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5-HT_{2A} receptors in rat sciatic nerves and Schwann cell cultures

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Abstract

Pharmacological approaches and optical recordings have shown that Schwann cells of a myelinating phenotype are activated by 5-HT upon its interaction with the 5-HT_{2A} receptor (5-HT_{2A}R). In order to further characterize the expression and distribution of this receptor in Schwann cells, we examined rat sciatic nerve and cultured rat Schwann cells using probes specific to 5-HT_{2A}R protein mRNA. We also examined the endogenous sources of 5-HT in rat sciatic nerve by employing both histochemical stains and an antibody that specifically recognizes 5-HT. Rat Schwann cells of a myelinating phenotype contained both 5-HT_{2A}R protein and mRNA. In the healthy adult rat sciatic nerve, 5-HT_{2A}Rs were evenly distributed along the outermost portion of the Schwann cell plasma membrane and within the cytoplasm. The most prominent source of 5-HT was within granules of the endoneurial mast cells, closely juxtaposed to Schwann cells within myelinating sciatic nerves. These results support the hypothesis that the 5-HT receptors expressed by rat Schwann cells *in vivo* are activated by the release of 5-HT from neighboring mast cells.

Introduction

The involvement of immune responses has been described for numerous demyelinating peripheral neuropathies (Robbins & Cotran, 1979; Hartung *et al.*, 1993). Most of these responses occur at later stages of nerve injury, when Schwann cells have already demyelinated, and involve non-resident macrophages that infiltrate the site of injury (Perry & Brown, 1992; Griffin *et al.*, 1993). At present, relatively little is known about what mechanisms alert the immune system of neural injury, thereby triggering demyelination and macrophage infiltration.

Endoneurial mast cells reside in healthy peripheral nerves (Gamble & Goldby, 1961; Majeed, 1994), and are strong candidates for initiating the immune responses that participate in demyelination (Dines & Powell, 1997). Activation of mast cells causes degranulation and subsequent release of several substances including cytokines (Galli *et al.*, 1991), proteases (Woodbury *et al.*, 1981; Huang *et al.*, 1998), nerve growth factor (Levi-Montalcini *et al.*, 1995, 1996), and—in rodents—5-HT (Olsson, 1965). Likely targets of 5-HT release are receptors expressed by resident Schwann cells (Yoder *et al.*, 1996, 1997a, b) and local vascular cells (Peroutka, 1984; Ullmer *et al.*, 1995). While evidence for 5-HT receptors in peripheral nerve vasculature has been well documented using multiple approaches, most of the evidence for 5-HT receptors in Schwann cells is pharmacological (Yoder *et al.*, 1996).

In previous studies, we reported that quiescent, myelinating rat Schwann cells respond to bath application of 5-HT by releasing calcium from inositol 1, 4, 5-triphosphate sensitive intracellular stores (Yoder *et al.*, 1996, 1997a). Further pharmacological studies showed that treatment with ketanserin

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(a competitive antagonist for 5-$HT_{2A/2C}$ receptors) or spiperone (a competitive antagonist for 5-HT_{2A}Rs) reversibly blocked calcium release in response to 5-HT, suggesting the presence of 5-HT_{2A}Rs at the Schwann cell plasma membrane (Yoder *et al.*, 1996). Using an anti idiotypic antibody able to bind 5-HT_{2A}, 5-HT_{1B} and 5-HT_{2C} receptors, we also found 5-HTR immunoreactivity in healthy and regenerated peripheral nerves, when Schwann cells displayed a myelinating phenotype (Yoder *et al.*, 1996, 1997a, b).

In this study, we utilized specific molecular probes to map the expression of 5-HT_{2A}R in sciatic nerves and to determine whether these results corroborated our previous pharmacological data. We examined both cultured rat Schwann cells and rat sciatic nerves using a mRNA probe and a monoclonal antibody, both specific to 5-HT_{2A}R. Schwann cells of a myelinating phenotype were found to express both 5-HT_{2A} receptor and message. Furthermore, we examined rat sciatic nerves for 5-HT, and determined that the prominent source was within granules of endoneurial mast cells.

Methods

CHEMICALS

Triton X-100 and Tween 20 were obtained from Sigma (St. Louis, MO). PBS, FBS, DMEM, antibiotics and trypsin/EDTA were obtained from Gibco (Grand Island, NY). Normal goat serum was obtained from Jackson Immuno Research (West Grove, PA). ^{32}PdCTP for random priming was obtained from NEN (Boston, MA). Diaminobenzidine tetrahydrochloride (DAB) was obtained from Sigma (St. Louis, MO). Durcupan resin was obtained from Electron Microscopy Sciences (Fort Washington, PA).

ANTIBODIES

Rabbit anti S100β was purchased from Charles River Pharmaceutics (Southbridge, MA). Fluorescein isothiocyanate (FITC) conjugated-donkey anti-rabbit IgG was obtained from Jackson Immuno Research (West Grove, PA). Mouse anti 5-HT_{2A}R G186-1117 (Wu *et al.*, 1998) was obtained from PharMingen (San Diego, CA). Rabbit anti 5HT was purchased from Chemicon (Temecula, CA).

