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Nogo Receptor mRNA Expression in Intact and Regenerating CNS Neurons

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The expression of mRNA for Nogo-66 receptor (NgR) in unoperated adult rats and mice, and rats with nerve grafts placed in the thalamus and cerebellum to stimulate axonal regeneration, was investigated by *in situ* hybridization. NgR was strongly expressed in neurons of the neocortex, hippocampal formation, and amygdaloid nuclei and dorsal thalamus and moderately expressed in the red nucleus and vestibular nuclei. NgR mRNA was expressed in cerebellar deep nuclei and more strongly by granule cells than by Purkinje cells. Large regions of the forebrain, including the striatum, thalamic reticular nucleus, hypothalamus, and basal forebrain showed little or no NgR expression. NgR was weakly expressed in spinal neurons and some primary sensory neurons. Nerve implantation into the brain did not affect NgR expression. Some regeneration-competent neurons expressed NgR but others did not. Thus NgR expression was not correlated with the ability of neurons to regenerate axons into nerve grafts although Nogo-66 was strongly upregulated by some cells in the distal stumps of injured sciatic nerves. Nogo-66 transcripts were strongly expressed by many classes of CNS neurons and less strongly in white matter.

INTRODUCTION

Injuries to peripheral nerves of adult mammals are followed by vigorous axonal regeneration which often leads to functional recovery. In marked contrast, axons in the central nervous system (CNS) normally show at most only abortive axonal sprouting following injury, which therefore results in permanent functional defi-

cits. Some types of CNS neuron can, however, regenerate axons into segments of peripheral nerve grafted into the brain or spinal cord (Aguayo, 1985), but whereas all types of neuron with axons in peripheral nerve trunks can apparently regenerate, CNS neurons show large variations in their propensities for regeneration into nerve grafts (Benfey *et al.,* 1985; Morrow *et al.,* 1993; Woolhead *et al.,* 1998; Anderson *et al.,* 1998; Anderson and Lieberman, 1999).

Variations in the regenerative ability of neurons are found in all parts of the brain where grafts have been implanted (Anderson and Lieberman, 1999). When grafts are implanted into the thalamus, many types of CNS neuron are axotomized yet more than 90% of the CNS axons which regenerate into and through the grafts originate from one nucleus, the thalamic reticular nucleus (TRN), the neurons of which project to dorsal thalamic nuclei. Few thalamocortical projection neurons (in the dorsal thalamus) regenerate their axons (Benfey *et al.,* 1985; Morrow *et al.,* 1993; Vaudano *et al.,* 1995). When nerve segments are implanted into the neostriatum, some of the small population of striatal cholinergic interneurons regenerate their axons into the grafts but the striatal projection neurons do not do so; neurons in the substantia nigra pars compacta give rise to most of the axons which successfully regenerate into grafts in this region (Woolhead *et al.,* 1998). Projection neurons in the cerebral cortex rarely regenerate into nerve grafts in the thalamus, neostriatum, or spinal cord (Anderson and Lieberman, 1999). Perhaps the most clear-cut model for studying differential axonal regeneration is provided by nerve grafts implanted into the cerebellum. Such grafts are invaded by axons from the deep cerebellar nuclei and precerebellar nuclei in the brain stem but never by the axons of Purkinje cells

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or other neurons within the cerebellar cortex (Dooley and Aguayo, 1982; Vaudano *et al.,* 1993, 1998; Chaisuksunt *et al.,* 2000). It is not clear why CNS neurons show such variations in regenerative capacity (Anderson and Lieberman, 1999) but differential sensitivity to growth-inhibitory molecules is one possible explanation.

The suggestion that CNS myelin might have inhibitory effects on axonal regeneration was made by Berry (1982) and the possibility systematically investigated by Schwab and Caroni (Caroni and Schwab, 1988a,b; Caroni *et al.,* 1988; Schwab and Caroni, 1988), who found two strongly inhibitory myelin proteins with relative molecular masses of 35,000 and 250,000. They produced a monoclonal antibody named IN-1, which recognized these proteins and blocked the ability of oligodendrocytes and myelin to inhibit neurite growth in culture. When introduced into the CSF of adult rats after spinal cord injury, IN-1 was reported to induce a subpopulation of corticospinal neurons to regenerate their axons around the injury site (Schnell and Schwab, 1990) and the rats showed evidence of functional recovery (Z'Graggen *et al.,* 1998). IN-1 treatment also caused the upregulation of some growth-associated genes by Purkinje cells (Zagrebelsky *et al.,* 1998).

The molecule recognized by IN-1, named Nogo, is a member of the reticulon family of membrane-associated molecules (Chen *et al.,* 2000; GrandPre *et al.,* 2000; Prinjha *et al.,* 2000). Three different transcripts (A, B, and C) were originally described as being formed from the gene, coding for three proteins: Nogo-A (the largest), Nogo-B, and Nogo-C (the smallest). Nogo-A probably corresponds to the 250-kDa protein recognized by IN-1. Nogo-A is found in CNS myelin and is highly expressed by oligodendrocytes but not by Schwann cells. A 66-residue putative extracellular domain sequence (Nogo-66), common to all three forms, inhibits axonal extension and induces the collapse of growth cones. It is now clear, however, that several other transcripts containing the Nogo-66 sequence are formed from the same gene (see Table 1 and Discussion). Recently, a leucine-rich, GPI-linked cell surface protein (NgR) of 473 amino acids has been identified as a putative Nogo-66 receptor. This molecule binds Nogo-66 with high affinity and its expression has been shown to be sufficient to confer sensitivity to Nogo-66 on otherwise insensitive cells (Fournier *et al.,* 2001).

If Nogo-66 is a major factor/molecule responsible for the failure of axonal regeneration in the CNS, its receptor should be expressed by all classes of neuron which do not regenerate after brain and spinal cord injury. Furthermore, since two classes of neuron, Purkinje cells and corticospinal neurons, which are reluctant to regenerate axons into peripheral nerve grafts, are reported to express NgR strongly (Fournier *et al.,* 2001), it is of interest to see if neuronal expression of NgR is generally correlated a low propensity to regenerate axons into grafts or whether the expression of NgR changes after graft implantation.

In this study we have examined the expression of NgR in the brain and spinal cord of adult rats and mice and in animals with peripheral nerve implants in the thalamus and cerebellum using two probes which recognize the 3′ and 5′ ends of the molecule. In addition we have compared NgR expression with that of its ligand, Nogo-66. The results do not suggest that Nogo-66 and NgR are alone likely to explain the absence of regeneration in the CNS.

RESULTS

NgR Expression in the Intact CNS

No signal was detected with the sense probes (Fig. 1). Both NgR antisense probes produced similar patterns of NgR mRNA distribution (Figs. 2–5). No differences were detected in the patterns of NgR signal in mouse and rat brains. NgR was expressed in gray matter but not in white matter. NgR was very strongly expressed in neocortical neurons in the forebrain of adult rats and mice (Figs. 2, 3, and 8), including large cells in the motor areas. The size and location of the cells expressing NgR in the neocortex suggest that they included pyramidal projection neurons. There was also strong NgR expression in the hippocampus, dentate gyrus (Figs. 4 and 5), and piriform cortex. Neurons in the amygdaloid complex showed strong expression of NgR mRNA (Fig. 2).

However, in other regions of the brain a striking pattern of differential expression of NgR was found (Figs. 4–8). In the diencephalon, the medial habenular nuclei showed very strong expression of NgR mRNA (Figs. 2, 4, and 5) and neurons in many nuclei within the dorsal thalamus showed strong NgR mRNA expression (Figs. 2, 4, and 5). However, neurons in the ventral lateral geniculate nucleus showed no signal for NgR (not shown) and there was no apparent NgR expression in the TRN (Fig. 5). Expression of NgR was also very low in the hypothalamus (Fig. 2).

In sections through the midbrain (Fig. 6), moderate NgR expression was found in neurons in the red nucleus and weaker expression in the substantia nigra pars compacta. Neurons in the septal nuclei and basal forebrain exhibited only very weak or background levels of signal for NgR mRNA (Fig. 7). The differential expression of NgR was particularly marked in the rostral forebrain: there was little or no NgR mRNA detect-

able in the neostriatum (Fig. 8) although neurons in the adjacent cerebral cortex displayed strong hybridization signals.

