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Review Article

The Cholinergic Kingdom Revisited

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Abstract

Acetylcholine is recognised as a neurotransmitter substance, but it is also a ubiquitous messenger which acts in an autocrine and paracrine manner in a great variety of living organisms from bacteria to plants, from lower invertebrates to human beings. In vertebrates, the majority of cholinergic cells are non-neuronal. These cells give cholinergic signals to neighbour cells in tissues like skin, epithelia, endothelia, placenta, gut, lung, kidney, spermatozoids, lymphocytes, cancer cells, etc. These processes in the non-neuronal cholinergic system are becoming of prime pharmacological and toxicological importance. Cholinergic neuro-neuronal synapses are characterised by a moderately rapid initial nicotinic signal, followed by a complex trail of late responses. They are found in the autonomous nervous system, and also in several places of the central nervous system. Ultra-rapid cholinergic synapses such as neuromuscular junctions and nerve-electroplaque junctions of electric fish are able to transmit nerve impulses at a high frequency; the duration of individual impulses in these synapses has therefore to be very brief. Several mechanisms curtail the duration of individual impulses. One is present in the presynaptic membrane (the voltage-gated K^+ current), the second is in the membrane of synaptic vesicles (the low affinity Ca^{2+}/H^+ vesicular antiport), and the third is highly active in the synaptic cleft (the asymmetric, collagen-tailed, molecular form acetylcholinesterase). Emphasis is also laid on mediatophore, a proteolipid complex localised in the active zones of the presynaptic membrane. Mediatophore is crucially involved in the mechanism of Ca^{2+}-dependent and quantal release of transmitter. These observations force one to reconsider the classical "vesicle hypothesis", as an explanation for acetylcholine release.

ABBREVIATIONS

Ach: Acetylcholine; AChE: Acetylcholinesterase; mAChRs and nAChRs: Muscarinic and Nicotinic ACh Receptors; ANS and CNS: Autonomic and Central Nervous System; ChAT: Choline Acetyltransferase; EPP: Endplate Potential or Electroplaque Potential; EPSP: Excitatory Postsynaptic Potential: MEPPs: Miniature Endplate or Miniature Electroplaque Potentials; NEJ: Nerve-electroplaque Junctions of Electric Organs; NMJ: Neuromuscular Junction; VAChT: Vesicular Acetylcholine Transporter

INTRODUCTION

Acetylcholine (ACh), a classical neurotransmitter, is also a ubiquitous messenger substance which is produced in a great variety of non-neuronal cells. Discovered in the beginning of the XXth century, our knowledge of cholinergic signalling has recently exploded, opening the field to unexpected findings of prime pharmacological and toxicological importance. Significant amounts of ACh and of its synthetizing enzyme choline acetyltransferase (ChAT) have been detected in unicellular organisms such as bacteria and algae, in plants, as well as in non-neuronal cells of invertebrates [1,2]. Just to give an example: Colhoun and Smith [3], discovered in 1960 that the honeybee royal jelly contains a surprisingly high concentration of ACh (5-8mM). Recently Wessler [4], reported that exposure of bees to a low neo-nicotinoid concentration significantly reduced ACh concentration in the royal jelly, thereby affecting the colony reproduction. Actually, the devastating effects of neo-nicotinoids on a great variety of invertebrates are the object of an intense research. At concentrations lower than that which kills the adult insects, these compounds perturb their reproduction, their nest behaviour, their thermoregulation and other functions [5]. Although the field of cholinergic signalling in primitive organisms, in plants and in invertebrates is of great importance, the present review will be restricted to cholinergic mechanisms occurring in vertebrates

IN VERTEBRATES THE VAST MAJORITY OF CHOLINERGIC CELLS ARE NOT NEURONS

ACh is synthesised and stored in a variety of non-neuronal cells, from which the mediator is released and acts in an autocrine and/or paracrine manner. Non-neuronal cholinergic cells were identified in placenta, cornea, skin, gut, lung, kidney, heart,

bones, vascular endothelium, spermatozoids, cancer cells, airway epithelium, fibroblasts, lymphocytes, etc [1,2,4,6-8].

ACh in the vascular endothelium

ACh is produced in a subset of endothelial cells. ACh synthesis and release are induced when the epithelial membrane is submitted to frictional forces (shear stress) or under the action of vasoactive agents. ACh in this case is directly liberated from endothelial cells, probably via organic cations transporters. 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), which provoke relaxation of the subjacent smooth muscle cells (Figure 1A) [2,9-11]. This muscarinic vasodilatation is not the only manifestation of the cholinergic system in blood vessels. Sensitive nicotinic receptors (nAChRs), mainly of the α7 type, are also present in the vascular endothelium and mediate a powerful angiogenic action (Figure 1B) [12]. By this way, nicotine stimulates angiogenesis at clinically relevant concentrations, i.e., at plasma concentrations similar to those generated by moderate smoking. Stimulation of angiogenesis exerts positive effects in certain cases: wound healing, osteogenesis, limb ischemia, post-infarction capillary development. But angiogenesis can worsen affections like macular degeneration in the retina, atherosclerosis, restenosis, and particularly several forms of cancer (lung, colon, and breast) [8,13,14].

Cholinergic immune cells

In the spleen, lymphocytes (mainly T-cells; and to a lesser extent, B-cells) express ACh and ChAT. Upon contact with antigen-presenting cells, T-cell synthetize and release ACh, activating neighbouring cells in an autocrine and paracrine manner. In most of the cases, their activation has a pro-inflammatory action, regulating cytokine and antibody production. Nicotinic receptors are also expressed in the membrane of immune cells, but their activation results in an anti-inflammatory action [15-17]. Thus, autocrine and paracrine ACh action in immune cells represents an efficient physiological modulator of both the inflammatory and the anti-inflammatory reactions.

ACh in the musculo-skeletal system

Osteoblasts, osteoclasts, primary bone cells, mesenchymal stem cells, tenocytes, fibroblasts express ChAT; they synthesise and release ACh in response to specific signals. Thus cholinergic mechanisms seem to be involved in affections like osteoporosis, tendinosis of the Achilles' tendon and rheumatoid arthritis. Smocking has been linked to a decrease in the bone mass and a reduced capacity for fracture healing, an effect which may result from a nicotinic down regulation of osteoblasts and up regulation of osteoclasts.

