Retinal Axon Regeneration in the Lizard *Gallotia galloti* in the Presence of CNS Myelin and Oligodendrocytes

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ABSTRACT Retinal ganglion cell (RGC) axons in lizards (reptiles) were found to regenerate after optic nerve injury. To determine whether regeneration occurs because the visual pathway has growth-supporting glia cells or whether RGC axons regrow despite the presence of neurite growth-inhibitory components, the substrate properties of lizard optic nerve myelin and of oligodendrocytes were analyzed in vitro, using rat dorsal root ganglion (DRG) neurons. In addition, the response of lizard RGC axons upon contact with rat and reptilian oligodendrocytes or with myelin proteins from the mammalian central nervous system (CNS) was monitored. Lizard optic nerve myelin inhibited extension of rat DRG neurites, and lizard oligodendrocytes elicited DRG growth cone collapse. Both effects were partially reversed by antibody IN-1 against mammalian 35/250 kD neurite growth inhibitors, and IN-1 stained myelinated fiber tracts in the lizard CNS. However, lizard RGC growth cones grew freely across oligodendrocytes from the rat and the reptilian CNS. Mammalian CNS myelin proteins reconstituted into liposomes and added to elongating lizard RGC axons caused at most a transient collapse reaction. Growth cones always recovered within an hour and regrew.

Thus, lizard CNS myelin and oligodendrocytes possess nonpermissive substrate properties for DRG neurons—like corresponding structures and cells in the mammalian CNS, including mammalian-like neurite growth inhibitors. Lizard RGC axons, however, appear to be far less sensitive to these inhibitory substrate components and therefore may be able to regenerate through the visual pathway despite the presence of myelin and oligodendrocytes that block growth of DRG neurites. *GLIA* 22:61–74, 1998.

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INTRODUCTION

Glia cells surrounding central nervous system (CNS) fiber tracts in mammals and birds exert a mostly growth-inhibitory influence on axons and prevent axon regeneration after lesion (for review: Schwab et al., 1993; Schwab and Bartholdi, 1996). Axon regrowth is blocked by astrocytes and invading fibroblasts forming the glia scar (Reier and Houlé, 1988), as well as by CNS myelin and oligodendrocytes (Schwab et al., 1993). The latter possess molecules that interfere with axon growth, including the so-called neurite growth inhibitors (NI; Caroni and Schwab, 1988a), two proteins of 35 and 250

kD. Growth cones collapse upon contact with myelin, oligodendrocytes, or myelin-derived proteins (Bandtlow et al., 1990, 1993; Fawcett et al., 1989; Moorman and Hume, 1993; Schwab and Caroni, 1988) and this collapse-inducing activity of neurite growth inhibitors is partially neutralized by IN-1, an antibody against the

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35/250 kD proteins (Caroni and Schwab, 1988b). Axons can cross oligodendrocytes when IN-1 is added to neuron-glia cocultures (Bastmeyer et al., 1991; Caroni and Schwab, 1988b). Furthermore, application of IN-1 allows lengthy regrowth of some axons in the rat spinal cord (Schnell and Schwab, 1993; Weibel et al., 1994). Moreover, the mammalian myelin/oligodendrocyte-associated inhibitors can act across species boundaries and cause growth cone collapse of axons from a wide variety of neurons, including axons from cold-blooded vertebrates such as fish and frogs (Bastmeyer et al., 1991; Lang et al., 1995).

However, myelin and oligodendrocytes in the visual system of amphibians and fish—in which axons regenerate successfully (Gaze, 1970)—possess growth-permissive substrate properties, and the outcome of a series of experiments with neurons from rats, chicks, and amphibians speaks for the absence of neurite growth inhibitors from these pathways (Bastmeyer et al., 1991; Carbonetto et al., 1987; Vanselow et al., 1990; Wanner et al., 1995). CNS myelin and oligodendrocytes in the *Xenopus* spinal cord, where axonal regeneration fails to occur (Forehand and Farel, 1982), however, do possess mammalian-like neurite growth inhibitors (Lang et al., 1995).

These and other studies suggest a correlation between success and failure of axonal regeneration and absence or presence of neurite growth inhibitors on oligodendrocytes and CNS myelin. They also suggest that these inhibitors were recruited by oligodendrocytes during evolution, perhaps at the transition from urodeles to anuran amphibians (Lang et al., 1995). This predicts the presence of mammalian-like neurite growth inhibitors in species that—by criteria of evolution—are between fish, frogs, and birds and mammals, i.e., reptiles, and one might expect that their CNS axons would fail to regenerate. A recent study on the Ornate Dragon Lizard *Ctenophorus ornatus*, however, has shown that retinal ganglion cell (RGC) axons regenerate in lizards when the optic nerve is severed, but they failed to restore a retinotopic map so that the animals remained functionally blind (Beazley et al., 1997). The regenerating RGC axons grew along the normal pathways, suggesting that the glial cell environment—in contrast to the prediction above—is permissive rather than inhibitory to axon growth (Beazley et al., 1997). Alternatively, the axons that regenerate could be insensitive to nonpermissive substrate properties and inhibitors, as has been proposed to be the case with embryonic neurons (Bedi et al., 1992; Li and Raisman, 1993; Li et al., 1995). This view has recently been supported by testing the response of embryonic RGC and DRG neurites to myelin-associated inhibitors (Bandtlow and Lösinger, in press). Moreover, the cellular environment may change in consequence of the injury and acquire growth-permissive properties (Bedi et al., 1992; Perry et al., 1987; Sivron et al., 1994).

To address these problems, we have analyzed in this study the substrate properties of CNS myelin and oligodendrocytes in the visual pathway of the lizard

Gallotia galloti. We also tested whether growth cones of lizard RGC axons are sensitive to neurite growth inhibitors of mammals.