Neurons in the granule cell layer of the cerebellar cortex strongly expressed NgR (Figs. 9 and 10). It was often difficult to identify Purkinje cells in the hybridized sections, since the intensity of NgR signal they exhibited appeared to be variable and was in most cases considerably weaker than the signal in the granule cell layer. When sections passed obliquely through the cerebellar cortex it was possible to identify NgR-positive Purkinje cells (arrows in Fig. 10). Neurons in the deep cerebellar nuclei showed moderate levels of NgR signal (asterisks in Fig. 9), as did neurons in various brain stem nuclei, including nuclei of the reticular formation, the pontine nuclei, nucleus prepositus hypoglossi, and the vestibular nuclei (not shown).

In the cervical and lumbar spinal cord, NgR expression was generally low but there was distinct signal from motor neurons (Fig. 11). Some DRG neurons showed NgR mRNA signal (Fig. 12), but at much lower levels than neurons in the cerebral cortex or cerebellar cortex. Neurons in the superior cervical ganglion also showed variable levels of NgR expression (not shown) similar to those found in DRG neurons. Thymus was NgR negative but there was moderate to strong NgR expression in heart muscle.

NgR Expression Following Axotomy and/or Nerve Graft Implantation

Following the implantation of a tibial nerve graft into the thalamus, there was no apparent expression of NgR mRNA in neurons within the TRN (Fig. 13), which are known to regenerate axons in such circumstances, i.e., there was no expression in the TRN on the side with the graft or on the contralateral side. There was also no consistent change in NgR expression by the dorsal thalamic neurons following graft implantation although in some sections from one animal, but not in other animals, a few neurons in the dorsal thalamus around the graft appeared to have upregulated NgR mRNA to a moderate extent. In none of the rats with a tibial nerve graft in the cerebellum was there any apparent change in NgR expression, i.e., neurons in the deep cerebellar nuclei close to the graft tip and neurons in the cerebellar cortex retained NgR expression, although the former are known to regenerate their axons into grafts but the latter fail to do so.

Expression of Nogo Encoding mRNA

There were no obvious differences in the distribution of signal with the radiolabeled probe to Nogo-66 and the two Dig-labeled probes (for Nogo-66 and the conserved 3′ region which includes Nogo-66, see Table 1) and the transcripts detected will subsequently be termed Nogo. No signal was detected using the sense probes (Fig. 14). The most striking observation was that Nogo mRNA was more strongly expressed in many, but not all, classes of neurons than in the white matter (Figs. 15–20). Strong Nogo mRNA expression was found in the neurons of the neocortex and hippocampal formation and many thalamic nuclei (Figs. 15, 17, and 18), including the TRN (arrows in Fig. 17). In the neostriatum, Nogo mRNA was widely expressed but most strongly by scattered large neurons whose size and distribution resembled that of the cholinergic interneurons (Figs. 15 and 16). Nogo mRNA was present in neurons in the septal nuclei (not shown) and vestibular nuclei (Fig. 19). In the cerebellum, Nogo mRNA was strongly expressed by Purkinje cells (Figs. 19 and 20) and neurons of the deep nuclei (Fig. 19) but weakly, if at all, by granule cells. In the spinal cord Nogo was strongly expressed by many neurons including motor neurons (Figs. 21 and 22). Strong signals for Nogo were detected in dorsal root ganglia (Fig. 23). Nogo was also expressed in the visual system (Figs. 24 and 25). In the retina, Nogo was expressed by cells in the outer nuclear layer, the inner nuclear layer, and the ganglion cell layer (Fig. 25). Nogo mRNA was expressed at low levels by scattered cells in the intact optic nerve (Fig. 24). Nogo expression was not detected in undamaged adult rat sciatic nerve by *in situ* hybridization (Figs. 29 and 31) but it was obviously upregulated in the distal stump of transected sciatic nerve, 3 days following injury (Figs. 26–28 and 30). Detailed examination revealed that, in addition to a generalized low-level upregulation (Fig. 32), there were scattered cells in the distal stump which showed a strong expression of Nogo mRNA (Figs. 26–28 and 30). RT-PCR was used to confirm that Nogo-66 transcripts were present in RNA extracted from injured sciatic nerve 3 days after transection (Fig. 33). In two rats with a nerve graft in the thalamus, Nogo-66 expression was not apparently altered in the neurons of the TRN, which regenerate axons into such grafts, but was upregulated by cells close to the graft–brain interface (Fig. 34). As is normally the case, the structure of grafts in the thalamus was not well preserved in fresh-frozen cryostat sections and it was difficult to be certain if Nogo was also upregulated in the nerve under these conditions.
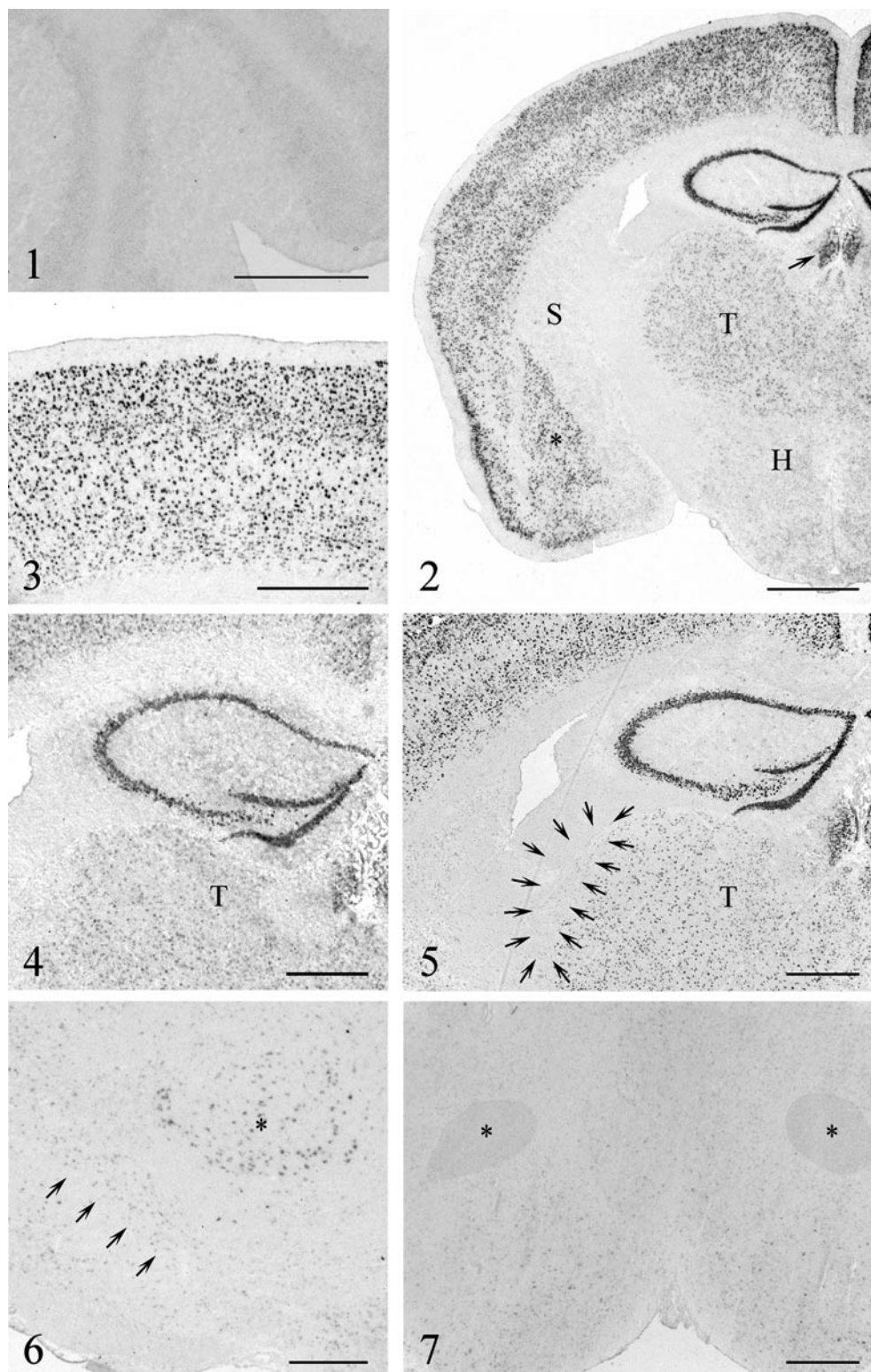
FIG. 1. Cerebellum of adult mouse reacted with sense NgR 3′ probe. No signal can be seen; compare with Fig. 9. Bar, 250 μm.
FIG. 2. Coronal section through the forebrain of an adult mouse, hybridized for NgR (3′ probe). Strong NgR signal can be seen in the neocortex and hippocampal formation, amygdaloid nuclei (*), medial habenular nucleus (arrow), and dorsal thalamus (T). The hypothalamus (H) and the small region of neostriatum visible (S) show little or no NgR signal. NgR signal is absent from white matter. Bar, 1 mm.

