Research report

Expression and localization of atrial natriuretic peptide and its receptors in rat spiral ganglion neurons

Fei Sun a,b,1, Ke Zhou a,1, Shu-juan Wang a, Peng-fei Liang a, Yong-xiang Wu c, Guo-xia Zhu d, Jian-hua Qiu a,*, Miao-zhang Zhu b,∗∗

a Department of Otolaryngology/Head and Neck Surgery, Xijing Hospital, Fourth Military Medical University, Xi’an, Shaanxi Province 710032, China
b Department of Physiology, School of Basic Medical Sciences, Fourth Military Medical University, Xi’an, Shaanxi Province 710032, China
c Department of Otorhinolaryngology, Head and Neck Surgery, No. 474 Hospital, Urumchi, Xinjiang Province 830011, China
d Department of Otolaryngology, Xi’an No. 4 Hospital, Xi’an, Shaanxi Province 710004, China

A R T I C L E   I N F O
Article history:
Received 17 January 2013
Received in revised form 30 March 2013
Accepted 1 April 2013
Available online 8 April 2013

Keywords:
Spiral ganglion neuron
Atrial natriuretic peptide
Natriuretic peptide receptor-A
Natriuretic peptide receptor-C
Rat

A B S T R A C T
Spiral ganglion neurons (SGNs) are the primary auditory neurons in the inner ear, conveying auditory information between sensory hair cells and the central nervous system. Atrial natriuretic peptide (ANP), acting through specific receptors, is a regulatory peptide required for a variety of cardiac and neuronal functions. While the localization of ANP and its receptors (NPR-A and NPR-C) in the inner ear has been widely studied, there is only limited information regarding their localization in cochlear SGNs and their regulatory roles during primary auditory neurotransmission. Here we have investigated the presence of ANP and its receptors in the cochlear spiral ganglion of the postnatal rat using immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis. ANP and its receptors are expressed in the cochlear SGNs at both the mRNA and protein level, and co-localize in the cochlear SGNs as shown by immunofluorescence. Our research provides a direct evidence for the presence and synthesis of ANP as well as its receptors in the cochlear SGNs, suggesting a possible role for ANP in modulating the neuronal functions of SGNs via its receptors.

© 2013 Elsevier Inc. All rights reserved.

1. Introduction
Spiral ganglion neurons (SGNs) are the primary auditory neurons in Rosenthal’s canal within the modiolus of the cochlea, where they play a prominent role in the auditory system of conveying the auditory signals perceived by the sensory hair cells to the cochlear nucleus located in the brainstem (Nayagam et al., 2011; Rusznak and Szucs, 2009). They comprise two subpopulations of neurons, the large type I neurons, representing approximately 90–95% of the afferent auditory neurons, and the small type II neurons, innervating the inner and outer hair cells respectively. Numerous factors including noise trauma, ototoxic drugs, infection, aging and genetic disorders can cause hair cell death, which leads to degeneration of SGNs, an important component of sensorineural hearing loss (Shepherd and Hardie, 2001). Understanding the expression, function and signaling interactions of the neurotransmitters, neuromodulators or other regulatory substances which affect neuronal physiology and neurotransmission in primary auditory neurons, may offer insights into the mechanisms underlying normal and pathological states of hearing, and provide important clues for effective prophylactic and therapeutic treatment for hearing impairment.

Atrial natriuretic peptide (ANP), the first member of the natriuretic peptide family with potent natriuretic, diuretic, and vasorelaxant activity, is primarily synthesized and secreted by the cardiac atria (de Bold et al., 1981). ANP exerts its actions through binding to specific high affinity receptors on the surface of target cells and it plays an important role in the regulation of cardiovascular homeostasis, maintaining blood pressure and extracellular fluid volume (Levin et al., 1998; Potter et al., 2006, 2009). ANP activates the transmembrane guanylyl cyclase (GC) natriuretic peptide receptor-A (NPR-A, or GC-A) to catalyze the synthesis of cyclic guanosine monophosphate (cGMP), which mediates the most known effects of natriuretic peptides. The natriuretic peptide clearance receptor (NPR-C) clears ANP from the circulation through receptor-mediated internalization and degradation (Levin et al., 1998; Potter et al., 2006). In addition to its well-characterized cardiac roles, recent studies have reviewed the presence and

Abbreviations: ANP, atrial natriuretic peptide; cGMP, cyclic guanosine monophosphate; CNS, central nervous system; GC, guanylyl cyclase; NPR-A, natriuretic peptide receptor-A; NPR-C, natriuretic peptide receptor-C; RT-PCR, reverse transcription-polymerase chain reaction; SGNs, spiral ganglion neurons.

