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Historical landmarks in the histochemistry of the cholinergic synapse: perspectives for future researches

Philippe ANGLADE[1] and Yamina LARABI-GODINOT[2]

[1] 28 bis Allée Piketty, Saint-Fargeau-Ponthierry, 77310 Saint-Fargeau-Ponthierry, France and [2] Centre Alexandre Koyré, Département Hommes, Nature, Sociétés, Muséum National d'Histoire Naturelle, 57 rue Cuvier, 75005 Paris, France

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ABSTRACT

Nearly one hundred years ago, acetylcholine (ACh) was proposed as a chemical agent responsible for nerve transmission at the synapse, the junction area between one neuron and its target cell. Since it has been proved that ACh played, indeed, a major role in the functioning of the nerve system in the vertebrates, cholinergic nerve transmission became a basic field of study in neuroscience. The birth of histochemistry and its ulterior developments allowed *in situ* localization of the molecular agents related to the functioning of the cholinergic synapse. This report presents historical landmarks in the histochemistry of major cholinergic agents (acetylcholinesterase, nicotinic acetylcholine receptor, choline acetyltransferase, and ACh), a domain which has greatly contributed to the knowledge of the nerve system. It is emphasized that despite extraordinary progresses made in this field, basic problems, such as *in situ* localization of ACh, still remain to be solved.

The concept of synapse was proposed more than one hundred years ago by C.S. Sherrington that referred to a particular property of the junctional zone between neurons or between neurons and their effector cells (4, 90, 91). Since, at that time, the strength of resolution of the light microscope was much too limited, the structure of the nervous junction remained a controversial subject. In the "reticular" theory, the nerve system was considered as a whole entity made of cells amalgamated by anastomoses or diffuse networks of nerve fibrils (47). On the contrary, the "neuron" theory proposed a gathering of independent neurons interconnected by particular contacts (87). Moreover, for decades, it remained impossible to answer the alternative proposed by E. Du Bois Reymond in 1848, according which the mode of transmission of the nerve influx was either electrical or chemical (34). Nevertheless, a series of physiological works led to suppose that nerve transmission occurred by means of the release of a chemical substance from the pre-synaptic neuron toward a receptive area of the post-synaptic cell (28, 33, 40).

A decisive breakthrough was made in 1921 by the experiments of O. Loewi (70) who demonstrated the chemical nature of the nerve transmission in the frog. Loewi stimulated the vagus nerve of an isolated heart and observed the stop of the heart beats. He collected the physiological solution coming out from the heart and perfused a second isolated heart with this solution. The second heart ceased to beat without any previous stimulation of the vagus nerve. Loewi concluded that a substance was released from the vagus nerve terminals to act on the heart muscle tissue. Acetylcholine (ACh) was identified as the chemical substance acting in the experiments of Loewi (see 90). It was also recognized as the neurotransmitter of postganglionic parasympathetic fibres, preganglionic autonomic fibres, motor nerve terminals, and probably of some pathways of central

Address correspondence to: Philippe Anglade, 28 bis Allée Piketty, Saint-Fargeau-Ponthierry, 77310 Saint-Fargeau-Ponthierry, France
Tel: +33-1-01 60 65 75 15
E-mail: philippe-anglade@orange.fr

nervous system (29, 90). Thus, ACh was considered as one of the two classical neurotransmitters (the other being noradrenaline) of the peripheral nerve system of the vertebrates.

The concept of synapse received a clearly defined morphological support from the work of R. Couteaux (1947) on the myoneural synapse. Indeed, using the Janus green B dye, Couteaux observed the "subneural apparatus", a specialized membranous structure attached to the sarcoplasmic side of the "synaptic gutter" in front of the nerve terminal (19, 101). This observation revealed a discontinuity at the neuromuscular junction. It substantiated previous data showing that post-ganglionic nerve fibres were not affected by degeneration of pre-ganglionic nerve fibres (9, 30). The structure of the synapse was definitively elucidated by the first images of the nerve junctions obtained by electron microscopy, between 1954 and 1956. Indeed, these pictures showed the presence of a "synaptic" space between pre and postsynaptic cells (31, 85, 89). In the synaptic area, vesicles of 30 to 50 nm in diameter were regularly observed in the nerve terminals close to the cytoplasmic membrane of the presynaptic neuron. These synaptic vesicles offered a morphological basis to the quantal release of neurotransmitters from nerve terminals detected by electrophysiological recordings (42). Thus, synaptic vesicles were regarded as the site of the storage and the release of neurotransmitter quanta (32). These observations definitely proved that the nerve system was composed of single cells, separated from each other by a synaptic extra-cellular space in which—according to Loewi's experiments—the chemical transmitter must be released from the nerve terminal.

