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Angiotensinergic neurotransmission in the peripheral autonomic nervous system

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1. ABSTRACT

Angiotensin (Ang) II has for long been identified as a neuropeptide located within neurons and pathways of the central nervous system involved in the control of thirst and cardio-vascular homeostasis. The presence of Ang II in ganglionic neurons of celiac, dorsal root, and trigeminal ganglia has only recently been described in humans and rats. Ang II-containing fibers were also found in the mesenteric artery and the heart together with intrinsic Ang II-containing cardiac neurons. Ganglionic neurons express angiotensinogen and co-localize it with Ang II. Its intraneuronal production as a neuropeptide appears to involve angiotensinogen processing enzymes other than renin. Immunocytochemical and gene expression data suggest that neuronal Ang II acts as a neuromodulatory peptide and co-transmitter in the peripheral autonomic and also sensory nervous system. Neuronal Ang II probably competes with humoral Ang II for effector cell activtation. Its functional role, however, still remains to be determined. Angiotensinergic neurotransmission in the autonomic nervous system is a potential new target for therapeutic interventions in many common diseases such as essential hypertension, heart failure, and cardiac arrhythmia.

2. INTRODUCTION

Chemical neurotransmission is a central mechanism of interneuronal communication by which neurons exchange information, maintain functional networks, and control homeostatic and integrative functions of the body. Neurons collecting information from the periphery (afferent) or communicating it centrifugally (efferent) usually generate electrical activity capable of releasing chemical neurotransmitters either non-specifically or at highly specialized axonal sites to activate target cells or to modulate their function. The vast majority of homeostatic organ functions in the body is under direct control of the autonomic nervous system and autonomic reflexes linking afferent with efferent neuronal pathways at the level of the spinal cord or prevertebral and visceral ganglia. These reflexes furthermore are under the control of higher regulatory sympathetic centers in the brain (1). Acetylcholine and noradrenaline are the classical neurotransmitters also released by autonomic pre- and postganglionic nerve endings. There are also many different neuropeptides produced by neurons and co-released with these transmitters which substantially increase the available spectrum of molecules for

neurochemical signaling. Together they define a characteristic chemical profile of each ganglionic neuron depending on its location and functional commitments. Neuropeptides usually function as signal modulators and influence the conditions of chemical neurotransmission at pre- and postjunctional synaptic membrane sites. Nevertheless, they may also activate their target cells directly via specific cell surface receptors (2). Neuropeptides represent a steadily increasing field of research with a promising potential concerning new therapeutical applications.

This review will focus on the role of angiotensin II (Ang II) as an emerging new neuropeptide co-transmitter in the peripheral aoutonomous nervous system and its pathophysiological implications with special respect to the heart. Ang II is an octapeptide and the main effector hormone of the plasmatic or humoral renin-angiotensin system. It is an important regulator of arterial vasomotion, renal function, salt and water balance, and cellular growth in the cardiovascular system with pleiotropic physiological effects (3). Its presence and local generation in the brain has furthermore been associated with salt appetite, central sympathetic nervous activity, blood pressure, and baroreflex sensitivity (4). The recent immunocytochemical detection of Ang II within sympathetic postganglionic neurons and also dorsal root ganglionic (DRG) neurons implicates a new functional role of Ang II as a putative neuropeptide transmitter in the peripheral autonomic nervous system including viscero- and somato-sensory afferent fibers (5, 6). We here provide an outline of the still limited knowledge concerning angiotensinergic neurotransmission in these fibers based on findings from our laboratory and other published observations.

3. PEPTIDERGIC NEUROTRANSMISSION

Some general aspects of neuropeptide co-transmission are summarized beforehand that appear relevant to the functional understanding of the immunocytochemical, morphological and protein expression data concerning Ang II. Various excellent in-depth reviews addressing specific topics of ongoing neurotransmitter and neuropeptide research are available and will help with additional informations (7-11). Neuropeptides are short peptides containing between 3 and ~100 amino acids produced within neurons. They are usually co-released together with a principal neurotransmitter of low molecular weight such as noradrenaline (MW 169) or acetylcholine (MW 246). The main synaptic transmitter effect is mediated by the small neurotransmitter molecule. Neuropeptides instead are primarily co-transmitters and modulators of chemical neurotransmission. Nevertheless, they may have also direct neurotransmitter functions, for instance in the brain. Neuropeptides may furthermore exert a trophic effect on target cells and stimulate neuronal growth. The release of neuropeptides may occur at highly specialized synaptic sites but there may also be non-synaptic axonal release, for instance in blood vessels where peptide-containing fiber varicosities are frequently located at a relevant distance from their target cells (12).

The de-novo production of neuropeptides is usually achieved by gene expression of specific precursor mRNA in the neuronal pericaryon followed by synthesis of neuropeptide precursor protein in the endoplasmatic reticulum. After processing and maturation neuropeptides are packed into membrane vesicles and transported into the axon periphery where they are stored locally before beeing released upon electrical activity and intracellular Ca^{2+}-signals. Neuropeptides and small molecular weight neurotransmitters are stored in separate synaptic vesicles with a characteristic electron microscopical appearance. The exocytotic release of neuropeptides is finally achieved by fusion of these vesicles with the axon membrane. Neurons may control the release of primary neurotransmitters and neuropeptides differentially by adjusting their firing frequency or changing their firing patterns. The postjunctional effects of neuropeptides are determined by the molecular structure of the synapse, the pre- and postjunctional receptor types, and finally the amount of neuropeptides released.

