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Review

Non-neuronal regulation and repertoire of cholinergic receptors in organs

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Abstract

Many studies on the cholinergic pathway have indicated that cholinergic receptors, which are widely expressed in various cells, play an important role in all body organs. In this review, we present the concept that cholinergic responses are regulated through a neuronal or non-neuronal mechanism. The neuronal mechanism is a system in which acetylcholine binds to cholinergic receptors on target cells through the nerves. In the non-neuronal mechanism, acetylcholine, produced by neighboring cells in an autocrine/paracrine manner, binds to cholinergic receptors on target cells. Both mechanisms subsequently lead to physiological and pathophysiological responses. We also investigated the subunits/subtypes of cholinergic receptors on target cells, physiological and pathophysiological responses of the organs via cholinergic receptors, and extracellular factors that alter the subtypes/subunits of cholinergic receptors. Collectively, this concept will elucidate how cholinergic responses occur and will help us conduct further experiments to develop new therapeutic agents.

Keywords: cholinergic receptors; neuronal regulation; non-neuronal regulation; organ; target cells.

Introduction

A growing body of evidence indicates that various cells express cholinergic receptors (1), suggesting that cholinergic signaling plays a key role in physiological functions. Cholinergic receptors are divided into nicotinic and muscarinic acetylcholine receptors (AChRs). Nicotinic AChRs belong to the receptors of ligand-gated ion channels (2), which have a wide variety of subtypes, including complex combinations of 16 subunits in mammals (α1, α2, α3, α4, α5, α6, α7, α9, α10, β1, β2, β3, β4, γ, δ, and ε). Muscarinic AChRs belong to class 1 heptahelical G-protein-coupled receptors, which have five distinct subtypes (M1, M2, M3, M4, and M5) (3).

Recent studies have shown that the regulation of cellular function by cholinergic receptors is accomplished in two different ways (1). The first type of regulation is characterized by binding of acetylcholine (ACh) derived from cholinergic nerves to cholinergic receptors on target cells. We designate this system as 'neuronal regulation'. This system which constitutes functional connections between neurons and target cells is partly composed of chemical synapses on interneurons or motor endplate. The second type of regulation is characterized by binding of ACh derived from neighboring cells, which produce and release ACh in an autocrine/paracrine manner, to cholinergic receptors on target cells. We designate this type of regulation as 'non-neuronal regulation'. In neuronal regulation, although it is well known that ACh regulates the function of organs via muscarinic receptors, convincing evidence has been published in recent years that not only muscarinic receptors but also nicotinic receptors on target cells play a vital role in functional cholinergic regulation (1). In non-neuronal regulation, ACh-producing cells, which contain choline transporters to uptake choline, choline acetyltransferases (ChATs) to synthesize ACh, vesicular ACh transporters (VAChTs) to translocate ACh, and acetylcholinesterase (AChE) to degrade ACh into choline and acetate, and target cells possess both muscarinic and nicotinic receptors. Several researchers have demonstrated that an abundant majority of mammals including human cells synthesize ACh (1). By contrast, the repertoire of cholinergic receptors expressed on target cells are diverse and are altered by extracellular factors associated with proliferation, differentiation, stress, and others (1). It is important to unravel the influence by various cytokines against cholinergic responses.

In this review, we discuss each organ or system which is regulated in a non-neuronal manner in separate sections. This review also focuses on the following issues: the regulation of cellular function by cholinergic receptors; subtypes/subunits of cholinergic receptors on target cells regulated by endogenous cholinergic ligands, including ACh and other ligands; physiological and pathophysiological responses of the organs via cholinergic receptors; extracellular factors that alter the subtypes/subunits of cholinergic receptors.

Vascular system

This section focuses on endothelial cells in the vascular system. Since Parnavelas et al. found the immunocytochemical localization of ChAT in endothelial cells of small brain vessels, several investigations have conducted experiments to clarify the expression of the non-neuronal cholinergic system in endothelial cells (4, 5). Although there is no evidence that the endothelium, the cell layer that forms the inner lining of blood vessels, is innervated by cholinergic nerves, endothelial cells possess a cholinergic system, suggesting that ACh exerts physiological effects via AChRs in endothelial cells. Nitric oxide (NO), a mediator of vascular smooth muscle relaxation, is released by the vascular endothelium in response to ACh through muscarinic AChRs on the endothelial cell membrane (6).