An essential step in the understanding of the functioning of the cholinergic synapse was the observation of quick inactivation of "the vagal substance" released after nerve stimulation (71). It was suggested that enzymatic degradation of the transmitter (supposed to be ACh) was essential in the fine regulation of nerve transmission (38). Extensive studies in the myoneural synapse demonstrated that the activity of this enzyme, acetylcholinesterase (AChE), was mainly located in the innervated zone of the muscle, outside of the nerve terminal (20, 43, 74). Thus, the histochemistry of the cholinergic synapse was born in 1949 when Koelle and Friedenwald (61) proposed acetylthiocholine, an artificial substrate of AChE, to localize AChE activity *in situ*.

Choline acetyltransferase (ChAT), the enzyme responsible for ACh synthesis by acetylation of choline, was discovered few years after AChE (81). Thus, the cholinergic synapse became an important field of research to understand the basic function of nerve transmission. This is all the more so as the neuromuscular junction, endowed with well-characterized structures and huge amounts of cholinergic agents, was particularly favourable to such studies. Thus, it is not surprising that the first "receptive substance" of a neurotransmitter to be isolated and characterized was the nicotinic acetylcholine receptor (25).

Since cholinergic synapse is a highly specialized structure, the *in situ* localization of the molecules acting as cholinergic agents was essential to understand the functioning mode and the regulation of cholinergic nerve transmission. Therefore, after the pioneering work of Koelle and Friedenwald (61), histochemistry of cholinergic synapse became a basic domain in neuroscience. In this report, landmarks in the histochemical localization of the cholinergic agents will be presented with the aim of pointing out key problems that remain to be solved.

ACETYLCHOLINESTERASE HISTOCHEMISTRY

In situ localization of AChE activity: a long chain of progress

After the introduction of sharp visualization of nerve cell bodies and processes by impregnation of metallic salts by Golgi in 1885 (46), no decisive progress in the histological methods took place during more than fifty years. Then, a new field of research, "histochemistry", was opened by G. Gomori and H. Takamatsu in 1939 (see 72, 79). It is interesting to note that Gomori and Takamatsu were working independently of each other, one in the U.S.A., the other in Japan. These two pioneers laid the basis for *in situ* localization of enzymatic activity. In one word, the product of an enzymatic reaction was precipitated by metallic ions to form insoluble metallic salts at the site of enzymatic activity (see 72, 79). The first "histochemical" staining successfully introduced in neuro-histology was the staining of AChE activity (61). This method was based on the use of acetylthiocholine, an artificial substrate of cholinesterases. Thiocholine was formed as the reaction product and precipitated at the enzymatic site by copper ions. Thus, the final precipitate of copper-thiocholine (converted in cupric sulphide, a black precipitate) revealed *in situ* the sites of AChE activity, providing careful controls of the diffusion of reaction products and of the specificity for AChE (for review see 44).

In order to limit the diffusion of the precipitates

of the histochemical reaction, Couteaux and Taxi modified Koelle's method and obtained a satisfactory localization of AChE activity in the neuromuscular junction (23). The pattern of the histochemical staining corresponded to the subneural apparatus visualized by Janus green B (19). Thus, these findings confirmed the biochemical data indicating that AChE activity was outside of the nerve terminals (20).

The method of Koelle and Friedenwald was extensively used for the localization of AChE activity in many tissues and species. Nevertheless, the final precipitate of the Koelle and Friedenwald reaction remained hardly visible under electron microscope. Lehrer and Ornstein (1959) proposed a new method for the observation of AChE activity at light and electron microscopic level (67). This "diazo coupling" method was based on the use of α-naphtyl acetate as an enzymatic substrate and hexazonium pararosanilin to form an "organic" precipitate of α-naphtol-diazonium at the enzymatic site. This ingenious technique suffered from a certain lack of specificity and artefacts (44). Nevertheless, the method of Lehrer and Ornstein allows the detection of AChE activity on tissues already processed for silver nitrate impregnation of nerve fibres (109). Moreover, the non metallic organic compounds present in the medium of the reaction of Lehrer and Ornstein remain stable during immunoperoxidase staining. Thus, this technique was used to localize simultaneously AChE activity and neuropeptide immunoreactivity in the rat myenteric plexus (3).

Karnovsky and Roots proposed, in 1964, the localization of AChE activity by a metallic precipitate visible at light and electron microscopic levels (58). The method of Karnovsky and Roots was based on the ability of thiocholine, the reaction product in the Koelle and Friedenwald method, to reduce ferricyanide into ferrocyanide. Then, ferrocyanide could precipitate with copper ions to form the insoluble salt of copper ferrocyanide (Hatchett's brown). This method allowed reliable observation of AChE activity at ultra-structural level (44). Alternatives have been proposed for the localization of AChE activity such as the use of thiolacetic acid as substrate of the enzyme (for review see 44). However, obtaining the precipitate of Karnovsky and Roots has become the most common way to detect AChE activity. Among the derivatives of the Karnovsky and Roots method, it is worth noting the generation of an osmiophilic polymer from the 3,3'-diaminobenzidine (DAB) monomer by the oxidative power of copper ferrocyanide (48). The method of Hanker permitted to shorten the incubation time of the enzymatic reaction and, thus to reduce the artifactual diffusion of the precipitates (48).

