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Sympathetic Nervous System Research Developments

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SYMPATHETIC NERVOUS SYSTEM RESEARCH DEVELOPMENTS

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PREFACE

This new book presents the lastest research on the sympathetic nervous system (SNS) which is a branch of the autonomic nervous system. It is always active at a basal level (called sympathetic tone) and becomes more active during times of stress. Its actions during the stress response comprise the fight-or-flight response. Like other parts of the nervous system, the sympathetic nervous system operates through a series of interconnected neurons. Sympathetic neurons are frequently considered part of the peripheral nervous system (PNS), although there are many that lie within the central nervous system (CNS). Sympathetic neurons of the spinal cord (which is part of the CNS) communicate with peripheral sympathetic neurons via a series of sympathetic ganglia. Within the ganglia, spinal cord sympathetic neurons join peripheral sympathetic neurons through chemical synapses. Spinal cord sympathetic neurons are therefore called presynaptic (or preganglionic) neurons, while peripheral sympathetic neurons are called postsynaptic (or postganglionic) neurons.

At synapses within the sympathetic ganglia, preganglionic sympathetic neurons release acetylcholine, a chemical messenger that binds and activates nicotinic acetylcholine receptors on postganglionic neurons. In response to this stimulus, postganglionic neurons principally release noradrenaline (norepinephrine). Prolonged activation can elicit the release of adrenaline from the adrenal medulla. Once released, noradrenaline and adrenaline bind adrenergic receptors on peripheral tissues. Binding to adrenergic receptors causes the effects seen during the fight-or-flight response. These include pupil dilation, increased sweating, increased heart rate, and increased blood pressure.

Chapter 1 - Life on earth has evolved in the presence of natural and ubiquitous magnetic fields. So it is not surprising that biological systems can respond to a wide range of static magnetic fields (SMF): weak-intensity SMF (microtesla level), including the geomagnetic field, moderate-intensity SMF (millitesla level), and strong-intensity SMF (tesla level). It has been estimated that some of these physiological responses seem to be mediated through the nervous system. For weak-intensity SMF effects, a neuroanatomical, electrophysiological and behavioral analysis of magnetoreception or magnetic compass orientation revealed that some neurons in the central nervous system and sympathetic nervous system have been activated by magnetic stimuli in some vertebrates and invertebrates. In several experimental studies on the SMF effects, pharmacological procedures and experimental animals have been used to assess the sympathetic responsiveness to SMF. It has been reported that continuous exposure to moderate-intensity SMF for several weeks can depress or suppress the action of sympathetic agonists (norepinephrine, phenylephrine, and dobutamine) and a sympathetic antagonist

(reserpine) on hemodynamics, blood pressure and/or behavioral activity by modulating sympathetic nerve activity or baroreflex sensitivity in animals. There is growing evidence to suggest that SMF of various intensities could alter the autonomic function in humans. Thus, this review describes the reported SMF effects (and non-effects) on the sympathetic nervous system of animals and humans.

Chapter 2 - Obesity and obesity-related cardiovascular disease are rapidly growing public health problems. Heightened sympathetic nerve activity is a well-established observation in obesity and hypertension. There is evidence that human obesity and hypertension have strong genetic as well as environmental determinants. Reduced energy expenditure and resting metabolic rate are predictive of weight gain, and the sympathetic nervous system participates in regulating energy balance through thermogenesis. The thermogenic effects of catecholamines in obesity have been mainly mediated via the β2 and β3-adrenergic receptors in humans. Further, β2-adrenoceptors importantly influence vascular reactivity. Genetic polymorphisms of the β-adrenoceptor gene have been shown to alter the function of several adrenoceptor subtype and thus to modify the response to catecholamine. Among β2-adrenoceptor polymorphisms, Arg16Gly, Gln27Glu, and Thr164Ile are considered the most functionally important.β2-adrenoceptor genes have been studied in relation to obesity. Genetic variations in the β3-adrenoceptor, such as the Try64Arg variant, are also associated with both obesity and hypertension. However, the precise relationships of the polymorphisms of β2- and β3-adrenoceptor genes with sympathetic nervous system activity, obesity and hypertension have not been fully clarified.

The authors findings show that: 1) β2-adrenoceptor polymorphisms are associated with heightened sympathetic nerve activity, and predict the future onset of obesity and hypertension in nonobese individuals, 2) β2-adrenoceptor polymorphisms accompanied by heightened sympathetic nerve activity and abdominal obesity, predict weight loss resistance during a weight loss program, and also predict rebound weight gain, 3) β2-adrenoceptor polymorphisms are linked to blunted leptin-mediated sympathetic nerve activation, leptin-resistance and resultant obesity, 4) β2-adrenoceptor polymorphisms are related to insulin-resistance, in both nonobese and obese normotensive individuals, and 5) β3-adrenoceptor polymorphism is directly linked to obesity and hypertension, but only in obese individuals.

The purpose of this article is to provide a synthesis of the current findings on this topic. The influence of the sympathetic nervous system and β2- and β3-adrenoceptor polymorphisms in hypertension, obesity and obesity-related hypertension will be explored through a literature review, matched against the authors own findings. Relevant studies of the β2- and β3-adrenoceptor genes in relation to obesity and hypertension were investigated through: 1) electronic search of PubMed (1990 to December 2006, NCBI interface) limited to human studies published in the English language and 2) searching the references lists of all included articles in order to ensure that all relevant material was obtained.

Chapter 3 - The small GTPase Rho and its downstream effector Rho-kinase are implicated in various cellular functions and in the pathogenesis of hypertension. The authors recently published a series of studies demonstrating that Rho/Rho-kinase in the brainstem is involved in central cardiovascular regulation via the sympathetic nervous system. Rho/Rho-kinase activity in the brainstem is greater in spontaneously hypertensive rats and in another type of hypertensive rat model (caused by chronic nitric oxide synthase inhibition) compared to normotensive Wistar-Kyoto rats. Inhibition of Rho-kinase in the brainstem by

microinjection of Rho-kinase inhibitors (i.e., Y-27632 or hydroxyfasudil) or an adenovirus vector encoding a dominant-negative Rho-kinase decreases arterial pressure, heart rate, and renal sympathetic nerve activity and augments baroreflex function, which might be due to enhanced glutamate sensitivity. The magnitude of the effects of Rho-kinase inhibitions was greater in hypertensive rat model compared to normotensive rat model. In addition, ovariectomy increase hypertension through Rho-kinase activation in the brainstem in female spontaneously hypertensive rats. Finally, the pressor response induced by central angiotensin II is mediated by activation of the Rho/Rho-kinase pathway via angiotensin II type 1 receptors. In this review, the authors describe the authors series of studies and novel pathophysiological implications of Rho/Rho-kinase in the brainstem related to the neural mechanisms of hypertension.

Chapter 4 - In general, it is considered that the sympathetic nervous system plays an important role in the generation of pain. Concerning low back pain, there is growing evidence that sympathetic afferents is key point. Thus, to elucidate the pathomechanisms of discogenic low back pain, sympathetic afferent discharges originating from lumbar disc via L2 root were investigated neurophysiologically. Using 31 Lewis rats, sympathetic afferent units were recorded from L2 root connected with only rami communicante to lumbar sympathetic trunk. The L5/6 disc were mechanically probed, electrically stimulated to evoke action potentials, and finally were applied with the chemicals for inflammation. The authors could not get any units in the L5/6 discs with mechanical stimulation, while identified 42 units belonged to mostly A-delta fibers with electrical stimulation. In some experiments with inflammation, response to mechanical probing to L5/6 disc was recognized. This suggests that mechanical stimulation to lumbar disc may not always produce pain, while inflammatory changes may cause lumbar disc to become sensitive to mechanical stimuli, resulting in nociceptive information is transmitted as discogenic low back pain to spinal cord through lumbar sympathetic trunk.

On the other hand, sympathetic efferents seem to be closely related to lumbar radicular pain. Therefore, to investigate the pathological role of the sympathetic nervous system in lumbar radiculopathy, the left L5 root of Sprague-Dawley rats was tightly ligated proximal to the DRG as a lumbar radiculopathy model. Postoperatively, bilateral DRGs and roots (L4 and L5) were removed, frozen and sectioned. Immunostaining was then performed with antibodies to tyrosine hydroxylase (TH) according to the ABC method. To quantify sympathetic nerve fibers, the authors counted TH-immunoreactive fibers in the DRG using a light microscope with the squares of the micrometer graticule. In the root constriction group, TH-immunoreactive fibers were more abundant in ipsilateral L5 DRGs as well as L4 compared with contralateral DRGs. In the sham and control groups, TH-immunoreactive fibers were scarce in both sides of L4 and L5 DRGs. Constriction of the lumbar root induced more sympathetic nerve sprouting in ipsilateral DRGs than contralateral DRGs. These findings suggest that the sympathetic nervous system may be closely related to a trigger of radicular pain, acting as efferents by sympathetic sprouting into DRGs.

Chapter 5 - Sympathetic neurons contain a different palette of neurotransmitters including classical neutransmitters (catecholamines and acetylcholine), neuropeptides and small molecules such as NO, CO.

The majority of the principal ganglionic sympathetic neurons are noradrenergic and show a positive immunocytochemical reaction to tyrosine hydroxylase (TH), i.e., a key enzyme in catecholamine synthesis. TH appears during embryonic development and the percentage of

TH-positive neurons remains virtually identical during ontogenesis. In sympathetic neurons of most species, noradrenaline is often colocalized with neuropeptide Y (NPY). NPY could also be demonstrated in noradrenergic neurons during embryogenesis. The percentage of neurons containing NPY decreases during early development and increases further after birth with age up to the period of adulthood.

A small number of postganglionic sympathetic neurons contains enzyme of acetylcholine synthesis (ChAT) and some neuropeptides, such as somatostatin (SOM), vasoactive intestinal (poly) peptide (VIP), calcitonin gene-related peptide (CGRP) and galanin (GAL). Acetylcholine-containing sympathetic neurons in most cases colocalize VIP and/or CGRP.

Increased levels of SOM, VIP, CGRP, GAL were reported in sympathetic ganglia during prenatal development. The number of cells containing the above mentioned peptides significantly decreases in prenatal period and after birth onwards. The proportion of VIP- and cholinergic neurons in paravertebral ganglia exhibits additional periods of increasing and decreasing in postanatal development.

In some species (cats, human) NO also acts as a neurotransmitter in the sympathetic ganglia. The proportion of NO-containing cells also changes during postnatal development. In kittens, in the stellate ganglion their number increases during first 30 days of life and decreases later. The content of NO-containing neurons in other ganglia is rather small and doesn’t change during the development.

Some combination of neurotransmitters can be revealed only in non mature animals. For example, combination NPY and SOM, TH and ChAT are not found in adults.

Thus, the expression of the spectrum of neurotransmitters in sympathetic ganglionic neurons of mammals is subject to changes during pre- and postnatal development. Different target-derived factors and multiple target independent intrinsic and extrinsic factors influence the expression of neurotransmitters. The pattern of development depends on the species and the type of ganglion.

Chapter 6 - In this study the authors investigated whether pre-ejection period (PEP), number of nonspecific skin conductance responses (ns.SCRs) and skin conductance level (SCL) quantify sympathetic nervous system (SNS) activity in a comparable way. Physiological data were obtained from 39 human subjects (23 males) with a mean age of 22.0 years (SD = 2.3) during exposure to seven different mental and physical stressors, and during subsequent recovery periods. Compared to pre-test resting baseline recordings significant decreases in PEP and parallel increases in the number of ns.SCRs and the SCL were found for stressors known to increase SNS activity. The between and within subjects correlations between ns.SCRs and SCL were significant and multilevel analysis showed that 43% of the variance in these skin conductance measures overlapped. Between and within subjects correlations between PEP and both skin conductance measures were not significant. This suggests that SNS activity is reflected differently by the heart and the skin. The authors conclude that SNS activity studies, when possible, should include both PEP and skin conductance measurements.

Chapter 7 - The sympathetic nervous system (SNS) plays a key role in the maintenance of homeostasis in different systems of the body. Beside its physiological function such as the regulation of cardiovascular functions, sympathetic efferent fibers can also contribute to immunoregulation. Under pathological conditions, the SNS is involved in the regulation of Th1-mediated autoimmune processes and can influence the course of neuroimmunological diseases, such as multiple sclerosis.

More recently, different structures within the sympathetic nervous system (SNS) have been reported to be targets for autoimmune processes, leading to a severe dysfunction of the SNS. Autoantibodies against beta-adrenoceptors are involved in the pathogenesis of some cases of dilated cardiomyopathy. These autoantibodies have been reported to be agonistic at the beta-adrenoceptor, leading to an overstimulation and, subsequently, to a dilatation of cardiomyocytes. Moreover, autoimmunity against SNS has been reported in autonomic neuropathies in type1 diabetes mellitus or in dysimmune neuropathies.

Complex regional pain syndrome (CRPS, sympathetic reflex dystrophy) is an etiologically unclear syndrome including pain and trophic disturbances after limb trauma or operation. Different studies showed a sympathetic dysfunction in CRPS, both locally and in the central nervous system. Very recently, the finding of autoantibodies against sympathetic nervous system structures has not only provided an explanation for the SNS dysfunction in these patients. It also led to the hypothesis of an autoimmune etiology of CRPS, which may have an important impact on the future treatment of CRPS patients.

Chapter 8 - Introduction: Respiratory nasal mucosa fulfils all functions efficiently for conditioning the inspired air. Physiologic and pathologic mechanisms in nasal mucosa are partially controlled by neural regulation. Beside autonomic neurotransmitters some neuropeptides as well as Nitric oxide (NO) seem to influence glandular secretion and the tone of nasal vasculature. In addition endothelial produced substances like Endothelin and also NO influences the vascular tonus.

This study was performed to identify the different kinds of nerve structures and neuronal transmitters of the sympathetic as well as the parasympathetic and sensory nervous system in human nasal mucosa. The morphologic results should elucidate the mechanisms of nasal swelling and secretion.

Material and methods: Tissue samples of 80 human inferior turbinates were taken during nasal surgery. Serial sections were cut and incubated with antibodies either to neuron-specific enolase (NSE), neurofilament (NF), tyrosine hydroxylase (TH) or to Vasoactive Intestinal Peptide (VIP), Calcitonin Gene-Related Peptide (CGRP), Neuropeptide Y (NPY) and brain Nitric Oxide Synthase (bNOS). In addition, acetylcholinesterase (AChE) and Nicotinamide-Adenine Dinucleotide Phosphate (NADPH)-diaphorase – histochemistry were performed. Finally, all sections were evaluated and documented through bright field microscopy. Additionally, electron microscopic researches were performed.

Results: Nasal vasculature and seromucous glands are controlled by a dense innervation pattern. While all larger arterial vessels show a mixed autonomic innervation, sympathetic nerve fibres seem to predominate in veins. These results were confirmed electron microscopically. Furthermore, immunoreactive nerve fibres were demonstrated around the acini, ducts and in the periglandular connective tissue of glands. A dense network of cholinergic nerve fibres could be detected around the acini. VIP was found in contact to arteries, arterioles, cushion veins as well as acinus cells. NPY is a co-transmitter in sympathetic nerve fibres, acts as a vasoconstrictor and was demonstrated in contact to arterial vessels. The sensory neuropeptide CGRP build a dense nerval network in the subepithelial connective tissue and around arterial vessels and glandular cells. Arteries and capillaries showed a distinctly developed nitric innervation. A high coexistence of NADPH-d in parasympathetic nerves could be detected.

Conclusion: Immunocytochemical, histochemical and electron microscopical methods allow a detailed marking of nerve supply in human nasal mucosa. General innervation could

be shown by using antibodies to neuron-specific enolase and neurofilament. The localization of neurons with different neurotransmitters and neuropeptides in the perivascular and periglandular tissue confirms the direct neural control of the diverse nasal functions. The detection of bNOS-and NADPH-d-positive structures around arteries and glandular cells suggests that NO takes an additional part in the control of nasal functions. The stronger innervation of arteries and cushion veins underlies their central position in the regulation of nasal airway flow.

It could be shown, that beside immunologic mechanisms also the dense network of sensory, sympathetic and parasympathetic nerve fibres act as protection of nasal respiratory mucous membranes from external and internal influences.

Chapter 9 - Currently it is estimated that there are approximately 1.4 million traumatic brain injuries (TBI) a year in the United States. Of the 1.4 million, 235,000 are hospitalized with 40% of in those individuals suffer severe TBI. In severe TBI structural damage occurs to the brain in the form of primary injury related to intraparenchyamal hemorrhages, diffuse axonal injury, contusions, epidural hematomas and subdural hematomas. Secondary injury amplifies the damage to the brain and is related to edema, reduction of cerebral blood flow or anoxia, release of excitatory amino acids, and formation of free radicals.

The physiological changes and clinical presentation varies from individual to individual and is directly related to location of injury and extent of injury. Alterations in level of conscious is the most common clinical presentation of TBI but range from changes in motor activity, speech, cognitive function, heart rate and rhythm, respiratory patterns and cranial nerve function.

In severe brain injury 15-35% of individuals will demonstrate clinical presentation of sympathetic storming, a form of abnormal sympathetic output or control. Presentation includes episodes of hypertension, tachycardia, tachypnea, diaphoresis, posturing, dystonia and hyperthermia. The clinical presentation varies from episode to episode in an individual and from individual to individual. Diagnosis is based on clinical exam and medical management is related to control of symptoms.

This section will integrate current literature while reviewing the proposed neuropathophysiology of sympathetic storming, clinical presentation, differential diagnosis, the adverse effects of sympathetic storming, and medical management.

Chapter 10 - GABA-containing fibers have been observed in the rodent sympathetic trunk. Most of them terminate in the superior cervical ganglion (SCG). Previous studies suggested that these fibers originated from GABA-immunopositive small intensely fluorescent (SIF)-like cells in the cervical sympathetic chain. However, no direct evidence of the origin of GABA-containing fibers had been obtained. The authors recently performed a series of experiments to clarify the origin of these fibers, and showed that some SPNs located in the most rostral part of the intermediolateral nucleus (IML) were GABAergic, and send their axons to the SCG. Although some SIF-like cells showed GABA immunoreactivity, they did not express glutamic acid decarboxylases. They are not likely to produce GABA but accumulate GABA released from adjacent fibers because they often showed close proximity to GABA-immunopositive varicose fibers, and completely disappeared after transection of the ventral roots of C8 to T4 segments. There is no evidence of GABAergic DRG neurons which send axons to the SCG. These observations clearly indicated that the majority of GABAergic fibers in the SCG originated in the most rostral part of the IML. In addition to

these data, the authors discuss the characteristics of GABAergic SPNs and function of GABAergic fibers in the SCG.

Chapter 11 - The sympathetic nervous system is an important modulator of cardiovascular function that contributes to the development and maintenance of cardiovascular disease. The effects of the sympathetic nervous system are mediated via the release of neurotransmitters and neuropeptides from nerve terminals innervating blood vessels and the heart. The mechanisms underlying the development and/or maintenance of cardiovascular sympathetic innervation are not well understood. This article will consider how neurovascular interactions affect vascular sympathetic innervation. In vivo and in vitro models for studying neurovascular interactions will be described. The effects of vascular cells (smooth muscle and endothelial cells) on sympathetic axon growth, axon guidance, target recognition, synapse formation, neurotransmitter/neuropeptide expression and neurotransmitter release will be considered. The mechanisms underlying these effects will also be discussed. Specifically, the role of diffusible mediators (nerve growth factor, artemin, vascular endothelial growth factor) and contact-dependent mediators (ephrins, semaphorins, integrins) will be considered. Results obtained from in vivo and in vitro models will be presented. Future directions and clinical implications will be discussed.

Chapter 12 - [^{3}H]noradrenaline ([^{3}H]NA) release (field stimulation parameters: 2Hz, 1 ms, 60V for 3 min) was measured from the isolated main pulmonary artery of the rabbit in the presence of uptake blockers (cocaine, $3x10^{-5}$M and corticosterone, $5x10^{-5}$M) and after blocking MAO with pargyline ($1.2x10^{-4}$M).

This release of [^{3}H]NA was abolished by TTX (10^{-7}M), even if the charge carrier through the fast Na^+-channel was mainly Li^+ (113 mM; $[Na^+]_o$: 25 mM). The release was also fully inhibited by block of the voltage-sensitive Ca^{2+}-channels (VSCCs) with combined application of the selective and irreversible 'N-type' VSCC-blocker ω-conotoxin-(ω-CgTx) GVIA (10^{-8}M) and the 'non-selective' VSCC-blocker neomycin ($3x10^{-3}$M). Correlation was obtained between the extent of VSCC inhibition and the NA-release potentiating effect of the preferential pre-synaptic α_2-receptor blocker yohimbine ($3x10^{-7}$M). When the release of NA was fully blocked (ω-CgTx GVIA + neomycin), yohimbine was ineffective. Under these conditions, i.e. in the absence of functioning VSCCs, Na^+-loading (Na^+-pump inhibition by 'K^+-free' perfusion; 45 min) was required to elicit NA-release again in response to nerve-stimulation. $0K^+_o$-solution also increased the spontaneous outflow of [^{3}H]NA. The nerve-evoked release of labeled NA in 'K^+-free' solution was abolished by TTX and by removal of Ca^{2+} from the external medium (+ 1mM EGTA). The release of NA was also significantly inhibited by Li^+-substitution of Na^+, or when the preferential reverse Na^+/Ca^{2+}-exchange blocker KB-R7943 ($3x10^{-5}$M) was applied. KB-R7943 also decreased the resting outflow of NA. In Na^+-pump inhibited nerves, and in the absence of functioning VSCCs, yohimbine further enhanced the nerve-evoked release of NA, while agonists of α_2-receptors (l-NA or clonidine, 10^{-6}M) inhibited it. The yohimbine-induced enhancement of neurotransmitter release was blocked by TTX and by Ca^{2+}_o-removal (+ 1mM EGTA). Similarly, Li^+-substitution or KB-R7943-application caused significant inhibition. In $0K^+_o$-solution, the fast Na^+-channel activator veratridine (10^{-5}M) further enhanced both the resting and the nerve-evoked release of NA. The veratridine-induced potentiation of neurotransmitter release was blocked by Ca^{2+}-free, 1 mM EGTA-containing solution and significantly inhibited by KB-

R7943-application. This latter was dependent on the pre-perfusion period with KB-R7943-containing solution, being greater if longer periods were used.

It is concluded, that physiological stimuli may reverse Na^+/Ca^{2+}-exchange when the VSCCs are blocked and when the nerves are Na^+-loaded. Pre-synaptic α_2-receptors may regulate reverse Na^+/Ca^{2+}-exchange as well as VSCCs.

Chapter 13 - Abnormal activity of the sympathetic nervous system is involved in the pathogenesis of protracted pain syndromes. The term sympathetically maintained pain is applied to those neuropathic pain cases that respond to sympatholytic maneuvers.

The sympathetically maintained pain concept has strong and ample foundations in the animal model. After nerve injury, sympathetic sprouting at the dorsal root ganglia becomes apparent and form basket-like structures around large-diameter axotomized sensory neurons; sympathetic stimulation can activate such neurons repetitively.

It has been proposed that this pathogenesis is operative in cases of complex regional pain syndrome type I (formerly known as reflex sympathetic dystrophy). Emerging evidence suggests that the sympathetic nervous system also plays a key role in the development of the complex generalized pain syndrome named fibromyalgia.

Evidence of sympathetic dysfunction in fibromyalgia was gained through the use of novel non-linear methods (heart rate variability analysis).

Chapter 14 - Sympathetic surgery has been performed for over a century. Its indications include a large variety of disorders e.g. angina pectoris, exophthalmic goiter, spastic paralysis, Raynaud's syndrome, reflex sympathetic dystrophy, peripheral occlusive vascular disease etc. However, they are now almost abandoned due to unpredictable and poor surgical outcome. Currently, hyperhidrosis is the most common indications owing to the high successful rate, low recurrence and good patient's satisfaction. The only problem remain unsolved is the troublesome post-operative side effect of compensatory hyperhidrosis, which on occasion causes regret for the procedure. Certainly, the modification of the surgical procedure e.g. ramicotomy, clipping etc. do largely purpose on lowering the rate of the side effect and increase the patient's satisfaction.

During the recent decade, the authors reviewed and analysed over a thousand operated patients and acknowledged that the etiology of the so called "compensatory hyperhidrosis" might be based on the level of ganglion interruption. More than that, the authors proposed a mechanism of compensatory hyperhidrosis and suggested it should be replaced by the term "reflex sweating".

Lin classification for sympathetic surgery is another new idea reported in 2001. With this classification, the authors can perform sympathetic block on a definite ganglion in a specific sympathetic disorder.

Through these several decades, the instruments are getting more and more advanced and refined so that the procedure can be performed in safe and effective condition. Besides, the post-operative pain is reduced and the cosmetic is excellent.

The future goal for sympathetic surgery is to stop the unwanted sweating, inducing no compensatory hyperhidrosis - the idea of "single determinant ganglion" for each sympathetic disorder.

In addition to palmer hyperhidrosis, sympathetic surgery has recently been extended to facial blushing, facial sweating and others, with satisfactory results. More than that, a certain percentage of essential hypertension is due to sympathetic over-activity. The authors treated a small number of such cases using sympathetic blockade. They responded well and the results

were published in 2005. The authors should further follow-up and perform larger studies in this area for the actual etiology and its relation with dopamine.

EXPERT COMMENTARIES

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DEVELOPMENT OF SYMPATHETIC FUNCTION TESTS

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Abstract

The autonomic nervous system innervates every organ in the body. With its thoracolumbar sympathetic and craniosacral parasympathetic pathways, and by means of a complex neural network, it controls numerous vital functions and maintains the internal physiologic homeostasis. Sympathetic and parasympathetic preganglionic and postganglionic function is regulated by specific neurotransmitters (acetylcholine and noradrenaline). Autonomic disease may occur at any age, resulting in hypo-activity or hyper-activity of cardiovascular, vasomotor, sudomotor, gastrointestinal, urinary, sexual and ocular systems.

The investigation of autonomic nervous system is of paramount importance as shown by the establishment of dedicated autonomic laboratories in the recent years.

Non-invasive quantitative autonomic function tests have been developed; they are recognized as a significant, highly specialised, section of clinical neurophysiology and as a fundamental extent of clinical examination and are currently used both in clinical and research settings. The main purposes of autonomic laboratory investigations are to detect normality or abnormality of autonomic function; to assess the degree of autonomic dysfunction and to determine if autonomic dysfunction is primary or secondary; to quantify the extent of autonomic dysfunction for staging, monitoring, and treating; to localize the abnormality to the central or peripheral levels; to confirm whether the sympathetic or parasympathetic or both systems are involved: determining which physiologic organ system such as parasympathetic cardiac (cardiovagal), sympathetic cardiovascular (adrenergic), sympathetic sudomotor (cholinergic) or other systems are involved guides further investigations, prognosis and management strategies [1,2]. Autonomic function tests assist observing the severity and distribution, and monitor the course, of autonomic failure. A battery of tests to assess cardiovagal, sympathetic adrenergic and cholinergic systems is

available [3]; to quote some routinely used, heart rate response to deep breathing and Valsalva ratio to evaluate cardiovagal function; beat-to-beat blood pressure recordings of the Valsalva manoeuvre to assess adrenergic function; sympathetic skin response (SSR) and quantitative sudomotor axon reflex test (QSART) to examine sudomotor cholinergic function.

Autonomic function tests support diagnosis of localised and generalised autonomic diseases or provide information for differential diagnosis of neurological diseases.

In many individual cases of neurological disorders when clinical features might not help identify the disease, like in the early stages of multiple system atrophy (MSA) and Parkinson's disease (PD), autonomic laboratory investigations are of fundamental importance. Abnormal SSR can be found more frequently in patients with MSA (69%) than in idiopathic PD (7.7%), along with a lower R-R interval variability (RRIV) during deep breathing and the Valsalva manoeuvre [4]. These differences in SSR and RRIV between MSA and PD, reflecting a more widespread and severe involvement of the autonomic nervous system in MSA, may facilitate differentiating between the two conditions and support the exclusion of misdiagnosed MSA patients from clinical trials. SSR latency can be significantly longer and amplitude significantly smaller in MSA than in idiopathic PD; similarly, cardiovascular function tests can show significantly greater abnormalities of sympathetic and parasympathetic activity in MSA than PD [5].

A very important role of the sympathetic nervous system is to regulate the function of the skin, which is essential for the constancy of the internal milieu and for its adaptation during perturbations in the environment. The sympathetic postganglionic neurons in the skin innervate blood vessels, eccrine sweat glands and erector pili muscles [6].

Skin blood flow, vasomotor tone and vasomotor responses to various stimulations or manoeuvres can be measured by using laser Doppler flowmetry [7,8]. This method utilises fibre optics to direct a laser light at the skin surface; the red blood cells in the skin capillaries reflect the light back to the recording element of the probe. The Doppler shift effect caused by the movement of the red blood cells relative to the probe alters the wavelength of the reflected light. This altered wavelength allows calculating an arbitrary perfusion rate (perfusion units) rather than the absolute flow, which is displayed as a real time trace. With this technique, vasoconstriction is shown as a reduction in perfusion units relative to a baseline perfusion.

Evaluation of sympathetic vasomotor function by the use of a laser Doppler is not included in the battery of routine autonomic tests and remains investigational; nevertheless, it has been used widely in clinical research works.

Studies investigating sympathetic cutaneous vasomotor function have been performed in patients with autonomic impairment due to different neurological diseases. In progressive supranuclear palsy (PSP) and PD skin vasomotor reflex recordings (from the index finger by using a laser Doppler flowmeter) to stimuli such as deep inspiration, mental arithmetic, bilateral leg elevation, and tactile stimulation of the hand, have suggested that a combination of cardiovascular and skin sympathetic tests my be useful for distinguishing between PSP and PD [9].

Using a similar method, vasoconstriction, manifested as skin vasomotor response (SkVR) to physiological stimuli activating the sympathetic system, has been studied in patients with MSA [8]. Despite its limitation with regards to the measurement of changes in relative blood flow, the laser Doppler technique has been shown to be helpful in the differentiation between MSA from pure autonomic failure (PAF) [10]: SkVRs to inspiratory gasp, mental arithmetic, bilateral leg elevation and cutaneous cold are all decreased in PAF and significantly impaired

compared to healthy control subjects for inspiratory gasp and cutaneous cold and to MSA for inspiratory gasp.

The measurement of cutaneous vasomotor function to physiological stimuli, by using the laser Doppler has also been investigated in patients with complete spinal cord injury (SCI) [11], showing that, compared to healthy controls, SCI patients with high (C6- T5) and low (T6-T11) lesions have similar skin blood flow at baseline in the pulp of the index finger. Differences can be seen in the vasoconstrictor responses to the physiological activating stimuli: SCI patients with high lesions demonstrate significantly diminished SkVRs to inspiratory gasp, mental arithmetic, tactile stimulation of hands, cutaneous cold and deep breathing compared to controls; in contrast, SCI with low lesions show preserved SkVRs following all stimuli. The technique utilized in this study allows non-invasive recording of localised, peripheral blood flow. The findings indicates that sympathetic innervation to the hand is impaired in SCI with high lesions, reflecting an impairment of skin sympathetic adrenergic pathways descending in the cord; these results may correlate with the sympathetic skin cholinergic pathway; nevertheless a discrepancy between adrenergic and cholinergic function may indicate sparing of one but not the other pathway, depending on the lesion.

It is noteworthy that SkVRs are highly localised to the palm and the sole areas [12]: the use of this technique is limited to those areas and does not allow exploitation in other areas, such as the trunk. Although it would not aid the detection of segmental sympathetic innervation or denervation due to its confinement to palms and soles, the technique has a great implication in indicating a certain degree of localised skin sympathetic impairment.

Skin blood flow measurement with laser Doppler flowmetry in patients with diabetic neuropathy has been investigated in a number of studies aimed to demonstrate the usefulness of detecting autonomic neuropathy in patients with diabetes mellitus [13, 14, 15]. There is a suggestion that skin blood flow monitoring at the index finger pulp before and after sympathetic stimulation can be regarded as a reproducible parameter of sympathetic vasomotor control [12]. Measurement of vasoconstrictor responses to deep inspiration in the foot facilitates detecting peripheral sympathetic failure in patients with non-insulin-dependent diabetes mellitus [14].

The above-mentioned laser Doppler procedures to study vasoconstriction offer a means towards better understanding of sympathetic involvement at various stages in neurological diseases. A relatively less explored technique, which uses laser Doppler flowmetry, is cutaneous axon-reflex vasodilatation.

Various skin stimuli, such as mechanical, thermal, electrical and chemical, can evoke a sensory cutaneous axon-reflex vasodilatation, which is part of the “Lewis triple response”: immediate reddening, flare (spreading area of redness) and wheal (local oedema) [16]. Sensory cutaneous axon-reflex vasodilatation following intradermal injection of histamine has been used in clinical practice. In brachial plexus injuries, preganglionic (proximal to the dorsal root ganglion) lesions have been considered to be associated with a poor prognosis when showing a normal response to the histamine test, consisting of cutaneous vasodilatation with presence of wheal and flare; whilst postganglionic lesions have been thought to carry a better prognosis after nerve repair when showing an abnormal response to the histamine test, consisting of absent flare [17].

Axon reflex vasodilatation following intradermal injection of histamine has been investigated in trunk dermatomes above and below the level of lesion of patients with chronic spinal cord injury (SCI) observing a diminished vasodilatation in dermatomes below the level

of lesion [18]. A non-invasive method to study axon- reflex vasodilatation in patients with chronic SCI has been developed: heat-provoked skin vasodilatation recorded by laser Doppler flowmetry using thermostatic laser Doppler probes [19,20]. These probes permit recording of skin blood flow while heating the underlying skin surface. Two independent mechanisms mediate vasodilatation occurring during local heating of nonglabrous skin [21]: an initial vasodilatation, which is a neurogenic phase mediated by axon-reflexes with its peak occurring in a few minutes followed by a brief nadir; a secondary vasodilatation phase, mediated by local production of endothelial nitric oxide, which reaches its plateau in about 30 minutes [21]. The sympathetic nerves modulate the neurogenic vasodilatation phase, which is initiated by the activity of sensory nerves [22].

Using local heating of the skin, the neurogenic mediated axon-reflex phase of vasodilatation has been observed to be significantly diminished on the foot, below the level of lesion, in complete SCI [19]. Using a physiological stimulus, this finding is consistent with previous reports on histamine induced axon-reflex vasodilatation in SCI [23, 18]. A significant difference in the skin vasodilator response to local skin heating in trunk dermatomes below the level of lesion in complete SCI with high lesions (C6-T5) as compared to SCI with low lesions (T6-T11) has also been demonstrated [20]. Sympathetic vasomotor function in innervated and denervated areas of the trunk in complete SCI can be evaluated using heat-provoked vasodilatation; this method can be used to aid classification of the sympathetic component of a spinal cord injury and can be proposed as a useful means to detect changes in spinal autonomic function after therapeutic intervention in spinal cord injury.

Development of sympathetic function tests such as vasoconstriction and vasodilatation recording by laser Doppler should be put forward to investigating the autonomic involvement of different neurological diseases. Laser Doppler techniques being non-invasive and very informative merit further development to promote and establish their application and relevance in clinical and scientific settings.

An important area to be explored is the potential for the skin vasomotor function testing to be used as a non-invasive and safe clinical drug trial outcome measure.

Established autonomic function tests have already been used as outcome measure in clinical trials: cardiovascular assessments, including recording responses to orthostatic test, deep breathing, Valsalva manoeuvre, hyperventilation, handgrip, cold pressor test with sphygmomanometer or an automated device (Dinamap, Finapres or tonometer); beat to beat heart rate variation at rest and during deep breathing; lower body negative pressure; sudomotor function assessments like the SSR and QSART; pupillometry (ClinicalTrials.gov). There is uncertainty on whether vasoconstrictor and vasodilatation measurements can be included as quantitative tools to measure clinical trial outcomes. However, a double- blind placebo-controlled clinical trial of recombinant human brain-derived neurotrophic factor (rhBDNF) in patients with insulin-treated diabetes mellitus with diabetic polyneuropathy used axon reflex vasodilatation measured with a laser Doppler devise after intradermal injection of capsaicin into the right lateral calf [24].

Techniques to study vasomotor function as an index of sympathetic system activity in different neurological diseases merit further development. The advantage of the laser Doppler techniques, being non-invasive and quantifiable, deserves consideration for being introduced as an outcome measure in clinical trials.

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INFLUENCE OF ANDROGENIC STEROIDS ON CARDIOVASCULAR FUNCTION

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Introduction

Epidemiological research has consistently demonstrated that men are much more affected by cardiovascular diseases than women [1]. Among the factors determining this difference emerges the proatherogenic effect of androgens; however, the reasons why cardiovascular diseases are more prevalent [2] and severe in adult men than in premenopausal women are not completely clear (**Table 1)**.

Table 1. Coronary heart disease (CHD) mortality. Differences between male and female.

Age	Sex ratio of CDH mortality
(Years range)	(male: female)
35-44	6.8
45-54	5.3
55-64	3.3
65-74	2.3
> 75	1.6

For a long time it was reported that estrogen replacement protected premenopausal women against cardiovascular diseases, but the Women`s Health Initiative Randomized Controlled Trial has challenged former studies because a higher risk of cardiovascular disease has been observed with regular use of estrogen replacement therapy [3] in postmenopausal women.

Influence of Sexual Hormones on Plasma Lipids

During infancy, heart size is about the same in both boys and girls. This pattern changes in adolescence, the male heart increasing in size, most probably under the influence of testosterone. Prepuberal testosterone plasma concentration is 20-40 ng/dL in boys and girls, rising in adult men to 300 – 1000 ng/dL, these values being sustained by a daily testosterone synthesis of 2.5 – 11.0 mg/day.

Before puberty, male and female lipoprotein levels do not differ significantly; however, when sexual maturity is reached, male subjects show a decrease of HDL-C and an increase of LDL-C and triglycerides [4], these substances remaining thus throughout life, and being most probably related to the surge of plasma testosterone.

Obesity introduces metabolic and cardiovascular disturbances in men; when men suffer visceral obesity, a negative correlation is found between plasma testosterone concentration and lipid levels of triglycerides, total cholesterol and LDL-C [5].

Role of Androgens in Atherosclerosis

Atherosclerosis manifestations appear in men 10 years earlier than in women, and this difference has been explained by the anti-atherogenic effect of female sex hormones, in particular estradiol. This fact in turn has been associated in women to their higher levels (as compared to men) of HDL-C and lower concentrations of LDL-C, lipoprotein (a) (Lp_a) [6], fibrinogen and homocysteine levels.

In several male cohort studies, other researchers have found no correlation between coronary events and testosterone blood levels [7, 8] and an absence of correlation with the weak androgenic compound dehydroepiandrosterone sulphate (DHEAS) plasma concentration [9]; this is probably one of the reasons why this substance was no longer promoted as a cardiovascular protector. These facts point to the idea that coronary atherothrombosis is not linked to androgenic plasma levels.

In controlled clinical trials, other researchers have found that male patients with coronary heart disease treated with testosterone showed less severe and frequent angina pectoris painful crises and fewer events of exercise-induced ST-segment depression [9, 10]. These findings mean androgenic treatment offers some degree of cardiovascular protection

However, it is worth mentioning that when androgenic steroids are self-administered at very high doses, as it happens in young athletes, myocardial infarction is observed early in them [11], and this cardiovascular event may be related to atherothrombotic lipid changes promoted by such unphysiological doses of androgenic substances. These changes include a decrease of plasma HDL-C, particularly of subfractions HDL-2 and HDL-3, as it

has been observed in healthy men receiving 200 mg testosterone per week for three months [12, 29].

Effects of Testosterone on Animal Models of Atherosclerosis

Rabbits fed with a cholesterol-enriched diet and treated with testosterone showed no change in their aortic cholesterol content [13]. These experiments demonstrate that pharmacological doses of testosterone do not accelerate atherogenesis when administered on a short-term basis, but this cannot be compared to using it over an extended period of time, as seen in some athletes or in hypogonadal men.

Similar findings were observed when rabbits were treated with the androgenic steroid stanozolol [14].

In a different kind of experiments, female monkeys [15] and rabbits [16] received a long-term testosterone treatment that induced atheromatous lesions in their coronary arteries and aorta.

Thrombogenic Effects of Testosterone

In a double-blind, placebo-controlled trial carried out in healthy men, 200 mg of testosterone administered twice a week, for two weeks, increased thromboxane A_2 receptor density in platelets and their aggregability [17]. This experiment indicates a thrombogenic effect of testosterone when administered in high doses, as employed by physical culturist athletes. This prothrombotic action of testosterone makes blood more vulnerable to clot-forming in the presence of already formed atheromatous arterial lesions. It remains to be seen if much lower doses do not promote this effect.

Apparently, more physiological testoterone concentrations can be achieved for hypogonadism treatment with new galenic formulations such as transdermal patches, invisible gels, or mucoadhesive bucal testosterone sustained release tablets [30].

Correlation between Testosterone Plasma Levels and Some Components of the Metabolic Syndrome

In a clinical study conducted in 50 hypoandrogenic men who had higher than normal body mass index (BMI); waist-hip ratio; systolic blood pressure; insulin plasma levels; triglycerides; and LDL-C and apo-B, associated to lower HDL-C and apoA-I concentrations, researchers found a negative correlation between plasma testosterone and insulin and triglyceride levels.

Many of the aforementioned changes form part of the metabolic syndrome. In this study, the negative correlation persisted between testosterone and both insulin and triglyceride plasma levels when the findings were adjusted for BMI and waist-hip ratio [18]. It is remarkable that abdominal obesity, hypertension, hypertrigliceridemia, hyperinsulinemia and low HDL-C levels negatively correlate with lower androgenic steroid plasma levels. In a more recent study, mortality risk increased in men older than 40 years, when their serum total

testosterone was lower than 250 ng/dL [19]. All these findings indicate that testosterone exerts some kind of cardiovascular protecting action.

Hyperandrogenism in Cases of Polycystic Ovary Syndrome

Androgenic hormone excess is observed in premenopausal women with polycystic ovary syndrome; however, clinical findings in this disease are rather different compared to exogenous administration of androgenic steroids because polycystic ovary syndrome is accompanied by the presence of high estradiol synthesis through peripheral aromatase activity.

These women show virilization, obesity, insulin resistance, low HDL-C and higher triglycerides and LDL-C levels [20], but do not show hypertension. Another difference of this hyperandrogenic condition is that these patients have increased plasma levels of endorphin and inhibin [21], complicating the pathophysiology of this disease.

Effect of Androgenic Steroids on Blood Pressure

Blood pressure increases upon long-term testosterone [21], methylandrostendiol [22], or nandrolone [23] administration. Several mechanisms are involved in the genesis of hypertension observed in rodents receiving androgenic steroids. The following ones have been documented: increased vasopressor action of noradrenaline [24]; a less effective prejunctional α_2 adrenergic negative feedback mechanism, a process that is modulated by testosterone levels, the deficit of which contributes to higher noradrenaline release and stimulation of α_1 postsynaptic arterial receptors; and endothelial dysfunction involving decreased tonic release of nitric oxide from arteries as it was demonstrated in isolated rabbit aorta [25].

Some other mechanisms of hypertension production by androgenic steroids include plasma renin activity increase, which leads to more angiotensin II synthesis [26] and reduced hepatic aldosterone clearance [27]; both mechanisms contribute to increase kidney sodium reabsorption and extracelular volumen expansion; and finally, as it was recently discovered in our laboratory, testosterone and nandrolone administered to male rats increase sodium sensitivity of the brain anteroventral third ventricle area which increases sympathetic tone in the cardiovascular system [23] **(Figure 1)**

Testosterone administration does not increase plasma endothelin-1, a potent vasoconstrictor peptide, when administered to males with hypogonadism. Actually, endothelin-1 slightly (no significant change) decreased from 0.99 ± 22 to 0.76 ± 0.25 fmol/mL of plasma after a 6-month treatment [28].

It is worth mentioning that in spontaneously hypertensive rats, blood pressure decreases after gonadectomy and increases significantly upon dihydrotestosterone administration [29], revealing that an androgenic mechanism underlies this hypertensive condition. However, many other well known mechanisms operate to increase blood pressure in this rat strain.

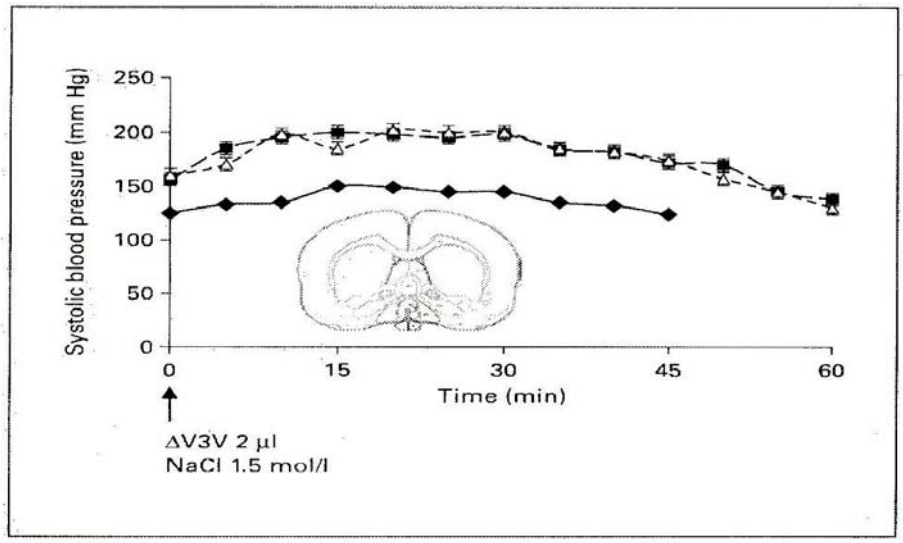

Figure 1. Change of systolic blood pressure after 2 µl microinjection of hypertonic 1.5 mol/l NaCl into ΔV3V in control (♦), testosterone-treated (■) and nandrolone-treated (Δ) rats. Symbols represent the mean and SEM of 10 experiments per group. ** $p<0.01$ vs. control. Inserted figure of brain cut indicates (▼) site of micro-injection.

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IRRATIONAL UTILIZATION OF THE SYMPATHETIC BLOCKERS IN THE THERAPY OF HYPERTENSION IN OBESE SUBJECTS

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The autonomic nervous system controls the metabolic balance, so that the vegetative influences affect the storage and the consumption of energy [12, 13]. Indeed, an administration of a sympathetic blocker reduces the energy expenditure in experimental models of sympathetic activation [6].

Food intake is accompanied by an increase in the sympathetic discharge [4], so that an activation of the sympathetic nervous system is included among factors influencing the satiety [14].

On the other hand, an increment of body weight causes an increase of the sympathetic discharge. This increase is mentioned among factors inducing the hypertension in obese subjects [16].

The hypertension and the obesity are largely diffused in the industrialized world, so that the therapeutic strategies of these pathologies (which are frequently associated) assume social relevance.

Since food intake activates the sympathetic nervous system, the hypertension associated with the obesity is due to an elevated sympathetic discharge, which is correlated to an excessive food consumption [1].

The increase in sympathetic activity in overfed subjects could be a compensatory mechanism that tries to limit excessive increment of body weight. A reduction of this compensatory elevated discharge can induce further growth of body weight.

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The sympathetic blockers, like the diuretics, are pharmacological substances diffusely utilized in the therapy of hypertension. Although some authors have recently criticized the use of sympathetic blockers as first-line therapy for hypertension [2, 11], these blockers are considered as drugs of primary choice.

The utilization of sympathetic blockers may interfere with the above described compensatory mechanism of energy expenditure and the efficiency of dietetic therapy may be significantly reduced. On the other hand, a possible increment of body weight, as side effect, in lean subjects should be considered, when the sympathetic blockers are utilized in the therapy of hypertension in patients with normal body weight.

Furthermore, patho-physiological mechanisms of the obesity involve alterations of the sympathetic nervous system in accordance with the "Mona Lisa Hypothesis" (an acronym for "most obesities known are low in sympathetic activity") [3]. The use of sympathetic blockers reduces the sympathetic control on energy expenditure with a further exacerbation of the obesity.

Since a high percentage of hypertensive obese patients benefit from a reduction of body weight, the dietetic therapy is a principal therapeutic tool in the management of hypertension in obese subjects.

The importance of body weight reduction in the therapy of hypertension should address the choice of drugs, which should not interfere with the sympathetic activity.

Body weight is a balance between food intake and energy consumption. An increase in food intake and/or a decrease in energy expenditure leads to an increment of body weight. The blockers of the sympathetic activity exert not only influences on energy expenditure, but also on food intake.

An increase of the sympathetic discharge after food intake causes an elevation of body temperature. This rise limits food intake, in accord to the "thermostatic hypothesis" of food intake [15]. The use of the sympathetic blockers can reduce the satiety, inducing to a larger intake of food.

On the other hand, the "thermostatic hypothesis" integrates other hypotheses on the control of food intake [8]. Indeed, blood level of glucose ("glucostatic hypothesis") influences the sympathetic activity with associated modification of body temperature and food intake [10]. Leptin, a fundamental peptide of the "lipostatic hypothesis", reduces food intake with an increase in sympathetic discharge and body temperature [5, 7]. Hormones secreted by stomach and intestine ("gastrointestinal hypothesis") affect the sympathetic discharge and body temperature [9]. Thus, the effects of the sympathetic blockers on the above described mechanism should be considered.

In conclusion, this comment emphasizes the irrational utilization of the sympathetic blockers in the therapy of hypertension, especially in obese subjects and invite to a reflection on more opportune pharmacological choice in the hypertension management.

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SHORT COMMUNICATION

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THE CONTROL OF BODY WEIGHT AND PHYSIOPATHOLOGY OF OBESITY: THE ROLE OF THE SYMPATHETIC NERVOUS SYSTEM

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Abstract

The control of body weight is exerted by numerous factors, including the influences of the autonomic nervous system. The aim of this paper is to emphasize the role played by the sympathetic activity in the regulation of body weight that is a result of food intake and energy expenditure. The sympathetic nervous system is able to influence both eating behavior and energy consumption, and it is a key factor in the control of physiological or pathological body weight. The sympathetic branch of the vegetative nervous system is retained a fundamental factor in all models of the control of food intake, especially in the "thermoregulatory hypothesis". Alterations of the sympathetic tone and, in general, of the vegetative asset are retained the cause of obesity. On the other hand, being overweight increases the sympathetic discharge that contributes to induce pathologies related to abnormal body weight. In a strategy to reduce the incidence of obesity in the industrialized world, the role of the sympathetic nervous system should be emphasized to address the research in the investigation of the influence of the autonomic nervous system in the control of body weight.

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Introduction

Since the incidence of body weight superior to normal values is increasing in the industrialized word, the World Health Organization considers obesity as a pandemia in rich populations. Investigation into the mechanisms that control body weight give growing relevance to the possibilities of new strategies to reduce the incidences of overweight and obesity, which are frequently associated with metabolic and cardiovascular diseases.

Body weight is a result of a balance between food intake and the consumption of energy. Systems that control food intake and/or energy expenditure are able to influence body weight. Several substances are able to influence food intake. The "glucostatic hypothesis" emphasizes the role of blood glucose, considering that an increase in glucose blood level induces a reduction of food intake. Leptin, a peptide secreted by white adipose tissue, acts on the hypothalamic areas inducing a reduction of food ingestion, supporting the "lipostatic hypothesis" of food intake. Gastro-intestinal hormones also lower food intake; this influence is known as the "hypothesis of gastro-intestinal control of food intake". The autonomic nervous system is involved in the control of eating behavior through influences exerted on the production and loss of heat. Thus, the control of body temperature is strictly associated with the control of body weight; this is in accord with the "thermoregulatory hypothesis" of food intake. On the other hand, the metabolic balance is controlled by the autonomic nervous system, so that the vegetative influences affect the storage and the consumption of energy.

The aim of this article is to report our evidences which demonstrate that modifications in the sympathetic discharge affect thermogenesis and food intake, so that body weight can be affected by changes in the sympathetic activity.

Experimental Demonstrations

First Demonstration

The food intake, firing rate (FR) of the sympathetic nerves to interscapular brown adipose tissue (IBAT), IBAT and colonic temperatures (T_{IBAT} and T_C), were monitored in 24h-fasting male Sprague-Dawley rats for 90 min after food presentation. Prostaglandin E_1 (500 ng) or saline was injected into the lateral cerebral ventricle immediately before food presentation. Two other groups of control animals were tested with the same procedure of intracerebroventricular injection, but food was not presented. The results (see figure 1) showed that food intake was significantly lower in the group receiving prostaglandin E1 before food presentation in comparison to the group receiving saline. FR, T_{IBAT} and T_C were significantly higher in the rats receiving prostaglandin E_1 with respect to rats receiving saline.

These findings illustrate direct evidence indicating that an increase in the sympathetic discharge due to prostaglandin E1 is related to a decrease in food intake [18]. The simultaneous measurement of the sympathetic firing rate and food intake is the nicest demonstration of the feed-back between the sympathetic nervous system and food intake. It is clear that the sympathetic activity rises before food intake terminates. This implies that the rise in sympathetic discharge also serves as endogenous satiety signal.

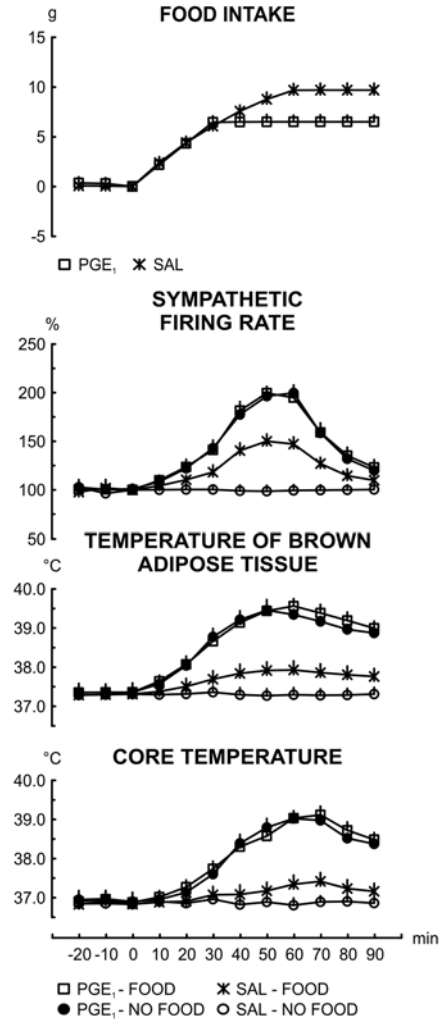

Figure 1. Food intake, sympathetic discharge, temperature of brown adipose tissue and core temperature in rats receiving prostaglandin E_1 (PGE_1) or saline (sal) with presentation (FOOD) or not presentation (NO FOOD) of food at time 0. PGE_1 or saline was injected into the lateral cerebral ventricle at time -3min.

Other substances with primary hyperphagic effect, as neuropeptide Y or galanin, induce a reduction of the sympathetic discharge and a decrease in body temperature [2, 7, 20, 22]. Conversely, substances with a primary hypophagic effect cause an increase in the sympathetic activity. For example, leptin induces reduction of food intake [21, 24], along with an increase in the firing rate of the sympathetic nerves to IBAT and a rise in T_{IBAT} [9, 11].

Second Demonstration

The effect of intraperitoneal injection of lysine acetylsalicylate was tested on 1) food intake and 2) the sympathetic and thermogenic changes induced by lesion of the lateral

hypothalamus. Food intake, FR of the nerves innervating IBAT, and T_{IBAT} and T_C were monitored in male Sprague-Dawley rats lesioned in the lateral hypothalamus. These variables were measured before and after an intraperitoneal injection of lysine acetylsalicylate. The same variables were also monitored in 1) lesioned rats with intraperitoneal administration of saline, 2) sham-lesioned animals with intraperitoneal injection of lysine acetylsalicylate, and 3) sham-lesioned rats with intraperitoneal injection of saline. The results (see figure 2) show that lysine acetylsalicylate modifies the aphagia by increasing food intake and also reduces the enhancements in FR, T_{IBAT}, and T_C induced by the lateral hypothalamic lesion.

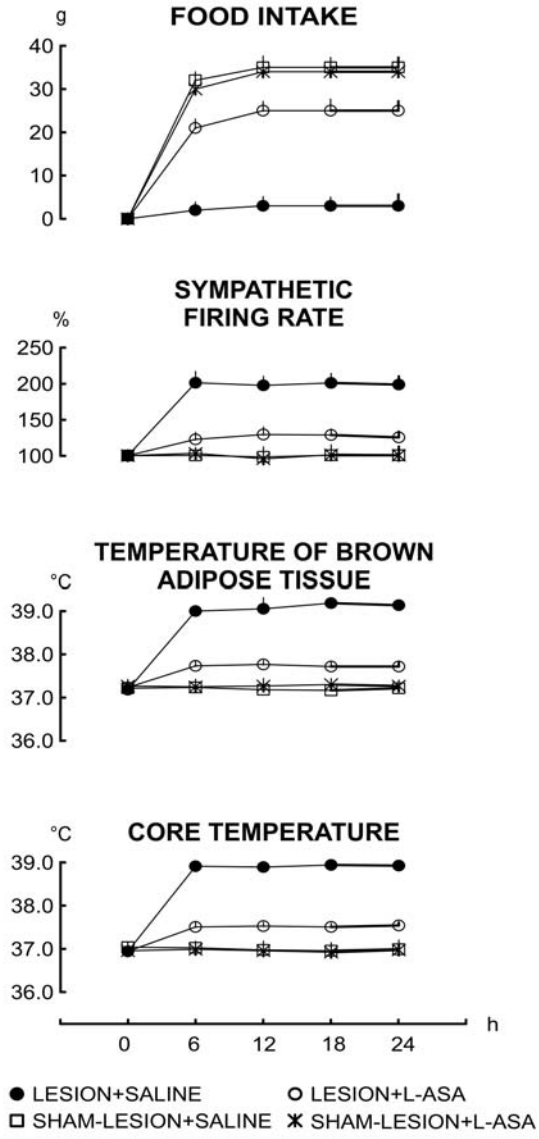

Figure 2. Food intake, sympathetic discharge, temperature of brown adipose tissue and core temperature in sham- or lesioned rats receiving lysine acetilsalicylate (L-ASA).

The findings of this experiment demonstrate that the inhibition of prostaglandin synthesis with reduction of temperature is able: 1) to reduce the sympathetic discharge of nerves to IBAT, and 2) to modify the aphagia in lesioned rats [14]. The electrolytic lesion in the lateral hypothalamus regulates body weight at a lower level. The lesioned rats lose body weight at a faster rate than sham-lesioned controls subjected to the same degree of food deprivation [10]. The sympathetic activation and rise in body temperature are specific for a lesion of the lateral hypothalamus [25]. It has been accepted that the mechanism for increasing heat production following lateral hypothalamic lesion includes activation of non-shivering thermogenesis due to IBAT [12]. The significant role of IBAT in the hyperthermia induced by this lesion is confirmed by these findings. Throughout our experiment, we report direct evidence of increased sympathetic tone in nerves innervating IBAT after lateral hypothalamic lesion, in agreement with our previous findings [13] and those of other authors [1]. These experiments confirm that an increase in the sympathetic activity reduces food intake, in accord with the findings of Bray [3]. On the other hand, our data show that an inhibitor of prostaglandin synthesis can modify the aphagia induced by the lesion of the lateral hypothalamus, throughout a reduction of the sympathetic discharge and body temperature.

Third Demonstration

This experiment evaluated the effects of ventromedial hypothalamus lesions on the thermogenic changes that follow food intake. Two groups of six Sprague-Dawley male rats were used. Under anesthesia with pentobarbital, the animals of the first group received lesions at the ventromedial hypothalamus, and animals of the second group received sham lesions. Body weight and food intake were monitored daily until the experimental procedure began. Twenty days after lesion, oxygen consumption, firing rate of sympathetic nerves to IBAT, and IBAT temperature were monitored for 45 min both before and after 5 g food intake in 24 h fasted rats of both groups. The results (see upper panel of the figure 3) showed that the lesion produced hyperphagia. FR of nerves to IBAT, IBAT temperature, and oxygen consumption (see other panels of the figure 3) increased after food intake in sham-lesioned rats. This increase was significantly reduced in the lesioned rats.

These findings indicate that the sympathetic nervous system is involved in the control of postingestional thermogenesis and reduction of this thermogenesis occurs when the ventromedial hypothalamus is lesioned [17]. A long-term reduction of postingestional thermogenesis may contribute to the obesity induced by this hypothalamic lesion through a decrease in energy expenditure induced by reduced sympathetic activity. Since there is a close relationship between the sympathetic activity and food intake [3], a reduction in the sympathetic response after food intake could induce an increase of total amount of ingested food. This reduction may be another factor in the induction of obesity due to ventromedial hypothalamic lesions. In other words, the increased body weight may be caused by a reduction of satiety signals and a decrease in postingestional energy expenditure.

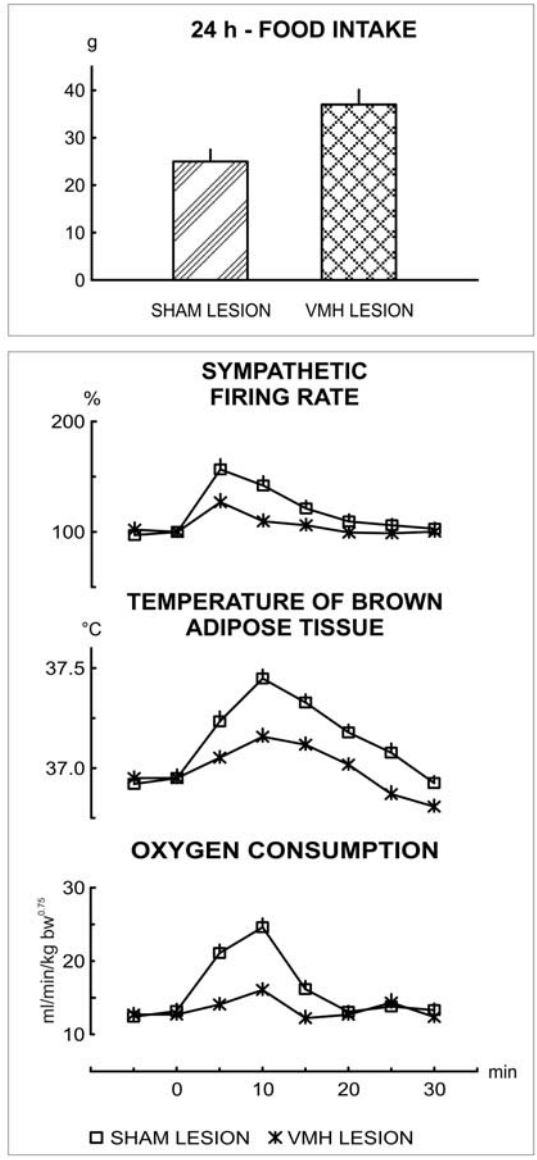

Figure 3. Food intake of sham- and lesioned animals (upper panel). In other panels, sympathetic discharge, temperature of brown adipose tissue and oxygen consumption in sham- and lesioned rats receiving food at time 0.

Fourth Demonstration

The food intake, FR of the sympathetic nerves to IBAT, T_{IBAT} and T_C were monitored in 24h-fasting male Sprague-Dawley rats for 15 h after food presentation. Orexin A (1.5 nmol) was injected into the lateral cerebral ventricle 6 h before food presentation while FR, T_{IBAT} and T_C were also monitored. The same variables were controlled in rats receiving orexin A at the same time of food presentation. Two other groups of control animals were tested with the same procedure, but orexin A was substituted by saline. The results (see figure 4) showed that food

intake was significantly lower in the group receiving orexin A 6 h before food presentation in comparison to all the other groups. FR, T_{IBAT} and T_C were significantly higher in the rats receiving orexin A with respect to rats receiving saline.

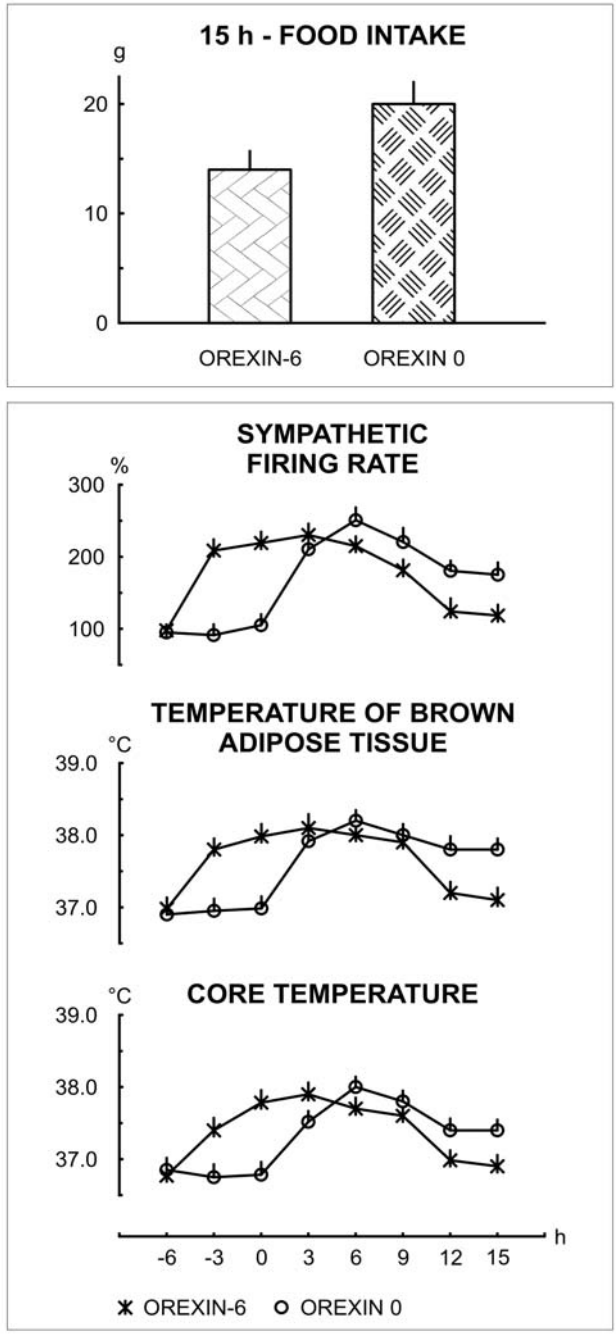

Figure 4. Food intake, sympathetic discharge, temperature of brown adipose tissue and core temperature in rats receiving orexin 6 hours before (OREXIN-6) or at same time (OREXIN 0) of food presentation (time 0).

This experiment demonstrates that an increase in the sympathetic discharge and body temperature due to orexin A reduces food intake, when the presentation of food is delayed [19]. This indicates that the sympathetic activity is involved in the control of hunger or satiety signals.

Fifth Demonstration

This study analyzed the vegetative modulation, expressed as heart rate variability (HRV) power spectral analysis, in lean and obese women at pre-menopausal or post-menopausal age to reveal possible differences in menopause-related autonomic activity in lean and obese subjects. Sedentary women (n=40) were divided in four groups: pre-menopausal lean and

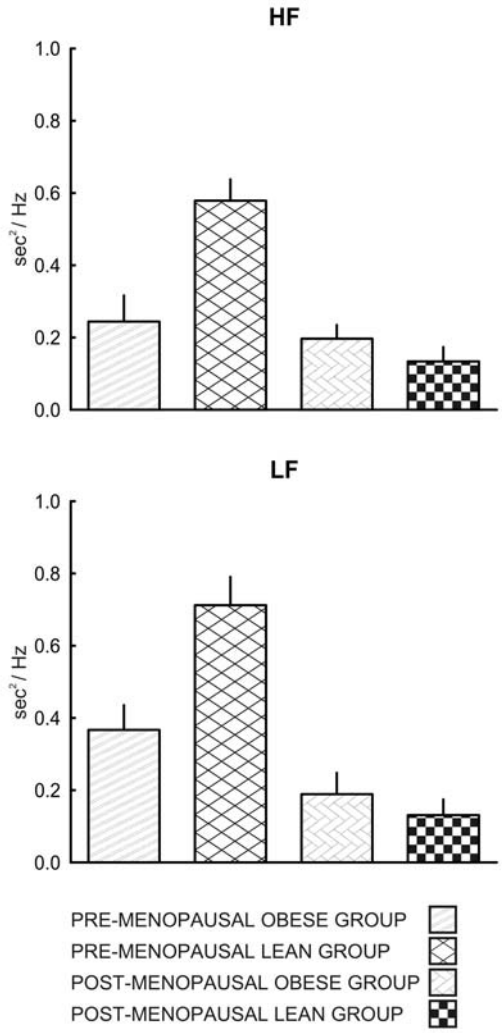

Figure 5. High-frequency (HF) and low-frequency (LF) power in pre- and post-menopausal lean or obese women.

obese women, post-menopausal lean and obese subjects. The HRV-power spectrum was evaluated on a 5-min long ECG recording. The absolute values of the spectrum were summed in the following frequencies: a low frequency (0.04-0.15 Hz; LF), and high frequency (0.15-0.40; HF) range. LF and HF were values used to estimate the sympathetic and parasympathetic activity. LF and HF values of pre-menopausal obese women were lower than values of lean women. The menopause induced a same decrease in LF and HF values in lean and obese subjects, so that no difference was found in post-menopausal groups (see figure 5).

The present experiment indicates a reduction of the vegetative modulation in obese young women and the reduction of the autonomic control regards both the sympathetic and parasympathetic components [16]. The reduction of the sympathetic branch could be an important factor in the maintenance of obesity in pre-menopausal age. Indeed, a reduction in the sympathetic activity could be related to a low energy expenditure, so that a reduced energetic cost could explain the higher body weight in pre-menopausal women. This vision is in accordance with the "Mona Lisa Hypothesis", an acronym for "most obesities known are low in sympathetic activity" [4].

In this experiment, the autonomic activity of post-menopausal women is lower than that of pre-menopausal subjects. The same reduction of the vegetative control was found in both post-menopausal groups, so that there was no difference between lean and obese women. This indicates that the modifications of the autonomic modulation cannot be included among factors related to obesity in post-menopausal subjects.

The age-related decline in the vegetative control has been considered an important factor in the reduction of resting energy expenditure in the older women. Indeed, suppression of sex hormones to post-menopausal levels reduces resting energy expenditure in young healthy women, through a reduction of autonomic nervous activity [6]. This result is also useful in the interpretation of the relationship between the sympathetic nervous system and food intake in young and older subjects. It has demonstrated a significant influence of sympathetic activity on eating behavior [5], also through an increase in thermogenesis [15, 19]. Many experimental evidence has demonstrated that an increase in sympathetic and thermogenic activity reduces food intake. Therefore, the obesity can be due to an increase in food intake associated to a reduced activity of the sympathetic nervous system and thermogenesis.

Although food intake was not measured, the results of the present experiment are consistent with the hypothesis that a reduction in autonomic activity could play a determinant role in the increase in food intake and in the induction or maintenance of obesity of pre-menopausal women.

Discussion

The above-reported demonstrations indicate that the sympathetic nervous system can be considered a fundamental factor in the regulation of food intake and body weight. The sympathetic influence on this regulation is exerted by an influence on body temperature; this is in accord with the "thermoregulatory hypothesis" of food intake. The consequences of this hypothesis is that subjects with a high set-point of body temperature and/or low sympathetic activity are induced to eat a high quantity of food to elevate the sympathetic discharge and body temperature. Conversely, subjects with a low thermal set-point and/or a high sympathetic tone need to introduce a lower quantity of food to reach a pre-fixed thermal set-

point. On the other hand, alterations of post-prandial thermogenesis due to a reduced response of sympathetic activation can play an important role in inducing obesity. In the other words, subjects with a low post-prandial sympathetic activation need to introduce a higher quantity of foot to reach a prefixed body temperature. Our findings support the "Mona Lisa hypothesis" [4], which suggests that most types of obesity are due to an alteration of the sympathetic activity.

On the other hand, being overweight increases the sympathetic discharge that contributes to induce pathologies related to abnormal body weight [8, 23]. Researches should be addressed to reveal further aspects of the control exerted by the vegetative nervous system on body weight, so that additive approaches could be used for the prevention and therapy of obesity.

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RESEARCH AND REVIEW STUDIES

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Chapter 1

EFFECTS OF STATIC MAGNETIC FIELDS ON SYMPATHETIC NERVOUS SYSTEM IN ANIMALS AND HUMANS

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Abstract

Life on earth has evolved in the presence of natural and ubiquitous magnetic fields. So it is not surprising that biological systems can respond to a wide range of static magnetic fields (SMF): weak-intensity SMF (microtesla level), including the geomagnetic field, moderate-intensity SMF (millitesla level), and strong-intensity SMF (tesla level). It has been estimated that some of these physiological responses seem to be mediated through the nervous system. For weak-intensity SMF effects, a neuroanatomical, electrophysiological and behavioral analysis of magnetoreception or magnetic compass orientation revealed that some neurons in the central nervous system and sympathetic nervous system have been activated by magnetic stimuli in some vertebrates and invertebrates. In several experimental studies on the SMF effects, pharmacological procedures and experimental animals have been used to assess the sympathetic responsiveness to SMF. It has been reported that continuous exposure to moderate-intensity SMF for several weeks can depress or suppress the action of sympathetic agonists (norepinephrine, phenylephrine, and dobutamine) and a sympathetic antagonist (reserpine) on hemodynamics, blood pressure and/or behavioral activity by modulating sympathetic nerve activity or baroreflex sensitivity in animals. There is growing evidence to suggest that SMF of various intensities could alter the autonomic function in humans. Thus, this review describes the reported SMF effects (and non-effects) on the sympathetic nervous system of animals and humans.

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Introduction

All living organisms have been continuously exposed to natural magnetic fields. So it seems likely that the exposure can cause biological effects, although the precise effects provoked are not well known. It has been argued that the controversial or inconsistent effects of static magnetic fields (SMF) reported so far are primarily due to the diversified responses of living organisms as well as SMF of various intensity ranges: weak-intensity SMF (microtesla [μT] level), including the geomagnetic field (GMF; typically around 50 μT; ranging 20–90 μT), moderate-intensity SMF (millitesla [mT] level), and strong-intensity SMF (tesla [T] level). The nervous system is considered to be one of the systems most sensitive to various magnetic fields, including SMF and electromagnetic fields (EMF) [1], and it has been estimated that some of these responses seem to be mediated through the central nervous system (CNS) and sympathetic nervous system (SNS). With particular reference to mT level, this is a field strength that would benefit from increased investigation because SMF therapy could be useful for circulatory diseases, including ischemic pain and hypertension, primarily due to the modulation of blood flow or blood pressure (BP), partly through the nervous system.

However, there might be safety concerns on magnetic resonance imaging (MRI) for diagnostic imaging and magnetic levitation for transportation using T level SMF. Therefore, knowledge of SMF effects on the SNS is extremely important considering human health, and the relationship between the SNS-related diseases (e.g., hypertension) and stress response (e.g., oxidative stress). Several attempts have been made to explore the parameters of the SNS when animals and humans have been exposed to SMF. In this review, we introduced and focused on a number of recent studies, including our own, describing the SMF effects on the SNS.

Weak-Intensity

For μT level SMF, including the GMF, a neuroanatomical, electrophysiological and behavioral analysis of magnetoreception or magnetic compass orientation revealed that some neurons in the CNS and SNS have been activated by magnetic stimuli in some vertebrates and invertebrates. The weak SMF can affect the orientation and navigation behaviors in different kinds of living organisms through various sensors, receptors, neural processing, and mechanisms [2–34]. Demonstration of magnetoreception in genetic model organisms, the thale cress (*Arabidopsis thaliana*) [25, 26], the fruit fly (*Drosophila melanogaster*) [28, 29] and the zebrafish (*Brachydanio rerio*) [30], opens a possibility to genetically identify the magnetoreceptor and its downstream signaling cascade of mechanisms.

Various methodological approaches have been attempted to detect neurophysiological or neurobehavioral responses to the GMF. Němec *et al*. (2001) [21] investigated magnetoreception by combining two established methodological approaches: a behavioral test designed to assess magnetic compass orientation in Zambian mole rat (*Cryptomys anselli*) and a neuroanatomical technique. This technique enables immunocytochemical visualization of a transcriptional regulatory protein, c-Fos, as a marker of neuronal activity. They showed that the superior colliculus of mole rats contains populations of neurons that are responsive to magnetic stimuli and that it is involved in the neural processing of magnetic information.

Nishi *et al*. (2004) [31] examined the magnetosensitivity of the Japanese eel (*Anguilla japonica*) using conditioning and electrocardiogram (ECG). Marine eels, river eels and farmed eels were conditioned to an imposed magnetic field parallel to the fish body, which was placed along the earth's west-east axis. A resultant magnetic field was produced by the combination of the GMF and the imposed magnetic field. After 10–40 conditioning runs, the ECG results indicated that all the eels exhibited a significant conditioned response (slowing of the heart beat) to a 192,473 nanotesla (nT) magnetic field and even to a 12,663 nT magnetic field, respectively. These field intensities were equivalent to 5.92 times and 0.38 times of the horizontal GMF (32,524 nT) at the laboratory. The west-east vector of the imposed magnetic field (12,663 nT) was combined with that of the GMF and produced a horizontal resultant magnetic field of 21° easterly. The ECG results showed that the Japanese eels are magnetosensitive to nT fields whether they are at sea, in the river or in the farm. The results on the Japanese eel were different from those on the other kinds of eels that showed no magnetic sense in the American eel (*Anguilla rostrata*) and the European eel (*Anguilla anguilla*).

The Lohmann research group (2003, 2004, 2006) [32–34] has investigated the magnetosensitivity of the marine mollusk (*Tritonia diomedea*) using electrophysiological analysis and neuroanatomical techniques. They found that four identifiable neurons responded with enhanced electrical activity to changes in earth-strength magnetic fields, whereas two additional neurons were inhibited by magnetic stimuli. They speculated that the neurons (neural cells) might play a key role in detecting magnetic fields, in processing magnetic field information, in generating a motor response that involves crawling along a specific magnetic heading, or suppressing behavior that might otherwise impede orientation. Furthermore, they investigated the function of Pd5 neurons (the largest magnetically responsive cells) and suggested that Pd5 neurons could control or modulate the ciliary activity involved in crawling during orientation behavior [34].

One of the most promising mechanisms for explaining magnetic compass is "a radical pair mechanism" [12–15]. The radical pair mechanism for magnetic compass has been proposed by Ritz *et al*. (2000, 2004) [12, 13] as a biophysical mechanism. This mechanism implies that *in vivo* magnetoreception involves radical-pair processes which are governed by anisotropic hyperfine coupling between (unpaired) electron and nuclear spins. One particularly interesting aspect of this mechanism is a link between photosensitivity (photoreception) and magnetoreception: "non-visual" magnetoreception is based on photoreceptor in the visual system and in the pineal gland [12, 13]. From behavioral, physiological and theoretical studies, the possible sources of free radicals have been recently identified in certain blue light receptors and circadian proteins, cryptochromes [22–26] (flavin-tryptophan [24] and flavin adenine dinucleotide [FAD]-tryptophan [25, 26] are involved in their intermediate radical pair systems) and a photopigment, melanopsin (an opsin based photopigment) [27]. However, there has been no report that a specific neurotransmitter in the nervous system is part of the radical-pair system involved in magnetoreception. Several experimental results obtained from various animal species have indicated that this magnetic compass mechanism seems to be primarily limited to avian magnetic compass localized in the retina of migratory birds. In contrast to the magnetic compass mechanism of birds, that of rodents does not involve radical-pair processes. Instead, it seems to be based on a fundamental different principle, which probably involves magnetite and magnetically sensitive chemical reactions, as reviewed by Thalau *et al*. (2006) [15].

Studies on neurophysiology of biogenic magnetite and magnetite-based receptors have been well reviewed by Kirschvink *et al*. (2001) [11]. Magnetite is intracellular single-domain crystals of a super paramagnetic iron oxide mineral (Fe_3O_4) in candidate magnetoreceptor cells. The magnetite has been found and/or the natural remnant magnetization has been measured primarily by using superconducting quantum interference device (SQUID) magnetometer, confocal laser-scanning microscopy, and/or atomic and magnetic force microscopy in a wide range of organisms, such as bacteria (magnetotactic bacteria), algae, mollusks (e.g., chiton teeth), water fleas, lobsters, insects, fishes, amphibians, reptiles, birds and mammals including humans. In magnetotactic batretia, a torque on magnetite chains (magnetosomes) produces a magnetic moment large enough to rotate the cells passively into alignment with the GMF, much like a compass needle [2]. Using an electrophysiological technique, Semm and Beason (1990) [19] firstly obtained clear recordings of responses to weak magnetic stimuli in a migratory bird, bobolink (*Dolichonyx oryzivorus*). They provided electrophysiological evidence for a link between magnetoreception and the trigeminal nerve (TN) system. They found that single units in the superficial ophthalmic (SO) branch and ganglion cells of the TN system responded to changes in the GMF intensity as small as 200 nT, or 0.4% variation in background strength. They also showed that the firing rates of units increased as the logarithmic function of field intensity, and that units fired in phase with a weak sinusoidal magnetic stimulus at very low frequencies. Apparently, however, the units locked on to one phase of the wave cycle and not to its anti-phase. Walker *et al*. (1997) [9] observed a similar response in rainbow trout (*Oncorhynchus mykiss*). They reported that units in the SO branch of the TN system responded to either the onsets or offsets of step changes in magnetic intensity, but not to both. These results point to a common locus of magnetic field detection in vertebrates.

If magnetite-containing cells are used in magnetoreception, it is reasonable to predict that they should be linked to magnetically responsive nerves [11]. Nerve-tracing studies in the trout [9, 10] have used Di-I (a fluorescent lipophilic dye) placed on the cut ends of the SO branch of the TN at the site where electrophysiological recordings of responses to magnetic stimulation were performed. The Di-I migrated in both anterograde and retrograde directions along myelinated and unmyelinated fibres in the TN. Posterior to the orbit, the SO branch joined other branches of the TN, terminating in cell bodies of the anterior ganglion. From the ganglion, labeled nerve tracts entered the anterior dorsal area of the medulla oblongata. Anterior to the orbit, the SO branch has rami that innervate the skin, surround the olfactory nerve and olfactory capsule, and that surround as well as penetrate the olfactory capsule. Fine branches of the SO also penetrated the olfactory lamellae from the top and the base. The top branches terminated in finer processes at the distal end of the olfactory lamellae. Diebel *et al*. [10] then used the crystal and magnetic properties of single-domain magnetite to identify candidate magnetoreceptor cells in the nose despite the small size (<100 nm) and extreme rarity (<5 ppb by volume) of the magnetite crystals. The length of the magnetite crystal chains was 1 μm long (ranging 0.5–1.5 μm) giving a magnetic to thermal energy ratio of 4, which is appropriate for magnetoreception [4]. The multi-lobed cells containing magnetite particles were 10–12 μm long and were consistently located near the basal lamina of the olfactory epithelium. The location of the magnetite crystals chains within each cell suggests that a mechanical linkage could transduce its movement in response to external magnetic fields into changes in the membrane potentials of the cell. This may be achieved by opening

mechanically-activated transmembrane ion channels, and the biophysical properties of such a system seem plausible and are well understood [4, 7, 8, 18].

The above-mentioned magnetite hypothesis posits that crystals of the magnetic mineral magnetite transduce magnetic field energy into physical forces that can be detected by the nervous system, where magnetite-based receptors or specific mechanoreceptors should exist [18]. However, higher-order neural processing often gives rise to behavioral outputs that do not closely mirror the properties of receptors [18]. There is a missing link in this process.

The GMF itself is often categorized as a SMF because it seems to be apparently static on the physiological time scale. However, it is not temporally stable and has natural spatiotemporal variation. In this regard, the GMF is not a real SMF (it is a quasi SMF) but it is considered to be essential to living organisms as a natural, pervasive, and ubiquitous weak magnetic field. It has been reported that the natural variation in the local geomagnetic activity or geomagnetic storms might be related to the occurrence of sudden death in seizure and epilepsy patients [35], sudden infant death syndrome [36], depression [37] and suicide [38]. These reports have been increasing but, at present, there is controversy between the articles and this tends to weaken the effects of events and findings. Much of the skepticism surrounding the effects of the GMF on clinical phenomena might be due to complicated factors, including the uncertainty of the variable dosimetry of exposure, the variable magnetic sensitivity in humans tested in the laboratory, and the implicated physiological mechanisms. The mechanisms by which changes in geomagnetic activity cause alterations in human physiology and behavior are still uncertain. The postulated mechanisms imply an interaction between geomagnetic disturbance and melatonin regulation in the brain. The pineal gland represents the largest source of melatonin in the CNS and SNS and it may also function as a receptor for detecting geomagnetism in humans. In both humans and rats, there is evidence that geomagnetic disturbance is significantly associated with a decrease in melatonin release from the pineal gland [39, 40]. The possibility arises that the occurrence of a geomagnetic storm may result in decreased melatonin release, thereby causing some mental disorders, neurological disorders, and abnormal behaviors.