CELL CULTURE

Schwann cells were isolated from neonatal (P1) Sprague-Dawley rat sciatic nerves according to the methods of Brockes *et al.* (1979) and maintained in culture as previously described (Yoder *et al.*, 1996). Cells for RNA isolation were grown to subconfluence and then fed with growth medium containing a lower percentage of FCS (1%) 24 hours prior the extraction. This treatment increases the percentage of cells displaying functional 5-HT receptors (Yoder *et al.*, 1997a).

OLIGONUCLEOTIDES

Amplification primers were obtained through Gibco BRL (Gaithersburg, MA), deprotected and desalted by reverse phase chromatography. The oligonucleotide sequences encoded rat 5-HT_{2A} residues 14-37 (5′-GTGAAGACAATATCTCTCTGAGCT-3′) and residues 408-425 (5′-GTATGGGTACCGGTGGCC-3′). The specificity of each RT-PCR product was checked by direct sequencing of the amplified product (sequencing was performed at the UCSD Center for AIDS Research support).

RNA ISOLATION, RT-PCR, AND TRANSCRIPT ANALYSIS

Sciatic nerves for RNA isolation were prepared from one month-old male Sprague-Dawley rats as previously described (Mi *et al.*, 1995) and total RNA was purified according to a modified version of a published protocol (Chomczynski and Sacchi, 1987).

cDNA templates for RT-PCR were prepared using the cDNA cycle kit by Invitrogen (Carlsbad, CA). Total RNA was treated with RNase free-DNase I (Boehringer Mannheim, Indianapolis, IN) to remove genomic DNA; the poly A+ fraction was isolated by two sequential treatments with Oligotex beads (Qiagen, Valencia, CA). The PCR was carried out in a mini thermo cycler (MJ Research Inc., Watertown, MA). DNA fragments were separated in 1.5% agarose gel. The separated DNA fragments were visualized using an UV transilluminator and photographed using IS-1000 Digital Imaging System (Alpha Innotech Co., San Leandro, CA).

Twenty μg of total RNA were run on 1.2% agarose-formaldehyde gels, transferred to Hybond N+ membrane (Amersham Biosciences, Piscataway, N.J.), and immobilized by UV cross-linking. A cocktail of restriction fragments from the plasmid pAM (Bam HI-Xba I, 1928bp; Xba I-Pst I, 888bp; Pst I-Pst I, 904bp) was labeled by random priming. Prehybridization and hybridization were performed at 50°C in 0.5 M Na_2HPO_4, 1 mM EDTA, 5% SDS, 25% formamide, 1% BSA. $2{\cdot}10^6$ cpm/ml of the randomly primed probe were added to the hybridization solution and the blot was incubated for 16–24 hours on a shaker at 50°C. The blot was then washed at high stringency and exposed to X-ray film (Kodak, Rochester, N.Y.) between two intensifying screens at -80°C for 1 week.

IN SITU HYBRIDIZATION (ISH)

Probes

The sequence delimited by residues 1–240 of the rat 5-HT_{2A} cDNA, was amplified by PCR, gel purified with the QIAEXII kit (Qiagen, Valencia, CA) and ligated into the riboprobe vector pPMG (PharMingen, San Diego, CA). The presence of the right insert was checked by sequencing. To prepare the ribonucleotide probes, the recombinant plasmid DNA was linearized with EcoRI (to give the antisense riboprobe) or Hind III (to give the sense riboprobe), gel purified and used as template in the *in vitro* transcription reaction, performed in the presence of digoxigenin-UTP, ribonucleotides and T7 (antisense riboprobe) or SP6 (sense riboprobe) RNA polymerase.

Protocol

The procedure used for ISH on cultured cells was previously reported (Tang *et al.*, 1997). ISH on rat sciatic nerves was performed as follows. Rat sciatic nerves from 1-month old rats

were dissected, embedded and quickly frozen in liquid nitrogen. Twelve μm thick sections were collected on Superfrost Plus slides (Fisher Scientific), fixed in 4% paraformaldehyde and dehydrated by incubating for 2 min in 50%, 75%, 95%, and 100% ethanol. Air-dried sections were stored at -70°C in an airtight box. Prior to hybridization, sections were allowed to reach room temperature, re-hydrated in PBS, treated with proteinase K (1 μg/ml in TE) and post-fixed in 4% paraformaldehyde. The following steps were performed in order to facilitate accessibility of 5-HT$_{2A}$ mRNA, to reduce non specific binding of the riboprobe and to reduce background due to endogenous peroxidase: 10 min in 3% H_2O_2, 2 min in 0.5% Triton X-100 (in PBS), 5 min in 0.5 N HCl and 10 min in acetylation buffer (583 μl triethanolamine, 125 μl acetic anhydride in 50 ml depc treated-water). Each step was followed by two brief washes in PBS. Fifty μl of a pre-hybridization solution (containing 50% formamide, 5X SSC, 5X Denhardt's reagent, 50 μg/ml ssDNA, 25 μg/ml tRNA) lacking the probe were applied to each sample.

