did not prevent or reduce the shrinkage of layer V pyramidal neurons. In the present study, this observation is confirmed by comparing the intact monkeys (green violin plots) to the control antibody-treated monkeys (blue violin plots) and to the anti-Nogo-A antibody-treated animals (red violin plots) (Fig.5C). Irrespectively of the treatment given, all but one of the SCI monkeys exhibited an interhemispheric difference, with CS neurons on the contralesional hemisphere significantly smaller than those on the ipsilesional hemisphere. For monkey Mk-CH, no difference in the median soma size was observed. However, for this animal as for all other monkeys, the proportion of largest CS neurons dropped (somatic area > 2250 μm^2). In parallel, and unlike what was observed in the other animals, the proportion of the smallest neurons dropped too, thereby keeping the median value stable. The reduced proportion of small neurons may be the consequence of the poor quality of the SMI-32 immunostaining obtained with the tissue derived from Mk-CH. In addition, among the intact monkeys, Mk-IC showed a low but barely significant interhemispheric difference in cell soma area. In this case, the violin plots are very similar, suggesting that the difference in size is indeed limited.

To clarify whether the combination of the anti-Nogo-A antibody and BDNF affects the shrinkage of layer V pyramidal neurons in MI, the same analysis was conducted in the group of five monkeys treated with the combined treatment (Fig. 5C; yellow violin plots). In all five monkeys, the soma cross-sectional areas of SMI-32 positive pyramidal neurons located in the layer V of M1 were significantly reduced in the contralesional hemisphere as compared to the ipsilateral one indicating that the axotomized CS cells shrank (Fig. 5C; yellow animals) (Mann-Whitney two tailed test $p<0.001$). Thus, the addition of BDNF to the anti-Nogo-A antibody treatment did not prevent or diminish the shrinkage of the soma of the axotomized CS neurons.

Figure 5

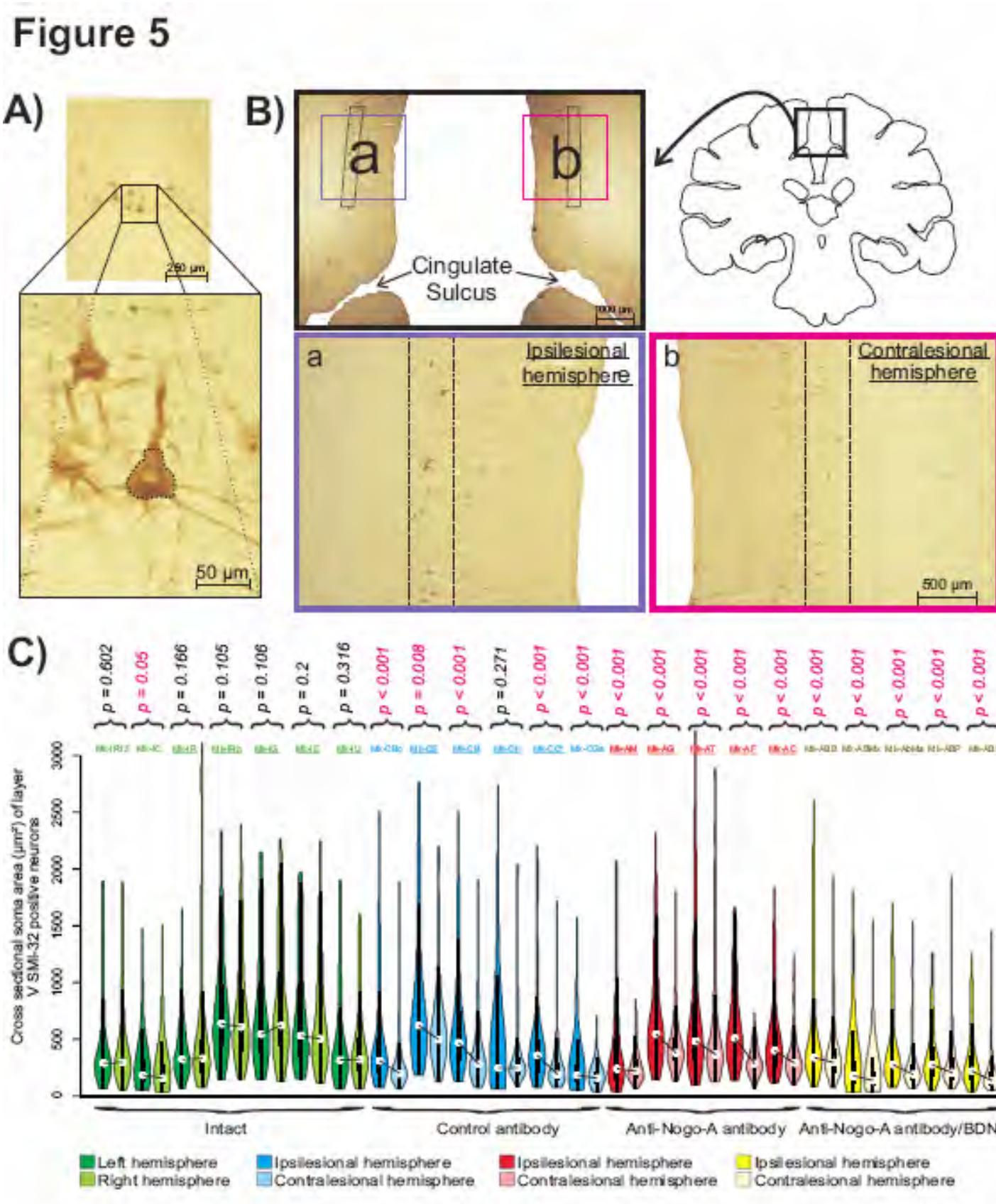

Figure 5: Relationship between the soma sizes of the CS neurons located in the layer V of M1 and the different treatments. <u>Panel A</u>: Photomicrograph showing the typical appearance of the CS neurons SMI-32-positive located in the layer V of M1. Around the soma, the cells have a typical thick apical dendrite and numerous dendrites projections. The black dashed outline

illustrates the criteria applied in order to define the soma cross-sectional silhouette: a nucleus easily identifiable and the end of the soma situated 20 µm from the nucleus Panels B: Photomicrograph showing the part of M1 corresponding to the hindlimb representation on both hemispheres SMI-32-stained. The SMI-32 reveals typically the pyramidal neurons located in the layer III and V. The size of CS soma cells was evaluated in the layer V of M1 in both hemispheres SMI-32-stained in a territory of about equivalent length at the identical dorsoventral location in the same histological brain section (left box: doted lined). In all lesioned monkeys and at low magnification, the layer V of the hemisphere affected by the spinal cord lesion (contralesional side) appears characteristically less densely stained than on the ipsilesional one. However, at higher magnification (boxes a and b), the pyramidal cells become more visible. Panel C: Violin plot illustrating the somatic cross-sectional areas of SMI-32 positive neurons in layer V of M1 for four groups of monkeys: intact monkeys in green, control antibody-treated monkeys in blue, anti-Nogo-A antibody-treated monkeys in red and anti-Nogo-A-antibody/BDNF-treated monkeys in yellow. Note that the animals that were already reported have their name underlined. Each hemisphere of one animal is represented by one violin plot, where the left and the ipsilesional hemispheres are shown with a dark colors and the right and the contralesional hemispheres are shown with bright colors. The violin plot is the combination of a box plot and a kernel density plot: while the median value of each violin representation is indicating with a white circle, the top and the bottom of the black box in each violin correspond to the 75 and the 25 percentile values respectively. The top and the bottom extremities of each vertical black line in the violin correspond on each side to the 90 and 10 percentile values respectively. Finally, the global shape of the violin corresponds to the soma cross-sectional areas values distribution. For a better comprehension, a black line links the two median of one animal in order to visualize easily the median values difference among the two hemispheres of one animal. For each monkey, the *p* value of the Mann-Whitney two tailed test is indicated above the name of each animal and whose values that are statistically significant are put in pink. All but one lesioned monkey exhibited a significant inter-hemispheric difference of cross-sectional soma area. On the other hands, the intact animals have the tendency to have similar soma size among of their two hemispheres. Thus, it appears that neither the anti-Nogo-A antibody alone nor the combination of the anti-Nogo-A antibody with BDNF prevent the soma shrinkage due to the spinal cord lesion.

Discussion

We report here that in the macaque monkeys subjected to an unilateral cervical cord section, the intrathecal infusion of a combination of an anti-Nogo-A antibody and of BDNF has no impact on functional recovery, nor on sprouting of CS fibers in the C3 segment. This differs from what was observed in monkeys treated with an anti-Nogo-A antibody alone. On the other hand, the shrinkage affecting layer V pyramidal neurons in the contralesional M1 is unaffected by either treatments. Our results therefore suggest that in the macaque, the addition of BDNF to a treatment based on anti-Nogo-A antibody hinders the processes leading to an improved functional recovery and to axonal sprouting which are induced by the anti-Nogo-A antibody treatment alone.

Nogo-A is a major neurite growth inhibiting molecule (Fawcett 02;Gonzenbach et al 10;Schwab 10). Following a spinal cord injury in rats or primates, treating the lesioned animals with antibodies directed against Nogo-A promotes sprouting and regeneration of CS fibers and improves functional recovery (Bregman et al 95;Brosamle et al 00;Fouad et al 04;Freund et al 06;Freund et al 09;Freund et al 07;Liebscher et al 05;Schnell and Schwab 90;Thallmair et al 98). In previous investigations (Freund et al 06;Freund et al 09), we have reported that treating SCI monkeys with a control antibody leads to a lower level of functional recovery than that observed when treating SCI animals with an antibody neutralizing Nogo-A. The present investigation adds behavioral data for two animals treated with a control antibody. The two control antibody treated animals reached levels of recovery in the same range as anti-Nogo-A treated animals, a situation not encountered previously (Fig. 2 and 3). Nevertheless, the statistical analysis of this enlarged populations (eight control antibody-treated and eight anti-Nogo-A antibody-treated animals) still leads to the conclusion that treating lesioned animals with an antibody neutralizing Nogo-A improves functional recovery. In previous investigations (Freund et al 06;Freund et al 09), we found and reported that the control antibody treated animals present a strong correlation between the size of the lesion and the level of recovery. The two new animals added in the present report fell outside of the range predicted by the previous reports, and new calculations including these data points lead to the conclusion that for control treated animals too, the correlation between the size of the lesion and the level of recovery is absent, at least for the lesion sizes encountered in this study. The reasons accounting for this observation are unclear. Variations in the exact rostro-caudal level of the lesion could contribute, but other factors, as for instance the extent of section of different descending pathways, could also participate.

The most striking observation arising from the present study is the disappearance of the beneficial effects of an anti-Nogo-A antibody treatment on the post-lesion functional recovery when BDNF was added to the treatment. In addition, the combined treatment failed to stimulate the sprouting of CS fibers in segments placed rostrally to the lesion as was observed by using the anti-Nogo-A antibody alone. However, some caution should be exercised before assigning this observation to the effects of BDNF. Indeed, while two animals that have received the BDNF (Mk-ABB and Mk-ABP) have lesions of dimensions comparable with those of control antibody treated animals, the three other animals that received BDNF presented a larger volume of lesion than the control antibody treated animals.

