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The Non-Neuronal Cholinergic System in Health and Disease

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Key Words

Non-neuronal cholinergic system · Acetylcholine · Nicotinic receptors · Muscarinic receptors · Choline acetyltransferase · Vesicular acetylcholine transporter · Organic cation transporter · Acetylcholinesterase

Abstract

Acetylcholine (ACh) is not only a neurotransmitter but is an ancient molecule that can be released by and act on non-neuronal cells. In these cells the system of ACh-synthesizing enzymes, transporters, receptors and degrading enzymes is termed the non-neuronal cholinergic system (NNCS). There is increasing evidence that the NNCS is dysregulated in various diseases and can have an influence on their pathology. However, for many organ systems not much is known about the expression and function of the NNCS. Thus, this review focusses on the role of the NNCS in different organ systems in health and disease. Dysregulation of ACh synthesis and release, mutations or polymorphisms in genes encoding NNCS components, and auto-antibodies against NNCS components are common factors influencing disease progression. Pharmacological agents targeting the NNCS are already successfully in clinical use for some disorders, indicating that interfering with this system is very promising and more research is needed to elucidate the role of the NNCS in different tissues and pathological states.

Introduction

The term cholinergic system is commonly used for the well-known neurotransmitter acetylcholine (ACh) and the system of synthesizing enzymes, transporters, receptors and enzymes for degradation. ACh was the first neurotransmitter that has been identified in 1926 by Otto Loewi; for that he and Sir Henry Dale received the Nobel prize in 1936. In the following years most effort was put into exploring the neuronal cholinergic system where ACh was identified as neurotransmitter of the postganglionic parasympathetic system, the preganglionic neurons of the sympathetic system, in sweat glands of the human axilla, and in several areas of the brain. However, in view of phylogenesis, the non-neuronal cholinergic system (NNCS) already existed in non-neuronal cells like bacteria, algae and protozoa before the nervous system was developed (reviewed in Wessler et al. [1]). After rediscovery of the NNCS in the 1990s, the research was focussed on the distribution, the functions, the molecular components and its involvement in pathological conditions. One of the best-reputed organs in this regard is the skin. But besides the skin, the NNCS was found in many organ systems and for some a role in disorders has already been described. Cholinergic receptors seem to be expressed by nearly all cell types; however, the expression of the receptors alone is not an indication of the NNCS, but it is characterized by its ability to synthesize and re-

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lease ACh. Cells that exhibit solely cholinergic receptors can serve as effector cells for non-neuronally as well as neuronally synthesized and released ACh. Thus, the presence of an NNCS has to be classified before considering its involvement in organ-specific disorders. The same molecular components that are necessary for synthesis and release of ACh in neurons are often identified in non-neuronal cells as well. In neurons and most non-neuronal cells, ACh is synthesized by the enzyme choline acetyltransferase (ChAT) from choline and acetyl coenzyme A [1, 2]. It was shown that ACh can also be produced by the enzyme carnitine acetyltransferase (CarAT) in some non-neuronal cells, e.g. skeletal muscle cells and the urothelium [3, 4]. The rate-limiting step in ACh synthesis is the re-uptake of the essential nutrient choline. In the nervous system this is mediated by the high-affinity choline transporter-1 (CHT1) [5]. In some non-neuronal cells CHT1 is also present [6–8], whereas in others the re-uptake of choline is performed by choline transporter-like proteins (CTL1–5) [9, 10] or organic cation transporters (OCTs). In neurons ACh is stored and released by the vesicular acetylcholine transporter (VAChT) [11], whereas in non-neuronal cells VAChT is only expressed cell type specifically [12]. In cells that do not express VAChT, ACh is usually not stored but directly released via OCTs [13, 14]. Further, the mediatophore [15], a protein of 220 kDa consisting of 15-kDa proteolipid subunits of the vacuolar H^+-ATPase, is also discussed to be responsible for ACh exocytosis [15]. Extracellular ACh exerts its effect on a variety of different nicotinic and muscarinic ACh receptors. Nicotinic ACh receptors (nAChRs) are ligand-gated cation channels consisting of 5 subunits. Nine α-subunits and 4 β-subunits are known. The subunits α_2–α_7 are combined in heteropentamers with β-subunits, whereas α_9 and α_{10} can form α-heteropentamers and α_7 and α_9 can assemble to homopentamers [16]. Interestingly, some nAChR compositions, e.g. the homopentameric α_7-nAChR, were shown not only to have ion channel functions but to serve as metabotropic receptor [17, 18]. Recently, members of the lymphocyte antigen-6/urokinase-type plasminogen activator receptor superfamily were identified as endogenous allosteric modulators of nAChR, with SLURP (secreted lymphocyte antigen-6/urokinase-type plasminogen activator receptor-related peptide)-1 and -2 being one of the most characterized members [19].

Muscarinic ACh receptors (mAChRs) are G-protein-coupled receptors subdivided into inhibitory receptors (M2 and M4) and excitatory receptors (M1, M3 and M5). M2 and M4 mAChR couple preferentially to $G_{i/9}$ affecting the adenylyl cyclase activity and inhibiting non-selective cation channels, transient receptor potential channels and potassium channels [20, 21]. M1, M3 and M5 mAChR couple to $G_{q/11}$ and increase intracellular calcium via generation of inositol 1,4,5-trisphosphate and 1,2-diacylglyerol [20, 21]. The degradation of ACh into choline and acetate is catalyzed by the enzymes acetylcholinesterase (AChE) and the less specific butyrylcholinesterase.

A schematic drawing of the NNCS with the ACh synthesizing, releasing, and degrading machinery as well as ACh receptors and their modulators is depicted in figure 1.

Modulation of the neuronal cholinergic system, e.g. by AChE inhibitors, is a common treatment for neuronal diseases like e.g. Alzheimer's disease. For some diseases targeting the NNCS with cholinergic drugs is a well established treatment. However, in many non-neuronal tissues not much is known about the expression and function of the NNCS. The aim of this review is to give a short overview on the involvement of the NNCS in several pathological conditions and on the occurrence of the NNCS in healthy tissue and its basic functions there.

