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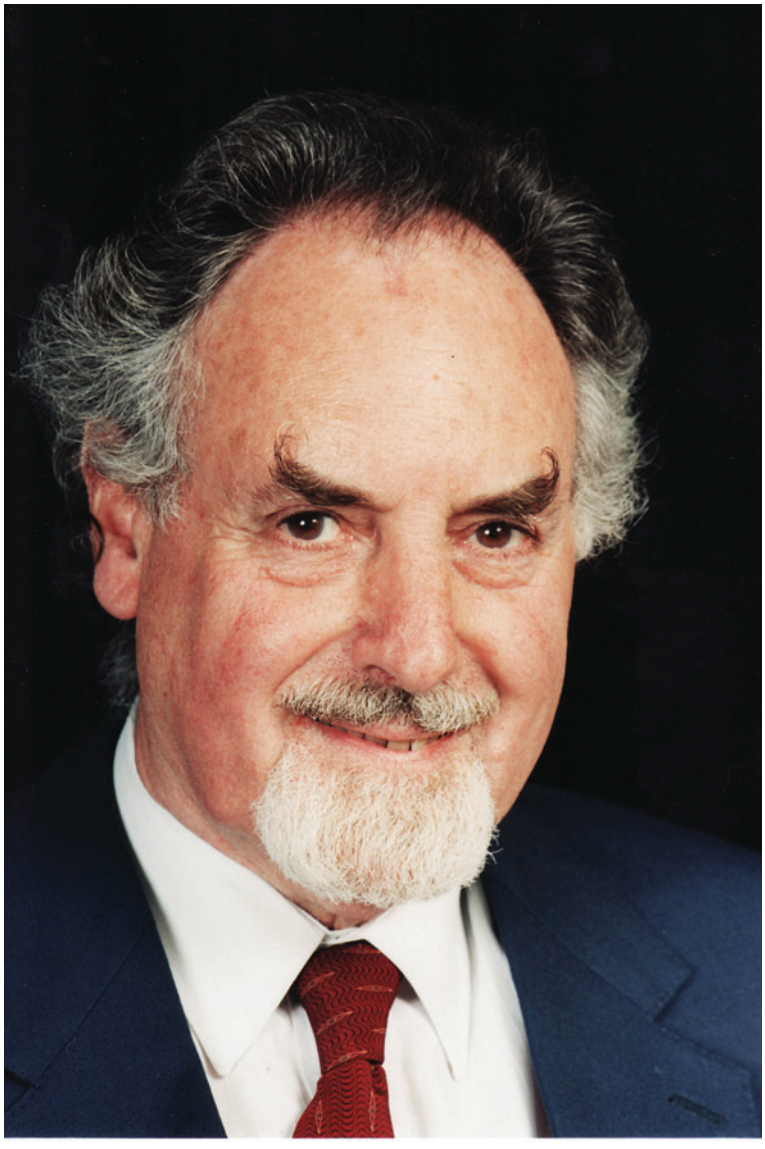

Autonomic Neurotransmission: 60 Years Since Sir Henry Dale

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Key Words

ATP, brain stem, cotransmission, neuropeptides, pathophysiology, receptors

Abstract

In the early twentieth century, Sir Henry Dale and others described brilliant studies of autonomic neurotransmission utilizing acetylcholine and noradrenaline. However, within the past 60 years, new discoveries have changed our understanding of the organization of the autonomic nervous system, including the structure and function of the nonsynaptic autonomic neuroeffector junction, the multiplicity of neurotransmitters, cotransmission, neuromodulation, dual control of vascular tone by perivascular nerves and endothelial cells, the molecular biology of receptors, and trophic signaling. Further, it is now recognized that an outstanding feature of autonomic neurotransmission is the inherent plasticity afforded by its structural and neurochemical organization and the interaction between expression of neural mediators and environmental factors. In this way, autonomic neurotransmission is matched to ongoing changes in demands and can sometimes be compensatory in pathophysiological situations.

INTRODUCTION

Sir Henry Dale was a great scientist, often described as the founder of pharmacology. He and a number of other outstanding figures in the early part of the twentieth century, including Langley, Magnus, Gaskell, Feldberg, Elliot, Loewi, and Von Euler, were particularly interested in the chemical mechanism of signaling between nerves and between nerves and muscles (see 1). They concentrated on short-term interactions that led to the classical view that the autonomic nervous system (ANS) consisted largely of antagonistic components of cholinergic parasympathetic and adrenergic sympathetic nerves. For nearly 50 years, most responses to autonomic nerves were interpreted entirely in terms of the two chemical transmitters, acetylcholine (ACh) and noradrenaline (NA). However, following the discovery of nonadrenergic, noncholinergic (NANC) nerves in our laboratory in the early 1960s, researchers recognized a multiplicity of autonomic neurotransmitter substances, beginning with purines and serotonin and followed by more than 16 neuropeptides. Nitric oxide (NO) and endothelin have also been recognized as autonomic transmitters (see 2, 3). At first sight, this formidable list of transmitters seems to be an unmanageable elaboration, but the situation has become much clearer since the introduction of the cotransmitter hypothesis (4).

I began my studies in the early 1950s using a combination of the well-established method of silver and methylene blue staining of nerve fibers and cells and isolated organ pharmacology to try to understand the nervous control of the gastrointestinal tract. These methods had serious limitations, and I wondered whether a more fruitful approach might be to start at the cellular level and build up to organ and then whole animal physiology.

With the encouragement of Professor W. Feldberg at the National Institute of Medical Research at Mill Hill, Ralph Straub and I developed the sucrose gap method for recording correlated changes in the membrane potential and tension in smooth muscle (5). Later, under the guidance of Edith Bülbring at Oxford, the sucrose gap became a valuable tool for assessing the actions of neurotransmitters and related compounds on smooth muscle (6, 7). After working with C. Ladd Prosser in the United States to explore a wide variety of potential autonomic nerve–smooth muscle preparations for studies on the electrophysiology of neurotransmission, I moved to Australia, where Mollie Holman and I used the guinea pig vas deferens and were the first to record junction potentials in smooth muscle (8). To interpret these electrophysiological studies fully, we clearly needed to know the precise geometry of the nervous environment of individual smooth muscle cells. We therefore extended our studies to include two additional methods: the Falck-Hillarp method for fluorescence histochemical localization of adrenergic nerves, and with the help of Neil Merrillees, electron microscopy (9). The combination of these methods allowed us to put forth models of the structure and function of the autonomic neuromuscular junction in both visceral and cardiovascular systems (10).

The pioneer investigators of autonomic control mechanisms and neurotransmission focused on several concepts that gave a brilliant lead and a framework for research in the field. A disadvantage, however, was that researchers, perhaps under the influence of the much admired physical scientists, created laws or principles for some of the concepts. Hence, later researchers, if their data did not fit this framework, tended to hide these anomalies under the carpet. Accordingly, it became progressively more difficult to challenge these principles, and they remained deeply entrenched for more than 60 years. During the past decade or so, many anomalies came to light with the availability of new techniques, and it became clear that the classical picture of autonomic neurotransmission needed drastic revision and that a new framework was required. My own view is that the philosophy of the evolutionary biologist is more appropriate than that of the physical scientist in developing a new framework. We need to recognize the variety of available mechanisms and to enjoy seeing how nature utilizes various combinations of mechanisms to solve particular problems encountered by

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different organs in different species during evolution. This approach seems preferable to taking a rigid view that tries to fit data about a particular tissue into a general principle or, equally misleading, that rejects a general principle because data about a particular tissue are not consistent with that principle.

DEFINITION OF THE AUTONOMIC NEUROEFFECTOR JUNCTION

The autonomic neuromuscular junction differs in several important respects from the better-known skeletal neuromuscular junction (see 11, 12). It is not a synapse with the well-defined prejunctional and postjunctional specializations established for the skeletal neuromuscular synapse or ganglionic synapses. A model of the autonomic neuroeffector junction has been proposed on the basis of combined electrophysiological, histochemical, and electron-microscopical studies. The essential features of this model are that the terminal portions of autonomic nerve fibers are varicose (**Figure 1*a*** and ***b***) and that the transmitter is being released *en passage* from varicosities during conduction of an impulse, although excitatory and inhibitory junction potentials (**Figure 2**) are probably elicited only at close junctions (as little as 20 nm in visceral organs, but up to 1–2 μm in large blood vessels). Furthermore, the effectors are muscle bundles rather than single smooth muscle cells, which are connected by low-resistance pathways (gap junctions or nexuses) that allow electrotonic spread of activity within the smooth muscle bundle (13) (**Figure 3*c***). In blood vessels, the nerves are confined to the adventitial side of the media muscle coat, and this geometry appears to facilitate dual control of vascular smooth muscle by perivascular nerves and by endothelial relaxing and contracting factors (**Figure 3*b***). Neuroeffector junctions do not have a permanent geometry with postjunctional specializations, but rather the varicosities are continuously moving and their special relation with muscle cell membranes changes with time, including dispersal and reformation of receptor clusters (14). For example, varicosity movement is likely to occur in cerebral blood arteries, where there is a continuously increasing density of sympathetic innervation during development and aging, and in hypertensive vessels or those that have been stimulated chronically in vivo, where there is an increase in innervation density of up to threefold. Although there are many examples of prejunctional thickenings of nerve membranes in varicosities associated with accumulations of small synaptic vesicles, which represent sites of transmitter release (see **Figure 1*c***), there are no convincing demonstrations of postjunctional specializations such as membrane thickening, folding, or absence of micropinocytic vesicles. This is in keeping with the view that even close junctions might be temporary liaisons.

It is likely that a given impulse will evoke transmitter release from only some of the varicosities that it encounters. Some substances stored and released from nerves do not act directly on effector muscle cells but alter the release and/or the actions of other transmitters; these substances are termed neuromodulators. Many other substances (e.g., circulating neurohormones; locally released agents such as prostanoids, bradykinin, histamine, and endothelin; and neurotransmitters from nearby nerves) are also neuromodulators in that they modify the process of neurotransmission both by prejunctional modulation of transmitter release and by postjunctional modulation of transmitter action. Many cotransmitters are also neuromodulators. The wide and variable cleft characteristic of autonomic neuroeffector junctions makes them particularly amenable to the mechanisms of neural control mentioned above. Several neurotransmitters/neuromodulators are trophic molecules, with mitogenic or growth-promoting/-inhibiting properties.