DISCUSSION

NgR (receptor for Nogo-66) was expressed by neurons in the neocortex and the hippocampus of adult rodents, but not in white matter, confirming previous observations (Fournier *et al.,* 2001). However, we have found more extensive expression of NgR in the cerebellar cortex than previously reported and, in addition, we have shown that NgR is differentially expressed in various regions of the forebrain. In particular, neostriatal neurons and neurons in the ventral lateral geniculate nucleus and TRN appear not to express NgR mRNA. Motor neurons express NgR weakly and most other neurons in the spinal cord express it not at all. Primary sensory neurons vary in NgR expression, although none express it strongly, and others not at all. If NgR is the principal neuronal receptor by which Nogo inhibits axonal regeneration in the CNS, the marked differences in NgR expression by different classes of neuron would appear to limit the range of neurons likely to be susceptible to the growth-inhibitory effects of Nogo-66. In particular, the binding of Nogo-66 to neuronal NgR is not likely to be a mechanism by which ascending axons in the spinal cord are inhibited from regenerating. In addition, we have shown that the Nogo-66 coding sequence mRNA is most strongly expressed by neurons rather than by CNS glia and is also strongly expressed by some cells in injured peripheral nerves.

The differences between our observations on the localization of NgR expression in the cerebellar cortex and those previously published are difficult to explain; we found that NgR expression in Purkinje cells was variable and generally much weaker than that in the granule cell layer, whereas Fournier *et al.* (2001) showed a line of Purkinje cells expressing NgR without apparent expression in granule cells. Fournier *et al.* (2001) used a 1-kb probe to the 5′ end, whereas we have used two separate probes to sequences at the 5′ and 3′ regions of the molecule, both of which produce similar results. Curiously, we found that in the cerebellar cortex, strong expression of mRNA for Nogo-66 was restricted to Purkinje cells, giving an appearance similar to that previously published for the distribution of NgR.

Implications of the Distribution of NgR and Nogo-66 for the Regulation of Axonal Sprouting and Regeneration in the Brain and Spinal Cord

It has been widely assumed that Nogo is a protein predominantly expressed by oligodendrocytes, capable of causing the collapse of growth cones with which it comes into contact (Schwab, 1990). Growth cone collapse is assumed to be the result of the binding of Nogo to its receptor on the surface of axons (Fournier *et al.,* 2001). NgR expression by cerebral cortical neurons and Purkinje cells may explain why they are so poor at regenerating axons in damaged white matter. Antibodies to Nogo have been shown to enhance the regeneration of injured corticospinal axons and enhance their sprouting rostral to the lesion (Schnell and Schwab, 1990; Z′Graggen *et al.,* 1998). Antibodies to Nogo also stimulate both axonal sprouting and the expression of some growth-related genes by Purkinje cells (Zagrebelsky *et al.,* 1998). There is little evidence that the forebrain neurons which fail to express NgR, such as neostriatal cells and ventral lateral geniculate nucleus neurons, have an unusually high capacity for regenerating their axons in the CNS, but such information would have been difficult to obtain. However, axonal sprouting is particularly extensive around grafts of smooth muscle in the neostriatum (Tew *et al.,* 1998).

The expression of NgR by neocortical pyramidal neurons in the motor cortex, neurons in the magnocellular part of the red nucleus, and neurons in the lateral vestibular nuclei, all of which contribute to important long descending tracts in the spinal cord, is compatible with the hypothesis that Nogo-66 expressed in the spinal cord is a major cause of the failure of regeneration of descending axons following spinal injury. In contrast, the generally weak expression of NgR by DRG neurons may explain why antibodies against Nogo failed to

FIG. 3. Neocortex from an adult mouse hybridized with NgR 5′ probe. The pattern of NgR signal is very strong in neurons of all layers, but not in glia of the molecular layer or subcortical white matter. Bar, 500 μm.

FIGS. 4 and 5. Sections through the hippocampal formation and dorsal thalamus (T) from adult mice, hybridized with the 5′ probe (Fig. 4) and the 3′ probe (Fig. 5). The pattern of NgR signal obtained with the two probes is identical. Strong signal can be seen in the hippocampus and dentate gyrus and medial habenular nuclei and in the dorsal thalamus, but not in the TRN (between arrows in Fig. 5) or subcortical white matter. Bar, 500 μm.

FIG. 6. Transverse section through the crus cerebri of an adult rat hybridized with the 3′ NgR probe. Neurons in the red nucleus (*) show moderate levels of NgR signal but those in the substantia nigra pars compacta (arrows) show only very weak signal. Bar, 500 μm.

