

Chemical transmission and the cholinergic synaptic vesicles

Introduction

The object of this article is to give some a glimpse of the discovery of cholinergic transmission. At the start of the 20th century there was little knowledge on chemical transmitters and it was really not until 1950 that chemical transmission was generally accepted. During the 1950s improvement in physiological, morphological and chemical techniques allowed one to visualize, detect and isolate the synaptic vesicles. But it took a long time and intense discussion before it was generally accepted that they were involved in the release of transmitters. In this paper some, but not all, of the important progress is outlined. I have concentrated on cholinergic transmission since that has been most closely linked to the “quanta” and vesicular release. It is not my intention to write an extensive review, but to shed some light on some of the important and controversial findings.

I have not dealt with the impressive work done on vesicular protein and vesicular transporters during in the last 20 years. But that may be another story as Kipling said.

The forefathers

It started back in 1877 when E. Du Bois Raymond suggested that “of known natural processes that might pass on excitation, only two are in my mind worth talking about: either there exists at the boundary of the contractile substances a stimulatory secretion in the form of a thin layer of ammonia, lactic acid or some other powerful stimulatory substance or the phenomenon is electrical in nature.” During the first part of the 20th century as will be seen later, there was a vigorous debate regarding the two alternatives.

In 1901 it was known that adrenaline, crystallized from an extract of adrenal glands, reproduced the effects of sympathetic stimulation. In a note to the Physiological society T.R. Elliot, a young research fellow, wrote “Adrenaline might be the chemical stimulant liberated on each occasion when the impulse arrives at the periphery.” J.N. Langley, particularly known for his studies on receptors for nicotine, wrote in 1906 “And this seems in its turn to require that the nerve impulse should not pass from nerve to muscle by an electric discharge, but by secretion of a special substance at the end of the nerve.” These were some of the first statements supporting chemical transmission. At the time, however, many physiologists and pharmacologists believed that the adrenaline having been released originated from the responding muscle.

Is acetylcholine a neurotransmitter?

The important progress in cholinergic research came with the identification of effects of “Vagusstoff.” In his autobiographic sketch O. Loewi tells the story of how he discovered the “Vagusstoff.” in 1920. He woke up the night before easter Sunday, jotted down a few lines on a paper and went back to sleep again. The next morning it occurred to him that something important had been written down, but it was unreadable. The next night, at three o’clock he woke up again and the idea returned. He immediately got up, went to the laboratory and performed a simple experiment on the frog heart. Two frog hearts were attached and filled with Ringer solution, the vagus nerve of the first heart was stimulated and the Ringer solution passed on to the second heart without stimulation. The second heart reacted as if it was stimulated with the vagus nerve.” The Vagusstoff was later identified as acetylcholine (ACh) and it is surprising that it was preserved in the solution without an acetylcholinesterase (AChE) inhibitor present, in high enough concentration to give a response. But it could be the long stimulation (for minutes), the low temperature and the low AChE activity of the frog muscle which allowed acetylcholine to be recovered in the Ringer solution. O. Loewi enjoyed a good story and there are some doubts about the dates in the story, but everybody accepts the facts. He repeated the experiment several times and demonstrated it also at the International Congress of Physiology in Stockholm 1926.

But ACh was not yet accepted as a neurotransmitter. Three tools became important for further studies on ACh during the 1930-1950 : 1, the use of eserine, an important AChE inhibitor; 2, the dorsal muscle of the leach for detection of small amounts of ACh (Minz, 1932); and 3, the less sensitive, but more stable rectus abdominis muscle of the frog, for detection of ACh. It was the subsequent work by H.H. Dale, W. Feldberg and D.L. Brown that substantiated the role of ACh as a transmitter of the peripheral nervous tissue. Dale also separated the effects of acetylcholine into muscarinic and nicotinic effects. W. Feldberg as a refuge from Nazi-Germany worked in Dale’s laboratory and had brought with him the dorsal leach muscle method for detection of ACh. Stimulation of the cholinergic input in the presence of eserine allowed one to detect a large increase in released ACh. In Berlin he had shown that stimulation of the vagus nerve produced ACh that was only detectable in the presence of eserine (Feldberg and Krayer, 1933). Later, Feldberg working with Dale showed that on vagal stimulation ACh appeared in the blood of the stomach of the dog (1934). They later established that ACh was released from several different peripheral synapses. H.H. Dale and O. Loewi received the Nobel Price in 1936 for their work. P.R. Lewis told me as an example

of transmitter work at the time that two neurochemists after a heavy game of tennis decided to acid-boil their socks to try to find the transmitter of sweating. There is, however, no record of this courageous experiment in the literature!