Samples were pre-hybridized at 48°C for 1 hour in a humid chamber. At the end of the pre-hybridization step, samples were rinsed in 5X SSC and prepared for the hybridization step: 50 μl of pre-hybridization solution, containing a 1:10 dilution of the Digoxigenin labeled sense or antisense riboprobe (previously denatured at 65°C for 10 min) were applied to each slide, covered with a glass coverslip and sealed with rubber cement. Samples were hybridized overnight at 48°C in a humid chamber. The post-hybridization washes were performed in SSC wash buffer at high astringency (2X to 0.1X). Samples were treated with RNase to remove any unhybridized mRNA. Detection of the hybridized riboprobes was performed with a sheep anti-digoxigenin F(ab) antibody (Boehringer Mannheim, Indianapolis, IN) followed by incubation with a biotinylated donkey anti sheep antibody and a final hybridization with streptavidin, horseradish peroxidase conjugated (Boehringer Mannheim, Indianapolis, IN).

Slides were incubated for 10 min in 0.1 mg/ml 3-3' diaminobenzidine (DAB) containing 0.008% hydrogen peroxide, rinsed in TE, dehydrated in an ascending series of ethanol concentrations followed by delipidization with ascending series of xylene concentrations and mounted in Permount (Fisher). The samples were viewed on an EDGE Stereomicroscope (Edge Scientific Instrument Co. LLC, Santa Monica, CA) using an oil immersion planapochromatic objective lens, (Zeiss 40X, 1.00 NA).

WESTERN BLOT ANALYSIS

Protein lysates from brain, sciatic nerve and cultured Schwann cells were prepared as follows: freshly isolated specimens were homogenized in 0.32 M sucrose, 10 mM HEPES pH 7.0, 0.1 mM EDTA, containing a cocktail of protease inhibitors, sonicated and centrifuged at 4°C. The supernatant was mixed with an equal volume of 2$\times$ sample buffer (Novex, San Diego, CA) and an aliquot of each sample was heated at 100°C for 2 min, cooled at room temperature, fractionated on a 4–20% gradient Tris-Glycine gel (Novex, San Diego, CA) and blotted onto Immobilon P membrane (Millipore, Bedford, MA). The filter was blocked and hybridized with a 1:1,000 dilution of G186-1117. The immunoreactivity was visualized with an ECL kit (NEN, Boston, MA) according to the manufacturer's protocol.

IMMUNOCYTOCHEMISTRY

Immunolocalization was performed using methods similar to those previously described (Mi *et al.*, 1995). For fluorescent labeling the fixed, teased apart and permeabilized sciatic nerves were incubated in antibody (G186-1117) diluted 1:100 overnight at 4°C. Nerves were incubated in FITC-conjugated donkey anti-mouse IgG antibody (Vector Laboratories Inc., Burlingame, CA) diluted 1:75 in PBS with 5 μg/ml propidium iodide for 1 hour. After washing, the nerves were mounted in Gelvatol and imaged using an MRC-1024 laser-scanning confocal system (BioRad, Cambridge, MA) coupled to a Zeiss Axiovert 35 M microscope. To label 5-HT, we used a polyclonal antibody (Chemicon, Temecula, CA) diluted 1:200, and prepared the tissues according to the supplier's indications. Nerves were incubated in a goat anti rabbit, horseradish peroxidase conjugated (Calbiochem, La Jolla, CA) for 1 hour. After extensive washing, nerves were incubated for 5 min in a DAB solution and mounted as previously described. Neutral Red and Toluidine Blue stainings were performed as previously described (Schueller *et al.*, 1967; Ibeachum, 1979).

For electron microscopy nerves were incubated in antibody (G186-1117) diluted 1:1000 overnight at 4°C. Control nerves were incubated in antiserum that was blocked by preincubation with the specific blocking peptide (obtained from PharMingen, San Diego, CA) for 2 hours and otherwise treated identically. Nerves were then washed in buffer and incubated in biotinylated goat anti-rabbit IgG for 1 hour at 4°C. Following washes in PBS, the nerves were incubated in an avidin-biotin complex (Vector Laboratories Inc., Burlingame, CA) for 1 hour, washed again in PBS and reacted for 6 minutes in 0.05 mg/ml DAB with 0.01% H_2O_2. After washing, the nerves were post fixed with 1% OsO_4 in PBS for 1 hour, rinsed in double distilled water, dehydrated in an ethanol series and embedded in Durcupan resin. Some nerves were pretreated with 1mg/ml collagenase and 1mg/ml hyaluronidase in rat Ringer's for 1 hour at room temperature prior to fixation. Ultrathin (80 nm) sections were cut using a diamond knife (Diatome U.S., Fort Washington, PA) and an Ultracut E ultramicrotome (Leica Inc., Deerfield, IL) and mounted on uncoated copper grids. Thin sections were imaged at 80 Kev using a JEOL 100CX or 2000EX electron microscope (Jeol USA Inc., Peabody, MA).