Digestive, urinary and respiratory systems

In the digestive, the urinary and the respiratory systems, ACh is liberated by non-neuronal cells in addition to the well-known release from the parasympathetic nerve terminals. There is good evidence that non-neural ACh is involved in several diseases, like gastro-intestinal reflux, ulcers, colon cancer, ulcerative colitis, Crohn's disease, acute kidney injury, overactive bladder syndrome, asthma, lung cancer, cystic fibrosis, etc. In these systems, nAChRs are expressed in addition to mAChRs. This can explain why smocking often affects in different manner the course and the gravity of the above-mentioned affections.

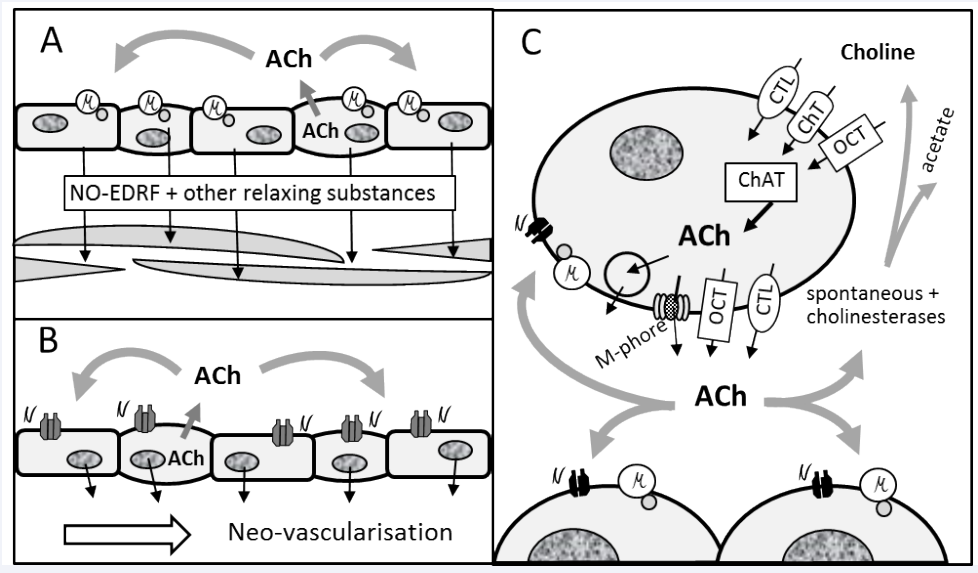

Figure 1 Cholinergic mechanisms in non-nervous tissues. A. Muscarinic vasodilatation. Acetylcholine (ACh) is produced by and released from a subset of endothelial cells and acts on muscarinic receptors localised on the membrane the whole of endothelial cells present in the area. The latter secrete nitric oxide (NO-EDRF) and other relaxing substances, causing relaxation of the vascular smooth muscle fibres, and thereby vasodilatation. B. In a similar autocrine/paracrine signalling process, ACh activates sensitive nicotinic receptors, which starts mitosis and differentiation of endothelial cells, favouring processes leading neo-formation of blood vessels (angiogenesis or neo-vascularisation). C. Diagrammatic representation of the mechanisms involved in ACh synthesis, release, signalling and hydrolysis in non-nervous cholinergic systems. ChT: choline transporter proteins; CTL: Choline transporter-like proteins; OCT: organic cation transporters; M-phore: mediatophore, a 15-kDa proteolipid complex described in Part 4.

ACh and reproduction

It has long been known that the human placenta contains substantial amounts of ACh. Cholinergic signalling has been reported to regulate blood flow, vascularisation and nutrient transport in the placenta. Actually cholinergic signalling is at work in virtually all parts of the female and male reproductive systems. In the male, ACh is involved in the motility of spermatozoids and in the process of oocyte fertilisation.

Skin

Keratinocytes in the skin synthesise ACh in high amounts. Auto and paracrine secretion of ACh in the skin is assumed to regulate the intimate connection of keratinocytes, their proliferation, differentiation, apoptosis, adhesion and migration. It is not surprising therefore that a strong implication of the cholinergic system has been recognised in several skin diseases.

Non-neuronal ACh in the nervous system

Acetylcholine is also released from non-neuronal cells in the peripheral and central nervous system. Birks, Katz and Miledi [18], were surprised to record miniature endplate potentials at the denervated frog neuromuscular junction several days after complete disappearance of the motor nerve terminals. This activity resulted from the release of ACh from the Schwann cells which covered the junction at this stage. In the central nervous system, ACh and ChAT have been more recently detected in astrocytes and in the microglia.

ACh synthesis, release and hydrolysis in the extra-neural cholinergic systems

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 [8,19]. The supply of external choline in non-nervous cells is supported by the high-affinity transporter-1 (CHT1), but also by other mechanisms, such as choline transporters-like proteins (CTLs) or by organic cation transporters (OCTs; Figure 1C). Most non-nervous cholinergic cells do not contain typical synaptic vesicles or the vesicular acetylcholine transporter (VAChT). Therefore, ACh is believed not to be stored in these cells, but directly released after synthesis, via transporters like OCTs or CTLs. In T lymphocytes, there is a clear evidence that ACh is directly released by mediatophore [20], an homo-oligomer proteolipid composed of a subunit which is the product of the ATP6VOC gene. It also a component of several proteolipid complexes involved in membrane function (see Part 4).

ACh being a ubiquitous messenger molecule, it is not surprising that the ACh-degrading enzyme acetylcholinesterase (AChE) can be detected in various amounts in virtually all tissues. For instance, it has been shown in the pancreas that epithelial cells are able to synthesize end secrete AChE [21]. 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 hand. Now most muscarinic and some 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 impulses), the non-nervous system works in a more tonic manner, controlling in time and space slow local changes of ACh concentration [22]. On the other hand, spontaneous hydrolysis may also contribute to ACh removal, since ACh is a labile compound, especially in neutral or alkaline environment.

