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ScienceDirect
Neuropeptides 43 (2009) 81–92
Neuropeptides
www.elsevier.com/locate/npep

Type-1 angiotensin receptors are expressed and transported in motor and sensory axons of rat sciatic nerves

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Received 7 October 2008; accepted 9 January 2009
Available online 23 February 2009

Abstract

Angiotensin II (Ang II) and its type-1 receptor (AT_1) 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 AT_1-like binding sites and intense expression of AT_1 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 ^{125}I-Ang II binding that could be specifically blocked by the AT_1 antagonist, losartan. Immunohistochemistry with an AT_1-antibody validated by Western blots also showed patches of AT_1-reactivity in nerve. These patches were localized around large myelinated axons with faint immunoreactivity in their lumens. Sixteen hours after nerve ligation there was no change in the patches or hot spots, but luminal AT_1-reactivity increased dramatically in a narrow zone immediately above the ligature. With double ligation there was a pronounced accumulation of AT_1 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 AT_1 indicated that many AT_1-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 AT_1 accumulations in both motor and sensory fibers. We conclude that somatic sensory and motor neurons of the rat export substantial quantities of AT_1 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

1. 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 (AT_1) 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

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0143-4179/$ - see front matter © 2009 Elsevier Ltd. All rights reserved.
doi:10.1016/j.npep.2009.01.001

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, 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 AT_1 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 AT_1-positive fibers of sciatic nerve are primarily motor or sensory in nature.

2. Methods

2.1. Animals

Adult male Sprague–Dawley rats (200–250 g, 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.

2.2. Sciatic nerve ligation and retrograde neuronal labeling

Rats were anesthetized with sodium pentobarbital (45 mg/kg, i.p.), supplemented with smaller doses (5 mg/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), 5–10 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 hours 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 ^{3}H-tyrosyl-Ang II (52.5 Ci mmol, New England Nuclear, Waltham, MA) 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 ^{3}H-Ang II was then slowly injected into the SCG on one side (50 nCi in 10 μl 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 ^{3}H-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.

2.3. Immunohistochemistry

Sciatic nerves were dissected from euthanized rats and flash frozen on dry ice. Later, frozen 16-μm 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-μm sections were then prepared and incubated overnight at 4 °C with primary antibodies. The primary AT_1 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 h 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 diamino-benzidine. Stained slides were mounted, coverslipped, and examined with a standard