As established in a study by Cooke and Ghebremariam, nicotine exerts angiogenic effects via nicotinic AChRs (7). They also described that the selective α7 antagonist α-bungarotoxin completely and reversibly inhibited endothelial network formation in an *in vitro* angiogenesis model, and that the pharmacological inhibition of α7 significantly exhibits attenuated angiogenic response to ischemia and inflammation *in vivo*. However, this response, which is associated with the abrogation of α7 in $\alpha7^{-/-}$ mice, was reduced in comparison with the pharmacological inhibition of α7, possibly owing to developmental upregulation or compensation of other nicotinic AChRs. Pharmacological inhibition of nicotinic AChRs containing β2 tended to slightly reduce network formation *in vitro* because of their high affinity with the α3β2 or α4β2 subtype, indicating that this alternative pathway could be present in $\alpha7^{-/-}$ mice.

Other studies demonstrated that endothelial cells express functional α3, α5, β2, β4, α7, and α9 (8, 9). In skin keratinocytes and bronchial epithelial cells, α3 seems to be involved in the maintenance of the flat shape of the cells or the bronchial surface (10). The presence of the cholinergic system in keratinocytes and endothelial cells supports the hypothesis that self-stimulation of α3 represents a general cellular mechanism for the maintenance of the integrity of both the external and internal surfaces.

The expression of the muscarinic AChR subtypes in endothelial cells differs in each tissue (11). Genetic studies suggest that M3 mediates the vasodilatory actions of ACh in several vascular beds (12). However, M1 has been suggested to mediate cholinergic dilation in human pulmonary vasculature (13). Yamada et al. reported that vasodilation elicited by ACh is abolished in cerebral arteries isolated from $M5^{-/-}$ mice, whereas the effects of ACh on coronary and carotid arteries remain intact (14). Evora et al. reported that endothelium-dependent vasodilatation to hypothermia is mediated by M1 in systemic and coronary arteries (15). Taken together, these results indicate that endothelial cells in the vascular system are regulated by non-neuronal regulation and contain functional nicotinic and muscarinic AChRs.

Skin

In this section, the cholinergic effects on keratinocytes, fibroblasts, and melanocytes in the skin are discussed. Grando et al. found that normal human keratinocytes possess ChAT and AChE, and synthesize, store, release, and degrade ACh, the cholinergic system is involved in basic functions of the skin through an autocrine/paracrine manner (10, 16). Recent studies have demonstrated that keratinocytes, fibroblasts, and melanocytes express one or more functional elements of the cellular cholinergic system (1, 10). In the epidermis, the repertoire of cholinergic receptors demonstrates temporally and spatially diverse patterns. During cell maturation, keratinocytes respond to ACh via different combinations of AChRs at each developmental stage. Immature keratinocytes respond to ACh predominantly via the α3β2 or α3β4 subtype with or without α5, M2, and M3; transitional keratinocytes have more α5 containing α3, and also express α9 as well as M4 and M5; mature keratinocytes possess mainly α7 and M1 (10). From the viewpoint of spatial distribution, within the epidermis, α3, α5, β2, and β4 are expressed in the basal cell layer and in a single-cell layer in the stratum granulosum; α9 is expressed in the basal and lower spinous layers. α7, α10, and β1 are selectively detected in the upper spinous and granular layers. With regard to muscarinic AChRs, M1 and M4 are found in the suprabasal layers, whereas M2, M3, and M5 are restricted to the lower layers. In the outer root sheath of the hair follicle, all AChRs, except α9, β1, and M4, are found in the basal cell layer, whereas α9, M4, and M5 are restricted to the central cell layer. α5, β1, β2, M1, M2, M3, and M4 are strongly expressed in the inner root sheath (10).

α7 nicotinic AChRs in keratinocytes control homeostasis and terminal differentiation. The phenotypic abnormalities in the epidermis of $\alpha7^{-/-}$ mice were consistent with retention hyperkeratosis, which is a morphologic manifestation of delayed epidermal turnover (10). $\alpha7^{-/-}$ keratinocytes also demonstrated changes in the gene expression of α3, α5, α9, and α10, suggesting that ACh signaling in these cells is rerouted to alternative cholinergic pathways. These changes are partially compensated by redirection of cholinergic signaling via α3 in keratinocytes associated with immature cells, and via α9 in keratinocytes that are coupled to the regulation of apoptotic secretion. Long-term blocking of α3, α9, and M3 signaling pathways results in cell-cell detachment and changes in the expression levels of E-cadherin, β-catenin, and γ-catenin, suggesting that α3, α9, and M3 play distinct roles in cholinergic control of keratinocyte adhesion.