Results

IDENTIFICATION OF 5-HT$_{2A}$ RECEPTOR TRANSCRIPT IN RAT SCHWANN CELLS USING RT-PCR AND NORTHERN BLOT ANALYSIS

5-HT$_{2A}$ transcripts were identified in cultured rat Schwann cells and in sciatic nerve by RT-PCR analysis of Poly A+ RNA (Fig. 1A). We used one pair of primers, delimiting the area between nucleotide 14 and nucleotide 425 of the rat cDNA sequence. A band of the expected size was identified in the brain positive control (lane 1), in the cultured Schwann cell (lane 2) and in the sciatic nerve (lane 3) samples. No amplification occurred if the reverse transcriptase reaction was omitted (lane 4), indicating that no genomic DNA was left in the sample. We directly sequenced the amplified

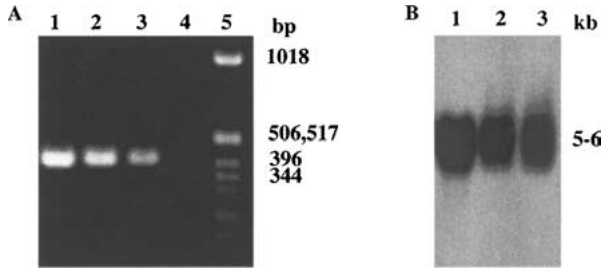

Fig. 1. *RT-PCR and Northern Blot Analysis*. (A) RT-PCR analysis of the N-terminus of rat 5-HT_{2A}. Rat brain cDNA (lane 1), Schwann cell cDNA (lane 2), sciatic nerve cDNA (lane 3) and rat Schwann cell RNA (lane 4) were amplified in 35 cycles of PCR performed with a sense and an antisense primer delimiting the region corresponding to 14 to 425 nucleotides from the start codon. (B) Northern Blot analysis of rat 5-HT_{2A}. Twenty μg of total RNA from rat brain (lane 1), sciatic nerve (lane 2) and cultured Schwann cells (lane 3) were run on a 1% agarose-formaldehyde gel, blotted to Hybond N + membrane and probed with a 5-HT_{2A} specific probe. The blot was washed at high astringency and exposed to X-ray film (Kodak) between two intensifying screens at -80°C for 1 week.

fragments and found that they matched the corresponding sequence in the rat 5-HT_{2A} cDNA.

A cocktail of restriction fragments from the plasmid pAM (Bam HI-Xba I, 1928bp; Xba I-Pst I, 888bp; Pst I-Pst I, 904bp) was used to probe a Northern blot (Fig. 1B) of total RNA from adult rat brain (lane 1), sciatic nerve (lane 2) and cultured rat Schwann cells (lane 3). The Northern analysis revealed in each sample one band of 5–6 kb, a much larger area than the coding region, which is known to be approximately 1.4 kb long. This was previously reported for the rat (Garlow *et al.*, 1994), mouse (Yang *et al.*, 1992), and the human (Chen *et al.*, 1992) 5-HT_{2A} transcripts.

DISTRIBUTION OF 5-HT_{2A} RECEPTOR TRANSCRIPTS IN RAT SCIATIC NERVE AS REVEALED BY ISH

The distribution of 5-HT_{2A} transcripts in rat cultured Schwann cells was examined by ISH (Figs. 2A–F). The antisense digoxigenin-UTP-labeled riboprobe, specific for rat 5-HT_{2A} mRNA, strongly stained the cytoplasm of cultured rat Schwann cells (Figs. 2A and C). Staining for S100β, a Schwann cell marker, was used to verify the purity of our cultures and the cellular identity of the 5-HT_{2A}-positive cells (Figs. 2B, C, E and F). The sense riboprobe used as a negative control produced no appreciable staining (Figs. 2D and F).

The antisense digoxigenin-UTP-labeled riboprobe was also employed in ISH on sciatic nerve sections, where it revealed signals predominantly associated with myelinated nerve fibers (Figs. 2G, H and J). At a higher magnification (100x), the typically bipolar morphology of Schwann cells could be easily identified (Fig. 2J). The sense riboprobe gave no appreciable signal (Fig. 2I). The distribution of the transcript was mainly

