Acetylcholine, a Ubiquitous Signalling Substance

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By

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Cambridge Scholars Publishing

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This book first published 2021

Cambridge Scholars Publishing

Lady Stephenson Library, Newcastle upon Tyne, NE6 2PA, UK

British Library Cataloguing in Publication Data
A catalogue record for this book is available from the British Library

ISBN (10): 1-5275-6721-4
ISBN (13): 978-1-5275-6721-4

This book is dedicated to Annemarie
and to our children and grandchildren

We cannot change the reality according to our ideas, but we can change our ideas according to reality, some people can, at all events.

—Fridjof NANSEN, neurobiologist, explorer, Nobel Peace Prize

Whenever a new discovery is reported to the scientific word, they say first: “It is probably not true.” Thereafter, when the truth of the proposition has been demonstrated beyond question, they say: “Yes, it may be true, but it is not important.” Finally, when sufficient time has elapsed to fully evidence its importance, they say: “Yes, surely it is important, but it is no longer new.”

—Attributed to Michel de MONTAIGNE

Eppure si muove !

—Attributed to GALILEO

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ACKNOWLEDGEMENTS

I am very grateful to:

The Fonds National Suisse pour la Recherche Scientifique (Switzerland), the FNRS (France), the British Council (UK) and the Universities of Geneva, Lausanne, Paris and Cambridge;

Maurice Israël and his colleagues (Gif-sur-Yvette, France) for a long-lasting and friendly collaboration;

All my co-workers who enthusiastically collaborated in this research from the 1960s until now. Their names can be found as my co-authors in the list of references, among them particular gratitude to Jean Falk-Vairant and Dominique Muller, two brilliant scientists who died too soon in accidents.

INTRODUCTION

Classically, acetylcholine (ACh) is recognised as a neurotransmitter substance, conveying nerve impulses in synapses of the central and the peripheral nervous systems; but ACh is much more than that. By taking into account ancient – but often forgotten – observations and recent reports from the now exploding research in the field, one can realise that ACh is present everywhere in living organisms. Acetylcholine is a ubiquitous signalling substance, which is at work in all phyla of the evolutionary tree. High ACh concentrations are encountered in the fluid of nettle hairs, in bamboo shoots, in the royal jelly of bees and in the human placenta! In vertebrates, many non-neural cells produce ACh in response to specific signals, mediating or modulating a great variety of processes such as cell division, migration or motility, the synthesis of specific proteins, angiogenesis, antibody production and secretion of different substances including ACh itself. We are just beginning to realise the importance of cholinergic signalling in the development and differentiation of tissues. This field is of a great practical significance, since drugs which interfere with cholinergic mechanisms not only perturb the function of synapses but exert their effects in many unexpected places in nature, including in microorganisms, plants, invertebrates and non-neural tissues of vertebrates. This should be taken into account for evaluating the consequences of acute and chronic nicotine consumption, and also for making allowances for the effects of neonicotinoids, anticholinergics and several other pesticides which are largely spread out in our environment.

The mechanisms of nerve impulse transmission in cholinergic synapses have also been fundamentally revisited, particularly concerning ACh release. Time is a crucial parameter in this connection. While cholinergic signalling between non-neural cells is a relatively slow process, transmission reaches the millisecond range in neuromuscular junctions and in their homologues, the nerve-electroplaque junctions of electric fish. A glimpse at recent findings concerning rapid cholinergic processes will certainly pave the way towards fascinating discoveries in the near future.

Acetylcholine has been at the crossroads of famous discoveries in the past, discoveries which were sometimes the subject for vivid controversies. Some of these are evoked in the present monograph, for instance: a) the importance of non-neuronal ACh (Chapters 1–3), a subject which is largely

omitted in classical textbooks; b) the quarrel between the tenants of the reticular hypothesis and those of the neuronal hypothesis, a question which was settled by observations partly performed in experiments using the neuromuscular junction (Chapter 5); c) cholinergic synapses of the autonomic nervous system were also at the centre of the debate between the humoral hypothesis and the electrotonic hypothesis for synaptic transmission (Chapter 4); d) many questions were raised by the discovery that transmitter release is quantal, and still more by the demonstration that the ACh quantum is composed of ACh subquanta (Chapter 5); e) exocytosis of the synaptic vesicle content has long been taken as the only possible mechanism explaining quantal ACh release (this turned out to become almost a dogma). This view should be revisited criticised and the pivotal role of the mediatophore proteolipid complex in quantal release recognised (Chapter 8); f) Chapter 10 describes the crucial role played by synaptic vesicles in rapid calcium buffering and later extrusion from nerve terminals.