Despite the substantial progress made in the accuracy of the localization of AChE activity from the original Koelle and Friedenwald method, histochemistry of AChE steadily suffered from artifactual deposition of precipitates (see 44). In order to overcome this problem, S. Tsuji, a pupil of Couteaux, undertook a series of chemical analyses of the reactions devised by Koelle and Friedenwald and by Karnovsky and Roots. The work of Tsuji brought out basic data on the reaction mechanisms actually taking place in the histochemical medium. Tsuji demonstrated that the reaction of Koelle and Friedenwald did not occur when iodide was replaced by another halogen anion, such as chloride, and that the histochemical precipitate actually was cuprous thiocholine *iodide* (see 98). Moreover, analyzing the medium of Karnovsky and Roots, he showed the unexpected presence of the precipitate taking place in the Koelle and Friedenwald reaction, in addition to cupric ferrocyanide ($Cu^{++}_2\ Fe^{++}(CN)_6$), the well-known precipitate of Karnovsky and Roots. From these data, Tsuji found that cuprous thiocholine iodide reacted with $Fe^{+++}\ (CN)_6$ to form $Cu^{+}_3Fe^{+++}(CN)_6$ in the medium of Karnovsky. Thus, the reaction of Karnovsky and Roots actually resulted in the formation of two precipitates, $Cu^{++}_2Fe^{++}\ (CN)_6$ and $Cu^{+}_3Fe^{+++}(CN)_6$ (98). A further analysis revealed that these two products may be transformed in each other by alternating intra-molecular oxide-reduction (104). The theoretical soundness of this chemical analysis of the histochemical reactions did not markedly improve, at first, the accuracy of the localization of AChE activity. Nevertheless, AChE activity was localized in the synaptic cleft of the neuromuscular junction (98). A few years after, in 1978, McMahan *et al.* demonstrated that AChE activity was associated with the basal lamina of the myoneural synapse (73). This definitely showed that the subneural apparatus observed in 1947 by Janus green B (19) and in 1952 by AChE histochemistry (23) corresponded to the folds of the basal lamina of the sarcoplasmic membrane.

The visualization of AChE activity was significantly improved when H. Tago *et al.* proposed in 1986 a new method based on the peroxidase-like activity of the metallic precipitates (Hatchett's brown) formed in the reaction of Karnovsky and Roots (95). Tago's staining is based on the oxidation of DAB by O_2 liberated from H_2O_2 by the peroxidase-like activity of Hatchett's brown. This technique benefit-

ed from the strong catalytic activity of $Cu^{++}{}_2Fe^{++}(CN)_6$ and $Cu^{+}{}_3Fe^{+++}(CN)_6$ and of the high staining density of oxidized DAB. Therefore, Tago *et al.* obtained a sharp and intense localization of AChE in various areas of the rat brain using only a diluted medium of Karnovsky and Roots. This technique improved the accuracy of visualization of AChE activity. It became an adequate tool to detect AChE activity, especially in the central nervous system where the huge amounts of nerve elements had previously remained a puzzling question for histochemical detection of AChE. Tsuji further investigated the chemical basis of the catalytic activity of Hatchett's brown used in Tago's method (100). He showed that the peroxidase-like activity of the precipitates of Karnovsky and Roots obtained in Tago's reaction corresponded to their oxidase-like activity previously demonstrated by Hanker *et al.* (48). Slightly modifying Tago's method, Tsuji obtained in the rat nigral neurons a localization of AChE activity in the intracellular organites with a high resolution (100) (Fig. 1a and b).

New perspectives for AChE histochemistry

AChE activity was localized in the cell bodies of various types of non cholinergic neurons (93, 96). This suggested that these neurons, though they did not synthesize ACh, were endowed with ACh receptors. For that reason, they were called "cholinoceptive" neurons. Since histochemical staining of AChE activity did not allow the unequivocal identification of the cholinergic neurons, AChE histochemistry felt in disrepute during a while. However, the localization of AChE activity recovered primal importance when were established the non cholinergic functions of cholinesterases in basic phenomena, such as cell-cell interaction, cell division, cell differentiation and neural development (see 8). A series of investigations confirmed this new field of research, such as evidence for dendritic release of AChE in the rat substantia nigra (2), morphogenic role for AChE in axonal outgrowth (12), induction of AChE during apoptosis (114). The discovery of the role of AChE in the pathogenesis of Alzheimer disease could be of primal therapeutic importance. Until now, Alzheimer disease is most commonly treated by AChE inhibitors in order to compensate for the symptoms due to the loss of cholinergic neurons (see 88). However, AChE seems to play a major, non cholinergic, role in the pathogenesis of the disease, by favouring the aggregation of beta-amyloid peptide and modulating tau-phosphorylation (18, 66, 92). Therefore, the proposition of new AChE inhibitors specifically targeting beta-amyloid deposition and abnormal tau-phosphorylation, may lead to improvement in the cure of Alzheimer disease (18, 66, 92). Recently devised methods may be useful in this domain, such as the use of backscattered electron imaging and X-ray mapping to observe extra-neuronal AChE activity in fine neural networks (108).