Release of a neurotransmitter causes a transient change in the membrane potential of the postjunctional cell (2). If the result of a single pulse is a depolarization, the response is called an excitatory junction potential (EJP) (**Figure 2*a***). EJPs summate and facilitate with repetitive stimulation, and at a threshold depolarization there is generation of an action potential, resulting in

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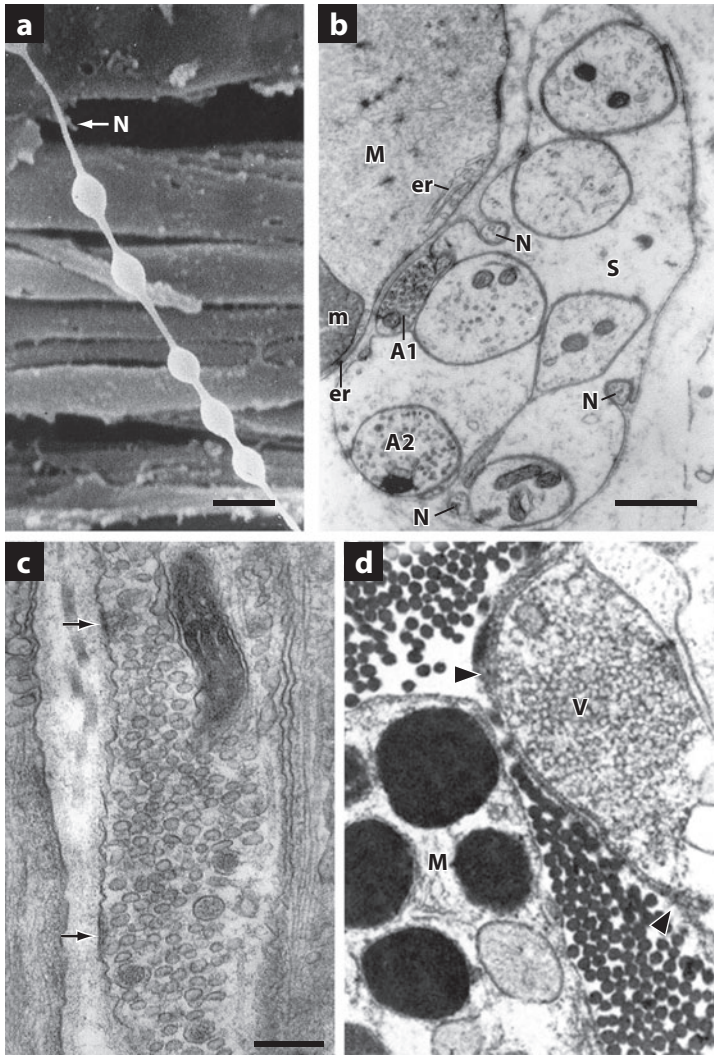

Figure 1

(*a*) Scanning electron micrograph of a single terminal varicose nerve fiber lying over smooth muscle of small intestine of rat. Intestine was pretreated to remove connective tissue components by digestion with trypsin and by hydrolysis with HCl. Scale bar = 3 μm. (Reproduced from Reference 101 with permission from Springer.) (*b*) A medium-sized intramuscular bundle of axons within a single Schwann cell (S). There is no perineurial sheath. Some axons, free of Schwann cell processes, contain synaptic vesicles (e.g., A1 and A2). For nerve profile A1, there is close proximity (approximately 80 nm) to smooth muscle (M) with fusion of nerve and muscle basement membranes (m, mitochondria; er, endoplasmic reticulum). Most of the axons in bundles of this size have few vesicles in the plane of section, but they resemble the vesicle-containing axons of the larger trunks in that they have few large neurofilaments. The small profiles (N), less than 0.25 μm in diameter, are probably intervaricosity regions of terminal axons. Scale bar = 1 μm. (Reproduced from Reference 9 with permission from Rockefeller University Press.) (*c*) Autonomic varicosities with dense prejunctional thickenings and bunching of vesicles, probably representing transmitter release sites (*arrows*), but there is no postjunctional specialization. Scale bar = 0.25 μm. (Reproduced from Reference 102 with permission from Elsevier.) (*d*) Ultrathin section of rabbit middle cerebral artery showing mast cells (M) separated by a distance of less than 200 nm from a nerve (V, varicosities; arrowheads, basement membranes). Magnification, x 29,374. (Reproduced from Reference 16 with permission from Elsevier.)

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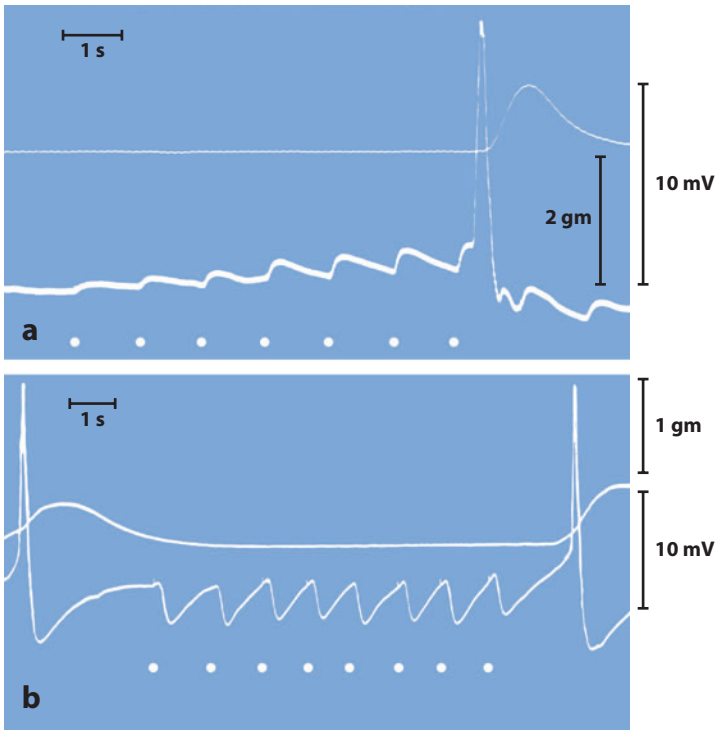

Figure 2

Transmission at autonomic neuromuscular junctions. Changes in membrane potential (*bottom trace*) and contraction (*top trace*) recorded with a sucrose-gap method. The junction potentials recorded with this method are qualitatively similar to those recorded with intracellular microelectrodes. (*a*) Excitatory junction potentials (EJPs) recorded in smooth muscle of the guinea pig vas deferens in response to repetitive stimulation of postganglionic sympathetic nerves (*white dots*). Note both summation and facilitation of successive EJPs. At a critical depolarization threshold, an action potential is initiated that results in contraction. (*b*) Inhibitory junction potentials (IJPs) recorded in smooth muscle of the atropinized guinea pig taenia coli in response to transmural repetitive stimulation (*white dots*) of the intramural nerves remaining after degeneration of the adrenergic nerves by treatment of the animal with 6-hydroxydopamine ($250\,mg\,kg^{-1}$ intraperitoneally for 2 successive days) 7 days previously. Note that the IJPs in response to repetitive stimulation result in inhibition of spontaneous spike activity and relaxation. (Reproduced from Reference 103 with permission from Wiley-Blackwell.)

a mechanical contraction. If the result of a single pulse of neurotransmitter release is a hyperpolarization, then the response is called an inhibitory junction potential (IJP) (**Figure 2*b***). IJPs prevent action potential discharge in spontaneously active smooth muscle and thus cause relaxation.

Many nonexcitable effector cells are innervated, albeit transiently, by nerves. This is because, as described above, the autonomic neuroeffector junction is not a fixed structure with postjunctional specialization, but rather, when varicosities form close relationships with effector cells, these cells are in effect innervated. The evidence is reviewed in detail by Burnstock (12). For many years, researchers did not consider that cells of the immune system were innervated because neural boutons could not be found on their surface membranes. However, close contact of nerve varicosities with immune cells constitutes innervation, albeit of a transient nature. Also, there is increasing recognition that nerves can influence the immune system, and the field of neuroimmunology is growing (15). Cells of the immune system consist of a large family, including lymphocytes, mast

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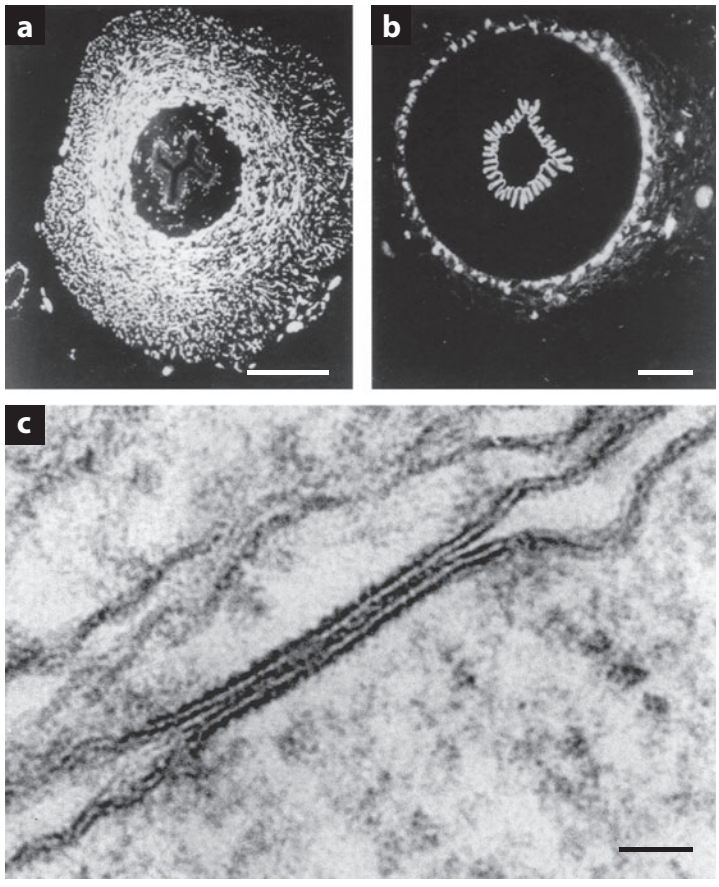

Figure 3

Comparison between the sympathetic innervation of the densely innervated vas deferens of the guinea pig (*a*) and the rabbit ear artery (*b*) in which the NA-containing fibers are confined to the adventitial-medial border. The inner elastic membrane shows a nonspecific fluorescence (autofluorescence). (*a* and *b* reproduced from Reference 64 with permission from Springer.) (*c*) A gap junction between two smooth muscle cells grown in tissue culture. Scale bars = 500 μm (*a*), 50 μm (*b*), and 50 nm (*c*). (Reproduced from Reference 104 with permission from Rockefeller University Press.)

cells, macrophages, neutrophils, eosinophils, thymocytes, dendritic and hematopoietic cells, as well as microglia and osteoclasts. Both sympathetic and sensory nerves innervate immune organs and release their cotransmitters in the vicinity of immune cells, which express receptors for these transmitters. Mast cells were the first immune cell type shown to be innervated (see 16) (**Figure 1*d***). Epithelial cells in airways, liver, kidney, gut, gall bladder, adipose tissue, eye, and uterus express multiple receptors to neurotransmitters involved in cytosolic calcium regulation of chloride and fluid secretion, sodium transport, and ciliary and mucociliary clearance. Endocrine cells are also innervated by sympathetic, parasympathetic, and sensory nerves (see 12). In the microvasculature, it is likely that transmitters released from varicosities in the perivascular nerve plexus would act on endothelial receptors.