* Corresponding author. Tel.: +86 029 84775381; fax: +86 029 83224749.
** Corresponding author. Tel.: +86 029 84774574.
E-mail addresses: qiuju@fmmu.edu.cn (J.-h. Qiu), zhuzm@fmmu.edu.cn (M.-z. Zhu).
1 These authors contributed equally to this work.

0361-9230/5 – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.brainresbull.2013.04.001
functionality of ANP in several systems, including kidney, adrenal, lung, adipose tissue, vascular smooth muscle, retina and the central nervous system (CNS) (Cao and Yang, 2008; Levin et al., 1998; Potter et al., 2006, 2009). Importantly, signaling by ANP has been linked to different neuronal functions in the CNS (Cao and Yang, 2008).

The presence of ANP and/or its receptors in the inner ear of rodents is well-documented (Chen et al., 1994; Furuta et al., 1995; Koch et al., 1992; Krause et al., 1997; Lampecht and Meyer zum Gottesberge, 1988; Meyer zum Gottesberge et al., 1991, 1995; Meyer zum Gottesberge and Lampecht, 1989; Qiao et al., 2011; Seebacher et al., 1999; Suzuki et al., 1998; Yoon and Anniko, 1994; Yoon and Hellstrom, 1992). These investigations suggest that ANP may play a role in the regulation of the fluid and electrolyte balance in the inner ear as a local hormone. There is some evidence that ANP receptors are localized to the cochlear modiolus of the guinea pig (Lampecht and Meyer zum Gottesberge, 1988) and rat spiral ganglion (Furuta et al., 1995). However, no published work provides further information regarding the localization and function of ANP and its receptors in the SGNs. In our current study, we investigated the presence of ANP and its receptors (NPR-A and NPR-C) in the rat SGNs by immunohistochemistry, reverse transcription-polymerase chain reaction (RT-PCR) and Western blot analysis.

2. Materials and methods

2.1. Animals and tissue preparation

All tissues were obtained from postnatal day 7 (P7) Sprague-Dawley rats (provided by the Laboratory Animal Center of the Fourth Military Medical University, Xi’an, China). The care and use of all animals in the study were carried out in accordance with the institutional guidelines of the Fourth Military Medical University. After cryoanesthesia, the rats were decapitated and the skulls were opened midsagittally. With the aid of a dissecting microscope, the cochleae were removed from the temporal bone, washed in ice-cold Hank’s Balanced Salt Solution (HBSS; Invitrogen, USA) and collected for further use. In order to separate the modiolus tissues containing most of the spiral ganglia from the more peripheral tissue (the spiral ligament, stria vascularis and the organ of Corti), careful dissection was made at the border between the spiral ganglion and the spiral limbus (adapted from Whilton et al., 2006).

2.2. RT-PCR analysis

The modiolus tissues were transferred to DNase/RNase-free microcentrifuge tubes containing RNalater RNA Stabilization Reagent (Qiagen, USA). The total RNA was isolated from the homogenates using an RNasy Protect Mini Kit (Qiagen) following the manufacturer’s instructions. The RNA quality was determined with a spectrophotometer (BioPhotometer plus; Eppendorf, Germany). Total RNA obtained was reverse transcribed into complementary DNA (cDNA) with a PCR thermocycler (MJ Mini Personal Thermal Cycler; Bio-Rad Laboratories, USA) by using RevertAid™ First Strand cDNA Synthesis Kits (Fermentas, USA). The PCR reaction consisted of 4 min at 94 °C, followed by 35 cycles of 94 °C (30 s), 55 °C (30 s), 72 °C (30 s), and ending with 10 min extension at 72 °C. The amplified products were separated on a 1% agarose gel, stained with ethidium bromide, and visualized on a UV transilluminator (Gel Doc™ XR+ system; Bio-Rad). For each experiment, cardiac tissue was used as a positive control, while water was used as a negative control. All primer sequences are as follows: ANP (forward 5'-CCGCTGCCAGAGATAAACAGC-3', reverse 5'-CTCGAGGAGGTAACTTCAA-3'), NPR-A (forward 5'-CTTCACGCCTGATCTGG-3', reverse 5'-ATCCCTCCAGGTGACTGTTG-3'), NPR-C (forward 5'-TGACACCATTCGGAAATCA-3', reverse 5'-CATCTCGGTAAGAAGCTTTGTA-3'), GAPDH (forward 5'-GTCGGTTGTAACCGATTGG-3', reverse 5'-CTTGGCTCGGGTATCAT-3'). All PCR experiments were performed in at least triplicate.

