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A HISTORIC PERSPECTIVE ON THE CURRENT PROGRESS IN ELUCIDATION OF THE BIOLOGIC SIGNIFICANCE OF NON-NEURONAL ACETYLCHOLINE

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Abstract

The "5th International Symposium on Non-neuronal Acetylcholine: from Bench to Bedside" was held on September 27–29, 2019 in Hyatt Regency, Long Beach, CA, USA. Approximately 50 scientists from 11 countries over 6 continents participated in this meeting. The major topics included an overall biologic significance of non-neuronal acetylcholine (**ACh**) and the roles of the non-neuronal cholinergic systems in mucocutaneous, respiratory, digestive, immunologic, endocrine, cardiovascular, musculoskeletal and kidney diseases, and cancer. This meeting facilitated continued work to advance the fundamental science and translational aspects of the interdisciplinary studies on non-neuronal ACh. The progress made has opened a new chapter in the field of cholinergic pharmacology, and advanced our knowledge beyond regulation of individual cell- and tissue-types, defining a new paradigm of selective pharmacological regulation of vital function of practically all types of non-neuronal cells. It is now clear that the autocrine and paracrine control of non-neuronal cells by non-neuronal ACh is implemented through synergistic, additive, and reciprocal effects triggered by two different cholinergic receptor classes. Each biologic effect of ACh is determined by a unique combination of cholinergic receptors subtype expressed at each stage of cell development and differentiation. The plasticity of the non-neuronal cholinergic system helps adjust homeostasis to new environmental conditions.

The "5th International Symposium on Non-neuronal Acetylcholine: from bench to bedside" was held on September 27–29, 2019 in Hyatt Regency, Long Beach, CA, USA (https://sites.uci.edu/2019nihsymposium). Approximately 50 scientists from 11 countries over 6 continents participated in this meeting (Figure 1). The major topics included an overall biologic significance of non-neuronal acetylcholine (**NNACh**) and the roles of the

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non-neuronal cholinergic systems in mucocutaneous, respiratory, digestive, immunologic, endocrine, cardiovascular, musculoskeletal and kidney diseases, and cancer. During last two decades, the scientific community working in the field of NNACh has continuously grown, expanding our knowledge on the biologic and pathologic aspects of the non-neuronal cholinergic system. The scientists exploring the biologic roles of NNACh gathered in 2002 in San Francisco, USA, for a first international symposium on NNACh, followed by meetings held in Mainz, Germany (2007), Groningen, the Netherlands (2011), Giessen, Germany (2014) and now in Long Beach, USA. The articles of participants of the 5th International Symposium on NNACh covering a large variety of biologic and medical aspects of non-neuronal cholinergic systems are published in the current virtual special issue of *International Immunopharmacology*. Together with previous four symposium proceedings (ie, Life Sciences 2003, Vol. 72, No. 18–19; Life Sciences 2007, Vol. 80, No. 24–25; Life Sciences 2012, Vol. 91, No. 21–22 and International Immunopharmacology 2015, Vol. 29. No. 1), the present virtual special issue of *International Immunopharmacology* provides the most comprehensive summary of the history and current progress in the development of the field of NNACh.

The fact that ACh is present in bacteria, blue-green algae, yeast, fungi, protozoa and primitive plants [1, 2] indicates that ACh has been acting as a signaling molecule in non-neuronal cells for about 3 billion years, whereas its neuronal function spans only a relatively short period of about ½ billion years. The discovery of ACh outside the neural system was followed by the discoveries in the non-neuronal locations of the metabolizing enzymes choline acetyltransferase and acetylcholinesterase (**AChE**), muscarinic and nicotinic classes of cholinergic receptors, (**mAChRs** and **nAChRs**), choline and ACh transporters, and, most recently, of non-canonical endogenous ligands, such as members of the Ly6 protein family. It is therefore currently well-established that ACh is a ubiquitous molecule in life that, in addition to neurotransmission, plays important roles in various aspects of cell biology and homeostasis outside the neural system.