A model of the autonomic neuromuscular junction has been proposed on the basis of combined electrophysiological, histochemical, and electron-microscopical studies described earlier for both visceral (**Figure 4*a***) and vascular (**Figure 4*b***) smooth muscle.

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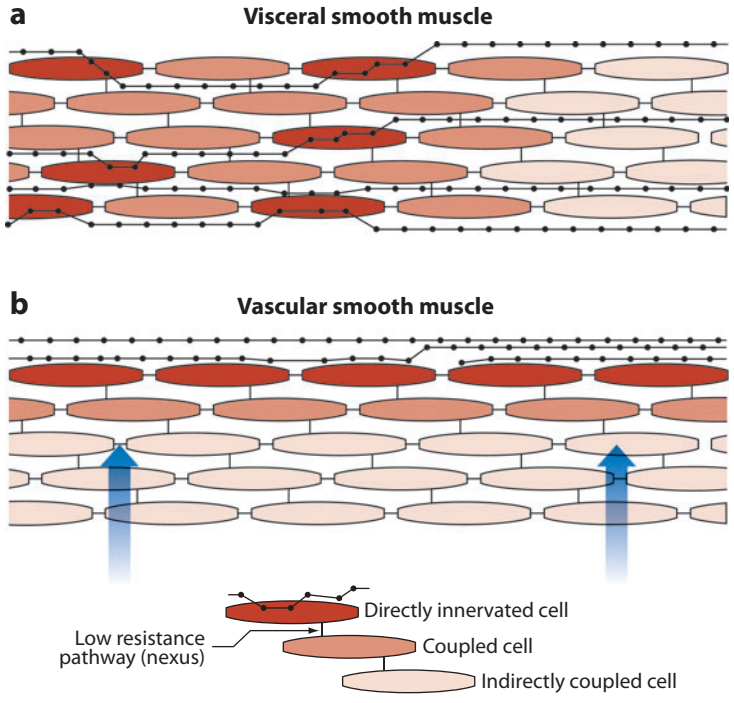

Figure 4

(*a*) Schematic representation of the innervation of visceral smooth muscle. Directly innervated cells (*dark red*) are those directly activated by a neurotransmitter released from nerve varicosities (*black dots*); coupled cells (*pale red*) are those where junction potentials spread from directly innervated cells. When a sufficient area of the muscle effector bundle is depolarized, a propagated action potential will activate the indirectly coupled cells (*pink*). (*b*), Schematic representation of control of vascular smooth muscle by perivascular varicose nerves in the adventitia (*black dots*) and endothelial factors (*blue arrows*). (Modified from Reference 64 with permission from Springer.)

THE MULTIPLICITY OF NEUROTRANSMITTERS IN AUTONOMIC NERVES

A neurotransmitter is a chemical substance released from nerves upon electrical stimulation. Neurotransmitters bind to specific receptors on adjacent effector cells to bring about a response, thus acting as chemical messengers of neural activation. In early studies, acceptance of a substance as a neurotransmitter required satisfaction of the following criteria: (*a*) the presynaptic neuron synthesizes and stores the transmitter; (*b*) the transmitter is released in a calcium-dependent manner; (*c*) there is a mechanism for terminating the activity of the transmitter, either by enzymatic degradation or by cellular uptake; (*d*) local exogenous application of the substance mimics its effects following release owing to electrical nerve stimulation; and (*e*) agents that block or potentiate the endogenous activity of the transmitter also affect local exogenous application in the same way. Recent studies of autonomic neurotransmission have revealed a multiplicity of neurotransmitters in the ANS. Neurally released substances, including monoamines, amino acids, neuropeptides, ATP, NO, and carbon monoxide (CO) have been identified (see **Table 1**). Because NO does not conform to the constraints of the criteria outlined above, although it certainly acts as a rapid chemical messenger in the ANS, a reappraisal of the criteria for defining a neurotransmitter has

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Table 1 Neurotransmitters/neuromodulators claimed to be present in the autonomic nervous system. (Updated and reproduced from Reference 22 with permission from Oxford University Press.)

Noradrenaline (NA)
Acetylcholine (ACh)
Adenosine 5′-triphosphate (ATP) and other nucleotides
Nitric oxide (NO)
Carbon monoxide (CO)
5-Hydroxytryptamine (5-HT)
Dopamine (DA)
γ-Aminobutyric acid (GABA)
Glutamate (GLU)
Neuropeptides
Neuropeptide Y (NPY)/pancreatic polypeptide (PP)
Enkephalin (ENK)/endorphin (END)/dynorphin (DYN)
Vasoactive intestinal polypeptide (VIP) and related peptides PHI and PHM
Pituitary adenylate cyclase-activating peptide (PACAP)
Substance P (SP)/neurokinin A (NKA)/neurokinin B (NKB)
Calcitonin gene-related peptide (CGRP)
Somatostatin (SOM)
Galanin (GAL)
Gastrin releasing peptide (GRP)/bombesin (BOM)
Neurotensin (NT)
Cholecystokinin (CCK)/gastrin (GAS)
Angiotensin II (AgII)
Adrenocorticotrophic hormone (ACTH)
Secretoneurin
Endothelin (ET)

been proposed (17), taking into account evidence for nonvesicular, Ca^{2+}-independent release of some classical neurotransmitters and the intracellular site of NO action.

Noradrenaline and Acetylcholine

In the past 60 years, researchers have made advances in understanding the metabolism of the classical transmitters. The synthesis of NA is catalysed by three enzymes, tyrosine hydroxylase (TH), L-dopa decarboxylase, and dopamine-β-hydroxylase (DBH) (18). NA exists in the neuronal cytosol but is stored in small and large dense core vesicles together with chromogranins and DBH. Following electrical stimulation, the vesicular contents are released by exocytosis, in a Ca^{2+}-dependent manner, into the extracellular space. Upon interaction with specific receptors, the action of NA is rapidly terminated by reuptake into the nerve varicosity or into non-neuronal cells, where it is metabolized by the intracellular enzymes monoamine oxidase (MAO) and catechol-*O*-methyltransferase (see **Figure 5*a***).

The synthesis of ACh from choline and acetyl coenzyme A is catalyzed by choline acetyltransferase (ChAT) and takes place in the neuronal cytoplasm. ACh is then pumped into small

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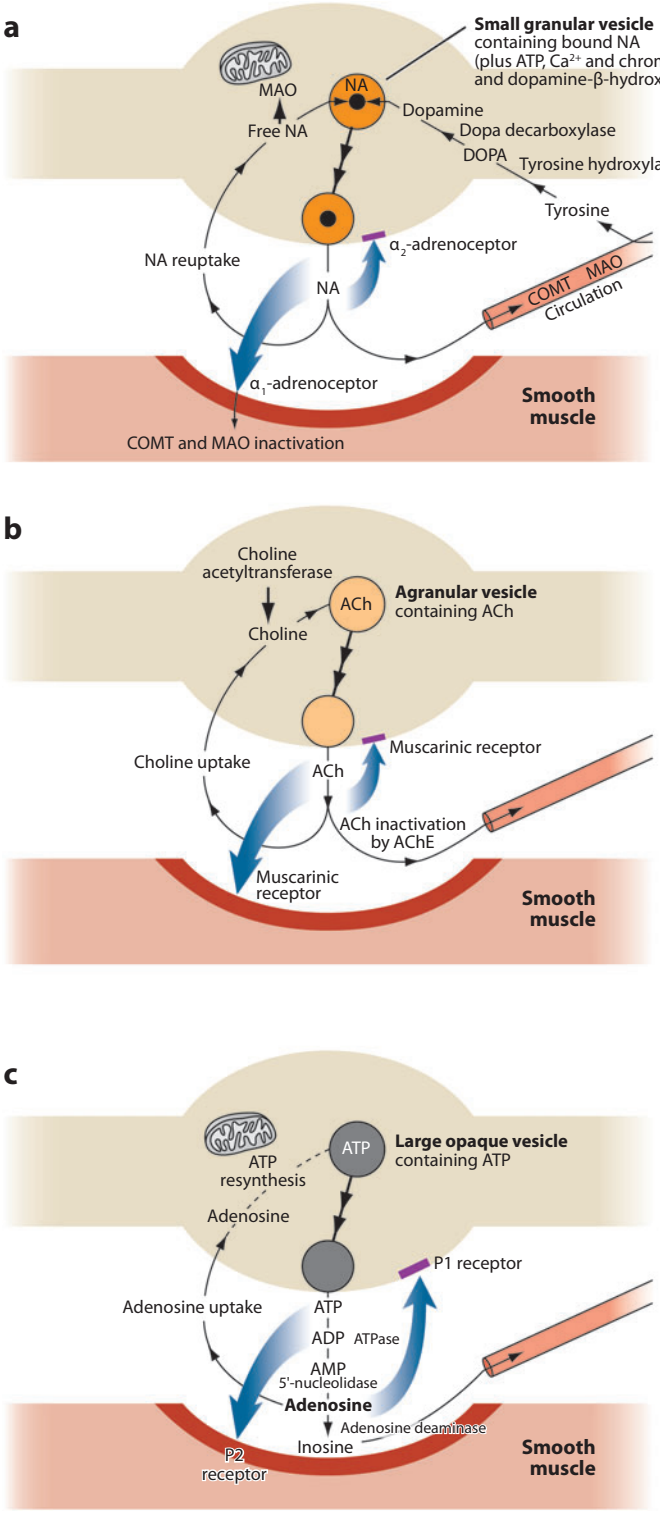

Figure 5

Simplified schematic representation of synthesis, storage, release, receptor activation, and neurotransmitter inactivation at neuromuscular junctions. (*a*) Noradrenergic neurotransmission; (*b*) cholinergic neurotransmission; (*c*) purinergic neurotransmission. (Modified from Reference 19 with permission from the American Society for Pharmacology and Experimental Therapeutics.)

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agranular vesicles, which have a specific ACh transporter in their membranes, and stored until Ca^{2+}-dependent exocytotic release upon electrical stimulation. ACh released during neurotransmission is inactivated by hydrolysis caused by the action of acetylcholinesterase (AChE), which is localized on both pre- and postsynaptic membranes. The choline that results from this breakdown is recycled by transport back into the nerve varicosities by a metabolically driven high-affinity choline uptake mechanism for resynthesis and vesicular storage of ACh. Choline uptake into the presynaptic terminal is the rate-limiting factor for ACh synthesis. A schematic of cholinergic autonomic neurotransmitters is present in **Figure 5*b***.