BDNF, like the other neurotrophic factors, has been shown to contribute to neuronal survival, axonal growth, synaptic plasticity and neurotransmission. The receptor of BDNF, *trkB*, is expressed in normal and axotomized CS neurons of rodents and of primates (Giehl et al 01;Lu et al 01;Ohira et al 99;Okuno et al 99;Zhang et al 07) and grafting BDNF/NT-3-secreting cells within the sites of a C7 hemisection in primates promotes regeneration and hinders the atrophy of the CS soma cells (Brock et al 10). In addition, rats subjected to a high unilateral lesion of the CST and treated with an anti-Nogo-A antibody show compensatory sprouting within the spinal cord which is associated with an increase in gene expression of BDNF (Bareyre et al 02). These studies suggest that combining the neutralization of Nogo-A to applications of BDNF could stimulate axonal regeneration and/or sprouting of CS neurons and thereby provide a structural basis for improving functional recovery.

Application of BDNF to lesioned CST neurons in rats promotes collateral spouting of the axotomized CS axons rostrally to the lesion (Hiebert et al 02;Vavrek et al 06). Likewise, overexpression of BDNF in the rat sensorimotor cortex together with overexpression of NT-3 at the lumbar spinal level significantly amplifies axonal sprouting for the injured CST compared to that induced by the NT-3 alone (Zhou and Shine 03). Moreover, injections of BDNF-hypersecreting human mesenchymal stem cells rostrally and caudally to a thoracic lesion in adult rat result in an improved locomotor recovery and an increased sprouting of CST and serotonergic fibers, as well as to an increase of CS neurons cell survival in M1 cortex (Sasaki et al 09). In the same way, other studies have shown that the delivery of BDNF protect injured CS neurons and can improve functional recovery (Giehl and Tetzlaff 96;Hammond et al 99;Jakeman et al 98;Kim and Jahng 04;Namiki et al 00). However, as various studies also fail to observe regenerating effects of BDNF on CS neurons, the effects of BDNF on these neurons remain ambiguous. For instance, in adult rats, transplants of fetal spinal cord and BDNF administrated two weeks after SCI do not significantly increase the amount of spared CS axons rostrally and caudally to the lesion whereas transplants combined

with NT-3 do (Iarikov et al 07). Likewise, BDNF-secreting grafts elicited no growth or limited growth responses of CS nerve fibers in adult rats after SCI and fail to improve functional recovery of primary motor functions (Lu et al 01;Nakahara et al 96;Shumsky et al 03;Tobias et al 03).

Very little is known on the effects obtained by combining neurotrophic factors to antibodies neutralizing Nogo-A and the few investigations dealing with that topic have also led to ambiguous results. For instance, in neonatal rats, the regeneration of injured optic nerve fibers is improved by associating BDNF with an antibody neutralizing Nogo-A (Weibel et al 94;Weibel et al 95). Similarly, the combined application of an antibody neutralizing Nogo-A with the ciliary neurotrophic factor (CTNF) enhances axonal regeneration of transected retinal ganglions axons (Cui et al 04). In contrast, whereas sprouting of CS axons was enhanced by combining an anti-Nogo-A antibody with injections of NT-3 in young adult rats subjected to spinal cord injury, no effects were observed with the combination of the same antibody with injections of BDNF (Schnell et al 94). As we failed to detect sprouting of CS fibers in segments positioned rostrally to the lesion, our observations are in line with this last report. Because the behavioral test used in our experiment mainly assesses manual dexterity which is known to depend on the integrity of the CST, the absence of sprouting may account for the limited functional recovery that we observed. As exogenous applications of neurotrophins, including BDNF, are known to act differentially on different categories of neurons such as raphespinal, coeruleospinal or CS axons (Bregman et al 97), it is not excluded that other motor attributes which do not rely heavily on CS projections, may improve as a result of the combined treatment.

In summary, our findings demonstrate that in spinal cord injured macaque monkeys, the combination of BDNF and the anti-Nogo-A antibody thwarts the effects acquired when the anti-Nogo-A antibody was given alone. In fact, the animals treated only with the anti-Nogo-A antibody show a significant axonal sprouting of their axotomized CS axons at spinal level, associated with functional recovery of their manual dexterity. However, when combined with BDNF less recover was obtained as well as axonal sprouting of CS neurons. Thus, on the contrary to the neurotrophic factor NT-3, it appears that the association of both BDNF and an anti-Nogo-A antibody thwarts the positive outcomes obtained by the anti-Nogo-A antibody applied alone. Presently, it is not known how these two molecules, which individually lead to neurite growth, negatively interact and block their respective intrinsic beneficial effects.

Abbreviations

SCI = spinal cord injury

BDA = Biotinylated Dextran Amine

CNS = Central Nervous System

CST = Corticospinal tract

CS = Corticospinal

HNOGO antibody = Anti-Nogo-A antibody

BDNF = Brain-derived neurotrophic factor

CTNF = Ciliary neurotrophic factor

NT-3 = Neurotrophin 3

NgR = Nogo receptor

TrkB = Receptor tyrosine kinase B

M1 = Primary motor cortex

PMd = Premotor cortex

DRG = Dorsal root ganglia

PBS = Phosphate-buffered saline

ABC = Avidin-biotin complex

DAB = Diaminobenzidine tetrahydrochloride

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General Discussion

We conducted investigations in adult macaque monkeys subjected to an unilateral cervical cord lesion performed at the level C7/C8 which interrupts most of the corticospinal (CS) axons. Our aim was to establish the effects of the administration of two types of treatments (the anti-Nogo-A antibody treatment alone and a combination of this treatment with the brain-derived neurotrophic factor (BDNF), on neuronal and axonal plasticity as well as on the amount of functional recovery. Whereas the anti-Nogo-A antibody treatment has already been shown, in a previous series of experiments, to promote regenerative sprouting of corticospinal (CS) axons around the lesion and into the denervated monkey's spinal cord in addition to enhance the functional recovery (Fouad et al 04;Freund et al 06;Freund et al 09;Freund et al 07), other effects of this treatment remain however to be clarified. On the other hand, the effects of the combinatorial treatment anti-Nogo-A antibody/BDNF on both the behaviour and the anatomy of spinal lesioned non-human primates were totally unknown before the present investigation.

Using the SMI-32 staining (i.e. an antibody directed against a nonphosphorylated neurofilament protein that labels pyramidal cells), the first investigation of the present work has examined into three groups of monkeys (i.e. intact monkeys, injured monkeys treated with a control antibody and injured monkeys treated with a anti-Nogo-A antibody), the effects of an anti-Nogo-A antibody treatment on soma shrinkage observed into pyramidal neurons located in layer V of the primary motor cortex (M1). Most or even all of these neurons were probably axotomized CS neurons. Indeed, CS neurons are exclusively located in the cortical layer V (Murray and Coulter 81) and those located in the mesial motor cortex normally project to the lumbar segments, that is caudally to the level of the spinal lesion. In a initial study conducted on lesioned monkeys treated only with a control antibody, it has been observed that most of the pyramidal CS neurons positive to the SMI-32 staining in the layer V of the hindlimb area of M1 survived to their axotomy but that their soma shrank subsequently to the spinal cord injury (SCI) (Wannier et al 05).It thus seems that SCI in non-human primates does not lead to the death of the CS neurons in the cortex, an observation that is in line with other studies (Bronson et al 78;Davison 37;Lassek 42;Nielson et al 10;Tower 40), but these results contradict those obtained by other studies conduced both in rodents (Hains et al 03) and primates (Holmes and May 09;Levin and Bradford 38;Pernet and Hepp-Reymond 75;Wohlfarth 32), who concluded a subsequent cell loss following a lesion of the CST.

Nevertheless, following SCI it appears that soma shrinkage remains the terminal issue for the axotomized CS neurons who have survived. Because antibodies directed against Nogo-A exhibit promising results, anatomically as well as behaviourally, on monkeys subjected to SCI (Fouad et al 04;Freund et al 06;Freund et al 09), the question was addressed whether this type of treatment can prevent soma shrinkage of CS neurons after SCI.

First, the present investigation has confirmed that unilateral cervical lesion is not followed in monkeys by a significant elimination of the layer V pyramidal neurons in M1. Second, though the anti-Nogo-A antibody treatment promoted sprouting at the spinal cord level, it appears that it did not avoid or reduce the CS soma cells shrinkage caused by the lesion. Indeed, when given intrathecally, the anti-Nogo-A antibody treatment does not influence remote cortical areas affected by the spinal cord lesion, like for instance the neurons located in the cortical layer V of M1 in the contralesional hemisphere. For that reason, the question was addressed about how the effects obtained through anti-Nogo-A antibody treatment could be enhanced and the possibility to combine it with other types of treatments that have shown also promising results on injured CST was considered. According to the classical neurotrophin hypothesis, which argues that the survival of neurons is mediated by their access to survival-promoting substances (Lewin and Barde 96), the neurotrophins present good possible candidates. Survival substances like neurotrophic factors interact with receptors at axonal endings and produce consequently a signal which is retrogradely transported towards the soma. Indeed, it has recently been shown that the neurotrophic factor “nerve growth factor” (NGF) is endocytosed with its receptor from the surface of axon tips forming endocytotic vesicles retrogradely transported towards the nucleus through the axon (Delcroix et al 04;Schwab 10). Thus, a supply of such neurotrophic factors could possibly prevent soma shrinkage after SCI. This hypothesis was tested in a further investigation also presented in this work, namely by infusing BDNF at the lesion site. More precisely, it was chosen in the last investigation presented in this work, to associate the anti-Nogo-A antibody treatment with an exogenous intrathecal infusion of BDNF with the purpose to prevent the soma shrinkage of the axotomized CS neurons.

Another possibility of the inefficacity of the anti-Nogo-A antibody treatment to prevent subsequent soma shrinkage following SCI could be attributed to anatomical features. In fact, the present work has shown that large neurons shrink more than neurons of smaller size. It has been demonstrated previously in an anatomical study that monkey’s neurons that send their axons in lumbosacral enlargement have significant larger cell diameter than neurons that project at cervical level (Murray and Coulter 81). Moreover, a study using rat rubrospinal neurons as model has shown that the degree of soma shrinkage depended on both the duration

of survival and the proximity of the lesion (Wang et al 02). Therefore, it would be very interesting to analyse, under similar conditions, monkeys subjected to lumbosacral lesions. Finally, another option that could be considered to optimize the treatment is to inject anti-Nogo-A antibodies also at cortical levels. In fact, in this study, the treatment was given only intrathecally at spinal level and it has been shown that the penetration of the anti-Nogo-A antibody treatment was less complete in the brain than in the spinal cord (Weinmann et al 06). Therefore, it remains possible that the anti-Nogo-A antibody was efficient only at spinal level because too weak concentrations of the anti-Nogo-A antibody treatment reach cortical levels.