It has been reported that a geomagnetic storm could modulate biogenic monoamines and neurotransmitters in the CNS and SNS. The CNS and SNS are well known to play a pivotal role in modulating the threshold for seizures or epilepsy, because endogenous norepinephrine is an anticonvulsant neurotransmitter, and blockade of α- and β-adrenoreceptors potentiates seizures [41, 42]. In addition, the serotonin-dopamine interaction in the CNS and SNS is critical for fast-onset action of antidepressant treatment [43]. Therefore, in the case of seizures and depression, the increased geomagnetic activity may suppress the CNS and SNS activity thereby leading to the occurrence of seizures and depression.

It is known that cardiac sympathetic afferent reflexes contribute to increases in sympathetic outflow and that sympathetic activity can antagonize arterial baroreflex function (reflexes initiated by receptors in the aortic arch that alter peripheral vasomotion). Gmitrov (2005) [44] investigated the effects of geomagnetic activity on arterial baroreflex in conscious rabbits (*Oryctolagus cuniculus*) by measuring BP and microcirculation. Baroreflex sensitivity (BRS) was assessed as an indicator of cardiac autonomic regulation on the basis of the heart rate (HR)/mean arterial BP relationship during a vasodilator (sodium nitroprusside) or a vasoconstrictor (phenylephrine) treatment. The results indicated that on days with intense geomagnetic activity, both microcirculation and BRS were decreased. This study further suggests that the GMF directly modifies microcirculatory responses related to BRS. These

findings may have serious implications for individuals with ischemic diseases during periods of intense geomagnetic activity or geomagnetic storms.

McKay and Persinger (2006) [45] reported that artificially generated weak magnetic field patterns do not affect maze performance in normal rats (*Rattus norvegicus*), but disrupt that in the seized rats normalized with ketamine, a non-competitive N-methyl-D-aspartic acid (NMDA) antagonist. They hypothesized that complex neuroelectromagnetic patterns supported by specific spatial configurations of neurons underlie the generation of behaviors. When the pattern of neuronal connectivity is changed, as occurs during limbic epilepsy, neuroelectromagnetic patterns change in parallel to sustain behavioral outputs. Thus, a testable prediction of the neuromatrix concept is that the apparently "normal" behaviors of animals with markedly reorganized neuroelectromagnetic patterns are vulnerable to specific stimuli that are ineffective when applied to a normal population. Because the rats treated with ketamine after being induced to seize with pilocarpine, an imidazole alkaloid, exhibit behaviors indistinguishable from those of control populations despite structural damage in the brain, they represent an ideal population in which to examine this hypothesis. The ketamine-treated pilocarpine-seized rats and normal rats were exposed continuously either to complex sequence magnetic fields or to control conditions during the acquisition of a radial arm maze task for 8 days. After 14 days of subsequent exposure to frequency-modulated magnetic fields (7–500 nT), during which time there was no training, the seized rats exposed continuously to the two types of magnetic fields exhibited conspicuously slower response durations per arm than the seized rats exposed to control conditions or the normal rats exposed continuously to either the two types of magnetic field or control conditions. The results suggested that the behaviors of the seized rats with damaged brains may be seriously disrupted by the appropriate pattern of exogenous weak magnetic fields.

Compared with the GMF, the effects of a zero magnetic field have been assessed by magnetic shielding. The visual system of the fruit fly (*Drosophila melanogaster*) was investigated after exposure to a zero magnetic field [46]. Adults from pupae maintained in a zero magnetic field for 20 h used for the electroretinogram. A significant increase in sensitivity of neural cells from the first optic ganglion was indicated.

Prato *et al*. (2005) [47] reported the effects of a magnetically shielded environment on morphine, an opioid agonist, induced analgesia. Mice (*Mus musculus*) were placed in a Mu-metal (a nickel-iron alloy)-lined box (magnetically shielded condition) or an opaque Plexiglas box (sham condition) for 1 h/day for 10 consecutive days. Nociception was measured as the latency time to a foot lift/lick in response to an aversive thermal stimulus of 51°C before and immediately after the exposure. It was shown that mice can detect and will respond to the repeated absence of the ambient magnetic fields, with the maximum analgesic response occurring over 4–6 day exposure and returning to baseline thereafter. The effects were robust, independent of pre-exposure and intermittent testing, and seem to be opioid related, since the results obtained on day 5 were similar to those from morphine and were abolished with naloxone, an opioid antagonist.

In summary, concerning the effects of weak-intensity SMF, despite recent advances, however, magnetoreceptors (specific chemoreceptors or mechanoreceptors) have not identified with certainty in any animal including humans, and the mode of transduction for the magnetic sense has not been clarified [18]. In addition, the underlying mechanisms of the effects, including geomagnetic disturbance, on living organisms remain unknown. The studies on a zero magnetic field are needed to assess the significant effects of weak-intensity SMF.

Moderate-Intensity

Increased knowledge of the influence of SMF on hemodynamic function may have significant therapeutic potential [48]. For example, SMF therapy could be useful for circulatory diseases, including ischemic pain and hypertension, primarily due to the modulation of blood flow or BP through the nervous system. With regard to the relationship between the hemodynamics and nervous system, it is well known that the vasoconstriction is mediated mainly through sympathetic activity and adrenergic pathways. In contrast, the vasodilation is mediated mainly through parasympathetic activity and cholinergic pathways and, more specially, the acetylcholine-induced vasodilation is induced by endothelium-derived nitric oxide (NO). Therefore, considering the physiological relationship, the reported SMF effects on blood flow and/or BP could be induced through the nervous system.

Several attempts have been made to explore the parameters of microcirculation and microvasculature when tissue and/or blood vessels have been exposed to moderate-intensity SMF. These studies have been well reviewed by McKay *et al*. (2007) [48]. For example, the rabbit ear chamber (REC) offers the advantages of superior optical quality [49]. Due to the longer duration of an individual measurement, we have exclusively utilized REC to investigate SMF effects on microcirculation using microphotoelectric plethysmography (MPPG) monitoring system [49]. The MPPG provides relative changes in microcirculation in cutaneous tissues based on the light absorption of hemoglobin. The REC is a round-table chamber made of acryl resin for disk with an observing table and three holding pillars, a sustaining ring, and a glass window. The methods for installation of REC and its availability to the bioelectromagnetic research have been published in detail [50–53]. BP in a central artery contralateral to that of an ear lobe having the REC, fixed on the microscope stage, was monitored by a BP monitoring system.

Using these methods, Ohkubo and Xu (1997) demonstrated that cutaneous microcirculation was modulated by mT level SMF: biphasic effects of a 1, 5, and 10 mT SMF on cutaneous microcirculation were found in conscious rabbits [50]. A 10 min exposure to the SMF induced changes in vasomotion in a non-dose-dependent manner in this range. When the initial vessel diameter was less than a certain value, the SMF exposure caused an increase in vessel diameter (vasodilation). In contrast, when the initial diameter was greater than a certain value, the SMF exposure caused a decrease in vessel diameter (vasoconstriction). Based on these results, it would appear that the initial state of the vessel is of importance when considering SMF effects on microcirculation and microvasculature.

Likewise, this observation using REC is reflected in the following studies: biphasic effects (activation/inhibition) of a 10 min exposure to a 1 mT SMF on cutaneous microcirculation were found in conscious rabbits treated with vasoactive agents [52]. When high vascular tone was induced by norepinephrine to cause vasoconstriction, the SMF exposure led to increased vasomotion and caused vasodilation. In contrast, low vascular tone was induced by acetylcholine to cause vasodilation, the SMF exposure led to decreased vasomotion and caused vasoconstriction.

Gmitrov *et al*. (2002) [53] investigated changes in blood flow within the cutaneous tissue of the rabbit ear lobe using REC and MPPG. It was found that a SMF exposure (250 mT for 40 min) led to a 20–40% increase in microcirculation in pentobarbital-anesthetized rabbits. Blood flow was significantly increased starting 10 min into the exposure through to 20 min post-exposure compared with sham exposed animals. In a similar experiment, the effects of a

SMF (350 mT for 40 min) on microcirculation and the arterial baroreflex of conscious rabbits were investigated [54]. The SMF significantly increased the BRS, HR, mean arterial BP, and blood flow. Verapamil, a Ca^{2+} channel blocker, decreased the BRS. Vasodilation occurred both after the SMF exposure and verapamil treatment, applied separately. The highlight of the findings was that when the SMF and verapamil were applied simultaneously, the BRS and microcirculation were unaffected. It was suggested that the verapamil counteracted the SMF and that the site of action of the SMF on the microcirculation was the Ca^{2+} channels.

Other studies without using REC have also been well reviewed by McKay *et al*. (2007) [48]. Similar findings were reported for the microvessels of rat skeletal muscle: an acute SMF exposure (70 mT for 15 min) had a restorative effect on microvascular tone in pentobarbital-anesthetized rats [55]. When vessels had high tone (constricted), the SMF acted to reduce tone, and when vessels had low tone (dilated), the SMF acted to increase tone. This response was amplified when the vessels had an initial diameter of less than 30 μm. In contrast, separate analysis of arteriolar and venular diameters in mouse dorsal skinfold chambers revealed that a more prolonged SMF exposure (60 mT for up to 7 days) significantly abrogated the luminal diameter expansion observed in sham treated networks: SMF exposed arteriolar and venular diameters were significantly reduced compared with sham exposed vessels [56].

The SMF effect on skin blood flow was investigated within the loaded or unloaded skin in ketamin-xylazine-anesthetized rats using wavelet analysis [57]. SMF intensity in the locally exposed trochanter area was 30 mT and the duration of exposure was 40 min. Skin was loaded by locally applied pressure of 13.3 kPa (100 mmHg) for 24 h within 4 consecutive days to the trochanter area. The results showed that SMF significantly enhanced blood flow amplitude in the loaded skin, whereas SMF did not induce significant change in the blood flow amplitude in the unloaded skin. This suggests that the enhancing effect of SMF on blood flow amplitude might counteract the reduced vasomotion by prolonged loading.

In our series of later experiments [58–65], our previous works on cutaneous microvasculature in conscious animals have been extended. These studies focused on BP changes associated with the SMF exposure. For instance, when BP was increased using a nitric oxide synthase (NOS) inhibitor (vasoconstrictor), exposure to a SMF (5.5 mT for 30 min) caused a significant decrease in BP during and post-exposure, and led to vasodilation [58]. This led to a significant increase in blood flow, measured using MPPG, after 10 min exposure through to 40 min post-exposure. Alternatively, when BP was decreased using a Ca^{2+} channel blocker (vasodilator), the SMF caused a significant increase in BP during and post-exposure, and led to vasoconstriction. This led to a significant decrease in blood flow for 10 min during the exposure. Furthermore, it is postulated through theoretical calculations that the applied SMF can be converted into a changing magnetic field in the baroreceptor region by means of the carotid artery pulsation [62]. Therefore, it is speculated that the changing magnetic field, i.e., the magnetic field modulated by the pulse rate, may influence the activity of baroreceptor and baroreflex function.

The ability of a SMF (5.5 mT for 30 min) to alter BP was again tested on conscious rabbits with pharmacologically induced hypertension [59]. Norepinephrine or a NOS inhibitor was used to induce vasoconstriction. For the group that received the norepinephrine, the SMF increased the mean blood flow in the ear lobe (measured by MPPG) after 10 min exposure through to 50 min post-exposure. Likewise, for the group that received the NOS inhibitor, the SMF increased the mean blood flow after 20 min exposure through to 20 min post-exposure.

The SMF also reduced both the norepinephrine-induced and NOS inhibitor-induced high BP 60–100 min post-exposure. When a SMF-exposed group with no pharmacological treatment was compared to a sham exposure group with no pharmacological treatment, no significant differences were found.

These effects were further tested on spontaneously hypertensive rats (SHR) [60, 62, 65]. Young prehypertensive SHR were continuously exposed to gradient SMF (1–180 mT) for up to 14 weeks. When the SHR were exposed to the SMF, BP and/or plasma concentration of NO metabolites (NO_x), angiotensin II and/or aldosterone were reduced. Young SHR are known to have increased levels of NO_x (SHR = 17.9 μM > normotensive rat = 10.1 μM), likely due to the upregulation of NOS. For example, exposure to a 5 mT SMF for 6 weeks significantly reduced the concentration of NO_x (exposed SHR = 13.1 μM < non-exposed SHR = 17.9 μM). The SMF also reduced angiotensin II and aldosterone during 3–6 weeks. However, until the 9th week of exposure, irrespective of the longer duration of exposure, all significant antihypertensive effects of SMF disappeared, due to the development of hypertension in young SHR.

The homeostatic effects of a SMF (25 mT for 12 weeks) were again reinforced [63] when reserpine, an indole alkaloid, was used to induce hypotension and deplete catecholamine reserves in conscious rats. The SMF exposure significantly reduced the effect of the reserpine, reducing the hypotension caused by the drug. A 10 mT SMF did not have any effect. It is concluded that a 25 mT SMF could potentially reduce hypotension *in vivo*.

The combined effects of a SMF (12 mT for 10 weeks) and two different sympathetic agonists were investigated in conscious rats [64]. The two different sympathetic agonists, an α1-adrenoceptor agonist, phenylephrine and a β1-adrenoceptor agonist, dobutamine, induced hypertension and different hemodynamics: phenylephrine increased BP and decreased HR, skin blood flow, skin blood velocity, and the number of rearing responses; dobutamine increased BP and HR, increased skin blood flow and velocity, and the number of rearing responses. Continuous neck exposure to the SMF alone for up to 10 weeks induced no significant changes in any of the measured cardiovascular and behavioral parameters. The SMF exposure for at least 2 weeks (1) significantly depressed phenylephrine effects on BP, skin blood flow and velocity, and rearing activity; (2) significantly depressed dobutamine effects on BP, skin blood flow and velocity, and suppressed dobutamine-induced increase in the rearing activity. These results suggest that continuous neck exposure to a 12 mT SMF for at least 2 weeks may depress or suppress sympathetic agonists-induced hypertension, hemodynamics, and behavioral changes by modulating sympathetic nerve activity.

The SMF effects on blood velocity were assessed in pentobarbital-anesthetized mice [66]. Peak blood velocity in the tibialis anterior muscle of mice was measured using a fluorescence epi-illumination system (a fluorescence microscope, charge-coupled device camera, video time generator, tape recorder, and display monitor). It was reported that whole body exposure to a 1 mT SMF for a duration of 10 min led to a 20–45% increase in blood velocity over a period of 45 min post-exposure. No significant increase was noted during the exposure period. When the mice were exposed to a 10 mT SMF, blood velocity was increased by 15% immediately after the initiation of the SMF and 45% immediately after the end of exposure. A 0.3 mT SMF did not have any effect. These results suggest that a 1 mT SMF may be the threshold for altering hemodynamics. This study clearly demonstrates that various microcirculatory effects are possible depending on the SMF parameters used.

It was reported that blood brain barrier (BBB) permeability in rats was increased for 1 h after exposure to a 150 mT SMF for 23 min [67]. As for the relationship between the SNS and BBB, it is known that stimulation of the sympathetic nerves to the brain tends to make the resistance vessels able to withstand a higher BP, i.e., to prevent BBB dysfunction and overperfusion in acute hypertension [68, 69]. Therefore, considering the relationship, the reported SMF effects on BBB could be induced through the SNS.

In another animal study, however, no circulatory effects have been described. Steyn *et al*. (2000) [70] examined the effect of a SMF on blood flow to the metacarpus of horses and found that in horses, exposure to a 27 mT SMF for 48 h did not change blood flow to the portion of the metacarpus underneath the magnetic wrap.

Several clinical studies on the SNS or blood flow of healthy human subjects have been carried out using permanent magnets [71–76]. For instance, an acute SMF exposure (100 mT for 36 min) resulted in no significant change in skin blood perfusion [71]. Both laser Doppler flowmetry and imaging indicated that no differences in microcirculation existed between groups that received either 36 min of sham or SMF exposure. Perfusion measurements were made before and during the exposure. These authors emphasized that the lack of SMF effect may have been a result of studying healthy subjects with “normal, unstressed circulation”. Similarly, no significant effect of an 85 mT SMF on human skin blood flow was found using laser Doppler flowmetry [72]. When subjects took a deep and rapid inspiration, sympathetic reflexes led to transient vasoconstriction in the skin microvasculature (inspiratory gasp reflex). The SMF exposure for 20 min did not affect the magnitude of this vasoconstriction. Although this vasoconstriction deviates from “normal” resting conditions, the authors suggested that the extent that a tissue/vessel deviates from normality may affect the SMF effect. In another experiment, however, Mayrovitz and Groseclose (2005) [73] did find an effect of a 400 mT SMF on skin microcirculation of human subjects. A sham magnet was placed under the 2nd finger and another placed under the 4th finger for a period of 15 min. Next, a sham magnet was again placed under the 4th finger and an active magnet (of either polarity) was placed under the 2nd finger for 15 min. This process was repeated for another 15 min using a magnet of the opposite polarity under the 2nd finger. A significant reduction in skin blood flow was reported after three 15 min exposure intervals using magnets of either polarity. Reversing the polarity of the magnet had no effect.

An acute SMF exposure (60 mT for 1 h) resulted in no significant change in the SNS or skin blood velocity [76]. This study is the first randomized, double-blinded, placebo-controlled, cross-over experiment to test the physiological effects in humans. Each subject was required to rest on the control or magnetic mattress for one hour. After one hour, variables were measured and the subjects then performed three interventions known to increase sympathetic outflow and act as noxious stimuli. The interventions used were (1) isometric handgrip, (2) post-exercise muscle ischemia, and (3) cold pressor test. The SMF did not alter pain perception, cardiovascular, or sympathetic responses to these three distinct pain conditions inducing physiological stressors in humans.

As above-mentioned in the effects of geomagnetic disturbances on humans, these clinical studies on higher SMF effects in the mT range have also reported inconsistent, inconclusive and controversial results. These discrepancies might be due to the differences between the studies in terms of the intensity of SMF, the duration of exposure, the exposure point, the variable magnetic sensitivity in humans tested in the laboratory and the implicated physiological mechanisms.

Table 1. Summary of Moderate-Intensity SMF Effects on Hemodynamics and/or Blood Pressure (BP)

Increased hemodynamics and/or BP (n = 7)	Effective exposure conditions
Prato *et al.* (1990) [67]	150 mT; 23 min; rats
Xu *et al.* (1998) [51]	180 mT; 1–3 weeks; rabbits
Xu *et al.* (2000) [66]	1, and 10 mT; 10 min; mice
Gmitrov *et al.* (2002) [53]	250 mT; 40 min; rabbits
Gmitrov (2004) [54]	350 mT; 40 min; rabbits
Okano *et al.* (2005a)[a] [63]	25 mT; 2–12 weeks; rats
Li *et al.* (2007) [57]	30 mT; 40 min; rats
Decreased hemodynamics and/or BP (n = 7)	**Effective exposure conditions**
Okano and Ohkubo (2003a)[b] [59]	5.5 mT; 30 min; rabbits
Okano and Ohkubo (2003b)[c] [60]	10, and 25 mT; 2–9 weeks; SHR
Okano and Ohkubo (2005b)[c] [62]	180 mT; 1–8 weeks; SHR
Okano and Ohkubo (2007)[b] [64]	12 mT; 2–10 weeks; rats
Okano *et al.* (2005b)[c] [65]	5 mT; 2–8 weeks; SHR
Mayrovitz and Groseclose (2005) [73]	400 mT; 45 min; humans
Morris and Skalak (2007) [56]	60 mT; 4–7 days; mice
Biphasic effect (n = 5)	**Effective exposure conditions**
Ohkubo and Xu (1997) [50]	1, 5, and 10 mT; 10 min; rabbits
Okano *et al.* (1999) [52]	1 mT; 10 min; rabbits
Okano and Ohkubo (2001) [58]	1 mT; 30 min; rabbits
Okano and Ohkubo (2005a) [61]	5.5 mT; 30 min; rabbits
Morris and Skalak (2005) [55]	70 mT; 15 min; rats
No effect (n = 6)	**Exposure conditions**
Steyn *et al.* (2000) [70]	27 mT; 48 h; horses
Mayrovitz *et al.* (2001) [71]	100 mT; 36 min; humans
Mayrovitz *et al.* (2005) [72]	85 mT; 20 min; humans
Hinman (2002) [74]	50 mT; 15 min; humans
Martel *et al.* (2002) [75]	80 mT; 30 min; humans
Kuipers *et al.* (2006) [76]	60 mT; 1 h; humans

[a]Initial state of subjects: pharmacologically induced hypotension.
[b]Initial state of subjects: pharmacologically induced hypertension.
[c]Initial state of subjects: spontaneously hypertensive rats (SHR).

In summary, significant or nonsignificant circulatory system responses to moderate-intensity SMF (mT level) have been recently reviewed in experimental animals and/or humans: the SMF exposure between 1 mT and 400 mT for anywhere between 10 min and 8 weeks (e.g., antihypertensive period for young SHR) could affect cutaneous microcirculation, hemodynamics and/or arterial BP [48, 77–79] (Table 1). Seven of a total of 25 studies in the

last two decades report either an increase in blood flow, or an elevation in BP. In contrast, seven of the 25 studies indicate either a decrease in blood flow or a reduction in BP. Five studies suggest that the SMF exposure could trigger either vasodilation or vasoconstriction depending on the initial tone of the vessel. The remaining six studies report no effect.

In particular, our series of studies [51–53, 58–66], as shown in Table 1, have demonstrated that cutaneous microcirculation, hemodynamics and/or BP were modulated by mT level SMF in pharmacologically treated animals and genetically hypertensive animals, while there was no significant change in normal, conscious animals when the initial state of the vessel was not identified. Therefore, we concluded that significant bioresponses to therapeutic signals occur when the state of the target tissues or cells is far from the homeostasis [80].

Saunders (2005) commented that most of these studies were undertaken in the context of the potential therapeutic SMF effects on various disorders. Further studies with some independent replications are required even if the SMF effects on both blood flow and BP indicate possible medical applications [77].

The mechanisms of SMF effects in the mT range on blood flow and/or BP could be mediated by suppressing or enhancing the action of biochemical effectors, thereby biphasically inducing homeostatic effects. The potent mechanisms of SMF effects have often been linked to NO pathway, Ca^{2+}-dependent pathway, SNS (e.g., BRS and the action of sympathetic agonists or antagonists), and neurohumoral regulatory system (e.g., production and secretion of angiotensin II and aldosterone), as reviewed by McKay *et al*. (2007) [48].

In order to detect SMF-induced functional changes in the CNS and SNS, Rosen and Lubowsky (1987) [81] examined the effects of a 120 mT SMF on the excitability of striate cortex in adult cats (*Felis catus*). The visual evoked response was used as a measure of cortical excitability. The SMF induced a significant decrease in both amplitude and variability of the evoked response. This effect began more than 50 sec after the SMF was turned on and persisted, even after the SMF was turned off, for several minutes. This phenomenon appears to be due to action of the SMF at the synapse rather than on axonal conduction. In addition, Rosen and Lubowsky (1990) [82] examined the effects of a 123 mT SMF on spontaneous discharge frequency and discharge pattern of principle cells in the cat's lateral geniculate body (LGB). They showed that the SMF induced gradual onset and prolonged time course of changes in LGB cell activity. The results suggest either an alteration in the synaptic ionic environment or in neurotransmitter availability. It has been postulated that the SMF could produce a partial realignment of diamagnetically anisotropic molecules within the cell membrane, thereby distorting ion-specific channels sufficiently to alter their function.

Veliks *et al*. (2004) [83] performed an investigation to identify the SMF effects on HR and heart rhythm as physiological indicators using an ECG. Ketamine-xylazine-anesthetized rats were exposed to a 100 mT SMF for 10 min. It was found that the SMF exposure evoked changes in both HR and heart rhythm in 80% of the subject animals.

As described above, most of *in vivo* animal experiments involving the SMF effects on physiological indicators have been performed under anesthesia. The choice of anesthetic agents used by researchers is variable and each anesthetic agent alters the physiological indicators in a slightly different manner. However, little or no experiments have been carried out to determine whether the mechanisms of anesthetic action are affected in any way by the SMF. McKay *et al*. (2007) [48] proposed that it may be useful to test the SMF effects using different anesthetics and determine whether there are any differences in the results.

McLean *et al*. (2003) [84] examined the combined effects of field intensity and a maximum spatial magnetic flux gradient (field intensity and field flux gradient, 10.5 mT and 0.48 T/m, or 5.27 mT and 0.24 T/m; 30 min exposure) on audiogenic seizures of DBA/2 mice. Two strains of DBA/2 mice were subjected to auditory stimulation that resulted sequentially in wild running, loss of righting, clonus, tonic hindlimb extension, and death in 80–95% of animals in different experiments. The incidence of seizure stages in groups of animals pretreated with a SMF, an anticonvulsant, phenytoin (PHT), or both was compared with the incidence in sham-exposed control mice. Depending on magnetic flux density and duration of exposure, seizure severity decreased significantly, but not completely, in both strains. A SMF had some anticonvulsant effects when employed alone. More robust effects were seen in combination with PHT. The exposure to a 10.5 mT with 0.48 T/m was not superior to a 5.27 mT with 0.24 T/m in reducing seizure in one strain of DBA/2 mice. These findings suggest that there is a limit on the extent to which the nervous system is capable of responding to pretreatment with the SMF studied here.

Norepinephrine is an important endogenous anticonvulsant neurotransmitter in general [41, 42] and is deficient in genetically epilepsy-prone rats with audiogenic seizures [85, 86]. Taking the neuronal mechanisms of seizures into consideration, SMF may suppress seizures through noradrenergic neurotransmitter in the CNS and SNS. Further investigation is required to clarify the anticonvulsant effects and elucidation of mechanisms of action induced by SMF.

Klimovskaia and Maslova (1981) reported that an acute SMF exposure (400 mT for 1 h) caused increases in the blood content of epinephrine, norepinephrine and acetylcholine, and in the adrenal content of epinephrine in rats [87]. Stimulation of the midbrain reticular formation led to a significant increase of the blood concentration of catecholamines and acetylcholine. After the exposure, the stimulatory effects of the reticular formation on the adrenergic system diminished and on the cholinergic system remained elevated.

Satow *et al*. (2001) [88] observed that the effects of a 650 mT SMF on muscle tension in the neuromuscular preparation of the sartorius muscle of bullfrog (*Rana catesbeiana*). The muscle tension development was obtained by stimulation of the sciatic nerve or of the sartorius muscle itself for 30 min. The muscle tension was higher in test conditions than in control conditions, whereas a decrease in muscle tension was observed in both conditions throughout the experimental period. The results indicated that the SMF prevented the decrease in muscle tension. From the relationship between the SNS and muscle tension, it is suggested that sympathetic outflow to a resting muscle was augmented with the increase in muscle tension [89]. Therefore, it seems plausible that the SMF effects on muscle tension could be mediated through the SNS. For the SMF effects on muscles, Abdelmelek *et al*. (2006) [90] found that a 128 mT SMF (1 h/day for 5 consecutive days) induced an increase in norepinephrine content in rat gastrocnemius muscles, whereas a 67 mT SMF did not. The results suggested that a SMF could induce a stimulatory effect on the SNS contributing to the regulation of muscle tension.

It has been proposed that several physical transduction mechanisms induced by SMF could affect action potential (AP) propagation, neurotransmitter release and/or specific ion channels. The SMF effects on AP propagation and excitation recovery in nerve have been reviewed [79, 91, 92].

Azanza (1989) [93] measured the AP in isolated neurons of land snail (*Helix aspersa*) under exposure to SMF ranging 3–260 mT for several minutes and found a SMF of 116 mT and 260 mT mimiced the inhibitory and excitatory actions of caffeine, an amphetamine, on

neurons in Ca^{2+}-dependent manner: bursting cells in the parietal ganglion, which were inhibited by caffeine, were also inhibited by SMF; in silent cells in the visceral ganglion, similar parallelism was observed between the excitatory action of caffeine and the SMF action. The results with caffeine corroborate that changes in Ca^{2+} kinetics underlie the electrophysiological membrane changes observed in neurons exposed to the SMF. Further experiments with individual neurons have demonstrated that exposure to a SMF of 260 mT produced an increase of intracellular Ca^{2+} and that the SMF effect could be blocked by terbium binding to the Ca^{2+} channels in the cell membrane [94]. This experiment also showed that the SMF effect on neurons depends from the Ca^{2+} ions flow into the cytosol. In addition, Azanza and del Moral (1996) [95] measured the AP in isolated neurons of the land snail under exposure to SMF of 70–700 mT. A decrease in the spike depolarization voltage was observed, and it is postulated to be due to desensitization of the membrane Na^{+}-K^{+}-ATPase pumps through an anisotropic diamagnetic reorientation.

A SMF in the 10 mT range blocks sensory neuron AP, which suggests that the SMF could alleviate pain [96–98]. McLean *et al*. (1995) [96] showed that the maximally effective region (MER) coincided with regions in which the gradient is predominantly perpendicular to the local field vector in the case of a square array of four cylindrical permanent magnets of alternating polarity. Placing cultured neurons at effective distances over the MER resulted in reversible, time-dependent blockade of electrically stimulated AP of cultured mouse dorsal root ganglion cells without altering resting membrane potentials. McLean *et al*. (2001) [98] found the responses of neurons to the pain-producing substance capsaicin were also blocked by SMF reversibly over the MER.

Balaban *et al*. (1990) [99] reported the influence of a SMF on bioelectric properties of snail neurons. Identified cells of a land snail (*Helix lucorum*) received exposure to a 23, 120, and 200 mT SMF for 20 min. Resting potentials and input resistances were measured. Controls were instituted for temperature changes and for mechanical and other sources of artifact. Resting potentials did not change with the SMF exposure. Input resistances decreased significantly in normally silent cells during the exposure, but increased significantly in spontaneously active cells. The magnitudes of changes were monotonically related to the SMF strength. Changes in excitatory postsynaptic potentials (EPSP) were observed during the exposure. Elimination of perineuronal glia by proteolytic enzymes abolished the SMF effects.

Trabulsi *et al*. (1996) [100] measured the EPSP in a mouse hippocampal slice after exposure to a SMF of up to 10 mT for 20 min. They observed biphasic effects at 2–3 mT and depression of EPSP at 8–10 mT. It has been suggested that changes in intracellular Ca^{2+} concentration were responsible for these effects.

Ye *et al*. (2004) [101] investigated whether exposure to a SMF affects the passive properties of neurons that mediate tail-flip escape behavior in red swamp crayfish (*Procambarus clarkia*). A permanent magnet was placed under the isolated nerve cord of crayfish to experience SMF ranging 4.74–43.45 mT intensity for various period of time (20 sec–3 h). An intracellular electrode was impaled on the axon of the lateral giant neuron (LG) of the last abdominal ganglion of crayfish to record the evoked AP and EPSP. The SMF exposure increased the amplitude of AP in the LG depending upon both the field intensity and exposure duration. The changes in AP by field exposure are likely to be mediated by the increasing level of intracellular Ca^{2+} in the LG because the chelating of intracellular Ca^{2+} would block the SMF effect, while the injection of Ca^{2+} into the LG could mimic the SMF effect. The SMF exposure also increased the input resistance of the LG membrane. Therefore,

the magnitude of the EPSP in LG evoked by electrical shock on the sensory nerves was found to be enhanced after the exposure. The results showed that some passive membrane properties of neurons were affected by the SMF exposure. It is suggested that the increase in magnitude of evoked AP and EPSP might be related to an increase in the sensitivity of the LG neuron. It is also suggested that "an exposure volume window" exists for biological reaction resulting from the SMF exposure. The product values of SMF intensity multiplied by the exposure duration (mT × min) represent the SMF volume experienced by the nerve cord. It was found that the AP amplitude was enhanced only when the product values was ranging 14.48–7821.

Rosen (1992) [102] reported the SMF effect on acetylcholine release at the neuromascular junction. The frequency of miniature end-plate potentials (MEPP) was recorded from the mouse phrenic nerve-diaphragm preparation. In the presence of a spatially uniform 120 mT SMF for 100 sec, statistically significant changes in MEPP frequency were observed. There was a modest increase in frequency at and below 34°C and a prominent decrease in frequency above 35°C. The abrupt temperature-dependent changes in MEPP frequency response to SMF reflect analogous changes in acetylcholine release from the presynaptic nerve terminals leading to a phase transition-induced alternation in function of the presynaptic membrane. This effect was not seen in the absence of Ca^{2+} in the perfusate. The SMF reduced acetylcholine release in an intensity-dependent manner (in particular, ranging 80–120 mT) at 35°C. These results suggest that, at its phase transition temperature, the diamagnetic anisotropy of the presynaptic membrane is sufficient to influence acetylcholine release by altering the function of the transmembrane Ca^{2+} transfer mechanism.

In addition, Rosen (1996) [103] investigated the SMF effect on voltage-activated Ca^{2+} channel function in cultured rat pituitary tumor GH3 cells. A 120 mT SMF was applied for 150 sec. Reversible decreased activation in Ca^{2+} channel function was observed to be temperature-dependent. The results indicated that these changes were a result of a functional disruption of the intramembranous portion of the Ca^{2+} channel, primarily due to the magnetically induced membrane deformation. Furthermore, Rosen (2003a) [104] examined the SMF effect on voltage-activated Na^{+} channels in GH3 cells using the whole-cell patch-clamp technique. Exposure to a 125 mT SMF for 150 sec on voltage-gated Na^{+} channel kinetics induced a slight shift in the current-voltage relationship, a 5% reduction in peak current, and an increase in the activation time constant, τ_m, during and at least 100 sec after the exposure. Significant changes were only observed at 35°C and 37°C. It was suggested that the temperature dependence factor that affected this process was probably due to the greater ease with which the liquid crystal membrane was deformed. The results suggested that the changes might be due to the reorientation of diamagnetic anisotropic molecules in the membrane. This is an extension of a similar mechanism proposed by Azanza and del Moral (1994): the SMF could affect on lipid bilayers by operating the SMF interaction with structures with anisotropic diamagnetic susceptibility [105]. Hinch *et al*. (2005) [92] theoretically estimated the SMF effects on AP propagation and excitation recovery in nerve using the Hodgkin-Huxley and one-dimensional cable equations. At a field level of 125 mT, which was the same condition previously used by Rosen (2003a) [104], however, they could not predict major changes in the electrical functioning of neurons. In contrast, observations by Hughes *et al*. (2005) [106] and by Petrov and Martinac (2007) [107] appear to support the proposed mechanism showing that the SMF effect on "mechanosensitive ion channels" may be mediated by changes in membrane properties due to anisotropic diamagnetism of lipid molecules.

Wieraszko (2000) [108] studied the effect of 2–3 mT SMF applied for 20 min on the evoked potential response in mouse hippocampal slices. The results showed both an alteration of the evoked potential and an effect on the influence of dantrolene, an inhibitor of intracellular Ca^{2+} channels. However, N-methyl-d-aspartate (NMDA) antagonists had no effect. It is suggested that the mechanism of field sensitivity may well therefore be related to the release of intracellular Ca^{2+} rather than to the influx of Ca^{2+} through the activation of transmembrane ion channels. The Ca^{2+} can be released from the endoplasmic reticulum via the ryanodine-sensitive receptors, or IP3 (inositol triphosphate) receptors.

Sonnier *et al*. (2000) [109] found no significant effect from the SMF exposure at a 0.1, 0.5, and 7.5 mT, applied for 5 sec, on resting potential in cultured neuroblastoma cells. They also used the patch-clamp technique to measure transmembrane Na^+, K^+ currents in neuroblastoma SH-Sy5Y cells exposed to SMF of up to 7.5 mT [109]. The SMF exposure did not result in detectable changes in any of the AP parameters.

Coots *et al*. (2004) [111] reported the effect of a 500 mT SMF on spinal cord conduction in guinea pigs (*Cavia porcellus*) by measurement of compound-evoked potentials. There was no significant change in response latency during the exposure period but there was a small, statistically significant, decrease in amplitude. The maximum effect was evident 1–2 min after the field was turned on with return to baseline within 1 min after the field was turned off. These results may be explained by a conduction block in the small axon subpopulation due to the SMF effect on voltage-activated Na^+ channels. The relative selectivity of the field is believed to occur because of the relatively greater number of Na^+ channels present in smaller axons.

Shen *et al*. (2007) [112] reported the effect of a spatially uniform SMF on the voltage-gated K^+ channel currents in trigeminal root ganglion (TRG) neurons. To evaluated the SMF effect on two types of voltage-gated K^+ channel (VGPC) currents: I(K,A) and I(K,V), whole-cell patch-clamp experiments were conducted on acute dissociated rat TRG neurons. The results suggested that exposure to a 125 mT SMF for 15 min could influence the inactivation kinetics of these two VGPC currents by altering the inactivation rate and velocity, but not the activation properties. These findings supported the hypothesis that biological membrane would be deformed by SMF and the physiological characteristics of ion channels on the membrane would be influenced. The proposed mechanism underlying the different SMF effects on the I(K,A) and I(K,V) inactivation might be attributable to the different distortion of ion channels between their inner and outer terminals.

For the SMF effect on neural gene expression in the CNS, Hirai *et al.* (2006) [113] identified Ntan1 (amidohydrolase for N-terminal asparagine) as a magnetism response gene by differential display screening in cultured rat hippocampal neurons. In addition, Goto *et al*. (2006) [114] examined the SMF effect on Ntan1 mRNA expression in cultured mouse hippocampal neurons. They found that Ntan1 mRNA expression was significantly increased transiently about two-fold 12 h after 15 min exposure to a 100 mT SMF. When embryonic 12-day-old or newborn mice were successively exposed to a 100 mT SMF for 2 h, four times per day until the postnatal 7 days, Ntan1 mRNA was significantly increased about 1.5–2-fold in the hippocampus *in vivo*. The mice exposed to the SMF under the same condition showed significantly decreased locomotor activity. These results suggest that the SMF exposure affects higher order neural functions through modulation of genes expression.

In humans, sensitivity to magnetic fields some 20 times larger than the GMF has been demonstrated in drug resistant epileptic patients and has some clinical value. Interictal

(between seizure) firing rates in the hippocampus of temporal lobe epileptic patients were increased by fields of the order of a few millitesla [115–118].

There are a number of important dosimetry issues that could exhibit significant effects on the SNS and CNS. It has been shown that it is more appropriate to consider biological responses to SMF through the hypothesis of intensity windows, instead of intensity-response dependence [119]. Otherwise, most of the experimental studies showing positive results have been carried out using gradient magnetic fields: the gradient component of SMF at the target site might be responsible for the physiological responses *in vivo* [61, 62] and *in vitro* [96–98, 120–123]. For example, the *in vitro* effects of gradient fields on AP generation [96–98], myosin phosphorylation [120] and endothelia tubular formation [121–123] have been found mostly in the absolute field gradient range of more than 1 mT/mm (1 T/m) in the target tissues or cells. However, these hypotheses have not been proven, and the effects and underlying mechanisms remain elusive. In particular, to reveal and clarify the effects and mechanisms of spatial magnetic flux gradient, it is necessary to carry out the experiments comparing the spatially homogeneous and inhomogeneous SMF.

SMF effects have been observed either during the exposure period, the post-exposure period, or both periods. However, it is most difficult to take accurate measurements during the exposure period due to interference of noise (background signals). McKay *et al*. (2007) [48] recommended that more accurate measurements during the exposure period may provide helpful information as to when a significant biological effect occurs. In addition, reversibility or irreversibility of induced effects has seldom been investigated and reported.

Strong-Intensity

Most of the studies on tesla level SMF, mainly related to MRI systems, have been carried out, and it has been reported that in MRI-related studies using the strength up to 8 T, the SMF strength itself does not directly induce significant physiological changes in cardiovascular and circulatory parameters [48, 77–79, 124–127]. Theoretical calculations have demonstrated that the SMF strength below 10 T cannot be a direct effect of Lorentz forces acting on the induced currents in the blood vessels [128] and calcium ions [129].

For example, whereas skin temperature decrease in urethane-anesthetized rats exposed to a high spatial magnetic flux gradient (maximum intensity, 8 T; exposure duration, 5–20 min) have been observed [130, 131], this observation can be explained in terms of the effects of the SMF on air convection and water vapor [131]. That is, spatial magnetic flux gradients (135–140 T^2/m) push the diamagnetic water molecules towards the magnetic bore of a superconducting magnet, in which the animal was placed. This increases the movement of water molecules in the air around the animal body and the vaporization rate. The heat of vaporization leads to a decrease in skin temperature. The decrease in the blood flow in the skin microcirculation is assumed to be a secondary change to the decrease in skin temperature.

Effects of strong SMF on subjective complaints have been evaluated. Working in the inhomogeneous stray magnetic fields outside of the bore of the MRI has been linked to an increase in subjective complaints, such as dizziness, nausea, tiredness, headaches, etc [132, 133]. de Vocht *et al*. (2003, 2006b) carried out volunteer studies on the stray field of a 1.5 T SMF [134, 135] and a 3.0 T SMF [135]. They suggested that eye-hand coordination, visual

contrast sensitivity, visual tracking tasks, and working memory are negatively affected during the exposure. In the recent extensive study (2007) [136], they suggested that exposure to the stray field of a cylindrical, whole-body 7 T MRI scanner, without radiofrequency (RF) energy or switched gradient magnetic fields, affects the visual sensory domain and, to a lesser extent, eye-hand coordination. The results of the exposure assessment of healthy volunteers were as follows: for the high exposure level condition, the exposure level at the head of a seated volunteer was approximately 1.5–2.0 T, depending on the height and posture of each volunteer. This reduced to approximately 0.8–1.0 T if a volunteer leaned forward during the head movements and 500–800 mT when a volunteer was doing a neurobehavioral test. The medium exposure level was approximately 800 mT when the volunteer was seated in the chair. The SMF exposure at the low exposure level was approximately 2 mT. The root mean square (rms) time-varying magnetic field, d*B*/d*t*, for one volunteer performing the standardized head movements at the position closest to the magnet bore, was measured to be approximately 300 mT/sec. This was reduced to 150 mT/sec at 52 cm away from the bore of the magnet. The magnitude of the effects seems to depend on the magnitude of the dynamic field component generated by the additional head movements and not on the strength of the stray field itself. They thought that the effects should be related to the actual performance of staff working in the vicinity of an MRI scanner, and they proposed that exposure guidelines should take into account the effects of moving in magnetic fields as well as the SMF strength.

As with the effects of moderate-intensity SMF on BBB permeability, it was also reported that exposure to a high SMF applying for MRI could change BBB permeability [137]. Prato *et al*. (1994) [137] investigated the effects of a high SMF applying for MRI on BBB permeability in rats using a radioactive tracer, ^{153}Gd-DTPA. The results indicated that exposure to a SMF of 1.5 T and 1.89 T alone for 45 min, without radiofrequency-specific absorption rate (RF-SAR) and temporal gradient, increased BBB permeability. In functional MRI (fMRI) of brain activity, use of the blood oxygen level-dependent (BOLD) signal is common [138]. The differences in magnetic properties of oxygenated and de-oxygenated hemoglobin, mainly within the localized blood flow or microcirculation, are used to produce a signal. When fMRI is used to measure related changes in blood flow, there is a possibility that the SMF itself is causing, confounding, or contributing to, the change [48].

There have been several reports on the neurobehavioral effects of strong-intensity SMF generated by a superconducting magnet or a resistive electromagnet on rats and mice [139–144]. Using a conditioned taste aversion technique, it was shown that exposure to a 9.4 T SMF for 30 min suppressed rearing and induced tight circling [139], and induced brainstem expression of c-Fos [140]. Following this report, similar experiments were carried out: rats were exposed to a SMF of 7 T and 14 T [141] and 4–19.4 T [142]. In these reports, rearing was suppressed after exposure to 4 T or higher; circling was observed after exposure to 7 T or higher. Conditioned taste aversion was acquired after exposure to 14 T or higher. Exposure to + 14 T SMF induced counter-clockwise circling, while exposure to - 14 T SMF induced clockwise circling. The rat's orientation in the SMF is a key factor for the direction of the circling. Similar results were obtained with mice [143]. It has been suggested that the neural activation response might be responsible for promoting conditioned taste aversion learning. Furthermore, exposure to a 14.1 T SMF for 5 min decreased the amount of glucose and saccharin solution consumed by water-deprived rats over 10 min [144]. The suppression of drinking by the SMF is consistent with the acute effects of other aversive stimuli, such as whole-body rotation, on short-term ingestion.

For the effects of strong-intensity SMF on adrenergic and cholinergic systems, Klimovskaia and Maslova (1982) [145] reported that a chronic SMF exposure (1.6 T for 1 month) increases blood and brain epinephrine, norepinephrine and acetylcholine in mice transiently during the 3rd week of exposure.

The effect of a 1 T SMF on peripheral nerve regeneration was evaluated in rat sciatic nerve [146]. The results demonstrate that the SMF exposure (12 h/day for 4 consecutive weeks) has no statistically significant effect on nerve regeneration as determined by myelinated axon counts and electrophysiologic studies. Also, the SMF has no influence on axonal growth or nerve conduction. The effect of a 1.5 T SMF on nerve conduction was also investigated using an MRI system in a partially active nerve in bullfrog (*Rana catesbeiana*) [147]. The AP and nerve impedance measurements for 20 msec at the onset of the SMF exposure indicated that the strength of 1.5 T SMF had no effect on nerve conduction. Therefore, it is concluded that nerve conduction in damaged nerve would not be affected by the SMF exposure.

For safety concerns, based on advanced studies of the EMF effects on oxidative stress reactions, the potential hazardous effects of SMF on living organisms are that SMF could increase the activity, concentration and lifetime of free radicals, which might cause an oxidative stress, genetic mutation or apoptosis [148, 149]. Although the relationship between the oxidative stress and activation of the SNS (in particular, hypertension) has been implicated [150, 151], the underlying mechanisms of generation of free-radical species induced by SMF exposure, for example, reactive oxygen species (ROS), such as superoxide anion and hydrogen peroxide, and reactive nitogen species (RNS), such as NO, have not been clarified.

Conclusion

The findings presented in this review suggest that the SNS as well as CNS are modulated by SMF of different intensities and spatial magnetic flux gradients, some with excitatory effects, some with inhibitory action, some with both, and others with none. Reversibility or irreversibility of induced effects has seldom been investigated and reported. The recent data related to the mechanisms of action of SMF are diverse and multifold but elusive. The proposed mechanisms of the magnetic sense have a missing link in the neural processing of the magnetic receptors, the transducers and the behavioral outputs. The SMF effects on the clinical relevance and the pathogenesis have not been resolved. The putative underlying mechanisms of generation of ROS and RNS induced by SMF have not been clarified, although the relationship between the oxidative stress and hypertension has been implicated. Further studies are necessary to evaluate the mechanisms of action of SMF on the nervous system in more detail. On the basis of these findings, it seems plausible that our understanding of SMF effects on the nervous system will continue to grow as a focus of increasing attention and importance.

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Chapter 2

OBESITY AND OBESITY-RELATED HYPERTENSION: ROLE OF THE SYMPATHETIC NERVOUS SYSTEM AND β-ADRENOCEPTOR POLYMORPHISMS

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Abstract

Obesity and obesity-related cardiovascular disease are rapidly growing public health problems. Heightened sympathetic nerve activity is a well-established observation in obesity and hypertension. There is evidence that human obesity and hypertension have strong genetic as well as environmental determinants. Reduced energy expenditure and resting metabolic rate are predictive of weight gain, and the sympathetic nervous system participates in regulating energy balance through thermogenesis. The thermogenic effects of catecholamines in obesity have been mainly mediated via the β2 and β3-adrenergic receptors in humans. Further, β2-adrenoceptors importantly influence vascular reactivity. Genetic polymorphisms of the β-adrenoceptor gene have been shown to alter the function of several adrenoceptor subtype and thus to modify the response to catecholamine. Among β2-adrenoceptor polymorphisms, Arg16Gly, Gln27Glu, and Thr164Ile are considered the most functionally important.β2-adrenoceptor genes have been studied in relation to obesity. Genetic variations in the β3-adrenoceptor, such as the Try64Arg variant, are also associated with both obesity and hypertension. However, the precise relationships of the polymorphisms of β2- and β3-adrenoceptor genes with sympathetic nervous system activity, obesity and hypertension have not been fully clarified.

Our own findings show that: 1) β2-adrenoceptor polymorphisms are associated with heightened sympathetic nerve activity, and predict the future onset of obesity and hypertension in nonobese individuals, 2) β2-adrenoceptor polymorphisms accompanied by heightened sympathetic nerve activity and abdominal obesity, predict weight loss resistance during a weight loss program, and also predict rebound weight gain, 3) β2-adrenoceptor polymorphisms are linked to blunted leptin-mediated sympathetic nerve activation, leptin-resistance and resultant obesity, 4) β2-adrenoceptor polymorphisms are related to insulin-

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resistance, in both nonobese and obese normotensive individuals, and 5) β3-adrenoceptor polymorphism is directly linked to obesity and hypertension, but only in obese individuals.

The purpose of this article is to provide a synthesis of the current findings on this topic. The influence of the sympathetic nervous system and β2- and β3-adrenoceptor polymorphisms in hypertension, obesity and obesity-related hypertension will be explored through a literature review, matched against our own findings. Relevant studies of the β2- and β3-adrenoceptor genes in relation to obesity and hypertension were investigated through: 1) electronic search of PubMed (1990 to December 2006, NCBI interface) limited to human studies published in the English language and 2) searching the references lists of all included articles in order to ensure that all relevant material was obtained.

Introduction

Hypertension, obesity and obesity-related hypertension are major and growing health problems. Both are defined as risk factors for cardiovascular events. Obesity and hypertension are intimately associated, however the pathophysiological mechanisms by which obesity leads to hypertension remain uncertain. Understanding these processes might, perhaps, underpin more effective weight loss programs for patients with obesity-related hypertension, and provide a rational basis for drug treatment of obesity-related hypertension. Additionally, knowledge of this type might perhaps explain why not all subjects who succeed in weight loss have blood pressure reductions [1].

A number of clinical and epidemiologic observations show that hypertension and obesity are associated with heightened sympathetic nervous system activity. More than 1000 manuscripts including about 200 reviews on the relationships between sympathetic nervous system activity and hypertension, and 146 manuscripts including 44 reviews between sympathetic nervous system activity and obesity were found in PubMed from the 1980s to March 2007. The number of articles demonstrates strong interest in the role of the sympathetic nervous system in hypertension, obesity and obesity-related hypertension.

Recently, there is evidence that human hypertension and obesity have strong genetic as well as environmental determinants [2-4]. Masuo *et al.* have reported that heightened sympathetic nervous system activity determined by genetic backgrounds predict subsequent blood pressure elevations (hypertension) and weight gain (obesity) in originally nonobese subjects [5-7]. These findings show that the genetic background, especially β2- and β3-adrenoceptor polymorphisms are associated with sympathetic nervous system activity, and are important in the pathogenesis of hypertension and obesity. Combining β adrenoceptor polymorphisms with “hypertension” and “obesity” when searching for manuscripts on PubMed, produced only 6 manuscripts [8-12]. A search for “the β adrenoceptor polymorphisms” and “obesity” and “sympathetic nervous system activity” revealed only 2 manuscripts [7, 12] on PubMed. These numbers show that the interrelationships of the β adrenoceptor polymorphisms with sympathetic nervous system activity, hypertension, obesity and obesity-related hypertension have been little researched.

The purpose of this article is to provide a synthesis of the current findings on this topic. The contribution of the sympathetic nervous system and β2- and β3-adrenoceptor polymorphisms to the development of hypertension, obesity and obesity-related hypertension will be explored through a literature review, matched against our own findings.

Methods of Assessment of Sympathetic Nervous System Activity

The activity of sympathetic nervpous system can be studied mainly by three techniques: (1) biochemical methods that measure plasma norepinephrine concentration and norepinephrine spillover rates, (2) microneurography in sympathetic muscle nerve fibres, and (3) power spectral analysis of heart rate. Plasma norepinephrine concentration is the most commonly used global indicator of sympathetic nervous system activity in clinical studies, however limitations have to be kept in mind. Plasma norepinephrine is a function of multiple processes including norepinephrine production, release, reuptake and clearance from plasma. The use of forearm venous measurements has been criticized since they may primarily reflect metabolism in the organ drained rather than in the whole body. Biochemical methods that estimate the concurrent processes of norepinephrine release and removal from arterial blood are thought to give a more robust index of whole-body and regional sympathetic nervous system activity.

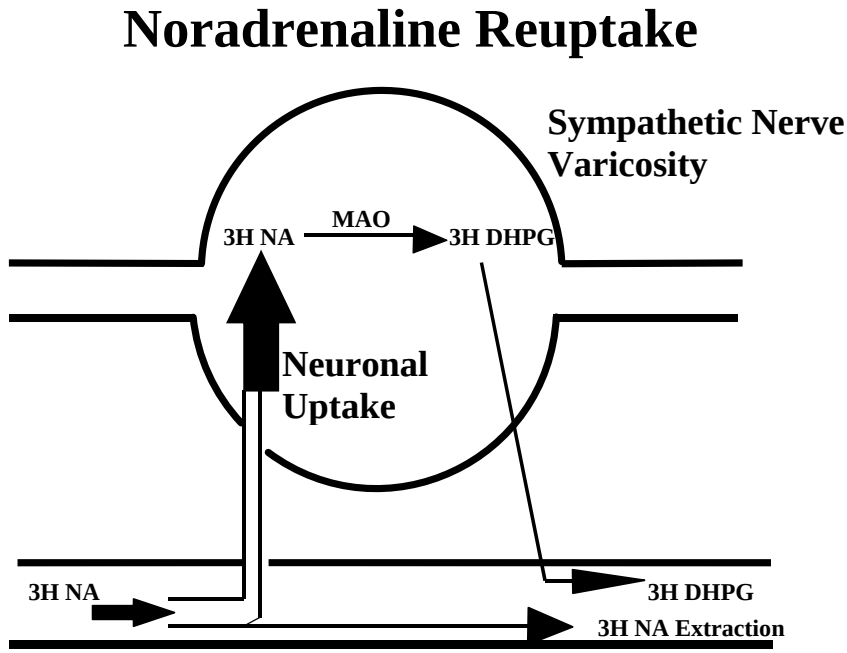

Figure 1. Measurement for norepinephrine spillover to plasma

The spillover method utilises radiotracer-derived measurements of the rate at which norepinephrine released from sympathetic nerve endings enters the plasma component (Figure 1). An important limitation of norepinephrine spillover is that it reflects not only release but also uptake processes that occur before the tracer reaches the circulation. In our experience, plasma norepinephrine concentrations from forearm venous sampling correlate closely with whole-body norepinephrine spillover measured by the isotope dilution technique (Figure 2. unpublished data) and muscle sympathetic nervous activity using microneurography [13, 14]. This suggests that forearm venous plasma norepinephrine levels are still practical for population-based studies, although current gold standard techniques for

the measurement of sympathetic nervous system activity in human include the procedures of microneurography and whole-body or regional norepinephrine spillover [15, 16].

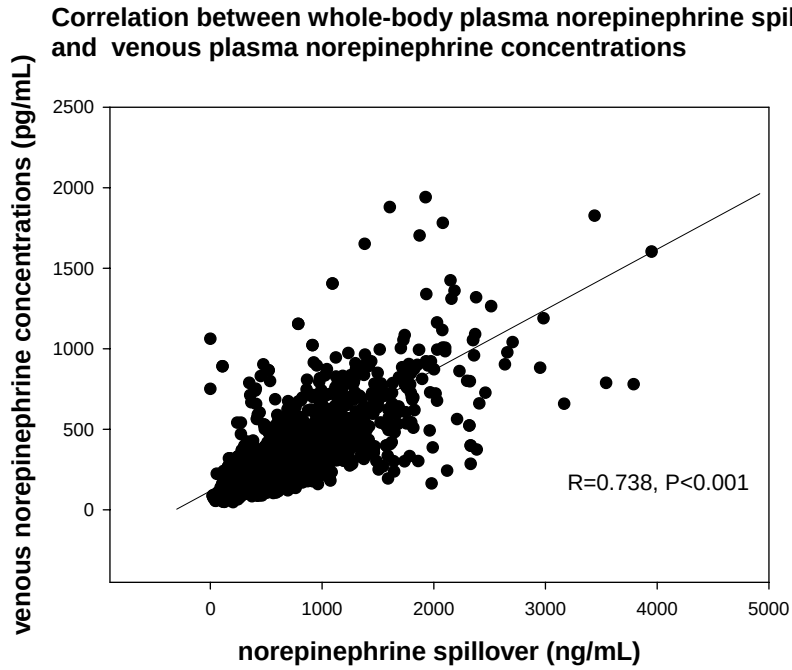

Figure 2. Strong correlation between whole-body plasma norepinephrine spillover and venous plasma norepinephrine levels (unpublished data)

Microneurography is a direct measure of peripheral postganglionic nerve firing directed towards blood vessels within skeltal muscle. The method requires percutaneous insertion of tungsten microelectrodes into a peripheral nerve (usually the peroneal or radial nerves) to record multi-unit spontaneous sympathetic action potentials. Recently the technique has been refined to measure single-unit recordings. Both norepinephrine spillover and microneurography require a high level of technical skill and equipment and are thus limited to small sample sizes.

Organ-specific differences in norepinephrine release and spillover rates have been demonstrated [17, 18]. Similarly, Anderson *et al.* [19] have shown differences in muscle sympathetic nervous activity between arms and legs using microneurography. Several investigators have shown a discrepancy between the whole-body plasma norepinephrine spillover and muscle sympathetic nerve activity measured by microneurography at the peroneal nerve, because sympathetic outflow to the skeletal muscle vasculature contributes approximately 20% to whole-body norepinephrine spillover [20]. Differences in multi-unit and single-nerve recording of the muscle sympathetic nerve activity have been reported in patients in essential hypertension [21].

Heart rate is used sometimes as an index of sympathetic nervous system activity [22, 23]. In 243 subjects consisting of normotensive and hypertensive subjects, and lean and obese subjects, heart rates, plasma norepinephrine concentrations and efferent postganglionic muscle sympathetic nerve activity (microneurography in the peroneal nerves) were measured

in the supine position. Supine heart rate was strongly correlated with both plasma norepinephrine and muscle sympathetic nerve activity, suggesting that supine heart rate can be regarded as a marker of sympathetic nervous system activity. However, the limitation for using heart rate as a marker of sympathetic nervous system activity has been well documented, because it is a function of both sympathetic nervous system activity and parasympathetic nerve activity.

Other indirect measures include autonomic reflex testing, autonomic blockade, and power spectral analysis of heart rate. Power spectral analysis is based on sophisticated mathematical models to determine the balance between sympathetic and vagal nerve activity on the heart. One criticism of this technique is that it has poor correlation with cardiac norepinephrine spillover [24].

Heightened Sympathetic Nervous System Activity in Hypertension

Results of many studies show that plasma norepinephrine and 24-hour urinary norepinephrine excretion are elevated in hypertensive patients compared with age-matched normotensive subjects [25-27]. Hypertensive patients [28] and borderline hypertensive patients [29] have elevated muscle sympathetic nerve activity measured by microneurography, and renal norepinephrine spillover indicative of increased renal sympathetic nerve activity [17]. Another method to evaluate sympathetic nerve activity in hypertensive patients involves examination of the depressor responses to sympathetic nervous antagonism, with blocking agents such as beta-blockers or central acting sympathetic suppressants, such as clonidine. The level of plasma norepinephrine predicts the degree of depressor response after acute administration of a beta-blocker or clonidine in essential hypertensive patients, suggesting a relationship exists between decreased sympathetic nervous system outflow and blood pressure reduction. Masuo *et al.* [30] have shown in a longitudinal study that higher baseline plasma norepinephrine levels in normotensive subjects predict future blood pressure elevations over a 10-year period independent of changes in body weight. Borderline hypertensive patients, who were strictly matched for age and body mass index (BMI) with normotensive subjects, had persistently elevated levels of plasma norepinephrine throughout the study. These observations indicate that heightened sympathetic nervous system activity plays an important role in the development and maintenance of hypertension.

Heightened Sympathetic Nervous System Activity in Obesity

Reduced energy expenditure and resting metabolic rate are predictive of weight gain (obesity). The sympathetic nervous system participates in regulating energy balance through thermogenesis [31]. Therefore, theoretically reduced sympathetic nervous system activity should be found in obesity. However, short-term overfeeding is known to activate the sympathetic nervous system in rats and humans [32, 33], thereby stimulating thermogenesis as a compensatory mechanism to limit further weight gain. In conditions of chronic high caloric consumption, shifts in body fat distribution and the onset of insulin resistance further

increase sympathetic nervous system activity. The evidence for increased sympathetic nervous system activity in established obesity will now be reviewed.

(1) Cross-sectional (epidemiological) studies in obesity

The Normative Aging Study of 752 non-diabetic, middle-aged men showed that subjects with the greatest body mass index (BMI) or greatest waist-to-hip ratio had the highest urinary norepinephrine excretion [34]. Grassi *et al*. [35] investigated muscle sympathetic nerve activity in 10 obese normotensive subjects versus aged matched lean normotensive subjects. Blood pressure levels at baseline were similar between lean and obese subjects, but muscle sympathetic nerve activity was significantly greater in obese subjects. Rumantir *et al.* [17] found that renal norepinephrine spillover was increased in both normotensive and hypertensive obese subjects, while cardiac norepinephrine spillover was significantly reduced in obese normotensive individual. These observations show that heightened sympathetic nervous system activity is closely linked to obesity. Further, Alvarez *et al.* [36,37] reported that visceral, but not abdominal subcutaneous, fat is associated with elevated muscle sympathetic nerve activity, demonstrating that fat distribution is important for heightened sympathetic nervous system activity.

(2) Longitudinal studies (weight loss studies and weight gain studies)

Several studies of longitudinal design have examined the effect of body weight changes (weight loss or weight gain) on sympathetic nervous system activity. Elevations of sympathetic nervous system activity during weight gain have been based on plasma norepinephrine concentrations in humans [38], muscle sympathetic nerve activity in humans [39, 40], and renal sympathetic nerve activity measured by microneurography in rats. Higher baseline plasma norepinephrine concentrations in originally nonobese subjects predicted subsequently significant weight gain (≥10% of BMI at entry period) and greater increases in plasma norepinephrine compared to those who had stable body weight [38, 41]. Conversely, during weight loss, there is a fall in blood pressure, which correlates with a reduction in plasma norepinephrine [1, 42-44], muscle sympathetic nerve activity [44-46] and whole body norepinephrine spillover [44]. These findings from weight change (weight gain or weight loss) studies with a longitudinal design would provide strong evidence for a close linkage of high sympathetic nervous system activity with obesity.

Sympathetic Nervous System Activity in Obesity-Related Hypertension

It is known that established obesity or being overweight, plus the process of gain in body weight, are commonly associated with hypertension [47-49]. In humans, sympathetic nervous system overactivity accompanies both obesity and hypertension. Thus, one could speculate that stimulated sympathetic nervous system activity might be a key factor for obesity-related hypertension. Insulin resistance, elevated plasma leptin (the adipocytes hormone), and stimulated the renin-angiotensin system which may contribute to sympathetic nervous system overactivity in obesity and hypertension [38, 46, 50-58].

(1) Epidemiological and longitudinal studies in obesity-related hypertension

Masuo *et al.* [59] have reported in 912 young, non-diabetic, Japanese men with a wide range of BMI that obesity (BMI) and heightened sympathetic nervous system activity (seen in plasma norepinephrine levels) were both significantly correlated with blood pressure levels in multiple regression analysis. Furthermore, higher plasma norepinephrine levels predicted future weight gain-related blood pressure elevation (obesity-related hypertension) in originally nonobese, normotensive men [38]. They have also examined plasma norepinephrine in a weight loss program of 6 months in 113 obese subjects. The subjects were subdivided into weight loss-sensitive to blood pressure reduction and weight loss-resistant to blood pressure reduction. Decreases in plasma norepinephrine levels were significantly less in subjects who failed to achieve blood pressure reduction despite weight loss. Plasma norepinephrine levels before weight loss were significantly greater in subjects who failed to decrease their blood pressure compared to those who succeeded in blood pressure reduction. Weight loss resistance and rebound weight gain after significant weight loss were also associated with higher baseline levels of plasma norepinephrine in another study [60]. These investigations demonstrate that 1) heightened sympathetic nervous system activity is associated with obesity-related hypertension, and that 2) heightened sympathetic nervous system activity might predict a failure to succeed in weight loss or in weight loss-induced blood pressure reduction.

(2) Insulin resistance and/or hyperleptinemia associated with heightened sympathetic nervous system activity are related to obesity or obesity-related hypertension, or mechanisms of obesity-related hypertension

(i) Insulin resistance

It is widely recognized that insulin resistance or hyperinsulinemia relates to hypertension and obesity [61, 62]. Anderson *et al.* [63] reported that acute increases in plasma insulin within the physiological range elevated sympathetic neural outflow in normal humans. A cross-sectional analysis in 512 normotensive subjects demonstrated that subjects with hyperinsulinemia had higher levels of plasma norepinephrine and higher blood pressure levels than subjects with normal insulin levels [64]. These findings show a close relationship between sympathetic nervous system activity and insulin levels. The sympathetic nervous system activity may be a prime mover for obesity-related hypertension, and insulin resistance may be an ancillary factor for weight gain-related blood pressure elevation [30, 65]. In contrast, many investigators have indicated that insulin resistance may play a major role in obesity-related hypertension, and sympathetic nervous system activity accompanies with insulin resistance [44, 50, 54].

(ii) Hyperleptinemia or leptin resistance

Leptin, a 16-kDa protein derived principally from adipose tissue, has been implicated in body weight homeostasis (weight loss) by reducing appetite and by increasing energy expenditure through sympathetic nerve stimulation to thermogenic tissue [56, 57, 66]. Agata *et al.* [67] have shown a positive relationship between plasma leptin and blood pressure levels in the Japanese population with essential hypertension. Eikelis *et al.* [68] have shown the existence of a strong correlation between plasma leptin concentration and renal norepinephrine spillover in men with widely differing adiposities. Intravenous infusion of

leptin to rats is accompanied by activation of sympathetic activity in the kidneys and hind limb vasculature with an increase in heart rate [66]. Subcutaneously administrated leptin leads to increases in energy metabolism, resting metabolic rate and sympathetic nervous system activity in both obese and lean subjects [69, 70]. These observations show the close relationships between plasma leptin, sympathetic nervous activity, and obesity-related hypertension.

Human obesity appears to generally be associated with leptin resistance (hyperleptinemia). The recent concept is that leptin resistance is selective to the metabolic effects of leptin, sparing its sympathoexcitatory actions [56, 57].

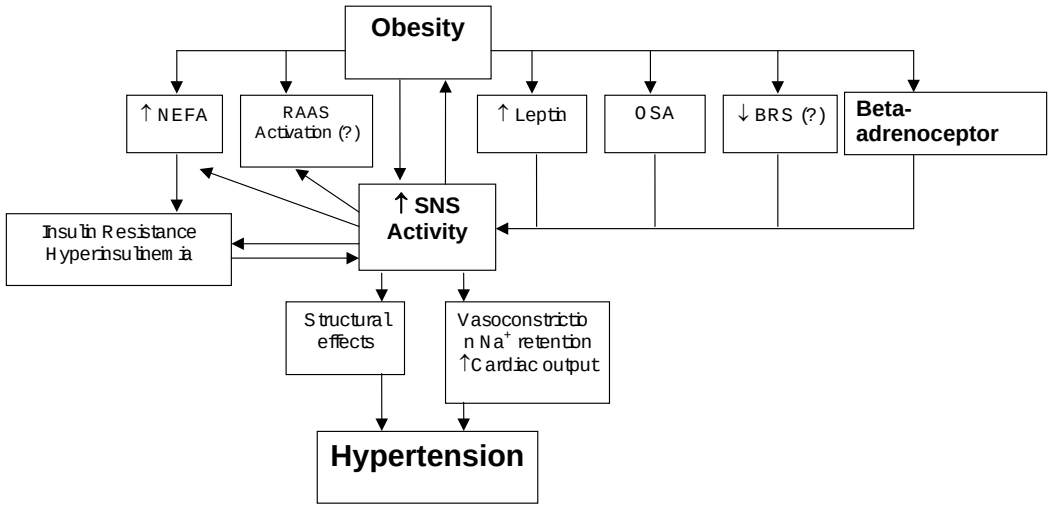

Figure 3. Potential pathophysiological mechanisms by which obesity may contribute to hypertension {modified figure from reference 48]. RAAS, renin-angiotensin-aldosterone system; SNS, sympathetic nervous system; OSA, obstructive sleep apnea; BRS, baroreflex sensitivity

In summary, sympathetic nervous system activity is closely linked with both plasma insulin and leptin levels [41, 58, 64, 71] (Figure 3). The relationships and interactions between sympathetic nervous system activation, insulin resistance and hyperleptinemia, especially in obesity or in metabolic syndrome, ware well documented in many reviews [51, 53, 54, 66].

(3) Confounding variables affecting sympathetic nervous system activity measurements (Table 1)

In 1992 Young and Macdonald [72] analysed 43 studies addressing plasma norepinephrine and epinephrine as indices of sympathetic nervous system activity in obesity. In their review, they noted that 11 studies found higher mean levels of plasma norepinephrine in obese compared with lean subjects, whereas 19 studies found no differences in mean plasma norepinephrine between lean and obese subjects. Conflicting data on the sympathetic nervous system activity in obesity as well as obesity-related hypertension have been observed, with high and normal levels, but not low levels. Table 1 shows the variables which may account for the discrepancies in sympathetic nervous system activity in hypertension, obesity, and obesity-related hypertension. .

Table 1. Confounding variables considered to cause the discrepancy of sympathetic nervous system activity measurements

Variables [reference number]	Findings in the studies
Gender [73-75]	Men have higher SNS activity compared to women.
Age [76, 77]	Elderly normotensive subjects have higher SNS activity compared to young normotensive subjects.
Ethnic differences [31, 78]	The relations of SNS activity with metabolic rate (energy expenditure) are different between the Caucasians and Pima Indians.
Genetic background of hypertension and obesity [2, 3, 79-81]	Subjects with a positive family history of hypertension or obesity have higher SNS activity compared to those without family histories.
Presence of obesity and hypertension [2, 3, 80]	Obese and hypertensive subjects have heightened SNS activity compared to lean and normotensive subjects.
Insulin resistance [30, 34, 41, 50, 62, 63, 82]	Subjects who are insulin resistant have heightened SNS activity.
Fat distribution [36, 37, 48, 83, 84]	Visceral fat, but not subcutaneous fat, is associated with heightened SNS activity and beta-adrenoceptor mediated lipolysis. Body fat is a major determinant of SNS activity.
Obstructive sleep apnea [85-87]	Subjects carrying OSA have heightened SNS activity.
Sodium intake [25, 88, 89]	Low sodium diet (NaCl <5g daily) elevates SNS activity compared to regular sodium diet (NaCl 7-12g daily) or high sodium diet (NaCl >15g daily).
Food consumption [90-92]	High fat diet stimulates SNS activity.
Posture [25]	Upright posture stimulates SNS activity compared supine posture.
Medications [92-95]	Angiotensin converting enzyme inhibitor inhibit SNS activity. Angiotensin II receptor blockers inhibit SNS activity. Beta-adrenergic blocker does not change SNS acvtivity. Caicium channel blockers stimulate or does not change SNS activity.

SNS, sympathetic nervous system; OSA, obstructive sleep apnea.

Role of β-Adrenergic Receptor Polymorphisms in Obesity and Obesity-Related Hypertension

The sympathetic nervous system plays an important role in the regulation of energy expenditure. A large part of the sympathetic nervous system -mediated energy expenditure takes place in skeletal muscle, via the coupling of catecholamines with β2-adrenoceptors. Catecholamines are also powerful regulators of lipolysis and act via β1-, β2-, β3- (stimulatory) and α2- (inhibitory) adrenoceptor subtypes in adipose tissue, where their role becomes especially important during both exercise and energy restriction, when increased need for fat as a fuel exists. Stimulation of β-adrenergic receptors by the sympathetic nervous system is a significant physiological modulator of pre- and postprandial energy expenditure [96-98] and total daily energy expenditure [90, 91].

Recent studies show that β-adrenoceptors are polymorphic. Single nucleotide polymorphisms might have functional consequences in terms of receptor activity and regulation and hence may contribute to the pathophysiology of hypertension and obesity.

β1-Adrenoceptor Polymorphisms

The β1-adrenoceptor is predominantly expressed in cardiac myocytes and adipose tissue, where its activation leads to increased heart rate and contractility and stimulation of lipolysis, respectively. The β1-adrenoceptor is a candidate gene for obesity because of its role in catecholamine mediated energy homeostasis. In obese individuals, the degree of weight loss during a very low calorie diet has been shown to correlate with changes in β1-adrenoceptor protein concentration in adipose tissue [99]. The two most common β1-adrenoceptor polymorphisms are Ser49Gly and Arg389Gly, with relative allele frequencies of 0.85/0.15 and 0.70/0.30 respectively. A population cohort of 761 women showed that women carrying the Gly49 genotype had greater increases in BMI over15 years compared to those with the Ser49 genotype [100]. Conversely, the distribution of the Arg389Gly polymorphism is similar in lean and obese subjects, suggesting that it has no important influence on human obesity [101]. Although earlier small case-control studies demonstrated an increase in the risk of hypertension in Arg389 homozygotes [102, 103], a recently published study comprising 3981 normotensive and 2518 hypertensive patients failed to replicate this association [104].

β2-Adrenoceptor Polymorphisms

The β2-adrenoceptor is the dominant lipolytic receptor in white human adipose tissue [98] and in skeletal muscle [97]. It also plays an important regulatory role in the peripheral vasculature. Genetic polymorphisms of the β2-adrenoceptor have been associated with obesity, diabetes mellitus and hypertension. The most common polymorphisms are Arg16Gly, with an allele frequency of 0.40/0.60 and Gln27Glu, with an allele frequency of 0.55/0.45. The Thr164Ile polymorphism is rare, occurring in only 3 to 5% of the general population in Caucasians population.

Table 2. Arg16Gly β2-adrenoceptor polymorphisms: associations with obesity, hypertension, obesity-related hypertension, and type2 diabetes (DM)

Authors	Year	Populations	Subjects	Associations with the polymorphism
Large V et al. [107]	1997	Swedish	140 Caucasian women with a wide range of obesity	obesity
The Quebec Family Study [112]	2000	Canada	Caucasian men and women	obesity, hyperlipidemia
Hayakawa T et al. [113]	2000	Japanese	210 Japanese men from a population	No association with obesity
Jia H et al. [114]	2000	USA	Caucasians (298 hypertensive vs 298 normotensive subjects)	No association with hypertension
Xie HG et al. [115]	2000	USA	Black and white Americans (including normotensive and hypertensive subjects)	No associations with hypertension
Candy G et al. [116]	2000	England	Black African men (including 192 hypertensive and 123 normotensive men)	No association with hypertension
Cockcroft JR et al. [117]	2000	Caucasian	127 young normotensive men	forearm vascular responses (hypertension)
Meirhaeghe A et al. [8]	2000	French	1195 middle-aged Caucasian from the urban population	obesity, if subjects carry Gln27Gln
Kato N, et al [118]	2001	Japanese	842 hypertensive and 633 normotensive subjects	BP levels (hypertension) in normotensives
Bengtsson K et al. [119]	2001	Swedish	Hypertensive patients with and without type 2 DM	hypertension in subjects with DM
The Bogalusa Heart Study [120]	2002	USA	1151 Caucasian and Black-Africans childhood (including boys and girls)	weight gain in males
Kim SH et al. [121]	2002	Korean	type 2 DM patients	obesity, DM, hyperlipidemia
Chang TJ et al. [122]	2002	Taiwanese	type 2 DM patients	type 2 DM
Van Rossum CT et al. [123]	2002	Dutch	286 subjects with a significant weight gain Over 7 years including men and women	weight gain in men, but not in women
The HERITAGE family study [124]	2003	Canada	sedentary black and white women	lower fat in obese white women
Pereira AC et al. [6]	2003	Brazilian	1576 ethnically mixed population (including men and women)	systolic BP, BMI
The Olivetti heart study [125]	2004	Italian	993 middle-aged men regardless of BP levels or BMI	No association with obesity or hypertension
Tafel J et al. [126]	2004	Germany	extremely obese children	No association with obesity
Ellsworth DL et al. [127]	2005	USA	Black and white American men and women	BMI (obesity) in only men
Trombetta IC et al. [128]	2005	Brazilian	Brazilian healthy women	hypertension (blunted forearm vasodilation response)
Masuo K et al. [6]	2005	Japanese	nonobese, normotensive men	weight gain, BP elevation, obesity-HT
Masuo K et al. [12]	2005	Japanese	nonobese, normotensive men	insulin resistance
Masuo K et al. [129, 130]	2006	Japanese	normotensive men (including nonobese and obese men)	weight gain, blunted leptin-sympathetic axis
Gjesing AP, et al. [137]	2007	Dutch	7808 white subjects	No association with hypertension or obesity

BP, blood pressure; BMI, body mass index; HT, hypertension; DM, diabetes mellitus

Table 3. Studies showing associations between Gln27Glu, β2-adrenoceptor polymorphisms, obesity, hypertension, obesity-related hypertension, and type2 diabetes (DM).

Authors [reference number]	Year	Populations	Subjects	Associations with the polymorphism
Large V et al. [107]	1997	Swedish	Caucasian women with a wide range of obesity	obesity
Echwald SM et al. [131]	1998	Danish	Caucasian juvenile-onset obese men	No association with obesity
Hellstrom L et al. [132]	1999	Swedish	Caucasian men and women	obesity only in women
Kortner B et al. [133]	1999	German	Caucasian with morbid obesity	No association with obesity
Xie HG et al. [115]	2000	USA	Black and white Americans	No associations with hypertension
The Quebec Family Study [112]	2000	Canada	Caucasian men and women	obesity, hyperlipidemia
Hayakawa T et al. [113]	2000	Japanese	210 Japanese men from a population	No association with obesity
Candy G et al. [116]	2000	England	Black African men (including 192 hypertensive and 123 normotensive men)	No association with hypertension
Meirhaeghe A et al. [8]	2000	French	1195 middle-aged Caucasian from the urban population	obesity in men
Kato N, et al [118]	2001	Japanese	842 hypertensive and 633 normotensive subjects	BP levels (hypertension) in normotensives
Kawamura T et al. [134]	2001	Japanese	Japanese-Americans	No association with obesity or DM
Ukkola O et al. [135]	2002	USA	12 pairs of twins, Caucasians	weight gain (obesity)
Kim SH et al. [121]	2002	Korean	patients with type 2 DM	obesity, DM, hyperlipidemia
Gonzalez-Sanchez JL et al. [136]	2003	Spanish	666 Caucasian based study (including men and women)	obesity only in men
The HERITAGE family study [119]	2003	Canada	sedentary black and white men	lower fat in obese white men
Pereira AC et al. [6]	2003	Brazilian	1576 ethnically mixed population (including men and women)	No association with systolic BP or BMI
The Olivetti heart study [125]	2004	Italian	993 middle-aged men (regardless of BP levels or BMI)	No association with obesity or hypertension
Tafel J et al. [126]	2004	Germany	extremely obese children	No association with obesity
Masuo K et al. [7]	2005	Japanese	nonobese, normotensive men	BP elevation
Trombetta IC et al. [128]	2005	Brazilian	Brazilian healthy women	hypertension (blunted forearm vasodilation response)
Gjesing AP, et al. [137]	2007	Dutch	7808 white subjects	No association with hypertension or obesity

BP, blood pressure; BMI, body mass index, DM, diabetes mellitus; NIDDM, non-insulin dependent diabetes mellitus

Table 4. Studies showing associations between Trp64Arg, β3-adrenoceptor polymorphisms, obesity, hypertension, obesity-related hypertension, and type2-diabetes mellitus (DM)

Authors [reference number]	Year	Populations	Subjects	Associations with the polymorphism
Clement K et al. [145]	1995	French	185 subjects with morbid obesity and 94 subjects with normal weight	increased capacity of weight gain
Widen E et al. [146]	1995	Finns	335 subjects including 207 non-DM and 128 patients with NIDDM	insulin resistance
Fujisawa T et al. [147]	1996	Japanese	patients with NIDDM	type 2 DM, weight gain (obesity)
Shiwaku K et al. [148]	1998	Japanese	moderate overweight men	No association with obesity
Kurokawa N et al. [133]	2001	Japanese	meta-analysis in 6582 subjects	BMI (obesity)
Ochoa MC et al. [149]	2004	Spanish	185 obese and 185 nonobese children	BMI (obesity)
Ellsworth DL et al. [127]	2005	USA	1179 African-Americans and white-Americans	BMI (obesity)
Masuo K et al. [7]	2005	Japanese	nonobese, normotensive men	BP elevation
Masuo K et al. [130]	2006	Japanese	55 obese normotensive men	weight gain (obesity), BP elevation (hypertension)

BP, blood pressure; BMI, body mass index; DM, diabetes mellitus; NIDDM, non-insulin dependent diabetes mellitus

Table 5. Important confounding variables considered to cause the discrepancy of the relationships between β-adrenoceptor polymorphisms and phenotypes of obesity and hypertension

Variables [reference number]	Findings in the studies
Severity of obesity [4, 126, 145, 149, 152]	In lean subjects, β2-AR polymorphisms linked to obesity and obesity-related hypertension, but in obese subjects, β2- and β3-AR polymorphisms relates to obesity and obesity-related hypertension. Morbid obesity is linked with β3-AR polymorphisms, but overweight or mild obesity is not associated with Those.
Gender differences [136]	Interaction between β1- and β2-AR polymorphisms with changes in BMI was observed in men only, while in women an interaction between beta1- and β3-AR polymorphisms was observed in a longitudinal over a 24-year period large cohort study.
Ethnic difference [153]	Distributions of β-AR polymorphisms are different in 8 different ethnic population.
Haplotype [6, 127, 128, 150, 151, 154]	Functions expressed of β-AR polymorphisms are different due to the other β-AR polymorphisms.

AR, adrenoceptor; BMI, body mass index

Studies of agonist stimulation in cultured cells demonstrate that Gly16 receptors have a greater reduction in numbers or enhanced down-regulation when compared with Arg16, whereas the Glu27 receptor is resistant to down regulation when compared with the Gln27 variant [105]. A number of clinical studies have investigated the impact of these polymorphisms on vascular responsiveness [106, 107]. Gratze et al. [108] found that young normotensive white men homozygous for the Gly16 allele had higher blood pressure and lower peripheral vasodilation after infusion of the β2- agonist salbutamol. Similar results were obtained by Hoit et al. [109] using the agonist terbutaline. On the other hand, three studies investigating isoprenaline induced increase in the limb blood flow found that volunteers homozygous for Gly16 exhibited larger vasodilatory responses than did volunteers homozygous for Arg16 [110]. Conflicting results have also been published with regards to the effects of genetic variants on the sympathetic nervous system modulation of energy expenditure. Bell et al. [111] reported that the response of resting energy expenditure to non-specific β-adrenoceptor stimulation (with isoproterenol infusion) was not different between the 3 genotypes of Arg16Gly. Stob et al. [112] showed that individuals carrying the Arg16Arg variant of the β2-adrenoceptor gene have a reduced thermogenic response to selective β2-adrenoceptor activation.

Associations of β2-adrenoceptor polymorphisms with obesity and hypertension have been reported in many epidemiological studies but results are also discordant (summarised in Tables 2 and 3).

β3-Adrenoceptor Polymorphisms

The β3-adrenoceptor, which is mainly expressed in adipose tissue differs from the β2-adrenoceptor in two ways: it has a lower affinity for catecholamines, and it resists desensitisation (i.e. down-regulation). These characteristic differences might lead to the different effects of catecholamine on β2-adrenoceptor and β3-adrenoceptor. β3-adrenoceptor stimulates the mobilization of lipids from the white fat cell and increases thermogenesis in brown fat cell. Decreased function of β3-adrenoceptor in white adipose tissue could slow lipolysis and thereby cause the retention of lipids in fat cells. Slow lipolysis may contribute strongly to visceral obesity in human, and treatment of obese animal models with selective β3-adrenergic agonists reduces fat stores most effectively [138-140]. Many epidemiological studies have shown the strong relationships between β3-adrenoceptor polymorphisms (mainly Trp54Arg), obesity, metabolic syndrome, and hypertension [138, 139, 141-144] (Table 4).

(4) Confounding variables affecting the relations of β-adrenoceptor polymorphisms with obesity and hypertension (Table 5)

Tables 2, 3, and 4 show the discordant contributions of β-adrenoceptor polymorphisms to obesity, hypertension, or type2 diabetes (metabolic syndrome). Table 5 summarizes factors which might explain the discrepancy of published data. Further, haplotypes of polymorphisms have strong influence on β-adrenoceptor function in each polymorphism [6, 127, 128, 150, 151].