ATP

The purine nucleotide, ATP, was the first substance found to best satisfy the criteria for a neurotransmitter in NANC nerves (19). There is now substantial evidence to support widespread purinergic signaling in both neuronal and non-neuronal systems (20). ATP is a transmitter at neuroeffector junctions (see **Figure 5*c***), at synapses in peripheral autonomic ganglia, and in the brain and spinal cord (21). ATP is also a major signaling molecule in the enteric nervous system (22) and on sensory nerves, where it is involved in both physiological reflexes and nociception (23). ATP is synthesized in nerve terminals and is stored in vesicles. After its release and activation of postjunctional P2X ion channel receptors, ATP is rapidly broken down to ADP, AMP, and adenosine by ectonucleotidases. Adenosine is transported into neurons and non-neuronal cells via a nucleoside carrier high-affinity uptake system and either phosphorylated to ATP and reincorporated into physiological stores or broken down by adenosine deaminase to inosine, which is inactive and leaks into the circulation (see **Figure 5*c***). Extracellular adenosine acts on prejunctional P1 receptors to inhibit the release of transmitter. In addition to ATP, there is now evidence that small amounts of other nucleotides such as ADP, AMP, GTP, UTP, and diadenosine polyphosphates are stored in synaptic vesicles and may play neuromodulatory signaling roles in the nervous system.

Neuropeptides

Peptides involved in neurotransmission in the ANS are a large and diverse group (**Table 1**). Like the classical neurotransmitters, they are stored in vesicles and are released upon depolarization to act on specific receptors to produce an effector response. However, by virtue of their structure, there are important differences from classical neurotransmission in their mode and site of synthesis and in their inactivation after release: Namely, they are synthesized and packaged into vesicles in the nerve cell body, which are then transported to terminal nerve varicosities, and there are no mechanisms for reuptake and recycling of neuropeptides after receptor activation (24). Neuropeptides are stored in large electron-dense cored vesicles and released by exocytosis. The regulation of neuropeptide neurotransmission is quite different from the classical neurotransmitters because replacement of neuropeptides after release is dependent on new synthesis in the nerve cell body and axonal transport, a relatively slow process compared with local synthesis in nerve terminals by enzymatic activity and replacement by efficient reuptake mechanisms. Neuropeptide release is more easily exhausted by repeated or prolonged stimulation. The action of neuropeptides is terminated mainly by metabolism by proteolytic enzymes but also by internalization and degradation of the receptor-bound peptide. A few key ectoenzymes, including endopeptidase 24.11 and angiotensin-converting enzyme, are thought to account for the degradation of most neuropeptides. The regulation of expression of these ectopeptidases is another level of modulation of peptidergic neurotransmission.

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Nitric Oxide

NO has been added to the list of putative neurotransmitters in the ANS. NO is not stored within neurons because it can travel freely through membranes. Furthermore, NO does not act on extracellular receptors on the postjunctional membrane of the target, but rather at intracellular sites (see **Figure 6**). NO is synthesized in a reaction in which L-arginine is converted to L-citrulline by nitric oxide synthase (NOS) and exists in three main isoforms, types I–III. Type I is constitutively expressed in autonomic neurons (see 25). Synthesis of NO is stimulated during transmission by a Ca^{2+}-dependent mechanism rather than being released from intracellular stores. Once NO has been synthesized, it can diffuse freely through membranes to the postjunctional target to act on intracellular guanylate cyclase, leading to relaxation. Because it is a free radical, NO is unstable. Thus, to end NO-dependent responses there is no need for the mechanisms required for other neurotransmitters, such as degradative enzymes or reuptake. NO binds readily to the heme group of hemoglobin, which can thus inhibit NO-dependent responses.

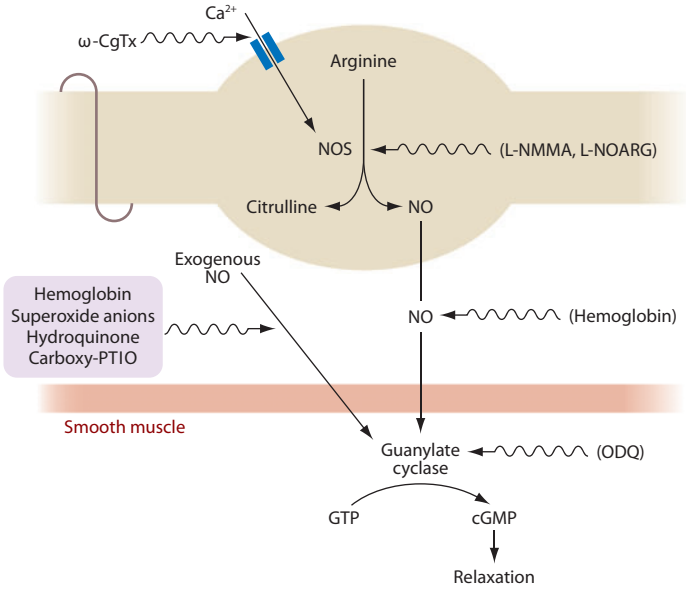

Figure 6

Current model for the nitrergic neurotransmission process. The arrival of an action potential at the terminal region opens voltage-operated Ca^{2+} channels, allowing calcium to enter the neuronal cytoplasm and activate NOS. The enzyme converts L-arginine to L-citrulline, with the concomitant production of NO. The NO rapidly diffuses out of the nerve cell, across the gap, and into the postjunctional cell (usually smooth muscle), where it binds to the heme group of soluble guanylate cyclase and consequently activates the conversion of GTP to cyclic GMP. Nitrergic transmission may be inhibited by ω-conotoxin (ω-CgTx; inhibits calcium channel), L-N^G-monomethyl-arginine and L-N^G nitro-arginine (L-NMMA and L-NOARG; inhibits NOS), hemoglobin (traps NO in the junctional gap), or 1H-[1,2,4]-oxadiazolo[4,3-a]quinoxalin-L-one (ODQ; inhibits soluble guanylate cyclase). Exogenous NO mimics the relaxant effect of nitrergic stimulation and is similarly inhibited by the presence of hemoglobin. However, several substances (superoxide anions, hydroquinone, carboxy-PTIO) strongly inhibit the relaxation to exogenous NO but have little effect on nitrergic responses, raising doubts about whether NO is released from the nerve as a free radical or in some other form. (Reproduced from Reference 105 with permission of Elsevier.)

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Other Neurotransmitters

Several other neurotransmitters have been identified in autonomic nerves (see 26). 5-Hydroxytryptamine (5-HT) is an indolamine synthesized from tryptophan via 5-hydroxytryptophan by two enzymes, tryptophan hydroxylase and L-aromatic amino acid decarboxylase. Neuronal synthesis of 5-HT has been demonstrated in myenteric neurons, but it can also act as a false neurotransmitter after it is taken up and released from sympathetic nerves. After release, it is catabolized by MAO-A to 5-hydroxyindoleacetaldehyde and subsequently to 5-hydroxyindoleacetic acid. Dopamine has also been identified as a sympathetic neurotransmitter.

GABA, glutamate, and dopamine, classical neurotransmitters in the central nervous system (CNS), are also autonomic neurotransmitters. GABA plays a role in enteric neurotransmission via excitatory $GABA_A$ and prejunctional inhibitory $GABA_B$ receptors. The GABA synthesizing and catabolizing enzymes (glutamate decarboxylase and 4-aminobutyrate:2-oxoglutarate transaminase, respectively), GABA, and high-affinity GABA uptake sites have all been localized in gastrointestinal tissue. CO could behave, analogous to NO, as a neuronal messenger. There is also evidence for endothelin as a transmitter in perivascular nerves in cerebral blood vessels (27).

COTRANSMISSION

For many years, our understanding of neurotransmission has been dominated by Dale's Principle, the concept that one neuron releases only a single transmitter. This idea arose from a widely adopted misinterpretation of Dale's 1935 suggestion that the same neurotransmitter was stored in and released from all terminals of a single sensory neuron. It was only in 1957 that Eccles introduced the term Dale's Principle and the notion that neurons utilize a single transmitter, which dominated thinking until the mid-1970s. At this time, several lines of evidence emerged that were inconsistent with this principle, and I introduced the concept of cotransmission in 1976 (4). It is now known that individual neurons in both peripheral and central nervous systems contain and can release a large number and variety of substances that are capable of influencing target cells (see 21). The precise combinations of neurotransmitters (and neuromodulators) contained in individual neurons and their projections and central connections, termed their chemical coding, have been defined in studies of the enteric nervous system (28). Ultrastructural studies of the enteric nervous system offered the first suggestion that there were several different cotransmitters in autonomic nerves; at least nine distinguishable types of axon profiles showing different combinations of vesicle types were described in the guinea pig myenteric plexus (29).

Sympathetic Nerves

The first evidence for sympathetic nerve cotransmission involving ATP with NA came from studies I made with Che Su and John Bevan when I was on sabbatical leave in California in 1971.We showed that stimulation of periarterial sympathetic as well as enteric NANC nerves led to release of ATP from guinea pig taenia coli (30). Later, the possibility that ATP might be coreleased with NA in chemical transmission from sympathetic nerves supplying the guinea pig seminal vesicle (31) and the cat nictitating membrane (32) was raised. The most extensive evidence for sympathetic cotransmission, however, came from studies of the vas deferens, initially by Westfall and his colleagues in West Virginia (33) and later from a number of laboratories that established sympathetic cotransmission in a variety of different blood vessels (34, 35). NA and ATP are coreleased in varying proportions, depending on the tissue and species and also on the parameters of stimulation. Short bursts at low frequency particularly favor the purinergic component, whereas longer

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periods of nerve stimulation favor the adrenergic component, and neuropeptide Y (NPY) release is optimal with high frequency intermittent bursts of stimulation. In the rabbit ear artery the purinergic component is relatively small, whereas in intestinal submucosal arteries the responses to sympathetic nerve stimulation are mediated solely by ATP, with NA acting as a prejunctional modulator via α_2-adrenoceptors (36). The effects of ATP and NA released as sympathetic cotransmitters are generally synergistic. NA and ATP can depress sympathetic neurotransmission by prejunctional modulation via α_2-adrenoceptors, or predominantly, via P1 receptors following extracellular breakdown of ATP to adenosine, but also via P2 receptors in some vessels. In most tissues, including the vas deferens and many blood vessels, NPY does not act as a genuine neurotransmitter, having little direct postjunctional action, but rather acts as a neuromodulator, often by prejunctional attenuation of NA and ATP release and/or postjunctional potentiation of responses to adrenergic and purinergic components of sympathetic nerve responses. A schematic showing sympathetic cotransmission is shown in **Figure 7*a***. Other substances localized within sympathetic nerves include 5-HT, which is taken up by sympathetic nerves and released as a false transmitter. Opioid peptides are also widely distributed in sympathetic neurons where their main function appears to be prejunctional inhibitory actions on sympathetic neurotransmission.

In the adult animal, there are both structural and pharmacological indications that the distinction between adrenergic and cholinergic neurons in some organs of some species is not as rigid as previously supposed (see 37). For example, nerves, characterized as adrenergic because of a predominance of small granular vesicles, may stain heavily for AChE. The denervated nictitating membrane appears to be reinnervated by preganglionic cholinergic fibers, which then develop adrenergic features. Cholinergic vasodilation of uterine arteries appears in late pregnancy, when adrenergic vasoconstriction is diminished. Under certain conditions in vitro, a single sympathetic neuron may at different times release NA, ACh, or a mixture of these two transmitter substances. These multipotential cells require nerve growth factor (NGF) to survive, and they respond to NGF with an increased production of both ChAT and TH.