Our results show the nonpermissive substrate properties of CNS myelin and oligodendrocytes in lizards that block axonal growth of rat neurons and indicate the existence of mammalian-like neurite growth inhibitors. However, the sensitivity of lizard RGC axons to these nonpermissive substrates and to mammalian neurite growth inhibitors appears reduced compared with that of mammalian axons. This apparently allows lizard RGC axons to regenerate through nonpermissive territories.

MATERIALS AND METHODS

Animals

Adult lizards (*Gallotia galloti*) and fertilized eggs were collected on the island of Tenerife under licence and maintained in compliance with local legislation. Lizards were kept in large holdings tanks fitted with heaters and overhead lighting and fed on a mixed diet of commercially available cat food as well as a variety of live insects and fruit. Eggs were placed in boxes containing moist vermiculite and kept in an incubator at 22°C.

Optic Nerve Transections

The lizards were anesthetized on ice. An incision was made along the margin of the largest supraocular osteodermal plaque, to expose the optic nerve. The optic nerve was transected about 1 mm from the eye using iridectomy scissors, and care was taken not to injure the blood vessels supplying the retina. Following optic nerve transection, the osteodermal plaque was put back in place, and the lizards were returned to their holding tanks. Recovery was rapid, and the animals behaved normally. The lizards were left to recover for up to 12 months after the operation.

Cell and Tissue Culture

For glial cell cultures, lizard embryos of developmental stages 37 or older (after Dufaure and Hubert, 1961) were removed from their egg cases in sterile L15 medium and decapitated; the optic nerve and spinal cord were then dissected free. The tissue was cut into small pieces, transferred to 0.25% trypsin in Hanks' CMF (Sigma), and incubated at 37°C for 30 min by gentle agitation at 10 min intervals. Following trypsin digestion, the tissue was spun down, and L15 medium with 20% fetal calf serum (FCS) was added. The tissue chunks were triturated with a Pasteur pipette with a rounded tip (about 20 cycles). Remaining tissue fragments were allowed to settle, and the supernatant containing the dissociated cells was collected. Fresh

medium was added to the tissue and the trituration repeated. The resulting cell suspension was centrifuged (5 min, 900 rpm) and the pellet taken up in culture medium, consisting of F12/Dulbecco's modified Eagle's medium (DMEM) (1:1), supplemented with 5% FCS, 0.4% methyl cellulose (MC), 15 mM HEPES, 50 µg/ml bovine transferrin, 5 µg/ml bovine insulin, 100 µM putrescine, 30 nM sodium selenite, 20 nM progesterone, 15 nM triiodothyronine (all from Sigma), and 50 µg/ml gentamycin (Gibco). The glial cells were plated on polylysine/laminin-coated coverslips at a density of about 10^4 cells/coverslip and grown at 28°C in the presence of 5% CO₂.

Rat oligodendrocytes were obtained through a procedure modified after McCarthy and de Vellis (1980). Briefly, mixed glial cell cultures were prepared from cortices of neonatal rat pups and grown for 7–9 days in F12/DMEM medium containing 10% FCS. Oligodendrocytes and their precursors were then dislodged by shaking the primary cultures horizontally overnight (200 cycles/min, 37°C), pelleted, and resuspended in supplemented F12/DMEM medium. They were grown on polylysine/laminin-coated coverslips at a density of about 10^4 cells/coverslip.

Dorsal root ganglia (DRGs) were prepared from neonatal rat pups. They were dissected in L15 medium, freed of connective tissue and nerve roots, and cut into small pieces on a McIlwain tissue chopper. The fragments were resuspended in L15 containing 20% FCS and passed repeatedly through a Pasteur pipette with a rounded tip to dissociate the tissue mechanically. Remaining tissue aggregates were allowed to settle for 10 min, and the suspended cells were centrifuged briefly, taken up in L15 culture medium (modified after Mains and Patterson, 1973) supplemented with 5% FCS and 100 ng/ml nerve growth factor (NGF; Sigma), and cultured at 37°C.

Explants of adult lizard retina were prepared as described previously for goldfish (Bastmeyer et al., 1991; Lang et al., 1995; Wanner et al., 1995). Briefly, the retinae were dissected free from the sclera and flat-mounted with the ganglion cell layer up on nylon filters (Amersham). The retinae were then cut into strips 400 µm wide on a McIlwain tissue chopper and cultured, with the RGC layer facing the substrate, in modified L15 medium with 5% FCS at 28°C, in the presence of 5% CO₂.

For coculture assays of glial cells and axons, DRG neurons or retinal explants were added to lizard or rat glial cell cultures after 7 days in vitro (DIV). In functional assays, supernatant containing the monoclonal antibody (MAb) IN-1 specific for myelin-associated neurite growth inhibitors (NI; Caroni and Schwab, 1988b) was added to the cocultures diluted 1:3 in culture medium, as was the control MAb O4, specific for sulfatide (Sommer and Schachner, 1981).

To determine the extent to which preadsorption with lizard CNS myelin would reduce the neutralizing effect of IN-1-containing hybridoma supernatant, this super-

natant was incubated with lizard CNS myelin (50 mg myelin/ml supernatant) overnight at 4°C under gentle agitation. Then the myelin was removed by centrifugation and the hybridoma supernatant passed through sterile filtration and applied to rat oligodendrocytes (in coculture with DRG neurites) at a dilution of 1:3 in culture medium.

Cocultures of glial cells and neurons were subsequently observed under time lapse videomicroscopy, or analyzed after 3 DIV following fixation and immunostaining.