AChE is the intriguing well-known molecule

Following the purification and the crystallization of AChE from the electric organ of electric eel by Leuzinger *et al.* in 1968 (68), an antiserum raised against electric eel AChE allowed the first immunocytochemical localization of AChE (10). At the same time, the polymorphic structure of AChE was discovered by Massoulié *et al.* (77) and extensively studied for several decades (for review see 75, 76). Since AChE is expressed as a set of molecular forms of different quaternary structure and mode of anchoring (see 37), a wide range of antibodies have been raised, in many species, against monomers, dimers, and tetramers that participate to the whole structure of the protein. Owing to the success of histochemical detection of AChE activity, immunocytochemical localization of AChE was only scarcely used by neuro-anatomists. Nevertheless, immunocytochemical investigations have recently suggested that AChE might play a cholinergic role (*i.e.* role in the functioning of the cholinergic system) other than quick degradation of ACh during nerve transmission. Indeed, an immunocytochemical staining of rat nigral neurons revealed AChE in structures identified as dendritic spinules (54). Since dendritic spinules are associated with synaptic plasticity (41) and metabolic exchange with glial processes (6), these data could point towards the putative contribution of AChE to the remodelling of cholinergic synapses.

CONTRIBUTION OF HISTOCHEMISTRY TO THE STUDY OF NICOTINIC ACETYLCHOLINE RECEPTOR

At the beginning of the 20th century, in Cambridge, T.R. Elliott, a student of the physiologist J.N. Langley, observed that adrenaline had an effect similar to sympathetic nerve stimulation. From his finding, Elliott clearly introduced the concept of chemical receptors in these terms: "The point at which the stimulus of the chemical excitant is received, and transformed into what may cause the change of tension of the muscle fibre, is perhaps a mechanism developed out of the muscle cell in response to its union with the synapsing sympathetic

fibre, the function of which is to receive and transform the nervous impulse." (40). At the same time, in Germany, P. Ehrlich proposed the "side chain theory", in which he introduced the notion of chemoreceptors, cell constituents on which bind specific drugs (39). However, the receptor of a neurotransmitter remained a concept until the characterization of the nicotinic ACh receptor (nAChR) was performed in the laboratory of J.P. Changeux. It is, thus, interesting to recall that it had been suggested that AChE might be the putative receptor for ACh before it was established that they were really two different molecules (26).

In 1970, the isolation of postsynaptic membrane fractions of the electric organ of an electric eel allowed Changeux's group to obtain closed vesicles excitable by cholinergic agonists (60). Then, C.Y. Lee, coming from Taiwan, gave the opportunity to Changeux and his collaborators to use an irreversible blocking agent of nAChR, the α-bungarotoxin, a polypeptide purified from the Cobra snake. By means of the snake neurotoxin and immune serum directed against the toxin, nAChRs were characterized (25) and localized on the electroplax, the modified postsynaptic membrane of the electric fish (13). A fluorescent dye conjugated to α-bungarotoxin was used to localize nAChR in the vertebrate skeletal muscle (1). Further investigations enabled a large-scale purification of nAChR from Torpedo electric organ (94). Electron microscopic observations revealed the pentameric structure of nAChR endowed with a hole at the centre of the molecule (17). Using molecular biological techniques, the group of S. Numa revealed the primary structure of nAChR (82). The nAChR protein contains a binding site for ACh and a cationic channel. The opening of the ion channel is coupled with the binding of ACh (for review see 24, 111). The structure of nAChR has been recently analysed at high resolution (112).

Despite the extensive study of nAChR by cellular and molecular biology, almost unexplored problems remain. Clustering of nAChRs in the postsynaptic membrane might play an important role in the regulation of the nerve transmission at the neuromuscular junction (see 62). The receptor associated protein RAPsyn (see 49) and cytoskeleton molecules are probably involved in the interactions between sarcoplasmic membrane and cytoskeleton (62). Thus, the trans-membranous and cytomatrix-related part of nAChR was visualized under electron microscope, using the immunochemical avidity of immunoglobulin G for d-tubocurarine, a quaternary ammonium compound binding to nAChR (49). By this method, it was observed intra-cytoplasmic extensions associated to the nAChR-like profiles. Since these filamentous structures overlengthed the nAChR-RAPsyn complex, they might include putative cytoskeletal proteins related to the receptor complex. Moreover, intra-sarcoplasmic internalization of nAChR induced by α-bungarotoxin has been recently demonstrated by fluorescent dye conjugated to the complex α-bungarotoxin-nAChR (65). Thus, endocytosis of nAChR might be involved in the modulation of the nerve transmission at the neuromuscular junction and in disorders of the central nervous system.

