Chapter 4
Neuropeptides and Angiogenesis

Leena Pradhan-Nabzdyk and Christoph Nabzdyk

Abstract Neuropeptides are one of the most conserved proteins across different species and are ubiquitously expressed in different organs. In the peripheral nervous system, neuropeptides are secreted by the sensory and autonomic nerves and participate in a wide range of functions including immune surveillance, cardiovascular homeostasis, regulation of endocrine function, cytokine and growth factor release, and importantly angiogenesis. Neuropeptides including neuropeptide Y, substance P, calcitonin gene-related peptide, vasoactive intestinal peptide, and somatostatin (SS) are some of the neuropeptides that have been investigated regarding their role in modulating the vascular system and angiogenesis. All of these neuropeptides are pro-angiogenic except SS, which has anti-angiogenic properties. This chapter aims to present up-to-date evidence on the various mechanisms of action of the aforementioned neuropeptides and their clinical implications.

Keywords Neuropeptides • Wound healing • Cancer • Ischemia

L. Pradhan-Nabzdyk, Ph.D. (✉)
Department of Surgery, Division of Vascular and Endovascular Surgery,
Beth Israel Deaconess Medical Center, Harvard Medical School,
330 Brookline Avenue, Dana Building 805, Boston, MA 02215, USA
e-mail: lpradhan@bidmc.harvard.edu

C. Nabzdyk
Department of Surgery, Tufts Medical Center, Boston, MA USA

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4.1 Introduction

Neuropeptides are some of the most conserved and abundant peptides in vertebrates and function as neurotransmitters or neuromodulators or both within the central nervous system (CNS) and peripheral nervous system (PNS). Neuropeptides released from the PNS participate in major physiological functions ranging from cardiovascular homeostasis and gastrointestinal motility to immune cell trafficking. Neuropeptides including neuropeptide Y (NPY), substance P (SP), calcitonin gene-related peptide (CGRP), vasoactive intestinal peptide (VIP), and somatostatin (SS) have emerged as important regulators that affect the vascular system by modulating the vascular tone, angiogenesis, and vascular remodeling [1–10]. Most of these neuropeptides are pro-angiogenic, with the exception of SS, which has anti-angiogenic properties. Numerous studies have investigated the role of neuropeptides in angiogenesis, but there has been limited success in the development of neuropeptide-based therapies targeting angiogenesis. While some of the neuropeptides or neuropeptide-based therapeutics is in the pipeline for treatment of other diseases, so far, SS analogues are the only neuropeptide-based therapies used for modulating/inhibiting angiogenesis in cancer. This suggests that the ubiquitous and complicated neuropeptide signaling system in the vasculature needs a more in-depth understanding. Additionally, delivery of these peptides and their receptor agonists/antagonists to the target organ also poses various challenges.

This chapter aims to provide the most up-to-date information on the widely studied angiogenic neuropeptides, NPY, SP, CGRP, VIP, and SS. The reader has to note that this is not an exhaustive review but rather a concise summary that includes those neuropeptides that have received the most attention for their role in modulating angiogenesis.

4.2 Neuropeptide Y

NPY is a 36-amino acid peptide and is one of the most abundantly and ubiquitously distributed neurotransmitters in the CNS and PNS [11]. In the CNS, it is mainly expressed in the hypothalamus, [12] and in the PNS, it is found in sympathetic nerves, where it is stored either alone in small vesicles or in combination with catecholamines in larger vesicles [13, 14]. Moreover, NPY is also produced by vascular smooth muscle cells, endothelial cells (ECs), vas deferens cells, and pancreatic acinar cells [12, 15].

NPY signals through six subtypes of G protein-coupled receptors (GPCRs) including NPY1R, NPY2R, NPY3R, NPY4R, NPY5R, and NPY6R [16]. NPY regulates vascular tone by inducing vasoconstriction [17], stimulates vascular smooth muscle cell growth and hypertrophy of ventricular cardiomyocytes [18, 19], and is also involved in immune cross talk [20]. Signaling through NPY1R,

NPY2R, and NPY5R is implicated in diabetes, heart failure, hypertension, peripheral arterial disease, and feeding disorders [21]. Furthermore, these receptors are involved in angiogenesis [22], calcium homeostasis [21], renin-angiotensin-aldosterone system (RAAS), and protein kinase C (PKC) activation of diabetes and heart failure [23].