Neuropeptides are ligands of specific membrane-bound G-protein coupled receptors while low-molecular weight transmitters usually activate ligand-gated ion channels. Since different neuropeptides are packed non-specifically into the same vesicles and then are released together, their junctional effects are directly dependent on their intravesicular concentrations which in turn are determined individually by neuronal production and gene expression rates. Interestingly, there is no synaptic re-uptake mechanism for neuropeptides as for the other low-moleular weight transmitters. After their liberation neuropeptides instead are either degraded enzymatically or cleared by diffusion which explains their extracellular longevity and also rather slow effects on target cells. This characteristic allows neuropeptides also to address distant, non-innervated target cells by interstitial diffusion. Neuropeptide transmitter effects therefore are usually tonic and long-lasting compared to the low molecular weight transmitters with a short-acting characteristic.

There is a great biochemical diversity and anatomical variation of neuropeptide expression and co-localization in the brain (7). Neuropeptide co-existence in the brain has for instance been described for substance P and noradrenaline or neuropeptide Y (NPY) and gamma-aminobutyric acid (13, 14). Neuropeptides are also widely distributed in the peripheral nervous system including spinal lower motor neurons, sensory DRG neurons and neurons of autonomic nervous system (15-17) Calcitonin gene-related peptide (CGRP) and substance P (SP) furthermore are typically expressed in somato-sensory or viscero-afferent neurons located in the dorsal root ganglion (DRG) while postganglionic sympathetic fibers preferentially show co-expression of noradrenaline (NA) with NPY (18, 19).

4. ANGIOTENSIN II AS A NEUROTRANSMITTER: EVIDENCE FROM THE BRAIN

The renin-angiotensin system and its principal effector Ang II have initially been described as a humoral

peptide generating system in plasma. Angiotensinogen (Agt) is the high-molecular weight precursor of Ang II and represents a ~55-60 kDa glycoprotein belonging to the serpin superfamily of proteins. It is mostly synthesized in the liver from where it is constitutively released into the blood stream (20). Angiotensinogen is cleaved at its N-terminus by renin, an aspartyl proteinase, released by the kidney in a regulated manner to generate Ang I. Ang I is further processed by angiotensin converting enzyme (ACE) to produce Ang II (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe) as the biologically active peptide (MW 1046). ACE is an ectoenzyme present on cell surfaces (21). A variety of other angiotensins and Ang fragments such as Ang (1-7) may result from alternative enzymatic pathways (22). Although angiotensinogen is the only known physiological substrate of renin, it is not the only enzyme capable of cleaving Agt (23). The main cellular effects of Ang II are mediated by specific Ang receptor subtypes AT_1 and AT_2.

Besides the liver, many other tissues are capable of expressing Agt, and to a lesser extent also renin thereby contributing to local Ang I and Ang II concentrations independently from plasma-derived components. One of the first such local renin-angiotensin systems investigated was the brain renin-angiotensin system. Ang II in the central nervous system (CNS) has been associated with central sympathetic outflow, blood pressure and baroreflex control, thirst, neuroendocrine secretion, and mood (4, 24). Immunocytochemical studies have consistently confirmed the presence of immunoreactive Ang II in different areas of the brain, in neuronal somata, and also in connecting fiber pathways (25, 26). Furthermore, all components necessary to generate Ang II from its precursors are expressed in brain tissues either at the mRNA or protein level including Agt, renin and ACE.

Angiotensinogen, the Ang II precursor molecule, was found to be expressed mainly in brain glial astrocytes but it was also detected in single brain neurons. Experimental studies have furthermore demonstrated its mRNA expression and secretion as a protein by cultured neuronal and glial cells (27-30). Although initial reports seemed to support the presence and function of renin as a relevant Ang I generating enzyme in the brain (31, 32), subsequent molecular studies finally have detected renin mRNA only in very low abundance (33). Its intraneuronal presence and pivotal role for intraneuronal Ang II generation has not yet received unequivocal support by experimental data and this issue still remains debated (34). The anatomical distribution of renin mRNA expression in the brain for instance does not fit well with regions where Agt is highly expressed or where cardiovascular functions are regulated by Ang II such as the subfornical organ or the rostral ventrolateral medulla (33, 35-37). In transgenic mice expressing reporter genes under the control of long Agt and renin promoter constructs these transgenes were mainly expressed only in adjacent cell populations (38). Furthermore, the glial- or neuron-specific ablation of secreted renin in another transgenic mouse model had no effect on blood pressure and other biological functions supporting the notion that secretory renin is not a central Ang I generating enzyme in the brain. A nonsecretory intracellular renin isoform may thus be the only renin isoform expressed in the brain with a putative physiological role (39, 40).

There is also abundant expression of ACE in various parts of the brain. Nevertheless, ACE is an ectoenzyme and may therefore contribute only to extracellular Ang II generation (41). In one report, ACE protein has been identified in neuronal membrane fractions together with muscarinergic receptors but not in synaptic vesicles (42). Apart from Ang I and Ang II, a variety of other Ang peptides such as Ang III, Ang IV, Ang (1-7) and Ang (1-9) have been isolated from brain tissue with a documented or supposed physiological function (43, 44). Concerning these fragments there is increasing evidence that peptidases different from ACE are involved in their generation, for instance angiotensin converting enzyme type 2 (ACE2) or aminopeptidases A and N (22, 45). The failure to convincingly demonstrate all classical steps to generate Ang II within neurons has therefore led to alternative models explaining the presence of Ang II within neurons. One is extracellular uptake of Ang II from the interstitium, for instance by Ang receptor internalization but this is a very slow and limited mechanism. It may not replete Ang II losses by co-transmission efficiently (46). Alternatively, there may be a renin-independent generation of Ang II from angiotensinogen involving possibly cathepsin D, cathepsin G and tonin which are abundantly present in the brain (23, 47-49).