FIG. 7. Section through the basal forebrain of an adult rat hybridized with the 3′ NgR probe showing only background levels of signal. Septal nuclei (*). Bar, 500 μm.
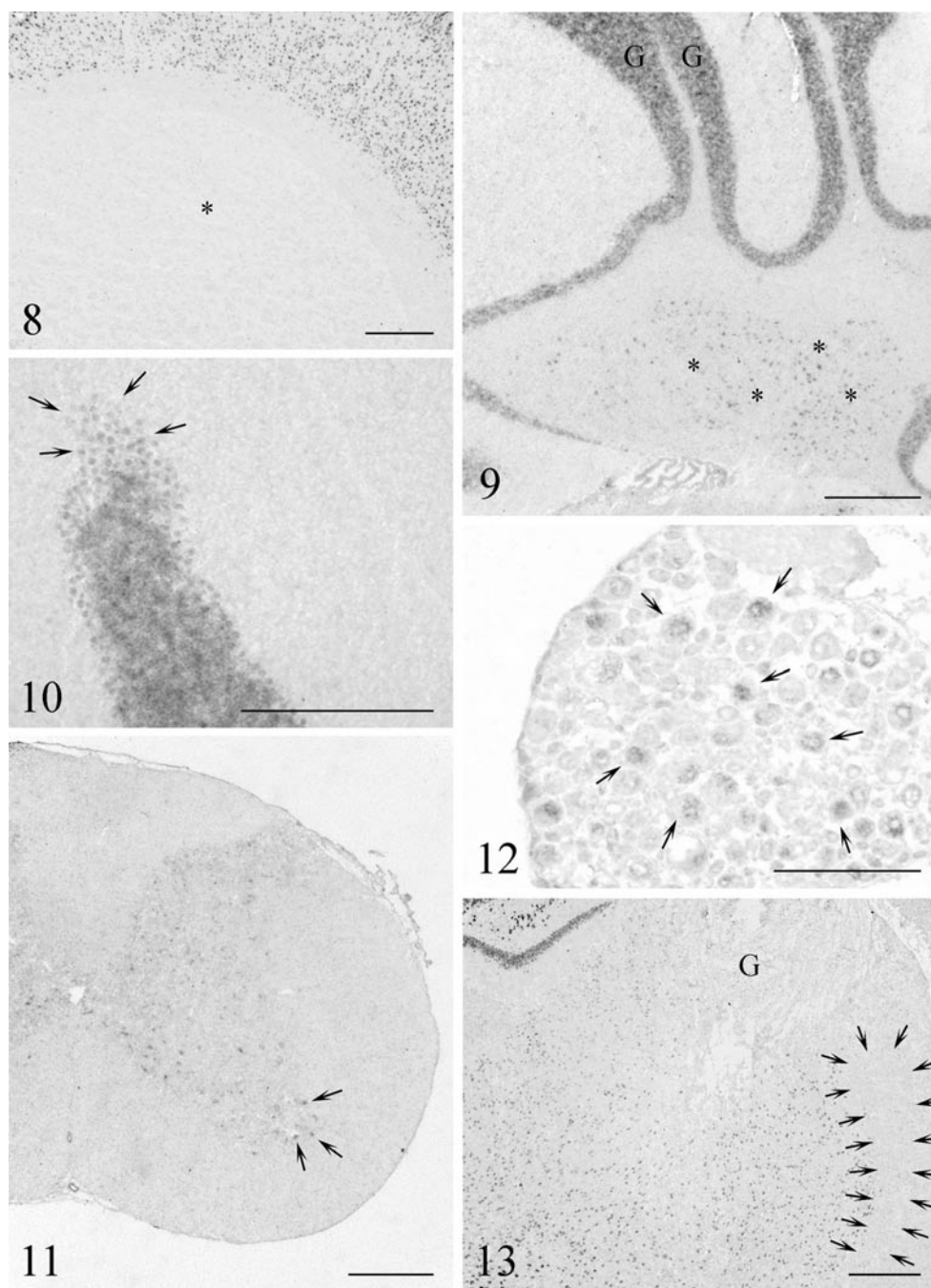
FIG. 8. Section through neocortex and neostriatum of an adult rat, hybridized with 3′ probe. Note the virtual absence of NgR signal from the caudatoputamen (*) and the subcortical white matter, in contrast to the strong signal from the neocortical neurons. Bar, 500 μm.
**FIG. 9.** Section through the cerebellum from an adult rat hybridized with 3′ NgR probe. Note the strong signal from the granule cell layer (G) and moderate signal from neurons in the deep nuclei (*). Bar, 500 μm.
FIG. 10. Higher power image of a section through rat cerebellar cortex. The oblique section through the cortex allows NgR signal from the Purkinje cells (arrows) to be seen, although it is always weaker than that from the granule cell layer. Bar, 500 μm.
FIG. 11. Transverse section through the cervical spinal cord from an adult rat hybridized with the 3′ NgR probe. There is generally little NgR mRNA detectable in the spinal cord although motor neurons (arrows) show a distinct but weak signal. Bar, 500 μm.
FIG. 12. L5 DRG from an adult rat hybridized with the 3′ NgR probe. A subpopulation of primary sensory neurons (e.g., at arrows) shows NgR signal. Bar, 250 μm.
FIG. 13. Coronal section through the thalamus from a rat 3 days after the implantation of a tibial nerve graft (G). Neurons in the TRN (between arrows) still show no NgR signal although they are known to regenerate into such grafts. Bar, 500 μm.
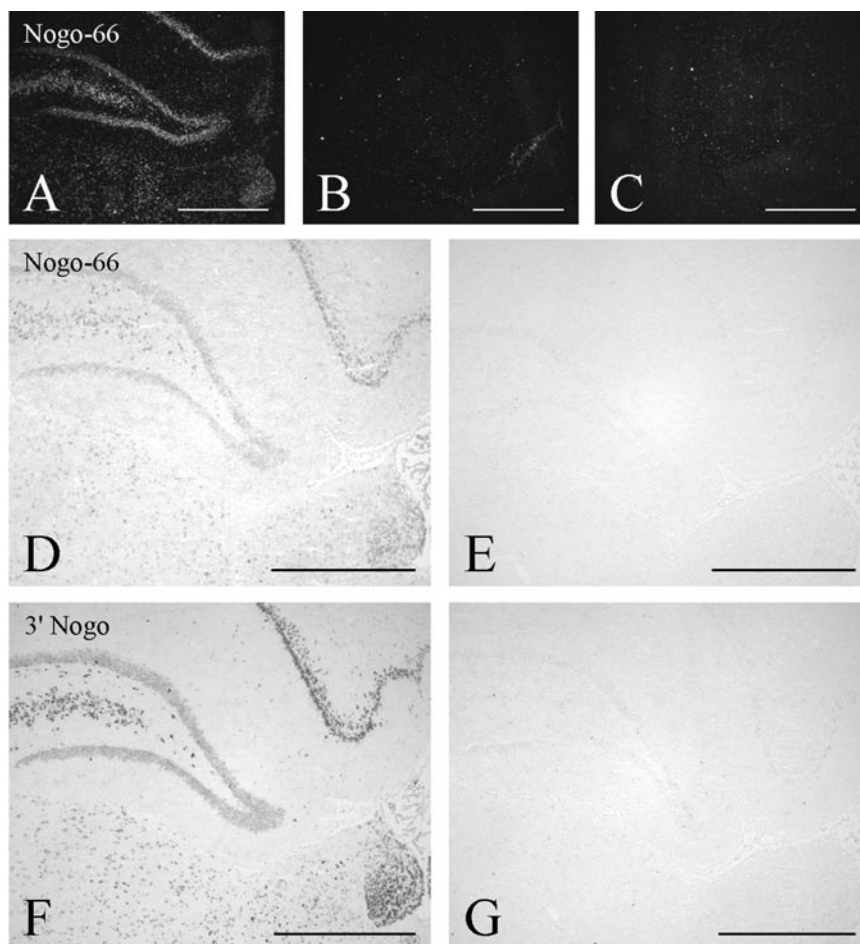
FIG. 14. Specificity of Nogo probes. Sections through the hippocampal formation and dorsal thalamus reacted with three antisense probes, and their controls. In A and B sections were probed with ^{35}S-labeled antisense Nogo-66 DNA oligonucleotides and viewed with dark-field illumination. There is strong signal from the labeled probe in A but in B excess unlabeled probe has been added as a competitive control to detect nonspecific binding. In C, ^{35}S-labeled sense probe has been used and viewed with dark-field illumination. There is no signal from either of the control sections. In D and E sections were reacted with antisense (D) and sense (E) Dig-labeled Nogo-66 riboprobes. The section treated with sense probe (E) shows no signal. In F and G sections were reacted with Dig-labeled antisense (F) and sense (G) 3′ region Nogo Dig-labeled riboprobes. Strong signal is present in the section treated with antisense (F) but no signal is detected by the sense probe (G). Bar, 500 μm.
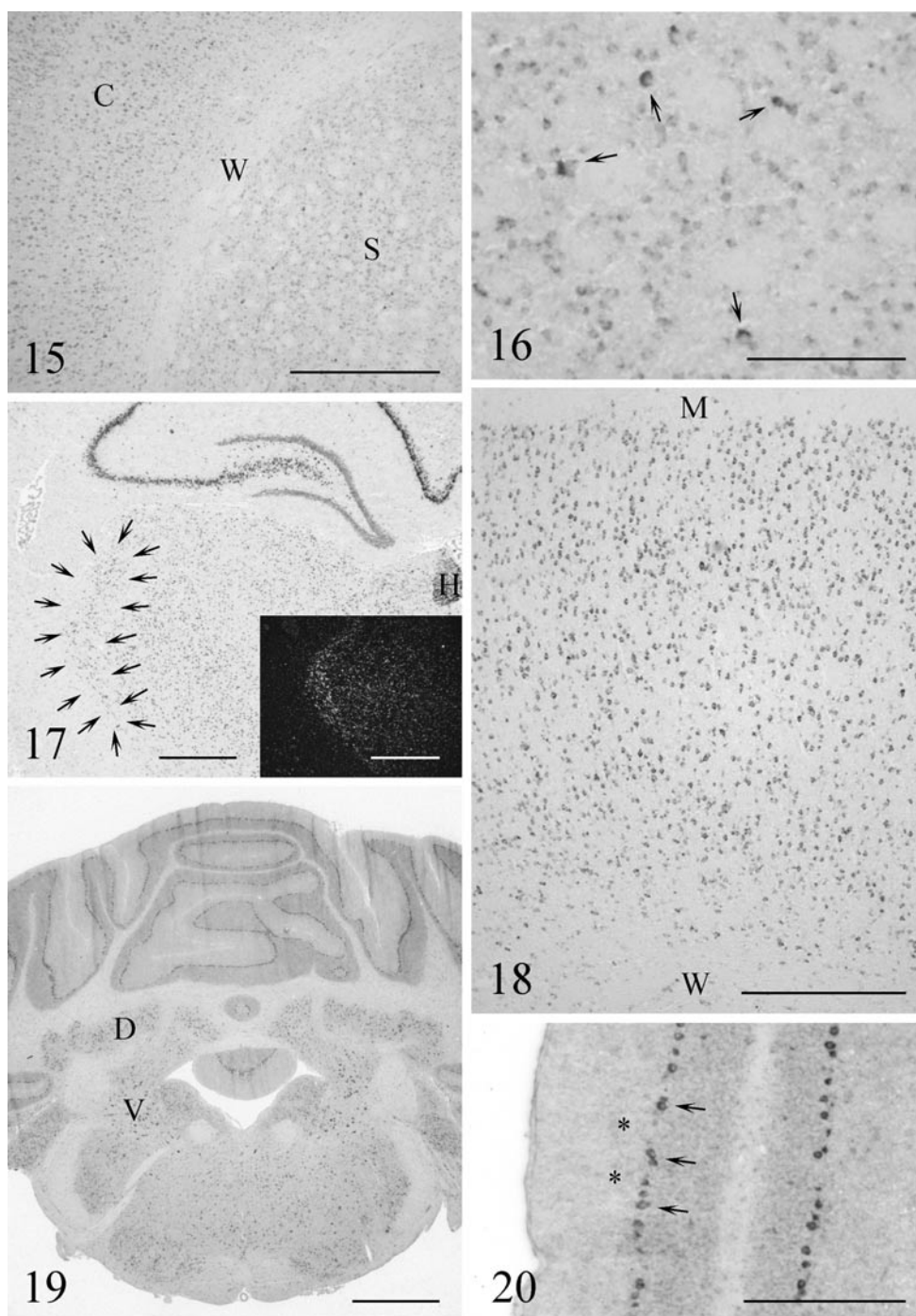
improve regeneration of DRG axons into the spinal cord from nerve grafts (Oudega *et al.,* 2000), although embryonic chick DRG neurons do respond to Nogo-66 *in vitro* (Fournier *et al.,* 2001). Indeed, the very low or absent expression of NgR by most neurons in the spinal cord, other than motor neurons, would suggest that, in general, the binding of Nogo-66 to neuronal NgR is unlikely to provide an explanation of the failure of regeneration of ascending tracts following spinal injury. Other factors must also be involved in limiting regeneration in the spinal cord. Furthermore, the prominent expression of Nogo-66 by neocortical neurons and Purkinje cells also introduces the possibility that antibodies to Nogo may stimulate axonal regeneration by di-

rect binding to neuronal cell bodies, although it is not clear how Nogo would transduce a signal into the cytoplasm.