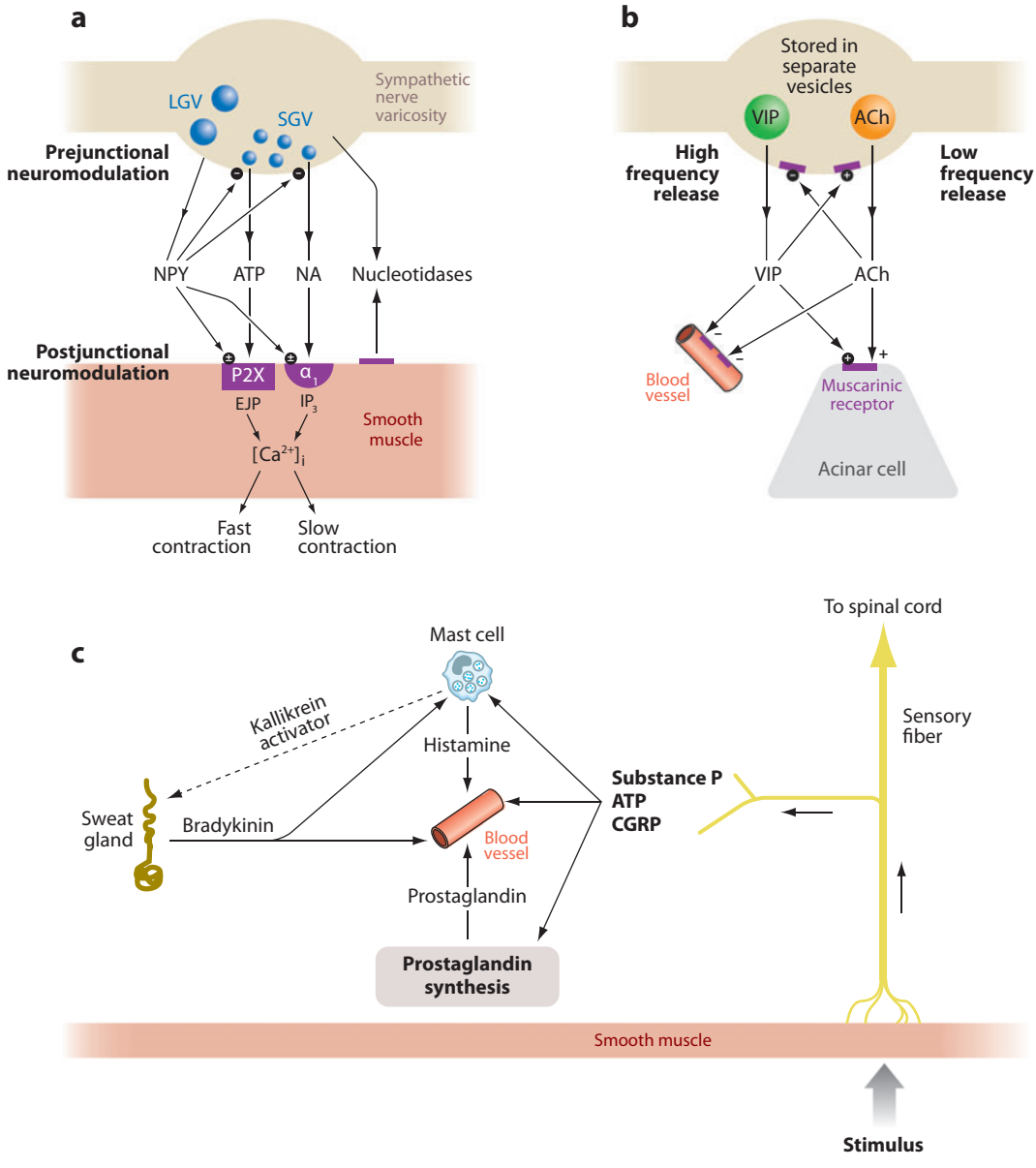
Parasympathetic Nerves

ACh, vasoactive intestinal polypeptide (VIP), ATP, and NO are cotransmitters commonly synthesized in and released from parasympathetic nerves (38). As with sympathetic cotransmission, the relative functional importance of the cotransmitters in parasympathetic neurotransmission is variable in different tissues and species. For example, NO may be the main mediator of neurogenic vasodilation in cerebral vessels, whereas VIP may be of more importance during neurogenic vasodilation in the pancreas. The coordinated roles of VIP and ACh in parasympathetic neurotransmission were demonstrated in an elegant study by Lundberg (see 38) of the cat exocrine salivary gland innervation (**Figure 7*b***). VIP has since been shown to have a direct vasodilatory action in the submandibular gland in man. Pituitary adenylate cyclase-activating polypeptide also seems to be present in VIP-containing parasympathetic nerves. NOS is often colocalized with ACh and VIP in parasympathetic nerves innervating blood vessels. Postganglionic nerves from pelvic ganglia containing VIP, ACh, and NOS project to the urethra, colon, and penis. In the rodent bladder, ATP is a major excitatory cotransmitter with ACh in parasympathetic nerves. However, only a small purinergic component is present in human bladder, except in pathological conditions (see below).

Sensory-Motor Nerves

The motor function of sensory nerves, whereby antidromic impulses down collatoral fibers result in local release of sensory neurotransmitters, is widespread in autonomic effector systems and

forms an important physiological component of autonomic control (39). To distinguish these nerves from the other subpopulation of afferent fibers that have an entirely sensory role and have terminals containing few vesicles and a predominance of mitochondria, they were termed sensory-motor nerves. Substance P (SP) and calcitonin gene-related peptide (CGRP) are cotransmitters in many unmyelinated, primary afferent nerves. They often coexist in the same large granular vesicles in capsaicin-sensitive nerve terminals, although their proportion varies with species. ATP is contained in a subpopulation of sensory-motor fibers (see **Figure 7*c***) and is also established as a cotransmitter with glutamate in small primary afferent sensory nerves in the spinal cord that mediate nociceptive signals (23).



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Other neuropeptides and transmitters have been localized in sensory-motor nerves. For example, in the human urinary bladder, VIP, cholecystokinin (CCK), and dynorphin are present with SP and CGRP in the afferent projections to the lumbosacral spinal cord. In the guinea pig, dorsal root ganglion (DRG) neurons containing SP, CGRP, CCK, and DYN project to the epidermis and small dermal blood vessels. NOS and endothelin have been localized in subpopulations of primary sensory neurons of trigeminal and DRG. There are increasing examples in the literature of cross talk between sensory-motor, sympathetic, and parasympathetic nerves.

Intrinsic Neurons in Gut, Heart, Bladder, and Airways

Many intrinsic neurons localized within autonomic neuroeffector tissues are part of the parasympathetic system. There are also intrinsic neurons derived from neural crest tissue that is different from that which forms sympathetic and parasympathetic neurons, including many in the gut and probable subpopulations in the heart and airways. However, studies of the latter have been largely neglected, primarily because of the difficulty in visualizing these cells and distinguishing intrinsic from extrinsic nerve fibers.

The most extensive system of intrinsic neurons is in the myenteric and submucous plexuses of the gastrointestinal tract. These enteric neurons contain numerous neuroactive substances of which the majority are involved in neurotransmission or neuromodulation at the ganglion level and/or have a trophic role; some are involved in neuromuscular transmission. The chemical coding of enteric neurons, particularly of neuropeptides, has been examined in detail, particularly in the guinea pig (40). ATP, NO, and VIP mediate NANC inhibitory neuromuscular transmission in the gut in varying proportions, depending on the region. ACh and SP are cotransmitters in enteric excitatory neurons. Intramural sensory neurons, identified as Dogiel Type II AH neurons in both myenteric and submucous plexuses, express P2 receptors and are involved in reflex control of motility and nociception (see 21).

There are many intrinsic neurons in the heart, particularly in the right atrium. The neurochemical makeup of the intrinsic cardiac ganglia is heterogeneous and includes a variety of neurochemical markers (see 41, 42). For example, subpopulations of atrial intrinsic neurons from newborn guinea pigs immunostain for NPY, 5-HT, heme oxygenase-2, and NOS, and these neurons probably also

Figure 7

(*a*) Schematic of sympathetic cotransmission. ATP and NA released from small granular vesicles (SGV) act on P2X and α_1 receptors, respectively, on smooth muscle. ATP acting on inotropic P2X receptors evokes excitatory junction potentials (EJPs), increase in intracellular calcium ($[Ca^{2+}]_i$), and fast contraction; occupation of metabotropic α_1-adrenoceptors leads to production of inositol triphosphate (IP_3), increase in $[Ca^{2+}]_i$, and slow contraction. Neuropeptide Y (NPY) stored in large granular vesicles (LGV) acts, after release, both as a prejunctional inhibitory modulator of ATP and NA release and as a postjunctional modulatory potentiator of ATP and NA actions. Soluble nucleotidases are released from nerve varicosities, and are also present as ectonucleotidases. (Reproduced from Reference 106 with permission from Neville N. Osborne.) (*b*) A classic transmitter (ACh) coexists with vasoactive intestinal polypeptide (VIP) in parasympathetic nerves supplying the cat salivary gland. ACh and VIP are stored in separate vesicles; they can be released differentially at different stimulation frequencies to act on acinar cells and glandular blood vessels. ACh is released during low-frequency stimulation to increase salivary secretion from acinar cells and to elicit some minor dilatation of blood vessels in the gland. At high stimulation frequencies, VIP is released to produce marked dilatation of the blood vessels in the gland and to act as a neuromodulator, postjunctionally on the acinar gland to enhance the actions of ACh, and prejunctionally on the nerve varicosities to enhance the release of ACh. ACh also has an inhibitory action on the release of VIP. (Reproduced from Reference 107 with permission from Neville N. Osborne.) (*c*) Diagram showing the basis of the axon reflex in the skin, leading to vasodilation and inflammation. It is suggested that calcitonin gene-related peptide (CGRP), substance P (SP), and adenosine 5′-triphosphate (ATP) are released during antidromic activation of sensory collaterals. (Adapted from Reference 108 with permission from The Nature Publishing Group.)

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utilize ACh and ATP. On the basis of electrophysiological data, researchers have suggested that, rather than the original assumption of a simple nicotinic parasympathetic relay station, the intrinsic neural circuitry may sustain local intrinsic reflexes in the heart. Most airway intrinsic neurons contain ChAT, but NOS and VIP are also found in these neurons in humans, and VIP, somatostatin, NPY, and CGRP are expressed in tracheal ganglia (43). Intrinsic ganglia in the human urinary bladder wall contain a number of neuroactive substances (VIP, NOS, NPY, ATP, galanin, and occasionally TH); in the bladder neck, a few intrinsic neurons contain enkephalin and SP. Intramural ganglia containing NPY and VIP have been identified in human urethra.

