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Intraneuronal angiotensinergic system in rat and human dorsal root ganglia

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Abstract

To elucidate the local formation of angiotensin II (Ang II) in the neurons of sensory dorsal root ganglia (DRG), we studied the expression of angiotensinogen (Ang-N)-, renin-, angiotensin converting enzyme (ACE)- and cathepsin D-mRNA, and the presence of protein renin, Ang II, Substance P and calcitonin gene-related peptide (CGRP) in the rat and human thoracic DRG. Quantitative real time PCR (qRT-PCR) studies revealed that rat DRG expressed substantial amounts of Ang-N- and ACE mRNA, while renin mRNA as well as the protein renin were untraceable. Cathepsin D-mRNA and cathepsin D-protein were detected in the rat DRG indicating the possibility of existence of pathways alternative to renin for Ang I formation. Angiotensin peptides were successfully detected with high performance liquid chromatography and radioimmunoassay in human DRG extracts. *In situ* hybridization in rat DRG confirmed additionally expression of Ang-N mRNA in the cytoplasm of numerous neurons. Intracellular Ang II staining could be shown in number of neurons and their processes in both the rat and human DRG. Interestingly we observed neuronal processes with angiotensinergic synapses en passant, colocalized with synaptophysin, within the DRG. In the DRG, we also identified by qRT-PCR, expression of Ang II receptor AT_{1A} and AT_2-mRNA while AT_{1B}-mRNA was not traceable. In some neurons Substance P and CGRP were found colocalized with Ang II. The intracellular localization and colocalization of Ang II with Substance P and CGRP in the DRG neurons may indicate a participation and function of Ang II in the regulation of nociception. In conclusion, these results suggest that Ang II may be produced locally in the neurons of rat and human DRG and act as a neurotransmitter.

Keywords

Renin; angiotensin system; Angiotensin II; Neurotransmitter; Neuronal angiotensin; Sensory system

1. Introduction

The renin–angiotensin system (RAS), with circulating angiotensin II (Ang II) as its active principle, is a key regulatory factor in blood pressure control and fluid balance [16,46]. Ang II effects are produced by stimulation of selective receptor types, the AT_1 and AT_2 receptors. While humans express a single type of AT_1 receptors, rodents express two AT_1 receptor subtypes, the AT_{1A} and AT_{1B} receptors [9,54,63]. Formation of circulating Ang II is mediated

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through cleavage of the precursor angiotensinogen (Ang-N) by the enzyme renin to produce the decapeptide angiotensin I (Ang I). In turn, Ang I is cleaved by angiotensin converting enzyme (ACE) to generate Ang II [8]. However, more recent studies suggest significant roles for Ang II related peptides in addition to those of Ang II [16], the presence of multiple local organ and cellular RAS systems where Ang II plays multiple regulatory roles [46] and alternative pathways for Ang II formation, in particular for the intracellular RAS [30,31].

In the nervous system, Ang II is involved in the regulation of multiple functions, including sympathetic and hormonal control [2,51–53,65]. In addition, there is substantial evidence that Ang II contributes to regulate the central and peripheral components of sensory systems [3,4, 15,49,51,57,64,68]. Ang II may exert local regulatory effects in dorsal root ganglia (DRG) [28,68], structures containing cell bodies of primary afferent neurons that are involved in the regulation of blood pressure and in many sensory modalities [17,67].

Neuronal localization of RAS components has been reported in bilaterally ovariectomized rat DRG [7], in normotensive rat and human sympathetic coeliac ganglia [45] as well as in normotensive rat and human trigeminal ganglia [23]. Recent studies characterized the presence and transport of Ang II AT_1 receptors between the DRG and the spinal cord [47,62]. In the DRG, Ang II may act directly and/or modulating the effects of additional local peptidergic systems. Of particular interest is Substance P, a regulator of sensory transmission in the trigeminal ganglion and other DRG [13,19,20,33]. This peptide has been proposed as an important neurochemical mediator of certain kinds of noxious peripheral stimuli [3]. Substance P is colocalized with Ang II in neurons of trigeminal ganglia [23], whereas other studies show that Substance P release is modulated by Ang II [12,27]. Calcitonin gene-related peptide (CGRP) found in the sensory neuron is significantly involved in the regulation of cardiovascular function through inhibitory modulation of sympathetic nervous activity [32].

Here we address the intracellular localization of RAS components in rat and human DRG, and the relationship of Ang II with Substance P and CGRP expression in these sensory tissues.

2. Materials and methods

2.1. Rat and human dorsal root ganglia and sampling procedures

We purchased 8-week-old, male Wistar Kyoto (WKY) rats (approximately 200 g body weight) from the Central Animal Facilities of the University of Bern. Adequate measures were taken to minimize pain or discomfort, in accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and in accordance with Animal Protocols approved by the Animal Care and Use Committee, NIMH, NIH, USA. Rats were anesthetized intraperitoneally with 100 mg/kg thiopentane sodium and were perfused transcardially with 150 ml Ringer solution containing 1000 U heparin at 37 °C followed by 300 ml 2% freshly prepared formaldehyde at 4 °C. Thoracic dorsal root ganglia (DRG) were carefully removed and incubated by immersion fixation in 2% formaldehyde for 28 h at 4 °C. Subsequently, tissues were immersed for 14 h in phosphate-buffered saline (PBS-Dulbecco) containing 18% sucrose at 4 °C, embedded with M-1 embedding matrix (Thermo Shandon). Then 30 μm thick sections were cut on a cryostat (at −15 °C) and subsequently used as free-floating sections for immunocytochemistry. For some experiments after perfusion and immersion fixation the rat ganglia were embedded in paraffin. Paraffin sections, 7 μm thick, were used for immunocytochemical as well as for *in situ* hybridization experiments.

For extraction of total RNA, rats were shortly anesthetized with halothane and subsequently sacrificed by decapitation. For extraction of total RNA, fresh rat thoracic DRG, liver, lung, adrenal glands and kidneys were dissected and instantly transferred into RNA later (Ambion), frozen in liquid nitrogen, and then processed for total RNA extraction (Ambion).

For specific measurement of different angiotensin peptides separated by high performance liquid chromatography (HPLC) prior to highly sensitive radioimmunoassay, rat thoracic DRG were rapidly removed, rinsed with cold Ringer solution, blotted by filter paper and wet weight was measured. The ganglia were frozen in liquid nitrogen and stored at −70 °C.

Human thoracic DRG were procured from adult human individuals for whom a permit for clinical autopsy (informed written consent by next of kin) had been obtained according to state law, in accordance with the Code of Ethics of the World Medical Association (Declaration of Helsinki). Human DRG were fixed by immersion in freshly prepared 2% formaldehyde for 3 days and then used for cryosectioning or embedded in paraffin. To perform HPLC-RIA the same method as described for rats was used.

2.2. RNA isolation and quantitative real time PCR (qRT-PCR)

Fresh rat DRG were dissected as mentioned above and instantly transferred into RNA later (Ambion), frozen in liquid nitrogen, and then processed for total RNA extraction (Ambion). RNA integrity was confirmed for each sample on the Agilent Bioanalyzer using the RNA 6000 Nano kit (Agilent Technologies). 1 μg of total RNA was reverse transcribed using Superscript II (Invitrogen) and random hexamers according to the manufacturer's protocol. For qRT-PCR, reverse transcribed material corresponding to 40 ng RNA was amplified with the TaqMan assays described below in 25 μl Universal PCR Master Mix, No AmpErase UNG on the SDS 7000 (Applied Biosystems) using the standard thermal protocol. Average values and standard deviations of relative mRNA levels of each sample, normalized to relative 18S rRNA levels, are from four measurements and were calculated using the relative differences. Values were expressed as percent of values obtained from liver for Ang-N and cathepsin D, kidney for renin, lung for ACE, and adrenal gland for angiotensin receptors.