The lack of NgR expression in the substantia nigra pars compacta and basal forebrain suggest that NgR is not a promising candidate for therapeutic intervention in Parkinson's and Alzheimer's diseases, although Nogo-66-coding sequences are expressed by these cells.

NgR, Nogo, and Axonal Regeneration into Nerve Grafts

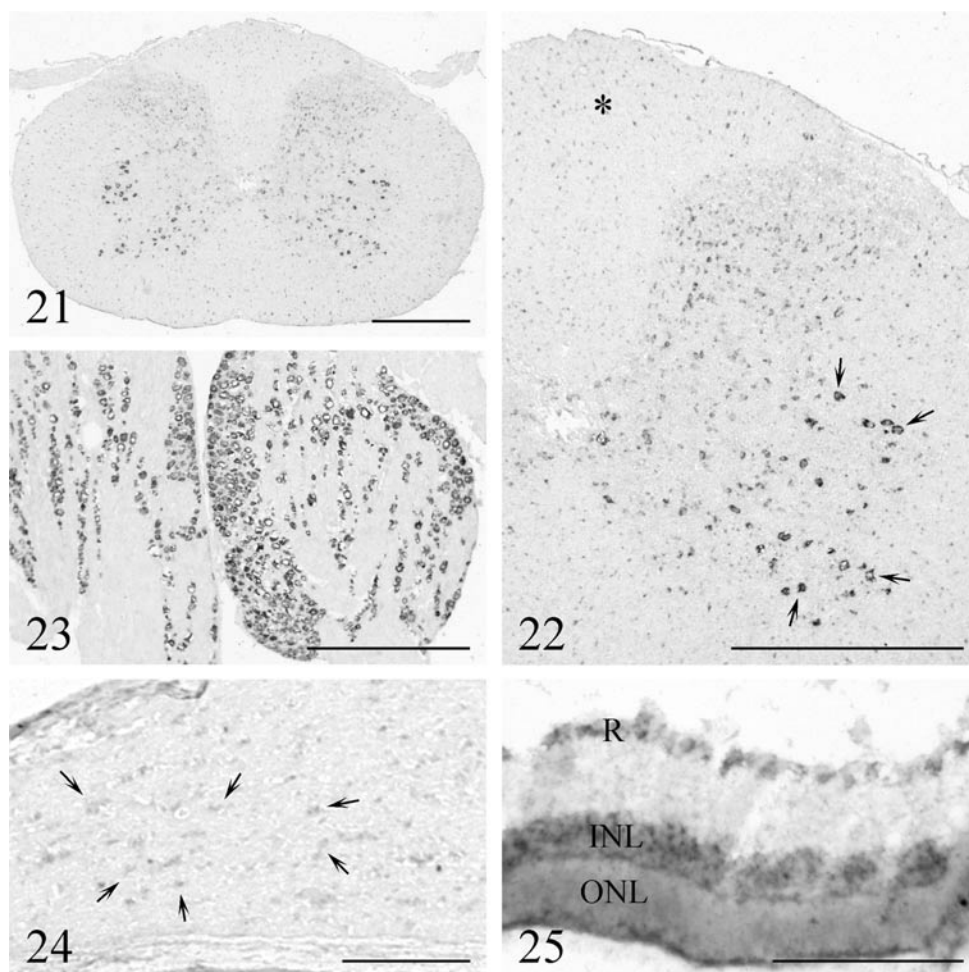Corticospinal neurons, Purkinje cells, and hippocampal neurons all express NgR and all show remarkably

FIG. 21. Cervical spinal cord from adult rat reacted with the probe to 3′ region Nogo. Signal can be seen in many neurons in the gray matter, but is strongest in motor neurons. At this magnification it is difficult to resolve any signal in white matter. Bar, 500 μm.

FIG. 22. Higher power image of cervical spinal cord from adult rat reacted with the probe to 3′ region Nogo. Signal can be seen in many neurons in the gray matter, but is strongest in motor neurons (arrows). A distinct signal is also present in the white matter of the dorsal columns (*). Bar, 500 μm.

FIG. 23. Lumbar dorsal root ganglia reacted with the probe to 3′ region Nogo. Very strong signal is present in primary sensory neurons but not in the Schwann cells.

FIG. 24. Optic nerve from adult rat, reacted with the probe to 3′ region Nogo. A weak signal can be detected in scattered glial cells (arrows). Bar, 100 μm.

FIG. 25. Retina from adult rat, reacted with the probe to 3′ region Nogo. Strong signal can be seen from the ganglion cell layer (R), inner nuclear layer (INL), and outer nuclear layer (ONL). Bar, 100 μm.

little propensity for regenerating axons into peripheral nerve grafts in the brain (Anderson and Lieberman, 1999). Indeed, corticospinal axons do not grow into nerve grafts even when they have been stimulated to regenerate by NT-3 generated within the grafts (Blits *et al.,* 2000), and sprouting Purkinje cell axons in trans-

FIG. 19. Section through the cerebellum and brain stem of an adult rat reacted with the probe to 3′ region Nogo. Strong signal can be detected from Purkinje cells, neurons in the deep cerebellar nuclei (D) and vestibular nuclei (V). Bar, 1 mm.