Conversion of electrical to chemical transmission

There were several opponents, particularly physiologists, to chemical transmission in the late 30s and 40s, and the most significant of them were John C. Eccles. Although H. Dale and JC Eccles were opponents, they maintained a good personal relationship. The conversion of electrical to chemical transmission is well told by Eccles himself (Eccles, 1976). Eccles main objection seems to be that transmission was too fast, both in ganglions as well as for the neuromuscular junction, to be chemical and therefore had to be electrical. He also argued that eserine had an effect directly on neurotransmission. The results fitted his conclusion on electrical transmission when he used extracellular recordings. Work, however, with B. Katz and S. Kuffler (1942) on the neuromuscular junction, they demonstrated the endplate potential and showed that eserine prolonged the time course of the endplate potential. This partly converted him to chemical transmission. However, in sympathetic ganglion he did not find the same effect of eserine on single or two impulses, but only on tetanic stimulation (Eccles 1944). This is due to the fact that AChE here is not localized to the same extent in the synaptic cleft and diffusion of ACh is more important. But in 1949 he had accepted that in the peripheral nervous system transmission was chemical, but there were still problems with synapses in the brain.

With intracellular recording well established, Eccles went on to show that inhibitory synapses in CNS was also chemically mediated and assumed that this was the case also with excitatory synapses in the brain. "Since the experimental evidence has falsified the Golgi-cell hypothesis of inhibition and left the chemical transmitter hypothesis as the only likely explanation, it suggests further that excitatory synaptic action is also mediated by a chemical transmitter" (Brook et al, 1952). He immediately reported this back to H. Dale who congratulated him on his excellent report and added slightly ironically: "Your new-found enthusiasm is certainly not going to cause us any embarrassment." Dale later wrote that the conversion of Eccles to chemical transmission in the spinal cord was like the conversion of Saul on the road to Damascus "when the light shone and the scale fall from his eyes" (Eccles, 1976). (Eccles did, however, not change his name; author's comments). John Eccles got the Nobel Price in 1963 for his contribution to chemical neurotransmission.

The discovery of quanta of ACh and isolation of the synaptosome

The three techniques that were important for the observation of quanta of acetylcholine and the hunt for the possible correlation between quanta and the synaptic vesicles were: intracellular registration, electron microscopy and subcellular fractionation.

In 1952 Fatt and Katz found something quite unexpected. The endplate region was not at rest but displayed electrical activity in the form of spontaneous subthreshold activities at an apparent normal synapse. They were about 1/100 of a normal endplate response to a motor nerve impulse. They suggested that the miniature endplate potential (MINEPs) were packages of acetylcholine released in some way from the synaptic membranes. The response was increased in size and duration by an acetylcholinesterase inhibitor, prostigmine, and blocked by curare (blocker of ACh receptor) showing that in effect the compound could be ACh (Fatt and Katz, 1952). Del Castillo and Katz (1954) expanded this further and also suggested that release of ACh occurred at specific sites where there was a calcium-dependent lock and key relationship between the two membranes. Interestingly, Fatt and Katz (1952) found a large increase in discharge of MINEPs when the muscle was exposed to a 50% increase of the osmotic pressure. A similar technique is used presently to increase synaptic release, possibly restricted to vesicles localised close to the presynaptic membrane (Rosemund and Stevens, 1996). For his discovery B Katz was awarded the Nobel Prize in 1970.

Soon after the electron microscope became available, several scientists described round granular structures 200-400 Å to be present inside the nerve terminals and some of them close to the synaptic gap. This first description of the synaptic vesicles occurred in a series of abstracts (De Robertis and Bennet, 1954; Palade 1954; Palay 1954; Engstrom and Sjöstrand 1954). It will not be fair to claim one of them as the discoverer.