4.3 NPY and Angiogenesis

In recent years, the contribution of NPY-associated angiogenesis to cardiovascular disease, wound healing, and cancer has received a lot of attention [1, 2, 24–26]. The first report that described the sympathetic co-transmitter NPY, as a pro-angiogenic molecule, was presented by Zukowska et al. and was based on work in human umbilical vein endothelial cells (HUVECs). The authors reported that not only the receptors NPY1R and NPY2R but also NPY itself and the enzyme, dipeptidyl peptidase (DPPIV) that converts NPY_{36} to the angiogenic $NPY_{3–36}$, are expressed in these cells. Of the NPY receptors, NPY1R, NPY2R, and NPY5R are involved in angiogenesis. So far the only known mechanisms by which NPY leads to angiogenesis are release of vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), and NO [26, 27].

NPY1R antagonism is shown to inhibit angioplasty-induced atherosclerotic-like vascular remodeling, without affecting ischemic revascularization; NPY2R activation is shown to stimulate ischemic angiogenesis. NPY5R enhances the effect of both NPY1R and NPY2R [6, 25]. NPY1R is normally implicated in pathologies of the cardiovascular system including cardiac hypertrophy and vasoconstriction, whereas NPY2R and NPY5R are known to ameliorate ischemia and impaired wound healing. In a recent swine study of chronic myocardial ischemia, treatment with exogenous $NPY_{3–36}$ increased capillary and arteriole formation along with upregulation of NPY1R, NPY2R, NPY5R, VEGF, endothelial nitric oxide synthase (eNOS), phospho-eNOS (p-eNOS) on Ser1177, and PDGF and a downregulation of anti-angiogenic factors endostatin and angiostatin in the NPY-treated group [26]. This suggests that NPY signaling occurs in a feed-forward pro-angiogenic manner where it enhances the entire NPY signaling system by upregulating its own receptors.

NPY has been demonstrated to have a role in the angiogenesis phase of wound healing in both cutaneous and tendon healing by acting through NPY2R and NPY5R [1, 28, 29]. In fact, in NPY2R knockout mice, angiogenesis and thereby wound healing are disrupted [8].

In cancer, NPY and its receptors are expressed in both the tumor cells and the tumor vasculature. In human neuroblastoma tissues, NPY is predominant in the tumor cells whereas NPY2R is expressed in both tumor and ECs, making NPY2R a promising target for neuroblastoma therapy [30]. Similarly, prostate tumors and neural crest-derived tumors release NPY that can facilitate tumor vascularization [31, 32]. These studies demonstrate an important role for NPY in the treatment of wound healing, ischemic revascularization, and cancer.

4.4 Substance P

SP is an 11-amino acid neurotransmitter and neuromodulator widely distributed in the CNS and PNS [13, 33]. SP is released from C-fiber sensory nerves, in primary sensory neurons and neurons intrinsic to the gastrointestinal, respiratory, and genitourinary tracts [34, 35]. SP binds to three G protein-coupled receptors, NK1R, NK2R, and NK3R, out of which NK1R is its high-affinity receptor that is present on a variety of cell types including immune cells, keratinocytes, ECs, neurons, and glial cells [36]. SP is involved in several physiological processes, including maintenance of cardiovascular tone, smooth muscle activity, vomiting reflex, defensive behavior, and stimulation of salivary secretion [9, 37]. SP has been shown to be mitogenic towards smooth muscle cells, fibroblasts, and ECs [1, 35, 38–40]. SP has shown to affect different pathways in different cell types including activation of phospholipase C producing a net rise in intracellular (Ca^{2+}) and cAMP via AC [41, 42]; induction of cytokines, tumor necrosis factor-alpha (TNF-α), interleukin (IL)-6, IL-8, IL-1β, and IL-2, from T lymphocytes, macrophages, and neutrophils [43]; activation of protein kinase B (Akt) pathway in dopaminergic neurons [44]; and induction of transforming growth factor-beta (TGF-β) from fibroblasts [45, 46].