Using the same staining and under the same conditions, a second investigation presented in this work has evaluated the presence of SMI-32 positive fibers into the scar tissue in three groups of injured/treated monkeys (injured monkeys treated with a control antibody treatment, injured monkeys treated with the anti-Nogo-A antibody treatment and injured monkeys treated the anti-Nogo-A antibody treatment combined with a BDNF infusion). The scar tissue formed after CNS injury is thought to be a major factor that limits axonal regeneration and an acute disruption of the scar tissue has been shown to improve nerve fibers growth after SCI (Bradbury et al 02;Fawcett and Asher 99;Grimpe and Silver 02;Moon et al 01). However, this study shows that scar tissue degeneration is not ultimately necessary to observe axonal regeneration. In fact, a significant colonization of positive SMI-32 nerve fibers was observed into the lesion site of each lesioned monkey, even in control antibody treated animals. Several studies have already shown that following SCI some category of axons are able to colonized spontaneously the scar tissue in relatively high density in certain cases (Brook et al 98;De Castro, Jr. et al 05;Fenrich and Rose 09;Inman and Steward 03;Linda et al 92;Matthews et al 79;Wallace et al 87;Wang et al 96). However to our knowledge, it has never been reported in lesioned adult macaque monkeys that axons positive to SMI-32 staining, possess also the ability to penetrate successfully the scar tissue. We provide converging indirect evidence supporting the idea that a large proportion of these axons originate from motoneurons. This raises the question, why these SMI-32 nerve fibers can invade effortlessly the scar tissue whereas other nerve fibers of the CNS, like CS axons, fail to enter the lesion territory in the same conditions? It will be then interesting to define more precisely which types of nerve fibers possess this particular ability and which mechanisms are associated to this particular type of regrowth.

Furthermore, it was demonstrated in this investigation that neither the anti-Nogo-A antibody treatment alone or its combination with BDNF have enhanced the number and/or the length of the SMI-32 positive fibers visible into the scare tissue. Nevertheless it remains possible that

the intrathecal infusion of BDNF could have an effect on these fibers but that this effect was not observed in our analysis because it may have been only transient. In fact, it has been demonstrated in adult rats subjected to SCI that the number of injured BDNF immunoreactive motoneurons increased after the injury and peaked 3 days postoperation (Qin et al 06). However, after these 3 days, the peak of cells decreases progressively to finally recover, 7 days post-operation, the number of motoneurons of the sham operated animals. In addition, other studies have demonstrated that following both spinal cord compression and lumbar ventral root transection the level of BDNF mRNAs increases also in motoneurons to reach a maximum level 1 day post-operation and remained elevated 14 days in case of the lumbar ventral root transection and 3 days following spinal cord compression (Ikeda et al 01;Li et al 06). Though opposite results, i.e. a down-regulation of BDNF expression, were also observed on injured lumbar motoneurons 30 minutes, 4 and 12 hours following a spinal cord hemisection (Gulino et al 04), all these studies mention that the BDNF effect remains only visible few days post-operation. Thus, it remains possible that the time window used to infuse BDNF in this investigation and/or to perform the analysis were not optimal to observe some BDNF effects. Likewise, it remains conceivable that the BDNF concentrations could be inappropriate. Thus, it remains plausible that the intrathecal infusion of BDNF could have effects on the fibers observed into the scar tissue but because the monkeys were not sacrificed in the early period postoperation, these possible effects would have then disappeared at the time of the anatomical assessments. It would then be interesting to show, under the same conditions and/or under different BDNF concentrations and/or different timing, whether monkeys' fibers visible into the lesion tissue will be enhanced or not by the application of BDNF. It would be also interesting to test other types of treatments. In fact, it remains possible that these fibers are sensible to other substances like other neurotrophic factors, such as NT-3 for instance.

Recently, Schwab has proposed an interesting theory concerning opposite mechanisms established between Nogo-A and neurotrophins at the level of growth cone (called "ying-yang mechanism") (Schwab 10). According to this author, growth inhibitors such as Nogo-A and growth stimulators such as neurotrophins are both internalized in a ligand-receptor complexes and transported retrogradely towards the soma, but whereas Nogo-A complex inhibits the transcription machinery, neurotrophins complex appears to produce right the opposite effect. It is believed that this "ying-yang mechanism" may regulate the stability and the plasticity of both developing and adult CNS. Thus, this theory shows that Nogo-A and the neurotrophic factors, are in some way related together. This relation was underlined in the last investigation

presented in this work in spite the fact that the results presented in this last study show synergic effects rather than antagonist effects as proposed by the “ying yang theory”.

The latter investigation has examined in injured adult monkeys the effects of the combination of the anti-Nogo-A antibody treatment and BDNF on different parameters:

1. On the amount of functional recovery,
2. On the amount of CS sprouting located rostrally to the lesion at cervical level C3
3. On CS neuronal soma shrinkage

About the different neurotrophic factors and their effects on spinal injured animals, a lot of contradictions exist in the literature. For BDNF, numerous investigations have failed to observe regenerative effects. For instance, Novikova et al.(2000, 2002) have reported that in adult rats subjected to SCI, intrathecally delivery of BDNF was ineffective on the survival and on the atrophy of ascending spinocerebellar neurons of the Clarke nucleus. However, the administration of NT-3 alone showed neuroprotective effect and the association of these two neurotrophic factors abolished the effect of NT-3 while these types of neurons expressed both *trkB* and *trkC* receptors (i.e. the receptors related to these two neurotrophins) (Novikova et al 00a). This group has also observed that long-term infusion of BDNF plus NT-3 decreased significantly the sprouting of rubrospinal fibers to the lesion site and diminished the regenerative response of intrinsic spinal cord neurons (Novikova et al 02). Similar results were obtained by Bradbury et al: while the intrathecal infusion of BDNF was inefficient on the cell atrophy and the number of surviving cells on axotomized spinal cord neurons, NT-3 infusion shows opposed results while both *trkB* and *trkC* receptors are expressed in the neurons of interest (Bradbury et al 98). Sayer et al. reported that injection of neurotrophins, such as BDNF, did not prevent adult rats axotomized CS nerve fibers to degenerate following SCI in contrast to the sensory axons (Sayer et al 02). In addition, a number of reports have now demonstrated the failures of regenerating CS neurons via the transplantation of cells genetically modified to secrete BDNF. In adult rats, transplants of fetal spinal cord and BDNF administrated two weeks after SCI do not significantly increase the amount of spared CS axons rostrally and caudally to the lesion whereas transplants plus NT-3 exhibit opposite results (Iarikov et al 07). Likewise, BDNF-secreting grafts elicited no growth or limited growth responses of CS nerve fibers in adult rats after SCI and did not lead to a recovery of the primary motor function (Lu et al 01;Nakahara et al 96;Shumsky et al 03;Tobias et al 03). Thus, it appears that the application of BDNF alone or combined with other neurotrophic factors like NT-3 is not always followed by the regeneration of the injured CS cells. Some attempt to explain these results involves the theory that several neurotrophins can antagonistically regulate other neurotrophins, but this hypothesis can not account for all

experimental observations (Giehl et al 01). On the other hand, other studies have reported positive results when using BDNF as treatment. Zhou et al. have shown that overexpression of BDNF in the rat sensorimotor cortex near the CST soma cells together with overexpression of NT-3 at the lumbar spinal level significantly amplifies the axonal sprouting of the injured CST compared to that induced by the NT-3 alone (Zhou and Shine 03). Likewise, after a thoracic spinal cord injury, the application of BDNF to the cell bodies of the lesioned CST neurons in rats promotes collateral spouting of the axotomized CS axons rostrally to the lesion (Vavrek et al 06). Hibert and al. have shown that the infusion of BDNF on the motor cortex promotes sprouting of CS axons rostrally to the injury site but failed to produce regeneration into peripheral nerve grafts (Hiebert et al 02). Another study mentioned that injections of BDNF-hypersecreting human mesenchymal stem cells rostrally and caudally to a thoracic lesion in adult rat results in structural changes in brain and spinal cord associated with improved locomotor recovery and an increase of sprouting of the CST, as well as serotonergic projections, in addition to an increase of CS neurons cell survival in M1 cortex (Sasaki et al 09). In the same way, other studies have shown that the delivery of BDNF protects injured CST neurons and can improve functional recovery (Giehl and Tetzlaff 96;Hammond et al 99;Jakeman et al 98;Kim and Jahng 04;Namiki et al 00). An additional report has demonstrated that the exogenous application of different neurotrophins, including BDNF, increase the growth of raphespinal, coeruleospinal and CS axons (Bregman et al 97).

Therefore, the choice of combining the infusions of both the anti-Nogo-A antibody and of the BDNF molecules represents a real challenge regarding the complexity and the disparities of the results published in the literature. In the spite the fact that some studies have reported negative results when using BDNF as treatment, it appears that BDNF could however be a good candidate to be combined with the anti-Nogo-A antibody treatment. In fact, it has been demonstrated that rats subjected to a high unilateral lesion of their CST and treated with anti-Nogo-A antibodies show stable compensatory sprouting within the spinal cord associated with an augmentation in gene expression related to growth-associated molecules, including BDNF, which had been the most upregulated gene observed (Bareyre et al 02). Therefore, this study suggests that there are at least two mechanisms involved in the compensatory reorganization of the injured CNS that are possible to thwart in order to stimulate the axonal regeneration: first, prevent the axonal outgrowth inhibition via the neutralization of Nogo-A using antibodies and, second, stimulate the growth of lesioned nerve fibers via growth-related factors in order to reinnervate their denervated targets. Furthermore, it has been demonstrated that the receptor of BDNF *trkB*, is expressed in normal and axotomized CS neurons in particular in the layer V of M1 in rodents, and also in primates (Giehl et al 01;Lu et al 01;Ohira et al 99;Okuno et al 99;Zhang et al 07). Indeed, the receptor tyrosine kinase *trkB* mediates all functions of BDNF via its activation through its binding to this neurotrophic factor of multiple intracellular signaling pathways (Huang and Reichardt 03). Moreover, a recent study performed on primates has shown that implants of autologous BDNF/NT-3-secreting cell grafts placed within the sites of a C7 hemisection lesions promote regeneration of axons locally into the lesion and reduce the atrophy of the CS soma cells (Brock et al 10). However these studies are in contradiction with other reports, claiming that the expression of BDNF is most prominent in noncorticospinal cells located in the layer V or that the *trkB* receptor is present on corticospinal soma and apical dendrites but not on their axonal projections (Giehl et al 98;Lu et al 01). Nevertheless, taking together, these studies have motivated our choice to combine the anti-Nogo-A antibody treatment to BDNF because it appeared to be promising alliance: this association could not only enhance the axonal sprouting at the spinal level but could in addition stimulate cortical regeneration and prevent or reduce the neuronal shrinkage observed in a previous study (Beaud et al 08).

