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Spinal cord injury induces expression of RGS7 in microglia/macrophages in rats

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Abstract

RGS proteins regulate G protein-mediated signalling pathways through direct interaction with the Gα subunits and facilitation of GTP hydrolysis. An RGS subfamily consisting of RGS 6, 7, 9, and 11 also interacts with the G protein β subunit $G\beta_5$ via a characteristic Gγ-like domain. Thus far, these complexes were found only in neurons, with RGS7 being the most widely distributed in the brain. Here we confirm the expression of RGS7 in spinal neurons and show as a novel finding that following an experimental spinal cord injury in rats, expression of RGS7 is induced in a subpopulation of other cells. Immunofluorescent confocal microscopy using a series of cell specific antibodies identified these RGS7 positive cells as activated microglia and/or invading peripheral macrophages. To rule out interference from the adjacent neurons and confirm the presence of RGS7-$G\beta_5$ complex in inflammatory cells, we performed immunocytochemistry, RT-PCR, Western blot, and immunoprecipitation using microglial (BV2) and peripheral macrophage (RAW) cell lines. Expression of RGS7 mRNA and protein are nearly undetectable in non-stimulated BV2 and RAW cells, but remarkably increased after stimulation with LPS or TNF-α In addition, RGS7-positive cells were also found in the perinodular rim in the rat spleen. Our findings show that RGS7-$G\beta_5$ complex is expressed in immunocompetent cells such as resident microglia and peripheral macrophages following spinal cord injury. This expression might contribute to the post-traumatic inflammatory responses in the central nervous system.

Introduction

G-protein coupled receptors are the largest family of cell-surface receptors and mediate numerous functions essential for maintaining homeostasis of the organism. Heterotrimeric G proteins consist of an α, β, and γ subunit. The Gα subunit binds guanine diphosphate (GDP). Receptor-mediated activation of the Gα subunit facilitates the release of bound GDP allowing guanine-triphosphate (GTP) to bind. This causes the Gα subunit to dissociate from the tightly associated βγ-complex (Birnbaumer, 1992). The active Gα-GTP complex as well as free βγ, modulate effector enzymes and ion channels, altering the intracellular concentration of second messengers. This activation lasts until hydrolysis of GTP occurs.

The rate of GTP hydrolysis is controlled by a family of accessory proteins called regulators of G protein signalling (RGS) that act as GTPase activating proteins (GAP) for the Gβ subunits (Berman *et al*., 1996; Hunt *et al*., 1996; Watson *et al*., 1996). The RGS family consists of approximately 30 members and share a conserved RGS box, a 120-amino acid domain which is responsible for the binding of Gα and GTP hydrolysis. RGS7 is a member of the neuron-specific RGS subfamily (RGS 6, 7, 9, 11). This subfamily is characterized by the presence of two additional structural domains. The first is the Gγ-like (GGL) domain which interacts with $G\beta_5$ (Snow *et al*, 1998; Levay *et al*., 1999). The second is the DEP domain which is also found in a number of signalling molecules, but its function is yet unknown (Ponting & Bork, 1996). The structure and functional activity of $G\beta_5$ is markedly different from the other four $G\beta_{1-4}$ isoforms. $G\beta_5$ was previously only detected in mammalian brain and retina (Watson *et al*., 1994; Watson *et al*., 1996; Cabrera *et al*., 1998; Makino *et al*., 1999; Witherow et al. 2000; Zhang et al. 2001). *In vitro*, $G\beta_5$ associates Gγ subunits, but *in vivo* it is only found in a tightly associated complex with GGL-containing RGSs, with dimerization being necessary to stabilize both $G\beta_5$ and RGS (Cabrera *et al*., 1998; Chen *et al*., 2000; Witherow *et al*., 2000; Zhang & Simonds 2000; Chase *et al*., 2001; Zhang *et al*., 2001).

Spinal cord injury (SCI) initiates a robust immune response characterized in part by the synthesis of cytokines and chemokines as well as the coordinated infiltration of peripheral leukocytes (Acarin *et al*, 2000; Schwab & Bartholdi, 1996; Bartholdi & Schwab, 1997; Klusman & Schwab, 1997; Popovich *et al*, 1997; Bethea *et al*., 1998; Bethea *et al*., 1999; Hayashi *et al*., 2000). SCI-induced inflammation may result in a further deterioration of functional outcome due to the development of scar tissue and necrosis or apoptosis of neurons and oligodendrocytes. However, the exact mechanism, and consequences of a SCI-induced inflammatory response with activation of central nervous system (CNS) resident microglia and recruitment of blood-born inflammatory cells is not fully understood at this time.

Previous studies have demonstrated that RGS7 may play a role in tumour necrosis factor-α (TNF-α)-mediated immune responses

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within the CNS (Benzing *et al*., 1999). TNF-α prevents proteasome-dependent degradation of RGS7 via a stress-activated protein kinase p38-mediated mechanism (Benzing *et al*., 1999).

In this study, we investigated if SCI alters RGS7 expression in the spinal cord. We demonstrate that RGS7 and $G\beta_5$ are not only expressed in neurons but following injury this expression also occurs in microglia/macrophages. These data suggest that RGS7 may be an important mediator of SCI-induced activation of the post-traumatic inflammatory cascade.

Materials and methods

Experimental paradigm

Adult female Fischer rats underwent moderate contusive SCI as previously described (Bethea *et al*., 1998; Bethea *et al*., 1999). Spinal cords were harvested 1, 3, 7, and 14 days after surgery. At each time point, six animals were killed; the normal non-injured control group consisted of six animals. Spinal cords were dissected and tissues prepared for histological, immunohistochemical, and quantitative protein studies.

Surgery

All surgical procedures were approved by the Department of Veterinary Resources of the University of Miami. The rats were anaesthetized with 1% halothane (in 60 : 40 oxygen : nitrogen). As antibiotic prophylaxis penicillin G was given. A laminectomy was performed on the 8th thoracic vertebra (T8) under a stereomicroscope. A moderate spinal cord contusion was performed using the NYU impactor weight-drop device (Basso *et al*., 1996; Bethea *et al*., 1998; Bethea *et al*, 1999). The vertebral column was stabilized using clamps on T7 and T9 spinal processes, and a 10-g weight rod was dropped from 12.5 mm height onto the exposed dorsal aspect of the spinal cord. The rod was immediately removed, and the muscles and the skin were closed. SCI data (e.g. impact velocity and compression depth) were recorded and analyzed (NYU spinal cord contusion system, impactor software version 7.0) as described previously (Basso *et al*., 1996). The analysis of the impact data showed a successful contusion in all cases.

Immunohistochemistry

For immunohistochemical studies, the rats were euthanized with an overdose of rat cocktail (ketamine 42.8 mg/mL, xylazine 8.6 mg/mL, acepromazine 1.4 mg/mL) and pericardially perfused with a 0.1 M phosphate buffer (PB) solution containing heparin (50 000 IU/L), followed by ice-cold 4% paraformaldehyde in 0.1 M PB, pH 7.4. The spinal cords and spleens (control tissue for immunocompetent cells) were dissected and post-fixed overnight in the same fixative. All tissue samples were cryo-preserved with buffered 25% sucrose. Spinal cords were divided into cervical, thoracic, and lumbar segments and stored at 4 °C until further processing. Serial free-floating sagittal sections were cut frozen on the sliding microtome at 40 μm thickness throughout the thoracic, cervical, and lumbar spinal cord. The sections were stored at –20° in cryo-protective solution (30% sucrose and 30% ethylene glycol in 0.1 M PB) until further processing.