Focused on presynaptic mechanisms the present monograph is not directly devoted to the description of proteins characteristically involved in cholinergic signalling (choline transporters, choline acetyltransferase, vesicular ACh transporter, muscarinic or nicotinic ACh receptors, cholinesterases, etc.). These have been the object of several excellent reviews, which will be mentioned in passing. Rather, emphasis will be placed here on the general functions of cholinergic signalling, and on the presynaptic mechanisms and regulations prevailing in slow and in ultra-rapid cholinergic systems.

ABBREVIATIONS

ACh	Acetylcholine
AChE	Acetylcholinesterase
AmPy	Aminopyridine
ANS	Autonomic nervous system
BoTx	Botulinum toxin
ChAT	Choline acetyltransferase
CNS	Central nervous system
EDRF	Endothelium-derived relaxing factor
EPP	End-plate potential or electroplaque potential
EPSP	Excitatory postsynaptic potential
IMP	Intramembrane particle
mAChR	Muscarinic acetylcholine receptor
MEPP	Miniature end-plate potential or miniature electroplaque potential
$Na^{+/}K^{+}$ ATPase	Sodium-potassium-adenosine triphosphatase
nAChR	Nicotinic acetylcholine receptor
NEJ	Nerve-electroplaque junction
NMJ	Neuromuscular junction
NO	Nitric oxide
RyR	Ryanodine receptor
SNAP	Synaptosomal-associated protein
SNARE	Soluble N-ethylmaleimide-sensitive-factor-Attachment protein REceptor
TMP, TDP, TTP	Thiamine mono-, di- and triphosphate
VAChT	Vesicular acetylcholine transporter
VAMP	Vesicle-associated membrane protein
VIP	Vasoactive intestinal peptide

CHAPTER 1

ACETYLCHOLINE: A UBIQUITOUS MESSENGER

Acetylcholine in primitive organisms

In 1914, Sir Henry Dale noticed that certain batches of rye ergot contained a substance causing a profound fall in blood pressure in cats. He asked his chemist colleague Ewins to see whether he could isolate this compound. Ewins (1914) identified the substance as acetylcholine, and concluded that ACh is a normal constituent of ergot and not a contamination "due to fermentative or other changes taking place during the preparation of the extract". The same year, Dale (1914) published his seminal paper describing the muscarinic and nicotinic actions of ACh. Later on, Oury and Bacq (1938), who failed to detect the presence of ACh in fresh ergot extracts, informed Dale about their results. The latter answered that the batch used by Ewins was rather putrefied; it was discarded, being unsuitable for therapeutic use. The exact source of that ergot contamination was never determined but, since ACh was subsequently shown to be produced by *Lactobacillus plantarum, Pseudomonas fluorescens* and other microorganisms, one of these was most probably involved in Ewins' analysis (Burgen 1995).

Nowadays, due to the great improvements in analytical techniques, the presence and the synthesis of ACh have been documented in representative forms of life of the whole evolutionary tree, i.e. in bacteria, in archaea and eukarya (Kawashima et al. 2007). Also, unicellular organisms, for instance *Trypanosoma rhodesiense,* produce ACh (Bülbring et al. 1949). Therefore, the synthesis of ACh occurred at the very early steps of the evolutionary process. Substantial amounts of ACh have been found in *Bacillus subtilis*, while other strains (*Escherichia coli, Staphylococcus aureus*) had a lower, although significant, ACh content. Acetylcholine synthesis in primitive bacteria and archaea seems to be supported by an enzyme distinct from choline acetyltransferase (ChAT), since it is not inhibited by bromoacetylcholine (Horiuchi et al. 2003).

In fungi, ACh was detected in the *Lactarius blennius* (Oury and Bacq 1938) and later in many other species, such as yeast (*Saccharomyces cerevisiae*) and the shiitake mushroom (*Lentinus edodes*). In the latter, the yield of ACh is higher in the stem than in the cap (Horiuchi et al. 2003).