The following TaqMan assays were used for qRT-PCR at a final concentration of 250 nM TaqMan probe and 900 nM of each primer:

Angiotensinogen

Forward primer 5′-CACGACTTCCTGACTTGGATAAAGA-3′

Reverse primer 5′-CTGCGGCAGGGTCAGA-3′

TaqMan probe 5′-FAM CCTCGGGCCATCC G MGB-3′ manufactured as Assays-by-Design (RATG-EJ3) by Applied Biosystems

Renin Assay-on-demand Rn00561847_m1 from Applied Biosystems

ACE Assay-on-demand Rn00561094_m1 from Applied Biosystems

Cathepsin D Assay-on-demand Rn00592528_m1 from Applied Biosystems

AT$_{1A}$ Assay-on-demand Rn01435427_m1 from Applied Biosystems

AT$_{1B}$ Assay-on-demand Rn02132799_s1 from Applied Biosystems

AT2

Forward primer 5′-GTGGGAAGCTCAGTAAGCTGATTTA-3′

Reverse primer 5′-GTCAGAGACTCCCAATCCTTACAC-3′

TaqMan probe 5′-FAM ACACTGGCAACTAAAAGA MGB-3′ manufactured as Assays-by-Design (RATG-EJ3) by Applied Biosystems

18S rRNA Predeveloped Assay Reagent 431-9413E from Applied Biosystems

2.3. In situ hybridization

2.3.1. DIG-labelled RNA probe preparation—By using an appropriate cDNA template for Ang-N [35], a 403 bp long fragment corresponding to nucleotides 221–623 was generated by digestion with restriction enzymes *EcoRV* and *Bam HI*. The obtained fragment was cloned between *Stu I* and *Bam HI* into pBluescript I KS+ (Stratagene). Digoxigenin-labelled probes were prepared using the DIG-RNA-labelling mix (Roche) according to the manufacturer's protocol. T7 RNA polymerase was used to generate antisense riboprobe using the *Hind III* linearized template, and the sense strand (used as a control) was generated by T3 RNA polymerase using the same template linearized with *Bam HI*. The RNA concentration was estimated comparing dot-blot tests and NanoDrop measurements. Filter hybridization with RNA extracts from DRG (RNaqueous-4PCR kit from Ambion) was used for binding tests.

2.3.2. Procedure for in situ hybridization—For *in situ* hybridization, 6 μm thick paraffin sections from rat DRG were rehydrated in a graded alcohol series (2 times Xylol for 10 min, EtOH 100% (2 times), 96%, 70%, 50%, each step 5 min) using DEPC-treated H_2O for the dilution of all reagents and solutions. The sections were equilibrated in proteinase K buffer (100 mM Tris, 50 mM EDTA, pH 7.5) for 5 min and then treated with proteinase K (19 μg/ml) at 37 °C for 2 min, after a wash with DEPC-treated H_2O. Following a wash in DEPC-water, sections were post-fixed with freshly prepared 4% formaldehyde for 5 min, followed by two subsequent washes in DEPC-water for 5 min each. Sections were then incubated in prehybridization solution (Sigma) at 45 °C for 2 h, followed by incubation with heat denatured sense and antisense riboprobes (5–10 ng/μl) in 30 μl hybridization mix (Sigma) for 48 h at 45 °C in a humid chamber (saturated with 2× SSC). Subsequently, the sections were incubated with 2× SSC for 30 min at room temperature, followed by 1 h in 2× SSC at 45 °C and 1 h in 0.1× SSC at 45 °C. Sections were equilibrated for 5 min with buffer 1 (100 mM Tris, 150 mM NaCl, pH 7.5), then incubated with alkaline phosphatase-coupled anti-digoxigenin antibody (Roche) 1:500 diluted in buffer 2 (10× blocking solution diluted with buffer 1) for 2 h at room temperature, followed by two 5 min washes with buffer 1. Finally, after 5 min equilibration with buffer 3 (100 mM Tris, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5), the color reaction containing nitro blue tetrazolium (NBT) and 5-Bromo-4-chloro-3-indolyl phosphate (BCIP) in buffer 3 was completed according to manufacturer's protocol (Sigma).

2.4. Immunocytochemistry

For immunocytochemical incubations for rat and human DRG, a protein G purified murine monoclonal antibody against Ang II (Mab-Trap G II column, Amersham Sciences) was used at a concentration of 0.3 μg/ml in buffer solution. This is a self generated monoclonal antibody against the synthetic peptide to Ang II. The specificity for the monoclonal antibody against Ang II (4B3) has been previously documented and it produced the same staining as a polyclonal antibody against Ang II in rat adrenal glands [14]. For immunization of the mice, Ang II peptide was cross-linked with glutaraldehyde to keyhole limpet hemocyanin. In dot-blot assay, the monoclonal antibody against Ang II (4B3) showed total cross reactivity with Ang III (2–8), Ang 3–8, Ang 4–8 and Ang 5–8. It showed no cross reaction with human plasma Ang-N, Ang I (1–10) and Ang 1–7. Pre-absorption was performed by using a batch procedure with the synthetic Ang II peptide that was covalently linked through its N-terminus to CH-Sepharose 4 B. The gel was loaded into a glass column and the monoclonal anti Ang II antibody solution effluent was used for immunocytochemical pre-absorption controls. Further controls were done with mouse non-immune serum and for the secondary goat anti-mouse Cy3 (GAM[Cy3]) antibody without the primary antibody. All these additional controls showed absolute no staining within the sections. The pre-absorbed monoclonal antibody after low pH-elution revealed the same staining as the Mab-Trap G II purified antibody.

Substance P was investigated with a mouse monoclonal antibody to the synthetic peptide Substance P from Novus Biologicals (SP-DE4-21; 0.5 μg/ml) and renin with a murin monoclonal antibody to renin (SWANT, Belinzona; 1:700). Goat anti-mouse immunoglobulins (GAM^{Cy3}; 1:600, Jackson ImmunoResearch) pre-incubated with rat serum prior to immunocytochemistry were used as secondary antibodies.

For colocalization studies we used a chicken anti-CGRP polyclonal antibody (GenTex; 1:500), for synaptophysin a rabbit monoclonal antibody (EPITOMICS; 1:1200) and for cathepsin D a rabbit monoclonal antibody (EPITOMICS; 1:500). The secondary antibodies for these studies were Cy5-conjugated goat anti-chicken IgG (Jackson ImmunoResearch; 1:250) respectively Cy5-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch; 1:300).

Sections were incubated free floating for 36 h at 4 °C with the primary antibodies, washed and incubated with the secondary antibodies for 90 min at room temperature and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). After washing, cryosections were mounted on gelatin-coated slides and air-dried. The stained sections were embedded with Glycergel (DAKO) and cover-slipped. The same procedure as for free-floating cryosections has been used for paraffin sections mounted on glass slides. Pictures were taken by a normal fluorescent light microscope (LEICA DM 6000B) using a digital camera or by a confocal laser scanning microscope (LEICA SP2 with AOBS).

2.4.1. Post in situ hybridization immunocytochemistry—Paraffin sections of rat DRG underwent *in situ* hybridization incubation and staining. Afterwards the same sections were processed for immunocytochemistry as mentioned above.

2.4.2. Colocalization studies for Ang II, Substance P, CGRP, cathepsin D and synaptophysin—Directly adjacent free-floating cryosections of rat and human DRG were immunostained for Ang II and Substance P or in the same section for CGRP, cathepsin D and synaptophysin, as mentioned above. Pictures were taken by a normal fluorescent light microscope (LEICA DM 6000B) using a digital camera or by a confocal laser scanning microscope (LEICA SP2 with AOBS).

