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Type-1 Angiotensin Receptors are Expressed and Transported in Motor and Sensory Axons of Rat Sciatic Nerves

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Abstract

Angiotensin II (Ang II) and its type-1 receptor (AT₁) occur in neurons at multiple locations within the organism, but the basic biology of the receptor in the nervous system remains incompletely understood. We previously observed abundant AT₁-like binding sites and intense expression of AT₁ immunoreactivity in perikarya of the dorsal root ganglion and ventral horn of the rat spinal cord. We have now examined the receptor in rat sciatic nerve, including the dynamics of its axonal transport. Ligand-binding autoradiography of resting nerve showed “hot spots” of ¹²⁵I-Ang II binding that could be specifically blocked by the AT₁ antagonist, losartan. Immunohistochemistry with an AT₁-antibody validated by Western blots also showed patches of AT₁-reactivity in nerve. These patches were localized around large myelinated axons with faint immunoreactivity in their lumens. Sixteen hr after nerve ligation there was no change in the patches or hot spots, but luminal AT₁-reactivity increased dramatically in a narrow zone immediately above the ligature. With double ligation there was a pronounced accumulation of AT₁ immunoreactivity proximal to the upstream ligature and a very slight accumulation distal to the second ligature. This asymmetric pattern of accumulation, confirmed by quantitative receptor binding autoradiography, probably reflected axonal transport rather than local production of receptor. Retrograde tracing and stereological analysis to determine the source of transported AT₁ indicated that many AT₁-positive fibers arise in the ventral horn, and a larger number arise in dorsal root ganglia. A corresponding result was obtained with double-label immunohistochemistry of ligated nerve, which showed AT₁ accumulations in both motor and sensory fibers. We conclude that somatic sensory and motor neurons of the rat export substantial quantities of AT₁ into axons, which transport them to the periphery. The physiologic implications of this finding require further investigation.

Keywords

neuropeptide receptors; axonal transport; nerve ligation; retrograde fluorescence tracing; dorsal root ganglia; spinal cord ventral horn; choline acetyltransferase; calcitonin-gene-related peptide

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Introduction

Angiotensin II (Ang II), an octapeptide involved in homeostasis of fluids and electrolytes, is produced systemically and locally from angiotensinogen in many tissues, including kidneys, adrenal glands, blood vessels, and heart (Allen et al., 1990; Barnes et al., 1993; Edwards and Aiyar, 1993; Lenkei et al., 1997; Montiel et al., 1993; Saavedra et al., 1993). Ang II is also found along with its receptors in nervous tissue including the brain. Recently, abundant expression of type-one angiotensin receptors (AT₁) has been observed in a majority of the neurons in dorsal root ganglia (DRG), in the neuropil of the dorsal horn, and in ventral horn neurons of the rat spinal cord (Ahmad et al., 2003; Tang et al., 2008). These locations represent the origins or terminations of motor or sensory fibers in peripheral nerve. Many neuropeptides, as well as trophic factors and their receptors, are conveyed within nerve fibers by axoplasmic transport (Altar et al., 1997; Donnerer et al., 1993; Schwartz, 1979; Wamsley, 1992; von Bartheld et al., 1996; von Bartheld et al., 2001), a process critical for neuronal survival. In fact, early studies showed that binding sites for Ang II would accumulate at a crush site on rabbit vagus nerve (Diz and Ferrario, 1988). Some investigators have interpreted this phenomenon as an increased local production of AT₁ in response to injury and inflammation. The present work with rat sciatic nerve was undertaken to confirm the more likely alternative hypothesis that Ang-II receptors are in constant flux along nerve fibers and will accumulate at points where axonal flow is interrupted. We also sought to determine whether the AT₁-positive fibers of sciatic nerve are primarily motor or sensory in nature.

Methods

Animals

Adult male Sprague-Dawley rats (200-250g, Harlan, Madison, WI) were handled in accord with the *NIH Guide for Care and Treatment of Laboratory Animals* under protocols approved by the Mayo Institutional Animal Care and Use Committee.