The evidence for intraneuronal Ang II as a neuropeptide transmitter comes from these morphological and cytochemical investigations but also numerous functional studies with targeted intranuclear or intraneuronal injections of Ang II or using pharmacological inhibitors (reviewed in 30, 50, 51). The experimental electrical stimulation of the subfornical organ projecting to the paraventricular nucleus (PVN) for instance was shown to induce a long-duration excitatory response in PVN neurons which was abolished by losartan, a specific AT_1 receptor blocker, while the short duration electrical response instead persisted. The results support a dual neurotransmitter release with Ang II responsible for the observed tonic excitatory component sensitive to losartan (52). There is no doubt that Ang receptors are expressed on CNS neurons (24). One immunohistological study co-localized Ang II intraneuronally with gamma-aminobutyric acid, an inhibitory low-molecular weight neurotransmitter (53). Ang II has also been detected in CNS nuclei where also noradrenergic neurons are located (54). The traditional criteria for the classification as a neurotransmitter, however, are not yet completely fulfilled by Ang II and several required elements are lacking (50). The criteria have initially been set up for classical low-molecular weight neurotransmitters and therefore may not all apply to neuropeptides. Nevertheless, the published data are consistent with a neuromodulatory and also direct neurotransmitter function of neuronal Ang II in the brain while this notion is now becoming increasingly accepted.

5. NEURONAL ANGIOTENSIN II EXPRESSION IN THE PERIPHERAL AUTONOMIC NERVOUS SYSTEM

5.1. Sympathetic postganglionic fibers

The presence of Ang II containing neurons and fibers in the peripheral autonomic nervous system and in dorsal root or trigeminal ganglia has only recently been reported by our laboratory which, to the best of our knowledge, has otherwise not yet been described (5, 6, 55, 56). In the past, just intraneuronal "binding sites" for Ang II were known with little additional information from cultured pig fetal cervical ganglionic neurons (57, 58). This is somewhat surprising since the innervation and the local renin-angiotensin systems of circulatory organs have already been extensively studied. Current information on this novel autonomic angiotensinergic innervation therefore is still limited.

Our laboratory initially relied on a polyclonal affinity-purified Ang II-antibody to study neuronal pathways in the brain (59). To avoid the many disadvantages of polyclonal antibodies mouse monoclonal antibodies against Ang II, and AT_1 and AT_2 receptors suitable for immunocytochemical investigations were subsequently developed (60). The mouse anti-Ang II antibody (4B3) detects Ang II specifically. It does not crossreact with angiotensinogen, Ang I or Ang (1-7) but recognizes also Ang (2-8), (3-8), (4-8) or (5-8) indicating a C-terminal specificity (56). With this antibody, rat and human celiac ganglia were studied for the presence of Ang II-positive cell bodies and fibers (5). The celiac ganglion harbors efferent postganglionic neurons of the sympathetic nervous system that innervate abdominal viscera and blood vessels. They receive their preganglionic input from neurons residing preferentially in the zona intermedia of the spinal cord (1).

Postganglionic sympathetic neurons have a typically noradrenergic phenotype and mostly express also NPY as a co-transmitter. In rat ganglionic specimens also a few cholinergic neurons have been described. Some of them co-localize also vasoactive intestinal polypeptide (VIP) or CGRP. Sympathetic neurons may furthermore express nitric oxide synthase, proenkephalin, and SP (61-63). In addition, the immunocytological studies in rat and human celiac ganglia from our laboratory identified a great number of celiac ganglionic neurons and intraganglionic nerve fibers containing Ang II. Mesenteric resistance arteries were similarly found to display a dense plexus of Ang II containing fibers in the adventitial layer. These Ang II-positive fibers often showed synaptic varicosities suggestive of postganglionic sympathetic fibers with a noradrenergic phenotype. Ang II could possibly be liberated from such angiotensinergic fibers as a co-transmitter and activate vascular smooth muscle cells (VSMCs) directly. It could also modulate noradrenergic neurotransmission. Ang receptors are present on smooth muscle cells and mediate vasoconstriction (64). NPY is known to potentiate NA-induced vasoconstriction by postjunctional Y1 receptors (65). Neuronal Ang II co-transmission could therefore like NPY enhance NA induced vasoconstriction via pre- or postjunctional AT1 receptors.

Some indirect evidence for ganglionic Ang II release has been obtained in vitro from cultured and electrically stimulated stellate and cervical ganglia which produced an increase of Ang II concentrations in the culture medium presumably caused by neuronal liberation (66). AT1 receptors have also been identified on sympathetic neurons (67). Ang II can directly activate subpopulations of postganglionic sympathetic neurons and facilitate NA release from peripheral sympathetic nerve terminals via such prejunctional AT1 receptors. Ang II stimulation at the ganglionic level may directly induce junctional catecholamine release from sympathetic nerve endings (68-70). In the rat mesenteric artery bed, Ang II experimentally enhanced NPY overflow induced by electrical nerve stimulation. The effect could be blocked by specific AT1 and also AT2 receptor blockers (71). It is therefore an attractive hypothesis to assume that some of these puzzling effects could be mediated by neuronal Ang II release. A hypothetical scenario for Ang II co-transmission in sympathetic postganglionic nerve endings is shown in Figure 1.