Myelin Preparation

Myelin of lizard optic nerve, goldfish CNS, and rat or bovine spinal cord was prepared as previously described (Bastmeyer et al., 1991; Wanner et al., 1995). The tissue was rapidly dissected free in ice-cold phosphate-buffered saline (PBS) and transferred to homogenization buffer [10 mM TRIS-HCl, 2 mM CaCl₂, 1 mM EDTA, 25 µg/ml aprotinin, 25 µg/ml leupeptin, 5 µg/ml pepstatin, 50 mM 2,3-dehydro-2-deoxy-N-acetylneuraminic acid, 5 mM iodoacetamide, and 1 mM spermidine (all from Sigma)]. After homogenization, fractions enriched in myelin were obtained through centrifugation at 23,000 rpm for 12 min at 4°C in a sucrose step gradient (15%, 25%, and 50% sucrose for lizard and rat myelin, 20% and 50% sucrose for goldfish myelin, all in homogenization buffer; modified after Colman et al., 1982). Myelin-enriched fractions were washed three times at 4°C in PBS containing protease inhibitors and the protein content determined by the Pierce-BCA method. The myelin fractions were used in outgrowth assays immediately after preparation.

Outgrowth Assay on Myelin

Freshly prepared myelin fractions were resuspended in PBS, centrifuged (30 min, 4,000 rpm) on polylysine-coated coverslips (100 µg myelin/cm²), with neonatal rat DRG neurons plated on top at low density (about 200 cells/cm²), and grown at 37°C. The myelin/polylysine-coated coverslips were preincubated (15 min, 37°C) with hybridoma supernatants containing MAb IN-1 against NI proteins. Controls were MAb O4, or hybridoma medium only [Iscove's minimum essential medium (MEM) with 5% FCS]. Hybridoma supernatants and antibodies remained present in the culture medium (1:3 dilution) throughout the culture period. After 36 h, cultures were fixed and subjected to immunostaining with an antiserum to L1 that recognizes DRG neurons. Labeled cells were recorded on videotape using an SIT camera, as described in Wanner et al. (1995). Only neurons with distinct processes longer than one cell diameter were included in measurements of neurite length.

Collapse Assay

Detergent extraction of CNS myelin proteins and their reconstitution into liposomes has been described in detail previously (Bandtlow et al., 1993; Rubin et al., 1995; Wanner et al., 1995). Myelin from bovine spinal cord was prepared as described above and osmotically shocked with ice-cold water. Myelin preparations were subsequently extracted with ice-cold 60 mM CHAPS, 20 mM TRIS (pH 8), 100 mM Na₂SO₄ in the presence of 1 mM PMSF, 1 mM EDTA, 2.5 mM iodoacetamide, 2 µg/ml aprotinin, 1 µg/ml pepstatin, and 1 µg/ml leupeptin. Extracted myelin proteins (200–500 µg) were combined with a tenfold excess of egg lecithin (grade 1, Lipid products). The mixture was put on ice for 1 h before liposomes were allowed to form by dialysis against PBS for 48 h at 4°C. Buffer was changed 3–4 times, and the last step was dialysis against modified L15 medium. For time lapse analysis of growth cone reactions, explants of lizard retinæ as well as rat DRG neurons were prepared as described above and cultured in 400 µl modified L15 with 5% FCS but without methylcellulose at 28°C and 37°C, respectively, on polylysine/laminin-coated coverslips.

Liposomes were applied to the cultures with a microliter syringe (Hamilton) directly into the medium. Growth cones were video-recorded for 10–15 min before adding the liposomes. Only growth cones that were motile were included in the experiment.

Time Lapse Videomicroscopy and Image Processing

Prior to time lapse video-recordings, cultures were transferred to bicarbonate-free medium. Growth cone reactions were observed under a Zeiss Axiovert microscope equipped with a heatable stage set at 28°C (for lizard RGC axons) or 37°C (for rat DRG neurites). Contacts of individual DRG growth cones and oligodendrocytes as well as growth cone responses in the collapse assay were monitored using a video camera (Newvicon) and the time lapse sequences stored on videotape after image processing (Hamamatsu DVS 3000). Single images were obtained from a video graphic printer (UP-860CE, Sony). Some of the photographs presented in this work were edited using the Adobe Photoshop software.

Immunohistochemistry

Immunostaining of cultured glial cells was performed as described previously (Lang and Stuermer, 1996). Briefly, cells were fixed in methanol (5 min, –20°C) followed by immersion into 4% paraformaldehyde (5 min, room temperature). After extensive washes in PBS, the cells were incubated with primary antibodies diluted in PBS containing 1% bovine serum albumin (BSA) for 2 h at 37°C. This was followed by at least

three washes in PBS and incubation (2 h, 37°C) with the appropriate secondary antibodies, which were donkey-anti-mouse, donkey-anti-rabbit, and rabbit-anti-rat IgG (heavy + light chain), conjugated to Cy3 (dilution 1:1,000). For double or triple stainings, these were combined with FITC- or AMCA-conjugated goat-anti-mouse and goat-anti-rabbit IgG antibodies (dilution 1:100, all secondary antibodies from Jackson Immuno Research). Finally, the cell cultures were rinsed thoroughly with PBS and coverslipped with Mowiol (Hoechst) containing n-propylgallate as anti-fading agent. Primary antibodies were MAb A2B5 recognizing O-2A progenitor cells in mammals (Eisenbarth et al., 1979, supernatant diluted 1:3), anti-GalC (Ranscht et al., 1982, 1:20) as well as O1 and O4 specific for myelin glycolipids (Sommer and Schachner, 1981, undiluted supernatants), rat MAb 6C2 raised against guinea pig myelin basic protein (MBP; gift of C. Linington, supernatant diluted 1:5), and rabbit antisera to myelin-associated glycoprotein (MAG; gift of M. Schachner, 1:500) as well as proteolipid protein (PLP; gift of C. Linington, 1:400). The antibodies A2B5, O1, O4, and GalC were added to cultures prior to fixation (30 min, room temperature) to stain live glial cells. To stain cultured neurons, either MAb SMI-31 specific for phosphorylated neurofilaments (Sternberger-Meyer, dilution 1:500) or a rabbit antiserum to the cell adhesion molecule L1 (gift of F. Rathjen, dilution 1:1,000) were used on methanol/paraformaldehyde-fixed cells.