Sciatic nerve ligation and retrograde neuronal labeling

Rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.), supplemented with smaller doses (5mg/kg, i.p.) as needed. The skin of the left thigh was shaved and disinfected with 1.75% iodine. Next the sciatic nerve was exposed and tied with autoclaved silk suture (5-0 gauge), 5 to 10 mm above the bifurcation of its tibial and peroneal branches (in certain experiments, a second ligature was placed 2-3 mm distal to the first one). The skin incision was closed with sterile sutures. Sixteen hr later, the rats were euthanized with sodium pentobarbital (250 mg/kg, i.p.). The ligated nerves, along with controlateral controls, were dissected, frozen on dry ice, and kept at - 80 °C until sectioning for immunohistochemistry or autoradiography.

For retrograde fiber tracing, sciatic nerves in rats anesthetized and prepared as described above were transected at mid-thigh, and each proximal stump was inserted into a Silastic tube filled with 7 µl of 2% Fluorogold ("FG", Biotium, Hayward, CA). The tube, sealed at its distal end, was affixed to the surrounding skeletal muscles with tissue glue ("Nexaband S / C", Abbott Laboratories). After 3 days, a period found optimal for labeling in preliminary experiments, rats were euthanized with pentobarbital. The L-4 segment of the lumbar spinal cord was then dissected for histochemical analysis, along with the associated dorsal root ganglion (DRG) identified by tracing sciatic nerve roots back to their entries.

Other transport experiments utilized ³H-tyrosyl-Ang II (52.5 Ci mmol, New England Nuclear, Waltham MA) was injected into superior cervical ganglia (SCG) or applied to sciatic nerve. Rats were anesthetized with pentobarbital and prepared for sterile surgery as above, and ganglia or nerves were exposed through small skin incisions in the neck or thigh. In some rats ³H Ang

II was then slowly injected into the SCG on one side (50 nCi in 10 μ l of 0.9% NaCl). The tracer solutions contained Dextran Blue as marker dye, and injections were deemed successful when they resulted in blue ganglia with minimal leakage. In other rats the sciatic nerve was transected and the proximal stump was introduced into a Silastic tube prepared with the same ^3H -Ang II solution. Skin incisions were closed with sterile sutures, as above, and animals were closely observed until euthanasia for signs of infection (not seen). Subsequently, samples of nerve, ganglion, and spinal cord were digested in 2 ml of NCS tissue solubilizer (Amersham), and radioactivity was determined by scintillation counting in Optima Gold fluor.

Immunohistochemistry

Sciatic nerves were dissected from euthanized rats and flash frozen on dry ice. Later, frozen 16- μ m sections were cut and thaw-mounted on slides for further processing. L4 spinal cord segments and DRG samples were obtained from rats perfused with 500 ml of phosphate-buffered saline (PBS), 0.1 M, pH 7.4, followed by 500 ml of 4% paraformaldehyde in PBS. These tissues were post-fixed for 2 h, and immersed overnight in 20% sucrose-PBS at 4° C. Floating 30- μ m sections were then prepared and incubated overnight at 4° C with primary antibodies. The primary AT₁ antibody (Novus 18801-50) was a rabbit antiserum at 1:50 dilution which we had found to label a single band at 45 kDa in Western blots of rat spinal cord (Tang et al., 2008) and sciatic nerve (Tang and Brimijoin, unpublished data). Mouse antibody to choline acetyltransferase (ChAT) from Dr. B.K Hartmann was applied at 1:50 dilution; mouse antibody to calcitonin-gene-related peptide (CGRP) from Santa Cruz (SC-7448) was applied at 1:500; and goat anti-myelin basic protein antibody (SC-13912) was applied at 1:1600. Sections were rinsed and incubated with fluorescein- or rhodamine-conjugated secondary antibodies for 3 hours at room temperature. Other sections for “ABC staining” were incubated with biotinylated secondary antibodies at 1:150 (Vector Labs, Burlingame, CA) for 2 h at room temperature, processed with avidin-coupled horseradish peroxidase, and developed with diaminobenzidine. Stained slides were mounted, coverslipped, and examined with a standard Zeiss microscope or a LSM 510 confocal fluorescence imaging system.