Sympathetic fibers finally may release neurotransmitters from non-synaptic varicosities located at a distance from their target cells. The released transmitters then reach their many cellular targets simultaneously via interstitial diffusion (12). The neuronal plexus of arteries furthermore is located within the adventitia bordering the smooth muscle cell layer where the fibers do not penetrate. Therefore, not all VSMCs are directly innervated. Neuronal Ang II release may in such situations directly compete with humoral Ang II for the same Ang receptors on target cells. One possible physiological role of this Ang II co-transmission could be to provide a rapid increase of local Ang II concentrations on demand compared to a slower response mediated by humoral Ang II depending on renal renin secretion. Neurogenic Ang II release could thus help control Ang II-dependent effects at precise anatomical locations for instance the sinu-atrial node or in single coronary arteries while Ang II signals mediated by plasma renin are nonspecific in this respect. As a hypothesis this concept nevertheless still remains to be verified. However, it receives strong support from a theoretical explanatory model based on preexisting experimental data. The model predicts that in small resistance vessels abluminal rather than intraluminal Ang II is important for maintaining vasoconstrictor tone (72).

5.2. Sensory-afferent neurons in dorsal root and trigeminal ganglia

Numerous Ang II-positive neurons were found in rat and human DRG and trigeminal ganglia (6, 55). These ganglia contain pseudounipolar neurons with afferent input from the viscera and the cerebral vasculature. Ang II-containing neurons were detected among both large and small size neuronal cell populations together with Ang II-positive surrounding fibers. Some of these fibers showed beaded varicosities. The intensity of neuronal angiotensin staining was not uniform indicating some variability of intracellular Ang II-concentrations. There was also co-expression of CGRP or SP in a subgroup of Ang II-positive neurons including small size ganglionic neurons with typically nociceptive properties (73). About 25% of the investigated rat trigeminal neurons (n=826) were positive for Ang II and 9,3% of these also co-localized SP. Ang II-positive

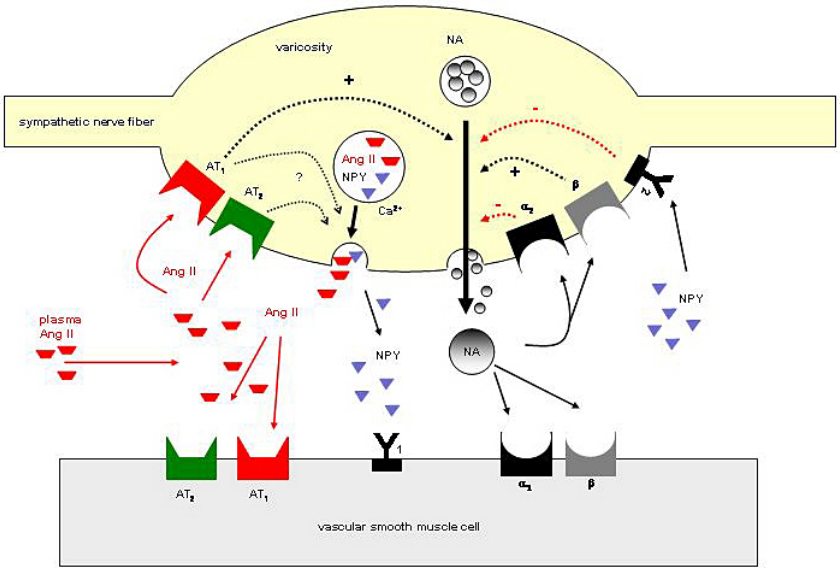

Figure 1. Hypothetical scenario of neuronal Ang II co-transmission at a sympathetic postganglionic neuro-effector junction with a vascular smooth muscle cell. Ang II and NPY are stored in the same synaptic vesicles and then co-released upon an in tracellular Ca2+-signal to activate pre- and postjunctional angiotensin and NPY receptors. Neuronal Ang II competes locally with Ang II of plasmatic or humoral origin. NA release is regulated by prejunctional adrenoceptors and NPY receptors. The role of prejunctional angiotensin receptors concerning Ang II and NPY co-release is still unclear. Ang II, angiotensin II; NPY, neuropeptide Y; NA, noradrenaline; Y1 and Y2, NPY receptors type 1 and type 2; AT1 and AT2, Ang II receptors type 1 and type 2; alpha, beta1, beta2 are adrenergic receptor subtypes.

terminals could furthermore be identified projecting centrally to the spinal trigeminal tract.

Other co-existing neuropeptides in DRG neurons show a great chemical diversity (74, 75). Both CGRP and SP may be released by peripheral sensory-afferent terminals (76, 77). Of note, CGRP may produce a direct vasodilatatory effect in arteries and antagonize NA and NPY induced vasoconstriction (78, 79). Conversely, NPY and Ang II were both shown to be modulators of CRGP release from sensory vasodilator nerves (80, 81). The presence of Ang II in sensory DRG and trigeminal ganglionic neurons and its co-localization with CGRP or SP strongly suggests a neuromodulatory or co-transmitter role of Ang II also in sensory afferent fibers.