The discoveries of cholinergic molecules started in 1864 when Oscar Liebreich prepared a lipid-free extract from brain that he called “protagon” [3]. Using alkaline extraction, he obtained from protagon a basic compound that he named “neurin”. Two years later, Adolf von Bayer crystalized neurin, identified it as ACh and synthesized it, referring to it as “acetylneurin” [4, 5]. ACh had only a chemical interest until it was found to be biologically active. In 1906, Reid Hunt and René de M. Taveau discovered that ACh in small doses decreases blood pressure [6]. The story of NNACh started in 1914 when Arthur J. Ewins extracted ACh from ergot, the fungus *Claviceps purpurea,* and identified it as a blood pressure decreasing agent [7]. Later in 1914, Henry Hallett Dale characterized physiologic effects of ACh, compared to choline, and identified differences between muscarinic and nicotinic effects of ACh [8]. In collaboration with Harold Dudley, he first isolated ACh from animal body in 1929 [9]. In that study, the spleen extract containing ACh showed similar effects on the blood pressure of cat and on the intestinal muscle tone of rabbit. Since Dale and Dudley used tissue extract from ox and horse spleens that do not have vagal innervation, the discovery of the biologic significance of NNACh preceded that of neuronal ACh. Within next few years, using various methods of extraction, NNACh was

found in extracts of animal blood, eye, bladder, skin, testis, fat, kidney, trachea, lung, uterus, vagina, placenta, adrenal gland, salivary glands, esophagus, stomach, pancreas, liver, intestinal muscle and mucous membrane, and some other tissues and organs (reviewed in [10, 11]). However, the following decades witnessed a rapid progress in elucidating role of ACh in neurotransmission, while the NNACh was almost forgotten [12]. For his work on the role of ACh in chemical neurotransmission, Dale was awarded the Nobel prize in physiology or medicine in 1936, shared with the Austrian pharmacologist Otto Loewi. Using sophisticated frog heart preparations, Loewi found that electric stimulation of vagus nerve attached to the heart elicits the release of a substance named “Vagusstoff” that inhibits the heart contractility [13]. Later on, Loewi and Navratil pharmacologically identified Vagusstoff as ACh [14]. Dale introduced the terms “cholinergic” and “adrenergic” to describe nerve fibers which transmit their actions by releasing at their endings ACh and a substance related to adrenaline, respectively [15].

The interest to NNACh as to an important biologic modulator has been slowly growing from the early 1970s, owing to the discoveries of functional cholinergic receptors in non-neuronal cells. In 1969, Sarah Tjioe and C. Paul Bianchi identified “muscarinic sites” in frog ventricle [16], which might represent mAChRs of both cardiomyocytes and nerve terminals. During the following decade, mAChRs were found in the chronologic order in retina [17], lymphocytes [18], spleen [19], adrenal gland [20], stomach [21], parotid gland [22], lacrimal gland [23] and colonic epithelium [24]. In 1977, Engel et al [25] first reported about expression of nAChRs outside the neural system. The authors demonstrated specific binding of the canonical nicotinic ligand α-bungarotoxin to thymic epithelial cells. In early 1990th, classic nAChRs were found in nonmuscle and non-neuronal human cell lines, including small cell lung carcinoma, adenocarcinoma, and several other tumor cell lines [26, 27].

In 1983, Paul Layer identified the presence of both AChE and butyrylcholinesterase at very early times in the embryo [28]. This was in keeping with and further elaborated on the earlier report by Ulrich Drews about the presence of “embryonic cholinesterase” that showed activity in epithelial cells independent from innervation [29]. It also became evident that in addition to regulating ACh level, thus playing a role of the “bottleneck” of cholinergic functioning (coined by Paul Layer), AChE exhibits a large variety of non-enzymatic activities in both neuronal and nonneuronal cells (reviewed in [30, 31]).

Subsequent reports convincingly demonstrated that ACh and cholinergic enzymes and receptors are ubiquitously present in human body (reviewed in [32–34]). Soon after characterization of non-neuronal cholinergic enzymes and receptors, Prof. Kummer’s group first demonstrated expression of the high-affinity choline transporter, CHT1, in human and rat skin [35], and Prof. Schallreuter’s group that of the vesicular ACh transporter in human melanocytes and keratinocytes [36]. More recently, Prof. Skok’s group reported the presence of nAChRs on the mitochondrial outer membrane coupled to regulation of cell survival [37]. Among the recently discovered components of the non-neuronal cholinergic systems are non-canonical endogeneous ligands of nAChRs, such as secreted mammalian Ly-6/urokinase plasminogen activator receptor-related protein (**SLURP**)-1 and -2 and some others proteins (reviewed in [38]).