2.3. Western blot analysis

Total protein extract was prepared from the modiolus tissues using a lysis buffer containing 20 mM Tris–HCl, 150 mM NaCl, 0.5% sodium deoxycholate, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, 1 mM phenylmethylsulphonyl fluoride, and a protease inhibitor cocktail (Sigma, USA). The samples were then clarified, sonicated and centrifuged at 14,000 rpm for 4°C for 10 min. The protein concentration in the supernatant was determined with a spectrophotometer (Nanodrop 2000c; Thermo Fisher Scientific, USA). Protein samples (50 μg) were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto a polyvinylidene fluoride membrane (PVDF; Millipore, USA) using a Trans-Blot SD Semi-Dry Transfer Cell (Bio-Rad, USA). The blots were incubated for 1 h in blocking buffer containing 5% nonfat dry milk in PBS-T (0.1% Tween 20 in PBS) and then incubated overnight at 4°C with primary antibodies (rabbit anti-ANP, goat anti-NPR-A and goat anti-NPR-C, all 1:500; Santa Cruz, USA) diluted in blocking buffer. The blots were washed in PBS-T and incubated for 2 h at room temperature with the appropriate peroxidase-conjugated secondary antibodies (1:1000, Santa Cruz), and developed using enhanced chemiluminescence reagent (Thermo Fisher Scientific). Immunoreactive bands were visualized on a chemiluminescence system (FluorChem FC2; Alpha Innotech, USA). For each experiment, a total protein extract from cardiac tissue was used as a positive control. All Western blot experiments were performed in at least triplicate.

2.4. Immunohistochemical analysis

The cochlear tissue sections were processed for immunofluorescence as follows. Briefly, the cochleae were fixed with 4% paraformaldehyde (PFA) in 0.1 M phosphate buffer (PB, pH 7.2) overnight at 4°C, and cryoprotected in 10% sucrose for 4 h, followed by 30% sucrose overnight. All cochleae were then embedded in Tissue-Tek OCT compound (Sakura Finetek, USA) and frozen at −20°C. The sections were sectioned into 16μm-thick modiolar cross-sections using a cryostat microtome (CM1850; Leica, Germany) and mounted on poly-L-lysine-coated slides. After washing with 0.01 M phosphate buffered saline (PBS, pH 7.4), all specimens were blocked with 5% normal donkey serum (NDS, Jackson ImmunoResearch, USA) in PBS containing 0.1% Triton X-100, all 1 h, and incubated overnight at 4°C with polyclonal mouse anti-Tubulin β-III (TuJ1) antibody, polyclonal rabbit anti-ANP antibody, and either polyclonal goat anti-NPR-A or anti-NPR-C antibody (all 1:250; Santa Cruz) in antibody solution (1% NDS, 0.1% Triton X-100 in PBS). After washing, the samples were treated with Alexa Fluor 594-conjugated donkey anti-mouse IgG (1:800; Invitrogen), Alexa Fluor 647-conjugated donkey anti-goat IgG and DyLight 405-conjugated donkey anti-rabbit IgG (both 1:500; Jackson ImmunoResearch) in antibody solution at room temperature for 1 h. Each experiment also included a control where the primary antibody was omitted. After rinsing, the samples were mounted with Vectashield antifade mounting medium (Vector Laboratories, USA) and examined under a spectral scanning laser confocal microscope (FV1000; Olympus, Japan). All images were saved as TIFF files using Olympus confocal software FV10-ASW 1.7a and processed with Adobe Photoshop CS5 (Adobe Systems, USA) for adjustments of brightness and/or contrast.
3. Results

