European Journal of Neuroscience, Vol. 27, pp. 326–333, 2008 doi:10.1111/j.1460-9568.2007.06006.x

Lamina-specific restoration of serotonergic projections after Nogo-A antibody treatment of spinal cord injury in rats

Angela Müllner,*,† Roman R. Gonzenbach,† Oli Weinmann, Lisa Schnell, Thomas Liebscher and Martin E. Schwab
Brain Research Institute, University of Zurich and Department of Biology, Swiss Federal Institute of Technology (ETH), Winterthurerstrasse 190, CH-8057 Zurich, Switzerland

Keywords: 5-HT, functional recovery, plasticity, raphe-spinal, regeneration, sprouting

Abstract

Blocking the neurite growth inhibitor Nogo-A by neutralizing antibodies improves functional recovery after partial spinal cord injury. In parallel, regeneration and sprouting of cortico- and rubrospinal projections are increased and may partially explain the enhanced functional recovery. The serotonergic raphe-spinal tract, which plays a key regulatory role for spinal motor circuits, has not been analysed in detail with regard to its response to Nogo-A function blocking antibody treatment after spinal cord injury. We studied the effect of 2 weeks of intrathecal Nogo-A antibody application after partial thoracic spinal cord injury on the lamina-specific restitution of the serotonergic (5-HT) raphe-spinal projections to the mid-lumbar grey matter. Nine weeks after the lesion, the number of 5-HT fibres in Rexed's laminae 4 and 7 and the number of 5-HT-positive varicosities on motoneurons in lamina 9 returned to their lamina-specific preinjury levels in Nogo-A antibody-treated rats. By contrast, control antibody-treated animals showed only a moderate increase in 5-HT fibre density in the respective laminae, and the number of 5-HT-positive varicosities on motoneurons remained low. Our results suggest that the Nogo-A antibody-induced recovery of descending serotonergic projections to the grey matter is lamina-specific and molecular cues must be present to guide the growing axons to the correct target areas. This appropriate restitution of the serotonergic innervation below the lesion site probably contributes to the impressive recovery of motor function.

Introduction

Spinal cord injury (SCI) leads to the destruction of ascending and descending axonal tracts that control motor and sensory as well as autonomic functions. The consequences are devastating: patients suffer from permanent, often lifelong motor and sensory disabilities below the site of injury, combined with impaired basic vital functions. Functional recovery is restricted because axons in the central nervous system (CNS) regenerate poorly. Nogo-A is a well-known inhibitory component of CNS myelin that limits the regenerative capacity of axons (Schwab, 2004). Its presence on oligodendrocytes in the adult mammalian CNS plays an important role for the lack of substantial functional recovery after an injury to the spinal cord.

Inactivation of Nogo-A by function-blocking antibodies leads to an increased regeneration of lesioned corticospinal tract (CST) fibres in adult rats, marmosets and macaque monkeys below the site of injury (Schnell & Schwab, 1990; Fouad *et al*., 2004; Liebscher *et al*., 2005; Freund *et al*., 2006). Also, Nogo-A antibody treatment of spinal cord injured rats enhances compensatory sprouting of spared corticospinal fibres (Thallmair *et al*., 1998). Most importantly, this increased plasticity of lesioned and spared corticospinal projections is accompanied by improved recovery on the functional level: treated animals recover faster and to a larger extent from SCI-induced functional deficits than control animals. Treated rats, for instance, perform better in behavioural tasks when tested for the ability to walk on a beam, to swim or to grasp food pellets.

However, simple locomotor behaviours such as walking or swimming do not depend on the CST. Its selective destruction leads generally to transient, minor deficits in the walking pattern (Armstrong, 1986; Metz *et al*., 1998; Muir & Whishaw, 1999). Other descending tracts such as those originating from the brainstem (rubro-, vestibulo- and reticulospinal tract and descending monoaminergic projections) as well as the intricate intraspinal circuitries play a more important role for locomotion than the CST (Raineteau & Schwab, 2001). These tracts are phylogenetically older than the CST and may have different propensities for sprouting and regeneration.

Neutralization of Nogo-A by antibodies or interfering with Nogo-A signalling leads to increased sprouting of serotonergic fibres caudal to an injury (Bregman *et al*., 1995; GrandPre *et al*., 2002; Li & Strittmatter, 2003). These existing reports did not consider different serotonergic (5-HT) fibre types or distinguish between different subregions of the spinal cord. A thorough investigation of the exact pattern and the extent of the recovery of the serotonergic system is therefore still lacking.

5-HT fibres are unequally distributed in the spinal cord grey matter, but fibre densities for the different areas are tightly regulated and specific for each lamina (Steinbusch, 1981; Marlier *et al*., 1991). Here, we investigate the recovery of the serotonergic projections to the lumbar spinal cord in detailed immunohistochemical analyses for the dorsal horn, the intermediate grey and the ventral horn in Rexed's laminae 4, 7 and 9, respectively. We observed a nearly complete and lamina-specific restoration of 5-HT fibre projections 9 weeks after

Correspondence: Dr R.R. Gonzenbach, at *present address below.
E-mail: gonzenbach@hifo.uzh.ch

**Present address*: Brain Research Institute, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland.

†A.M. and R.R.G. contributed equally to this paper.

Received 26 February 2007, revised 19 October 2007, accepted 21 November 2007

spinal cord lesion in the Nogo-A antibody-treated animals, but not in the control antibody-treated animals. This restoration of the descending serotonergic raphe-spinal tracts most certainly contributes to the substantial recovery of motor functions.

Materials and methods

Animals and treatments

The animals used in the present immunohistochemical study were adult 2-month-old (160–180 g) Lewis rats; they were kept in groups of 4–6 animals in standardized cages (type 4, Macrolon) on a 12-h-light 12-h-dark cycle on a standard food regimen with water *ad libitum* (for details see Liebscher *et al*., 2005). All experiments were performed according to the guidelines of the Veterinary office of the conton of Zurich, Switzerland and approved by its Commission for Animal Research. In brief, a T-shaped lesion that included the dorsal, dorsolateral, ventral and ventromedial areas of the spinal cord was made at thoracic level T8 under Hypnorm/Dormicum anaesthesia (Hypnorm: 120 μL/200 g body weight; Janssen Pharmaceutics, Beerse, Belgium; and Dormicum: 0.75 mg/200 g body weight; Roche Pharmaceuticals, Basel, Switzerland). The size and shape of the lesions are shown schematically in Fig. 1F.

The mouse monoclonal antibody 11C7 was raised against a peptide corresponding to the rat Nogo-A sequence amino acids 623–640 and the antibody 7B12 against prokaryotically produced recombinant Nogo-A fragment amino acids 1–979 (Oertle *et al*., 2003). Both antibodies were monospecific for Nogo-A on Western blots (Oertle *et al*., 2003; Dodd *et al*., 2005) and there was no cross-reactivity with other Nogo variants (Oertle *et al*., 2003; Dodd *et al*., 2005). Their function-blocking capacity has been confirmed *in vitro* and *in vivo* (Wiessner *et al*., 2003; Liebscher *et al*., 2005; Weinmann *et al*., 2006).