2.5. Measurement of angiotensin peptides

Ang-(1–8) octapeptide (Ang II), Ang-(2–8) heptapeptide, Ang-(3–8) hexapeptide and Ang-(4–8) pentapeptide were measured in rat and human DRG by RIA after prior separation by HPLC [40]. Measurement of true angiotensin was applied also for Ang-(1–10) decapeptide (Ang I), and peptide extraction from tissue was adapted for additional freeze–thaw procedures to ensure the lysis of cells [39,41]. Briefly, DRG were homogenized in 1.5 ml water, shock frozen in ethanol/dry ice, and thawed in a 37 °C water bath. The freeze–thaw procedure was repeated twice. Samples were sonicated for 10 min at 37 °C, centrifuged at 3500 *g* and the supernatant in an albumin-coated polypropylene tube was dried at 37 °C under nitrogen. The residue was dissolved in 2.2 ml Tris buffer (0.1 M, pH 7.5) containing bovine serum albumin (5 g/l). Solid-phase extraction on phenylsilylsilica, HPLC and radioimmunoassay were performed according to the routine procedures using very sensitive antisera for Ang II and Ang I with cross-reactivities of 53% for Ang-(2–8) heptapeptide as well as for Ang-(3–8) hexapeptide and 52% for Ang-(4–8) pentapeptide [39,41]. Recoveries for the different angiotensin peptides and for ir-Ang II were consistently above 67%. Therefore, no corrections were made for recovery losses. Results are presented as fmol Ang per gram wet weight. Detection limits were variable according to the various weights of individual ganglia. Instead of homogenates, controls of 1.5 ml water were extracted and quantitated with every analysis and any traces of Ang peptides found in these "blanks" were subtracted to obtain final ganglial concentrations.

3. Results

3.1. Quantitative determination of RAS components in the rat and human dorsal root ganglia

3.1.1. Quantitative real time RT-PCR—We detected significant amounts of Ang-N-, ACE- and cathepsin D-mRNA in the rat DRG (Fig. 1a, c and d, respectively). No renin mRNA was detected in the same DRG extracts (Fig. 1b).

Receptor studies revealed AT_{1A} receptor mRNA expression in rat DRG (Fig. 2a) whereas no expression of AT_{1B} receptor mRNA could be discovered (Fig. 2b). Also the presence of AT_2 receptor mRNA could be shown (Fig. 2c).

3.1.2. HPLC and RIA of angiotensin peptides—Quantitative measurement of Ang II and related peptides in individual DRG was possible for human DRG, but not for the tiny rat DRG (detection limit too high). Ang II and related peptides (Ang 1–10, Ang 2–8, Ang 3–8 and Ang 4–8) were extracted, separated by HPLC and quantitated by RIA as described in Materials and methods. Results of 4 human DRG are summarized in Table 1. They are expressed as fmol/g wet weight. Ang I was found to be twice and three times above detection limit in two DRG but absent in the remaining DRG. Ang II was consistently found at 1.3-, 9-, 13- and 42-fold above detection limit, respectively. No metabolites of Ang II were found (all below detection limits).

In rat DRG, only after pooling of six individual samples, merely Ang II was detected at 1.3-fold above detection limit (data not shown).

3.2. Localization of Ang-N mRNA and Ang II in rat dorsal root ganglia

3.2.1. In situ hybridization—Ang-N mRNA was detected in most, if not all the neurons studied in the rat DRG with the antisense probe, indicating intracellular localization of Ang-N mRNA (Fig. 3b). No staining was produced using the sense probe as a control (Fig. 3a).

3.2.2. Immunocytochemistry—Ang II immunoreactivity was present in many, but not all, neurons of the rat DRG, with a cytoplasmic localization and a variable intensity (Fig. 4).

In the human DRG we detected Ang II immunoreactivity in the cytoplasm of many neurons showing the intracellular localization of Ang II (Fig. 5a) and in their projections (Fig. 5b) with free-floating cryosections (Fig. 5c and d; Supplementary material, movies 1, 2 and 3).

3.2.3. Post in situ hybridization immunocytochemistry—We used rat DRG paraffin sections for *in situ* hybridization followed by immunocytochemistry to determine the intracellular colocalization of Ang-N mRNA (Fig. 6a) and Ang II (Fig. 6b). We found that most but not all the Ang-N mRNA positive neurons were stained with the antibody recognizing Ang II as indicated by "*".

3.2.4. Colocalization studies for Ang II with Substance P, CGRP, synaptophysin, renin and cathepsin D—In directly adjacent free-floating cryosections of rat DRG, we detected a number of neurons revealing intracellular colocalization of Ang II with Substance P (Fig. 7). A higher number of neurons expressed immunoreactive Ang II (Fig. 7a) than immunoreactive Substance P (Fig. 7b). Double staining in the same section of human DRG, discovered colocalization of Ang II with CGRP (Fig. 8c), Ang II with synaptophysin (Fig. 9c) and Ang II with cathepsin D (Fig. 10c). Interestingly the staining for the protein renin was negative.

4. Discussion

The major findings of our study are the demonstration and quantitation of intraneuronal Ang II in rat and/or human sensory DRG and the presence of Ang-N, ACE- and cathepsin D-mRNA, Ang II and related peptides, Ang II AT_{1A} and AT_2 receptor mRNAs; the neuronal colocalization of Ang-N mRNA and Ang II, and the neuronal colocalization of Ang II and Substance P in rat DRG.

We have detected significant amounts of Ang II in both the rat and human DRG, a clear localization of intraneuronal Ang II. In addition, we detected mRNA for the Ang II precursor Ang-N, and for ACE, in the rat DRG. Furthermore, combination of *in situ* hybridization and immunohistochemistry revealed colocalization of Ang-N mRNA and Ang II immunoreactivity in most DRG neurons of the rat. The coexistence of Ang II and mRNA for Ang-N, the only known precursor for Ang I production and Ang II in the same neurons demonstrates that Ang-N uptake from the circulation may not be necessary for the local intraneuronal formation of Ang II. While in the brain, Ang-N is localized and transcribed in multiple cell types, predominantly in astrocytes but also in neurons and cerebral endothelial cells [22,59,69,70], our study demonstrates the presence of Ang-N mRNA specifically in the cytoplasm of neuronal cell bodies of the DRG, and not in surrounding cells.

It is of note that Ang-N mRNA in addition to its colocalization with neuronal Ang II was present in neurons without certain Ang II immunoreactivity. It is possible that in some neurons Ang II may be produced in amounts too low to be detected with our immunocytochemical methods. Alternatively, Ang-N may participate, in some neurons, in functions unrelated to the RAS system [37,56].

Surprisingly, and in contradiction to a previous report in bilaterally ovariectomized rat DRG [7] we did not detect mRNA for renin, the enzyme classically considered necessary for cleaving Ang-N into the Ang I precursor of Ang II [46] in our wild type rat DRG samples. Further the detection of the renin protein by immunocytochemistry with human DRG was negative in spite of strong positive immunocytochemical staining for renin in the jugxtaglomerular cells with the human kidney. Instead, we showed immunocytochemical staining for the cathepsin D-protein and found substantial expression of cathepsin D-mRNA. The levels are higher in comparison with those found in rat liver and kidney, but lower in comparison to those recently reported in rat coeliac and trigeminal ganglia [23,45]. Cathepsin D is a protease capable of cleaving Ang-N into angiotensin I [55], a non-renin alternative pathway for Ang II formation. Our results are compatible with an alternate pathway for Ang II synthesis in DRG sensory neurons. Such an alternate pathway for neuronal Ang II generation would allow to maintain the hypothesis that kidney renin is the main, if not the only source of renin in mammals [66]. Nevertheless, local Ang II formation through a renin-dependent pathway may still occur, if circulating renin and (pro)renin are taken up and activated by the recently discovered renin-receptor [8], but the presence of (pro)renin receptors in DRG has not been reported so far.