CHOLINERGIC NEURO-NEURONAL SYNAPSES

Acetylcholine operates as a neurotransmitter in a majority of synapses of the autonomic nervous system (ANS), but only in a minority of neuro-neuronal synapses of the central nervous system (CNS). The following features characterize cholinergic neuro-neuronal transmission:

a) the time course of the initial nicotinic Excitatory Post-Synaptic Potential (EPSP) is moderately rapid; with a rise-time of 5-50 ms and a decay time taking several tens of milliseconds;

b) the initial EPSP is followed by a complex trail of late responses involving a variety of different mechanisms, some of them being muscarinic, other nicotinic, purinergic, adrenergic or peptigergic;

c) the synaptic cleft is in neuro-neuronal synapses is narrow (10-20nm);

d) acetylcholinesterase is not concentrated in the synaptic cleft of neuro-neuronal synapses; in the CNS the predominant molecular form of AChE is the G4 form, while in the ANS all the AChE molecular forms are present, the most abundant being the G4 form, which is either membrane bound or secreted;

Sympathetic nervous system

The general organisation, morphology, development and physiology of sympathetic ganglia can be found in classical textbooks and monographies [23-26]. Figure 2A shows the localisation of AChE in the superior cervical ganglion of the rat. Most of the AChE histochemical reaction is present in the endoplasmic reticulum of the neurons and on nuclear membrane. Glial cells do not show significant reaction. Intracellular traces recorded in a sympathetic ganglion in response to stimulation of preganglionic nerve fibres are seen in Figure 2B. The nicotinic EPSP has a rise-time of approximately 7 ms; its decay takes several tens of milliseconds. Upon a preganglionic stimulation of graded intensity, the neuron responds either by a lone EPSP, or by several EPSPs which add up and trigger action potentials. The different delays from the stimulus artefact reveal that several afferent fibres with distinct excitability and conduction time converge to the same neuron. Figure 2C shows extracellularly recorded EPSPs taken from a ganglion where nicotinic transmission was partly inhibited by mecamylamine (25μM). The compound EPSP is preceded by a brief presynaptic action potential [27]. Unexpectedly, the time-course of the initial EPSP is not significantly prolonged when AChE is inhibited by 10μM eserine. The unavoidable conclusion is that the action of ACh of the initial nicotinic EPSP in ganglionic synapses is not abbreviated by AChE [28,29]. This is in line with the fact that

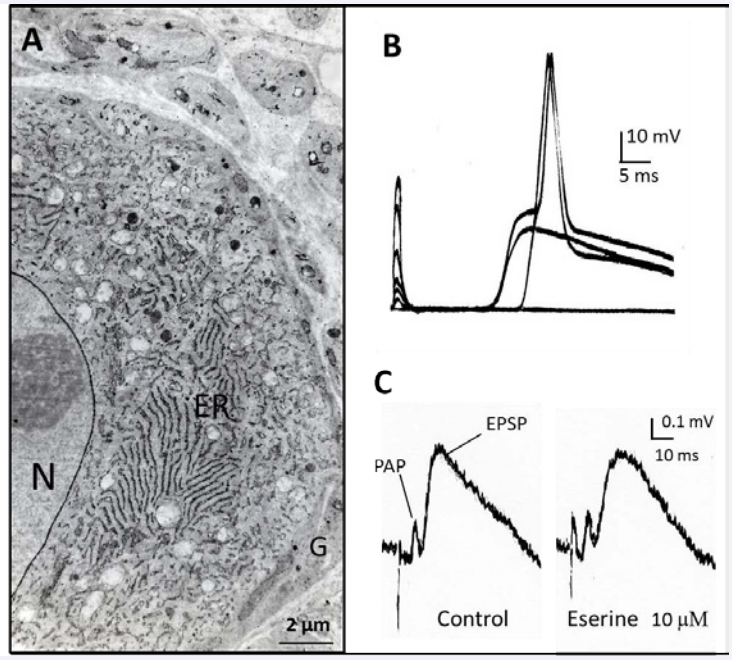

Figure 2 A. AChE localisation in a neuron of the rat sympathetic ganglion. Most of the activity is present in endoplasmic reticulum of neurons (ER) and on the nuclear membrane. Glial cells (G) do not show significant AChE histochemical reaction. AChE in ganglia is present as the globular G4 form, which is secreted from the neurons. B. Excitatory postsynaptic potentials and action potentials recorded intracellularly from a neuron of the same preparation in response to stimuli of increasing intensity applied to the preganglionic nerve. The different delays after stimulus artefact result from different conduction times in the preganglionic nerve fibres. The initial nicotinic EPSP lasts for several tens of ms. C. Extracellular recording in a ganglion where transmission was partly blocked by mecamylamine (25µM). PAP: presynaptic action potential [27]. At the difference of NMJ and NEJ, the anticholinesterase eserine does not prolong the EPSP time course.

AChE is not concentrated in the cleft of neuro-neuronal synapses, at the difference of the picture found in NMJs (see Part 4).

Parasympathetic nervous system

In contrast to sympathetic ganglia, the **parasympathetic ganglia** are located relatively far from the central nervous system, at the proximity of the innervated structures, often in the wall of the target organ. A classic example is the parasympathetic ganglion lying in the interatrial septum of the frog heart. The preganglionic fibres (which originate from the vagus nerve) branch extensively for innervating several ganglion cells. These cells are monopolar, and on each of them 10-20 presynaptic boutons, usually deriving from a single vagal fibre, make synapses which are mainly axo-somatic synapses. Spontaneous miniature potentials were recorded in parasympathetic synapses of the frog heart and the quantal nature of the EPSP demonstrated. Cholinesterase inhibitors (tensilon, eserine) had a variable action on different cells with respect to nerve-evoked and ACh-evoked potentials. In a few cases, the duration of the initial nicotinic EPSP is prolonged but generally the EPSP time-course is not affected [30,31].