The rats were randomly divided into the following experimental groups: lesion + anti-Nogo-A antibody 7B12 treatment ($n = 14$), lesion + anti-Nogo-A antibody 11C7 treatment ($n = 11$), lesion + control mouse IgG treatment ($n = 15$) and unlesioned animals ($n = 7$). The lesioned animals received 2 weeks of intrathecal infusion with the respective antibodies starting immediately after the spinal cord injury. An intrathecal catheter (32 gauge; Recathco, Allison Park, PA, USA) was inserted from L2 and pushed up to T10, to deliver continuously the monoclonal antibodies from an osmotic minipump (5 μL/h, 3.1 μg IgG/μL, Alzet 2ML2; Charles River Laboratories, Les Oncins, France) into the cerebrospinal fluid for 2 weeks. Analgesics (Rimadyl; 5 mg/kg, s.c., Pfizer AG, Zürich, Switzerland) were given perioperatively. To prevent bladder infections, the antibiotic Baytril (5 mg/kg body weight, SC; Bayer AG, Leverkusen, Germany) was given once a day for 1 week, starting preoperatively. The pumps and intrathecal catheters were removed after 2 weeks under isoflurane anaesthesia. Three untreated animals were killed 3 days after the lesion to investigate the initial extent of 5-HT fibre loss after SCI. Another group of rats ($n = 7$) did not undergo surgery and was used to analyse 5-HT fibre density in intact animals. The animals were number-coded and randomly mixed in the cages. The experimenters were blind with regard to treatment throughout the experiment.

Tissue collection

Animals were deeply anaesthetized with pentobarbital (450 mg/kg, i.p.; Abbott Laboratories, Cham, Switzerland) and perfused transcardially with 100 mL of Ringer's solution containing 100 000 IU/L heparin (Liquemin, Roche, Basel, Switzerland) and 0.25% $NaNO_2$ followed by 300 mL of 4% phosphate-buffered paraformaldehyde containing 5% sucrose. The brains and spinal cords were dissected, postfixed overnight at 4 °C in the same fixative and cryoprotected in a phosphate-buffered 30% sucrose solution for another 2 days. Cross-sections of 50 μm were cut on a vibratom, collected in 0.1 M phosphate-buffered saline and then prepared for immunohistochemistry, using the semifree floating technique (Herzog & Brosamle, 1997).

Immunohistochemistry

Conventional protocols for 5-HT immunohistochemistry failed to detect fine regenerated serotonergic fibres. We therefore developed an improved protocol for the detection of serotonergic fibres. The optimized protocol included antigen retrieval by microwave irradiation and tyramide signal amplification, modifying a method established by Loup *et al*. (1998). This led to increased sensitivity and reduced background staining.

Microwave irradiation

Sections were postfixed a second time on slide (4% paraformaldehyde, 0.1% glutaraldehyde, 0.1% saturated picric acid) for 20 min to prevent 5-HT washout. The slides were then washed in 0.1 M phosphate buffer (PB) three times until no yellow colour was visible and then quenched with ethanol peroxide (50% ethanol plus 0.3% hydrogen peroxide in ddH_2O) for 10 min. The slides were then transferred into 0.2% sodium borohydride for 10 min to reduce aldehyde and keto-groups (Clancy & Cauller, 1998). After washing in 0.1 M PB three times for 20 min, the slides were incubated in citrate buffer (0.1 M, pH = 4.5) overnight at 4 °C. The slides were then placed in a household microwave oven and irradiated twice at 600 W for 30 s in citrate buffer.

Incubation with primary and secondary antibody

The sections were blocked with 0.5% Top Block gelatine (Sigma, St Louis, MO, USA) in 0.3% TBS-TX (50 mM Tris, 0.875% NaCl, 0.3% Triton X-100, pH = 8.0) for 20 min, and incubated for 4 days at 4 °C with the primary rabbit antibody against 5-HT (1 : 8000, rabbit, Immunostar) in 0.3% TBS-TX (pH 8) and 4% normal goat serum. After incubation, the sections were washed twice in 0.1 M PB for 10 min. The Avidin Biotin Blocking Kit (Vector Laboratories, Burlingame, CA, USA) was used for further blocking: the slides were blocked for 5 min in avidin (four drops of avidin in 1 mL 0.1 M PBS), washed three times in 0.1 M PBS for 2 min, blocked in biotin (eight drops of biotin in 1 mL 0.1 M PBS) and washed again in 0.1 PBS. They were then incubated for 45 min with the secondary, biotinylated goat antirabbit antibody (1 : 300, 0.3% TBS-TX, pH 8, 2% normal goat serum, Sigma).

Tyramide signal amplification

After washing three times in 0.3% PBS-TX, the sections were blocked in a modified TNB buffer (0.5% Top Block in 0.3% TBS-TX) for 30 min and then incubated in ABC elite complex (Vector Laboratories) for 45 min. Thereafter, the slides were washed three times for 5 min in 0.1 M PBS/0.05% Tween 20. They were then incubated for 5 min in biotinylated tyramide (stock solution: 50 mM borate buffer, pH 8, 0.25% NHS-LC-biotin, 0.075%) diluted 1 : 100 in 0.1 M PBS/0.05% Tween 20 with 0.006% H_2O_2, washed twice in 0.1 M PBS for 5 min, and again blocked in modified TNB buffer for 30 min. Diluted ABC elite complex (1 : 3) was added for 10 min, followed by washing three times in PBS. Sections were then preincubated in 0.05% diaminobenzidine tetrahydrochloride (DAB; Sigma) in 0.05 M Tris, pH 8.0, followed