4.5 SP and Angiogenesis

The vasodilatory effect of SP was evident long before nitric oxide (NO) was recognized as the endothelium-derived relaxation factor (EDRF) [47]. Only later, it was demonstrated that SP-induced vasodilation was indeed mediated by NO via the NK1R [48, 49]. The first evidence indicating the role of SP in EC proliferation and neovascularization was demonstrated by Ziche and colleagues in vitro in HUVECs and in vivo in an avascular cornea rabbit model [40]. Similar to NPY, SP-mediated angiogenesis via NK1R contributes to wound healing, tumor angiogenesis, and ischemic angiogenesis [1, 50–53]. SP plays a major role in cutaneous wound healing by participating in the inflammatory and angiogenic phases [35]. In vitro studies indicate that SP treatment leads to proliferation and tube formation of dermal microvascular ECs in normal and hyperglycemic conditions [1, 54, 55]. Moreover, inhibition of the enzyme neprilysin that breaks down SP also enhances angiogenesis [56]. In cutaneous wound healing, NO release is the only known mechanism through which SP has been shown to lead to angiogenesis. In addition to cutaneous wound healing, SP has also shown to aid in tendon healing after injury by augmenting angiogenesis [51, 57].

SP and NK1R are implicated in different cancers including brain tumors, melanomas, and breast cancer [58–60]. NK1R receptors are upregulated in brain tumors, and therefore NK1R antagonists that cause tumor cell apoptosis and also inhibit tumor vascularization hold great promise in treating these tumors [58, 61]. Aprepitant,

the NK1R antagonist approved for the treatment of chemotherapy-induced nausea and vomiting, has shown some promising results in inhibiting cell growth of different melanoma cell lines [62]. However, at this time aprepitant has not been approved for any cancer treatment. The genes that encode SP, preprotachykinin 1, and NK1R and NK2R have shown to be upregulated in breast cancer, and NK1R antagonists have shown to inhibit breast cancer cell growth in in vivo models [60, 63]. Currently there are no studies targeting breast tumor angiogenesis with NK1R antagonists.

In a recent mouse hind limb ischemia study, SP has shown to mobilize NK1R-expressing progenitor cells from bone marrow and promote reparative angiogenesis [50]. In the same study, authors showed that patients with acute myocardial infarction had high circulating levels of SP and NK1R-positive cells that co-express progenitor cell antigens, which are abundant in infarcted hearts, but interestingly not in hearts that developed an infarct after transplantation [50].

These studies demonstrate that in addition to its role as a sensory neuropeptide, SP and its receptors also play an important role in wound healing, ischemic revascularization, and cancer.

4.6 Calcitonin Gene-Related Peptide

CGRP is a 37-amino acid peptide generated in the CNS and PNS from the alternate splicing of calcitonin gene mRNA in a tissue-specific manner [64]. In the PNS, CGRP is expressed in the nerves innervating the skin, gut, pancreas, heart, and the vasculature. It is co-localized with SP in the sensory nervous system and, similar to SP, is a potent vasodilator [65].

CGRP receptor is a heterodimer of calcitonin receptor-like receptor (CRLR), a GPCR, which is linked to receptor activity modifying protein (RAMP)1, RAMP2, or RAMP3 [66]. CRLR-RAMP1 is specific for CGRP while CRLR-RAMP2 and RAMP3 can also bind adrenomedullin, a related peptide [66]. CGRP is known to be involved in vasodilation, nociception, glucose uptake, and the stimulation of glycolysis in skeletal muscles [67]. Binding of CGRP to its receptors leads to an increase of cAMP and activation of protein kinase A (PKA), phospholipase C beta (PLCβ1), mitogen-activated protein kinase (MAPK), and production of NO [68].