Central nervous system

The first description of cholinergic neuro-neuronal synapses in the CNS was the motoneurons/Renshaw-cells synapses in the spinal cord [32]. The Renshaw-cells are spinal interneurons which receive a cholinergic nicotinic activation from motoneuron collaterals; Renshaw cells exert a GABA-ergic inhibition on a large pool of motoneurons. The system therefore realizes a neuronal negative feedback loop in the pathway controlling muscle activity. The initial response recorded from Renshaw cells in response to a single volley applied to motoneuron collaterals lasts a few tens of milliseconds and has a complex time course, displaying two peaks, generated via two populations of nicotinic ACh receptors. These nicotinic responses are followed by two glutamatergic excitatory currents produced by AMPAR and NMDA receptors, respectively, and by other complex late responses [33,34]. The initial nicotinic EPSP recorded from Renshaw cells is not prolonged when AChE is inhibited or absent. Interestingly, in the absence of G4-AChE these synapses can adapt themselves to the increased local ACh concentration [35]. This adaptation may be supported by a muscarinic auto-inhibition of ACh release [36], and by down-regulation of receptors.

There are of course many other examples of cholinergic neuro-neuronal synapses in the ANS and the CNS. Significant differences can be found from ganglion to ganglion with respect to animal species, cytological organisation, physiological working, but they have in common the properties enumerated above, particularly a moderately rapid speed of transmission and a low – or no - AChE concentration in the synaptic cleft.

PART 3. ACH RELEASE FROM "FREE" AXON TERMINALS. DIFFUSE TRANSMISSION

In addition to the axon endings forming real synapses, a significant number of cholinergic boutons and varicosities surrounding target cells is non synaptic, i.e., "free", without any close contact with a neurons or another cell. This, of course, is the

characteristic of ACh signalling in the organs innervated by the post-ganglionic fibres of the parasympathetic nervous system, but such a "diffuse" transmission seems to be an important cholinergic mechanism in the CNS [22,37].

Parasympathetic terminal innervations

It has long been known that the transmitter action on the organs that are the targets of the para-sympathetic system is not a brief, point-to-point process. The postganglionic axons issued from parasympathetic neurons do not form real synapses in target cells. In most places, they give terminals and varicosities which are "free", releasing ACh in a given area, for activating or modulating various processes like secretions, smooth muscle contraction, modulation of cardiac frequency, regulation of metabolic pathways, control of gene expression, etc. Such cholinergic neuronal - but non-synaptic - signalling has been called diffuse transmission. Innervation of the target organs is loose, with bunches of terminals and varicosities spraying ACh over a relatively vast territory. The time lasting between ACh release and the end of the cellular effects ranges from seconds to minutes, and even more. In others words, the cholinergic signalling is not phasic, but tonic at these places. ACh there should regarded more as a *local hormone,* than as a neurotransmitter [26].

Central nervous system

Due to marked advances made in techniques for identifying and characterising cholinergic nerve terminals, it turned out that volume transmission is also a major cholinergic mechanism operating in the CNS. Indeed, systematic examination of a large number of cholinergic terminals, boutons and varicosities at the ultrastructural level, led to the conclusion that most of them lack the cytological specialisations that are the hallmark of synapses (close apposition of pre- and postsynaptic membranes, local membrane thickenings) [22,37].

Another important-and unexpected - picture emerged from the molecular identification of different type of ACh receptors. Many ACh receptors in the brain and other tissues are not localised on the postsynaptic membranes, as could be expected. Actually, the great majority of receptors, either muscarinic (mAChRs) or nicotinic (nAChRs) are present in extra-synaptic areas of neurons: on dendrites, on axons and nerve terminals, as well as on the membrane of a great number of non-neuronal cells [38-40].In most instances the messages conveyed by ACh in the brain are slow. For instance, cortical neurons respond to a brief iontopheretic application of ACh by a delayed excitation which can last several seconds [41]. Presynaptic muscarinic AChRs at several places inhibit the release of various transmitters, including ACh itself [36], while activation of presynaptic or pre-terminal nicotinic AChRs stimulates the release of transmitters [38-40]. For instance, nicotine application to hippocampus mossy fibres causes a delayed and prolonged release of glutamate. The process occurs without membrane depolarisation and without dissipation of the vesicular proton gradient [42,43].

Also the direct determination of ACh concentration in the extracellular space of different brain areas supports the concept that ACh there acts more as a local hormone than as a rapid point to point neurotransmitter. In most places, cholinergic signalling appears mainly as a tonic process, where rather slow changes in ACh concentration are controlled in space and time by an extra-synaptic AChE [37,44].

PART 4. ULTRA-RAPID CHOLINERGIC TRANSMISSION

The present chapter will deal with the mechanisms of transmission in neuromuscular junctions (NMJs) of vertebrate fast twitch fibres [45], and with their homologs, the nerve-electroplaque junctions (NEJs) of electric fish. The electric organs are indeed modified neuromuscular systems. In *Torpedo* species, the electric discharge of the fish is made of the synchronised summation of a huge number of pure postsynaptic potentials [46-48]. The mechanisms of cholinergic transmission of nerve impulses is very similar in NMJs and NEJs; many features make them different from those operating in cholinergic neuro-neuronal synapses:

a) the time course of the nicotinic postsynaptic response, the End-Plate Potential (EPP), is extremely brief, lasting no more than 2-4 ms;

b) nerve impulses can be transmitted repetitively at high frequencies, 100 Hz or even higher; thus the mean interval between two impulses can be of the order of ten ms;

c) the synaptic cleft in neuromuscular and nerve-electroplaque junctions is wider than is other synapses (50-75 nm); this "primary" cleft is open to "secondary" invaginations of the postsynaptic membrane;

d) a specific, collagen-tailed, form of acetylcholinesterase (mainly A12-AChE) is highly concentrated in the primary and secondary clefts, attached to the basal lamina;

e) The ACh quanta characterizing transmitter release are extremely large in these junctions (~10'000 ACh molecules); they are composed of subunits or sub-quanta (~1'000 molecules).