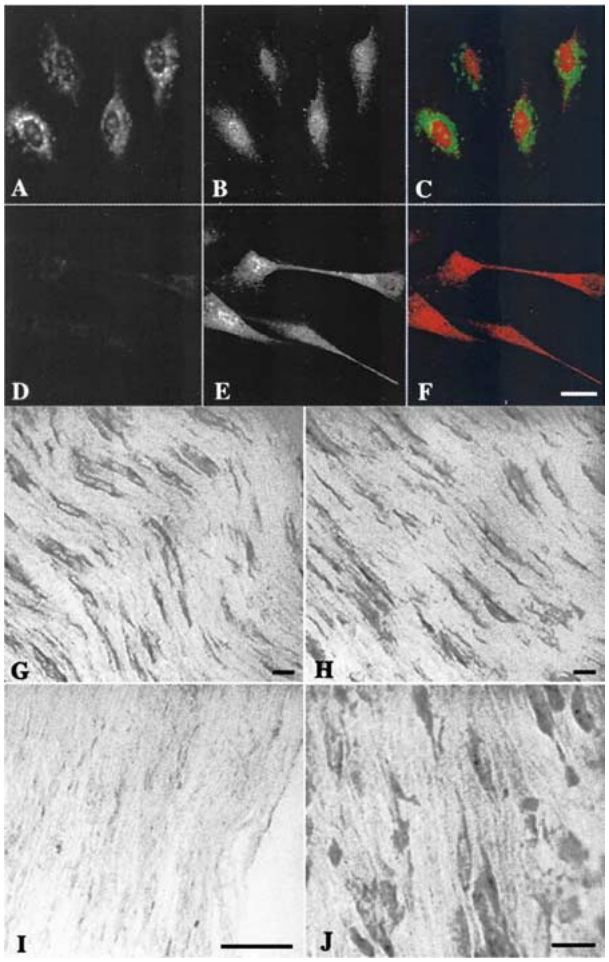

Fig. 2. *ISH experiments on cultured Schwann cells and longitudinal sections of sciatic nerve*. Cultured cells (A–F) or nerve sections (G–J) were hybridized with an antisense riboprobe specific for 5-HT_{2A} (A–C, G, H and J) or, as a negative control, with the corresponding sense riboprobe (D–F and I). Cultured rat Schwann cells were co-labeled for S100β (B, C, E and F), a Schwann cell marker. Panels (C) and (F) show the double labeling for S100β (in red, C and F), and antisense (in green, C) or sense (F) riboprobes. Magnification of a section of sciatic nerve hybridized with the antisense riboprobe displays the typical longitudinal shape of Schwann cells (J). Scale bar, (A–F), 20 μm; (G, H and J), 10 μm; (I), 100 μm.

cytoplasmic, and resembled that of other proteins expressed by Schwann cells in the sciatic nerve (Kuhn *et al.*, 1993; Gillen *et al.*, 1996).

IDENTIFICATION OF 5-HT_{2A}R PROTEIN IN RAT SCHWANN CELLS USING WESTERN BLOT ANALYSIS

We examined the expression of the 5-HT_{2A}R protein using a receptor-specific monoclonal antibody, G186-1117 (Fig. 3). G186-1117 is specific to 5-HT_{2A}R and does not cross-react with other 5-HT receptors (Wu *et al.*, 1998). When used in Western blot analysis on protein lysates from cultured rat Schwann cells (lane 2) and rat sciatic nerve (lane 3), G186-1117 labeled one major

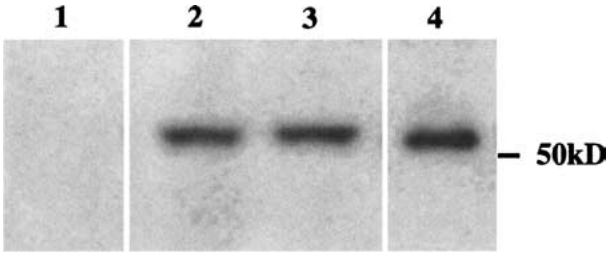

Fig. 3. *Western Blot Analysis of 5-HT_{2A}.* Whole cell lysates from rat brain (lane 1 and 4), cultured rat Schwann cells (lane 2) and adult rat sciatic nerves (lane 3) were western blotted with G186-1117, a monoclonal antibody specific to 5-HT_{2A}R (lane 2, 3, 4). No G186-1117 was hybridized to lane 1 (negative control).

band slightly higher than 50 kDa, that co-migrated with an immunoreactive band from rat brain lysate (lane 4). This finding is in agreement with the expected receptor size of 53-55 kDa. Negative control experiments performed using the secondary antibody alone revealed no detectable bands (lane 1).

IMMUNOHISTOCHEMICAL DISTRIBUTION OF 5-HT_{2A}R PROTEIN IN RAT SCIATIC NERVE AS REVEALED BY LIGHT AND ELECTRON MICROSCOPY

Healthy rat sciatic nerves were dissected and teased apart until individual fibers were sufficiently dissociated from one another to allow antibody penetration. G186-1117 was used to examine the localization of 5-HT_{2A}Rs in adult rat sciatic nerve by immunofluorescence staining and laser-scanning confocal microscopy (Fig. 4). The fluorescent labeling was found on the outer layer of the Schwann cell membrane and in the Schwann cell cytoplasm. While staining was observed at the nodes of Ranvier (Fig. 4, arrow), it was not restricted to these locations. This labeling pattern resembles the pattern observed with the 5-HTR anti-idiotypic antibody (Yoder *et al.*, 1997b). Negative controls, performed by omitting the primary antibody and by using a pre-immune IgG2A mouse antibody, showed little or no staining (data not shown).