However, surprisingly and contradicting the assumption made from the "ying-yang theory" (Schwab 10), whereas anti-Nogo-A antibody treatment infused alone has shown functional and structural improvements on lesioned monkeys (Freund et al 06;Freund et al 09;Freund et al 07), its combination with the neurotrophic factor BDNF appears to eliminate the improvements obtained by the infusion of the anti-Nogo-A antibody treatment alone. In addition, as for the anti-Nogo-A treatment alone, the combinatorial treatment did not prevent the soma shrinkage of the pyramidal neurons observed in the layer V of the hindlimb area of the primary motor cortex (M1). Thus, it appears in this study that treating spinal injured non-human primates simultaneously with the anti-Nogo-A antibody treatment and BDNF counteracted the positive effect obtained by the anti-Nogo-A antibody treatment alone and that the supply of neurotrophic factor at spinal level such as BDNF did not change the fate of the axotomized CS neurons. To our knowledge, this is the first time that such conflicting relationship between antibodies directed against a myelin-growth inhibiting molecule and a neurotrophic factor is demonstrated both anatomically and behaviourally. These results are in contradiction with whose published by other research groups who tried in rodents also to combine the anti-Nogo-A antibody treatment with neurotrophic factors such as BDNF or NT-3 and have obtained growth improvements (Cui et al 04;Schnell et al 94;Weibel et al 94;Weibel et al 95). In fact, it has been demonstrated in a study conducted in SCI young adult rats that, whereas sprouting of CS axons was enhanced by anti-Nogo-A antibody treatment combined with injections of NT-3, no effects were observed when anti-Nogo-A antibody treatment was combined with injections of BDNF (Schnell et al 94). On the other hand, it has been demonstrated in neonatal rats with injured optic nerve fibers that the regeneration of this tract was improved subsequently to a treatment that associated the anti-Nogo-A antibody treatment and BDNF (Weibel et al 94;Weibel et al 95). Thus, it appears that diverse types of neurotrophins can interact in a different way with the Nogo-A molecule and that the level of development of the subject who received the treatment may play a role for achieving or not regeneration. In addition to interact with myelin-growth inhibitors such as Nogo-A, it has also been suggested that several neurotrophic factors like for instance BDNF and NT-3 may reciprocally regulate their specific actions, even antagonistically (Giehl et al 01). Therefore, it seems that molecules that could have a beneficial effect separately on recovery may not show synergic responses when combined together. Because the situation appears complex, it is primordial to continue research on this matter in different animal models, even in non-human primates. It will then be very interesting to understand which common links have Nogo-A and BDNF and which are the mechanisms that conduct BDNF to interfere with Nogo-A blocking.

Though some investigations have already shown that both BDNF and Nogo-A use the co-receptors p75 and LINGO-1, evidence of linked cross pathways between these two molecules was presented in a recent publication. In fact, Chytrova and co-workers have shown that BDNF abolished the effects of both myelin-growth inhibitors, MAG and Nogo-A, via the inactivation of the RhoA signaling pathway thereby explaining how BDNF has the ability to overcome growth inhibition (Chytrova et al 08). Thus, it appears that the biochemical responses generated by Nogo-A and BDNF are linked but supplementary investigations are needed to understand fully this relationship.

Another factor that could contribute to explain the observed results in our investigation is the effects related to the timing of the treatment administration. Indeed , several studies have shown that the temporal pattern of the treatment must be adequate in order to optimize its efficiency (Coumans et al 01;Novikova et al 00b;Novikova et al 02). In rodents, the effects on both regeneration and functional recovery were reduced if the anti-Nogo-A antibody treatment was delayed until eight weeks after the axotomy of the CS axons (Von Meyenburg et al 98). Recently, it has been shown that BDNF induces neurite outgrowth in human neuroblastoma cells treated with retinoic acid by inducing phosphorylation of NgR1 via CK2-like activity but fails to elicit outgrowth in dorsal root ganglia (DRG) neurons (Takei 09). Therefore, this investigation shows that several experimental manipulations of the CNS environment successfully elicit regeneration in certain types of neurons but fail to promote regeneration of other types of cells like in our case the CS neurons, suggesting that cell-intrinsic mechanisms limiting the regeneration could be present in certain critical class of neurons. Likewise, another investigation has shown that following SCI, BDNF contents change in a cell-specific fashion (Dougherty et al 00). Taken together, these investigations demonstrate that a cellular spatiotemporal pattern exists among the BDNF responses. Furthermore, using neurotrophins, the question of the efficacy related to the concentrations was addressed because in this study, high doses of BNDF were infused into the spinal lesion during one month. We chose the quantity of BDNF infused in accordance with some related publications. In fact, as compared to other neurotrophins, BDNF is one of the neurotrophic factors for which the range of effective concentrations were best characterized. A study has measured in the cerebrospinal fluid of a control population a BDNF concentration reaching approximately 45 pg/ml (Ilzecka and Stelmasiak 02). In monkey, a protective effect of BDNF on axotomized nigrostriatal dopaminergic neurons has been reported when a total of 6.5 µg of the neurotrophic factor was infused during 14 days intrathecally, via osmotic minipump into the cisterna magna (Takeda 95). In the sheep, intrathecal infusion of 500 µg/day of BDNF is well supported, not toxic and leads to a BDNF concentration of 1.2 µg/ml in the cerebrospinal

fluid in proximity of the infusion site (Dittrich et al 96). Taking in to account the volume differences between the sheep and the macaques, calculations suggest that the quantities provided in our study are suitable to reach pharmacologically active concentrations. However, our results show that the doses of BDNF used in this study inhibit not only the axonal regeneration by thwarting the effects of the anti-Nogo-A antibody at the spinal level but fail in addition to prevent or reduce the soma shrinkage associated to the axotomy of CS neurons at the cortical level.

In contrary to the assumption made in the "yin-yang theory", neurotrophic factors can also produce synergic affects of Nogo-A, raising the questions:

1. Why BDNF was counteracting the growth promoting action of the anti-Nogo-A antibody in our treatment?
2. Is BDNF always counteracting on the growth promoting action of an anti-Nogo-A antibody? In particular, could this effect become agonistic and additive, under certain conditions as different concentrations for instance?
3. How do other neurotrophic factors such as NT-3, improve the growth promoting effects of an anti-Nogo-A antibody treatment, (Schnell et al 94)?

Finally, because the last investigation in the present thesis has assessed the motor capacity of monkeys subjected to a unilateral cervical cord lesion (using the "modified Brinkman board test"), it is essential to discuss the possible role of indirect CS projections in the observed functional recovery. It has been shown in monkeys that 5-10% of the total amount of the CS axons are undecussated descending nerve fibers (Jankowska and Edgley 06;Lacroix et al 04;Rouiller et al 96;Yoshino-Saito et al 10). Thus, in the present work, in all animals subjected to the unilateral cervical cord lesion this amount of nerve fibers is spared and provides an indirect pathway to access to the spinal circuits placed caudally to the lesion. Indeed, it has been shown that though axonal regeneration after CNS injury is limited, partial injury performed in mammalian spinal cord is some time followed by extensive recovery and it is believed that the spared axons could play a role in this observed recovery (Fawcett et al 07). Moreover, a recent publication has shown that monkeys lesioned at C7 level exhibit extensive spontaneous plasticity of their CS axons associated to substantial improvements in both hand functions and locomotion related to the CS spared axons (Rosenzweig et al 10). According to this study, non-human primates exhibit a more extensive spontaneous recovery as compared to rodents, who recover to a lesser extent in similar conditions because, stimulated by the lesion, more spared CS axons sprout and cross the midline rostrally to the lesion. Furthermore, it has been demonstrated that a second hemisection located more rostrally and on the opposite side to the initial lesion eliminates in both humans and non-

human primates the spontaneous recovery observed when the subjects sustain to only one hemisection (Nathan and Smith 73;Turner 91). Thus, it seems that following incomplete spinal cord lesions, the spared uncrossed axons may contribute to the functional recovery. This could explain why some of the animals treated with the control antibody and subjected to large lesions exhibit such substantial recovery. However, it has been demonstrated in this work that the spinal infusion of an anti-Nogo-A antibody treatment alone but not combined with BDNF can improve this functional recovery. To understand fully the situation and what role plays BDNF, a population of monkeys subjected to the same conditions but treated only with BDNF remains to be analysed. Nevertheless it is the first time that a report conducted in adult injured monkeys shows obviously at both anatomical and behavioural level counteracting interactions between the neutralization of myelin-growth inhibitor Nogo-A and the neurotrophic factor BDNF.

In conclusion, though a lot of questions remain to answered, some key pieces of this complicated puzzle that represent the challenge of generating therapies for treating SCI appear slowly and provide progressively clarifications into the comprehension of both the developing and the adult CNS functioning. The results obtained by the experimentations on non-human primates for the use of anti-Nogo-A antibody as a treatment for SCI has contributed to the initiation of a clinical study that uses a human Nogo-A antibody (ATI-355; Novartis) for treating patients with acute lesions in the spinal cord (ClinicalTrials.gov: NCT00406016) (Schwab 10). But much more data are still needed to fully understand the situation and to allow one day the generation of a successful treatment for SCI.

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Annexes

Manuscript 4:

A case of polymicrogyria in macaque monkey: impact on anatomy and function of the motor system.

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A case of polymicrogyria in macaque monkey: impact on anatomy and function of the motor system

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Abstract

Background: Polymicrogyria is a malformation of the cerebral cortex often resulting in epilepsy or mental retardation. It remains unclear whether this pathology affects the structure and function of the corticospinal (CS) system. The anatomy and histology of the brain of one macaque monkey exhibiting a spontaneous polymicrogyria (PMG monkey) were examined and compared to the brain of normal monkeys. The CS tract was labelled by injecting a neuronal tracer (BDA) unilaterally in a region where low intensity electrical microstimulation elicited contralateral hand movements (presumably the primary motor cortex in the PMG monkey).

Results: The examination of the brain showed a large number of microgyri at macro- and microscopic levels, covering mainly the frontoparietal regions. The layered cortical organization was locally disrupted and the number of SMI-32 stained pyramidal neurons in the cortical layer III of the presumed motor cortex was reduced. We compared the distribution of labelled CS axons in the PMG monkey at spinal cervical level C5. The cumulated length of CS axon arbors in the spinal grey matter was not significantly different in the PMG monkey. In the red nucleus, numerous neurons presented large vesicles. We also assessed its motor performances by comparing its capacity to execute a complex reach and grasp behavioral task. The PMG monkey exhibited an increase of reaction time without any modification of other motor parameters, an observation in line with a normal CS tract organisation.

Conclusion: In spite of substantial cortical malformations in the frontal and parietal lobes, the PMG monkey exhibits surprisingly normal structure and function of the corticospinal system.

Background

Polymicrogyria is a developmental malformation of the cerebral cortex, characterized by multiple small gyri with abnormal cortical lamination [1]. PMG can be unilateral or bilateral and its extent varies from focal PMG in otherwise normal brain to diffuse PMG with multiple other brain abnormalities. The spectrum of clinical manifestations ranges from normal individuals, with only selective

impairment of cognitive function [2] and no or easily controlled epilepsy, to patients with severe encephalopathy and intractable epilepsy [3]. Motor and cognitive deficits such as a delay in development [4], or congenital contractures [5] are commonly described in patients suffering from PMG. Microscopically, two histological types of PMG were recognized: a simplified four layered form and an unlayered form [6]. The two types of PMG may coexist in contiguous cortical areas [7]. Recent report provides evidence that PMG areas are functional [8].

The present report describes a case of spontaneously occurring PMG in a macaque monkey for which tracing of corticospinal projections had been obtained. Moreover, the animal was involved in a study on the mechanisms of bimanual coordination, and its PMG was discovered after sacrifice. The first goal of the present report was to present in more details the general morphological traits of the PMG brain. More specifically, we sought to establish which brain regions and how the laminar pattern of the cerebral cortex have been affected by the PMG. In human patients with a unilateral PMG, the CS tract originating from the affected hemisphere presented an altered structure in DTI and fMRI investigations [9]. The second aim of the present case report in monkeys was to evaluate whether the cortical malformations affected the characteristics of the corticospinal projections. For this purpose, the anterograde tracer Biotinylated Dextran Amine (BDA) was injected unilaterally in the electrophysiologically identified hand representation of the presumed primary motor cortex. Finally, the motor capacity of the PGM macaque was compared with that of a normal macaque monkey, both trained to perform the same motor task, namely a modified version of the so-called "reach and grasp drawer" task [10].

Results

The PMG monkey described in this study is the only case of cortical malformation ever observed in our laboratory.