Chernyavsky et al. demonstrated the relationship between AChRs and epithelialization by using a gene-targeting technique (17, 18). The pivotal role of M4 in facilitating keratinocyte crawling locomotion has been convincingly clarified in experiments in which M4 expression was either knocked down with siRNA or completely abolished in $M4^{-/-}$ mice. Studies with anti-M3 siRNA and $M3^{-/-}$ mice demonstrated that reduced or absent signaling through M3 increases keratinocyte migration. Available data indicate that ACh regulates keratinocyte migration through both stimulatory and inhibitory signaling pathways, involving different populations of cell surface AChRs. α9 directly regulates cell-matrix and cell-cell adhesion. In contrast to *in vitro* experiments, no obvious defects in cell-extracellular matrix or cell-cell adhesion have been observed in $\alpha9^{-/-}$ mice, suggesting that chronic abrogation of α9 signaling is compensated through alternative cholinergic pathways.

Mammalian secreted lymphocyte antigen-6/urokinase-type plasminogen activator receptor-related peptide-1 (SLURP-1) is a positive allosteric ligand for α7 (19). Arredondo et al. have demonstrated that recombinant SLURP-1 binds to α7 on human keratinocytes and induces proapoptotic activity (20). SLURP-1 acts as an epidermal modulator essential for keratinocyte homeostasis. Human epidermal and oral keratinocytes also produce and secrete a novel cholinergic peptide, SLURP-2 (19). The biological effects of SLURP-2 on keratinocytes are predominantly mediated by α3 receptors, which contain heteropentameric nicotinic AChRs and are involved in the inhibition of terminal differentiation and apoptosis (21). This is in marked contrast to the binding mode exhibited by recombinant SLURP-1, which has a higher affinity for α7. When keratinocytes were exposed to carbachol, α7 worked together with M1 to exert its effect on the directionality of crawling locomotion. These results suggest that synergic cooperation between nicotinic AChRs and muscarinic AChRs is required for keratinocyte reorientation toward the concentration gradient of ACh (22).

Under different *in vitro* and *in vivo* conditions, dermal fibroblasts can express the α3β2 or α3β4 subtype with or without α5, α7, α9, M2, M4, and M5 coupled to the regulation of calcium influx (10). Other studies indicated that carbachol and pilocarpine act on skin fibroblasts via M1 and M3 (23, 24). Skin melanocytes express α1, α3, α5, α7, β1, β2, γ, and δ in nicotinic AChRs and all muscarinic AChRs (19). Collectively, keratinocytes, fibroblasts, and melanocytes in the skin are subject to non-neuronal regulation and contain functional nicotinic and muscarinic AChRs.

Respiratory system

In this section, we discuss smooth muscle cells, epithelial cells, fibroblasts and tumor cells in the respiratory system. Smooth muscles are innervated by cholinergic nerves to induce contraction. The expression of nicotinic AChRs in airway smooth muscles is still under debate (25). Dorion et al. reported that nicotinic AChRs in murine airway smooth muscles are of α4 and α7 (26). Their results clarified that relaxation of tracheal smooth muscles could be directly mediated by α4. However, an indirect effect via NO produced by airway epithelial cells is also suggested. α6, β2, and β4 were detected in airway and vascular smooth muscle cells derived from the monkey (27). The functional expression of muscarinic AChRs is definitive (28). Airway smooth muscle contraction in humans is regulated by the parasympathetic nerves via both M2 and M3 (29). The contraction of airway smooth muscle is mediated by M3, but activation of postsynaptic M2 is also likely to contribute to this response. ACh leads to binding to prejunctional M2, which mediates the inhibition of ACh release.

Klapproth et al. have reported that ChAT, high affinity choline transporter (CHT1), and its product ACh are present in many non-neuronal cells in the lung and airways (30). Using immunohistochemistry and RT-PCR, Proskocil et al. have demonstrated that lung bronchial epithelial cells express ChAT, VAChT, and CHT1 (31). It is well established that bronchial epithelial cells have all the necessary components for the production, storage, secretion, and degradation of ACh, which acts as an autocrine/paracrine molecule (32). α1, α3, α4, α5, α7, α9, β1, β2, β4, δ, and ε nicotinic AChRs, and M1 and M3 AChRs, are expressed in the human airway epithelium (1). The α3α5β2 subtype in bronchial epithelial cells plays a vital role in wound repair, which is significantly improved by nicotinic AChR agonists such as nicotine and ACh. By contrast, wound repair is delayed in the presence of nicotinic AChR antagonists of the α3β2 subtype, such as mecamylamine, α-conotoxin MII, and κ-bungarotoxin. These observations demonstrate that the α3α5β2 subtype actively contributes to the modulation of cell shape in the respiratory tract (33). Regeneration of the airway epithelium after epithelial desquamation injury in $\alpha7^{-/-}$ mice is delayed and characterized by transient hyperplasia of basal cells. α7 is involved in the differentiation of the respiratory epithelium and is essential for regeneration for airway remodeling (34).