The traditional description of cholinergic transmission in NMJs is as follows. Acetylcholine is synthesized in the cytosol of motor nerve terminals by choline acetyltransferase (ChAT) from choline (which is taken up by a high-affinity membrane transporter) and acetate (which is provided as acetyl-coenzyme-A from the glucose metabolism). ACh is then transported via the vesicular ACh transporter (VAChT) into the synaptic vesicles, where it is stored in a very compact manner, being bound with ATP to a glycosaminoglycan intra-vesicular matrix [49]. Invasion of the terminal by an action potential triggers entry of Ca^{2+} ions which reach a high concentration during a brief time in "micro-domains" localised near the inner face of the presynaptic membrane. This "calcium spark" provokes the abrupt fusion of synaptic vesicles which deliver their ACh content into the synaptic cleft by a process called exocytosis. In this "vesicle hypothesis", one quantum is expected to correspond to the Ach content of one vesicle. The released transmitter crosses the cleft and activates nicotinic receptors on the postsynaptic membrane. ACh is then eventually hydrolysed by the junctional acetylcholinesterase (AChE).

While several features of this description have been clearly verified during the past decades; important discrepancies were raised, however, by experiments designed to investigate the crucial steps of this process. *i*) The procedures used to isolated synaptic vesicles (homogenisation, freezing-thawing) represent a very drastic stimulation; nevertheless vesicular ACh survives these treatments. This was not expected for a transmitter pool which would be "immediately available for release"[50]. In contrast, cytosolic ACh (20-30mM, [51-53]) is immediately destroyed by these procedures. *ii)* In cholinergic – and also in other synapses-it is the pool of transmitter which was the most recently synthesised which is the first to be released. Moreover, the cytosolic pool of Ach was found to be used and renewed during repetitive nerve stimulation before vesicular ACh which behaves as a reserve pool, being consumed only after prolonged activity [47,53,54]. *iii*) The morphologic change observed in the presynaptic membrane during the very moment of transmitter release (and under the most physiological conditions) is a very brief (2-3 ms) increase in the number of intramembrane particles [55-58]. As for the number of vesicle openings in the presynaptic membrane, it also augments as a function of stimulation and of Ca^{2+} entry, but in most cases this occurs after the precise moment of release, especially at a period of time when the Ca^{2+} ions entered during activity are massively expelled from the terminals [55,57,59-63]. ACh release is supported by a proteolipid complex forming clusters at the presynaptic active zones of NEJs, NMJs and other synapses. This complex was discovered and called mediatophore by Maurice Israël and colleagues. When reconstituted in liposomes, in oocytes or in deficient cell lines, mediatophores mimic the physiological ACh release, including the Ca^{2+}-dependency, the production of ACh quanta and the fleeting occurrence of intramembrane particles in the presynaptic membrane. Mediatophores can deliver ACh quanta in the absence of synaptic vesicles and of vesicle-associated proteins [56,64-67].

These are some of the features which force one to reconsider the "vesicle hypothesis" as the only explication of quantal ACh release. Thereafter, we want to briefly report some additional observations which, we hope, will help to rewrite this chapter (Figure 3).

Three mechanisms which shorten the duration of individual impulses

Time is a crucial parameter in this type of synapses. To be fast, transmission must rely on ultra-rapid physicochemical reactions. Now, these are characterized by a low affinity, since the velocity of a chemical reaction is inversely related its affinity. "Time is gained at the expense of sensitivity", as elegantly formulated by Bernard Katz [70]. Figure 3 illustrates three mechanisms which are particularly well expressed in ultra-rapid cholinergic synapses. Inhibition of each of these mechanisms *i*) prolongs the time course of an individual EPP and *ii*) compromises repetitive transmission at high frequencies [71].

The first "curtailing" mechanism relies on **voltage-gated K^+-channels** which are exceptionally efficient in motor and electromotor nerve terminals for abbreviating the duration of presynaptic action potentials [68,72-74]. At the arrival of a nerve action potential, the axon is abruptly depolarised, due to opening of voltage-operated Na^+-channels. Then, the nerve is rapidly repolarised (quite before inactivation of the Na^+-channels) by a voltage-operated outward K^+ current (the so-called delayed rectifier), which rapidly rises to a maximum and then declines slowly [75]. At NMJs and NEJs, inhibition of the delayed rectifier (by 4-aminopyridine for example) dramatically potentiates the duration of ACh release in individual impulses [76-79]. The EPP is enormously extended, due to prolongation of the inward Ca^{2+}-current; it is converted into a giant EPP, which abates only after 500-600ms (Figure 3A). A long delay at rest is then required before a second giant potential could be triggered. Repetitive activity is therefore compromised [68].

The second "curtailing" process is illustrates in Figure 3B. It is **the vesicular Ca^{2+}/H^+ antiport**, which abbreviates the presynaptic Ca^{2+} spark. Gonçalves et al. [80,81], discovered that calcium is sequestrated into mammalian brain synaptic vesicles via a low-affinity Ca^{2+}/H^+ antiport ($K_{0.5}$ = 217µM; maximal activity at 500-600µM Ca^{2+}). This antiport is able to work in milliseconds. It efficiently curtails the duration of the Ca^{2+} spark during transmission of an individual impulse. When the vesicular Ca^{2+}/H^+ antiport is blocked, the duration of ACh release is significantly lengthened, resulting in a EPP of a longer time course The effect is clearly presynaptic since the amount of transmitter released by an individual stimulus is increased [82]. Working at low affinity, the vesicular Ca^{2+}/H^+ antiport is faster than the other processes which converge towards reducing the Ca^{2+} concentration in nerve terminals (diffusion, protein binding, Ca^{2+}/Na^+ exchange, Ca^{2+}-pumps in the plasmalemma, in the reticulum and in synaptic vesicles [83-87]. Most of the latter processes being high-affinity reactions, they are therefore poor candidates for a fast repetitive involvement during the very moment of transmission.

The molecular counterpart of the vesicular Ca^{2+}/H^+-antiport was recently elucidated: Synaptotagmin-1 (Syt-1) is essential for its activity. Synaptotagmin-1 is a vesicular protein interacting with membranes upon low-affinity Ca^{2+}-binding. It has been claimed to play a major role in excitation-release coupling, by synchronizing calcium entry with fast neurotransmitter release. Experiments carried out with Syt-1-positive and Syt-1 negative cell lines showed that Synaptotagmin-1 is absolutely required for the expression of the vesicular Ca^{2+}/H^+-antiport [88].