1) Cortical structure

The topographical analysis of the PMG monkey brain showed an abundance of small gyri, affecting mainly the frontal and parietal lobes. In the PMG monkey, the structure of the sulci were hardly identifiable on both hemispheres (Fig. 1A and 1C), and did not display the arrangement normally observed (Fig. 1B and 1D). In contrast, the ventral aspects of the brain, particularly the occipital and the temporal lobes appeared macroscopically normal (Fig. 1E), with individual sulci exhibiting a pattern closely resembling that observed in normal monkeys (Fig. 1F). To tentatively represent the extent of cortical malformation, the border of the affected territories were superimposed on the healthy brain of Mk-IU in panels B and D (dashed line).

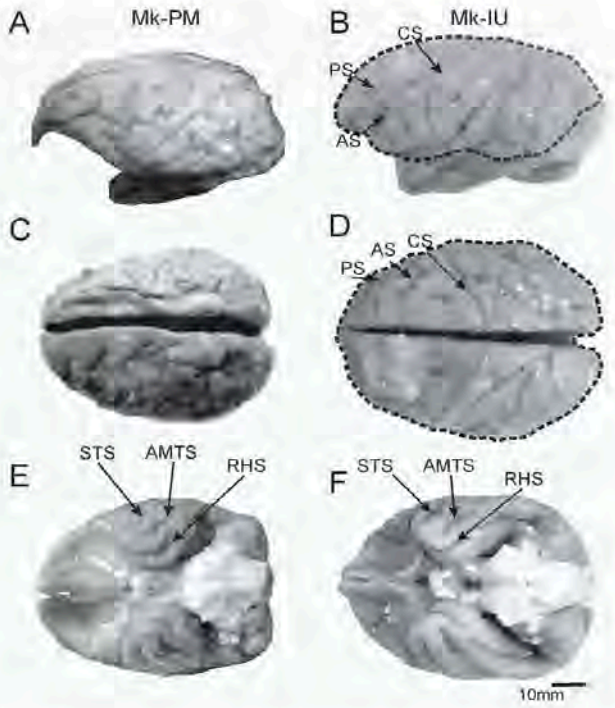

Figure 1

macroscopic views of the brain. Macroscopic appearance of the brain of the PMG monkey Mk-PM (A, C, E) and of a normal monkey Mk-IU (B, D, F) in lateral (A and B), dorsal (C and D) and ventral (E and F) views. Comparison of panels A and C with B and D shows a clear excessive number of small gyri in Mk-PM, and a loss of the normal topography of the brain, such as the disappearance of the normally well defined arcuate (AS), central (CS) or principal (PS) sulci. Nevertheless, the topographical organization of the ventral part of the PMG brain (E) is closer to that of the normal brain (F), as the superior temporal (STS), the anterior middle temporal (AMTS) or the rhinal sulci (RHS) are clearly identifiable. The extent of the cortical malformation in Mk-PM was projected over the healthy brain of Mk-IU (dashed line).

The cortical surface of the brain in the frontal and parietal lobes presented a disorganized aspect with numerous small gyri (Fig. 2A, B) delimited by intermingled invaginations (Fig. 3A arrow). In large regions of the frontal, parietal and temporal lobes, only layer I was clearly identifiable in Nissl stained sections, the remaining cortex failing to present a well characterized laminar organization (Fig. 2D). In ventral regions, a normal laminar organization in 6 layers was preserved (Fig. 2E). In some sulci, the cortical surface was not covered with a pial lamella, and the layers I of the two facing banks were fused (Fig. 3B). Locally, the normal laminar organization was disrupted in Nissl stained sections (Fig. 3C) as well as in SMI-32 stained sections (Fig. 3D). Indeed, in the PMG monkey, the SMI-32 positive neurons were often located at a dis-

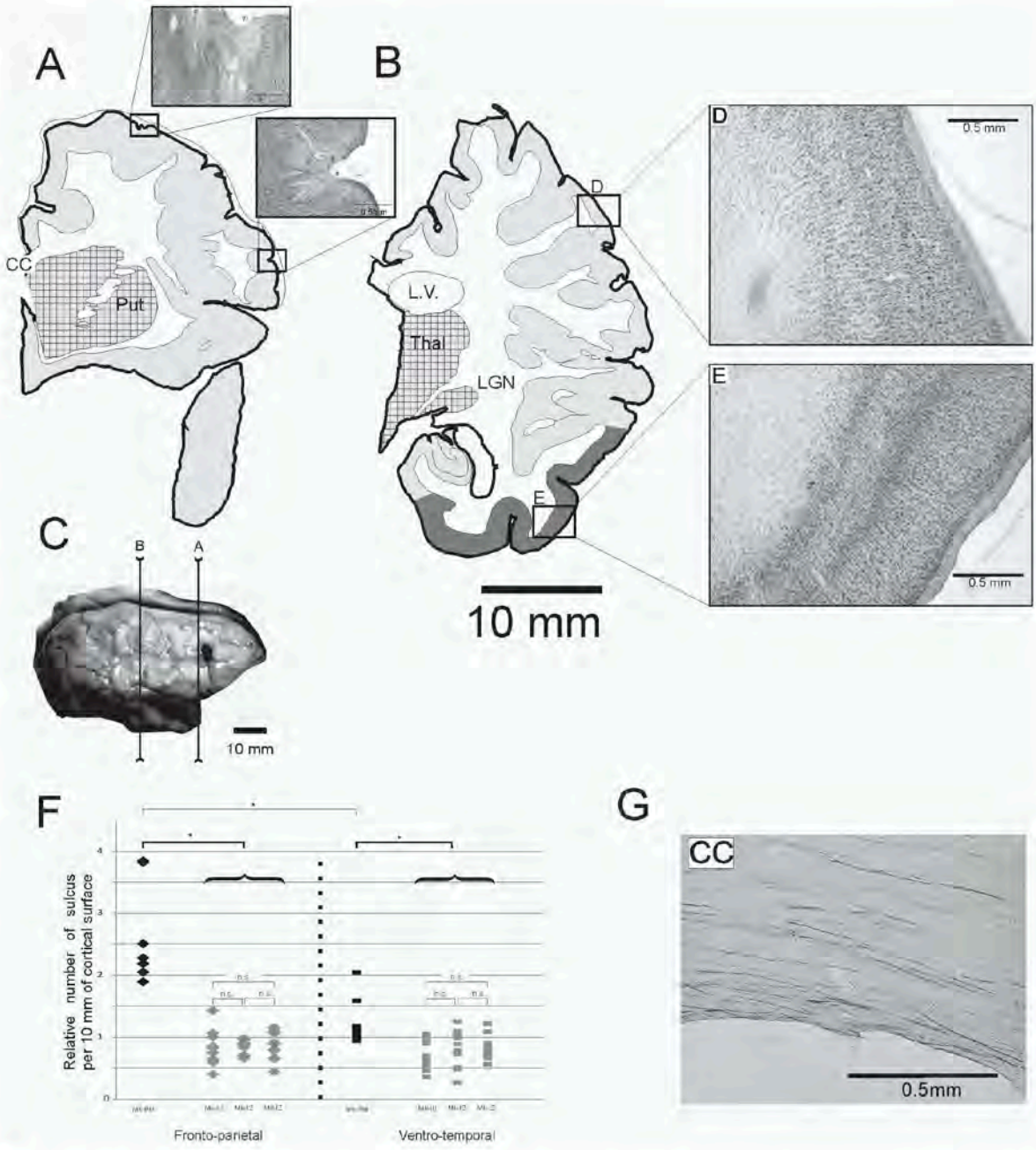

Figure 2

Reconstructions of frontal Nissl-stained sections in PMG monkey. **Panels A and B:** Location of cortical malformation on coronal sections in the right hemisphere at two rostrocaudal levels. Sub-cortical structures, such as the thalamus (Thal.), the lateral geniculate nucleus (LGN), the putamen (Put) or the corpus callosum (CC) are visible and they do not present conspicuous abnormalities, but the lateral ventricle (L.V.) is enlarged, including two enlarged photo micrograph of the cortex showing the microscopic cortical ectopic sulci (#), where the layer I is clearly distinguishable. Light grey: unlayered cortex. Dark grey: normally layered cortex: Squared: subcortical nuclei **Panel C:** Photograph of the cerebral cortex in Mk-PM with the position of the sections depicted in panels A and B. **Panels D and E:** The cortical organization in layers is lost in the parietal lobe (D) but normal in the inferior temporal lobe (E). **Panel F:** Diagram showing the distribution of the number of sulci per cortical length (number of sulcus per 10 mm) in the PMG monkey (black) and in the three control monkeys, Mk-IU, Mk-I2 and Mk-IZ (grey). Diamonds represent the results obtained in the frontoparietal region and bars in the ventro-temporal region. N.s is for non-statistically significant. **Panel G:** Photomicrograph of the Corpus Callosum (CC) in the right hemisphere of Mk-PM showing large quantity of BDA stained fibers. Scale bar: 500 microns.

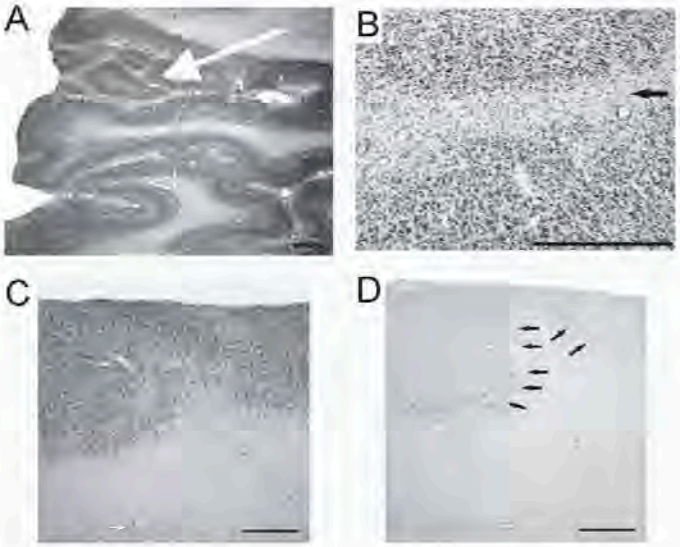

Figure 3
Abnormalities in cortical organization. Panel A: Photomicrograph of a Nissl stained section demonstrating the disorganization of the microgyrial cortex. The layered organization is irregular and anomalous, with the formation of small islets (arrow). **Panel B:** Photomicrograph showing the lack of lumen and of pia between two facing portions of cortex in a sulcus, resulting in the fusion of both layers I in a unique strip of white matter (black arrow). **Panel C:** Photomicrograph of a Nissl stained section showing the absence of well defined laminar organization. **Panel D:** Photomicrograph of a SMI-32 stained section adjacent to the section presented in panel C. Note that the SMI-32 positive neurons occupy positions in the cortex which vary strongly with regard of its distance to the cortex surface (black arrows). White arrows in panels C and D indicate the same blood vessels. Scale bars: 500 microns.

tance from the surface that varied over a short rostro-caudal interval, and were not always oriented perpendicularly to the brain surface (Fig. 3D).

In the cortical region where ICMS elicited movements of the hand and where the laminar organization was identifiable, large pyramidal neurons were observed in Nissl and in SMI-32 stained material (Fig. 4A and 4C, black arrows) at a depth corresponding to layer V in normal monkeys (Fig. 4B and 4D, black arrows). However, in normal monkeys, numerous layer III pyramidal neurons were also SMI-32 positive (Fig. 4D, white arrow), whereas only few were recognizable in the PMG monkey (Fig. 4C).