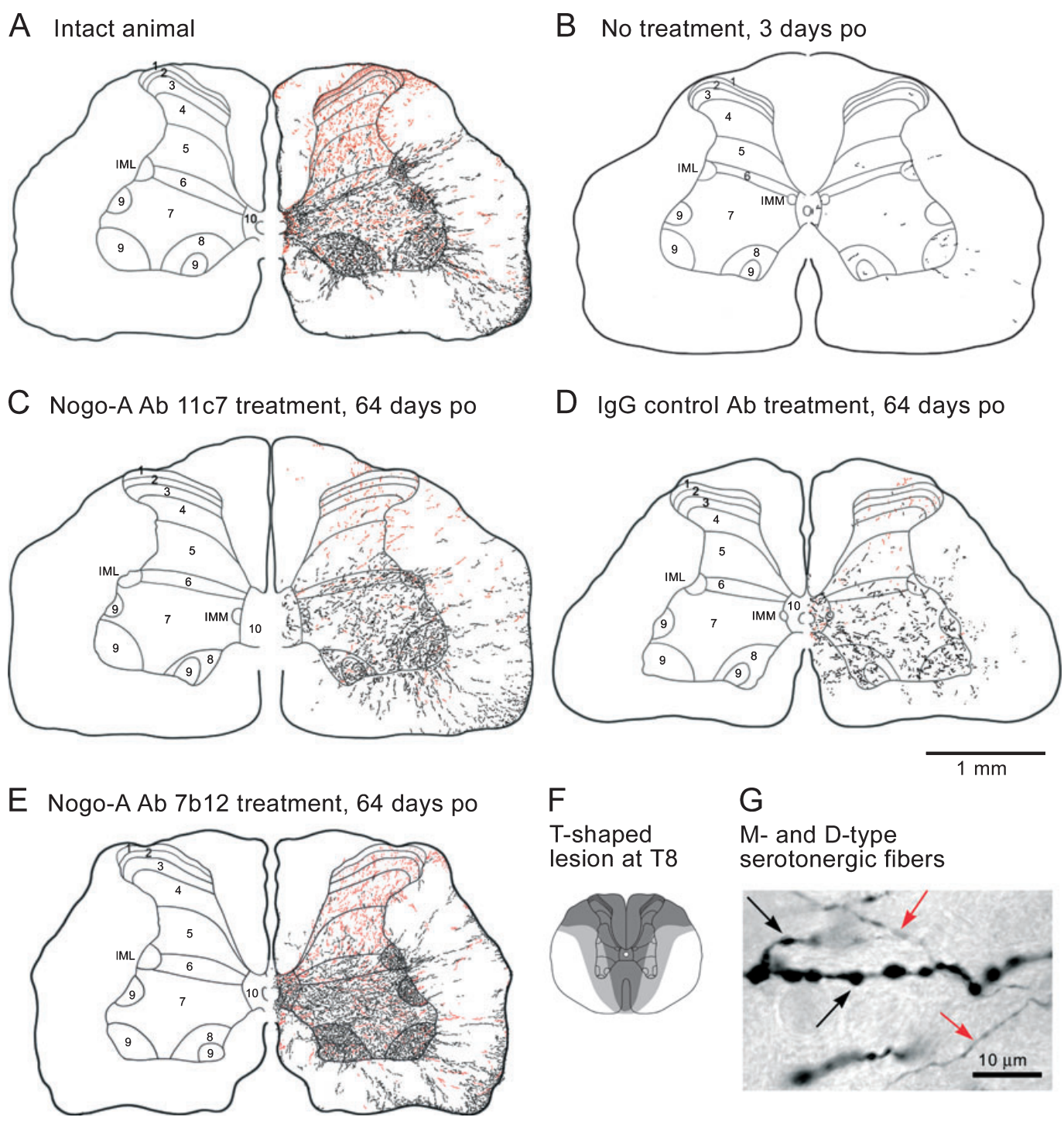

FIG. 1. Camera lucida reconstructions of 5-HT-positive fibres at lumbar segment L3 on spinal cord cross-sections (50 µm). Fine D-fibres with small varicosities are depicted in red. Thick M-fibres with large varicosities are depicted in black. (A) Intact rat: the density of serotonergic fibres in the grey substance differs between different layers. The 5-HT fibre density is highest around the central canal, intermedio-lateral column and around the motoneuron pools. (B) Three days after a large, but incomplete spinal cord lesion at T8 (see F): very few 5-HT-positive fibres remain caudal to the lesion, indicating that the lesion interrupted most parts of the descending raphe-spinal tracts. (C–E) Spinal cord injured rats that were treated intrathecally for 2 weeks with either control IgG antibody (D) or monoclonal anti-Nogo-A antibody 11C7 (C) and 7B12 (E) and killed 9 weeks after the SCI: the number of 5-HT-positive fibres increased slightly 9 weeks after the T-lesion in the control antibody-treated animal (D). In contrast, in the Nogo-A antibody 11C7- and 7B12-treated rats (C and E), the number of 5-HT-positive fibres increased dramatically. The distribution of thin and thick serotonergic fibres in the anti Nogo-A antibody-treated animals is similar to that of the intact animal. Left side of reconstructions: spinal cord laminae according to (Rexed, 1954). (F) Scheme of a spinal cord cross-section indicating in grey the area which is affected by the spinal cord T-lesion. Dark grey indicates the extent of the primary lesion; light grey approximately delineates the area of secondary tissue destruction. Note the substantial white matter area that was left intact by the T-lesion. These regions contain intact fibre tracts and may form bridges of intact tissue for regenerating fibres. (G) Serotonergic D- and M- fibres at high magnification. M-fibres (black arrows) have a larger diameter (1–5 µm) than D-fibres (less than 1 µm, red arrows) and bear spherical varicosities, giving them a beaded appearance. D-fibres have small pleomorphic varicosities.

by incubation in the reaction solution (0.05 M Tris, pH 8.0, 0.05% DAB and 0.001% H_2O_2) for 15 s. The reaction was stopped by ice-cold 0.05 M Tris (pH 6.0) for 30 s. Finally, sections were washed in 0.1 M PB and ddH_2O, air-dried overnight, dehydrated and coverslipped. Control experiments for antibody specificity included replacement of the primary antibody with nonimmune serum, or use of secondary antibody alone. No specific staining was seen in either case.

Quantification of regenerated 5-HT fibres

Sections were analysed under an Olympus brightfield and epifluorescence microscope (Vanox-T). Photomicrographs were taken with an Axiocam digital camera. All quantifications were done at the lumbar level L3 of the spinal cord using the AxioVision software (Version 4.4, Zeiss, Jena, Germany).

Quantification of 5-HT fibres in laminae 4 and 7

5-HT-positive fibres were counted in Rexed's laminae 4 and 7 on four alternate sections and on both the left and the right side of the spinal cord for each animal and each chosen lamina.

5-HT fibres in grey matter have a very irregular course, going in and out of the section plain. We therefore quantified them by counting the number of 5-HT-positive fibre segments in each layer. Each 5-HT-positive fibre segment was counted independently, irrespective of fibre diameter or length. A segment was defined as a continuous 5-HT-positive fibre that is not interrupted by branches.

Adjacent Nissl-stained sections were used to determine the borders of laminae 4 and 7. The fibre segment count was then divided by the surface area of the respective lamina to calculate the average relative fibre density (number of 5-HT-positive fibre segments/mm^2) for each lamina.

Identification of M- and D-type 5-HT fibres

Large varicose M-fibres were differentiated from thin D-fibres based on two criteria: (i) M-fibres had a diameter of 1–5 μm, significantly larger than that of D-fibres (less than 1 μm); and (ii) large M-fibres had spherical varicosities giving them a beaded appearance whereas D-fibres had minute pleomorphic varicosities that were granular or fusiform in shape. These criteria were first introduced by Kosofsky & Molliver (1987) for 5-HT fibres in the rat cortex. The two fibre types are shown in Fig. 1G.

Quantification of initial 5-HT fibre loss 3 days after SCI

To determine the overall fibre loss after the T-lesion, all 5-HT-positive fibres were counted on four alternate L3 spinal cord cross-sections and on both sides of the spinal cord for each animal (untreated, intact animals: $n = 7$; untreated, spinal cord injured animals: $n = 3$).

Quantification of 5-HT-positive varicose appositions on lamina 9 motoneurons

The criteria for 5-HT-positive appositions on motoneurons were a clear swelling of the 5-HT-containing varicosities and no discernible gap between the presynaptic swelling and the adjacent motoneuron cell body at high magnification (Voss *et al*., 1990; Fyffe, 1991). The number of appositions was related to the cell body perimeter, which was measured with the AxioVision software, and expressed as number of 5-HT appositions per 1000 μm of cell surface. For quantification, six motoneurons were randomly chosen on each side of the cord on four consecutive sections for each animal (24 motoneurons per rat).