Using immunoelectron microscopy, 5-HT_{2A}R immunoreactivity was found to be uniformly distributed on the outer Schwann cell membrane (Figs. 5A and 5B). Preabsorption with the peptide immunogen abolished staining (Fig. 5D). Near nodes of Ranvier, reaction product was observed on the outer Schwann cell membranes and on the microvilli extending from the Schwann cells, adjacent to the nodal membrane (Fig. 5C). No staining of the nodal axolemma itself was observed.

DISTRIBUTION OF 5-HT IN THE RAT SCIATIC NERVE

We employed a polyclonal antibody recognizing 5-HT to investigate the cellular location of this neurotransmitter in rat sciatic nerves. 5-HT-like immunoreactivity

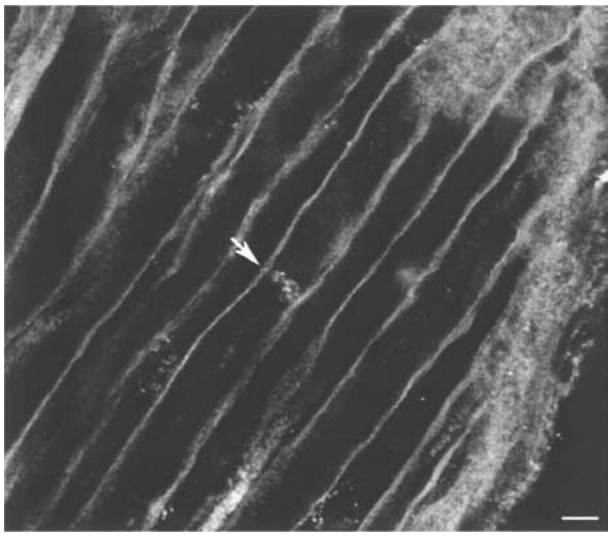

Fig. 4. *Confocal microscopy of a rat sciatic nerve section immunolabeled with G186-1117.* Sciatic nerves were isolated from adult rats, teased apart and prepped as described in the Methods. The immunolabeling was performed using G186-1117, followed by a FITC-conjugated secondary antibody. The specimen was viewed with a Bio-Rad MRC-1024 laser-scanning confocal system. Arrow points at a Node of Ranvier. Scale bar, 5 μm.

was found to be restricted to granule-containing cells within the peripheral nerve (Figs. 6A–F). To determine if these corresponded to mast cells, we subsequently stained the same sections in a solution of Neutral Red (Ibeachum, 1979) or Toluidine Blue (Schueller *et al.*, 1967). These histological stains label the mast cell cytoplasm in dark red (Neutral Red, Figs. 6B and E) or deep purple (Toluidine Blue, Figs. 6C and F), leaving the nucleus virtually unstained. As shown in Figure 6, the granule-containing cells that stained positively for 5-HT, were also stained by Neutral Red and Toluidine Blue. Thus, endoneurial mast cells appear to be the main source of 5-HT in rat sciatic nerves, as previously reported by other methods (Olsson, 1965, 1966).

Discussion

This study directly demonstrates the expression by rat Schwann cells of transcripts for 5-HT_{2A}R and of the 5-HT_{2A} receptors themselves. We performed our analysis both at the RNA level and at the protein level, and examined the distribution of the 5-HT_{2A} receptor with both LM and EM. Using a combination of histochemical stains and immunolabeling, we also determine that endoneurial mast cells are the most probable source of 5-HT available to activate 5-HT_{2A} receptors on myelinating Schwann cells in rat sciatic nerves.

We detected the messenger for 5-HT_{2A}R by RT-PCR and Northern blot analysis of RNA isolated from cultured Schwann cells and sciatic nerves. The size of the transcript identified in the Northern blot was larger