FIG. 20. A higher power image of cerebellar cortex from adult rat reacted with the probe to 3′ region Nogo. A line of Purkinje cells (arrows) showing strong signal for Nogo can be seen, with weak signal from cells in the granule cell layer and background levels in the molecular layer (*). Compare with NgR expression in Fig. 9. Bar. 250 μm.
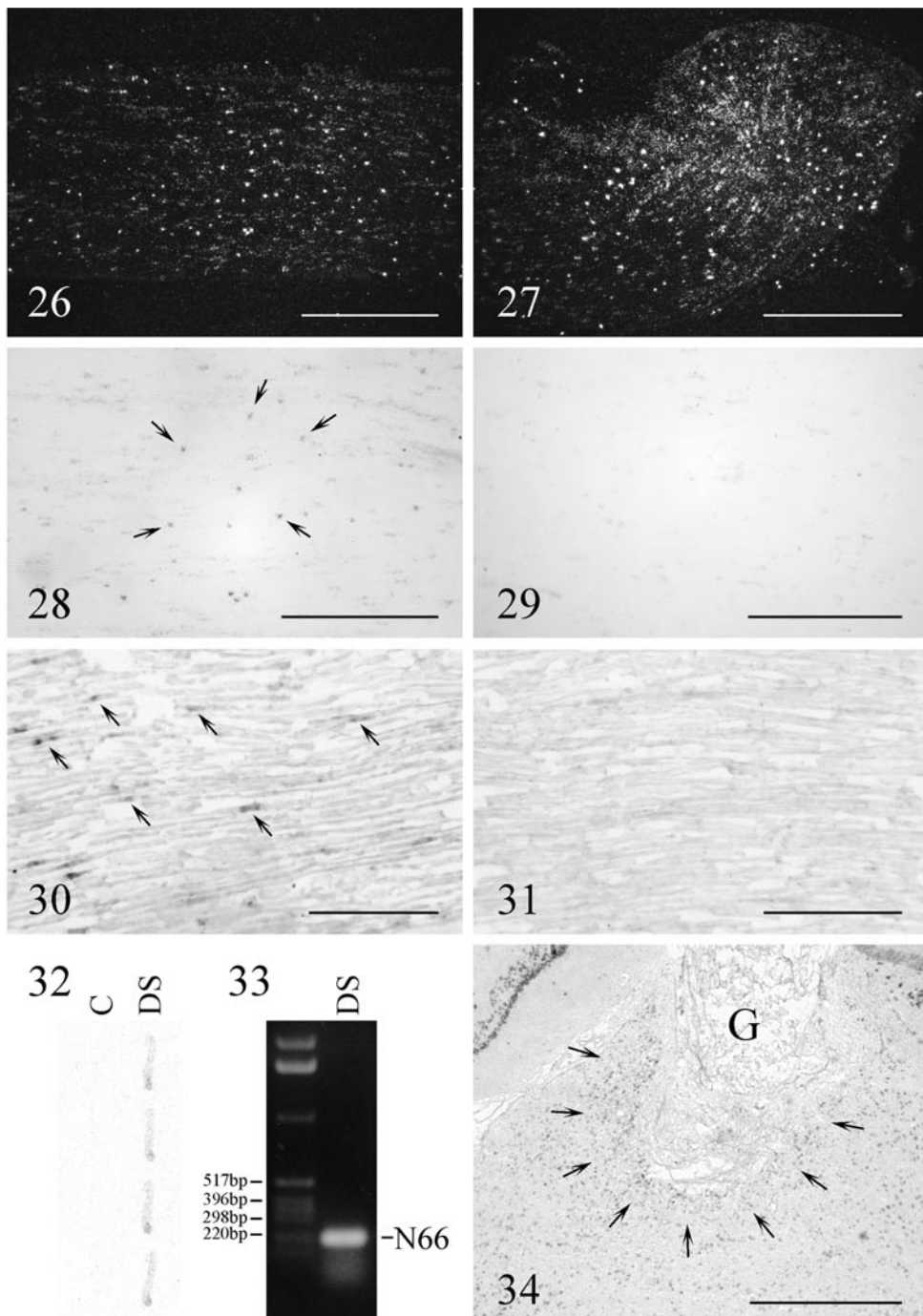
FIGS. 26–33.   Detection of Nogo in adult rat peripheral nerve.
FIGS. 26 and 27.   Dark-field images of distal stump of sciatic nerve 3 days after transection, reacted with the radioactive Nogo-66 probe. A low level of signal is present from many cells but is strong on scattered cells, particularly near the cut end of the nerve (Fig. 27). Bar, 500 μm.
FIG. 28.   Bright-field image of the section seen in Fig. 26. Some cells showing highest levels of signal are arrowed. Bar, 250 μm.
FIG. 29.   Bright-field image of contralateral (uninjured) sciatic nerve. No signal for Nogo-66 is detectable. Bar, 250 μm.
FIGS. 30 and 31.   Bright-field images of sections of sciatic nerve 3 days after injury (Fig. 30) and contralateral to the injury (Fig. 31), reacted with the probe to 3′ region Nogo. Strong signal can be seen from scattered cells in the injured nerve (e.g., arrows) but not in the intact nerve (Fig. 31). Bar, 100 μm.
FIG. 32.   Film, after 3 days exposure, showing sections of sciatic nerve from adult rat treated with the radioactive probe to Nogo-66. Strong signal is apparent from sections of the distal stump of the injured nerve (DS) 3 days after transection, but not from sections of the intact nerve (C) which are consequently undetectable in the figure.

genic mice overexpressing GAP-43 and L1 turn away from nerve grafts (Zhang *et al.,* unpublished observations). This suggests that there may be factors in peripheral nerves which can repel some classes of CNS axons. In contrast, neurons in the TRN do not express NgR mRNA, even in animals with a peripheral nerve graft in the thalamus, and they are among the most successful CNS neurons at regenerating their axons into nerve grafts (Benfey *et al.,* 1985; Morrow *et al.,* 1993; Vaudano *et al.,* 1998). NgR mRNA expression is also absent or at very low levels in the basal forebrain and substantia nigra pars compacta, and neurons in these regions are also among the CNS neurons which are capable of regenerating axons into nerve grafts (Woolhead *et al.,* 1998). Most neurons in the dorsal thalamus around the nerve grafts, which normally fail to regenerate axons, express NgR, although they did not usually show a detectable upregulation of NgR in response to axotomy. It is, therefore, tempting to speculate that it is Nogo-66, or another molecule acting through NgR, which limits the ability of these neurons to regenerate their axons into grafts. Nogo-66 mRNA was not detected in peripheral nerves in some previous investigations (GrandPre *et al.,* 2000; Josephson *et al.,* 2001). However, Nogo-B was found in sciatic nerve by Chen *et al.* (2000) and we have found that Nogo-66-encoding sequences are upregulated in injured sciatic nerve, which makes it feasible that Nogo could play a part in inhibiting the regeneration into nerve grafts of axons from those neurons which express NgR. Nonetheless, neither neostriatal neurons nor neurons in the ventral lateral geniculate nucleus express NgR, yet neither is good at regenerating their axons into nerve grafts in the brain (Vaudano *et al.,* 1995; Woolhead *et al.,* 1998). Similarly, neurons in the deep cerebellar nuclei are good at regenerating axons into nerve grafts (Vaudano *et al.,* 1993, 1998; Chaisuksunt *et al.,* 2000) although they express NgR mRNA both in the intact state and after graft implantation into the cerebellum. It seems that NgR–Nogo-66 interactions are unlikely to be the only explanation of the specificity of CNS axonal regeneration into nerve grafts. However, it remains possible that the neuronal expression of NgR may limit the ability of some CNS axons to sprout in the brain and spinal cord, and that both NgR expression and other factors, e.g., neu-

rotrophin sensitivity, may determine whether or not the sprouts can elongate through nerve grafts.

It is now clear that Nogo-66-coding sequences are found in at least seven transcripts of the reticulon 4 gene produced in many types of cells (see Table 1). It is not possible to say which transcripts were detected in the present study. Our findings on the distribution of Nogo transcripts are broadly similar to those published previously by Josephson *et al.* (2001) but extend the observations to include cerebellum and injured peripheral nerve. The stronger expression of Nogo transcripts in neurons than in glia in our study may be the result of the detection of transcripts coding for derivatives of the gene, other than Nogo-A, which is probably most strongly expressed in oligodendrocytes Chen *et al.* (2000). However, Josephson *et al.* (2001) also detected signal from many neurons using a Nogo-A-specific probe (a pan-Nogo probe gave a stronger signal from neurons in human, but not rat tissue). The extent to which the protein products of the transcripts found in neurons and nonneuronal cells of peripheral nerves are expressed at the cell surface or are otherwise available for detection by growth cones is also unclear. Nonetheless, the distribution Nogo transcripts in neurons opens the possibility that NgR could be involved in interactions between neurons. The increased expression of Nogo around peripheral nerve grafts in the brain, i.e., at an injury site, is in contrast to the previously reported absence of upregulation at injury sites in the spinal cord (Josephson *et al.,* 2001) and would seem to merit further study. Nogo-66 in this region obviously has the potential to deter NgR-expressing axons from regenerating into the grafts.

Thus there is no simple correlation between the ability of CNS neurons to regenerate axons within the CNS, or into nerve grafts, and their expression of NgR or Nogo mRNAs, except that cortical neurons which express NgR most strongly are known to respond to the IN-1 antibody. However, it is possible that Nogo-66 could also act through other, unidentified receptors, which could extend the range of neurons sensitive to its growth-inhibitory activity. It is also possible that the differential expression by CNS neurons of receptors for other inhibitory molecules produced in the CNS or by injured peripheral nerves may be involved both in limiting axonal regeneration in the brain and spinal cord

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FIG. 33. RT-PCR for Nogo-66 from RNA extracted from the distal stump of adult rat sciatic nerve, 3 days after transection. A single strong band for Nogo-66 (~0.2 kb) is present after 35 PCR cycles. Sample was run against a 1-kb DNA molecular weight marker (Gibco BRL) on a 1% agarose gel.

FIG. 34. *In situ* hybridization with the probe to 3′ region Nogo on a section of the thalamus of an adult rat 16 days after the insertion of a peripheral nerve graft (G). Increased signal can be seen from cells in the thalamus near the graft/brain interface. Bar, 500 μm.