Acetylcholine in plants

The presence of ACh is also documented in the tissues of most plant species, ranging from the evolutionary old ferns, horsetails and mosses to *Anthophyta* and *Coniferophyta*. Emmelin and Feldberg (1947) reported that the reaction of the human skin to the sting of the nettle (*Urtica urens*) is brought about by histamine, ACh and other substances, which are concentrated in the fluid of nettle hairs. Acetylcholine potentiates the itching sensation caused by histamine and in addition, ACh itself provokes a burning pain. High ACh amounts have been found in eggplants (*Solanum melongena*) and in bamboo shoots (*Phyllostachys bambusoides*). The ACh content of the top of a bamboo shoot (almost 3 µmol/g) is higher than that found in the majority of animal cholinergic tissues (approximately 1 µmol/g in the *Torpedo* electric organ). The fact that the ACh yield is particularly high in the rapidly growing parts of plants suggests that cholinergic signalling plays a major role in cell division and in development, as demonstrated for tomatoes (Di Sansebastiano et al. 2014). Regulation of water homeostasis and photosynthesis has also been attributed to cholinergic mechanisms, which are also involved in plant fertilisation (Roshchina 1998). Like in the animal kingdom, ChAT seems to be the main enzyme responsible for ACh synthesis in plants (Sastry and Sadavongvivad 1978; Smallman and Maneckjee 1981; Wessler et al. 1998; Horiuchi et al. 2003; Kawashima et al. 2007).

Conclusion

ACh is a ubiquitary signalling messenger. In unicellular organisms, cholinergic signalling seems to be involved in the phase of rapid division. Similarly, in pluricellular organisms ACh is expressed in cells engaged in multiplication, growth and differentiation. In most primitive organisms and in plants, the enzyme responsible for ACh hydrolysis might be different from acetylcholinesterase. Research in progress will elucidate the types of ACh receptors which are implicated in these systems.

CHAPTER 2

EXTRANEURONAL ACETYLCHOLINE SIGNALLING

It was from the spleen of an ox and a horse that Dale and Dudley (1929) extracted and identified ACh in an animal organ for the first time. In the following years, people supposed that the existence of ACh in the spleen was due to the presence of nerve fibres of the parasympathetic system. This is not the case, however, since recent investigations showed that ACh is produced in the spleen by immune cells, and not by neuronal structures (Kawashima and Fujii 2000). We presently know that a great variety of cholinergic cells exists in non-neuronal tissues, such as placenta, cornea, skin, gut, lung, kidney, heart, bones, vascular endothelium, spermatozoids, airways, fibroblasts, lymphocytes and cancer cells. The scientific community first considered these findings more or less as curiosities, but owing to significant improvements of cellular and molecular approaches, the non-neuronal cholinergic system is becoming a domain of very high physiological, pathological and toxicological importance (Sastry and Sadavongvivad 1978; Wessler et al. 1999; Kawashima et al. 2007; Wessler and Kirkpatrick 2008; Beckmann and Lips 2013).

Cholinergic signalling in the vascular endothelium

Blood vessels are not innervated by cholinergic fibres of the parasympathetic nervous system; nevertheless, it has long been known that ACh and muscarine provoke a vasodilation. The dilemma was elucidated when it was demonstrated that this muscarinic vasodilation disappears when the vascular endothelium is removed (Furchgott and Zawadzki 1980), and that a subset of endothelial cells do contain ACh and ChAT. When the endothelial membrane is submitted to frictional forces (shear stress) or under the action of vasoactive agents, ACh is synthesised in – and released from – these cells. Once liberated, ACh acts in an autocrine and paracrine manner via muscarinic receptors (mAChRs), prompting the surrounding

endothelial cells to secrete relaxing substances, principally EDRF-NO (endothelium-derived relaxing factor, identified as nitric oxide). The latter compounds provoke relaxation of the subjacent smooth muscle cells, and thereby vasodilation. Acetylcholine in this case is directly liberated after synthesis, probably via organic cation transporters (Fig. 2-1, A and C). The process of flow-mediated vasodilation has been investigated in great detail by Wilson et al.; it is of great physiological and pathological importance (Parnavelas et al. 1985; Kirkpatrick et al. 2003; Wessler and Kirkpatrick 2008).