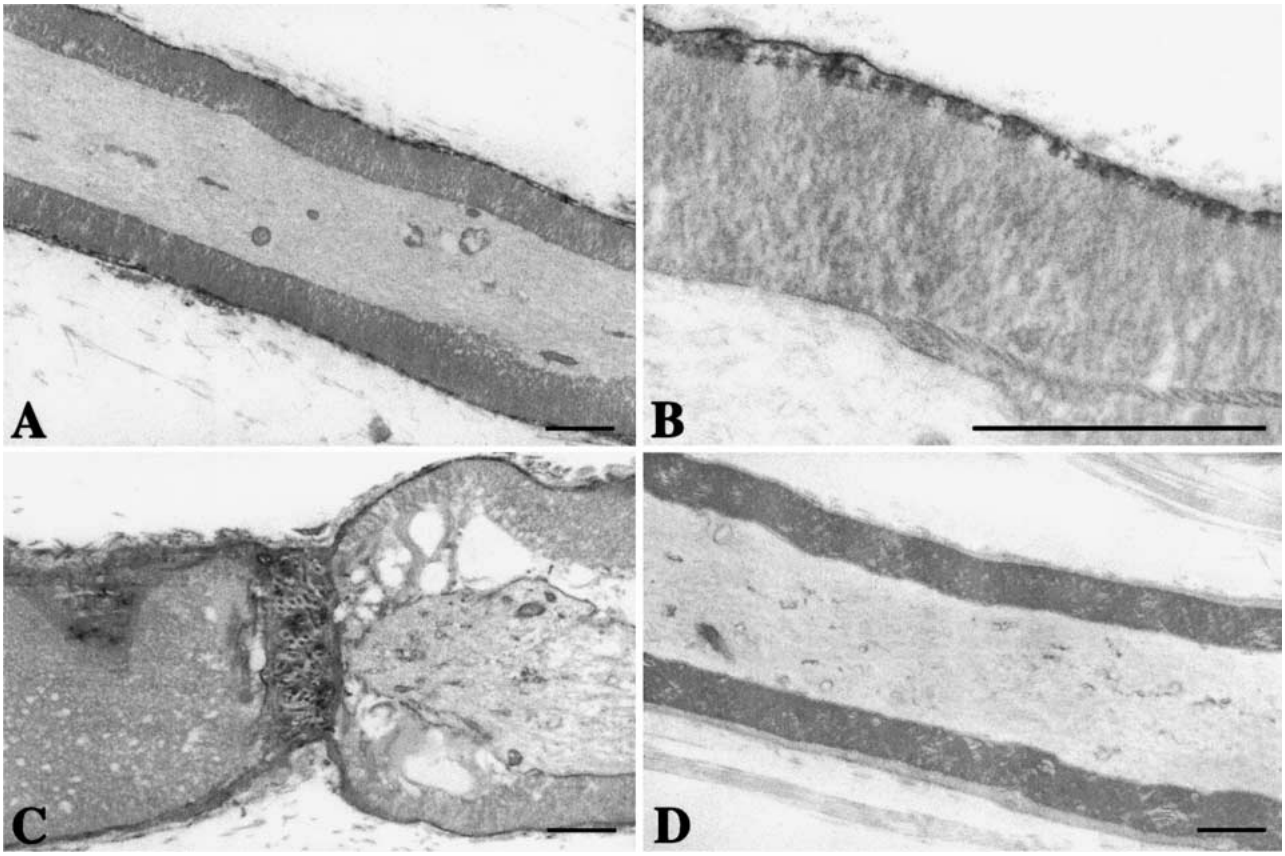

Fig. 5. *Immunoelectron microscopy of rat sciatic nerve sections stained with G186-1117*. DAB reaction product is disbursed uniformly on the outer Schwann cell membrane, as shown in panel A and, at higher magnification, in panel B. At nodes of Ranvier, immunoreactivity is found on the nodal microvilli and outer Schwann cell membrane (panel C). No immunoreactivity is seen on the nodal membrane itself. No staining is visible in control preparations incubated in antibody preabsorbed with the peptide antigen (panel D). Scale bar, 1 μm.

than the 1.4 kilobases predicted by the length of the coding region for 5-HT_{2A}R. The discrepancy in size may indicate that large untranslated areas exist in the up- or downstream regions of the transcript. Similar data have also been presented for mouse (Yang *et al.*, 1992), rat (Garlow *et al.*, 1994) and human (Chen *et al.*, 1992) 5-HT_{2A} mRNA. The role played by these areas could be of controlling the transcription of the 5-HT_{2A} message, probably by providing docking sequences for specific transcriptional factors. The Northern blot and RT-PCR data that we present do not rule out the possibility that other cellular components of peripheral nerves express 5-HT_{2A} transcripts. 5-HT_{2A}Rs are present on endothelial cells, as previously shown (Martin, 1994). The engagement of these receptors by 5-HT may alter the permeability of local vasculature and prime the tissue for the arrival of inflammatory cells (Martin, 1994).

To further corroborate at the RNA level the hypothesis that Schwann cells express 5-HT_{2A}R, we analyzed cultured rat Schwann cells and sections of sciatic nerve by ISH using a 5-HT_{2A}-specific antisense riboprobe. Our results clearly showed 5-HT_{2A} transcript mainly localized throughout the cytoplasm of Schwann cells, with a distribution similar to what was showed for other transcript species expressed in peripheral nerves (Kuhn *et al.*, 1993; Gillen *et al.*, 1996).

The monoclonal antibody G186-1117 gave us the advantage of a higher specificity for 5-HT_{2A}R over the less specific anti-idiotypic antibody used in our previous work. The anti-idiotypic polyclonal antibody was generated by immunization with antibodies to 5-HT and was produced for use as an agonist (or antagonist) through its interaction with the ligand binding regions of 5-HT receptors (Tamir *et al.*, 1991). On the other hand, G186-1117 was generated using a glutathione S-transferase-human 5-HT_{2A}R fusion protein, containing the amino acids 1-78 of the N-terminus region of the receptor (Wu *et al.*, 1998). G186-1117 reacted with a 55 kD band corresponding to the predicted size of 5-HT_{2A}R in lysates from rat brain and no cross-reactivity with 5-HT_{2B}R and 5-HT_{2C} receptor was detected (Wu *et al.*, 1998). G186-1117 was originally tested in immunohistochemical labeling of rat brain sections and cultured rat Schwann cells (Wu *et al.*, 1998). In the present study, we labeled sections of rat sciatic nerve with G186-1117 and found 5-HT_{2A}R to distribute evenly along the outer