At this time subcellular fractionation had become an important tool in cell biology. De Duve had isolated lysosomes, and Blaschko et al (1955) and Hillarp et al (1955) had isolated the adrenaline rich chromaffin granules. Four years after the observation of vesicles, Hebb and Whittaker (1958) at Babraham, Cambridge, isolated a particle containing ACh and its synthesising enzyme choline acetyltransferase. They used differential centrifugation to separate particles according to size and density gradient centrifugation to separate the large so called mitochondrial fraction into 3 sub fractions. At the time using a poor electron

microscope, Whittaker (1959) saw lumps of vesicles in the fraction. But the fraction had too low sedimentation rate to contain isolated synaptic vesicles. Later, after fixation under iso-osmotic conditions, electron microscopy showed particles with intact membranes with vesicles (Gray and Whittaker, 1962). V.P. Whittaker tells: I shall never forget the day march 30th of 1960 when I met George Gray at Cambridge station and he said as we walked to my car: You have isolated vesicles, Victor, but inside pinched off nerve terminals.” The paper was submitted to the Journal of Physiology, but rejected for not being physiological! This depressed the authors and it took some time before they submitted it to it to the Journal of Anatomy, London. It is one of the most cited articles of that journal (Gray and Whittaker, 1981). Interestingly, it may come as a comfort to readers and a warning to editors, that an analysis has shown that of 100 highly cited articles, 10 had problems with getting the article published. At the same time De Robertis and associates (1962a) also published a similar finding using similar technique in Journal of Neurochemistry. This started a race and heated discussion, which lasted for many years, between the two laboratories separated by the Atlantic Ocean. To my mind this tended to keep the isolated fractions “clean” and the results reliable.

One Saturday morning during a leisurely bath, Whittaker named these detached nerve terminals “synaptosomes”. This word has entered the Oxford English Dictionary and all languages in which neurobiological research is published. I hereby recommend a bath to all biochemist that need to find a suitable name for their protein or particle.

The race for the isolation of the synaptic vesicles

In his 1959 paper, Whittaker had shown that occluded ACh could be released after treating the particles with hypo-osmotic shock. It is therefore not surprising that both De Robertis and Whittaker in between 1962-64 reported the isolation of synaptic vesicles by hypo-osmotic shock. After hypo-osmotic shock of the synaptosomes, De Robertis used differential centrifugation to isolate particles sedimenting at 20000g x 20mins and recovered from rat brain both acetylcholine and choline acetyltransferase in his vesicular fraction (De Robertis et al., 1962b, 1963). On the other hand Whittaker (1963, 1964) with density gradient centrifugation of hypotonically treated synaptosomes from guinea pig brain obtained only acetylcholine in his vesicle fraction and choline acetyltransferase in the cytoplasm. As expected the location of choline acetyltransferase became the focus of a heated discussion. R. McCaman (1965) working as a visitor in De Robertis laboratory suggested that species

differences (rat vs guinea pig) could explain the different results. He found that choline acetyltransferase from rat was bound to vesicles and that the enzyme from guinea pig was more soluble. It seemed odd that such a fundamental property as storage and synthesis of acetylcholine should be different in the two species. Fonnum (1967 and 1968), in Whittaker's laboratory, suggested that the change in pH and ionic strength, as a consequence of the hypo-osmotic shock, could explain an artificial binding of the enzyme to membranes. Also the enzyme was bound to all membranes not specifically to vesicles. He was able to bind and release the enzyme from membranes by changing pH and ionic strength. In agreement the species differences found by McCaman could be explained from the different external cationic charge of choline acetyltransferase from different species (Fonnum, 1970). The conclusion was that Whittaker's vesicles were more pure.

The sequence of events should be that choline was taken up into the synaptosomes by a high affinity procedure (Marchbanks, 1968b), acetylated by choline acetyltransferase and the product ACh taken up into vesicles. At this time there was no clear evidence of uptake of ACh into the vesicles in spite of several different careful approaches (Marchbanks, 1968a). As will be apparent later, the uptake of acetylcholine into vesicles is much slower than that of other transmitters and uptake into brain vesicles have only recently been claimed.