In the arterial wall sympathetic efferent and sensory fibers are closely apposed (79). With this anatomical vicinity there may exist complex neurotransmitter interactions and yet unknown effects on arterial vasomotion depending on the efferent or afferent nature of Ang II co-transmission. Rat DRG neurons express AT1A receptors and synaptic Ang II liberation could therefore modulate sensory fiber activity by presynaptic Ang receptor feed-back (82). Ang II co-released with CGRP or SP from sensory fibers could furthermore play a pathogenetic role in neurogenic inflammation and nociception (83, 84). Another speculative function of neuronal Ang II relate to the modulation of sensory receptor thresholds in the fiber periphery. In the renal pelvis inflammatory model, Ang II was found to modulate the responsiveness of pelvic mechanosensory afferents and to block substance P release from renal sensory nerves by inhibiting PGE2-mediated activation of intracellular cAMP (76).

5.3. Evidence for renin-independent angiotensin II generation in peripheral ganglionic neurons

To further support the putative role of Ang II as a neurotransmitter we attempted to provide experimental evidence for an independent intraneuronal Ang II synthesis in ganglionic neurons. By in-situ hybridization methodology we were able to demonstrate neuronal Agt mRNA expression in single neurons of rat and human celiac, dorsal root and trigeminal ganglia (5, 6, 55). Using a hybrid methodology Agt mRNA expression could be co-localized with immunoreactive Ang II in the same neurons. Interestingly, a few neurons expressing Agt mRNA showed no Ang II staining suggesting that there is at least one additional regulated step necessary to achieve intraneuronal Ang II generation which may be an enzyme of variable expression or activity. Furthermore, we found measurable amounts of Ang II and other Ang peptides in extracts from human spinal and rat trigeminal ganglia. Although these quantitative data do not differentiate between cellular sources, they clearly document the presence of true Ang II peptide in support of our immunocytochemical findings.

Moreover, there was detectable mRNA expression of angiotensinogen, cathepsin D (CatD) and ACE in rat celiac, spinal, and trigeminal ganglia by quantitative polymerase chain reaction. In contrast, we failed to detect any appreciable amounts of renin mRNA which was below detection level in all samples despite sensitive measurement conditions. There was also detectable AT1A and AT2 receptor mRNA expression in rat DRG. The molecular data did not differentiate between ganglionic cell types but the absence of detectable renin mRNA expression was obvious in all samples. These findings strongly argue against renin as a significant Ang I forming enzyme in adult ganglionic neurons.