In 1993, localization of ACh has been found for the first time in human peripheral blood mononuclear leukocyte fraction consisting of mainly T and B cells [39]. In 1995 and thereafter, the discoveries of choline acetyltransferase (**ChAT**) mRNA and enzyme expression in T cells and B cells definitely proved that immune cells have the ability to synthesize ACh by ChAT (reviewed in [40]). Interestingly enough, these findings indicate that ACh isolated from the spleen by Dale and Dudley [9] is derived from non-neuronal immune cells including T and B cells, because the spleen is not innervated with cholinergic nerve (reviewed in [41, 42]). ChAT expression in T and B cells, dendritic cells and macrophages was further confirmed in ChATBAC-eGFP transgenic mice [43] and ChAT-Cre-tdTomato mice [44]. Expression of various subtypes of both mAChRs and nAChRs in T and B cells, dendritic cells and macrophages has been demonstrated by detecting mRNAs for respective ACh receptors with RT-PCR (reviewed in [45]).

All five M1-M5 mAChR subtypes are expressed in almost all immune cells at various levels. Studies in mAChR knockout mice revealed that M1/M5 mAChRs up-regulate TNF-α, IFN-γ and IL-6 production in spleen cells, leading to an elevation of serum antigen specific IgG1 [46]. Immune cells also express neuronal type nAChRs as pentamers comprised of two to five distinct subunits (ie, α2-α10, and β2 and β3) forming ligand-gated ion channels. Expression of various ACh receptor types may vary depending on immunological status of the subjects and respective immune cells, such as infection [47, 48]. Among the nAChR subtypes expressed in immune cells, the role of α7 nAChR in the regulation of inflammatory and immune responses has drawn attention, in part, because stimulating α7 nAChRs on macrophages suppressed the synthesis and release of TNF-α, thereby protecting mice from lethal endotoxin shock induced by lipopolysaccharide [49]. On the basis of these findings, Tracey [50] proposed the “inflammatory reflex” pathways in which sensory input evoked by infection or injury travels through the afferent vagus nerve and cytokines to integrative regions in the brainstem, and after processing, the efferent vagus nerve carries the outbound signals terminating in the spleen. However, reflecting the lack of the direct vagal innervation of the spleen, the “inflammatory reflex” pathways have been modified to acknowledge that neural signals are relayed by ACh synthesized in a subset of CD4+ T cells to α7 nAChRs on macrophages within the spleen [51]. Taken together, these findings demonstrate that ACh synthesized in T cells plays critical roles in regulation of inflammatory responses.

Recent study by Mashimo et al [52] revealed divergent roles of α7 nAChRs in antigen-presenting cells (**APCs**) and T cells in regulation of naïve CD4+ T cell differentiation by demonstrating that: 1) α7 nAChRs of APCs down-regulate T cell differentiation by inhibiting antigen processing and thereby interfering with antigen presentation; and 2) α7 nAChRs of T cells up-regulate differentiation into regulatory T cells and effector T cells. Although α7 nAChRs are also expressed almost ubiquitously in immune cells, their structure and function in immune cells are yet to be defined. Activation of α7 nAChRs, α9 nAChRs and α9α10 nAChRs in monocytes inhibits pro-inflammatory IL-1 release via ATP-induced ion current at ATP receptor P2X7 and inflammasome activation [53–55]. In addition to ACh and nicotine, phosphocholine, Creactive protein and phosphocholine-modified lipooligosaccharides also serve as agonists for α7 nAChRs, α9 nAChRs and α9α10 nAChRs to inhibit the ionotropic function of P2X7R and modulate

ATP-induced IL-1β release [53–55]. These findings suggest that α9 nAChRs and α9α10 nAChRs are involved in down-regulation of innate immunity [53]. However, in murine experimental autoimmune encephalomyelitis (**EAE**) model, it has been shown that α9 subunits of nAChRs in peripheral immune cells mediate exacerbation of disease severity and inflammatory responses in the CNS, but that α10 subunits of nAChRs are not involved in EAE exacerbation [56, 57]. Altogether, these findings suggest that α7 nAChRs, α9 nAChRs and α9α10 nAChRs are potential targets of therapeutic ligands to modulate inflammation and immune responses.