Other transmitters in vesicles

It should be noted that the demonstration of ACh in vesicles were highly helped from the fact that any leaked ACh was hydrolysed during the isolation of vesicles and therefore did not contaminate the soluble fraction. Subsequent work on the vesicular localisation in brain of serotonin, noradrenalin, dopamine and adrenaline was always affected by the soluble pool of transmitter (De Robertis, 1966, Whittaker, 1966). The soluble pool of free amino acids such as glutamate and GABA completely overshadowed any vesicular localisation (Mangan and Whittaker 1966). An interesting study was performed by Krnjevic and Whittaker (1965). They added extract of vesicles on to neurons to see if there were some unknown active compounds in the vesicles. These were early days in vesicle research, but they found some depression and excitation, probably due to free amino acids, of cortical neurons in vesicle fractions and synaptosome extracts.

De Robertis also published several papers on the localisation of biogenic amines and GABA in synaptosomes, but he later went on to study membranes and receptors. Whittaker on the other hand developed the concept of vesicular release further.

The cholinergic animal, Torpedo

ACh is present in very high doses in the electric organ of electric fishes such as the Torpedo. This has been known for many years and an interesting story of activities on Torpedo before the war is given by Whittaker (1998). The first attempts to isolate vesicles from the electric organ of Torpedo in the same way as from brain was not a great success (Sheridan et al, 1966). M. Israel (Israel et al., 1968 and 1970), decided to try to isolate vesicles directly from the electric tissue. He homogenized the tissue vigorously in iso-osmotic solution (0.3M Sucrose-0.2M NaCl) and in this way liberated the vesicles. He thereby avoided the step of synaptosomes, and by using differential and gradient centrifugation he isolated cholinergic vesicles directly from the homogenate. An important point was that vesicles isolated under iso-osmotic conditions leaked less than after hypo-osmotic shock and this vesicle fraction contained much more of acetylcholine than possible for mammalian species. This finding was an important step forward in the study of cholinergic vesicles. Alternatively, Torpedo vesicles could also be extracted by crushing the tissue in liquid nitrogen and extracting particles in 0.3M sucrose-0,2M NaCl and separating the vesicles on a continuous gradient (Whittaker et al, 1972). In both cases choline acetyltransferase from the electric organ was recovered in the soluble fraction.

Further improvements in vesicle isolation from Torpedo included the isolation of high content of vesicles using density gradient during zonal rotor centrifugation (Whittaker et al, 1972) and very pure vesicles could be obtained after chromatography with controlled glass beads (Nagy et al., 1976). The latter technique was used extensively later when one was isolating specific proteins of the vesicles from mammalian brain. The cholinergic vesicles were also shown to contain ATP similar to as had been found previously for the catecholaminergic vesicles (Dowdall et al., 1974).

Release of acetylcholine from vesicles

An important question was what happens to the vesicles after release. To everybody's surprise the early findings from different preparations showed that the newly synthesised ACh was released first. This results was shown by stimulation of the tissue in the presence of

labelled choline and was found in superior cervical ganglion (Collier, 1969), hemidiaphragm (Potter, 1970), brain cortex surface (Chakrin et al, 1972) and the electric organ of Torpedo (Dunant et al., 1971). These findings indicated also that there was a fraction more active than the general vesicular population that was responsible for the release. M. Israel defined this fraction as acetylcholine “libre” (Marchbanks and Israel, 1972). It is an open question to me whether this fraction contains extremely labile vesicles which are in close contact to the presynaptic membrane or cytoplasmic acetylcholine. The work of Israel and coworkers lead to the isolation of a protein called mediatorphore which is identical to the c part of proton ATPase V_0 fraction. Transfection of mediatorphore and choline acetyltransferase into cells that are not involved in release, showed calcium depended and quantal release of acetylcholine (Bloc et al., 1999). The discovery of this protein has caused more indignation than that of most other synaptic proteins. But it has been suggested recently that V-type proton ATPase could also be in some way involved in the trafficking of different types of vesicles (Marshansky and Futai, 2008)

The Göttingen (Whittaker and coworkers) laboratory went on to describe biochemical heterogeneity of synaptic vesicles after stimulation and release of the electric organ of the Torpedo (Zimmermann and Whittaker, 1977). Stimulation of the electric tissue (0.1Hz, 1800 pulses) in the presence of both labelled choline and adenosine followed by vesicles isolation by zonal density gradient centrifugation showed that in addition to the normal peak of vesicles at 0.4 M sucrose there appeared a denser peak of highly labelled acetylcholine and ATP which indicated more recently synthesized transmitters (Zimmerman and Bokor, 1979). The denser vesicles are 25% smaller than the normal vesicles. Similar heterogeneity could be demonstrated by purifying the vesicles on chromatography on glass beads (Giompres et al, 1981). This could correspond to the two pools of vesicles we discuss today namely the readily releasable pool and the reserve pool.