Integumentary System

The integument is composed of the skin and its derivates, e.g. glands, hair and nails. The most significant cell types of the skin are keratinocytes that are able to synthesize ACh in high amounts. A single keratinocyte synthesizes and releases a mean of 2×10^{-17} mol ACh and 7×10^{-19} mol ACh per minute, respectively [22]. Keratinocytes also express members of both classes of ACh receptors, the necessary transporters for an efficient auto-/paracrine cholinergic loop, and the degradation enzyme AChE. Skin fibroblasts and melanocytes exhibit molecular components of the NNCS; however, most information about the NNCS is available from keratinocytes. The skin NNCS is assumed to regulate the intimate connection of keratinocytes, proliferation, differentiation, apoptosis, adhesion and migration [23]. Thus, in healthy skin the NNCS is of great impact. Under pathological conditions it is involved in several diseases.

Atopic dermatitis is a chronic, inflammatory, pruritic skin disorder in which ACh levels are elevated 14-fold in the superficial epidermis and upper dermis and 3-fold in the underlying dermis and hypodermis [23, 24]. Allergens, infections, environmental pollutants and emotional stress trigger the clinical onset of disease with main fea-

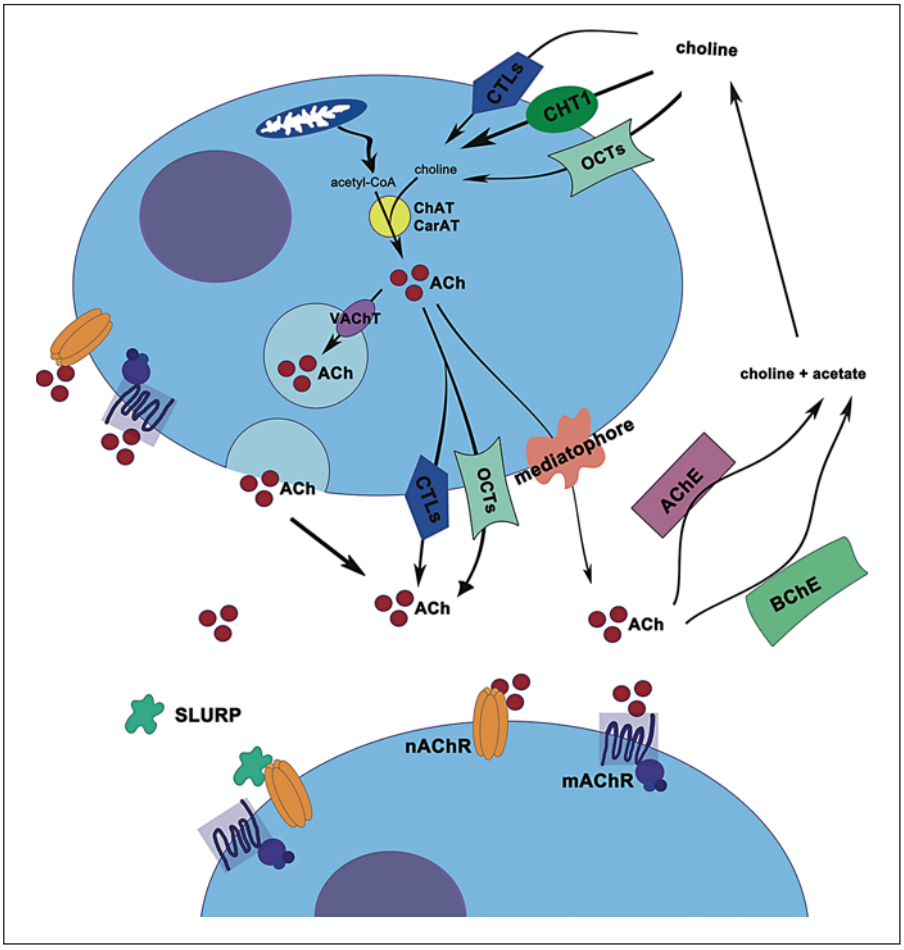

Fig. 1. Schematic drawing summarizing the components of the NNCS involved in the synthesis, release, degradation and signalling of non-neuronal ACh. BChE = Butyrylcholinesterase.

tures being eczematous skin lesions [25]. In a mouse model the histopathology of atopic dermatitis could be completely prevented by pretreatment with corticotropin-releasing hormone which is released in response to cholinergic stimulation [26]. These results indicate that ACh and the cholinergic system are involved in the regulation of atopic dermatitis [27].

Vitiligo is a chronic, non-infectious disease that is characterized by patchy loss of skin pigmentation, most prominent on the face, hands and wrists. The affected melanocytes contain a high amount of H_2O_2. AChE is regulated by H_2O_2. High concentrations of H_2O_2 (10^{-3} mol/l) inhibit AChE, whereas low concentrations of H_2O_2 (10^{-6} mol/l) activate the enzyme by increasing the maximum reaction velocity by >2-fold [28]. AChE is deactivated by H_2O_2-mediated oxidation of the amino acid residues Trp(432), Trp(435) and Met(436), leading to an alteration of the active site His(440) and subsequently to the inhibition of the enzyme [28]. This mechanism should be taken into account for the regulation of the skin NNCS because H_2O_2 can be generated in the millimolar range via UV radiation [23].

Mal de Meleda is a rare autosomal skin disorder that is characterized by a palmoplantar keratoderma. An underlying mutation in the ARS B gene (EMBL AC: X99977) leads to a lack in the non-canonical α_7-nAChR ligand SLURP-1 [19], which enhances the amplitude of the ACh-evoked macroscopic current [29]. These data suggest that SLURP-1 and the non-neuronal cholinergic pathway provide the fine-tuning of keratinocyte functions [23].