Immunofluorescence double staining

Immunofluorescence staining was performed using the protocol described by Loup *et al*. (1998). The sections were incubated overnight with primary antibodies against 5-HT (1 : 2000, rabbit, Immunostar) and GAP-43 (1 : 250, mouse, Sigma). They were then incubated with affinity-purified goat antimouse and antirabbit secondary antibodies (Jackson Immunoresearch, West Grove, PA, USA) coupled to FITC or TRITC, respectively. Control sections were run without primary antibody and gave no specific staining. The sections were analysed using a Zeiss LSM 410 microscope. Imaris software (Bitplane, Zurich, Switzerland) was used for image processing.

Camera lucida reconstructions

A representative 50-μm-thick spinal cord cross-section from level L3, stained for 5-HT, was projected onto one plane. Different types of serotonin-positive fibres were colour coded: thick M-fibres with large varicosities were depicted in black, thin D-fibres were depicted in red.

Statistical evaluation

Data were analysed using the Kruskal–Wallis test for analysis of variance by ranks, followed by Student's *t*-test.

Results

To give an overall impression of the extent and specificity of the serotonergic reinnervation of the spinal cord after SCI, we made camera lucida reconstructions of representative cross-sections of the lumbar spinal cord (Fig. 1). For quantitative analysis we separately counted the number of 5-HT-positive fibres in Rexed's lamina 4 in the dorsal horn and in the intermediate grey of lamina 7. In the ventral horn, we counted the number of serotonergic varicosities that were closely apposed to motoneurons in Rexed's lamina 9 (Fig. 2).

Camera lucida reconstructions

In the intact, uninjured adult rat spinal cord (Fig. 1A) the density of 5-HT fibres was high in the grey substance, especially around the central canal in lamina 10, in lamina 1 and 2, in the intermedio-medial column, in the intermedio-lateral column and around motoneurons (lamina 9). The 5-HT fibre density in laminae 3–7 was intermediate, and it was low in the surrounding white matter. There, 5-TH fibres were mainly found in the more medial parts of the lateral and ventral funiculi, corresponding to the respective descending raphe-spinal tracts. We distinguished thick M-fibres with large varicosities and thin D-fibres with minute varicosities according to a classification that was established for serotonergic fibres in the cerebral cortex of the rat by Kosofsky & Molliver (1987) (Fig. 1G). These fibres are depicted in different colours in Fig. 1A–E: M-fibres in black and D-fibres in red. Thin D-fibres were predominant in the dorsal horn whereas thick M-fibres were more abundant in the ventral horn (Fig. 1A) (Ridet *et al*., 1993).

The T-shaped spinal cord lesion at T8 completely destroyed the dorsal and dorsolateral parts of the spinal cord and partly damaged ventral and ventromedial areas. Accordingly, the serotonergic raphe-spinal tracts were massively but not completely destroyed using this lesion paradigm: 3 days after the lesion we detected only very few serotonergic fibres (4.3 ± 3.8%) caudal to the injury site at L3 than in intact animals (100%) (Fig. 1B). Nine weeks after the SCI, control