Sympathetic Nervous System Activity and β2- and β3-Adrenoceptor Polymorphisms in Obesity, Hypertension, and Obesity-Related Hypertension (Figure 3)

Many studies have been examined to determine the associations of the β2- or β3-adrenoceptor polymorphisms with BMI (obesity) and blood pressure (hypertension) as mentioned above. Figure 3 shows the potential pathophysiological mechanisms including the β-adrenoceptor polymorphisms by which obesity may contribute to hypertension [53]. Only on study has included measurements of sympathetic nervous system activity [7].

Masuo et al. [7] have longitudinally clarified the relevance of β-adrenergic receptor (Arg16Gly and Gln27Glu of β2-arenoceptor and Trp64Arg of β3-adrenoceptor) polymorphisms related to weight gain (obesity) and blood pressure elevations (hypertension) in 160 originally nonobese normotensive Japanese men. The Gly16 allele was related to greater weight gain and blood pressure elevations, and Glu27 and Trp64 alleles are linked to only blood pressure elevations. The originally nonobese, normotensive subjects carrying the Gly16 allele of Arg16Gly, β2-adrenoceptor polymorphisms accompanying with entry high plasma norepinephrine levels linked to weight gain (obesity) and blood pressure elevations (hypertension) and weight gain-induced blood pressure elevation (obesity-related hypertension).

In a weight loss study over a 24-month period, the Gly16 allele of Arg16Gly, the β2-adrenoceptor, was associated with resistance to long-term (24 months) significant weight loss, and the Glu27 allele was linked to resistance to short-term (6 months) weight loss [11]. Nonobese normotensive men carrying the Gly16 allele of Arg16Gly, the β2-adrenoceptor polymorphisms had higher frequency of HOMA index elevation as an index of insulin resistance, which is generally observed in hypertension and obesity [12, 30]. These studies are strong evidence for the linkage between the β2-adrenoceptor polymorphisms accompanying with heightened sympathetic nervous system activity, obesity, hypertension, obesity-related hypertension, and insulin resistance.

The slopes between plasma leptin versus plasma norepinephrine levels, was much lower in subjects carrying the Gly16 allele of Arg16Gly, β2-adrenoceptor, compared to those without the Gly16 allele, suggesting that β2-adrenoceptor polymorphisms might relate to blunted leptin-stimulated sympathetic activity and result in lower thermogenesis [129, 130]. These findings support the previous investigations that the β2-adrenoceptor polymorphism (the Gly16 allele of Arg16Gly) leads to lower thermogenesis through lower stimulations on sympathetic nervous system activity .

Renal injury evidenced as proteinuria predictes the development of cardiovascular disease [155]. Obese subjects and hypertensive patients have higher risk for renal injury. Many investigators have reported that plasma norepinephrine and heightened sympathetic nerve activity predict survival and incident cardiovascular events in large cohort longitudinal studies [156]. The subjects carrying the Gly16 allele, β2-adrenoceptor polymorphism associated with high plasma norepinephrine at entry link to significant elevations in blood urea nitrogen, creatinine and creatinine clearance rate over a 5-years period in nonobese, normotensive individuals without significant weight gain or blood pressure elevations. The

observation demonstrates that the β2-adrenoceptor polymorphisms might also predict future renal function deterioration through high sympathetic nervous system activity [157].

It should be noted that this series of studies are the first simultaneously to investigate both the β2- and β3−adrenoceptor polymorphisms and sympathetic nervous system activity (observed in plasma norepinephrine levels). The β2-adrenoceptor polymorphism (Arg16Gly) accompanied by heightened sympathetic nervous system activity (high plasma norepinephrine) is strongly linked to hypertension, obesity, obesity-related hypertension through blunted sympathetic nerve activation, and several status (i.e. insulin resistance, renal injury) related with obesity or hypertension in a Japanese male cohort.

Summary

The role of the sympathetic nervous system, β2- and β3-adrenoceptor polymorphisms on hypertension, obesity and obesity-related hypertension are discussed through a literature review, matched against our own findings. Relevant studies of the β2- and β3-adrenoceptor genes in relation to obesity and hypertension were investigated. Relevant studies of sympathetic nervous system activity or β-adrenoceptor polymorphisms (mainly β2- and β3-adrenoceptor polymorphisms) might contribute to the onset and maintenance of obesity, hypertension, and obesity-related hypertension, however, few studies have been performed to evaluate the relationship between β2- and β3-adrenoceptor polymorphisms and sympathetic nervous system activity during the same study. A better understanding for the relationships of genetic background (polymorphisms) with sympathetic nervous system activity as the cause for blood pressure elevation and weight gain might help for clinical implications (treatment) for obesity-related hypertension. Further, to clarify the pathogenesis and mechanisms of obesity-related hypertension may lead to prevention of obesity-related hypertension. Further studies are needed to clarify these relationships and the mechanisms of obesity-related hypertension,

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Chapter 3

ROLE OF RHO/RHO-KINASE IN THE BRAINSTEM IN CARDIOVASCULAR REGULATION VIA THE SYMPATHETIC NERVOUS SYSTEM

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Abstract

The small GTPase Rho and its downstream effector Rho-kinase are implicated in various cellular functions and in the pathogenesis of hypertension. We recently published a series of studies demonstrating that Rho/Rho-kinase in the brainstem is involved in central cardiovascular regulation via the sympathetic nervous system. Rho/Rho-kinase activity in the brainstem is greater in spontaneously hypertensive rats and in another type of hypertensive rat model (caused by chronic nitric oxide synthase inhibition) compared to normotensive Wistar-Kyoto rats. Inhibition of Rho-kinase in the brainstem by microinjection of Rho-kinase inhibitors (i.e., Y-27632 or hydroxyfasudil) or an adenovirus vector encoding a dominant-negative Rho-kinase decreases arterial pressure, heart rate, and renal sympathetic nerve activity and augments baroreflex function, which might be due to enhanced glutamate sensitivity. The magnitude of the effects of Rho-kinase inhibitions was greater in hypertensive rat model compared to normotensive rat model. In addition, ovariectomy increase hypertension through Rho-kinase activation in the brainstem in female spontaneously hypertensive rats. Finally, the pressor response induced by central angiotensin II is mediated by activation of the Rho/Rho-kinase pathway via angiotensin II type 1 receptors. In this review, we describe our series of studies and novel pathophysiological implications of Rho/Rho-kinase in the brainstem related to the neural mechanisms of hypertension.

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Introduction

Recent advances in molecular biology have elucidated the important role of small GTP-binding proteins (G proteins), such as the Rho family, in mediating various cellular functions [1,2]. Rho-kinase is a RhoA target protein [3] and its substrate has been identified. They include the myosin binding subunit of myosin light chain phosphatase [4], ERM family (ezrin, radixin, moesin) [5], adducin [6], Na^+-H^+-exchanger [7], and LIM-kinase [8]. Recent studies suggest that the Rho/Rho-kinase pathway is involved in the pathogenesis of hypertension. For example, Rho-kinase inhibition reduces arterial pressure in various hypertensive models [9,10,11,12]. In addition, Rho-kinase inhibition reduces the increased forearm vascular resistance in patients with hypertension [13]. Thus, the Rho/Rho-kinase pathway plays a role in peripheral mechanisms of hypertension and Rho/Rho-kinase inhibitor is reported to have therapeutic potential for cardiovascular diseases [14,15]

RhoA and Rho-kinase are also distributed in the central nervous system [16,17], and the Rho/Rho-kinase pathway is involved in the maintenance of dendritic spines [18], neurite remodeling [19], and axon outgrowth in vitro[20]. These morphological changes are actin-dependent and are regulated by Rho/Rho-kinase [21].

Dendritic spines form the postsynaptic contact sites for the majority of excitatory synapses in the central nervous system. Recent studies suggest that morphological changes in dendritic spines occur rapidly and are associated with synaptic transmission and sensitivity to glutamate, an excitatory neurotransmitter [21-23]. Brains are enriched in the GTPase-activating protein, p250GAP, which co-exists with RhoA in dendritic spines, and is involved in *N*-methyl-D-aspartate (NMDA) glutamate receptor activity-dependent actin reorganization in the dendritic spines [24]. Furthermore, RhoA interacts with glutamate receptors at the level of the postsynaptic density in dendritic spines and regulates dendritic spine actin in a Rho-kinase dependent manner. Such RhoA-dependent dendritic spine actin regulation is rapidly modulated in response to α-amino- 3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA) or NMDA stimulation [25]. Therefore, the Rho/Rho-kinase pathway is thought to possibly have an important role in the establishment and maintenance of synaptic transmission, particularly in excitatory synapses. Furthermore, there are structural differences in dendritic spines in the nucleus tractus solitarii (NTS) in the brainstem between Wister-Kyoto rats (WKY rats) and spontaneously hypertensive rats (SHR) [26]. The NTS has an important role in regulating arterial pressure and sympathetic nerve activity [27]. The NTS forms excitatory synapses and receives signals through afferent fibers from arterial baroreceptors, chemoreceptors, cardiopulmonary receptors, and other visceral receptors [28]. Thus, the NTS plays an important role in the integration of the cardiovascular system. These findings led to the hypothesis that the Rho/Rho-kinase pathway in the brainstem, particularly in the excitatory synapses of the NTS, affects synaptic transmission via the modulation of glutamate sensitivity. Therefore, we examined the role of the Rho/Rho-kinase pathway in the brainstem in central cardiovascular regulation. In this article, recent advances in the research on the importance of the Rho/Rho-kinase pathway in central cardiovascular regulation and in the pathogenesis of hypertension are summarized.

RhoA and Rho-Kinase Protein Expression and Rho/Rho-Kinase Activity in the NTS

The NTS tissues were obtained by punching out the NTS from coronal slices (2-mm thick) of the brainstem with an 18-gauge needle (inside diameter: 950 μm). The RhoA and Rho-kinase protein levels in the NTS were confirmed by Western blot analysis using the rabbit IgG polyclonal antibody to RhoA (1:1000, Santa Cruz Biotechnology) and mouse IgG monoclonal antibody to Rho-kinase (1:1000, Transduction Laboratories). RhoA activity was examined by the translocation of RhoA protein in the membrane [29] and Rho-kinase activity was examined by the phosphorylation of the ERM family, which are target proteins of Rho-kinase [5], using rabbit IgG to antiphosphorylated ERM family members : moesin (Thr 558), ezrin (Thr 567) , and radixin (Thr 564), which are target proteins of Rho-kinase [5]. The expression of RhoA in the membrane fraction was significantly higher in SHR than in WKY rats. There were no significant differences in the expression of Rho-kinase protein between SHR and WKY rats. The extent of the phosphorylated ERM family, which reflects the Rho-kinase activity, was significantly higher in SHR than in WKY rats. These results indicated that the activity of the Rho/Rho-kinase pathway in the NTS was greater in SHR than in WKY rats [30]. Activation of the Rho/Rho-kinase pathway in the NTS was also demonstrated in another hypertensive model caused by chronic inhibition of nitric oxide (NO) synthesis [31]. Therefore, activation of Rho/Rho-kinase pathway in the NTS is thought to play an important role in the central mechanisms of hypertension.

Effects of Rho-Kinase Inhibition in the NTS on Cardiovascular Regulation

As pharmacologic inhibitors of Rho-kinase, fasudil [32] and Y-27632 [9] inhibit Rho-kinase activity in a competitive manner with ATP [33]. Hydroxyfasudil, a major active metabolite of fasudil, has a more specific inhibitory effect on Rho-kinase [34]. The Ki values (μM) of hydroxyfasudil and Y-27632 are 0.17 and 0.14 for Rho-kinase, 18 and 26 for protein kinase C, and 140 and >250 for myosin light chain kinase, respectively [9, 34].

Therefore, we used Y-27632 and hydroxyfasudil as specific Rho-kinase inhibitors and examined the effects of these Rho-kinase inhibitors in the NTS on cardiovascular regulation in WKY rats as normotensive rats, and SHR and WKY rats treated with the NO synthase inhibitor (N^{ω}-nitro-L-arginine methyl ester [L-NAME]) as hypertensive rats in our studies. L-NAME was administered to WKY rats in their drinking water (1 mg/mL) for 2 weeks [31].

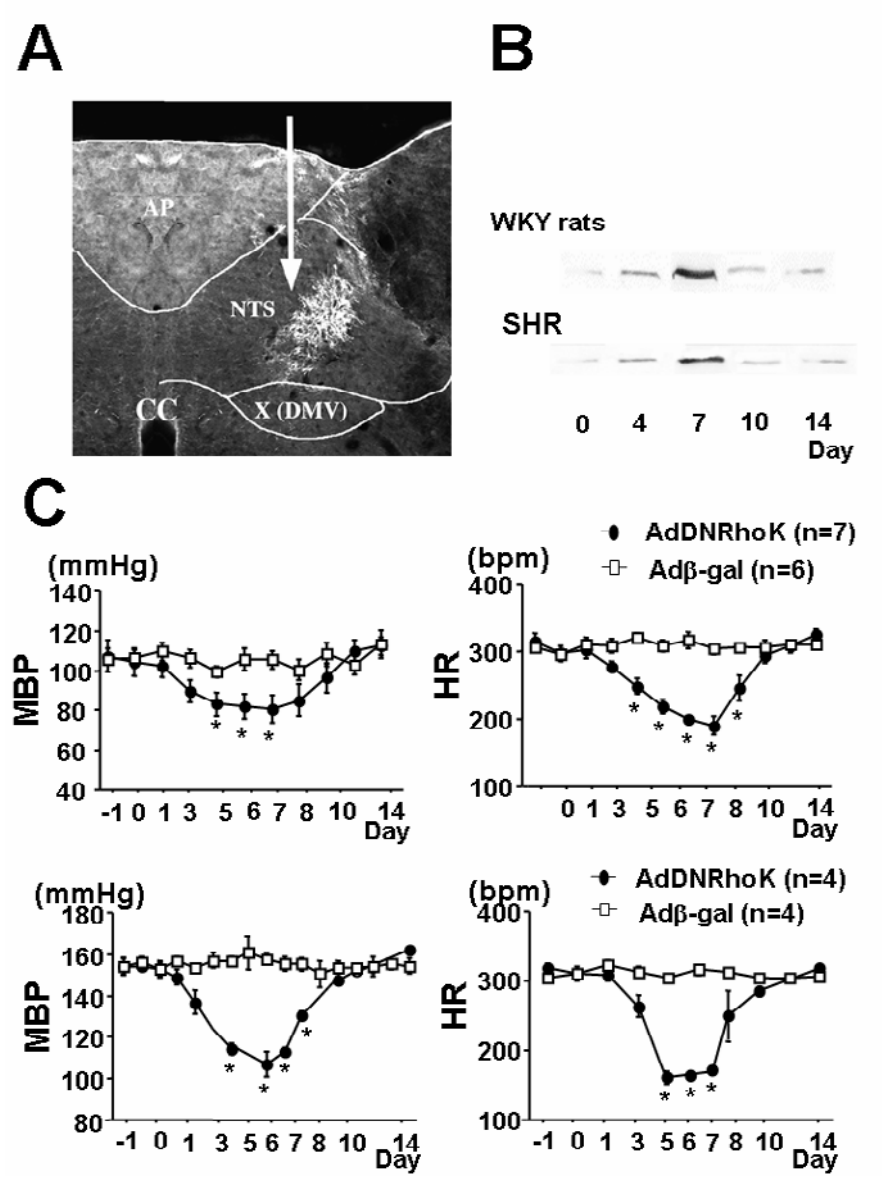
Microinjection of Y-27632 into the NTS elicited a dose-dependent decrease in arterial pressure and heart rate (HR) in both normotensive and hypertensive rats [30,31]. The magnitude of the decreases in arterial pressure and HR, however, was significantly greater in hypertensive rats than in normotensive rats. Furthermore, microinjection of Y-27632 into the NTS decreased renal sympathetic nerve activity, as a marker of sympathetic nerve activity, and the magnitude of the decreases in renal sympathetic nerve activity were significantly greater in hypertensive rats than in normotensive rats. Microinjection of hydroxyfasudil into the NTS also decreased arterial pressure and HR, similar to Y-27632 [35]. These results indicate that inhibition of Rho-kinase activity in the NTS decreases arterial pressure and HR via the sympathetic nervous system.

Effects of Rho-Kinase Inhibition in the Rostral Ventrolateral Medulla (RVLM) on Cardiovascular Regulation

Microinjection of hydroxyfasudil into the RVLM did not alter arterial pressure and HR in WKY rats. In SHR, however, microinjection of hydroxyfasudil into the RVLM slightly increased arterial pressure [35]. Because we performed only acute experiments regarding the effects of Rho-kinase in the RVLM on arterial pressure regulation, further studies are needed to clarify the role of Rho-kinase in the RVLM.

Effects of Rho-Kinase Inhibition by the Gene Transfer of Dominant Negative Rho-Kinase on Cardiovascular Regulation

Rho-kinase has a catalytic (kinase) domain in its N-terminal domain, a coiled-coil domain in its middle portion, and a putative pleckstrin-homology domain in its C-terminal domain. The Rho-binding domain of Rho-kinase is located in the C-terminal portion of the coiled-coil domain and Rho-kinase activity is enhanced by binding GTP-Rho [3]. The kinase activity-deficient form or the C-terminal fragments that lack the kinase activity should theoretically serve as the dominant-negative form of Rho-kinase [36]. The Rho-binding domain, a dominant-negative Rho-kinase mutant driven by the cytomegalovirus promoter and containing a c-myc tag, was prepared through homologous recombination between cotransfected pJM17 and shuttle plasmids in 293 cells. Integration of the transgene into the adenoviral genome was determined by polymerase chain reaction and restriction analysis. An adenoviral suspension containing 1×10^8 plaque forming units/ml was microinjected into four sites into the NTS over 20 minutes in both WKY rats and SHR [30]. Transfection of adenovirus vectors encoding dominant-negative Rho-kinase (AdDNRhoK) was confirmed by immunohistochemistry and Western blot analysis for c-myc, a marker of AdDNRhoK (Figure 1A and B). The expression of c-myc was increased and peaked at day 7 after AdDNRhoK transfection. The magnitude of the increase in c-myc expression did not differ between WKY rats and SHR. To confirm the inhibition of Rho-kinase activity by AdDNRhoK transfection, we examined the phosphorylation of the ERM family and α-adducin, which are target proteins of Rho-kinase, in the NTS. The extent of ERM phosphorylation was greater in SHR than in WKY rats before the gene transfer. After AdDNRhoK transfection, phosphorylation of ERM family and α-adducin was significantly reduced in both WKY rats and SHR. Further, we used β-galactosidase (Adβgal) as a control for the gene transfer in this study [37,38]. Figure 1C shows the time course of arterial pressure and HR before and after AdDNRhoK or Adβgal transfection into the NTS. Arterial pressure and HR were monitored in awake free-moving rats using radio-telemetry system [37,38]. Arterial pressure and HR were significantly decreased 5 to 7 days after AdDNRhoK transfection, but not after Adβgal transfection in both WKY rats and SHR. The time course of the changes in arterial pressure and HR corresponded to the time course of c-myc expression. The magnitude of the decrease in arterial pressure and HR was significantly greater in SHR than in WKY rats (mean arterial pressure: -52±3 mmHg versus -33±4 mmHg, $P<0.05$, HR: -161±6 bpm versus -120±13 bpm, $P<0.05$). Twenty-four hour urinary norepinephrine excretion, a marker of sympathetic nerve activity, measured at day 7 after the gene transfer, was decreased after AdDNRhoK



*P<0.05 versus Adβgal-transfected rats
(Adopted and modified from Ito et al., Circ Res 2003;92:1337-1343)

Figure 1. **A:** Laser scanning microscopy image of a section of the medulla stained with anti-c-myc antibody, an AdDNRhoK tag protein (high intensity, visualized with FITC-conjugated fluoroprobe). **B:** Representative Western blot analysis demonstrating the expression of c-myc, an AdDNRhok tag protein, in the medulla containing the NTS of WKY rats and SHR. **C:** Time course of mean arterial pressure and HR in Adβgal-transfected and AdDNRhoK-transfected rats before and after the gene transfer in WKY rats and SHR.

transfection. Furthermore, 24-hour urinary norepinephrine excretion before the gene transfer was higher in SHR than in WKY rats, and the magnitude of the decrease was greater in SHR than in WKY rats (-0.80±0.12 μg/dl versus -0.48±0.07 μg/dl, n=6 for WKY rats, n=4 for SHR, P<0.05). These results suggest that inhibition of Rho-kinase in the NTS by AdDNRhoK

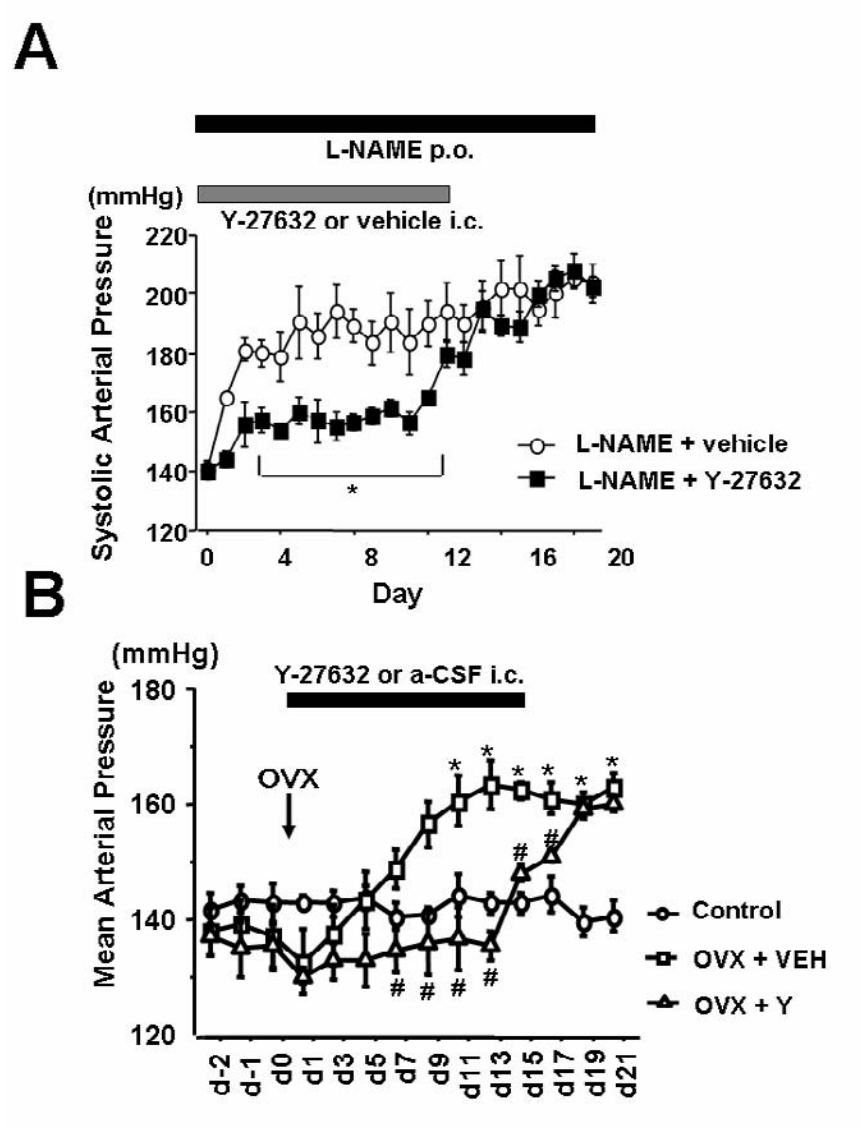
transfection decreases arterial pressure, HR, and sympathetic nerve activity in awake. In addition, the effects of Rho-kinase inhibition were significantly greater in SHR than in WKY rats. These results indicate that activation of Rho/Rho-kinase pathway is involved in the central mechanisms underlying hypertension via the sympathetic nervous system.

In addition, we examined the baroreflex control of HR by changing arterial pressure with intravenous infusion of phenylephrine or sodium nitroprusside [39]. The maximum gain of baroreflex control of HR was attenuated in SHR compared with WKY rats before the gene transfer. The transfer of AdDNRhoK significantly augmented the maximum gain in both WKY rats and SHR. The extent of this augmentation, however, was greater in SHR than in WKY rats. After treatment with metoprolol, the maximum gain was significantly decreased in AdDNRhoK-transfected rats, but not in control rats. In contrast, after treatment with atropine, the maximum gain was greater in AdDNRhoK-transfected rats compared with control rats, although it was decreased in both groups. These results indicate that inhibition of Rho-kinase in the NTS augments baroreflex control of HR in both WKY rats and SHR, probably because of a cardiac sympathoinhibitory effects.

Effects of Rho-Kinase Inhibition in the Brainstem on Arterial Pressure Elevation Caused by NO Synthase Inhibition

Inhibition of NO synthase produces hypertension in many species [40,41]. Although this hypertension was initially attributed to the inhibition of endothelial NO synthesis, numerous studies suggest that inhibition of neuronal NO also has an important role in arterial pressure regulation [42,43]. Previous studies demonstrated that the Rho/Rho-kinase pathway is activated in blood vessels of L-NAME-treated rats and, in the peripheral circulation, Rho-kinase is apparently involved in the mechanisms of L-NAME-induced hypertension [12]. There is evidence that L-NAME crosses the blood-brain barrier when administered orally [44] and the sympathetic nervous system is involved primarily in the maintenance, rather than initiation, of L-NAME-induced hypertension [45]. Furthermore, activation of the renin-angiotensin system in the NTS via angiotensin II type 1 receptors is involved in L-NAME induced hypertension [46].

Figure 2A shows the time course of systolic arterial pressure from the beginning of treatment with L-NAME along with continuous intracisternal infusion of Y-27532 or vehicle [31]. L-NAME was administered to WKY rats in their drinking water (1 mg/mL) for 2 weeks. Y-27632 or vehicle was administered intracisternally for 2 weeks with a mini-osmotic pump from the beginning of L-NAME treatment. The concentration of Y-27632 was 5 mmol/L and the mini-osmotic pump infusion rate was 0.25 μL/h. Systolic arterial pressure was measured using the tail-cuff method for 21 days. Systolic arterial pressure increased in the L-NAME-treated rats with vehicle intracisternal infusion. Y-27632 intracisternal infusion, however, significantly attenuated the increase in systolic arterial pressure. It is unlikely that nonspecific effects were caused by the surgical procedure in this study because continuous intracisternal infusion of vehicle using the same devices did not suppress the arterial pressure elevation and arterial pressure increased to a level similar to that in rats treated with L-NAME and vehicle after discontinuing treatment with Y-27632.



(n=4 for each, #*P*<0.05 versus ovariectomy with vehicle infusion)
(Adopted and modified from Ito et al., Hypertension 2004;43:156-162 and Hypertension 2006;48:651-657)

Figure 2. **A:** Time course of systolic arterial pressure from the beginning of the treatment with L-NAME along with continuous infusion of Y-27632 or vehicle intracisternally for 2 weeks with a mini-osmotic pump. (n=4 for each, **P*<0.05 versus L-NAME with vehicle). **B:** Time course of mean arterial pressure after ovariectomy with continuous infusion of Y-27632 or vehicle intracisternally for 2 weeks with a miniosomotic pump.

Effects of Rho-Kinase Inhibition in the Brainstem on Arterial Pressure Elevation Caused by Ovariectomy

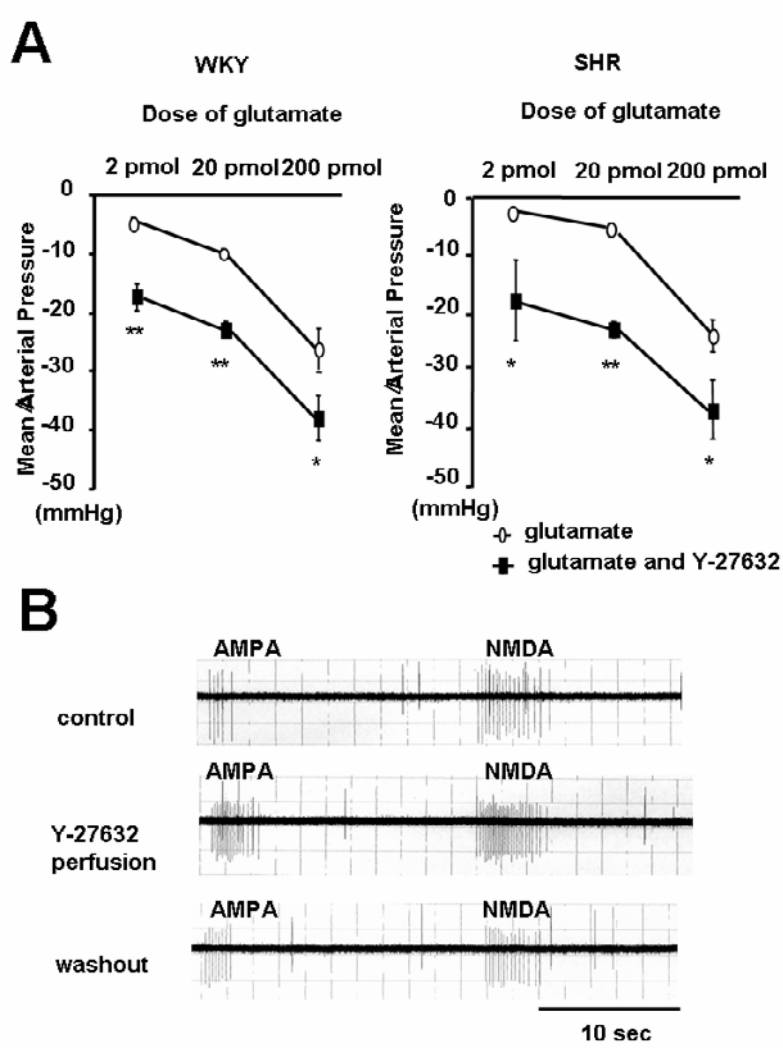
The incidence of cardiovascular disease is lower in premenopausal women than in age-matched men [47,48] and postmenopausal women [49]. The decreased protective effects

against cardiovascular disease in postmenopausal women in thought to be due to endogenous ovarian estrogen depletion. Estrogen and estrogen receptors are present in the brainstem where the vasomotor centers, such as the NTS and VLM, are located [50]. Medullary injections of exogenous estrogen decrease arterial pressure, HR, and renal sympathetic nerve activity and enhance reflex control of the HR in male rats, as well as in ovariectomized female rats [50], suggesting that estrogen has beneficial effects on autonomic functions [52]. In neurons, estrogen regulates the formation of excitatory synapses on dendritic spines [53]. Estrogen treatment increases spine number and synaptic density in ovariectomized adult female rats [54]. These findings led to the hypothesis that the effects of endogenous estrogen on central cardiovascular regulation involve alterations in Rho-kinase activity in central cardiovascular centers.

To determine whether ovariectomy affects arterial pressure via the Rho/Rho-kinase pathway in the brainstem, we performed bilateral ovariectomy in 12-week-old female SHR [55]. Arterial pressure and HR were monitored in awake free-moving rats using a radio-telemetry system [30,37]. Y-27632 or vehicle was administered intracisternally for 2 weeks with a mini-osmotic pump immediately after performing bilateral ovariectomy. The concentration of Y-27632 was 5 mmol/L and the mini-osmotic pump infusion rate 0.25 μL/h. Arterial pressure and HR were significantly increased in ovariectomized rats. Intracisternal infusion of Y-27632 significantly attenuated the increase in arterial pressure and HR. After discontinuing Y-27632 treatment, arterial pressure and HR increased (Figure 2B). These results indicate that the depletion of endogenous estrogen by ovariectomy, at least in part, induces hypertension in female SHR via activation of the Rho/Rho-kinase pathway in the brainstem.

Effects of Rho-Kinase Inhibitor on Glutamate Sensitivity in NTS Neurons

We examined the effects of Y-27632 on the responses to glutamate injection into the unilateral NTS of WKY rats and SHR [56]. Microinjection was performed with a micropipette connected to a Hamilton microsyringe. We used 3 doses of glutamate (2, 20, and 200 pmol; 0.1, 1.0, and 10 mmol/L in 20 nL). In the Y-27632 coinjection study, we used a 2-barrel micropipette. One side of the pipette was filled with glutamate and the other side with Y-27632 (40 pmol; 0.5 mmol/L in 80 nL) or vehicle (artificial cerebrospinal fluid). Glutamate was injected 60 seconds after Y-27632 injection. In addition, to avoid the possibility of differences in the amount of drug spread, a microdialysis probe with an injection line (MI-A-I-12-01; Eicom) connected to a syringe pump was used in another study. In this study, NMDA (0.5 mmol/L; infusion speed 2 μL/min for 5 minutes) was infused through a microdialysis probe [57] and Y-27632 (5 mmol/L; injection speed 0.02 μL/min for 5 minutes) was injected through the injection line with syringe pump. Furthermore, we performed single-unit recordings of NTS neurons and examined the effects of Y-27632 on the neuronal activity of NTS neurons for the iontophoretic application of AMPA or NMDA [58].



(Adopted and modified from Ito et al., Hypertension 2005;46:360-365)

Figure 3. **A:** Effect of Rho-kinase inhibition on glutamate sensitivity in the NTS of WKY rats. (n=5 for each, **P*<0.05, ***P*<0.01 versus injection of only glutamate). **B:** Effects of Y-27632 on neural activity in the NTS evoked by iontophoretically- applied NMDA or AMPA. Example of raw neurograms indicating the increased neuronal activity after Y-27632 perfusion.

Figure 3A shows the effects of unilateral injection of glutamate into the NTS of WKY rats and SHR. Unilateral microinjection of glutamate into the NTS decreased arterial pressure in a dose-dependent manner in WKY rats and SHR. When lower doses of glutamate were microinjected, the magnitude of the decrease in arterial pressure was significantly reduced in SHR compared with WKY rats. The percent change in arterial pressure was significantly greater in WKY rats than in SHR for all doses of glutamate examined. The magnitude of the arterial pressure decreases evoked by unilateral glutamate injection after Y-27632 injection into the NTS was significantly greater compared with glutamate injection alone in both strains. The magnitude of the augmentation, however, was significantly greater in SHR than in WKY rats (glutamate dose 2 pmol: 1.8±0.2 versus 4.0±0.3; 20 pmol: 1.7±0.3 versus

3.1±0.3; 200 pmol: 1.2±0.1 versus 1.4±0.2; data are expressed as the relative ratio of the percent change compared with only glutamate injection, which was assigned a value of 1; $P<0.05$; n=5 for each). The perfusion of NMDA unilaterally into the NTS through a dialysis probe decreased arterial pressure. The magnitude of the decrease in arterial pressure induced by NMDA with Y-27632 injection was significantly greater than that by NMDA perfusion alone. Furthermore, perfusion of Y-27632 increased neuronal activity evoked by NMDA and AMPA (NMDA 1.22±0.19 versus 1.78±0.19 spikes/s; n=12 for each, $P<0.05$; AMPA 0.98±0.09 versus 1.33±0.11 spikes/s; n=12 for each, $P<0.05$, Figure 3B). These results suggest that inhibition of Rho-kinase activity in the NTS enhances glutamate sensitivity in WKY rats and SHR and might improve impaired glutamate sensitivity in SHR.

Role of Rho/Rho-kinase pathway in the brainstem in angiotensin II induced hypertension

The renin-angiotensin system is one of the major pathways involved in central cardiovascular regulation. Previous reports suggest that angiotensin II contributes to the neural mechanisms of hypertension [46,59]. Inhibition of Rho-kinase activity in vascular smooth muscle suppresses angiotensin II-induced cardiovascular effects [60]. Intracisternal infusion of angiotensin II increased arterial pressure and co-intracisternal infusion of Y-27632 attenuated these angiotensin II-induced effects [61]. These results suggest that the Rho/Rho-kinase pathway in the brainstem is involved in angiotensin II-induced hypertension.

Methodological Considerations

It is possible that the drugs and adenovirus vector injections into the NTS spread to other brain regions where the effects of Rho-kinase might also be exerted. Therefore, we confirmed the extent of spread of a dye that was injected with the same volume (80 nl) as used for the Y-27632 injection and confirmed the restriction of drugs spread into the NTS [35]. Furthermore, we performed the immunohistochemical analysis for c-myc, a tag protein of DNRhoK. As shown in Figure 1A, the spread of the adenovirus vector was also restricted to the NTS. Thus, it is unlikely that the effects of Y-27632 or AdDNRhoK on arterial pressure regulation were produced by the spread of the injected compounds to regions other than the NTS.

Y-27632 or hydroxyfasudil was used as a specific Rho-kinase inhibitor. As mentioned above, these drugs are selective enough to inhibit Rho-kinase activity. Whether the effects of these drugs on arterial pressure are due to inhibition of Rho-kinase activity alone, however, has not been clarified. Therefore, we performed a gene transfer study with AdDNRhoK to obtain more specific effects of Rho-kinase on cardiovascular regulation.

In the gene transfer study, we used the adenovirus vector. Although adenovirus is most effective for transfection, adenovirus infection might cause inflammation and cytotoxicity [62]. The extent of ED-1 positive cell infiltration, a marker of inflammation, did not differ between AdDNhoK and Adβgal. The transfection of Adβgal did not alter arterial pressure. These results suggest that any inflammation and cytotoxicity caused by adenovirus infection did not affect arterial pressure [63].

The NTS is a relatively large nucleus involved in many physiological processes. The NTS contains heterogenous neurons, including neurons not related to cardiovascular control. In fact, in the single-unit recording study, Rho-kinase inhibition increased the response of the recorded neurons to NMDA or AMPA, but the magnitude of the augmentation differed in each recorded neuron. Therefore, it is possible that some of the recorded neurons do not contribute to baroreflex function. Furthermore, the NTS receives chemoreceptor afferents [28], which cause an opposite arterial pressure response [27]. Therefore, in the microinjection study, we attempted to identify the depressor areas in the NTS evoked by microinjecting a small amount of glutamate. It is difficult, however, to completely distinguish the cardiovascular from the other functional portions of the NTS.

In addition, because the NTS is rich in blood vessels, it is possible that inhibition of Rho-kinase activity in the NTS changes arterial pressure by increasing local blood flow. Therefore, we microinjected another vasodilator, hydralazine, into the NTS and confirmed that hydralazine did not alter arterial pressure, indicating that the effects of the Rho-kinase inhibitor in the NTS on arterial pressure regulation were not caused by its local vasodilator effects (data not shown).

The RVLM locates close to the caudal VLM [27]. Thus, we were particularly careful to avoid the caudal VLM. To identify the RVLM, we used the following criteria in this study: (1) an increase in arterial pressure occurred immediately after glutamate injection; (2) the change in arterial pressure was more than 30 mmHg.

Intracisternal infusion of Y-27632 might affect many areas, including the cardiovascular center. Naturally, it is not possible to identify the specific areas affected by intracisternally infused drugs, and intracisternal infusion of Y-27632 might also alter neuronal activity in areas other than the NTS, such as the RVLM. We demonstrated, however, that there were greater effects of Y-27632 on arterial pressure–when injected into the NTS compared to the RVLM. Therefore, intracisternal infusion of Y-27632 might alter the neuronal activity mainly in the NTS.

Possible Mechanism(s) Involved in Arterial Pressure Regulation by Rho-Kinase in the Brainstem

Recent studies demonstrated that 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors (statins) upregulate endothelial nitric oxide synthase (NOS) expression and activity [64,65]. Statin effects are mediated by the Rho/Rho-kinase pathway [66], and they negatively regulate endothelial NOS expression [64]. NO in the NTS or the RVLM decreases arterial pressure and HR by decreasing sympathetic nerve activity [37,38]. Therefore, we examined the endothelial NOS and neuronal NOS expression after AdDNRhoK transfection, and found that neuronal and endothelial NOS expression levels are slightly decreased but with a bare significance [30]. However, it was established concepts that inhibition of Rho-kinase upregulates endothelial NOS expression. Therefore, the results of in this study indicate that the effects of Rho-kinase in the NTS on cardiovascular regulation are due to mechanisms not involving NOS and that the reduced NOS expression might be due to hypotension-induced compensation. Further studies should be needed to clarify the relations between Rho/Rho-kinase pathway and NOS in the NTS. Our series of studies suggests that one of the mechanisms of arterial pressure regulation mediated by the Rho/Rho-kinase pathway might

be the changing of glutamate sensitivity in the excitatory synapses. In neurons, glutamate stimulation induces a transient increase of Rho/Rho-kinase activity [67]. The physiological significance of activating the Rho/Rho-kinase pathway after glutamate stimulation is not clear. In previous studies in our laboratory, Rho-kinase inhibition in the NTS induced hypotension and Rho-kinase inhibition in the RVLM induced hypertension. From these findings, we hypothesized that Rho-kinase inhibition affects glutamate sensitivity because glutamate in the NTS induces hypotension, but in the RVLM, hypertension. Therefore, we confirmed that Rho-kinase inhibition in the NTS augmented the responses to glutamate both indirectly and directly.

Possible Mechanism(s) Involved in Hypertension Caused by Rho-Kinase Activation in the Brainstem

The magnitude of the arterial pressure alterations by Rho-kinase inhibition in the NTS was significantly greater in SHR than WKY rats. In hypertensive rats, Rho-kinase activity in both the NTS and the whole brainstem was significantly greater compared with the normotensive rats. Furthermore, Rho-kinase activity in the brainstem was already enhanced in immature SHR (4 week- old) compared with age-matched WKY rats (data not shown). Therefore, the Rho/Rho-kinase pathway was activated in the brainstem probably due to activation of the upstream portion of the Rho/Rho-kinase pathway, such as angiotensin II, and activation of Rho-kinase affected mainly the excitatory synapses. In the RVLM, the main neurotransmitter is γ-amino butyric acid (GABA) and GABA in the RVLM induces hypotension. On the other hand, glutamate in the RVLM induces hypertension. In the RVLM, GABA might be a more powerful neurotransmitter compared with glutamate because a previous report demonstrated that increased NO production in the RVLM induces hypotension instead of the release of not only GABA but also glutamate [38]. In hypertensive rats, Rho-kinase activity might be also increased in the RVLM and thereby decrease glutamate sensitivity. The increase in Rho-kinase activity in excitatory synapses, such as in the NTS, decreases glutamate sensitivity and attenuates the GABAergic inputs into the RVLM. Because the decrease in GABAergic inputs rather than the decreased sensitivity for glutamate in the RVLM might strongly affect arterial pressure regulation via the sympathetic nervous system, Rho-kinase activation in the brainstem including the RVLM might produce hypertension. Therefore, the activation of Rho-kinase in the brainstem contributes to the central mechanisms of hypertension.

Furthermore, activation of Rho-kinase in the NTS might contribute to one of the mechanisms for resetting the baroreflex function in hypertensive rats. As described previously, inhibition of the Rho/Rho-kinase pathway with AdDNRhoK augments baroreflex function. In this study, we examined the effects of arterial pressure reduction itself on baroreflex function [37]. Although the effects of arterial pressure reduction by hydralazine on baroreflex function and Rho-kinase activity in the NTS were weaker than in AdDNRhoK, treatment with hydralazine (hydralazine hydrochloride in drinking water: 0.6 mg/mL) in SHR significantly increased the maximum gain of baroreflex control of HR and decreased Rho-kinase activity in the NTS. These results indicate that an arterial pressure decrease might attenuate inputs from baroreflex afferents and decrease glutamate stimulation in the NTS. The decrease in glutamate stimulation in the NTS might lead to attenuation of Rho-kinase activity and increased glutamate sensitivity in the NTS and then improve baroreflex function,

particularly in SHR. Conversely, arterial pressure elevation might augment inputs from baroreflex afferents and increase glutamate stimulation in the NTS. The glutamate stimulation might lead to Rho-kinase activation and decreased glutamate sensitivity in the NTS and impair the baroreflex function, and then arterial pressure might be maintained at a higher level, i.e., resetting the set point (Figure 4). There are some reports that augmented input from cardiac sympathetic afferents inhibits the baroreflex in rats [68] and baroreceptor inputs at NTS synapses might affect baroreflex function [69]. These findings support our hypothesis.

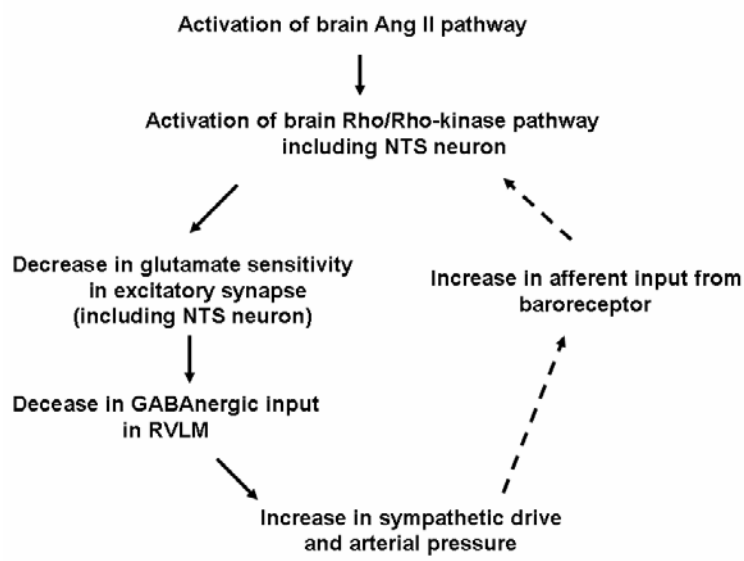


Figure 4. Hypothesis of the role of Rho-kinase in the brainstem in arterial pressure regulation.

In addition, we examined the activity of Rho-kinase in the brainstem in myocardial infarction (MI) in mice as a heart failure model. Although the study is preliminary, Rho-kinase in the brain tends to be activated in the MI mouse (data not shown). Even though the role of Rho-kinase in the brainstem of MI mouse has not been clarified, the Rho/Rho-kinase pathway might be involved in the activation of the sympathetic nervous system during heart failure.

The detailed mechanisms of cardiovascular regulation by Rho-kinase in the brainstem are not clear. Further studies are needed to clarify the mechanisms underlying our observations and the involvement in other cardiovascular diseases, such as heart failure.

Conclusion

The results of these studies indicate that the Rho/Rho-kinase pathway in the brainstem contributes to arterial pressure regulation via the sympathetic nervous system, and activation of this pathway is involved in the neural mechanisms of hypertension.

Acknowledgments

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Chapter 4

SYMPATHETIC AFFERENT AND EFFERENT EFFECTS ON THE LOW BACK AND RADICULAR PAIN

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Abstract

In general, it is considered that the sympathetic nervous system plays an important role in the generation of pain. Concerning low back pain, there is growing evidence that sympathetic afferents is key point. Thus, to elucidate the pathomechanisms of discogenic low back pain, sympathetic afferent discharges originating from lumbar disc via L2 root were investigated neurophysiologically. Using 31 Lewis rats, sympathetic afferent units were recorded from L2 root connected with only rami communicante to lumbar sympathetic trunk. The L5/6 disc were mechanically probed, electrically stimulated to evoke action potentials, and finally were applied with the chemicals for inflammation. We could not get any units in the L5/6 discs with mechanical stimulation, while identified 42 units belonged to mostly A-delta fibers with electrical stimulation. In some experiments with inflammation, response to mechanical probing to L5/6 disc was recognized. This suggests that mechanical stimulation to lumbar disc may not always produce pain, while inflammatory changes may cause lumbar disc to become sensitive to mechanical stimuli, resulting in nociceptive information is transmitted as discogenic low back pain to spinal cord through lumbar sympathetic trunk.

On the other hand, sympathetic efferents seem to be closely related to lumbar radicular pain. Therefore, to investigate the pathological role of the sympathetic nervous system in lumbar radiculopathy, the left L5 root of Sprague-Dawley rats was tightly ligated proximal to the DRG as a lumbar radiculopathy model. Postoperatively, bilateral DRGs and roots (L4 and L5) were removed, frozen and sectioned. Immunostaining was then performed with antibodies to tyrosine hydroxylase (TH) according to the ABC method. To quantify sympathetic nerve fibers, we counted TH-immunoreactive fibers in the DRG using a light microscope with the squares of the micrometer graticule. In the root constriction group, TH-immunoreactive fibers were more abundant in ipsilateral L5 DRGs as well as L4 compared with contralateral DRGs. In the sham and control groups, TH-immunoreactive fibers were scarce in both sides of L4

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and L5 DRGs. Constriction of the lumbar root induced more sympathetic nerve sprouting in ipsilateral DRGs than contralateral DRGs. These findings suggest that the sympathetic nervous system may be closely related to a trigger of radicular pain, acting as efferents by sympathetic sprouting into DRGs.

Introduction

In general, it is considered that the sympathetic nervous system plays an important role in the generation of pain. Concerning low back pain, there is growing evidence that sympathetic afferents is key point. Lumbar intervertebral discs are recognized to be one of the major sources of low back pain. Elucidating sensory innervations of lumbar intervertebral disc and adjacent tissues is an important key to resolving the puzzling mechanisms of the discogenic low back pain. It is generally accepted that lumbar intervertebral discs are innervated segmentally with sinuvertebral nerves branching from the lumbar spinal nerve fibers and the rami communicantes of the corresponding levels.[1, 2, 3] Meanwhile it has been recently demonstrated in rats that the sinuvertebral nerves proceed into paravertebral sympathetic trunk through the rami communicantes.[2, 4] Several immunohistological studies [5, 6, 7] using retrograde neurotracer have shown that L5/6 intervertebral discs and adjacent tissues in the rat were innervated from L1 and L2 dorsal root ganglia (DRG) neurons through paravertebral sympathetic trunk.

Similarly, it has been reported in neurophysiologic studies that mechanosensitive afferent units with receptive fields in the retroperitoneal space including ventral aspects of lumbar intervertebral disc and psoas muscles can run through paravertebral sympathetic trunk. [8, 9] In addition, these units responded to mechanical and chemical stimuli such as KCl, hypertonic NaCl solution and bradykinin, which may indicate the involvement of nociceptive units. Regarding transmission of nociceptive information through sympathetic afferents, in the gastrointestinal system it has been demonstrated that sympathetic C fiber afferents with low and high threshold have the distinct ability to encode nociceptive information such as excessive distention and ischemia. [10] Collectively, these studies indicated that sensory information including nociceptive stimulation from lower lumbar intervertebral discs innervated by the sinuvertebral nerves is conducted by paravertebral sympathetic trunk via the rami communicantes and further, passing through L1 and L2 DRG into the spinal cord. Thus, to elucidate the pathomechanisms of discogenic low back pain, it should be investigated that neurophysiological characteristics of sympathetic afferent discharges originating from lower lumbar intervertebral discs and adjacent tissues pass through L2 dorsal roots.

On the other hand, lumbar radicular pain seems to be closely related to sympathetic efferents. Mechanical compression of the nerve root and the chemical reactions induced by herniated disc containing nucleus pulposus have been suggested as the cause of lumbar radicular pain, but the pathological mechanism is still unclear[11-22]. While neuropathic pain following peripheral nerve injury is considered to show that there are two types of pain state: sympathetically maintained pain (SMP) and sympathetically independent pain (SIP). SMP is a condition in humans for which pain can be attenuated by sympathectomy or sympathetic nerve block[23-25]. In animal models of neuropathic pain caused by peripheral nerve injury, immunohistological studies were performed to confirm that the sympathetic nervous system is involved in generating this pain state. McLachlan et al. reported that sympathetic nerve fibers were increased in the corresponding dorsal root ganglion (DRG) after sciatic nerve

ligation, sprouted to DRG somata and formed basket-like structures around large-diameter axotomized sensory neurons. The release of norepinephrine from sprouting sympathetic nerve endings activated primary afferent DRG neurons with α−adrenoceptor, resulting in central sensitization. This abnormal sympathetic-somatosensory interaction seems to lead to SMP[26-28]. Likely, the sympathetic nervous system is currently considered to be involved in causing radicular pain in humans. There are some reports that sympathetic block is effective for relieving radicular pain in clinical situations[29, 30]. In basic studies using animal models, however, the efferent influence of the sympathetic nervous system on radicular pain has not been fully explored. Therefore, to clarify a relationship the sympathetic nervous system and radicular pain, it is investigated that in the lumbar radiculopathy model, the distribution of the sympathetic nerves around DRG neurons using immunohistochemical analysis.

Analysis of Sympathetic Afferents

Neurophysiological Examination

Thirty-one adult male Lewis rats were sedated and anesthetized. Activity from peripheral receptive fields from L2 dorsal and ventral rami was abolished by cutting L2 dorsal and ventral rami, respectively. At this point, the L2 root was connected with only rami communicante to the lumbar sympathetic chain. A laminectomy from L1 to L6 was performed to expose L2 dorsal nerve root and the dorsal aspect of L5/6 intervertebral disc. After cutting the dura, a pool was formed from skin flaps, and the spinal cord and nerve roots were immersed in warm (37°C) mineral oil to prevent nerve roots from drying. The left L2 dorsal root was detached from the spinal cord and draped over one or two bipolar platinum recording electrodes to examine afferent units. The L2 root was then split to record from a smaller number of units for neurophysioogical study (Figure 1).

For mechanical stimulation, lumbar structural elements including the dorsal aspects of L5/6 intervertebral discs and adjacent facets and the dorsal side of psoas muscles were mechanically probed with blunt glass rods and a 25 ga needle. Once the receptive fields were identified, the mechanical threshold was measured with calibrated nylon filaments. Separately, the test probing on bone lamina was performed to insure no artifact responded to lumbar spinal motion. For electrical stimulation, if the disc and adjacent tissues did not respond to any mechanical stimulation, electrical stimulation with a bipolar electrical stimulator (1 to 20 V) was applied to the dorsal aspects of L5/6 discs and adjacent tissues to evoke action potentials and obtain latency. Later, based on the latencies and the distance between recording electrode and electrical stimulating point, conduction velocity (CV) was estimated. Finally, the chemical application with 2% carrageenan (0.1 ml) to L5/6 disc was performed to produce inflammation. The change of discharge rate and the response of L5/6 disc to mechanical probing were observed and recorded.

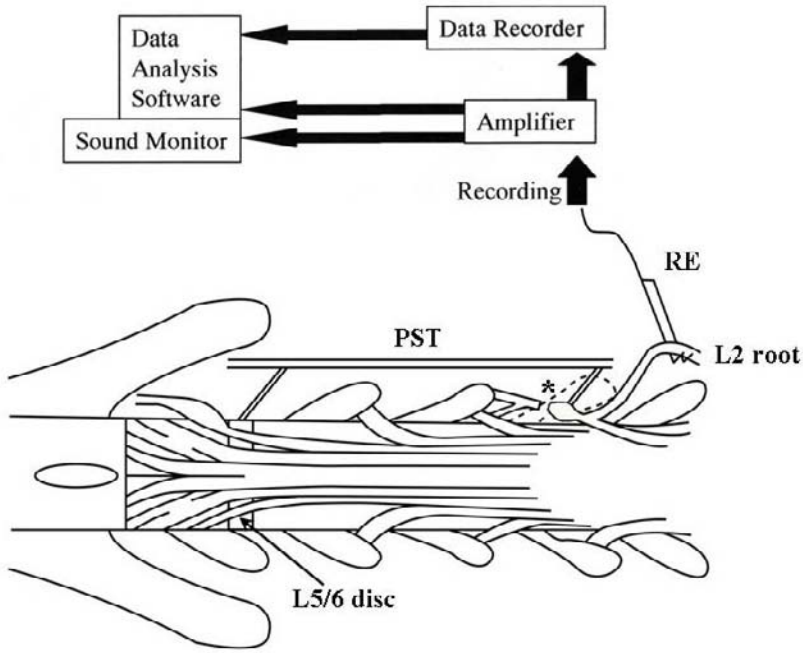

Figure 1. Experimental schema of the recording of sympathetic afferent units. Afferent impulses from L2 dorsal rootlets were amplified and recorded on an FM tape recorder. L2 spinal nerve roots connected only with rami communicante to lumbar paravertebral sympathetic trunk. RE: Recording electrode, PST: Paravertebral sympathetic trunk. *: Cutting L2 dorsal and ventral rami. (Reprinted with permission from Takebayashi T et al: J Bone Joint Surg 88B: 554-7, 2006)

Results

For mechanical stimulation, psoas muscle units were observed in about 40% (12/31) of the experiments. No systematic attempt was made to evaluate quantitatively the mechanical thresholds because of the variable location of the receptive fields. Only three units were identified at the DRG itself, whose discharge pattern was sporadic and seemed not to be directly influenced by mechanical stimulation (Figure 2). Consequently, we could not identify any mechanically responding units in the L5/6 intervertebral discs. Meanwhile, spontaneous irregular bursting discharges, not responsive to any mechanical stimulation, were sometimes observed. These may be visceral sympathetic afferents (Figure 3). Since any distinct receptive field in discs and adjacent tissues was not detected, intervertebral discs were electrically stimulated for evoking action potentials of the units. Over all we identified 42 units with conduction velocities, 7.86 ± 4.9 m/s (2.56 to 21.6). Most of these conduction velocities belonged to A-delta fibers with thin myelinated axons (Figure 4). In 19 experiments (out of 31), the inflammation with carrageenan was tested. In only five experiments out of 19, when the L 5/6 intervertebral disc was stimulated mechanically, the responses were observed regardless of no responses before inflammation (Figure 5).

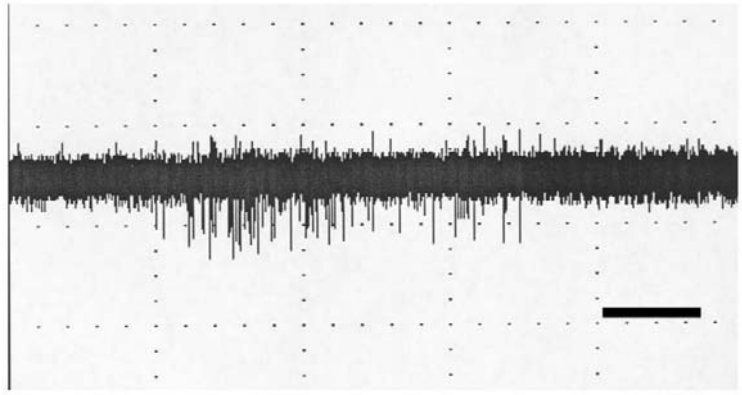

Figure 2. Response of DRG units to mechanical stimulation. The discharge pattern was sporadic and seemed not to be directly influenced by mechanical stimulation. Black bar indicates 1 second. (Reprinted with permission from Takebayashi T et al: J Bone Joint Surg 88B: 554-7, 2006)

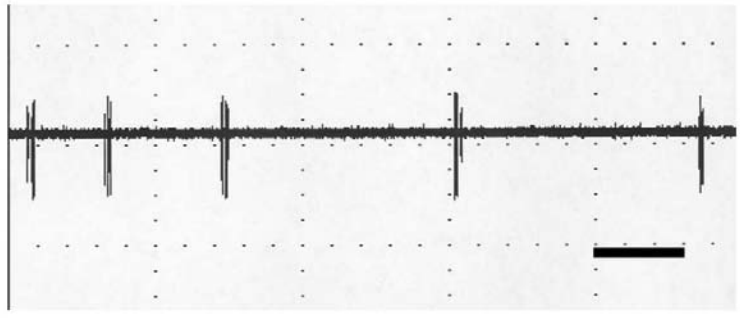

Figure 3. Spontaneous irregular bursting discharge being insensitive to any mechanical stimulation. Black bar indicates 2 seconds. (Reprinted with permission from Takebayashi T et al: J Bone Joint Surg 88B: 554-7, 2006)

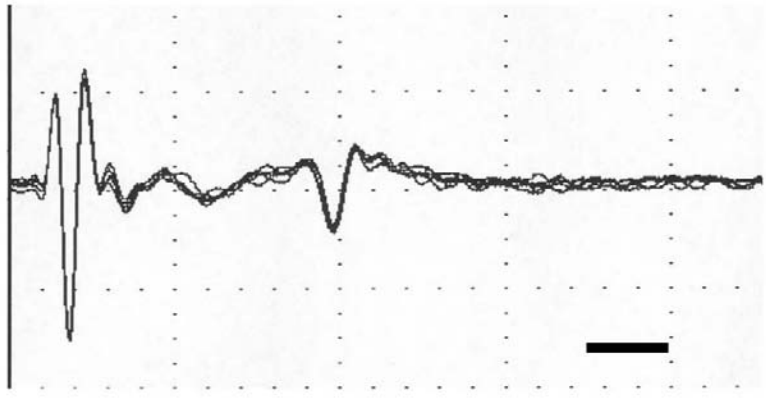

Figure 4. Action potential evoked by electrical stimulation to L5/6 intervertebral disc. Black bar indicates 1msec. (Reprinted with permission from Takebayashi T et al: J Bone Joint Surg 88B: 554-7, 2006)

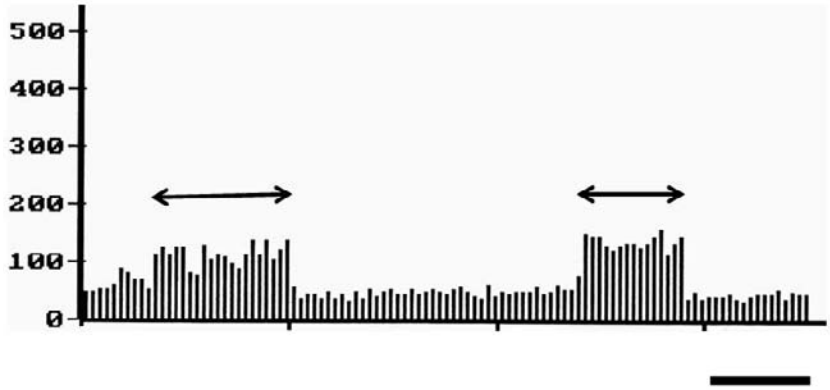

Figure 5. Mutiunit discharge rate changed in histogram in response to mechanical stimulation after inflammation. Black bar shows the interval from the onset to the end of the mechanical stimulation. Black bar indicates 5 seconds and arrow indicates mechanical stimulation. (Reprinted with permission from Takebayashi T et al: J Bone Joint Surg 88B: 554-7, 2006)

Analysis of Sympathetic Efferents

Making Lumbar Radicular Pain Model

Male Sprague-Dawley rats (200-250g) were used. In lumbar root constriction group (n=20), the left L5 spinal root was exposed and ligated tightly with 8-0 nylon suture just proximal to the DRG as radicular pain model. While in sham group (n=20), the left L5 nerve root was exposed only without the ligation. Non-operated rats were used as a control group (n=10).

Behavioral Testing

Behavioral testing was performed before and after the operation on 3, 7, 10, 14, 21, 28 days. Mechanical stimulation was applied 30 times on the hind paw with Semmes-Weinstein monofilaments (1.2g). Mechanical threshold was presented as withdrawal frequency of the rat hind paw. Noxious radiant heat as thermal stimulation was applied likely and withdrawal latency was measured. We compared three groups with the withdrawal frequency and latency, calculated the difference of ipsilateral and contralateral side.

Immunohistochemistrical Examination

Bilateral DRGs and spinal roots (L4 and L5) were removed, frozen and sectioned on a cryostat (8-10μm). They were then immunostained with rabbit antibodies to transmitter-synthesizing enzyme tyrosine hydroxylase (TH) according to the ABC methods. TH is an important enzyme synthesized by sympathetic nerve fibers and transforms tyrosine, the precursor of noradrenaline, into dopa. Therefore, antibodies to TH are frequently used for assessing the presence of sympathetic nerve fibers. To quantify the extent of sympathetic nerve fiber presence, TH-immunoreactive (TH-IR) fibers in the DRG using a light microscope were counted.

Results

The lumbar radicular pain model showed pain-related behavioral, mechanical allodynia and thermal hyperalgesia, which were markedly observed compared with sham or control group [31] (Figure 6, 7). In ipsilateral hind paw, significant increases in withdrawal frequency and decreases in withdrawal latency were measured from 3 days after the operation and maintained up to 28 days. The increases in withdrawal frequency were flattened at 7 days after the operation, and the decreases in withdrawal latency were flattened at 3days after the operation. No significant differences were seen between two kinds of Semmes-Weinstein monofilaments.

In the lumbar radicular pain model, TH-IR fibers were more abundant in ipsilateral L5 DRGs as well as L4, compared with contralateral DRGs. However, they could not be found around the DRG neuron soma, but were present around the myelin sheaths in the DRG. In the sham and control groups, TH-IR fibers were scarce in both sides of L4 and L5 DRGs. The mean number of the micrometer graticule squares containing TH-IR fibers in sections of the L5 DRG at the ipsilateral side (left side) was 23.6±6.1 in the lumbar radicular pain model, 7.2±2.2 in the sham group and 4.8±1.9 in controls. TH-immunoreactive fibers increased significantly in ipsilateral DRG of the lumbar radicular pain model. Likely, TH-IR fibers of the ipsilateral L4 DRG increased significantly in root constricted DRG although the reaction was less strong than in L5 DRG (Figure 8).

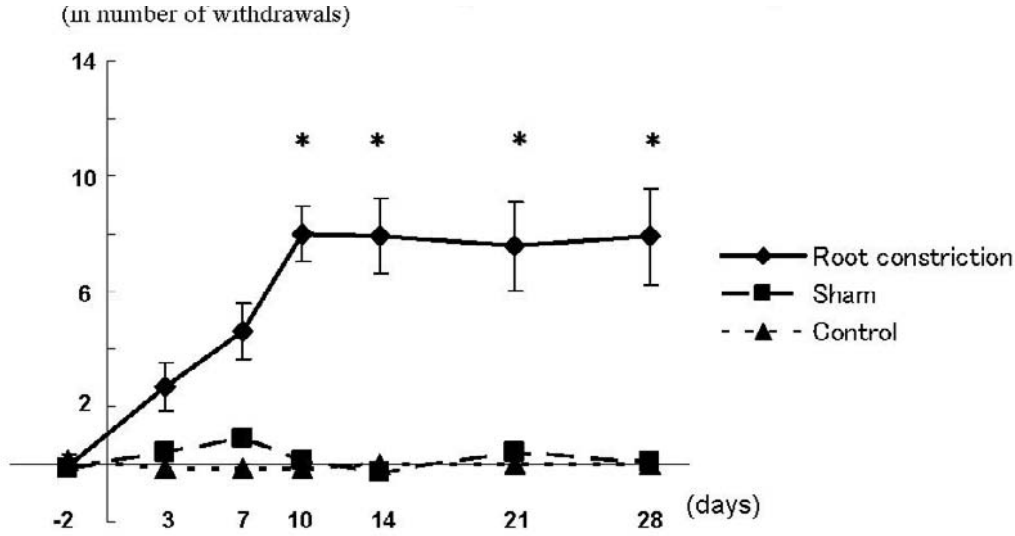

Figure 6. The mechanical sensitivity of rat hindpaw in lumbar root constriction, sham-operated, and control group. Time course of the difference in withdrawal frequency to mechanical stimulation for the ipsilateral versus the contralateral hindpaws. The root constriction group demonstrated a significant increase in tactile sensitivity up to 28 days after surgery compared with the withdrawal difference of preoperative response. (*$p<0.05$) (Reprinted form with permission from Kirita T, et al: Electrophysiological changes in dorsal root ganglion neurons and behavioral changes in a lumbar radiculopathy model. Spine 32: E65-72, 2007)

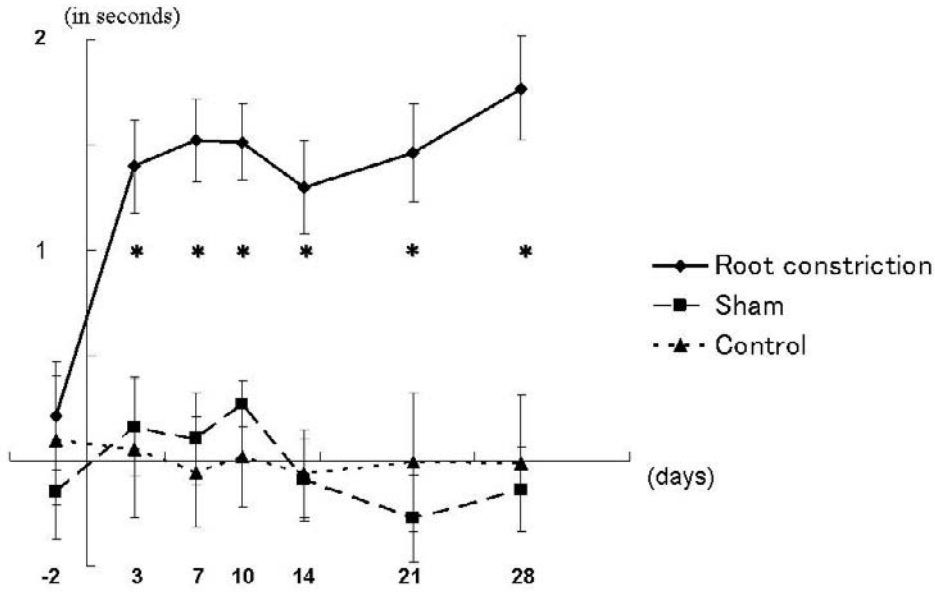

Figure 7. The thermal sensitivity of rat hindpaw in lumbar root constriction, sham-operated, and control group. Time course of the difference in withdrawal latency to noxious heat stimulation for the ipsilateral versus the contralateral hindpaws. The animals in the root constriction group demonstrated statistically significant higher thermal sensitivity than those in the preoperative difference. (*p<0.05) Each point and vertical bar represents the mean and SEM. (Reprinted form with permission from Kirita T, et al: Electrophysiological changes in dorsal root ganglion neurons and behavioral changes in a lumbar radiculopathy model. Spine 32: E65-72, 2007)

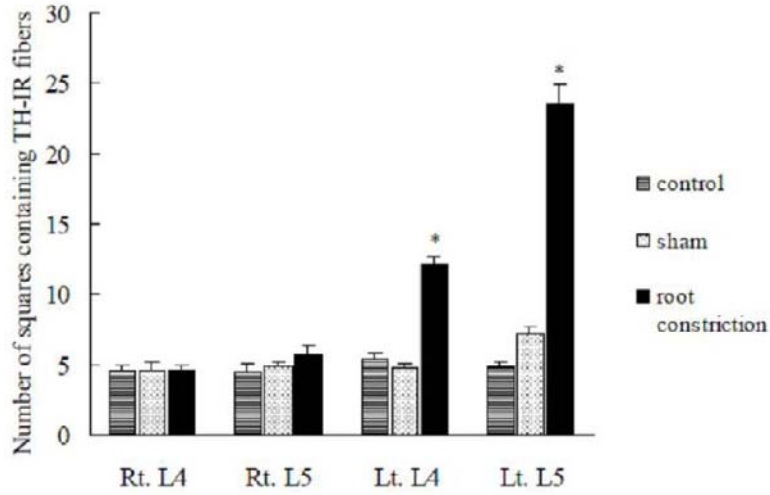

Figure 8. Mean numbers of squares containing TH-immunoreactive fibers at bilateral L4 and L5 DRG. TH-immunoreactive fibers were increased significantly in root constricted ipsilateral L4 and L5 DRG (*p < 0.05). There was no significant difference in ipsilateral L4 and L5 DRGs among root constriction group, sham group, and control group. Bars indicate SEM. (Reprinted form with permission from Mizuno S, et al: Effects of Sympathetic Nerves on Lumbar Radicular Pain. J Bone Joint Surg Br in press)

Discussion

Low Back Pain Related to Sympathetic Nervous System

In general, it is considered that the sympathetic nervous system plays an important role in the generation of pain[26-28, 32-34]. Concerning low back pain, there is growing evidence that sympathetic afferents play a key role. Lumber intervertebral discs have been considered as one of the major source of low back pain. Nakamura et al. reported a study in 33 patients in which injection of L2 nerve roots with lidocaine relieved back pain originating at the lower lumbar discs[35]. They proposed a sympathetic afferent pathway that was supported by a neuroanatomical study in the rat[26] and suggested that low back pain is a type of visceral pain. Based on neural tracer studies[37], it is also known that afferents from the lower lumbar disc have pathways to L2 DRG and are innervated by the lumbar sympathetic trunk. Also, it have been reported that electrical stimulation of the lumbar sympathetic trunk could experimentally cause low back pain, [38] and conversely lumbar sympathetic block reduced low back pain.[39] Our results also showed that afferent signals originating from L5/6 intervertebral disc passed through lumbar sympathetic trunk into L2 roots and further into the spinal cord. These data suggest that lumbar discogenic pain is closely connected with sympathetic afferent system.

Our study demonstrated that L5/6 intervertebral discs units were not responsive to mechanical stimulation under normal conditions, meanwhile after inflammation via carrageenan application, some of these mechanically insensitive afferents (MIA) responded to mechanical stimulation. MIAs that are insensitive to mechanical stimuli under normal conditions and responsive to the stimuli under pathological conditions such as inflammation are distributed in joint, [40] viscera[41] and cornea.[42] The receptors of MIA s fiber are called “silent nociceptors” and approximately half of A-delta and 30% of C-fiber nociceptors are silent nociceptors. [43-46] These silent nociceptors have been reported in the digestive system related to the autonomic nerve fibers partially passing through sympathetic chain. The afferent fibers innervating the colon became sensitized to mechanical stimuli during inflammation.[47] Therefore, the receptors of the lumbar intervertebral discs may be silent nociceptors, which are activated under inflammation and modulate nociceptive information.

Kuslich et al. [48] reported in local anesthetized low back surgeries that approximately two thirds of symptomatic patients had significant pain when the affected disc annulus fibrosus was mechanically stimulated, which strongly suggested the reproduction of discogenic low back pain. Meanwhile, the fact that the remaining one third of these patients had no pain is an interesting finding to be reckoned with. Similarly, it has been demonstrated that in discography in a group of asymptomatic patients only 10 % felt pain from the procedure.[49] The valid explanation for these clinical phenomena is necessary to characterize sensory information originating from lower lumbar intervertebral discs and being transmitted to the central nervous system. Recently, nerve growth has been found in granulation tissue in the fissures of degenerated discs. [50] Are some degenerated lumbar discs innervated with more abundant sensory fibers than others? The possibility of “silent nociceptors” may be a reasonable explanation for those clinical phenomena that mechanical stimulation to lumbar disc may not always produce pain. Inflammatory changes may cause silent nociceptors of discs to become responsive to mechanical stimuli, and this nociceptive information is transmitted as discogenic low back pain to the spinal cord through the sympathetic trunk. Still it remains to be resolved how disc degeneration not associated with

inflammation is correlated with low back pain. Further analysis using a chronic model with degenerated discs will be needed.

On the other hand, sympathetic efferents also play a role in low back pain. Neuroanatomical and immunohistochemical studies have revealed sympathetic neurons in the intervertebral disc and adjacent tissue[51, 52]. Based on neurophysiological studies, Roberts has proposed that the sympathetic system induces pain by activating A-β afferents that excite wide dynamic range (WDR) neurons in spinal cord pain pathways[23]. Peripheral sensory nerves appear to have an increased concentration of αadrenergic receptors after nerve injury[53]. Elevated levels of norepinephrine may lead to phenotypic changes in nociceptors and ultimately to central sensitization[54]. Evidence for a role of beta adrenergic receptors in sympathetically-maintained pain was demonstrated by Cunha et al. by attenuation of inflammation-induced hyperalgesia through local injection of propranolol and atenolol[55]. Gillette et al. recorded from the lateral dorsal horn in cats while stimulating lumbar spine tissue and electrically stimulating the sympathetic chain[56]. They reported that there were two patterns of response to sympathetic stimulation: entrained responses that appeared to be due to direct stimulation of afferents in the sympathetic trunk, and non-entrained responses that were more variable in relation to a stimulus pulse and appeared to be due to interaction between the noradrenergic sympathetic efferents and the primary afferents in the spinal tissue. These latter responses were blocked by the alpha-adrenergic antagonist, phentolamine. These results are consistent with the hypothesis proposed by Roberts that sympathetic efferents stimulate myelinated sensory fibers innervating low back, leading to stimulation of pain-producing WDR neurons in the dorsal horn of the spinal cord[23].

Lumbar Radicular Pain Related to Sympathetic Nervous System

Regarding lumbar radicular pain, the effectiveness of sympathetic nerve block has been demonstrated clinically, not only for chronic pain such as causalgia, but also for lumbar radicular pain. Takahashi et al. reported that sympathetic nerve block was still effective one month later in 59% of patients with leg pain due to lumbar spinal canal stenosis who did not have pain relief after epidural and lumbar root block[29]. Yabuki et al. also reported that sympathetic nerve block was effective in about 40% of patients for relief of pain from radicular-type lumbar spinal canal stenosis[30]. In animal models, however, the influence of the sympathetic nervous system on radicular pain was hitherto unexplored.

In basic research using rats with peripheral nerve injury, McLachlan et al. reported that extensive sympathetic nerve fibers increased in corresponding DRGs and their adjacent peripheral nerves after sciatic nerve ligation, and sprouted to DRG somata, forming basket-like structures around large-diameter axotomized sensory neurons[28]. The pain behavior such as allodynia and ongoing pain are correlated with the density of sprouting and the number of bascket formations in the DRG. In other words, the sympathetic nervous system connected directly to DRG neuron somata where sympathetic efferent signals were transmitted to secondary afferent neurons through DRG neurons. Therefore, sympathetic sprouting related to nerve injury is a representative phenomenon in neuropathic pain.

In our radicular pain model, in which the nerve root was injured proximal to the DRG, sympathetic nerve fibers sprouted not to somata but to myelin sheaths in DRG neurons. This implies that the sympathetic excitation must have indirectly influenced the DRG neurons. Considering these pain models, the difference on the way of sympathetic axons invade the DRG

in different animal models (neuropathic pain and radicluar pain) may be attributed to the differential dependency on neurotrophins such as NGF and neurotorphin-3, and different sprouting patterns of connection to the DRG may lead to clinical symptoms of differing severity; i.e., neuropathic pain, a refractory disease, or radicular pain, with reversible symptoms.

In the present study, TH-immunoreactive fibers were found to be increased not only at the corresponding L5 DRG but also the ipsilateral L4 DRG. It is considered that sympathetic sprouting is caused by many factors such as nerve growth factor (NGF), leukemia inhibitory factor (LIF) and interleukin (IL)-6 [57-61]. In particular, NGF is regarded as the most important factor to facilitate sprouting of sympathetic nerve fibers to the DRG following peripheral nerve injury, because the sympathetic neurons that sprout to form baskets express the high affinity NGF receptor, TrkA[62, 63]. In addition, anti-NGF treatment can reduce injury-induced basket formation[64, 65]. Meanwhile, several compelling lines of evidence suggest that the uninjured L4 spinal nerve is the main route through which impulses evoked in the periphery are transferred to the spinal dorsal horn in L5 spinal nerve ligation models[66, 67]. Likely, in our rat model, the mechanisms underlying sympathetic sprouting in the adjacent intact L4 DRG to root constriction is not clear. One possible hypothesis is that NGF may be synthesized in the L5 spinal nerve distal to the ligation site, transported peripherally via spinal roots, diffused into the L4 spinal nerve, and transported retrogradely to the L4 DRG neurons. Another one is that sympathetic sprouting may involve the activation of satellite glia in the involved L4 DRG.

As previously described, the sympathetic efferents as sprouting in the DRG neurons may attribute to development and persistence of lumbar radicular pain through the release of norepinephrine from sprouting sympathetic nerve endings leading excitation of DRG neurons by activating α-adrenoceptor. While intrathecal administration of norepinephrine has reversely antinociceptive effect by inhibiting A-delta-fiber- and C-fiber mediated sensory transmission to substantia gelatinosa (SG) neurons in the spinal dorsal horn through the activation of α-adrenoceptor. [68] The presence of adrenoceptors in DRG and spinal dorsal horn neurons has been demonstrated [69], suggesting a role of norepinephrine at presynaptic and postsynaptic sites in the modulation. Therefore, the norepinephrine may have different modulation of pain sensation depending on site of its releasing to DRG or spinal dorsal horn. Because the sympathetic nervous system is autonomous and can not be self-control, the effects of administration of the norepinephrine on lumbar radicular pain may be beyond anticipation. Although speculating that the sympathetic nervous system play an important role in generating radicular pain, more comprehensive understanding of sympathetic system should be needed to treat lumbar radicular pain.

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Chapter 5

DEVELOPMENT OF NEUROTRANSMITTER CONTENT IN SYMPATHETIC GANGLIA

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Abstract

Sympathetic neurons contain a different palette of neurotransmitters including classical neutransmitters (catecholamines and acetylcholine), neuropeptides and small molecules such as NO, CO.

The majority of the principal ganglionic sympathetic neurons are noradrenergic and show a positive immunocytochemical reaction to tyrosine hydroxylase (TH), i.e., a key enzyme in catecholamine synthesis. TH appears during embryonic development and the percentage of TH-positive neurons remains virtually identical during ontogenesis. In sympathetic neurons of most species, noradrenaline is often colocalized with neuropeptide Y (NPY). NPY could also be demonstrated in noradrenergic neurons during embryogenesis. The percentage of neurons containing NPY decreases during early development and increases further after birth with age up to the period of adulthood .

A small number of postganglionic sympathetic neurons contains enzyme of acetylcholine synthesis (ChAT) and some neuropeptides, such as somatostatin (SOM), vasoactive intestinal (poly)peptide (VIP), calcitonin gene-related peptide (CGRP) and galanin (GAL). Acetylcholine-containing sympathetic neurons in most cases colocalize VIP and/or CGRP.

Increased levels of SOM, VIP, CGRP, GAL were reported in sympathetic ganglia during prenatal development. The number of cells containing the above mentioned peptides significantly decreases in prenatal period and after birth onwards. The proportion of VIP- and cholinergic neurons in paravertebral ganglia exhibits additional periods of increasing and decreasing in postanatal development.

In some species (cats, human) NO also acts as a neurotransmitter in the sympathetic ganglia. The proportion of NO-containing cells also changes during postnatal development. In kittens, in the stellate ganglion their number increases during first 30 days of life and decreases later. The content of NO-containing neurons in other ganglia is rather small and doesn't change during the development.

Some combination of neurotransmitters can be revealed only in non mature animals. For example, combination NPY and SOM, TH and ChAT are not found in adults.

Thus, the expression of the spectrum of neurotransmitters in sympathetic ganglionic neurons of mammals is subject to changes during pre- and postnatal development. Different target-derived factors and multiple target independent intrinsic and extrinsic factors influence the expression of neurotransmitters. The pattern of development depends on the species and the type of ganglion.

Introduction

Sympathetic ganglia consist of neurochemically and functionally distinct populations of neurons, characterized by a specific projection pattern and a set of neutransmitters (Lindth et al., 1989; Gibbins, 1992; Morris et al., 1998; Gibbins et al., 2003).

In sympathetic principal ganglionic neurons (PGN) of most species, noradrenaline is often colocalized with neuropeptide Y (NPY) (Gibbins, 1992, Klimaschewski et al., 1996). Apart from this major group of catecholaminergic neurons, a small but significant number of postganglionic sympathetic neurons contains acetylcholine. Cholinergic neurons can be identified by specific markers, such as choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAChT) (Ernsberger and Rohrer, 1999). Acetylcholine-containing sympathetic neurons in most cases colocalize VIP and/or CGRP. Additionally, a small population of PGN contains somatostatin (SOM), substance P and galanin (GAL) in rats, guinea pigs and humans (Klimaschewski et al., 1996).

It has been shown that the expression of the spectrum of neurotransmitters in sympathetic PGN is subject to changes during pre- and postnatal development (Cochard et al., 1979; Baetge et al., 1990; Tyrrell and Landis, 1994; Ernsberger and Rohrer, 1996; Roudenok, 2000; Roudenok and Kuhnel, 2001; Masliukov and Timmermans, 2004). Some combination of neurotransmitters can be revealed only in non mature animals. For example, combination NPY and SOM, TH and ChAT are not found in adults (Anderson et al., 2001). Some neuropeptides, such as VIP and SOM, are known to play an important role in the development of the nervous system and sympathetic ganglia in particular. Subsequent development and maintenance of phenotypic features of different classes of PGN, particularly their neurochemical phenotype, have been attributed variously to target-derived factors or to multiple target independent intrinsic and extrinsic factors. Indeed, it is likely that these factors are not the same for all sympathetic ganglia (Patterson and Landis, 1992; Pincus et al., 1992; Rao and Landis, 1993; Forehand, 1995; Hall and MacPhedran, 1995; Ernsberger and Rohrer, 1996, 1999; Francis and Landis, 1999).

My purpose in this chapter is to describe some recent approaches in our knowledge about the neuroplasticity of sympathetic neurons during postnatal ontogenesis.

Embryonic Development of Sympathetic Ganglia

All peripheral autonomic neurons arise from neural crest cells that migrate away from the neural tube (Weston, 1963). Some neural crest cells are multipotent and give rise to multiple types, including neurons and glia (Frank and Sanes, 1991; Stemple and Anderson, 1992 Hall and Landis, 1991; Duff et al., 1991). Neural crest progenitors are multipotent with respect to neuronal phenotype (Baroffio et al., 1988).