The muscarinic vasodilation is not the only type of cholinergic signalling in blood vessels. Sensitive nicotinic receptors (nAChRs), mainly of the $\alpha 7$ type, are also present in the vascular endothelium and mediate a powerful angiogenic action (Heeschen et al. 2001). They modulate key processes such as endothelial cell survival, proliferation, migration and tube formation (Fig. 2-1B). Importantly, nicotine stimulates angiogenesis at clinically relevant concentrations, i.e. at plasma concentrations similar to those generated by moderate smoking. While stimulation of angiogenesis exerts positive effects in certain situations (wound healing, osteogenesis, limb ischaemia, post-infarction capillary development), angiogenesis can worsen other disorders, (age-related retina maculopathy, atherosclerosis, restenosis, and particularly lung, colon, breast and other cancers. In the latter conditions, it might be useful to develop substances impairing angiogenesis by inhibiting this nicotinic pathway. However, the drugs available now (mecamylamine, hexamethonium, etc.) would obviously provoke an intolerable trail of unwanted effects (Kawashima et al. 1989; Cooke and Ghebremariam 2008; Beckmann and Lips 2013).

Digestive, urinary and respiratory systems

In these tissues ACh is liberated by a variety of non-neural cells, in addition to the well-established release from the parasympathetic nerves; the neuronal and the non-neuronal processes seem to be coordinated in some way. Non-neural ACh has been reported to play a role in numerous conditions such as gastrointestinal reflux, ulcers, colon cancer, ulcerative colitis, Crohn's disease, acute kidney injury, overactive bladder syndrome, asthma, lung cancer and cystic fibrosis. In these systems, nicotinic receptors are involved in addition to muscarinic receptors. This may explain why smoking often affects the course and the gravity of certain diseases in different ways. For instance, smoking clearly worsens symptoms in Crohn's disease, but may exert a protective action in the case of ulcerative colitis, reducing mucosal inflammation (de Jonge and Ulloa 2007). Further research

will certainly provide new and fruitful knowledge about the role of non-neuronal cholinergic cells in these domains (Beckmann and Lips 2013).

During the recent Covid-19 pandemic, preliminary observations suggested that chronic smokers and non-smokers have not the same susceptibility to be infected by the virus (Miyara et al. 2020). This might be explained in the following way. Chronic exposure of the respiratory system to nicotine would lead to a re-structuration of mucosa cells, and the new cells would not present the same number of virus-binding proteins. Other explanations are, however, plausible and these observations have to be confirmed.

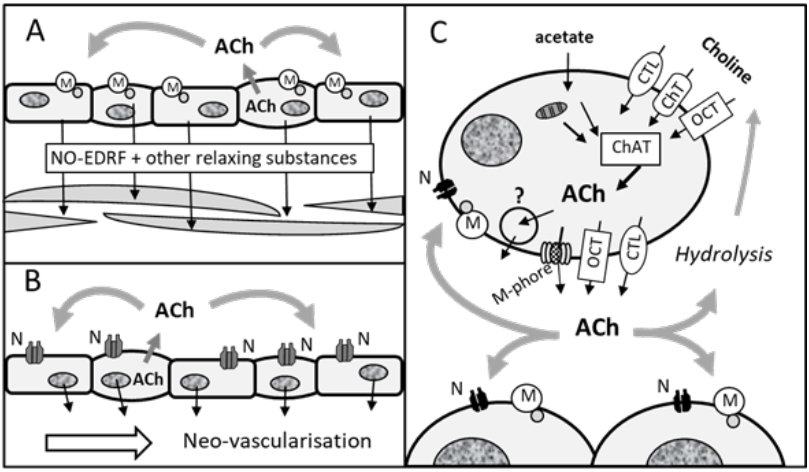

Figure 2-1: Cholinergic mechanisms in non-nervous tissues
A. Muscarinic vasodilation. Acetylcholine (ACh), produced by – and released from – a subset of endothelial cells, acts on muscarinic receptors localised on the membrane of neighbour cells. The latter secrete nitric oxide (EDRF-NO) and other relaxing substances, causing relaxation of the vascular smooth muscle fibres, and thereby vasodilation. **B**. In a similar autocrine/paracrine process, ACh activates sensitive nicotinic receptors, a process which initiates mitosis and differentiation of endothelial cells, favouring processes leading to the neoformation of blood vessels (angiogenesis or neovascularisation). **C**. Diagrammatic representation of the mechanisms involved in ACh synthesis, release, signalling and hydrolysis in the non-nervous cholinergic system. ChT, choline transporter protein; CTL, choline transporter-like proteins; OCT, organic cation transporters; M-phore, mediatophore. References and further explanations are in the text.