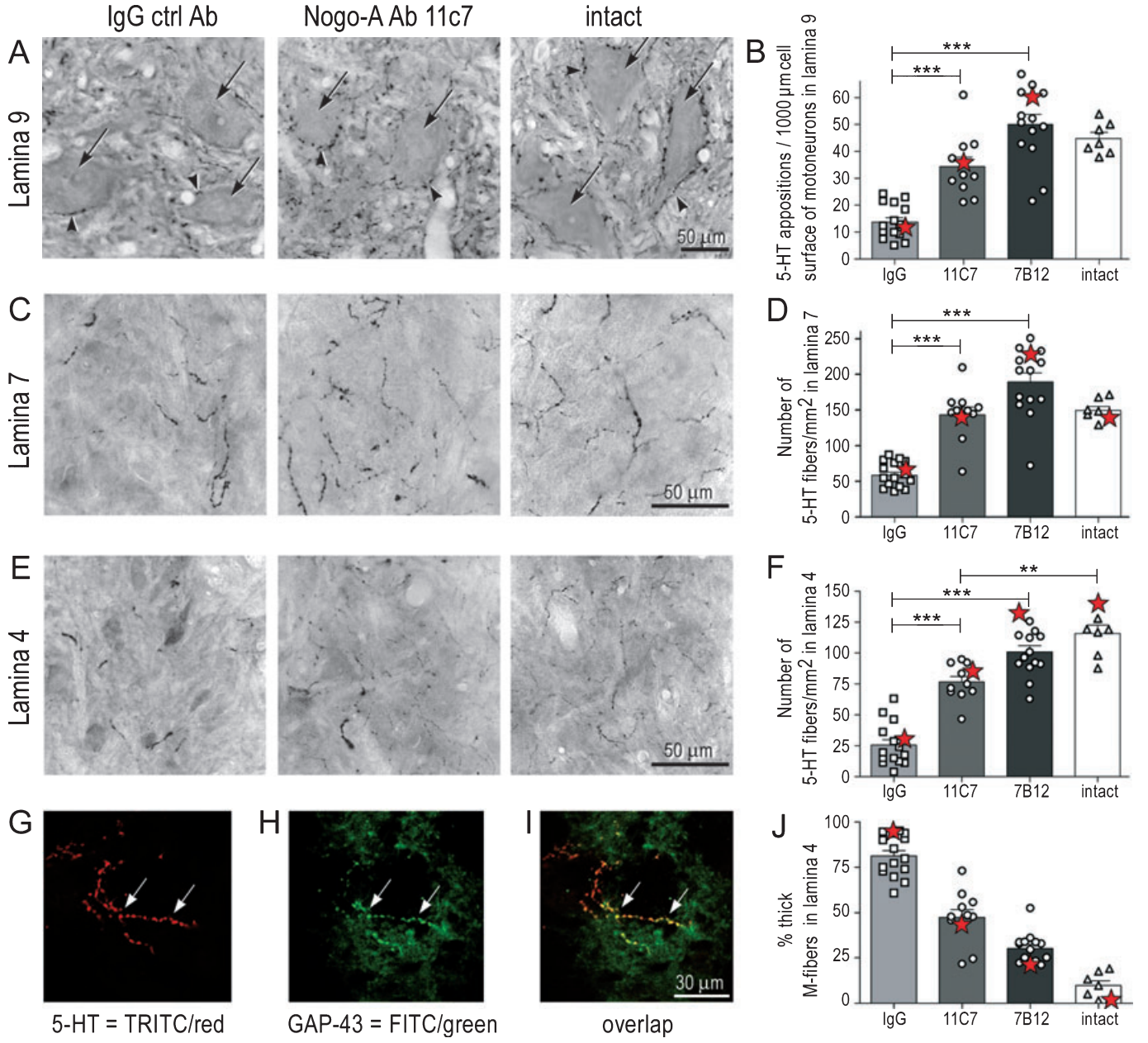

FIG. 2. Layer-specific recovery of the serotonergic innervation in intact rats and in Nogo-A antibody 11C7- or 7B12- or IgG control antibody-treated spinal cord injured rats 9 weeks after partial spinal cord injury. (A) 5-HT-immunoreactive bouton-like varicosities (arrowheads) in close apposition to lamina 9 motoneurons (arrows). (B) Number of 5-HT-positive, bouton-like appositions on motoneurons relative to motoneuron circumference. The number of these presumptive serotonergic synaptic terminals on motoneurons is significantly higher in Nogo-A antibody-treated rats than in control antibody-treated rats (anti-Nogo-A monoclonal antibody 7B12 -treated rats, n = 14; anti-Nogo-A monoclonal antibody 11C7-treated rats, n = 11; control antibody-treated rats, n = 15; 24 motoneurons were counted per animal; $P < 0.0001$ for both 7B12 treatment vs. control IgG and 11C7 treatment vs. control IgG). In 7B12-treated animals the recovery was complete and comparable with intact animals (n = 7). (C) 5-HT-positive fibres in Rexed's layer 7. (D) 5-HT-positive fibres per mm^2 in layer 7. The density of serotonergic fibres is much higher in the Nogo-A antibody-treated groups than in the control antibody-treated group and comparable with that in intact, untreated rats ($P > 0.0001$ for both 7B12 and 11C7 vs. control IgG treatment). (E) 5-HT-positive fibres in Rexed's layer 4. (F) The differences between the control IgG-treated group and the anti-Nogo-A antibody-treated groups were significant ($P < 0.0001$). The 7B12-treated animals reached 5-HT fibre densities that were similar to those of intact animals. For D and F adjacent Nissl-stained sections were used to determine the area of laminae 7 and 4, respectively. (G–I) Immunohistochemical staining for 5-HT in red (G) and GAP-43 in green (H) in an 11C7-treated animal; overlap in shown I. Most 5-HT fibres were GAP-43 positive in lesioned animals, regardless of treatment. In intact animals, GAP-43 was absent in 5-HT-positive fibres (not shown). (J) Percentage of thick M-type 5-HT fibres in lamina 4. In intact animals, fine D-type 5-HT fibres dominate in lamina 4 and only very few M-type 5-HT fibres are found. In control antibody-treated rats most 5-HT fibres that have re-grown into lamina 4 were thick M-fibres. In contrast, anti-Nogo-A antibody treatment led to a lower proportion of M fibres in the dorsal horn, approaching the situation seen in intact animals. **$P < 0.001$, ***$P < 0.0001$ determined by Kruskal–Wallis test for analysis of variance by ranks, followed by Student's t-test. Error bars indicate SEM, scale bars are 50 µm (A, C, E) or 30 µm (I). Rectangles (control IgG treated), dots (anti-Nogo-A antibody treated) and triangles (intact) represent values of single animals. Red stars in B, D, F and J correspond to the animals whose camera lucida reconstructions are shown in Fig. 1A, C, D and E, respectively.

IgG-treated animals showed a substantial but limited recovery of the serotonergic projections (Fig. 1D). In contrast, anti-Nogo-A antibody-treated animals showed an extensive recovery of the serotonergic projections to the lumbar spinal cord (Fig. 1C and E). The observations in surviving rats at 9 weeks were quantified for the spinal cord laminae 4, 7 and 9.

Quantification of 5-HT-positive varicosities on layer 9 motoneurons

The serotonergic innervation of motoneurons was analysed by counting the number of 5-HT-positive varicosities that were closely apposed to the cell body surface of motoneurons (Fig. 2A). These close appositions presumably correspond to the well-known axo-somatic serotonergic synapses on motoneurons (Alvarez *et al*., 1998). Figure 2B shows the density of 5-HT-positive varicosities relative to motoneuron cell body surface. The control antibody-treated rats ($n = 15$) showed an average of 13.7 ± 1.6 appositions per 1000 μm cell body surface compared with 34.4 ± 3.4 appositions in the anti-Nogo-A antibody 11C7-treated group ($n = 11$) and 50 ± 3.7 in the anti-Nogo-A antibody 7B12-treated group ($n = 14$). The differences between the control antibody-treated group and the groups that received either of the anti-Nogo-A antibodies were highly significant (unpaired *t*-test, $P < 0.0001$). There was no significant difference in the number of close serotonergic appositions on motoneurons of intact animals (44.8 ± 2.2, $n = 7$) and of spinal cord-injured 7B12-treated animals. Taking these data together, Nogo-A antibody treatment led to recovery of the serotonergic innervation of motoneurons to levels comparable with those in intact rats, whereas control antibody-treated animals reached only low levels of motor neuron reinnervation.

Intermediate grey: number of serotonergic fibres in a motor interneuron layer (lamina 7)

To assess the restoration of 5-HT fibres in an area that potentially contains many motor interneurons, including those of the central pattern generator, we counted the number of serotonergic fibres in lamina 7 (Fig. 2C and D). Intact animals had a 5-HT fibre density of 149.9 ± 5.6 fibres/mm^2 in lamina 7. Lesioned animals that received control IgG reached a density of 58.8 ± 4.6 fibres/mm^2, whereas Nogo-A antibody-treated animals had a fibre density that was comparable with that of intact animals (11C7, 143.6 ± 10.7; 7B12, 189.7 ± 12.8). The effect of the Nogo-A antibody treatment (11C7 and 7B12) on 5-HT fibre density in lamina 7, compared with control antibody treatment, was highly significant (unpaired *t*-test, $P < 0.0001$). By contrast, the fibre density in the control group remained low. Nogo-A antibody treatment thus led to a complete recovery of the 5-HT fibre density in lamina 7, reaching preinjury densities. Of note, the 5-HT fibre density in lamina 7 was higher (by 50%) than that of the sensory lamina 4.

Dorsal horn: number of serotonergic fibres in a sensory input layer (lamina 4)

In Rexed's lamina 4 of spinal cord injured rats, 5-HT fibre density remained low after treatment with the control antibody (25.6 ± 4.4 fibres/mm^2, Fig. 2E and F). 5-HT fibre density was much higher in Nogo-A antibody-treated animals, reaching 76.7 ± 4.4 fibres/mm^2 (unpaired *t*-test, $P < 0.0001$) in the 11C7-treated group and 100.9 ± 5.2 fibres/mm^2 (unpaired *t*-test, $P < 0.0001$) in the 7B12-treated animals. There was no significant difference between the lesioned, anti-Nogo-A antibody 7B12-treated and the unlesioned group (115.8 ± 6.8 fibres/mm^2, unpaired *t*-test, $P = 0.10$), suggesting complete restoration of 5-HT innervation in lamina 4. However, 11C7-treated animals showed a slightly lower 5-HT fibre density than the unlesioned animals (unpaired *t*-test, $P < 0.001$, compared with control IgG).