LOCALIZATION OF CHOLINE ACETYLTRANSFERASE

The enzyme of ACh synthesis, ChAT, has been evidenced as early as 1943 by Nachmansohn and Machado who succeeded in the first enzymatic acetylation of choline. It was further demonstrated that ChAT catalyzed the synthesis of ACh from the substrates choline and acetyl coenzyme A (69). Twenty years later, *in situ* localization of ChAT activity was proposed by using lead salts to form a lead mercaptide precipitate with co-enzyme A, a product of the enzymatic reaction (16, 59). However, these methods recurrently suffered from a lack of specificity for ChAT (see 53).

The efforts towards the puzzling localization of ChAT activity ceased when reliable ChAT antibodies were proposed for immunocytochemical detection of the protein. ChAT catalyses ACh synthesis in the nerve terminals from acetyl coenzyme A and from choline produced in the synaptic cleft after ACh degradation and taken up by high-affinity transporters of the presynaptic membrane (see 64). Thus, the localization of ChAT in the nerve endings is an essential criterion to identify the cholinergic synapses.

Cholinergic neurons and pathways of the central nervous system were stained by specific ChAT antibodies and immunoperoxidasic or immunofluorescent procedures, in many species (for review see 51, 83). Comparisons between the staining of AChE activity and ChAT immunoreactivity showed that numerous neuronal cell bodies with AChE activity were devoid of ChAT immunostaining. This confirmed the already established idea of the presence of non cholinergic "cholinoceptive" neurons. New perspectives were opened by ChAT immunocytochemistry when ChAT was localized in vascular endothelial cells of rat brain (86). These data demonstrated that ACh was synthesized and stored