Cholinergic immune cells

As already mentioned, the ACh content of the spleen (Dale and Dudley 1929) cannot be attributed to parasympathetic fibres. There is a population of immune cells which locally synthesise and liberate ACh. While ACh and ChAT are expressed predominantly in T-cells, they are also present, although in lower amounts, in B-cells, dendritic cells, mast cells, granulocytes and macrophages. Upon contact with antigen-presenting cells, T-cells synthesise and release ACh, activating neighbouring cells in an autocrine and paracrine manner. The different types of muscarinic receptors (M1 to M5) are present on immune cells. In most cases, their activation has a pro-inflammatory action, regulating cytokine and antibody production. Nicotinic receptors (mainly $\alpha 7$) are also expressed in the membrane of certain immune cells. Their activation might result, in contrast, in an anti-inflammatory action. A cholinergic anti-inflammatory reflex has been described as a pathway implicating afferent vagal fibres, a central nervous system (CNS) relay and sympathetic efferent fibres ending in the spleen (Kawashima and Fujii 2000; Rosas-Ballina et al. 2011; Fujii et al. 2017). Therefore, autocrine and paracrine ACh release in immune cells represents an efficient physiological modulator of either the inflammatory or the anti-inflammatory reactions.

Acetylcholine in the skin and the corneal epithelium

Skin keratinocytes are able to synthesise ACh in high amounts; they also express ACh receptors of both classes. Auto and paracrine secretion of ACh in the skin is assumed to regulate the intimate connection of keratinocytes – their proliferation and differentiation – and processes like apoptosis, cell adhesion and migration. It was not surprising therefore that strong disfunctions of the cholinergic system were recognised in several skin diseases. In atopic dermatitis, for instance, the ACh yield of the superficial epidermis is increased 14-fold. The cutaneous cholinergic system might also be involved in vitiligo, Mal de Meleda, psoriasis, pemphigus, palmoplantar pustulosis and certain types of cancer. Epidemiological studies revealed that smoking is a risk factor for cutaneous squamous cell carcinoma, whereas it may lower the risk in Kaposi's sarcoma.

The ACh content of corneal epithelium exceeds that found in neural tissues. Cholinergic signalling is essential for the maintenance of the corneal epithelium, and especially for its repair in case of wounds or other injuries. Application of ACh, cholinomimetic drugs and cholinesterase inhibitors accelerates corneal re-epithelialisation. In these cells, muscarinic and

nicotinic signalling pathways are not antagonists but might cooperate in the repair processes, which involve survival, multiplication and migration of epithelial cells (Chernyavsky et al. 2014). Interestingly, like in neuromuscular systems, external acetate is used by the corneal epithelial cells as a direct precursor for ACh synthesis (Fitzgerald and Cooper 1967).

Non-synaptic ACh in the myoskeletal system

Osteoblasts, osteoclasts, primary bone cells, mesenchymal stem cells, tenocytes, fibroblasts express ChAT and produce ACh. They synthesise and release ACh in response to specific signals. In the myoskeletal system, muscarinic and nicotinic receptors are present in many places other than the neuromuscular junctions. Cholinergic signalling plays a role during development for the establishment of nerve–muscle connections (Chow and Poo 1985). ACh is also involved in a number of pathological conditions, including osteoporosis, tendinitis of the Achilles tendon and rheumatoid arthritis. Smoking seems to be linked to a decrease in the bone mass and a reduced capacity for fracture healing, an effect which might result from a nicotinic downregulation of osteoblasts and up regulation of osteoclasts (Beckmann and Lips 2013; Eimar et al. 2013).

Striated muscle fibres contain a globular form of AChE, in addition to the asymmetric, collagen-tailed form, described in Chapter 11. The globular AChE of muscle cells is predominantly the tetrameric form (G4-AChE), which fulfils a distinct functional role and is located in perijunctional regions (Gisiger and Stephens 1988; Bernard et al. 2011). The abundance of G4-AChE varies according to the muscle twitch properties (it is high or low, in fast and slow muscles, respectively). Moreover, the amount of G4-AChE increases in proportion to the level of fast-muscle activity. From a functional point of view, the globular G4-AChE (which does not abbreviate the time course of individual fast EPPs) controls the ambient concentration of ACh created by its diffusion out of the junctions upon repetitive stimulation. G4-AChE is therefore expected to protect nicotinic receptors from desensitisation (Gisiger and Stephens 1988; Jasmin and Gisiger 1990; Gisiger et al. 1994; Descarries et al. 1997; Rossi et al. 2000; Dunant and Gisiger 2017).