Normalization of the M/D 5-HT fibre ratio in lamina 4

In the dorsal horn of intact animals, including lamina 4, thin D-fibres dominate and thick M-fibres are scarce (Figs 1A and 2J). To determine whether the recovered serotonergic fibres would be more like M- or D-fibres, we determined the M/D fibre ratio in lamina 4. Nine weeks after spinal cord lesion, control IgG-treated animals had a significantly increased number of M-fibres in the dorsal horn compared with uninjured animals (81.4 ± 2.9% vs. 9.8 ± 2.8% M-fibres; unpaired *t*-test, $P < 0.0001$). By contrast, anti-Nogo-A antibody treatment led to a lower proportion of M-fibres in lamina 4 (47.5 ± 4.4% M-fibres after 11C7 treatment and 30.2 ± 2.2% M-fibres after 7B12 treatment), approaching the situation in the intact spinal cord.

Colocalization with the axon growth marker GAP-43

To investigate whether the increase of 5-HT fibres was due to newly grown fibres, we examined whether 5-HT-positive fibres in the lumbar spinal cord (L3) also expressed the growth-associated protein GAP-43. Immunofluorescence double staining revealed a consistent colocalization of 5-HT (Fig. 2G) and GAP-43 (Fig. 2H) in many fibres in the injured spinal cords (overlap in Fig. 2I), regardless of treatment. By contrast, GAP-43 was absent in 5-HT fibres of intact animals (data not shown) (Alonso *et al*., 1995).

Discussion

In the present study, we demonstrate that 2 weeks of intrathecal infusion of monoclonal anti-Nogo-A antibodies after partial thoracic SCI leads to a nearly complete and lamina-specific restoration of serotonergic raphe-spinal fibres in the grey matter of the lumbar spinal cord.

The origin of the serotonergic fibres in the CNS are the raphe nuclei in the brainstem (Dahlstrom & Fuxe, 1964). The descending spinal projections mainly originate from the medullary raphe pallidus, the raphe obscurus and the raphe magnus nuclei. The raphe obscurus and pallidus nuclei predominantly project to the ventral horn (Bowker *et al*., 1982; Rajaofetra *et al*., 1992), where they form conventional synapses on motoneurons and interneurons. They also project to the intermediate grey where they terminate on interneurons and on sympathetic preganglionic neurons in the intermediolateral cell column (Rajaofetra *et al*., 1992; Allen & Cechetto, 1994; Jacobs *et al*., 2002). Their axons are thick with large varicosities and are referred to as M-type 5-HT fibres (Kosofsky & Molliver, 1987). The serotonergic projections from the raphe magnus nucleus project mainly to Rexed's lamina 1 and 2 of the dorsal horn. Direct synaptic contacts on dorsal horn neurons are scarce and serotonin seems to be mostly released into the extracellular space at nonsynaptic sites (Ridet *et al*., 1993). These fibres are very fine and have minute varicosities. They are referred to as D-type 5-HT fibres (Kosofsky & Molliver, 1987). The distribution of serotonergic fibres in the spinal grey matter is tightly regulated and specific for each lamina (Steinbusch, 1981; Marlier *et al*., 1991). We found and average density of 150 fibres/mm^2 in lamina 7 and 115 fibres/mm^2 in lamina 4.

Descending raphe-spinal projections play a key regulatory role for the spinal motor circuits and can modulate their function in many ways: 5-HT facilitates the expression of plateau potentials in motoneurons (Hounsgaard & Kiehn, 1985, 1989; Hounsgaard *et al*., 1988; Hultborn & Kiehn, 1992); it induces fictive locomotion in the *in vitro* neonatal rat spinal cord preparation (Cazalets *et al*., 1992) and facilitates spinal reflexes; and it modulates sensory transmission, which in turn can activate the motor circuits. Overall, 5-HT activates and facilitates motor functions (Schmidt & Jordan, 2000; Jacobs *et al*., 2002). After SCI, when all or most serotonergic input below the site of injury is lost, application of serotonin or serotonergic agonists or transplantation of serotonergic cells can improve motor functions (Barbeau & Rossignol, 1991; Feraboli-Lohnherr *et al*., 1997, 1999; Fong *et al*., 2005), suggesting that appropriate serotonergic reinnervation of the de-afferentiated spinal cord is crucial for the recovery of motor functions.

We investigated the serotonergic projections to the lumbar level L3, which in intact animals is extensively innervated by 5-HT fibres and contains neurons of the spinal motor circuits for the hindlimbs (Cazalets *et al*., 1995; Cowley & Schmidt, 1997; Kiehn, 2006).

We found that the anti-Nogo-A antibody-mediated recovery of the serotonergic projections below the lesion site was very precise in two aspects. First, the reinnervation was highly lamina specific: in Rexed's laminae 4 and 7, the lamina-specific preinjury levels of fibre densities (115 fibres/mm^2 in lamina 4, and 150 fibres/mm^2 in lamina 7) were correctly re-established after anti-Nogo-A antibody treatment. Control antibody-treated animals showed a lower degree of 5-HT fibre recovery, far below preinjury levels. Second, the distribution of thick and thin (M- and D-type) 5-HT fibres in Nogo-A antibody-treated rats was more similar to the preinjury distribution than in control IgG-treated animals: In the intact spinal cord, lamina 4 receives serotonergic innervation mainly by D-fibres and few M-fibres. This ratio was disturbed after SCI in the control animals, in which more M-fibres recovered instead of D-fibres. In the anti-Nogo-A antibody-treated rats, however, the ratio of M- to D-type 5-HT fibres in lamina 4 was similar to the physiological ratio found in intact rats (Fig. 2J).

Bouton-like varicosities of 5-HT-immunoreactive fibres were present throughout laminae 7 and 9 and suggest that the newly grown fibres make synaptic contacts onto interneurons and motoneurons and integrate into the local spinal circuitry. In fact, in the anti-Nogo-A antibody-treated rats, the density of axo-somatic 5-HT boutons on large motoneuron somata also returned to the level of that of intact animals (Fig. 2B).

It is possible that considerable remodelling and growth of 5-HT fibres continued after the 2 weeks of treatment, i.e. in the absence of Nogo-A function-blocking antibodies. However, one mechanism of action of Nogo-A antibody treatment is down-regulation of Nogo-A from the cell surface by internalization of the Nogo-A/antibody complex (Weinmann *et al*., 2006). Whether and how fast surface Nogo-A returns to pretreatment levels after the cessation of Nogo-A antibody application is not known. For that reason, the treatment effect may outlast the antibody treatment period.

Two hypotheses may explain the precise, lamina-specific serotonergic reinnervation of the grey matter. Local guidance cues may be present or re-expressed in the injured and denervated adult spinal cord to direct re-growing or sprouting 5-HT fibres accurately to the correct targets in the respective laminae. The identification of such molecular signals, which could mediate the correct rewiring of plastic neurons, remains a major challenge. Ongoing proteomic and genomic studies suggest that growth and guidance factors are altered after spinal cord injury and Nogo-A antibody treatment and may guide correct rewiring of the injured CNS (Bareyre *et al*., 2002; and our unpublished results). Alternatively, sprouting axons may connect randomly to nearby neurons. In an activity-controlled second step, wrong and meaningless projections could be pruned with only meaningful connections being maintained (Bareyre *et al*., 2002; Maier & Schwab, 2006). In both cases, inactivating Nogo-A by function-blocking antibodies decreases the myelin-derived inhibition of growing axons and thereby reveals and reinforces mechanisms that allow the injured CNS to reorganize meaningfully.