4.7 CGRP and Angiogenesis

Similar to SP, CGRP was found to be a potent vasodilator that acted through an endothelial-dependent mechanism which later was identified to be NO [69, 70]. The first connection between CGRP and angiogenesis was identified in a rat ischemic skin flap partial denervation study where angiogenesis preceded reinnervation with CGRP positive nerves [71]. In a subsequent study, the same group

showed that in fact CGRP at least partially contributed towards angiogenesis [72]. Similar to SP, CGRP-associated angiogenesis is involved in wound healing, ischemic revascularization and tumor vascularization. In a rat knee-joint model intra-articular CGRP injection increased endothelial cell proliferation while inhibition of CRLR-RAMP1 attenuated EC proliferation in capsaicin-induced knee-joint synovitis [73]. In a model of rat hind limb ischemia, CGRP levels were elevated in the ischemic tissue, and over-expression of CGRP enhanced blood flow recovery and increased capillary density in ischemic hind limbs most likely via activation of AMP-activated protein kinase (AMPK) [74]. In vitro, CGRP induced p-eNOS in HUVECs at Ser1177 and Ser633 in a time-dependent manner, and these effects were abolished by AMPK inhibition [74]. In addition to AMPK and eNOS, CGRP has also shown to enhance angiogenesis in cutaneous and gastric mucosal healing via VEGF [75, 76]. In CGRP knockout mice, tumor growth and tumor-associated angiogenesis of implanted Lewis lung carcinoma (LLC) cells along with downregulation of VEGF expression in tumor stroma were significantly reduced compared with those in wild-type mice [77].

CGRP is very similar to SP in its angiogenic profile and can serve as an important target for wound healing and cancer.

4.8 Vasoactive Intestinal Peptide

VIP is a 28-amino acid peptide that is expressed all throughout the CNS including the hypothalamus, PNS, intestines, pancreas, urogenital tract, thyroid, and adrenal glands [78].

VIP affects a wide range of biological activities including vasodilation and smooth muscle relaxation, stimulation of pepsinogen secretion by the chief cells of the gut, secretion of water and electrolytes into the intestines, enhancement of glycogen metabolism in the cerebral cortex, regulation of embryonic growth, promotion of neuronal survival, and modulation of the immune system as well as mammalian circadian rhythm [78]. VIP exerts its biological effects through its receptors, vasoactive intestinal polypeptide receptor 1 (VPAC1) and VPAC2 which are GPCRs. Receptor activation leads to modulating the activity of phospholipase D (PLD) and also increased cAMP and intracellular calcium levels [78].

4.9 VIP and Angiogenesis

Similar to SP and CGRP, VIP was identified as a vasodilator in a canine model where VIP was injected in the glandular artery of the submandibular gland [79]. Later it was demonstrated that VIP-induced vasodilation is also mediated via l-arginine-NO pathway [80]. VIP-associated angiogenesis is implicated in tumor vascularization with some studies demonstrating its protective role in ischemic injury. Although

VIP is shown to aid in wound healing, most of this effect is attributed to its role in bronchial epithelial cell proliferation [81] and keratinocyte proliferation [82, 83] but not through angiogenesis. In addition to increasing EC proliferation, VIP is also known to increase EC migration [84]. In a model of acute cerebral ischemia, a single dose of intracerebroventricular injection of VIP at the beginning of reperfusion led to increased number of ECs and microvessels at the boundary of the ischemic lesion. VIP further significantly increased VEGF levels in the ischemic hemisphere as well as VEGF receptor flt-1 and flk-1 immunoreactivity in Ecs [85, 86].

In the human small lung cancer cell line H446 VIP administration increased c-fos and VEGF mRNA expression that was reversed by c-fos antisense oligodeoxynucleotide [87]. In a mouse xenograft model in which human prostate cancer cells were transplanted, VIP administration resulted in increased tumor growth along with increased VEGF expression [88]. In another study, the same group showed that VIP induces VEGF mRNA expression via c-fos, which in turn is induced by calcium signaling in human prostate LNCaP cells [89]. In a colon carcinoma model, systemic VIP treatment reduced angiogenesis within tumor masses by cAMP-dependent mechanism [90].