We first used RT-PCR to demonstrate whether the transcripts of ANP and its receptor were expressed in the spiral ganglion of rat cochlea (Fig. 1). As shown in Fig. 1A, the RT-PCR products derived from mRNA of ANP, NPR-A and NPR-C were detected in the cochlear modiolus tissue as single bands of the expected size (120 bp, 116 bp and 107 bp), and they were also detected in positive control samples obtained from the cardiac tissue. GAPDH (150 bp) was used as an endogenous housekeeping control gene. Control reactions omitting reverse transcriptase (−) or cDNA template (negative control) gave no reaction product.

We subsequently used SDS-PAGE and Western blotting to confirm whether the protein products were also present. As shown in Fig. 1B, the principal immunoreactive bands around 17 kDa, 130 kDa and 65 kDa were detected in modiolus tissue with ANP, NPR-A and NPR-C antibodies respectively, consistent with the expected bands detected in the rat cardiac tissue. Antibody against GAPDH (35 kDa) was used as a loading control.

Having demonstrated that ANP and its receptors are expressed in the rat spiral ganglion at both the protein and mRNA level, we went on to investigate their cellular localization using immunohistochemistry (Fig. 2). ANP was found to co-localize with both NPR-A and NPR-C in the perikarya of β-III Tubulin positive SGNs (Fig. 2B–E and H–K respectively, as indicated by arrows). We observed heterogeneity in the levels of immunoreactivity of ANP and its receptors, with less co-localization in a sub-population of SGNs (as indicated by arrowheads). There were however no distinct differences in intensity of immunostaining of ANP and its receptors between β-III Tubulin positive neurons in apical and basal turns of the cochlea. No immunoreactivity was observed in the negative controls (Fig. 2F, L).

4. Discussion

In this study, we have demonstrated that ANP and its receptors (NPR-A and NPR-C) are expressed in the rat spiral ganglion at the mRNA and protein levels using RT-PCR and Western blotting. We have further demonstrated the co-localization of ANP with both NPR-A and NPR-C in the cochlear SGNs by immunohistochemistry. Our research provides a direct evidence that ANP and its receptors are expressed in the primary auditory neurons of rat cochlea, suggesting a possible role for ANP in modulating the neuronal functions of SGNs via its receptors.

As a potent cardiac hormone, ANP primarily exerts its actions via cell-surface GC-coupled receptors, NPR-A, with ligand binding catalyzing increased production of intracellular cGMP. As a second-messenger, cGMP in turn modulates the activity of downstream effectors including protein kinases, ion channels and phosphodiesterases, ultimately mediating local antihypertrophic and antiinflammatory actions, as well as various physiological effects on blood pressure and volume homeostasis, neuronal excitability, transepithelial ion transportation, and the sensory transduction pathways underlying olfaction and vision (Potter et al., 2006, 2009). In contrast, NPR-C lacks GC activity and was originally considered to be a clearance receptor without any physiological role. However, it is now known to be coupled to inhibitory G proteins, which inhibit adenylyl cyclase/cAMP signal transduction and activate phospholipase-C to elicit its own physiological functions (Anand-Srivastava and Trachte, 1993; Rose and Giles, 2008).