When compared to normal monkeys (Mk-IU, Mk-I2 and Mk-IZ), the frequency of sulci measured on coronal sections in the fronto-parietal as well as in the ventro-temporal cortical regions was significantly higher in the PMG monkey ($p < 0.05$, Mann and Whitney with Bonferroni correction for multiple comparisons). Furthermore, in the PMG monkey, the frequency of sulci in the fronto-parietal cortex was nearly twice as high than that of the ventro-temporal region (Fig. 2F; $p < 0.05$, Mann-Whitney). No statistically significant difference was observed in the three normal monkeys. These differences do not reflect changes in the volume of the cortex, as at a comparable rostro-caudal position, the measured distance between the corpus callosum and the external part of the lateral fissure is similar among all animals.

In contrast to the few SMI-32 positive neurons detected in layer III of the presumed M1 area of the PMG-monkey, injections of BDA in this cortical region in the left hemisphere stained a large number of fibers in the corpus callosum (Fig. 2G) and several retrogradely labelled neurons were found in the right hemisphere in the frontal lobe. This observation suggests that a significant number of pyramidal neurons in lamina III are present, but do not express the neurofilament recognized by the SMI-32 antibody. As the cortical structure of the PMG brain is also disturbed in the contralateral side, it was not possible to assess the exact areas where projections were terminating and where callosal neurons were stained.

The analysis of the cross-sectional area of SMI-32 positive pyramidal cells in the putative layer V of M1 showed a statistically significant difference of somatic size between the left and the right hemispheres of the PMG monkey, a difference that was not observed in normal monkeys (Fig. 5). However, the somatic size of SMI-32 stained pyramidal cells in the PMG monkey is comprised in the range found in normal monkeys. As M1 is not well defined in the PMG monkey, it is difficult to ascertain that the measures were done on neurons placed in equivalent regions. The relative number of SMI-32 positive cells in layer V was not different among all monkeys.

2) The corticospinal (CS) projection

2a) Crossed versus uncrossed CS projections

In normal macaque monkeys, after a unilateral BDA injection in M1, 85-95% of the CS fibers were found in the opposite dorsolateral funiculus whereas 5-15% were in the ipsilateral dorsolateral and ventral funiculi (Fig. 6B). In the PMG monkey, BDA injections were placed in a cortical territory where ICMS elicited contralateral hand movements (Fig. 6A). This territory was later found to contain large pyramidal neurons. The proportions of BDA labelled CS axons in the contralateral and ipsilateral cervical white matter of the PMG monkey were 95% and 5%, respectively (Fig. 6C), thus comprised within the range found in normal monkeys.

2b) Density of CS axonal arbors in the grey matter

The macroscopic structure and general histology of the spinal cord of the PMG monkey was normal. When compared to three normal monkeys, the PMG monkey exhibited a comparable CS arborization density in the grey

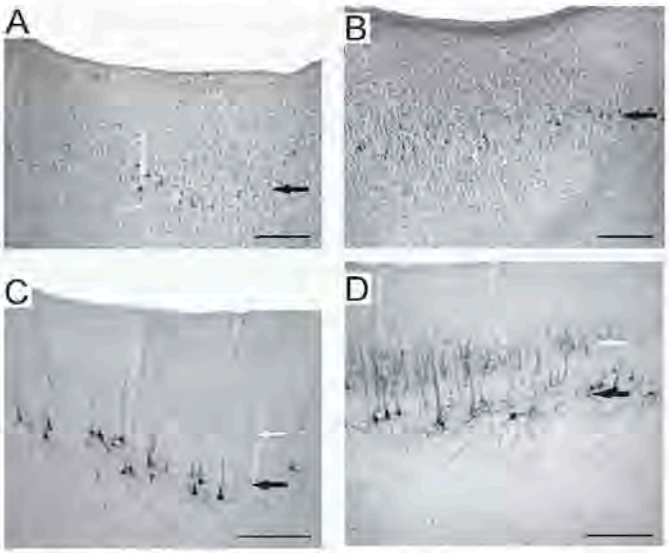

Figure 4
Cytoarchitecture of the micro-excitable cortex. Photomicrographs of motor cortex of the right hemisphere in the PMG monkey (A: Nissl staining; C: SMI-32 staining) and in the normal monkey (B: Nissl staining; D: SMI-32 staining). Black arrows show the location of pyramidal cells in layer V, whereas the open arrows show the location of layer III. Note the absence of layer III SMI-32 stained neurons in Mk-PM. Scale bars: 500 microns.

matter at C5 (Fig. 6D; Table 1). The projection pattern of CS axonal arbors within the grey matter did not differ with respect to density nor to spatial distribution, as the CS arbors in the PMG monkey terminated mainly within the intermediate zone (Rexed laminae IV-VII), contralateral to the side of BDA injection (Fig. 6B, C), as in normal monkeys.

2c) CS axons crossing the midline at C5

We examined the same material to determine whether BDA-labelled CS axon collaterals crossed the midline in the grey matter (see methods). The number of CS axons crossing the midline at C5 in each monkey was normalized to the total number of labelled CS axons, in the white matter. On average, the PMG monkey exhibited a higher number of midline crossing CS axons, comprised within the range found in normal monkeys (Fig. 6E).

3) Magnocellular part of the red nucleus (RNm)

In the RNm of the PMG monkey, numerous neurons presented large vesicles (Fig. 7), a histological feature not observed in normal monkeys. We also investigated whether the number and somatic size of SMI-32 positive RNm neurons differed in the PMG monkey. The mean number per section and somatic size of SMI-32 stained RNm neurons was 131 16.1 μm^2 (+- SD) +- 11.5. These values are in the ranges obtained in normal monkeys (Mk-IR: n = 174, 19.3 +- 9.7; Mk-IE: n = 114, 12.8 +- 9.9; Mk-IZ: n = 93, 13.5 +- 7.8; Mk-IRh: n = 120, 14.7 +- 9.7).

4) Intracortical microstimulation and manual dexterity data

During the two years of behavioral training and single neuron recording, no deficit in learning and executing the demanding reach and grasp drawer task was detected in the PMG monkey, the pathology being discovered post-mortem.

Intracortical microstimulation (ICMS) was conducted in the PMG monkey to confirm that single unit recordings had been obtained from the hand area of M1. The general organization of this region in the PMG monkey corresponded to that of normal monkeys: ICMS along tracks located laterally to the hand area elicited contractions of facial muscles whereas proximal movements (shoulder muscles) were elicited along tracks located medially to the hand area (Fig. 8). The ICMS thresholds to elicit joint movements were in the range observed in normal monkeys, typically at current intensities smaller than 10 microamps (Fig. 8B).

The extracellular single neuron activities recorded in motor areas of the left hemisphere while the PMG monkey performed the reach and grasp drawer task were similar to those obtained in normal monkeys. Epileptic seizures were never observed.

After 7 to 10 months of training, the PMG monkey reached a success rate of 90 to 95% in the reach and grasp drawer task, a performance similar to that of the normal monkey (MK-IU). On the other hand, the reaction times (RTs) were significantly longer in the PMG monkey to perform the unimanual task with the fastest hand, as compared to the normal monkey (Mk-IU) (Fig. 9A Student's t-test, $p < 0.0001$). The PMG monkey also showed significantly longer RTs with the fastest hand as compared to two other monkeys (Mk-I4 and Mk-I5) involved in a former version of the reach and grasp drawer behavioral task, as reported previously [11] (Fig. 9A). The longer RT is not associated to motor impairment as in three out of four measures of motor capacity in the behavioral task (reaching times, pulling time, grasping time; Fig. 9B), the PMG monkey was significantly faster than the intact monkey (Mk-IU).

Discussion

The present case of brain malformation in a macaque monkey can be diagnosed as a PMG according to previously reported criteria, namely disorganized cortical gyri in excessive number in both hemispheres and local loss of laminar organization in the frontal and parietal cortices [4]. Accord-

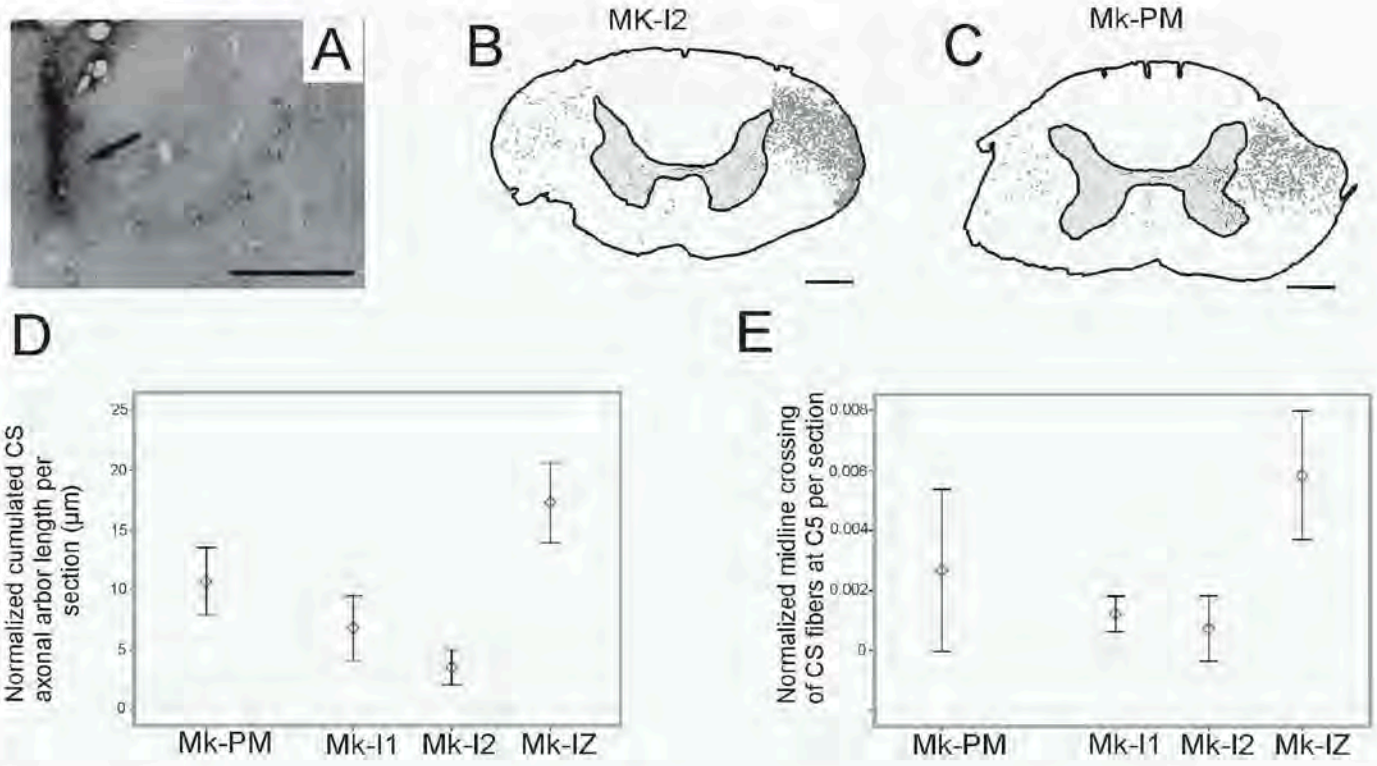

Figure 6

Corticospinal projections in PMG monkey. **Panel A:** Site of BDA injection in M1 hand area of the left hemisphere (arrow), close to identified layer V pyramidal neurons. **Panels B and C:** Reconstructions of BDA stained corticospinal (CS) fibers in coronal sections of the cervical spinal cord at the C5 level, as a result of BDA injection in the right motor cortex of a normal monkey (B) and in the left motor cortex of the PMG monkey (C); scale bar 1 mm. For better visual comparison of both reconstructions, the reconstruction in panel C was drawn with the left spinal side on the right side of the drawing. Grey dots indicate the location and distribution of the CS axons in the white matter. In both monkeys, most fibers were found in the dorsolateral funiculus (DLF) contralateral to the injection site, the rest running along the dorsolateral and ventral funiculi ipsilateral to the injection site. In comparison to normal monkeys, slightly fewer CS fibers were found in the grey matter ipsilateral to the injection side in the PMG monkey. **Panel D:** Normalized cumulated axonal arbor length of corticospinal projections in the cervical grey matter in the PMG monkey (black) and in three normal monkeys (grey). **Panel E:** Number of midline crossing CS fibers at cervical level C5. The number of fibers crossing the midline was normalized by dividing it by the total number of labelled CS fibers present in the white matter (see methods for detail).