TABLE 1

Splice Variants of Reticulon 4 Containing the Nogo-66 Sequence

Name	GenBank Accession No.	Exon 1	Exon 2	Exon 3	Exon 4	Exon 5	Exon 6	Exon 7	Exon 8	Exon 9	Exon 10	Exon 11	mRNA length	Amino acid length	Sequence submitted by	Source (human tissue)
Nogo-A	AJ251383	Y	N	Y	Y	N	Y	Y	Y	Y	Y	Y	3579	1192	Prinjha *et al.*	None specified
Reticulon 4a	AF148537	Y	N	Y	Y	N	Y	Y	Y	Y	Y	Y	4632	1192	Zhou *et al.*	None specified
KIAA0086	AB020693	Y	N	Y	Y	N	Y	Y	Y	Y	Y	Y	4053	1192	Ohara *et al.*	Adult male brain
Rtn-xL	AB040462	Y	N	Y	Y	N	Y	Y	Y	Y	Y	Y	4166	1192	Eguchi *et al.*	Fetal brain
Testis Specific Rtn (Rtn-T)	AF333336	N	Y	Y	Y	N	Y	Y	Y	Y	Y	Y	3491	986	Sha *et al.*	Adult testis
Nogo-A short form	AF320999	Y	N	N	Y	N	Y	Y	Y	Y	Y	Y	2883	960	Jin and Ju	None specified
Brain my043	AF063601	N	N	N	Y	N	Y	Y	Y	Y	Y	Y	2481	647	Mao *et al.*	Fetal brain
MGC clone:15807 IMAGE:3505850	BC016165	Y	N	Y	N	N	Y	Y	Y	Y	Y	Y	1784	392	NIH-MGC	Kidney: renal cell adenocarcinoma
Nogo-B	AJ251384	Y	N	N	N	N	Y	Y	Y	Y	Y	Y	1122	373	Prinjha *et al.*	None specified
Reticulon 4b	AF148538	Y	N	N	N	N	Y	Y	Y	Y	Y	Y	2235	373	Zhou *et al.*	None specified
ASY cell-death-inducing gene	AB015639	Y	N	N	N	N	Y	Y	Y	Y	Y	Y	2052	373	Yutsudo	Fibroblast
Foocen-m	AF132047	Y	N	N	N	N	Y	Y	Y	Y	Y	Y	2276	373	Ito and Schwartz	Placenta
Rtn-xS	AB040463	Y	N	N	N	N	Y	Y	Y	Y	Y	Y	1709	373	Eguchi *et al.*	Fetal brain
MGC clone:10125 IMAGE:3901353	BC010737	Y	N	N	N	N	Y	Y	Y	Y	Y	Y	1485	373	NIH-MGC	Pancreas: epitheloid carcinoma
MGC clone:13655 IMAGE:4082756	BC012619	Y	N	N	N	N	Y	Y	Y	Y	Y	Y	1654	373	NIH-MGC	Brain: glioblastoma
Nogo-C	AJ251385	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	600	199	Prinjha *et al.*	None specified
Reticulon 4C	AF087901	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	1617	199	Zhou *et al.*	None specified
NSP C Homolog	AF077050	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	1785	199	Song *et al.*	Pituitary CD34+
NSP C Homolog	AF125103	N	N	N	N	Y	Y	Y	Y	?	Y	Y	1798	199	Zhang *et al.*	Hematopoietic stem/progenitor cells
SP1507 Unknown mRNA	AF177332	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	1700	199	Gu *et al.*	None specified
MGC clone:1239 IMAGE:3139770	BC001035	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	1160	199	NIH-MGC	Placenta: choriocarcinoms
MGC clone:14766 IMAGE:4291127	BC007109	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	1079	199	NIH-MGC	Skeletal muscle
MGC clone:24257 IMAGE:3933041	BC014366	N	N	N	N	Y	Y	Y	Y	Y	Y	Y	1698	199	NIH-MGC	Brain

Note. The human reticulon 4 gene has been mapped to chromosome 2p14 → 13 (Yang *et al.,* 2000). It comprises 11 known exons and is spread over nearly 80 kb of chromosomal DNA. Differential splicing of the gene gives rise to at least seven different mRNAs containing the Nogo-66 sequence. For some of these molecules there are multiple encoding sequences deposited in the NCBI database with variations confined to the first and last exons—probably because the sequencing process does not reliably read the ends of the transcripts. The dilysine ER retention signal, –KRKAE, is found at the C-terminus (exon 11) of all spice variants. The Nogo probes we have used for *in situ* hybridization, to the Nogo-66 encoding sequence located within exons 6 and 7, and the 3′ Nogo region spanning exons 6–11 have the capacity to detect any of the splice variants listed. Splice variants containing the Nogo-66 sequence are widely expressed in normal and neoplastic human tissues, implying that this gene is multifaceted and has functions other than inducing neuronal growth cone collapse. Nogo-A has a specific domain (exons 3 and 4) which also displays neurite outgrowth inhibitory activity, through an unknown mechanism. However, the testis specific reticulon (Rtn-T) also contains these two exons and has been postulated to play a role in testicular development. N.B. Several anomalies were noted in performing homology searches of proteins predicted by transcript sequences within the different groups: Nogo-A has amino acid residue P instead of S, in contrast to all other transcripts within the group, at position 458. ASY cell-death-inducing gene has amino acid residue C instead of S, in contrast to all other transcripts within the group, at position 107. NSP C homolog (AF125103) has amino acids LQCSCYL instead of FSVPVIY, in contrast to all other transcripts within the group, at positions 156–162. Furthermore, exon 9 was not detected in a Blast search of the transcript sequence against the human genome (denoted by '?'). Whether these aberrations are due to sequencing errors or natural human genetic variation is not clear. NIH-MGC, National Institutes of Health–Mammalian Gene Collection project. NSP, neuroendocrine-specific protein.

and determining which axons can regenerate into nerve grafts. In any case, it seems unlikely that the conventional interpretation of Nogo function (that it is predominantly a myelin protein comprising the major axonal-growth inhibitory influence in the CNS) is correct, because Nogo-66 mRNA is more strongly expressed by neurons than by glial cells (Josephson *et al.,* 2001; the present study), Nogo interacts with two mitochondrial proteins (Hu *et al.,* 2001) and Nogo-B is a proapoptotic protein (Li *et al.,* 2001). Finally, Nogo-66 may be added to the list of putative growth-inhibitory molecules/epitopes upregulated in injured peripheral nerves, a list which also includes tenascin C (Martini, 1994) and chondroitin sulfate proteoglycans (Zuo *et al.,* 1998).

EXON 1 2 3 4 5 6 7 8 9 10 11

900Kb 910Kb 920Kb 930Kb 940Kb 950Kb 960Kb 970Kb 980Kb 990Kb

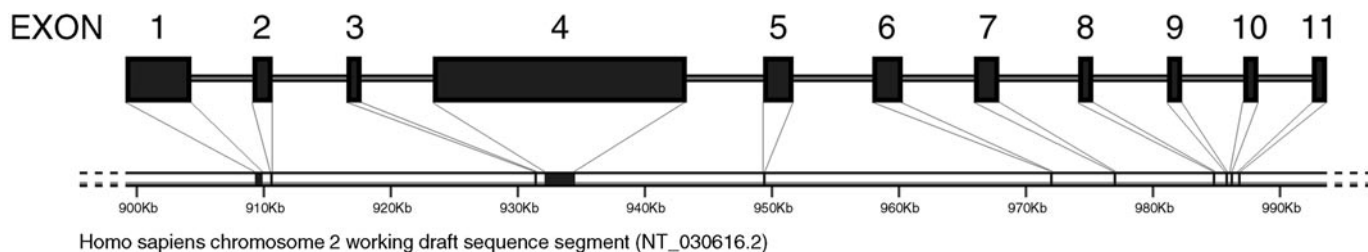Homo sapiens chromosome 2 working draft sequence segment (NT_030616.2)

FIG. 35. Diagram showing the reticulon 4 gene, to be used in conjunction with Table 1.

EXPERIMENTAL METHODS

All surgical procedures were approved by the UCL ethical committee and licensed by the home office. For nerve grafting experiments, adult female Sprague–Dawley rats were anaesthetized with halothane, nitrous oxide, and oxygen mixture. In 7 rats, segments of the left tibial nerve were removed and one end was implanted through a craniotomy, using coordinates taken from the atlas of Paxinos and Watson (1986), into the left thalamus and in 3 other rats the grafts were implanted with their proximal end in the region of the cerebellar deep nuclei. Animals were killed 3 days ($n = 1$), 2 weeks ($n = 4$), and 16 days ($n = 2$) after graft implantation in the thalamus and 3 days ($n = 1$) and 4 weeks after graft implantation into the cerebellum. In 6 animals the left sciatic nerve was transected in the thigh, and the animals were killed after 3 days. The grafted and injured animals and 2 unoperated male C57 black mice and 12 unoperated female Sprague–Dawley rats were killed by decapitation while deeply anesthetized. Samples of brain, spinal cord, optic nerves, retina, the L4 and L5 dorsal root ganglia, the sciatic nerves, the superior cervical ganglion, thymus, and heart were fresh-frozen in Tissue Tek, cooled by dry ice.