Cryosections (10–30 µm) of lizard brain and optic nerve as well as of the rat spinal cord were cut on a Reichert-Jung cryostat, dried onto Vectabond (Vector Laboratories) treated glass slides, and fixed by immersion into methanol (5 min, –20°C). For immunostaining of myelin proteins, sections were fixed in Clarke's solution (95% ethanol with 5% glacial acetic acid for 25 min at 4°C; Rubin et al., 1994). After several washes with PBS, the sections were incubated (2 h, 37°C) with primary antibodies, which were rat MAb 6C2 to MBP, rabbit antisera to MAG and PLP, MAb SMI-31 (dilutions as above), and MAb IN-1 specific for NI proteins (undiluted supernatant). Following extensive washes with PBS, sections were exposed (2 h, 37°C) to the appropriate secondary antibodies [donkey-anti-mouse, donkey-anti-rabbit, and rabbit-anti-rat IgG (heavy + light chain), Cy3-conjugated, all diluted 1:1,000]. After rinsing in PBS, the sections were counterstained with the nuclear dye DAPI (Sigma) and coverslipped with Mowiol. Alternatively, primary antibodies bound to cryosections were incubated with biotinylated secondary antibodies and visualized by the reaction of bound avidin-horseradish peroxidase (HRP) complex with diaminobenzidine (DAB) according to the Vectastain (Vector Laboratories) method.

RESULTS

Antibodies against the major myelin marker proteins of birds and mammals (MAG, MBP, and PLP) recognize

myelinated fiber tracts in the lizard CNS, including the visual pathway. This is exemplified by anti-MBP immunostaining of the lizard brain (Fig. 1a). In corresponding sections the same myelinated tracts were IN-1 immunoreactive (Fig. 1b), suggesting that lizard CNS myelin contains the same major constituents typical of warmblooded vertebrates and amphibians and that it may also possess mammalian-like neurite growth inhibitors.

To test whether myelin and IN-1 immunoreactivity are present during retinal axon regeneration, the distribution of myelin marker proteins and neurofilaments was examined using the relevant antibodies on horizontal sections through the normal and lesioned optic nerve/tract (Fig. 2). Aside from retinal axons in the normal optic nerve/tract and the eye-side portion of the lesioned nerve, the neurofilament antibody SMI-31 recognized the regenerating retinal axons that appeared in the brain-side optic nerve/tract as late as 2 months after optic nerve transection (Fig. 2d). SMI-31-positive regenerating axons were not present 1 month after optic nerve transection but were identified in increasing numbers between 2 and 12 months after optic nerve lesion (not shown). Before and at the time when regenerating RGC axons were found, myelin persisted in the brain-side portion of the optic nerve and optic tract. Anti-MBP (as well as antibodies to MAG and PLP; data not shown) labeled these regions of the experimental side, at the same intensity as the uninjured myelinated axons in the control side (Fig. 2b). The eye-side part of the optic nerve, however, was MBP negative at 1–12 months after optic nerve transection. The MBP-labeled regions also exhibited IN-1 immunoreactivity in the experimental and control nerve (Fig. 2c), suggesting that regenerating RGC axons must have grown despite the persistence of myelin and (by immunostaining criteria with IN-1) the presence of neurite growth inhibitors.

To evaluate the substrate properties of lizard CNS myelin and oligodendrocytes directly, DRG neurons from neonatal rats were used since they are known to be sensitive to myelin- and oligodendrocyte-associated inhibitors (Bandtlow et al., 1993). The length of DRG neurites on myelin substrates derived from the lizard optic nerve, the rat CNS (an inhibitory substrate; Schwab and Caroni, 1988), or the goldfish CNS (known to possess permissive substrate properties; Wanner et al., 1995) was measured and compared with that on polylysine as a control. DRGs and their neurites were visualized by an antibody against L1, 36 h after plating. DRGs extended many and long neurites on polylysine as well as on goldfish CNS myelin. However, neurite length was markedly reduced on myelin of the lizard optic nerve and rat CNS (Fig. 3). When myelin was pretreated with IN-1, however, the length of neurites on lizard optic nerve myelin increased by about 50% and by more than 100% on rat CNS myelin (Fig. 3B). This increase in neurite length on IN-1-treated lizard optic nerve myelin was statistically significant ($\alpha < 0.005$ in Student's *t*-test). Control MAb O4 had no effect on

Fig. 1. IN-1 immunoreactivity in myelinated fiber tracts of the lizard CNS. Adjacent parasagittal sections were exposed to (a) MAb against MBP to visualize myelinated fiber tracts and (b) MAb IN-1. The distribution of MBP and IN-1 immunoreactivity is co-extensive, including the optic nerve (ON) and tract, and RGC axon fascicles in the upper layers of the optic tectum (OT). T, telencephalon; C, cerebellum; SC, spinal cord. Scale bar: 2.0 mm.

neurite length. Also, in the course of earlier experiments (Bandtlow et al., 1990; Lang et al., 1995; Wanner et al., 1995), the possibility that MAb IN-1 as such or when bound to a polylysine substrate would serve to promote growth of DRG neurites was excluded.

These results imply that myelin of the lizard optic nerve has nonpermissive substrate properties for rat DRG neurons, much as rat CNS myelin (Schwab and Caroni, 1988). MAb IN-1 partially neutralizes the growth-inhibiting properties of lizard optic nerve myelin. However, this neutralization is less efficient than that observed with rat CNS myelin (here and Caroni and Schwab, 1988b).