Taken together, our results suggest local Ang II generation and storage in the DRG similar to earlier published evidence for the rat and human sympathetic coeliac and sensory trigeminal ganglia [23,45]. Our findings suggest that in the DRG, Ang II may be a principal effector for neuronal functions and that a non-renin enzyme like cathepsin D may cleave Ang-N to form Ang I. Neuronal Ang II demostrated to occur intracellularly may take part in an angiotensinergic system, i.e. Ang II may act as a neurotransmitter.

We detected the expression of Ang II receptor mRNA in our DRG samples. The physiological receptor for Ang II is the AT_1 receptor type [25]. In rodents, there are two AT_1 receptor subtypes, differentially expressed and regulated [25]. AT_{1A} receptors are widely distributed and their stimulation carries out most of the physiological effects of Ang II in rodents. AT_{1B}

receptor distribution is more restricted, principally to the pituitary and adrenal glands and the hippocampus in adult rodents [5,26,34]. The AT_{1A} and AT_{1B} receptor subtypes cannot be distinguished pharmacologically because of the high homology of their translated regions and similar agonist affinities; however they can be identified by qRT-PCR or *in situ* hybridization with the use of selective primers or probes for untranslated, non-homologous regions [5,26, 34]. Using specific primers with qRT-PCR, we detected AT_{1A} mRNA, but not AT_{1B} mRNA expression in our rat DRG samples. Receptor expression in DRG is an indication that Ang II, either circulating or locally formed, may exert effects in this tissue. Our results confirm previous observations of high AT_{1A} expression in selective DRG neurons, as determined by *in situ* hybridization [47], and high AT_1 receptor immunoreactivity in DRG neurons [62].

In our samples we detected low expression of Ang II AT_2 receptor mRNA, a receptor type whose function is still controversial [10,50,52,53]. Expression of AT_2 receptor mRNA in DRG, as reported recently by *in situ* hybridization and receptor binding, was very variable and much lower than that of AT_{1A} receptor mRNA [47]. Nevertheless, the presence of specific AT receptors in DRG increases the likelihood of a neuronal function of Ang II.

We have detected Ang II-like immunoreactivity not only in neuronal cell bodies but also in neuronal projections within the rat DRG, and intense immunoreactivity in fibers with synapses en passant, colocalized with synaptophysin, in the human DRG. There, neuronal Ang II may act as a neurotransmitter at presynaptic sites to regulate sensory transmission, a function similar to that demonstrated in the sympathetic nervous system and in sensory system of trigeminal ganglia [23,45]. The presence and importance of presynaptic Ang II receptors in sympathetic nerves, spinal cord motor neurons, the neuromuscular junction and central sensory fibers have long been recognized [21,42,44,58].

Although the functions of Ang II in the DRG system have not been extensively studied and therefore are not fully clarified, our results are not without clinical interest. Ang II has been proposed to be involved in the regulation of sensory information, and in particular nociception [24,36,48,49,61]. Hence, we attempted to determine whether Ang II was associated with other nociceptive-controlling neuropeptides in the DRG. We selected Substance P, a neuropeptide firmly established as a key regulator of sensory transmission, and in particular nociception, in the DRG [1,11,38,43,60]. A relationship between Substance P and Ang II was proposed earlier, since Ang II was shown to regulate Substance P release [12,15,27]. We found that Ang II was colocalized with Substance P in DRG neurons, supporting the hypothesis of a close integration between these two systems.

Sensory denervation exacerbates the development of hypertension and impairs renal excretory function when a suppressor dose of Ang II is given. These results indicate that activation of sensory nerves, either by Ang II or by other hormonal or hemodynamic factors, plays a compensatory role in promoting urine and sodium excretion and attenuating elevated blood pressure initiated by Ang II [68]. Thus, the role of Ang II in sensory function may also be related to its well-known regulation of cardiovascular function. CGRP found in the DRG neurons, has shown to be significantly involved in the regulation of cardiovascular function [29,32]. Recently, it has been reported that, AT1 receptor activation by Ang II leads to a decrease in the release of CGRP from DRG neurons of spontaneously hypertensive rats, however AT1 receptor blockers reverse decrease in the CGRP levels [18]. In our studies, angiotensinergic synapses en passant and intracellular colocalization of Ang II with CGRP in human DRG support the involvement of Ang II in the release of CGRP from sensory neurons and their role in regulation of cardiovascular function.

Synaptophysin is a well known marker used for the detection of synaptic vesicles [6]. Colocalization of Ang II with sypatophysin in human DRG confirms the presence of Ang II in the ganglionic synapses en passant.

