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EDITORIAL

Vesigate Hypothesis of Neurotransmitter Release Explains the Formation of Quanta by a Non-Vesicular Mechanism

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Introduction

The discovery that the transfer of signals between neurones may be mediated by the quantal release of neurotransmitter at nerve terminals and the morphological identification of synaptic vesicles led to the formulation of the vesicular hypothesis for transmitter release (Del Castillo and Katz 1957). This intellectually very attractive theory states that transmitters are released through exocytosis of synaptic vesicles. It not only gives an explanation of the quantal character of synaptic transmission but also an explanation of how quanta are formed and stored in the nerve terminal, ready for release. Moreover, it gives a unitary view on the secretion process in nerve and gland cells (for recent reviews see Ceccarelli and Huribut 1980, Vizi 1984, Cooper and Meyer 1984, Thesleff 1986, Whittaker 1986, Van der Kloot 1988, Heuser 1989, DeCamilli and Jahn 1990, Almers 1990, Valtorta *et al.* 1990).

However, despite the large amount of work done, no decisive direct evidence has been brought forward that validates this hypothesis of neurotransmitter release by neuronal cells. Observations presented below and in several review articles (Israel *et al.* 1979, Marchbanks 1979, Tauc 1979, 1982, Vizi 1984, Cooper and Meyer 1984, Israel and Manaranche 1985, Tauc and Baux 1985, Dunant 1986) cannot easily be explained by the vesicular hypothesis and justify the elaboration of an alternative explanation of the release mechanism.

In this brief review, we deal essentially with cholinergic synapses, the only system about which enough experimental information is available (for the release of other neurotransmitters or neuromodulators see Uvnäs 1985, Nicholls 1989, DeCamilli and Jahn 1990, Almers 1990, Maycox *et al.* 1990) and we propose a working hypothesis which attempts to explain experimental data by attributing the roles of quantum formation and of neurotransmitter extrusion from the cytoplasmic compartment to a presynaptic membrane mechanism, termed vesigate (Tauc 1979, 1982).

Dynamics of acetylcholine compartments with acetylcholine release

A prerequisite to the formation of a tenable alternative release hypothesis is the existence of a non-vesicular (e.g. cytoplasmic) compartment that contains a sufficient concentration of the neurotransmitter. Cytoplasm is the site of ACh synthesis (for review see Tuček 1985). The "free" cytoplasmic ACh represents 30 % to 50 % of the total ACh content (Dunant *et al.* 1974, Tauc 1977, Baux *et al.* 1979) and can be detected electrophysiologically (Fig. 1). In the terminal, it is estimated to be at a concentration between 10 to 50 mM (Israel *et al.* 1979, Morel *et al.* 1978, Katz and Miledi 1977, Poulain *et al.* 1986a).