The components of non-neuronal cholinergic systems such as mAChRs and nAChRs are also the targets for both effectors of autoimmunity and novel therapeutic approaches in patients with relevant autoimmune diseases (reviewed in [58–60]). Autoantibodies reacting with the M2 mAChR are found in patients with dilated cardiomyopathy, Chagas heart disease and arrhythmic disorders. Both autoantibodies and autoreactive T cells against the M3 mAChR subtype expressed in salivary and lachrymal glands are found in patients with Sjögren's syndrome. Autoantibodies to certain subtypes of both mAChRs and nAChRs expressed on oral and cutaneous keratinocytes are found in patients with pemphigus, a potentially lethal autoimmune mucocutaneous blistering disease. Recent data presented at the 5th International Symposium on NNACh implicated anti-M3 mAChR antibody in determining the level of intraepidermal split just above the basal cells in patients with pemphigus vulgaris [61]. Although the presence of autoantibodies against keratinocyte mAChRs in pemphigus patients has been known for more than 25 years [62], specific targeting of the M3 mAChR subtype was discovered only recently in proteomic studies [63, 64]. Most recently, it has been demonstrated that the titer of anti-M3 mAChR antibody correlates with disease activity in pemphigus patients and declines with therapy [65, 66]. The pathogenic significance of autoimmunity against keratinocyte M3 mAChR helps explain therapeutic activity of the AChE inhibitor pyridostigmine bromide (Mestinon) in pemphigus [67].

The involvement of cholinergic signaling in the generation of cancer was among the topics of the 5th International Symposium on NNACh. Already in 1991, Soreq and coworkers discussed a possible role of cholinesterases in tumorigenesis [68]. Based on the observation that the enzymes AChE and butyrylcholinesterase, both limiting the cellular effects of ACh, are mutated, amplified and/or aberrantly expressed in a variety of human tumor types, the authors considered a proliferative effect of cholinergic signaling in tumor cells. Correspondingly, organophosphorous poisons have shown some tumorigenic effects in humans [68]. In the same year, Gutkind et al [69] reported that stimulation of M1, M3 and M5 mAChRs induced foci of transformation in proliferating cultured cells. These effects occurred dose-dependently and the authors concluded that mAChRs may operate as conditional oncogenes.

Meanwhile, our knowledge about cellular effects of ACh has substantially increased. NNACh, via auto- and paracrine pathways, was found to be involved in the regulation of the cell cycle, proliferation, differentiation, migration, apoptosis and angiogenesis (reviewed in [32, 34, 70, 71]). Cholinergic signaling has been investigated in multiple tumors, i.e., cancers of the lung, female breast, colon, stomach, pancreas, prostate gland and the

hematopoietic system. NNACh was shown to promote cancer cell proliferation as auto-stimulating growth factor in some tumors [72–74].

The deleterious effects of nicotine in context of lung cancer are well known and described in detail elsewhere [71, 75, 76]. For example, nicotine increases the survival of cancer cells, reduces apoptosis, facilitates spreading and metastasis of some tumor cells ([77] and also reviewed in [76, 78]). In human colon cancer, significant differences in the expression pattern of cholinergic signaling components ChAT, AChE, α7 nAChR and its endogenous peptide ligand modulator SLURP-1 were found between normal and tumor tissues [79]. Moreover, the extent of the difference in the expression pattern corresponded to the prognosis factors [79]. Using a human colon cancer cell line, the proliferative effects of a very low concentration of nicotine (1 nM) have been reported, and the opposite effect was observed after blockade of the synthesizing enzyme ChAT [72].