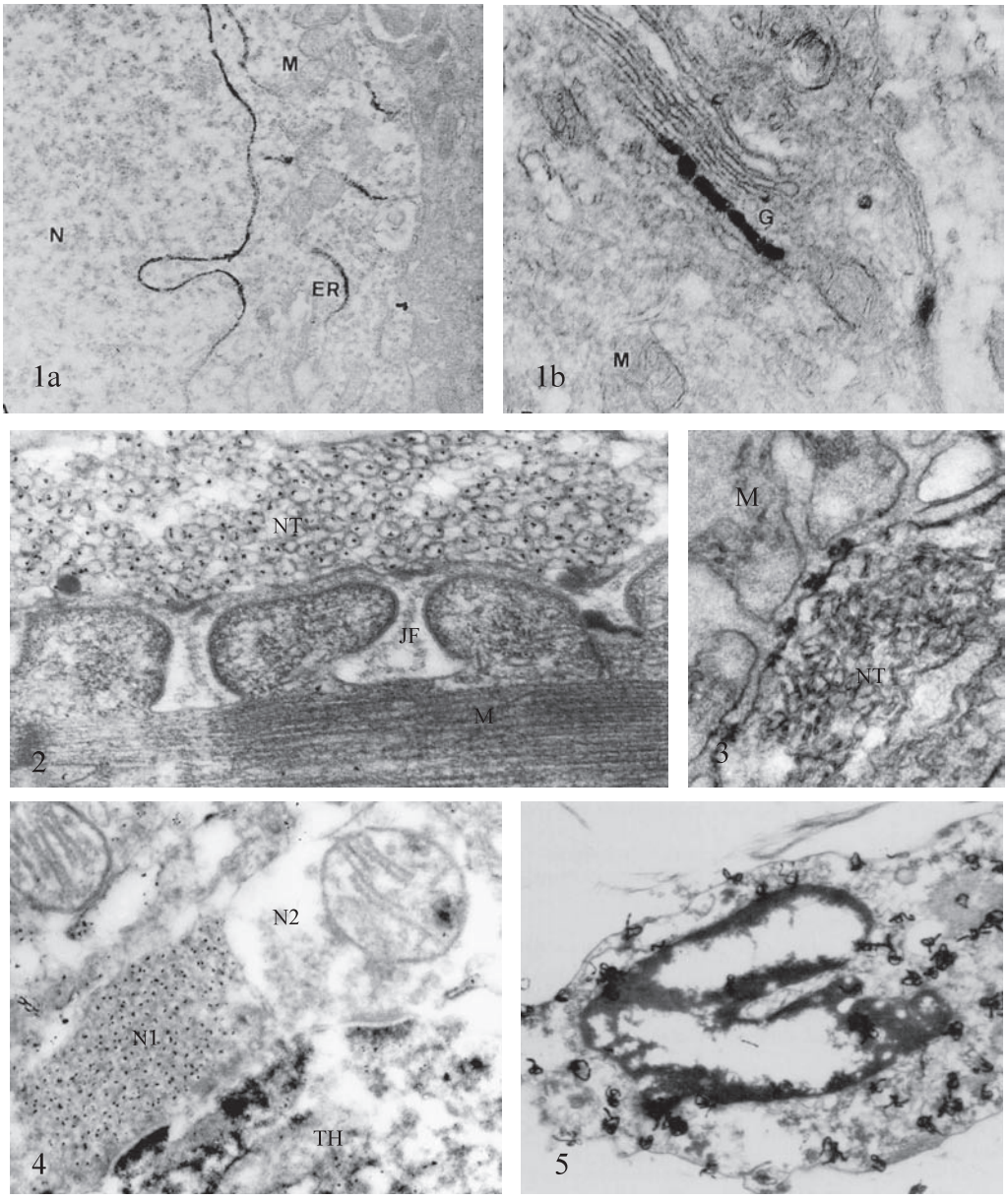

Fig. 1 AChE activity in a dopaminergic neuron of the rat substantia nigra observed at electron microscopic level by the method of Tago performed without metallic impregnation. 1a: The dark precipitates of DAB oxidized by cupric ferrocyanide are finely localized in the endoplasmic reticulum (ER) and in the nuclear envelop. M: mitochondria, N: nucleus. (× 15,000) 1b: AChE activity is visible in the cisternae of the Golgi apparatus (G). M: mitochondria. (× 51,000), (reproduced from the figures of ref. 100).

Fig. 2 Ultrastructural localization of acetylcholine-like cations in frog neuromuscular junction at 25°C (resting state). Point-like precipitates of acetylcholine-like cations are observed in the synaptic vesicles of the nerve terminal (NT). JF: junctional fold of the synaptic cleft, M: muscle cell. (× 55,000)

Fig. 3 Ultrastructural localization of acetylcholine-like cations in frog neuromuscular junction at 0°C. The diffusion of acetylcholine-like cations in the synaptic cleft is reduced at low temperature, which enables the visualization of pairs of diffuse precipitates beneath the sites of the active zone of the nerve terminal (NT). M: muscle cell. (× 65,000)

Fig. 4 Ionic fixation of acetylcholine-like cations after tyrosine hydroxylase (TH) immunostaining in the substantia nigra of post-mortem human brain. Two nerve endings (N1 and N2) are observed in synaptic contact with a TH-immunoreactive dendritic process of a dopaminergic neuron (TH) stained by dark DAB precipitates. Point-like precipitates of acetylcholine-like cations are present in the synaptic vesicles of N1 and absent in the synaptic vesicles of N2. (× 46,000)

Fig. 5 Ionic fixation applied to frog neuromuscular junction after loading with [^{3}H] choline and brief tetanic stimulation. The cytoplasm of a fibrocyte situated in the vicinity of the neuromuscular junction is intensely labelled with silver grains whereas the nucleus is almost devoid of autoradiographic label. (× 11,000), (reproduced from the figures of ref. 106).

in the endothelial cells of small blood vessels. This suggested that ACh might play, through local vasodilatation, a protective role against the hypoxia related to ischaemia.

Amino acid sequence of ChAT was elucidated after the isolation of the complete sequence of a cDNA encoding porcine and rat ChAT (11, 14). This allowed the subsequent *in situ* hybridization studies that have revealed the expression of ChAT mRNAs in neurons of the central nervous system (83, 97). However, the putative localization of ChAT mRNAs in the nerve terminals has not, so far, been analyzed by *in situ* hybridization. Beyond a difficult technical tuning, the cause might be that ChAT mRNAs are mostly located in the neuronal cell bodies and not in the synaptic areas.

The knowledge of cholinergic systems was greatly improved by the development of ChAT immunocytochemistry in the central nervous system. However, for a while, reliable specific neuronal staining by ChAT antibodies could not be satisfactorily obtained in the peripheral nervous system (see 113). Thus, immunocytochemical localization of the high-affinity choline transporter was proposed as a specific detection of the cholinergic nerve endings in the heart (50). An answer to this problem came when it was evidenced that a variant of ChAT mRNA coded an isoform of the ChAT protein which was preferentially expressed in the peripheral nervous tissue (see 113). This ChAT isoform was called "peripheral" type by opposition to the well-known "common" type already localized in the central nervous system. Immunocytochemical investigation showed that the common and the peripheral types of ChAT were differentially expressed in subclasses of enteric neurons (27). Moreover, these two types of ChAT follow different intracellular translocation (78). They were recently localized in the nerve cells and fibres of the rat heart (113). These data raise the important question whether ChAT might play different physiological roles through its different isoforms.