Morphological studies were also used to a large extent to study the interaction between synaptic vesicles and the presynaptic membranes. Zimmermann and Whittaker (1974) found a decrease in both ACh (90%) and ATP (90%), vesicle number (50%) and size (25%) after stimulating the electric lobe of the Torpedo in situ. Stimulation of rat diaphragm, frog neuromuscular junction or electric tissue showed a reduction in vesicular number or in vesicular diameter (Korneliussen, 1972; Ceccarelli et al, 1973). Hubbard and Kwanbunbumpen (1970) found that in neuromuscular junctions soaked in solutions

containing 20 mM KCl, the number of vesicles in the terminal decreased markedly. Particularly worthy of note is the finding that the vesicles touching the membrane were markedly reduced in number. Black spider widow venom (Latrotoxin), which abolishes synaptic vesicles in the presence of extracellular calcium, was shown by Ceccarelli et al. (1979) to completely inhibit neurotransmission, indicating the importance of synaptic vesicles.

Uptake of acetylcholine

It took a long time before anybody was able to show an uptake of ACh into vesicles. The first indication of an active uptake of ACh was carried out on PC12 cells (Greene and Rein, 1977). The first characterisation of the uptake was done by Toll and Howard (1980) in the same type of cells. The active uptake of labelled ACh into Torpedo vesicles were achieved by several groups (Michaelson and Angel, 1981; Diebler and Morot Gaudry, 1981; Anderson et al., 1982). They showed that the uptake occurred by a proton-pump and that the uptake depends primarily on a change in pH. This is similar to what has been found with other low molecular transmitters (Anderson et al., 1982). The transporter molecule was later obtained from the famous worm *Caenorhabditis elegans*. There is a question whether this worm deserves the Nobel Prize. The transporter had great similarity to VMAT (Roghani et al., 1994; Varoqui et al., 1994). Since there are almost no data of ACh uptake from vesicles isolated from laboratory species, it seems that the activity may be slow. It is perhaps a question whether we still lack a stimulating factor for the transporter.

One way of showing the importance of vesicular release is to see if inhibitors of acetylcholine vesicle uptake or knock out vesicular transporter of acetylcholine will inhibit chemical transmission. One of the earliest inhibitors used was Botulinium toxin which already in 1954 was shown to inhibit mineps (Brooks, 1954). Work much later has characterized the mechanism of botulinium toxin and shown that it prevents exocytosis of the vesicles. Back in 1970 Marshall showed that AH 5183 (2-(4-phenyl piperidino)cyclohexanol) (a putative inhibitor of vesicular release) produced neuromuscular block of slow onset in rapidly stimulated nerve-skeletal muscle preparations of the rat, chicken and cat (Marshall, 1970). It took 14 years before this was further investigated, showing that probably none of us believed his conclusion at the time! Today it is the best known inhibitor of vesicular uptake of ACh and is widely used. Treatment of PC12 cells with AH 5183 at low concentrations (40 nM to 40 microM) inhibited the loading of newly synthesized ACh into storage vesicles, but it had

little effect on choline uptake, ACh synthesis, or the vesicular content of previously loaded acetylcholine (Melega and Howard, 1984). Inhibition of ACh uptake into vesicles also reduced the quantal size at frog muscular junction (Van Der Klot, 1984). Parsons (2000) in particular carried out a series of studies on the mechanism of the inhibition of vesicular ACh uptake by vesamicol (AH 5183). Recently a knock-out of ACh vesicular transporter has been developed. The homozygote animal dies at birth from respiratory failure. The heterozygote animal shows decreases in ACh and quantal size showing the link between vesicles and quanta of ACh (Prado et al, 2006).

Concluding remarks.

Science moves forward at often a slow rate. It has taken us 100 years to accept chemical transmission, ACh as a transmitter and the involvement of vesicles in transmitter release. The slow rate is not necessarily all bad because it has allowed objective criticism, new ways of carrying out experiments, improved techniques and confirmation, and challenges of results by other people and other approaches.