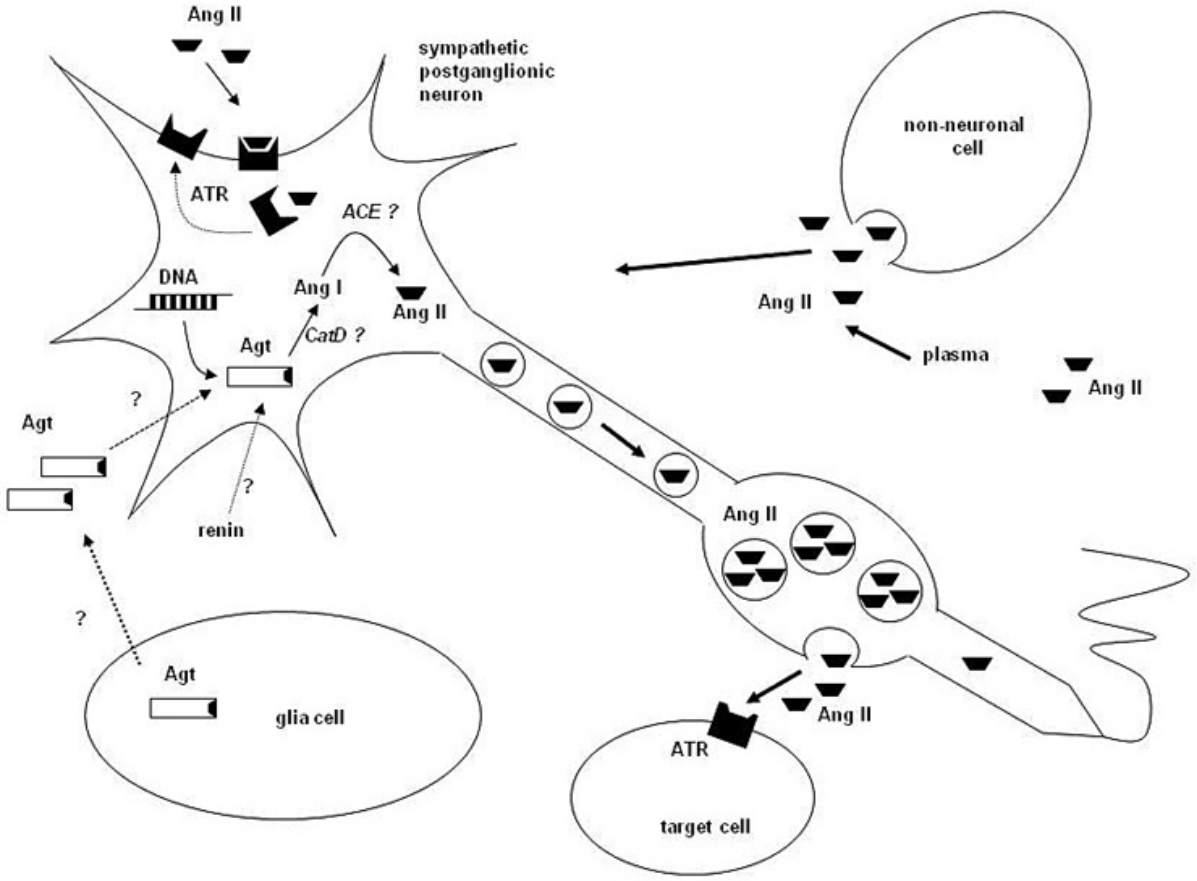

Figure 2. Intraneuronal Ang II generation in ganglionic neurons from Agt by a renin-independent pathway involving possibly cathepsin D and ACE. Agt and Cat D are coexpressed by ganglionic neurons. There may hypothetically also be Agt generation and release by non-neuronal (glia) cells with subsequent neuronal uptake from the interstitium. Some extracellular Ang II could also be taken up by binding to surface Ang II receptors followed by internalization. Extracellular angiotensinogen, renin and Ang II sources may be local non-neuronal cells or plasma. ACE, angiotensin converting enzyme; Agt, angiotensinogen; Ang II, angiotensin II; CatD, cathepsin D; ATR, angiotensin receptor.

Therefore, other enzymes such as CatD might be involved during intraneuronal Agt processing. Cleavage of Agt by CatD generates Ang I (23). CatD protein expression was co-localized immunocytochemically with Ang II in human DRG neurons (6). Various other Agt-cleaving or Ang-processing enzymes such as cathepsin G which generates Ang II directly from Agt or ACE2 are candidate enzymes but have not yet been investigated by us (23, 85). Our results finally are in contrast with *in-vitro* data from cultured fetal neurons, or neuroblastoma tumor cells where renin expression or supposed enzymatic renin activity have previously been reported (58, 86). There may be a phenotypic switch during pre- or postnatal development characterized by the suppression of renin expression in favor of other Ang II generating pathways.

As discussed for the brain, there is finally also the possibility of neuronal uptake of Ang II and other components from the interstitium to explain the immunocytochemical results. However, the presence of Ang II together with its precursor Agt strongly suggests that sympathetic postganglionic and DRG neurons are capable of generating Ang II themselves as summarized in Figure 2. This conclusion is further supported by similar data obtained in the carotid body, and also stellate and inferior cervical ganglia (66, 87). In fact, one important question for future research would be to find out which enzymatic steps lie in between these two molecules. Our data so far indicate that ganglionic neurons operate a renin-independent Ang II generating pathway that responds to regulatory stimuli essentially different from those controlling renal renin release.

6. ANGIOTENSIN II IN AUTONOMIC NERVOUS FIBERS OF THE HEART AND INTRINSIC CARDIAC NEURONS

The heart receives a rich autonomic innervation of both sympathetic and parasympathetic origin. Sympathetic postganglionic neurons reside mainly in the superior, middle and cervico-thoracic ganglion while parasympathetic preganglionic projections to the heart are

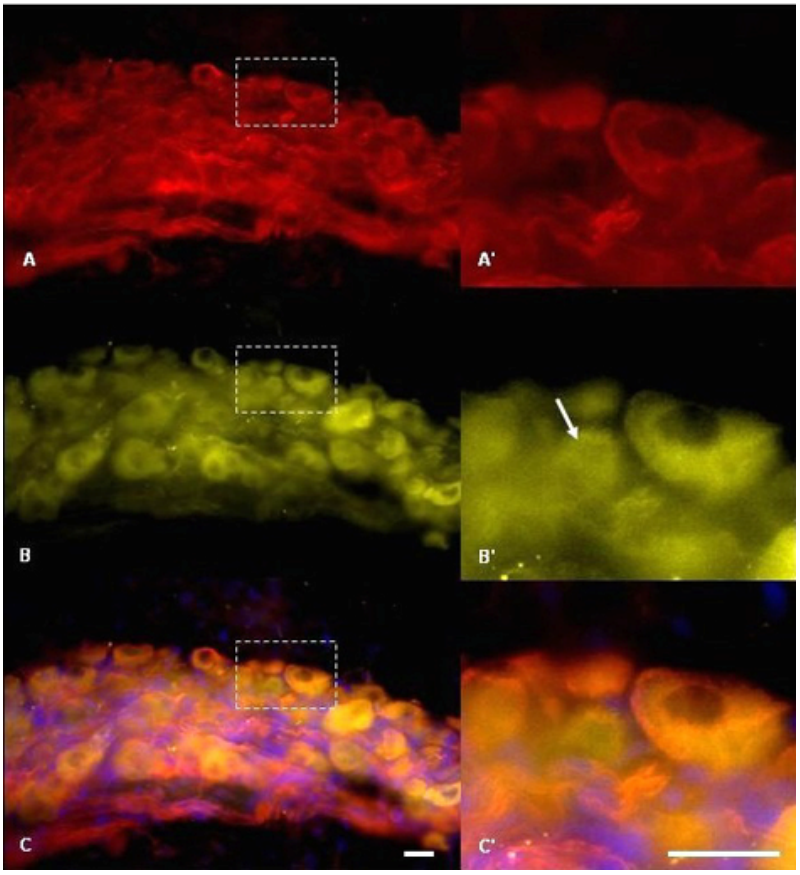

Figure 3. Double-staining of rat atrial ganglionic neurons for Ang II (panels A and A', red) and dopamine beta-hydroxylase, a noradrenergic marker (panels B and B', green). Numerous Ang II-positive pericarya are seen presenting also a noradrenrgic cophenotype (merged pictures, panels C and C', co-localization yellow). Panels A' to C' show a magnified region as indicated by the frames in panels A-C. Note also the presence of numerous Ang II-containing nerve fibers and a noradrenergic cell body not co-localizing Ang II (arrow). Cell nuclei are stained in blue by 4'-6-diamidino-2-phenylindole. Scale bar represents 20 μm.