Psoriasis is an immune-mediated non-contagious skin disorder with typical generation of plaques. The nAChR modulator SLURP-2 is 3.8- and 2.8-fold upregulated in psoriatic lesional skin in comparison to normal skin and psoriatic non-lesional skin, respectively [30]. SLURP-2 binds predominantly to the ligand binding sites of nAChR subunits α_3 of keratinocytes, thus being involved in the

pathophysiology of psoriasis through regulation of differentiation and apoptosis [31].

Pemphigus is a rare auto-immune disease with typical creation of blisters caused by auto-antibodies against about 50 antigens, e.g. desmogleins and ACh receptors (M1, M2, M4, M5 mAChR and nAChR subunits α_3, α_9 and α_{10}) [32]. Because of this diversity of auto-antibodies, several hypotheses about the causes of pemphigus were formulated. Grando et al. [23] proposed a multiple-hit hypothesis with an interplay of antigens regulating cell shape and cell-to-cell adhesion. However, non-neuronal auto-/paracrine ACh seems to be involved in the pathology of pemphigus. Administration of the angiotensin-converting enzyme inhibitor captopril increased the AChE concentration significantly, leading subsequently to a reduction of ACh and might therefore induce generation of the typical pemphigus blisters by acantholysis [33].

Palmoplantar pustulosis occurs mostly in smokers where blisters appear on the palms and soles. Abnormalities in the expression of ChAT, AChE and nAChR subunits α_7 and α_3 were determined [34] and hence concluded that smoking and the NNCS are involved in this cutaneous inflammatory disease [23, 35].

The majority of epidemiological studies demonstrated an association of smoking and the incidence of certain types of *skin cancer*. Smoking is a risk factor for cutaneous squamous cell carcinoma [36] whereas it lowers the risk of classic Kaposi's sarcoma [37]. However, tobacco-derived nitrosamines stimulate the α_7-nAChR expressed by oral keratinocytes and subsequently lead to activation of STAT (signal transducer and activator of transcription)-3 and STAT-2 via the Ras/Raf-1/MEK1/ERK pathway and Janus kinase (JAK)-2 pathway, respectively [38]. Thus, the α_7-nAChR is a promising molecular target for therapeutic intervention.

Multiple cells and processes are involved in *cutaneous wound healing*. One step with distinct participation of keratinocytes is the epithelialization. Chernyavsky et al. [39] demonstrated that SLURP-1 slows keratinocyte migration but enhances anti-inflammatory activity whereas SLURP-2 increases the outgrowth of keratinocytes dose-dependently and has a lesser anti-inflammatory effect than SLURP-1. Thus, distinct nAChR are able to stimulate wound repair via regulation of keratinocyte migration and inflammation.

Respiratory System

The respiratory system consists of different organs (e.g. nose, nasopharynx, trachea and lung) that are involved in the transport and exchange of the respiratory gases oxygen and CO_2. The luminal surface of the airways is covered by the respiratory epithelium consisting of at least 12 cell types. Airway fibroblasts and inflammatory cells are also involved in airway pathophysiology. Moreover, autonomic nerve fibres occur close to airways, bronchial and tracheal smooth muscle and submucosal glands. Thus, it can hardly be discriminated between effects caused by the neuronal or the non-neuronal cholinergic system. However, it is well known that neuronally released ACh triggers mucus secretion and bronchoconstriction via muscarinic receptors that limit airflow [40]. nAChR were also determined in the airways, e.g. on fibroblasts, immune cells and epithelial cells [41]. Stimulation of the cholinergic receptors on epithelial cells leads to enhanced proliferation [42, 43] and improved cell survival [43]. Cleaning the airway surface from inhaled particles is facilitated by mucociliary transport which is driven by ciliary epithelial cells. ACh is known to regulate the ciliary beat frequency via different mAChR. While activation of M3 mAChR stimulates ciliary-driven particle transport, signalling through M2 mAChR inhibits this process [44].

ACh itself has also been determined in rat tracheal epithelium (2.8 ± 0.5 nmol/g) as well as in human bronchial epithelium (23 ± 6 pmol/g) [45]. Moreover, additional evidence was provided for the synthesizing enzyme ChAT and acetylcholine transporters like OCT, CHT1 and VAChT (reviewed in Kummer et al. [12]). But some of these molecular components were restricted to one or only few cell types of the respiratory epithelium. Thus, the respiratory NNCS is considered to be cell type specific, a fact that complicates the attribution to pathological conditions.

Asthma and Chronic Obstructive Pulmonary Disease

Peribronchiolar fibrosis is observed in small airways of patients with asthma and chronic obstructive pulmonary disease (COPD). A key step of the formation of peribronchiolar fibrosis is the transition of fibroblasts into secretory active myofibroblasts that are identified by the increased expression of α-smooth muscle actin and collagen type-1 [46]. This takes place under chronic inflammatory conditions. Recently it has been shown that the remodelling into myofibroblasts could be reversed by administration of the anticholinergic aclidinium bromide, by silencing M1, M2 or M3 mAChR mRNA or by

degradation of ACh using AChE treatment [47]. Thus, the NNCS is involved in one of the key steps of COPD and asthma consolidation. Furthermore, the NNCS was shown to be downregulated in an acute allergic airway inflammation animal model [48]. The immunomodulatory effects of ACh in asthma and COPD have been reviewed in detail by Gwilt et al. [49]. In brief, ACh is assumed to inhibit the early asthmatic reaction by preventing mast cell degranulation but stimulates the lymphocyte-driven inflammation by enhancing lymphocyte survival and proliferation. In COPD, ACh stimulates the inflammatory actions of epithelial cells and lymphocytes leading to an increase in pro-inflammatory cytokines. In general, in the respiratory system, ACh seems to be pro-inflammatory in lymphocytes and epithelial cells, anti-inflammatory in mast cells and macrophages and can exert both effects in monocytes [49].