The third "curtailing" mechanism is well-known. It relies on the action of **the junctional acetylcholinesterase**. This specific form of acetylcholinesterase is an asymmetric collagen-tailed form, mainly A12-AChE, which is anchored to the basal lamina occupying the primary and the secondary cleft [89-94]. In the absence or after inhibition of junctional AChE, the EPP is prolonged, repetitive transmission is compromised despite of the fact that the amount of delivered per impulse is strongly diminished (Figure 3C). The A12-AChE dramatically abbreviates neurotransmission in neuromuscular and nerve-electroplaque junctions, due to the following features: *i*) The A12-AChE is localised in the centre of the NMJ junction, where it is positioned between the active zones of the motor nerve ending and the nicotinic receptors bound to the postsynaptic membrane boarding the primary cleft [95]; *ii*) it undergoes substrate inhibition for ACh concentrations exceeding 1mM [96]; *iii*) the A12-AChE has a very high rate of ACh hydrolysis, beyond 10^3

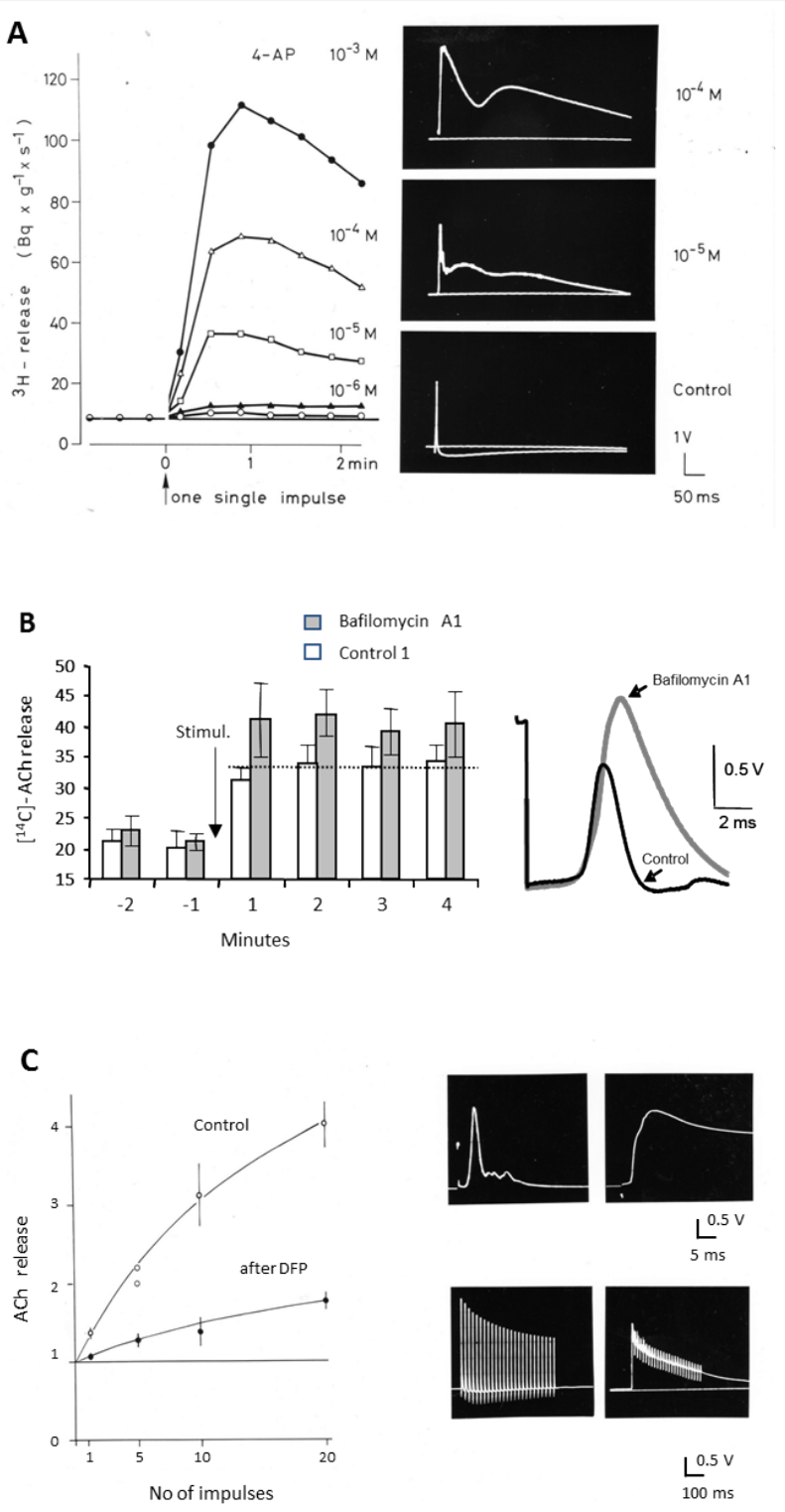

Figure 3 Inhibition of three mechanisms which curtail the duration of transmission of individual impulses in rapid cholinergic synapses.
In **A**, the presynaptic K^+-current (delayed rectifier) is inhibited by increasing concentrations of 4-amino-pyridine. The duration of the EPP responding to a single impulse is enormously prolonged, due to the huge prolongation of ACh release [68].
In **B**, Increase in the amplitude and duration of a single EPP after inhibition of the vesicular Ca^{2+}/H^+ antiport. Bafilomycin, a blocker of the V-ATPase, suppresses the driving force of the antiport, slowing down the sequestration of Ca^{2+} into the synaptic vesicles. As a result the amount of ACh released by a single impulse is increased. (Significance: $P<0.01$, tested with Anova) [69].
In **C**, increase in the duration of the EPP when acetylcholinesterase has been inactivated by pre-treatment with DFP (di-iso-propyl-pyrophosphate). The EPP is enormously prolonged in spite of a strong reduction of the amount of ACh released per impulse. The synapses lost their capability to fire at a high frequency (here 20 impulses in 1 s).[68]. All these experiments were made on the nerve-electroplaque junction of *Torpedo.*