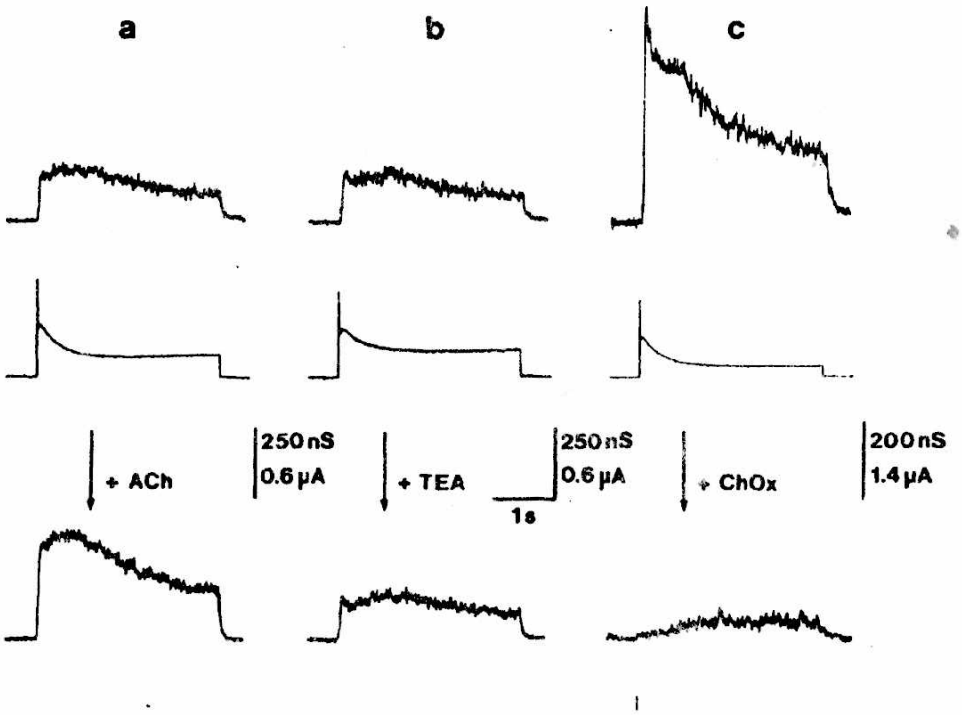

Fig. 1

K^+ current as an experimental index of changes in presynaptic acetylcholine concentration.

In each of the three panels, the upper recording shows control long duration responses for a different postsynaptic neuron of Aplysia, induced by presynaptic depolarization to +5 mV (in *a*) or to +10 mV (in *b* and *c*). The second line shows the control presynaptic current.

In the lower half of panel (*a*), after presynaptic injection of acetylcholine (50 nA for 10 min), the size of the postsynaptic response was enhanced due to an increase in acetylcholine release. Because acetylcholine is a quaternary ammonium derivative, the presynaptic current (K^+) for the same depolarization is reduced.

In the lower half of panel (*b*), presynaptic injection of TEA (50 nA for 5 min) induced a similar decrease in the presynaptic K^+ current as in (*a*) without modifying the postsynaptic response.

In the lower half of panel (*c*), when choline oxidase was bath applied, long presynaptic stimulations (6 series of 4 stimulations of 1 min to +10 mV) led to a lack of available acetylcholine and subsequently to depression of the postsynaptic response. The presynaptic K^+ current was increased.

In all presynaptic current traces, the spike represents the early transitory K^+ current, IA.

Using radioactive precursors, newly synthesized ACh has been shown to be preferentially released upon stimulation (Collier 1969, Potter 1970) which is contrary to what would be expected if ACh is released from a stable preformed store. In the *Torpedo* electric organ, stimulation increases first the turnover of ACh in the cytoplasmic compartment whereas vesicular ACh is unchanged for several minutes (Dunant *et al.* 1974, Dunant 1986). Under conditions where re-synthesis of ACh cannot occur, stimulation of the ACh release leads to a clear decrease of cytoplasmic ACh up to near exhaustion while the vesicular ACh is essentially preserved (Israel and Lesbats 1981). This indicates that, during intense stimulation, there is little or no exchange between cytoplasmic and vesicular ACh compartments. A parallel evolution of free ACh in the terminal and of the amplitude of the evoked postsynaptic synaptic response has been observed in the *Torpedo* electric organ (Corthay *et al.* 1982).

Interesting results have been obtained using monoethylcholine, a compound that is taken up by nerve terminals at the neuromuscular junction, acetylated in the cytoplasm and released as a false transmitter, acetylmonoethylcholine (AMECh). The analysis of miniature endplate currents showed that both transmitters present in the terminal, ACh and AMECh, mixed immediately and were co-released within the same quantum (Large and Rang 1978). Since a rapid exchange between cytoplasmic and vesicular ACh has not been detected in any biochemical study (Israel *et al.* 1979 and the above reviews) this means that quanta of mixed false and true transmitters came from the cytoplasmic compartment.

Stoichiometry of the co-release of acetylcholine and other substances

Vesicles contain substances other than the transmitter; a stoichiometric release of all soluble contents of vesicles would be expected during exocytosis. ATP is present in cholinergic vesicles (Volknandt and Zimmermann 1986) and the co-release of ATP and ACh was observed using brain synaptosomes (White *et al.* 1980) and the *Torpedo* electric organ (Meunier *et al.* 1975). The ATP to ACh ratio was 1:5 in the vesicles and 1:45 in the perfusate (Morel and Meunier 1981). Possibly, this difference could result from the presence of presynaptic ATPase (Unsworth and Johnson 1990), but in other experiments it was shown that application of botulinum neurotoxin blocks the release of ACh but not of ATP (White *et al.* 1980, Marsal *et al.* 1987). These experiments are not consistent with the view that the whole vesicle content is released by exocytosis.