In conclusion, our results support the hypothesis of intraneuronal Ang II formation in the rat and human DRG, and are compatible with an interaction of Ang II with Substance P and CGRP. We hypothesize that these peptides interact in the DRG in the management of sensory, and particularly nociceptive information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi: 10.1016/j.regpep.2010.03.004.
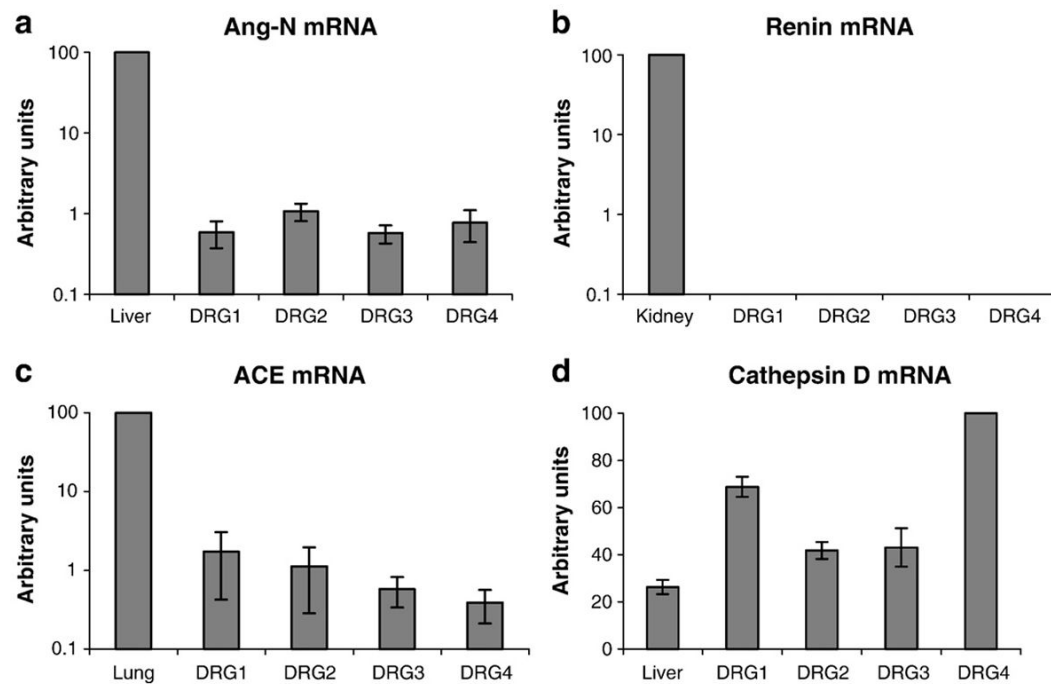
Fig. 1.
Determination of relative levels of Ang-N-, renin-, ACE- and cathepsin D-mRNA by qRT-PCR in rat thoracic DRG. Reverse transcribed total RNA extracts of rat thoracic DRG were studied by qRT-PCR for the presence of Ang-N mRNA (a), for renin mRNA (b), for ACE mRNA (c) and cathepsin D-mRNA (d) and values expressed as percent of those obtained from liver, kidney and lung as described in Materials and methods. Total RNA extracts from liver (a), kidney (b), lung (c) and liver (d) were used as reference samples expression. All relative mRNA values are normalized to 18S rRNA levels. Average values and standard deviations of four qRT-PCR individual measurements are shown.
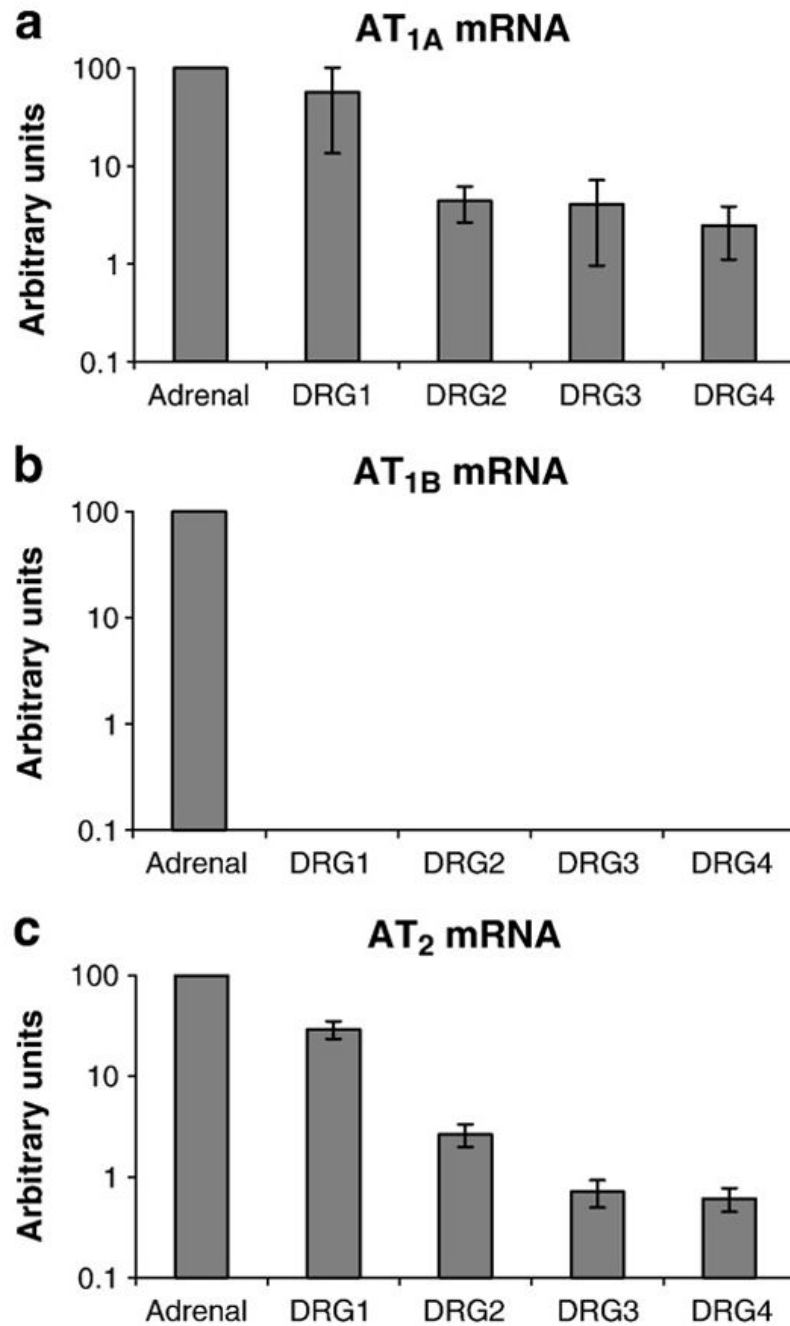
Fig. 2.