Sympathetic precursor cells derived from neural crest first appear along the thoracic dorsal aorta at E10-E11 and in the cervical region at E12, where they begin to form ganglia (Cochard et al., 1978, 1979; Rothman et al., 1978; Teitelman et al., 1979; Rubin, 1985a). Soon after this, at E13.5 precursor cells are committed to either a neuronal or non-neuronal fate (Hall and Landis, 1991). Most sympathetic neuronal precursors undergo a terminal division between E12 and E 16 (Hendry, 1977). Neurotransmitter characteristics of mature sympathetic neurons are not detectable until ganglion formation (Allan, Newgreen, 1977; Teitelman et al., 1979). The maturation of sympathetic ganglia proceeds along a rostral-to-caudal developmental gradient.

Many sympathetic neurons first contact their target tissues during embryogenesis; the timing of this event appears variable and is partially dependent on the distance of the target from the ganglion. Sympathetic fibers are detected in the iris at E15 and are likely to reach closer targets before that (Rubin, 1985a, b). Distal targets, including pineal gland and sweat glands, are contacted after birth (Landis and Keefe, 1983; Li and Walsh, 1991). Since sympathetic neurons in ganglia are generated during several days, neurons at different developmental stages are present in embryonic ganglia. Although initial presynaptic and postsynaptic contacts are established before birth, major expansions in dendritic arborizations and preganglionic and postganglionic terminal plexuses occur after birth (Black and Mytilineou, 1976; Smolen and Raisman, 1980; Voyvodic, 1987).

Noradrenaline Synthesis in the Developing Sympathetic Ganglia

The largest part of PGN contains noradrenaline as their main neutransmitter. Noradrenaline is synthesized from tyrosine into several steps. The enzyme tyrosine hydroxilase (TH) converts the amino acid l-tyrosine into 3,4-dihydroxyphenylalanine which is then decarboxylated to dopamine by DOPA decarboxylase. Finaly, dopamine is converted to noradrenaline by the action of dopamine β-hydroylase (DBH) (Cooper et al., 1996).

Catecholamines are detectable as early as at 3.5 embryonic day (E3.5) in chick sympathetic ganglia (Enemar et al., 1965; Vogel, Weston, 1990). TH appears slightly earlier than the product catecholamines (chick E3, Cochard et al., 1979; Rothman et al., 1980). Ganglia along the entire chick sympathetic chain have detectable catecholamines by E4 (Shirley et al., 1996).

During rat embryonic development, TH and DBH expression as well as catecholamine fluorescence are found simultaneously at E11-12.5 showing a rostral-to-caudal gradient of differentiation (Cochard et al., 1978, 1979; Teitelman et al., 1979; Hall and Landis, 1991; Ernsberger, Rohrer, 1996). TH expression is observed to start after the precursors of the sympathetic neurons have reached the dorsal aorta indicating that the signals received during migration at this location may play an important role in the induction of the noradrenergic transmitter phenotype (Cochard et al., 1978, Stern et al., 1991; Groves et al., 1995; Ernsberger, Rohrer, 1996).

After birth, the largest number of sympathetic neurons in the para- and prevertebral ganglia of mammals contains this enzyme. In the rat and mouse stellate ganglion, the percentage of TH-positive neurons remains virtually identical during ontogenesis and varies from 85% to 95% without statistically significant differences between age groups (Masliukov and Timmermans, 2004; Masliukov et al., 2005, 2006; Maslyukov et al., 2006).

In humans, TH-positive neurons are observed at 24-27 weeks of fetal development. Their number varied from 85% to 90% in para- and prevertebral ganglia of fetuses and newborns. The proportion of TH-IR cells slightly increases until 95% in adults during further development. In aged humans, the number of TH-IR neurons decreases till 75-85% in both para- and prevertebral ganglia (Roudenok, 2000).

In guinea-pig, four neurochemically distinct populations of sympathetic neurons with moderate TH-immunoreactivity (IR) were identified within the coeliac ganglion from late embryonic stages of development: 1) neurons expressing NPY-IR but not SOM-IR; 2) neurons with immunoreactivity for both NPY and SOM, a combination not found in coeliac ganglia of adult guinea pigs; 3) neurons expressing SOM-IR but not NPY-IR; and 4) neurons expressing only TH-IR and expressing neither neuropeptide (Anderson et al., 2001).

Coeliac ganglion neurons of guinea-pig expressing TH-IR, but neither NPY-IR nor SOM-IR occurrs at all developmental ages. The relative size of this population of TH-IR neurons decreases significantly with time both in the medial region and the lateral region (Anderson et al., 2001).

NPY Expression during Ontogenesis

NPY is a vasoconstrictor (Lundberg et al., 1990; Owan, 1990) and inhibits secretion in a variety of tissues (Dunning et al., 1987; Walker et al., 1991). It also inhibits the release of noradrenaline, with which it is colocalized (Lundberg et al., 1990; Bleakman et al., 1992). Almost all NPY-positive neurons in adult rat and mice are TH-immunoractive (Masliukov and Timmermans, 2004; Masliukov et al., 2005, 2006). A subpopulation of NPY-immunoractive PGN in paravertebral ganglia of guinea-pigs and dogs was shown to colocalize VIP (Heym and Lang, 1986; Gibbins and Morris, 1997).

In avians, the initial expression of NPY follows the expression of catecholaminergic properties of neurons (Garcia-Arraras et al., 1992). In bullfrog sympathetic ganglia, NPY expression begins after adrenergic differentiation (Stofer and Horn, 1990).

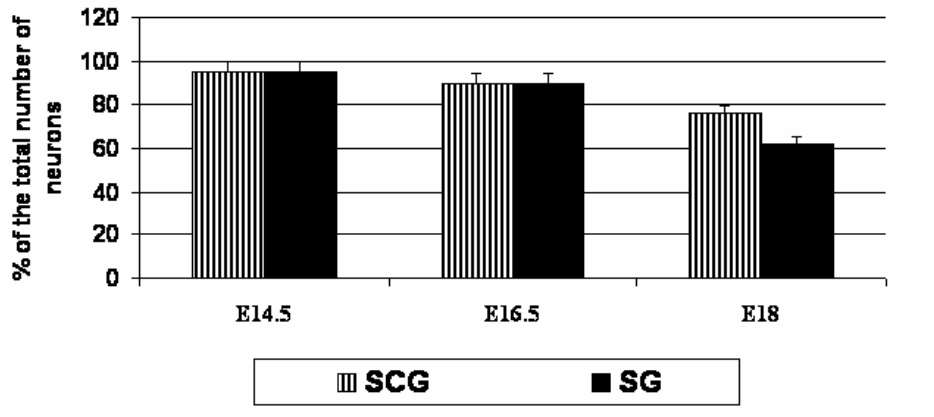

Figure 1. Percentage of NPY-IR neurons in the stellate and superior cervical ganglion of rats during early embryonic development (Adopted from Tyrrell and Landis, 1994).

By contrast, in rats, NPY IR is first detected in sympathetic ganglia at E12.5. When it first appears, NPY-IR is present in almost all TH-IR cells and the peptide immunofluorescence is faint throughout the cytoplasm. During the next 3 days, the proportion of TH-IR cells with peptide-IR remains relatively constant while the intensity of peptide immunofluorescence increases. At E16.5, the immunofluorescence intensity of NPY-IR begins to appear heterogeneous; some cells are more brightly immunoreactive than others. As development proceeds, the proportion of cells with detectable NPY-IR decreases significantly from approximately 90% in both the superior cervical ganglion and the stellate ganglion at E16.5 to 76% at in the SCG and 62% in the stellate ganglion at E18 (Fig. 1). By PO, the proportion of neurons with NPY-IR in the superior cervical ganglion and in the stellate ganglion is slightly more than 50% (Tyrrell et al., 1991; Tyrrell and Landis, 1994).

In guinea-pig, at Carnegie stages 14–15, NPY-IR is observed in many of the most rostrally located cells, whereas only weak NPY-IR is present in cells at levels caudal to the developing stomach. By stages 16-17, NPY-IR cells are first identified in the caudally located paravertebral chains and in the prevertebral-adrenal regions. NPY-IR is found in cells

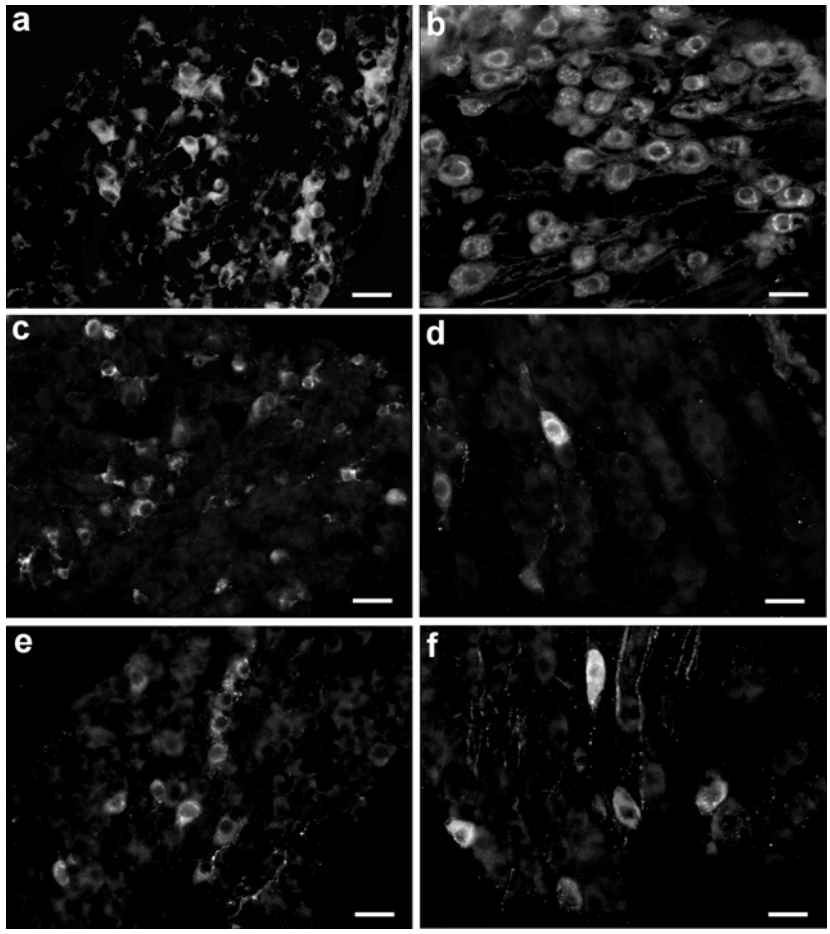

Figure 2. NPY-IR (a, b), SOM-IR (c, d) and VIP-IR (e, f) neurons in the stellate ganglion of newborn (a, c, e) and 30-day-old (b, d, f) rats. Bar: 30 μm (Adopted from Masliukov et al., 2006).

with either moderate or intense TH-IR. From late embryonic period, as many as 80% of TH-immunoreactive neurons have NPY-IR. The proportion of TH-immunoreactive neurons with NPY-IR decreased significantly to 55% by midfetal stages and stayed constant through the remainder of fetal growth (Morris et al., 2001).

In the developing celiac ganglia of guinea-pig, at late embryonic stages, a similar proportion of neurons expresses detectable NPY-IR in medial and lateral regions of the ganglion. From early fetal stages onward, a proportion of NPY-IR neurons located laterally increases significantly greater than medially located cells (Anderson et al., 2001).

After birth, the proportion of NPY-IR neurons in the stellate ganglion of rats and mice is not constant but increases during the early postnatal period from the moment of birth until the second month of life (Fig. 2, 3). Meanwhile, the number of NPY-IR neurons is higher in the rat than in the mouse stellate ganglion in all ages in postnatal ontogenesis. Only single NPY-IR neurons immunonegative for TH are observed in the stellate ganglion of rodents (Masliukov, Timmermans, 2004; Masliukov et al., 2005, 2006).

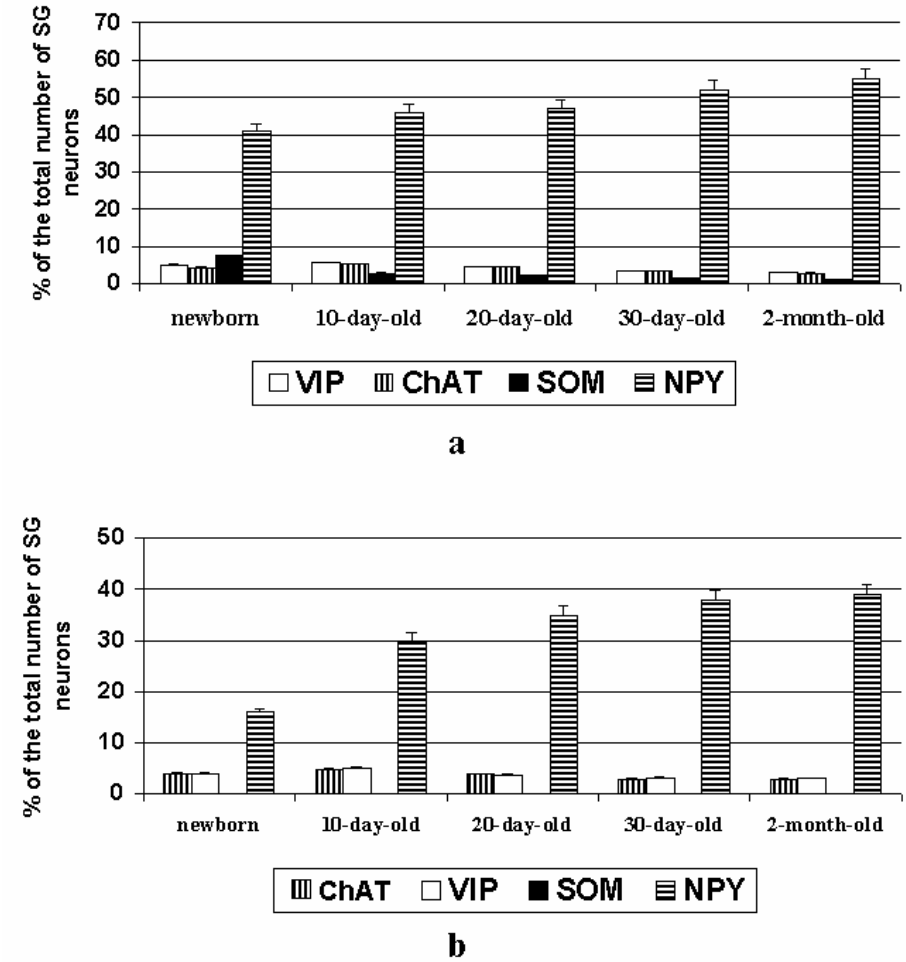

Figure 3. Percentage of NPY-, ChAT-, VIP- and SOM-IR neurons in the rat (a) and mouse (b) stellate ganglion (SG) at different ages (Adopted from Masliukov et al., 2005, 2006).

In the para- and prevertebral ganglia of human fetuses at 24-27 weeks only a small (from 1.5% in celiac ganglia up to 7% in SG) population of NPY-IR nerve cells are observed. At the moment of birth, the number of NPY-IR ganglionic neurons substantially increases and reaches 27% in mesenteric ganglia and 41% in the stellate ganglion. During the development, the percentage of NPY-containing cells continues to increase and becomes 63% in mesenteric ganglia and 73% in the stellate ganglion. In aged humans, the number of NPY-IR neurons decreases till 37% in mesenteric ganglia and 48% in the stellate ganglion. (Roudenok, 2000b).

During the embryonic development, NPY phenotype results from a complex combination of regulatory cues; neuronal phenotype is not due solely to environmental cues or lineage restrictions alone, but arises from multiple interactions (Hall and MacPhedran, 1995).

Cholinergic Cells during the Development

Acetylcholine is synthesized from choline and acetyl coenzyme A by the cytosolic enzyme choline acetyltransferase (ChAT). Acetylcholine-containing PGN in most cases colocalize vasoactive intestinal (poly)peptide (VIP) and/or calcitonin gene-related peptide (CGRP) (Lundberg et al., 1979; Landis and Fredieu, 1986; Heym et al., 1993).

ChAT activity in lumbosacral sympathetic ganglia is not detectable at E4, weak at E5 and increases from E6 to E8 in the chick (Marchisio and Consolo, 1968; Ernsberger and Rohrer, 1999). In mouse, ChAT activity is observed at E13 and increases between E16 and E17 as well as after birth (Coughlin et al., 1978).

Cholinergic neurons, containing vesicular acetylcholine transporter (VAChT) first appear in the rat stellate ganglion at E 14.5. Later, during several days the number of VAChT-IR neurons substantially reduces and only several percentage of neurons contains VAChT to the moment of birth (Schäfer et al., 1997).

However, first cholinergic fibers appear at the target-organs after birth. Initially, they contain TH. Fibers innervate periosteum exhibit cholinergic markers at postnatal day 4 (Asmus et al., 2000). In sweet glands they appear at postnatal day 11 (Ernsberger and Rohrer, 1999).

In the rat and mouse, the number of ChAT-IR neurons increases from the moment of birth until 10 days of postnatal life and then declines for the other ages studied (Fig. 3). The majority of ChAT-IR cells in newborn rats are also TH-IR. The proportion of these cells rapidly decreases in the first ten days of animals' life. Nevertheless, a small population of ChAT-IR neurons in the stellate ganglion of 2-month-old rats and mice exhibites TH-IR.

In humans, a small number of ChAT-positive neurons and fibers is detectable at 24-27 weeks of fetal development. These neurons are observed only in para- but not in prevertebral ganglia. The proportion of ChAT-neurons in the stellate ganglion increases from 1% in fetuses to 5% in children and declines further to 2% in adults. (Roudenok et al., 1999; Roudenok and Kuhnel, 2001).

VIP-IR Neurons during the Development

In mammals, the main targets of these cholinergic, VIP/CGRP-containing PGN appear to be sweat glands. In cats and dogs, but not in rodents or primates, cholinergic VIP-containing

neurons were found to innervate arterial vessels in skeletal muscle as well (Lundberg et al., 1979; Guidry and Landis, 2000).

VIP is differentially expressed in the superior cervical ganglion and stellate ganglion of rats throughout development. VIP-IR neurons are never observed in embryonic or neonatal superior cervical ganglion, many VIP-IR cells and fibers are present in stellate ganglia after E 14.5. At E 14.5 the intensity of immunofluorescence varied considerably between cells and many immunoreactive cells were faintly labeled. VIP expression is maximal at a time when neuroblasts are proliferating (Hendry, 1977; Schütz et al., 1998). Following the initial appearance, the proportion of VIP-IR cells declines rapidly, and by PO, only 3.5% of the neurons within the stellate ganglion possesses VIP-IR. When VIP-IR initially appears in embryonic stellate ganglia at E14.5 and E16.5, however, it is colocalized with TH-IR. By E14.5, approximately one-third of TH-IR cells in the stellate ganglia expresses VIP-IR (Tyrrell, Landis, 1994).

We also observed VIP-IR neurons in the stellate ganglion of all rats and mice after birth (Fig. 2, 3). Most of the ChAT-IR cells in the stellate ganglion of rats were also VIP-IR. In accordance with previous observations on ChAT IR, the proportion of VIP-positive neurons was maximal in 10-day-old animals and then decreased up to 60 days of age. Some of the VIP-IR neurons were also TH-IR. During further development, the proportion of neurons exhibiting VIP and TH as well as ChAT and TH dramatically decreased from newborn to 10 days of life and further declined slowly later on (Masliukov, Timmermans, 2004; Masliukov et al., 2005, 2006).

Instead of expression of cholinergic markers, immunoreactivity for VIP, present in cholinergic sympathetic fibers innervating adult rodents sweat glands, is not detectable during the first postnatal week. VIP-IR is demonstrated first at postnatal day 10 in some glands and at day 14 in all glands (Landis et al., 1988; Ernsberger and Rohrer, 1999).

In humans, many VIP-IR neurons are observed in fetuses at 24-27 weeks (26% in celiac ganglia and 75% in stellate ganglion). At the moment of birth, the number of VIP-positive neurons substantially declines. The degree of decreasing is more in the stellate ganglion where 19% of neurons are VIP-IR in comparison with coeliac ganglia (12% of neurons are VIP-IR). During the childhood, the percentage of VIP-IR cells continues to decrease and reaches 10.5% in the stellate ganglion and only 1% in mesenteric ganglia. In adults, the percentage of VIP-IR greatly reduces to 1% in the stellate ganglion and remains the same 0.5-1% in mesenteric ganglia. In aged humans, the number of VIP-IR neurons increases till 5% in mesenteric ganglia and 11% in the stellate ganglion. (Roudenok et al., 1999; Roudenok and Kuhnel, 2001).

CGRP-Containing Cells in the Development

CGRP is found in a population of noncatecholaminergic, mostly VIP and sometimes also SP-immunoreactive PGN in guinea-pig, rat and cat that probably project to sweat glands (Landis and Fredieu, 1986; Kummer and Heym, 1988; Lindth et al., 1988; Klimaschewski et al., 1996).

As well as VIP-IR, CGRP-IR in cholinergic sympathetic fibers is not detectable during the first postnatal week. CGRP-IR is first detected at day 14 (Landis et al., 1988; Ernsberger and Rohrer, 1999).

In humans, pattern of the CGRP-IR neurons development was quite similar to the ontogenesis of VIP-IR neurons. Also, the large number of CGRP-IR neurons is present in fetuses at 24-27 weeks (31% in celiac ganglia and 49% in stellate ganglion). At the moment of birth, the number of CGRP-IR neurons substantially declines (10% in celiac ganglia and 21% in the stellate ganglion). During the childhood, the percentage of CGRP-IR cells continues to decrease and reaches 2% in celiac ganglia and 9% in the stellate ganglion. In adults, the percentage of CGRP-IR is rather low (0.1% in celiac ganglia and 0.5% in the SG). During ageing, the number of CGRP-IR neurons increases till 3.5% in celiac ganglia and 6% in the stellate ganglion. (Roudenok 2000a; Roudenok and Kuhnel, 2001).

SOM Containing Cells in Ontogenesis

Somatostatin (SOM), a tetradecapeptide, has been found in various species, in neuronal and non-neuronal cells (Hökfelt et al., 1975, 1977; Alumets et al., 1977; Patel and Reichlin, 1978). In adults, only few TH-positive neurons in the superior cervical ganglion of rat and man contain SOM-IR (Hökfelt et al., 1977; Léránth et al., 1980; Järvi et al., 1987). Larger numbers of SOM-IR cells have been observed in the guinea pig and pig superior cervical ganglion, and in some adrenergic prevertebral sympathetic neurons of the guinea pig and rat (Hökfelt et al., 1977; Léránth et al., 1980).

During embryonic development of chicks and quail first SOM-IR cells appears at E4. Like catecholamine-containing cells, SOM-containing cells appear as small clusters of cells with a bilateral distribution lateral to the dorsal aorta. From E7-E8 their number decreases and does not change significantly in future (García-Arrarás et al., 1984, 1986; Maxwell et al., 1984; New and Mudge, 1986).

In the rat, no SOM-IR cells are found at E12.5 in PGN. At E16.5, the vast majority of cells in the superior cervical ganglion are expressed SOM (Katz et al., 1992). In the stellate ganglion, the highest density of SOM-containing neurons is observed in newborns (Fig. 2, 3). Later, the degree of SOM IR substantially decreases and is constant as from 10 days of life. The most of the VIP-positive neurons are also SOM-reactive at birth, after which the number of neurons containing both peptides decreases. VIP- and SOM-positive cells do not contain NPY in any of the age groups studied. (Masliukov and Timmermans, 2004; Masliukov et al., 2006).

In contrast, in the mouse SG, SOM-IR is expressed only in single cells from the moment of birth till adulthood (Masliukov and Timmermans, 2004; Masliukov et al., 2005; Maslyukov et al., 2006).

In the guinea-pig, no cells with SOM-IR are seen at early embryonic stages (Carnegie stages 14–15). Only occasional SOM-IR cells are found in the developing prevertebral-adrenal regions. These cells have lower levels of immunoreactivity compared with SOM-IR neurons at late embryonic stages and also contain NPY-IR. No SOM-IR cells are found in the paravertebral regions. However, cells with intense SOM-IR occur in the gut from stage 16–17 embryos. At late embryonic stages, very few neurons express SOM-IR anywhere in the ganglion. In contrast to the NPY-IR neurons, the relative size of the population of Som-IR neurons increases dramatically in the medial region, whereas it remains low and unchanged laterally. TH-IR neurons containing both NPY-IR and SOM-IR are rare throughout the development of the coeliac ganglion and decreases to be almost completely absent from

neonatal and adult ganglia. However, at late embryonic stages, 75% of neurons with SOM-IR also contain NPY-IR in both medial and lateral regions of the ganglion. The proportion of SOM-IR neurons with NPY-IR decreases dramatically over time, so that, by neonatal stages, <2% of SOM-IR neurons in the medial ganglion also express NPY-IR (Anderson et al., 2001).

In humans, SOM-IR neurons are found in great number even in fetuses at 24-27 weeks (56% in celiac ganglia and 49% in stellate ganglion). At the moment of birth, the number of SOM-positive neurons substantially decreases and reaches 26% in mesenteric ganglia and 11% in the stellate ganglion. Later, the percentage of SOM-IR cells continues to decrease more slowly and becomes 18-21% in mesenteric ganglia and 7-9% in the stellate ganglion of children and adults. In aged humans, the number of NPY-IR neurons decreases till 6.5% in mesenteric ganglia and 2% in the stellate ganglion (Roudenok and Kuhnel, 2001).

GAL-IR Neurons during the Development

GAL transiently expressed by sympathoblasts in the 4-day chick embryo. Within next two days (E5 and E6), the number of GAL-IR neurons increases. At E10-11 the most, if not all, cells expressing GAL-IR also express immunoreactivity to the catecholamine synthesizing enzyme. During this early embryonic period, almost TH, SOM and GAL are coexpressed in sympathoblasts before their differentiation into PGN (Garcia-Arrarás and Torres-Avillán, 1999). Further, the percentage of GAL-containing cells decreases and at E15-E19 GAL-IR restricted to only SIF cells (Barreto-Estrada et al., 1997).

The rat stellate ganglion is found to harbor a small population of GAL-IR neurons, whose number is less than 1% of all stellate ganglion neurons. Only a few GAL-IR neurons are found in rats up to 10 days of life, after which their number increases several times to reach a maximal value in 30-day-old animals and then declined again to the level observed in newborns. No GAL-IR neurons are present in normal condition in the mouse stellate ganglion (Masliukov and Timmermans, 2004).

SP-IR Neurons in Ontogenesis

SP belongs to a family of closely related peptides, called tachykinins or neurokinins. SP-IR has been demonstrated in few PGN in the superior cervical ganglion of cat and dog (Darvesh et al., 1987; Kummer and Heym, 1988)

In the rat, SP-IR neurons appears in the superior cervical ganglion at 14 day of the embryonic development. Small numbers of SP-IR ganglion cells were seen up to the fifth postnatal day after which SP-containing PGN are no longer observed (Virta and Uusitalo, 1993).

Development of NO-Containing Neurons

Nitric oxide (NO), acts as a neurotransmitter in the autonomic nervous system (Grozdanovic et al., 1992; Ceccatelli et al., 1994; Maifrino et al., 2006). NO is generated by the enzyme

nitric oxide synthase (NOS) in a reaction that converts the amino acid L-arginine to L-citruline. It has become apparent that histochemical techniques to demonstrate NADPH-diaphorase enzymatic activity detect the presence of NOS in neurons. It has been suggested that only intensely stained NADPH-d-positive neurons are involved in NOS production (Hope et al., 1991; Santer and Symons, 1993).

NOS-positive cells are absent in the superior cervical ganglion and stellate ganglion in adult rats and mice (Grozdanovic et al., 1992; Santer and Symons, 1993). In contrast, large number of these neurons is found in the cat SG (Anderson et al., 1995; Klimaschewski et al., 1996).

NO may colocalize with acetylcholine and one or more neuropeptides. In the cat, NOS is detected in 99% of presumably sudomotor neurons exhibiting CGRP and VIP immunoreactivity and in 70% of presumably muscle vasodilatator neurons containing VIP but not CGRP (Anderson et al., 1995).

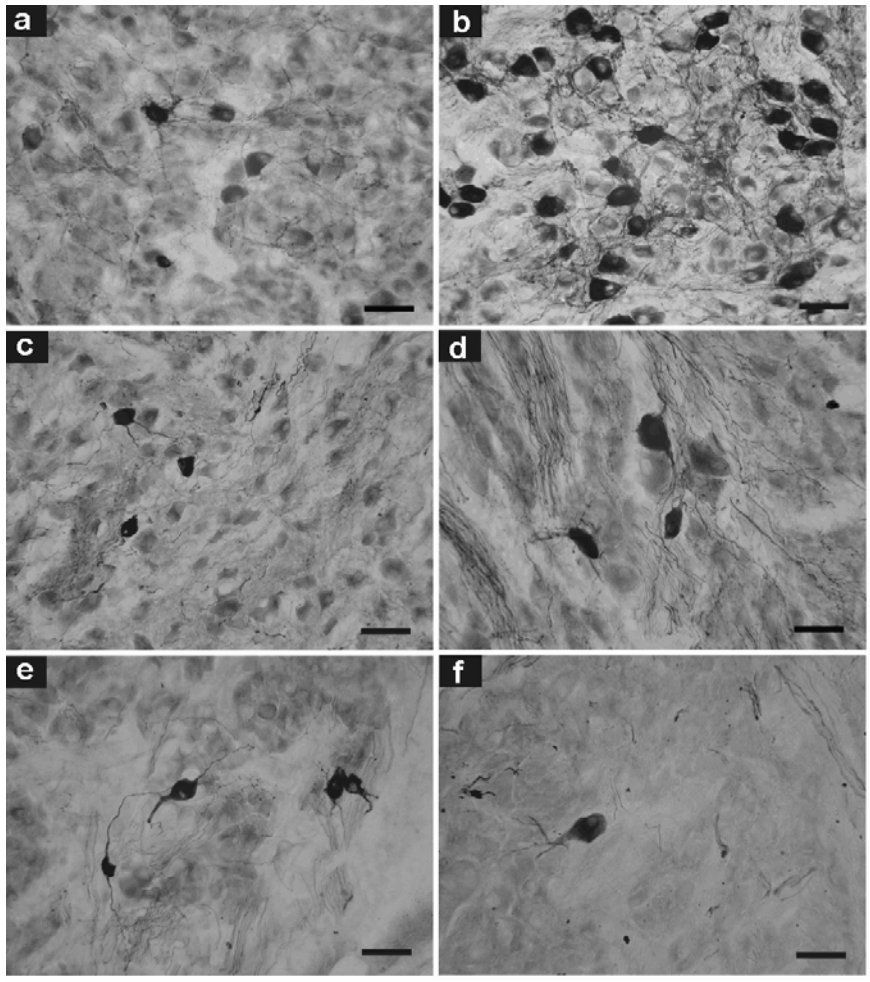

Figure 4. NADPH-d-positive neurons in the stellate ganglion (a, b), superior cervical ganglion (c, d), coeliac ganglion (e, f) of newborn (a, c, e) and 20-day-old (b, d, f) kittens. Bar, 60 μm.

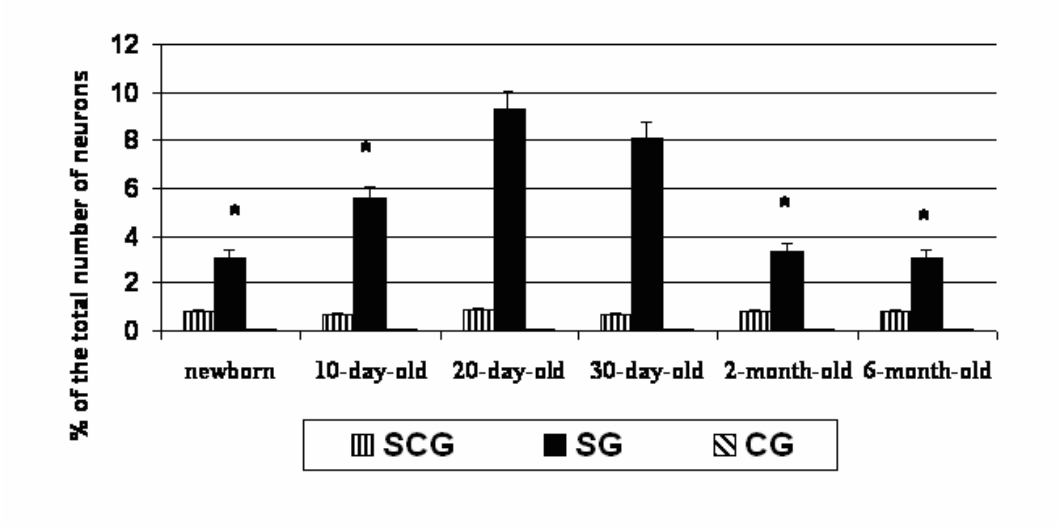

Figure 5. Percentage of NADPH-d-positive neurons in the stellate ganglion (SG), superior cervical ganglion (SCG) and coeliac ganglion (CG) of kittens at different ages (*P<0.05, comparison was made between 20-day-old and other age groups).

In kittens, the largest number of stained cells is located in the stellate ganglion in comparison to the superior cervical ganglion and celiac ganglia (Fig. 4, 5). In newborn kittens, the proportion of NADPH-d-positive cells is rather small in the stellate ganglion. The number of these neurons increases during the development, becomes maximal in 20-day-old and 30-day-old animals and declines further. The proportion of NADPH-d-positive cells becomes constant in two-month-old and older kittens (Masliukov et al., 2003).

The occurrence of NADPH-d-positive neurons in 10-day-old kittens in the stellate ganglion is restricted to the lateral part of the ganglion. The location area of these neurons on ganglion sections increases in first twenty days (Masliukov et al., 2003).

In the superior cervical ganglion is observed a small percentage of stained neurons. Only single NADPH-d-positive cells per ganglion are found in the coeliac ganglia. In the superior cervical ganglion and coeliac ganglia, the stained cells are located diffusely throughout the ganglion. The percentage of NADPH-d-positive cells doesn't change during the development in the superior cervical ganglion and coeliac ganglia.

Factors Influencing the Development of Sympathetic Neurons

Different target-dependent and target-independent factors influence the development of sympathetic neurons with different neurotransmitter properties. The bone morphogenetic proteins (BMPs) derived from the dorsal aorta play a central role for the differentiation of sympathoadrenal cells (Reissmann et al., 1996, Shah et al., 1996; Ernsberger, 2001). Differentiation of sympathoadrenal cells is promoted by a cross-regulatory network of transcription factors, which is at least in part activated by BMPs. This includes the mammalian achaete–scute homolog 1 MASH-1, the paired homeodomain proteins Phox2a and Phox2b, Hand2 (dHAND) and GATA 2/3 (Guillemot et al., 1993; Howard et al., 2000; Pattyn et al., 1999; Lim et al., 2000; Stanke et al., 1999; Huber, 2006).

Biochemical and immunological analysis of conditioned media showed the presence of several differentiation-inducing factors. The induction of TH is stimulated by co-culture with primary smooth, skeletal or cardiac muscle but not by co-culture with fibroblasts or glial cells (Iacovitti et al., 1989). Heart-cell-conditioned medium contained at least three distinct factors, one increasing acetylcholine levels and VIP-like signals while decreasing catecholamine levels, the second inducing only VIP-related peptides and the third affecting neither acetylcholine levels nor VIP-like signals but somatostatin expression (Nawa and Patterson 1990).

Ciliary neurotrophic factor (CNTF) (Saadat et al. 1989), cardiotrophin-1 (Pennica et al. 1995) and oncostatin M (Rao et al. 1992) treatment increases ChAT activity and VIP expression in cultures of superior cervical ganglion neurons from postnatal rats.

However, the factors involved in the control of the early embryonic VIP expression in the stellate ganglion are presently unclear. This early expression of VIP in this ganglion is not due to CNTF- or LIF-like cytokines (Ernsberger and Rohrer, 1999). Thus, VIP expression may be regulated differently in different populations of sympathetic neurons, which may be due to properties intrinsic to the ganglion or differences in the environment.

After axotomy, sympathetic neurons begin to express substance P, and decrease expressing neuropeptide Y. Additionally, two peptides, VIP and galanin, are induced in axotomized sympathetic neurons (Zigmond, 1997). The loss of nerve growth factor does produce phenotypic changes in these neurons (Zhou and Rush, 1996; Ruit et al., 1990). Also, leukemia inhibitory factor (LIF) plays a large role in the switch in neuropeptide phenotype and in galanin expression (Zigmond, 1997; Klimaschewski, 1997).

In vitro, activin A, a member of the transforming growth factor β, but not LIF or related cytokines, induces CGRP in newborn sympathetic neurons (Fann and Patterson, 1994).

Not only chemical factors but functional activity can also switch neurotransmitter properties. Preganglionic nerve activity in vivo or membrane depolarization in vitro can reduce substance P and SOM content in developmentally older rat superior cervical ganglia (Kessler et al., 1981; Kessler and Black, 1982).

Conclusions

The various neurotransmitter phenotypes are regulated independently of each other, as well as from morphological properties. There are common features in the neurotransmitter development of sympathetic neurons, for example, early TH, NPY, SOM and VIP expression during early embryonic development. Species differences in the ontogenesis of neurotransmitter phenotypes are also observed. Target-derived factors and multiple target independent intrinsic and extrinsic factors determine the expression of neurotransmitters.

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Chapter 6

SYMPATHETIC NERVOUS SYSTEM ACTIVITY IN THE HEART AND THE SKIN: ARE THEY COMPARABLE?

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Abstract

In this study we investigated whether pre-ejection period (PEP), number of nonspecific skin conductance responses (ns.SCRs) and skin conductance level (SCL) quantify sympathetic nervous system (SNS) activity in a comparable way. Physiological data were obtained from 39 human subjects (23 males) with a mean age of 22.0 years (SD = 2.3) during exposure to seven different mental and physical stressors, and during subsequent recovery periods. Compared to pre-test resting baseline recordings significant decreases in PEP and parallel increases in the number of ns.SCRs and the SCL were found for stressors known to increase SNS activity. The between and within subjects correlations between ns.SCRs and SCL were significant and multilevel analysis showed that 43% of the variance in these skin conductance measures overlapped. Between and within subjects correlations between PEP and both skin conductance measures were not significant. This suggests that SNS activity is reflected differently by the heart and the skin. We conclude that SNS activity studies, when possible, should include both PEP and skin conductance measurements.

Introduction

Various non-invasive indicators of sympathetic nervous system activity are in use in psychophysiological studies, including heart rate frequency measures, impedance-derived

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measures and skin conductance measures. Few studies though have examined the correspondence between these various indices of sympathetic activity. In the present chapter, we will report on the comparison between pre-ejection period, an index of sympathetic nervous system activity at the heart, and two skin conductance measures indicative of sympathetic nervous system activity at the skin.

Cardiac sympathetic nervous system (SNS) activity can be non-invasively obtained by thoracic impedance cardiography (Cacioppo et al., 1994). In impedance cardiography a high frequency alternating current is introduced across the thorax by two electrodes. Two inner and parallel electrodes measure the changes in the impedance of the enclosed thorax column (dZ), which is largely a function of aortic blood flow. The impedance cardiogram (ICG) is defined as the first derivative of the pulsatile changes in transthoracic impedance (dZ/dt). From the ICG the pre-ejection period (PEP) can be derived as the time interval between the onset of ventricular depolarization and the opening of the semilunar valves. Changes in PEP reliably index β-adrenergic inotropic drive to the left ventricle as shown in laboratory studies manipulating β-adrenergic tone by epinephrine infusion (Schachinger, Weinbacher, Kiss, Ritz, & Langewitz, 2001; Mezzacappa, Kelsey, & Katkin, 1999; Svedenhag, Martinsson, Ekblom, & Hjemdahl, 1986), adrenoceptor blockade (Schachinger et al., 2001; Winzer et al., 1999; Harris, Schoenfeld, & Weissler, 1967), exercise (Krzeminski et al., 2000; Smith et al., 1989; Miyamoto et al., 1983), or emotional stress (Berntson et al., 1994; Sherwood, Allen, Obrist, & Langer, 1986; Newlin & Levenson, 1979). The PEP has been shown to be a stable individual characteristic. The test-retest reliability coefficients generally range between .45 and .83 when measured over a few weeks to 3 year or longer (Goedhart, Kupper, Willemsen, Boomsma, & de Geus, 2006; Burleson et al., 2003; Matthews, Salomon, Kenyon, & Allen, 2002; Willemsen et al., 1998) and substantial heritability of PEP (57 %) has been reported (de Geus, Kupper, Boomsma, & Snieder, in press; Kupper, Willemsen, Boomsma, & de Geus, 2006).

Skin SNS activity can be non-invasively measured by the activity of the sweat glands. The eccrine sweat glands are innervated by efferent neurons from the sympathetic axis of the autonomic nervous system that use acetylcholine as their neurotransmitter. Changes in SNS activity modulate the conductance of an applied current to the skin (mostly palmar) and are reflected in the resulting changes in electrodermal activity. Blockade studies have shown that a cholinergic antagonist (atropine) strongly reduces sweat gland activity (Foster & Weiner, 1970). The primary function of most eccrine sweat glands is thermoregulation. However, the eccrine glands located on the palms and soles of the feet have been thought of as being more concerned with grasping behaviour than with evaporative cooling (Edelberg, 1972), and it has been suggested that these glands are more responsive to emotional stimuli than to thermal stimuli (Dawson, Schell, & Filion, 2000). Electrodermal activity incorporates both slow tonic shifts in basal skin conductance level (SCL) and more rapid phasic transient events, that is, skin conductance responses (SCRs), which are also referred to as galvanic skin responses (GSRs) (Dawson et al., 2000; Boucsein, 1992; Fowles, 1986; Venables & Christie, 1980). The frequency of the nonspecific SCRs (ns.SCRs) reflects an important psychophysiological trait which is termed electrodermal lability (Dawson et al., 2000; Lacey & Lacey, 1958; Mundy-Castle & McKiever, 1953). Both SCL and ns.SCRs have been shown to be influenced by emotional stress (Dawson et al., 2000; Boucsein, 1992). The test-retest reliability coefficients over time periods encompassing one day to a year for SCL levels (both during rest and during periods of stimulation) ranged from .40 to .85 and for ns.SCRs correlations

ranged from .40 to .76 (Schell, Dawson, Nuechterlein, Subotnik, & Ventura, 2002; Vossel & Zimmer, 1990; Schell, Dawson, & Filion, 1988; Iacono et al., 1984; Freixa i Baque, 1982). Moderate heritability estimates between .40 and .50 have been found for electrodermal lability (Crider et al., 2004; Lykken, Iacono, Haroian, Mcgue, & Bouchard, 1988).

Based on the physiological underpinnings above, both PEP and skin conductance are widely used as non-invasive measures of within and between subject differences of SNS activity in psychophysiology (Popma et al., 2006; Vrijkotte, van Doornen, & de Geus, 2004; Kronholm, Hyyppa, Jula, & Toikka, 1996; Jacobs et al., 1994; Cacioppo et al., 1994; Sherwood et al., 1990). However, only very few studies have empirically tested whether PEP and skin conductance are correlated across subjects or how PEP and skin conductance covary within subjects during exposure to different stressors engaging the sympathetic nervous system. A study by Kelsey (1991) found a positive relation between electrodermal lability and PEP reactivity to stress. The subjects with a high frequency of ns.SCRs exhibited greater myocardial reactivity than did the subjects with low frequencies of such responses. However, this relation reflected a comparison between frequency of ns.SCRs at baseline and PEP reactivity during the presence of a stressor. No mention was made of the relation between ns.SCRs and PEP levels at rest or during the tasks. Tomaka, Blascovich and Swart (1994) measured reactivity (compared to the last minute of the rest period preceding each task) of both ns.SCRs and PEP to two different mental arithmetic tasks. They found higher SCR reactivity during reading aloud task while PEP reactivity was higher during the silent task. In this case, PEP and SCR did not seem to follow the same pattern.

The current study was designed to allow a more extended comparison of PEP, ns.SCRs and SCL at rest, during different types of mental and physical stress tasks, and during subsequent recovery. The questions we want to address are whether SNS activity shows the same pattern in the heart and in the skin and to what extent PEP, ns.SCRs and SCL are exchangeable in within and between subject designs. We expected a positive correlation between ns.SCRs and SCL and a significant negative linear relation between PEP and these two skin conductance measures, both between and within subjects.

Methods

Subjects

Thirty-nine university students (23 males) between 18 and 28 years (mean = 22.0 years, SD = 2.4) were recruited, who had no overt somatic or psychiatric disease, did not taking cardio-active or psychotropic medication and were not severely obese (BMI <30). The study was approved by an ethics committee, and all subjects provided written informed consent. At the end of the second test day participant received 40 euro.

Protocol

This study is part of a double-blind randomized controlled trial testing the effects of dexamethasone versus placebo on cardiovascular reactivity. Here we only report on the data collected after the administration of a placebo. All women not taking OC (n = 2) were tested

in the follicular phase (days 1-11) of their menstrual cycle according to self-report. Women taking OC were not restricted to a specific phase of the menstrual cycle.

Procedure

The subjects were asked to refrain from alcohol- or caffeine-containing beverages the evening before the test day and in the morning before coming to the laboratory. Testing always took place exactly 3 hours after spontaneous awakening. The experimental session was conducted in a dimly lighted, sound-attenuated cabin, with the subjects facing a video screen at 90 cm. Subject were attached to the electrocardiogram (ECG), the impedance cardiogram (ICG) and the EDA recording devices of the BioPac data-acquisition system (BioPac systems Inc., Santa Barbara, CA). This will be discussed in more detail later on.

The various experimental conditions were explained to the subject and the mental and physical stress tasks were briefly practiced. The actual experiment started by asking the subjects to sit quietly and relax for a pre-test 10 minutes resting baseline. Next, the following conditions were presented in a fixed order: Stroop colour word task (4 min), recovery1 (3 min), tone avoidance task (4 min), recovery2 (3 min), lying (2 min), standing (2 min), recovery3 (2 min), hand grip test (2 min), cold pressure test (1 min), recovery4 (3 min), and the step test (2 min). After the step test a final post-stress resting condition of 13 minutes concluded the physiological recordings.

Mental and Physical Stress Tasks

Stroop colour word (SCW). Subjects were presented with one slide per second on a computer screen which had the name of a colour printed in a contrasting coloured ink. Participants were requested to verbally identify as fast as possible the colour of the ink, not the name of the colour.

Tone avoidance task (TA). An ‘x’ was shown briefly (500 ms) in one of the corners on the screen and the subjects were asked to respond as fast as they possibly could by pressing the button opposite to this corner on a four-button response panel (e.g. ‘x’ shown in the top left-hand corner, press the bottom right-hand button). Incorrect or too slow responses were punished with a loud noise burst (1000 Hz, 85 dB) that lasted 500 msec. Reaction time had to be shorter than a maximal response period, that was initially set to 550 msec, and was thereafter continuously adapted to the performance of the subject (Willemsen, de Geus, Klaver, van Doornen, & Carroll, 1996).

Postures. Subjects were asked to lie down for 2 minutes, followed by standing for 2 minutes.

Hand grip (HG). During the practice part of the experiment, maximum grip strength in the dominant hand was established with a hand grip dynamometer. During the actual hand grip test subject squeezed at 30% of their maximum voluntary contraction for a period of 2 minutes.

Cold pressor (CP). The subjects were asked to submerge their dominant hand up to the wrist joint in a bucket of ice water of 3–5 ˚C and to hold the fingers in a relaxed position. After exactly 60 seconds the hand was removed from the bucket.

Harvard step test (ST). Subjects were asked to stand comfortably upright before a standard gym bench of exactly 45 cm height. They were asked to step up the bench every two seconds for 2 minutes (60 steps). Timed verbal commands ensured that the appropriate step frequency was maintained.

Physiological Assessments

The ECG and ICG were recorded using seven pregelled Ag/AgCl spot electrodes (UltraTrace, ConMed, USA) in a configuration shown in Figure 1. The electrodes were connected to the ECG100C and NICO100C BioPac modules using extension leads. For each experimental condition the PEP (in msec) was scored as the interval from the R-wave peak, minus a fixed interval of 48 msec (Lozano et al., 2007; Willemsen et al., 1996; Sherwood et al., 1990) to the B-point, which signals opening of the aortic valves.

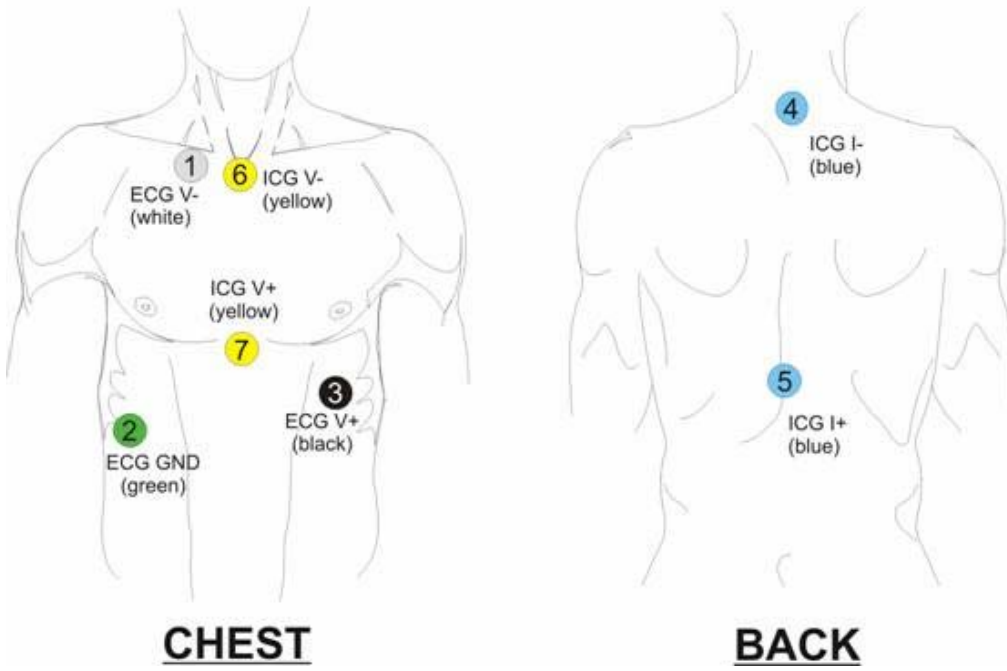

Figure 1. Location of the seven ECG and ICG electrodes.

Skin Conductance information was collected using a pair of Ag/AgCl (unpolarizable) electrodes (Ø = 6 mm). To ensure sufficient electrode–skin contact, isotonic electrode paste was used (0.5% saline in a neutral base). The electrodes were attached with a Velcro strap to the distal phalanx of the index and middle finger (Scerbo, Freedman, Raine, Dawson, & Venables, 1992) of the non-dominant hand with the leads connected to the GSR100C BioPac module (Figure 2). The skin conductance is measured with the 0.5 V constant voltage

method. The fluctuating current conducted through the skin of the subject represents the conductance signal. Recorded data was processed to obtain ns.SCRs and SCLs for every period of interest. The SCL was defined as the mean level of skin conductance and the ns.SCRs as the number of phasic increases in conductance of at least .05 µmho per minute.

Figure 2. Location of the two skin conductance electrodes.

Analyses

With an independent t-test in SPSS 13.0 (SPSS Inc., Chicago, USA) we first tested whether sex differences influenced baseline PEP, ns.SCRs and SCL scores. We also examined the effect of age on PEP, ns.SCRs and SCL by Pearson correlation analysis. Repeated measures ANOVA was used to test for the effect of condition (rest 1, SCW, recovery1, TA, recovery2, lying, standing, recovery3, HG, CP, recovery4, ST, and rest 2) on PEP, ns.SCRs and SCL. To test for significant reactivity, pre-planned contrast compared the pre-test resting baseline levels of PEP, ns.SCRs and SCL to the levels obtained in the stress conditions. Finally, Pearson correlations were computed between PEP, ns.SCRs and SCL separately for each of the conditions (between-subject correlations) and separately for each of the subjects across all conditions (within-subject correlations).

If a significant relationship between two variables was found, we used multilevel analysis to examine the effect of individual differences on the intercept and slope of the regression between the variables. Multilevel analysis is a general method of analyzing data with a hierarchical or clustered structure (Snijders & Bosker, 1999). In our data the different stress tasks are clustered within subjects. For this reason, we used multilevel analysis to examine the relationship between PEP on ns.SCRs and SCL, allowing us to investigate the effect of individual differences on the intercept and slope of the regression between the variables. Models with random slope and/or intercept (restricted models) were compared to the unrestricted model. An unrestricted model (also called null model) is one that contains a

dependent variable and a level-1 random intercept. We compared the models on the basis of their explained variance and their fit. A model describes the data better than a previous model when it explains more variance in the dependent variable *and* when the fit is significant better. Explained variance was computed with the following formula suggested by Kreft and de Leeuw (1998): (unrestricted error – restricted error)/ unrestricted error. The deviance fit test, or likelihood ratio test, was used to compare the fit of two models. This test is based on the difference between the deviance statistics of the two models, which has a chi-square distribution with degrees of freedom equal to the difference in the number of parameters estimated in the models being compared. Finally, sex and age were added as potential predictors in the level 2 model to see whether these variables could account for the variance of the random intercept and slope.

Results

For the baseline, the average PEP score was 119.49 ms (*SD* = 12.22), the average number of ns.SCRs was 1.64 (*SD* = 1.37), and the average SCL was 11.43 µmho (*SD* = 3.92). An independent samples *t* test was conducted to see if there were any differences between sexes. No significant sex differences were found (PEP, t (37) = -.29, p = .77; ns.SCRs, t (37) = .99, p = .33; SCL, t (37) = .34, p = .74). The computed correlation of PEP, ns.SCRs and SCL with age revealed no significant effect of age on the three measures (r = -.03, r = -.02, and r = -.05, respectively).

Table 1. Mean scores (SD) for PEP, ns.SCRs and SCL separately per condition

Conditions	PEP(msec)	ns.SCRs (freq)	SCL (µmho)
Rest 1	119.49 (12.22)	1.64 (1.37)	11.43 (3.92)
Stroop	116.62 (12.53)*	4.85 (2.69)*	13.58 (4.01)*
Recovery 1	119.28 (12.27)	1.35 (1.27)*	11.92 (3.51)*
Tone Avoidance	117.13 (13.20)*	4.69 (2.65)*	13.61 (3.76)*
Recovery 2	120.31 (11.07)	1.30 (1.23)*	11.99 (3.52)*
Lying	107.79 (8.99)*	.86 (1.00)*	12.44 (3.55)*
Standing	116.82 (11.79)*	2.13 (1.31)*	12.55 (3.64)*
Recovery 3	118.97 (11.19)	2.15 (1.71)*	12.76 (3.73)*
Hand grip	118.97 (12.20)	3.50 (2.60)*	14.28 (3.83)*
Cold Pressor	119.49 (12.59)	2.40 (2.10)*	13.97 (3.70)*
Recovery 4	120.72 (10.89)	1.59 (1.32)	12.63 (3.45)*
Step test	70.40 (7.89)*	10.57 (4.06)*	16.20 (3.24)*
Rest 2	117.54 (10.73)	2.21 (1.78)*	12.88 (3.71)*

* Significant difference at p <.05 level with rest 1.

The means and standard deviations for PEP, ns.SCRs and SCL per condition are presented in Table 1. For all three variables significant effects of condition were found, F (12, 23) = 120.72, p = .00 for PEP, F (12, 25) = 32.22, p = .00 for ns.SCRs, and F (12, 27) = 15.51, p = .00 for SCL. As expected, ns.SCRs and SCL were found to increase significantly over baseline levels during conditions known to increase SNS activity, i.e. Stroop colour word task, tone avoidance task, standing up, hand grip, cold pressor and the step test. In the supine condition, which is expected to decrease SNS activity, ns.SCRs indeed decreased but SCL slightly increased. During the recovery periods both ns.SCRs and SCL decreased in comparison to previous task levels, but in the course of the experiment, and lasting to the post-stress resting condition, a slow increase in ns.SCRs and SCL above the pre-test baseline was seen. PEP significantly decreased in response to the Stroop and tone avoidance task, to standing and to the step test. No changes in PEP were seen during hand grip and cold pressor and unexpectedly, PEP decreased in response to lying down. PEP systematically returned to the baseline level during all recovery periods.

The Figures 3, 4 and 5 display the scatterplots between PEP, ns.SCRs and SCL for all data points. Note that these figures contain both within and between subject variance. PEP and ns.SCRs (r = -.52), PEP and SCL (r = -.30), and ns.SCRs and SCL (r = .42) were significantly correlated, but the correlations between PEP and the two measures of skin conductance can be largely ascribed to the step test condition, where the shortest PEP and highest ns.SCRs and SCL co-occur.

Table 2. Between-subject correlations between PEP, ns.SCRs, and SCL separately per condition

Conditions	$r_{\text{PEP-ns.SCRs}}$	p	$r_{\text{PEP-SCL}}$	p	$r_{\text{ns.SCRs-SCL}}$	p
Rest 1	-.12	.49	-.16	.34	.51	.00
Stroop	-.03	.85	-.14	.41	.55	.00
Recovery 1	-.21	.20	-.15	.37	.43	.01
Tone Avoidance	-.15	.38	-.15	.37	.49	.00
Recovery 2	-.29	.08	-.24	.14	.34	.04
Lying	-.27	.10	-.27	.09	.24	.14
Standing	-.16	.33	-.13	.44	.36	.02
Recovery 3	-.27	.10	-.24	.14	.53	.00
Hand grip	-.15	.37	-.36	.03	.33	.05
Cold Pressor	-.43	.01	-.23	.17	.24	.14
Recovery 4	-.19	.26	-.25	.12	.41	.01
Step test	.39	.02	-.07	.69	.05	.79
Rest 2	-.22	.17	-.28	.08	.53	.00

Bold: significant at p < .05 level.

To separate the between and within subject components of the covariance in our measures we first computed between subject correlations separately within each of the 13

experimental conditions. This is shown in Table 2. Moderate correlations ($0.34 < r < 0.55$) were found between the two measures of skin conductance, except during lying, cold pressor and the step test. However, PEP was largely uncorrelated with ns.SCRs and SCL.

Table 3. Within-subject correlations between PEP, ns.SCRs, and SCL for all conditions.

Subject	$r_{PEP\text{-}ns.SCRs}$	*p*	$r_{PEP\text{-}SCL}$	*p*	$r_{ns.SCRs\text{-}SCL}$	*p*
1	-.26	.39	-.11	.72	.56	.05
3	.20	.53	.51	.09	.77	.00
5	-.77	.00	-.64	.02	.87	.00
6	-.74	.00	-.54	.06	.91	.00
7	-.62	.02	-.47	.11	.66	.02
8	-.73	.01	-.62	.02	.89	.00
9	-.37	.24	-.29	.35	.89	.00
10	-.92	.00	-.73	.00	.82	.00
11	-.85	.00	-.70	.01	.74	.00
12	-.82	.00	-.44	.13	.73	.01
13	-.86	.00	-.56	.05	.67	.02
14	-.88	.00	-.71	.01	.59	.03
15	-.81	.00	-.46	.11	.69	.01
16	-.92	.00	-.35	.24	.39	.19
17	-.86	.00	-.65	.02	.87	.00
18	-.50	.08	-.38	.20	.91	.00
19	-.94	.00	-.36	.23	.40	.18
20	-.48	.10	-.72	.01	.87	.00
21	-.51	.08	-.23	.46	.78	.00
22	-.68	.01	-.43	.15	.75	.00
23	-.61	.03	-.66	.01	.78	.00
24	-.91	.00	-.81	.00	.83	.00
25	-.84	.00	-.73	.00	.78	.00
26	-.87	.00	-.66	.01	.65	.02
28	.75	.01	.41	.18	.67	.01
29	-.96	.00	-.46	.11	.57	.04
30	-.31	.30	-.15	.63	.91	.00
31	-.23	.44	-.29	.34	.64	.02
32	-.83	.00	.49	.09	-.34	.26
33	-.75	.00	-.08	.80	.64	.02
34	-.81	.00	-.79	.00	.96	.00
35	-.70	.01	-.77	.00	.82	.00
36	-.68	.01	-.59	.03	.74	.00
41	-.55	.07	-.41	.19	.80	.00

Table 3. Continued

Subject	$r_{PEP\text{-}ns.SCRs}$	*p*	$r_{PEP\text{-}SCL}$	*p*	$r_{ns.SCRs\text{-}SCL}$	*p*
42	-.41	.16	-.55	.05	.83	.00
43	-.75	.00	-.02	.96	.49	.09
45	-.62	.02	-.61	.03	.90	.00
47	-.80	.00	-.84	.00	.80	.00
48	-.46	.12	-.49	.09	.93	.00

Bold: significant at $p < .05$ level.

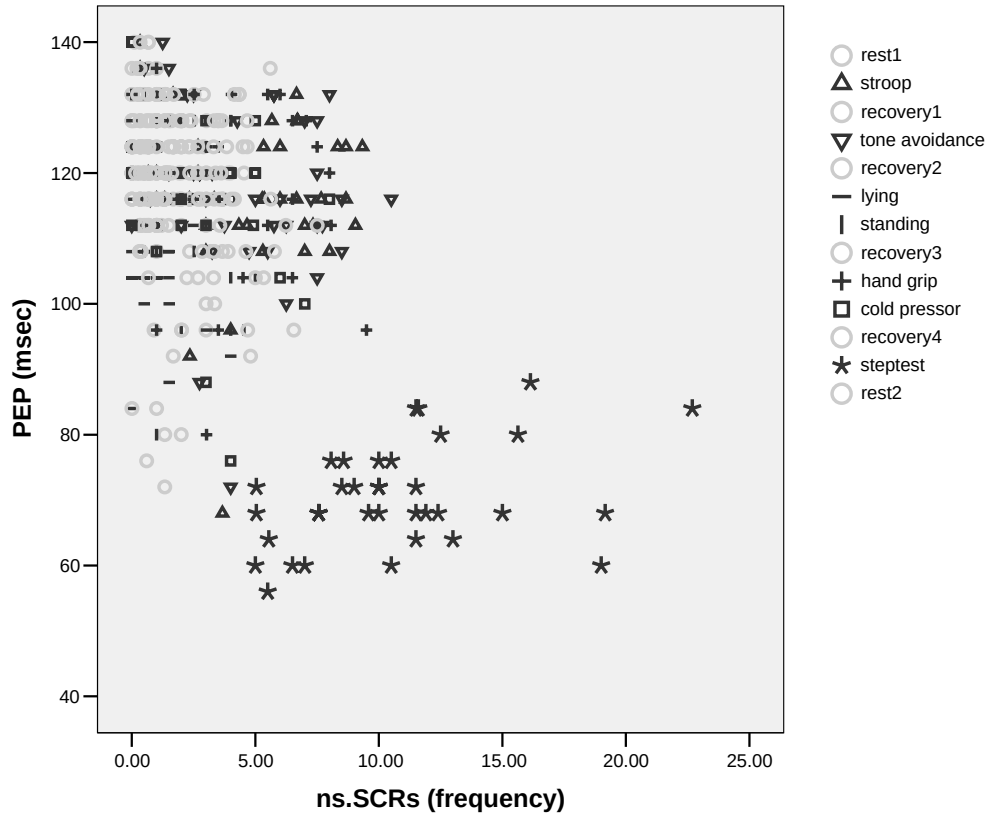

Figure 3. Scatterplot of the number of ns.SCRs per minute and the PEP for all subjects in all conditions.

Within-subject correlations across the 13 experimental conditions are shown in Table 3. The mean within-subject correlation between ns.SCRs and SCL was .72, between PEP and ns.SCRs -.63, and between PEP and SCL -.43. Though within-subject correlations were generally high between ns.SCRs and SCL, large individual differences (r ranged from -.96 to .75) were found in the within-subject correlation between PEP and ns.SCRs and between PEP and SCL. Figure 3 and Figure 4, however, strongly suggest that the relation between PEP and ns.SCRs and between PEP and SCL entirely depended on the exercise condition. When we recomputed the within-subject correlations after exclusion of the step test data, only two significant correlations remained between PEP and ns.SCRs, and only one between PEP and

SCL (Table 4). The correlation between the two measures of skin conductance also decreased but remained significant overall (mean within-subject r = .59).

To further examine the relationship between ns.SCRs and SCL, we used a multilevel analysis. The regression coefficients of the tested models are shown in Table 5. The random intercept model consisting of ns.SCRs being predicted by SCL explained 37.9 % of variance in ns.SCRs and had a significant better fit than the 'empty' model, $\chi^2(1) = 159.57$, $p < .001$. The random slope model explained 38.6 % of variance in ns.SCRs and had a significant better fit than the 'empty' model as well, $\chi^2(1) = 171.80$, $p < .001$. Finally, the extended linear model with a random intercept and a random slope, explained 43.2 % of the total variance in ns.SCRs and had a better fit than both previous models, $\chi^2(2) = 21.81$, $p < .001$ and $\chi^2(2) = 9.58$, $p = .008$ respectively. Figure 6 shows the regression lines per individual for the final model. Sex and age could not predict slope or intercept differences. When we reran the multilevel analysis after exclusion of the step test data, comparable results were found. Again, the best fitting model was the model with random intercept and slope, and this model explained 42.5 % of the variance in ns.SCRs.

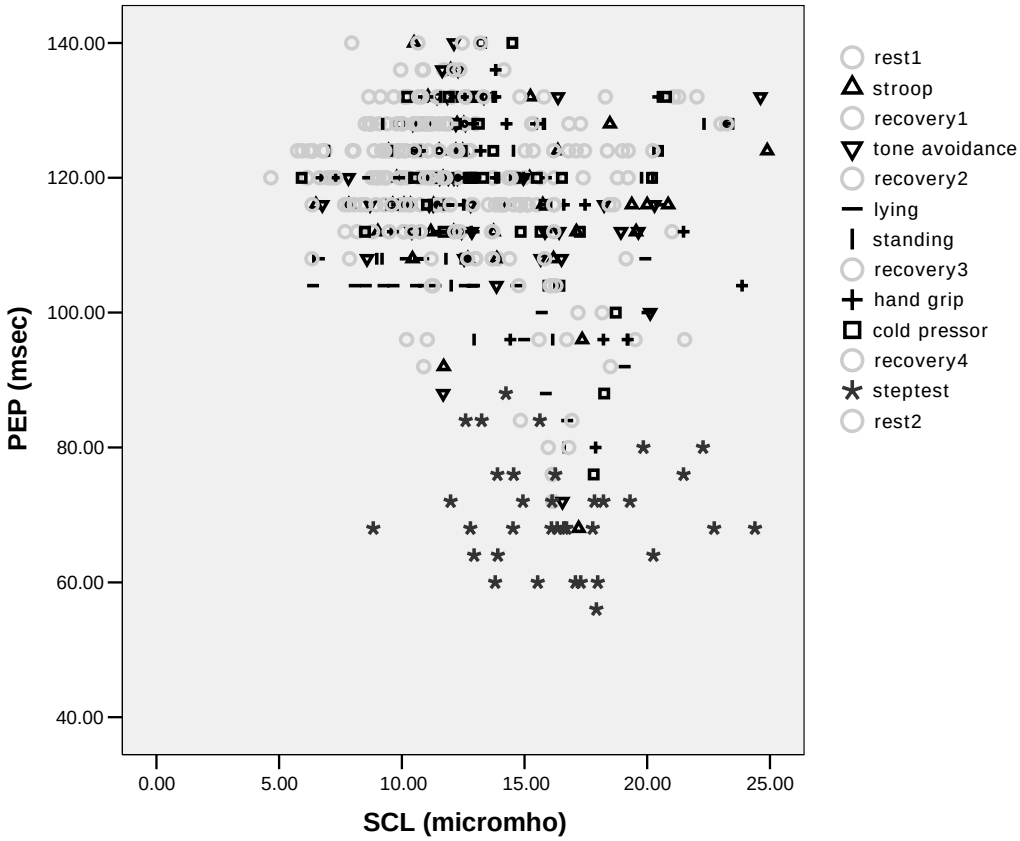

Figure 4. Scatterplot of the SCL and the PEP for all subjects in all conditions.

Table 4. Within-subject correlations between PEP, ns.SCRs, and SCL without step test data.

Subject	$r_{PEP\text{-}ns.SCRs}$	*p*	$r_{PEP\text{-}SCL}$	*p*	$r_{ns.SCRs\text{-}SCL}$	*p*
1	.37	.24	.46	.14	.49	.10
3	.20	.53	.51	.09	.67	.02
5	-.18	.57	-.03	.94	.76	.00
6	.53	.07	.45	.14	.89	.00
7	.15	.64	.32	.31	.47	.12
8	.02	.96	-.27	.39	.85	.00
9	-.37	.24	-.29	.35	.89	.00
10	.31	.33	-.16	.72	.32	.32
11	-.35	.26	-.48	.11	.55	.06
12	-.67	.02	-.22	.49	.66	.02
13	.15	.66	-.10	.76	-.01	.97
14	-.56	.06	-.73	.01	.49	.11
15	.11	.74	.25	.44	.49	.10
16	.48	.12	.00	1.00	.18	.58
17	-.28	.39	-.09	.79	.78	.00
18	.22	.50	.12	.72	.90	.00
19	-.12	.71	-.01	.98	.19	.56
20	-.12	.71	-.16	.62	.86	.00
21	.21	.52	.25	.44	.77	.00
22	-.12	.72	.14	.66	.66	.02
23	.27	.41	-.18	.58	.58	.05
24	.02	.95	-.21	.52	.16	.61
25	-.06	.86	-.19	.55	.40	.19
26	.13	.68	-.05	.88	-.05	.89
28	.75	.01	.41	.18	.76	.00
29	-.45	.14	-.42	.18	.73	.01
30	.22	.49	.26	.42	.91	.00
31	.17	.61	.01	.98	.60	.04
32	-.21	.52	-.22	.50	.39	.21
33	.20	.53	.44	.16	.84	.00
34	.04	.89	.01	.98	.86	.00
35	-.06	.87	.19	.56	.50	.10
36	-.01	.97	-.05	.88	.54	.07
41	-.55	.07	-.41	.19	.40	.20
42	.13	.68	.35	.27	.78	.00
43	-.03	.94	.25	.44	.71	.01
45	.09	.77	-.01	.99	.82	.00
47	-.10	.77	-.37	.24	.38	.23
48	-.07	.83	-.04	.90	.91	.00

Bold: significant at $p < .05$ level.

Table 5. Results of multilevel analysis

	Empty model		**Random intercept**		**Random slope**		**Random intercept and slope**		**Random intercept and slope, sex and age**	
	Coefficients(SE)	*p*	*Coefficients(SE)*	*p*	*Coefficients(SE)*	*p*	*Coefficients(SE)*	*p*	*Coefficients(SE)*	*p*
Fixed effects										
Intercept (γ_{00})	3.05 (.21)	.00	-7.93 (0.82)	.00	-7.50 (0.67)	.00	-8.92 (.99)	.00	-5.80 (.85)	.00
SCL (γ_{10})			0.85 (.05)	.00	0.85 (0.06)	.00	.97 (.09)	.00	.56 (.24)	.00
Sex									-.01 (.05)	.82
Age									.00 (.01)	.63
	Variance (SD)	*p*	*Variance (SD)*	*p*	*Variance (SD)*	*p*	*Variance (SD)*	*p*		
Random effects										
Level 1 residual (R_{ij})	9.82 (3.13)	NA[a]	6.10 (2.47)	NA[a]	6.02 (2.45)	NA[a]	5.58 (2.36)	NA[a]	2.13 (1.46)	NA[a]
Level 2 residuals										
Intercept (U_{oj})	0.80 (0.89)	.00	5.67 (2.38)	.00			16.81 (4.10)	.00	14.48 (3.81)	.00
Slope (U_{1j})					0.02 (0.17)	.00	.16 (.40)	.00	.14 (.37)	.00
Deviances	2478.35		2318.78		2306.55		2296.97		1728.31	
Estimated parameters	3		4		4		6		8	
Explained variance			37.9 %		38.6 %		43.2 %		43.3 %	

[a] The p values are not given because HLM software does not provide a significance test for level 1 residuals.

Conclusion

Both PEP and skin conductance measures are extensively used as indices of SNS activity. In accordance, we found that PEP generally decreased and ns.SCRs and SCL increased during mental and physical stressors known to engage the SNS. The between- and within-subject correlations between the two measures of skin conductance were overall significant, and results showed that around 43% of the variance in ns.SCRs could be explained by SCL when allowing for individual differences. This suggests that these parameters both reflect SNS activity but that the overlap is imperfect and each skin conductance measure therefore also reflects different components of SNS activity. More surprising results were obtained for the association between PEP and the skin conductance measures. The between-subject correlations between PEP and the two measures of skin conductance were weak or absent during each of the stressful tasks. Within subjects, a significant relation was found between changes in PEP and the two measures of skin conductance, but this was entirely due to a short period of moderately intense exercise, which led to a strong decrease in PEP and a strong increase in ns.SCRs and SCL in most subjects.

The absence of a between-subject correlation between PEP and ns.SCRs and between PEP and SCL was unexpected, though results do correspond with the only other study which compared skin conductance and PEP responses to stress (Tomaka, Blascovich, & Swart, 1994). Between-subject differences in absolute PEP have been shown to closely reflect individual differences in ß-adrenergic inotropic drive (Cacioppo et al., 1994). In a study of 10 female undergraduate students a high correlation was found between absolute PEP and heart period increases in response to sympathetic blockade. In further support, a significant inverse correlation between a subjects' absolute PEP and their plasma adrenaline level was found (Levi, Ratti, Cardone, & Basagni, 1982). At the same time, between-subject differences in electrodermal activity have been shown to correlate to individual differences in the number of sympathetic action potentials in peripheral sympathetic nerves. Within normal ranges of ambient room temperature and subject thermoregulatory states, there was a high correlation between bursts of skin sympathetic nerve activity and SCRs (Wallin, 1981). In addition to measuring SNS activity, between-subject differences in both PEP and skin conductance have been shown to be reliable and stable over time (Goedhart et al., 2006; Schell et al., 2002). So, why did we not find between-subject correlations between these measures?

The most likely explanation is uncorrelated individual differences in the responsiveness of the effector systems. SNS activity changes contractility, and with that PEP, through the effects of noradrenergic fibers acting on β_1- and β_2-receptors on the left ventricle (Kelsey, 1991; Sherwood et al., 1990). Hence, the effect of a fixed amount of cardiac sympathetic activity on contractility (and PEP) depends on the sensitivity of these β-adrenergic receptors, which is known to vary strongly between individuals (Brodde, Bruck, & Leineweber, 2006). Likewise, the effect of a fixed amount of skin sympathetic activity on sweat gland activity (and skin conductance) depends on the number of sweat ducts per area of skin. These also show large individual differences (Sato & Sato, 1983). In other words, a subject with a low β-receptor sensitivity and a high number of sweat glands may have long PEP and high SCL, whereas another subject with identical SNS activity but high β-receptor sensitivity and a low number of sweat glands may have a shorter PEP but lower SCL.

More alarming than the absence of a between-subject correlation is the absence of a within-subject correlation. Individual differences in sweat glands or receptor sensitivity should not prevent PEP and skin conductance to correlate across different levels of SNS activation *within* the same individual. Yet, such correlations were not found, although the addition of more powerful engagement of the SNS by the step test did induce a correlation in most subjects. It is unclear why the changes in PEP and ns.SCRs and in PEP and SCL across the other stressors were uncorrelated. One explanation is that the changes in PEP across the stressors are not solely influenced by sympathetic activity, but by changes in preload and afterload effects as well (Lewis, Rittgers, Forester, & Boudoulas, 1977). These latter two are usually not that prevalent during experiments where subjects sit down in a lab during the whole experiment (Sherwood et al., 1990) but during conditions that induce a large change in mean arterial pressure or end-diastolic filling, such effects could have influenced PEP independently from true changes in SNS activity. This seems supported by findings for PEP in the supine and cold pressor conditions. Lying down increases preload and reduces afterload. Whereas we would expect SNS activity to be lower in a supine position, PEP in fact became shorter, and this likely reflects decreased afterload. In contrast, SNS activity can be expected to increase during the stressful cold pressor test, but in fact PEP was unchanged from resting level. This is likely due to the strong increase in mean arterial pressure that is reflexively induced by cold stress. Since ns.SCRs and SCL are not affected by preload and afterload effects, this may have led to a discrepancy between PEP and skin conductance measures.

The within subject correlation may further have been compromised by a gradual increase in skin conductance level and spontaneous frequencies during the experiment which may not reflect a true increase in SNS activity. Although sweat gland activity (filling of the sweat ducts) appears to be the main determinant of both skin conductance measures (Dawson et al., 2000), sweat on the skin (corneal hydration) is thought to play a role as well, especially for SCL (Boucsein, 1992; Fowles, 1986). This also might explain the proportion if unexplained variance between ns.SCRs and SCL. Sweat duct activity is a volatile process mainly due to recent sympathetic nerve activity, but corneal hydration is a much slower process, probably building up during the experiment independently from changes in sympathetic activation. Since corneal hydration occurs independently from changes in sympathetic activation this may have added to the discrepancy between skin conductance measures and PEP.

Finally, differences in adrenal catecholamine release in response to the various stressors may have acted to reduce the PEP – skin conductance correlation. Ventricular β_1- and β_2-receptors do not respond solely to noradrenaline released from the cardiac sympathetic nerve, they are also highly sensitive to circulating catecholamines. Many of the stressors used are known to increase circulating levels of adrenaline and noradrenaline (Schachinger et al., 2001; Kjaer, Secher, & Galbo, 1987) which will co-determine PEP responses. In contrast, sweat gland activity is controlled by sympathetic cholinergic fibers acting on muscarinergic type 3 receptors (Kelsey, 1991; Shields, MacDowell, Fairchild, & Campbell, 1987). Although these also receive additional hormonal input (Wallin, 1981) they will not be sensitive to circulating noradrenaline or adrenaline.

Apart from the methodological explanations above, the absence of a relation between PEP and skin conductance may also reflect true differences in the activation of the various branches of the SNS during stressful tasks. Such differentiation is known to occur from previous studies using direct recording of skin and muscle sympathetic activity (Wallin,

1981) that showed that sympathetic activity might not have a uniform effect on all effector organs. Baroreflex engagement may be a powerful source of differences in vascular/cardiac versus skin SNS activity. Unlike cardiac SNS activity , skin SNS activity is not influenced by the baroreflexes (Wilson, Cui, & Crandall, 2001; Vissing, Scherrer, & Victor, 1994; Bini, Hagbarth, & Wallin, 1981; Wallin, Sundlof, & Delius, 1975). Task induced changes in baroreflex activity, therefore, will affect PEP but not SCL and ns.SCR. The well-known individual differences in baroreflex sensitivity (Riese et al., 2006; Tank et al., 2001) may also partly account for the low between-subject correlations.

We conclude that PEP and skin conductance respond to stress tasks in a manner compatible with increased SNS activity but that their response is largely uncorrelated. The heart and the skin, therefore, seem to reflect different aspects of sympathetic nervous system activity. Since all three measures are responsive to SNS activity and have already shown their use in psychophysiological testing we conclude that, whenever possible, PEP, ns.SCR and SCL should all be measured.

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Chapter 7

THE SYMPATHETIC NERVOUS SYSTEM IN AUTOIMMUNITY – TARGET AND IMMUNE MODULATOR

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Abstract

The sympathetic nervous system (SNS) plays a key role in the maintenance of homeostasis in different systems of the body. Beside its physiological function such as the regulation of cardiovascular functions, sympathetic efferent fibers can also contribute to immunoregulation. Under pathological conditions, the SNS is involved in the regulation of Th1-mediated autoimmune processes and can influence the course of neuroimmunological diseases, such as multiple sclerosis.

More recently, different structures within the sympathetic nervous system (SNS) have been reported to be targets for autoimmune processes, leading to a severe dysfunction of the SNS. Autoantibodies against beta-adrenoceptors are involved in the pathogenesis of some cases of dilated cardiomyopathy. These autoantibodies have been reported to be agonistic at the beta-adrenoceptor, leading to an overstimulation and, subsequently, to a dilatation of cardiomyocytes. Moreover, autoimmunity against SNS has been reported in autonomic neuropathies in type1 diabetes mellitus or in dysimmune neuropathies.

Complex regional pain syndrome (CRPS, sympathetic reflex dystrophy) is an etiologically unclear syndrome including pain and trophic disturbances after limb trauma or operation. Different studies showed a sympathetic dysfunction in CRPS, both locally and in the central nervous system. Very recently, the finding of autoantibodies against sympathetic nervous system structures has not only provided an explanation for the SNS dysfunction in these patients. It also led to the hypothesis of an autoimmune etiology of CRPS, which may have an important impact on the future treatment of CRPS patients.

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1. Introduction

The autonomic nervous system (ANS) and the immune system are two major systems to maintain homeostasis and the integrity of the body. However to fulfil this aims, a cross talk between both systems is necessary. The ANS is clearly divided into three components, being the sympathetic (noradrenergic), the parasympathetic (cholinergic) and the enteric nervous system in the wall of the gut. Almost all tissues and organs of the body are innervated by the ANS and the function of this system is mainly not under conscious control. The sympathetic nervous system (SNS) in generally prepares the body for stress. Most cell bodies of the sympathetic preganglionic fibers are located in the lateral horns of the spinal chord segments Th1-L2. The preganglionic fibers enter the sympathetic ganglia, which are located anterolateral to the spine or prevertebral. From these ganglia, postganglionic fibers are sent to all innervated organs. Acetylcholine acts via nicotinic receptor in preganglionic synapses and in sweat glands sympathetic nerve endings. Most nerve terminals of postganglionic fibers release noradrenaline, which can bind to different receptors, mainly α- and β-adrenoceptors. The adrenal medulla, in which the chromaffine cells are innervated preganglionic (nicotinic) sympathetic nerve terminals, releases systemically mainly adrenaline and less noradrenaline (approximate ratio 4:1).

A. Influence of the SNS on the Immune System

Whereas the sympathetic innervation of heart, blood vessels and other organs has been established a long time ago, the innervation of lymphoid organs and a cross talk between the these two systems has first been shown in the 1970s and 1980s, mainly by the work of Besedovsky and co-workers [Besedovsky et al. 1975]. Postganglionic sympathetic nerve fibers enter the thymus and the majority is ending in the thymic cortex and the corticomedullary junction, whereas the sympathetic innervation of the medulla is sparse [Williams & Felten 1981, Felten 1985]. Different cell populations in the thymus, such as thymocytes and mast cells, but also thymic epithelial cells, carry β-adrenoceptors on their surface [Vizi et al. 1995, Kurz et al. 1997]. Other lymphoid organs (spleen, lymph nodes and tonsils) show also sympathetic innervation [review in Elenkov et al. 2000]. Most immune cells with exception of T helper (h) 2 cells express β-adrenoceptors [Khan et al. 1986, Maisel et al. 1990] and adrenergic signalling via the sympathetic nervous system influences thymocyte development, lymphocyte traffic and circulation [review in Elenkov et al. 2000]. Additionally, noradrenaline has different effects on type 1 and type 2 cytokine expression, leading to a shift towards Th2 [Elenkov et al. 1996, Sanders et al. 1997]. Interestingly, some cell types, such as dendritic cells (DC) can react differently, depending on the adrenergic receptor expressed on the surface: β2-adrenergic stimulation inhibits DC functions, whereas the expression and stimulation of alpha-adrenergic receptors stimulates DC [Maestoni 2006]. However, different studies show that, apart from noradrenaline (NA), neuropeptide Y (NPY) is an important neurotransmitter of postganglionic sympathetic nerve terminals innervating lymphoid organs. NPY signalling can suppress human NK cell activity [Nair et al. 1993] and NPY modulates T-cell adhesion processes [Levite et al. 1998].

B. Effects of Immune-Regulatory Molecules on the SNS

Immune cells release a variety of interleukins (IL) and other factors, that can directly influence the nervous system, firstly shown by the influence of IL-1 on hypothalamic noradrenergic neurons and on the NA content in the spleen [Besedovsky et al. 1983, 1986]. Interferon-α (IFN-α) or IL-1β administration induces an increase of sympathetic activity in the spleen [Katafuchi et al. 1991], whereas Tumor-necrosis-factor-α (TNF-α) seems to inhibit noradrenergic transmission in some hypothalamic regions [Elenkov et al. 1992]. In the enteric nervous system, TNF-α induces hyperpolarisation of neurons and inhibits the NA release [Hurst & Collins 1994, Rehn et al. 2005]. Interestingly, immune factors do not only influence activity states of the sympathetic nervous system. Recently, it has been shown, that the chemokine CXCL9 promotes the survival of rat primary sympathetic neurons [Uwabe et al. 2005]

The cross-talk between the two systems, SNS and immune system, seems to influence the function of the autonomic nervous system generally during inflammatory diseases. Vice versa; the SNS is obviously able to modulate immune functions in different ways. However, this physiological cross-talk can also contribute to pathological conditions such as autoimmunity. In autoimmune diseases, the SNS can be the target of an autoimmune attack, but can also contribute to the development of autoimmunity by influencing autoreactive cells. The following review will describe both ways, the SNS can be involved in autoimmunity.