In injured animals most serotonergic fibres were GAP-43 positive, regardless of treatment, corroborating that they had newly grown after the spinal cord lesion (Fig. 2G–I). In intact animals 5-HT did not colocalize with GAP-43. We have prveiously shown that intact animals treated with anti-Nogo-A antibodies transiently expressed growth factors (BDNF, VEGF) and growth-related proteins (actin, myosin, GAP-43) and that neurites showed a transient growth response in the spinal cord (Bareyre *et al*., 2002) and the cerebellum (Buffo *et al*., 2000). Correspondingly, treating intact animals with anti-Nogo-A antibodies might lead to a transient sprouting response of serotonergic fibres.

In conclusion, our results show that acute intrathecal anti-Nogo-A antibody application for 2 weeks leads to a lamina-specific and almost complete restoration of lumbar serotonergic projections to laminae 4, 7 and 9 in spinal cord injured adult rats.

Acknowledgements

A.M. was supported by an ENS fellowship from the European Neurological Society. R.R.G. received a fellowship from the Foundation Louis-Jeantet de Médecine. The study was also supported by grants from the Swiss National Science Foundation (31-63633.00), the NCCR Neural Plasticity and Repair of the Swiss National Science Foundation, the Spinal Cord Consortium of the Christopher Reeve Foundation (Springfield, NJ, USA, Project MSC 2005(1)), the Transregio-Sonderforschungsbereich Konstanz-Zurich (Project 34150609) and the Neuron Network of Excellence, European Consortium for Research in Neurodegenerative Diseases (Sixth Framework Programme). We thank Novartis for supply of highly purified antibodies. Special thanks to J. Scholl and R. Schneider for help with animal care and tissue handling, and to E. Hochreutener and R. Schoeb for help with preparation of the figures.

Abbreviations

5-HT, 5-hydroxytryptamine; CNS, central nervous system; CST, corticospinal tract; SCI, spinal cord injury.

References

Allen, G.V. & Cechetto, D.F. (1994) Serotoninergic and nonserotoninergic neurons in the medullary raphe system have axon collateral projections to autonomic and somatic cell groups in the medulla and spinal cord. *J. Comp. Neurol.*, **350**, 357–366.

Alonso, G., Ridet, J.L., Oestreicher, A.B., Gispen, W.H. & Privat, A. (1995) B-50 (GAP-43) immunoreactivity is rarely detected within intact catecholaminergic and serotonergic axons innervating the brain and spinal cord of the adult rat, but is associated with these axons following lesion. *Exp. Neurol.*, **134**, 35–48.

Alvarez, F.J., Pearson, J.C., Harrington, D., Dewey, D., Torbeck, L. & Fyffe, R.E. (1998) Distribution of 5-hydroxytryptamine-immunoreactive boutons on alpha-motoneurons in the lumbar spinal cord of adult cats. *J. Comp. Neurol.*, **393**, 69–83.

Armstrong, D.M. (1986) Supraspinal contributions to the initiation and control of locomotion in the cat. *Prog. Neurobiol.*, **26**, 273–361.

Barbeau, H. & Rossignol, S. (1991) Initiation and modulation of the locomotor pattern in the adult chronic spinal cat by noradrenergic, serotonergic and dopaminergic drugs. *Brain Res.*, **546**, 250–260.

Bareyre, F.M., Haudenschild, B. & Schwab, M.E. (2002) Long-lasting sprouting and gene expression changes induced by the monoclonal antibody IN-1 in the adult spinal cord. *J. Neurosci.*, **22**, 7097–7110.

Bowker, R.M., Westlund, K.N., Sullivan, M.C. & Coulter, J.D. (1982) Organization of descending serotonergic projections to the spinal cord. *Prog. Brain Res.*, **57**, 239–265.

Bregman, B.S., Kunkel-Bagden, E., Schnell, L., Dai, H.N., Gao, D. & Schwab, M.E. (1995) Recovery from spinal cord injury mediated by antibodies to neurite growth inhibitors. *Nature*, **378**, 498–501.

Buffo, A., Zagrebelsky, M., Huber, A.B., Skerra, A., Schwab, M.E., Strata, P. & Rossi, F. (2000) Application of neutralizing antibodies against NI-35/250 myelin-associated neurite growth inhibitory proteins to the adult rat cerebellum induces sprouting of uninjured purkinje cell axons. *J. Neurosci.*, **20**, 2275–2286.

Cazalets, J.R., Borde, M. & Clarac, F. (1995) Localization and organization of the central pattern generator for hindlimb locomotion in newborn rat. *J. Neurosci.*, **15**, 4943–4951.

Cazalets, J.R., Sqalli-Houssaini, Y. & Clarac, F. (1992) Activation of the central pattern generators for locomotion by serotonin and excitatory amino acids in neonatal rat. *J. Physiol.*, **455**, 187–204.

Clancy, B. & Cauller, L.J. (1998) Reduction of background autofluorescence in brain sections following immersion in sodium borohydride. *J. Neurosci. Methods*, **83**, 97–102.

Cowley, K.C. & Schmidt, B.J. (1997) Regional distribution of the locomotor pattern-generating network in the neonatal rat spinal cord. *J. Neurophysiol.*, **77**, 247–259.

Dahlstrom, A. & Fuxe, K. (1964) Localization of monoamines in the lower brain stem. *Experientia*, **20**, 398–399.

Dodd, D.A., Niederoest, B., Bloechlinger, S., Dupuis, L., Loeffler, J.P. & Schwab, M.E. (2005) Nogo-A-B, and -C are found on the cell surface and interact together in many different cell types. *J. Biol. Chem.*, **280**, 12494–12502.

Feraboli-Lohnherr, D., Barthe, J.Y. & Orsal, D. (1999) Serotonin-induced activation of the network for locomotion in adult spinal rats. *J. Neurosci. Res.*, **55**, 87–98.

Feraboli-Lohnherr, D., Orsal, D., Yakovleff, A., Gimenez y Ribotta, M. & Privat, A. (1997) Recovery of locomotor activity in the adult chronic spinal rat after sublesional transplantation of embryonic nervous cells: specific role of serotonergic neurons. *Exp. Brain Res.*, **113**, 443–454.

Fong, A.J., Cai, L.L., Otoshi, C.K., Reinkensmeyer, D.J., Burdick, J.W., Roy, R.R. & Edgerton, V.R. (2005) Spinal cord-transected mice learn to step in response to quipazine treatment and robotic training. *J. Neurosci.*, **25**, 11738–11747.

Fouad, K., Klusman, I. & Schwab, M.E. (2004) Regenerating corticospinal fibers in the Marmoset (*Callitrix jacchus*) after spinal cord lesion and treatment with the anti-Nogo-A antibody IN-1. *Eur. J. Neurosci.*, **20**, 2479–2482.

Freund, P., Schmidlin, E., Wannier, T., Bloch, J., Mir, A., Schwab, M.E. & Rouiller, E.M. (2006) Nogo-A-specific antibody treatment enhances sprouting and functional recovery after cervical lesion in adult primates. *Nat. Med.*, **12**, 790–792.

Fyffe, R.E. (1991) Spatial distribution of recurrent inhibitory synapes on spinal motoneurons in the cat. *J. Neurophysiol.*, **65**, 1134–1149.