Cell counting and stereology

FG-labeled and / or AT₁-positive spinal cord motor and DRG neurons were imaged with an LSM 510 confocal imaging system and subjected to stereological analysis essentially as described by Hart and Terenghi (2004). From each L4 spinal cord segment, an exhaustive series of 30- μ m cross sections was cut. One in every three sections was processed for AT₁ immunohistochemistry and for FG-tracer analysis, which counted every stained and / or labeled ventral horn neuron with a visible nucleus. Multiplying the number of sections by the average numbers of positive neurons per section yielded preliminary estimates of the totals per segment. On the sensory side, an exhaustive series of 30- μ m sections was prepared from each DRG. In this case, however, cell counts were performed on every section, in a single randomly chosen field, ignoring neurons whose nucleus touched the left and lower borders of the selected fields (again, only neurons with visible nuclei were counted). The resulting values were used to determine the average number of neurons per mm² on that section. These numbers were multiplied by the section area, as measured by calibrated video microscopy with NIH image (version 1.57). The sum of the values for all sections yielded a preliminary estimate of the number of labeled and stained neurons in each ganglion. To correct for over-counting, preliminary estimates were multiplied by the factor, $F = T / (T + d)$, where T is section thickness (30 μ m) and d is average nuclear diameter (Hart and Terenghi, 2004). For an empirical estimate of d, we measured cross sectional area (A) in 100 randomly chosen neuronal nuclei, assumed approximate circularity, and applied the formula, $d = 2(A \cdot \pi)^{0.5}$. Resulting values of 24 μ m and 16.8 μ m, for cord and DRG respectively, yielded correction factors of 0.56 and 0.64.

Ang II receptor binding

Autoradiography was used to determine the location and abundance of Ang II binding sites in sciatic nerve with ligand selectivity characteristic of AT₁ receptors. Consecutive 16- μ m sections of fresh frozen samples were cut at -20°C, thaw-mounted on poly-L-lysine coated slides (Erie Scientific, Portsmouth, NH), dried overnight in a desiccator at 4°C, and stored at -80°C until used. Binding studies were then performed as described previously (Tsutsumi and Saavedra, 1991). In brief, sections were pre-incubated 15 min at room temperature in 10 mM sodium phosphate buffer, pH 7.4, containing 120 mM NaCl, 5 mM Na₂EDTA, 0.1 mM bacitracin (Sigma-Aldrich, St. Louis, MO) and 0.2% protease-free bovine serum albumin (Sigma). Slides were then incubated for 2 hr at room temperature in fresh buffer with 0.5 nM of [¹²⁵I]-Sarcosine¹-Ang II (Peninsula Laboratories, Belmont, CA) iodinated to 2176 Ci/mmol by the Peptide Radioiodination Center, Dept. Pharmacology, Univ. Mississippi. Adjacent sections were incubated as above but in the presence of 5 μ M unlabeled Ang II (Peninsula Laboratories). Radioactivity displaced by the excess of unlabeled agonist was our measure of specific binding to Ang II receptors in general. To determine binding to receptor subtypes, additional sections were incubated with 0.5 nM of [¹²⁵I]-Sarcosine¹-Ang II in the presence of the selective AT₁ antagonist, losartan (10 μ M; Dupont-Merck, Wilmington, DE, USA) or the selective AT₂ antagonist, PD 123319 (10 μ M, Sigma), under conditions chosen to give maximal specific displacement (Tsutsumi and Saavedra, 1991).

After incubation, slides were rinsed four times for 1 min each in fresh ice-cold 50 mM Tris-HCl buffer (pH 7.5), dipped in ice-cold water, and dried under a stream of air. Dried sections were exposed to Kodak BioMax MR film (Eastman Kodak, Rochester, NY) together with [¹⁴C] micro-scales (American Radiolabeled Chemicals, St. Louis, MO). Exposure times varied, depending on the observed binding, to obtain images within the linear portion of the standard curve. Films were developed for 4 min in ice-cold GBX developer (Eastman Kodak), fixed in Kodak GBX for 4 min at room temperature, and rinsed in running water for 15 min. Finally, using a program based on "NIH Image" (Scion Image, Scion Corporation), we normalized the [¹²⁵I] autoradiograms to the [¹⁴C] standards and converted the optical densities to values of fmol/mg protein (Miller and Zahniser, 1987; Nazarali et al., 1989).