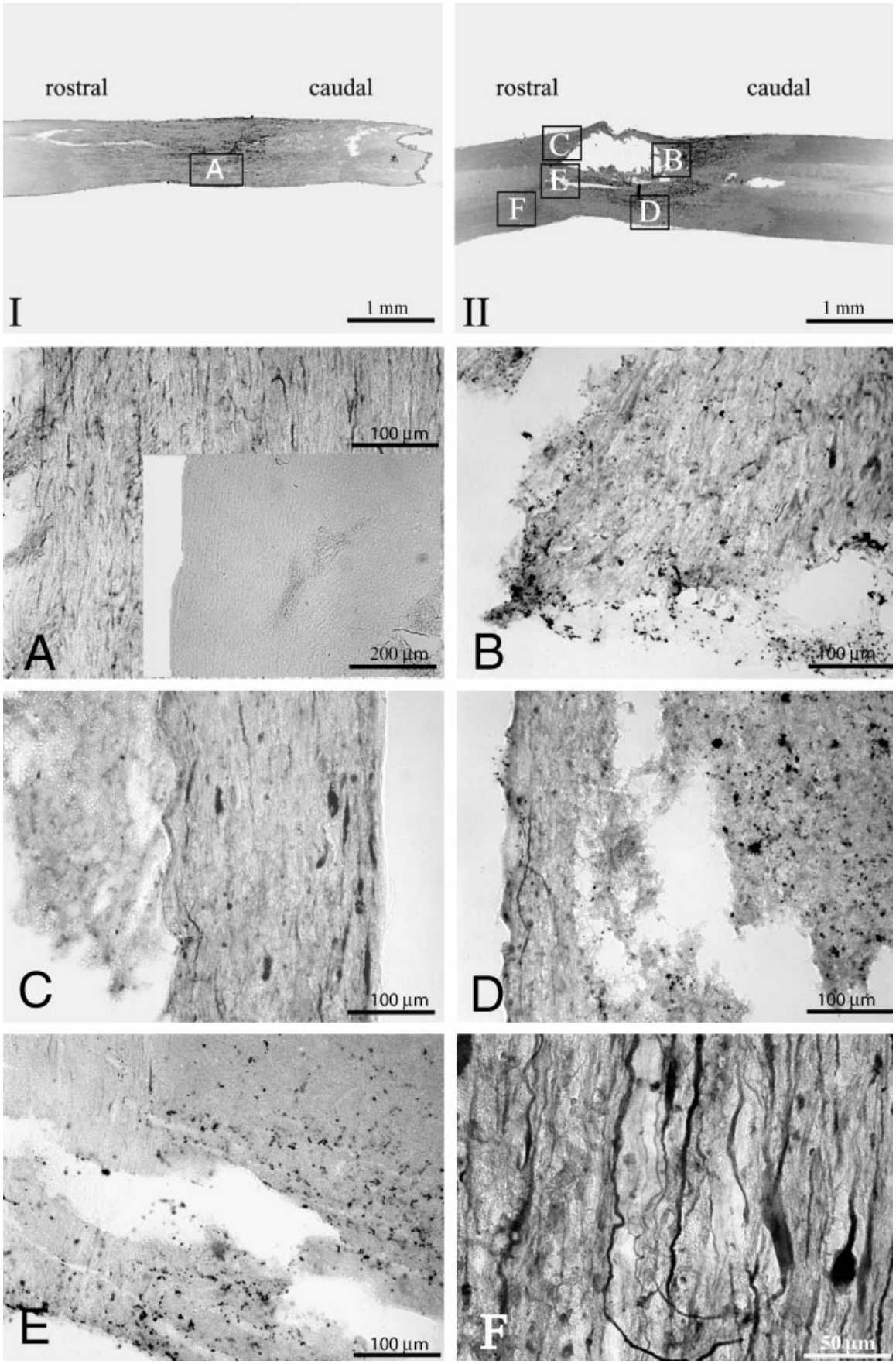
Purified polyclonal rabbit anti-RGS7 and $G\beta_5$ antibodies were raised against specific peptides, as previously described (Cabrera *et al*., 1998; Levay *et al*., 1999; Witherow *et al*., 2000). Antibody specificity was tested by peptide blocking experiments. As a negative control, the primary antibody was omitted.

To detect the expression of RGS7 protein in the rat spinal cord, serial sections were stained with the anti-RGS7 antibody. Endogenous peroxidases were quenched with 0.3% hydrogen peroxide for 30 min and nonspecific antigens were blocked with a mixture of 5% normal goat serum and 2% normal rat serum. Permeabilization of the cell membranes was achieved by adding 0.4% Triton X-100 (TX) (Sigma Chemicals, St Louis, MO) to preincubation and incubation solutions. All solutions were made in saline PB (PBS). After preincubation, sections were incubated with anit-RGS7 antibody (1 : 5000) overnight at 4 °C. After several washes in PBS containing 0.4% TX, tissue sections were incubated for 1.5 h with secondary antibodies (biotinylated goat anti-rabbit IgG) 1 : 200, followed by a series of rinses and 1.5 h incubation with Avidin Biotin Complex (ABC *Elite*) 1 : 100 (biotinylated IgG and Vectastatin ABC *Elite* standard kit, Vector Laboratories, Burlingame, CA). Reaction was developed with nickel intensified DAB (0.05% of 3,3-diaminobenzidine (DAB, Sigma Chemicals), 2.5% nickel ammonium sulphate in 0.05 M acetate, 0.002 M imidazole buffer at pH 7.2) and 0.001% hydrogen peroxide.

Immunofluorescence

To demonstrate the localization of the RGS7 protein in a variety of cell types in non-injured and injured spinal cord, alternative serial sections were double labelled with anti-RGS7 and various neuronal, glial, and immune cell markers. All sections were quenched in 2% sodium para-periodate (Sigma Chemicals) to reduce nonspecific background and enhance fluorescent signal, blocked with preimmune serum and permeabilized with TX (PBS + 5% normal goat serum + 2% normal rat serum + 0.4% TX). Sections were incubated with the anti-RGS7 antibody 1 : 100 overnight at 4 °C. To demonstrate the RGS7-$G\beta_5$ complex in cell cultures, anti-$G\beta_5$ antibodies were used at 1 : 200 (the sensitivity of these antibodies was reduced in tissue, therefore we used it only for *in vitro* immunocytochemistry). Following several rinses in PB, sections were incubated with goat anti-rabbit IgG conjugated to Alexa 488 (green) (Molecular Probes, Eugene, Oregon). After first antibody labelling, tissue was divided into series labelled for neuronal, glial, and immune cell markers. Neurons were stained with mouse monoclonal antineuronal nuclei (NeuN) 1 : 200 (Chemicon International, Temecula, CA). To visualize the cytoskeleton of neurons, mouse monoclonal anti-β-tubulin III (TuJ1) 1 : 300 (Sigma Chemicals) and anti-neurofilament M (145 kDa) 1 : 200 (Chemicon International). Astrocytes were labelled with a mouse monoclonal anti-glial fibrillary acid protein (GFAP) 1 : 400 (Chemicon International), oligodendrocytes and schwann cells with mouse monoclonal anti-CNPase antibody 1 : 400 (Sigma Chemicals) and RIP 1 : 40 (hybridoma developed by S. Hockfield, obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA). To demonstrate the presence of microglia and peripheral phagocytic macrophages, the following antibodies were used: mouse monoclonal anti-rat ARU 0151 1 : 400 against cytoplasmatic ED-I (Biosource International, Camarillo, CA) and mouse monoclonal anti-rat OX-42 1 : 400 against CD 11 membrane protein Cd3 receptor (PharMingen, San Diego, CA). Secondary antibodies were conjugated with Alexa 594 (red).

The slides were studied under Zeiss Axioplan 2 Fluorescence and Zeiss confocal LSM 510 microscopes (Carl Zeiss, Thornwood, NY). The image assembly (Adobe Photoshop) and illustration (Adobe Illustrator) software programs were obtained from Adobe Systems, Inc., San Jose CA. Confocal scanning settings were not changed nor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was the staining intensity enhanced by subsequent picture processing for each individual antibody.

Analysis of RGS7 expression in spinal cord

For Western blot studies, animals were anaesthetized in a chamber with halothane and decapitated. The cervical, thoracic, and lumbar spinal cords were removed by hydrostatic pressure and the cords were frozen instantly on dry ice. All samples were stored at –80 °C until further processing.

The tissue was homogenized in the buffer containing 20 mM Tris pH 7.5, 100 mM NaCl, 1 mM DTT and 1 mM PMSF at the total protein concentration of 5 mg/mL then 20 µL were mixed with 20 µL of 2× SDS PAGE sample buffer resolved on a 10% SDS gel, transferred to a nitrocellulose filter, probed with RGS7 antibody and developed using enhanced chemoluminiscent as described previously (Witherow *et al*., 2000). The film was scanned and analyzed by Scion Image (Imagequant Software). For quantitative comparison of RGS7 levels in different regions after injury or in control, the samples were subjected to serial dilutions in order to achieve a linear dependence of ECL signals from the amount of protein loaded on the gel; each dilution was analyzed in duplicate. All Western blots were normalized to β-actin.