To investigate whether lizard oligodendrocytes, like their mammalian counterparts (Bandtlow et al., 1990; Fawcett et al., 1989), induce growth cone collapse, they were isolated from embryonic optic nerve or spinal cord and allowed to differentiate *in vitro*. Cultured lizard oligodendrocytes displayed features characteristic of differentiating oligodendrocytes of mammals. They developed from A2B5-immunoreactive precursor cells (Raff et al., 1983) with few processes (not shown) into highly branched oligodendrocytes, formed membrane sheets, and were immunoreactive with O4 and O1 antibodies, anti-GalC, anti-MAG, anti-MBP, and anti-PLP (Fig. 4). Highly branched oligodendrocytes were cocultured with rat DRG neurons on laminin as substrate. Growth cones approaching and making contacts with lizard oligodendrocytes were monitored using time lapse video recordings (Fig. 5).

Fig. 2. Persisting IN-1 immunoreactivity in the brain-side optic nerve and optic tract after optic nerve transection and during axon regeneration. Adjacent horizontal sections of the uninjured left and lesioned right optic nerve/tract, 3 months after optic nerve transection. The lesion site is marked by arrowheads. **a**: Phase contrast; sections were immunostained with MAbs **(b)** 6C2 against MBP, **(c)** IN-1 against myelin-associated inhibitors, and **(d)** SMI-31 against neurofilaments. MBP and IN-1 immunoreactivity are present throughout the normal optic nerve/tract and in the brain-side portion of the lesioned optic nerve and tract. The eye-side portion of the optic nerve is free of MBP and IN-1 immunoreactivity. **d**: Staining by SMI against neurofilament demonstrates the presence of many RGC axons in the normal optic nerve and in the eye-side portion of lesioned optic nerve, and also shows regenerating RGC axons in the brain-side optic nerve and tract. Scale bar: 500 μ m.

Upon contact with lizard optic nerve—as well as with spinal cord-derived oligodendrocytes—DRG growth cones collapsed and retracted in more than 70% of all encounters (Fig. 5B). Moreover, when these cocultures were fixed and immunolabeled after 3 DIV, highly branched oligodendrocytes were surrounded by neurites that apparently had avoided crossing the cells. This situation is similar to that observed with DRG neurons and rat oligodendrocytes (see Fig. 7A; Schwab and Caroni, 1988) or frog spinal cord oligodendrocytes (Lang et al., 1995). In the presence of MAb IN-1, collapse frequency in encounters of DRG neurites and lizard oligodendrocytes was reduced to about 25% (Fig. 5B). IN-1 specifically affected the interaction of DRG neurites with oligodendrocytes and had no visible effect on neurite outgrowth and elongation (see also Bandtlow et al., 1991; Lang et al., 1995). This shows that lizard optic nerve oligodendrocytes have nonpermissive substrate properties for rat DRGs, which is consistent with the outcome of experiments with lizard optic nerve myelin as a substrate. The finding that MAb IN-1

reduces the frequency of DRG growth cone collapse suggests that mammalian-like neurite growth inhibitors are present on lizard oligodendrocytes. Compared with its effect in mammals, the efficacy with which IN-1 neutralizes neurite growth inhibitors of lizard CNS myelin and oligodendrocytes is low, perhaps because of species-specific differences in the relevant epitope. When the hybridoma supernatant containing IN-1 was adsorbed with lizard CNS myelin and then applied to sections and rat oligodendrocytes, a small but significant reduction in staining intensity and neutralization of the growth-inhibiting effect of rat oligodendrocytes was observed. Myelinated tracts in rat spinal cord white matter were stained after exposure to the hybridoma supernatant, but the staining intensity was weaker after preadsorption (Fig. 6a,b). Moreover, when the hybridoma supernatant after preadsorption with lizard CNS myelin was applied to rat oligodendrocytes cocultured with DRG neurites, the number of oligodendrocytes being crossed by DRG neurites was reduced when compared with cocultures containing non-preadsorbed hybridoma supernatant (Fig. 6c). This difference is small but statistically significant (χ -square test, $\alpha = 0.004$), and thus confirms that lizard CNS myelin possesses neurite growth inhibitors related to those of the mammalian CNS.

Whether lizard RGC growth cones would collapse when encountering rat oligodendrocytes was investigated in another coculture experiment. The growth pattern of rat DRG neurons in coculture with rat oligodendrocytes served as control. Figure 7A (b,d) shows highly branched rat oligodendrocytes immunostained with anti-PLP. Rat DRG neurites in coculture with these cells and visualized by MAb SMI-31 were seen around the perimeter of the oligodendrocytes, but axons did not cross them (Fig. 7A, a). Lizard RGC axons (also stained by SMI-31), however, did grow across the oligodendrocytes (Fig. 7A, c,d), and growth cones are visible on the oligodendrocyte surface. Lizard RGC growth cones behaved similarly when contacting either rat or lizard oligodendrocytes. In time lapse recordings, collapse was rarely observed, and most growth cones freely crossed the oligodendrocytes (Fig. 7B). These observations strongly suggest that lizard RGC axons are far less sensitive to the inhibitory components of rat and lizard oligodendrocytes than rat DRG neurites, or most growth cones tested so far (but see Discussion).