Results

AT₁ in resting sciatic nerve

Receptor binding autoradiography showed punctate or patchy ¹²⁵I-Ang II binding in the resting sciatic nerve. This binding was blocked by the AT₁ antagonist, losartan, but not by the AT₂ antagonist, PD 123319 (Fig.1). Staining of longitudinal nerve sections by a specific AT₁ antibody exhibited a generally similar pattern of localization, though the distribution of AT₁ immunoreactivity was less obviously discontinuous (Fig. 2A). In cross-sections, occasional rings of intense AT₁ immunoreactivity partly surrounded large axonal profiles (Fig.3A), and faint immunoreactivity was sometimes detected in the axonal lumens. Double immunohistochemistry with antibody for myelin basic protein indicated that the brightest staining was outside the axon proper, possibly associated with Schwann cells (Fig. 3B, C).

Axonal Transport of AT₁

Given previous observations of high AT₁ expression in neuronal cell bodies of the DRG and spinal cord (Tang et al., 2008), the association of AT₁ immunoreactivity with peripheral nerve fibers suggested an ongoing process of axonal transport. To test this possibility, double ligations were placed on sciatic nerves in vivo for 16 hr. Specific AT₁-like immunoreactivity increased to a major extent on the proximal side of the "upstream" ligature and to a much lesser extent on the distal side (Fig. 2 C, D). Cross-sections clearly showed that the increased immunoreactivity was axoplasmic, i.e., in the interior of the axon (Fig 3 D, E, F). These two

results are strongly indicative of rapid axonal transport. Furthermore, AT₁ immunoreactivity proximal to the first ligation was far more intense than it was proximal to the second ligation, or distal to either of the ligations (compare Fig. 2 C with D, E, & F). The accumulation of AT₁ binding sites therefore followed the same pattern of marked spatial asymmetry that would be expected from the interruption of an ongoing flow of receptor (Fig. 4). In fact, a quantitative analysis of the binding data indicated that AT₁ binding sites increased by more than an order of magnitude in the immediate vicinity of the proximal ligature, as compared with unligated nerve or with segments isolated between ligatures (Fig. 5). We concluded that AT₁ protein was indeed undergoing axonal transport, mostly in the anterograde direction but possibly, to a lesser extent, also retrograde.

To extend these findings we attempted to determine whether exogenous Ang II could be captured by neurons and conveyed back toward nerve cell bodies along with its receptor. For that purpose, sciatic nerves of three rats were transected on one side, ligatures were placed 12 mm above the cut, and the proximal stumps were exposed in situ to solutions containing ³H-Ang II. After 48 hr, the treated nerves and untreated contralateral nerves were harvested to assess the content and distribution of ³H peptide (see Methods). In all cases, radiolabel remained in the immediate vicinity of the proximal stump and there was no evidence of accumulation below the ligature (Fig 6). Also, 7 days after treatment of unligated nerve, there was no accumulation of label in DRG (L1 to L5 level) on the side exposed to ³H-Ang II (not shown). Negative results were likewise obtained when ³H-Ang II was injected into superior cervical ganglia and samples (T3 to C8 spinal cord segments and DRG) were harvested 2 or 7 days later (6 rats each). In each case there was no excess radiolabel on the treated side (not shown). Therefore, little or no exogenous Ang II appeared to be captured and transported by AT₁-bearing axons under any of these conditions. If transport did occur, the amount of peptide conveyed was below the limit of radiochemical detection in our system (approximately 10⁻¹⁵ mol).