2. Sympathetic Regulation of Autoimmune Processes

Autoimmune diseases are thought to be the result of the tolerance breakdown of the immune system to the own tissues of the body. They can appear as organ-specific autoimmune diseases, such as thyroiditis or multiple sclerosis or involving different organs and tissue types as seen in inflammatory rheumatic disorders. The exact physiopathology of autoimmune diseases is still not understood. Pathogenic autoantibodies, mainly of the IgG class can be observed in some diseases, others seem to be mainly T-cell mediated and the observed autoantibodies are only a diagnostic hallmark in the serum. However, CD4+ Th1 cells are widely suspected to have a key role in the physiopathology of autoimmune diseases. The production of proinflammatory cytokines by these cells augments and maintains the autoimmune process, leading n the end to tissue destruction.

As described in the introduction, the communication between SNS and immune system can influence both systems. Noradrenaline is able to suppress Th1-mediated immune processes [Elenkov et al. 1996, Sanders et al. 1997]. Interestingly, a similar effect of the SNS in autoimmune diseases could also be shown: treatment with the β2-agonist salbutamol suppresses the collagen-induced autoimmune arthritis, which is typically associated with a Th1 immune response [Malfait et al. 1999]. Treatment with beta-agonists in this animal model obviously promotes anti-inflammatory cytokines (IL-4, TGF-β) and suppresses the production of proinflammatory cytokines [Cobelens et al. 2002]. In another animal model of autoimmune disease, experimental autoimmune encephalomyelitis (EAE), an increased severity of EAE after chemical sympathectomy could be demonstrated [Chelmicka-Schorr et al. 1988]. Additionally, beta2-agonist treatment can also suppress the autoimmune process in EAE [Wiegmann et al. 1995]. However, sympathetic signalling is not only mediated by

catecholamines, but also by other neuropeptides, such as NPY. This neurotransmitter acts via specific receptors (NPY receptors Y1-Y5) and has also modulatory effects on immune functions. The secretion of IFN-γ is suppressed and, parallel, IL4 production is increased in murine lymphocytes in vitro [Kawamura et al. 1998, Levite et al. 1998].

In paraneoplastic (tumour-associated) neurological syndromes with autonomic neuropathy, a disturbance in the IgG subclass distribution including IgG4 down-regulation can be observed. Since these patients have severe disturbances of the autonomic nervous system, which, vice versa induces disturbances of the immune system [Blaes et al. 2002].

It seems, that SNS neurotransmission into the immune system, both catecholaminergic and NPY-mediated, generally lead to a shift from Th1 towards Th2. This is supported by different observations. It has been suspected, that a hypoactive SNS can contribute to multiple sclerosis or rheumatoid arthritis, in both of which Th1-mediated autoimmunity is observed. Actually, it could be found that sympathetic skin responses in multiple sclerosis are decreased and, in EAE the noradrenaline content in the spleen is already reduced in preclinical stages [Karaszewski et al. 1990, Mackenzie et al. 1989]. Additionally, hypoactive SNS has been observed in Lewis rats, an animal which is prone to develop autoimmune diseases, such as arthritis or EAE. Vice versa, Fisher rats, having a hyperactive SNS are resistant against the induction of these diseases [review in Wilder 1995].

In conclusion, the activation of the SNS can possibly suppress Th1-mediated, but augments Th2-mediated autoimmunity, what may be a potential pharmacological target in autoimmune diseases.

3. Autoimmunity Against the Sympathetic Nervous System

A. Autoimmune Autonomic Neuropathy

Isolated autonomic neuropathies are rare disorders. Since a variety of symptoms, such as orthostatic hypotension, cardiac arrhythmia, gastrointestinal dysmotility, sweating disturbances or erectile dysfunction affecting different organ systems, the definite diagnosis is sometimes difficult. It has been known for decades, that autonomic failure can be a n associated symptoms in neurodegenerative disorders, such as Parkinson´s disease. Pure or predominant autonomic neuropathy can be caused by many diseases, such as diabetes, amyloidosis, toxic agents or hereditary [review in Freeman 2003]. The main clinical syndrome of patients with a subacute autonomic neuropathy is the development of acute padysautonomia including orthostatic hypotension, anhidrosis and gastrointestinal dysfunction [Suarez et al. 1994]. An autonomic neuropathy as a distinct autoimmune neuropathy was first described by Vernino et al. [1998]. The majority of these patients had autoantibodies against the α3 subunit of the nicotinic acetylcholine receptor (nAChR). The α3 nAChR subtype is involved in the fast synaptic transmission through both sympathetic and parasympathetic ganglia [review in Skok 2002]. The autoantibodies against the α3 nAChR have been shown to be associated with cancer-related (paraneoplastic) and non-paraneoplastic pure autonomic neuropathy, but not in other diseases associated with autonomic neuropathies or healthy controls [Vernino et al. 1998, Vernino et al. 2000]. The same group could demonstrate that immunization with the recombinant α3 subunit of nAChR (residues 1-205) induces autonomic neuropathy in rabbits [Lennon et al. 2003]. Beginning at week 4, the

rabbits developed autoantibodies against the ganglionic acetylcholine receptor, and parallel, the food intake began to fall, the rabbits showed weight loss. As a sign of sympathetic denervation, some of the animals showed sustained ptosis. Autopsy showed signs of intestinal pseudo-obstruction and megacystis [Lennon et al. 2003]. However, not only active immunization could induce autonomic neuropathy. Passive transfer of rabbit IgG containing ganglionic AChR antibodies into mice induced transient dysautonomia including gastrointestinal dysmotility, reduced heart rate variability and impaired catecholamine response to stress [Vernino et al. 2004]. The successful establishment of an autonomic neuropathy in animals by active and passive immunization with the α3 nAChR does not only prove the autoimmune pathogenesis of the disease, but also provides a model for the study of autonomic disturbances.

B. Autoimmune Diseases with Autonomic Disturbances

Different autoimmune diseases can be associated with disturbances of the autonomic nervous system and some of them are associated with autoantibodies against well characterised autoantigens (Tab.1). In dysimmune neuropathies, life-threatening autonomic disturbances can frequently be observed. Moreover, autonomic neuropathies have been described in inflammatory rheumatic diseases, such as lupus erythematosus or Sjögren´s syndrome. Moreover, paraneoplastic (tumour-associated) neurological syndromes (PNS) can have severe autonomic symptoms, involving both sympathetic and parasympathetic nervous system.

Polyradiculitis Guillain-Barré (GBS) is the prototype of a dysimmune neuropathy, mostly developing as a inflammatory neuropathy after bronchopulmonary or gastrointestinal infection and concomitant cross-reactive autoimmunity against peripheral nervous system structures. Clinically, a variety of autonomic disturbances, such as cardiac arrhythmia, labile blood pressure and others can be observed. The diagnosis of autonomic involvement includes different autonomic function tests, such as valsalva ratio and R-R interval variation during rest and deep breathing for parasympathetic autonomic function, and, for the sympathetic function, blood pressure responses to sustained handgrip and active standing [Lyu et al. 2002a]. For severe GBS patients, which are mechanically ventilated, an increased daily systolic blood pressure variation has been described as a marker of autonomic nervous system involvement [Pfeiffer et al. 1999]. These studies show a severe autonomic involvement in about 25% of GBS patients. However a mild autonomic impairment including both sympathetic hyper- and hypoactivity, can be observed in almost all GBS patients depending on the autonomic function tests used [Pfeiffer et al. 1999, Asahina et al. 2002, Lyu et al. 2002a]. In chronic inflammatory demyelinating neuropathy, a mild autonomic neuropathy is detectable in up to 50%, and even in experimental allergic neuritis, an animal model of dysimmune neuropathy, a sympathetic involvement occurs in about half of tested animals [Wang et al. 2001, Lyu et al. 2002b]. Interestingly, the exact pathogenesis of autonomic involvement is widely unclear. GBS can occur both as a demyelinating or axonal neuropathy, and most autoantibodies described in GBS are directed against structures of the myelin sheath [Willison & Yuki 2002]. It could be shown, that the IgG fractions of GBS patients have blocking effects in an vitro model of the neuromuscular junction [Buchwald et al. 1998] and this effect can be decreased by pooled intravenous immunoglobulin fractions (Buchwald et al.

2002). In contrast, binding of GBS IgG to autonomic structures or in vitro effects of GBS IgG on autonomic neurons have not been described yet.

Paraneoplastic neurological syndromes (PNS) are nervous system disorders, that are associated with a tumour, but are not caused by a local effect of the tumour or its metastases. Classical PNS are sensory neuropathy, subacute cerebellar degeneration and limbic encephalitis. These syndromes are not specifically paraneoplastic, but they are frequently associated with an underlying tumour [Dalmau & Graus 1997]. In about 50-70% of the PNS patients, autoantibodies against neuronal structures can be found [Review in Blaes et al. 2000]. These antineuronal autoantibodies react also with autoantigens expressed in the tumour, indicating a cross-reactive autoimmune reaction against onconeuronal antigens in the pathogenesis of PNS [Dalmau et al. 1992]. In some of these patients, autonomic disturbances are described, mainly as gastrointestinal pseudoobstruction, leading to recurrent gut motility disturbances [Blaes et al. 2002]. However, in our own patients, about 1/3 exhibit autonomic disturbances, mostly gastrointestinal pseudoobstruction, but also orthostatic hypotension and other cardiovascular problems, indicating an involvement of the SNS in these syndromes. The central nervous system PNS are mainly T-cell mediated diseases [Albert et al. 1998, Bernal et al. 2002]. However, in peripheral nervous system PNS, the role of T-cells and autoantibodies is not completely understood. Most patients with PND autonomic disturbances have anti-Hu-, anti-CRMP5 or anti-Yo antibodies. Anti-Hu antibodies bind to a group of RNA-binding proteins expressed in neuronal and tumour cell nuclei and are mostly associated with chronic pseudoobstruction and small cell lung cancer (SCLC). Anti-CRMP5 antibodies bind to a cytosolic phosphoprotein expressed in a subset of oligodendrocytes in the central nervous system and some Schwann cells and sensory neurons in the peripheral nervous system. The patients have chronic gastrointestinal pseudoobstruction and SCLC or thymoma. We and others could show, that IgG of patients with PNS and anti-Hu, a specific antineuronal autoantibody, can induce apoptosis in primary cultures of gut myenteric plexus neurons [Schäfer et al. 2000, de Giorgio et al. 2003]. However, a specific effect on SNS neurons has not been established yet.

In another classical PNS, the Lambert-Eaton myasthenic syndrome (LEMS), muscle weakness and autonomic disturbances, including sweating disturbances, gastrointestinal motility disturbances are the main symptoms. About 60% of the LEMS patients develop a small cell lung cancer (SCLC) and autoantibodies against P/Q-type voltage-gated calcium channels (anti-VGCC) are detectable in almost all LEMS patients [O`Neill et al. 1988, Motomura et al. 1997]. *In vitro* studies could demonstrate, that the IgG of LEMS patients impair the transmitter release from parasympathetic and sympathetic neurons through down-regulation of voltage-gated calcium channels [Waterman et al. 1997].

Inflammatory rheumatic diseases, such as systemic lupus erythematosus (SLE), scleroderma (SSc) or Sjögren´s syndrome are autoimmune diseases involving different tissues and organ systems. Both central and peripheral nervous system involvement has been described in all these diseases. Using a set of standardised autonomic function tests, dysautonomia can be found in 15-30% of SLE and SSc and 3-60% of Sjögren´s syndrome [Straub et al. 1996, Kovasc et al. 2004, Mori et al. 2005]. However, regarding single autonomic functions, such as cardiovascular abnormalities, orthostatic dysregulation, gastrointestinal or urogenital dysfunctions, the prevalence of autonomic disturbances varies between 0-90% [review in Straub et al. 2005]. Therefore, it is necessary to have clear criteria defining an autonomic neuropathy. It has been proposed, that the diagnosis of autonomic

neuropathy should only be made, if at least two out of five autonomic function tests are abnormal [Ziegler et al. 1992]. In most clinical studies, involvement of the sympathetic part of the autonomic nervous system in rheumatic disease-associated autonomic neuropathy could be demonstrated [review in Straub et al. 2005]. The physiopathology of autonomic dysfunction in rheumatic diseases is unclear, although an autoimmune process against structures of the autonomic nervous system may be a logical assumption. However, in contrast to other autoimmune autonomic neuropathies, direct autoimmunity against the sympathetic nervous system has not been demonstrated yet in these patients [Schnell et al. 1996, Blaes et al., unpublished data]. Only in patients with scleroderma, an influence of autoantibodies on the gut myenteric plexus neurons could be demonstrated [Eaker et al. 1999]. The lacking detection of specific autonomic nervous system autoantibodies may be due to the problem of a variety of high-titer autoantibodies against ubiquitous autoantigens, such as antinuclear antibodies (ANA). An additional pathogenetic factor may be the influence of proinflammatory cytokines. IL-6 administration induces sympathetic dysfunction in humans and TNF-α inhibit noradrenergic transmission in some hypothalamic regions [Elenkov et al. 1992, Torpy et al. 2000]. Since both cytokines are elevated in inflammatory rheumatic diseases, these factors may contribute to the development of autonomic neuropathy.

Different authors have described autoantibodies against sympathetic nervous system in type 1 diabetes [Zanone et al. 1993, Schnell et al. 1996]. These autoantibodies bound to neuronal cells in sympathetic ganglia and adrenal medulla. In type 1 diabetes, an autoimmune reaction against different autoantigens in the pancreatic islet cells, such as anti-glutamic acid decarboxylase or anti-islet cell antigen (ICA) antibodies can be detected. It has never been excluded, that the autoantibodies against sympathetic tissues in diabetes patients result from a cross reaction between the neuroendocrine differentiated islet cells and sympathetic neurons. Additionally, a specific autonomic nervous system autoantigen has never been identified. However, there are different results supporting the hypothesis, that autonomic neuropathy in type 1 diabetes is caused by an autoimmune process against sympathetic and other autonomic nervous system structures. The detection of autoantibodies in these patients was associated with autonomic neuropathy, determined by standardised cardiovascular function tests [Zanone et al. 1993, Schnell 1996]. Moreover, it has been shown, that IgG fractions of patients exhibiting autoantibodies against sympathetic nervous system tissue induces cytotoxic effects in neuroblastoma cells [Zanone et al. 2003]. Therefore, clinical and experimental data suggest that autoimmunity against sympathetic neurons is one cause for autonomic neuropathy in type 1 diabetes.

C. Autoimmunity against Beta-Adrenoceptors

In 1976, Sterin-Borda et al. reported, that antibodies of patients with Chagas disease, an infectious disorder caused by Trypanosoma cruzi, have a positive chronotropic effect on isolated rat atrial preparations. They also found, that the effect was blocked by beta-antagonists and postulated the existence of beta-agonistic autoantibodies in Chagas heart disease [Sterin-Borda et al. 1976]. In 1980, autoantibodies against the β2-adrenergic receptor were described in allergic rhinitis and asthma (Venter et al. 1980) and this finding was confirmed by another group [Wallukat & Wollenberger 1991]. Additionally, Wallukat and

co-workers demonstrated agonistic effects of IgG fractions from allergic asthma and dilated cardiomyopathy (DCM) on beta-adrenoceptors in neonatal rat heart myocytes [Wallukat & Wollenberger 1987] and postulated the existence of anti-β1-adrenoceptor autoantibodies (β1-AAB) in DCM patients. This finding was confirmed by Limas et al. [1989] and one year later, epitope mapping revealed the first and second extracellular loops of the β1-adrenoceptor as main antigenic epitope in patients with DCM and β1-AAB [Magnusson et al. 1990, Wallukat et al. 1995].

Since incidence and mortality of DCM is still high, the disease and its underlying physiopathology has a great clinical impact. Three factors are discussed in the physiopathology of DCM: a genetic predisposition, viral infections and immune mechanisms. As mentioned above, the finding of β1-AAB led to an autoimmune hypothesis in at least a part of idiopathic DCM. In the recent years there are several lines of evidence, that a subgroup of DCM is autoimmune and caused by β1-AAB. The β1-adrenoceptor obviously plays a key role in the development of DCM. Physiologically, stimulation of this G-protein coupled receptor by catecholamines activates the adenylylcyclase-protein kinase A (PKA) pathway. Activated PKA then phosphorylates L-type calcium channels, inducing Calcium influx in the cells from outside the cells and subsequently from the sarcoplasmatic reticulum. The increase in intracellular Calcium is responsible for the positive inotropic (increase in contractility) and chronotropic (increase in beating frequency) effects of the catecholaminergic stimulation. However, the PKA and an additional activated molecule, the G-protein coupled protein kinase 2 (GRK2) both phosphorylates the β1-adrenoceptor, leading to a desensitisation and internalisation of the receptor as a negative regulation [review in Wallukat 2002]. Pharmacological overstimulation of the receptor by beta-agonists has been shown to increase both processes, receptor desensitisation and internalisation, and to lead to cytotoxic effects on cardiomyocytes which may contribute to the pathogenesis of DCM [Karliner et al. 1986, Zhang et al. 2005]. Anti-β1-adrenoceptor autoantibodies isolated from patients sera by immunoadsorption also exhibit complement-dependent cytotoxic effects on cardiomyocytes in vitro and positive chronotropic effects [Chen et al. 2006]. Moreover, it has been shown, that the chronotropic effect induced by β1-AAB is mediated via activation of the adenylate cyclase / PKA pathway [Chen et al. 2006]. Interestingly, although β1-AAB do not bind to β1-adrenoceptor-agonists binding site, they can produce similar effects [Wallukat et al. 1995]. Recently, it could be shown, that immunisation of inbred rats with a peptide from the second extracellular loop of the β1-adrenoceptor induced agonistic binding β1-AAB´s and clinical cardiomyopathy in these animals, indicating, that the β1-AAB as a sole factor is able to induce cardiomyopathy [Jahns et al. 2004]. In another study, Whistar Fur rats were immunised with a peptide of the second extracellular loop of the β1-adrenoceptor monthly over one year, inducing not only the production of agonistic β1-AAB, but also clinical signs of DCM [Buvall et al. 2005]. Additionally the adrenoceptors kinase GRK2 was up-regulated indicating desensitisation of the β1-adrenoceptor [Buvall et al. 2005].

The protozoan parasite Trypanosoma cruzi causes a chronic infection, Chagas, in which cardiomyopathy including cardiac arrhythmias is one major clinical problem [Elizari et al. 1993]. After detection of β1-AAB autoantibodies in these patients, it has been discussed, whether Chagas heart disease is also autoimmune-mediated [Sterin-Borda et al. 1976, Elies et al. 1996]. β1-AAB in Chagas heart disease react also with an epitope on the second extracellular loop of the β1-adrenoceptor, but interestingly cross-react with an epitope on the M2 muscarinic acetylcholine receptor [Elies et al. 1996]. Moreover, it could be demonstrated,

that the β1-AAB show also cross-reactivity against Trypanosoma cruzi ribosomal P proteins [Kaplan et al. 1997]. Immunisation with a peptide of the Trypanosoma cruzi ribosomal P2beta protein induces specific autoantibodies, which induce electrocardiographic changes in immunised mice including supraventricular tachycardia [Lopez Bergami et al. 1997, Lopez Bergami et al. 2001]. More recently Labovsky et al. [2007] demonstrated binding of β1-AAB from patients with Chagas heart disease to the native human β1-adrenoceptor and could also show, that these IgG fractions induce cAMP accumulation in β1-transfectd COS-7 cells. These results indicate that a cross-reactive autoimmune process between a protozoan protein and the human β1-adrenoceptor contributes to the development of Chagas heart disease.

D. Complex Regional Pain Syndrome

Complex regional pain syndrome (CRPS, M.Sudeck) is a severe chronic pain syndrome, associated with serious trophic disturbances, frequently occurring after a limb trauma. Two forms can be distinguished: CRPS1 without and CRPS 2 with associated nerve injury. Patients exhibit a severe neuropathic pain syndrome, which is often combined with autonomic disturbances, such as edema, impaired skin blood flow, sudomotor dysfunction, and disturbed hair and nail growth in the affected limb. It can be observed in about 0.2-0.5% of all limb trauma patients and about 35-50% of the cases will become chronic, often leading to complete loss of function one arm or leg. Two important pathological features have been found in CRPS: a neurogenic inflammation at the site of the CRPS and a disturbance of the local and central sympathetic nervous system. Neurogenic inflammation may be responsible fro different clinical symptoms in CRPS resembling inflammation, such as pain, edema, increased skin temperature and blood flow. This may be due to the release of neuropeptides mainly, calcitonin gene-related peptide (CGRP) and substance P (SP), both of which are able to induce inflammatory changes in the affected tissue. The proinflammatory effects of these neurotransmitters include increase in vascular permeability, vasodilatation and activation of macrophages [Lembreck et al. 1979, McEwan et al. 1958, Kimball et al. 1988]. CGRP can induce TNF-α and, vice versa, TNF-α can stimulate CGRP expression and secretion from rat trigeminal ganglion neurons [Yaraee et al. 2003, Bowen et al. 2006]. This positive-regulated loop can not only maintain the neurogenic inflammation. TNF- α has been shown to be involved in local inflammatory processes in CRPS, especially mechanical hyperalgesia at the affected site [Huygen et al. 2002, Maihofner et al. 2005]. However, the reason for both was unclear yet. Several lines of evidence suggest a key role of a sympathetic nervous system dysfunction in CRSP. In the most CRPS patients, skin temperature on the affected limb is increased in the acute stage of the disease, whereas in chronic stages and a smaller part of the acute CRPS skin temperature is decreased. These patients often have increased sweating, and the pattern of vasoconstrictor hypoactivity and sudomotor hyperactivity suggest a dysfunction of central nervous system sympathetic structures [Birklein et al. 1998]. It has also been shown, that the noradrenergic control of blood vessels is reduced in the affected limb of CRPS patients [Drummond et al. 1991]. These data may explain a part of the trophic and autonomic disturbances in CRPS, but the sympathetic nervous system has also a local impact on the pain development in these patients. After nerve injury, α-adrenoceptors are up-regulated on nociceptive afferents and agonistic stimulation lead to higher response of the fibers [Koltzenburg et al. 1994, Ali et al. 1999]. In the recent years, an involvement of the

immune system in CRPS has been discussed by different authors: CRPS is associated with distinct HLA alleles, which is not a proof for an immune-mediated disease, but a frequent observation in autoimmune diseases [Van de Beek et al. 2003]. Changes in cytokine patterns with an increase in proinflammatory cytokines such as TNF-α and Interleukin-6 have also been described in CRPS both locally and systemic [Maihofner et al. 2005, Schinkel et al. 2006]. Disturbances in the cytokine distribution towards an inflammatory pattern have been described even in the cerebrospinal fluid of CRPS patients [Alexander et al. 2005]. Additionally, one group found an increased reactivity of peripheral blood monocytes to proinflammatory cytokines in CRPS patients [Hartrick 2002]. Recently, autoantibodies against autonomic nervous system structures could be demonstrated in CRPS [Blaes et al. 2004, Fig.1]. This autoantibodies bind mainly to the surface of autonomic neurons and therefore, makes them more likely to be pathogenic [Blaes et al., in press]. The finding of nervous system autoantibodies in CRPS was more recently confirmed by another group [Goebel et al. 2005]. Interestingly, the same authors could demonstrate abnormalities in motor function tests in mice with passive transferred CRPS IgG [Goebel et al. 2005]. An autoimmune hypothesis in CRPS may well explain many of the symptoms, although it does not explain, why the disease is locally restricted. However, this hypothesis is also supported by case reports about positive effects of intravenous Immunoglobulin treatment in CRPS patients [Goebel et al. 2005]. IGIV is mainly effective in B-cell (autoantibody-) mediated autoimmune diseases, such as myasthenia gravis, Guillain-Barré syndrome and others [Dalakas 2004]. One possible explanation for the induction of autoantibodies in CRSP may be an infectious agent, leading to a cross-reaction with nervous system structures. This has been proven in some other autoimmune diseases including Chagas heart disease and polyradiculitis Guillain-Barré after a Campylobacter jejuni-infection [Kaplan et al. 1997, Willison & Yuki 2002]. Recently, a increased seroprevalence for Campylobacter could be found in CRPS patients, and. More recently, we could demonstrate an increased seroprevalence of parvovirus B19 IgG in CRPS [Goebel et al. 2005, Gross et al. 2007]. Parvovirus B19 has also been described in association with different autoimmune phenomena [Holm et al. 1995, Lhote & Guillevin 1995]. Therefore, identification of the underlying autoantigens and characterisation of possible functional effects of CRPS IgG is necessary to prove the immune hypothesis of CRPS.

4. Conclusions

The cross-talk between the sympathetic nervous system and the immune system plays a key role in physiologic and pathological conditions of both systems. In the seventies and eighties, basic research revealed the influence of the sympathetic nervous system on the immune system, including the sympathetic innervation of immune organs and the discovery of adrenoceptor expression of immune cells. The basic effect of sympathetic neurotransmission in the immune system seems to be a shift from Th1 towards Th2, therefore decreasing the severity of Th1-mediated autoimmune processes. In the recent years, sympathetic nervous system has been discovered to be a target in different autoimmune diseases. In inflammatory rheumatic diseases, Guillain-Barré syndrome or in CRPS, sympathetic nervous system can be part of a more generalised autoimmune process, whereas in dilatative cardiomyopathy or Chagas heart disease, the autoimmunity is specifically directed against a sympathetic nervous

system receptor, the β1-adrenoceptor. Future research will include the identification of not yet identified autoantigens in some of the above mentioned diseases and the elucidation of pathogenic autoimmunity should lead to substantial changes in treatment regimen in some more diseases involving the sympathetic nervous system.

Table 1. Autoantibodies associated with sympathetic dysfunction and / or autonomic neuropathy

Autoantibody	Antigen	Associated disease(s)	
Anti-m2AChR	Muscarinergic AChR (M2 subunit)	Cardiomyopathy in Chagasic disease	Cross-reaction between Trypanosoma proteins and the human receptor
Anti-m3AChR	Muscarinergic AChR (M3 subunit)	Sjögren´s_Syndrome	Autoantibodies inhibit saliva secretion *in vitro* as a possible model for sicca syndrome
Anti-α3nAChR	α3-subunit of the nicotinic AChR	Subacute autonomic neuropathy	Can also be observed in paraneoplastic autonomic neuropathy
Anti-β1-adrenoceptor	2nd extracellular loop of the β1-adreno-ceptor	Dilatative cardiomyopathy Chagas heart disease	Cross-reaction between Trypanosoma proteins and the human receptor
Anti-VGCC	Voltage-gated, presynaptic P/Q-type calcium channels	Lambert-Eaton-myasthenic syndrome (LEMS)	55% associated with tumour, most of them SCLC
Anti-Hu	Group of RNA-binding proteins (HuC, HuD, Hel-N1)	paraneoplastic neurological syndromes including AN	90-95% association with SCLC
Anti-CRMP5	Collapsin-response mediator protein 5	Paraneoplastic neurological syndromes including AN	SCLC / thymoma
Anti-Yo	Yo protein	Paraneoplastic cerebellar degeneration, including AN	Zink-finger protein, expressed in tumour and Purkinje cells
CRPS-autoantibodies	unknown	Complex-regional pain syndrome (M. Sudeck)	Binding to the surface of peripheral neurons

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Chapter 8

BASIC AUTONOMIC AND SENSORY INNERVATION PATTERN OF HUMAN NASAL MUCOSA

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Abstract

Introduction: Respiratory nasal mucosa fulfils all functions efficiently for conditioning the inspired air. Physiologic and pathologic mechanisms in nasal mucosa are partially controlled by neural regulation. Beside autonomic neurotransmitters some neuropeptides as well as Nitric oxide (NO) seem to influence glandular secretion and the tone of nasal vasculature. In addition endothelial produced substances like Endothelin and also NO influences the vascular tonus.

This study was performed to identify the different kinds of nerve structures and neuronal transmitters of the sympathetic as well as the parasympathetic and sensory nervous system in human nasal mucosa. The morphologic results should elucidate the mechanisms of nasal swelling and secretion.

Material and methods: Tissue samples of 80 human inferior turbinates were taken during nasal surgery. Serial sections were cut and incubated with antibodies either to neuron-specific enolase (NSE), neurofilament (NF), tyrosine hydroxylase (TH) or to Vasoactive Intestinal Peptide (VIP), Calcitonin Gene-Related Peptide (CGRP), Neuropeptide Y (NPY) and brain Nitric Oxide Synthase (bNOS). In addition, acetylcholinesterase (AChE) and Nicotinamide-Adenine Dinucleotide Phosphate (NADPH)-diaphorase – histochemistry were performed. Finally, all sections were evaluated and documented through bright field microscopy. Additionally, electron microscopic researches were performed.

Results: Nasal vasculature and seromucous glands are controlled by a dense innervation pattern. While all larger arterial vessels show a mixed autonomic innervation, sympathetic nerve fibres seem to predominate in veins. These results were confirmed electron

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microscopically. Furthermore, immunoreactive nerve fibres were demonstrated around the acini, ducts and in the periglandular connective tissue of glands. A dense network of cholinergic nerve fibres could be detected around the acini. VIP was found in contact to arteries, arterioles, cushion veins as well as acinus cells. NPY is a co-transmitter in sympathetic nerve fibres, acts as a vasoconstrictor and was demonstrated in contact to arterial vessels. The sensory neuropeptide CGRP build a dense nerval network in the subepithelial connective tissue and around arterial vessels and glandular cells. Arteries and capillaries showed a distinctly developed nitric innervation. A high coexistence of NADPH-d in parasympathetic nerves could be detected.

Conclusion: Immunocytochemical, histochemical and electron microscopical methods allow a detailed marking of nerve supply in human nasal mucosa. General innervation could be shown by using antibodies to neuron-specific enolase and neurofilament. The localization of neurons with different neurotransmitters and neuropeptides in the perivascular and periglandular tissue confirms the direct neural control of the diverse nasal functions. The detection of bNOS-and NADPH-d-positive structures around arteries and glandular cells suggests that NO takes an additional part in the control of nasal functions. The stronger innervation of arteries and cushion veins underlies their central position in the regulation of nasal airway flow.

It could be shown, that beside immunologic mechanisms also the dense network of sensory, sympathetic and parasympathetic nerve fibres act as protection of nasal respiratory mucous membranes from external and internal influences.

Key words: Innervation - Human nasal mucosa – Sympathetic nervous system – Parasympathetic nervous system – Nitric oxide - Immunocytochemistry - Neuropeptides

Introduction

Respiratory nasal mucosa fulfils all functions for conditioning the inspired air. The periodic nasal cycle and pathologic functional disturbance of the endonasal swelling tissue influence the passage of nasal airways. Secretion of seromucous glands and extravasations of ingredients of the blood vessels are essential for the mucocilliary transport. Nasal vasculature and seromucous glands are exposed to complex mechanisms influenced by external as well as internal stimuli.

Nasal mucosa offers a high density of vessels. They show unique morphologic features which are seldom found in other human organs. The arterioles run parallel to the bone of the nasal turbinate and are formed of a thick musculature. Smaller arteries branch off in the direction of the epithelium. There are formed loop-like, because the have to stretch during the swelling mechanism. Capillary beds are mainly found around seromucous glands and under the epithelium. They show fenestrations which are directed to the glands and to the epithelium. It is postulated, that the major part of the nasal secretion is passing over these capillary caps to the glands and to the surface of nasal mucosa. Also arteriovenous anastomoses are described however we never found these structures during our ultrastructural or microscopic researches in human nasal mucosa (11, 19). Capillaries disembogue in a spaciously venous system, which is situated in the deeper regions of the mucosa. These veins are cavernous with a thin wall and show subendothelial muscular conglomerations which are protruding into the lumen. Cauna therefore named them cushion veins (12). These structures are said to serve as a kind of plug to slow down the blood flow out of the nasal mucosa (46). The subendothelial muscular bolsters also present another particularity - so called intervascular muscle fibres (18). It is postulated, that during the contraction phase of all

smooth muscle cells in the lamina propria mucosae they are pulled out of the vascular lumen and therefore the venous blood flow increases (46).

In the subepithelial layer of human nasal mucosa an extensive complex of seromucous glands are found under a small capillary region (29, 30, 33). Seromucous glands are important components of human nasal mucosa and participate in the protection of the lower respiratory tract by humidification of the inhaled air. They produce a gel-colloidal solution. The glandular secretion contains nonspecific antibacterial and antiviral substances such as lactoferrin, lysozyme, glucosidases, interferons and peptidases. Furthermore immunoglobulins IgA, IgE und IgG were found in the glandular secretion (5, 6). Due to their subepithelial location and the specific composition of glandular secretion the glands take part in the defence against infection of the lower respiratory tract.

The innervation patterns are relevant for understanding the control of the different physiological and pathophysiological functions of nasal mucosa.

Previous studies on nasal glands have demonstrated the predominant influence and the stimulating function of the parasympathetic system (13, 23, 60) and ascribed an inhibitory function to the sympathetic system (24).

Beside the classical neurotransmitters acetylcholine and noradrenaline some neuropeptides are responsible to have modulatory function in nasal mucosa (5,6, 21,40,41,50,53). Vasoactive Intestinal Peptide (VIP) and Calcitonin Gene-Related Peptide (CGRP) have special importance for the coinnervation of nasal structures (5,6).

Vasoactive Intestinal Peptide consisting of 28 amino acids was first detected in the gastro-intestinal and urogenital tract and later found in the nasal mucosa of rabbits, cats and guinea pigs (53).

VIP has been detected in a variety of different tissues and the co-localization with Acetylcholine could be proved (6, 21, 53). VIP is a postganglionic-parasympathetic neurotransmitter and a potent vasodilatator (6, 37). VIP appears to influence the glandular secretion (6, 38, 53) and has protecting qualities for the epithelium (4).

Calcitonin Gene-Related Peptide (CGRP) consisting of 37 amino acids and has been found in sensory neurons (4, 5, 7, 61).

It is regarded as a long-acting vasodilatator in humans (5, 59, 61). Corresponding effects of CGRP was assign to receptors of pancreas, the cardio-vascular system as well as in the cerebellum and spinal cord (61).

In bronchial mucosa CGRP is an active agent in allergy and could lead to bronchoconstriction (5). In vivo investigations with CGRP in the nasal mucosa of cats and guinea pigs have shown an increased plasma extravasation and glandular stimulation (17, 49).

Sympathetic nerves contain noradrenaline and Neuropetide Y (NPY). NPY is a potent long-lasting vasoconstrictor (50) and is more effective than oxymetazoline at reducing nasal obstruction and nasal hypersecretion following allergen challenge. This neuropeptide is co-released with noradrenaline, although with a slower onset and a longer duration of vasoconstriction (58).

In addition blood vessels and glands are influenced by endothelial and humoral factors. During the different type of rhinitis sensory neuropeptides and inflammatory mediators take part in the pathomechanisms and can lead to a so called neurogenic inflammation of nasal mucosa.

The neurotransmitter nitric oxide (NO) has been proved to be a cotransmitter to acetylcholine in parasympathetic nerve fibres of the central and periphery nervous system, and can modulate cholinergic effects in the vascular system and glands (9, 43)

Endothelial produced NO can trigger vasodilatation through relaxation of the vessel musculature (28, 39). Immunological effects due to influencing macrophages activity as well as antiviral and antibacterial properties are also described.

Three isoforms of NO synthetases (NOS) can be distinguished, two constitutive Ca^{2+} - dependent NOS (NOS I located in nerves and NOS III in the endothelium), and one Ca^{2+} - independent inducible iNOS (NOS II) (15, 45). Constitutive NOS releases a basal amount of NO under physiological conditions. In contrast to this an increased expression of inducible NOS is found after incubation with lipopolysaccharides and/or cytokines in epithelium, glands, leucocytes and the vascular endothelium (43, 55). The endothelial NOS appear to have a substantial influence on the basic regulation mechanisms of the vessels.

This study was performed to identify the different kinds of nerve structures and neuronal transmitters of the sympathetic as well as the parasympathetic and sensory nervous system in human nasal mucosa by using histochemical and immunocytochemical techniques. In contrast to previous studies using silver-intensivation-methods (52) and immunofluorescence-techniques (21) with immunocytochemical and electron microscopic methods a precise assignment of nerval structures and their neurotransmitters can be demonstrated. The morphologic results should elucidate the mechanisms of nasal swelling and secretion.

Materials and Methods

Sampling of nasal mucosa: Specimens of inferior turbinates were taken from 80 patients (45 men, 35 women; age range: 22 to 59 years; mean age: 35.3y) who required surgery due to nasal obstruction. Tissue samples of nasal mucosa which had been pathologically altered by chronic rhinosinusitis were not included in the investigation. Patients with history of allergic or idiopathic rhinitis or acute as well as chronic rhinosinusitis and patients with topic application of glucocorticosteroides and vasoconstrictors were excluded. Allergy tests (skin prick test, blood tests for IgE) were taken on all patients.

For the first morphological evaluation, sections were stained with Mayer`s hemalum and tissue with inflammatory cell infiltration was excluded.

Preparation for immunocytochemistry: Paraffin sections were rehydrated and the cryosections were air-dried, fixed in ice cold acetone and air dried again. After blocking of endogenous peroxidase by immersion in 0.002% hydrogen peroxide solution in phosphate buffered saline (PBS), the sections were preincubated with normal horse or goat serum in PBS. Incubation in the incubation cupboard with the following antibodies was then performed:

- on paraffin sections the antibody against Neurofilament (NF, monoclonal, DAKO/ Hamburg, Germany) for 1 hour at room temperature
- on paraffin and cryosections the anti-NSE-antibody (polyclonal, DAKO/ Hamburg, Germany) for 1 hour at room temperature
- on paraffin and cryosections the anti-Tyrosinhydroxylase (TH)-antibody (monoclonal, Boehringer/ Mannheim, Germany) for 2 hours at room temperature

- on cryosections the anti-VIP (polyclonal, Peninsula/ USA) for 1 hour at room temperature
- on paraffin and cryosections the anti-CGRP-antibody (polyclonal, Peninsula/ USA) for 2 hours at room temperature
- on paraffin and cryosections the anti-NPY-antibody (polyclonal, Peninsula/ USA) for 2 hours at room temperature
- on cryosections the brain-NOS-antibody (polyclonal, Transduction Lab., Lexington/ USA) for 1 hour at room temperature
- on cryosections the endothelial-NOS-antibody (polyclonal, Transduction Lab., Lexington/ USA) for 18 hours at room temperature.

Biotinylated anti-rabbit IgG at a dilution of 1:200 were applied. Finally, the immunocomplexes were visualized by the Avidin-Biotin-Complex (ABC) method and chromogen 3-amino-9-ethylcarbazol or diaminobenzidine. At the end of the immunocytochemical procedures, counterstaining with Mayer`s hemalum was performed.

Preparation for histochemistry: Tissue samples for histochemistry were fixed in 4% phosphate-buffered paraformaldehyde solution for 2 hours at 4^0C and cryoprotected in sucrose solution, snap-frozen in liquid nitrogen and stored at -20^0C. Sections of 8 to 15µm thickness were cut.

Parasympathetic structures were identified by Acetylcholinesterase-histochemistry first described by Karnovsky and Roots (27). For this procedure, frozen sections were washed with sodium acetate buffer and incubated in acetylthiocholine solution for 1 hour. After the slides were rinsed in sodium acetate buffer, they were incubated in ammonium sulfite.

These slides were then washed in sodium nitrate buffer and incubated in silver nitrate for 1 minute. Finally, they were rinsed with sodium nitrate buffer.

Reactivity to Nicotinamide-Adenine Dinucleotide Phosphate (NADPH)-diaphorase was demonstrated by a modified histochemical method of Vincent and Kimura (57). Frozen sections were immersed in a solution consisting of nitroblue tetrazolium (0.1mg/ml), ß-NADPH (1.0mg/ml) and Triton-X 100 (0.3%) in phosphate buffer. Control sections were prepared by omitting ß-NADPH. For NADPH-d-AChE double-staining procedure sections were incubated with sodium citrate (0.1mol), cupric sulfate (0.03mol), potassium-ferricyanide (0.005mol), tetraisopropyl pyrophosphamide and acetylcholine iodide dissolved in sodium hydrogen-maleate buffer (o.1mol, pH6.0).

Finally, all sections were mounted with Kaiser`s glycerin gelatine, evaluated, and documented through bright field microscopy.

Preparation for electron microscopy: The specimens were immersed in a solution of 3 % phosphate-buffered glutaraldehyde (according to Schultz-Karlsson). After a brief washing procedure in buffer and dehydration the specimens were incubated in unicryl overnight at 4^0C. After polymerization serial ultrathin sections (70nm) were cut (Reichert-Jung Ultracut E, Vienna, Austria) and then placed on nickel grids.

Transmission electron microscopy: After washing in phosphate buffer they were refixed in a solution of 1% osmium acid, and after a renewed buffer washing we performed dehydration in an ascending acetone series. A penetration phase with Durcupan or Araldit was followed

by heat polymerisation in which the specimens were caused to harden. After preparing semithin cuts and staining with toluidine blue, we selected appropriate tissue areas of inferior turbinate for the electron microscopy. Finally, the preparations were cut ultra thin (Reichert-Ultracut). After double-contrasting with uranylacetate and lead citrate ultrastructures were photodocumented using a transmission electron microscope (EM 902 A Zeiss, Jena, Germany).

Preparation for immunoelectron microscopy: The specimens were immersed in a solution of 2% phosphate-buffered paraformaldehyde and 0.1 % glutaraldehyde. After a brief washing procedure in buffer and dehydration the specimens were incubated in unicryl overnight at 4^0C. After polymerization serial ultrathin sections (70nm) were cut (Reichert-Jung Ultracut E, Vienna, Austria) and then placed on nickel grids.

Electron microscopic immunocytochemistry: For detection of nNOS (NOS I), sections were incubated at 4^0C overnight with a polyclonal rabbit antibody against neuronal NOS (Transduction Laboratories, Lexington, KY, USA). Furthermore, the tissues were treated with a rabbit polyclonal antibody against endothelial NOS eNOS (NOS III) (Transduction Laboratories, Lexington, KY, USA) under the same conditions. After rinses in phosphate-buffered saline (PBS) biotinylated secondary antibodies (VECTOR, Burlingame, CA, USA) were applied for 2 hours. The immunocomplexes were visualized by an immunocytochemical staining technique using a 10nm gold-labeled streptavidin-immunogold-complex (AMERSHAM, Auroprobe EM Streptavidin G10, Buckingshamshire, UK). The sections were stained with uranyl acetate or with lead citrate. Immunostained structures were photodocumented using a transmission electron microscope (EM 902 A Zeiss, Jena, Germany).

Control experiments: Controls consisted of omitting the primary antibodies in the staining procedure and incubating the sections with buffer or a nonimmune serum (DAKO Diagnostika, Hamburg, Germany) diluted to the same concentration as the corresponding antibody. No specific immunoreactions were observed.

The study was approved by the Institutional Review Board of the MLU Halle Wittenberg and the LMU Munich, Germany and was in accordance with the Declaration of Helsinki.

The authors attested that we have no commercial associations that might pose a conflict of interest in connection with these findings. We have no relevant financial interests in this article.

Results

By using immunocytochemical, histochemical and electron microscopical methods the basic innervation pattern and the different neurotransmitters of human nasal mucosa could be demonstrated. Through an additional counterstaining with Mayer`s hemalum as well as haematoxylin eosin staining (Fig. 1) nerve structures could be contrasted with the surrounding anatomical structures.

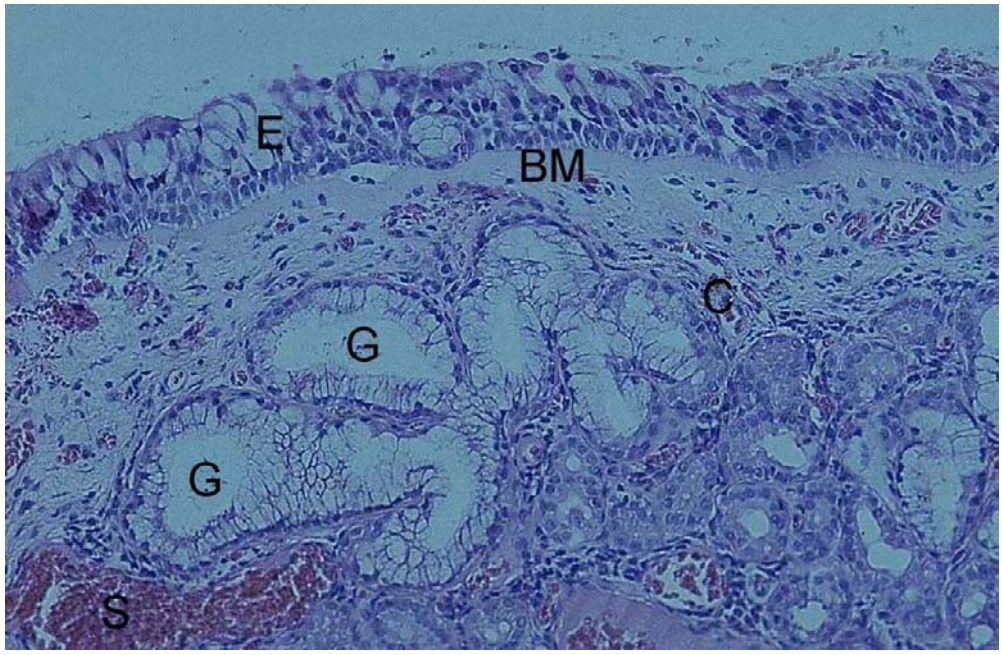


Figure 1. Human inferior turbinate; overview of the epithelium (E) and the subepithelial region with seromucous glands (G). Under the basal membrane (BM) capillaries and venous sinusoids (S) could be found. Paraffin section, haematoxylin eosin staining. Original magnification x 80

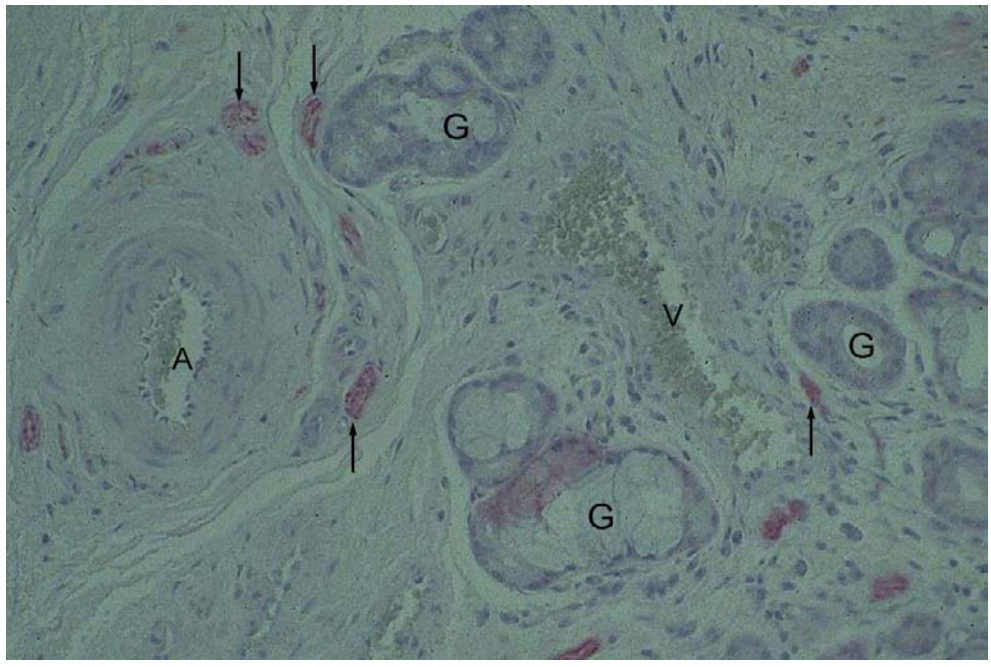


Figure 2. Human inferior turbinate; nasal vasculature and seromucous glands are controlled by a dense innervation pattern. Localization of nerve fibres (arrows) in the adventitia of arteries (A) and around seromucous glands (G) by using a NSE antibody. Venous sinusoid – V. Paraffin section, ABC method. Original magnification x 150

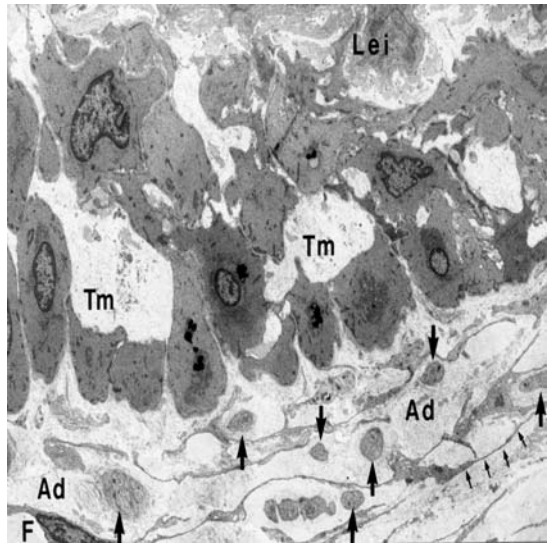


Figure 3. Longitudinal cut nasal mucosa artery wall: *Lei* - Lamina elastica interna; *Tm* – Tunica media; *Ad* – Adventitia; *F* – fibroblast; *small arrows* – fibroblastic extensions with circular running axons (*big arrows*); Transmission electron microscopy. Original magnification x 3.600

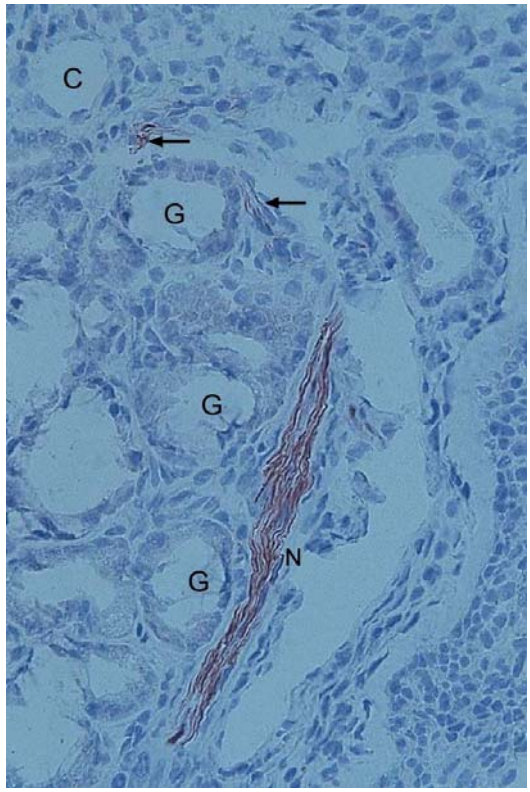


Figure 4. Immunocytochemical localization of nerve fibres (N, arrows) in direct contact to seromucous glands (G). Capillary – C. Anti-NF-antibody; Paraffin section, ABC method. Original magnification x 150

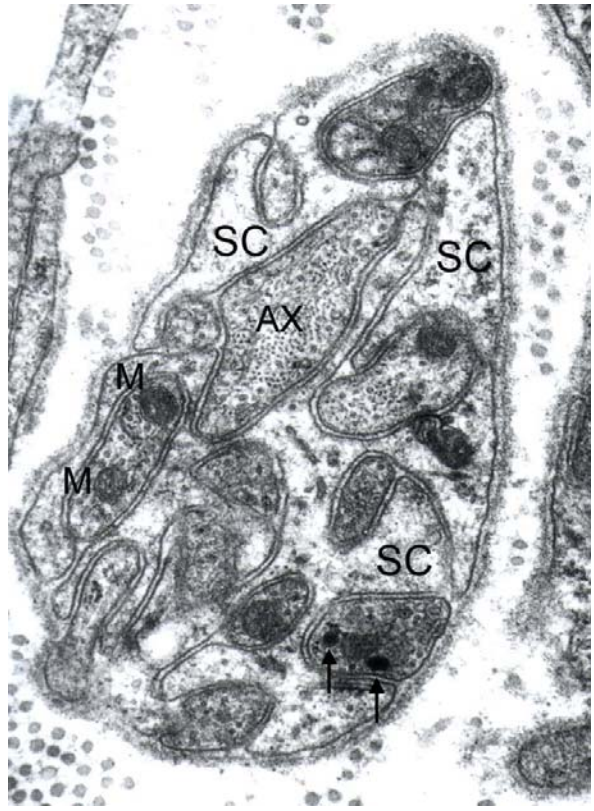


Figure 5. Electron microscopy of a nonmyelinated nerve fibres in the periglandular connective tissue. Note the axons (AX) included in Schwann cells (SC). Mitochondrias (M) and dense core vesicles (arrows). Transmission electron microscopy. Original magnification x 20.000

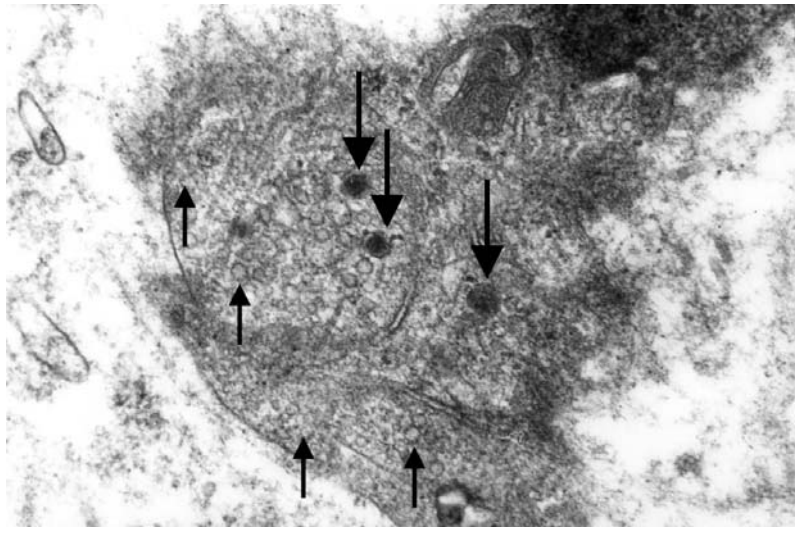


Figure 6. Neuro-glandular synapse with dense core vesicles (long arrows). Furthermore, empty cholinergic vesicles (short arrows) were demonstrated. Transmission electron microscopy. Original magnification x 30.000

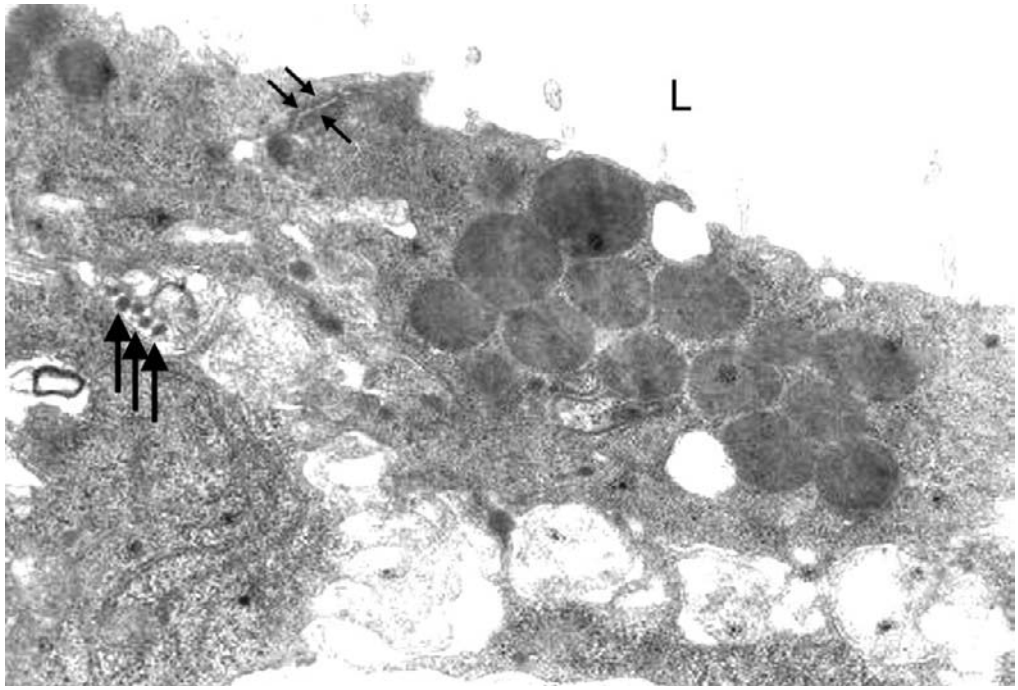


Figure 7. Lumen (L) of acinus cells of seromucous glands with droplets. Intracellular connection with desmosomes (small arrows). Neuro-glandular synapse (big arrows) with dense core vesicles. Transmission electron microscopy. Original magnification x 20.000

Nasal vasculature and seromucous glands are controlled by a dense innervation pattern (Fig.2, scheme 2-4). While all larger arterial vessels show a mixed autonomic innervation, sympathetic nerve fibres seem to predominate in veins. These results were confirmed electron microscopically (Fig. 3). Furthermore, immunoreactive nerve fibres were demonstrated around the acini, ducts and in the periglandular connective tissue of glands (Fig. 4).

Electron microscopically axons which are surrounded by “Schwann cells” show the typical structure of peripheral nerves with neurofibrils, neurotubuli and numerous mitochondrias (Fig. 5). Numerous synaptic electron empty vesicles and dense core vesicles could be observed regularly (Fig. 6). In several regions of seromucous glands, neuroglandular synapses could be found (Fig. 7). Cholinergic vesicles were detected electron empty. The dense core vesicles contain neuropeptides.

Cholinergic Innervation

Using the Acetylcholinesterase-histochemistry techniques according to Karnovsky and Roots it was possible to visualize cholinergic nerve structures. The brown-coloured reaction products could be seen in thick nerval bundles and in parasympathetic neural networks around the acinus cells and glandular duct system (Fig. 8). A dense network of cholinergic nerve fibres could be detected around the acini.

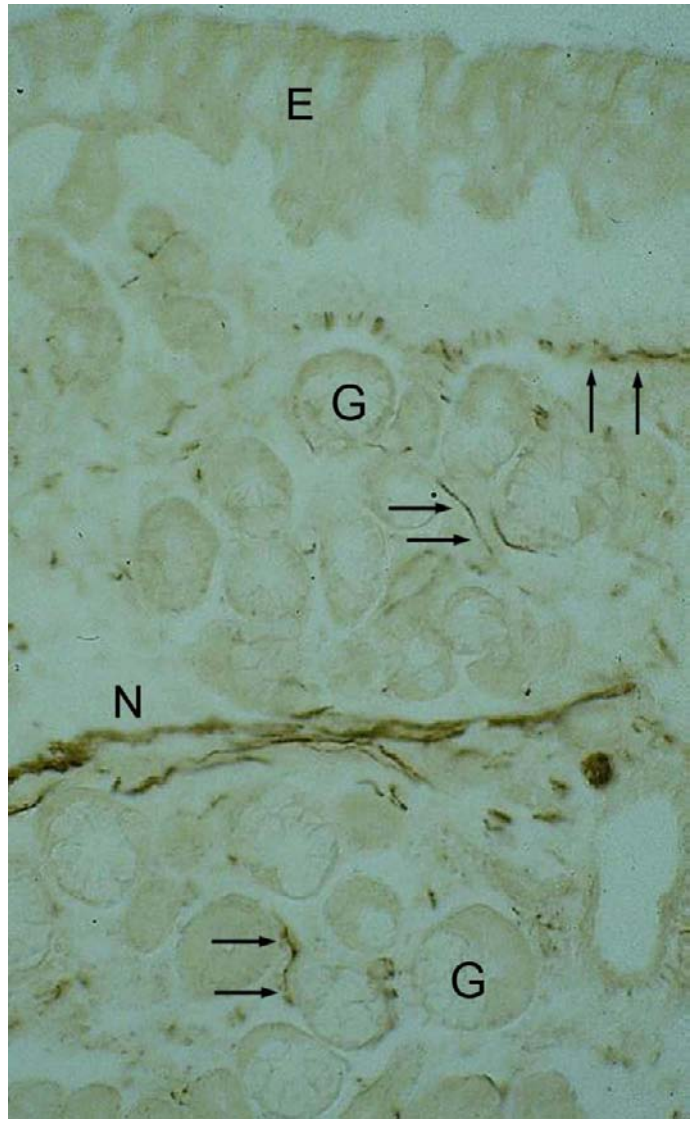


Figure 8. Human inferior turbinate; epithelium (E) and subepithelial region demonstrating AchE-reactive nerve fibres. Thick nerve bundles (N) and fine cholinergic fibres (arrows) around acinus cells (G). Cryosection, ABC method. Original magnification x80

Adrenergic innervation

By using the monoclonal antibody to TH, noradrenergic structures could be seen in arterial vessels of nasal mucosa (Fig. 9). In contrast to the rich sympathetic nerve supply of arteries, the veins showed a poor presence of noradrenergic neural structures. Occasional noradrenergic structures could be seen near the glandular acini.

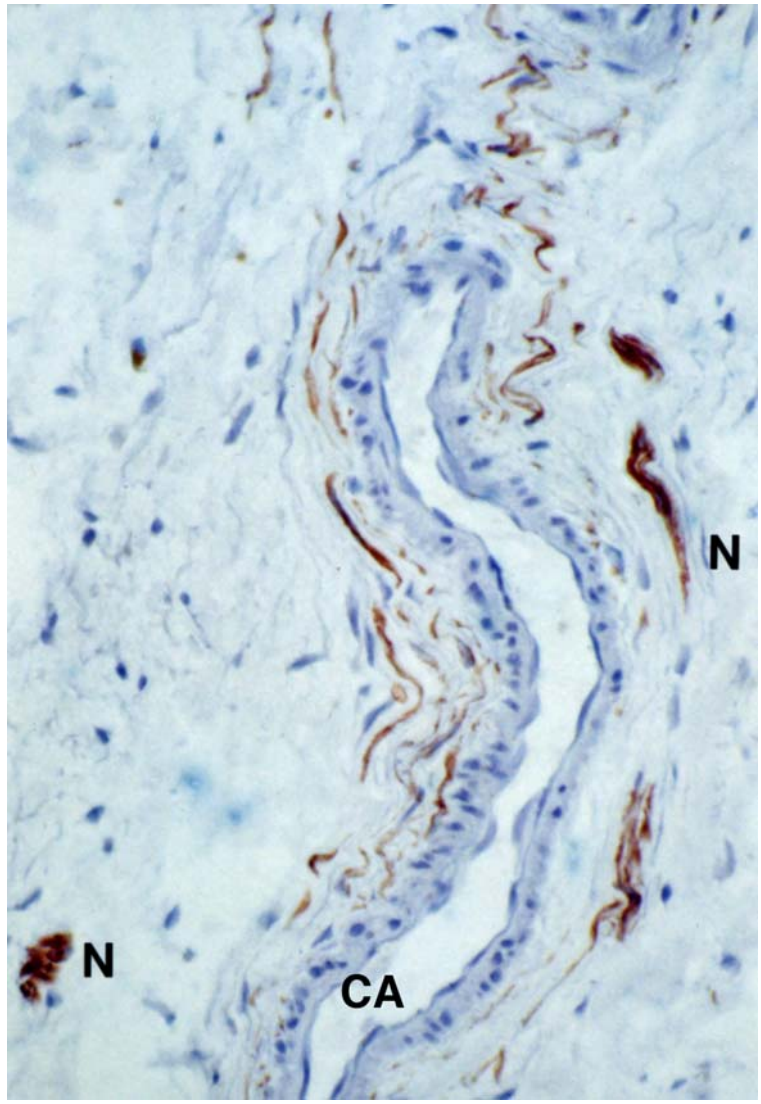


Figure 9. Longitudinal-section of a climbing artery (CA): Longitudinal running TH-immunoreactive nerve fibres (*red*) and nerve bundles (*N*) are seen in the adventitia of arteries (*A*). A solitary adrenergic nerve is seen at left bottom of the picture (*N*). Cryosection, ABC method. Original magnification x320

VIP

In thick nerve bundles units of VIP-immunoreactive fibres could be demonstrated. Extending from these VIP-containing nerves immunopositive fibres were found around and in contact to arteries, arterioles and cushion veins. Furthermore, VIP was localized near to acinus cells as well as in the region of the ducts of the seromucous glands (Fig. 10). VIP could be localized immunoelectron microscopically in dense core vesicles of periglandular axons (Fig. 11).

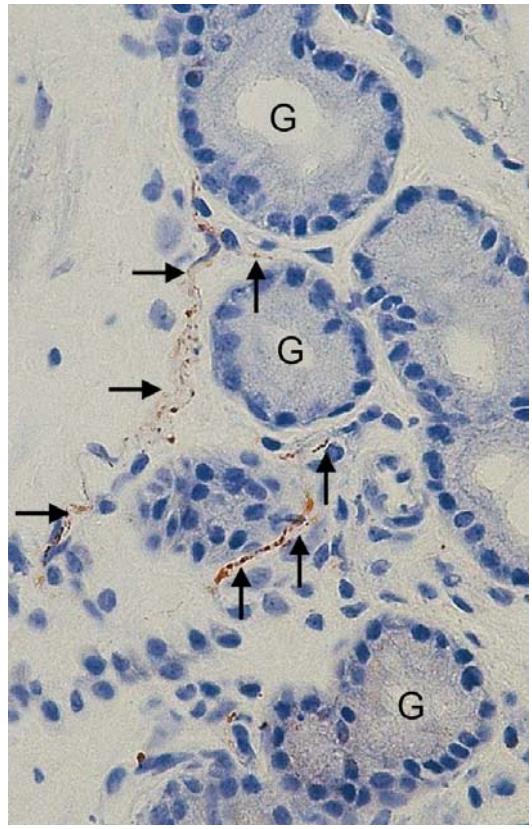


Figure 10. Glandular region (G) of nasal mucosa. Longitudinal section of a nerve bundle (arrows) with some VIP-immunoreactive axons and periglandular nerve fibres (arrows). Paraffin section, ABC method. Original magnification x200

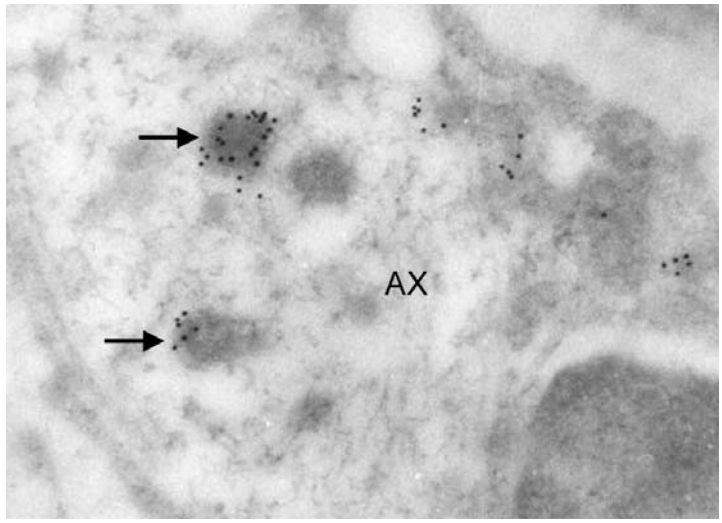


Figure 11. Immunoelectron microscopic detection of VIP in dense core vesicles (arrows) of periglandular axons (AX). Transmission electron microscopy. Original magnification x 30.000

CGRP

The sensory neuropeptide CGRP build a dense nerval network in the subepithelial connective tissue and around arterial vessels and glandular cells.

Unlike VIP-containing nerves CGRP-positive nerve fibres were found in interstitial spaces but not in contact with seromucous glands. Inside the nerve fibres fine varicosities were seen (Fig. 12).

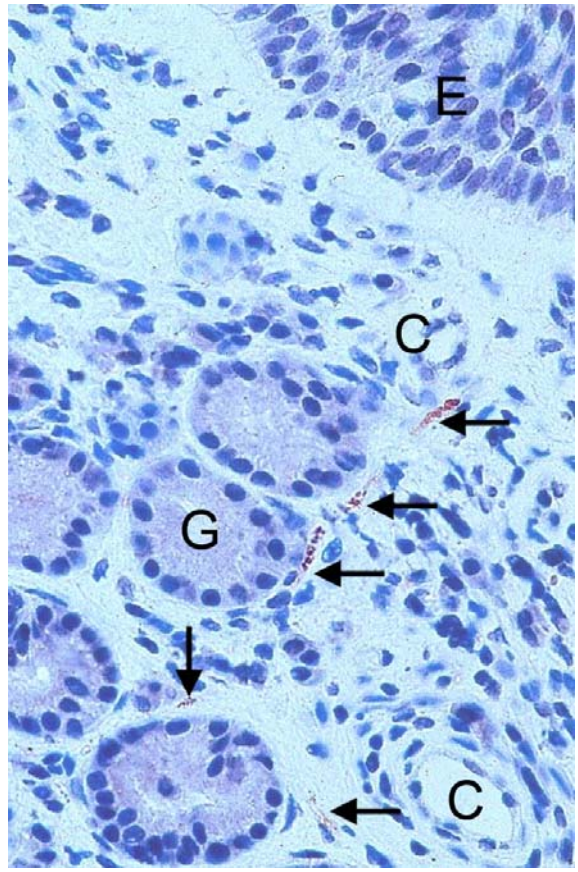


Figure 12. Under the epithelium (E) immunocytochemical staining of CGRP containing axons. CGRP-immunoreactive fibres (arrows) in the periglandular tissue (G). Capillary – C. Cryosection, ABC method. Original magnification x180

NPY

NPY is a co-transmitter in sympathetic nerve fibres, and was demonstrated in the adventitia of thick arterial vessels (Fig. 13).

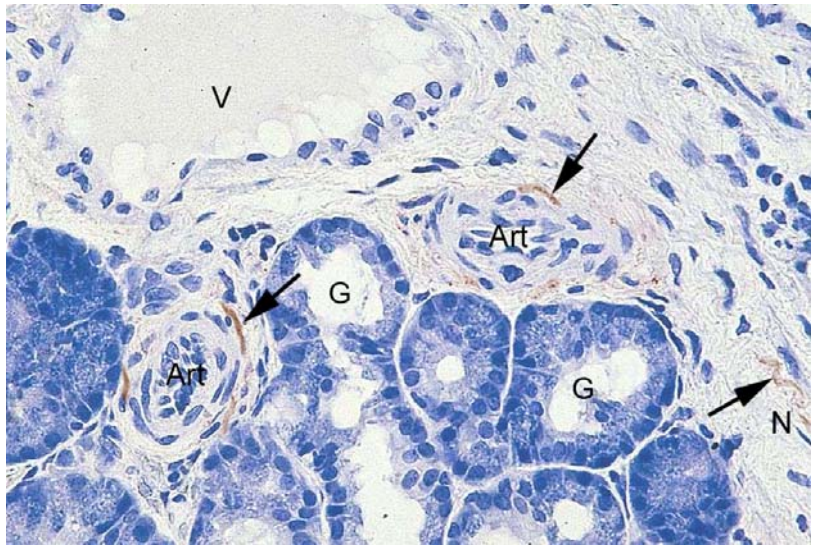


Figure 13. Cross section of an artery (Art). Immunocytochemical detection of NPY in a nerve bundle (N) and nerve fibres (arrows) of the arterial adventitia. Note the dense innervation pattern. Glands-G; venous sinusoids-V. Paraffin section, ABC method. Original magnification x 100

Nitric Innervation

Arteries and capillaries showed a distinctly developed nitric innervation. A high coexistence of NADPH-d in parasympathetic nerves could be detected.

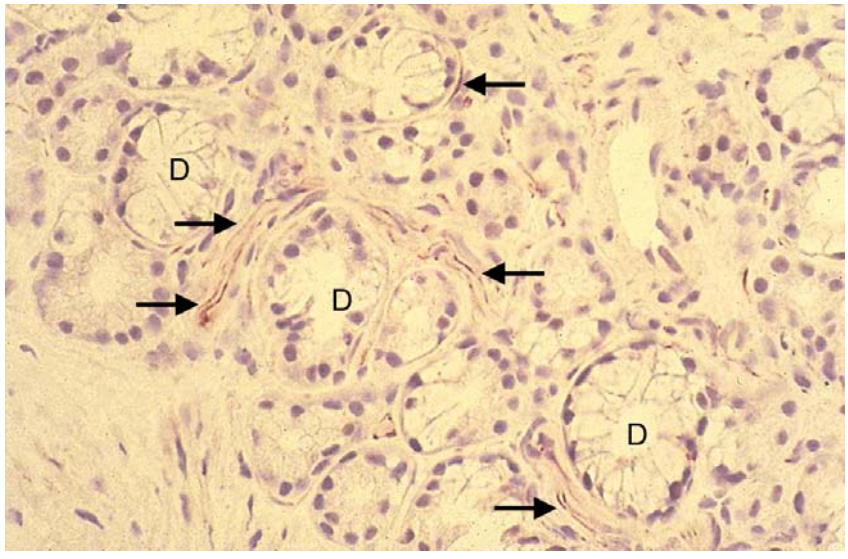


Figure 14. Immunocytochemistry of brain nitric oxide synthetase (bNOS)-positive nerve fibres (arrows) between acinus cells of seromucous glands (G). Cryosection, ABC method, Original magnification x180

With the bNOS-immunocytochemistry NO synthesis could be demonstrated in thick nerve bundles as well as in subepithelial and arterial nerve fibres. Between the acinus cells fine NOS-positive fibres were marked (Fig. 14). Additionally positive eNOS-immunoreactions were found in the endothelial cells of periglandulary capillaries (Fig. 15), postcapillary venules and in endothelial cells of arterioles.

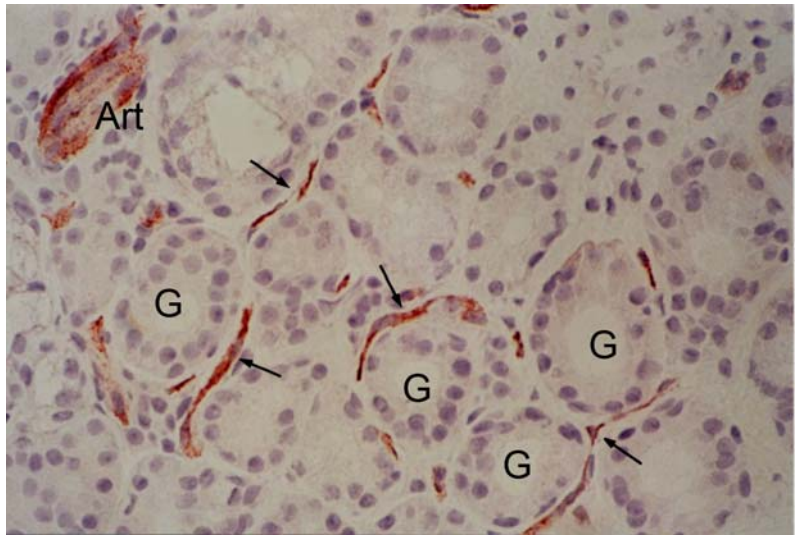


Figure 15. Detection of endothelial nitric oxide synthetase (eNOS) in the endothelium of capillaries (arrows) around seromucous glands (G) and in the endothelium of a longitudinally cut small artery (Art). Cryosection, ABC method. Original magnification x400

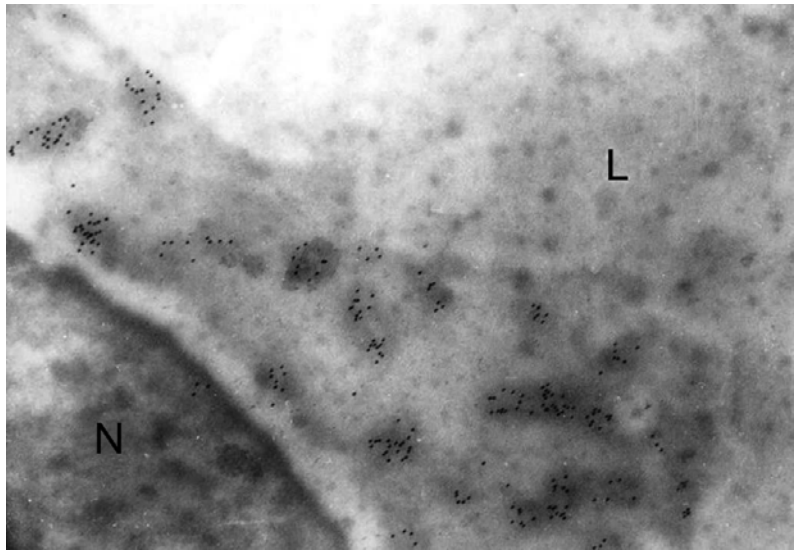


Figure 16. Immunoelectron microscopical detection of NOS III in the endothelium of capillaries in the periglandular tissue. Strong immunoreactions in the cytoplasma. A smaller number of gold particles (arrows) are visible within the nucleus (N). No immunoreactivity is seen within the lumen (L) of blood vessel. Original magnification: x30.000

With immunoelectron microscopic methods a powerful accumulation of endothelial NOS was identified in the endothelium of subepithelial and periglandular capillaries as well as in the endothelium of arterioles and arteries (Fig. 16).

NADPH-d-AChE-Double Staining

The NADPH-d-AChE-double staining procedure revealed a coexistence of both enzyme activities in axons of the lamina propria of the respiratory nasal mucosa. AChE (brown colour) was also located in neurons that did not show NADPH-d staining (blue colour). A fine network of NADPH-d-AChE-positive fibres could be seen around seromucous glands (Fig. 17).

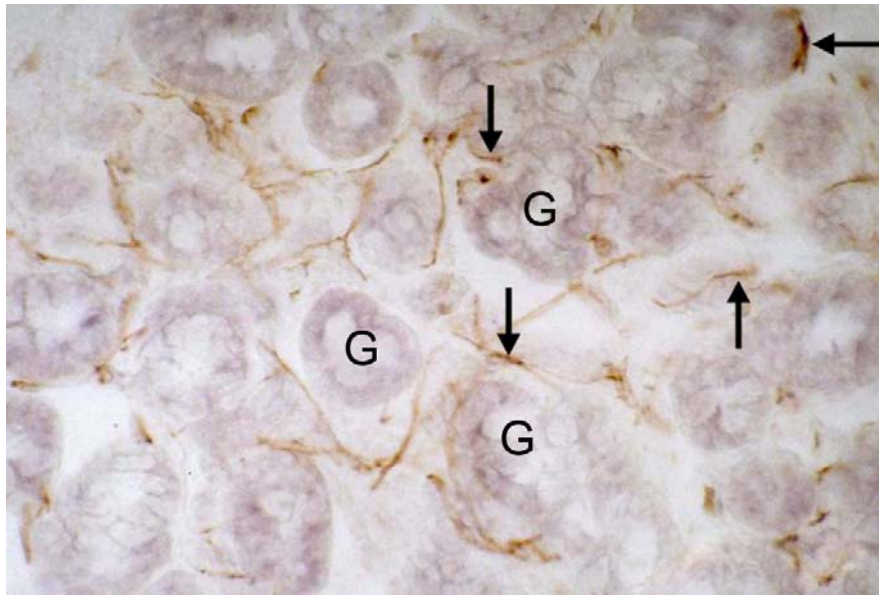
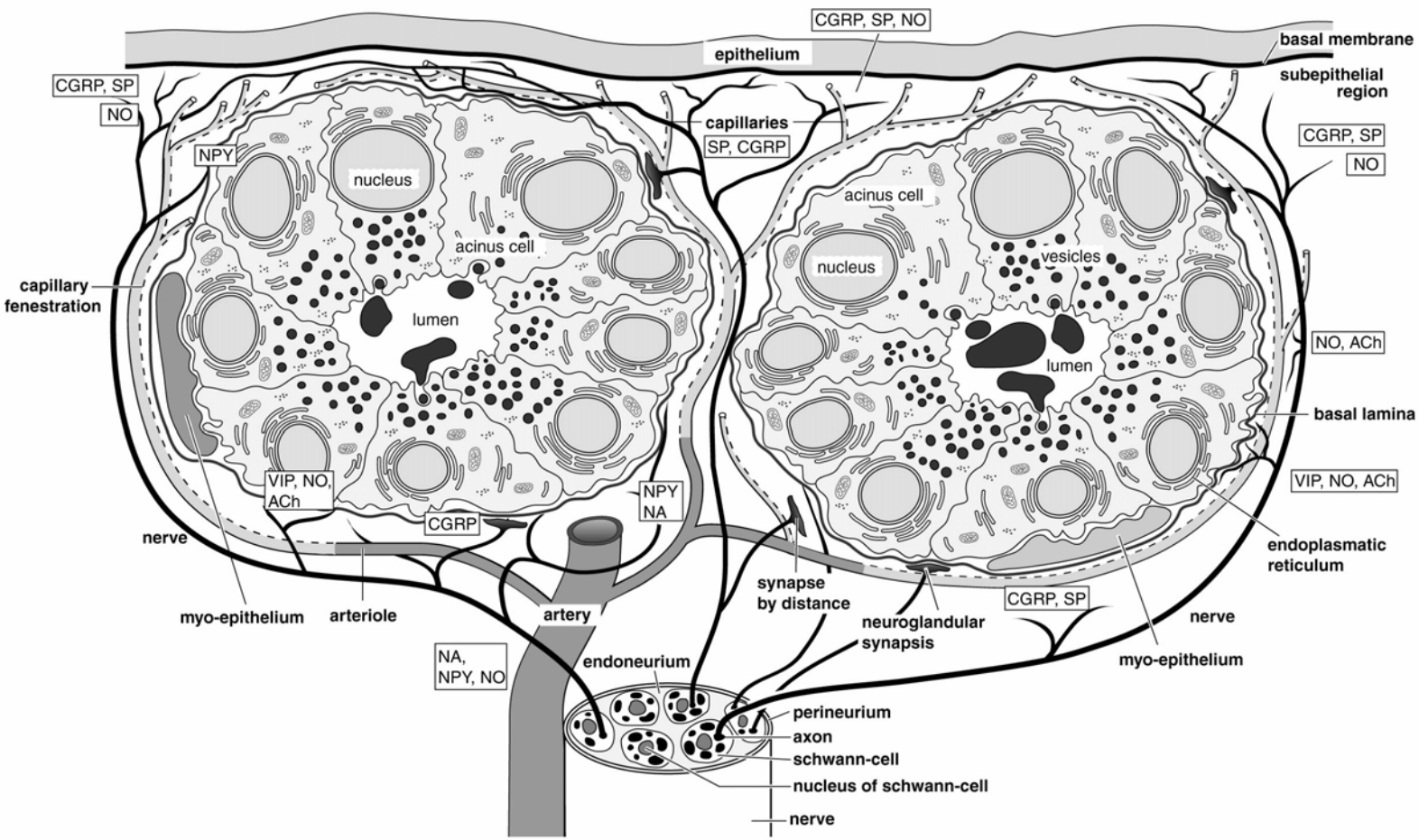


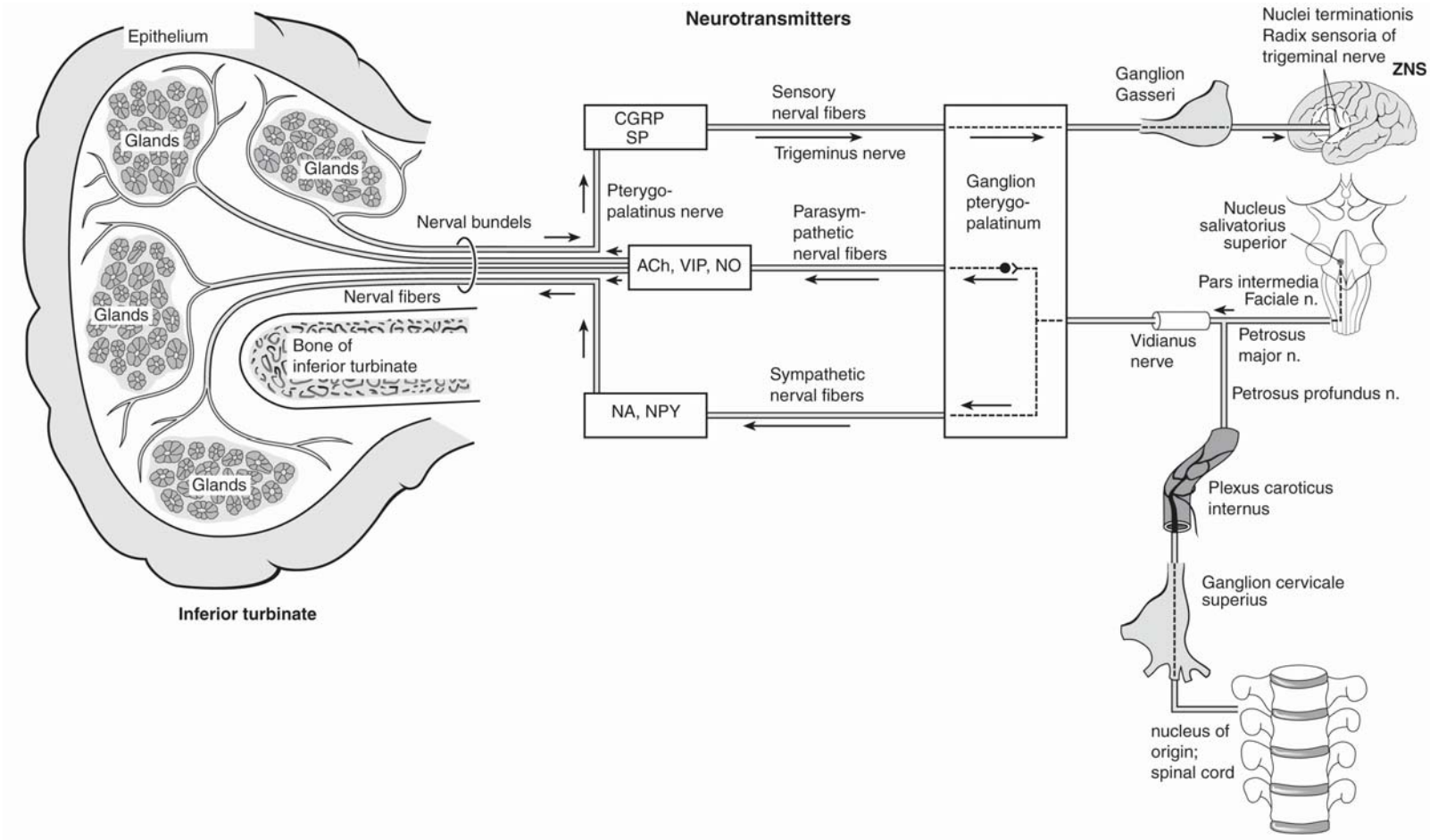
Figure 17. NADPH-d-AChE-double staining of periglandular nerve fibres (arrows). Fine network of AChE - (brown, thin arrows) and NADPH-d stained (blue, thick arrows) fibres around acini of seromucous glands (G). Histochemistry. Original magnification x320.