HISTOCHEMISTRY OF ACETYLCHOLINE

The precursory works of R. Hunt pointed out the major role of ACh in the organism functioning. Hunt found that extracts of suprarenal glands free from adrenaline lowered the blood pressure. At first, choline was suspected to be responsible for this effect. However, the extensive analysis published by Hunt and Taveau, in 1906, showed that ACh had a much more potent lowering action on blood pressure than choline (52). In 1914, Dale observed that ACh could reproduce the effect of parasympathetic nerve stimulation. He, then, supposed that ACh might be the putative substance released from parasympathetic nerves (28). This was confirmed more than 20 years after, when ACh was shown to be released not only from parasympathetic nerves but also from preganglionic sympathetic fibres and motor nerve endings (29). Thus emerged the principle (called Dale's principle) that noradrenaline and ACh were, respectively, responsible for the complementary and antagonistic effect of the sympathetic and parasympathetic nerves. At that time, ACh was also considered as a probable agent of the chemical nerve transmission taking place in the central nervous system. However, probably owing to its quick hydrolysis and its problematic *in situ* fixation, ACh could not be satisfactorily detected and localized in the tissues.

The key problem of *in situ* localization of ACh remained out of the investigations until the pioneering studies of Tsuji initiated around 1980. The pupil of Couteaux understood that molybdic and tungstic heteropolyanions might precipitate ACh *in situ*, favouring their stability by suitable experimental conditions. Thus, neuromuscular junction of the frog cutaneous pectoris muscle was treated by phosphomolybdic or silicotungstic acid (STA), at low pH, and processed for ultrastructural observation. In that case, point-like precipitates were regularly observed in the synaptic vesicles of the cholinergic nerve terminals, whereas only background staining was seen in the cells of muscular and connective tissue (102, 105) (Fig. 2). Tsuji interpreted the point-like staining as vesicular ACh precipitated *in situ* by STA. He called this method "ionic fixation of ACh-like cations" since STA has the property to precipitate quaternary ammoniums, such as choline or ACh, after ionic interaction.

Ionic fixation was applied at low temperature (0 to 4C°) in resting and excited frog neuromuscular junction in order to reduce the diffusion of ACh-like cations in the synaptic cleft. Tsuji obtained, in these conditions, spot-like precipitates in the synaptic space just at the level of the "active zone" described by Couteaux and Pécot-Dechavassine (21, 22) (Fig. 3). These images displayed twin spots at the supposed sites of the double rows of exocytotic vesicles, and laminar precipitates extending in the folds of the subneural apparatus (99). These data substantiated the concept of the active zone of the nerve terminals. Indeed, ionic fixation—at low temperature or during nerve stimulation—evidenced that in the

neuromuscular junction an *intense activity* was related to the structures of the active zone. However, the stringent treatments of the cellular tissues (low temperature, nerve stimulation, low pH of ionic fixation) caused poor preservation of the ultra-structures and some of the precipitates were rather questionable.

At the same time was proposed a hopeful devise of immunocytochemical localization of ACh (45). Indeed, an antibody was specifically raised against ACh conjugated to an immunogenic molecule. The antibody bound *in situ* was detected by immunoperoxidase procedure. However, reliable staining could not be obtained from this method. This encouraged Tsuji to pursue his research on the histochemical localization of ACh. Thus, Tsuji and Ohoka gauged the specificity of the ionic fixation for ACh by *in vitro* experimental models and ultrastructural observation of cholinergic and non cholinergic nerve terminals in the frog heart. They evidenced a relative specificity of ionic fixation by STA for ACh compared to the other neurotransmitters (84): only ACh, choline, and to a less extent, serotonine can be precipitated by STA in the range of the concentrations found in the cellular tissues. Moreover, electron microscopic studies revealed punctiform precipitates in small clear vesicles of presumed cholinergic terminals, whereas they were not found in the large dense-cored vesicles of the presumed noradrenergic varicosities. However, soluble proteins were precipitated at low concentrations by STA. This remained a question, although the pattern of point-like staining in the synaptic vesicles corresponded to the distribution of the cholinergic vesicles.

Further works permitted to precise both the limits and the relevance of ionic fixation for a specific detection of cholinergic nerve terminals. By using this method, a plasticity of the nerve afferents to nigrostriatal dopaminergic neurons was evidenced in Parkinson's disease, although the cholinergic nature of all the stained terminals could not be assessed (5). Moreover, ionic fixation seemed more specific for ACh in peripheral nervous system (84, 105) than in the brain where the concentrations of neurotransmitters are the highest. Since, until now, ionic fixation by STA is the one and only method allowing *in situ* insolubilization of ACh, further works must be performed in order to unequivocally characterize the compounds of the precipitates observed under electron microscope.

The soundness of this method for future researches came from its successful application to choline autoradiography (see 7). Indeed, STA enables to limit the diffusion of [^{3}H] choline captured by cholinergic neurons and allows an intense and reliable autoradiographic labelling of cholinergic pathways (107). This is all the more interesting as ionic fixation is compatible with immunocytochemical reaction (5, 107) (Fig. 4). In the same way, an important capture of [^{3}H] choline was evidenced in fibrocytes surrounding the frog motor end plates (106) (Fig. 5)

Using the strong power of STA to precipitate the quaternary ammonium compounds, a binding avidity of immunoglobulin G for ACh was evidenced on nitrocellulose membrane (110). This may be a clue of a putative property of immunoglobulin G to bind ACh, perhaps related to the phenomena of autoimmunity against ACh.