Acetylcholine and reproduction

Cholinergic signalling is at work in virtually all parts of the female and male reproductive systems. It has long been known that ACh is present in the human placenta, where it regulates blood flow, vascularisation and

nutrient transport. In the male, ACh is involved in the motility of spermatozoids. Also, the process of oocyte fertilisation seems to be initiated by ACh release from the spermatozoid head. Conditions such as tubal ectopic pregnancy, pre-eclampsia and different forms of infertility might be associated with troubles affecting cholinergic signalling in the reproductive system (Wessler and Kirkpatrick 2017).

The royal jelly of honey bees (*Apis mellifera*) contains a huge concentration of ACh (approximately 8 mM), which is very well conserved because of its acidic environment (pH 4.0) (Colhoun and Smith 1960). The jelly food destined for working bee larvae is also rich in ACh. This food is produced by nursing bees whose hypopharyngeal glands are equipped with choline acetyltransferase. In experiments on artificial larval breeding, increasing the ACh content of the jelly increased the brood survival rate, while decreasing the ACh content markedly affected the survival rate. Now, exposure to neonicotinoids reduced the jelly ACh content and had adverse effects on brood development (Wessler et al. 2016). Therefore, non-neuronal ACh plays also a crucial role in the reproduction and development of invertebrates. There is no doubt that the action of pesticides on the non-neuronal cholinergic systems damages the insect populations more severely than direct killing.

Cholinergic cell lines

Since ACh is an ubiquitary messenger, present in virtually all tissues of the organism, it is not surprising that several cell lines, particularly embryonic pluripotent and cancer cells, express one or several mechanisms involved in cholinergic signalling. A classic example is the neuroblastoma-glioma hybrid cell line NG108-15, which can establish functional cholinergic junctions with embryonic muscle cells in culture (Hamprecht 1977; Nirenberg et al. 1983; Kimura et al. 1992; Israël et al. 1998). Certain cell lines have provided a fantastic tool for dissecting the molecular steps of synapse formation and cholinergic signalling. Mechanisms such as voltage-dependent calcium entry, high-affinity choline uptake, ACh synthesis, vesicular ACh transport, vesicular Ca^{2+}/H^{+} exchange, Ca^{2+}-dependent quantal ACh release, have been induced or blocked by activating or inactivating expression of the corresponding protein complexes. Using cell lines which are deficient in one of these elements, it has been possible to "rescue" the lacking function by adding the appropriate molecule (see examples in Chapters 9 and 10). There is good evidence that these approaches will likely open the way for cell or gene therapies in conditions such as neurodegenerative diseases or cancers (Higashida et al. 2017).

ACh synthesis, release and hydrolysis in the non-neuronal cholinergic system

Like in neurons, ACh is synthesised in most non-neuronal cells by choline acetyltransferase (ChAT) from choline and acetyl coenzyme A. However, ACh might also be synthesised by carnitine acetyltransferase in certain cells under particular conditions (Tucek 1978; Beckmann and Lips 2013). The supply of external choline to non-neuronal cells is supported by the high-affinity transporter-1 (CHT1), and by other mechanisms, such as choline transporter-like proteins (CTLs) or organic cation transporters (OCTs; see Fig. 2-1C). In the corneal epithelium, external acetate is taken up and used for ACh synthesis. This is probably also the case for other non-neuronal cells. It should be noted that most non-neuronal cholinergic cells do not contain synaptic vesicles or the vesicular acetylcholine transporter (VAChT). Moreover, botulinum toxins do not alter ACh release from cholinergic skin cells (Wessler and Kirkpatrick 2008). Therefore, in these cells ACh is not stored in vesicles, but directly liberated after synthesis via transporters such as OCTs or CTLs. In T-lymphocytes, Fujii et al. (2012) demonstrated that ACh release is supported by the mediatophore proteolipid complex (see Chapter 9).

As acetylcholine is a ubiquitous messenger molecule, it is not surprising that the ACh-degrading enzyme acetylcholinesterase (AChE) was detected in various amounts in virtually all tissues. For instance it has been shown that epithelial cells of the pancreas are able to synthesise and release AChE (Bendayan and Gisiger 2001). This explains the great mismatch that has been observed between the localisation of AChE on one side, and that of the sites releasing ACh on the other side. Now, most muscarinic and nicotinic receptors in the non-nervous cholinergic system are sensitive receptors, detecting low ACh concentrations. At the difference of rapid synapses which function in a phasic manner (they generate high-frequency trains of brief impulses), the non-nervous system works in a rather tonic manner, controlling in time and space slow local changes in ACh concentration (Dunant and Gisiger 2017). On the other hand, spontaneous hydrolysis may also contribute to ACh removal from the tissues, since ACh is a labile compound in a neutral or basic environment.