Functional classification of AT₁-positive fibers

In principle the fibers engaged in transporting AT₁ might be either sensory or motor. A baseline experiment with immunoperoxidase staining confirmed previous observations of abundant AT₁-positive neurons in the L4 DRG (Fig. 7) and in L4 ventral horn (not shown). To determine which type of neuronal perikarya represent the main source of the transported AT₁ immunoreactivity and specific binding sites in sciatic nerve, we utilized retrograde tracer labeling with the fluorescent marker, FG. This marker, which efficiently labels motor, sensory, and autonomic neurons (Velandia et al., 2002) was applied to sciatic nerve stumps in the thigh. Three days were allowed for the marker to reach the cell bodies of motor and sensory neurons in spinal cord and DRG. L4 spinal cord and corresponding ganglia were then harvested for immunohistochemistry and observation of tracer fluorescence. Neurons labeled with both FG and AT₁ were considered to represent the origin of AT₁-bearing fibers in sciatic nerves. We found many such double-labeled neurons in the ventral horn of the spinal cord as well as in the DRG (Fig. 8). Therefore, the AT₁-positive fibers in sciatic nerve must be a mixture of motor and sensory axons. For a quantitative analysis, we separately counted FG-positive and FG-AT₁ double-positive neurons in spinal cord and DRG. Since both spinal motor neurons and DRG neurons project single fibers to the periphery, it is valid to assume that each FG-positive motor or DRG neuron represented one motor or sensory fiber in the sciatic nerve. Likewise, we took each doubly positive motor or sensory cell body to represent an AT₁-positive motor or sensory fiber in the sciatic nerve. The data from this experiment showed that a sizeable majority of the FG-positive cell bodies were also AT₁-positive—75% of them in ventral horn and 65% in DRG (Fig. 9). Also worth noting, tracer-labeled perikarya were 74% more numerous in the L4 DRG than in the L4 ventral horn, and double-labeled perikarya were 51% more numerous.

Because the efficiency of labeling might differ in sensory and motor systems one should not draw rigid quantitative conclusions from the tracer study. Nonetheless, these results were supported by an additional experiment in which cryostat sections of sciatic nerve, prepared after 16 hr ligation, were subjected to immunohistochemistry for AT₁ and the motor axon marker, ChAT, or the sensory axon marker, CGRP. Each marker co-localized with AT₁ in some fibers. No formal attempt was made to obtain quantitative data. Nonetheless, axonal profiles positive for both AT₁ and CGRP were more numerous than those positive for AT₁ and ChAT (Fig. 10).

Discussion

In this study, ligand-binding autoradiography revealed moderate levels of AT₁-type Ang II receptors in unperturbed rat sciatic nerve, corroborated by immunohistochemistry that also showed isolated patches of marked AT₁-reactivity. This resting immunoreactivity appeared near the outer margins of large axons, but in locations that did not correspond closely to axolemmal membranes or to myelin (as identified by staining for myelin basic protein). Definitive identification requires additional experiments but we tentatively ascribe these natural hot spots to Schwann cells.

Nerve ligation changed the picture substantially. When nerves were ligated for a few hours, AT₁-like binding sites (but not AT₂ sites) increased sharply, and AT₁-immunoreactivity accumulated within the axonal lumens. This effect was more substantial and much faster than the local increases of AT₁ and AT₂ mRNA that have been noted several days after a sciatic nerve crush lesion (Gallinat et al., 1998). Our rapid time course argues strongly for a mechanism involving interruption of axonal transport rather than induction of local synthesis. Additional reasons why the accumulation of AT₁ almost certainly involved axonal transport are: 1) it localized within axons; 2) it was vastly greater on the proximal side than on the distal side of the ligature; and 3) it was weak or absent at a second ligature placed a few mm distal to the primary one. The results do not imply that Schwann cells or other peripheral nerve elements fail to produce such receptors in response to injury. Nonetheless, they confirm that, under normal physiologic conditions, spinal neurons express AT₁ widely but not AT₂, actively distribute the receptor into their axons, and move it rapidly towards their terminals. This conclusion is fully supported by previous situ hybridization and other studies indicating widespread AT₁ expression in neuronal cell bodies of rat DRG and spinal ventral horn (Ahmad et al., 2003; Pavel et al., 2008; Tang et al., 2008). Our finding that fluorescent tracer molecules from the sciatic nerve can travel upwards along axons to label these AT₁-positive perikarya is further support for the concept of a dynamic system for production and dispersion of Ang II receptors in multiple elements of the peripheral nervous system. The quantitative distribution of the labeled perikarya, more numerous in DRG than in ventral horn, matches the qualitative pattern of co-localization with fiber-type specific markers in ligated nerve. From all these observations we conclude that AT₁ must play some physiological role in both sensory and motor systems but may be especially important in the former.