Lung Cancer

Approximately 80–90% of all lung cancer cases are considered to be associated with cigarette smoke [50]. Nicotine was shown to induce proliferation of cancer cells and seems to even inhibit the apoptotic effect of chemotherapeutics [18, 51]. Both small cell lung carcinoma cells as well as non-small cell lung carcinoma cells are able to synthesize and release ACh [52]. Recently, it was shown that in small cell lung carcinoma cells, ACh synthesis is positively linked to CTL4 expression [53], while the choline transport to non-small cell lung carcinoma adenocarcinoma cells can be mediated by CTL1 and CTL2 [54]. Grozio et al. [55] demonstrated that inhibition of α_7-nAChR signalling with α-cobratoxin led to enhanced apoptosis of adenocarcinoma cells in culture as well as in established tumour xenografts in vivo. Further, M3 mAChR seems to be a promising target for the inhibition of tumour growth, as it was shown that various antagonists for this receptor inhibited growth of lung cancer cells in vitro and in vivo [56]. Interestingly, an increased risk for lung cancer is associated with single nucleotide polymorphisms on chromosome 15q25.1, containing genes coding for nAChR subunits α_3, α_5 and β_4 [57, 58] and with single nucleotide polymorphisms in the gene for nAChR subunit α_9 [59]. The exact function of these single nucleotide polymorphisms and how these can be used therapeutically is still unknown and has to be elucidated in further research studies.

Cystic Fibrosis

The highly prevalent genetic lung disorder cystic fibrosis is caused by a mutation of the cystic fibrosis transmembrane conductance regulator (CFTR) chloride channel [60]. ACh represents an important regulator of epithelial ion and water movements. In patients suffering from cystic fibrosis, the ACh content was markedly reduced [61]. Interestingly, mAChR and α_7-nAChR seem to be regulators of CFTR [62, 63]. Activation of α_7-nAChR enhances CFTR-mediated chloride secretion, and CFTR and α_7-nAChR were shown to be associated in a macromolecular complex within lipid rafts at the apical membrane of the airway epithelium [63]. Additionally, pro-inflammatory cytokine production in response to innate immune activation was inhibited by activation of α_7-nAChR expressed on tracheal epithelial cells [64].

Cardiovascular System

Both, the neuronal cholinergic system as well as the NNCS were shown to be present in the cardiovascular system that consists of heart and blood vessels. Adult heart cardiomyocytes are able to synthesize, transport and secrete ACh [65], whereas in newborn rats ACh is only released by autonomic nerve fibres [66]. The same age-dependent expression pattern was observed for ChAT [65] that showed regional differences in its activity [67]. While in the rat ventricles only 50% of the ChAT activity observed for the atria could be detected, the highest activity of ChAT was determined in the region of the sino-atrial node [67] where ACh is responsible for reducing pacemaker activity [65]. Moreover, CHT1 [65], VAChT [65], OCT [14], nAChR [68] and mAChR [69, 70] were found in cardiomyocytes and the heart, as well as in several vascular beds [71–74] and endothelial cells [70, 75–77]. In endothelial cells ChAT and ACh itself were also detected [75]. Angiogenesis, the sprouting of new blood vessels, is indispensable for normal growth and homeostasis; however, when dysregulated it contributes to the pathogenesis of many disorders like e.g. cancer, arthritis, obesity and cardiac diseases.

Nicotine is a potent stimulator of angiogenesis, which is meditated mainly via α_7-nAChR and involves mitogen-activated protein kinase and transcription factor nuclear factor-κB activation [77]. This pathway is independent of vascular endothelial growth factor and fibroblast growth factor, the most important mediators of angiogenesis. Endothelial cells were shown to upregulate α_7-nAChR under conditions associated with angiogenesis. The α_9-nAChR on the other hand appears to oppose this pathway [78]. Further, application of the reversible AChE inhibitor donepezil promoted angiogenesis, indicating that it could

be used as therapeutic tool for diseases with pathologically reduced angiogenesis like cardiac diseases [79].

Cardiac diseases like cardiac ischaemia, hypertrophy, heart failure and arrhythmias are still among the world's leading causes for death [80]. Heart functions are regulated by the autonomic nervous system interacting with the M3 mAChR which is involved in physiological and pathological conditions. Moreover, M3 mAChR promotes cardioprotection via suppression of specific miRNA in myocardial ischaemia [81]. Ischaemic heart disease is a complex disorder of great clinical impact that is often associated with well-known cardiovascular risk factors including hypertension, hyperlipidaemia, diabetes, atherosclerosis, heat failure, and aging [80].

Atherosclerosis is the major cause of heart attack, stroke and peripheral arterial disease [82] and is characterized by arterial wall thickening due to accumulation of lipids. Apolipoprotein E plays an important role in lipid metabolism, and its deficiency was shown to induce atherosclerosis in mice. In this murine model of atherosclerosis, administration of nicotine was shown to promote disease [83].

Digestive System

The digestive system consists of various organs such as the pharynx, oesophagus, stomach, small and large intestines, and glandular organs like the liver, pancreas and salivary glands.

Nguyen et al. [84] showed that in oesophageal epithelia ChAT, AChE as well as different nAChR subunits can be found. Reduced ACh concentrations and signalling can lead to a reduction in lower oesophageal sphincter function and subsequently to *gastro-oesophageal reflux disease* [85]. Additionally, a recent study on cats revealed that the NNCS could also contribute to gastro-oesophageal reflux disease by e.g. modulating cell-cell contacts, as the expression of components necessary for ACh synthesis and release, namely CHT1, ChAT, VAChT and OCT1, was altered in the oesophageal mucosa under pathological conditions [86].

The digestive tract is abundantly innervated by cholinergic neurons [87]; thus, many effects can be contributed to neuronal rather than non-neuronal ACh. Smooth muscle cell contraction of the gastro-intestinal tract is mediated by M2 and M3 mAChR, the latter being less abundantly expressed, however functionally more relevant [88].

Gastric pepsinogen secretion is mainly driven by ACh signalling via M1 and M3 mAChR, as in gastric mucosa of M1/M3 mAChR double-deficient mice carbachol-induced pepsinogen secretion was abolished [89]. While M1 mAChR is only present on pepsinogen-producing chief cells, M3 mAChR can also be found on other cell types of the gastric gland [89]. Thus, M3 mAChR localized on parietal cells was reported to be mainly responsible for secretion of gastric acid [90]. Alterations in the cholinergic process of pepsinogen and gastric acid secretion can contribute to *gastric and duodenal ulcers* [91]. Accordingly, anticholinergic agents are successfully used as therapy for these conditions.