Conclusions and perspectives

The non-neural cholinergic system is a rapidly moving domain. It will certainly be found to operate in all organs of the body. The reader will find more information in selected reviews (Sastry and Sadavongvivad 1978;

Kawashima et al. 2007; Wessler and Kirkpatrick 2008; Beckmann and Lips 2013; Wessler and Kirkpatrick 2017). The “rediscovery” of the non-nervous cholinergic system opens avenues of an immense physiological and pathological importance. We know most of the acute effects of the toxic substances acting on muscarinic and nicotinic receptors but little is known about their long-term action. For instance, the chronic effects of nicotine on healthy or cancer cells need further investigation. Also, chronic exposition to low doses of pesticides such as neonicotinoids, carbamates or anticholinesterases should certainly provoke dysfunctions affecting the non-nervous cholinergic system. A recent report suggests that the non-neuronal cholinergic system was probably involved in the pathogeny of the Gulf War illness, a chronic multisymptom disorder which affected soldiers having been exposed to pyridostigmine, as a protective drug against nerve gas. By inhibiting AChE, pyridostigmine apparently caused a disruption of the gastrointestinal neuro-immune system, among many other unwanted effects (Hernandez et al. 2019).

CHAPTER 3

EXTRA-SYNAPTIC ACETYLCHOLINE SIGNALLING IN NERVOUS SYSTEMS

Diffuse cholinergic transmission in the autonomic nervous system

The first demonstrations of ACh being a mediator substance were performed using organs that are targets of parasympathetic postganglionic fibres, such as cardiac and smooth muscle cells, and secretory cells. (Loewi 1921; Bacq 1975; Burgen 1995; Brown 2006). Transmitter action in these tissues is not a brief and highly restricted point-to-point process. In most cases, the postganglionic axons issued from parasympathetic neurons do not form real synapses on target organs. They give bunches of terminals and varicosities which are "free", spraying ACh over a relatively vast territory where it activates or modulates cellular processes such as secretions, smooth muscle contraction, inhibition of cardiac frequency, regulation of metabolic pathways and control of gene expression. In these areas, the time elapsing between ACh release and the end of the cellular effects is by several orders of magnitude longer than in neuromuscular junctions, ranging from seconds to minutes, or more. There, ACh plays the role of a local hormone rather than that of a neurotransmitter (Cooper et al. 1996). Acetylcholinesterase (AChE) is usually present in tissues and in fluids surrounding the sites of ACh secretion. Therefore, the relatively slow time course of ACh action is determined by factors such as ACh diffusion from the releasing source, the cytological architecture of the tissue, receptor kinetics, the time course of second messenger processes and, of course, by AChE which controls the local ACh concentration in space and time. To be distinguished from true synaptic transmission, this mechanism has been called "bulk or diffuse transmission". It should be noted that diffuse transmission coexists in the autonomic system with a variety of different forms of signalling, including true cholinergic synapses (Burnstock 1979).

Quantal acetylcholine release from Schwann cells at the denervated neuromuscular junction

Birks, Katz and Miledi (1960b), working on denervated frog neuromuscular junction several days after complete disappearance of the motor nerve terminals, were surprised to record spontaneous discharges of miniature end-plate potentials. At this stage

> *the axon terminals have completely disintegrated and have been replaced in their synaptic position by the Schwann cells. […] Evidence is presented for the view that the miniature potentials at the denervated end-plates arise, like those in normal muscle, from quantal liberation of acetylcholine […] from a nearby source on the outside of the muscle fibre* (pp. 162, 166)

Compared to miniatures occurring in normal muscles, the renewed discharge differed about their size distribution and their response to various stimulating manoeuvres. The finding was groundbreaking at that time since it suggested that a substance regarded as a neurotransmitter can be secreted in a quantal manner by non-neuronal cells, and also because the Schwann cells contain scarcely any substructure resembling synaptic vesicles.

Since then, ACh production by glial cells has been confirmed in the central and peripheral nervous system. Choline acetyltransferase has been detected in astrocytes. Also, the activation of microglia was found to involve cholinergic mechanisms resembling those of T-lymphocytes activation (Wessler and Kirkpatrick 2008).