Cell culture

The mouse peripheral macrophages cell line RAW 264.7 and a mouse microglia BV2 cell line were maintained in high glucose Dulbecco's modified Eagle medium containing 10% fetal bovine serum, 100 µg/mL Penicillin G and 100 µg/mL Streptomycin (Gibco BRL, Grand Island, NY) at 37 °C and 5% CO_2. For fluorescent staining the cells were seeded on 8-well plastic chamber slides. For Western blot and polymerase chain reaction (PCR) cells were seeded (cell density: 500 000 per well) into 6-well dishes. RAW and BV2 cells were stimulated with 10 ng/mL TNF-α (R & D Systems, Minneapolis, MN) and 1 mg/mL lipopolysaccahride (LPS) (Sigma Chemicals) for 1, 6, 12, 24, and 48 h.

As rat sympathetic PC12 pheochromocytoma cells are known to express RGS7 and $G\beta_5$ (Zhang *et al*., 2001), this cell line was used as a positive control for immunocytochemistry. As an additional unbiased control for immunocytochemistry, human embryonic kidney (HEK) 293 T cells were transfected with RGS7 cDNA using a standard Lipofectamine protocol described previously (Witherow *et al*., 2000).

Detection of RGS7 mRNA by RT-PCR in RAW cells

The expression of RGS7 mRNA in naïve and LPS or TNF-α stimulated RAW cells was confirmed by reverse transcription and polymerase chain reaction (RT-PCR). RNA was isolated with Ultraspect™ (Biotecx Laboratories Inc., Houston TX) and treated with DNAase before RT. For the standard RT reaction 2 ng of RNA was used.

Primers set for sense (5′-CCATGGCTACTTCTTTCCCA-3′) and antisense (5′-CTAGCCTTGCCTTGTTTTGC-3′) (AlphaDNA, Montreal, Canada) were used. PCR conditions were as follows: 94° C for 30 s, 55° C for 45 s, and 72° C for 60 s for 35 cycles.

Immunoprecipitation

Immunoprecipitation with RGS7 antibody was carried out using Protein A Sepharose as described in detail previously (Witherow *et al*., 2000). RAW cells were stimulated with TNF-α (25 ng/mL)

TABLE 1. Semi-quantitative description of RGS7 expression after SCI

	RGS7 expression			
Segment	No injury	Day 1	Day 3	Day 7
Cervical	+	+	++	+
Thoracic	+	++	+++	+++
Lumbar	+	+	++	+

Semi-quantitative evaluation of the time-course of the RGS7 expression shows an increased intensity of the immunohistochemical DAB staining 1, 3, and 7 days after SCI (Fig. 1A–E). Criteria for the blinded evaluation were as follows. (+) Baseline immunoreactivity with staining of the grey and white matter. The shape of neurons in the grey can be differentiated. The white matter staining is characterized by a slight staining of the fibers mesh-work. (++) Increased intensity of the DAB staining of the neurons and also of the intercellular space in the grey matter. In the white matter, the majority of fibers stain distinctively with DAB, however, axonal end bulbs may not be seen. (+++) Appearance of RGS7 strongly positive cells in the grey and white matter adjacent to the lesion. The staining intensity of these cells is much higher compared to the DAB staining of neurons in the grey matter in uninjured and injured tissue. Intensively DAB positive axonal end bulbs are in the white matter.

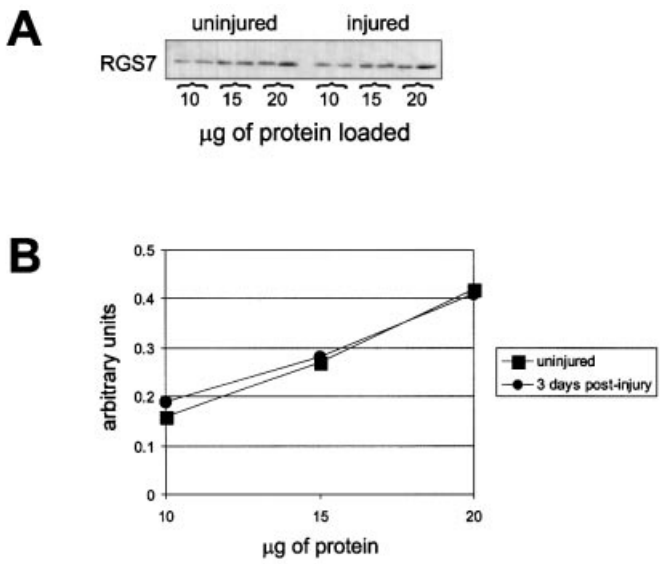
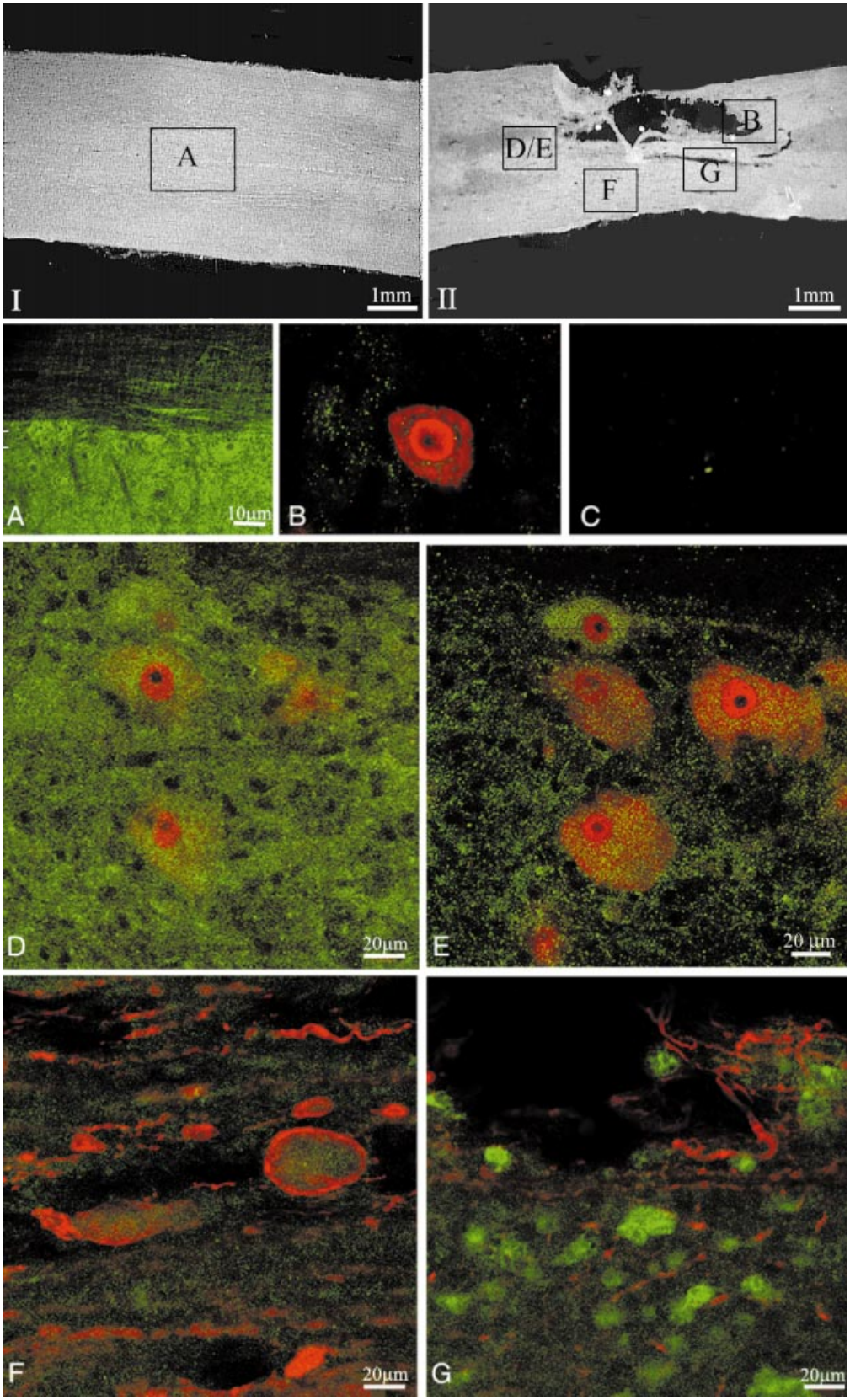


FIG. 2. Western blots of non-injured and injured spinal cords. (A) Western blots of non-injured and injured spinal cords (3 days post-injury) with increasing protein concentrations. (B) Quantitative analysis of the data presented in A each data-point was obtained in duplicate. The amount of RGS7 protein remains stable in non-injured and injured tissue. The figure is representative of five independent experiments.