Cholinergic receptors play an important role in the regulation of ion transport across the intestinal epithelial cell membrane [92]. Further, enhanced M3 mAChR signalling seems to increase the permeability to macromolecules in the mouse jejunum and thus leads to barrier dysfunction which can subsequently cause initiation and exacerbation of intestinal disease [93]. It was shown that ChAT is expressed by cells of the intestinal epithelium as well as by microfold cells covering Peyer's patches [94]. Isolated rat colonic crypt cells express ChAT and OCT, and non-neuronal ACh could be measured in concentrations of 1.8 and 11.9 nmol/g for the proximal and the distal colon, respectively [95]. In the rat mucosa, the release of ACh could be further induced by stimulation with the bacterial fermentation product propionate [95].

In *colon cancer* cells, an upregulation of M3 mAChR was observed [96] and it was shown that muscarinic agonists are able to stimulate colon cancer cell migration and invasion via induction of matrix metalloproteinase-1 [97]. Further, α_7-nAChR [98] and CTL1–5 [53] are present on colon cancer cells. Interestingly, knock-down of CTL4 can lead to suppression of cell growth as CTL4 expression is positively linked to ACh synthesis [53]. Like in other cancer cells, stimulation of α_7-nAChR by ACh or nicotine is able to induce cell proliferation and to inhibit apoptosis of colon cancer cells [99]. α_7-nAChR is negatively regulated by SLURP-1 that is also present in colon cancer cells as well as in the immune cells of the lamina propria and smooth muscle tissue of the colon [98, 100].

Inflammatory bowel diseases comprise disorders like ulcerative colitis as well as Crohn's disease. Patients with inflammatory bowel diseases show significantly lower AChE activity and correspondingly higher levels of miRNA-132, a post-transcriptional inhibitor of AChE [101]. Interestingly, patients with Crohn's disease or ulcerative colitis differ in their response to nicotine. While for Crohn's disease smoking clearly worsens the symp-

toms [102], for ulcerative colitis it seems to have protective effects and can reduce mucosal inflammation (reviewed in de Jonge and Ulloa [103]). Patients with ulcerative colitis show an enhanced expression of M2 mAChR and a downregulation of ChAT [104] and nAChR subunit α_3 [105] in the colon epithelium. The α_7-nAChR plays an important role in colitis as it was shown that α_7-nAChR-deficient mice suffered from increased severity of experimentally induced colitis [106]. However, these anti-inflammatory effects of α_7-nAChR stimulation are most probably mediated by resident immune cells in the intestine expressing this receptor [107]. Further, the nAChR subunit α_5 also seems to play a role in colitis as nAChR subunit α_5-deficient mice revealed an enhanced susceptibility to experimentally induced colitis [108].

The pancreas is a gland with endocrine and exocrine function. The endocrine release of hormones is facilitated by the islands of Langerhans and the exocrine release of digestive enzymes by pancreatic acinar cells. The exocrine function was shown to be mediated by M1 and M3 mAChR as carbachol-induced amylase secretion was significantly reduced in M1 and M3 mAChR single-deficient mice and completely abolished in M1/M3 mAChR double-deficient mice [109]. Further, nicotine can induce the proliferation of these cells [110]. Most interestingly, pancreatic stellar cells were shown to express ChAT and VAChT and are able to synthesize non-neuronal ACh, which then can induce amylase secretion in neighbouring acinar cells [111].

In the islands of Langerhans, M3 mAChR expressed on β-cells was demonstrated to be of great importance for the maintenance of blood glucose homeostasis as activation of this receptor enhances glucose-induced insulin release by β-cells and improves glucose tolerance [112]. In humans the cholinergic innervation of the islands of Langerhans is sparse; however, α-cells of the pancreas express ChAT as well as VAChT, thus non-neuronal ACh produced by these cells is able to enhance M3 mAChR signalling in neighbouring β-cells [113]. Transgenic mice overexpressing M3 mAChR were protected from detrimental metabolic effects like hyperglycaemia caused by a high-fat diet [112], indicating that enhanced signalling through M3 mAChR may be a useful treatment for *type 2 diabetes*.

Pancreatitis is an acute or chronic inflammation which occurs when pancreatic digestion enzymes are activated before reaching the small intestine. Its pathology appears to be aggravated by smoking [114], and pancreatic M3 mAChR was shown to be upregulated after induction of acute pancreatitis in the rat [115].

Urinary System

The urinary system consists of organs involved in the generation and excretion of urine and associated organic substances. Here we focussed on the urinary bladder and kidneys. Recently, proof was provided for an NNCS in the kidney comprising the synthesizing enzyme ChAT and the transporters VAChT and CHT1 [116]. ChAT was localized in the apical part of principal cells of the renal cortical collection ducts suggesting that ACh might be released into the lumen and act via an auto-/paracrine loop [116]. ACh enhances the excretion of ions and water, thereby promoting diuretic effects and hypotension [117, 118]. It is assumed that ACh acts directly on renal tubules, glomeruli and Bowman's capsule via binding to cholinergic receptors. Both, nAChR as well as mAChR were expressed in several renal regions [119–126]. Only few reports are available on the involvement of molecular components of the cholinergic system in renal pathologies. In contrast, in several bladder disorders antimuscarinic agents have been used for years as clinical first-line treatment. The autonomic nervous system regulates the urinary micturition. The parasympathetic system is responsible for the voiding, that is regulated via mAChR on the detrusor smooth muscle. In the healthy situation, M3 mAChR causes direct and M2 mAChR indirect contraction of detrusor muscle whereas M1 mAChR increases and M4 mAChR decreases the release of ACh and therefore might influence afferent nerve activity and stimulate the release of ATP and nitric oxide from umbrella cells [127]. In addition to the neuronal cholinergic system, the occurrence of a bladder NNCS has been described [4, 128, 129]. ACh could be measured in the abraded urothelium at concentrations of 0.22 ± 0.03 nmol/g wet weight in mouse and 8 and 14 pmol/g wet weight in human samples [4]. In contrast to the nervous system and several non-neuronal organ systems, ACh was not synthesized by ChAT but CarAT and not transported by VAChT and CHT1 but by OCT [4]. The presence of ChAT and CHT1 still remains controversial [128, 129]. However, despite this controversy, the NNCS is indisputably present in the urothelium and is accompanied by several nAChR and mAChR [130, 131] that were also analysed in bladder disorders.