Similar to NPY, SP, and CGRP, VIP has been shown to play a role in ischemic vascularization and in cancer angiogenesis; however, its role in the angiogenic phase of wound healing has not yet been investigated. Given the angiogenic profile of VIP, it would not be too far-fetched to surmise that VIP could promote wound healing.

4.10 Somatostatin

Somatostatin (SS) which was discovered in 1974 by Brazeau et al. [91] is a major inhibitory neuropeptide produced in the hypothalamus and the arcuate nucleus but also secreted by peripheral nerves in the gut [92, 93]. In fact, the gastrointestinal tract contains about 70 % of the total SS [94]. SS exists in three forms, the 14 amino acid, SS-14 (clinical analogues are made towards SS-14); the 28 amino acid, SS-28; and the 25 amino acid, SS-25 [95]. SS-28 and SS-25 are in fact SS-14 with a 14-amino acid and an 11-amino acid extension at the N-terminus, respectively [96]. SS-14 and SS-28 have similar physiological activities but bind to the receptors with different potencies, while not much is known about SS-25 [95].

SS exerts its effects via five different receptor subtypes (sst1–sst5) that are high-affinity membrane-bound GPCRs and are expressed throughout the CNS and on the pancreas, gut, pituitary, kidneys, thyroid, lung, and endothelial and immune cells [92, 93]. SS affects various tissues and is known to inhibit endocrine and exocrine secretions, intestinal motility, modulate neurotransmission, motor and cognitive functions, vascular contractility, and cell proliferation [92]. All five receptors are known to play a role in the anti-proliferative effect of SS. Binding of SS to sst2 and sst3 leads to apoptosis via p53-independent pathways. On the other

hand, binding of SS to sst1, sst2, sst4, and sst5 leads to cytostasis via different pathways including increase of $p21^{Cip1/Waf1}$, induction of $p27^{Kip1}$, hypophosphorylation of retinoblastoma (Rb) protein, inhibition of cyclin E/cdk2, inhibition of MAPK cascade, and inhibition of guanylyl cyclase [93]. Additionally, SS also inhibits cellular proliferation by inhibiting secretion of numerous growth factors such as growth hormone, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), insulin-like growth factors I and II (IGF-I and IGF-II), insulin-like growth factor binding protein, and PDGF [93].

4.11 Somatostatin and Angiogenesis

In contrast to all the aforementioned neuropeptides, one of the earliest studies investigating the role of SS on vasculature suggested that SS decreases blood flow in the gut [96]. Later on it was suggested that this was a direct effect of SS on vascular smooth muscle cells [97]. It is now known that the vasomodulatory effects of SS are confined to the splanchnic circulation and that SS decreases gut motility most likely by causing the release of vasoconstrictors such as endothelin-1 [98].

SS as an anti-angiogenic peptide has a therapeutic potential in the treatment of diseases such as cancer, proliferative retinopathy, and endometriosis where the goal is to inhibit angiogenesis. Using a chick embryo-chorioallantoic membrane (CAM) assay, Woltering and colleagues conducted one of the first studies indicating a role for SS as an anti-angiogenic neuropeptide, suggesting a role for SS in tumor angiogenesis [99].

sst2 is the most commonly involved receptor eliciting the anti-angiogenic effects of SS along with sst1 and sst5. SS is shown to have anti-angiogenic effect by suppressing the expression of pro-angiogenic factors such as VEGF and VEGFR2 [100], IGF-R1, and angiopoietin 2 (Ang 2) and its receptor Tie-2 [101] or by increasing the expression of anti-angiogenic factors such as thrombospondin-1 (TSP-1) [102].