The so-called collapse assay has been used successfully to monitor the reaction of individual growth cones when myelin proteins including the neurite growth inhibitors are reconstituted in liposomes and added to advancing growth cones (Bandtlow et al., 1993; Wanner et al., 1995). Bovine CNS myelin proteins reconstituted in liposomes elicit a rapid collapse reaction in rat DRG growth cones (Bandtlow et al., 1993; Wanner et al., 1995). When these liposomes were added to lizard RGC growth cones, most also collapsed and some even retracted. However, within 30–60 min, they redeveloped growth cones of normal morphology. Lamellipodia

A **B**

Fig. 3. Inhibition of neurite extension on a substrate consisting of reptilian CNS myelin and polylysine. **A:** (a) Rat DRG neurons extend many and long neurites on polylysine. (b) Neurite extension is inhibited on reptilian CNS myelin. (c) Goldfish CNS myelin allows growth of many and long DRG neurites. DRG neurons were visualized by staining with L1-antibodies. **B:** Quantification of DRG neurite lengths on substrates of polylysine and myelin. Striped bar, polylysine as the sole substrate; hatched bar, goldfish CNS myelin; open bars, lizard CNS myelin; solid bars, rat CNS myelin. HM, myelin treated with hybridoma medium only; O4, myelin treated with MAb O4; IN-1, myelin treated with MAb IN-1. The numbers of neurons are given on

top of each column, and the standard error of the mean is indicated. Neurite length is significantly reduced on lizard and rat CNS myelin as substrates compared with substrates consisting of polylysine and goldfish CNS myelin. In the presence of the control MAb O4, no increase in neurite length on lizard CNS myelin is observed. In the presence of IN-1 there is a more than 2-fold increase in neurite length on rat CNS myelin and a 1.5-fold increase on lizard CNS myelin. The increase in neurite length on IN-1-treated lizard myelin is statistically significant (bars marked *, $\alpha < 0.005$ by Student's *t*-test). Scale bar: 200 μ m.

and filopodia reappeared at the retraction bulbs and the growth cones began to re-elongate (Fig. 8A). These growth cones collapsed again upon renewed addition of

liposomes with myelin proteins and subsequently recovered. They thus resembled embryonic neurons from rats and birds in their behavior, which, in this very

Fig. 4. Characterization of lizard oligodendrocytes isolated from the CNS of lizard embryos. Oligodendrocytes develop a highly branched morphology and express myelin marker proteins as revealed by immunocytochemistry. They are (a) O4 and (b) GalC positive and express (c) MAG, (d) MBP, and (e) PLP. (f) Phase contrast image. Scale bar: 50 μ m.

collapse assay, show a transient collapse reaction and subsequently regrow (Bandtlow and Lösinger, in press). In contrast, most rat DRG growth cones did not recover over the 2 h observation period, and the neurites remained retracted (Fig. 8B).

This demonstrates that lizard RGC axons can respond to neurite growth-inhibitory proteins when they are exposed to these proteins in high concentrations. They can execute growth cone collapse, but they also possess cellular mechanisms for recovery, reformation of the growth cone, and regrowth.

DISCUSSION

This study demonstrates that retinal axons in adult lizards regenerate successfully despite the nonpermissive substrate properties of myelin and oligodendrocytes in the reptilian visual pathway, apparently because their sensitivity to the myelin/oligodendrocyte-associated growth-inhibiting components is less developed than in neurons from other adult or postnatal vertebrates.

In their ability to grow over oligodendrocytes—derived from their own CNS or from the rat—and by their recovery from myelin protein-induced collapse, regenerating RGC axons from lizards resemble embryonic axons from mammals and birds (Bandtlow and Lösinger, in press; Kobayashi et al., 1995).

Oligodendrocytes and myelin in lizards share morphological features and the major myelin proteins with

their mammalian counterparts, in vitro and in vivo (Knapp et al., 1987; Monzon-Mayor et al., 1990; Nadon et al., 1995; Yanes et al., 1992). That myelin and oligodendrocytes in the lizard visual pathway—normal and lesioned—represent nonpermissive and inhibitory substrates was evidenced by functional assays with rat DRG neurons, which are known to respond to myelin/oligodendrocyte-associated growth inhibitors (Bandtlow et al., 1993). Myelin derived from the lizard optic nerve (or from the entire CNS) in fact inhibits rat DRG neurite extension to the same extent as myelin derived from the rat CNS. Lizard oligodendrocytes elicit DRG growth cone collapse to the same extent as rat oligodendrocytes, and since application of MAb IN-1 partially neutralizes neurite growth inhibitors on lizard myelin and decreases the rate of oligodendrocyte contact-evoked collapse, we conclude that mammalian-like neurite growth inhibitors contribute to the nonpermissiveness of these glial cells for DRG neurites.

The results of the present study are in contrast to the view of Beazley and colleagues (1997), who recently demonstrated successful regeneration of retinal axons after optic nerve lesion in the lizard *Ctenophorus ornatus*, and who concluded: "It is clear that the lizard optic nerve rather than inhibiting axonal regeneration, is highly permissive" and "conductive to most axons attempting to regenerate."

The nonpermissive or inhibitory substrate properties of lizard CNS myelin and oligodendrocytes correlate with IN-1 immunoreactivity in myelinated CNS fiber tracts including the optic nerve. MAb IN-1, which was raised against a major growth-inhibitory myelin protein of the rat (Caroni and Schwab, 1988b), is not suited for the detection of these neurite growth inhibitors in Western blots (Lang et al., 1995, 1996; Wanner et al., 1995), and no other probes for demonstrating their presence are presently available. When applied to isolated glia cells in culture, bound IN-1 can be visualized on rat oligodendrocytes as staining of low intensity (Caroni and Schwab, 1988b; Lang et al., 1996), whereas significant immunostaining on cultured glial cells of nonmammalian species was not achieved (Lang et al., 1995). However, in previous immunostaining experiments on tissue sections through the nervous system of rats (Rubin et al., 1994) and amphibians (Lang et al., 1995), IN-1 selectively bound to myelinated fibers in the CNS, and myelin derived from immunopositive CNS fiber tracts proved to be a nonpermissive, growth-inhibiting substrate. Moreover, in *Xenopus*, IN-1 stains myelinated tracts of the hindbrain and spinal cord but does not label the *Xenopus* optic nerve and forebrain, nor myelin in the peripheral nervous system (Lang et al., 1995). This correlates with the nonpermissive, inhibitory substrate properties of CNS myelin and oligodendrocytes in the *Xenopus* hindbrain/spinal cord and the growth permissiveness of the corresponding structures and glial cells of the *Xenopus* optic nerve/forebrain (Lang et al., 1995) and peripheral nervous system (Lang and Stuermer, 1996). Since MAb IN-1 stains CNS myelin in so distantly related species as