Acknowledgement.

I want to express my thanks to professors Yves Dunant and Herbert Zimmermann for valuable comments and Professor SI Walaas for reading through the manuscript.

REFERENCES

- Anderson DC, King SC, Parsons SM. (1982). Proton gradient linkage to active uptake of acetylcholine by the Torpedo electric organ synaptic vesicles. *Biochemistry* 21, 3037-43.
- Blaschko H. Hagen P, Welch AD. (1955). Observations on the intracellular granules of the adrenal medulla. *J. Physiol.* 129, 27-49.
- Bloc A, Bugnard E, Dunant Y, Falk-vairant J, Israel M, Loctin F, Roulete E. (1999). Acetylcholine synthesis and quantal release reconstituted by transfection of mediatophore and choline acetyltransferase cDNAs. *Eur. J. Neurosci.* 11, 1523-34.
- Brooks VB. (1954). The action of botulinum toxin on motor-nerve filaments. *J. Physiol.* 123, 501- 515.
- Brooks LG, Coombs JS, Eccles JC. (1952). The recording of potentials from motoneurons with an intracellular electrode. *J. Physiol.* 117, 431-60.
- Ceccarelli B, Hurlbut WP, Mauro A. (1973) Turnover of the transmitter and synaptic vesicles at the frog neuromuscular junction. *J. Cell Biol.* 57, 499-524.

Ceccarelli B, Grohovaz F, Hurlbut WP (1979). Freeze-fracture studies of frog neuromuscular junctions during intense release of neurotransmitter. I. Effects of black widow spider venom and Ca^{2+} -free solutions on the structure of the active zone. *J. Cell Biol.* 81, 163-77.

Chakrin LW, Marchbanks RM, Mitchell JF, Whittaker VP. (1972). The origin of the acetylcholine released from the surface of the cortex. *J. Neurochem.* 19, 2727-36.

Collier B. (1969). Preferential release of newly synthesised transmitter from a sympathetic ganglion. *J. Physiol.* 205, 341-52.

Dale HH and Feldberg W. (1934). The chemical transmitter of vagus efferents to the stomach. *J. Physiol.* 81, 331-370.

Del Castillo J, Katz B. (1954). Quantal components of the endplate potential. *J. Physiol.* 124, 563-70.

De Robertis E. (1966). Adrenergic endings and vesicles from brain. *Pharmacol. Rev.* 18, 413-24.

De Robertis E and Bennet (1954). Submicroscopic vesicular components in the synapse. *Fed. Proc.* 13, 35.

De Robertis E, Pellegrino De Iraldi AG, Salganicoff L. (1962). Cholinergic and non-cholinergic nerve endings in rat brain. I. Isolation and subcellular distribution of acetylcholine and acetylcholinesterase. *J. Neurochem.* 9, 23-35.

De Robertis E, Rodriguez De Lores Arnaiz, Salganicoff L, Pellegrino De Iraldi A, Zieher LM. (1963). Isolation of synaptic vesicles and structural organization of the acetylcholine system within brain nerve endings. *J. Neurochem.* 10, 225-35.

Diebler MF, Morot Gaudry Y. (1981). Acetylcholine incorporation by cholinergic synaptic vesicles from *Torpedo marmorata*. *J. Neurochem.* 37, 467-475.

Dowdall MJ, Boyne AF, Whittaker VP. (1974). Adenosine triphosphate. A constituent of cholinergic synaptic vesicles. *Biochem. J.* 140, 1-12.

Du Bois Raymond E. (1877). *Gesammelte Abhandlung der allgemeinen Muskel- und Nervenphysik.* 2, 700.

Dunant Y, Gautron J, Israel M, Lesbats B, Manaranche R. (1971) Effect of the stimulation of numb-fish on free and bound compartmental acetylcholine. *C R Acad. Hebd. Seances Acad. Sci.* 273, 233-6.

Eccles JC. (1944). The nature of synaptic transmission in sympathetic ganglion. *J. Physiol.* 103, 27-54.

Eccles JC. (1976). From electrical to chemical transmission in the central nervous system. *Notes and records of the Royal Society London;* 30, 219-230.

Eccles JC, Katz B, Kuffler SW. (1942). The effect of eserine on neuromuscular transmission. *J. Neurophysiol.* 5, 211-230.