FIG. 1. Time-course of RGS7 expression (DAB staining) of non-injured and injured spinal cord. Low power magnification of uninjured (I) and injured tissue (II) containing boxed inserts, which indicate regions used in A–F. Neurons within the grey matter are stained weakly, whereas fibers of the white matter are stained intensely. Note that in the injured tissue some fibers are enlarged. These axonal swellings may represent post-traumatic swelling of fibers. Intensely DAB positive cells are seen adjacent to the lesion in the grey and white matter. A post-traumatic pial enhancement is seen beginning one day after injury. (A) Uninjured tissue, white matter, box: negative control, white matter. (B) One day post-injury, white and grey matter. (C) Three day post-injury, white and grey matter. (D) Seven days post-injury, white and grey matter. (E) Fourteen days post-injury, white and grey matter. (F) White matter three days post-injury, note swollen axonal end bulbs.

24 h before collection. RAW cells were homogenized in PBS containing protease inhibitor mixture (Roche Molecular Biochemicals, Indianapolis, IN). The lysates were spun in a table-top centrifuge at 4 °C for 20 min. The pellet was resuspended in PBS containing 1% Triton-X 100 for 30 min. The cleared lysate (500 µL) was mixed with the antibodies and 40 µL of Protein beads overnight.



The beads were washed with 20 mM Tris-HCl pH 7.4, 50 mM NaCl, 1 mM EDTA, and 1 mM EGTA and then eluted with 1% SDS. Samples were analyzed by Western blotting using $G\beta_5$ antibody.

Results

RGS7 expression following spinal cord injury

The grey and white matter of the uninjured spinal cord stained with the anti-RGS7 antibody. In the grey matter, cells with a morphology resembling motoneurons were immunopositive for RGS7. Typically, the staining pattern of these cells had a granular cytoplasmatic appearance. In the white matter, sharply defined neuronal fibers were RGS7 positive, few of these white matter fibers were running perpendicular entering the grey matter (Fig. 1A).

In the injured tissue, there was a marked increase in RGS7 expression in some cells. These cells were seen only in the injured tissue and were mainly located close to the lesion epicentre. Further, in the injured tissue several of the white matter fibers had axonal end bulbs (Fig. 1B–F). Semi-quantitative evaluation of RGS7 immunoreactivity revealed detectable RGS7 expression 1 day post-injury, with levels peaking between 3 and 7 days after SCI (Table 1).

Western blotting was performed to quantitatively evaluate RGS7 protein expression following SCI. The overall level of RGS7 in the homogenized tissue, as revealed by quantitative Western blot, did not change (Fig. 2). $G\beta_5$ levels in the same spinal cord segments also remained unchanged after injury (data not shown). Because progressive neuronal cell death occurs in the grey matter, the constant expression of RGS7 is likely due to either infiltrating cells from the periphery, induced expression in CNS cells not constitutively expressing RGS7, or a combination of both.

Cell type determination of RGS7 expression

Neurons are RGS7 positive in controls and SCI rats

To confirm that neurons express RGS7, we used double-labelling with the anti-RGS7 antibody and antibodies against neuronal markers (NeuN, antineurofilament, and anti-β-tubulin III (TuJ1)). Neuronal expression of RGS7 was confirmed with all three neuronal markers. The grey matter stained with a distinct granular meshwork-like pattern sparing the nuclei of motoneurons (Fig. 3A).

Using the laser confocal microscopy, RGS7 positive cells localized with NeuN positive neurons within the grey matter. Analysis of 1μm thick optical sections, revealed that the granular RGS7 staining was mainly colocalized on the surface of the neurons (Fig. 3B, D and E) and near the perinuclear membrane, but less intense within the cytoplasm and not in the nucleus. Double staining for neurofilaments and RGS7 showed that the RGS7 positive granular structures followed the nerve fibers. As in the DAB staining, enlarged antineurofilament positive axonal end bulbs were seen in the injured white matter, which colocalized with RGS7 (Fig. 3F and G). Confocal microscopy of anti-β-tubulin III (Tu J1) and RGS7 double-labelled tissue revealed that the RGS7 positive granula colocalized with the neuronal β-tubulin positive structures in uninjured and injured tissues. In the white matter, the more linear staining pattern could be seen where RGS7 positive granules surrounded β-tubulin positive fibers. In the grey matter, clusters of RGS7 positive meshwork in the extracellular space were seen mainly in the injured tissue, possibly representing granules in receptor or dendrites. The anti-β-tubulin III (TuJ1) staining confirmed the findings of the NeuN staining showing the granular RGS7 pattern mainly on the surface of the cell bodies and along the nerve fibers tracts.

Astrocytes and oligodendrocytes do not colocalize with RGS7

Astrocytes were stained with GFAP; oligodendrocytes were labelled with CNPase and RIP. RGS7 positive cells did not colocalize with astrocytes or with oligodendrocytes in uninjured and injured tissue in the confocal microscope (data not shown).

Microglia and peripheral macrophages are RGS7 positive following injury

Numerous intensely RGS7 positive cells appeared very close to the lesion, fewer cells were observed up to 2 mm away from the cavity in the injured tissue. As no antibody can distinguish between activated microglia and invaded peripheral macrophages, we used two different antibodies to distinguish the morphology of these immunocompetent cells. The OX-42 antibody recognizes complement CD 11 (C3b receptor) expressed by resting and activated resident microglia as well as peripheral macrophages (Carlson *et al*., 1998). ED-1 recognizes an intracellular antigen in activated microglia and macrophages. Resting microglia could be recognized by the distinct star-shape morphology, particularly with the OX-42 antibody. Activated phagocytic microglia are rounded and are therefore not easily distinguishable from peripheral macrophages (Carlson *et al*., 1998). In contrast to the ED-1 antibody, the OX-42 antibody stains also resting microglia; both antibodies stain activated and phagocytic microglia and peripheral macrophages (Streit *et al*., 1988).

In uninjured tissue, very few OX-42 positive cells were present and no ED-1 positive cells could be found. However after SCI, there were numerous, strongly OX-42 positive microglia with a ramified star-shaped morphology in the grey and white matter close to the lesion epicentre. Beginning at day 1, however, more pronounced at day 3, these activated microglia changed their originally stellate morphology to a more rounded appearance. At day 3, some very intense RGS7 positive cells appeared and colocalized with OX-42 and ED-1 positive cells; however, only a subpopulation of these immunocompetent cells stained for RGS7 (Fig. 4A–C and E–G) The colocalization was mainly observed in active phagocytic cells with a more round morphology, some of them also had intracellular lipid accumulation. However, some of the star-shaped OX-42 and stellate shaped ED-1 positive cells did also colocalize with RGS7. At day 7, the number of ED-1 positive cells increased dramatically, most of them with the round morphology.

FIG. 3. Co-localization of RGS7 (green) with neurons (red). (A) Granular RGS7 staining pattern in the grey matter with sparing of the nuclei in uninjured tissue. The intercellular space is filled with a meshwork of RGS7 positive structures, which possibly represent synapses. Note the fine laminar structures in the white matter. (B) In this NeuN double labelled neuron, RGS7 is mainly localized in the cytoplasm around the nuclear membrane, but not within the nucleus, day three. (C) Negative control, day three (no primary antibodies). (D and E) Different optical confocal layers show double-labelling for RGS7 and NeuN positive neurons in the grey matter. Note the granular staining pattern mainly on the surface of the neurons (top cell) compared to the other cells in which the optical layer of the confocal microscope layers through the cell body (lower cells), day three. (F) Grey matter adjacent to the lesion cavity. RGS7 positive extracellular meshwork and destroyed neurofilaments. Note the bright green RGS7 positive cells which do not colocalize with the neuronal markers (40×), day three. (G) RGS7 is expressed in the neurofilament positive axonal end bulbs in the injured white matter. Further, RGS7 positive granula enclose neurofilament positive nerve fibers, day three. B and C are at the same magnification as D and E.