A **B**

Fig. 5. DRG growth cones collapse upon contact with lizard oligodendrocytes. **A:** Selected image of a 3 h continuous time lapse videorecording. (a) To the left is a rat DRG neuron that gives rise to numerous neurites. DRG neurites whose growth cones have collapsed upon contact formation with the glial cell and the retraction bulbs are marked by arrowheads. None of the DRG neurites has crossed the glial cell. (b) This oligodendrocyte is O1 positive. **B:** Quantification of the reactions of rat DRG growth cones upon encountering lizard oligodendrocytes. Solid bars, collapse; striped bars, avoidance; open bars,

growth across the cell. ON, optic nerve-derived oligodendrocytes; SC, spinal cord-derived oligodendrocytes; IN-1, oligodendrocytes treated with MAb IN-1. The number of growth cones monitored in time lapse videorecordings is given on top of each bar, and their relative number is expressed in percent. Optic nerve- as well as spinal cord-derived lizard oligodendrocytes elicit growth cone collapse in more than 70% of the encounters. In the presence of MAb IN-1, the collapse frequency is reduced to about 25%, and the majority of the growth cones grow around the cell. Scale bar: 100 μ m.

amphibians and mammals and selectively in those CNS regions where functional tests confirm that inhibitors are present and neutralized by IN-1, IN-1 immunoreactivity in the lizard brain is likely to be indicative of the presence of mammalian-like neurite growth inhibitors in the CNS myelin of this species. The outcome of the present functional assays using lizard optic nerve myelin and oligodendrocytes as substrates for inhibitor-sensitive neurons, i.e., neonatal rat DRGs (Bandtlow et al., 1993; Wanner et al., 1995), lends further support to this view. MAb IN-1 improves the nonpermissive substrate properties of CNS myelin and oligodendrocytes from various species, as was evidenced by the response of inhibitor-sensitive neurons and 3T3 cells (Bandtlow et al., 1990; Bastmeyer et al., 1991; Caroni and Schwab, 1988b; Lang et al., 1995). This antibody, however, did not alter the growth of neurites or improve cell adhesion on other substrates, nor did it on its own serve as

an adhesive or growth-promoting substrate. These observations support the view that the observed effect of IN-1 results from specific neutralization of the neurite growth inhibitors.

The gain in DRG neurite length on IN-1-treated rat CNS myelin is about 2-fold and about 1.5-fold in case of IN-1-treated lizard CNS myelin. Furthermore, although the number of collapsing DRG growth cones upon contact with lizard oligodendrocytes was reduced to 25% in the presence of MAb IN-1, growth cones encountering IN-1-treated oligodendrocytes avoided the cells by growing around instead of crossing them. Hybridoma supernatant containing IN-1 lost some of this neutralizing activity with rat oligodendrocytes after preadsorption with lizard CNS myelin. The outcome of these tests suggests that inhibitors in the lizard are present but less efficiently blocked by MAb IN-1 than in mammals, perhaps because of species-specific

Fig. 6. Activity of IN-1 after preadsorption with lizard CNS myelin. **a, b:** Cross sections through the adult rat spinal cord immunostained by IN-1-containing supernatant (a) and after preadsorption with lizard CNS myelin (b). There is a slight reduction in staining intensity over myelinated tracts in spinal cord white matter after preadsorption (b, compared with a). **c:** Rat oligodendrocytes co-cultured with rat DRG neurites were exposed to O4 antibodies (O4), IN-1-containing hybridoma supernatant (IN-1), and IN-1-containing hybridoma supernatant after preadsorption with lizard CNS myelin [IN-1 (PA)]. The

number of oligodendrocytes (on top of each pair of bars) being avoided (black bars) or being crossed (white bars) was determined and expressed in percent. In cultures treated with IN-1 (PA) the number of oligodendrocytes that are crossed by DRG neurites is reduced compared with the number of oligodendrocytes being crossed when the non-preadsorbed (IN-1) supernatant is present. This difference (sets marked *) is statistically significant (χ -square test, $\alpha = 0.0004$). O4-treated cultures served as controls. Scale bar: 1 mm.

differences in protein sequence or structure (Lang et al., 1995) and supports the notion that neurite growth inhibitors are not the only molecules responsible for the nonpermissive substrate properties of lizard oligodendrocytes. MAG, present in the lizard CNS, has been proposed to block the growth of certain types of neurons (McKerracher et al., 1994; Mukhopadhyay et al., 1994), which is, however, not the case with neonatal rat DRG neurons. Other molecules that can interfere with neurite elongation are janusin (Pesheva et al., 1989) and certain proteoglycans (e.g., Dou and Levine, 1994). While these and other components may affect rat DRG growth cones, they apparently do not hinder the growth of lizard RGC axons across mammalian or lizard oligodendrocytes.

With myelin being a nonpermissive substrate (Schwab and Caroni, 1988), axon regeneration can be successful if myelin is rapidly removed from lesioned tracts. This occurs in the visual system of fish (Dowding et al., 1991) and amphibians (Lang et al., 1996; Wilson et al., 1992). In the reptilian visual system, the long-lasting persistence of MAG, MBP, and PLP immunoreactivity—which is temporally and spatially co-extensive with IN-1 immunoreactivity—indicates that the timely degradation and removal of myelin and its neurite growth inhibitors has not occurred, which, however, does not prevent regeneration of retinal axons through the lizard optic nerve. The ability of lizard RGC growth

cones to regenerate through nonpermissive myelin debris-containing territories, to extend along aberrant pathways into the intact contralateral optic nerve (unpublished observations) in vivo, and to grow across oligodendrocytes in culture supports the conclusion that these axons are less sensitive to these substrates and their inhibitors.