Elliot TR. (1904) On the action of adrenaline. *J Physiol.* 31, XX-XXI.

Engstrom H and Sjöstrand FS. (1954). The structure and innervation of the cochlear hair cells. *Acta Oto-laryngal.* 44, 490-4.

Fatt P and Katz B. (1952). Spontaneous subthreshold activity at motor nerve endings. *J. Physiol.* 117, 109-128.

Feldberg W, Kraye O. (1933). Das Auftreten eines azetylcholinartigen Stoffes in Herzvenen Blut von Warmblutern der Reizung der Nerve vagi. *Naunyn-Schmiedebergs Arch. Exp. Path. Pharmacol.* 172,170-193.

Fonnum F.(1967). The compartmentation of choline acetyltransferase within the synaptosome. *Biochem. J.* 103, 262-70.

Fonnum F. (1968). Choline acetyltransferase binding to and release from membranes. *Biochem. J.* 109, 389-98.

Fonnum F.(1970): Surface charge of choline acetyltransferase from different species. *J. Neurochem.* 17, 1095-100.

Giompress PE, Zimmermann H, Whittaker VP. (1981). Purification of small dense vesicles isolated from stimulated Torpedo electric tissue isolated by chromatography on glass bead column. *Neuroscience*; 6, 765-74

Gray EG, Whittaker VP. (1962). The isolation of nerve endings from brain: an electron-microscopic study of cell fragments derived by homogenization and centrifugation. *J Anat*; 6, 79-88.

Gray EG, Whittaker VP. (1981). This weeks Citation classic number. 1,13.

Greene LA , Rein G. (1977). Synthesis, storage and release of acetylcholine from a noradrenergic pheochromocytoma cell line. *Nature*; 268, 349-51.

Hebb CO, Whittaker VP. (1958). Intracellular distributions of acetylcholine and choline acetylase. *J Physiol* 142, 187-96.

Hubbard JI Kwanbunbumpen S. (1968). Evidence for the vesicular hypothesis. *J Physiol* 194, 407-20.

Korneliusson H. (1972). Ultrastructure of normal and stimulated motor endplates with comments on the origin and fate of synaptic vesicles. *Z Zellforsch Mikrosk Anat.*;130(1):28-57.

Krnjević K, Whittaker VP.(1965). Excitation and depression of cortical neurones by brain fractions released from micropipettes. *J Physiol.*;179(2):298-322

Israel M., Gautron J, Lesbats B. (1968). Isolation of the synaptic vesicles of the electric organ of the torpedo and localisation of acetylcholine at their level. C R Acad Hebd Seances Acad Sci D; 266, 273-5.

Israel M, Gautron J, Lesbats B. (1970). Subcellular fractionation of the electric organ of Torpedo Marmorata. J Neurochem; 17, 1441-50.

Langley JN. (1906). On nerve endings and excitable substances in cells. Proc.R Soc Med; 78B, 179-194.

Loewi, O (1960). An autobiographical scetch. Perspectives in biol and medicine., 4, 3-25.

Marchbanks RM. (1968a). Exchangeability of radioactive acetylcholine with the bound acetylcholine of synaptosomes and synaptic vesicles. Biochem J; 106, 87-95.

Marchbanks RM. (1968b). The uptake of (C14)choline into synaptosomes in vitro. Biochem J; 110, 533-41.

Marchbanks RM. Israel M. (1972) The heterogeneity of bound acetylcholine and synaptic vesicles

Marshall IG. (1970). Studies on the blocking action of 2-(4-phenylpiperidino)cyclohexanol (AH 5183). British J Pharmacol ; 38, 503-16.

MarshanskyV, Futai M. (2008). The Vtype H⁺.ATPase in trafficking, regulation and function. Curr Opin Cell Biol; 20, 415-426.

McCaman RE; Del Lores Arnaiz R, DeRobertis E. (1965). Species differences in subcellular distribution of choline acetylase in the CNS. A study of choline acetylase, acetylcholinesterase, 5-hydroxytryptophan decarboxylase, and monoamine oxidase in four species. J Neurochem; 12, 927-35.

Michaelson DM Angel I. (1981). Saturable acetylcholine uptake into purified synaptic vesicles. Proc.natl.Acad.Sci USA; 78, 2048-52.