The antitumor effect of SS could be direct by leading to tumor cell apoptosis or indirect by suppressing angiogenesis and inhibiting growth factors [103]. sst2 is commonly involved in cancers. Over-expression of sst2 in human pancreatic (capan-2) and lung cancer cells (A549) transplanted in mice inhibited the growth of both sst2-positive and sst2-negative xenografts by affecting the cellular levels of signaling molecules in apoptotic pathways, MAPK pathway, and angiogenesis [104]. SS analogue, octreotide, has been therapeutically used in cancer treatment, especially neuroendocrine tumors [105–107]. Octreotide has also shown beneficial effects in a mouse hepatocellular carcinoma model where it inhibited the incidence of second primary tumors, decreased lung metastasis, and prolonged the life span by decreasing intratumoral angiogenesis [108]. In a gastric cancer trial, compared to patients that received placebo, patients that received SS had significant decrease in serum VEGF level, which was partially dependent on the synthesis and degradation of the protein but not the transcription of mRNA

[109]. SS treatment has also demonstrated beneficial effects in prostate cancer, pancreatic adenocarcinoma models, and an ovarian cancer study [110–113].

In addition to cancer treatment, SS analogues are used for the treatment of refractory bleeding in gastrointestinal angiodysplasias [111, 114] and have also shown beneficial effects in models of endometriosis where receptors sst1, sst2, and sst5 are highly expressed [115]. SS is also used in treatment of proliferative retinopathy [116, 117] where another SS receptor agonist, non-peptide imidazolidine-2,4-dione (NISA), similar to octreotide, inhibited growth factor-induced EC proliferation, migration, and tube formation [118].

Unlike the other neuropeptides discussed, SS is the only one that is proven to be anti-angiogenic and the only one that has been successfully used as an anti-angiogenic agent in cancers. In addition, these data demonstrate that SS-based therapy can also be promising in the treatment of other diseases such as proliferative retinopathy and endometriosis.

4.12 Conclusions

Neuropeptides have a tremendous potential to be developed as therapies for treating myriads of diseases in which modulation of angiogenesis is essential. Thus in ischemia and impaired wound healing, therapies should aim at enhancing NPY, SP, CGRP, and VIP signaling and to inhibit SS signaling system (Fig. 4.1). Conversely in cancer, the goal ought to be to block the NPY, SP, CGRP, and VIP signaling system and to enhance SS signaling (Fig. 4.1). At time there is lack of in-depth understanding regarding the signaling mechanisms of some neuropeptides therefore, the common and overlapping pathways through which some of these neuropeptides signal should be further explored for their potential therapeutic use. Another area that needs investigation is delivery of neuropeptides, their analogues or receptor antagonists at the disease site. For example, one of the challenges in treating chronic

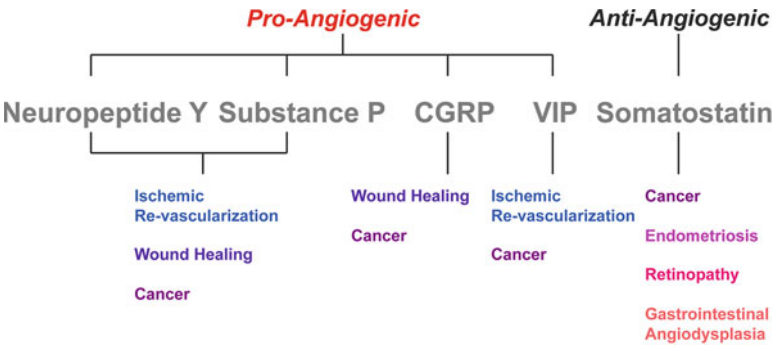

Fig. 4.1 Summary of the role played by neuropeptides in different angiogenesis-based functions and pathologies

wounds such as diabetic foot ulcers is that the wound microenvironment is very hostile with high expression of proteases, and therefore direct application of neuropeptides to the wound site is not feasible. Another example is in treatment of ischemic revascularization where a systemic delivery of neuropeptide and/or analogue is necessary. One way to overcome this problem is by inhibiting proteases that could cause endogenous neuropeptide degradation instead of systemic injection of neuropeptides that is not feasible. One such protease, neprilysin, that breaks down substance P has already received some attention. However, this too could pose a problem because systemic increase of neuropeptides has a high potential for side effects as these neuropeptides participate in several different physiological processes.

Overall, neuropeptides play an important role in angiogenesis and their ubiquitous nature emphasizes that they can affect angiogenesis in almost all organs. Thus further in-depth exploration is necessary to develop neuropeptide-based angiogenesis therapies and develop strategies for their efficacious delivery.

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