performs apparently as well as the normal monkey, both in terms of training curve and stabilized motor performance after training. However, the behavioral data derived from the reach and grasp drawer task showed that the PMG monkey (Mk-PM) had significantly longer RTs than the normal monkeys (Fig. 9A). Nevertheless, after initiation of the movement sequence, the time intervals between different movement components of the overall motor response did not show any systematic variation (longer or shorter). Indeed, the first reaching time (interval between movement onset and drawer knob grasping) in the unimanual task was longer in the PMG monkey but the pulling time (opening of the drawer), the second reaching time and the grasping time were slightly shorter (Fig. 9B). This observation of "normal" motor control in the PMG monkey is in line with a generally normal organization of its corticospinal tract (Fig. 6). Furthermore, "normal" motor control in the PMG monkey is also consistent with the electrophysiological data, namely the presence of low (normal) threshold ICMS effects observed in the presumed hand area of the primary motor cortex (Fig. 8). The latter ICMS data are also coherent with a normal density and appearance of large pyramidal neurons in layer V in the presumed motor cortex in the PMG monkey, as seen in SMI-32 staining (Fig. 4).

The significantly prolonged RTs in the PMG monkey may be associated to a deficit of attention. It has been shown

Table 1: Quantitative anatomical data for the CS tract tracing.

Monkey	Mk-PM PMG	Mk-I1 Intact	Mk-I2 Intact	Mk-IZ Intact
Volume of BDA Injected in M1 in μl	24	10	22.5	25.5
Number of BDA Injection sites	13	7	15	17
Survival time (in days) cumulated	41	21	45	51
Number of BDA-labelled CS axons at C5 in white matter	821	3133	1394	1884
% of uncrossed CS axons at C5	14.9	11.2	14.5	8.9
Normalized axon arbor length at C5 per section	10.71	6.88	3.58	17.32
Normalized number of axonal arbors crossing midline at C5	0.00268	0.00121	0.000717	0.00584

Survival time: number of days separating the injection of BDA in the contralesional M1 and the day of sacrifice of the animal.

that, in a delayed conditional task instructed with visual cue signals and requiring discrimination of a specific stimulus among irrelevant distracters, attention is under the control of top-down inputs from the lateral prefrontal cortex onto visual cortical areas [19]. The authors found a significant increase of RTs in the task after lesion of the lateral prefrontal cortex. In the present case, as the PMG involved the frontal lobe, the lateral prefrontal cortex may be affected, leading to a deficit of attention. Along this line, the disorganization of some cortical layers, and the decrease of the density of SMI-32 positive neurons in layer III (Fig. 4C), suggests that some cortico-cortical interactions may be abnormal in the PMG monkey.

Conclusions

Overall, these data suggest that the PMG pathology may have affected some cortico-cortical connections (crucial for attention), but not the corticospinal projection as indicated by normal motor control in a well trained behavioral task.

Methods

Animals

The data were derived from eleven young adults (2-9 years old) macaque monkeys (Macaca mulatta or fascicularis, of either sex, weighing from 3.0 to 9.0 kg, see Table 2). Monkeys Mk-IG, Mk-IE, Mk-IRh, Mk-IR, and Mk-IZ were involved in previously published studies [17,20]. Surgical procedures and animal care were conducted in accordance with the Guide for the Care and Use of Laboratory Animals (ISBN 0-309-05377-3; 1996) and approved by local (Swiss) veterinary authorities. Details on the sacrifice of the animals at the end of the experiments and on histological processing are available in Additional file 1.

Behavioral experiments

The PMG monkey (Mk-PM) and a normal monkey (Mk-IU) were enrolled in a conditional delayed bimanual dexterity task (see additional files 1 and 2 and additional Fig. S1), corresponding to a modified and more complex version of the so-called "reach and grasp drawer task" [10,21-23]. In order to locate the hand representation of the primary motor cortex (M1), an intracortical microstimulation (ICMS) mapping was performed, as described in detail earlier [13,24-28].

Tracing experiments

In the PMG monkey and three normal monkeys, a craniotomy provided access to the cerebral cortex, allowing injections of the tracer BDA at physiologically defined loci in the M1 hand area of one hemisphere. Under propofol anaesthesia (Disoprivan, 3 mg/kg/h, i.v.), the craniotomy was performed in those four monkeys to expose the stere-

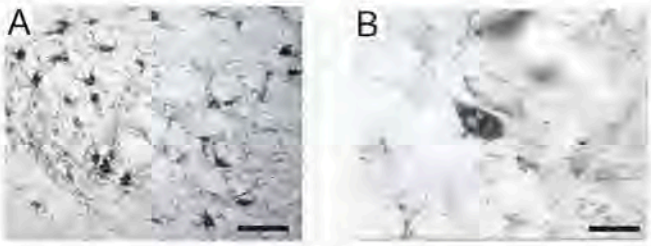

Figure 7
Abnormal vesicles in RNm neurons. SMI-32 staining of the red nucleus pars magnocellularis (RNm) in the PMG monkey. The general structure of the nucleus is comparable to that observed in normal monkeys (A), but abnormal accumulation of vesicles were observed in SMI-32 positive RNm neurons (B). Scale bars: (A) 200 μm; (B) 50 μm.

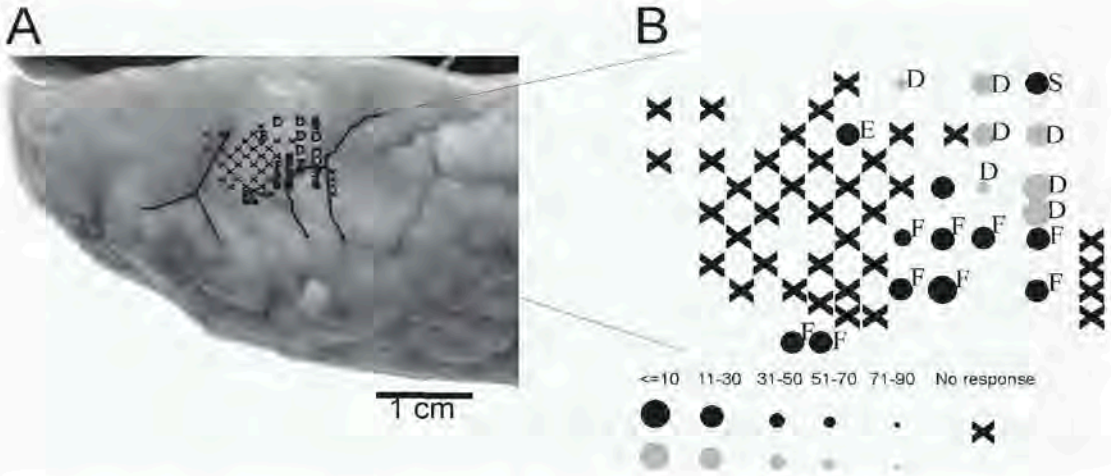

Figure 8
Functional organisation of M1 in the PMG monkey. **Panel A:** Left frontal lobe region of the brain of the PMG monkey with the map of the sites where intracortical microstimulation (ICMS) has been performed at a stereotaxic position corresponding to M1 in normal monkeys. Note the difficulty to identify the central sulcus and the precentral gyrus. **Panel B:** Map of the sites where ICMS has been performed, with indications on the body part of the observed movements and of the minimal current required to elicit the movement. D: digit, F: face, E: elbow and S: shoulder. X: No response at 80 microamps of stimulation intensity.

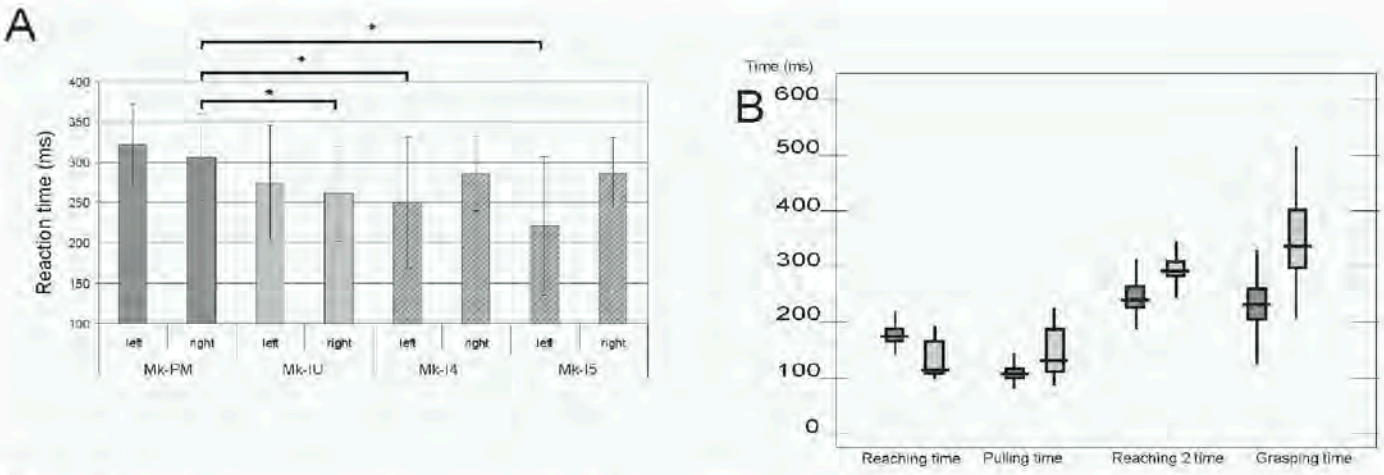

Figure 9
Behavior: reach and grasp drawer task. **Panel A:** Reaction times (RT) for each hands in the behavioral unimanual tasks for the 4 monkeys involved in the behavioral reach and grasp drawer task. In each case, the hand with which the PMG monkey (Mk-PM) exhibited the shortest average RTs (right) has been statistically tested against the fastest hand of the 3 normal monkeys (Mk-IU, Mk-I4, Mk-I5) using a Mann-Whitney test. **Panel B:** Movement times corresponding to the following movement components: First reaching time: from 'hand movement onset' to 'touch knob', Pulling time: from 'touch knob' to 'drawer fully open', Second reaching time: from 'grasping hand movement onset' to 'enter drawer's well', Grasping time: from 'hand penetrating in the well' to 'hand out of the well'.