M3 is expressed in airway epithelium and regulates cell proliferation through the release of a diffusible factor (35). M1 is also detected in the airway epithelium and induces cell proliferation (36). Ciliary beating of airway epithelial cells removes mucus and particles from the airways (37). A recent investigation on muscarinic AChR gene-deficient mice has elucidated that M3 stimulates cilia-driven particle transport, whereas M2 inhibits and M1 increases cilia-driven particle transport if M3 and M2 are missing, respectively. However, none of the muscarinic AChRs is essential for epithelial development (38).

Song et al. reported that all components of an ACh autocrine loop including ChAT, VAChT, and CHT1 are expressed, and α3, α5, α7, β2, β4, M3, and M5 were found in SCLC cells (39). They also demonstrated that lung cancers secrete ACh and that cholinergic signaling modulates growth as an autocrine growth factor, and the existence of this cholinergic autocrine loop in lung cancers suggests the importance of non-neuronal ACh in pathologic processes (39). These results suggest that smooth muscle cells undergo neuronal regulation, whereas epithelial cells, fibroblasts, and tumor cells in the respiratory system undergo non-neuronal

regulation. These cells contain functional nicotinic and muscarinic AChRs.

Bladder

This section focuses on bladder smooth muscle cells and urothelial cells. Bladder smooth muscles are innervated by cholinergic nerves. Whereas male $M3^{-/-}$ mice showed severe urinary retention, the urinary bladder of $M2^{-/-}$ mice is normal in both sexes (28), suggesting that M3, but not M2, is essential for voiding in males.

Recent studies suggest that the non-neuronal cholinergic system could contribute to the physiology and pathophysiology of human bladder function (40, 41). Yoshida et al. have demonstrated that ACh is measured by high-performance liquid chromatography and ChAT is detected by immunohistochemistry in the urothelium (40). Lips et al. detected ACh in a nanomolar range per gram of wet weight and the expression of carnitine acetyltransferase, VAChT, and polyspecific organic cation transporters in the urothelium (41). Murine urothelial cells express several subtypes of both muscarinic and nicotinic AChRs, which are differentially distributed among urothelial cell types (42). α9 and M2 are exclusively observed in umbrella cells, and α4, α7, α10, M3, M4, and M5 are also detected in the intermediate and basal cell layers. α5 is localized only in the basal cell layer. In the rat urothelium, α3, α5, α7, β3, and β4 are detected as functional receptors that affect micturition (43). The human urothelium carries multiple cholinergic receptor subtypes, all muscarinic AChRs and α7, α9, and α10, with predominant expression of α7, M2, and M3 (44). Taken together, these results suggest that bladder smooth muscle cells undergo neuronal regulation and contain functional muscarinic AChRs, whereas urothelial cells undergo non-neuronal regulation and contain functional nicotinic and muscarinic AChRs.

Reproductive system

Parts of the male reproductive tract such as efferent ductules and the epididymis receive cholinergic innervations (45). Pharmacological studies suggest that M1, M2, and M3 are expressed in the efferent ductless, caput, and cauda epididymis (46). M3 is exclusively expressed in the peritubular smooth muscle layer of the epididymis, and the carbachol-induced contractile response of the epididymal tubules is blocked by selective M1 and M3 antagonists. These results suggest that M3 plays a crucial role in smooth muscle contraction in the epididymis. Regulation of muscarinic AChR density by androgen has been shown in the prostate and urinary bladder (47). Maróstica et al. demonstrated that orchidectomy increased M1 in the caput and cauda epididymis, and testosterone replacement slightly modified this effect (48). Whereas orchidectomy downregulates the level of M2 mRNA in both the caput and cauda epididymal regions, castration significantly upregulates the amount of M3 mRNA in the caput region. This regulation is prevented by testosterone replacement in castrated rats, suggesting that testosterone and/or testicular factors play a role in the regulation of expression of muscarinic AChRs in the rat epididymis (48).

Although the ovary is innervated by the autonomous nervous system, whether the ovary receives cholinergic innervation is unclear (49). However, recent papers described that the ovary has a cholinergic system: granulosa cells were shown to possess ChAT and VAChT (49, 50). Fritz et al. have reported that M1, M3, and M5 are present in adult human and monkey ovaries, and that luteinized human granulosa cells express M1 and M5 (49). These findings suggest that ovarian ACh plays a role in the regulation of cell proliferation in developing follicles and in the modulation of steroid production (49). Taken together, these results suggest that smooth muscle cells in the epididymis undergo neuronal regulation and contain functional muscarinic AChRs, whereas the ovary at least undergoes non-neuronal regulation and contains functional muscarinic AChRs.