Conclusion

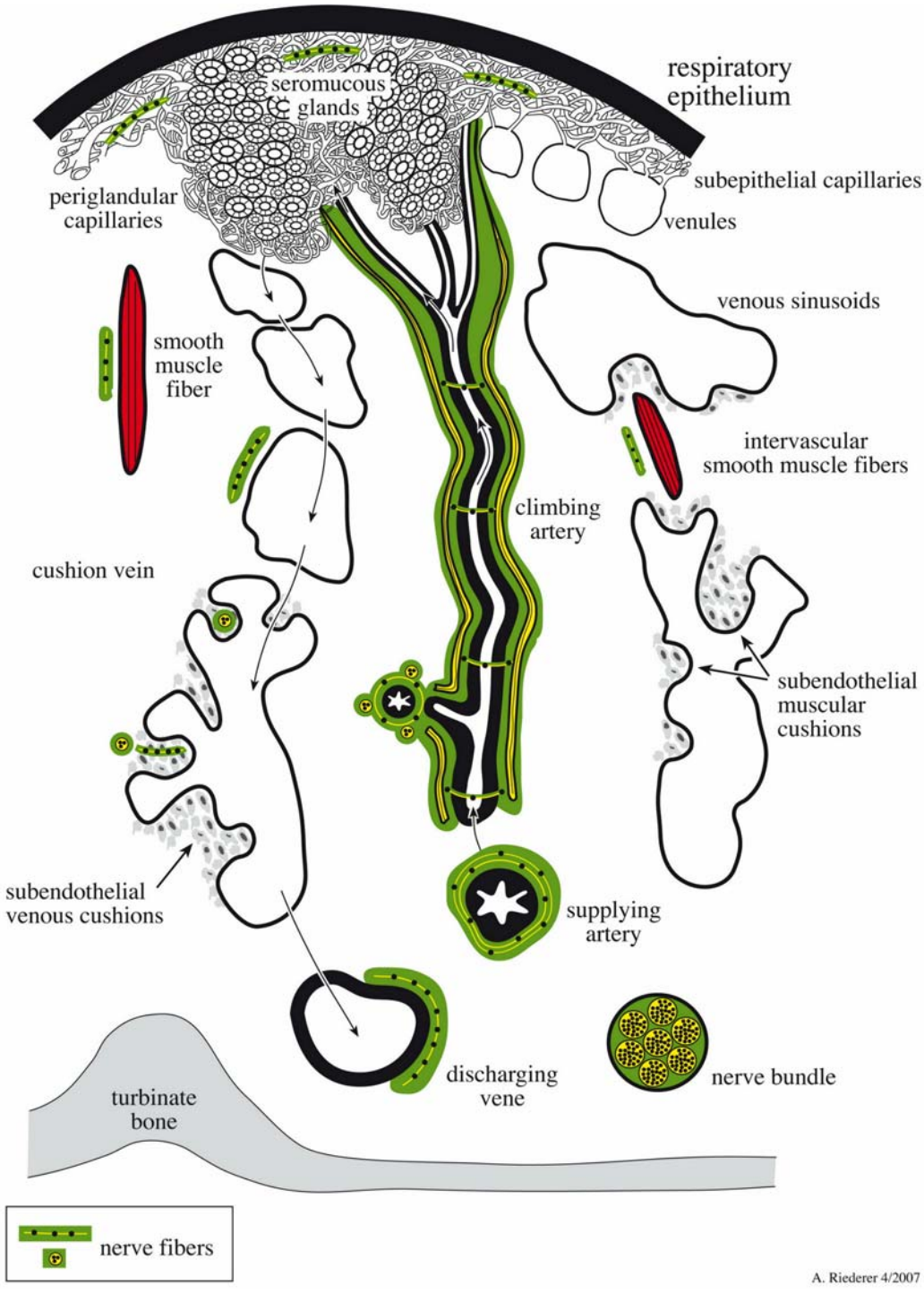
Immunocytochemical, histochemical and electron microscopical methods allow a detailed marking of nerve supply in human nasal mucosa. With immunocytochemical labelling of neurons a precise assignment of nerval structures to surrounding tissue could be achieved (29, 31, 42). General innervation could be identified by using antibodies to neuron-specific enolase (NSE), neurofilament (NF) as well as electron microscopy. The localization of neurons with different neurotransmitters and neuropeptides in the perivascular and periglandular tissue (scheme 1-4) confirms the direct neural control of the diverse nasal functions.



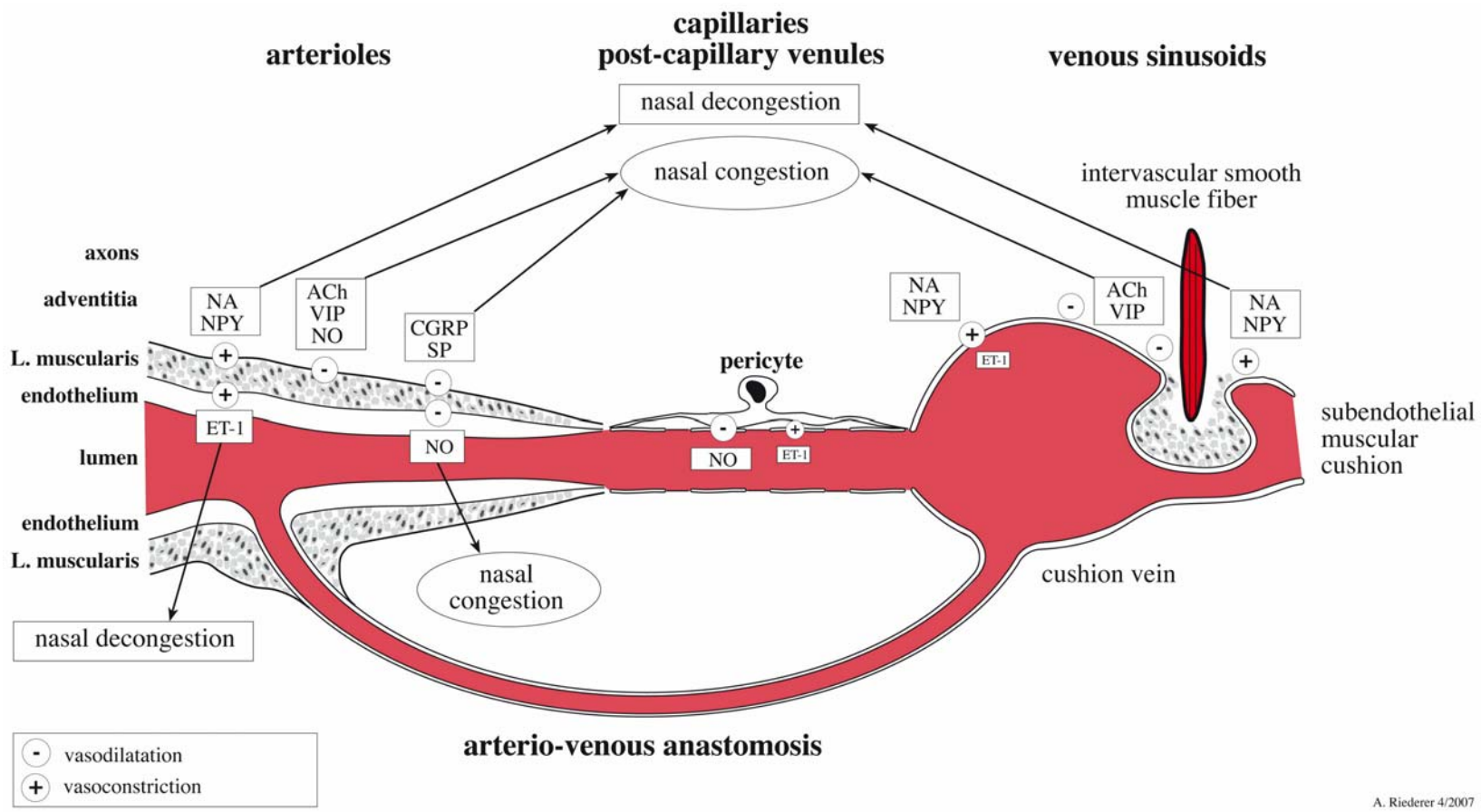
Scheme 1. Distribution of neurotransmitters and neuropeptides in the glandular region of human nasal mucosa.



Scheme 2. Neurotransmitters and neuropeptides of human nasal mucosa.



Scheme 3. Morphological scheme of human nasal mucosa and the distribution of the different vessels with their innervation pattern.



Scheme 4. Longitudinal section scheme of the vascular bed in human nasal mucosa. Neurotransmitter and endothelial transmitter distribution and their physiological actions.

Seromucous Glands

The intense detection of cholinergic nerves at the glands and their efferent ducts demonstrated the predominant influence of the cholinergic system on glandular functions. Cholinergic nerves have been shown in human nasal glands using histochemical techniques (23). Dahlström and Fuxe (1965) also verified cholinergic periglandular nerves with a fluorescence technique in different mammals (13). Grote identified only cholinergic nerval fibres in the nasal mucosa of rats by using histochemical and fluorescence methods (20). However at glandular ducts he did not find any AChE-containing nerves. Therefore these authors did not report a sympathetic influence. However other authors have described a sympathetic influence on the glandular innervation. By using fluorescence-microscopic techniques Änggard showed adrenergic nerves at the glands in cats (3). Baraniuk and Wolf could find a sympathetic participation on glandular innervation (4, 5, 59).

Submucosal glands are innervated mainly by parasympathetic nerves, but also few sympathetic structures were found near to acinus cells (4, 33) (scheme 1). Our findings support the thesis of a mixed parasympathetic-sympathetic innervation of the nasal glands in man (1, 48). The noradrenergic impulses could exert an inhibitory effect on the glandular secretion (24). According to Canning sympathetic nerves stimulation on seromucous glands is part of nasal inflammation (10). Our findings about sympathetic participation on glandular innervation could be reinforced electron microscopically

Nasal Vasculature

Most of the nerve structures are found in the arterial part of the human nasal vascular system, but in the subendothelial muscular bolsters of venous vessels also a high density of nerve fibres could be detected (scheme 3). Mainly arterial vessels showed reactions to antibodies directed against endothelial transmitters. Neuropeptides were detected predominately in arteries and arterioles (scheme 4).

According to the morphological findings the swelling mechanism and secretion of the human nasal mucosa is regulated by a dual (neuronal and endothelial) control of arteries. The special types of nasal veins however are mainly nerval controlled according to our findings. In our opinion swelling of nasal mucosa is achieved by a simultaneous relaxation of all smooth muscle cells in nasal mucosa (51), which leads to the dilatation of arteries and therefore to a higher precapillary pressure. Also the smooth muscle cells of venous vessels relax and dilated their lumen. The drainage of the vascular bed is reduced because the venous muscular bolsters fall into the lumen of the cushion veins. In this manner also the postcapillary pressure is increased. By raising the precapillary and postcapillary pressure fenestrated capillaries are expanding and the endothelial fenestrations can open. In addition, an active dilatation is discussed because of the detection of eNOS in the nasal capillaries. Thus liquid blood components can pass in the lamina propria mucosae.

Vice versa, a contraction of all smooth muscle cells leads to a contraction of the arteries and, consecutively, to a reduction of blood supply. Simultaneously the muscular bolsters are torn out of the lumen of venous sinuses and the blood drainage is increased. Nasal concha decongests (scheme 4). The liquid blood components are pressed transepithelially into the nasal lumen (46).

Neuropeptides

In addition to classical neurotransmitters neuropeptides could influence nasal vasculature and glandular functions (58). By using immunocytochemical methods and immuno-gold-silver staining Baraniuk found VIP-positive nerve structures in the area surrounding human nasal glands (6). Uddmann demonstrated VIP containing nerves near the nasal glands in rats, cats and guinea pigs with immunofluorescence techniques (54). With semiquantitative analysis of nerve fibres neuropeptide density Fischer were able to demonstrate a significant increase of VIP-positive fibres in patients with allergic rhinitis (16).

Yokoyama demonstrated VIP containing synapses electron microscopically in nasal glands of guinea pigs (60). VIP-binding sites in human nasal mucosa were demonstrated by own immunocytochemical investigations (29). According to Karhunen neuropeptides like VIP as well as CGRP was localized in dense core vesicles (26). The localization of VIP in periglandular nerves was confirmed and reinforced by the immunocytochemical and immunoelectron microscopic findings described in this paper.

Few immunoreactions to VIP-antibodies were found in nerve fibres of the adventitia of veins and arteries. VIP-reaction could be demonstrated also in the tunica media of venous and arterial vessels (40).

CGRP was seen in nerve fibres in the periglandular tissue, but not in direct contact with the acinus cells. However in the glandular duct system CGRP-containing neurons could be seen. It could be assumed that VIP and CGRP exert a direct influence on glandular secretion. These neurotransmitters could affect the composition and amount of glandular secretion (29, 40, 41). Baraniuk presumes a rather indirect effect of CGRP on the glands about a direct effect on blood vessels (5). In particular CGRP is a mediator in sensory nerve fibres (53). CGRP could take part in reception, modulation and transmission of external stimuli in axon reflexes (10, 59, 61). Through different chemical, thermal and tactile impulses by afferent and consecutive antidrome-efferent pathways the glands can be stimulated (5, 8). CGRP may influence the release of immunoglobulins, lactoferrin and lysozyme. In idiopathic rhinitis (formerly vasomotoric rhinitis) a neurogenous oedema may be caused by CGRP-mediated effect (7, 49, 59). CGRP plays a dominant role in neurogenic inflammation by increasing permeability and inflammatory cell chemotaxis (36). According to Eccles (2000) stimulation of sensory nerve endings caused reflex glandular secretion, sneezing, and sensations such as itching, irritation, pain, and pressure (14). These symptoms are triggered by stimulations of sensory neuropeptides containing neurons. In 1977 the nervus vidianus-transsection was postulated by Jahnke as a possible therapy for idiopathic rhinitis (24). Current in vivo-investigations in pigs and rats however may demonstrate a favourable effect of CGRP-receptor antagonists at chronic idiopathic rhinitis (47). Van Rijswijk et al. described a safe and effective treatment with capsaicin in idiopathic rhinitis (56). Capsaicin leads to a selective degeneration as well as desensitization of sensory neurons.

CGRP-immunoreactions were found in the adventitia of arteries and arterioles. Also in the subendothelial sensory nerves CGRP-locations were detected. However venous vessels showed no immunoreactions (41).

In human nasal mucosa NPY-positive nerve fibres were mainly located in the adventitia of arterial vessels. There were also NPY-immunoreactive arterioles near to the glands. Periglandular a lower density of immunoreactions could be observed. NPY-positive fibres could be detected in the subepithelial connective tissue and at glandular ducts. These results

indicate that NPY-containing nerve fibres innervating arteries as well as nasal glands. NPY play a significant role as a neuromodulator in the control of both vasculature and glandular secretion (53). The localisation of NPY in periglandular and periductal nerves confirms the direct influence of glandular functions. NPY-agonists may be a beneficial additional treatment of rhinitis to reduce nasal obstruction and mucus secretion. (32, 54)

Since NADPH-d histochemistry is generally considered an indirect marking procedure for all NOS isoforms, histochemical NO tracing could be confirmed by NOS immunocytochemistry.

Nerval cells can form NO-radicals by using neuronal enzymes of NO-synthetases that are effective as neurotransmitters (43). In the respiratory and olfactory epithelium of rats Kulkarni identified NADPH-d-positive nerves, but not in the human nasal mucosa (35). In the present investigations we applyed commercially available specific primary antibodies against isoforms of NOS. The detection of bNOS-and NADPH-d-positive structures around arteries and glandular cells suggests that NO takes an additional part in the control of nasal functions. Similarly to the ultrastructural results of Heinrich who studied the Corti organ of guinea pigs, strong NOS immunoreactions in the cytoplasm of capillaries and endothelial cells of arteries can be shown in the human nasal mucosa (22). Veins and venous sinusoids do not show any NOS immunoreactivity (45).

Nitric oxide (NO) could be demonstrated in both periglandular nerves and the endothelial cells of fenestrated capillaries surrounding acinus cells through NADPH-diaphorase-histochemistry and immunocytochemical identification of NOS. NO seems to influence particularly resistance and exchange vessels in respiratory nasal mucosa. Through the immunoelectron microscopic detection of NOS I in the already ultrastructurally observed nerve network of the seromucous glands and the arterial vessels, it can be assumed that NO takes part in modulation of nerval regulations (30). NO seems to influence the glandular secretion due to the powerful immunoreactivity in the periglandular and periductal axons as well as the cytoplasm of acinus cells under physiological conditions (25).

Due to the ultrastructural detection of NOS in perivascular nerves and in the endothelium, it can be assume that NO has a dual influence on nasal vessel tonus. Apart from modulation of the tonus of arterial vessels there could exists also a relaxing effect on capillary pericyts. Arterial dilatation causes an increase of precapillary pressure and a dilatation of the capillaries walls. Additionally, it can be postulated an active relaxation of pericyts, which results in the opening of the capillary fenestrations. Consecutively an increased plasma extravasation as well as an increased supply of serum particles for the seromucous glands can be found. Next to its effects as a neuromodulator in nerves near the glands, NO also seems to influence the function of the glands through the regulation of the periglandular blood flow (34).

In this study the cholinergic-nitrergic co-innervation of human nasal glands could be demonstrated by using the NADPH-d / AChE-histochemistry-double staining. It could be assumed that NO amplifies the stimulation of seromucous glands caused by Acetylcholine. Through a vasodilatatory effect of NO on periglandular capillaries, the so called endothelium-derived relaxing factor can lead to an extravasation of serum components. This could have an influence on glandular secretion. The nerval control of glandular functions can not be assigned only to the parasympathetic system. Rather, complex influences on the glands can be accepted also through neuropeptides and neuronal and endothelial Nitric Oxide in the human nasal mucosa. By using double staining methods the nitrergic coinnervation in cholinergic

nerves could be demonstrated. Additionally, with counterstaining of the surrounding tissue a precise assignment of nerval structures was possible.

It could be shown, that beside immunologic mechanisms also the dense network of sensory, sympathetic and parasympathetic nerve fibres (scheme 2, scheme 4) act as protection of nasal respiratory mucous membranes from external and internal influences.

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Chapter 9

CLINICAL PRESENTATION OF SYMPATHETIC DYSFUNCTION IN SEVERE TRAUMATIC BRAIN INJURY: SYMPATHETIC STORMING

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Abstract

Currently it is estimated that there are approximately 1.4 million traumatic brain injuries (TBI) a year in the United States. Of the 1.4 million, 235,000 are hospitalized with 40% of in those individuals suffer severe TBI. In severe TBI structural damage occurs to the brain in the form of primary injury related to intraparenchyamal hemorrhages, diffuse axonal injury, contusions, epidural hematomas and subdural hematomas. Secondary injury amplifies the damage to the brain and is related to edema, reduction of cerebral blood flow or anoxia, release of excitatory amino acids, and formation of free radicals.

The physiological changes and clinical presentation varies from individual to individual and is directly related to location of injury and extent of injury. Alterations in level of conscious is the most common clinical presentation of TBI but range from changes in motor activity, speech, cognitive function, heart rate and rhythm, respiratory patterns and cranial nerve function.

In severe brain injury 15-35% of individuals will demonstrate clinical presentation of sympathetic storming, a form of abnormal sympathetic output or control. Presentation includes episodes of hypertension, tachycardia, tachypnea, diaphoresis, posturing, dystonia and hyperthermia. The clinical presentation varies from episode to episode in an individual and from individual to individual. Diagnosis is based on clinical exam and medical management is related to control of symptoms.

This section will integrate current literature while reviewing the proposed neuropathophysiology of sympathetic storming, clinical presentation, differential diagnosis, the adverse effects of sympathetic storming, and medical management.

Introduction

Episodic autonomic dysfunction was first described by Wilson (1823) when he observed a patient with mass effect from a tumor displaying episodes of increase posturing. He termed the episode as a “tonic fit” and speculated that the increase in intracranial pressure created an imbalance within the autonomic nervous system leading to increased sympathetic output. Penfield (1929) was the first to describe the classic pattern of presentation of sympathetic storming, intermittent episodes of diaphoresis, hypertension, shivering and tachypnea. The individual had a tumor at the foramen of Monroe. Penfield concurred with Wilson that the pattern of clinical presentation was consisted with autonomic dysfunction and given the sporadic occurrence term the phenomena “diencephalic autonomic seizures”.

The majority of the literature are case study reports [6, 8, 10-13, 16, 19-21, 24, 27-30] with one retrospective study analyzing medications [5], one retrospective analyzing clinical features and management [4], general overview of the dysfunction [18, 22], analysis from rehabilitation perspective [3, 4, 5], and more recently from a critical care perspective ,[6, 11, 29]. Numerous names have been used for the dysfunction have been used within the literature since then and include dysautonomia [3-8], paroxysmal autonomic instability with dystonia [9], paroxysmal sympathetic storms [10-12], diencephalic autonomic seizures [2,13], central dysregulation [14], tonic decerbrate spasms [15], reversible diencephalic dysfunction [16], traumatic apallic syndrome [17], autonomic dysfunction syndrome, [18-22], acute midbrain syndrome [23], hypothalamic midbrain dysregulation syndrome [24], autonomic storm [25], sympathoadrenal response [26], acute hypothalamic instability [27], and sympathetic storming [28, 29]. All of these describe a similar phenomenon of apparent spontaneous episodes of hypertension (HTN), tachycardia, tachypnea, diaphoresis, posturing, dystonia and hyperthermia [1-31].

The term sympathetic storms characteristically describe the episodes as the episodes are sympathetic in origin and erratic in nature with unpredictable clinical presentation; episode to episode and individual to individual. Sympathetic storming has been associated with hydrocephalus, increased intracranial pressure and more commonly severe traumatic brain injury.

Proposed Theories of Pathophysiology

There has been much speculation in the mechanism of autonomic dysfunction though the precise mechanism remains debated. Theories range from seizure activity, autonomic dysregulation, autonomic release phenomenon, interruption of autonomic pathways and loss of cortical control.

Penfield (1926), given the intermittent episodic nature of the symptoms, felt that the symptoms were related to epileptic activity within the thalamus. This theory has been disproved as electroencephalography (EEG) does not demonstrate seizure activity during the episodes [3, 10, 12, 17, 28, 29].

Autonomic control is the result of input from feedback loops from multiple areas within the brain from cortical and subcortical structures, hypothalamus (posterior and preoptic area), thalamus, orbitalfrontal cortex, and amygdale [18, 22, 33]. The coordination of these areas allow for control of sympathetic and parasympathetic activity and injury any of these areas

could lead to dysfunction. Given the extreme state of storming and the spontaneous nature of the episodes one would suspect a more global dysfunction of the brain in the control of sympathetic activity.

Clinical Presentation

There is an initial catecholamine surge following extensive intracranial injury. Levels will generally return to normal within days. In severe TBI (Glasgow coma score (GCS) less than 8 (table 1 & 2) or Ranchos Los Amigos scale of IV or less) (table 3), there can be prolonging of this catecholamine response in the form of spontaneous episodic events or sympathetic storming. 9.3 - 33 % of individuals with severe TBI will demonstrate sympathetic storming [3, 6, 7, 18, 22,]. The onset of can occur within days or anytime during the recovery of the injury [6, 9, 18]. It is common for sympathetic storming to occur within the intensive care unit and has been associated with the discontinuation of sedation and paralytics [7, 28, 29].

Table 1. Glasgow Coma Scale

Best Verbal Response	Oriented	5
	Confused	4
	Inappropriate speech	3
	Incomrehensive speech	2
	No speech	1
Best Eye response	Opens eye spontaneously	4
	Opens eyes to voice	3
	Opens eyes to pain	2
	No eye opening	1
Best Motor Response -	Follows command	6
	Localizes	5
	Withdrawal to pain	4
	Flexor (decorticate)	3
	Extensor (decerebrate)	2
	No motor response	1

Table 2. Severity of traumatic brain injury

Mild traumatic brain injury	13-15
Moderate brain injury	9-12
Severe brain injury	3-8

Classic clinical presentation consists of sporadic spontaneous episodes of HTN, tachycardia, tachypnea, diaphoresis, posturing, dystonia and hyperthermia [2-30]. Rabinstein & Wijdicks, 2004, noted that the episodes generally occur 1-3 times daily with a single episode lasting up to 10 hours. The frequency sympathetic storming lessens over time though episodes have been reported to increase in intensity and duration [10, 13, 19].

Table 3. Ranchos Los Amigos Scale

Level	Description
I	No response to visual, verbal, tactile, auditory, noxious stimuli
II	Generalized response
III	Localized response
IV	Confused-agitated
V	Confused-inappropriate
VI	Confused-appropriate
VII	Automatic-inappropriate
VIII	Purposeful and appropriate
IX	Purposeful and appropriate (standby assistance on request)
X	Purposeful and appropriate (modified independent)

Hortung, 1980, made the diagnosis solely based on relief of symptoms with intravenous morphine. Strum, 2004, felt that storming was a diagnosis of exclusion and that there needed to be recurrent episodes of HTN, tachycardia and hyperthermia. Bageluy, et al (2005), required 5 of 7 clinical features be present (HTN, tachycardia, tachypnea, dystonia, posturing, hyperthermia, and diaphoresis) for the diagnosis.

Blackman, et al, (2005) outlined criteria for the diagnosis requiring recurrent episodes of one per day for three consecutive days in individuals with severe brain injury (GCS less than 8 or Ranchos Los Amigos scale of IV or less). He defined the clinical parameters as: HTN: systolic blood pressure (SBP) greater than 140 mm Hg, tachycardia: heart rate (HR) of 130/min or greater, tachypnea: respiratory rate (RR) greater than 40 breaths per minutes and hyperthermia: temperature 38.5 ° Celsius or greater.

Bagley, et al (2005) delineated sympathetic storming into phases. Phase 1: asymptomatic in individuals receiving sedation or paralytics; Phase 2: episodes of storming; Phase 3: cessation of diaphoresis, dystonia and spasms. The storming episodes generally began approximately one week after the injury, or when sedation is withdrawn, with a mean duration of 74 days [5].

Lemke (2007) define two phases, as the use of sedation and paralytics essentially limit the adrenergic activity and treat the symptoms. Phase 1: sympathetic disassociation with episodic storming (HTN, tachycardia, tachypnea, diaphoresis, dystonia, posturing, hyperthermia); phase 2: resolution of sympathetic disassociation or return of parasympathetic/sympathetic regulation, though the true end point of the phenomenon is difficult to define as it may be resolved prior to the discontinuation of medications.

Triggers, or activities that precipitate an episode, have been noted by nursing staff [28, 29]. A trigger may be perceived by the brain as stressor thus eliciting the episode. Triggers can be turning the patient, suctioning, sound or environmental stimulation. Pretreatment prior to an identified activity can assist in abating an episode or lessening the intensity. A common trigger is an infectious process, which exacerbate temperature surges amplifying the metabolic rate. Rapid workup to identify the infectious etiology should be completed with institution of appropriate antibiotic coverage. Lemke, 2007, noted that the presence of hyperthermia can intensify and prolong the episodes thus should be treated promptly with the goal of maintaining normothermia.

Differential Diagnosis

Catecholamine panels demonstrate elevation in norepinephrine levels though generally the diagnosis of sympathetic storming is a clinical diagnosis [17, 28 29, 30]. Computerized tomography (CT) and magnetic resonance imaging (MRI) fail to provide information to assist the clinician in making the diagnosis as no definitive lesion has been associated with sympathetic storming [2-31]. A common injury, diffuse axonal injury, has been linked with sympathetic storming though is not a criteria for diagnosis [6, 10, 22, 23, 27, 28, 31].

Multiple diagnoses can mimic the clinical picture of sympathetic storming. An expanding intracranial lesion, infection, central fever, malignant hyperthermia, thyroid storms, deep vein thrombosis, pulmonary emboli, neuroleptic syndrome, and drug/alcohol withdrawal can present with a similar clinical symptoms (table 4) [3, 4, 9,11, 18, 22, 25- 29].

The process of diagnosis requires the clinician to evaluate the individual for any of these conditions to assure proper treatment. Careful assessment of the patient will determine the need for further imaging, though any significant neurological change warrants a CT scan of the brain to assess for expanding lesion, edema or hydrocephalus. Elevation in intracranial pressure (ICP), related to mass effect or hydrocephalus, was once speculated to be a have been a trigger of the storming though current literature case reports demonstrate the increase in the ICP with the progression of the storming and not as preceding the episode [25,28, 29]. Overall the CT scan tends to be inconclusive in identifying the cause of the sympathetic storming.

If the CT scan is unremarkable, infection, DVT and central fever should be evaluated. Routine sputum, urine and blood cultures should be obtained to rule out infectious etiology. Central fever should be considered if cultures are negative and the classic presentation of sympathetic storming is not noted.

Ddimer should be done to evaluate for DVT. For a Ddimer is 3 or greater, lower extremity Dopplers should be done to evaluate for DVT. If respiratory instability is noted urgent helical CT of the chest and blood gases should be completed to rule out PE.

Initial history should be reviewed to determine if the individual is at risk for drug or alcohol withdrawal. The symptoms of withdrawal can be masked by the use of opiates, sedatives and paralytics in the ICU. In addition, if there has been prolonged use of high dose opiates and sedatives the symptoms may be related to the rapid discontinuation of the medications. Restarting the medications with a gradual taper can be done if there is concerned for withdrawal.

Thyroid panel evaluates for thyroid storming and careful evaluation of medications should be completed to rule out malignant hyperthermia or neuroleptic syndrome. Malignant hyperthermia generally occurs after the use of anesthetic agents, specifically succinylcholine. Common medications associated with neuroleptic syndrome include dopaminergic agents and metoclopramide (reglan).

Table 4. Differential diagnosis

Diagnosis	Clinical Presentation	Diagnostic evaluation	Treatment
Sympathetic storming	Spontaneous episodes of HTN (SBP > 140), tachycardia (HR $\geq$ 130/minute), tachypnea (RR > 40/minute), dystonia, posturing, hyperthermia (T $\geq$38.5° Celsius), diaphoresis	Labs – serum catecholamine levels Criteria for diagnosis: 1. Severe TBI (GCS 8 or less; Ranchos Los Amigos score IV or less) 2. Demonstrates 5 out of 7 classic symptoms 3. recurrent episodes of one per day for 3 consecutive days	Sedatives, opiate agonists, β antagonists, α antagonists, dopamine agonists, GABA-A agonists, GABA-B agonists
Hydrocephalus or expanding intracranial lesion	Decrease neurological response (decrease level of consciousness, posturing, non-reactive pupils, loss of brainstem reflexes), increased ICP, HTN, tachycardia or bradycardia, +/- fever	CT scan of brain	Treatment dependent on CT findings. Hydrocephalus – placement of external ventricular drain; serial lumbar punctures Intracranial lesion – Surgical evacuation of lesion, Medications - Mannitol, 23.4% saline bolus, sedation, paralytic agents
Infection Central fever	Fever, decreased neurological clinical exam, tachycardia, diaphoresis, dystonia	Labs: CBC, sputum, blood and urine cultures Radiographics: chest x-ray	Infection: treat with appropriate antibiotic. Central fever – acetaminophen, alternative methods to lower temperature
DVT/PE	Fever (may be low grade), decreased neurological clinical exam, tachycardia, localized edema & erythema at site of DVT, tachypnea, respiratory distress, variable changes in vital signs	Labs: Ddimer, Blood gases Radiographics: lower extremity dopplers, helical CT scan of chest	Anticoagulation, removal of sequential stockings, discontinuation of range of motion of affected limb
Neuroleptic syndrome	Fever, dystonia	Review of current medications Labs: creatine kinase level	Removal of causative agent Sedation, treatment of fever & HTN
Malignant hyperthermia	Fever, dystonia, tachycardia	Review of medications Labs: creatine kinase level	Removal of causative agent Treatment of fever
Thyroid storm	Tachycardia, HTN, tachpnea, dystonia, +/- fever,	Labs: thyroid panel	β blocker, methimazole
Drug/alcohol withdrawal	Agitation/restlessness, decreased neurological clinical exam, tachycardia, diaphoresis, HTN, +/- fever	Patient history	Narcotics, sedation (Ativan)

Table 5. Medications

Medication category	Dosage	Route	Action	Adverse effects	Literature support
Opiate agonist Oxycodone	5-10 mg every 4 hours scheduled; adjust dose per clinical exam	Enteric	Suppression of autonomic pathways/activity	Sedation	3, 7, 9, 18, 19, 22, 28, 29
Morphine	Enteric 10 -30 mg every 4 hours IV 5-20mg/70kg hours	Enteric IV	Suppression of autonomic pathways/activity	Hypotension, respiratory depression (IV route; high doses) Increases CSF pressure leading to potential increase in ICP	3, 4, 9-12, 19,
β antagonist Proprandolol (nonselective)	10-40 mg every 12 hours, increase as needed; doses up to 600mg/day	Enteric	Diminishes adrenergic response – reducing catecholamine related tachycardia and HTN	Bradycardia, hypotension Caution use with asthma & diabetes. Contraindicated in the presence of bradycardia & A-V conduction abnormalities	3, 4,9,10, 12, 18, 22, 24, 27-29
Labetolol Nonselective β and α 1 antagonist	100 mg two times daily; max daily dose 2400mg	Enteric	Diminishes adrenergic response – lowers HR & BP (β 2 effect > β1)	Contraindicated in patients with asthma and severe bradycardia. Caution in patients with diabetes.	7, 9, 11, 12,
Dopamine agonist bromocriptine	2.5 mg every 8 hours; increased as needed to 30-40 mg/day	Enteric	Increases central dopamine by activating post-synaptic dopamine receptors; hypothalamus effect of lowering temperature surges	Constipation, hypotension Caution to be used in patients with renal, hepatic dysfunction, & cardiovascular disease	3, 4, 9, 10, 12, 13, 19, 20, 22, 27-29
α antagonist clonidine	0.1 mg twice daily; maximum dosing 0.2mg every 8 hours	Enteric	Diminishes α outflow from CNS reducing circulating NE & EPI	Constipation Caution with rapid withdrawal as will cause rapid increase in catecholamine levels	3, 7, 9, 22, 24, 28, 29

Table 5. Continued

Medication category	Dosage	Route	Action	Adverse effects	Literature support
Muscle relaxant Dantrolene	25 mg daily to bid; maximum daily dose 400mg	Enteric	Reduction in skeletal muscle contraction by suppression of calcium	Respiratory depression, elevated liver enzymes	9, 10, 18, 22, 24,27-29
Dopamine agonist Chlorpromazine	0.1 mg every 15 minutes 10-25 mg every 6 hours	IV Enteric or IM	Acts at all levels of CNS, though precise mechanism is unknown. Possible suppression of hypothalamic vasomotor tone	Extrapyramidal side effects (tardive dyskinesis), lowers seizure threshold, hypotension , delayed renal failure	18, 22, 28, 29

Adverse Effects of Sympathetic Storming

Uncontrolled sympathetic storming can amplify the risk of secondary injury to an already injured brain [3, 18, 22, 28-30]. Prolonged episodes can lead to hypoxia of the brain related to reduction of tissue oxygenation and elevated metabolic needs. Increases in intracranial pressure can occur during the episode as a result of the persistent stimulation and hypoxia. Prolonged hyperthermia increases inflammatory changes, cell death, and intensifies metabolic needs of the brain.

Prolonged sympathetic storming accelerates metabolic rate increasing caloric needs as high as 200% [3, 18, 22, 28-30]. In addition, prolonged posturing will further elevate the metabolic rate and increase the risk of weight loss and muscle wasting. Prealbumin level and amount of weight loss aid will assist dietary in calculating the needed adjustments to caloric intake.

The increase in metabolic rate and diaphoresis can lead to excessive fluid loss and acute states of dehydration with hypernatremia and thickening of pulmonary secretions. Assessment of laboratory values (sodium, osmolarity, BUN, creatinine) is helpful in determining the extent of dehydration and the amount of needed water replacement.

Prolonged hypertension and tachycardia can place additional stress on already damaged vessels and lead to expansion of hemorrhage or increase cerebral edema and damage to the myocardium in the form of reversible hypokinesis with reduction of ejection fraction or microscopic damage. Neuromyocardial failure has been noted with increase β activity in catecholamine toxicity, though is more common in subarachnoid hemorrhage [29]. Autopsies of severely brain injured have demonstrated microscopic injury to the heart with contraction band necrosis [18, 22, 30].

Neurogenic pulmonary edema (NPE) can develop secondary to neuromyocardial failure or excessive α response [25, 29]. Both etiologies cause acute pulmonary inflammatory changes within the pulmonary system that lead to fluid overload creating stiffening and diminished oxygen transfer. Careful manipulation of oxygen concentration, tidal volumes, method of ventilation, antidiuretics and respiratory treatments is needed to maintain adequate oxygenation and reduce lung damage.

Medical Management

Goal of medical management aimed at diminishing the clinical symptoms or dampening the sympathetic system. Obtaining control is a matter of trial and error, though many clinicians have their mixture of medications that they utilize to attempt to control the episodes. Medications utilized include sedatives, opiate agonist, β antagonists, α antagonists, dopamine agonists, GABA-A agonist and GABA-B agonist (Table 5) [1-31].

Intravenous medications including opiate agonists (morphine, fentanyl) and GABA-A agonists (propofol and midazolam) are routinely used in the initial management of severe TBI. Resumption of any of these medications is not uncommon if sympathetic storming occurs in the ICU, though the presence of sympathetic storming does necessarily warrant ICU care. Therefore if normal gastric function is present the enteric route should be utilized. The enteric route allows scheduled dosing for better control of clinical symptoms, decreases cost,

decreases ICU length of stay and promotes transition to rehabilitation/long term care. If this is not possible, a continuous IV drip should be utilized to control of clinical symptoms and medications should be switched to enteric when possible.

A combination of an opiate agonist, a dopamine agonist and a nonselective β antagonist can be a starting point [4, 12, 13, 19, 26, 28, 29]. Oxycodone or enteric morphine, both opiate agonists, should be scheduled every four hours. Oxycodone 5-10 mg or Morphine 10 mg every 4 hours with as needed (PRN) dosing of 5 mg every 4 hours for break through activity. The dosing can then be adjusted based on the 24 usage of the PRN medication. Other opiate agonists can be used from codeine, methadone, morphine (IV), oxymorphine (naltrexone), oxycodone/acetaminophen (percocet), or hydrocodone/acetaminophen (loratab). Maximum daily dose of acetaminophen is 4 grams thus medication that contain acetaminophen should be avoided to allow intermittent use of acetaminophen for fevers. Methadone is a class II narcotic and is highly regulated within facilities thus its use is restricted. Intravenous morphine and fentanyl (duragesic), have demonstrates effectiveness in the treatment of sympathetic storming though is use is generally referenced within the ICU setting [25,26,30].

Bromocriptine (parlodel), a dopamine agonist, can be started at 1.25 - 2.5 mg every 8 hours to a maximum dose of 30-40 mg a day [13, 18, 20, 22, 25, 27- 29]. Bromocriptine increases available dopamine and is speculated to have a direct affect on the hypothalamus reducing temperature surges. In severe case of storming the maximum dose of 10 mg every hour can be the starting point with dose reduction after control is achieved.

Proprandolol (inderal), a nonselective β antagonist, can be initiated at a dose of 10 mg every 6 hours [18, 23, 26, 28, 29]. If severe tachycardia and HTN is noted the clinician may start with a higher dose as dosages up to 640mg a day reported [18, 22]. Atenolol (tenormin) and metoprolol (toprol), selective β1 adrenergic antagonists have not demonstrated the same effectiveness as proprandolol. Bradycardia is common side effect of proprandolol and continued use is clinician dependent. Care should be used in administering proprandolol in asthmatic and diabetic patients.

If this combination does not abate the episodes clonidine (α2 antagonist) or labetolol (nonselective β antagonist and α1 antagonist) can be added [18, 23, 28, 29]. Some clinicians will begin with labetolol with both β1, 2 and α1 properties though it has demonstrated variable effectiveness in controlling the episodes [11, 26].

Dantrolene (dantrium) can be used if there is persistent dystonia [18, 22, 23, 28, 29]. Dantrolene directly relaxes skeletal muscle with the suppression of calcium. Intrathecal baclofen (lioresal) has provided control of dystonia, though use has been limited to Europe [6, 8].

Chlorpromazine (thorazine), a dopamine antagonist, can be useful in lowering core temperature by suppressing hypothalamic vasomotor tone thus preventing piloerection. Chlorpromazine can be administered IV, intramuscular or enterically. Use of chlorpromazine can diminish overall symptoms though is rarely used clinically [4]. Interaction with dopaminergic pathways in the basal ganglia creates extrapyramidal side effects therefore long term use is not recommended. In severe hyperthermia, temperature greater than 40° Celsius 0.1 mg can be administered IV every 15 minutes to rapidly lower core temperature. Intravenous chlorpromazine should be given within the ICU setting and administered slowly as acute hypotension can occur. Intramuscular and enteric route is the common route with dosing of 25-50 mg every 4-8hours [18, 22].

Other medications that have been used with limited to no control of the episodes include atenolol, metoprolol, carbidopa/levodopa, phenytoin, phenobarbital, diazepam, and carbamazepine [3, 6, 7, 9, 10, 12, 21, 27-29].

Conclusion

Sympathetic storming appears be the aftermath of severe TBI and is a complex dysfunction of the sympathetic system and associated areas of the brain. Analysis of the clinical phenomenon and its treatment will continue as long as there is TBI. Research would provide insight into the pathway of dysfunction though reproducing the injury is difficult given there is not an isolated injury associated with the dysfunction.

Sedation and opiate agonist are mainstream treatment modalities within the critical care setting of severe TBI, though with the increase in reporting of the sympathetic storming in the ICU suggests the need to evaluate the method of withdrawal of these medications, variability in time frame the medications are utilized in the early stage of injury and the amount of sedation utilized.

Defining criteria for sympathetic storming is essential for accurate diagnosis and to allow further analysis. A combining the efforts of Baguley, et al (2005) and Blackman, et al (2006) would provide clear delineation needed for the diagnosis. The criteria for the diagnosis of sympathetic storming would require the individual have a severe TBI (GCS less than 8 or Ranchos Los Amigos score IV or less), 5 of 7 clinical features (HTN – SBP greater than 140, tachycardia – HR 130 or greater per minute, tachypnea – RR greater than 40 breaths per minute, dystonia, posturing, hyperthermia – temperature 38.5°Celsius or greater, and diaphoresis) and recurrent episodes of one per day for 3 consecutive days. Use of these criteria would not only assist the practitioner in making the diagnosis but provide a uniform method allowing further analysis of treatment and research. In addition, a longitudinal study incorporating critical care, acute and rehab would provide a clearer delineation of onset and duration.

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Chapter 10

GABAERGIC FIBERS IN THE ROSTRAL PARAVERTEBRAL SYMPATHETIC GANGLIA: DO THEY ORIGINATE FROM INTRINSIC NEURONS OR PREGANGLIONIC NEURONS?

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Abstract

GABA-containing fibers have been observed in the rodent sympathetic trunk. Most of them terminate in the superior cervical ganglion (SCG). Previous studies suggested that these fibers originated from GABA-immunopositive small intensely fluorescent (SIF)-like cells in the cervical sympathetic chain. However, no direct evidence of the origin of GABA-containing fibers had been obtained. We recently performed a series of experiments to clarify the origin of these fibers, and showed that some SPNs located in the most rostral part of the intermediolateral nucleus (IML) were GABAergic, and send their axons to the SCG. Although some SIF-like cells showed GABA immunoreactivity, they did not express glutamic acid decarboxylases. They are not likely to produce GABA but accumulate GABA released from adjacent fibers because they often showed close proximity to GABA-immunopositive varicose fibers, and completely disappeared after transection of the ventral roots of C8 to T4 segments. There is no evidence of GABAergic DRG neurons which send axons to the SCG. These observations clearly indicated that the majority of GABAergic fibers in the SCG originated in the most rostral part of the IML. In addition to these data, we discuss the characteristics of GABAergic SPNs and function of GABAergic fibers in the SCG.

Keywords: nerve transection, co-localization, anterograde tracing, retrograde tracing, immunohistochemistry, in situ hybridization

Abbreviations

SPN, sympathetic preganglionic neuron;

VAchT, vesicular acetylcholine transporter;

IML, nucleus intermediolateralis;

SCG, superior cervical ganglion;

STG, stellate ganglion;

DRG, dorsal root ganglion;

GAD, glutamic acid decarboxylase;

GFP, enhanced green fluorescent protein;

SIF, small intensely fluorescent;

nNOS, neuronal nitric oxide synthase

1. Introduction

It is now generally accepted that sympathetic postganglionic neurons receive inputs from not only cholinergic terminals but also other neurotransmitter / neuromodulator-containing ones. Some chemical compounds released from terminals function as excitatory or facilitatory transmitters (e.g. acetylcholine, substance P, etc.), while others act as inhibitory or suppressive transmitters (e.g. GABA, enkephalin, and dopamine). These inputs are derived not only from sympathetic preganglionic neurons (SPNs) in the spinal cord, but also from primary sensory neurons in the dorsal root ganglia (DRGs [1, 2]), enteric neurons in the gastro-intestinal tract [3] , and small intensely fluorescent (SIF) cells within the sympathetic ganglia [4]. It is also known that sympathetic postganglionic neurons receive both convergent and divergent inputs: a preganglionic fiber innervates 11-21 postganglionic neurons [5] and a postganglionic neuron receive inputs from 6-11 preganglionic neurons [6]. Therefore, the neural circuit of sympathetic ganglia is much more complex than previously thought. It is likely that many kinds of chemical inputs from various origins are integrated in sympathetic ganglia. It is very important for understanding the function of sympathetic ganglia to investigate both the chemical characteristics and origin of afferent fibers projecting to the ganglia.

The superior cervical ganglion (SCG) is located in the most rostral part of the sympathetic chain, and is the only sympathetic ganglion which projects to various organs in the head and face (e.g. blood vessels in the brain, pineal body, iris, salivary glands, cochlea, lacrimal gland, etc). Because these organs have quite unique roles in autonomic functions, the neural circuit of the ganglia is likely to be also specialized. Indeed, there is some evidence of unique properties of neural circuits of the SCG: A single preganglionic fiber innervates fewer postganglionic neurons than those in the stellate ganglion (STG) [5]; there is no segmental relationship between the segmental level of SPNs and rostro-caudal direction of postganglionic neurons innervated by the SPNs, while in the STG, there is a clear segmental relationship [7].

GABA is a major inhibitory neurotransmitter in the central nervous system. There are reports about the usage of GABA in the peripheral nervous system including the SCG [8–15], inferior mesentric ganglion [3], and sensory ganglia [16, 17]. In the SCG,

GABA-immunopositive fibers were observed [9, 10, 13, 14], GABA receptors were expressed [8, 12], GABA produced a large fall in cell input resistance [18], and stimulation of the cervical sympathetic trunk elicited postsynaptic potentials which were affected by a $GABA_A$ antagonist in postganglionic neurons [19]. Furthermore, GABA blocked long-term potentiation (LTP) [11], and long-term administration of GABA receptor agonists caused synaptogenesis [15, 20, 21]. These findings indicate that GABA acts as an inhibitory neurotransmitter in the SCG, as in the central nervous system, and also has plastic effects. Interestingly, GABA-immunopositive fibers are distributed in the rostral sympathetic trunk with a rostrally deviated gradient, and the number of GABA-immunopositive fibers reaches a maximum in the cervical sympathetic trunk [14]. These GABA-containing fibers make numerous axon baskets with varicosities mainly in the SCG [13]. These observations imply that GABA has a unique role in SCG-specific neural functions.

1.1. Morphological Observations of GABAergic Fibers in the SCG

There have been many morphological studies about GABAergic fibers in the SCG: Numerous GABA-immunopositive fibers were found in the SCG (Fig. 1a), and made axonal baskets [9, 13, 22–24]. The density of GABA-immunopositive baskets tended to be higher in the rostral part of the ganglion than the caudal part [22]. The majority of postganglionic neurons encircled by GABAergic fibers were NPY-negative [10, 23], and were not encircled by enkephalin-immunopositive axonal baskets [10]. Ultrastructually, GABA-immunoreactivity was observed in the nerve endings containing clear vesicles 40 nm in diameter [25]. Postembedding immunoelectron microscopy revealed immunoreactivity for GABA on small clear vesicles [25]. GABA-immunopositive terminals established asymmetric synapses [9, 25]. The majority of GABA-immunopositive terminals made asymmetric synapses upon dendritic or somatic spines [9]. Some GABA-immunopositive terminals made direct contact with dendritic shafts or soma of postganglionic neurons without synaptic specialization [9]. These results suggest that GABA is used for synaptic transmission to postganglionic neurons immunonegative for NPY. We recently reported that almost all terminals immunopositive for glutamic acid decarboxylase of 65 kDa (GAD65) and GAD of 67 kDa (GAD67), GABA-synthesizing enzymes, showed immunoreactivity for vesicular acetylcholine transporter (VAchT), a marker of cholinergic terminals [23, 24] (Fig. 1b, c).

1.2. A Hypothesis about the Origin of GABAergic Fibers in Previous Studies

To understand the role of GABA in the SCG, it is essential to clarify the origin of GABAergic fibers. Wolff and colleagues attempted to clarify this issue. They argued that, in the cervical sympathetic chain, which is composed of the SCG, STG, and cervical sympathetic trunk, SIF-like cells extended their axons and projected to the SCG [9, 14]. They showed that 1) GABA-immunopositive small cells are found in the cervical sympathetic chain [9, 14], 2) GABA-immunopositive fibers do not pass through the inner and outer carotid nerves, which contain postganglionic fibers and sensory fibers, but not preganglionic fibers [13], and 3) GABA-immunopositive fibers accumulate in the rostral part (the cervical sympathetic chain) of the sympathetic trunk [14]. They also argued that SPNs could not be the source of GABAergic fibers because a previous study [26] showed that GABA and

choline acetyl transferese, an acetylcholine synthesizing enzyme, did not co-localize in the spinal cord.

However, they did not provide direct evidence for the origin of GABAergic fibers: They did not show whether GABA-positive SIF-like cells had axons and innervate postganglionic neurons or not. Their data did not exclude that GABAergic preganglionic neurons send their axons to the SCG. Their discussion about the absence of GABAergic SPNs was not adequate because their argument was based upon the work of Kosaka and his colleagues [26], who did not investigate the thoracic spinal cord, or argue about SPNs. In fact, Kosaka and colleagues [26] used mid cervical spinal cord (around C5 level) which did not contain SPNs (Kosaka, personal communication). Therefore, it is possible that GABAergic fibers in the SCG originate from SPNs. Indeed, almost all GABA-containing terminals were immunopositive for VAchT (see above), and likely to be of spinal origin.

1.3. Three Candidates for the Source of GABA-containing Fibers

As discussed above, SIF-like cells and SPNs are candidates for the source of GABAergic fibers in the SCG. Although GABAergic enteric neurons innervate neurons in the inferior mesentric ganglion [3], collaterals of parasympathetic or enteric neurons are unlikely because neither the internal nor external carotid nerves contain GABA-immunopositive fibers [13]. Sensory neurons in the dorsal root ganglia (DRGs) may be the third candidate: there are some reports about the sensory collaterals in the SCG [1, 2, 27], and about GABA-containing sensory neurons [16,17,28]. In our recent studies [23,24], we performed a series of experiments to reveal the source of GABAergic fibers in the SCG. The results are summarized below.

2. Results

2.1. GABA-Immunopositive SIF-like Cells Did not Express GAD65 or 67

As previously reported [9, 14], GABA-immunopositive small cells were found in the SCG and STG in our study (Fig. 1d). These cells were fusiform or multipolar, and often in close proximity with GABA-immunopositive varicosities. These cells shared characteristics with the type 1 SIF-like cells described by Dobo and colleagues [9].

In contrast to the case of GABA-immunohistochemistry, no GAD67-immunopositive cell was found in the ganglia even when a sensitive immunohistochemical method (TSA method) was used. Furthermore, we performed in situ hybridization histochemistry for GAD65 and 67 mRNAs, and detected no signal in the SCG, STG, and cervical sympathetic trunk.

Interestingly, GABA-immunopositive SIF-like cells disappeared after the transection of ipsilateral ventral roots of C8 to T4 segments. These observations imply that SIF-like cells do not express GAD65 or 67, and some of them accumulate GABA released from neighboring GABAergic axons.

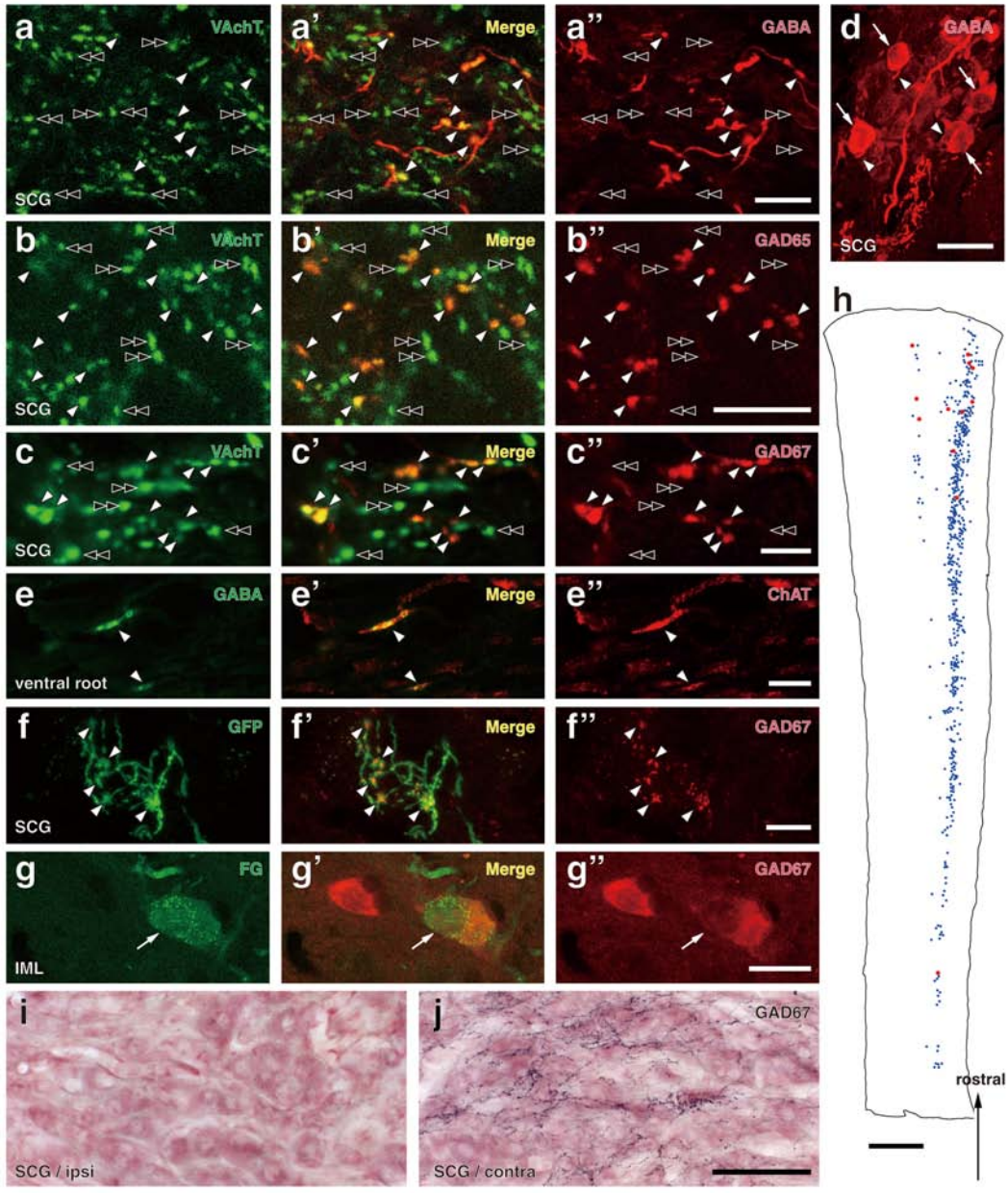

Figure 1. a: GABA-immunopositive varicosities (red) are immunopositive for vesicular acetylcholine transporter (VAchT; green) in the SCG. b: GAD65-immunopositive terminals (red) are immunopositive for VAchT (green). c: GAD67-immunopositive terminals (red) are immunopositive for VAchT (green). d: GABA-immunopositive SIF-like cells (arrows) often showed close proximity with GABA-immunopositive varicosities (arrowheads). e: Some choline acetyl transferase-immunopositive ventral root fibers (ChAT; red) showed immunoreactivity for GABA (green). The TSA method was used to enhance signals for GABA-immunoreactivity. f: After injection of Sindbis/palGFP virus into T1 IML, some labeled varicose fibers in the SCG (green) showed immunoreactivity for GAD67 (red). g: After injection of fluorogold (FG) in the SCG, some FG-positive neurons (green) showed signals for GAD67 mRNA (red). h: After injection of FG in the right SCG, FG-positive SPNs (blue dots) were distributed in C8-T5 segments of right IML. Some SPNs showed signals for GAD67 (red dots), and were located in the rostral portion of the IML. i, j: After transection of right C8-T4 ventral roots, almost all GAD67-immunopositive fibers disappeared in the ipsilateral SCG (i), while they remained unchanged in the contralateral ganglia (j). Bars: 5 μm in a, c, 10 μm in b, e, f, 20 μm in d, g, 100 μm in i, j, 1 mm in h.

2.2. GABA-containing Fibers Pass through the Ventral Roots, but not Dorsal Roots

Since there are some reports about GABAergic neurons in the DRGs [16,17,28], we then examined GABAergic properties in the ganglia. No cell body or fiber which was immunopositive for GABA or GAD67 was observed in the DRGs of C8 to T5 segments. Furthermore, no signal for GAD65 or 67 mRNA was detected in the ganglia. DRG neurons may express an undetectable amount of GAD.

GABA- and GAD67-immunopositive fibers were observed in the ventral roots of C8 to T5 segments, but not in the dorsal roots. All of these GABA-containing fibers showed immunoreactivity for choline acetyl transferase, a marker of cholinergic neurons (Fig. 1e). The numbers of GABA-containing fibers were larger in the ventral roots of C8 and T1 segments than those of T2-T5 segments. These results strongly suggest that GABA-containing motor neurons exists in the lower cervical and upper thoracic spinal cord.

2.3. GABAergic SPNs are Located in the Most Rostral Part of the Intermediolateral Cell Column

Since GAD65- and 67-immunopositive varicosities showed immunoreactivity for VAchT (Fig. 1b, c), some GABA-containing fibers in the ventral roots are likely to reach the SCG. We then injected Sindbis/palGFP virus [29], an excellent anterograde tracer, in the T1 intermediolateral nucleus (IML), and observed that some anterogradely labeled varicose fibers in the SCG were immunopositive for GAD67 (Fig. 1f). Furthermore, we examined whether retrogradely labeled SPNs express GAD67 mRNA or not. We injected fluorogold, a retrograde tracer, in the SCG. Three days later, animals were sacrificed, and fixed with 4% paraformaldehyde in 0.1M PB. Spinal cords of C8 to T5 segments were dissected, and processed for combined method of immunohistochemistry for fluorogold and in situ hybridization histochemistry for GAD67 mRNA. Signals for GAD67 mRNA were observed in the cell bodies of some retrogradely labeled SPNs (Fig. 1g). The morphology of these neurons was not distinguishable from that of GAD67-negative SPNs. About 3% of SPNs labeled with fluorogold expressed GAD67 mRNA. The majority of GAD67-expressing SPNs were located in the most rostral portion of the retrogradely labeled cell population (Fig. 1h). Since the SCG is supplied preganglionic fibers by the most rostrally located SPNs, we can say that the majority of GAD67-expressing SPNs are located in the most rostral part of the IML.

2.4. Almost All GABAergic Fibers in the SCG Originate from the Spinal Cord

The ratio of SPN fibers to all the GABA-containing fibers in the SCG may also be of interest. Since most of the SPNs projecting to the SCG are located in the spinal cord at the C8-T4 level [30], we transected the right ventral roots of C8 to T4 segments, and examined GABA and GAD67 immunoreactivity in the SCG and STG. Almost all GABA- and GAD67-immunoreactive fibers disappeared in the ipsilateral ganglia (Fig. 1i), while they were preserved on the contralateral side (Fig. 1j). This observation clearly indicated that almost all GABAergic fibers in the SCG originated in the spinal cord.

3. Discussion

3.1. Properties of GABAergic Preganglionic Neurons

As previously described, we performed combined method of immunohistochemistry for fluorogold and in situ hybridization histochemistry for GAD67 mRNA. Although the labeling with fluorogold was not able to visualize the entire cell morphology, primary dendrites, and sometimes higher branches, were clearly observed. We compared the morphology of GAD67-expressing SPNs and GAD67-negative SPNs, and found no obvious difference between them. However, it must be noted that there are not many intracellular labeling studies of SPNs (e.g. [31–33]). Therefore, we do not have enough data to argue about the morphological difference at the moment. To compare morphological properties between GABAergic and non-GABAergic SPNs, Golgi-like staining and quantitative analyses of the dendritic arbor of these neurons are needed.

GABAergic SPNs were mainly located in the rostral part of the IML. Interestingly, the rostral IML is known to have some unique properties: SPNs in this area receive much orexin [34] and much less serotonin than SPNs in more caudal segments [35]. In addition, SPNs immunopositive for neurokinin-1 receptor, which has high affinity with substance P, are fewer in T1 and T2 than in the more caudal segments [36]. These observations suggest that rostral SPNs are chemically coded. It is possible that the anatomical connection to this area is also unique: For example, (1) since orexin-containing neurons are located only in the lateral hypothalamus [37], direct innervation from the lateral hypothalamus may predominate in the rostral IML, (2) since substantial numbers of serotonin-containing fibers innervating SPNs originate from the medullary raphe [38], one of the sympathetic premotor nuclei, the rostral IML may have a weak association with this nucleus, and (3) since one of the sources of substance P in the IML is primary afferent fibers [39], sensory direct inputs are likely to be less important in the rostral IML than the caudal IML. Furthermore, these rostral SPNs innervate postganglionic neurons of the SCG in a unique manner as previously described [5,7]. These characteristics of rostral SPNs, which mainly project to the SCG, are likely to be associated with the functions of head- and face-specific organs (e.g. the pineal body, iris, salivary glands, cochlea, lacrimal gland, etc).

In a chemical aspect, there are some observations about the co-localization of GABA and other substances. It is known that about half of SPNs express nNOS. Interestingly, about half of GAD65-immunopositive terminals in the SCG showed neuronal nitric oxide synthase (nNOS) immunoreactivity [23]. If we assume that all SPNs make the same number of varicosities, the ratio of nNOS-positive GABAergic SPNs to all SPNs is likely not to be so different from that of nNOS-positive SPNs to all SPNs. Wolff's group suggested that GABA-containing terminals are not likely to contain Met-enkephalin because the ultrastructual morphology of these terminals was different [25]. Co-localization of vesicular glutamate transporters 1-3, markers of glutamatergic terminals, and GAD65 was not found in the mouse SCG [23]. There is some evidence that some SPNs utilize both glutamate and acetylcholine [23,40]. Thus, two major amino acid neurotransmitters in the central nervous system, glutamate and GABA, are also utilized in some SPNs, and each is used in different subpopulations of SPNs.

Dobo and colleagues [10] reported that GABA-immunopositive fibers mainly inner-

vated NPY-negative large postganglionic neurons. Since about half of all postganglionic neurons are NPY-positive vasoconstrictors [41], it can be postulated that half of all GAD-positive baskets would encircle NPY-positive postganglionic neurons if GABAergic fibers randomly choose the postganglionic target. However, only 10% of GAD-immunopositive baskets actually encircled NPY-positive postganglionic neurons [23], suggesting that GABAergic baskets do not prefer NPY-positive vasoconstrictor neurons. Because the majority of NPY-negative large postganglionic neurons are involved in the secretomotor pathway [41], and postganglionic neurons which are encircled by GABA-positive baskets exit the SCG through the internal carotid nerve [13], it may possible that postganglionic neurons encircled by GABAergic fibers regulate the activity of glands located in the cranium.

3.2. SPNs as GABAergic Projection Neurons

Our observations bring long-projecting GABAergic neurons to light. In a classical view, the majority of GABAergic neurons were regarded as interneurons like those in the neocortex. However, numerous studies reported GABAergic projection neurons such as Purkinje cells in the cerebellum, and neurons of strial-nigral pathway (for review, see [42]). These neurons extend their axons for several hundred micrometers to several millimeters. GABA-containing SPNs are interesting because their axons travel to the targets for several centimeters. It is interesting why "inhibitory" projection is needed in spite of the combination of excitatory projection and inhibitory interneurons. One can consider that omitting interneurons will save the number of neurons in a nucleus which receives projections (in this case, postganglionic neurons). Another implication is that these GABAergic projection neurons do not work as simple inhibitory neurons. The latter is more likely in this case because GABAergic SPNs contain not only GABA, but also acetylcholine, which works as an excitatory transmitter in autonomic ganglia. The function of these fibers will be discussed later.

3.3. The Role of Co-transmission of GABA and Acetylcholine

Our observations imply the co-release of excitatory (i.e. acetylcholine with nicotinic receptor) and "inhibitory" (i.e. GABA with $GABA_A$ receptor) neurotransmitters from some preganglionic fibers in the SCG because both acetylcholine receptors and $GABA_A$ receptors [8, 12] are expressed in the SCG.

Since the resting membrane potential is $-45 \sim -90$ mV [43] and the reversal potential for chloride ion is $-40 \sim -50$ mV in the SCG neurons [44], GABA depolarizes postganglionic neurons [18]. On the other hand, because the threshold for action potential production is 12.2 mV in the SCG neurons [43], activation of $GABA_A$ receptors inhibits action potential production in postganglionic neurons [18]. It seems strange that fibers from a single SPN co-release neurotransmitters which have opposite effects on action potential production.

Nevertheless, there are some reports about the co-release of neurotransmitters which have opposite effects via ionotropic receptors. In the neocortex, a subpopulation of GABAergic interneurons expresses vesicular glutamate transporter 3, a marker for glutamatergic neurons [45]. In the auditory brainstem of newborn rats, neurons which release

glutamate, GABA, and glycine are reported [46]. In this report, both GABA and glycine depolarize postsynaptic neurons, and play an important role in the maturation of neural circuits. It is also known that glutamatergic granule cells in the dentate gyrus of kindled animals release GABA [47]. In this case, GABA may weaken the hyperexcitation of neuronal circuits. Since we do not have enough data about the behavior of postsynaptic currents from postganglionic neurons innervated by GABAergic fibers, to record postsynaptic currents from these neurons will reveal the function of the co-release of GABA and acetylcholine.

It has also been reported that GABA strongly inhibits long-term potentiation via $GABA_A$ receptors in the rat SCG [11]. As noted above, GABAergic preganglionic fibers preferentially innervate NPY-negative secretomotor neurons, and associate with only a small number of NPY-positive postganglionic neurons. Therefore, postganglionic neurons innervated by GABAergic fibers, which may be associated with secretomotor functions, may be inhibited from producing long-term potentiation.

There is another view about the co-release of acetylcholine and GABA in the SCG: Acetylcholine works as an excitatory neurotransmitter via nicotinic receptors, while GABA works as a neurotrophic factor via $GABA_A$ and $GABA_B$ receptors. Wolff and colleagues published many papers about the plastic effect of GABA; long-term administration of GABA elicits the generation of dendritic spines and synapse-like structures [20], long-term administration of a $GABA_B$ agonist increases coated vesicles in dendrites but has no effect on the formation of vacant postsynaptic densities and spines [21]. Even now, it is not known whether this process really works *in vivo* or not. As noted above, GABAergic fibers preferentially encircle NPY-negative postganglionic neurons. Therefore, we can postulate that NPY-negative postganglionic neurons have more dendritic protrusions and synapses. However, no obvious difference was observed in the number of primary dendrites between NPY-positive and negative postganglionic neurons [48]. Furthermore, dendritic geometry is not dependent on ganglion cell activity or the presence of presynaptic innervation, but affected by the target size [49]. These data imply that preganglionic GABAergic innervation has no effect upon the dendritic morphology at least *in vivo*. Because these studies did not examine ultrastructual morphology, there remains a possibility that GABAergic innervation contributes to the fine morphological change of dendrites. To clarify this issue, a quantitative electron microscopic study is needed.

3.4. Future Directions

As discussed above, we can estimate role(s) of GABAergic fibers in the SCG, however, there is no direct evidence of targets which receive sympathetic postganglionic innervation related to the preganglionic GABAergic input, or that the LTP blockade phenomenon caused by stimulating GABAergic fibers exists. There also arises the question of whether the chloride equilibrium potential for cells surrounded by GABAergic terminal baskets differs from that for the other postganglionic neurons or not. The identification of postganglionic neurons richly innervated by GABAergic fibers, and morphological, electrophysiological, and pharmacological studies of these neurons will answer these questions.

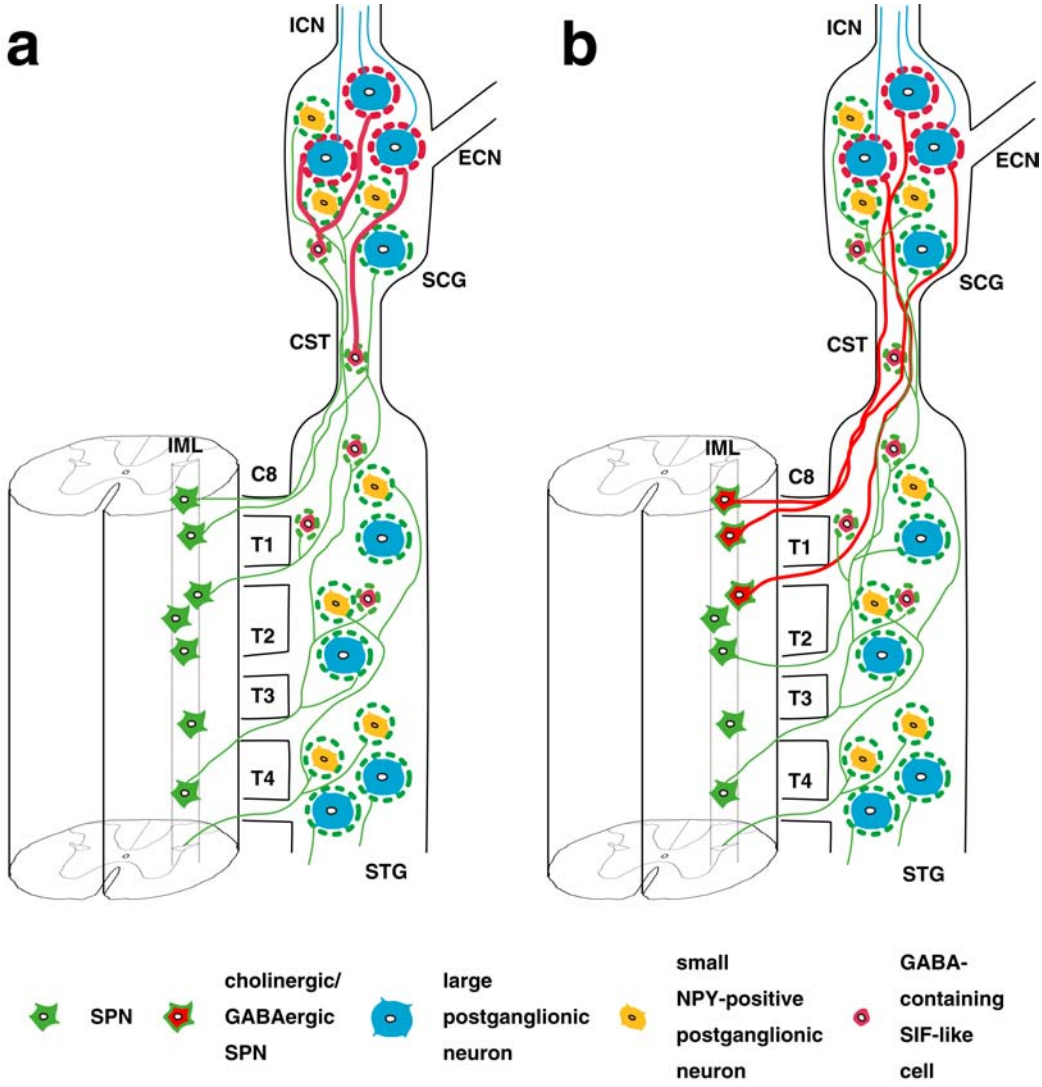

Figure 2. Schematic diagrams of GABAergic innervation in the rostral paravertebral ganglia. a: In the previous scheme ("feed-forward inhibition hypothesis"), proposed by Wolff et al [14], GABA-containing SIF-like cells in the k SCG, cervical sympathetic chain (CST), and STG innervate large NPY-negative postganglionic neurons lying rostral portion of the SCG. b: Observations in our studies [23, 24] clearly indicate that GABAergic SPNs in the most rostral part of the IML send their axons to the SCG via the ventral roots, STG, and CST, and innervate large NPY-negative postganglionic neurons.

4. Conclusion

Almost all GABAergic fibers in the SCG originated from the most rostral part of the IML, but not from SIF-like cells in the cervical sympathetic trunk, or primary sensory neurons in the DRGs. Therefore, the "feed-forward inhibition hypothesis" (Fig. 2a) proposed by a previous study [14] is discarded. These fibers are likely to innervate postganglionic neurons concerning secretory functions (Fig. 2b). GABA elicits depolarization but inhibits action

potential in these neurons. GABA also inhibits long-term potentiation and induces synapse formation.

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Chapter 11

SYMPATHETIC NEUROVASCULAR INTERACTIONS AND VASCULAR SYMPATHETIC INNERVATION

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Abstract

The sympathetic nervous system is an important modulator of cardiovascular function that contributes to the development and maintenance of cardiovascular disease. The effects of the sympathetic nervous system are mediated via the release of neurotransmitters and neuropeptides from nerve terminals innervating blood vessels and the heart. The mechanisms underlying the development and/or maintenance of cardiovascular sympathetic innervation are not well understood. This article will consider how neurovascular interactions affect vascular sympathetic innervation. In vivo and in vitro models for studying neurovascular interactions will be described. The effects of vascular cells (smooth muscle and endothelial cells) on sympathetic axon growth, axon guidance, target recognition, synapse formation, neurotransmitter/neuropeptide expression and neurotransmitter release will be considered. The mechanisms underlying these effects will also be discussed. Specifically, the role of diffusible mediators (nerve growth factor, artemin, vascular endothelial growth factor) and contact-dependent mediators (ephrins, semaphorins, integrins) will be considered. Results obtained from in vivo and in vitro models will be presented. Future directions and clinical implications will be discussed.

Introduction

The sympathetic nervous system is an important determinant of cardiovascular function that has been implicated in the development and/or maintenance of cardiovascular as well as other diseases [7,8,11,19,26,27,31,32,33,36,37,38,40,57,58,59,60,62]. The effects of the sympathetic nervous system are mediated via the release of neuropeptides and neurotransmitters from postganglionic sympathetic nerve terminals innervating target organs.

Alterations in cardiovascular sympathetic innervation are associated with disease. Increased vascular sympathetic innervation has been observed in several models of hypertension [19,38]. This increased innervation is thought to contribute to the development and maintenance of hypertension. Cardiac sympathetic innervation is lost following myocardial infarction [34] and cardiac transplants [4,5]. Reinnervation is limited, but when it occurs it improves cardiac function [4]. In diabetes, heterogeneous degeneration of distal portions of sympathetic axons and nerve terminals has been reported [58]. Vascular sympathetic innervation is unaffected in diabetes [33,62], but cardiac sympathetic innervation [58] and reinnervation of transplanted hearts [3] are decreased in diabetes. Loss of cardiac sympathetic innervation may contribute to sudden unexplained cardiac death in diabetic patients [58]. Loss of cardiac sympathetic innervation and accompanying orthostatic hypotension is observed in Parkinson's disease [31].

The function of cardiovascular sympathetic innervation is also altered in disease. Sympathetic activity is increased in hypertensive animals [36,40] and humans [32]. Sympathetic activity is also increased in obese animals [8] and humans [26,60]. Hart et al [33] reported that the content, uptake and release of norepinephrine were altered at sympathetic neurovascular junctions of diabetic rats. Speirs et al. [62] reported that the postsynaptic signaling was altered at sympathetic neurovascular junctions of diabetic rats. Sympathetic activity is also increased in heart failure [59].

This chapter will consider how vascular cells affect the growth and function of the postganglionic sympathetic neurons that innervate them. The effects of vascular cells (smooth muscle and endothelial cells) on growth, development and maintenance of sympathetic innervation (axon growth/axon guidance, target recognition/synapse formation) and function of sympathetic innervation (neurotransmitter/neuropeptide expression and neurotransmitter release) will be considered. Potential mechanisms underlying these effects will be discussed.

Sympathetic Neurovascular Interactions and the Growth, Development and Maintenance of Vascular Sympathetic Innervation

The growth, development and maintenance of vascular sympathetic innervation is a complex process. During de novo innervation during development or during reinnervation, sympathetic axons must grow towards blood vessels, recognize the vessels as their targets when they reach it, and then form functional synapses. Alterations in existing sympathetic innervation would involve axon growth or regression, and synapse formation or regression. Todd reported that innervated but not non-innervated blood vessels were reinnervated in an anterior eye chamber transplant model [65]. This study suggests that vascular cells from innervated arteries promoted the regrowth of sympathetic innervation, whereas vascular cells from the non-innervated arteries did not. Todd did not investigate how the innervated artery promoted the growth of sympathetic innervation. It is likely that diffusible factors would stimulate and guide the growth of axons to blood vessels. Once the axons reached the vessel, it is likely that contact-dependent mechanisms would be activated that would mediate target recognition and

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synapse formation. Several lines of evidence suggest that vascular cells affect multiple steps in these processes.

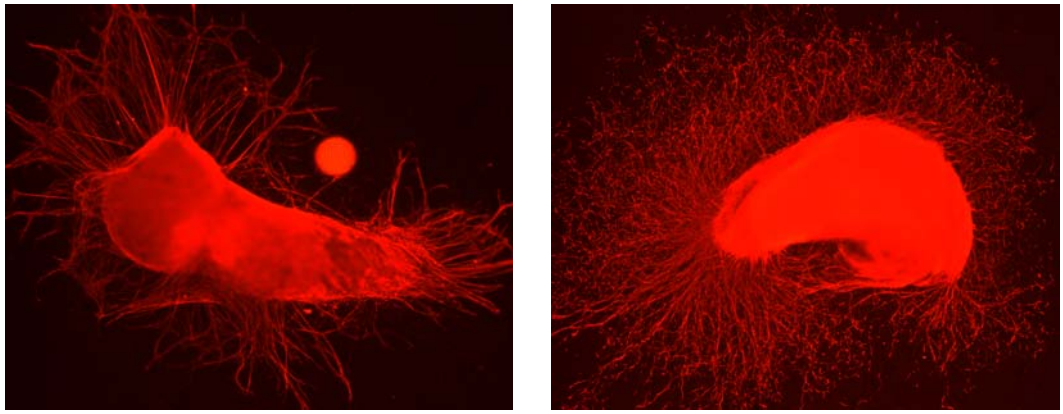

Figure 1. Vascular smooth muscle cells secrete factors that stimulate sympathetic axon growth. Explants of sympathetic superior cervical ganglia were grown for 72 hours in transwell cultures in the absence of NGF and in the absence (-VSM) and presence (+ VSM) of carotid artery vascular smooth muscle. Ganglia were fixed with formaldehyde and labeled with rabbit GAP43 primary antibody and corresponding fluorescent secondary antibody (donkey anti-rabbit AlexaFluor 555). In transwell cultures the neurons and vascular cells are 1 mm apart.

Figure 1 dramatically illustrates that cultured vascular smooth muscle (VSM) produce diffusible factors that stimulate the growth of sympathetic axons. In this experiment, explants of sympathetic ganglia containing postganglionic sympathetic neurons were grown in transwell cultures for 72 hours in the absence and presence of VSM from carotid arteries of adult rats. Growth in the presence of VSM (+ VSM) was greater than that in the absence of VSM (- VSM). In transwell cultures, the VSM are physically separated from the neurons by a distance of approximately 1 mm and thus the observed effect must be due to a soluble mediator. Organ culture studies also suggest VSM produce soluble factors that stimulate the growth of sympathetic axons. Femoral arteries grown in cultures with explants of sympathetic ganglia stimulated axon growth [15]. In these studies, as in the transwell studies, the neurons and arteries were separated by approximately 1 mm, indicating that the observed effect was mediated by a secreted factor.

VSM secrete several factors that are likely to modulate the growth of vascular sympathetic axons. Like all sympathetic targets, VSM produce nerve growth factor (NGF) [10]. NGF is required for the survival of postganglionic sympathetic neurons, but it is also required for appropriate sympathetic innervation of many targets [30]. NGF stimulates sympathetic axon growth in vitro [42] suggesting that VSM-derived NGF is likely to stimulate axon growth during sympathetic innervation or reinnervation of blood vessels. Studies of Glebova and Ginty [30], however, indicate that factors other than NGF are also involved in the growth of vascular sympathetic innervation.

Artemin is a diffusible factor produced by VSM that is likely to modulate vascular sympathetic axon growth. Artemin is a member of the glial-derived neurotrophic factor

family. Baloh et al. [2] reported that artemin was expressed in arteries of embryonic mice. Artemin-deficient mice have been reported to have abnormal sympathetic neuron migration and proximal axon growth [49]. These studies suggest that vascular-derived artemin plays a role in the early stages of the development of sympathetic ganglia. We have shown that artemin is produced by VSM at sympathetic neurovascular junctions in neonatal and adult animals [15], suggesting that vascular-derived artemin plays a role in the development and maintenance of vascular sympathetic innervation. We and others have shown that artemin stimulates sympathetic axon growth in vitro [15,68] and we have shown that inhibition of artemin reduces vascular stimulation of sympathetic axon growth in vitro [15]. Artemin's role in the growth of vascular sympathetic innervation in vivo remains to be established.

Vascular endothelial growth factor (VEGF) is another soluble factor produced by VSM that is likely to modulate sympathetic axon growth. We and others have shown that VSM produce secreted forms of VEGF [9,44]. We and others have found that VEGF promotes the growth of sympathetic axons in vitro [13,61]. Additional studies have demonstrated that VEGF may modulate sympathetic axon growth via two mechanisms. VEGF binds to neuropilin-1, which is also the binding receptor for semaphorin 3A [sema 3A; 48]. Sema 3A inhibits the growth of sympathetic axons towards a target by collapsing growth cones [13]. VEGF and sema 3A compete for binding to neuropilin-1 [48] and VEGF modulates sympathetic axon growth by inhibiting sema 3A [44]. Studies have shown that VEGF also has direct effects on sympathetic axon growth [44,61]. We have been studying the role of VEGF in the reinnervation of denervated femoral arteries and have found that inhibition of VEGF inhibits femoral artery reinnervation (unpublished observation). Thus, in vitro and in vivo data support a role for vascular-derived VEGF in promoting sympathetic axon growth and vascular sympathetic innervation.