ACh molecules/sec [91,97]. Accordingly, ACh molecules released from the active zones at almost molar concentrations [79,98], pass through the basal lamina while the anchored asymmetric AChE is temporarily inactivated by substrate inhibition [99]. ACh molecules reach the low affinity nicotinic receptors and ultimately, they are immediately destroyed when leaving them, since at that time the local ACh concentration has fallen and AChE has regained its full hydrolytic power, thereby cutting short the impulse within a 1-2ms. Consequently, reversible and irreversible cholinesterase inhibitors greatly lengthen the EPP decay phase in vertebrate NMJs and NEJs. The ACh concentration rises in the synapse, desensitising the nicotinic receptors and compromising high frequency transmission [68,100-102].

Quantal and sub-quantal ACh release in rapid cholinergic transmission

The quantal nature of rapid cholinergic transmission is a classic page of synapse physiology [102-104]. In a given NMJ, the number quanta forming an evoked EPP is correlated to the number of active zones present in this junction [105]. Refined experiments based on different approaches allowed to precise that one quantum results from the abrupt release of 6-10'000 ACh molecules [106,107]. Quantal ACh release in the nerve-electroplaque junction of the *Torpedo* electric organ is qualitatively and quantitatively identical to that in NMJs. In this junction, one quantum results from the synchronised liberation of 7-10'000 ACh molecules and was calculated to activate a little less than 1μm^2 of the postsynaptic membrane (1.3 quantum/μm^2), during 1-2ms [98,108].

Although the ACh quanta appeared remarkably constant in size and time course in many records, a population of much smaller MEPPs was discovered both in NMJs and NEJs [47,104]. Systematically improving signal-to-noise ratio, M. Kriebel and colleagues characterised these sub-MEPPs and proposed that the "classical" quantum in turn can be devolved into a number of a much smaller event, a sub-quantum resulting from the release of a packet of 1000 ACh molecules or less [109,110]. In the *Torpedo* electric organ, the devolution of ACh quanta into 10 sub-quanta has also been demonstrated, with values very close to those found in NMJs [98,111]. It is recalled the size of a synaptic vesicle is much larger in the electric organ (84nm) than in NMJs (45nm). Despite of this, quantal and sub-quantal sizes are almost identical in the two junctions. Direct estimation of the ACh content of a single vesicle could be performed using vesicles isolated from the electric organ. The value is very high, of the order of 200'000 ACh molecules [112]. Therefore, it is easy to calculate that there are enough ACh molecules in a single *Torpedo* vesicle to generate 20 quanta, or 200 sub-quanta.

Other rapid protagonists in neuromuscular and nerve-electroplaque transmission

The processes involved in rapid neurotransmission are characterised by a typical behaviour: abrupt exposition to the triggering agent (voltage change or ligand binding) causes fast activation soon followed by fast inactivation. Usually, prolonged exposition to the same agent provokes desensitisation. This is well documented for voltage-gated ion channels. They display a brief opening in response to a rapid potential change. Most of them undergo desensitisation if the electrical stimulus is maintained. Many ligand-gated channels, working at a high speed and low affinity, exhibit a similar behaviour**.** For instance, the nAChRs present in the NMJ postsynaptic membrane rapidly open in response to an abrupt jet of ACh. The mean open time of nAChRs is about 1 ms at NMJs; it is even shorter (0.6ms) in the *Torpedo* NEJ [113]. However, if ACh is not rapidly removed, nicotinic receptors will desensitize, that is, they will no longer open in response to ACh [114].

On the presynaptic side, the behaviour of mediatophores is quite similar. Mediatophore releases ACh quanta in response to a sudden elevation of $[Ca^{2+}]_i$, but desensitisation will occur if $[Ca^{2+}]_i$ remains elevated for several seconds or minutes [115]. It is recalled that mediatophore is a proteolipid complex forming clusters at the presynaptic active zones of NEJs, NMJs and other synapses. One mediatophore is a homo-oligomer composed of several copies of a 15-16 kDa proteolipid which is produced by the ATP6VOC gene, and which is therefore similar to the c-subunit of the membrane sector of V-ATPase. When reconstituted in liposomes, oocytes or deficient cell lines, mediatophore mimics the physiological ACh release, including the production of ACh quanta and the fleeting occurrence of intramembrane particles in the presynaptic membrane. The same proteolipid subunit is also involved in membrane fusion and several important cell functions [56,64-67].

Calcium buffering, vesicle-associated proteins and exocytosis

Thanks to the above-described Ca^{2+}/H^+ antiport, synaptic vesicles provide an efficient sink, rapidly clearing Ca^{2+} from the cytosol. Ancient observations *in situ* suggested that the more synaptic vesicles accumulate calcium, the more they lose vesicular ACh. If nerve stimulation is prolonged over the point of transmission failure, or low frequency stimulation is pursued during a long period of time, calcium progressively accumulates in nerve terminals (Figure 4A), while the level of vesicular ACh correlatively decreases [116,117]. This is explained by the important discovery that vesicular ACh and ATP are displaced by Ca^{2+} from binding sites provided by the intra-vesicular proteoglycan matrix [49]. In this view, vesicular ACh is delivered from the vesicles into the cytosol at active zones, preventing local exhaustion of the rapidly turning over, cytoplasmic pool of ACh.

As illustrated in Figure 4B calcium transiently accumulates in synaptic vesicles during a brief stimulation (for instance 12 s at 100 Hz).Calcium is subsequently cleared from vesicles, most probably by exocytosis (Figure 4C). Indeed, the density of vesicle openings in the presynaptic membrane(which do not increase at the very moment of synaptic transmission) clearly rises during the first 1-2 minutes following the train of stimulation, that is, just at the period when the rate of calcium clearance is high [62,63,116]. Transient Ca^{2+} accumulation in synaptic vesicles after activity was also demonstrated in other synapses. The fact that calcium might be delivered to the synaptic cleft via vesicle exocytosis was suggested for a CNS synapse as a way to refurnish the ions in this limited space of the cleft, where Ca^{2+} is probably exhausted in the course of an intense activity [118-120].