Determination of relative levels of Ang II AT_{1A} (a), AT_{1B} (b) and AT_2 (c) receptor mRNA in rat DRG. Reverse transcribed total RNA extracts of rat thoracic DRG were considered by qRT-PCR for the presence of Ang II AT_{1A}, AT_{1B} and AT_2 receptor mRNA as described in Materials and methods. Values are expressed as percent of those obtained from adrenal gland. All relative mRNA values are normalized to 18S rRNA levels. Average values and standard deviations of four qRT-PCR individual measurements are shown.
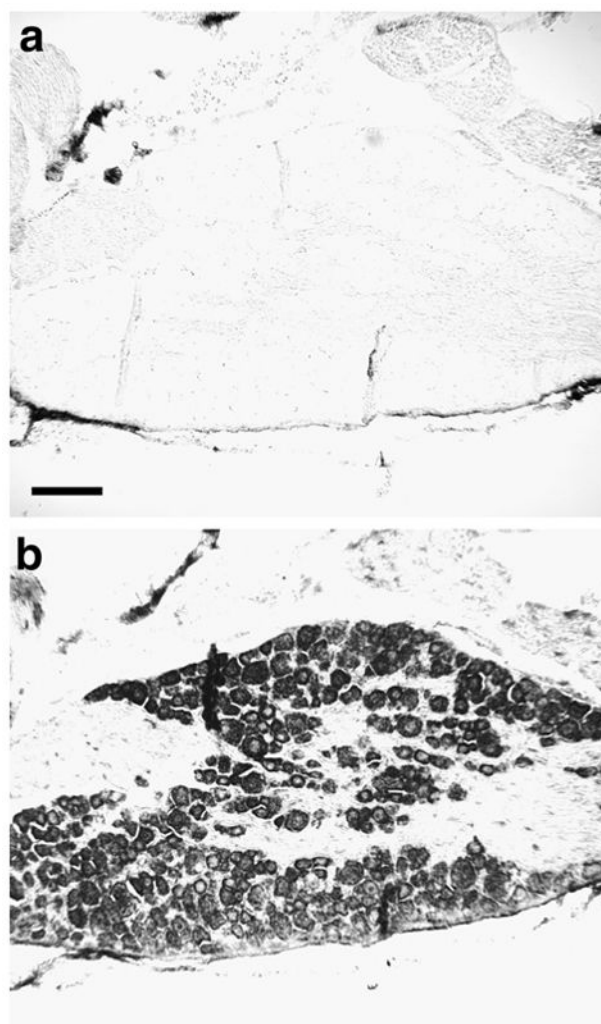
Fig. 3.
In situ hybridization in rat thoracic DRG for detection of Ang-N mRNA. 6 μm thick consecutive paraffin sections were processed as described in Materials and methods. a) Sense probe. b) Antisense probe, revealing positive staining in the cytoplasm of numerous neurons within the ganglion. Bar: 100 μm.
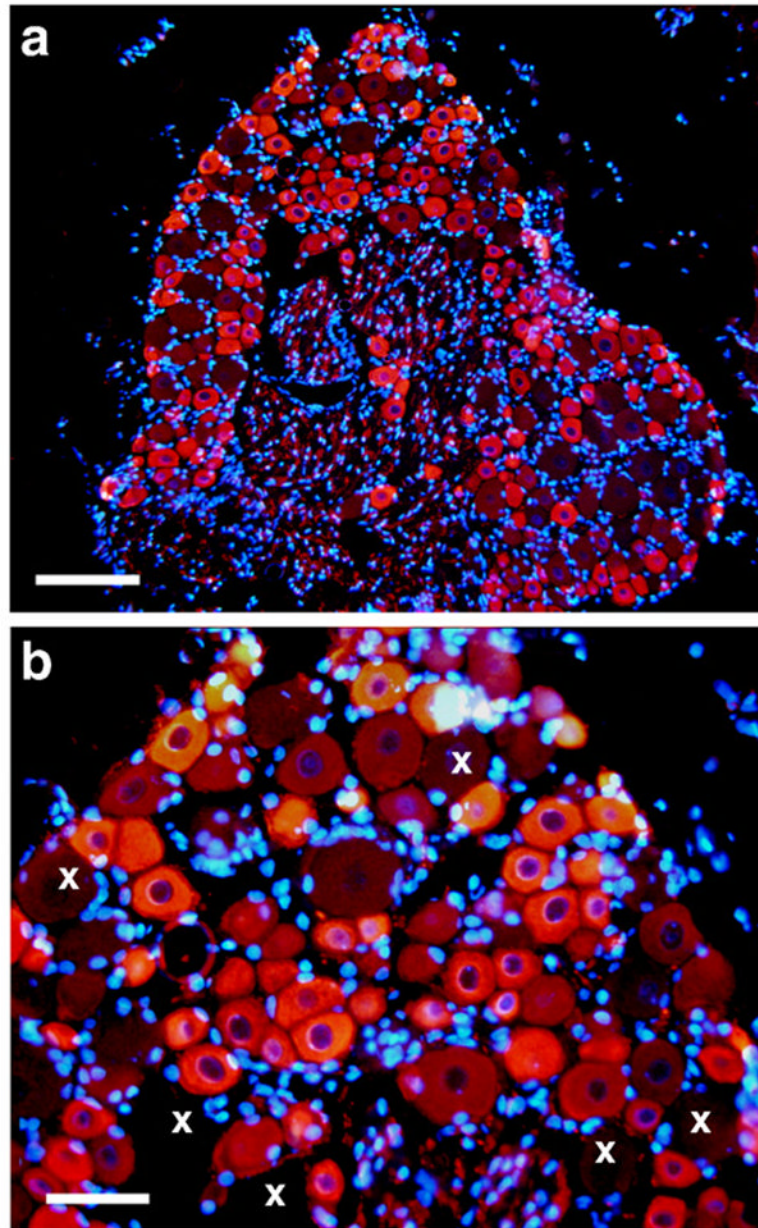
Fig. 4.
Angiotensin II immunocytochemistry in rat thoracic DRG. 30 μm thick free-floating
cryosections were prepared and incubated with our murine monoclonal anti Ang II antibody
[14], stained with GAM^{Cy3} in red and DAPI in blue for nuclei, as described under Materials
and methods. Note, the intensity of the staining for intracellular Ang II varies among the
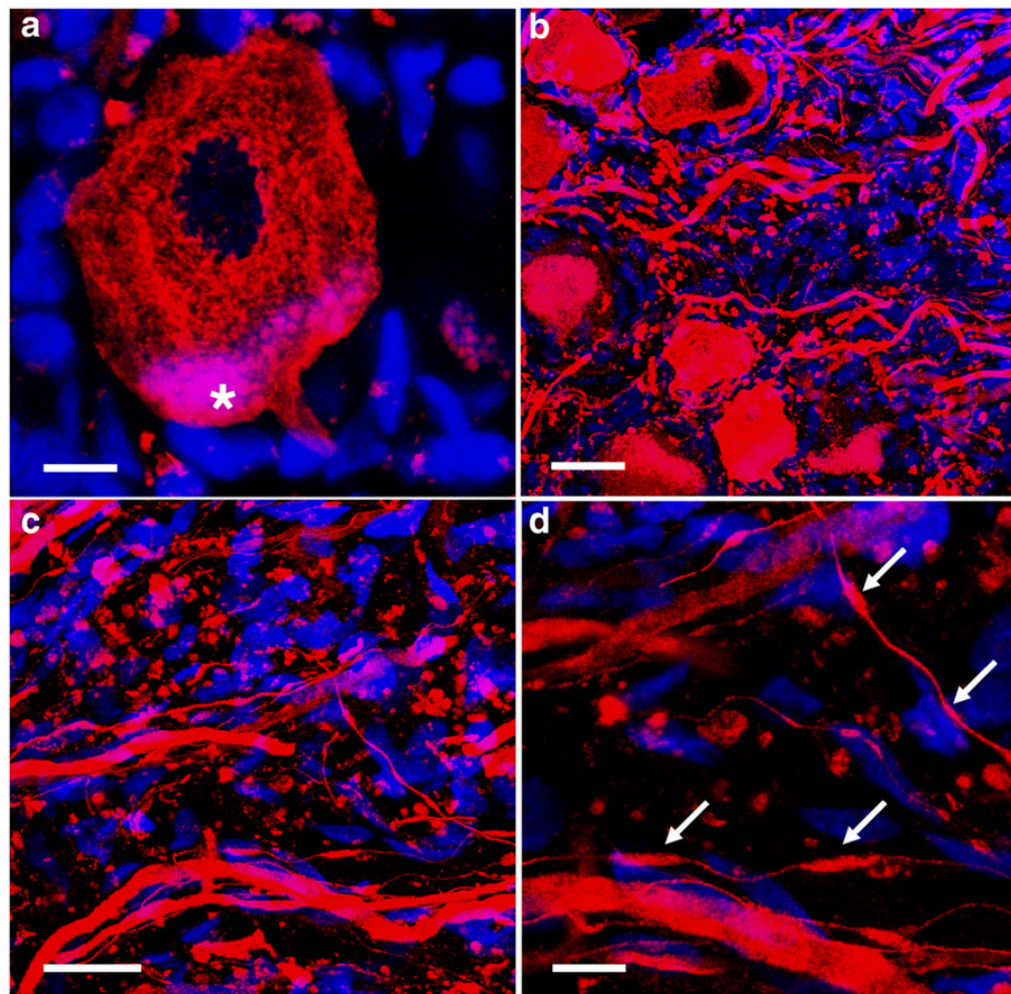neurons. Some neurons are not stained for Ang II (see x). Bars: 100 μm (a) and 50 μm (b).