provided by the vagus nerve. There are also numerous mechano-, chemo- and nociceptive afferents originating from the heart. Together, these fibers regulate cardiac function and indices involving complex autonomic reflexes (9, 88). Besides this extrinsic innervation, the heart possesses also an intrinsic autonomic neuronal system comprising more than 10^4 individual neurons located mainly in morphologically distinct ganglia and ganglionated nerve plexus in the epicardium (89). They constitute a widespread neuronal network of afferent, efferent and connecting circuit neurons with input also directly from intracardiac receptors. Parasympathetic preganglionic fibers primarily project to these intracardiac neurons while sympathetic preganglionic fibers may terminate on these intrinsic neurons or may directly innervate cardiac tissue without local relay neurons.

Many noradrenergic and also cholinergic fibers have been described in the endocardial, myocardial and epicardial layers of atria and ventricles with notable atrial to ventricle gradients and side differences (90, 91). Catecholaminergic fibers typically co-express NPY (9). Fibers positive for CGRP, VIP and somatostatin are less frequent in the heart. Immunocytological investigations of intracardiac ganglionic neurons revealed a similarly puzzling array of different neuropeptides (92-94). The vast majority of these neurons has been characterized as cholinergic but they may exceptionally display also a combined cholinergic and adrenergic phenotype (92, 95). The epicardial nerve plexus has recently gained much interest because of its role in cardiac rhythm control and during arrhythmia genesis (96).

As shown by our own studies, there is also an important cardiac innervation by Ang II-containing nerve fibers present in the epi- and myocardial layers of the atria and ventricles including the sinu-atrial and atrio-ventricular nodes (56). A proportion of these angiotensinergic fibers showed a noradrenergic phenotype or co-localized with synaptophysin. This is a membrane protein involved in vesicular maturation and exocytosis expressed for instance in sympathetic ganglionic neurons (97). However, there were also Ang II-positive fibers without a catecholaminergic or synaptophysin-expressing phenotype. Some were non-varicose. Many intrinsic ganglionic neurons similarly stained positive for Ang II and they mostly displayed a noradrenergic phenotype (Figure 3). *In-situ* hybridization revealed Agt mRNA expression in a

Table 1. Neuropeptide diversity in the central nervous system and in ganglionic neurons

Location	Neuropeptide	References
Brain	Angiotensin II	25, 26
	Angiotensin III	45
	Angiotensin IV	43
	Calcitonin gene-related peptide	54
	Substance P	13
	Neuropeptide Y	14
	Somatostatin	54
	Vasoactive intestinal polypeptide	54
	Vasopressin	26
	Neurotensin	54
	Thyrotropin-releasing hormone	54
	Beta-endorphin	54
Sympathetic postganglionic neurons	Calcitonin gene-related peptide	61, 62
	Substance P	61
	Neuropeptide Y	61-63
	Proenkephalin	62
	Vasoactive intestinal polypeptide	61
	Angiotensin II	5
Dorsal root ganglionic neurons	Calcitonin gene-related peptide	74, 75
	Substance P	74, 75
	Angiotensin II	6
	Galanin	74
	Somatostatin	74
	Vasoactive intestinal polypeptide	74
	Cholecystokinin	75
	Dynorphin	75
Intracardiac neurons	Angiotensin II	56
	Vasoactive intestinal polypeptide	92-94
	Neuropeptide Y	94
	Calcitonin gene-related peptide	92
	Substance P	94

subset of neurons. There was furthermore a significant Agt and CatD mRNA expression detected by polymerase chain reaction in cardiac tissue samples. Collectively, the findings document the ability of intrinsic cardiac neurons to generate Ang II from its Agt precursor while confirming our previous observations made in celiac and DRG neurons.

In view of the limited information available, the functional specifications and anatomical projections of these Ang II-positive intracardiac neurons still remains elusive. Nevertheless, the co-localization of Ang II with synaptophysin or dopamine beta-hydroxylase in varicose intracardiac fibers supports its presence in postganglionic sympathetic neurons with a similar neuromodulatory or neurotransmitter role as for NPY. Some intracardiac Ang II-containing fibers without NA or synaptophysin expression could finally represent sensory afferents belonging to intrinsic or extrinsic neurons (98). Our staining for the vesicular acetylcholine transporter (VAChT) finally visualized many varicose cholinergic fibers surrounding or contacting Ang II-positive neurons. They were most likely parasympathetic preganglionic fibers (90). Co-localization of VAChT and Ang II in neuronal somata was not seen but this issue is not yet settled. A comparison of neuropeptides in the brain and peripheral ganglia is shown in Table 1.

As in the vessel wall, neuronal Ang II release by intracardiac fibers may by this way contribute to local Ang II concentrations at the postjunctional Ang receptor independently from other Ang II sources. Neuronal Ang II liberation or spillover could furthermore represent a significant source for interstitial Ang II concentrations as appears to be the case for NPY (99,100). Pharmacological studies have repeatedly underscored the permissive or modulatory role of exogenous Ang II on junctional neurotransmitter release from cardiac sympathetic nerves (69, 101). Ang II co-transmission may have similar effects and interact with other neuropeptides to modulate noradrenergic neurotransmission and effector cell function. It could stimulate cardiomyoctes and VSMC in coronary arteries independently from plasma or interstitial Ang II. From an integrative viewpoint, Ang II release from intracardiac sympathetic fibers could be involved in the autonomic control of regional coronary blood flow. It could influence rhythm control by sinu-atrial node cells, or exert a proarrhythmic effect under certain pathophysiological conditions (102, 103). In this context Ang II as neuropeptide adds a new intriguing facet to the already known effects of Ang II effects in the heart. These functions, however, are still hypothetical and further studies are necessary to elucidate the pathophysiological role of this angiotensinergic cardiac innervation.