RECEPTORS: MOLECULAR BIOLOGY AND TRANSDUCTION MECHANISMS

With the development of molecular and cellular techniques, our knowledge of neurotransmitter receptors has greatly advanced since the time of Sir Henry Dale, and many receptor subtypes have been cloned and characterized pharmacologically. Receptors may be located on the plasma membrane, on the membrane of an organelle, or on the nucleus. Transduction of a signal mediated by the receptor in response to agonist activation is a key concept that differentiates receptors with a signaling role from binding sites.

There are four major criteria that should ideally be fulfilled before a receptor is accepted as functionally significant for inclusion in a pharmacological classification (see 44): (*a*) a protein sequence, (*b*) a defined link to a signal transduction mechanism, (*c*) endogenous receptor expression, and (*d*) established agonist and antagonist profiles. Most of the major neurotransmitter receptors (with the exception of NA) express both ionotropic (ligand-gated ion channel) and metabotropic (G protein–coupled) receptor subtypes (see 45; **Table 2**).

NA produces a variety of effects by interaction with a number of different adrenoceptor subtypes. Several subtypes of α_1-, α_2-, and β-adrenoceptors have been characterized and cloned. There are many regulatory systems inherent in the adrenergic machinery, including autoinhibition

Table 2 Comparison of fast ionotropic and slow metabotropic receptors for acetylcholine (ACh), γ-aminobutyric acid (GABA), glutamate, and 5-hydroxytryptamine (5-HT) with those proposed for ATP. (Updated and reproduced from Reference 45 with permission from John Wiley and Sons.)

	Receptors	
Neurotransmitter	**Fast ionotropic (ligand-gated ion channels)**	**Slow metabotropic (G protein)**
ACh	Nicotinic	Muscarinic
	Muscle type	M1 → M5
	Neuronal type	
GABA	$GABA_A$	$GABA_B$
Glutamate	AMPA	$mGlu_1$
	Kainate	↓
	NMDA	$mGlu_7$
5-HT	$5\text{-}HT_3$	$5\text{-}HT_{1A\text{-}F}$, $5\text{-}HT_{2A\text{-}C}$, $5\text{-}HT_4$, $5\text{-}HT_{5A\text{-}B}$, $5\text{-}HT_6$, $5\text{-}HT_7$
ATP	$P2X_1 \rightarrow P2X_7$	$P2Y_1$, $P2Y_2$, $P2Y_4$, $P2Y_6$, $P2Y_{11}$, $P2Y_{12}$, $P2Y_{13}$, $P2Y_{14}$

AMPA, 2-(aminomethyl) phenylacetic acid; NMDA, *N*-methyl-D-aspartate.

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of NA release via presynaptic α_2 receptors, regulation of NA synthesis, and adrenoceptor desensitization and supersensitivity, which is dependent on agonist exposure. A schematic of noradrenergic transmission is presented in **Figure 5*a***.

ACh acts on two different classes of receptors. Nicotinic receptors are ionotropic and consist of subunits that constitute multimeric ligand-gated Na^+ channels that mediate fast responses. In the ANS, nicotinic receptors (subtype N_2) are mainly found within ganglia. In contrast, muscarinic receptors are metabotropic and coupled with G proteins, have slower responses, and are widespread throughout autonomic effector tissues and smooth muscle.

Two major types of purine receptor were distinguished (46): P1 receptors are most sensitive to adenosine and are competitively blocked by methylxanthines; P2 receptors are most sensitive to ATP, and their occupation may lead to prostaglandin synthesis. Pharmacological, biochemical, receptor binding, and cloning studies have enabled subdivision of these two types of receptors (see 47). The four subtypes of P1 receptors are A_1, A_{2A}, A_{2B}, and A_3: A_1 receptors are preferentially activated by N^6-substituted adenosine analogues, and their occupation leads to decreased cyclic AMP levels, whereas A_2 receptors show preference for 5′-substituted compounds and cyclic AMP levels are increased; occupation of A_3 receptors does not lead to changes in adenylate cyclase. Researchers have identified selective agonists and antagonists for these P1 receptor subtypes. P2 receptors have been divided into two major families: a P2X receptor family, which are ligand-gated ion channel receptors mediating fast transmission, and a P2Y receptor family, which are G protein–coupled receptors mediating slower responses. Currently, seven P2X ($P2X_{1-7}$) subclasses and eight P2Y subclasses ($P2Y_{1,2,4,6,11,12,13,14}$) have been recognized (48). These include receptors that respond to the pyrimidine derivatives UTP and UDP ($P2Y_1$, $P2Y_4$, $P2Y_6$), as well as to ATP, and receptors that respond to adenine dinucleotide polyphosphates.

Neuropeptide receptors are G protein–coupled receptors that activate either adenylyl cyclase or phospholipase C as signal transducers (38). There are multiple 5-HT receptors ($5\text{-}HT_3$ ionotropic; $5\text{-}HT_{1,2,4,5,6,7}$ metabotropic) in sympathetic, parasympathetic, and sensory ganglia, and excitatory amino acids (see 49).

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DUAL CONTROL OF VASCULAR TONE BY PERIVASCULAR NERVES AND ENDOTHELIAL CELLS

For many years, the excitatory actions of catecholamines released from sympathetic perivascular nerves and from the adrenal medulla into the bloodstream, and opposed by ACh released from parasympathetic nerves, were considered responsible for neurohumoral control of the vasculature. New and improved techniques in immunohistochemistry, electron microscopy, electrophysiology, and pharmacology have led to a wealth of discoveries that have profoundly reshaped our understanding of the regulation of vascular activities (50). In particular, cotransmission and the seminal discovery that endothelial cells, which form the innermost layer of blood vessels, play a crucial role in the vasodilatory response of the vessel to ACh (51). The considerable interest in vascular control mechanisms arising from these studies has led to the concept of a dual regulation of blood vessel tone by nerves and the endothelium (52; **Figure 8**).

Perivascular Nerves

Neurotransmission takes place at vascular neuroeffector junctions where the closest junctional cleft varies between approximately 50 nm to as much as 2 μm in some large arteries. Neuromodulators may be circulating neurohormones; local agents, such as prostanoids, bradykinin, or histamine; or neurotransmitter substances released from nearby nerves or even the same nerve varicosity. Many

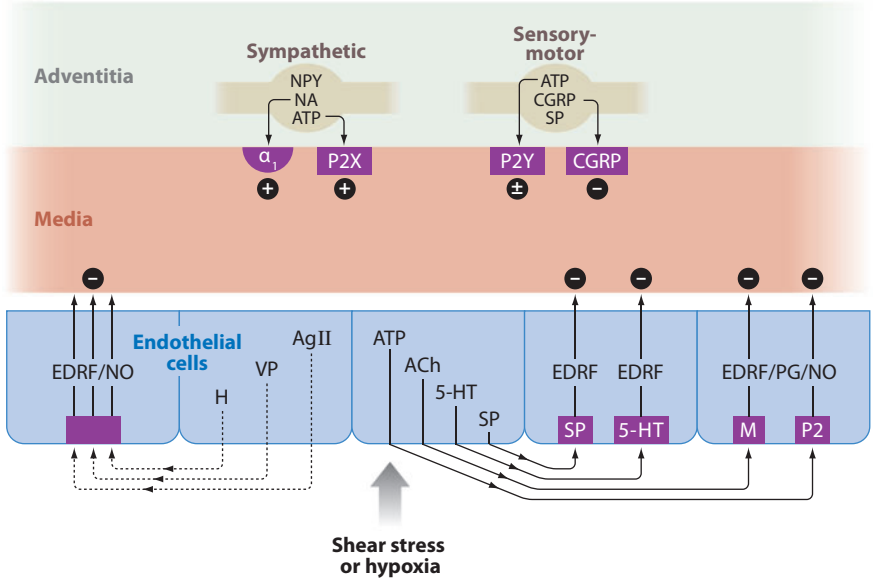

Figure 8

Regulation of vascular tone by perivascular nerves and endothelial cells. Neuropeptide Y (NPY), noradrenaline (NA), adenosine 5′-triphosphate (ATP), calcitonin gene-related peptide (CGRP), and substance P (SP) can be released from nerve varicosities in the adventitia to act on receptors in the media, causing vasoconstriction or vasodilation. ATP, ACh, 5-hydroxytryptamine (5-HT), and SP, released from endothelial cells by shear stress or hypoxia, act on their receptors on endothelial cells to cause a release of endothelium-derived relaxing factors (EDRF)/NO or prostaglandins (PG), which, in turn, act on the smooth muscle to cause relaxation. Angiotensin II (AgII), vasopressin (VP), and histamine (H) are also contained in, and may be released from, subpopulations of endothelial cells. In areas denuded of endothelial cells, vasoconstriction may be produced by circulating ATP and ACh via P2X and P2Y purinoceptors and muscarinic receptors (M) on the smooth muscle cells. (Modified from Reference 52 with permission from Oxford University Press.)

studies of sympathetic cotransmission, involving ATP and NA, have now been carried out on a number of different blood vessels (see 35). VIP released from parasympathetic nerves is a potent vasodilator of many vessels, notably penile vessels; it appears to play a major role in erection. It has been claimed that sensory-motor perivascular nerves use SP, CGRP, and ATP (see 39).

Little is known about the physiological roles or the pharmacology of intrinsic neurons of the heart because it is so difficult to study this in situ. However, our laboratory developed a novel culture preparation from the atria of newborn guinea pigs to study the intrinsic innervation of the heart under conditions of unequivocal extrinsic denervation (41). Some of these neurons show immunofluorescence for NPY, some for 5-HT, and some for variable mixtures of both transmitter substances. Projections of these neurons in situ form perivascular plexuses in small-resistance coronary vessels (53). Both NPY and 5-HT are potent vasoconstrictors of coronary vessels and may have synergistic actions. NOS is localized in a subpopulation of intrinsic cardiac neurons (42). Few studies have been carried out on the projections of intrinsic neurons to blood vessels in other organs, but intrinsic enteric neurons are known to supply some vessels in the gut and mesentery, and it is well known that monoamine-containing neurons in the brain contribute to the innervation of some cerebral vessels. NO appears to be a transmitter in cerebral and penile vessels and is perhaps released from local neurons.

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Endothelium

Since 1980, when Furchgott & Zawadzki (51) first reported that the vasodilation response to ACh requires the presence of an intact endothelium, the role of the endothelium in the regulation of vascular tone has attracted considerable interest (see 54). Action on endothelial receptors by a number of vasoactive substances stimulates the production of endothelium-derived relaxing factors (EDRF), endothelium-derived hyperpolarizing factor, and endothelium-derived constricting factors (EDCF) (see 50). These subsequently modify vascular tone by causing contraction or relaxation of the vascular smooth muscle. EDRF has been identified as NO (54), and the peptide endothelin is considered one of the constricting factors (55). There is considerable heterogeneity in the endothelium-dependent responses of mammalian blood vessels, with variations between arteries and veins and between different vascular beds. It is likely that such variations would be physiologically useful, particularly to ensure that the blood supply to the heart and brain are protected under a variety of different conditions.