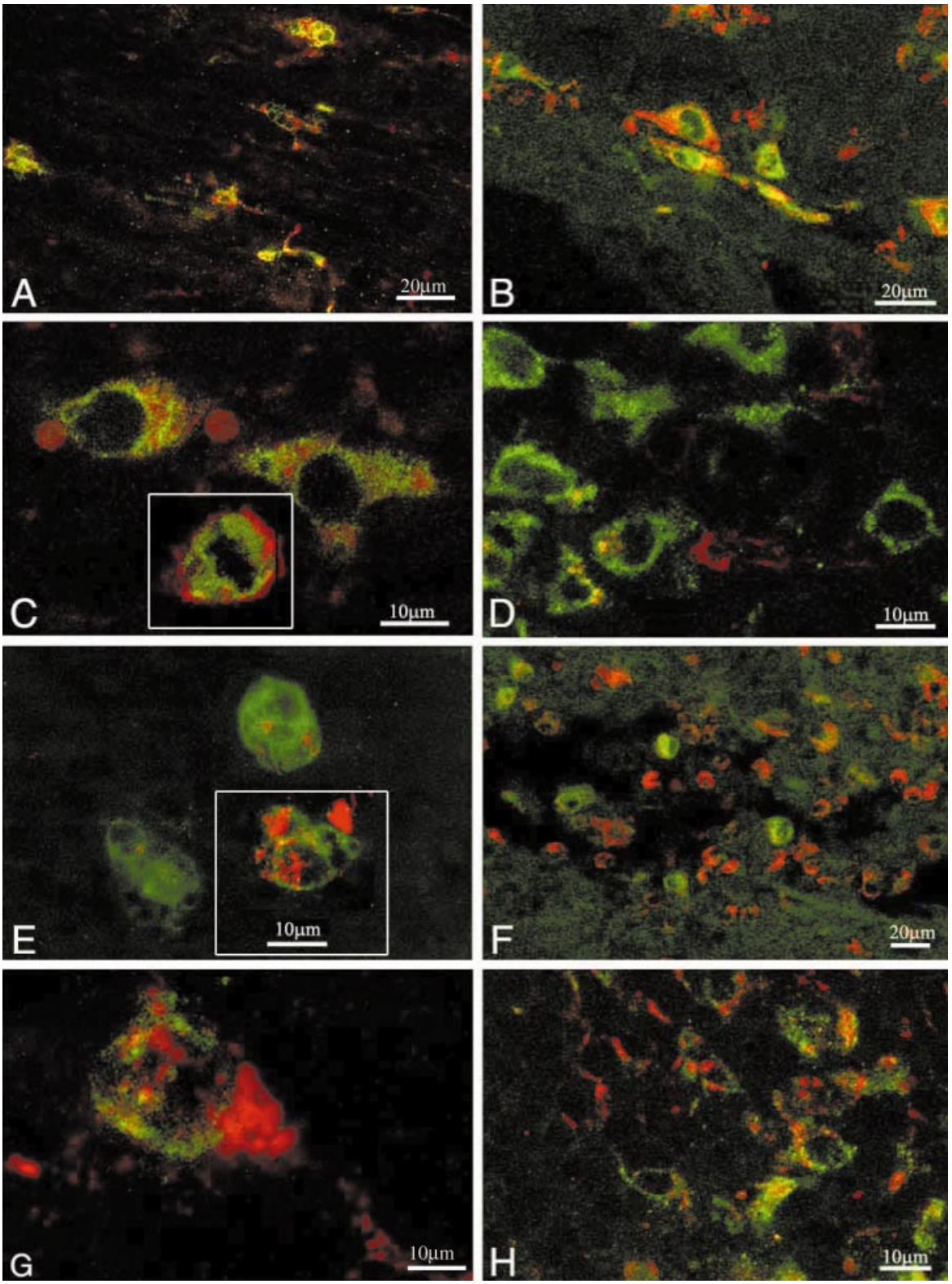


FIG. 4. Co-localization of RGS7 (green) with microglia/macrophages (red). (A–C) Co-localization of RGS7 and OX-42 immunocompetent cells 3 days post-injury; grey matter. Note the roundish shape of these colocalizing cells. Only a subpopulation of OX-42 cells colocalize with RGS7. (D) Co-localization in some of peripheral macrophages (OX-42 positive) and RGS7 positive cells in the perinodular rim in the spleen one-day after SCI. (E–G) Co-localization of RGS7 and ED-1 immunocompetent cells 3 days post-injury; grey matter. (H) Co-localization of a subpopulation of peripheral macrophages (ED-1 positive) and RGS7 in the subcapsular region of the spleen.