Fig. 5.
Ang II immunocytochemistry in human DRG. 30 μm thick free-floating cryosections were prepared and incubated with our murine monoclonal anti Ang II antibody [14], stained with GAM^{Cy3} in red and DAPI in blue for nuclei, as described under Materials and methods. Note, intracellular Ang II staining in neuronal cytoplasm (a) and projections (b). Autofluorescence of lipofuscin granules is marked with an asterisk (a). Additionally to thick fiber pathways there can be seen fine fibers with synapses en passant (c) and synapses en passant zoomed in panel c (d). Arrows are pointing to synapses en passant. The pictures were taken with a confocal laser scanning microscope. Bars: in (a) 10 μm; in (b) 50 μm; in (c) 25 μm and in (d) 10 μm. For better illustration of figures, see Movie 1 for panel a and see Movies 2 and 3 for panels c and d in the Supplementary material.
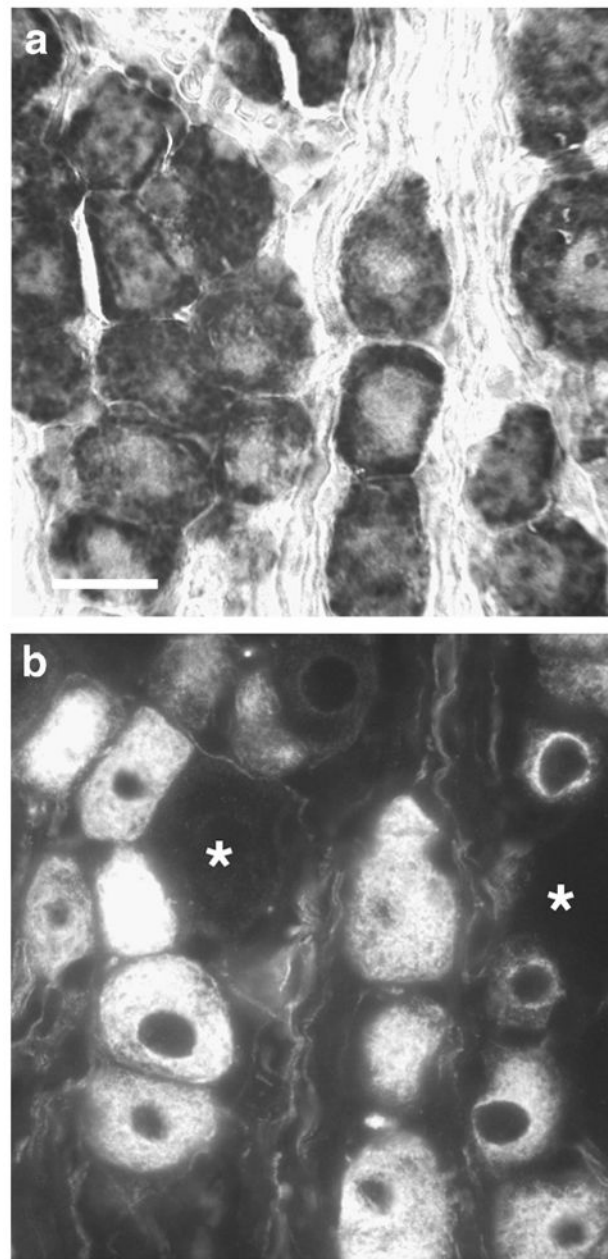
Fig. 6.
Intracellular colocalization of Ang-N mRNA and Ang II in the rat DRG. A 7 µm thick paraffin section was processed for *in situ* hybridization with the antisense probe to detect Ang-N mRNA (a) followed by immunocytochemistry for Ang II (b) as described in Materials and methods. Not all cells expressing Ang-N mRNA are colocalized with Ang II positive cells, see asterisks in (b). Bar: 20 µm.
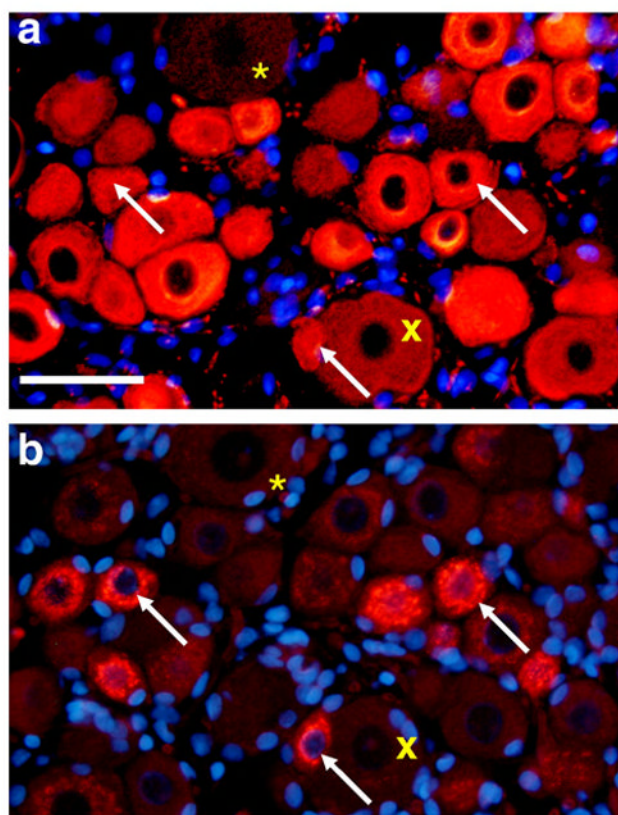
Fig. 7.
Colocalization of Ang II and Substance P in rat thoracic DRG. Directly adjacent, 30 μm thick free-floating cryosections were incubated with our murine monoclonal Ang II (a) or anti Substance P (b) antibodies and stained with GAM[Cy3] as secondary antibodies in red and DAPI in blue as nuclear stain, as described under Materials and methods. Arrows show colocalization, "x" indicates the absence of colocalization while asterisks indicate the absence of staining for Ang II as well as for Substance P. Bar: 50 μm.
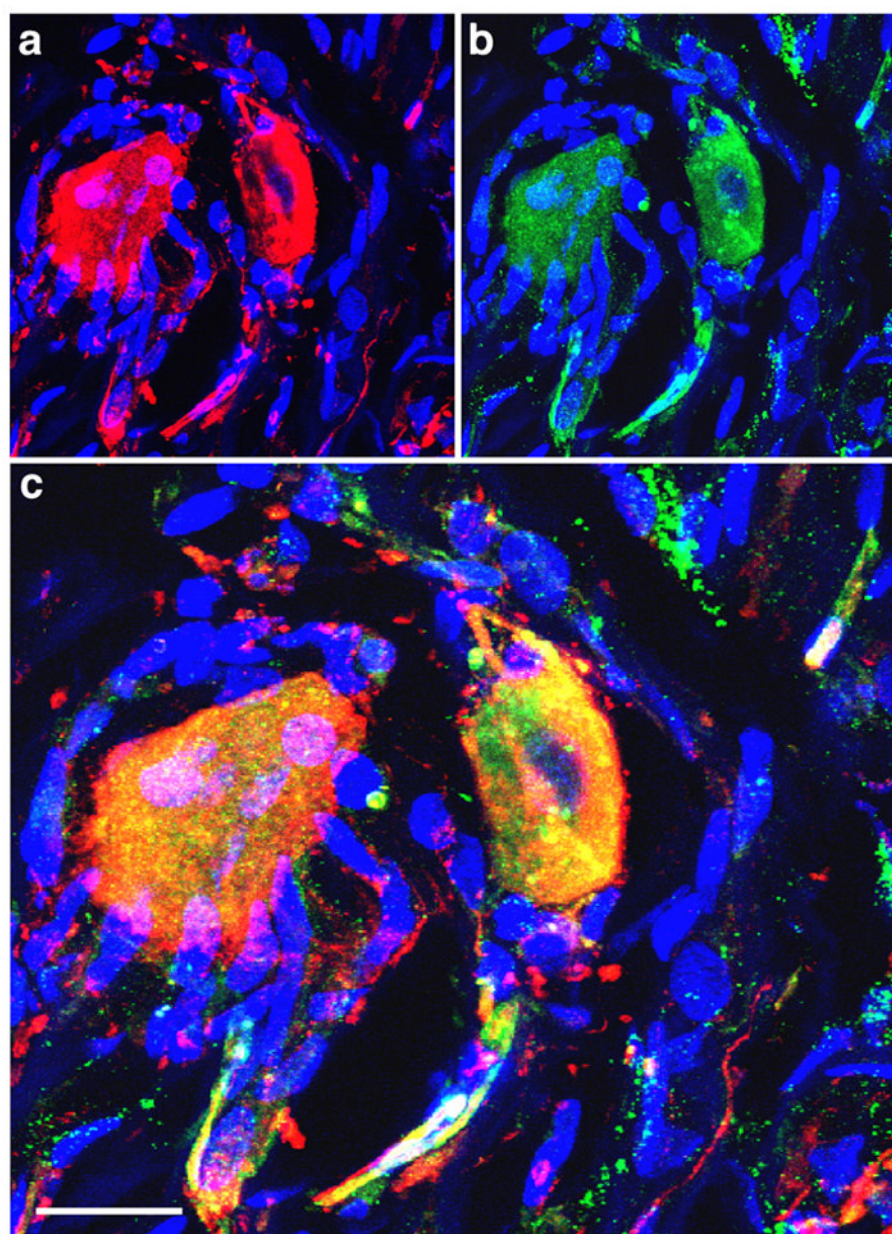
Fig. 8.
Colocalization of Ang II and CGRP in human DRG. Double staining in a free-floating