Melega WP. Howard BD. (1984) Biochemical evidence that vesicles are the source of the acetylcholine released from stimulated PC12 cells. Proc Natl Acad Sci USA; 81,6535-8

Minz B.(1932) Pharmacologische Untersuchungen am Blutegelpräparat, zugleich eine Methode zum biologischen Nachweis von Acetylcholine bei Anwesenheit anderer pharmakologischer wirksamer körpereigener Stoffe. J f exper.Pathol; 168, 292-304

Nagy A, Baker RR, Morris SJ, Whittaker VP. (1976) The preparation and characterization of synaptic vesicles of high purity. Brain Res;109(2):285-309.

Palade GE. (1954). Electron microscope observations of interneuronal and neuromuscular synapses. Anat Rec; 118, 335

Palay SL. (1954). Electron microscope study of the cytoplasm of neurons. *Anat Rec*; 118, 336.

Parsons SM. (2000). Transport mechanisms in acetylcholine and monoamine storage. *Fed proc*; 14, 2423-2434.

Potter LT. (1970). Synthesis, storage and release of (¹⁴C)acetylcholine from rat diaphragm muscles. *J Physiol*; 206, 145-66.

Prado VF, Martin-Silva C, de Castro BM et al., (2006) Mice deficient for acetylcholine vesicular transporter are myasthenic and have deficiencies in object and social recognition. *Neuron*; 51, 601-12.

Roghani A, Feldman J, Kohan SA, Shirzadi A, Gundersen CB et al. (1994). Molecular cloning of a putative vesicular transporter for acetylcholine. *Proc. natl. Acad. Scie. USA*; 91, 10620-24.

Sheridan MN, Whittaker VP, Israel M (1966). The subcellular fractionation of the electric organ of Torpedo, *Z Zellforsch Microsk Anat*; 74, 293-307

Toll L and Howard BD (1980) Evidence that an ATPase and protonmotive force function in the transport of acetylcholine into storage vesicles. *J biol.Chem.* 255, 1789-91.

Van Der Kloot W. (1986) 2-(4-phenylpiperidino)cyclohexanol (AH 5183) decreases quantal size at the frog muscular junction. *Plugers Arch*; 406, 83-5

Varoqui H, Diebler MF, Meunier FM, UsdinTB,, Bonner TI, Eiden LE, Erickson JD. Cloning and expression of the vesamicol binding protein from the marine array Torpedo Homology with the putative vesicular acetylcholine transporter UNC-17 from *Caenorhabditis elegans*. *FEBS lett*; 342, 97-102

Whittaker VP. (1959), The isolation and characterization of acetylcholine-containing particles from brain. *Biochem J.*;72:694-706

Whittaker VP. (1966). Catecholamine storage particles in guinea pig brain. *Pharmacol.Rev*18, 401-12

Whittaker VP, Michaelson IA, Kirkland RJ. (1964). The separation of synaptic vesicles from nerve-ending particles ('synaptosomes'). *Biochem J.*;90(2):293-303

Whittaker VP, Sheridan MN.(1965) The morphology and of isolated synaptic vesicles. *J Neurochem.*;12:363-72.

Whittaker VP, Essman WB, Dowe GH. (1972) The isolation of pure cholinergic synaptic vesicles from the electric organs of elasmobranch fish of the family Torpedinidae. *Biochem J.*;128(4):833-45

Zimmermann H, Bokor JT.(1979) 5'-triphosphate recycles independently of acetylcholine in cholinergic synaptic vesicles. *Neurosci Lett.*;13(3):319-24.

Zimmermann H, Whittaker VP. (1974). Effect of electrical stimulation on the yield and composition of synaptic vesicles from the cholinergic synapses of the electrical organ of Torpedo; a combined biochemical, electrophysiological and morphological studies. *J Neurochem*; 22, 435-50.

Zimmermann H, Whittaker VP. (1974). Different recovery rates of the electrophysiological, biochemical and morphological parameters in the cholinergic synapses of the Torpedo electric organ after stimulation. *J Neurochem*; 22(6),1109-14.

Zimmermann H, Whittaker VP.(1977). Morphological and biochemical heterogeneity of cholinergic synaptic vesicles. *Nature*; 267(5612):633-5.