Hematopoietic system

In the following section, we discuss the cholinergic effects on hematopoietic cells. Cells possess a cholinergic system and using gene arrays, ChAT and AChE were detected (51). α4, β2, and α7 are detected in murine bone marrow cells. Whereas the expression of α4 and β2 is downregulated in both erythroid and myeloid progenitor cells, α7 expression is upregulated in the mature phase. These results suggest that endogenous cholinergic ligands, such as ACh, regulate both myeloid and erythroid cells during development (52). Collectively, these results suggest that hematopoietic cells undergo non-neuronal regulation and contain functional nicotinic AChRs.

Immune system

The cholinergic effects on lymphocytes, mononuclear leukocytes, bone marrow-derived dendritic cells, and macrophages are discussed in this section. Several investigators have reported that ACh plays a vital role in immune response (53–55). The lymphocytic cholinergic system is activated by ACh via both muscarinic and nicotinic AChRs on T cells, B cells, dendritic cells, and macrophages, leading to the modulation of immune cell function. However, the origin of ACh is under debate because immune cells are not directly innervated. Tracey has found an efferent arm of the inflammatory reflex called the ‘cholinergic anti-inflammatory pathway’ through a series of seminal studies (55). The results showed that the vagus nerve can inhibit cytokines such as tumor necrosis factor-α (TNF-α) and thereby prevent tissue injury via macrophages (55). ACh released from the vagus nerve is known to act via α7 to suppress the synthesis and release of the proinflammatory cytokine TNF-α from macrophages, thereby suppressing inflammatory responses. Rosas-Ballina and Tracey have also demonstrated that the spleen is required for the interaction between immune cells and ACh (56).

Activation of a brain cholinergic network that depends on M1 increases the activity of the vagus nerve. The vagus nerve controls immune cell function in the spleen through a system of two serially connected neurons: one is a preganglionic neuron, which originates from the dorsal motor nucleus of the vagus nerve, and the other a postganglionic neuron, which originates from the ganglia of the celiac superior mesenteric plexus and travels along the splenic nerve. Rosas-Ballina and Tracey have hypothesized that norepinephrine release by the splenic nerve would act on β2-adrenergic receptors expressed on macrophages to attenuate TNF-α, and α7 expressed on neurons of the celiac superior mesenteric plexus would convey signals between the vagus and splenic nerve (56). They have also proposed the hypothesis that norepinephrine from splenic nerve terminals can induce the release of ACh derived from lymphocytes, which would then act on α7 expressed on macrophages (56). Moreover, another concept should be considered in the 'cholinergic anti-inflammatory pathway'. As suggested by Nance and Sanders, the stimulation of the efferent vagal nerve caused activation of the adrenal medulla via the sympathetic nervous system (57). In addition to this notion, Wessler and Kirkpatrick have proposed the noteworthy concept that neuronal ACh released from efferent and also from afferent fibers is involved and triggers the release of non-neuronal ACh from neighboring cells, passing the signal such as a wave within mucosal and parenchymatous tissues (1).

Lymphocytes contain all the components required to form a cholinergic system (55). Whereas lymphocytes contain ACh, AChE, CHT1, and AChRs, AChR expression was variable. Some recent studies have shown that all human thymocytes, mononuclear leukocytes, and human leukemic cell lines express neuron-type nicotinic AChRs, including α2, α3, α4, α5, α6, α7, α9, α10, β2, and β4, whereas no expression of muscle-type subunits has been observed. $CD8^+$ T cells from $M1^{-/-}$ mice cannot differentiate into cytolytic T lymphocytes. Several factors such as phytohemagglutinin, which activates T cell function, and CD11a, which is involved in cellular adhesion and co-stimulatory signaling and is also the target of the drug efalizumab, have been found to enhance lymphocytic cholinergic activity through upregulation of M5 (55). Moreover, both SLURP-1 and SLURP-2 are expressed in mononuclear leukocytes, dendritic cells, and macrophages, suggesting that these endogenous ligands could be involved in regulating lymphocyte function via the nicotinic AChR-mediated pathway (58).

In the mouse thymus, differential expression is observed. α2, α3, α4, α5, α7, β2, and β4 are expressed in thymic stromal cells, and α3, α5, α7, β2, and β4 are expressed in immature T cells. Peripheral T cells seem to express an unusual profile of α2, α5, and α7. These results demonstrate the important roles of nicotinic AChRs in the development of the neuroimmune network (59). Continuous exposure of isolated fetal murine thymus to nicotine increases the number of immature T cells while decreasing the number of mature T cells, suggesting that nicotinic AChRs are involved in the regulation of thymocyte development (60).