cryosection incubated with our murine monoclonal antibody for Ang II in red (a) and chicken
polyclonal antibody for CGRP in green (b); corresponding merged image (c) where yellow
color indicates colocalization of Ang II and CGRP in the same neurons and fibers. Secondary
antibodies were GAM[Cy3] respectively Cy5-conjugated goat anti-chicken IgG and DAPI in blue
as nuclear stain, as described under Materials and methods. Bar: 20 μm.
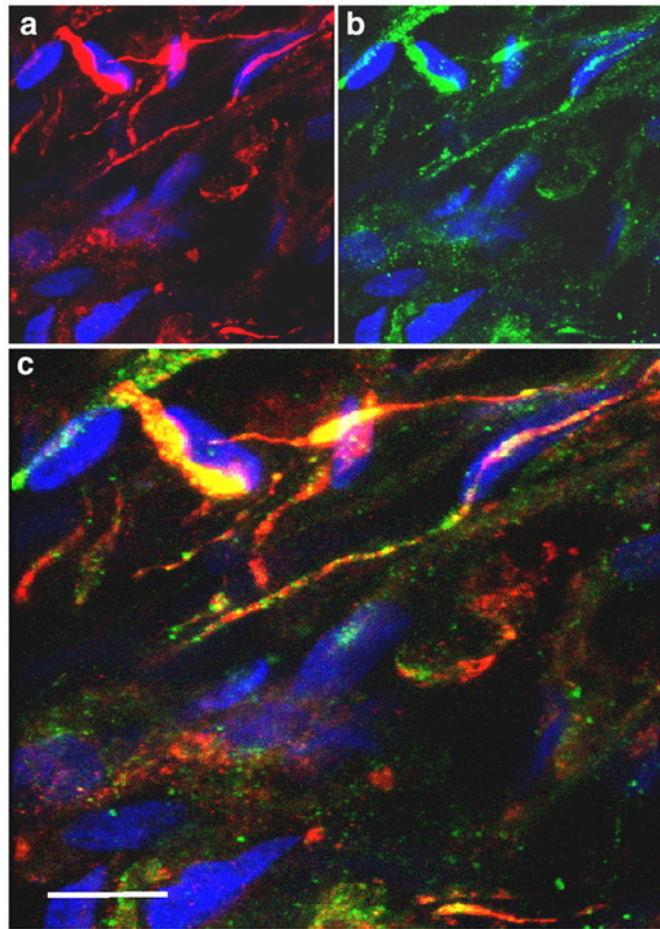
Fig. 9.
Colocalization of Ang II and synaptophysin in human DRG. Double staining in a free-floating cryosection incubated with our murine monoclonal antibody for Ang II in red (a) and a rabbit monoclonal antibody for synaptophysin in green (b); corresponding merged image (c) where yellow color indicates colocalization of Ang II and synaptophysin in the same fibers representing synapses en passant. Secondary antibodies were GAM^Cy3 respectively Cy5-conjugated donkey anti-rabbit IgG and DAPI in blue as nuclear stain, as described under Materials and methods. Bar: 10 μm.
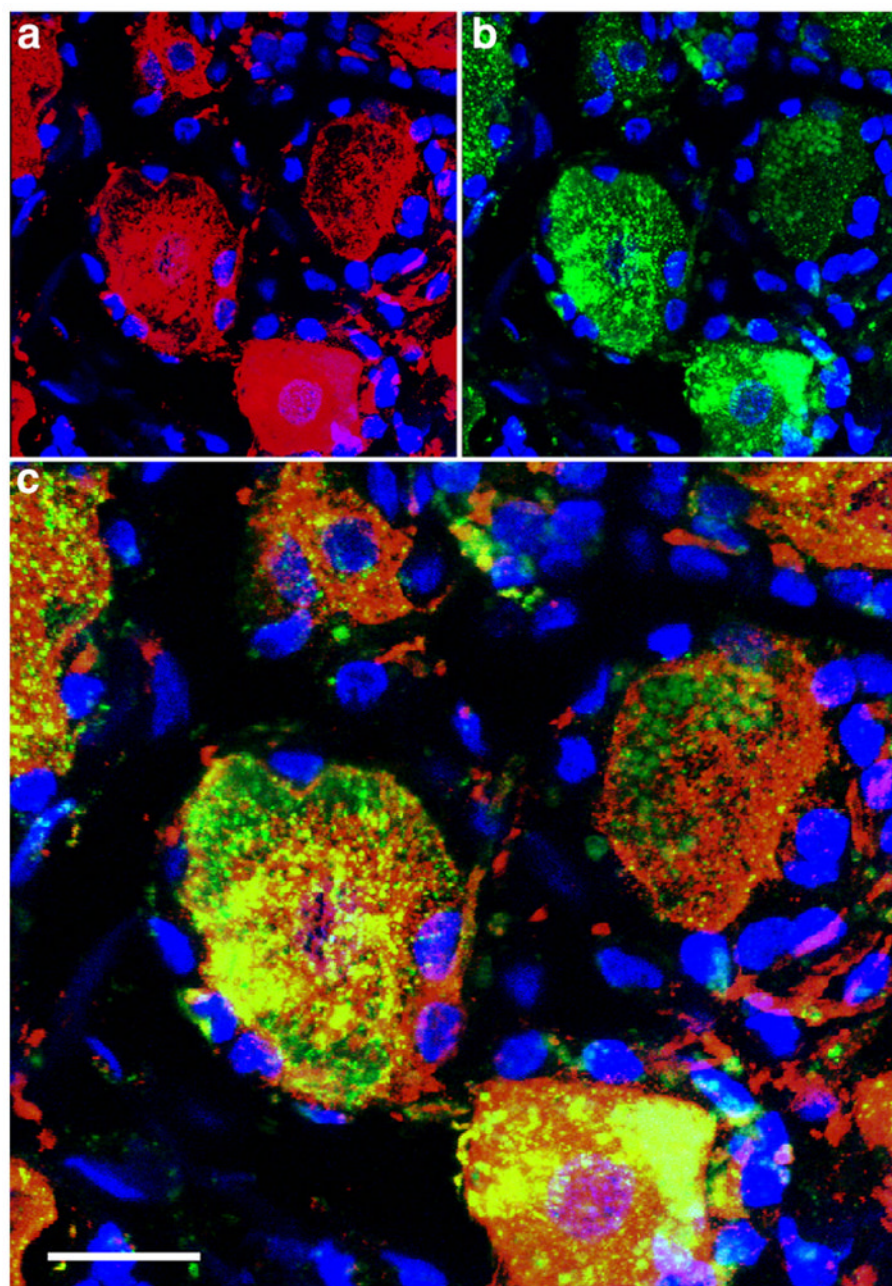
Fig. 10.
Colocalization of Ang II and cathepsin D in human DRG. Double staining in a free-floating cryosection incubated with our murine monoclonal antibody for Ang II in red (a) and a rabbit monoclonal antibody for cathepsin D in green (b); corresponding merged image (c) where yellow color indicates colocalization of Ang II and cathepsin D in the same neurons. Secondary antibodies were GAM^{Cy3} respectively Cy5-conjugated donkey anti-rabbit IgG and DAPI in blue as nuclear stain, as described under Materials and methods. Bar: 20 μm.

Table 1

Angiotensin peptides in human spinal ganglia (fmol/gram wet weight).

Spinal ganglia	Weight (mg)	Ang-(1–10)[a]	Ang-(1–8)[b]	Ang-(2–8)	Ang-(3–8)	Ang-(4–8)
1	200.4	<2.4	50.6 (<1.2)	<2.3	<2.3	<2.3
2	76.2	<6.3	4.1 (<3.2)	<6.0	<6.0	<6.2
3	132.6	7.2 (<3.6)	23.2 (<1.8)	<3.4	<3.4	<3.5
4	102.4	12.3 (<4.7)	20.2 (<2.3)	<4.3	<4.3	<4.4

Detection limit given in brackets.

[a] Angiotensin I.

[b] Angiotensin II.