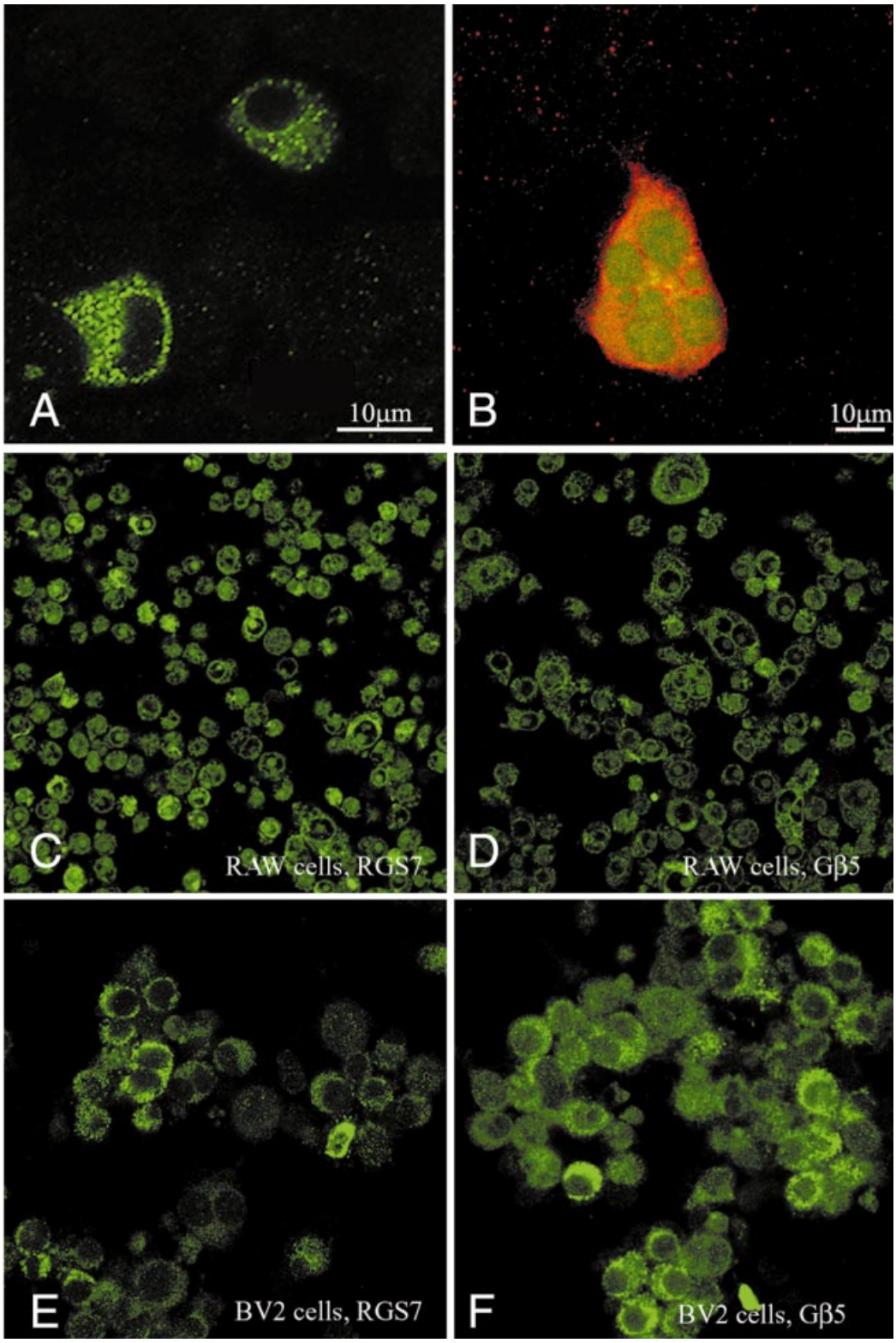


FIG. 5. *In vitro* immunocytochemistry of PC12, HEK, BV2, and RAW cells. Confocal microscopy. (A) Rat PC12 cells were used as positive controls for RGS7 staining. Note the granular RGS7 staining pattern of the cytoplasm and the nuclear membrane, however, only a weak staining of the nucleus was seen. (B) Cluster of RGS7 (red) transfected HEK 293 cells containing green fluorescent protein (green). (C) RAW cells stained for RGS7 48 h after LPS stimulation. (D) RAW cells stained for $G\beta_5$ 48 h after LPS challenge. (E) BV2 cells stained for RGS7 48 h after LPS stimulation. (F) BV2 cells stained for $G\beta_5$, 48 h after LPS stimulation. Calibration in A: 10 μm (A); 25 μm (C and E). Calibration in B: 10 μm (B); 25 μm (D and F).

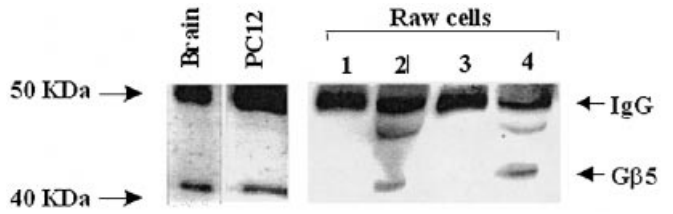


FIG. 6. RGS7-Gβ_5 complex in the soluble fraction of RAW cells. RAW cells (5×10^6), PC12 cells (5×10^6), and brain homogenate (100 μg protein) were immunoprecipitated with RGS7 antibody. The immunoprecipitates were immunoblotted with Gβ_5 antibody. Lanes 3 and 4 contain RAW cells incubated with TNF-α (25 ng/mL) 24 h before collection of the cells. Lanes 1 and 3 represent the membrane fraction, lanes 2 and 4 represent the soluble fraction.

Peripheral macrophages in the spleen are RGS7 positive

In the spleen, OX-42 and ED-1 positive immune competent cells were mainly localized in the perinodular rim, whereas RGS7 positive cells were also seen in the matrix between the nodules, but not in the nodules itself. As in the spinal cord, RGS7 appeared in a granular form within the cytoplasm. The majority of RGS7 positive cells do colocalize with either OX-42 or ED-1 (Fig. 4D and H).

In vitro *determination of RGS7 and Gβ$_5$ expression in microglia/macrophages*

As previous studies of RGS7 and Gβ_5 did not detect these molecules in non-neuronal tissues and cell lines (Watson *et al*., 1994; Watson *et al*., 1996; Liang *et al*., 2000; Witherow *et al*., 2000; Zhang *et al*., 2001), detection on RGS7 in activated microglia and peripheral macrophages was unexpected. We used a combination of immunocytochemistry, Western blotting, immunoprecipitation, and mRNA analysis to assess *in vitro* RGS7 and Gβ_5 expression in microglia and macrophages cell lines.

As a positive control rat PC12 pheochromocytoma were stained for RGS7 as they are known to express RGS7 (Zhang *et al*., 2001). Similar to the epifluorescence staining pattern, confocal image analysis revealed a granular pattern of the cytoplasm and the nuclear membrane. The nucleus and the nucleolus were only weakly RGS7 positive, similar to a recent report of describing a strong cytoplasmatic and a fainter as well as less consistent nuclear staining pattern in PC12 cells (Zhang *et al*., 2001) (Fig. 5A).

To confirm the specificity of immunocytochemistry using the anti-RGS7 antibody, wells with HEK 293 cells were randomly transfected with RGS7. As transfection marker, green fluorescent protein was used. After fixation, the cells were stained for RGS7. In an unbiased, blinded evaluation, all wells containing RGS7 transfected cells were recognized (Fig. 5B).

RGS7 expression in microglia/macrophages was confirmed *in vitro* with immunocytochemistry of RAW and BV2 cells. Non-stimulated RAW stained for RGS7 and Gβ_5. Following LPS and TNF-α (data not shown) stimulation for 48 h the number of positively RGS7 and Gβ_5 stained cells remained stable (Fig. 5C and D). The staining pattern of Gβ_5 was more homogeneous compared to the granular RGS7 staining pattern. Intracytoplasmic vacuoles were observed in some cells similar to the *in vivo* findings of phagocytotic inflammatory cells. Immunocytochemistry of BV2 cells also revealed the presence of RGS7 and Gβ_5 in microglia. Naïve BV2 cells positively stained for RGS7. However, 48 h following LPS or TNF-α stimulation, the number of RGS7 positive cells increased approximately 3-fold and the cells enlarged by a factor of 1.5. In LPS stimulated BV2 cells RGS7 staining was seen consistently in a very distinct granular matter in the cytoplasm, whereas only a faint nuclear staining pattern was observed (Fig. 5E and F). Non-stimulated BV2 cells stained weaker for Gβ_5 compared to RGS7. Following 48 h of LPS or TNF-α stimulation, the number of Gβ_5 positive cells increased approximately 2-fold. The cytoplasmatic staining pattern for Gβ_5 was more homogenous in contrast to the granular RGS7 staining pattern and the intensity of the nuclear staining pattern was increased compared to the RGS7 labelling.

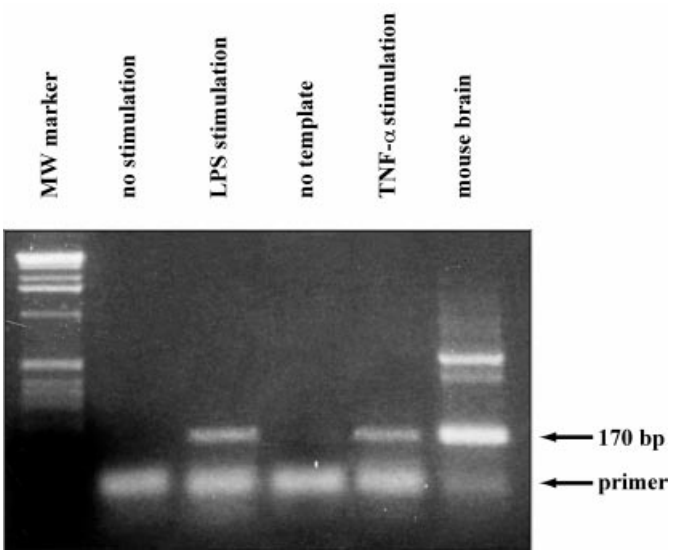


FIG. 7. LPS and TNF-α induction of RGS7 mRNA expression in RAW cells. A 170-bp product was detected in LPS and TNF-α stimulated RAW cells. No mRNA was detected in naïve non-stimulated cells. Mouse brain was used as positive control.

To confirm the results of the *in vitro* immunocytochemistry, Western blots and immunoprecipitation of RAW cell were performed. Western blotting demonstrated that non-stimulated RAW cells had distinct RGS7 and Gβ_5 bands. The RGS7 bands were blocked completely with RGS7 peptide. Peak expression of RGS7 was found 48 h after LPS stimulation, whereas the Gβ_5 band remained stable following LPS stimulation (data not shown). To confirm that RGS7 and Gβ_5 in RAW cells form a dimer, we immunoprecipitated cell lysates with RGS7 specific antibody and probed the immunoprecipitants with Gβ_5 antibody. The results shown in Fig. 6 demonstrate that Gβ_5 is tightly associated with RGS7 in a complex that is similar to one detected in the brain (Witherow *et al*., 2000; Zhang *et al*., 2000).

The presence of RGS7 mRNA in peripheral macrophages was confirmed with RT-PCR in LPS or TNF-α stimulated RAW cells (Fig. 7). However, we could not detect RGS7 mRNA in naïve non-stimulated cells.