"Volume transmission" in the central nervous system

Improved methods for identifying and characterising cholinergic nerve terminals revealed that diffuse transmission, also called "volume transmission", is a major mechanism operating in the CNS. In many places, cholinergic pathways (identified by their ChAT content) end in bunches of terminals which appear to be "free", without any close contact with some differentiated "postsynaptic" membrane. The mediator released from these terminals diffuses and exerts its effects in a relatively large volume of tissue. Systematic examination of numerous cholinergic terminals, boutons and varicosities at the ultrastructural level, led to the conclusion that the majority of them lack the cytological specialisations that are the hallmark of synapses, which are a close apposition and a thickening of the pre- and postsynaptic membranes (Descarries et al. 1997; Descarries 1998; Dunant and Gisiger 2017).

Another important – and unexpected – picture emerged from the molecular identification and the localisation of ACh receptors. A great number of receptors, either muscarinic (mAChRs) or nicotinic receptors (nAChRs) in nervous tissues are not restricted to postsynaptic membranes, as could be expected, but are present at various places such as extra-synaptic areas of neurons, dendrites, axons and nerve terminals, as well as in the membrane of non-neuronal cells, including glia and microvessels (Wonnacott 1997; Dani and Bertrand 2007; Lendvai and Vizi 2008). In most instances the messages conveyed by ACh in the brain are relatively slow. As an example, certain cortical neurons respond to a brief iontophoretic application of ACh by a delayed excitation which can last several seconds (Krnjevic et al. 1971). In many investigations, local changes in ACh concentration could be followed in various areas of the brain by biochemical assays, which are relatively slow techniques. This illustrates the fact that ACh in the brain acts at many places as a local hormone in addition to its action as a rapid point-to-point neurotransmitter (Greenfield and Smith 1979; Descarries et al. 1997).

Muscarinic and nicotinic autoreceptors modulate the release of ACh and other mediators. As a general rule, presynaptic muscarinic receptors depress the release process (Szerb and Somogyi 1973), while activation of presynaptic or preterminal nicotinic may stimulate release (Wonnacott 1997; Dani and Bertrand 2007; Lendvai and Vizi 2008). To give an example, nicotine application to hippocampus mossy fibres causes a delayed and prolonged release of glutamate, by a mechanism which does not include membrane depolarisation or dissipation of the vesicular proton gradient (Sharma et al. 2008; Bancila et al. 2009). Also, the fact that the variations of ACh concentration could be followed by biochemical assays in the extracellular space of different brain areas, supports the concept that ACh in the brain acts more often as a local hormone than as a rapid point-to-point neurotransmitter (Greenfield and Smith 1979; Descarries et al. 1997).

Therefore, the role of acetylcholinesterase in the brain is to contribute to this fine-tuning of local ACh concentration. As described in Chapter 11, the major molecular form of acetylcholinesterase (AChE) in CNS of birds and mammals is the globular forms, mainly the tetramer G4-AChE. It is relatively abundant on the membrane of cholinergic neurons (identified by their ChAT content), but it is also found in other types of neurons and other cells. The globular forms of AChE are attached to the plasma membrane; they are also secreted in the extracellular medium, but they are not densely concentrated in synaptic clefts.

Recent investigations showed that the organisation of cholinergic circuits in brain sectors is extremely complex. A characteristic example is the mammalian cerebral neocortex, which is densely innervated by cholinergic fibres issued from the neurons of the basal forebrain. This input reconfigures the local microcircuitry which also contains cholinergic elements, and is involved in behavioural transitions such as sleep to wakefulness, or distraction to attention. Cholinergic volume transmission in these places seems to predominate and there is evidence that the majority of psychotropic drugs interferes with volume cholinergic transmission (Zoli et al. 1999). However, volume transmission is combined with cholinergic synaptic transmission in a ratio which varies in different cortical areas and in different animal species (Muñoz and Rudy 2014; Colangelo et al. 2019).

Conclusion and perspectives

Diffuse or "volume" transmission represents a major mode of ACh action in the CNS. The mediator is released from bunches of cholinergic boutons and varicosities; it diffuses and exerts its effects on several cells in a relatively large volume. At these places, the local ACh concentration exhibits relatively slow variations, which result from the balance between the rate of release and the rate of hydrolysis by the membrane-bound or secreted AChE. (Greenfield and Smith 1979; Descarries et al. 1997; Lendvai and Vizi 2008). This should be kept in mind for the management of CNS conditions involving cholinergic mechanisms, such as Alzheimer's disease. It is also important for understanding short- and long-term effects of anticholinesterase intoxications and nicotine addiction.