The complex and multiple pathways in which ACh can be involved in the generation or promotion of cancer include mAChRs. Using gastric tumor cell lines, it was shown that ACh via M3 mAChRs and the epidermal growth factor receptor pathway stimulated tumor cell proliferation [80]. In whole animal studies with subcutaneously applied xenografted tumor models, M3 mAChR antagonists significantly reduced tumor growth and enhanced the cytotoxic effect of 5-fluorouracil [80]. Interestingly, surgical denervation of the stomach was followed by a reduced tumor incidence and a reduced progression of gastric cancer [81]. It is known that mAChRs are overexpressed in colon cancer compared to normal colon epithelial cells. Stimulation of these receptors mediates proliferation, migration and invasion of human colon cancer cells [82]. In a recently published review article, the role of M3 mAChR activation in promotion of colon cancer progression and dissemination has been summarized [83]. The downstream activation of several intracellular signaling pathways activated by M3 mAChR in colon tumor cells (i.e., the mitogen activated protein kinase/extracellular signal-related kinase; protein kinase C; p38 mitogen-activated protein kinase; phosphatidylinositol 3-kinase/Akt) and, additionally, the induction of metalloproteinases has been identified. In consequence of these activated signaling cascades, the transcription of genes and the expression of proteins are modified resulting in tumor cell proliferation, enhanced cell survival, migration and invasion [83].

Data about cholinergic signaling in breast cancer, the most frequent malignancy in women, was also presented at the 5th International Symposium on NNACh. The complexity of cholinergic signaling by different cellular effects mediated either by mAChRs or nAChR becomes evident in this tumor type, too. It appears that certain mAChRs are absent in normal breast tissue but are expressed in tumor tissue and stimulation of these receptors, at least in cultured breast tumor cells, reduced cell viability and migration [84, 85]. In contrast, nAChRs are present in normal and tumor breast tissue and their stimulation enhanced proliferation rate in a dose dependent manner, emphasizing the importance to consider consequences of nicotine consumption with respect to the effectiveness of anti-tumor therapy.

Discussions at the 5th International Symposium on NNACh strongly emphasized that basic science should be intensified to identify critical targets of cholinergic signaling in

carcinogenesis, because it is evident that cholinergic signaling is involved in this process. Analysis of cholinergic signaling in the so-called tumor stem cells should be among the focus of such research. Therewith, it will be a major challenge of science in the next decade to find out new therapeutic approaches targeting cholinergic signaling in tumor cells.

Thus, "5th International Symposium on NNACh from bench to bedside" facilitated continued work to advance the fundamental science and translational aspects of the interdisciplinary studies on NNACh. The progress made has opened a new chapter in the field of cholinergic pharmacology, and advanced our knowledge beyond regulation of individual cell- and tissue-types, defining a new paradigm of selective pharmacological regulation of vital function of practically all types of non-neuronal cells. It is now clear that the autocrine and paracrine control of non-neuronal cells by NNACh is implemented through synergistic, additive, and reciprocal effects triggered by two different cholinergic receptor classes: the ionic events, generated by ACh-dependent opening of nAChR channels and the metabolic events, due to ACh-binding to the G-protein coupled mAChRs as well as nAChR-dependent activation of signaling kinases [86]. Each biologic effect of ACh is determined by a unique combination of cholinergic receptors subtype expressed at each stage of cell development and differentiation. Simultaneous stimulation of mAChRs and nAChRs may be required to synchronize and balance ionic and metabolic events in a single cell, and a crosstalk between mAChRs and nAChRs may provide for a fine tuning of the signals emanating from CNS, endocrine glands and environmental stimuli. The plasticity of the non-neuronal cholinergic system helps adjust homeostasis to new environmental conditions.

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HIGHLIGHTS

1. The "5th International Symposium on Non-neuronal Acetylcholine: from Bench to Bedside" was held on September 27–29, 2019 in Hyatt Regency, Long Beach, CA, USA.
2. Acetylcholine is a ubiquitous molecule in life that, in addition to neurotransmission, plays important roles in various aspects of cell biology and homeostasis outside the neural system.
3. The plasticity of the non-neuronal cholinergic system helps adjust homeostasis to new environmental conditions.
4. A major challenge of science is to identify new therapeutic approaches targeting cholinergic signaling in non-neuronal cells.

Figure 1.
Participants of the Fifth International Symposium on Non-neuronal Acetylcholine, Long Beach, USA