7. POTENTIAL THERAPEUTIC IMPLICATIONS

Of the many potential and yet speculative therapeutical implications based on the concept of autonomic angiotensinergic cotransmission some examples are briefly mentioned here. First and foremost, the presence of an angiotensinergic neurotransmission in the peripheral autonomic nervous system constitutes a new paradigm to explain Ang II-related physiological and pharmacological effects. One option could be to develop pharmacological approaches that specifically interfere with Ang II co-transmission using already available drugs with new indications or modes of application (104). In the damaged

heart for example, clinical indications could be the suppression of atrial or ventricular arrhythmias. Arrhythmias are known to be facilitated by Ang II which is probably also of sympathetic origin. The reduction of sympathetic NA release by pharmacological inhibition of neuronal Ang II co-release could have a beneficial effect in this context (105, 106).

The reduction of neuronal Ang II release could be achieved by either the suppression of intraneuronal de novo Ang II synthesis or by inhibition of its processing, packaging into vesicles, transport, and synaptic release. This for instance could be achievec by targeted gene therapy or similar pharmacological approaches (107). Of particular interest could be the fact that intraneuronal Ang II generation is probably not renin dependent. Therefore, drugs that interfere with the alternative Ang II generating pathways might become of interest that selectively decrease neuronal Ang II production without interfering with the kidney and renin-dependent Ang II effects. Alternative strategies could aim at synaptic neuropeptide release or consist of the pharmacological enhancement of synaptic Ang II degradation. In this context, target specificity could be achieved for instance by antibodies with intrinsic angiotensinase activity designed to recognize angiotensinergic synapses selectively. Currently, vaccination against Ang II is under clinical investigation as an option to treat human hypertension and possibly heart failure (108). This approach might become a model also for other clinical applications.

Alternatively, neuronal ablation strategies could target local subpopulations of angiotensinergic nerves to diminish their activity in selected situations, for instance after myocardial infarction with a high incidence of arrhythmia (96). In this context, the topical application of drugs into the pericardium to selectively decrease angiotensinogen expression or to reduce angiotensinergic neurotransmission in the epicardial ganglionated plexus might be considered. Similarly, in situations where only a transient therapy would be of interest such as after cardiac surgery such strategies could be helpful.

Finally, essential hypertension and heart failure are frequent diseases associated with sympathetic overdrive and an excess neuronal catecholamine spillover. These diseases are examples where sympathetic co-release of Ang II might become a promising target for strategies to reduce the deleterious sympathetic overactivity pharmacologically (109). Similarly, neuroinflammatory reactions including migraine and nociception have been associated with neuropeptide release from sensory-afferent fibers. The pharmacological modulation of neuronal Ang II co-release could become a new therapeutic concept to reduce tissue damage and to prevent disease chronification (110). Although still hypothetical, these conditions clearly represent attractive indications where pharmacological interventions aiming at angiotensinergic co-transmission might gain future importance.

8. CONCLUSION

Recent immunocytochemical studies have demonstrated the presence of Ang II in neurons of the rat and human celiac, dorsal root and trigeminal ganglia. Ang II-containing fibers and intrinsic ganglionic neurons were also shown to innervate the heart and mesenteric resistance arteries. The available evidence suggests that Ang II acts as a neuropeptide and synaptic co-transmitter in the periphery of the sympathetic nervous system and also in primary somato-sensory and nociceptive neurons. Ganglionic neurons express angiotensinogen, the precursor of Ang II. They furthermore appear to generate Ang II by a renin-independent enzymatic intracellular pathway. Based on the known effects of humoral Ang II, there are many functional and pathophysiological roles that can hypothetically be attributed to this newly detected intraneuronal Ang. Additional immunocytological and functional studies however are necessary to confirm and to further elucidate the functional role of this peripheral angiotensinergic neurotransmission. Together, neuronal Ang II co-transmission may play an important role in the control of cardiovascular function. It is a promising new target for specifically tailored therapies in the context of many common diseases such as essential hypertension, heart failure, and cardiac arrhythmia.

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Abbreviations: ACE: angiotensin converting enzyme; ACE2: angiotensin converting enzyme type 2; Agt: angiotensinogen; Ang: angiotensin; AT_1: angiotensin receptor type 1; AT_2: angiotensin receptor type 2; CatD: cathepsin D; CGRP: calcitonin gene-related peptide; CNS: central nervous system; DRG: dorsal root ganglion; MW: molecular weight; NA: noradrenaline; NPY: neuropeptide Y; PVN: paraventricular nucleus; SP: substance P; VAChT: vesicular acetylcholine transporter. VIP: vasoactive intestinal polypeptide; VSMC: vascular smooth muscle cell.

Key Words: Angiotensin, Renin, Angiotensinogen, Sympathetic nervous system, Neurotransmission, Neuropeptide, Dorsal root ganglion, Celiac ganglion, Neuron, Heart, Review

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