Mononuclear leukocytes, bone marrow-derived dendritic cells, and macrophages express mRNAs encoding the five muscarinic AChRs and α2, α5, α6, α7, α10, and β2 (54). Interferon-γ differentiates EoL-1 into macrophages in association with the upregulation of M3 and M5 (61). Nicotinic AChRs are involved in the regulation of B lymphocyte development and activation (53). Whereas the α4(α5)β2 subtype is expressed in immature B lymphocytes of the bone marrow, the α7(α5β4) subtype is expressed in mature B lymphocytes in the spleen. Moreover, $\beta2^{-/-}$ mice have less serum IgG, IgG-producing cells, and natural IgG antibodies than their wild-type counterparts, and the absence of β2 leads to increased B lymphocyte activation and antibody immune response. ACh inhibited histamine release from mast cells via M1 (62). Kindt et al. reported α3, α5, and α10 expression in human skin mast cells (63). They also detected α3 and α5 in lesional skin area of patients with atopic dermatitis. These results suggest that immune cells undergo non-neuronal regulation and contain functional nicotinic and muscarinic AChRs.

Bone

Although the bone is almost completely made up of hard tissue, the periosteum, which surrounds the bone, is innervated by sympathetic nerve fibers (64), suggesting that osteoblasts lining the cortical bone are innervated. Shi et al. have demonstrated that M3 in the brain plays a vital role in the regulation of bone remodeling (65). These results supported the reports on an interaction between the neuronal system and bone metabolism. Recent studies indicate that osteoblasts containing a cholinergic system release and restore ACh (66, 67). mRNA of ChAT, CHT1, and VAChT were detected on osteoblasts. It has been speculated that central activation of M3 increases the sympathetic tone through the sympathetic nerves innervated into the periosteum, and neurons subsequently release norepinephrine, which directly acts on β2-adrenergic receptors expressed on osteoblasts. ACh derived from these cells acts on neighboring osteoblasts, resulting in increased bone formation.

The expression of AChRs in osteoblasts is still controversial. Walker et al. described the presence of α4 in human primary osteoblasts (68), and Katono et al. reported that nicotine increased the expression of α7 in a human osteosarcoma cell line (69). En-Nosse et al. have demonstrated that α3, α5, α9, α10, β2, β3, M2, and M5 are detected in human or murine osteoblastic cell lines (66). They also showed that α3 and α5 are upregulated in the presence of bone morphogenetic protein-2 (BMP-2) during healing of fractures. By contrast, we have reported that once ascorbic acid and β-glycerophosphate induce the differentiation of both murine primary osteoblasts and murine pre-osteoblastic cell lines, which express only β4, osteoblasts begin to express α1, α6, α7, β1, δ, ε, M1, M2, and M4 and produce ACh in the mature phase (67). In this model, ACh is believed to inhibit alkaline phosphatase activity via β4, and inhibitory signaling of differentiation by ACh is blocked by α6, α7, M2, and M4. Interestingly, α1, β1, δ, and ε are expressed in the muscular system but not in the nervous system, whereas α6 and

Table 1 Target cells, organs, and regulation.

Regulation	Target cells	Organs
Neuronal	Muscle cells	Respiratory system, gastrointestinal system, bladder, reproductive system (epididymis)
	Glandular cells	Gastrointestinal system
Non-neuronal	Endothelial cells	Vascular system
	Fibroblasts	Skin, respiratory system
	Keratinocytes, melanocytes	Skin
	Epithelial cells	Respiratory system, bladder
	Tumor cells	Respiratory system
	Luteinized granulosa cells	Reproductive system (ovary)
	Lymphocytes, dendritic cells, macrophages, mast cells	Immune system
	Osteoblasts	Bone
	Myeloid and erythroid cells	Hematopoietic system
	Adipocytes	White adipose tissue

α7 are expressed in the nervous system but not in the muscular system, suggesting that the nicotinic AChRs of mature osteoblasts exhibit properties of the nervous and muscle systems. More recently, Liu et al. also demonstrated that osteoblasts equip with muscarinic AChRs (70). Taken together, these results suggest that osteoblasts undergo non-neuronal regulation and contain functional nicotinic and muscarinic AChRs.