Discussion

This study is the first to demonstrate the up-regulated RGS7 expression and expression of the RGS7-Gβ_5 complex following SCI in activated microglia and peripheral macrophages. This finding is supported by *in vitro* immunocytochemistry, Western blotting, and RT-PCR detection in microglia and peripheral macrophage cell lines. We also demonstrate that RGS7 and Gβ_5 form a complex in macrophage cell lines. Furthermore, this study confirms the previous reports of RGS7 expression in neurons (Liang *et al*., 2000; Zhang *et al*., 2001). The time-course of RGS7 up-regulation after SCI is in

concord with the responses of the immunocompetent cells. These data suggest, that SCI-induced up-regulation of RGS7 in immune cells contributes to the modulation of the post-traumatic inflammatory reaction within the CNS.

RGS7 staining pattern following SCI

An increased intensity of the RGS7 staining was observed following injury, both in the grey and white matter, primarily in the lesion area. We did not detect a quantitative increase of RGS7 and $G\beta_5$ in Western blots in the solubilized fraction of injured compared to control spinal cord. These results may seem contradictory, but may be explained by the fact that the reduction of RGS7 due to neuronal cell death is replaced by an induction of RGS7 expression in microglia/macrophages, as the time-course of the RGS7 up-regulation after SCI is in concord with responses of the immunocompetent cells. Another explanation is that for Western blot experiments a 1.8-mm long spinal cord segment was homogenized and therefore the up-regulation of RGS7 in immune cells after SCI could not be detected quantitatively due to dilution by uneffected tissue.

Very small RGS7 positive structures could be seen in the intercellular space, possibly representing receptor or synapses staining on the membrane surface of the neuronal processes. It is conceivable that neurons under injury-induced stress release RGS7 positive material (degranulation) causing the increase of granular staining in the extracellular space.

Neurofilament positive axonal end bulbs which were found in the grey matter following injury may represent a secondary axotomy due to an axolemmal permeability change causing a focal swelling (Pettus & Povlishock, 1996). These distinct axonal end bulbs did colocalize with RGS7.

In accordance with earlier studies (Zhang *et al*., 2001), we also found that the nuclear staining pattern of $G\beta_5$ is more intense compared to RGS7, suggesting that $G\beta_5$ undergoes a nuclear translocation in neurons. The function of $G\beta_5$ and RGS7 in the nucleus of neurons remains unknown (Zhang *et al*., 2001). At present the only known function of $G\beta_5$-RGS7 complex is to act as GAP for $G\alpha$ subunits (Posner *et al*., 1999; Kovoor *et al*., 2000; Witherow *et al*., 2000; Keren-Raifman *et al*., 2001).

$G\beta_5$-RGS7 complex in microglia and macrophages

Microglia occupy approximately 13% of the entire glial cell population and are distributed diffusely throughout the whole CNS (Watanabe *et al*., 1999). After SCI, microglia/macrophages strongly express RGS7, especially when they are located within the penumbra. As no antibody is able to distinguish activated microglia and peripheral macrophages (Carlson *et al*., 1998), we confirmed our *in vivo* findings with *in vitro* study by immunocytochemistry, RT-PCR, and Western blotting, demonstrating expression of the RGS7-$G\beta_5$ complex in both microglia (BV2) and peripheral macrophage (RAW) cell lines.

Further, RGS7 positive cells were seen in peripheral immune tissue, such as the spleen. While this departs from the current belief that RGS7 is solely expressed in the CNS along with $G\beta_5$, it should also be noted that Jones *et al* (1998) reported that human $G\beta_5$ mRNA was also found in non-CNS tissue. Thus, while the current belief is that RGS7 and $G\beta_5$ always exists together as a dimer in the CNS due to their rapid degradation when expressed alone (Witherow *et al*., 2000), this finding suggests the dimer also exists in the peripheral immune tissue.

As microglia and peripheral macrophages both express injury-induced RGS7, it is tempting to speculate that this supports the theory of the mesodermal origin of microglia in contrast to the neuroectodermal origin of other glia cells (astrocytes and oligodendrocytes) and neurons (Streit *et al*., 1988).

Up-regulation mechanism

Numerous studies have shown that proinflammatory cytokines such as TNF-α are up-regulated after SCI (Bartholdi & Schwab, 1997; Bethea *et al*., 1999; Hayashi *et al*., 2000; Popovich, 2000; Taoka & Okajima, 2000). Up-regulation of proinflammatory cytokines and chemokines are involved in the activation of microglia and the recruitment of peripheral macrophages. In mice lacking TNF-α receptors, reduced microglia activation was found following traumatic brain injury (Bruce *et al*., 1996), suggesting that TNF-α plays an important role in microglia activation. TNF-α induced up-regulation of RGS7 may contribute to trauma induced inflammatory alterations in the CNS. The effect of TNF-α prevented degradation of RGS7 on post-traumatic scar formation is as yet unknown. SCI results in a glial reaction, leading to scar formation in which axonal regeneration fails and remyelination is also unsuccessful (Schwab & Bartholdi, 1996; Fawcett & Asher, 1999). Although the exact role of activated microglia/macrophages in scar formation after SCI is yet unclear, evidence suggests that microglia are likely to help CNS regeneration and that their presence in scar tissue is unlikely to contribute significantly to its inhibitory nature (Fawcett & Asher, 1999).

The RT-PCR studies show an increased expression of RGS7 mRNA following LPS and TNF-α challenge. A previous report showing TNF-α mediated up-regulation of RGS7 suggest that a phosphorylation event inhibits RGS7 degradation (Benzing *et al*., 1999). Functional specificity and temporal activity of RGS proteins depend on specific patterns of expression, transcriptional regulation, and subcellular localization. RGS7 protein levels are post-translationally regulated through rapid degradation by the ubiquitin-dependent proteasome pathway (Benzing *et al*., 1999; Kim *et al*., 1999). However, little is known about the upstream and downstream regulation of RGS7.

Future studies will investigate what effect of blocking RGS7 has on inflammation following SCI.

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Abbreviations

CNS, central nervous system; DAB, 3,3-diaminobenzidine; GAP, GTPase activating protein; $G\beta_5$, G protein β5 subunit; GDP, guanine diphosphate; GFAP, glial fibrillary acid protein; GGL, Gγ-like; GTP, guanine triphosphate; LPS, lipopolysaccharide; NeuN, neuronal nuclear; PB, phosphate buffer; PBS, phosphate buffered saline; PCR, polymerase chain reaction; RGS, regulators of G protein signalling; RT-PCR, reverse transcription and polymerase chain reaction; SCI, spinal cord injury; TNF-α, tumour necrosis factor-α; TX, triton X-100.

References

Acarin, L., Gonzalez, B. & Castellano, B. (2000) Neuronal, astroglial and microglial cytokine expression after an excitotoxic lesion in the immature rat brain. *Eur. J. Neurosci*, **12**, 3505–3520.

Bartholdi, D. & Schwab, M.E. (1997) Expression of pro-inflammatory cytokine and chemokine mRNA upon experimental spinal cord injury in mouse: an *in situ* hybridization study. *Eur. J. Neurosci.*, **9**, 1422–1438.

Basso, D.M., Beattie, M.S. & Bresnahan, J.C. (1996) Graded histological and locomotor outcomes after spinal cord contusion using the NYU weight-drop device versus transsection. *Exp. Neurol.*, **139**, 244–256.

Benzing, T., Brandes, R., Sellin, L., Schermer, B., Lecker, S., Walz, G. & Kim, E. (1999) Upregulation of RGS7 may contribute to tumor necrosis factor induced changes in central nervous function. *Nature Med.*, **5**, 913–918.

Berman, D.M., Wilkie, T.M. & Gilman, A.G. (1996) GAIP and RGS4 are GTPase-activating proteins for the Gi subfamily of G protein alpha subunits. *Cell*, **86**, 445–452.

Bethea, J.R., Castro, M., Keane, R.W., Lee, T.T., Dietrich, W.D. & Yezierski, R.P. (1998) Traumatic spinal cord injury induces nuclear factor-kappaB activation. *J. Neurosci.*, **18**, 3251–3260.