PERSPECTIVES

Histochemistry of the cholinergic synapse (see Fig. 6) is already a long story which greatly contributed to the extraordinary progress of neurobiology in the last sixty years. As reviewed here, new questions emerged from the extensive studies made in this domain. One of which is related to the old problem of chemical transmission of the nerve impulse. Indeed, the characterization of nAChR and the already advanced deciphering of its functioning and regulation brought a rather clear figure of the "receptive domain" of the synapse, suggested more than one hundred years ago. However, the mode of release of the neurotransmitter ACh from the nerve terminal remains to be established.

As mentioned above, as soon as they were observed under electron microscope, the synaptic vesicles close to the pre-synaptic membrane were considered as the morphological basis of the quantal release of ACh from the nerve terminals (32). It was proposed that one vesicle was the site of storage and release of a single quantum of ACh, and thus responsible for the minimum amplitude of the endplate potential (the miniature endplate potential) recorded at the resting neuromuscular junction (42). This concept, called vesicular theory of ACh release, became a basic notion in neuroscience. The vesicular theory was corrected, but not questioned in its principle, by the discovery of subunits of miniature endplate potentials (63). Thus, Motelica-Heino and Wernig (80) proposed the hypothesis of one vesicle corresponding to one "sub-miniature" endplate potential and one active zone to the miniature endplate potential described by Fatt and Katz in 1952 (42). However, though the quantal release of ACh has been universally recognized, a series of researches

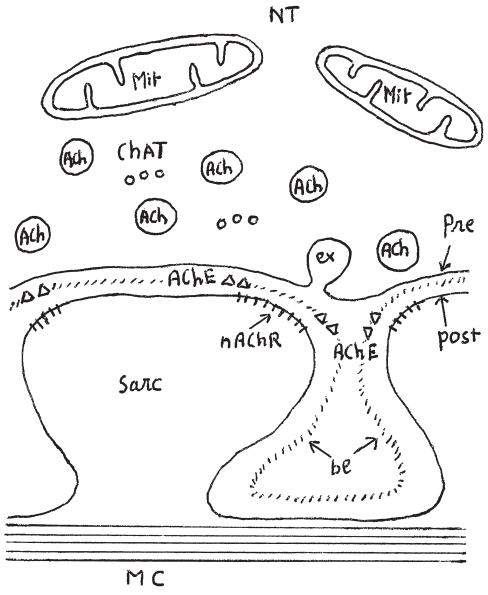

Fig. 6 Schematic drawing of the ultrastructure of a cholinergic synapse at the neuromuscular junction. ACh: acetylcholine in the synaptic vesicles, AChE: acetylcholinesterase (represented by small triangles), bl: basal lamina in the synaptic cleft, ChAT: choline acetyltransferase (represented by small circles), ex: exocytotic vesicle at the active zone, MC: muscle cell, Mit: mitochondria, nAChR: nicotinic acetylcholine receptors (represented by small rods), NT: nerve terminal, pre: presynaptic membrane, post: postsynaptic membrane, sarc: sarcoplasma.

have questioned the vesicular theory of neurotransmitter release.

Indeed, a "non vesicular" theory was proposed in 1979 by Israël *et al.* (57), based on the intriguing data showing that ACh newly synthesized in the cytoplasm was released before vesicle-bound ACh (36). A series of extensive studies were then performed on synaptosomes of Torpedo electric organ by the groups of M. Israël and Y. Dunant. They resulted in the purification of a membrane protein, called mediatophore, which can translocate ACh upon calcium activation (for review see 56). A plasmid encoding for mediatophore was introduced in cells deficient for ACh release, for ChAT and vesicular ACh-transporter genes. A quantal release of ACh evoked by calcium influx was observed in the cells transfected by the plasmid (56). Since the cells, previously loaded with ACh, were unable to synthesize and transport the neurotransmitter in vesicles, the quantal release of ACh was attributed to mediatophores concentrated in the cytoplasmic membrane (56). Moreover, the putative key role of mediatophore in ACh release was confirmed *in situ* by ultrastructural studies, such as immunocytochemical labelling of the mediatophore on the presynaptic membrane of the Torpedo electric organ (15) and freeze-fracture analysis of quick changes in the presynaptic intramembrane particles related to ACh release and exocytosis (35). From these whole data, a unifying hypothesis has been proposed for ACh release: the synaptic vesicles might control the micro-changes in the flux and the concentrations of calcium which would synchronize the quantal release of ACh through mediatophore—the smallest quantal unit of released ACh corresponding then to ACh translocated by one molecule of mediatophore (55). To progress in this basic question on the functioning of the cholinergic synapses, histochemistry might be helpful. Among other possibilities, the unequivocal identification of ACh after *in situ* precipitation by tungstic or molybdic heteropolyanions, would allow to know whether the neurotransmitter is present, or not, in the exocytotic synaptic vesicles of the cholinergic nerve terminals.

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