It is likely that other secreted vascular-derived factors promote the growth of sympathetic axons. VSM produce neurotrophin 3 [21] and hepatocyte growth factor [46,52], which stimulate the growth of sympathetic axons [43,55,64]. The role of these vascular-derived factors in promoting growth of vascular sympathetic innervation has not been investigated.

There is also evidence that vascular cells produce factors that inhibit sympathetic axon growth. Recent studies indicate that vascular endothelial cells (EC) express sema 3A that inhibits sympathetic axon growth [13]. These studies also suggest that it is a balance between axon growth stimulators and inhibitors that will determine whether a blood vessel is innervated or not. Sema 3A's role in the growth of vascular sympathetic innervation in vivo remains to be established.

Factors secreted by vascular cells would direct the growth of developing or regenerating sympathetic axons to a blood vessel. Figure 2 and 3 suggest that once the sympathetic axons reach the vessel, contact between VSM and postganglionic sympathetic neurons markedly affects the axons. In figure 2, explants of sympathetic ganglia (stained red for GAP43) were grown for 72 hours in the presence of 50 ng/ml NGF and in the absence (-VSM) and presence (+ VSM) of VSM (stained green for smooth muscle α actin) that made direct contact with the neurons. In the absence of VSM, axons extended uniformly and radially from the ganglia and the axons did not appear to bundle or fasciculate. In the presence of VSM, axons exhibited a "nodal" pattern of growth. The axons appeared to bundle and grow into and out of focal points or "nodes". This pattern was observed in 67% of the cultures grown in the presence of VSM. This effect was not observed (0%) in parallel transwell cultures in which the cells did

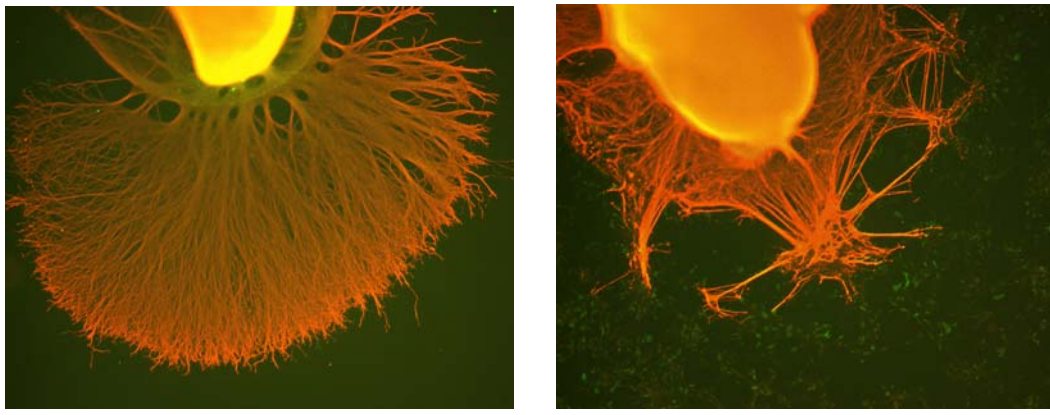

Figure 2. Contact between vascular smooth muscle cells and postganglionic sympathetic neurons alters axon growth. Explants of sympathetic superior cervical ganglia were grown for 72 hours in the presence of 50 ng/ml NGF and in the absence (- VSM) and presence (+ VSM) of carotid artery vascular smooth muscle cells. Ganglia and vascular smooth muscle cells were fixed with formaldehyde and labeled with rabbit GAP43 and mouse smooth muscle α actin primary antibodies and corresponding fluorescent secondary antibodies (donkey anti-rabbit AlexaFluor 555 and donkey anti-mouse AlexaFluor 488). In these cultures the neurons and smooth muscle cells were in contact or close proximity.

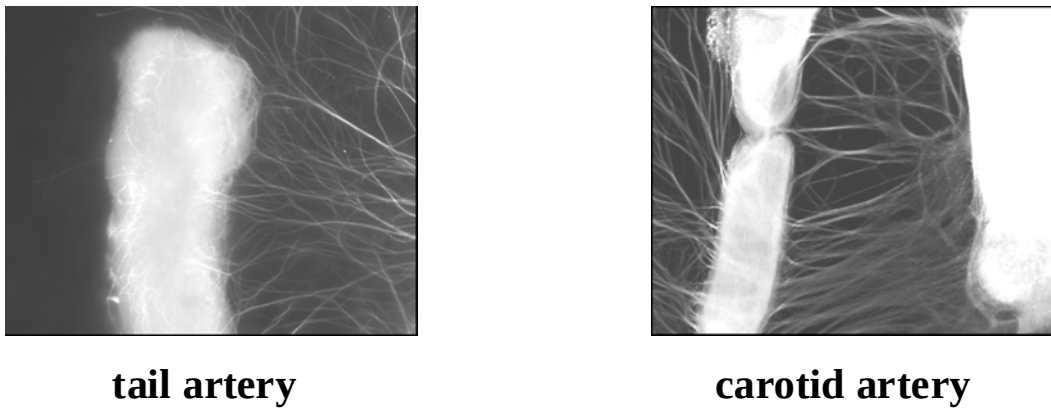

Figure 3. Vascular smooth muscle and sympathetic target recognition Explants of sympathetic superior cervical ganglia were grown for 7 days in the presence of 50 ng/ml NGF and in the presence of rat tail or carotid arteries. Ganglia and arteries were then fixed with formaldehyde and labeled with rabbit GAP43 primary antibody and corresponding fluorescent secondary antibody (donkey anti-rabbit AlexaFluor 555).

not make contact. In the representative experiment shown in figure 3, sympathetic ganglia were placed adjacent to but not touching adult tail or neonatal carotid arteries (neonatal carotid arteries, rather than adult carotid arteries, were used as they are closer in size to adult tail arteries). The ganglia/artery cultures were then grown for seven days in the presence of 50 ng/ml NGF. In the ganglia/tail artery culture the axons grew to the artery, but did not grow

past the artery. In the ganglia carotid artery culture, the ganglia grew to the artery and then continued to grow past the artery. These data indicate that contact between VSM and sympathetic axons provides a signal that alters the pattern of axon growth. It is not yet clear if the VSM provide a preferred substrate for the axons, if the axons recognize the VSM as a target, or if the VSM promote the formation of functional synapses. These questions are currently under investigation.

Ephrins are a family of membrane-bound proteins that, with their membrane-bound tyrosine kinase receptors, ephs, mediate contact-dependent cell-to-cell interactions [47,53]. These interactions have been implicated in the development of the nervous system as well as the vascular system [1,28,29,51]. In the nervous system ephrin/eph interactions guide axons, promote axon bundle formation, promote survival and axon outgrowth [29,51]. The role of eph/ephrin interactions at sympathetic neurovascular junctions has not been fully elucidated, but studies indicate that ephs and ephrins are expressed by postganglionic sympathetic neurons [29,62], ephs and ephrins are expressed by VSM [62], and that ephrins modulate the growth of sympathetic axons [29]. Eph/ephrin interactions and their role in the development of vascular sympathetic innervation are under investigation.

Integrins and their ligands are known to mediate many cell-cell and cell-matrix interactions. Several lines of evidence support a role for integrins as determinants of sympathetic innervation. The studies of Wingerd et al. [67] indicate that vascular cell adhesion molecule-1 (VCAM-1) binding to $\alpha 4/\beta 1$ integrin is required for the development of sympathetic innervation to the heart. These studies showed that VCAM-1 promotes axon growth and that VCAM-1 was expressed in VSM of coronary blood vessels. Thrombospondin-1 (TSP-1) binding to $\alpha 3\beta 1$ integrin has also been shown to promote neurite outgrowth from postganglionic sympathetic neurons [70]. Vascular cells produce both VCAM-1 [25,35,67] and TSP-1 [56]. When sympathetic axons approach blood vessels, neuronal integrins could interact with VCAM-1 on VSM and/or with TSP-1 on VSM or in matrix surrounding the blood vessel. These interactions could affect axon growth on the blood vessel by providing a preferred substrate for axon growth and/or a target recognition signal. These interactions may also influence synapse formation [20]. Future studies will provide a greater understanding of how integrins and integrin ligands at sympathetic neurovascular junctions affect vascular sympathetic innervation.

Sympathetic Neurovascular Interactions and the Function of Vascular Sympathetic Innervation

The primary function of postganglionic sympathetic neurons is to synthesize and release neurotransmitters and neuropeptides onto target organs in response to the preganglionic neurotransmitter, acetylcholine. Acetylcholine activates receptors on postganglionic neurons, resulting in depolarization and ultimately generation of action potentials that are conducted from somas in ganglia to nerve terminals in target organs. Depolarization of the nerve terminal results in increased intracellular calcium and neurotransmitter release. The function of postganglionic sympathetic neurons is thus determined by many factors including the expression and function of acetylcholine receptors, the expression and function of ion channels, and the expression, release, and transport of neurotransmitters and neuropeptides.

There is evidence that sympathetic neurovascular interactions affect these determinants of postganglionic sympathetic neuronal function.

The activity of postganglionic sympathetic neurons is determined primarily by input from preganglionic sympathetic neurons. Preganglionic neurons release acetylcholine, which activates nicotinic and muscarinic cholinergic receptors on postganglionic neurons. Studies in chickens and mammals suggest that sympathetic neurovascular interactions modulate the function of postganglionic sympathetic neurons by modulating cholinergic receptors. Devay et al. [17,18] clearly showed that sympathetic targets modulate nicotinic receptors in postganglionic sympathetic neurons. These investigators reported that the function and expression of nicotinic acetylcholine receptors in postganglionic sympathetic neurons innervating kidney were markedly different from those of neurons innervating cardiac cells [18]. Several groups of investigators reported that severing the connections between postganglionic sympathetic neurons and their targets altered nicotinic receptor expression in the neurons [16,69,71]. Yeh et al. [69] demonstrated that NGF partially inhibited this effect, suggesting that target-derived NGF was a determinant of cholinergic receptor expression. How sympathetic neurovascular interactions affect acetylcholine receptors in the sympathetic neurons, is an unexplored area that warrants investigation.

Acetylcholine released by preganglionic sympathetic neurons activates postganglionic sympathetic neurons by depolarizing these neurons. This signal is transduced, modulated and conducted to nerve terminals in target organs by ion channels. Many studies indicate that sympathetic neurovascular interactions are likely to affect the expression and function of ion channels in postganglionic sympathetic neurons that innervate blood vessels. In chicks, target tissues modulate potassium [22,23] and calcium currents [24]. In cultures of mammalian postganglionic sympathetic neurons, target tissues [50,66] modulate calcium channels, and NGF [41] and transforming growth factor β (TGF-β) [54], both of which are produced by VSM, modulate potassium channels .

The primary vasoactive neurotransmitter released by sympathetic neurons innervating the vasculature is norepinephrine. The primary vasoactive neuropeptide released by these neurons is neuropeptide Y. VSM have been reported to modulate the expression of tyrosine hydroxylase (TH), the rate limiting enzyme for the synthesis of norepinephrine, and NPY [14]. This effect has been shown to be mediated in part by VSM-derived leukemia inhibitory factor and NT-3. Other factors produced by vascular cells are also likely to modulate TH and/or NPY in postganglionic sympathetic neurons. NGF modulates NPY and TH [42]. TGF β is expressed by VSM and postganglionic neurons, is activated in sympathetic neurovascular cocultures [12], and modulates NPY in cultures of postganglionic sympathetic neurons [55]. The physiological or pathological significance of VSM modulation of sympathetic neurotransmitter/neuropeptide expression remains to be established.

The effective concentration of neurotransmitter/neuropeptide at sympathetic neurovascular junctions is determined by the release and subsequent reuptake of neurotransmitter/neuropeptide. In vitro studies indicate that sympathetic targets modulate neurotransmitter release [39,66]. Wakade et al. [66] reported that cardiac cells reduced norepinephrine release from postganglionic sympathetic neurons. Preliminary studies from my laboratory support the observations of Wakade et al. [66]. We found that VSM reduced agonist-induced norepinephrine release from postganglionic sympathetic neurons (data not shown). In vitro studies also suggest that VSM modulate reuptake of norepinephrine. NGF

and NT-3 [6], both of which are produced by VSM, have been reported to decrease the expression of the norepinephrine transporter gene. We have preliminary evidence that sympathetic neurovascular interactions modulate the function of the norepinephrine transporter. We found that uptake of tritiated norepinephrine in the presence of VSM ($1.95 \pm 0.26 \times 10^5$ cpm) was less than that in the absence of VSM ($2.91 + 0.17 \times 10^5$ cpm; $n = 9$; $p < 0.05$; unpaired t-test assuming unequal variances) in sympathetic neurovascular cultures. We have not determined the mechanism underlying this effect. These in vitro studies suggest an important role for sympathetic neurovascular interactions as determinants of sympathetic activity. Further studies are required to understand the in vivo significance of and mechanisms underlying the observed effects.

Conclusion

The studies described in this chapter provide compelling evidence that sympathetic neurovascular interactions modulate the growth, development and function of vascular sympathetic innervation. These studies are far from complete, however, and many unanswered questions remain. Vascular sympathetic innervation is an important determinant of cardiovascular function and altered vascular sympathetic innervation is implicated in many diseases [7,8,11,19,26,27,31,32,33,36,37,38,40,57,58,59,60,62]. Further studies are required to fully characterize the physiological role of and mechanisms underlying sympathetic neurovascular modulation of vascular sympathetic innervation. Further studies must also determine the role of sympathetic neurovascular modulation of vascular sympathetic innervation in the development and maintenance of disease.

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Chapter 12

REVERSE NA+/CA2+-EXCHANGE INDUCED TRANSMITTER RELEASE FROM NA+-LOADED PERIPHERAL SYMPATHETIC NERVES AND ITS REGULATION BY PRE-SYNAPTIC α2-RECEPTORS*

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Abstract

[^{3}H]noradrenaline ([^{3}H]NA) release (field stimulation parameters: 2Hz, 1 ms, 60V for 3 min) was measured from the isolated main pulmonary artery of the rabbit in the presence of uptake blockers (cocaine, $3x10^{-5}$M and corticosterone, $5x10^{-5}$M) and after blocking MAO with pargyline ($1.2x10^{-4}$M).

This release of [^{3}H]NA was abolished by TTX (10^{-7}M), even if the charge carrier through the fast Na^+-channel was mainly Li^+ (113 mM; $[Na^+]_o$: 25 mM). The release was also fully inhibited by block of the voltage-sensitive Ca^{2+}-channels (VSCCs) with combined application of the selective and irreversible 'N-type' VSCC-blocker ω-conotoxin-(ω-CgTx) GVIA (10^{-8}M) and the 'non-selective' VSCC-blocker neomycin ($3x10^{-3}$M). Correlation was obtained between the extent of VSCC inhibition and the NA-release potentiating effect of the preferential pre-synaptic α_2-receptor blocker yohimbine ($3x10^{-7}$M). When the release of NA was fully blocked (ω-CgTx GVIA + neomycin), yohimbine was ineffective. Under these conditions, i.e. in the absence of functioning VSCCs, Na^+-loading (Na^+-pump inhibition by 'K^+-free' perfusion; 45 min) was required to elicit NA-release again in response to nerve-stimulation. $0K^+_o$-solution also increased the spontaneous outflow of [^{3}H]NA. The nerve-evoked release of labeled NA in 'K^+-free' solution was abolished by TTX and by removal of Ca^{2+} from the external medium (+ 1mM EGTA). The release of NA was also significantly inhibited by Li^+-substitution of Na^+, or when the preferential reverse Na^+/Ca^{2+}-exchange blocker KB-R7943 ($3x10^{-5}$M) was applied. KB-R7943 also decreased the resting outflow of

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NA. In Na^+-pump inhibited nerves, and in the absence of functioning VSCCs, yohimbine further enhanced the nerve-evoked release of NA, while agonists of α_2-receptors (l-NA or clonidine, 10^{-6}M) inhibited it. The yohimbine-induced enhancement of neurotransmitter release was blocked by TTX and by $Ca^{2+}{}_o$-removal (+ 1mM EGTA). Similarly, Li^+-substitution or KB-R7943-application caused significant inhibition. In $0K^+{}_o$-solution, the fast Na^+-channel activator veratridine (10^{-5}M) further enhanced both the resting and the nerve-evoked release of NA. The veratridine-induced potentiation of neurotransmitter release was blocked by Ca^{2+}-free, 1 mM EGTA-containing solution and significantly inhibited by KB-R7943-application. This latter was dependent on the pre-perfusion period with KB-R7943-containing solution, being greater if longer periods were used.

It is concluded, that physiological stimuli may reverse Na^+/Ca^{2+}-exchange when the VSCCs are blocked and when the nerves are Na^+-loaded. Pre-synaptic α_2-receptors may regulate reverse Na^+/Ca^{2+}-exchange as well as VSCCs.

Key words: peripheral sympathetic nerves, [^{3}H]NA-release, Na^+-pump, Na^+/Ca^{2+}-exchange, voltage-sensitive Ca^{2+}-channels (VSCCs), pre-synaptic α_2-receptors, "negative feed-back auto-inhibition"

1. Introduction

Internal free Ca^{2+} ($[Ca^{2+}]_i$) plays a fundamental role in 'excitation-secretion coupling' of nerve terminals (Llinás et al., 1981, 1992; Augustine & Charlton, 1986; Augustine et al., 1987; Almers, 1990; Randall & Tsien, 1995; Dunlap et al., 1995; Stanley, 1997; Bennett, 1999). During the past years evidence has been accumulated that transmitter-release at low-frequencies of stimulation is triggered by Ca^{2+}-influx primarily through voltage-sensitive Ca^{2+}-channels (VSCCs) of the 'N-type' (Augustine et al., 1987; Hirning et al., 1988; Tsien et al., 1988; Lipscombe et al., 1989; Hille, 1994; Wright & Angus, 1996; Brain & Bennett, 1997; Smith & Cunnane, 1997). Other subtypes of VSCCs ('P/Q-', 'R-') are also involved in mediating a part of transmitter-release, however the importance of these channels is somewhat less (for review see: Waterman, 2000). Another possible pathway for Ca^{2+}-entry is through Na^+/Ca^{2+}-exchange working in reverse mode (Baker & Blaustein, 1968; Baker et al., 1969; Blaustein et al., 1991; Reuter & Seitz, 1968; for reviews see: Blaustein & Lederer, 1999; Philipson & Nicoll, 2000). This mechanism has gained much attention during the past years following experiments in cardiac muscle suggesting that without functioning VSCCs, the influx of Na^+ through tetrodotoxin-(TTX)-sensitive Na^+-channels can activate Ca^{2+}-entry through the exchanger (outward-current & hyperpolarisation), and result in subsequent Ca^{2+}-release from internal stores (Leblanc & Hume, 1990; Lipp & Niggli, 1994; Levi et al., 1994; Levi & Issberner, 1996; Wasserstrom & Vites, 1996; Lipp et al., 2002; but see: Sham et al., 1992, 1995; Bouchard et al., 1993a,b; Sipido et al., 1995; Cannell et al., 1995). The Na^+/Ca^{2+}-exchanger is expressed at high concentration in neurones (Yip et al., 1992; Furman et al., 1993; Juhaszova et al., 1996), and shows mainly pre-synaptic localisation (Luther et al., 1992; Reuter & Porzig, 1995; Fontana et al., 1995; Juhaszova et al., 1996, 2000; Blaustein et al., 2002; c.f. Blaustein & Lederer, 1999). This suggests that it may participate in the Ca^{2+}-dependent regulation of neurotransmitter release. However, properties of the exchanger is not ideal for this. In contrast to the plasma membrane Ca^{2+}-pump, the exchanger localises some distance from the vesicle docking sites (Juhaszova et al., 2000; Blaustein et al., 2002). The action potential (AP) duration in nerve terminal is less than a millisecond (Llinás et al., 1992),

thus much shorter than in cardiac muscle. The turnover rate of a single Na^+/Ca^{2+}-exchanger is also much less (~$5x10^3$ Ca^{2+} $(s)^{-1}$; Hilgemann et al., 1991; Hilgemann, 1996; Blaustein & Lederer, 1999) than that of a single open Ca^{2+}-channel (~10^7 $(s)^{-1}$; Hille, 1992), and, due to the electrogenic character of the exchanger ($3Na^+/1Ca^{2+}$), when the Ca^{2+}-entry mode is activated, the membrane is hyperpolarized, whereas presumably, the VSCCs are activated primarily on depolarisation (c.f. Blaustein & Lederer, 1999). Further, when the internal concentration of Ca^{2+} is elevated, as a result of VSCC(s) activation, the exchanger may will switch back to the Ca^{2+}-efflux mode (normal mode operation of Na^+/Ca^{2+}-exchanger (inward current & depolarisation; c.f. Philipson & Nicoll, 2000). Nevertheless Gleason et al. (1994, 1995) have reported in cultured retinal amacrine cells that after closure of VSCCs, prolonged depolarisation produces a second rise in $[Ca^{2+}]_i$ and transmitter release. The elevation in $[Ca^{2+}]_i$ was accompanied by an outward current, possibly mediated by reverse Na^+/Ca^{2+}-exchange.

In this chapter I describe a series of experiments carried out in our laboratory, which confirms the importance of reverse Na^+/Ca^{2+}-exchange in the evoked release of neurotransmitter. Namely, we measured labelled noradrenaline release from the sympathetic nerves of the isolated main pulmonary artery of the rabbit in response to physiological depolarising stimuli (2 Hz, 1 ms) and in the presence of uptake-blockers. Brief depolarising pulses could elicit transmitter release after pharmacological blockade of VSCCs but only if the nerves were Na^+-loaded (by Na^+-pump inhibition). The release of neurotransmitter is probably mainly due to reverse Na^+/Ca^{2+}-exchange activation since it was blocked either by TTX, or by $Ca^{2+}{}_o$-removal (+ 1mM EGTA). Similarly, the release of neurotransmitter was significantly inhibited when the charge carrier through the fast Na^+-channel was mainly Li^+ or when the preferential reverse Na^+/Ca^{2+}-exchange blocker KB-R7943 was applied. The rate of transmitter release (both the resting and the nerve-evoked) was directly correlated with the elevated level of Na^+ inside, since it was further enhanced when the fast Na^+-channels were additionally activated by veratridine. The veratridine-induced enhancement of NA-release was significantly inhibited by KB-R7943 and blocked by $Ca^{2+}{}_o$-removal. This portion of transmitter release (reverse exchanger mediated) was regulated by pre-synaptic α_2-receptors since it was further enhanced by yohimbine and inhibited either by l-noradrenaline or clonidine. TTX, Ca^{2+}-removal, Li^+-substitution or KB-R7943 application significantly reduced the yohimbine-induced potentiation of neurotransmitter release.

2. Methods

2.1. Rabbit Main Pulmonary Artery

The experiments have been carried out in the isolated main pulmonary artery of the rabbit (Starke et al., 1974; Török et al., 1992). Male albino rabbits (2-3 kg) were killed by cervical dislocation and exsanguinations through the carotid artery. The main pulmonary artery (weight: 40-80 mg) was dissected and placed into normal Krebs' solution which contained the monoamine oxidase inhibitor pargyline ($1.2x10^{-4}$M) and which was fully equilibrated with carbogene (5% CO_2 in O_2; pH: 7.4). The artery was opened longitudinally, fixed by two threads in such a way that the circular muscles were running between the threads, and placed into a loading bath (volume, 1.5 ml) for 30 min at 37 ^oC. The composition of normal Krebs'

solution was (mM): Na^+, 138.0; K^+, 5.9; Ca^{2+}, 2.5; Mg^{2+}, 1.2; Cl^-, 122.7; HCO_3^-, 25.0; $H_2PO_4^-$, 1.2; SO_4^{2-}, 1.2; glucose, 11.1. In 'K^+-free' solution, KCl (4.7 mM) and KH_2PO_4 (1.2 mM) were omitted with no substitution for KCl but substitution for KH_2PO_4 with equimolar concentration of NaH_2PO_4. Low Na^+-solution contained 25.0 or 26.2 mM Na^+ (in K^+-containing or 'K^+-free' solution), with NaCl being replaced by LiCl (113.0 mM). In Ca^{2+}-deficient, or Ca^{2+}-free solution $CaCl_2$ (2.5 mM) was reduced or omitted without ionic substitution. When Ca^{2+}-free solution was used, 1 mM EGTA was added. In this case the Ca^{2+}-gradient is probably reversed, since external Ca^{2+} goes down to about 10^{-9}M, thus Ca^{2+}-influx cannot occur either through VSCCs or reverse Na^+/Ca^{2+}-exchange.

2.2. Measurement of [^{3}H]noradrenaline Release

The method had been described previously (Borowski et al., 1977; Török et al., 1992). Briefly, after the artery had been re-warmed in Krebs' solution, 25 μl [^{3}H]noradrenaline ([^{3}H]NA; specific activity, 12.0 Ci $(mmol)^{-1}$) was added to the loading bath (final concentration of [^{3}H]NA, $1.39x10^{-6}$M) for 45 min (pargyline and the NA-stabilising agents ascorbic acid, $3x10^{-4}$M and disodium-edetate (Na_2EDTA), $3x10^{-5}$M were present). Subsequently, the artery was suspended in an organ bath (capacity, 2 ml) and perfused at a rate of 8 ml $(min)^{-1}$ with 800 ml medium, containing the neuronal uptake blocker cocaine ($3x10^{-5}$M) instead of pargyline. At the end of the washing period, the flow rate was reduced to 4 ml $(min)^{-1}$ and the extra neuronal uptake blocker corticosterone ($5x10^{-5}$M) was also added to the Krebs' solution for 30 min. In order to induce neurotransmitter release, field stimulation (2 Hz, 1 ms, 60 V) was used for 3 min (360 pulses) by means of two platinum electrodes fixed vertically on opposite sides of the tissue at the top and the bottom of the organ bath. The distance between the tops of the electrodes was 20 mm. Tetrodotoxin (TTX, 10^{-7}M) abolished the field stimulation-induced [^{3}H]NA-release and the post-synaptic contractile response indicating the nervous origin of liberated NA. Previously it was shown that in the presence of uptake blockers 86% of nerve-evoked released radioactivity is unmetabolised NA (Endo et al., 1977). Since pargyline was present at the beginning of our experiments, the spontaneous outflow of NA is probably unmetabolised too. On the basis of this assumption and knowing the specific activity of [^{3}H]NA, the release of labelled NA was calculated in pmoles $(min)^{-1}$ according to the method of Endo et al. (1977). The stimulation-induced [^{3}H]NA-release was calculated by subtraction of the resting outflow, determined immediately before stimulation, from the release obtained and up to 6 min after stimulation. Two control stimulation periods (S_1, S_2) were given and then modified Krebs' solution was used or drugs were added 3, 6 or 18 min before the third stimulation period (S_3) as required (see individual experiments). In most of the experiments ω-CgTx GVIA was pre-perfused 18 min before S_3, and was present during stimulation. Subsequently the toxin was washed out and a stimulation period was given (S_4). Neomycin was added to the perfusion solution 18 min before the fifth stimulation period (S_5) and was present during S_5 and the subsequent stimulation periods (in most of the experiments from S_6 to S_{10}. Reduction of external Ca^{2+} from 2.5 to 1.25, 0.75 or 0.25 mM was carried out 18 min before the stimulation periods (S_5, S_7 and S_9). Meantime (S_6 and S_8) and afterwards (S_{10}) normal Ca^{2+} was readmitted to the external medium. External K^+ (5.9 mM) was removed 18 min before S_6, and was readmitted 6 min before S_{10} (120 min 'K^+-free' perfusion). Li^+ (113.0 mM) was added to the Krebs'

solution 3 min before stimulation (S_3 or S_7), since longer periods increased the resting outflow of [^{3}H]NA. KB-R7943 was pre-perfused 6 or 18 min before stimulation (S_3 or S_7). Ca^{2+}-omission (+ 1 mM EGTA)- or TTX-, yohimbine-, l-noradrenaline (l-NA)-, clonidine-, ryanodine- and veratridine-addition to 'K^+-free' solution were carried out 18 min before S_7. The effects of drugs or ionic modifications were expressed as the ratio between the release of [^{3}H]NA (in pmol) evoked by stimulation-3, -5, -7 and the overflow evoked by stimulation-2 ($S_{3,\ 5,\ 7}/S_2$). In control experiments the ratio between S_3 and S_2 was 0.98±0.01 (n=24). The perfusate was collected in 3 or 6 min samples. At the end of the experiments the preparations were dissolved in 1 ml Soluene-350 (Packard) and the radioactivity of perfusate samples and tissues were determined using a liquid scintillation counter (Beckman, LS-5000 TA). The total initial [^{3}H]NA content of the arteries was 486.5±35.2 pmol (means±S.E.M. of 97 tissues; weight: 52.8±1.9 mg).

2.3. Drugs and Statistics

The following drugs were used: l-[7,8 ^{3}H]noradrenaline (specific activity, 12.0 Ci (mmol)$^{-1}$; Radiochemical Centre, Amersham, UK); pargyline-hydrochloride (Serva); cocaine-hydrochloride (Merck); corticosterone (Fluka); ascorbic acid (EGA); disodium-ethylenediaminetetraacetate (Na_2EDTA, Aldrich-Europe); tetrodotoxin (TTX, Calbiochem); ω-conotoxin-(ω-CgTx)-GVIA (Sigma, Alamone Labs Ltd., Jerusalem); KB-R7943 (2-[2-[4-(4-nitrobenzyloxy)phenyl]ethyl]isothiourea-methanesulphonate, Nippon Organon K.K., Osaka, Japan); ryanodine (Calbiochem); ethylene-glycol-bis-β-aminoethyl-ether)*N,N'*-tetraacetic-acid (EGTA, Sigma); yohimbine-hydrochloride (Serva); l-noradrenaline-hydrochloride (l-NA, Fluka); clonidine (Sigma); veratridine (Calbiochem); ryanodine (Calbiochem). The drugs were dissolved in Krebs' solution. Corticosterone was dissolved in propylene glycol (final concentration, 0.05%, v/v). KB-R7943 was dissolved in dimethyl-sulphoxide (DMSO, final concentration, 0.3%, v/v). Veratridine was dissolved in abs. ethanol or DMSO (final concentration, 0.1%, v/v). At the concentrations used, these solvents, separately or together did not affect neurotransmitter release. All of the chemicals used for preparing Krebs' solution were of analytical grade.

Means±S.E.M. are given. Data were compared using Student's paired or unpaired *t*-test and a one-way analysis of variance. Differences between means were considered significant if $P < 0.05$.

3. Results

3.1. The Effects of External Li^+ and KB-R7943 on [^{3}H]noradrenaline Release in the Presence of Functioning VSCCs

Two series of experiments were carried out in order to see (i), whether membrane depolarisation resulting from Na^+ substitution with Li^+ opens VSCCs in pulmonary artery sympathetic nerves as it does like in other tissues and (ii), how transmitter release is affected by the non-selective reverse Na^+/Ca^{2+}-exchange inhibitor KB-R7943.

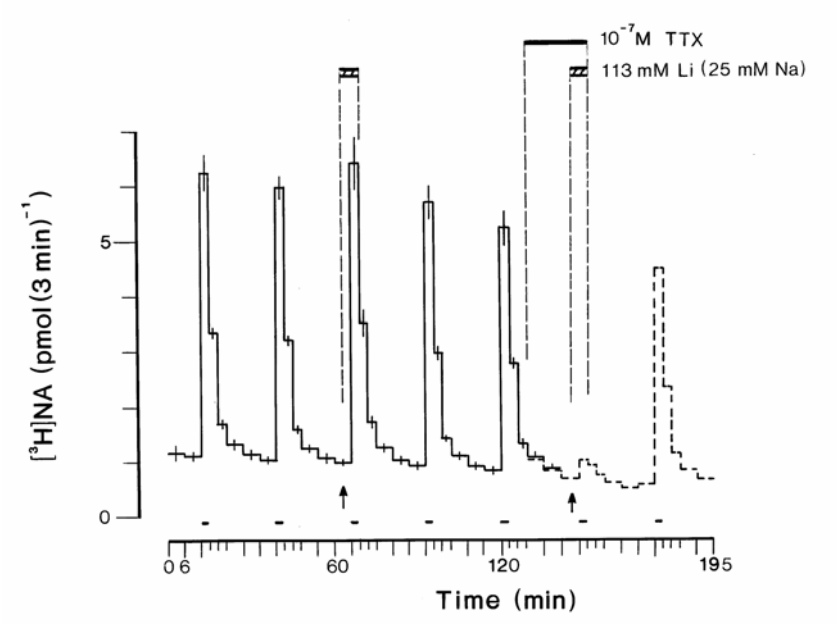


Figure 1. Low frequency-(2 Hz)-nerve stimulation-induced [^{3}H]noradrenaline-([^{3}H]NA)-release from the isolated main pulmonary artery of the rabbit in the presence of neuronal (cocaine, $3x10^{-5}$M) and extra neuronal (corticosterone, $5x10^{-5}$M) uptake blockers and the effect of external Li^+ (113 mM; $[Na^+]_o$: 25 mM) and tetrodotoxin (TTX, 10^{-7}M). Ordinate: [^{3}H]NA release in pmol (3 min)$^{-1}$; abscissa: time (min). Horizontal bars above the axis represent the stimulation periods (3 min in 27 min intervals). Dashed lines indicate the presence of Li^+ and TTX. Li^+ was pre-perfused 3 min before the third and sixth stimulation periods (S_3, S_6; arrows). TTX was applied 18 min before S_6. After two control stimulation periods (S_1, S_2), Li^+ (113 mM), did not significantly affect the stimulation-induced [^{3}H]NA-release. Subsequently normal Na^+ (138 mM) was readmitted and two stimulation periods were performed. Means ± S.E.M. of four identical experiments are shown. Note, that one of the experiments was continued (broken line), and TTX was added to Krebs solution 18 min before stimulation. TTX abolished the nerve-evoked release of [^{3}H]NA in Li^+-containing solution.

The nerve-stimulation induced [^{3}H]NA-release was slightly, but not significantly increased when most of the external Na^+ (113 mM) was substituted by Li^+ ($[Na^+]_o$: 25 mM; Fig. 1). The ratio of nerve-evoked release increased from 0.98±0.03 to 1.13±0.06 (n=4; P>0.05). In these experiments a short (3 min) pre-perfusion period was used with Li^+-containing solution, since longer periods increased the resting outflow of neurotransmitter (not shown). This latter is probably due to inhibition of the normal mode of operation of Na^+/Ca^{2+}-exchange (Reuter & Seitz, 1968; Baker & Blaustein, 1968; Baker et al., 1969) and/or inhibition of the delayed rectifier K^+-current (Kato et al., 1991), which is mainly responsible for AP-repolarisation of sympatehetic nerves (Boehm & Kubista, 2002). Fig. 1 also shows that the Li^+-evoked [^{3}H]NA-release was abolished when the fast Na^+-channels were blocked by TTX (10^{-7}M). It is known that Li^+ can carry the inward current through fast, TTX-sensitive Na^+-channels (Hodgkin & Keynes, 1957; Hille, 1992; Lipp & Niggli, 1994), thereby can depolarise the membrane, and the depolarisation opens VSCCs. The influx of Ca^{2+} may cause exocytotic release of NA.

We have previously shown that the preferential, but non-selective reverse Na^+/Ca^{2+}-exchange inhibitor KB-R7943 (Watano et al., 1996; Iwamoto et al., 1996), at a high concentration ($3x10^{-5}$M), increased the resting outflow of [^{3}H]NA immediately after its application (Török et al., 2004). Using a brief, 6 min pre-perfusion period, the spontaneous outflow of [^{3}H]NA increased from 0.45±0.06 to 0.59±0.07 pmol(3 min)$^{-1}$ (n=7). When longer pre-perfusion period was used (18 min), the resting outflow of labelled NA increased markedly (from 0.82±0.10 to 4.09±0.78 pmol(3 min)$^{-1}$ (n=4). This suggests that KB-R7943 can also release Ca^{2+} from internal stores, as it has been shown to do in cardiac and smooth muscles' (Iwamoto et al., 1995). However, KB-R7943, even in this high concentration (6 min pre-perfusion), failed to affect the peak release of [^{3}H]NA in response to field-stimulation (not shown; Török et al., 2004), suggesting, that the drug does not substantially inhibit the fast Na^+-channel in the sympathetic nerves of pulmonary arteries. However, KB-R7943 also inhibits certain K^+-channels (Matsuda et al., 2001), including the delayed rectifier (Tanaka et al., 2002). In pulmonary arteries KB-R7943 delayed the recovery of NA-release after stimulation (Török et al., 2004). Several effects may play a role in this phenomena, including inhibition of normal mode of operation of Na^+/Ca^{2+}-exchange (Iwamoto et al., 1996), Ca^{2+}-release from internal stores (Iwamoto et al., 1995), or inhibition of delayed rectifier K^+-channels (Tanaka et al., 2002). Although the drug also inhibits the 'L-type' VSCC (Watano et al., 1996), this channel is probably not involved in transmitter release of peripheral sympathetic nerves (Bennett, 1999).

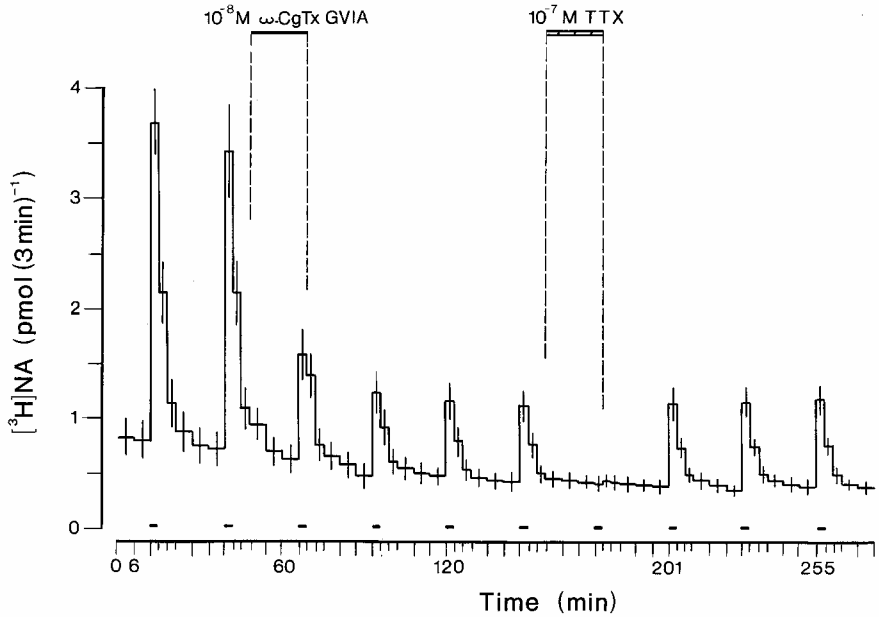
3.2. Abolition of [^{3}H]noradrenaline Release by Voltage-Sensitive Ca^{2+}-Channel-Blockers and the Lack of Transmitter-Releasing Effect of the Preferential α_2-Receptor Blocker Yohimbine

The 'N-type' voltage-sensitive Ca^{2+}-channel, first described in dorsal root ganglion neurones (Nowycky et al., 1985), plays a dominant role in triggering transmitter release in a number of pre-synaptic nerve terminals (Augustine et al., 1987; Hille, 1994). The 'N-type' Ca^{2+}-channel can be inhibited selectively and irreversibly by ω-CgTx GVIA (Kerr &Yoshikami, 1984; Perney et al., 1986; McCleskey et al., 1987; Tsien et al., 1988, 1995; Hirning et al., 1988; Gray & Olivera, 1988; Wagner et al., 1988; Smith & Augustine, 1988; Lipscombe et al., 1989; Stanley & Goping, 1991; Bertolino & Llinás, 1992; Williams et al., 1992; Olivera et al., 1994; Ellinor et al., 1994; Meir et al., 1999).

In pulmonary arteries a low concentration of ω-CgTx GVIA (10^{-8}M) significantly and irreversibly inhibited the nerve-evoked release of [^{3}H]NA (~60-70%; Fig. 2a,b). In 12 identical experiments the ratio of stimulation-induced release of [^{3}H]NA decreased from 0.96±0.03 (S_2/S_1) to 0.39±0.01 (S_3/S_2; %-inhibition: 59.4). Subsequently the toxin was washed out, however the release of [^{3}H]NA decreased further during the next stimulation period (ratio (S_4/S_2): 0.26±0.02; %-inhibition: 72.9). The 'residual release' of [^{3}H]NA after ω-CgTx GVIA treatment was reversibly blocked by TTX (10^{-7}M; Fig. 2a). The 'residual release' of [^{3}H]NA also proved to be external Ca^{2+}-dependent (Fig. 2b), and was fully inhibited by reducing $[Ca^{2+}]_o$ from 2.5 to 0.25 mM. These results suggest that depolarisation-induced Ca^{2+}-influx is required for 'residual release' to be observed. When a much higher

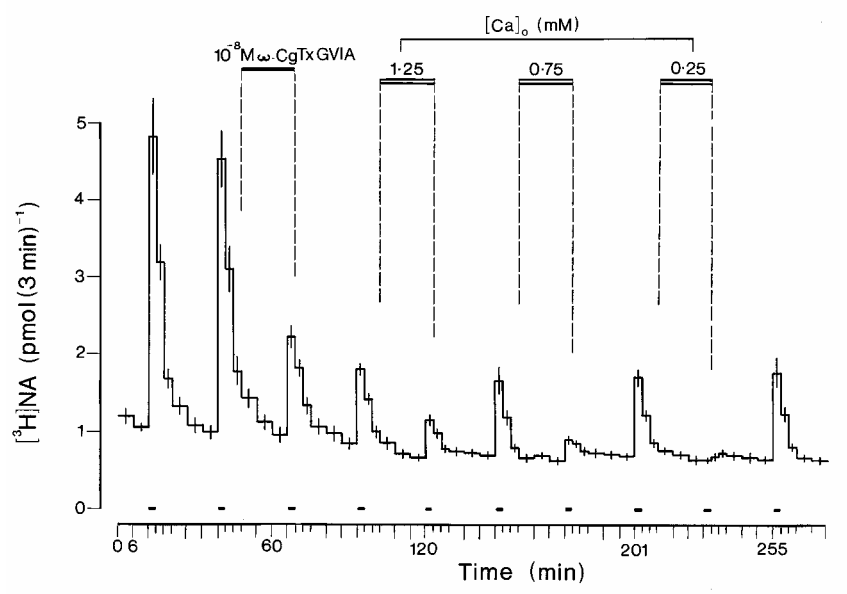
concentration of ω-CgTx GVIA was used (10^{-6}M; 60 min incubation period), the release of [^{3}H]NA was inhibited further (~80%; not shown), but still some 'residual release' remained, suggesting that other subtype(s) of VSCC(s) are probably activated. The 'P-/Q-type' VSCC-blocker ω-agatoxin IVA ($3x10^{-7}$M; 6 min pre-perfusion) was ineffective in inhibiting this 'residual release' of NA (not shown). It is possible that the 'R-type' VSCC is responsible for 'residual release' observed, however the effect of an 'R-type' selective Ca^{2+}-channel blocker such as SNX-482 has not been checked yet in pulmonary arteries.

In peripheral tissues and central neurones it has been shown that the amino-glycoside antibiotic neomycin, which has ototoxic effects in patients, inhibits 'N-type', 'L-type' and 'non-N-type' and 'non-L-type' high-voltage activated-(HVA)-Ca^{2+}-channels (Keith et al., 1992, 1993; Lampe et al., 1993). In pulmonary arteries neomycin concentration-dependently inhibited the stimulation-induced release of [^{3}H]NA (Fig. 3). $3x10^{-3}$M neomycin exerted nearly 75% inhibition of transmitter release. This concentration of neomycin fully inhibited the evoked-release of [^{3}H]NA after pre-treatment with a low concentration of ω-CgTx GVIA (10^{-8}M) (Figs. 3 and 4; see also Figs. 5-12). Desensitisation did not develop to the blocking effect of neomycin, i.e. the transmitter release did not increase when neomycin was present in the perfusion solution for four successive stimulation periods (not shown).



(A) After two control stimulation periods (S_1, S_2), ω-CgTx GVIA, pre-perfused 18 min before the third stimulation period (S_3), significantly inhibited the nerve-evoked release of [^{3}H]NA (ratio (S_3/S_2): 0.35±0.03; P<0.025). After washing, the release of [^{3}H]NA further decreased (compare S_4 with S_3). Note, that TTX, pre-perfused 18 min before S_7, reversibly abolished the 'residual release' of [^{3}H]NA (ratio: -0.03±0.02; *P*<0.0005). Further note, that the effect of ω-CgTx GVIA was irreversible. Means±S.E.M. of four identical experiments.

Figure 2. Continued on next page.



(B) ω-CgTx GVIA significantly and irreversibly inhibited the release of [^{3}H]NA on stimulation (ratio (S_3/S_2): 0.41±0.08; P<0.0125. Subsequently, $[Ca^{2+}]_o$ was reduced from 2.5 to 1.25(S_5), 0.75(S_7) and 0.25(S_9) mM respectively, as indicated. Between and after these stimulation periods normal Ca^{2+} was readmitted (S_6, S_8 and S_{10}). Note, that the 'residual release' of NA was reduced as external Ca^{2+} was decreased (ratios: 1.25 mM Ca^{2+} (S_5/S_2), 0.14±0.02; 0.75 mM Ca^{2+} (S_7/S_2), 0.11±0.02 respectively). 0.25 mM Ca^{2+} produced full inhibition (ratio (S_9/S_2): 0.01±0.01; *P*<0.0005). Further note, that on normal Ca^{2+}-readmission, the release of [^{3}H]NA returned to the preceding low levels. Means±S.E.M. of three identical experiments.

Figure 2. The effects of ω-conotoxin-(ω-CgTx) GVIA (10^{-8}M), TTX (10^{-7}M) and external Ca^{2+}-reduction on stimulation-induced [^{3}H]NA release from arteries in the presence of uptake blockers. Ordinate: [^{3}H]NA release (pmol (3 min)$^{-1}$); abscissa: time (min). Horizontal bars above the axis represent the stimulation periods. Dashed lines indicate the presence of ω-CgTx GVIA, TTX and reduction of $[Ca^{2+}]_o$.

It is well known that NA released from pre-synaptic nerve terminals inhibits its own further release by activation of pre-synaptic α_2-adrenoceptors, known as a "negative feed-back" mechanism (Langer, 1977, 1981, 1997; Langer & Vogt, 1971; Langer et al., 1977; Starke, 1977, 1981, 1987, 2001; Vizi, 1979). Centrally acting α_2-receptor agonists are widely used in hypertension. The "negative feed back" mechanism is mediated through a G-protein mediated transduction mechanism (Miller, 1990; Hille, 1994; Zhang et al., 1996; Herlitze et al., 1996; Shekter et al., 1997; Qin et al., 1997; Dolphin, 1998; Delmas et al., 1998; Owerholt & Prabhakar, 1999), and mainly due to inhibition of VSCCs (Horn & McAfee, 1980; Hirning et al., 1988; Lipscombe et al., 1989; Xu & Adams, 1993; Toth & Miller, 1995; Boehm & Huck, 1996; Brain & Bennett, 1997). Brain & Bennett (1997) succeeded in measuring Ca^{2+}-concentration changes ($\Delta[Ca^{2+}]_v$) in the sympathetic varicosities of mouse vas deferens preparations in response to short trains (5-impulse) at 5 Hz. Yohimbine (10^{-5}M), a preferential blocker of α_2-receptors, increased the amplitude of $\Delta[Ca^{2+}]_v$ (~54%), while clonidine (10^{-6}M, α_2-receptor agonist) decreased it (~55%).

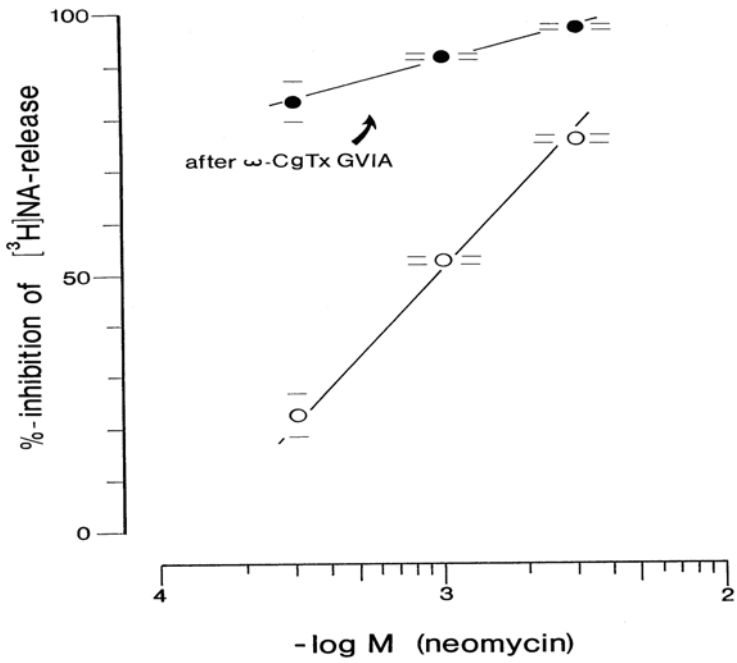


Figure 3. Concentration-response relationship of neomycin-induced inhibition of [^{3}H]NA release with (•-•) and without (o-o) ω-CgTx GVIA (10^{-8}M) pre-treatment. Ordinate: %-inhibition of [^{3}H]NA release; abscissa: -log M concentration of neomycin. Note, that in both cases, neomycin concentration-dependently inhibited the evoked-release of [^{3}H]NA, and the inhibition was more pronounced in ω-CgTx GVIA pre-treated arteries. Further note, that after ω-CgTx GVIA pre-treatment, $3x10^{-3}$M neomycin caused full inhibition. Both curves represent the means±S.E.M. of three identical experiments.

In rabbit pulmonary arteries, correlation was obtained between the extent of VSCC-inhibition and the [^{3}H]NA-release potentiating effect of yohimbine (Table 1). Yohimbine ($3x10^{-7}$M), in normal solution, i.e. with intact VSCCs, markedly potentiated the release of [^{3}H]NA on stimulation. The nerve-evoked release ratio of [^{3}H]NA increased from 0.97±0.01 (n=6) to 4.11±0.57 (n=4). When the nerve-evoked release of labelled NA was partly inhibited by a low concentration of ω-CgTx GVIA (10^{-8}M), yohimbine was still effective, but its potentiating effect on NA-release was much less than in normal solution (ratio: 0.77±0.05, n=3; ω-CgTx GVIA alone: 0.28±0.02 (ratio), n=4). And finally, when the release of NA was fully blocked by a combined administration of ω-CgTx GVIA plus neomycin ($3x10^{-3}$M), yohimbine was ineffective in increasing the release of NA on stimulation (Fig. 4, Table 1). In Fig. 4 it also can be seen that neomycin transiently increased the spontaneous outflow of [^{3}H]NA from arteries (see also Figs. 6b; 7a,b; 9; 10a,b; 12a,b,c). In GABA-ergic neurones, Martinez-Martos et al. (1997) have reported that neomycin activates fast, TTX-sensitive Na^+-channels, which may explain this phenomenon.

Table 1. The effects of yohimbine ($3x10^{-7}$M), l-noradrenaline (l-NA, 10^{-6}M), ω-conotoxin-(ω-CgTx)-GVIA (10^{-8}M) and neomycin ($3x10^{-3}$M) on nerve-evoked release of [^{3}H]noradrenaline

Treatment	Ratio of nerve-evoked* release of [^{3}H]NA	Significance (*P*)
1. control (S_3/S_2)	0.97 ± 0.01 (6)	
2. $3x10^{-7}$M yohimbine	4.11 ± 0.57 (4)	2/1 *P*<0.0005
3. 10^{-6}M l –NA	0.25 ± 0.03 (6)	3/1 *P*<0.0001
4. yohimbine + l-NA	0.67 ± 0.20 (5)	4/1 *P*<0.0025 4/2 *P*<0.0025 4/3 *P*<0.05
5. 10^{-8}M ω-CgTx GVIA	0.28 ± 0.02 (4)	5/1 *P*<0.0001
6. ω-CgTx GVIA + yohimbine	0.77 ± 0.05 (3)	6/1 *P*<0.0025 6/2 *P*<0.005 6/5 *P*<0.0005
7. ω-CgTx GVIA + l-NA	0.04 ± 0.02 (3)	7/1 *P*<0.0001 7/3 *P*<0.005 7/5 *P*<0.0005
8. ω-CgTx GVIA + yohimbine + l-NA	0.11 ± 0.01 (3)	8/1 *P*<0.0001 8/4 *P*<0.05 8/5 *P*<0.001 8/6 *P*<0.0005 8/7 *P*<0.05
9. ω-CgTx GVIA + $3x10^{-3}$M neomycin	0.05 ± 0.01 (3)	9/1 *P*<0.0001 9/5 *P*<0.0005
10. ω-CgTx GVIA + neomycin + yohimbine	0.07 ± 0.01 (3)	10/1 *P*<0.0001 10/2 *P*<0.0025 10/5 *P*<0.0005 10/6 *P*<0.0005 10/9 *P*>0.15

*Stimulation parameters: 2Hz, 1ms, 60V for 3 min (360 pulses).
Ratio of nerve-stimulation-evoked release was calculated as described by Endo et al. (1977).
Treatments No. 1-5: S_3/S_2; 6-10: S_7/S_2
Drugs were added to Krebs solution 18 min before stimulation
ω-CgTx GVIA was pre-perfused 18 min before S_3 and was present during stimulation (3').
Neomycin was applied 18 min before S_5 and was present during S_5, S_6, S_7 and S_8.
Number of experiments in parentheses

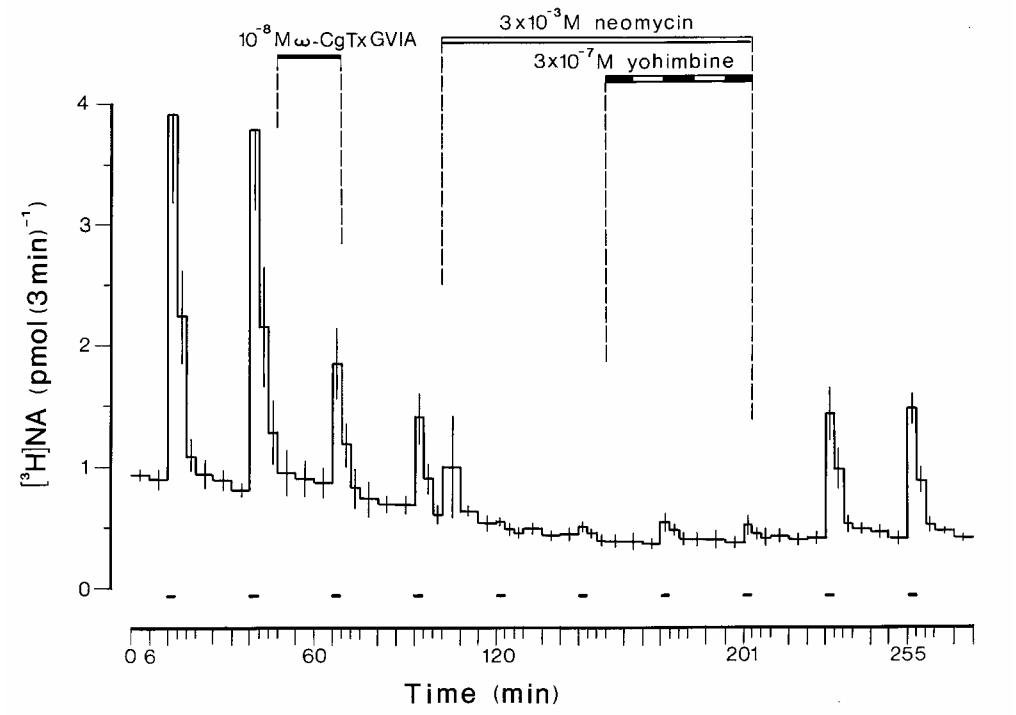


Figure 4. The ineffectiveness of yohimbine ($3x10^{-7}$M) in releasing [^{3}H]NA in ω-CgTx GVIA (10^{-8}M) pre-treated nerves in the presence of neomycin ($3x10^{-3}$M). Ordinate: [^{3}H]NA-release (pmol (3 min)$^{-1}$); abscissa: time (min). ω-CgTx GVIA, applied after two control stimulation periods significantly inhibited the nerve-evoked release of [^{3}H]NA (ratio (S_3/S_2): 0.27±0.03). Note, that the evoked release of [^{3}H]NA further decreased on ω-CgTx GVIA removal (compare S_4 with S_3). Subsequently, neomycin was perfused for four successive stimulation periods. Neomycin abolished the nerve-evoked release of [^{3}H]NA (ratios between S_5/S_2 and S_6/S_2: -0.03±0.003 and 0.01±0.005 respectively). Neomycin transiently increased the resting outflow of [^{3}H]NA. Yohimbine, pre-perfused 18 min before S_7 failed to induce NA-release. Means±S.E.M. of three identical experiments.

3.3. [^{3}H]noradrenaline Release Without Functioning VSCCs: Activation of Reverse Na^+/Ca^{2+}-Exchange in Na^+-Pump Inhibited Nerves and the Effects of TTX, Ca^{2+}_o-Removal, Li^+-Substitution or KB-R7943 Application

In order to investigate whether or not [^{3}H]NA-release can be elicited by fast Na^+-current in the absence of functioning VSCCs (e.g. in the presence of neomycin and after ω-CgTx GVIA treatment), the intracellular Na^+ concentration in sympathetic nerves of pulmonary arteries was increased by inhibiting the Na^+-pump. It is known that the Na^+-pump, similar to that of the exchanger, is an electrogenic and voltage-sensitive ion transport mechanism (stoichiometry: $3Na^+/2K^+$; Baker, 1966; Thomas, 1972; Akera & Brody, 1978; Glitsch, 1982, 2001; De Weer & Rakowski, 1984; Gadsby, 1984; Gadsby et al., 1985; De Weer et al., 1988; Rakowski et al., 1989, 1997; Gadsby & Nakao, 1989; Török, 1989; Blaustein, 1993; Glynn, 1993, 2002; Clausen, 2003). The two proteins, e.g. the pump and the exchanger, are co-

localised (Moore et al., 1993) and possibly functionally interact. It is interesting to note, that the primary ion transporter is the Na^+-pump and the secondary is the Na^+/Ca^{2+}-exchanger. If the pump is down regulated, the exchanger is up regulated, and the opposite, when the pump is up-regulated the exchanger is down regulated (Magyar et al., 1995; McDonough et al., 2002).

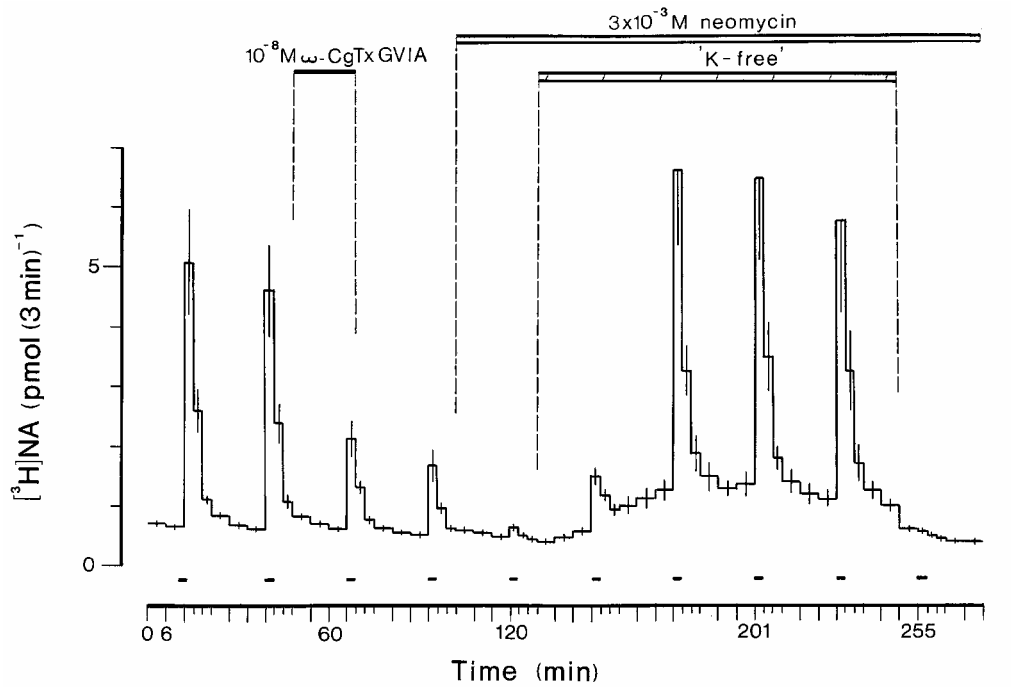
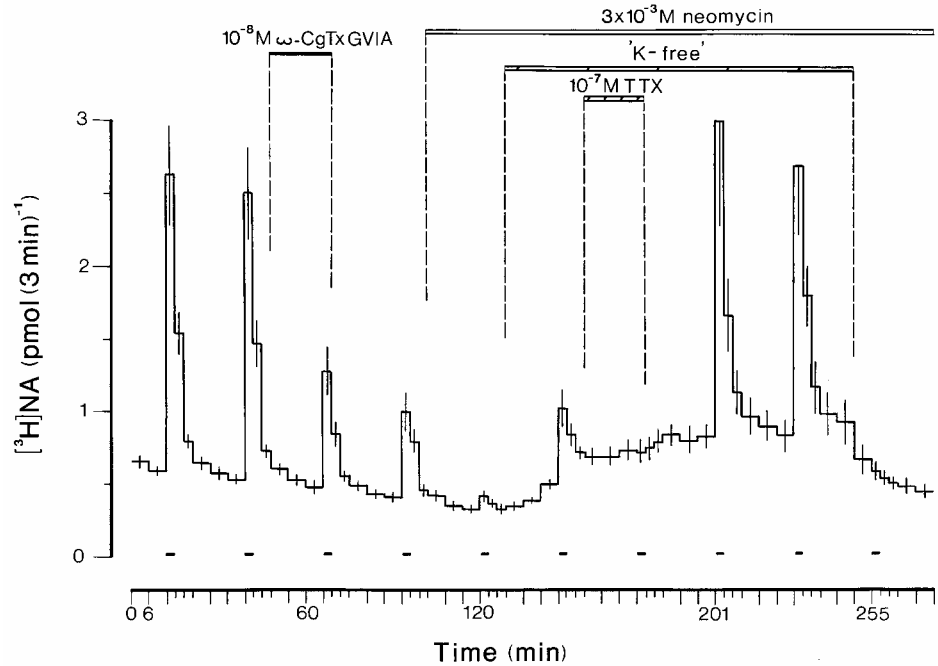


Figure 5. Restoration of [^{3}H]NA release by Na^+-pump inhibition ('K^+-free' perfusion) in the presence of neomycin ($3x10^{-3}$M) and after ω-CgTx GVIA (10^{-8}M) pre-treatment. Ordinate: [^{3}H]NA release (pmol (3 min)$^{-1}$); abscissa: time (min). Neomycin abolished the release of [^{3}H]NA after ω-CgTx GVIA pre-treatment (ratio (S_5/S_2): 0.03±0.02). Subsequently, $[K^+]_o$-removal, for four successive stimulation periods, increased both the resting and the nerve-evoked release of [^{3}H]NA (neomycin was present). The peak release of NA was obtained after 45 min of K^+-removal (second stimulation period in 'K^+-free' solution, S_7). After 120 min 'K^+-free' perfusion, external K^+ (5.9 mM) was readmitted 6 min before S_{10}. Note, that the resting outflow of NA, which had previously been elevated in 'K^+-free' solution was significantly decreased, and the nerve-evoked release of NA was abolished (ratio (S_{10}/S_2): -0.05±0.01). Means±S.E.M. of four identical experiments.

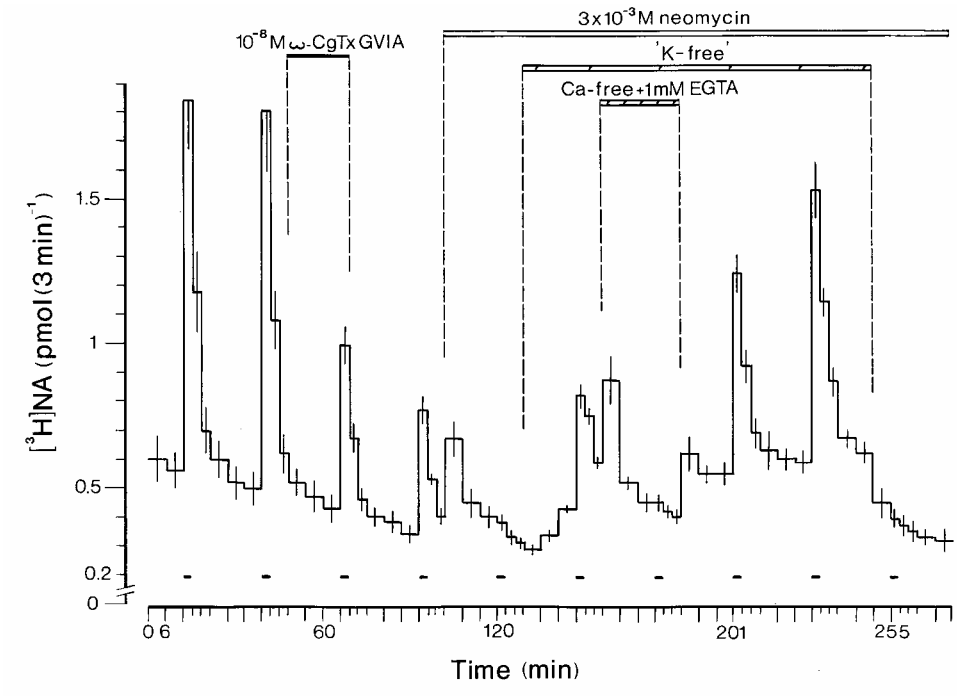
We have carried out some experiments in pulmonary arteries in which the Na^+-pump was inhibited by removal of K^+ from the external medium (Fig. 5). Previously the release of [^{3}H]NA was blocked by ω-CgTx GVIA (10^{-8}M) and neomycin ($3x10^{-3}$M). In 'K^+-free' solution, both the resting and the nerve-evoked release of [^{3}H]NA increased. The peak release of [^{3}H]NA was obtained after 45 min of K^+-removal (second stimulation period in $0K_o$-solution; ratio (S_7/S_2): 1.36±0.11, n=4; Table 2), subsequently it decreased slightly with time. In Na^+-pump inhibited nerves, the varicosities gain Na^+ and loose K^+. So that the internal arm of the exchanger is probably loaded with Na^+. Further, since the pump is electrogenic ($3Na^+/2K^+$), the membrane is probably depolarised (Thomas, 1972). The depolarisation is

either due to switching off the electrogenic contribution of the Na^+-pump, or through reduction of the transmembrane K^+-gradient. Depolarisation may activate the exchanger working in reverse mode (c.f. Blaustein & Lederer, 1999). Additional depolarisation, and Na^+-influx through voltage-sensitive Na^+-channels, may activate further the exchanger (reverse mode), eliciting Ca^{2+}-influx and NA-release. Three important points have to be listed here. (i) Since the exchanger is electrogenic ($3Na^+_i/1Ca^{2+}_o$), generating outward current, the net inward current necessary for the upstroke of the AP will need to be larger than in the absence of the exchanger. (ii) It is known, that external monovalent cations, such as K^+, Rb^+, Cs^+ and Li^+ increase the affinity of Ca^{2+}_o for the exchanger (Baker et al., 1969; Blaustein, 1977; Allen & Baker, 1986; Yasui & Kimura, 1990), even if the Ca^{2+}-binding is increased by reducing external Na^+ (Török & Powis, 1990). Since 'K^+-free' solution was used to load the nerves with Na^+, the exchanger mediated Ca^{2+}-influx was probably partly inhibited. (iii) In these experiments physiological, brief depolarising stimuli were used with 1 ms pulse duration. However, the gain of Na^+ is coupled to K^+-loss in Na^+-pump inhibited nerves. Therefore, it is possible that the duration of AP was somewhat longer than 1 ms, since less K^+ is available inside to carry the outward repolarising K^+-current(s) through K^+-channel(s). Summing up, the first two notes may decrease the influx of Ca^{2+} through the exchanger,



(A) ω-CgTx GVIA (10^{-8}M) significantly inhibited the nerve-evoked release of [^{3}H]NA and subsequently, neomycin ($3x10^{-3}$M), perfused 18 min before S_5, abolished it (ratio (S_5/S_2): 0.03±0.01). In the presence of neomycin external K^+ (5.9 mM) removal increased again the release of NA (ratio (S_6/S_2): 0.35±0.03). 18 min before the second stimulation period in 'K^+-free' solution (S_7), application of TTX abolished the release of labelled NA on stimulation. Note, that on TTX removal, the nerve-evoked release of NA increased again (S_8, S_9). Means±S.E.M. of six identical experiments.

Figure 6. Continued on next page.



(B) as A, except Ca^{2+}_o was removed and 1mM EGTA was added to the 'K^+-free' solution 18 min before S_7 (neomycin was present). In Ca^{2+}-free solution the resting outflow of NA decreased after a transient increase, and the nerve-evoked release was abolished. Note, that Ca^{2+}_o was absent during the stimulation period (3 min) and the subsequent 2x3 min collecting periods. Further note, that Ca^{2+} readmission increased both the resting and the stimulation induced release of NA. Means±S.E.M. of six identical experiments.

Figure 6. Inhibition of [^{3}H]NA-release in $[0K^+]_o$-solution by TTX (10^{-7}M) or by Ca^{2+}_o-removal (+1mM EGTA), in the absence of functioning VSCCs. Ordinate: [^{3}H]NA-release (pmol (3 min)$^{-1}$); abscissa: time (min).

while the third one may increase it. The question is which one is more pronounced.

In pulmonary arteries the following experimental results may support the suggestion that the reverse Na^+/Ca^{2+}-exchange is activated in 'K^+-free' solution when the VSCCs are previously been blocked:

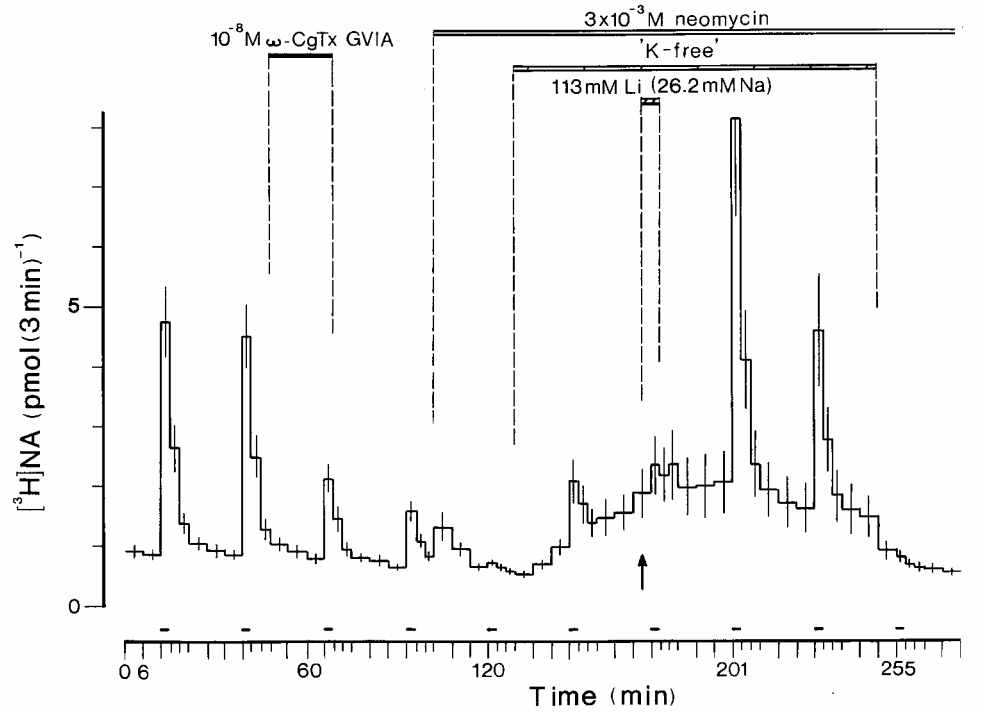
(i) The fast Na^+-channel blocker TTX (10^{-7}M) abolished the release of [^{3}H]NA on stimulation (ratio: 0.02±0.02, n=6; Fig. 6a, Table 2). Similarly, removal of external Ca^{2+} (+ 1 mM EGTA) had the same effect (ratio: -0.03±0.02, n=6; Fig. 6b, Table 2). In Ca^{2+}-free, 1 mM EGTA-containing solution the external Ca^{2+} goes down to about 10^{-9}M (Hubbard et al., 1968; Miledi & Thies, 1971), therefore the Ca^{2+}-gradient is probably reversed. Thus, Ca^{2+}-influx either through the exchanger or VSCC(s) cannot occur. Fig. 6b also shows that the resting outflow of [^{3}H]NA, which had already been elevated in $0K^+_o$-solution decreased when Ca^{2+}_o was removed and 1 mM EGTA was added. This suggests that the plasma membrane reverse Na^+/Ca^{2+}-exchanger is responsible, at least partly, for the spontaneous transmitter outflow increasing effect of 'K^+-free' solution.

Table 2. Na^+-pump inhibition-('K^+-free' solution; 45 min)-induced [^{3}H]noradrenaline release after blockade of VSCCs by ω-CgTx GVIA (10^{-8}M) plus neomycin ($3x10^{-3}$M), and the effects of TTX (10^{-7}M), Ca^{2+}-removal (+ 1 mM EGTA), external Li^+ (113 mM; $[Na^+]_o$: 26.2 mM), KB-R7943 ($3x10^{-5}$M), yohimbine ($3x10^{-7}$M), l-NA (10^{-6}M) and clonidine (10^{-6}M).

	Treatment	Ratio of nerve-evoked release of [3H]NA*	Significance (P)
1.	Control (S_3/S_2)	0.97 ± 0.01 (6)	
2.	10^{-8}M ω-CgTx GVIA + $3x10^{-3}$M neomycin	0.02 ± 0.01 (6)	2/1 P<0.0001
3.	'K^+-free' (45 min) in the presence of $3x10^{-3}$M neomycin and after 10^{-8}M ω-CgTx GVIA pre-treatment	1.36 ± 0.11 (4)	3/2 P<0.0001
4.	'K^+-free' in the presence of neomycin and after ω-CgTx GVIA pre-treatment + 10^{-7}M TTX	0.02 ± 0.02 (6)	4/3 P<0.0001
5.	'K^+-free' in the presence of neomycin and after ω-CgTx GVIA pre-treatment in Ca^{2+}-free + 1 mM EGTA sol.	- 0.03 ± 0.02 (6)	5/3 P<0.0001
6.	'K^+-free' in the presence of neomycin and after ω-CgTx GVIA pre-treatment + 113 mM $[Li^+]_o$#	0.21 ± 0.05 (5)	6/3 P<0.0001
7.	'K^+-free' in the presence of neomycin and after ω-CgTx GVIA pre-treatment + $3x10^{-5}$M KB-R7943##	0.38 ± 0.07 (6)	7/3 P<0.0005
8.	'K^+-free' in the presence of neomycin and after ω-CgTx GVIA pre-treatment + $3x10^{-7}$M yohimbine	3.56 ± 0.26 (5)	8/1 P<0.0001 8/3 P<0.001
9.	'K^+-free' in the presence of neomycin and after ω-CgTx GVIA pre-treatment + yohimbine + TTX	0.10 ± 0.03 (7)	9/3 P<0.005 9/8 P<0.0001
10.	'K^+-free' in the presence of neomycin and after ω-CgTx GVIA pre-treatment + yohimbine in Ca^{2+}-free + 1 mM EGTA sol.	- 0.07 ± 0.02 (5)	10/3 P<0.0001 10/8 P<0.0005
11.	'K^+-free' in the presence of neomycin and after ω-CgTx GVIA pre-treatment + yohimbine + $[Li^+]_o$#	0.37 ± 0.03 (5)	11/3 P<0.0001 11/8 P<0.0001
12.	'K^+-free' in the presence of neomycin and after ω-CgTx GVIA pre-treatment + yohimbine + KB-R7943##	0.54 ± 0.07 (5)	12/3 P<0.0005 12/8 P<0.0001
13.	'K^+-free' in the presence of neomycin and after ω-CgTx GVIA pre-treatment + 10^{-6}M l-NA	0.55 ± 0.07 (6)	13/3 P<0.001 13/8 P<0.0005
14.	'K^+-free' in the presence of neomycin and after ω-CgTx GVIA pre-treatment + 10^{-6}M clonidine	0.26 ± 0.06 (6)	14/3 P<0.0005 14/8 P<0.0005
15.	'K^+-free' in the presence of neomycin and after ω-CgTx GVIA pre-treatment + yohimbine + l-NA	0.72 ± 0.06 (5)	15/3 P < 0.005 15/8 P < 0.0005 15/13 P>0.35

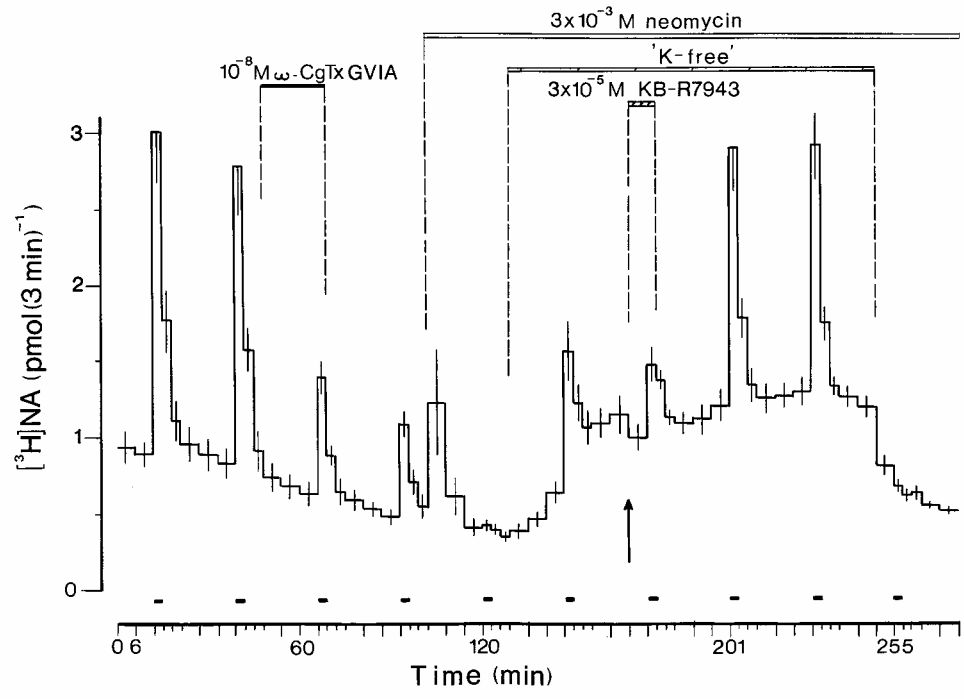
* Stimulation parameters: 2Hz, 1 ms, 60 V for 3 min (360 pulses).
Treatments number 2-15: S_7/S_2
ω-CgTx GVIA was pre-perfused 18 min before S_3 and was present during stimulation (3').
Neomycin was applied 18 min before S_5 and was present throughout.
External K^+ was removed 18 min before S_6 and was readmitted 6 min before S_{10}.
TTX, yohimbine, l-NA and clonidine were applied 18 min before S_7 and were present during stimulation (3').
$[Ca^{2+}]_o$ was removed (+ 1 mM EGTA) 18 min before S_7 and was absent during stimulation (3') and the subsequent 2x3'.
Li^+ was pre-perfused 3 min before S_7 and was present during stimulation (3').
KB-R7943 was pre-perfused 6 min before S_7 and was present during stimulation (3').

(ii) In Li^+-(113 mM; $(Na^+]_o$: 26.2 mM)-containing 'K^+-free' solution, the release of [^{3}H]NA was significantly inhibited (ratio: 0.21±0.05, n=5; Fig 7a (compare with Fig. 1); Table 2). External Li^+ can carry the inward current through fast, TTX-sensitive Na^+-channels, and thereby can depolarise the membrane (Hodgkin & Keynes, 1957; Hille, 1992; Lipp & Niggli, 1994), depolarisation opens VSCCs, however Li^+ inside cannot activate the exchanger working in reverse mode. Both for normal and reverse mode of operation of Na^+/Ca^{2+}-exchange, the requirement for Na^+ is absolute, i.e. $Li^+_{o/i}$ cannot substitute for $Na^+_{o/i}$ for exchange cycle (Baker et al., 1969; Miura & Kimura, 1989; Reuter & Porzig, 1995; Doering et al., 1998). It should be mentioned however that high concentrations of Li^+ (113 mM), may reactivated the Na^+-pump, since it is known that alkali metals, such as Rb^+, Cs^+ and Li^+, similar to that of K^+ can activate the Na^+-pump (c.f. Thomas, 1972; Török, 1989). However, this is unlikely in our experiments, when we used a short 3 min pre-perfusion period with Li^+-containing solution, since during the next stimulation period in 'K^+-free' solution, the release of [^{3}H]NA markedly increased (S_8 in Fig. 7a).



(A) Combined application of ω-CgTx GVIA (10^{-8}M) and neomycin ($3x10^{-3}$M) abolished the release of [^{3}H]NA on stimulation (ratio (S_5/S_2): -0.01±0.03). Subsequently, K^+_o-removal increased both the resting and the stimulation-induced release of [^{3}H]NA. Li^+-(113 mM)-perfusion, 3 min before S_7 (arrow), significantly inhibited the evoked release of NA. Note, that Li^+ was present during the 3 min stimulation period. Further note, that during the next stimulation period (S_8), the release of NA markedly increased. Means±S.E.M. of five identical experiments.

Figure 7. Continued on next page.



(B) as A, except KB-R7943 ($3x10^{-5}$M) was perfused in $0K^+_o$-solution 6 min before S_7 (arrow). After ω-CgTx GVIA pre-treatment, neomycin abolished the release of [^{3}H]NA on stimulation (ratio of S_5/S_2: -0.01±0.03). Subsequently, in 'K^+-free' solution, KB-R7943 (arrow) significantly inhibited the stimulation-induced release of [^{3}H]NA. Means±S.E.M. of six identical experiments.

Figure 7. Inhibition of [^{3}H]NA-release in Na^+-pump inhibited nerves ('K^+-free' perfusion) by external Na^+-substitution (Li^+: 113 mM; $[Na^+]_o$: 26.2 mM), or by KB-R7943 ($3x10^{-5}$M) application without functioning VSCCs. Ordinate: [^{3}H]NA-release (pmol (3 min)$^{-1}$); abscissa: time (min).

The preferential reverse Na^+/Ca^{2+}-exchange inhibitor KB-R7943 (Watano et al., 1996; Shigekawa & Iwamoto, 2001) also significantly inhibited the evoked release of [^{3}H]NA (ratio: 0.38±0.07, n=6; Fig. 7b, Table 2), when it was pre-perfused 6 min before stimulation. Fig. 7b shows that KB-R7943 also decreased the resting outflow of [^{3}H]NA. This latter phenomenon suggests that the plasma membrane reverse Na^+/Ca^{2+}-exchange is responsible, at least partly for the resting NA-release increasing effect of $0K^+_o$-solution. Further, the drug-caused inhibition of reverse Na^+/Ca^{2+}-exchange is probably more pronounced than its Ca^{2+}-releasing effect from internal stores. KB-R7943 has little effect on Na^+/K^+-ATPase, Na^+/H^+-exchange, sarcolemmal Ca^{2+}-ATPase and SR Ca^{2+}-ATPase (Iwamoto et al., 1996).

It is known that the reverse Na^+/Ca^{2+}-exchange requires internal Ca^{2+} for its activation ("positive feed-back" mechanism; Baker & DiPolo, 1984; Kimura et al., 1986; Hilgemann, 1990; DiPolo & Beaugé, 1993; c.f. Blaustein & Lederer, 1999), known as 'non-transported' Ca^{2+}. This Ca^{2+} may come from internal stores, possibly from the mitochondria by a $[Na^+]_i$-dependent Ca^{2+}-release mechanism (Na^+/Ca^{2+}-exchange; Gunter & Pfeiffer, 1990; Brierley et al., 1994), and/or it is provided by the plasma membrane exchanger (the Ca^{2+}-efflux mode is inhibited and the Ca^{2+}-influx mode is activated as $[Na^+]_i$ is elevated). This latter suggestion is

supported by the finding that the reverse Na^+/Ca^{2+}-exchange inhibitor KB-R7943 slightly decreased the resting outflow of NA in 'K^+-free' solution (Fig. 7b).