The present experiments complement our recent study combining in-situ hybridization and ligand-binding autoradiography (Pavel et al., 2008). That study showed high levels of Ang II receptor mRNA in DRG (primarily AT_{1A}) but low levels in spinal cord dorsal horn, while AT₁-like ligand binding was high in both locations. It also showed that selective dorsal rhizotomy caused substantial loss of AT₁ expression in dorsal horn. In addition the present study confirms our previous report (Tang et al., 2008) that a large proportion of DRG neurons exhibit immunohistochemical staining with a selective AT₁ antibody. Together, these findings imply that DRG neurons account for the dorsal horn's abundant expression of this receptor, which must be synthesized in the ganglia and delivered through central axonal projections.

Presynaptic receptors are important in modulating neurotransmitter release in both positive and negative fashion (Starke, 1981). Axonal transport is essential for these receptors to reach and incorporate into presynaptic nerve terminals, since the apparatus for protein synthesis in neurons is largely confined to cell bodies. Axons of the sciatic nerve are known to transport several classes of presynaptic neurotransmitter receptors, including muscarinic, nicotinic cholinergic receptors, adrenergic, and neuropeptide receptors (Gulya et al., 1989; Lundberg et al., 1978; Millington et al., 1985; Zarbin et al., 1983). Some of this transport also involves the neuropeptides and transmitters themselves (Brumovsky et al., 2002; Laduron, 1984, 1995; Lundberg et al., 1978). AT₁ clearly belongs in the group of transported presynaptic receptors. It was shown early on that Ang II enhances the release of norepinephrine from postganglionic sympathetic terminals (Endo et al., 1977). Later work demonstrated that this effect is mediated through AT₁ in the mouse heart and spleen (Cox et al., 1999) and the guinea pig enteric nervous system (Wang et al., 2005). Thus transported AT₁ serves as an excitatory presynaptic receptor in adrenergic neurons. AT₁ may serve as a presynaptic receptor in somatic motor systems as well, given its high expression in ventral horn neurons and substantial anterograde transport in sciatic nerve axons. In fact, recent work in frog neuromuscular junction (Augusto Oliveira et al., 2007) has shown that Ang II enhances the quantal content of stimulus-evoked acetylcholine release. Further work on this issue is warranted to explore the physiological implications of these observations and their relevance to neurotransmission in humans.

Axonal transport is important not only for delivery of materials from cell bodies to nerve terminals, but also for relaying trophic substances from target cells to neurons, ultimately affecting presynaptic transmitter release, postsynaptic receptor functions, and neuronal survival (Brimijoin, 1988). Certain neuropeptides transported in sciatic nerve, e.g., CGRP, have trophic effects on acetylcholine receptors and acetylcholinesterase at the neuromuscular junction (Fernandez and Hodges-Savola, 1994; Kashihara et al., 1989). Although Ang II has not been regarded as a neurotrophic factor, we deemed it of interest here to determine whether the trafficking of AT₁ might serve in part as a means of conveying “captured peptide” between nerve terminals and cell bodies. Our experimental results, however, do not favor such a concept. Thus, when applied to the proximal stump of transected sciatic nerve, exogenous, radiolabeled Ang II failed to move centrally in detectable quantities. Nor, when the labeled peptide was injected into SCG, which is heavily innervated by AT₁-expressing preganglionic neurons (Tang et al., 2008), was Ang II conveyed back to the spinal cord. Nonetheless, it remains likely that Ang II and AT₁ may be directly or indirectly involved in neurotrophic functions. For example there is evidence that Ang II, probably acting through AT₁, up-regulates the production of nerve growth factor (NGF) in vascular smooth muscle (Creedon and Tuttle, 1991; Jeffreson et al., 1995). Presynaptic receptors do not necessarily contribute to this process, but overall it seems clear that Ang II and AT₁ form one link in the complex web of phenomena that maintain function and structure in the peripheral nervous system.

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Fig. 1. Autoradiography for AT₁ on longitudinal sections (**A, B, C, D**) and cross sections (**E, F, G, H**) of resting sciatic nerve. ^{125}I (-Sarcosine¹)-Ang II was applied under four conditions: 1) no other ligand present (**A, E**); 2) losartan added to occupy AT₁ receptors (**B, F**); 3) PD123319 added to occupy AT₂ receptors (**C, G**); 4) unlabeled Ang II added to occupy both AT₁ and AT₂ receptors (**D, H**). Scale bar = 1 mm.

Fig. 2.