ANP and its receptors are also widely expressed in the CNS and peripheral sensory organs, including the retina. Their distributions in retinal ganglion cells (Xu et al., 2010), neuronal and glial elements of the CNS (Cao and Yang, 2008) have increasingly drawn our attention. Functionally, in addition to established roles for ANP in circuits regulating neuroendocrine and cardiovascular function as well as fluid-electrolyte balance, this peptide could potentially be involved in several types of neuronal functions such as neurotransmitter release and uptake, synaptic transmission, neural development and neuroprotection (Cao and Yang, 2008). First, ANP could regulate the release and uptake of many neurotransmitters. Centrally released ANP may inhibit osmotically evoked neurohypophysial hormone release through presynaptic inhibition of glutamate release from osmoreceptor afferents (Richard and Bourque, 1996). Further work showed that the inhibitory effect of ANP on norepinephrine and dopamine release is calcium-dependent (Kanwal et al., 1997; Vatta et al., 1994). Second, ANP could modulate neuronal activities through the elevation of cellular cGMP levels, including synaptic transmission from osmoreceptor afferents to the supraoptic nucleus (Richard and Bourque, 1996), as well as information processing in the spinal cord (Vles et al., 2000) and retina (Dixon and Copenhagen, 1997). Third, a transient shift of binding sites for ANP was observed in the rat brain during embryonic neurogenesis, indicating a possible role for ANP in neural development (Tong and Pelletier, 1990). Finally, several studies demonstrated the neuroprotective role of ANP, mediated by increased intracellular cGMP, which protected neurons against excitotoxic, metabolic and oxidative insults. ANP was found to protect retinal neurons from N-methyl-D-aspartate (NMDA) receptor-mediated glutamate neurotoxicity through a possible involvement of increased intracellular dopamine levels (Kuribayashi et al., 2006).
There is some evidence for the presence of ANP and its receptors in the inner ear, including ANP receptors in the cochlear stria vascularis and modioli, as well as the vestibular secretory epithelium (Lamprecht and Meyer zum Gottesberge, 1988; Meyer zum Gottesberge and Lamprecht, 1989), and ANP-immunoreactivity in the secretory and sensory area of the inner ear, especially in the outer hair cells, spiral ligament, spiral limbus and stria vascularis of the cochlea (Chen et al., 1994; Koch et al., 1992; Meyer zum Gottesberge et al., 1991; Yoon and Anniko, 1994; Yoon and Hellstrom, 1992). Moreover, several studies demonstrated the presence of ANP, NPR-A and NPR-C transcripts in the inner ear by RT-PCR, and detected NPR-A transcripts in the spiral ligament and spiral ganglion of the cochlea (Furuta et al., 1995; Krause et al., 1997; Qiao et al., 2011; Seebacher et al., 1999; Suzuki et al., 1998). These direct evidences for the existence of ANP and its receptors in the cochlear secretory and sensory compartments, suggest functions of ANP in the maintenance of the blood flow, fluid and electrolyte homeostasis in the inner ear. However, no study to date has determined the presence and function of ANP and its receptors in the SGNs. In our study, we have used RT-PCR, Western blotting and immunocytochemistry to confirm the presence of both ANP and its receptors at the mRNA and protein level in the cochlear spiral ganglion and have furthermore demonstrated for the first time that all three proteins are present in SGNs, the neuronal region of the cochlea. ANP may therefore play a role as a neuromodulator or neuropeptide to modulate the neuronal functions of SGNs through its cell-surface receptors in an autocrine way, or have an impact on the fluid and electrolyte balance via receptors localized in the secretory area of the inner ear in a paracrine way. Importantly, the modulatory effects of ANP on synaptic transmission, neural development and neuroprotection would make it become a promising pharmacological target that facilitates new and effective therapies for hearing impairment. We hypothesize that ANP may protect SGNs against noise trauma and concomitant excitotoxic, metabolic and oxidative insults, through the activation of GC-linked receptors and increased synthesis of cGMP. Furthermore, ANP may rescue SGNs from degeneration after hearing loss and encourage peripheral fibers to sprout toward cochlear implant electrodes after implantation. ANP may also ameliorate cochlear ischemia diseases manifested as tinnitus and sudden sensorineural hearing loss, by regulating microcirculation and synaptic transmission in the cochlea. Further investigations are necessary to gain insight into the physiological functions and the modulatory effects of ANP on the primary auditory neurons of cochlea mediated by its receptors during auditory neurotransmission. Moreover, studies investigating the expression of ANP and its receptors in embryonic and postnatal animals will provide valuable insights into their potential roles in auditory development.

Conflict of interest statement

The authors declared no conflict of interest.
Acknowledgements

This research was supported by grants from Major State Basic Research Development Program of China (973 Project, No. 2011CB504505), the National Natural Science Foundation of China (NSFC, No. 311010105008, 30930098, 30870902, 31271220, 81200737 and 81170911) and China Postdoctoral Science Foundation No. 2012MS12101. We thank Medjaden Bioscience Limited for assisting in the preparation of this manuscript.

References