Kidney and Bladder Disorders

Acute kidney injury often caused by sepsis leads to an increased risk of death that could be significantly decreased after administration of nicotine in an animal model [132]. Thus nAChR seems to act in an anti-inflam-

matory manner also in kidney diseases. It is still controversially discussed if the administration of nicotine improves *ischaemia-reperfusion injury* in the rat kidney via stimulation of α_7-nAChR [126, 133, 134]. However, administration of nicotine mediates the progression of *chronic kidney disease* via α_7-nAChR in an animal model and in human smokers where it is characterized by a progressive loss of renal function [124, 135, 136].

The *overactive bladder* syndrome is clinically characterized by symptoms of urgency, nocturia and occasionally incontinence [137]. The first-line treatment is administration of antimuscarinic agents that block more or less selectively muscarinic receptors and thereby reduce the bladder tone and increase the cytometric capacity [127, 138–140]. This improvement of overactive bladder symptoms is most anticipated during the storage phase where the afferent activity is altered and an involvement of the NNCS is controversially discussed [127, 140, 141].

Bladder outlet obstruction is characterized by an intravesical outlet obstruction that is often associated with detrusor overactivity and dramatic growth responses of muscles and ganglion cells through hyperplasia and hypertrophy (reviewed in Ochodnicky et al. [142]). Recently, the NNCS was shown not to be regulated on the mRNA level in this disease [143] whereas a downregulation was measured for the neuronal nAChR subunits α_{10} and α_5 (dorsal root ganglia L5–S2) [144]. Further, a mutation of the M3 mAChR is associated with the onset of bladder outlet obstruction [145]. However, the plasticity at the level of innervation leads to the assumption that the autonomic nervous system is most involved in bladder outlet obstruction, eventually in interaction with the NNCS [142].

Reproductive System

The female reproductive system comprises the vagina, cervix, uterus, oviducts and ovaries. The penis, testis and respective glands like the prostate gland belong to the male reproductive system. In females, production of non-neuronal ACh was shown for the human vagina [42], granulosa cells of the ovary [146], the placenta [147], and the oviduct [148]. Like the bladder, the uterus is richly innervated by cholinergic neurons, and contractions are mainly mediated via M2 and M3 mAChR [149]. The human placenta is not cholinergically innervated; however, non-neuronal ACh is released via OCT1 and OCT3 [147] and can regulate different physiological functions like blood flow, vascularization and nutrient transport via nAChR [150]. Further, M1–M4 mAChR are expressed in the human placenta [151]. In the oviduct, different nAChR and mAChR subtypes are expressed with M3 mAChR being mainly responsible for an increase in calcium signalling [152], which could also play a role in myogenic contractions.

Tubal ectopic pregnancy is a complication of pregnancy where the embryo implants outside the uterine cavity, in most cases in the oviduct. Smoking increases the risk for ectopic pregnancy, a mechanism that seems to be mediated via the α_7-nAChR [153]. Interestingly, during pregnancy α_7-nAChR was shown to be downregulated in the oviduct, possibly as a protective mechanism against this complication [152]. Generally, smoking during pregnancy is associated with an increased risk for various complications. Nicotine exposure was shown to result in low birth weights, cognitive dysfunctions as well as impaired lung function of newborns. With prenatal nicotine exposure expression of α_7-nAChR is enhanced in the neonatal lung [154]. Further, the risk of infant respiratory tract infections is supposed to be enhanced by in utero nicotine exposure, due to α_7-nAChR-mediated influence on alveolar macrophages [155].

Pre-eclampsia is an anti-angiogenic state associated with high blood pressure and proteinuria that can occur during pregnancy. Interestingly, nicotine exposure was shown to reduce the risk of pre-eclampsia by reducing the placental production of soluble vascular endothelial growth factor receptor 1 and inducing placental growth factor, thereby inducing angiogenesis [156]. Additionally, activation of α_7-nAChR seems to reduce placental cytokine production and thus further protects from pre-eclampsia [157].

In the male reproductive system, the presence of an NNCS was clearly shown for the testis, as ACh-synthesizing enzyme ChAT, transporters and ACh receptors were expressed in non-innervated testis parenchyma [158]. ChAT was also shown to be expressed in spermatozoa [159], and release of non-neuronal ACh was measured in the germinal epithelium [158]. For the prostate gland, activation of mAChR results in secretion of prostate fluids [160] as well as in contractile response which is being mediated via M3 mAChR in the mouse [161]. Further, prostate cells were shown to express the ACh-degrading enzymes AChE and butyrylcholinesterase [162].

Nicotine has negative effects on spermatogenesis and steroidogenesis; most interestingly, mice deficient for α_7-nAChR produce sperm with impaired motility [163] and mAChR agonists increase sperm motility in humans [164]. Many effects of ACh in the male reproductive tract such as vaso-activity, sperm transport, muscle contrac-

tion and cell secretion are mediated via mAChR [165]. However, more research in this area is needed to explore the exact functional influence of the NNCS and its possible role in reproductive system pathology.