White adipose tissue

Although white adipose tissue (WAT) is innervated by the sympathetic nervous system (71), no parasympathetic nerve markers in WAT of several species are detected (72). The existence of a cholinergic system in WAT has not been clarified. Parasympathetic neural activity increases the insulin sensitivity of WAT (73). $M3^{-/-}$ mice had impaired glucose homeostasis and insulin sensitivity, reduced food intake, and a significant elevation in basal and total energy expenditure owing to increased central sympathetic outflow and increased rate of fatty acid oxidation (74). More recently, Yang et al. have found that ACh reduces glucose uptake and lipolysis in WAT via M3 (75). Systemically administered nicotine induces lipolysis, in part, by directly activating nicotinic cholinergic receptors located in adipose tissue (76). In addition, Liu et al. detected the expression of functional nicotinic AChRs in adipocytes (77). α1, α2, α3, α4, α5, α6, α7, α9, α10, β1, β2, β3, β4, δ, and ε mRNAs were detected by RT-PCR in adipocytes, whereas the presence of α7 and β2 was demonstrated by immunocytochemistry. The existence of ACh derived from fat tissue in skin has been described by Wessler et al. (78). These results suggest that adipocytes undergo non-neuronal regulation and contain functional nicotinic and muscarinic AChRs.

Expert opinion

In each organ, cholinergic receptors exist as effectors that influence physiological response. We can summarize the target cells and organs based on two regulatory systems (Table 1). The regulatory system mediated by cholinergic pathways is classified into two patterns, as shown in Figure 1. Neuronal regulation is a system which cholinergic nerves regulate target cells (Figure 1A). In non-neuronal regulation, which is an autocrine/paracrine system, cholinergic ligands produced by neighboring cells binds to cholinergic receptors on target cells including endothelial cells (vascular system), epithelial cells (respiratory system and bladder), keratinocytes, fibroblasts (skin and respiratory system), melanocytes, tumor cells (respiratory system), luteinized granulosa cells, hematopoietic cells, adipocytes, immune cells, and osteoblasts (Figure 1B).

Based on *in vivo* experiments (65, 79), we speculate that non-neuronal cells which constitute the spleen or the periosteum, except for circulating immune cells, immune cells within the mucosa or circulating osteoblasts, can be regulated not by parasympathetic but by sympathetic neurons from the brain. It is conjectured that signals from brain through sympathetic nerve fibers affect immune cells or osteoblasts and then ACh released from these cells might affect themselves or neighboring cells via an autocrine/paracrine manner. With regard to WAT, we hypothesize that adipocytes in WAT could be involved in the same regulatory mechanisms via the sympathetic nervous system. Because genetic studies using knockout animals provide us with useful data, it is important to analyze the animals which lack the genes on nicotinic or muscarinic cholinergic receptors to validate this hypothesis. In this context, we propound a new concept that sympathetic nerves directly regulate target cells which equip with the non-neuronal cholinergic system. This concept seems to be interesting because cholinergic regulation could be modulated by sympathetic nerves via the cholinergic system. This system possesses the property including both neuronal (e.g., via nerve) and non-neuronal (e.g., via non-nerve) regulation. Although the structure between sympathetic neurons and target cells is unclear, we assume there might be an interaction such as synapse.

Although the non-neuronal cholinergic system has been explored in different organs, the way ACh acts on target cells without influence of cholinesterase still remains unknown. Kawashima and Fujii speculate that non-neuronal ACh