On the other hand, lizard RGC growth cones are not entirely insensitive to myelin-associated inhibitors since most collapse when contacted by liposomes containing myelin proteins enriched in neurite growth inhibitors, but they recover in less than an hour and continue to advance. Growth cone collapse elicited by neurite growth inhibitors was proposed to occur through receptors that upon ligand binding transduce signals into the cell (Bandtlow et al., 1993) and after collapse effect permanent growth cone arrest. Therefore it seems that reptilian RGCs possess fewer receptors for myelin-derived growth inhibitors, or the signaling cascade effecting permanent collapse and growth arrest (Bandtlow et al., 1993) is either incomplete or blocked.

A recent analysis of the reaction of embryonic rat DRG and chicken RGC neurons (Bandtlow and Löscher, in press) in similar collapse assays has shown that growth cones of embryonic neurons collapse but subsequently recover and grow. The neurite growth inhibitors induce elevation of the intracellular Ca^{2+} concentration in growth cones (Bandtlow et al., 1993),

Fig. 7. Lizard RGC axons grow across oligodendrocytes. **A:** Rat DRG neurons and lizard RGC axons were co-cultured with differentiated rat oligodendrocytes. Rat oligodendrocytes were immunostained by anti-PLP (b, d), and the DRG neurites (a) and lizard RGC axons (c) by the neurofilament antibody SMI-31. Rat DRG neurites avoided crossing rat oligodendrocytes (a, b), but lizard RGC growth cones (marked by arrowheads) do grow across these cells (c, d). **B:** Quantification of lizard RGC growth cone behavior upon contact with rat and lizard

oligodendrocytes. Solid bars, collapse; striped bars, avoidance; open bars, growth across the cell. Rat, rat brain oligodendrocytes; lizard, lizard optic nerve oligodendrocytes. The number of monitored growth cones is given on top of each bar, and their relative number is expressed in percent. The majority of lizard RGC growth cones grow across oligodendrocytes whether derived from the rat (60%) or the lizard (76%) CNS. Scale bar: 50 μ m.

which is, however, of much shorter duration in embryonic growth cones than in growth cones of older animals (Bandtlow and Löscher, in press). A long-lasting elevated Ca^{2+} concentration may therefore be needed to trigger downstream signaling cascades that effect permanent growth arrest (Bandtlow and Löscher, in press).

The ability of embryonic growth cones to recover after collapse may explain why embryonic axons are able of growing along heavily myelinated tracts in tissue slices of adult brains (Shewan et al., 1995) or after transplantation into the adult CNS (Davies et al., 1994; Li and Raisman, 1993; Li et al., 1995) and accounts for the observation that embryonic chick RGC axons can grow across rat oligodendrocytes in vitro (Kobayashi et al., 1995). Similar criteria may apply to regenerating RGC axons from adult lizards, but the underlying molecular mechanisms have not been explored. Receptors and intracellular signaling pathways appear to mature in

parallel with the onset of myelination in mammals and birds (Bandtlow and Löscher, in press). If lizard RGC axons acquire mature receptors and an efficient signal transduction machinery during development—which has not been examined—they obviously downregulate them upon injury and during regeneration. This contrasts to fish (Wanner et al., 1995), amphibians (Lang et al., 1995), and mammals (e.g., Bandtlow et al., 1990, 1993), in which the presence of fully mature receptors is evidenced by functional in vitro assays demonstrating that growth of regenerating RGC axons is arrested by the neurite growth inhibitors. On the other hand, the fact that lizard RGC axons do regenerate implies that the lesioned visual pathway also provides growth-promoting molecules, e.g., N-CAM (unpublished observations), and that lizard retinal axons (re)acquire the relevant receptors and signaling cascades for axon regrowth. Such growth-promoting cues may help override the influence of inhibitory molecules,

A **B**

Fig. 8. Collapse assay with myelin proteins from the mammalian CNS and lizard RGC growth cones. **A:** Selected images of continuous time lapse videorecordings showing lizard RGC growth cones (marked by arrowheads) before the addition of liposomes containing bovine CNS myelin proteins (1, 2). When liposomes are added and contact the growth cones (3), growth cones retract their filopodia and lamellipodia and form retraction bulbs. Within 30–60 min, growth cones reform (4). They collapse again (5) upon a renewed addition of liposomes and reform within the next hour (6). **B:** Quantification of growth cone behavior. Solid bars, collapse; open bars, transient collapse or no collapse. DRG, neonatal rat DRG neurons; RGC, lizard retinal ganglion cells. The number of growth cones whose behavior was monitored is given on top of each bar and their relative numbers expressed in percent. The majority of rat DRG growth cones (69%) collapse after liposomes with bovine CNS myelin proteins are added and do not recover over the 2 h observation period. The majority of lizard RGC growth cones (94%), however, show either no collapse or a transient collapse response. Scale bar: 50 μ m.

which otherwise interfere with growth cone elongation (Mohajeri et al., 1996).

In comparison with other lower vertebrate species, in which retinal axons regenerate spontaneously after optic nerve transection (Grafstein, 1986; Stuermer et

al., 1992), the onset and progression of RGC axonal regeneration in lizards is slow. Also, when segments of the retina are placed on growth-supportive substrates in vitro, few regenerating axons appear at 2 weeks after optic nerve transection, and maximum outgrowth oc-

curs between 2 and 3 months after nerve transection (unpublished observations). This speaks for a delayed activation of the cellular mechanism underlying axonal regeneration, which is paralleled by the persistence or delayed removal of myelin in the lesioned visual pathway.

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