Immune System

Already more than 80 years ago, ACh was reported to be present in ox blood (reviewed in Kawashima and Fujii [166]). However, only in 1993 were Kawashima et al. [167] able to convincingly demonstrate the presence of ACh in the human blood and that it was produced mainly by mononuclear leucocytes. The ACh-synthesizing enzyme ChAT is expressed predominantly by T cells (mainly by CD4+ T helper cells) but also by dendritic cells, granulocytes, macrophages and mast cells [168]. Upon contact with antigen-presenting cells activated T cells show an increase in ChAT activity and enhanced ACh synthesis via T cell receptor/CD3-mediated and CD11a-mediated pathways [168]. It is not clear whether in immune cells ACh can be stored in vesicles as VAChT was not detectable. Thus, it is more likely that newly synthesized ACh is directly released upon requirement [169]. Recently, Fujii et al. [170] suggested that ACh release in T cells is at least partly mediated via mediatophore. Immune cells are able to express mAChR as well as nAChR subtypes; however, expression patterns of these receptors seem to vary between individual subjects [171]. M1 mAChR was shown to play a role in the early differentiation of CD8+ T cells [172], and M5 mAChR is upregulated after activation of T cells [168]. Numerous studies revealed that mAChR activation has pro-inflammatory effects in different cell types. Accordingly, activated mononuclear leucocytes from M1/M5 mAChR-deficient mice produced significantly less pro-inflammatory cytokines [173]. Further, mAChR stimulation increases the number of leucocytes in splenic venous blood [174]. In contrast, activation of nAChR has been reported to have anti-inflammatory effects and especially the α_7-nAChR was thoroughly analysed regarding this point. In 2003, Wang et al. [175] were able to show that in macrophages α_7-nAChR stimulation leads to significant reduction in synthesis and release of tumour necrosis factor α.

The anti-inflammatory effect of α_7-nAChR stimulation was further confirmed in experimental *sepsis*, where α_7-nAChR-deficient mice showed higher levels of splenic and circulating pro-inflammatory cytokines after application of lipopolysaccharide [175]. Electrical stimulation of the vagus nerve leads to inhibition of inflammation and protection of septic shock [176], a phenomenon that is mediated via the α_7-nAChR [175] and termed as 'cholinergic anti-inflammatory pathway'. Since that work of Tracey and coworkers, the cholinergic anti-inflammatory pathway was extensively studied and broadly reviewed [177]. However, not only the α_7-nAChR can influence inflammation as the blockage of mAChR, especially M3 mAChR, appears to protect from lipopolysaccharide-induced *lung inflammation* [178]. Further, ChAT-expressing B cells were shown to reduce the recruitment of neutrophils during sterile lipopolysaccharide-induced endotoxaemia. Generally, the NNCS of immune cells seems to play a role in the regulation of immune system function.

Auto-Immune Diseases and Immunodeficiencies

Mice which develop auto-immune symptoms of *systemic lupus erythematosus* showed an increased ACh content in blood, thymus and spleen [179].

Sjögren's syndrome is a systemic autoimmune disorder mainly targeting the salivary glands and lacrimal glands, resulting in dry mouth and dry eyes. One important trigger for the pathogenesis of Sjögren's syndrome is the production of auto-antibodies against M3 mAChR acting as an antagonist for this receptor, resulting in suppression of aquaporin 5 trafficking and subsequent impairment of fluid secretion [180].

Spontaneously hypertensive rats develop an immune deficiency with decline in T cell function. These rats showed reduced ACh content as well as reduced ChAT expression in mononuclear leucocytes, probably due to the observed T cell deficiency [181].

Human immunodeficiency virus infection is characterized by an uncontrolled immune overstimulation which subsequently leads to a state of immunodeficiency. Thus, early therapies in dampening the overactivation of the immune system are needed. In a proof-of-concept, placebo-based study, the AChE inhibitor pyridostigmine was shown to reduce the T cell overactivation in human immunodeficiency virus patients [182].

Musculoskeletal System

The musculoskeletal system includes bones, muscles, cartilage, ligaments, tendons and joints.

Also in bone the presence of cholinergic components was clearly demonstrated. Inkson et al. [183] reported about the production of AChE in osteoblasts which is believed to be involved in cell-matrix interaction in the bone. Our group was able to show that osteoblasts express

all necessary components for the synthesis and release of ACh [184]. Further, nAChR and mAChR were identified also on primary bone cells, mesenchymal stem cells and osteoclasts [185].

Fracture Healing and Osteoporosis

Signalling via nAChR and mAChR is involved in bone mass turnover. In this regard the nAChR subunit α_2 and the M3 mAChR appear to play important roles as nAChR subunit α_2-deficient [186] and M3 mAChR-deficient mice [187, 188] both show an osteoporosis-like phenotype.

Smoking seems to be associated with a decrease in bone mass and a reduced capacity for fracture healing [185]. However, the effect of nicotine in this regard appears to be dose dependent as low nicotine concentrations induce osteoblast formation, high levels of nicotine on the other hand lead to desensitization of osteoblastic nAChR, downregulation of osteoblasts and upregulation of osteoclasts [185]. Generally, the effect of nicotine on bone metabolism is controversially discussed, and more research in this field is definitely needed.

In the skeletal muscle, expression of the ACh-synthesizing enzyme CarAT was found in denervated extensor digitorum longus muscles of the rat [3]. Further, proliferating myoblasts were shown to synthesize and release an ACh-like compound [189].

Tenocytes of patella [190, 191], Achilles [192] and plantaris [193] tendons express ChAT and VAChT as well as M2 mAChR, and their expression and activity were shown to be enhanced under pathological conditions such as *tendinosis*, a non-inflammatory degenerative disorder.

Nicotine influences chondrocyte differentiation [194, 195]. Besides this, not much is known about the NNCS in the cartilage. Unpublished data from our laboratory, however, revealed that there is marked expression of the NNCS in the cartilage of patients with osteo-arthritis and rheumatoid arthritis.

In 2008, Grimsholm et al. [196] were able to show that ChAT and the α_7-nAChR are present in the synovial tissue of the human knee joint. ChAT mRNA and protein were localized to fibroblast-like and macrophage-like cells as well as to some extent in blood vessel walls of the synovial sample, while α_7-nAChR was mostly found in the synovial intimal lining layer [197]. Recently, our group could show that different nAChR and mAChR as well as CarAT, transporters, and ACh-degrading enzymes are present in the synovial tissue of the human joint [198].