GrandPre, T., Li, S. & Strittmatter, S.M. (2002) Nogo-66 receptor antagonist peptide promotes axonal regeneration. *Nature*, **417**, 547–551.

Herzog, A. & Brosamle, C. (1997) 'Semifree-floating' treatment: a simple and fast method to process consecutive sections for immunohistochemistry and neuronal tracing. *J. Neurosci. Methods*, **72**, 57–63.

Hounsgaard, J., Hultborn, H., Jespersen, B. & Kiehn, O. (1988) Bistability of alpha-motoneurones in the decerebrate cat and in the acute spinal cat after intravenous 5-hydroxytryptophan. *J. Physiol.*, **405**, 345–367.

Hounsgaard, J. & Kiehn, O. (1985) Ca^{++} dependent bistability induced by serotonin in spinal motoneurons. *Exp. Brain Res.*, **57**, 422–425.

Hounsgaard, J. & Kiehn, O. (1989) Serotonin-induced bistability of turtle motoneurones caused by a nifedipine-sensitive calcium plateau potential. *J. Physiol.*, **414**, 265–282.

Hultborn, H. & Kiehn, O. (1992) Neuromodulation of vertebrate motor neuron membrane properties. *Curr. Opin. Neurobiol.*, **2**, 770–775.

Jacobs, B.L., Martin-Cora, F.J. & Fornal, C.A. (2002) Activity of medullary serotonergic neurons in freely moving animals. *Brain Res. Brain Res. Rev.*, **40**, 45–52.

Kiehn, O. (2006) Locomotor circuits in the mammalian spinal cord. *Annu. Rev. Neurosci.*, **29**, 279–306.

Kosofsky, B.E. & Molliver, M.E. (1987) The serotoninergic innervation of cerebral cortex: different classes of axon terminals arise from dorsal and median raphe nuclei. *Synapse*, **1**, 153–168.

Li, S. & Strittmatter, S.M. (2003) Delayed systemic Nogo-66 receptor antagonist promotes recovery from spinal cord injury. *J. Neurosci.*, **23**, 4219–4227.

Liebscher, T., Schnell, L., Schnell, D., Scholl, J., Schneider, R., Gullo, M., Fouad, K., Mir, A., Rausch, M., Kindler, D., Hamers, F.P. & Schwab, M.E. (2005) Nogo-A antibody improves regeneration and locomotion of spinal cord-injured rats. *Ann. Neurol.*, **58**, 706–719.

Loup, F., Weinmann, O., Yonekawa, Y., Aguzzi, A., Wieser, H.G. & Fritschy, J.M. (1998) A highly sensitive immunofluorescence procedure for analyzing the subcellular distribution of GABAA receptor subunits in the human brain. *J. Histochem. Cytochem.*, **46**, 1129–1139.

Maier, I.C. & Schwab, M.E. (2006) Sprouting, regeneration and circuit formation in the injured spinal cord: factors and activity. *Phil. Trans. R. Soc. Lond. B Biol. Sci.*, **361**, 1611–1634.

Marlier, L., Sandillon, F., Poulat, P., Rajaofetra, N., Geffard, M. & Privat, A. (1991) Serotonergic innervation of the dorsal horn of rat spinal cord: light and electron microscopic immunocytochemical study. *J. Neurocytol.*, **20**, 310–322.

Metz, G.A., Dietz, V., Schwab, M.E. & van de Meent, H. (1998) The effects of unilateral pyramidal tract section on hindlimb motor performance in the rat. *Behav. Brain Res.*, **96**, 37–46.

Muir, G.D. & Whishaw, I.Q. (1999) Complete locomotor recovery following corticospinal tract lesions: measurement of ground reaction forces during overground locomotion in rats. *Behav. Brain Res.*, **103**, 45–53.

Oertle, T., van der Haar, M.E., Bandtlow, C.E., Robeva, A., Burfeind, P., Buss, A., Huber, A.B., Simonen, M., Schnell, L., Brosamle, C., Kaupmann, K., Vallon, R. & Schwab, M.E. (2003) Nogo-A inhibits neurite outgrowth and cell spreading with three discrete regions. *J. Neurosci.*, **23**, 5393–5406.

Raineteau, O. & Schwab, M.E. (2001) Plasticity of motor systems after incomplete spinal cord injury. *Nat. Rev. Neurosci.*, **2**, 263–273.

Rajaofetra, N., Konig, N., Poulat, P., Marlier, L., Sandillon, F., Drian, M.J., Geffard, M. & Privat, A. (1992) Fate of B1–B2 and B3 rhombencephalic cells transplanted into the transected spinal cord of adult rats: light and electron microscopic studies. *Exp. Neurol.*, **117**, 59–70.

Rexed, B. (1954) A cytoarchitectonic atlas of the spinal cord in the cat. *J. Comp. Neurol.*, **100**, 297–379.

Ridet, J.L., Rajaofetra, N., Teilhac, J.R., Geffard, M. & Privat, A. (1993) Evidence for nonsynaptic serotonergic and noradrenergic innervation of the rat dorsal horn and possible involvement of neuron–glia interactions. *Neuroscience*, **52**, 143–157.

Schmidt, B.J. & Jordan, L.M. (2000) The role of serotonin in reflex modulation and locomotor rhythm production in the mammalian spinal cord. *Brain Res. Bull.*, **53**, 689–710.

Schnell, L. & Schwab, M.E. (1990) Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. *Nature*, **343**, 269–272.

Schwab, M.E. (2004) Nogo and axon regeneration. *Curr. Opin. Neurobiol.*, **14**, 118–124.

Steinbusch, H.W. (1981) Distribution of serotonin-immunoreactivity in the central nervous system of the rat-cell bodies and terminals. *Neuroscience*, **6**, 557–618.

Thallmair, M., Metz, G.A., Z'Graggen, W.J., Raineteau, O., Kartje, G.L. & Schwab, M.E. (1998) Neurite growth inhibitors restrict plasticity and functional recovery following corticospinal tract lesions. *Nat. Neurosci.*, **1**, 124–131.

Voss, M.D., De Castro, D., Lipski, J., Pilowsky, P.M. & Jiang, C. (1990) Serotonin immunoreactive boutons form close appositions with respiratory neurons of the dorsal respiratory group in the cat. *J. Comp. Neurol.*, **295**, 208–218.

Weinmann, O., Schnell, L., Ghosh, A., Montani, L., Wiessner, C., Wannier, T., Rouiller, E., Mir, A. & Schwab, M.E. (2006) Intrathecally infused antibodies against Nogo-A penetrate the CNS and downregulate the endogenous neurite growth inhibitor Nogo-A. *Mol. Cell Neurosci.*, **32**, 161–173.

Wiessner, C., Bareyre, F.M., Allegrini, P.R., Mir, A.K., Frentzel, S., Zurini, M., Schnell, L., Oertle, T. & Schwab, M.E. (2003) Anti-Nogo-A antibody infusion 24 hours after experimental stroke improved behavioral outcome and corticospinal plasticity in normotensive and spontaneously hypertensive rats. *J. Cereb. Blood Flow Metab.*, **23**, 154–165.