It is expected that operations which would impair the

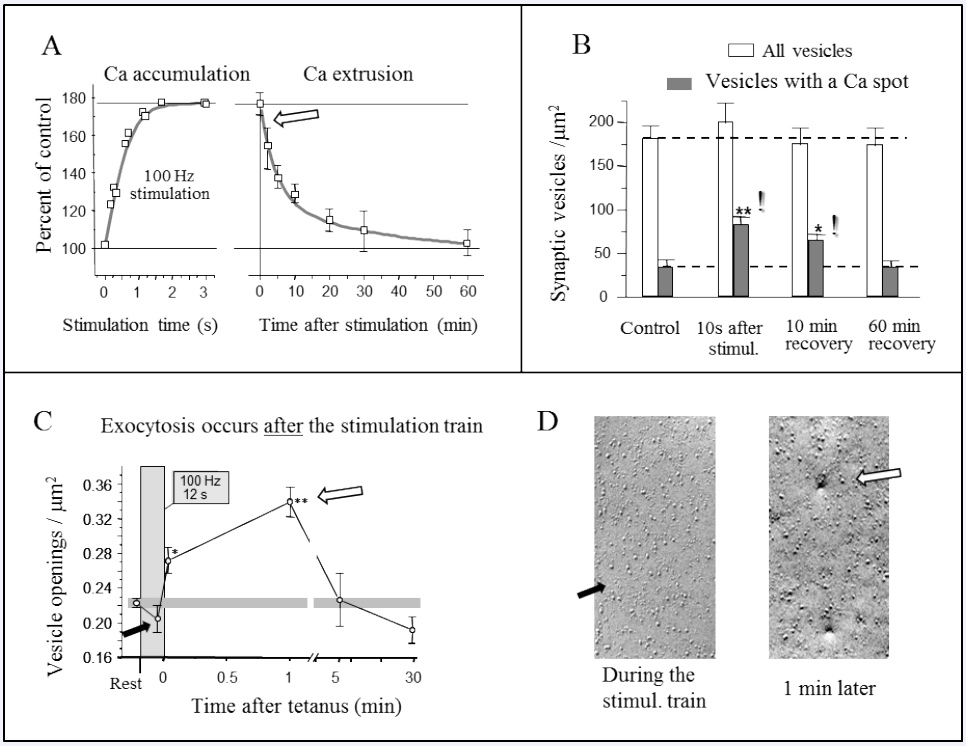

Figure 4 Transient accumulation of calcium into synaptic vesicles during a brief period of transmission, and its subsequent extrusion after the end of activity. A. Accumulation of calcium in the electromotor nerve terminals in the course of a brief titanic stimulation (3 s at 100 Hz) and the subsequent extrusion of the divalent during the minutes following the tetanus. B. It is in a significant fraction of synaptic vesicles that calcium transiently accumulates soon after stimulation at 100 Hz for 12 s. C and D. Number of vesicle openings in the presynaptic membrane during and after the same tetanic stimulation. The number of exocytosis images do not rises during the period of high frequency transmission but soon after the end of it, in parallel with the process of calcium extrusion from the nerve terminals [62,63,116].

mechanisms of calcium sequestration and clearance will lead to a steady augmentation of the intra-terminal Ca^{2+} concentration. The consequence will be: increase in the rate of spontaneous ACh release, decrease in the nerve-evoked release, disorganisation and desynchronization of the quanta and eventually block of transmission by desensitisation of mediatophores and/or voltage-gated Ca^{2+} channels. This is exactly what happens in synapses where Synaptotagmin-1 is absent or inhibited. A similar effect is observed when exocytosis is compromised by inactivation of vesicle-associated proteins (by clostridial toxins, for instance) or by other means [82,88,121-124], among many others. Thus, when exocytosis is blocked, transmitter release is not immediately blocked, but quantal release is strongly perturbed and desynchronised. Now, a desynchronised neuromuscular junction is totally inefficient.

From the above observation a new paradigm emerges for explaining ultra-rapid cholinergic transmission. *i*) The fast quantal transmitter release is supported by the synchronised activation of mediatophores. *ii*) Ca^{2+} is rapidly sequestrated via the low-affinity vesicular Ca^{2+}/H^{+} antiport (synaptotagmin), and exchanged against ACh and ATP in the intra-vesicular matrix. *iii*) After repeated activity, the accumulated calcium is eventually expelled by exocytosis [66,67,82].

CONCLUSIONS AND PERSPECTIVES

Until recently, acetylcholine was considered as a rather old-fashion domain, a vintage field. However, the development of refined molecular approaches brought a revival of our knowledge of cholinergic signalling. Although ACh has long been known to occupy important chapters in pharmacology and toxicology text-books, its interest has prodigiously increased over the last decades. ACh is now recognised as a ubiquitous messenger substance, involved in autocrine, paracrine as well as synaptic signalling. Just to give examples, every day we learn new facts concerning the non-synaptic actions of tobacco smoking and on the environmental long-term effects of pesticides such as anti-cholinesterases and neonicotinoids.

Also the mechanisms of neuro-neuronal and ultra-rapid cholinergic transmission have been thoroughly revisited. Old dogma need to be deconstructed and reconstructed. More attention should be devoted to the distinction between the ultra-rapid (low affinity) and slower mechanisms (high affinity). For instance, the high-affinity choline transporter is quite important to furnish the precursor at rest (and also for the scientist which want to label cholinergic neurons), but it is much too slow for supporting choline (and/or ACh) transport during transmission at a frequency of 100 Hz. Research should be focused on low affinity transporters in the case. Distinction between ultra-fast and slow mechanisms should also be applied to investigations on other neurotransmitter, such as glutamate or ATP, since which are more and more recognised to be also ubiquous signalling substances, able to work either at slow or at ultra-rapid speeds. Many fascinating fields remain to be explored.

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