In addition to ACh, endothelium-dependent vasodilation has been shown to occur in response to ATP, ADP, arachidonic acid, SP, neurokinin A, 5-HT, bradykinin, histamine, neurotensin, vasopressin (VP), angiotensin II (AgII), and thrombin (see 50). Different subtypes of the receptors to such vasoactive substances occur on the endothelium and on the vascular smooth muscle. For example, P2X receptors are present on the vascular smooth muscle and are acted on by ATP released from perivascular nerves to produce vasoconstriction; ATP can cause vasodilation via the P2Y and P2X receptors on the endothelial cells (see 56, 57).

It is unlikely that neurotransmitters can diffuse through the media and basal lamina of large blood vessels (without degradation) to act on endothelial receptors and produce vasodilation, although neurotransmitters may have a direct effect upon endothelium cells or microvessels. The possibility that endothelial cells may be the source of such substances was first proposed in 1985, when Parnavelas et al. (58) reported that ChAT, the enzyme responsible for the synthesis of ACh, could be localized in endothelial cells lining capillaries and small vessels in the rat cortex. Since this time, using the same technique of immunocytochemical staining combined with electron microscopy, ChAT, SP, 5-HT, VP, and AgII have been localized in endothelial cells from a variety of blood vessels (59). 5-HT, ATP, SP, and ACh were released after hypoxic perfusion of the Langendorf heart preparation from the rat (60). In the perfused rat hindlimb, increased flow caused the release of SP. After removal of the endothelium, increased flow no longer induced the release of SP, although denervation of the hindlimb vasculature of SP-containing nerves by capsaicin had no effect on flow-induced SP release (61).

It has been proposed that the endothelium mediates vasoconstriction via production of an EDCF in response to various chemical and physical stimuli, such as NA, thrombin, high extracellular potassium, hypoxia, and stretch (62). Endothelin and ATP, but not VIP, were released from isolated aortic endothelial cells exposed to increased flow. Receptors for endothelin were localized by autoradiography on cultured rat aortic smooth muscle cells, rat kidney, and human and porcine coronary arteries. A schematic model of the neural and endothelial factors involved in control of vascular tone is illustrated in **Figure 8**.

CENTRAL CONTROL OF AUTONOMIC FUNCTION

Previously, little attention was paid to the control of autonomic function by the CNS via sympathetic and parasympathetic pathways. There is now abundant evidence from the use of antidromic mapping; tracer mediators; and electrophysiological recordings from various areas of the brainstem, hypothalamus, and prefrontal cortex (see 63, 21) for CNS involvement with the regulation of

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cardiovascular, central chemoreception and respiration, gastrointestinal and urinogenital systems, as well as temperature regulation. There is also information accumulating about the transmitters involved in these regulatory mechanisms and their pharmacological manipulation in the CNS.

LONG-TERM (TROPHIC) SIGNALING AND PLASTICITY

Neurons possess the genetic potential to produce multiple neurotransmitters. The particular combination and quantity of neurotransmitters/neuromodulators expressed by neurons is partly preprogrammed and partly determined by trophic factors and hormones that modulate the expression or suppression of the appropriate genetic machinery. During a normal lifespan, there is constant potential for these factors to change, e.g., during development, pregnancy, hibernation, and altitude hypoxia. The plasticity of expression of neural substances coordinated to environmental cues allows rapid and precise matching of neurotransmission to altered demands.

In the late 1960s, we turned our attention to the question of long-term trophic interactions between autonomic nerves and cardiac and smooth muscle during development and wound healing (see 2, 64). Several types of systems were used for these studies, including perinatal development, anterior eye chamber transplants, regeneration of muscle minces in adult animals, and combined cultures of autonomic nerves and isolated smooth muscle cells, which were successfully grown for the first time in our laboratory by Mark et al. (65). The sequence of changes that occurred in joint nerve–smooth muscle cultures was similar to that described during normal development in vivo and in anterior eye chamber transplants of smooth muscle. Trophic factors released from nerves may be involved in the regulation of smooth muscle proliferation in vivo because sympathetic denervation of the ear artery in the young rabbit results in a reduction in the number of dividing cells compared with the control side (66). Levi-Montalcini et al. (67) first reported the influence of explants of autonomic effector organs on the growth of nerves from sympathetic ganglia in vitro involving NGF. NGF appears to affect cholinergic as well as adrenergic synthetic enzymes in immature sympathetic neurons (68). Little is known of the mechanisms involved in recognition of muscle cells by autonomic nerves, nor about the mechanism of rejection of further fibers that reach the cell after recognition and a long-lasting relationship is established (69).

Several neurotransmitters/neuromodulators are trophic molecules, with mitogenic or growth-promoting/-inhibiting properties. There is a growing interest in the trophic actions of purines (see 70). For example, extracellular ATP has been implicated as a mediator of programmed cell death, or apoptosis, and purine agonists prevent trophic changes caused by sympathetic denervation. Purines can act synergistically with growth factors to influence growth mitogenesis and morphogenesis. It has been suggested that extracellular ATP and ADP may play a physiological role in wound healing and act as a mitogenic agent in nervous and vascular systems (56).

A growing number of studies have been concerned with changes in autonomic nerves in development and aging (71, 72). Many of these studies addressed the innervation of blood vessels. For example, in a study of the changes in density of sympathetic adrenergic nerves in blood vessels of the rabbit, using image analysis quantitation, it was recognized that the pattern of change with age varied considerably between different vessels (73). There is a decrease in vasoconstrictor neurotransmitters and an increase in vasodilator neurotransmitters in cerebrovascular nerves in old age: In rat cerebral vessels, as in other vessels, the density of nerve fibers containing NA is reduced with age, and the density of CGRP and VIP-immunoreactive nerves is increased. In the gastrointestinal tract, there is extensive loss of enteric neurons and extrinsic sympathetic innervation in old age, and an increasing proportion of those remaining contain NOS (74). Age-related up- or downregulation of neurotransmitter receptor expression adds to overall changes in autonomic function (75).

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During pregnancy, the uterine wall undergoes considerable hypertrophy and hyperplasia with profound changes in innervation, particularly in the fetus-bearing regions. There is a progressive loss of NA-containing nerves innervating the uterus, leading to a disappearance of these sympathetic nerves in late pregnancy in parallel with a decrease in NPY- and VIP-containing nerves (see 26). There is also a switch from adrenergic to predominantly cholinergic responses in the uterine artery in late pregnancy (76).

In a series of studies on hibernation, we showed that changes in expression of transmitters and associated enzymes occurred in both the cardiovascular and visceral systems of hibernating hamsters (77–79). For example, NOS-positive and endothelin-positive endothelial cells were significantly lower than those observed in active, cold-exposed, or aroused animals, whereas there was an increase in TH- and NPY-immunoreactive perivascular nerves. There was a significant increase during hibernation of myenteric neurons immunoreactive to VIP, SP, CGRP, and cell bodies positive for TH, which are largely absent in control animals, whereas there was a significant decrease in the number of neurons expressing 5-HT. Interestingly, in an unpublished study, using microarray technology, we showed that, rather than the expected reduction in the number of mDNA transcripts, there was an increase, perhaps indicating the turn-on of genes serving an inhibitory role during hibernation, which are then rapidly turned off during arousal.

In recent years, the role of the ANS in adaptation to altitude hypoxia has become apparent (80). Chronic hypobaric hypoxia increases sympathetic activation and causes contraction of vascular smooth muscle, leading to a rise in pressure in the pulmonary arterial circulation, remodeling of the pulmonary vasculature, and development of pulmonary hypertension.

COMPARATIVE PHYSIOLOGY OF AUTONOMIC NEUROTRANSMISSION

Perhaps because of my early training in zoology, I have always found it valuable and fascinating to carry out comparative studies of the vertebrate ANS. Many fundamental problems can be more easily and profitably studied in primitive systems, and a knowledge of the pattern of evolution may give new insight into the mechanisms operating in man. Because of this predilection, I followed a tradition already established by my predecessor, J.Z. Young in the Chair of Anatomy at University College London. My colleagues and I examined the autonomic innervation of different organs in many different vertebrate species over a period of approximately 12 years. These and other data were brought together in a review entitled "Evolution of the Autonomic Innervation of Visceral and Cardiovascular Systems in Vertebrates" (81).

Cholinergic sympathetic postganglionic neurons, which have been shown to supply a number of visceral organs in mammals, are represented in much greater numbers in lower vetebrates. The nonadrenergic inhibitory fibers in the mammalian gut, many of which are under the control of preganglionic vagal nerve fibers, appear to be another example of a component of the ANS strongly represented in primitive vertebrates. The presence of a small number of adrenergic fibers in the vagus nerve in mammals has caused some confusion, but in view of the substantial component of sympathetic nerves that join the vagal trunk in lower vertebrates, this also appears to be a declining relic of the primitive state.

The presence of adrenergic terminal networks in ganglia in the bladder, heart, and gut in mammals appears to represent the emergence of presynaptic α-adrenoceptor neuromodulation as a new evolutionary trend, although it is found in rudimentary form in birds and reptiles. The presence of cholinergic vasodilator fibers in proximity to vessels in skeletal muscle beds appears to be a new evolutionary development unique to mammals. The confinement of perivascular nerves to the adventitial medial border associated with dual control of vascular smooth muscle by nerves

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and circulating catecholamines is a late evolutionary development because varicose nerve fibers occur within the media of many vessels in lower vertebrates.

Further features of lower vertebrates have disappeared in higher animals. Thus, the presence of intramural nerve cells containing NA in various organs appears to be an evolutionary development that took place in many early species (e.g., lizard gut, toad lung, frog bladder), but did not become established as a major feature of later evolution in mammals; no adrenergic cell bodies have been found in the mammalian gut (except in the proximal colon of guinea pig) and few in the heart and bladder. The influence of circulating catecholamines released from diffusely distributed chromaffin cells appears to play a much more significant role in lower than in higher vertebrates, in which direct nervous control is more sophisticated. Chromaffin tissue is widespread in cyclostomes and is closely associated with the segmentally arranged sympathetic ganglia in elasmobranchs and, to a large extent, in teleosts and amphibians.

This approach led me to consider the evolution of neurotransmitters. There are two essentially opposing views on the mechanisms by which new neurotransmitter systems evolve. One view is that neurons have a common phylohistogenetic origin and, owing to functional specialization, they differentiated into various chemical types in the course of evolution. An alternative view is that there were multiple origins of nerve cells, which meant wide diversity of neurotransmitters early in evolution and eventual reduction of the number of transmitters as a result of selection.