Rheumatoid arthritis (RA) is with 1% prevalence (female > male) one of the most common inflammatory joint diseases. Currently, disease-modifying antirheumatic drugs, like methotrexate, are still the most common treatment used for patients with RA; however, side effects can be severe. Recently, also anti-tumour necrosis factor-α treatment was successfully introduced as new therapeutic strategy, but unfortunately only in 26–42% of patients this treatment is effective [199]. Thus, there is still great need for new therapeutic strategies in RA, and the NNCS could be a promising target for pharmacological intervention.

In patients with RA, ChAT expression was markedly pronounced, probably because of a higher number of macrophage-like and fibroblast-like cells in the synovial samples [196]. There is increasing evidence for the role of synovial fibroblasts in the pathology of RA. In vitro studies on these cells revealed an anti-inflammatory effect for ACh and nicotine in these cells which was mediated via α_7-nAChR [200]. This anti-inflammatory effect of nicotine was also confirmed in vivo as administration of nicotine led to amelioration of experimentally induced arthritis in mice [201, 202]. Interestingly however, the effect of nicotine on arthritis seems to be strongly dependent on the mode and time point of application as well as the immune status of the cells [203]. The role of α_7-nAChR in experimentally induced arthritis is however controversially discussed as in studies with α_7-nAChR-deficient arthritic mice completely opposing results were obtained regarding disease progression by two different research groups [204, 205]. Thus, more extensive research is needed on the influence of the NNCS on inflammation and progression of RA.

It was shown that the cholinesterase inhibitor neostigmine has promising analgesic effects in inflamed joints [206]. However, there is one study where neostigmine was applied as analgesic agent intra-articularly to the rabbit knee joint, leading to increased infiltration of immune cells, cell hyperplasia and hypertrophy [207]. Also here, mode and time point of application may be relevant for the effects observed. Generally, analgesia is an important aspect for RA treatment. The activation of several nAChR was reported to have an antinociceptive effect [208, 209]. And also mAChR seem to play important roles in analgesia in arthritis [210], and especially M1 mAChR was involved in the modulation of inflammatory pain in the paw [211]. Thus, targeting the NNCS in RA could be beneficial not only in respect to inflammation but also regarding analgesic effects.

Table 1. NNCS components involved in pathological conditions, summarized for the different organ systems

Organ system	Components involved
Integumentary system	
Atopic dermatitis	ACh [23, 24]
Vitiligo	AChE [28]
Mal de Meleda	SLURP-1 and α_7
Psoriasis	SLURP-2 and α_3 [30, 31]
Pemphigus	M1, M2, M4, M5, α_3, α_9 and α_{10} [32]
Palmoplantar pustulosis	ChAT, AChE, α_7 and α_3 [34]
Skin cancer	α_7 [38]
Cutaneous wound healing	SLURP-1, SLURP-2 and nAChR [39]
Respiratory system	
Asthma and COPD	ACh [47, 49], AChE and M1–3 [47]
Lung cancer	ACh [52], CTL1 and CTL2 [54], CTL4 [53], α_7 [55], M3 [56]
Cystic fibrosis	ACh [61], mAChR [62], α_7 [63, 64]
Cardiovascular system	
Cardiac diseases	AChE [79], M3 [81]
Atherosclerosis	nAChR [83]
Digestive system	
Gastro-oesophageal reflux disease	ACh [85], ChAT, VAChT and OCT1 [86]
Gastric and duodenal ulcers	ACh, M1 and M3 [89–91]
Colon cancer	M3 [96, 97], α_7 [98, 99], CTL1–5 [53], SLURP-1 [98, 100]
Inflammatory bowel disease	AChE [101], M2 and ChAT [104], α_3 [105], α_7 [106], α_5 [108]
Type 2 diabetes and pancreatitis	M3 [112, 115], nAChR [114]
Urinary system	
Acute/chronic kidney disease	nAChR [132], α_7 [124, 135, 136]
Ischaemia-reperfusion	α_7 [126, 133, 134]
Overactive bladder syndrome	mAChR [127, 138–140]
Bladder outlet obstruction	M3 [145]
Reproductive system	
Tubal ectopic pregnancy and pre-eclampsia	α_7 [152–155, 157]
Impaired sperm motility	α_7 [163], mAChR [164]
Immune system	
Sepsis and endotoxaemia	α_7 [175], M3 [178]
Sjögren's syndrome and systemic lupus erythematosus	M3 [180], ACh [179]
Immunodeficiencies	ACh and ChAT [181], AChE [182]
Musculoskeletal system	
Fracture healing and osteoporosis	nAChR [185], α_2 [186], M3 [187, 188]
Tendinosis	ChAT, VAChT and M2 [190, 191]
Rheumatoid arthritis	ChAT [196], α_7 [200, 204, 205]

Conclusion

Taken together, non-neuronal ACh is produced by cells of various tissues and organ systems and acts in an autocrine and paracrine way on nAChR and mAChR present on the ACh-producing or neighbouring effector cells. In the non-diseased state the NNCS plays a role in many important biological and physiological processes like cell growth, adhesion, migration and differentiation. Thus, impairment or dysregulation of the NNCS and its functions can influence the pathogenesis and pathology of various diseases. Table 1 is summarizing the diseases mentioned in this review and the NNCS components involved in their pathology. In the last few years, studies on NNCS expression patterns and research on gene-deficient mice gave further insights into the diverse functions and importance of NNSC components in maintaining homeostasis. For some diseases like e.g. overactive bladder syndrome, pharmacological intervention of cholinergic signalling became the therapy of choice. However, there are tissue and organ systems for which the NNCS has not yet been extensively studied and/or

the effect of cholinergic signalling is still controversially discussed. Thus, more research on the presence, the expression pattern and distinct functions of the NNCS is definitely needed. Regarding the importance of NNCS functions and the impact of pharmacological intervention for some disorders, components of the NNCS are a promising target for the development of new therapeutic strategies.

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Disclosure Statement

The authors declare that there are no conflicts of interest.

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