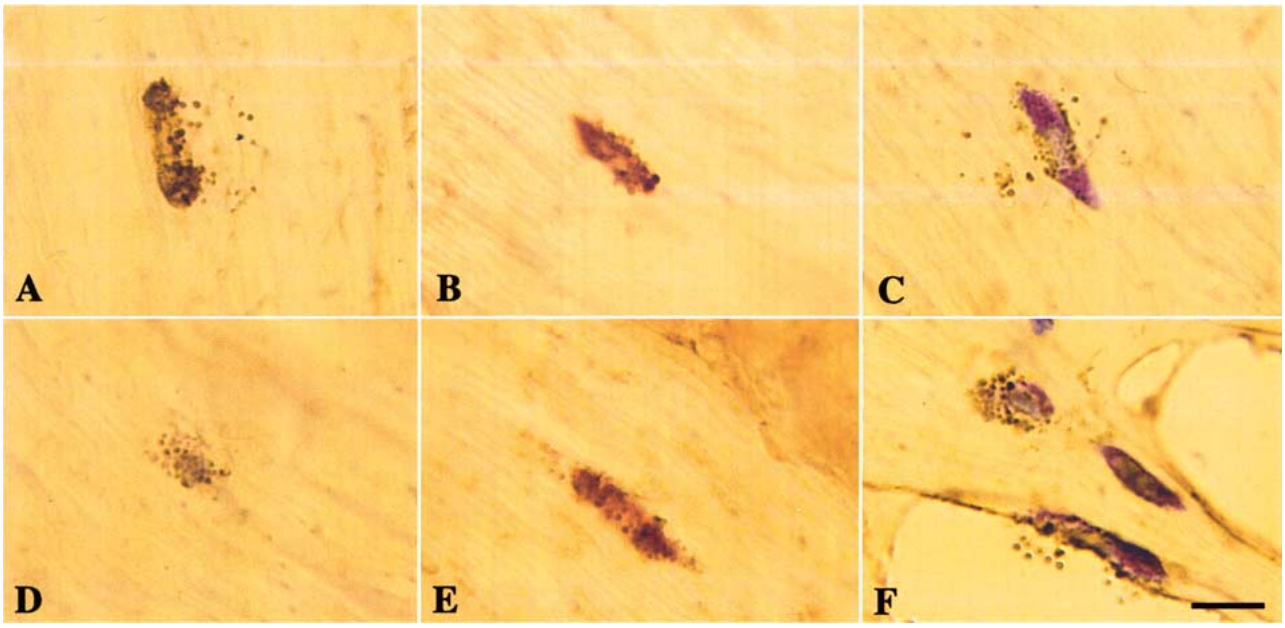

Fig. 6. *Endoneurial Mast Cells contain 5-HT*. A polyclonal antibody recognizing 5-HT was used to stain sections of rat sciatic nerve (A–F). The DAB reaction product was localized in the round granules of sparsely distributed cells (A and D). The same specimens were also stained with Neutral Red (B and E) or with Toluidine Blue (C and F), showing the typical dark red (Neutral Red) or metachromatic purple (Toluidine Blue) staining of mast cells. Panel B, C, E and F show both immunolabeling (in brown) and specific histological stains (in red-B, E- or purple-C, F). Scale bar, 20 μm.

cell membrane of Schwann cells in the nerve. 5-HT$_{2A}$R labeling at the nodes of Ranvier was observed in the Schwann cell microvilli but—as expected—not along the axolemma membrane. We previously obtained a similar staining pattern using the anti-idiotypic antibody, and suggested that "the distributed and nonspecific nature of the 5-HT receptor labeling in Schwann cells could reflect the distributed and dynamic nature of mast cell locations" (Yoder *et al.*, 1997b).

Our Toluidine blue and Neutral Red stainings showed mast cells to be located throughout the endoneurium of healthy nerves. Their granules contained 5-HT, as we showed in immunohistochemical labeling with a specific antibody. No 5-HT-like immunoreactivity was detected in the surrounding axons, suggesting that mast cells were the only source of 5-HT in the rat sciatic nerve. Similar findings have been obtained using a different method (Olsson, 1965, 1966; Anden & Olsson, 1967).

The participation of inflammatory cells (like macrophages) to nerve injury is typically prominent on a time course of days to weeks after injury (Griffin *et al.*, 1993). In contrast, mast cells resident in the endoneurium are able to degranulate within minutes after the insult, and thus are positioned to play a key role in the initial immune response following nerve injury. As the expression of serotonin receptors by Schwann cells occurs in healthy nerves and quickly downregulates following injury (Yoder *et al.*, 1997b), it could be that the detection by Schwann cells of mast cell-released serotonin triggers the early stages of demyelination. This possibility merits further investigation.

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