PATHOPHYSIOLOGY OF AUTONOMIC NEUROTRANSMISSION

There are often remarkable changes in the organization and neurotransmitter expression in the ANS as a result of pathophysiological situations such as trauma, surgery, and disease (82). Some of these changes are a compensatory advantage, and some lead to altered neural control of effector tissues, which is not beneficial. Manipulation of the ANS to encourage beneficial compensatory changes in nerve growth and the expression of neurotransmitters/neuromodulators and their receptors is an attractive means of therapeutic advance for autonomic dysfunction.

Central disorders of autonomic function include stroke, epileptic seizures, sympathetic hyperactivity in hypertension and insomnia, orthostatic hypotension, fever, vomiting, urine retention, pulmonary edema, neurogenic bladder, sexual dysfunction, and others (see 63). Detailed accounts of different types of autonomic failure can be found in Mathias & Bannister (83).

Chemical denervation and selected ganglionectomy studies have shown that loss of sympathetic or sensory innervation induces remarkable changes in the nerves that remain. These studies have been reviewed (26). For example, following chronic guanethidine sympathectomy (84), there is a striking increase in sensory innervation. This is attributed to increased availability of NGF (as there are no sympathetic nerves with which to compete), which promotes the growth of sensory nerves. In contrast, neonatal sensory denervation with capsaicin leads to sympathetic hyperinnervation of blood vessels. Immunosympathectomy by neonatal administration of antiserum to NGF leads to changes in expression of neuropeptides in the rat ileum. Denervation of extrinsic nerves to the gut provokes marked changes in expression of transmitters in enteric neurons and perivascular nerves. Similarly, following extrinsic denervation of the human respiratory tract by heart-lung transplantation, there are significant changes to the neurochemical makeup of the intrinsic neurons that remain. Following transection of preganglionic autonomic nerves or in spinal cord injury, there are marked changes in the nerves that remain. These manifest not only as nerve growth and changes in neurotransmitter expression but, remarkably, in reorganization of nerve pathways and their functions. The most dramatic examples of such plasticity occur in the urogenital tract (see 85, 86). Disorders of the urogenital tract can occur for a wide variety of reasons (see 87). Those involving the ANS range from trauma and diseases such as multiple

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sclerosis that affect the preganglionic autonomic neurons in the spinal cord to iatrogenic causes such as radical surgery or X-irradiation, which can result in local nerve damage, and, finally, to metabolic disorders such as diabetes. The most common cause of bladder dysfunction in males is obstruction owing to benign prostatic hypertrophy. During the course of obstruction, there is hypertrophy of the smooth muscle, and, in many cases, the bladder becomes hyperactive. Following hypertrophy, there is an increase in the size of autonomic postganglionic neurons in the rat major pelvic ganglion, and increased expression of α-adrenoceptors. Increased purinergic transmission has been demonstrated in patients with unstable obstructed bladders, interstitial cystitis, or neurogenic bladders. P2X receptors have been localized in nociceptive C-fibers, and ATP has the potential to contribute to bladder pain in inflammatory conditions as well as in micturition (88).

Reviews describing changes in expression of cotransmitters and receptors in diseased gut are available (40, 89, 90). VIP levels are reduced in the myenteric plexus and muscle layers of patients with idiopathic constipation, whereas levels of 5-HT are increased, and SP and NPY are normal. It has been known for many years that in the absence of enteric ganglia in the human colon, which occurs in Hirschsprung's disease, there is a striking hyperinnervation of the musculature by both adrenergic and cholinergic nerves (91). In Crohn's disease, there is an increase in VIP in the diseased intestine.

Changes in expression of autonomic transmitters and receptors have been described in disorders of the cardiovascular system (see 57); for example, irregularities of the sympathetic nervous system, renin-angiotensin system, and endothelial factors have been implicated in the development of hypertension. Sympathetic hyperinnervation and increased sympathetic activity have been reported in several vessels. Examination of sympathetic neurotransmission in the tail and mesenteric arteries of the spontaneously hypertensive rat (SHR) has revealed a greater cotransmitter role for ATP compared with NA. All three sympathetic cotransmitters, NA, NPY, and ATP, invoke a mitogenic response in human vascular smooth muscle cells, and there are indications that ATP regulates SHR smooth muscle cell proliferation via $P2Y_4$ receptors. Decreased sensorimotor nerve innervation may contribute to the development of hypertension. The potential role of endothelin in the genesis of hypertension has been extensively investigated as it is a potent vasoconstrictor, released from either endothelial cells or perivascular nerves (27). Impaired endothelium-mediated vasodilation has been described in atherosclerotic vessels (92). Diminished sympathetic nerve activity also occurs. Raynaud's phenomenon, in which there is inappropriate constriction of blood vessels to the digits on exposure to cold, is often an early event in the development of systemic sclerosis (93). There is early functional deficit of the vascular endothelium, but ANS dysfunction has also been linked with systemic sclerosis. Defective neurovascular sensory motor control is indicated by the reduction of CGRP-containing nerve fiber density around capillaries in dermal papillae.

Diabetes is the most common cause of autonomic neuropathy in humans. Diabetic autonomic neuropathy has been implicated in dysfunction of the cardiovascular, gastrointestinal, and urogenital systems (94). Studies of rats made diabetic by administration of streptozotocin (STZ) have provided a wealth of evidence for diabetes-induced changes in autonomic nerves throughout the vasculature and visceral organs. For example, both neurogenic and endothelium-dependent relaxation of erectile smooth muscle can be impaired in diabetic men with impotence and in animals with experimental diabetes. There is a similar loss of VIP and neurogenic relaxation in rats (95), and in patients with neurogenic impotence, there is also a significant reduction in NOS-containing nerve fibers in the corpus cavernosum. An early feature of autonomic nerve damage in diabetic gut may be a failure in release mechanisms resulting in accumulation of a neurotransmitter within the nerve (96). At a later stage, there is degeneration of nerve terminals resulting in loss of neurotransmitters. Not all autonomic nerves are affected in the same way by diabetes.

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For example, in rat cerebral vessels, there is a reduction of VIP and 5-HT but not NPY or NA in perivascular nerves in STZ-induced diabetes.

SOME FINAL SPECULATIONS

Although some remarkable findings have emerged in recent years that advance the early seminal discoveries of Sir Henry Dale and others, there are still enormous gaps in our understanding not only of details but also of the basic principles involved in autonomic neuroeffector transmission. For example, for more than 30 years there was resistance to the idea that there are transmitters other than NA and ACh in the autonomic system, and then in the following few years at least 12 putative transmitters were proposed. It will take time and many careful experiments before we know which of these, particularly the many neuropeptides, will survive the rigorous criteria required to establish them as neurotransmitters or cotransmitters. Some of them may be modulators rather than transmitters in the traditional sense; transmitters and modulators may coexist within the same autonomic nerve terminals and some may play trophic roles. In this connection, the question of how far we can relate the ultrastructure of intra-axonal vesicular components as an indication of transmitter type will need to be re-examined. The nature of interactions of different nerve components in integrated neural activity in local networks will also need to be resolved. Enteric plexuses in tissue culture appear to be a promising model for this type of study and the combination of electrophysiology, ionophoresis, and serial electron microscopy on single neurons may not only clarify the nerve interactions in the gut but also reveal neuronal mechanisms applicable to the even more complex interactions occurring in the CNS (see 97).

Much is still to be learned about neurotransmitter receptor chemistry and pharmacology, and one of the most outstanding questions in this field, largely neglected to date, concerns the multiple distribution of the receptors and their subtypes on autonomic effector cells and their interactions (see 98).

The status of vascular tone is the result of interactions between the neural and endothelial control mechanisms. It seems likely that spontaneous release of EDRF is responsible for a resting endothelial-mediated vasodilator tone, which is opposed by a resting vasoconstrictor tone mediated by sympathetic nerves. Under different physiological or pathophysiological circumstances, the balance may be altered so that one or the other may dominate. It seems likely that endothelial release of vasoactive substances may be of greater significance in the response of blood vessels to local changes in their environment, such as hypoxia and increased flow. In contrast, perivascular nerves may be more concerned with the integrative control of blood flow in the organism as a whole.

Studies of the trophic interactions of autonomic nerves and cardiac and smooth muscle are still in their infancy. Preparations in vitro may provide a particularly useful model, not only for the development of the autonomic neuromuscular system but also for questions relating to differentiation in general. For example, the powers of regeneration of autonomic nerves appear to be greater than those of motor nerves. The mechanism whereby nerves are attracted to effector cells and the way nerves recognize some muscle cells but not others can be fruitfully studied in vitro. Other intriguing problems can also be studied, like the mechanism underlying the dramatic transformation from vein to artery when venous transplants are made into the arterial circulation (99). The physiological role of NGF in the development of adrenergic nerves in perinatal growth and regeneration in adults is yet to be fully understood, and growth factors for cholinergic, purinergic, and other nerves are still to be identified.

As with all studies of basic mechanisms, time and energy ought to be devoted to bridging the gap with clinical practice. An understanding of the way the ANS works, develops, and adapts

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may contribute much to progress in the treatment of disease processes in conditions such as hypertension, impotence, incontinence, injury, irritable bowel syndrome, migraine, asthma, and diabetes. Treatment based on the assumption that only cholinergic and adrenergic transmitters are involved in autonomic control of effector muscles, and secretory or endocrine cells, may explain the stubborn resistance of such diseases to conventional treatments. The chronic administration of some drugs may produce irreversible damage to autonomic nerves (100): These effects need further study. Because the expression of cotransmitters and receptors changes so markedly with age, sex, and disease states, we cannot assume that drugs tested on young healthy male volunteers will be appropriate for a man of 80; thus the age, sex, and pathological history of individual patients will need to be taken into account in prescribing therapeutic strategies. Another possibility, although clearly not popular at present, is that the use of drug cocktails designed to match the precise coding of neurons projecting to a diseased or damaged area may be a more efficient strategy than using a single compound with widespread actions and then trying to find a dosage or procedure to avoid unwanted side effects. It is suggested that in neuropathological analysis, compensatory increases in expression of transmitter and density of nerves and receptors should be considered, as well as the more traditional approach of looking for damage or loss of nerves or downregulation of receptors. Studies of the mechanisms involved in the control of cotransmitter and receptor expression are now needed. Superimposed on the genetic programming of transmitter and receptor expression with development and aging there may be several different types of adaptive mechanisms involving growth factors, levels of activity in nerves, removal of inhibitory control, hormones, and semaphorins.

Finally, I suggest that the time has come to abandon the term adrenergic nerve (as well as cholinergic, peptidergic, purinergic, and nitrergic nerve) and speak rather of sympathetic nerves (also parasympathetic, enteric, and sensory nerves), with defined chemical coding when appropriate. However, I think that the term adrenergic or cholinergic transmission (or peptidergic or purinergic transmission) is still appropriate because it deals with a specific component of the transmission process, although interactions between the different transmitters via pre- and postjunctional modulatory mechanisms need to be clearly recognized. I hope that future studies will unravel the genetic and physiological mechanisms that control the expression of cotransmitters in aging and in various pathological conditions.

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DISCLOSURE STATEMENT

The author is not aware of any biases that might be perceived as affecting the objectivity of this review.

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