In summary, the above results suggest that the fast Na^+-channel(s) can admit enough Na^+ to reverse the exchanger, but only if the cytoplasmic Na^+-concentration had already been slightly elevated by Na^+-pump inhibition. Also, that the Na^+-channel- as well as the exchanger-proteins, and the vesicle docking sites are probably close to each other. This latter is theoretically likely, because of the small size of the varicosities.

3.4. Reverse Na^+/Ca^{2+}-Exchange and Transmitter Release Regulation by Pre-synaptic α_2- Receptors

Previously it has been shown in different preparations that the exchanger is regulated by adrenoceptors (arteries: Khoyi et al., 1991, 1993; Yamanaka et al., 2003; ventricular myocytes: Fan et al., 1996; Ballard & Schaefer, 1996; Shuba et al., 1998; Stengl et al., 1998; Perchenet et al., 2000; Viatchenko-Karpinski & Györke, 2001; Zhang et al., 2001, 2002; Woo & Morad, 2001; Pabbathi et al., 2002). Adrenoceptors regulate the gene expression of the exchanger as well (Menick et al., 1996; Reinecke et al., 1997; Satoh et al., 2000; Golden et al., 2000, 2001).

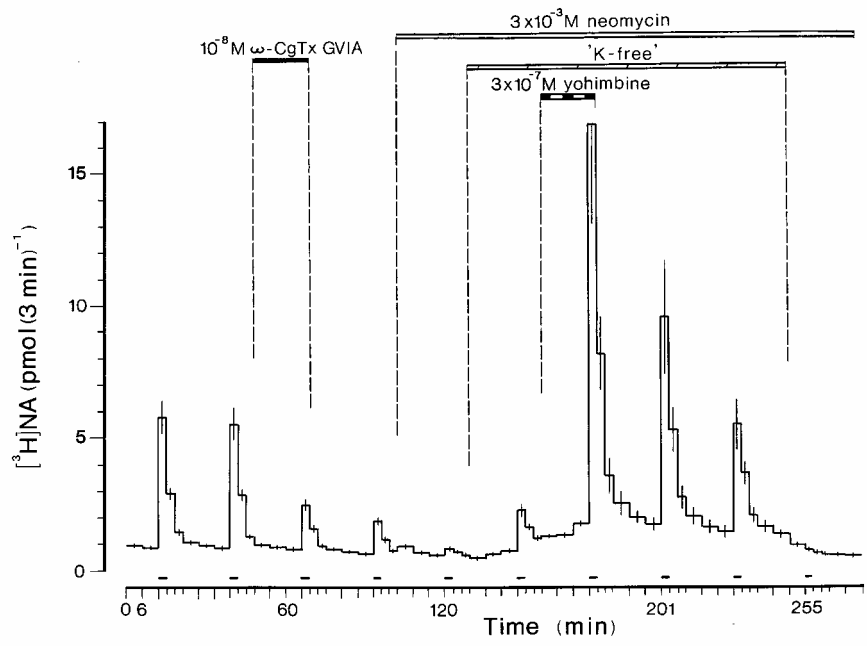


Figure 8. In the presence of neomycin ($3x10^{-3}$M) and after ω-CgTx GVIA (10^{-8}M) pre-treatment, yohimbine ($3x10^{-7}$M) enhanced potentiation of the stimulation-induced [^{3}H]NA-release by Na^+-pump inhibition ('K^+-free' perfusion). Compare with Figure 5. Ordinate: [^{3}H]NA-release (pmol (3 min)$^{-1}$); abscissa: time (min). ω-CgTx GVIA inhibited, while neomycin abolished the release of [^{3}H]NA on stimulation (ratio (S_5/S_2): -0.01±0.04). In $0K^+_o$-solution, the release of NA increased again. Yohimbine, pre-perfused 18 min before S_7, markedly further increased the release of NA on stimulation. Means±S.E.M. of five identical experiments are shown by vertical lines.

In Na^+-pump inhibited arteries, yohimbine ($3x10^{-7}$M) further potentiated the release of [^{3}H]NA in response to field stimulation in the absence of functioning VSCCs, i.e. in the presence of neomycin and after ω-CgTx GVIA pre-treatment (Fig. 8; Table 2). The opposite effect was obtained when the α_2-receptors were activated by clonidine (10^{-6}M; Fig. 9; Table 2) or by l-NA (10^{-6}M; Table 2).

The yohimbine-induced potentiation of nerve-evoked release of [^{3}H]NA was blocked by TTX (10^{-7}M; Fig. 10a, Table 2) or by $[Ca^{2+}]_o$-removal (Fig. 10b, Table 2), and significantly inhibited by Li^+-(113 mM)-substitution (Fig. 11a, Table 2) or by KB-R7943-($3x10^{-5}$M)-application (Fig. 11b, Table 2).

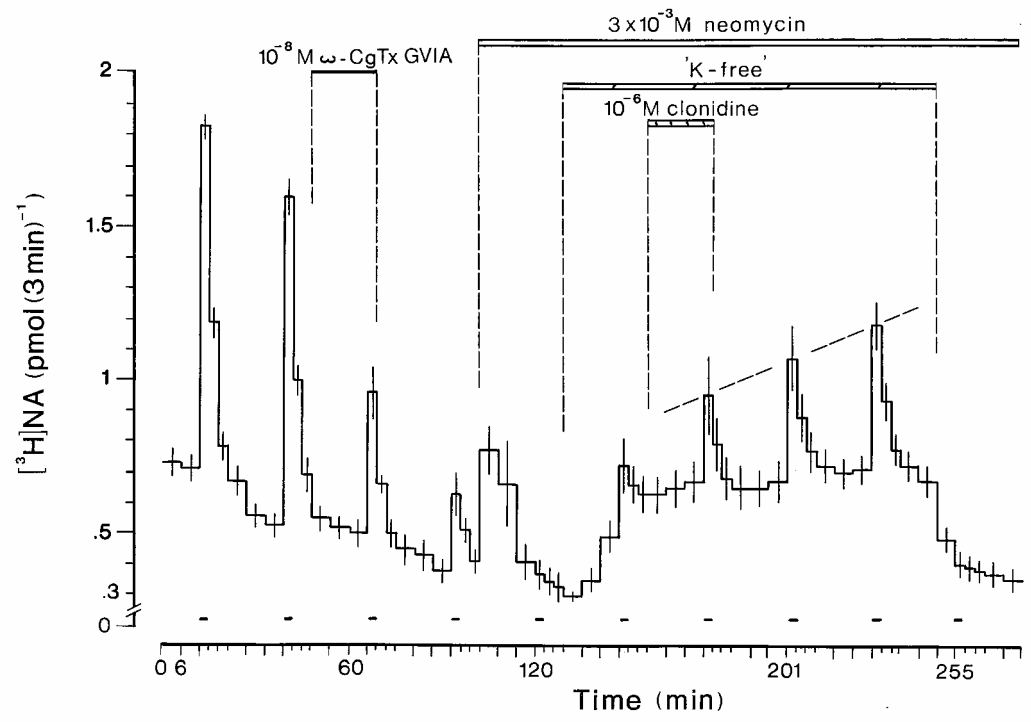
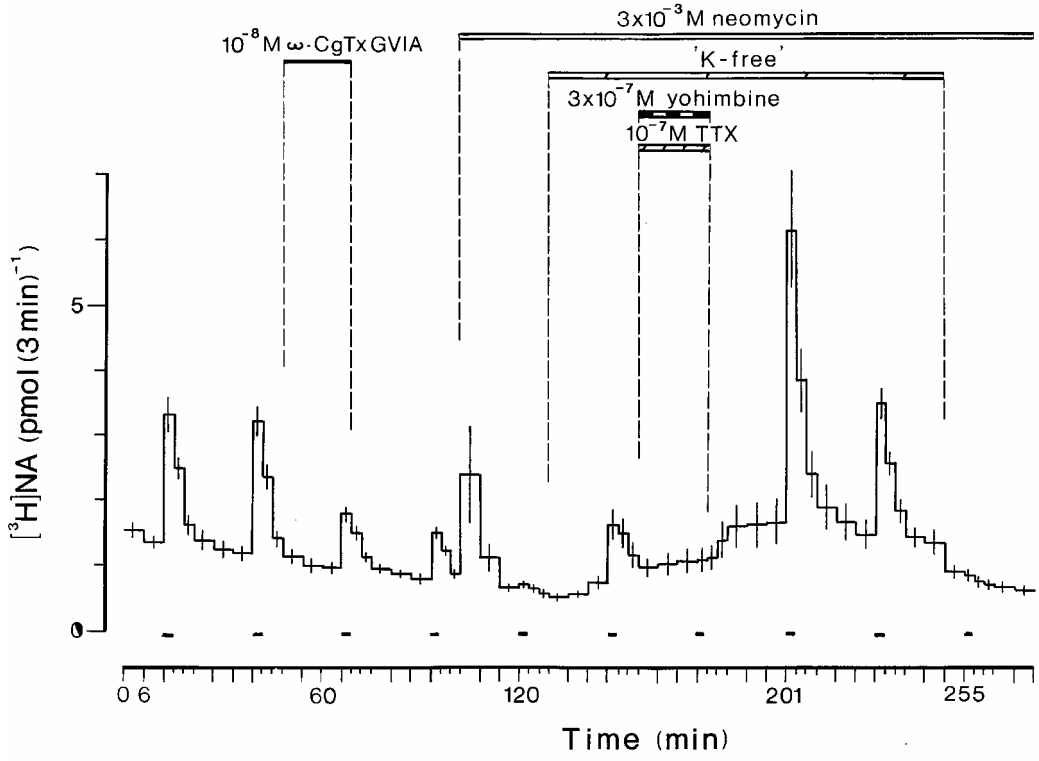


Figure 9. In the presence of neomycin ($3x10^{-3}$M) and after ω-CgTx GVIA (10^{-8}M) pre-treatment, clonidine-(10^{-6}M) inhibited the potentiation of the stimulation-induced [^{3}H]NA-release by Na^+-pump inhibition ('K^+-free' perfusion). Compare with Figure 5. and 8. Ordinate: [^{3}H]NA-release (pmol (3 min)$^{-1}$); abscissa: time (min). Neomycin, after a transient increase of resting outflow of [^{3}H]NA, abolished the evoked-release of neurotransmitter after ω-CgTx GVIA pre-treatment (ratio (S_5/S_2): -0.14±0.08). $0K^+_o$-solution increased again both the resting and the nerve-evoked release of [^{3}H]NA. Subsequently, clonidine was perfused 18 min before S_7. Note that clonidine significantly inhibited the nerve-evoked release of NA (ratio (S_7/S_2): 0.26±0.06). Further note, that after clonidine-removal the release of NA just slightly increased with time (ratios between S_8/S_2 and S_9/S_2: 0.46±0.05 and 0.49±0.06 respectively). Means±S.E.M. of six identical experiments are shown by vertical lines.

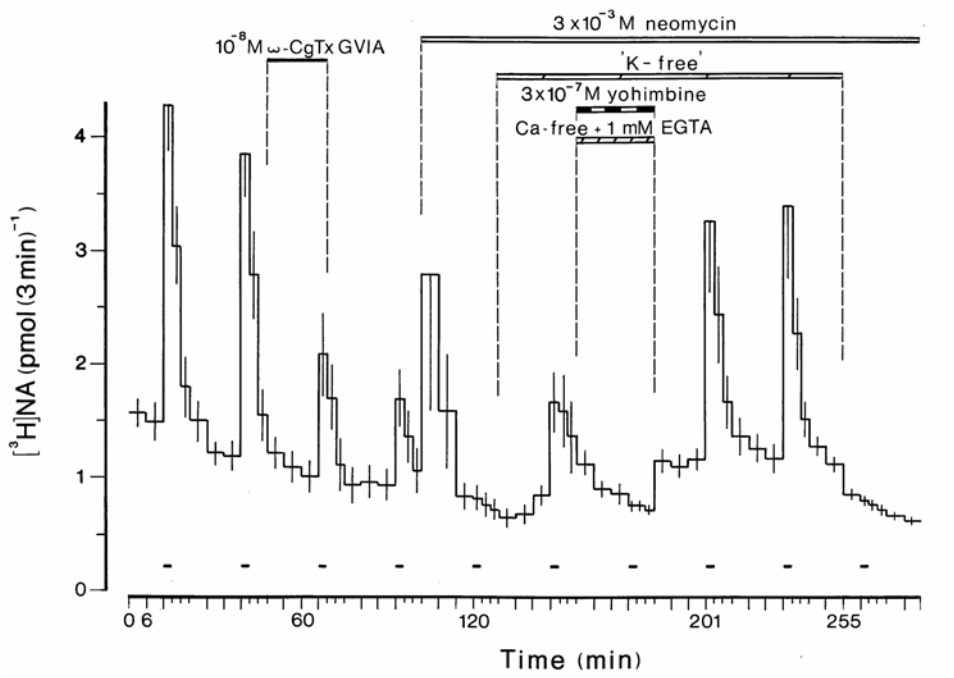
3.5. Potentiation of [^{3}H]noradrenaline Release by Veratridine in Na^+-Pump Inhibited Arteries and the Inhibitory Effect of KB-R7943

In 'K^+-free' solution, the fast Na^+-channel activator veratridine (10^{-5}M), which possibly activates the channel and/or blocks it in an open stage, further enhanced both the resting and the nerve-evoked release of [^{3}H]NA (Fig. 12a). Veratridine was pre-perfused 18 min before the second stimulation period in $0K^+_o$-solution (S_7). The resting outflow of [^{3}H]NA markedly further increased (from 0.37±0.17 to 1.61±0.62 pmol (3 min)$^{-1}$; Δpmol (3 min)$^{-1}$: 1.24±0.46; n=5). Subsequently, when the nerves were stimulated (S_7), the ratio of nerve-evoked release of [^{3}H]NA was further potentiated (2.66±0.23 pmol (3 min)$^{-1}$). This value was significantly higher to that obtained in the absence of veratridine (ratio: 1.36±0.11, Fig. 5; P<0.0125).



(A) ω-CgTx GVIA and neomycin fully inhibited the release of [^{3}H]NA on stimulation (ratio of S_5/S_2: -0.03±0.04). Note, that neomycin transiently increased the spontaneous outflow of NA. Subsequently, in $0K^+_o$-solution, yohimbine and TTX were pre-perfused together 18 min before S_7. TTX markedly inhibited the release of [^{3}H]NA. Means±S.E.M. of seven identical experiments are shown by vertical lines.

Figure 10. Continued on next page.



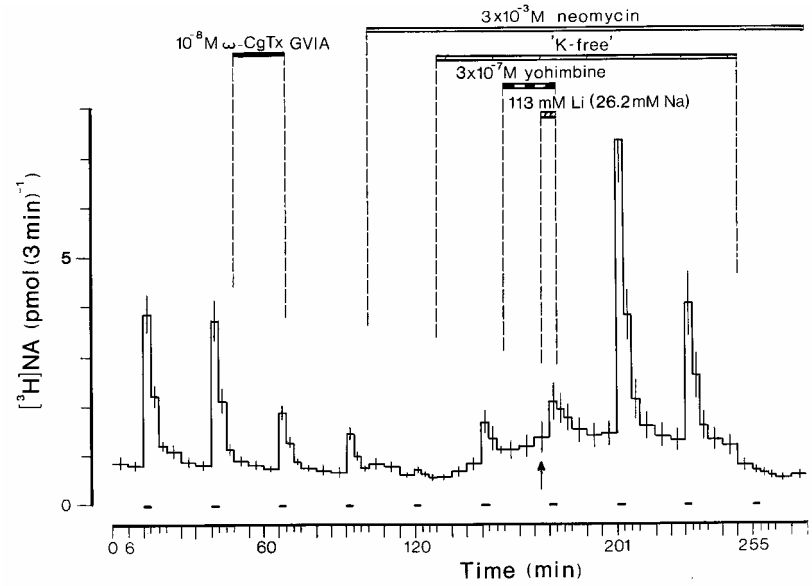
(B), as A, except yohimbine was pre-perfused in 'K^+-free' and in Ca^{2+}-free + 1 mM EGTA-containing solution 18 min before S_7. Previously, neomycin abolished the release of NA after ω-CgTx GVIA pre-treatment (ratio (S_5/S_2): -0.05±0.02). Note, that neomycin, transiently, but markedly increased the resting outflow of NA. In Ca^{2+}-free solution, yohimbine was ineffective in potentiating the release of NA. Note, that yohimbine was present and Ca^{2+} was absent during the 3 min stimulation period and the subsequent 2x3 min collecting periods. Further note that the resting outflow of NA also decreased in the absence of external Ca^{2+}. On Ca^{2+}-readmission however, the spontaneous outflow of NA increased again in 'K^+-free' solution. Means±S.E.M. of five identical experiments are shown by vertical lines.

Figure 10. Inhibition of yohimbine-($3x10^{-7}$M)-potentiated [^{3}H]NA-release in 'K^+-free' solution by TTX (10^{-7}M) or by $Ca^{2+}{}_o$-removal (+1 mM EGTA) in the absence of functioning VSCCs (10^{-8}M ω-CgTx GVIA + $3x10^{-3}$M neomycin). Ordinate: [^{3}H]NA-release (pmol (3 min)$^{-1}$); abscissa: time (min).

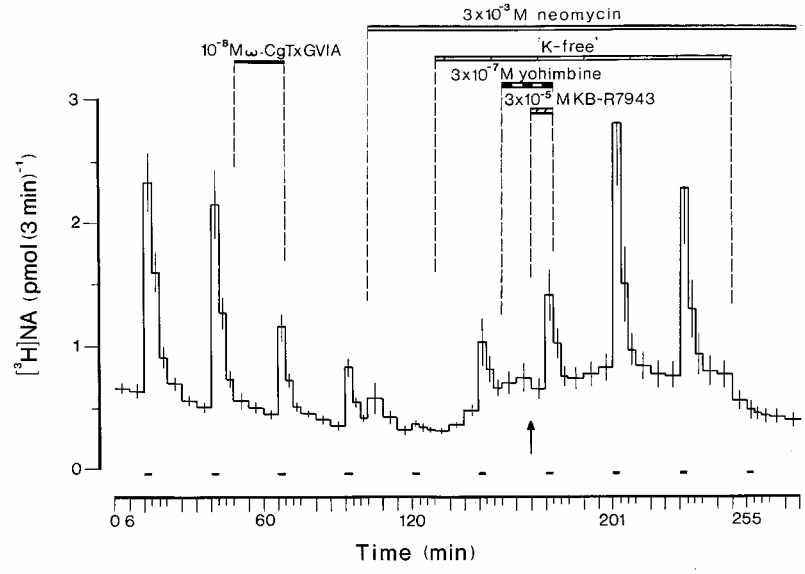
However, when KB-R7943 ($3x10^{-5}$M) was added to veratridine-containing 'K^+-free' solution 6 min before the stimulation period, the resting outflow of NA was not inhibited (Δ pmol (3 min)$^{-1}$: 1.37±0.14; n=6), but the nerve-evoked release was significantly reduced (ratio: 0.89±0.25, P<0.001; Fig. 12b). Further, both the resting and the nerve-evoked release of [^{3}H]NA were significantly inhibited when KB-R7943 was applied together with veratridine 18 min before S_7 (Fig. 12c). The resting outflow of NA just slightly increased (Δ pmol (3 min)$^{-1}$: 0.48±0.08, n=6; P<0.05), and the evoked-release was markedly inhibited (ratio: 0.36±0.09; P<0.0001).

The veratridine-induced potentiation of resting and nerve-evoked release of [^{3}H]NA was fully blocked by [Ca^{2+}[$_o$-removal with parallel addition of 1 mM EGTA (not shown).

The above results seem to confirm the previous suggestions, namely that the reverse Na^+/Ca^{2+}-exchange is mainly responsible for the resting-outflow increasing effect of 'K^+-free' solution, further, that the Na^+-channel, the exchanger and the transmitter-release sites are possibly close to each other, at least in these varicosities.



(A) ω-CgTx GVIA and the subsequently applied neomycin fully inhibited the evoked release of [^{3}H]NA (ratio (S_5/S_2): 0.02±0.01). Later, in $0K^+_o$-solution, yohimbine was perfused 18 min and Li^+ 3 min (arrow) before S_7. Note, that Li^+-substitution markedly inhibited the release of NA in yohimbine-containing solution on nerve-stimulation. Means±S.E.M. of five identical experiments are given.



(B) as A, except KB-R7943 was applied in yohimbine-containing 'K^+-free' solution 6 min before S_7 (arrow). KB-R7943 inhibited the evoked release of [^{3}H]NA (ratio (S_7/S_2): 0.54±0.07) and slightly decreased the resting outflow of NA as well. Compare with Figure 8. Means±S.E.M. of five identical experiments are given.

Figure 11. Inhibition of yohimbine-($3x10^{-7}$M)-potentiated [^{3}H]NA-release in 'K^+-free' solution by Li^+-(113 mM)-substitution or by KB-R7943 ($3x10^{-5}$M) application in neomycin ($3x10^{-3}$M)-containing solution and after ω-CgTx GVIA (10^{-8}M) pre-treatment. Ordinate: [^{3}H]NA-release (pmol (3 min)$^{-1}$); abscissa: time (min).

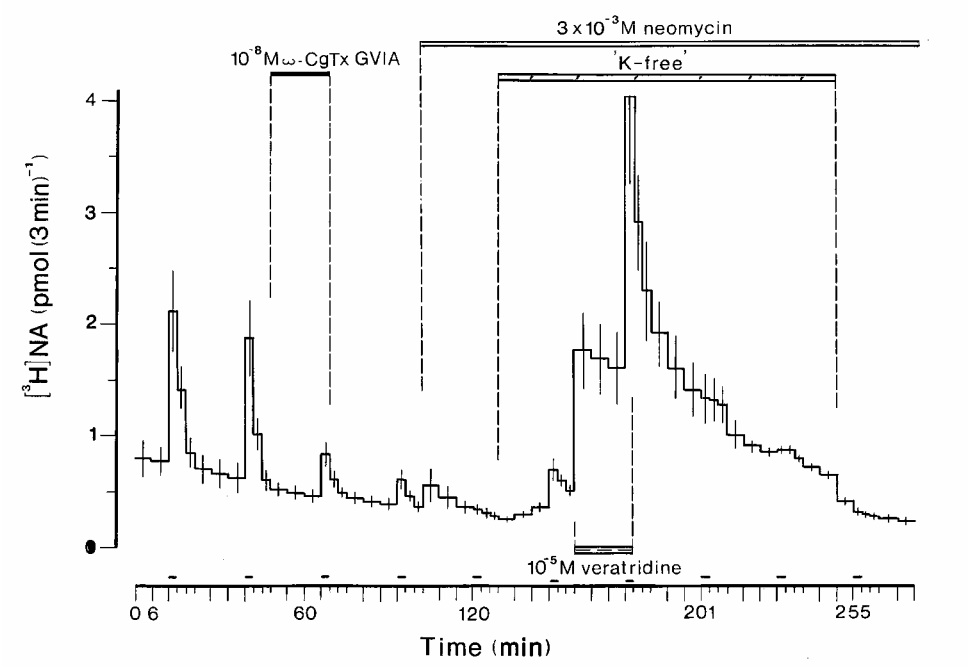
Conclusion

In the main pulmonary artery of the rabbit the 'N-type' voltage-sensitive Ca^{2+}-channel is mainly responsible for NA-release if physiological depolarising stimuli are used (2 Hz). Correlation was obtained between the extent of VSCC inhibition and the transmitter release potentiating effect of pre-synaptic α_2-receptor blocker yohimbine. When the release of NA was fully inhibited by a combined application of ω-CgTx GVIA and the 'non-selective' Ca^{2+}-channel blocker neomycin, yohimbine was ineffective. This confirms the original suggestion of others (Lipscombe et al., 1989; Brain & Bennett, 1997) that the α_2-receptor mediated 'negative feed-back' mechanism is mainly due to closure of VSCCs. It is known that neomycin, apart from its inhibitory effect on 'N-type', 'L-type' and 'non N-type' and 'non L-type' HVA Ca^{2+}-channels (Keith et al., 1992, 1993), also exerts inhibitory effect on Na^+/Ca^{2+}-exchange (Hilgemann, 1996; c.f. Blaustein & Lederer, 1999). Therefore, it is possible, that the 'residual release' of NA, which remains after ω-CgTx GVIA treatment (~20-30%), is due at least partly, to activation of reverse Na^+/Ca^{2+}-exchange (Török et al., 2004).

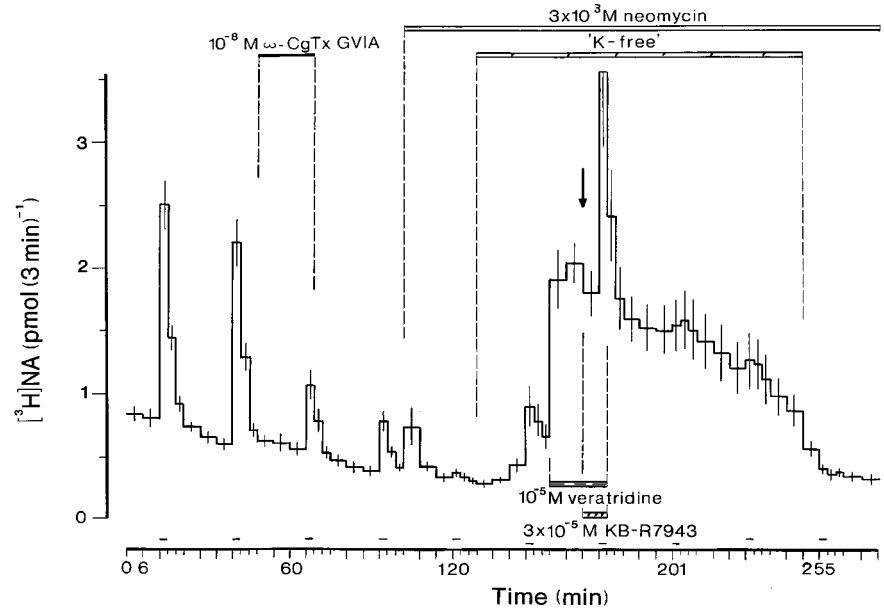
Na^+-loading (Na^+-pump inhibition) was required to increase again the nerve-evoked release of NA after blockade of VSCCs. This enhancement of evoked transmitter release was probably due to activation of reverse Na^+/Ca^{2+}-exchange, since it was abolished by TTX, Ca^{2+}-removal, Li^+-substitution, or by KB-R7943-application. This suggests that the fast Na^+-channel, the Na^+/Ca^{2+}- exchanger and the vesicle docking sites are close to each other.

It is possible however, that the depolarisation in 'K^+-free' solution recruits some so-called "dormant" HVA Ca^{2+}-channels. However this is unlikely in pulmonary arteries since KB-R7943 significantly reduced the nerve-evoked release of NA. KB-R7943 is able to block 'L-type' Ca^{2+}-channnel as well, but this channel is probably not involved in NA-release (Bennett, 1999). However the drug does not inhibit 'N-type' Ca^{2+}-current. There is no data whether or not other subtypes of Ca^{2+}-channels ('P/Q-'; 'R-') are inhibited by KB-R7943. If yes, these channels may not be activated by Li^+-depolarisation.

Theoretically it is possible, that similar to that of cardiac muscle (Leblanc & Hume, 1990), the exchanger mediated Ca^{2+}-influx may cause some additional release of Ca^{2+} from internal stores, contributing to the transmitter release obtained in these experiments. However, this is unlikely in pulmonary arteries, since large concentrations of ryanodine ($5x10^{-5}$M), slightly but not significantly, inhibited the release of NA in 'K-free' solution The ratios of [^{3}H]NA-release in the absence and presence of ryanodine were: 1.10±0.19 (n=5) and 0.82±0.15 (n=6) in two parallel series of experiments (not shown; %-inhibition ~25, P>0.05). In normal solution ryanodine was ineffective (unpublished observation). It should be mentioned however, that Smith & Cunnane (1996) could demonstrate a ryanodine effect in sympathetic nerves by using high frequency of electrical stimulation (10-50 Hz).

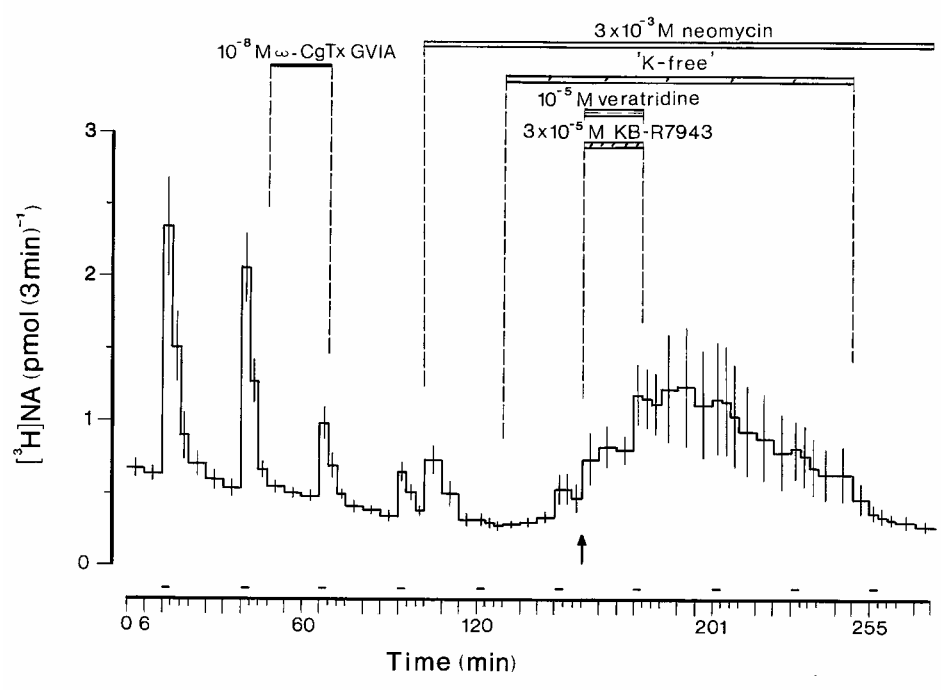


(A) Veratridine, pre-perfused 18 min before the second stimulation period in $0K^+_o$-solution (S_7) markedly further increased both the spontaneous outflow of [^{3}H]NA and the nerve-evoked release of neurotransmitter (ratio (S_7/S_2): 2.66±0.23). Means±S.E.M. of five identical experiments are shown by vertical lines.



(B), as A, except KB-R7943 was added to veratridine-containing 'K^+-free' solution 6 min before S_7 (arrow). Note, that KB-R7943 did not reduce significantly the resting outflow potentiating effect of veratridine, but markedly inhibited the evoked-release of NA (ratio (S_7/S_2): 0.89±0.27). Means±S.E.M. of six identical experiments are shown by vertical lines.

Figure 12. Continued on next page



(C), as A and B, except KB-R7943 was perfused together with veratridine in $0K^+_o$-solution 18 min before S_7 (arrow). Note, that KB-R7943 significantly inhibited both the resting- and the nerve-evoked release of [^{3}H]NA which had been potentiated by veratridine (ratio (S_7/S_2): 0.36±0.07). Means±S.E.M. of six identical experiments are shown by vertical lines.

Figure 12. Further potentiation of both the resting and the nerve-evoked release of [^{3}H]NA in 'K^+-free' solution by veratridine (10^{-5}M) in the presence of neomycin ($3x10^{-3}$M) and after ω-CgTx GVIA (10^{-8}M) pre-treatment and their inhibition by KB-R7943-($3x10^{-5}$M)-application. Ordinate: [^{3}H]NA-release (pmol (3 min)$^{-1}$); abscissa: time (min).

Pre-synaptic α_2-receptors regulate not only the VSCCs but possibly the exchanger as well. This suggestion was supported by the findings that in Na^+-loaded nerves, in which the VSCCs were previously been blocked, the nerve-evoked release of neurotransmitter was further enhanced by yohimbine and inhibited by l-NA or clonidine. The possible involvement of a second messenger has not been determined yet. The yohimbine-induced enhancement of transmitter release was significantly inhibited by TTX, Ca^{2+}-removal, Li^+-substitution and by KB-R7943 application. These results may give rise to the following possibility: in patients with enormously elevated blood pressure (e.g. in malignus or essential hypertension), the reverse Na^+/Ca^{2+}-exchange may also be involved, and α_2-receptor agonists may inhibit not only the VSCCs but also the reverse Na^+/Ca^{2+}-exchange.

Positive correlation was obtained between the degree of Na^+-loading and the depolarisation-induced enhancement of neurotransmitter release, i.e. the nerve-stimulation further potentiated the release of NA if the Na^+-channel activator veratridine was simultaneously present in $0K^+_o$-solution.

In conclusion, reverse Na^+/Ca^{2+}-exchange activation may occur physiologically, especially at high frequency of nerve APs, because of the small size of the nerve terminals.

Calculations based on the reversal potential ($E_{Na/Ca}$) and driving force ($\Delta V_{Na/Ca}$) of the exchanger show that a small increase of internal Na^+ (4-5 mM) is enough to reverse the exchanger. Further, similar to that of VSCCs, the reverse Na^+/Ca^{2+}-exchnage is probably also regulated by pre-synaptic α_2-adrenoceptors.

As for the therapy, an 'N-type' VSCC-inhibitor would be useful in hypertension to decrease blood pressure, further, a normal-mode selective Na^+/Ca^{2+}-exchange inhibitor in cardiac failure could be used to increase internal Ca^{2+}, since, unlike cardiac glycosides, it would not increase internal Na^+.

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Chapter 13

SYMPATHETIC NERVOUS SYSTEM AND CHRONIC PAIN SYNDROMES

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Abstract

Abnormal activity of the sympathetic nervous system is involved in the pathogenesis of protracted pain syndromes. The term sympathetically maintained pain is applied to those neuropathic pain cases that respond to sympatholytic maneuvers.

The sympathetically maintained pain concept has strong and ample foundations in the animal model. After nerve injury, sympathetic sprouting at the dorsal root ganglia becomes apparent and form basket-like structures around large-diameter axotomized sensory neurons; sympathetic stimulation can activate such neurons repetitively.

It has been proposed that this pathogenesis is operative in cases of complex regional pain syndrome type I (formerly known as reflex sympathetic dystrophy). Emerging evidence suggests that the sympathetic nervous system also plays a key role in the development of the complex generalized pain syndrome named fibromyalgia.

Evidence of sympathetic dysfunction in fibromyalgia was gained through the use of novel non-linear methods (heart rate variability analysis).

Introdution

The autonomic nervous system (ANS) is the main regulatory system of the body in charge of maintaining essential involuntary functions, such as the so-called *vital signs* – (blood pressure, pulse, respiration and temperature), among many other variables (1). Dysfunction of the autonomic nervous system has been associated to chronic pain syndromes. This chapter describes basic concept of the autonomic nervous system that are related to pain susceptibility. It also discusses experimental models linking autonomic dysfunction to chronic pain and finally presents emerging evidence suggesting that dysfunction of the sympathetic nervous system plays a key role in the pathogenesis of fibromyalgia .

Autonomic Nervous System. Basic Concepts Related to Pain Mechanisms

The ANS is the portion of the nervous system that controls the function of the different organs and systems of the body. It is "autonomic" because it works below the level of consciousness. One striking characteristic of this system is the rapidity and intensity of the onset of its action and its dissipation. The ANS is activated by centers located in the spinal cord, brain stem hypothalamus and thalamus. These centers also receive input from the limbic system and other higher brain areas. These connections enable the ANS to serve as the principal part of the stress response system in charge of the fight or flight reactions.

The ANS works closely with the endocrine system, particularly with the hypothalamic-pituitary-adrenal axis. The adrenal glands are rich in autonomic neurotransmitters. Another endocrine axis closely related to the ANS involves growth hormone secretion.

The peripheral autonomic system is divided into two branches; sympathetic and parasysmpathetic. These two branches have antagonistic actions on most bodily functions and thus their proper balance preserves homeostasis. The action of these two branches is mediated by neurotransmitters. Catecholamines are the sympathetic neurotransmitters whereas acethylcoline acts in the parasympathetic periphery (1).

The naturally occurring sympathetic catecholamines are norepinephrine, epinephrine and dopamine. The three substances act as neurotransmitters within the central nervous system. Norepinephrine acts also in peripheral postganglionic nerve endings and exerts its effects locally, in the immediate vicinity of its release (2), whereas epinephrine is the circulating hormone of the adrenal medulla and influences processes throughout the body. The major metabolic transformation of catecholamines involves methylation and oxidative deamination. Methylation is catalyzed by the enzyme catechol-O-methyltransferase (COMT), whereas oxidative deamination is promoted by monoamine oxidase (MAO). COMT enzyme gene has been the focus of interest due to its relationship with pain susceptibility in healthy women. There are several polymorphism in the COMT gene that are associated with a defective catecholamine-clearing enzyme. Women that posses these SNP are more susceptible to experience pain(3).

Clinical Assessment of Autonomic Nervous System Function

The function of the autonomic nervous system has been difficult to evaluate in clinical practice. This is a very complex system that can only be studied properly with non-linear instruments. Changes in breathing pattern, mental stress or even posture, alter immediately and completely the sympathetic/parasympathetic balance. So, this dynamic system could not be properly studied by "static" test such as levels of circulating neurotransmitters and less so by their urinary catabolites. Useful bedside maneuvers to assess ANS function have included measurements of supine and standing pulse and blood pressure. Sustained drops in systolic (>20 mmHg) or diastolic (>10 mmHg) blood pressure after standing for 3 minutes that are not associated with increase pulse rate of > 30 beats per minute suggest autonomic deficit (4).

Opportunely, two clinical research instruments have been recently introduced to aid in clinical research of cardiovascular autonomic function; heart rate variability analysis and tilt table test. These two methods have provided important clues in the pathogenesis of chronic

painful conditions. Recently heart rate variability analysis has been used as a feedback instrument in the treatment of chronic pain syndromes.

Heart Rate Variability Analysis

The method is based in the well known fact that the heart rate is not fixed, rather it varies from beat to beat constantly and at random. The harmonious effects of the sympathetic and parasympathetic branches of the ANS on the sinus node have different and discerning effects in this constant variability. Heart rate variability can be studied in the time domain where the basic units are milliseconds. Time domain mathematical calculations include, among others, the standard deviation of all R-R intervals duration in as well as the percentage of adjacent pairs of R-R intervals that differ by more than 50 milliseconds from each other in a given time period. The higher time domain variability indexes signify the more parasympathetic influx on the sinus node.

Heart rate variability can be also studied in the frequency domain using spectral analysis where the basic units are Hertz (cycles per second). Pharmacological and clinical studies have established that the high frequency band spectral power reflects parasympathetic activity on the heart, whereas low frequency band power is modulated mostly by sympathetic impulses. Since the two branches of the autonomic nervous system have antagonistic effects on the sinus node, the ratio low frequency band/high frequency band, is regarded as reflection of sympathetic activity (5).

This new method has several advantages. It is non-invasive so patients are subjected to no discomfort whatsoever. The equipment is portable thus recording can be accomplished while subjects perform their routine activities and lastly, the method is based on computerized calculations therefore it has boundless development potentials.

Tilt Table Test

This is another useful tool to study orthostatic intolerance and syncope. It is based on the physiological changes that occur after adopting upright posture with pooling of approximately 700 ml of blood in the lower parts of the body. In normal circumstances, the autonomic nervous system quickly compensates this relative volume loss by increasing vascular tone and cardiac output. This mechanism avoids hypotension and inadequate cerebral perfusion. Tilt table testing examines this response in a controlled environment. With passive orthostasis, additional stress is exerted on the sympathetic nervous system by blocking the influence of muscle contraction that could increase venous return. In the first step subjects are supine for 30 minutes. Then the subject is tilted up-right for 30 to 45 minutes at an angle of 60 to 80 degrees. Pharmacological stimulation with isoproterenol is sometimes used as an additional step.

The normal response to tilting consist of an increase heart rate in 10 to 15 beats per minute, elevation of diastolic blood pressure of about 10 mmHg and little change in systolic pressure. There are two types of abnormal responses: orthostatic hypotension defined as a reduction of systolic blood pressure of at least 20 mmHg or a reduction of diastolic blood pressure of at least 10 mmHg. This hypotension may induce syncope. Another type of

abnormal response is postural orthostatic tachycardia (POTS) that consists of a sustained increase of heart rate of at least 30 beats per minute or a sustained pulse rate of 120 beats per minute. Tilt table testing has been used mostly to study syncope in patients with no evidence of structural heart disease (6)

Autonomic Nervous System and Pain

For more than a century it has been assumed that abnormal activity of the sympathetic nervous system may be involved in the pathogenesis of protracted pain syndromes. This assumption was based mainly upon the observations that the pain is spatially correlated with signs of autonomic dysfunction and that blocking the efferent sympathetic supply to the affected region relieves the pain. This latter premise led to the clinical concept of *sympathetically maintained pain* that is applied to those neuropathic pain cases that respond to sympatholytic maneuvers (7). Sympathetically maintained pain has strong and ample foundations in the animal model, in contrast the clinical information sustaining this pathogenesis is mostly anecdotal and do not fulfill in most instances the strict evidence-based Medicine criteria. This paradox derives from the fact that most clinical evidence of sympathetically maintained pain comes from battle-field Medicine, that in most instances do not fulfill the evidence-based medicine criteria.

Animal Studies of Sympathetically Maintained Pain

Under normal circumstances primary afferent nociceptors do not have catecholamine sensitivity, however, under pathological conditions, particularly after trauma, a sympathetic-afferent interaction can be established both at the peripheral and central levels.

In a rabbit model, after peripheral nerve injury sympathetic stimulation and norepinephrine are excitatory for a subset of skin C-fibers nociceptors that express alpha-2 adrenergic-like receptors (8). Perhaps more germane to the pathogenesis of sympathetically maintained pain are the experimental models that have been extensively reproduced in which after nerve injury, sympathetic sprouting at the dorsal root ganglia becomes apparent and form basket-like structures around large-diameter axotomized sensory neurons; sympathetic stimulation can activate such neurons repetitively. (9) Another site of abnormal post-traumatic connections occurs in the dorsal horn of the spinal cord, where there is an A-fibers sprouting into the superficial layers thus provoking that tactile stimuli be felt as painful. This mechanism may explain the allodynia.

History of the Sympathetically Maintained Pain Concept in Clinical Practice

For over a century military physicians have reported cases of persistent burning pain in soldiers that sustained traumatic partial peripheral nerve injury. Typically in such cases, the pain is accompanied by hyperalgesia (defined as “an increased response to a stimulus which is normally painful”), allodynia (defined as “pain due to a stimulus which does not normally provoke pain”) and distal extremity swelling. Mitchell in 1865 coined the term *causalgia* (literally “burning pain”) to describe such cases (10). Leriche in 1916 reported that

sympathectomy dramatically relieves causalgia (11). This procedure was found to be effective in large series reports from different military physicians in different war events throughout the twentieth century including the recent Iran-Irak war (12).

Later on, it became apparent that sympathetically maintained pain can also affect civilians and that may develop after injuries not involving nerve trauma such as bone fractures, and also after diverse illnesses such as severe infection, stroke and myocardial infarction. Those cases also found relief with sympathetic blockade. Evans in 1946 coined the term *reflex sympathetic dystrophy* to diagnose such cases (13).

In 1994, the International Association for the Study of Pain, introduced the term "Complex Regional Pain Syndrome" (CRPS) to replace the terms causalgia and reflex sympathetic dystrophy. One of the arguments for the taxonomic change was the inconstant evidence of sympathetic hyperactivity in such instances. CRPS type 1 was introduced to substitute reflex sympathetic dystrophy and CRPS type 2 to be used instead of causalgia (14).

Controversy over the Sympathetically Maintained Pain Concept

The clinical observations in favor of the sympathetically maintained pain concept can be summarized as follows: a) Pain is spatially correlated with autonomic alterations, b) Sympatholytic maneuvers diminish the pain intensity and, c) Application of norepinephrine, the sympathetic neurotransmitter, rekindles the pain (7).

a) Pain is spatially correlated with autonomic alterations. In CRPS type I, temperature changes occur in the affected limb. In early stages vasodilation induce increased local temperature. Later the vasculature may develop sensitivity to cathecolamines possibly related to up-regulation of adrenoceptors, and decreased local temperature may supervene. Typically hyperhidrosis accompany the temperature changes.
b) Sympatholytic maneuvers diminish pain intensity. As described above, historically this has been the main argument in favor of the sympathetically maintained pain concept. Different techniques are used to accomplish this task: Surgical interruption of the sympathetic nerves proximal to the affected site, injection of local anesthetic around the sympathetic paravertebral ganglia that projects to the affected body part, regional intravenous application of adrenergic blocking agents such as guanethidine or reserpine or systemic intravenous infusion of phentolamine
c) Local application of catecholamines rekindles the pain. This phenomenon has been described in different types of neuropathic pain. After limb amputation, injection of epinephrine around the stump neuroma is intensely painful. Similar response has been observed in posttraumatic or postherpetic neuralgias (7).

A more physiological adrenergic stimulus has been recently used. Baron et al reported that whole body cooling induces endogenous catecholamines production and also worsen the pain and hyperalgesia in CRPS (15)

There is emerging genomic evidence that supports the sympathetically maintained pain concept. As mentioned before healthy individuals with specific variations in the COMT gene have a defective catecholamine-clearing enzyme. Such variations are also associated to increased pain susceptibility due to activation of the beta adrenergic receptors.

Arguments against the Sympathetically Maintained Pain Concept

Several authorities on the field seriously doubt the sympathetically maintained pain concept arguing the scarcity of controlled clinical studies supporting this idea, the lack of specificity of sympathetic blockade procedures that involve not only sympathetic fibers but also somatic nerves, and the lack of correlation between pain and sympathetic dysfunction. Another argument used against this concept is the prominent psychiatric component that some patients with this diagnosis display (16).

Sympathetic Nervous System Dysfunction in Fibromyalgia

There is accumulating evidence to suggest that abnormal activity of the sympathetic nervous system may play a key role in the pathogenesis of fibromyalgia. Bengtssong and Bengtssong published the first study in 1988 (17). It was a controlled therapeutic trial of stellate ganglion blockade. They reported marked improvement of regional pain and tenderness as response to this maneuver, in contrast to the lack of effect to a sham injection in the neck area. Subsequently Vaeroy et al using Doppler probes to measure skin blood flow in the hands, found that FM patients have less vasoconstrictory response to acoustic stimulation and cooling. They concluded that the cutaneous manifestations of FM previously interpreted as Raynaud's phenomenon should be reconsidered (18). These two seminal studies raised the possibility of sympathetic nervous system involvement in the pathogenesis of FM. Later on, Elam et al. recorded muscle sympathetic activity with microelectrodes placed at the peroneal nerve level (19). They found no exaggerated sympathetic activity in FM subjects, nevertheless these patients displayed less pronounced sympathetic activity as response to muscle contraction.

Since the publication of these seminal studies, little attention was paid to dysautonomia in FM until recently, with the introduction of heart rate variability and tilt table testing in the study of FM pathogenesis.

Heart Rate Variability Analysis and Tilt Table Testing in Fibromyalgia

Our group has used heart rate variability analysis to assess ANS function in patients with FM. Our first study was short term and it was intended primarily to define the response of the autonomic nervous system to a simple active orthostatic stress (to stand-up). Our main result showed that when compared to controls, patients with FM failed to increase low frequency ("sympathetic") band power as response to the up-right posture (20). This orthostatic sympathetic derangement has been confirmed by Kelemen et al (21) and by Raj et al (22) using the same method. Bou-Holaigah et al used tilt table testing to assess the response of FM patients to passive orthostatic stress. They found that during upright tilt 12 of 20 fibromyalgia patients (60%) but no controls had abnormal drop in systolic blood pressure of at least 25 mmHg and no associated increase in heart rate (23).

So this body of evidence strongly suggests that there is an orthostatic sympathetic derangement in patients with FM.

As technology and knowledge of heart rate variability evolved, we undertook a long-term study in patients with FM. This was intended to assess the circadian behavior of the ANS. Patients and controls wore a Holter monitor for 24 hrs. while performing their routine daily activities. Both time domain and frequency domain analyses demonstrated that patients with FM have changes consistent with relentless sympathetic hyperactivity during 24 hrs. This alteration was particularly evident at night (24). This sympathetic hyperactivity in FM has been confirmed by investigators from different parts of the world and represents perhaps the most consistent alteration found so far in FM.

So, it can be concluded that patients with FM display prominent dysautonomia when studied by means of heart rate variability analysis and/or tilt table test. This dysautonomia can be characterized as a sympathetic nervous system that is persistently *hyperactive* but *hypo-reactive* to stress. This apparent paradox (sympathetic hyperactivity with hypo-reactivity), nevertheless agrees with the basic physiological principle demonstrating that chronic hyperstimulation of the beta-adrenergic receptors leads to receptor desensitzation and down-regulation

The hypo-reactivity to stress concurs with the early reports of Vaeroy et al (18) and Elam et al (19). showing that FM patients have less peripheral sympathetic response to acoustic stimulation, cooling or muscle contraction.

Dysautonomia May Explain The Multisystem Features of FM

Fibromyalgia is a multisystem illness. The multicenter study that led to the ACR diagnostic criteria for FM, defined that besides its defining features (chronic widespread pain and tenderness at palpation in specific locations) patients with FM have significantly higher rates of diverse clinical manifestations when compared to patients with other rheumatic diseases. Such distinctive features are: sleep disorders, fatigue, paresthesias, headache, anxiety, sicca symptoms, Raynaud's phenomenon, morning stiffness and irritable bowel (27). So any valid theory attempting to explain the pathogenesis of FM should first give a coherent explanation for the presence of these disparate features in the same patient.

Autonomic nervous system dysfunction may explain the diverse clinical manifestations of FM . It is suggested that due to a ceiling effect, the hyperactive sympathetic nervous system of such patients becomes unable to further respond to different stressors, thus explaining the constant fatigue and morning stiffness these patients have. Relentless sympathetic hyperactivity may explain sleep disorders, anxiety, pseudo-Raynaud's phenomenon, sicca symptoms and intestinal irritability (28). Concurrent analyses of heart rate variability and polysomnography in subjects with FM have shown changes consistent with nocturnal sympathetic hyperactivity. Such changes coincided with increased nocturnal arousal-awakening episodes (29). In normal sleeping persons, electrocardiographic signs of sympathetic surge precede arousal/awakening episodes. This body of evidence suggests that in FM, sympathetic hyperactivity causes the excessive arousal/awakening episodes (29).

Fibromyalgia as a Sympathetically-Maintained Neuropathic Pain Syndrome

The defining FM features (widespread pain plus tenderness at palpation on specific anatomical points) as well as the paresthesias that these patients have, could be explained by the pathogenesis known as "sympathetically maintained pain" (30). The clinical manifestations of this type of neuropathic pain are characterized by its frequent post-traumatic onset and by the presence of stimuli-independent pain perception that is accompanied by paresthesias and allodynia. Such are precisely FM pain features: Different controlled studies have determined that subjects with FM have higher rates of physical or emotional trauma prior to the onset of their symptoms. FM is clearly a stimulus-independent pain state since there is not underlying structural damage, inflammatory signs are conspicuously absent. Most patients with FM have paresthesias as demonstrated in the original study that lead to the ACR criteria. In this study over 80 % of patients with FM had such sensory alteration. Simms and Goldenberg corroborated the extremely high prevalence of paresthesias (30). We used a questionnaire that is part of the Leeds Assessment of Neuropathic Symptoms and Signs Pain Scale. This instrument was developed to recognize neuropathic pain and set it apart from nociceptive pain. The questionnaire contains five items exploring 5 different domains within the paresthesia syndrome. Our study showed that the overwhelming majority of patients with FM give assenting responses to questions pertaining to dysesthetic, evoked, paroxysmal and thermal domains. This response rate was markedly different from that given by patients with active rheumatoid arthritis (31). Several groups of investigators have defined that the typical fibromyalgia tender points reflect a state of generalized hyperalgesia (32). Hyperalgesia in the absence of underlying tissue damage, strongly suggests a neuropathic etiology of the pain. It is interesting to notice that most FM tender points are located in the neck area, a zone with very rich sympathetic ganglia network. Nowhere else in the body are the sympathetic ganglia so near to the skin.

The clinical impression of FM as sympathetically-maintained neuropathic pain syndrome is now supported by experimental evidence. A prospective double-blind study demonstrated that 80% of subjects with FM have norepinephrine evoked pain compared with 30% of patients with rheumatoid arthritis or normal controls (33).

A prototype of sympathetically maintained pain syndrome is reflex sympathetic dystrophy, (nowadays named Complex Regional Pain Syndrome type I). There are important points of coincidence between reflex sympathetic dystrophy and FM. We propose that FM is a generalized form of reflex sympathetic dystrophy (34).

Management of Dysautonomia in Patients with Fibromyalgia

When discussing the management of dysautonomia in FM is important to first emphasize several points: The ANS is the main homeostatic system, therefore it regulates the function of most organs and systems of the body. As a consequence, a great variety of therapeutic modalities have secondary autonomic side-effects. Several drugs already tested in FM have clear autonomic consequences such as antidepressants and tranquilizing agents. It is also important to notice that the recognition of dysautonomia in FM is recent, therefore there are not controlled studies assessing the efficacy of drugs that directly affect autonomic neurotransmitters on the diverse manifestations of this painful syndrome. What will be

discussed in this section deals with theoretically promising therapies focused to correct autonomic abnormalities. Again is important to notice that, unless otherwise stated, these are unproven remedies that should first be tested by appropriate clinical research before considering them for clinical use (35).

Non-pharmacological Therapies

Since sympathetic hyperactivity is prevalent in FM, it seems wise to ask patients to avoid the intake of sympathomimetic products such as nicotine, caffeine containing soft drinks and coffee. Graded aerobic exercise has a proven benefit for FM symptoms (36), this type of exercise also improves resting vagal tone (37). Biofeedback, a mind-body therapy technique that improves sympatho/vagal balance when analyzed by heart rate variability studies (38) has been found to enrich self-efficacy for function as well as to improve tender point score in patients with FM (36).

As discussed before, many patients with FM have orthostatic hypotension. Based on what is recommended for patients with idiopathic orthostatic hypotension (39), we suggest patients with FM to have a liberal intake of water with high mineral contents. It is my impression that this simple therapy may improve patient's well being, particularly their chronic fatigue. The use of fitted stockings to the waist is another measure that may decrease blood pooling in the legs

Pharmacological Therapies

The use of medications should reserved for those cases in which the intensity of the symptoms markedly constrains their quality of life. It is clear that in this chronic illness with dramatic manifestations in diverse organs and systems of the body, polypharmacy should be avoided.

Mounting evidence suggests that FM patients lose their autonomic circadian rhythmicity, with nocturnal sympathetic hyperactivity, this alteration likely induce the sleep disorders. When insomnia is prominent, we recommend start with low dosages of sleep-inducing medications i.e. clonazepam 0.25 - 0.5 mg HS. When anxiety is also evident during the day we add clonazepam 0.125 mg after breakfast and lunch.

We seldom use tricyclic antidepressants unless there is associated depression. Although this type of medications has proven its short-term efficacy in FM when assessed by double blind studies, in our experience, side effects such as anxiety, dizziness and xerostomia limit its usefulness.

In view of the relentless sympathetic hyperactivity that these patients have, it seems logical to use adrenergic blocking agents. We have used, in selected cases (young patients with prominent autonomic manifestations such as palpitations, clammy hands, orthostatic tachycardia and/or profound fatigue) low dosages of the beta blocking agent propranolol (i.e. 10 mg t.i.d.). Alpha-adrenergic blocking agents would theoretically be useful in FM pain, since in sympathetically maintained pain syndromes, primary nociceptors start to express alpha adrenoceptors. Nevertheless current available alpha blocking agents have profound cardiovascular effects that probably will make them unfit for this type of clinical use.

Fludrocortisone with a starting dose of one mg per day may help to increase blood volume thus avoiding orthostatic hypotension. It should be noted however that a double-blinded study found that this medication given as monotherapy to patients with chronic fatigue syndrome failed to improve their symptoms (40)

Serotonin has similar chemical structure and co-localizes with nor-epinephrine in different receptor sites. Low dosages (5 mg) of tropisetron, a highly selective and competitive 5-HT3-receptor antagonist was found to be effective in FM in a double blind short-term (10 days) study. Higher dosages lost their therapeutic benefit, suggesting a bell-shaped dose response curve (41). Interestingly in a different study by the same research group in which they measured circulating cathecolamines, those patients with elevated basal dopamine tended to show a higher response rate to tropisetron (42).

In view of the fact that FM pain has been proposed to have a neuropathic etiology (31), drugs used in diverse types of neuropathies could theoretically have a beneficial effect for FM subjects. Gabapentin is being used as an off-label indication. We prescribe this compound in escalating dosages in patients with marked paresthesias, We start with 300 mg at night and slowly increase it to no more of 1500 mg in three divided doses. Xylocaine is a potent sodium channel blocker, Raphael et al used lignocaine intravenously in an open study. They injected up to 550 mg x day, per 6 days as an in-hospital treatment. Most of their patients reported pain relief that lasted a mean of 11 weeks (43). This provoking investigation should be further evaluated in controlled studies. It is worth noting that new types of antineuropathic medications are in the development period, such as sodium channel blockers with greater analgesic than anticonvulsant index, new GABA-enhancing drugs, and N-methyl-D-aspartate (NMDA) antagonists, among others (44). So, would be important to follow closely the developments in antineuropathic therapies as this knowledge may be also useful for FM treatment.

Conclusion

Dysautonomia provides a different perspective on the mechanisms that lead to FM. This new approach offers a coherent explanation for FM's multisystem symptoms including its psychological component. The proposal of FM as a sympathetically-maintained neuropathic pain syndrome may serve two purposes, on the one hand it validates FM pain as real and on the other it provides an opportunity to examine the unfolding neuropathic pain research knowledge and apply it to the understanding of FM.

Of course more research is needed to consolidate this paradigm

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Chapter 14

THE ALTERATIONS AND FUTURE RESEARCH IN SYMPATHETIC SURGERY

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Abstract

Sympathetic surgery has been performed for over a century. Its indications include a large variety of disorders e.g. angina pectoris, exophthalmic goiter, spastic paralysis, Raynaud's syndrome, reflex sympathetic dystrophy, peripheral occlusive vascular disease etc. However, they are now almost abandoned due to unpredictable and poor surgical outcome. Currently, hyperhidrosis is the most common indications owing to the high successful rate, low recurrence and good patient's satisfaction. The only problem remain unsolved is the troublesome post-operative side effect of compensatory hyperhidrosis, which on occasion causes regret for the procedure. Certainly, the modification of the surgical procedure e.g. ramicotomy, clipping etc. do largely purpose on lowering the rate of the side effect and increase the patient's satisfaction.

During the recent decade, the authors reviewed and analysed over a thousand operated patients and acknowledged that the etiology of the so called "compensatory hyperhidrosis" might be based on the level of ganglion interruption. More than that, we proposed a mechanism of compensatory hyperhidrosis and suggested it should be replaced by the term "reflex sweating".

Lin classification for sympathetic surgery is another new idea reported in 2001. With this classification, we can perform sympathetic block on a definite ganglion in a specific sympathetic disorder.

Through these several decades, the instruments are getting more and more advanced and refined so that the procedure can be performed in safe and effective condition. Besides, the post-operative pain is reduced and the cosmetic is excellent.

The future goal for sympathetic surgery is to stop the unwanted sweating, inducing no compensatory hyperhidrosis - the idea of "single determinant ganglion" for each sympathetic disorder.

In addition to palmer hyperhidrosis, sympathetic surgery has recently been extended to facial blushing, facial sweating and others, with satisfactory results. More than that, a certain percentage of essential hypertension is due to sympathetic over-activity. We treated a small number of such cases using sympathetic blockade. They responded well and the results were published in 2005. We should further follow-up and perform larger studies in this area for the actual etiology and its relation with dopamine.

Introduction

Sympathetic surgery was introduced in the late nineteenth century to treat a large variety of diseases [1-9]. During the twentieth century, most of these indications have gradually been discarded due to poor response or low success rate. In current practice, the most common indication is hyperhidrosis. The results, including success rate, recurrence, and patients' satisfaction, are reported to be good although the side effect, the so–called "compensatory hyperhidrosis", is still a matter of serious concern. Other rarer indications (e.g. control of pancreatic pain and Raynaud's syndrome) still need further studies. Moreover, essential hypertension is another field to be studied.

In addition to changes in indications, surgical techniques and instruments are improving and advancing, thereby reducing post-operative pain and resulting in better cosmetics.

Compensatory hyperhidrosis has long been discussed but is still unsolved. This troublesome post-operative side effect sometimes even causes patient to regret the procedure. To solve this problem, clipping technique, which is a reversible procedure, was introduced in 1998 [10]. To minimize the rate of compensatory hyperhidrosis, solution e.g. ramicotomy [11,12] was likewise introduced. However the recurrence rate remained high.

In 2001, the authors, unlike the current T_2 or T_{2-3} interruption, suggested T_4 interruption for palmar hyperhidrosis [13]. This was a great revolutionary change that controlled not only excessive sweating but also compensatory hyperhidrosis. Moreover, the authors reported a new classification for sympathetic surgery [14].

In 2006, the authors proposed a mechanism of compensatory hyperhidrosis [15], which explained some important clinical facts. The authors further suggested the use of the term "reflex sweating" instead of compensatory hyperhidrosis.

In the future, the direction of studies should point to (i) reducing the rate of reflex sweating; (ii) discovery of the determinant sympathetic ganglion for each disorder; (iii) the relationship between catecholamines and sympathetic nerve; and (iv) the role of sympathetic surgery in the treatment of essential hypertension.

Alterations in Surgical Techniques and Instruments

Sympathetic surgery was first introduced in the early twentieth century for the treatment of vasomotor disorders [16,17,18] and hyperhidrosis [19]. The former responded quite variably and the recurrence rates were high after sympathectomy [20]. Thus, it is now less commonly considered an indication. For hyperhidrosis, the reported [15,21,22,23,24,25] success rate and patients' satisfaction was high, while the recurrence rate was low. This is now the main indication.

The incidence of hyperhidrosis is particularly high in Asia [26]. The reported incidence among young people in Taiwan (authors' country) is approximately 0.3% [27]. Cloward [28] and Adar [29] both reported specific ethnic predisposition with a high incidence among Jews, North Africans, Temen, and the Balkans, although the reason was still unknown. Some authors [29] have thought that the warm climate is a related factor, but later reports [30-34] from Europe disproved such hypothesis. In the authors' series, family history is rare.

Excessive sweating causes a lot of trouble in daily academic and occupational activities, especially when the use of electronic equipment and computers are indispensable. Indoor climate control cannot solve the problem since the sweating is not temperature related. It also affects normal social activities, eventually causing withdrawal and depression.

Different methods of treatment have been designed, including iontophoresis and anti-cholinergic therapy [35]. However, the success rate is so low that these were almost discarded. Up to now, surgical treatment of sympathectomy remains the method of choice. Various surgical approaches, including the open thoracotomy [36], the dorsal approach [37,38], and the trans-axillary [39] approach have been described. However, they all have also been banned due to the relatively significant post-operative trauma and poor cosmetics.

In the current practice, endoscopic sympathetic nerve interruption is the best choice. It was first introduced by Hughes in 1962 [40]. In recent decades, it has been further refined, especially with new, minimally invasive techniques using more advanced instruments. In addition to less post-operative pain and excellent cosmetics, the endoscopic method is also safe and effective, with a high success rate [10,21,22,25]. Most patients are satisfied, especially with the introduction of the clipping method [13]. But the biggest problem - compensatory hyperhidrosis - remains a troublesome side effect, which can be so severe and annoying to cause regret for the operation.

Gossot [11] introduced ramicotomy to reduce the rate of compensatory hyperhidrosis. However, the longer operative time and the high recurrence rate did not lead to further acceptance. Ramicotomy is now rarely used. Another procedure was limited sympathectomy that was confined to T2 level only. Less compensatory hyperhidrosis was reported. However, recurrence after T2 sympathectomy was high because of Kuntz fibers [41]. According to the authors' proposed mechanisms [15], T_2 interruption should cause more serious compensatory hyperhidrosis than T_3, T_4, or T_5 interruption.

Alterations in Level of Interruption and the Concept of Reflex Sweating

Until 2001, Lin [13] reported T_4 sympathetic interruption for palmar hyperhidrosis that not only obtained a high success rate but also induced minimal compensatory hyperhidrosis. The discovery of T_4 interruption for palmar hyperhidrosis was accidental while utilizing the clipping instead of the cauterization or cutting methods. One of the advantages of the clipping method was its recognition of the interrupted level [42], which was easily detected through routine post-operative chest radiography. During the practice, the authors found a 4.4% rate of unilaterally clipping at the unintended level [43], which produced different outcomes between the two sides. For example, in some cases of facial sweating, T_3 was the target ganglion. However, because of misjudgement, one side T_3 and the contralateral T_2 clipping

was performed. As in some cases of palmar hyperhidrosis, in which T_4 was the target ganglion, post-operative one side T_3 and contralateral T_4 clipping were found. After thorough reviewing of those "erroneous" cases, we found that for palmar hyperhidrosis, T_4 interruption controlled excessive palm sweating effectively while keeping compensatory hyperhidrosis minimal.

Under this clinical experience, we realized that T_4 may be the more appropriate level of interruption for palmar hyperhidrosis. We subsequently performed bilateral T_4 sympathectomy for palmar hyperhidrosis in 84 cases [44] and the results were satisfactory. Recently, Neumayer [25] and Choi [24], who performed T_4 sympathectomy for palmar hyperhidrosis, reported a 3-8% rate of compensatory hyperhidrosis, which was much less than the 30-70% after T_2 or T_{2-3} sympathectomy [45].

This discovery not only revolutionarily changed the level of interruption for the treatment for palmar hyperhidrosis, but also inspired the philosophy for the mechanism of compensatory hyperhidrosis.

For a very long time, the change of pattern of locations of sweating after sympathectomy has been referred to as "compensatory hyperhidrosis". However, based on clinical facts and experience, the authors suggested that "compensatory hyperhidrosis" is a misnomer or a misused medical term because it might be attributable to a reflex response in the sweating center of the hypothalamus, and not a compensatory mechanism.

During the clinical practice covering over 1,000 cases of hyperhidrosis, the authors found that (i) different levels of sympathectomy result in different degrees of post-operative sweating (severe post-operative sweating was found with the T_2 procedure while almost no compensatory hyperhidrosis was noted in the T_4 or T_5 procedure); (ii) no change of sweating pattern after lumbar sympathectomy for plantar hyperhidrosis; (iii) sweating problem induced after sympathectomy for non-sweating sympathetic disorder e.g. facial blushing; and (iv) the amount of increase sweating not corresponding to the amount reduced after the sympathetic procedures. All of these may be due to a reflex response for which the authors advocate using the term "reflex sweating" instead of "compensatory hyperhidrosis".

How can the aforementioned clinical facts be explained? The neuro-anatomy of the sympathetic nervous system must be reviewed. The sympathetic nervous fibers originate from the intermediolateral horns of the spinal cord between T1 and L2. Each fiber is composed of pre- and post-ganglionic neurons. The post-ganglionic fibers arising from the ganglia in the sympathetic trunk, running through the grey ramus communicans and re-entering the corresponding spinal nerves, are distributed to the sweat glands.

In the sympathetic trunks, the fibers may go upward and downward before leaving and being distributed to the target organs. Therefore, the distributions overlap and are not necessarily to the same part of the body from the same spinal segment. The efferent fibers are accompanied by the afferent fibers. The autonomic nervous system functions the same as the neuro-endocrine system [46], through positive and negative feedback mechanisms, which mean that signals from the central control center (hypothalamus) to the target organs are efferent positive signals whereas the target organs send negative afferent signals to the central control center. The feedback mechanism of the sympathetic nerve is illustrated in figure 1 [15].

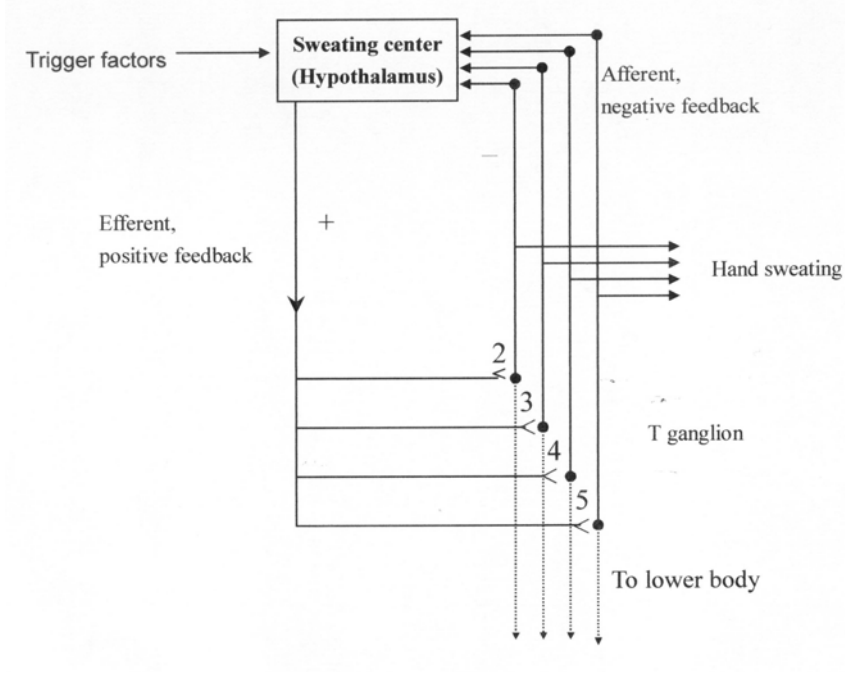

Figure 1. Schematic diagram showing the mechanism of central sweating.

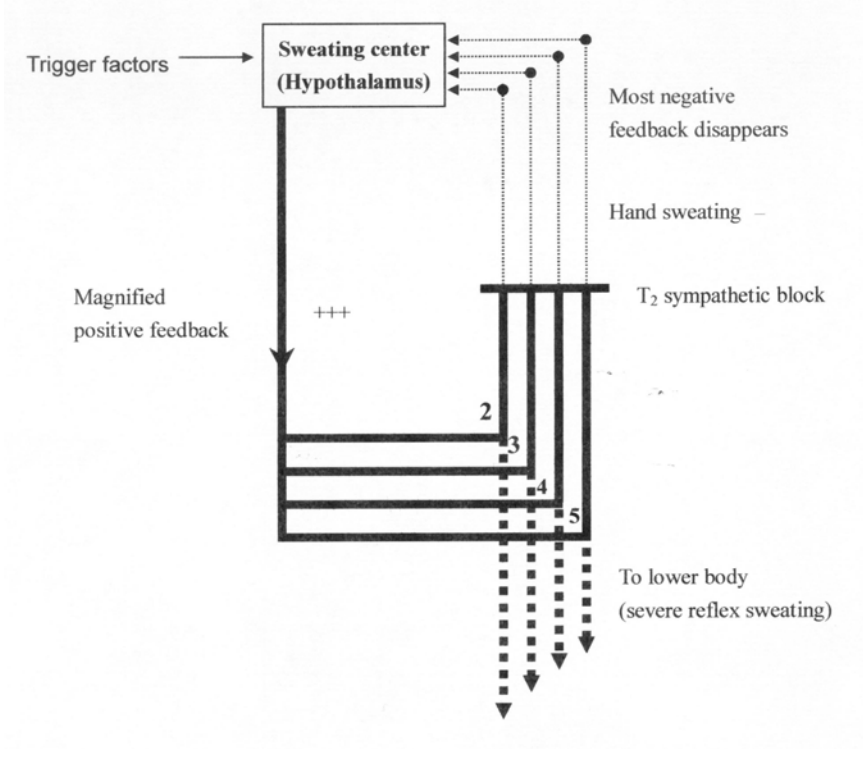

Figure 2. Schematic diagram showing the proposed mechanism of reflex sweating with T2 sympathetic block.

The hypothalamus sends positive efferent signals to the sweat glands and from the sweat glands, the negative afferent feedback signals are sent to and control the central center. In clinical practice, T_2 sympathectomy has the most severe compensatory hyperhidrosis, less in T_3, and least in T_4 or T_5. In case of sympathectomy, e.g. T_2 interruption (Fig.2) [15], most of the negative feedback signals to the central nervous system are stopped. Therefore, the efferent positive feedback signals to the sweat gland are strong and the compensatory hyperhidrosis is most severe. In T_3 blockade, fewer afferent negative feedback signals are interdicted so that parts of the efferent positive signals are blocked and the compensatory hyperhidrosis is less severe. For T_4 and even T_5 blockade (Fig.3)[15], much fewer afferent negative feedback signals are interrupted (more preserved) so that the efferent positive signals are much weaker and the reflex sweating is much less severe.

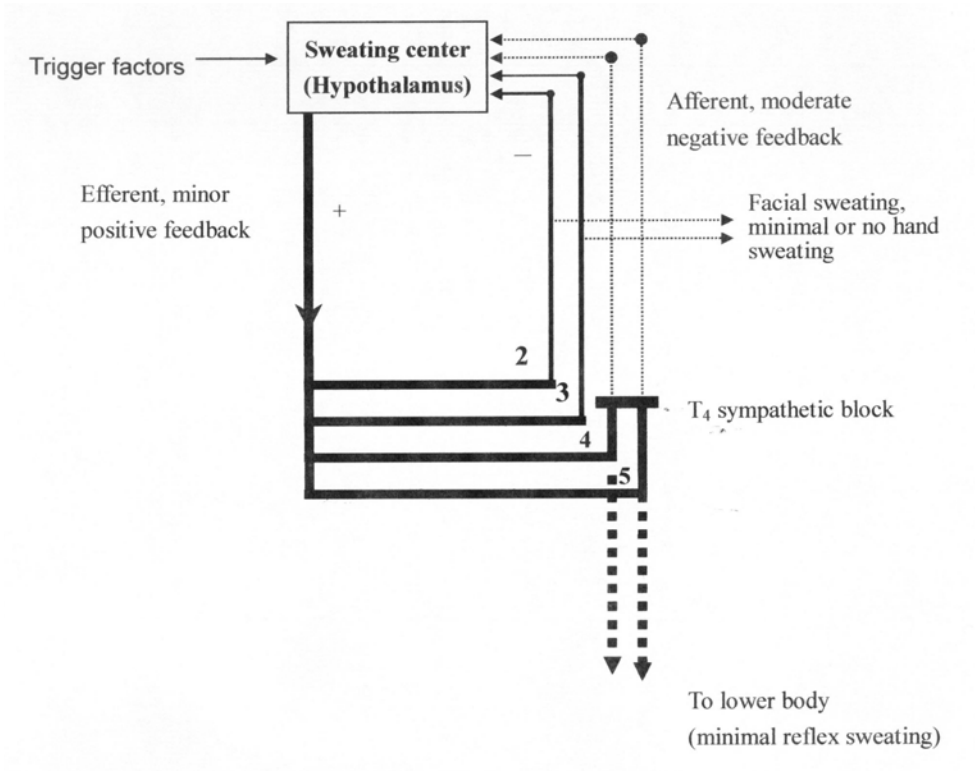

Figure 3. Schematic diagram showing the proposed mechanism of reflex sweating with T4 sympathetic block.

Reflex sweating always happens over the lower torso and never over the upper body because the nerve to the upper trunk has already been blocked. Currently, most surgeons still perform T_2 or $T_{2\text{-}3}$ sympathectomy for palmar hyperhidrosis [47]. The shifting to T_4 blockade for palmar hyperhidrosis in the near future must be anticipated. T_2 sympathectomy is performed for facial blushing but sometimes causes regret due to severe reflex sweating. The authors suggest the clipping method when the level of interruption is high, owing to its reversibility.

Based on the above findings and proposed mechanism of reflex sweating, we set up a new classification for sympathetic surgery [15]. In this classification, endoscopic block of T_2

is performed for facial blushing, T_3 for facial sweating, T_4 for palmar hyperhidrosis, and T_5 is for axillary sweating.

Alterations in Indications

Since the first sympathectomy was performed by Alexander in 1889 [1], the surgical procedure was used for various disorders, including angina pectoris [5] and exophthalmic goiter [3,7]. In the early 1900's, Raynaud's syndrome was a common indication [16,17,18], although it is now largely abandoned due to the variable response. Around the third decade of the twentieth century, spastic paralysis [6,8,48] was thought to be relieved with sympathetic nerve influence. However, this became obsolete because of the poor effect. Another less common indication was reflex sympathetic dystrophy, in which the success rate was not high [47]. Hypertension was once an indication [49] but only for a while. Certainly, the authors recently treated some cases with sympathetic hypertensive syndrome, which will be discussed later in this chapter. Peripheral occlusive vascular disease was successfully treated with angioplasty or arterial bypass so that sympathectomies were less commonly used. There were other rare indications, such as social phobia [50], long QT syndrome [47], and pancreatic pain [51], which all needed further studies.

By far the most common indication of sympathectomy is primary hyperhidrosis, including palmar hyperhidrosis, axillary hyperhidrosis, facial sweating [7], and plantar hyperhidrosis. Compared to the first three disorders, plantar sweating is less commonly reported. The most interesting thing is that in a certain percentage of patients who have undergone upper thoracic sympathectomy, their sole sweating simultaneously subsided. The reason is unknown and the mechanism is far from being fully understood. In recent years, facial blushing has become another new indication and the effect is reported to be good.

Alterations and Errors in Surgical Procedures

In the last decade, operative procedures in sympathetic surgery continued to be modified, from ganglionectomy (sympathectomy), sympathicotomy, ramicotomy to the clipping method. Modifications in surgical techniques are mainly to design an ideal method for lowering the rate of reflex sweating and increase patients' satisfaction.

In the current practice, the procedure is usually performed under general anesthesia with a single lumen tracheal intubation. The patient is placed supine with both arms abducted. Some doctors [47] use dual-lumen anesthesia with the patient in a modified lateral decubitus position. Either two 5-mm ports or a single 10mm-port is used. For the former, one port at the axilla and one at the mid-axillary line at the level of the nipple are inserted. For the single 10-mm port, it is inserted at the third intercostal space over the mid-axillary line. During sympathectomy, the pleura is opened along the sympathetic trunk. The upper and lower ends of the target ganglion are cut with a diathermy probe and the ganglion is removed.

For sympathicotomy, the procedure is the same as sympathectomy but without the removal of the ganglion. The ports are removed after the lung is fully inflated by the anesthesiologist. Sterile stripes are used for sealing both wounds. For the 10-mm port, one or two stitches are needed. Ramicotomy was introduced by Wittmeser [12] and Gossot [11] in

1997. Only the T_2 and T_3 rami are divided, which theoretically reduces the rate of reflex sweating. However, the procedure is more complex, with a longer operative time and a high recurrence rate.

For the advantage of reversibility in case the patient regrets the procedure, we [13] described the clipping method in 1998. The procedure is the same as sympathectomy except no diathermy is applied. Instead, endoscopic clips are applied over both ends of the target ganglion. The clips exert a force of approximately 150g [52]. Since a compressive force of more than 44g to the nerve fibers is enough to interrupt the nervous signal, the clips at both ends are effective for the purpose and works even when the clip is applied only on the upper end of the target ganglion [24]. The success, recurrence, and complication rates are the same as that of sympathectomy and sympathicotomy but with an advantage of reversibility.

In case the patient regrets the procedure due to intolerable reflex sweating, the clips can be removed under general or local anesthesia through the retained suture sling [53]. However, the reversible procedure should be performed within two weeks as Denny-Brown and Brenner [52] reported that compression for more than two weeks causes demyelination. In addition to the reversibility, changeability and immediate recognition of the clipping location are the other two advantages. There is still a 4.4% rate of clipping at the unintended level, even by an experienced surgeon [43]. Using diathermy, the surgeons will not know if they cut or remove the wrong ganglion because identification of the correct level is impossible. Even when the patients expresses the different feelings between the two sides, the surgeons rarely admit their misjudgement and only blame the temperamental nature of the patient. A Japanese group performed the procedure under fluoroscopy and advocated never making mistake [43]. The design is clever, however, there would be no lesson learnt when no mistake is made, i.e. we learn something during making mistake. The clipping level can be clearly seen on routine post-operative radiography, so that the different outcome between the two sides can be explained. Actually, the Lin classification [15] has been developed after reviewing such interesting cases. The clips also serve as a good marker for the pre-operative assessment for the reversible operation. The question of misjudgement is another lesson to be studied and up to now, there is no adequate explanation.

Recent Discoveries

In 2002 [54], the authors treated a 62-year-old female who suffered from craniofacial hyperhidrosis associated with a poor controlled hypertension of around 180/110mmHg, with a heart rate of about 105/min. She underwent aggressive medical treatment for her hypertension with three combined anti-hypertensives for 10 years, with poor results. She consulted with the author for treatment of craniofacial hyperhidrosis and bilateral T_3 endoscopic sympathetic block was performed. Post-operatively, her blood pressure was lowered to around 145/95mmHg. Afterwards, it was well controlled to around 125/80mmHg with intake of 5mg calcium channel blocker. After reviewing the literature, 30% to 40% of essential hypertension was found to be of sympathetic origin [55]. Some authors [56,57] had already published articles on the relationship between sympathetic tone with the heart and blood vessels.

Schachter [55] reported that the sympathetic nervous system is by far the most important effector pathway in blood pressure regulation. Neural control mechanism of blood pressure

regulation includes the ventrolateral and dorsomedial medulla in the brain stem (i.e. the baroreceptor reflex), while the hypothalamus is the higher center of blood pressure regulation. The entire baroreceptor reflex regulation is illustrated in Fig. 4. The baroreceptor in the arterial wall is stimulated when the vascular tone increases, which has a positive effect in the nucleus tractus solitarius. From the nucleus tractus solitarius, an inhibitory signal is sent to the rostral ventrolateral medulla, which causes the decrease in blood pressure. The influence of sympathetic surgery should also be related to the β-blocker effect [58]. Certainly, the exact mechanism remains unclear.

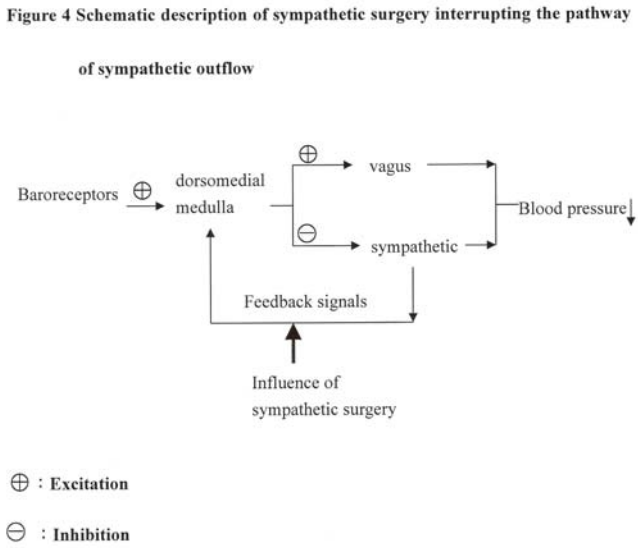

Figure 4. Schematic description of sympathetic surgery interrupting the pathway of sympathetic outflow.

We performed T_3 sympathectomy on a total of 17 patients who had craniofacial hyperhidrosis and hypertension. We noted that the patient who has stage II hypertension [59] with craniofacial hyperhidrosis and a heart rate of >100/min responded well to the sympathetic surgery. We called this triad “Sympathetic Hypertensive Syndrome” [54]. Also, males under 40 years of age responded better but the difference did not reach the level of statistical significance. Larger scale studies that include animal experiments and with long follow-up periods should be performed in order to sort out the actual physiologic effects of sympathectomy.

Future Prospects

Owing to the increased use of electronic devices as well as global warming, the population of patients with hyperhidrosis requiring treatment by sympathectomy will continue to increase. The problem of reflex sweating needs further studies and research. Although the authors have proposed an explanation to the post-operative changes in sweating pattern, the true etiology remains obscure. The main obstacle of the studies is the subjectivity and variability of the surgical outcome. There will also be gaps between animal experiments and actual clinical

practice. Large studies, including multi-centered, meta-analysis must be performed promptly. Although the clipping method provides a chance of reversibility, the ideal procedure of effectively reducing the excessive sweating but causes minimal reflex sweating is the final goal. The concept of "single determinant ganglion", like T_4 ganglion for palmar hyperhidrosis, has already been suggested by the authors. However, this still needs further advancement and the procedure must be more definite and refined.

Essential hypertension has long been researched, especially its relationship with catecholamines and its effect after sympathectomy. The authors reported some cases of hypertension combined with craniofacial hyperhidrosis (SHS) cured by sympathectomy. The actual treatment mechanism is still unknown. Also it is still equivocal whether essential hypertension can be treated with sympathectomy. Under what conditions can hypertension be treated with sympathetic surgery? Human studies are difficult to carry out. An aggressive but detailed and ethical protocol should be designed. If hypertension can be treated with surgery, not only is it going to be a major medical breakthrough, but also a great saving of the universal medical expenditure.

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