Table 2: List of monkeys included in the present study with identification code.

	Mk-PM	Mk-I1	Mk-I2	Mk-IZ	Mk-IU	Mk-IR	Mk-IE	Mk-IRh	Mk-IG	Mk-I4	Mk-I5
	PMG	**Intact**	**Intact**	**Intact**	**Intact**	**Intact**	**Intact**	**Intact**	**Intact**	**Intact**	**Intact**
Species	**mul.**	**fasc.**	**fasc.**	**fasc**	**mul.**	**mul.**	**mul.**	**mul.**	**mul.**	**fasc.**	**fasc**
Age at sacrifice (years)	5	3.75	7.75	8	9	5	5	6.5	8	2	2.5
Study	BIM + RT + SF	SCI study	SCI study +SF	SCI study + RN + SF	Cortex BIM + RT + SF	Cortex + RN	Cortex+ RN	Cortex+ RN	Cortex	RT	RT
ICMS	Yes	No	No	No	Yes	No	No	No	No	Yes	Yes

Monkey Mk-PM exhibited a brain with PMG whereas the other 10 monkeys had a brain with normal appearance.
Under species, "mul." is for macaca mulatta whereas "fasc." is for macaca fascicularis.
Under ICMS (intracortical microstimulation), "Yes" refers to the four monkeys subjected to ICMS sessions in M1.
BIM + RT: Bimanual reach and grasp task study: assessment of motor performance in the present reach and grasp drawer task (see additional file 1 Fig. S1) with measures of RT's.
RT: Assessment of RT in a former version of the reach and grasp drawer task[11];
SF: Measurement of the Sulcus frequency in the cortex.
RN: Red Nucleus investigation study [17]; Cortex: Cortical anatomical investigation study [20];
SCI: Spinal cord injury study: monkeys used as control intact monkeys in previous studies on spinal cord injury, used here for comparison.
Numbers in brackets correspond to the reference number in the manuscript.

otaxic area corresponding to the motor cortex. In intact monkeys, injections of BDA were placed in the rostral bank of the central sulcus, following the central sulcus going from lateral (hand representation) to medial (leg representation). In the PMG monkey, the BDA injections were performed at side where ICMS elicited hand movements. Based on our previous experience of tracing the CS tract with BDA in monkeys, the survival time after BDA injection was set to three to four weeks [25].

General morphology

To compare the frequency of sulci in the brain of the PMG monkey with that of normal monkeys, we counted the number of sulci in the frontoparietal and ventro-temporal lobes in the PMG monkey and in three control monkeys (Mk-IU, Mk-I2 and Mk-IZ), divided by the measured length of the corresponding cortical surface. The measurements were made on 5 coronal sections at 40× magnification, regularly distributed between the rostral end of the Nucleus Caudatus and the rostral end of the Lateral Geniculate Nucleus (LGN). On the rostral sections, where the lateral fissure is not present, the measure was obtained from the region delimited by the Corpus Callosum and the angle between the lateral and the ventral walls of the frontal lobe. A depression of the cortical surface was considered as a sulcus when we could identify a clear invagination of the layer I (Fig. 2A).

Measurement of CS axonal arborization

At cervical level C5, in the grey matter, the presence of BDA labelled CS axonal arbors was investigated on five coronal sections at 400× magnification. Using Neurolucida® software, each BDA-labelled axonal segment observed in the grey matter was traced and the cumulated axonal arbor length was then computed. As the number of BDA labelled CS axons varied across monkeys, the measures were normalized to the total number of BDA-labelled CS axons counted in the white matter at C5 level on three coronal sections (Table 2). Furthermore, on the five sections at C5 level, the numbers of CS fibers crossing the midline were counted, as previously reported [29]. For normalization, their cumulated number was divided by the total number of CS axons present at C5 level in the white matter.

Authors' contributions

ES sampled the behavioral data, performed the analysis of the BDA staining in the cervical cord, as well as the macroscopical analysis of the sulci in the brain. He also performed most of the statistical analysis of the data and part of the behavioral training. CJ was responsible for the behavioral training and electrophysiological recording in the monkeys as well as the analysis and the interpretation of the behavioral data. PF contributed to the BDA projections analysis wrote the first draft of the paper. PW carried out the morphological analysis of the cells and of their

distribution in the Red Nucleus. MLB made the morphological analysis of the cortical neurons in the presumed motor cortex. EMR participated to the design and the coordination of the study, the interpretation of the data, the surgical, histological and behavioral procedures. TW participated to the design of the study, the analysis of the histological data, the coordination of the comments and the replies to the reviewers received from the co-authors. All authors read and approved the final manuscript.

Additional material

Additional file 1

Figure depicting the behavioral task. Schematic representation of the sequences required for executing the complex behavioral task.
Click here for file
[http://www.biomedcentral.com/content/supplementary/1471-2202-10-155-S1.TIFF]

Additional file 2

Additional methods. Additional information about the procedures and legend of the additional file 1 figure S1.
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Curriculum Vitae

Name:	**Beaud**
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Home address:	Rue de la Carrière 20 CH – 1700 Fribourg 026/424.14.27. 076/574.05.92.
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Languages:	**French (langue maternelle)** English (professional competence for both oral and written communication) German (Gute mündliche und schriftliche Kenntnisse)

EDUCATION

2007-today	Doctoral student at Unit of Physiology, University of Fribourg Thesis advisors: Prof. Eric Rouiller & Dr Thierry Wannier *Topic: Anatomical organisation and functional recovery in spinal injured non-human primates treated with either an antibody directed against Nogo-A or a combination of this antibody with brain-derived neurotrophic factor (BDNF).*
2009	Course in Stereology in the Department of Anatomy at the University of Bern
2007	Course in Informatics for scientists at the University of Fribourg
2006-2010	Continuous formation from the personal specialized in animal experimentation

2006	Licence for animal experimentation (Module 1, CHUV, Lausanne)
2005-2007	Master of Science in Biology
2002-2005	Bachelor of Science in Biology
2002	Swiss Maturity Diploma College de Gambach in Fribourg (specific option biology/chemistry; complementary option psychology/pedagogy)

EXPERIENCE

2007-today	Teaching assistant in the Unit of Physiology for practical courses to medical and biomedical students
1997-2007	Saleswoman (in the dairy of Marly)
2002-2007	Teaching (course of supports and replacements)
1994-1997	Summer jobs (cleaning schools)

MEMBERSHIP IN PROFESSIONAL SOCIETIES

- Swiss Society for Neurosciences

PUBLICATIONS

PAPERS

***: shared first-authorship**

Beaud ML, Rouiller EM, Bloch J, Mir A, Schwab ME and Wannier T. After spinal cord injury, adult macaque monkeys exhibit numerous SMI-32 positive fibers into the scar tissue insensitive to the application of an anti-Nogo-A antibody alone or co-applied with BDNF. Neuroscience; in preparation.

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Schmidlin E*, Jouffrais C*, Freund P, Wannier-Morino P, **Beaud ML**, Rouiller EM and Wannier T. (2009) A case of polymicrogyria in macaque monkey : impact on anatomy and function of the motor system. BMC Neuroscience 10:155. [Original Paper]

Beaud ML*, Schmidlin E*, Wannier T*, Freund P, Bloch J, Mir A, Schwab ME and Rouiller E.M. (2008) Anti-Nogo-A antibody treatment does not prevent cell body shrinkage in the motor cortex in adult monkeys subjected to unilateral cervical cord lesion. BMC Neuroscience 9:5. [Original Paper]

ABSTRACTS

Beaud ML, Rouiller EM, Bloch J, Mir A, Schwab ME and Wannier T. BDNF interferes with a treatment neutralizing Nogo-A in adult macaques subjected to incomplete spinal cord injury. Forum of European Neuroscience Meeting, Amsterdam 2010. [Abstract]

Beaud ML, Schmidlin E, Freund P, Bloch J, Mir A, Rouiller EM and Wannier T. In adult macaque monkeys subjected to an incomplete spinal cord injury, numerous large SMI-32 positive fibers were observed into the lesion site. Forum of European Neuroscience Meeting, Geneva 2008. [Abstract]

Beaud ML, Wannier T, Schmidlin E, Freund P, Bloch J, Mir A, Schwab ME and Rouiller EM. Anti-Nogo-A treatment enhanced sprouting of corticospinal axons but did not prevent cell body shrinkage in the motor cortex in adult monkeys subjected to unilateral cervical cord lesion. Forum of European Neuroscience Meeting, Vienna 2006. [Abstract]

POSTERS PRESENTED TO SPECIAL INTERESTS MEETINGS

Beaud ML, Rouiller EM, Bloch J, Mir A, Schwab ME and Wannier T. Effect of a combinative treatment of Nogo-A antibody and BDNF on corticospinal (CS) neurons and axons in adult spinal cord injured macaque monkey. National Centers of Competence in Research Meeting, Berlingen 2010.

Beaud ML, Rouiller EM, Bloch J, Mir A, Schwab ME and Wannier T. BDNF interferes with a treatment neutralizing Nogo-A in adult macaques subjected to incomplete spinal cord injury. Swiss Society of Neuroscience Meeting, Lausanne 2010.

Beaud ML, Freund P, Schmidlin E, Bloch J, Mir A, Schwab ME, Rouiller EM and Wannier T. In adult macaque monkey subjected to an incomplete spinal cord injury, numerous large SMI-32 positive fibers penetrate the scar tissue. Swiss Society of Neuroscience Meeting, Fribourg 2009.

Beaud ML, Wannier-Morino P, Schmidlin E, Freund P, Belhaj-Saif A, Bloch J, Mir A, Schwab ME, Rouiller EM and. Wannier T. Effect of spinal cord injury on corticospinal (CS) and rubrospinal (RS) neurons in adult macaque monkeys treated with an antibody directed against Nogo-A. National Centres of Competence in Research Meeting, Berlingen 2008.

Beaud ML, Wannier-Morino P, Schmidlin E, Freund P, Belhaj-Saif A, Bloch J, Mir A, Schwab ME, Rouiller EM and. Wannier T. Anti-Nogo-A antibody treatment does not prevent cell body shrinkage in the motor cortex in adult monkeys subjected to unilateral cervical cord lesion National Centres of Competence in Research Meeting, Ittingen 2007.

Beaud ML, Wannier T, Schmidlin E, Freund P, Bloch J, Mir A, Schwab ME and Rouiller EM. Anti-Nogo-A treatment enhanced sprouting of corticospinal axons but did not prevent cell body shrinkage in the motor

cortex in adult monkeys subjected to cervical cord lesion. Swiss Society of Neuroscience Meeting, Basel 2006.

METHODS OF RESEARCH

Behavioural studies, electromyography, electroencephalography, evoked potentials, magnetic or electric transcranial stimulation, use of anterograde and retrograde tracers, immunohistochemistry, classical microscopy, stereology.

COMPUTER SKILLS

Very good knowledge of the MS-Windows System, of the MS-Office Programmes, as well as Corel Draw, Corel Photo-Paint, Matlab Dartfish, IrfanView, SPSS and Reference Manager Programmes.

REFERENCES

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