The NgR and Nogo-66 cRNA Sense and Antisense Riboprobes

Cloning of the mouse Nogo receptor. 5′ and 3′ segments of the mouse Nogo receptor (GenBank Accession No. AF283462) were cloned by PCR from a mouse single-stranded DNA library, using the proofreading polymerase *Pfu.* For the 5′ segment (bases 135–561), the primers ATC **GCT CGA G**GA AGC CGC TTC CAG TGC CCG AC and ACT G**AA GCT T**GC CGT GGA ACG TGG TAG GGT CC incorporating a *Xho*I and *Hin*dIII restriction endonuclease site, respectively, were used. For the 3′ segment (bases 784–1760), the primers ATC **GCT CGA G**AG TCT TGA CCG CCT CCT CTT and ACT G**AA GCT T**CC CGG AAC CCT GTA AAC ATG incorporating a *Xho*I and *Hin*dIII site, respectively, were used.

Cloning of rat Nogo-66. The Nogo-66 domain of rat Nogo-A (GenBank Accession No. AJ242961; bases 3328–3525) was cloned from a rat single-stranded DNA library by PCR using *Pfu* polymerase. The primers ATC **GCT CGA G**AG GAT ATA TAA GGG CGT GAT C and ACT G**AA GCT T**CT TCA GGG AAT CAA C TA AAT C incorporating a *Xho*I and *Hin*dIII site, respectively, were used.

Cloning of rat 3′ Nogo region. The 3′ region of rat Nogo-A (GenBank Accession No. AJ242961; bases 3222–3801) was cloned from a rat single-stranded DNA library by PCR using *Pfu* polymerase. The primers ACT **GCT CGA G**GG TGG TTG GTG CCA GCT TAT and ATC G**AA GCT T**CA CCC CCG TAA TCA AGT GAG incorporating a *Xho*I and *Hin*dIII site, respectively, were used.

All PCR products were ligated into the pSP72 vector (Promega) in the same orientation between the *Xho*I and *Hin*dIII sites. Sequences were verified using the ABI prism system.

Generation of Digoxigenin-Labeled Riboprobes

Antisense probes were generated by linearization of the pSP72 plasmid with *Xho*I, followed by *in vitro* transcription with T7 RNA polymerase. Conversely, sense probes were generated by linearization with *Hin*dIII, followed by *in vitro* transcription with SP6 RNA polymerase. Antisense and sense cRNA probes labeled with digoxigenin were generated according to the manufacturer's recommendations using an RNA labeling kit (Boehringer Mannheim, Germany).

In Situ Hybridization

In situ hybridization was carried out as described by Bartsch *et al.* (1992) and Zhang *et al.* (1995). In brief, cryostat sections of brain spinal cord and DRG were cut at a nominal thickness of 10 μm, thaw-mounted onto slides coated with 3-aminopropyltriethoxysilane, and fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) overnight at 4°C. After washing in PBS, sections were treated with 0.1 M HCl, washed in PBS, incubated in 0.1 M triethanolamine containing 0.25%

acetic anhydride, and then washed with PBS again, dehydrated in an ascending ethanol series, and air dried. Prehybridization was carried out at 37°C overnight with a mixture of prehybridization buffer/deionized formamide 1/1 (containing 50% formamide, 25 mM ethylenediaminetetraacetic acid (EDTA), 50 mM, pH 7.6 Tris–HCl, 2.5× Denhardt's solution, 0.25 mg/ml tRNA (Boehringer Mannheim), and 20 mM NaCl). The digoxigenin-labeled sense and anti-sense probes were prepared at a concentration of 3 μl/ml with hybridization buffer containing 50% formamide, 20 mM Tris–HCl (pH 7.50), 1 mM EDTA, 1× Denhardt's solution, 0.5 mg/ml tRNA, 0.1 mg/ml poly(A) RNA (Sigma), 0.1 M DTT, and 10% dextran sulfate. Hybridization was performed overnight at 63°C for the NgR(3′) and NgR(5′) probes and 62°C for the Nogo-66 and 3′Nogo probes. After hybridization, sections were washed in 0.2× standard saline citrate (SSC, containing 30 mM NaCl and 3 mM Na-citrate, pH 7.0) and then in 0.1× SSC/50% formamide at the hybridization temperature. Sections were equilibrated with buffer 1 (100 mM Tris–HCl, 150 mM NaCl, pH 7.5), incubated in modified buffer 2 (1% Boehringer blocking reagent, 0.5% BSA fraction from Sigma in buffer 1), and then incubated with alkaline phosphatase-coupled antibodies to digoxigenin (Boehringer Mannheim) at a dilution of 1:1500 in modified buffer 2 overnight at 4°C. Sections were washed in buffer 1, equilibrated in buffer 3 (100 mM Tris-base, 100 mM NaCl, 50 mM MgCl$_2$, adjusted to pH 9.5), and developed in the dark with buffer 3 containing 0.34 mg/ml 4-nitroblue tetrazolium chloride (Sigma), 0.175 mg/ml 5-bromo-4-chloro-3-indolylphosphate (Sigma), and 0.25 mg/ml levamisol (Sigma). Development was stopped by washing with buffer 4 (10 mM Tris–HCl, 1 mM EDTA, pH 8.0), following which the sections were mounted in DPX beneath glass coverslips. The specificity of the hybridization signal was verified by comparison with the sections processed with sense probe under identical conditions.

Preparation of ^{35}S-Radiolabeled Nogo-66 Oligonucleotides

Forty-five-mer antisense and sense DNA oligonucleotides (TGA ATG GGT GGC CTT CAT CTG ATT TCT GGA TAG CCT GGA TCA CGC and GCG TGA TCC AGG CTA TCC AGA AAT CAG ATG AAG GCC ACC CAT TC, respectively) were labeled with ^{35}S (Dupont NEN) using terminal deoxytransferase (Promega, UK). Levels of probe radioactivity were measured with a scintillation counter. Labeled DNA oligonucleotides were stored at −20°C, in 20 mM DTT.

Radioactive in Situ Hybridization

Tissue sections for radioactive *in situ* hybridization were cut, mounted, fixed, and dehydrated as described for the Dig-labeled ISH procedure.

Two nanograms of labeled probe was used per milliliter of hybridization buffer (50% formamide, 4× SSC, pH 7, 0.025 M sodium phosphate, pH 7, 0.001 M sodium pyrophosphate, 5× Denhardt's solution, herring sperm DNA, 0.1 mg/ml polyadenylic acid, 0.1 g/ml dextran sulfate, 20 mM DTT). For competitive cold controls, unlabeled 45-mer oligonucleotides were added to a concentration of 200 ng/ml hybridization buffer, inclusive of 2 ng/ml of the respective radioactive probe. One-hundred microliters of hybridization mixture containing relevant probes was added per slide. Incubation of slides was carried out in sealed chambers moistened with 2× SSC and 50% formamide. Hybridization was performed at 42°C overnight.

Four times 30-min high-stringency washes of the sections were performed using 1× SSC at 58°C. The sections were subsequently rinsed in 0.1× SSC at room temperature, before being dehydrated in 70% ethanol and 95% ethanol, for 1 min each. Sections were air-dried for 30 min and exposed to Biomax MR film (Kodak) for 3 days.

Emulsion Dipping

Slides were dipped in a 1:1 ratio of K.5 emulsion (Ilford) and 0.5% glycerol at 42°C and allowed to dry. Slides were transferred light tight boxes, containing silica gel, and stored at 4°C for 14 days, before undergoing development.

Slides were developed for 2 min in D19 (Kodak), rinsed in water, and fixed in Hypam (Ilford) for 4 min. The slides were then immersed in water for several hours before being stained with thionine, dehydrated, immersed in Histoclear, and coverslipped with DPX.

RT-PCR

Trizol (Gibco BRL) was used, as per the manufacturer's instructions, for the isolation of RNA from the distal stumps of axotomized peripheral nerve of three rats, 3 days after injury. Single-stranded DNA was generated using AMV-reverse transcriptase and oligo dT (Promega UK). This was used as template for PCR of the Nogo-66 domain using the primers described above. Fig. 33 shows the band obtained after 35 PCR cycles at 50°C annealing temperature.

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