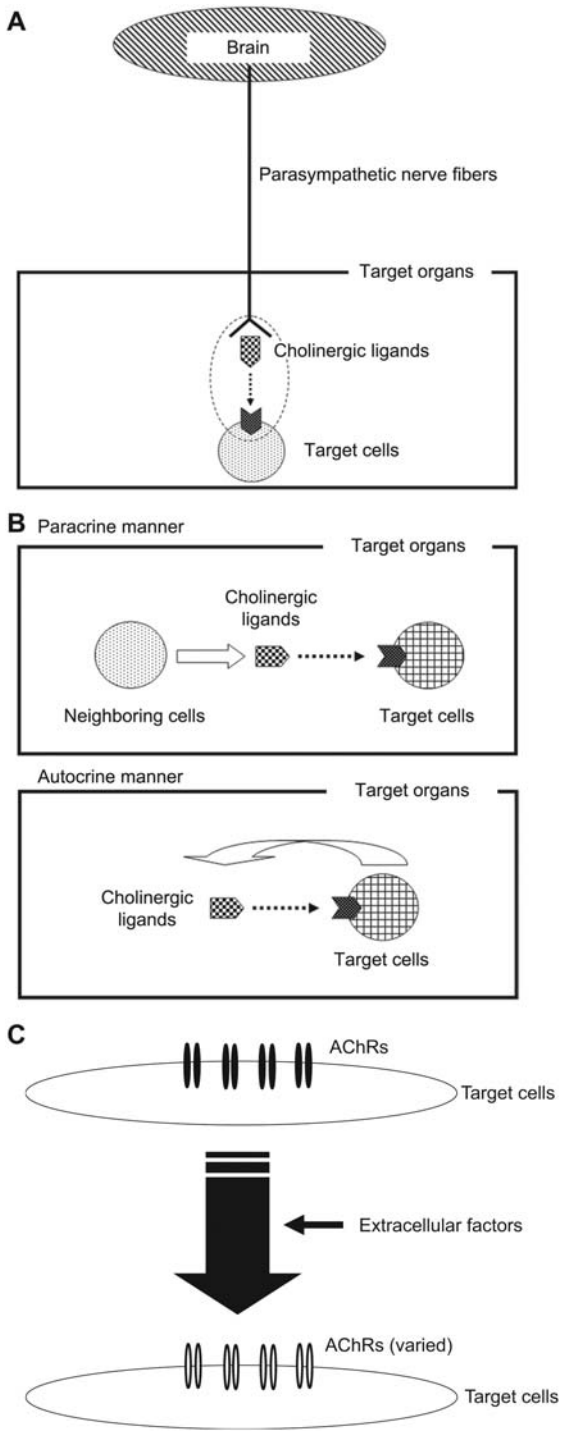

Figure 1 Cholinergic receptor regulation via a neuronal or non-neuronal manner and receptor modulation by extracellular factors. (A) Target cells are dominated by central nerves. (B) Target cells are dominated by neighboring cells or themselves. (C) Extracellular factors alter the repertoire of cholinergic receptors. Ellipses represent AChRs; white ellipses represent altered AChRs.

released from neighboring cells modulates via interaction through cell surface molecules (80). On the one hand, Kummer et al. postulate that there might be little effect of AChE in the airway epithelium because of AChE activity (32). Does a non-neuronal ACh exist *in vivo*? A recent seminal experiment which was conducted by Schlereth et al. has demonstrated the *in vivo* release of non-neuronal ACh in humans without a substantial contamination by neuronal ACh (81). This result strongly supports the evidence that ACh avoids the influence of AChE and subsequently acts on target cells in a non-neuronal manner *in vivo*. However, the mechanism whereby ACh avoids the effect of AChE and AChE degrades ACh is still unclear.

Not only ACh but also other cholinergic ligands play a pivotal role in physiological response. Both SLURP-1 and SLURP-2 are involved in the regulation of skin and immune systems (19, 58). It is possible that unknown endogenous ligands could exist in the living body.

Various extracellular factors affect the repertoire of cholinergic receptors (Figure 1C). For instance, during development, immature keratinocytes express α3, β2, β4, α5, M2, and M3, whereas mature keratinocytes express α7 and M1 (10). Other examples show that immature osteoblasts treated with BMP-2 express α3 and α5 (66), whereas immature osteoblasts treated with ascorbic acid and β-glycerophosphate express α1, α6, α7, β1, δ, ε, M1, M2, and M4 (67). Physiological responses elicited by the same cholinergic ligands differ between immature and mature osteoblasts. The existence of a crosstalk between nicotinic and muscarinic AChR signaling becomes clear. M1 synergizes with α7 to mediate the cholinergic control of cell shape and motility in keratinocytes (22). Nicotine, but not ACh, inhibits calcification during differentiation induced by ascorbic acid and β-glycerophosphate in osteoblasts, suggesting that muscarinic signaling inhibits nicotinic signaling (67, 82). From the viewpoint of the development in therapeutic drugs, it is an essential task to clarify how extracellular factors such as cytokine affect cholinergic receptors.

Outlook

Within ten years, all the organs which equip with the non-neuronal cholinergic systems and the action of ACh on target cells in the non-neuronal cholinergic regulation will be elucidated. Systemic comprehension of cholinergic responses needs to be validated for the development of therapeutic drugs targeted for each organ.

Highlights

The cholinergic pathway plays a vital role in the physiological functions of many organs. To advance this field, we should aim to reveal the upcoming problems in the near future.

- Do sympathetic neurons modify the activity of the non-neuronal cholinergic system directly or indirectly?
- How ACh avoids the effect of AChE and AChE degrades ACh in the non-neuronal cholinergic system?
- Do other endogenous ligands exist in the body?

- How various cytokines affect the expression of cholinergic receptors?

Conflict of interest statement

T.S., D.C., T.I., M.U., K.H., T.A., S.T., and T.Y. have no conflict of interest to disclose.

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