Selective AT₁ immunohistochemistry in resting and doubly-ligated sciatic nerves. Images were obtained by confocal microscopy. **A**, AT₁-like immunoreactivity in resting nerve. **B**, Absence of staining when primary antibody is omitted. **C** and **D**, AT₁ accumulations at the proximal and distal sides of the “upstream” ligature. **E** and **F**, AT₁ accumulations proximal and distal to the second ligature. Scale bar = 200 μm.

Fig. 3.

Double immunofluorescence staining of AT₁ and MBP in cross sections of resting and ligated sciatic nerve. Left panels are from resting nerve, middle panels are from the proximal side of a ligature and right panels are from the distal side. **A**, **D**, and **G** show expression of AT₁-like immunoreactivity; **B**, **E** and **H**) show immunoreactivity for MBP; **C**, **F** and **I** are the overlays of, respectively, **A** and **B**, **D** and **E**, **G** and **H**. Scale bar = 50 μm. Note pronounced immunoreactivity in axonal lumens proximal to the ligature (asterisked examples in **D**).

Fig. 4.

Ang II binding sites in double ligated sciatic nerve. After nerve ligation for 16 hr, [125 I]-Sarcosine¹-Ang II was applied to cryostat sections under four conditions: **A** no other ligand present; **B**) losartan added to occupy AT₁ receptors; **C**) PD123319 added to occupy AT₂ receptors; **D**) excess unlabeled Ang II added to occupy both AT₁ and AT₂ receptors. Shown are representative autoradiographs from sections of a single nerve. Scale bar = 1 mM.

Fig 5.

Quantitative autoradiography of AT₁ receptors in double-ligated sciatic nerve. P1 and D1 represent the proximal and distal sides of the first ligature; P2 and D2 are the proximal and distal sides of the second ligature; C is unligated control nerve. Values are expressed as fmol/mg protein and are means $\pm$ SEM from a group of 3 rats. AT₁ binding proximal to ligature 1 was significantly higher than at any other location ($P < 0.05$).

Fig 6. Labeled Ang II in nerve. Distribution of radiolabel in sciatic nerves from 3 rats, 2 days after exposing proximal nerve stumps in vivo to 50 nCi of ^3H -Ang II. Values are adjusted mean dpm in consecutive 3-mm nerve segments (exposure site, segment 9; ligation site, segment 5).

Fig. 7. Immunoperoxidase staining of L4 DRG. A) Representative image showing numerous large diameter cells, evidently sensory neuronal perikarya, with high levels of AT₁-like immunoreactivity. B) Section from same ganglion, processed identically except for omission of first antibody. Scale bar = 200 μm.

Fig. 8.

Retrograde tracer labeling of AT₁-expressing DRG and spinal cord motor neurons with axons in sciatic nerve. Shown are sections of L4 spinal cord ventral horn and the corresponding DRG prepared 7 days after introducing FG tracer into sciatic nerve. Confocal fluorescence images reveal FG itself or the rhodamine immunofluorescence signal from selectively bound AT₁ antibody. **A)** FG-positive motor neurons; **B)** AT₁-positive motor neurons; **C)** overlay of A and B; **D)** FG-positive DRG neurons; **E)** AT₁-positive DRG neurons; **F)** overlay of D and E. Scale bar = 100 μ m.

Fig. 9. Relative numbers of AT₁-expressing sensory and motor neurons with axons in sciatic nerve. FG-positive and FG+AT₁ doubly positive motor and sensory nerve cell bodies were counted after retrograde tracing from sciatic nerve. Shown are estimated total numbers of positively scored neurons in the L4 ventral horn and the corresponding DRG.

Fig. 10.

Co-localization of AT1 with sensory and motor markers. Cross sections of sciatic nerve proximal to a ligature placed 16 hr earlier were stained and imaged by confocal fluorescence microscopy with fluorescein detection of AT₁ immunoreactivity (green channel) and rhodamine detection of CGRP and ChAT immunoreactivity (red channel): **A)** AT₁; **B)** ChAT; **C)** AT₁-ChAT overlay; **D)** AT₁; **E)** CGRP; **F)** AT₁-CGRP overlay. Scale marker for all images, 20 μ m. Note isolated but clear cases of co-localization with ChAT and more extensive overlap with CGRP.