Bethea, J.R., Nagashima, H., Acosta, M.C., Briceno, C., Gomez, F., Marcillo, A.E., Loor, K., Green, J. & Dietrich, D. (1999) Systemically administrated interleukin-10 reduces tumor necrosis factor-α production and significantly improves functional recovery following traumatic spinal cord injury in rats. *J. Neurotrauma*, **16**, 851–863.

Birnbaumer, L. (1992) Receptor-to-effector signaling through G proteins roles for beta gamma dimers as well as alpha subunits. *Cell*, **71**, 1069–1072.

Bruce, A.J., Boling, W., Kindy, M.S., Peschon, J., Kraemer, P.J., Carpenter, M.K., Holtsberg, F.W. & Mattson, M.P. (1996) Altered neuronal and microglial responses to excitotoxic and ischemic brain injury in mice lacking TNF receptors. *Nature Med.*, **2**, 788–794.

Cabrera, J.L., de Freitas, F., Satpaev, D.K. & Slepak, V.Z. (1998) Identification of the $G\beta_5$-RGS7 protein complex in the retina. *Biochem. Biophys. Res. Commun.*, **249**, 898–902.

Carlson, S.L., Parrish, M.E., Springer, J.E., Doty, K. & Dossett, L. (1998) Acute inflammatory response in spinal cord following impact injury. *Exp. Neurol*, **151** 77–88.

Chase, D.L., Patikoglou, G.A. & Koella, M.R. (2001) Two RGS proteins that inhibit Galpha (o) and Galpha (q) signaling in *C. elegans* neurons require a Gbeta (59-like subunit for function. *Curr. Biol.*, **11**, 222–231.

Chen, C.K., Burns, M.E., He, W., Wensel, T.G., Baylor, D.A. & Simon, M.I. (2000) Slowed recovery of rod photoresponse in mice lacking the GTPase accelerating protein RGS9-1. *Nature*, **403**, 557–560.

Fawcett, J.W. & Asher, R.A. (1999) The glial scar and central nervous system repair. *Brain Res. Bull.*, **49**, 377–391.

Hayashi, M., Ueyama, T., Nemoto, K., Tamaki, T. & Senba, E. (2000) Sequential mRNA expression for immediate early genes, cytokines, and neurotrophins in spinal cord injury. *J. Neurotrauma*, **17**, 203–218.

Hunt, T.W., Fields, T.A., Casey, P.J. & Peralta, E.G. (1996) RGS10 is a selective activator of G alpha i GTPase activity. *Nature*, **383**, 175–177.

Jones, P.G., Lombardi, S.J. & Cockett, M.I. (1998) Cloning and tissue distribution of the human G protein β5 cDNA. *Biochim. Biophys. Acta*, **1402**, 288–291.

Keren-Raifman, T., Bera, A.K., Zveig, D., Peleg, S., Witherow, D.S., Slepak, V.Z. & Dascal, N. (2001) Expression levels of RGS7 and RGS4 proteins determine the mode of regulation of the G protein-activated K^+ channel and control regulation of RGS7 by $G\beta_5$. *FEBS Lett.*, **492**, 20–28.

Kim, E., Arnould, T., Sellin, L., Benzing, T., Comella, N., Kocher, O., Tsiokas, L. & Sukhatme, Walz, G. (1999) Interaction between RGS7 and polycystin. *Proc. Natl. Acad. Sci. USA*, **96**, 6371–6376.

Klusman, I. & Schwab, M.E. (1997) Effects of pro-inflammatory cytokines in experimental spinal cord injury. *Brain Res.*, **762**, 173–184.

Kovoor, A., Chen, C.K., He, W., Wensel, T.G., Simon, M.I. & Lester, H.A. (2000) Co-expression of Bbeta5 enhances the function of two Ggamma subunit-like domain-containing regulators of G protein signaling proteins. *J. Biol. Chem.*, **275**, 3397–3402.

Levay, K., Cabrera, J.L., Satpaev, D.K. & Slepak, V.Z. (1999) Gβ5 prevents in the RGS7–Gα interaction through binding to a distinct Gγ-like domain found in RGS7 and other RGS proteins. *Proc. Natl. Acad. Sci. USA*, **96**, 2503–2507.

Liang, J.J., Chen, H.H., Jones, P.G. & Khawaja, X.Z. (2000) RGS7 complex formation and colocalization with the Gbeta5 subunit in the adult rat brain and influence on Gbeta5gamma2-mediated PLCbeta signaling. *J. Neurosci. Res.*, **60**, 58–64.

Makino, E.R., handy, J.W., Li, T. & Arshavsky, V.Y. (1999) The GTPase activating factor for transduction in rod photoreceptors is the complex between RGS9 and type 5 G protein beta subunit. *Proc. Natl. Acad. Sci. USA*, **96**, 1947–1952.

Pettus, E.H. & Povlishock, J.T. (1996) Characterization of a distinct set of intra-axonal ultrastructural changes associated with traumatically induced alteration in axolemmal permeability. *Brain Res.*, **722**, 1–11.

Ponting, C.P. & Bork, P. (1996) Pleckstrin's repeat performance: a novel domain in G-protein signaling? *Trends Biochem. Sci.*, **21**, 245–246.

Popovich, P.G. (2000) Immunonological regulation of neuronal degeneration and regeneration in the injured spinal cord. *Prog. Brain Res.*, **128**, 43–58.

Popovich, P.G., Wei, P. & Stokes, B.T. (1997) Cellular inflammatory response after spinal cord injury in Sprayer–Dawly and Lewis rats. *J. Comp. Neurol.*, **377**, 443–464.

Posner, B.A., Gilman, A.G. & Harris, B.A. (1999) Regulators of G protein 6 and 7. Purification of complexes with gbeta5 and assessment of their effects on g protein-mediated signaling pathways. *J. Biol. Chem.*, **274**, 31087–31093.

Schwab, M.E. & Bartholdi, D. (1996) Degeneration and regeneration of axons in the lesioned spinal cord. *Physiol. Rev.*, **76**, 319–370.

Snow, B.E., Krumins, A.M., Brothers, G.M., Lee, S.F., Wall, M.A., Chung, S., Mangion, J., Arya, S., Gilman, A.G. & Siderovski, D.P. (1998) A G protein gamma subunit-like domain shared between RGS11 and other RGS proteins specifies binding to Gbeta5 subunits. *Proc. Natl. Acad. Sci. USA*, **95**, 13307–13312.

Streit, W.J., Graeber, M.B. & Kreutzberg, G.W. (1988) Functional plasticity of microglia: a review. *Glia*, **1**, 301–307.

Taoka, Y. & Okajima, K. (2000) Role of leucocytes in spinal cord injury in rats. *J. Neurotrauma*, **17**, 219–229.

Watanabe, T., Yamamoto, T., Abe, Y., Saito, N., Kumagai, T. & Kayama, H. (1999) Differential activation of microglia after experimental spinal cord injury. *J. Neurotrauma*, **16**, 255–265.

Watson, A.J., Aragay, A.M., Slepak, V.Z. & Simon, M.I. (1996) A novel form of the G-protein β-subunit $G\beta_5$ is specially expressed in the vertebra retina. *J. Biol. Chem.*, **271**, 28154–28160.

Watson, A.J., Katz, A. & Simon, M.I. (1994) A fifth member of the mammalien G-protein β-subunit family. *J. Biol. Chem.*, **269**, 22150–22156.

Watson, N., Linder, M.E., Druey, K.M., Kehrl, J.H. & Blumer, K.J. (1996) RGS family members: GTPase-activating proteins for heterotrimeric G-protein alpha-subunits. *Nature*, **383**, 172–175.

Witherow, D.S., Wang, Q., Levay, K., Cabrera, J.L., Chen, J., Willars, G.B. & Slepak, V.Z. (2000) Complex of the G protein subunit $G\beta_5$ with the regulators of G protein signaling RGS7 and RGS9. *J. Biol. Chem.*, **275**, 24872–24880.

Zhang, J.H., Barr, V.A., Mo, Y., Rojkova, A.M., Liu, S. & Simonds, W.F. (2001) Nuclear localization of G protein β_5 and regulator of G protein signaling 7 neurons and brain. *J. Biol. Chem.*, **276**, 10284–10289.

Zhang, J.H. & Simonds, W.F. (2000) Copurification of brain G-protein beta5 with RGS6 and RGS7. *J. Neurosci.*, **20**, 1–5.