Existence of subminiature endplate potentials

One very strong objection to the vesicular theory is the discovery of subminiature endplate potentials (S-MEPPs). A quite large population of "small" miniature potentials was found which are about 7 to 15 times smaller than "normal" MEPPs at the neuromuscular junction (Kriebel and Gross 1974, Erxleben and Kriebel 1988) and in the *Torpedo* electric organ (Müller and Dunant 1985). Release of a full sized ACh quantum would then result from the synchronous release of 7 to 15 subquanta. The existence of subunits is incompatible with the vesicular hypothesis, even by proposing that "normal" size MEPPs result from the

simultaneous release of several vesicles (Tremblay *et al.* 1983) because *i)* vesicles contain far more ACh than is needed to produce a S-MEPP; *ii)* during long stimulations the number of quanta released exceeds the number of "readily releasable" vesicles located adjacent to the presynaptic membrane along the active zone (Katz and Miledi 1979). Thus one vesicle apparently cannot correspond to one physiological unit.

Morphological and molecular correlates of acetylcholine release

The release of a large quantity of transmitter in quantal form must leave a trace on the membrane. According to the vesicular hypothesis, the presence of "omega shapes" or of "dimples" on electronmicrographs of the presynaptic terminal membrane are considered to indicate the opening of vesicles whereby a quantum of ACh is supposed to be ejected into the synaptic cleft (Couteaux and Pécot-Dechavassine 1970, for reviews see Ceccarelli *et al.* 1973, Heuser 1989). However, it has not been possible to clearly demonstrate a correlation between transmitter release and vesicle number (for a review see Ceccarelli and Huribut 1980).

A rearrangement of intramembrane particles associated with Ca^{2+}-dependent evoked ACh release was observed in stimulated preparations of the *Torpedo* (Israel *et al.* 1981, Müller et. al. 1987). The same ultrastructural modifications have been observed using reconstituted proteoliposomes free of synaptic vesicles, included in the membrane, and containing the "mediatophore", a protein found to translocate ACh (Israel *et al.* 1981,1988, Birman *et al.* 1986). These observations suggest that a membrane-bound macromolecule may be responsible for the extrusion of ACh. The possible role of the "mediatophore" in quantal release will be discussed shortly.

Effects of the modification of acetylcholine cytoplasmic compartment on acetylcholine release

If releasable ACh comes from the cytoplasmic pool, selective modification of this pool should immediately affect transmitter release. In *Aplysia*, the enhancement of presynaptic ACh content by injection of ACh or choline into a presynaptic neurone, leads rapidly to an increase in transmitter release (see Fig. 1) (Tauc and Baux 1985, Poulain *et al.* 1986a,b). Selective destruction of cytoplasmic ACh by injecting exogenous acetylcholinesterase into the presynaptic neurone leads to blockade of ACh release (Tauc *et al.* 1974, Tauc and Baux 1985, Baux and Tauc 1983). The same results have been obtained when ACh synthesis was prevented by a lack of its precursor, choline, after extra-or intracellular application of choline oxidase (see Fig. 1) (Poulain *et al.* 1986a,b). In these experiments, transmission could be restored by an injection of carbachol (a non-hydrolysable analogue of ACh) (Baux and Tauc 1983) or ACh (Poulain *et al.* 1986a,b).

An important observation was that the size of the released quanta was independent of the intraterminal ACh concentration (Tauc and Baux 1985, Baux and Tauc 1983, Poulain *et al.* 1986a,b). This confirms that only full-sized quanta can be released (Katz 1969) and agrees with the idea that ACh is not released through a channel as was later confirmed by Young and Chow (1987).

Intracellular pharmacology of acetylcholine release

If ACh is considered to be released neither by exocytosis of synaptic vesicles nor by extrusion through a channel, a hypothetical mechanism for release requires that ACh would be "concentrated" at specific membrane structures binding cytoplasmic ACh with high affinity. It is reasonable to assume that these "presynaptic intracellular ACh receptors", if they exist, must have pharmacological properties similar to those of the classical postsynaptic ACh receptors. Accordingly, ACh or carbachol are good intrasynaptic agonists since, when introduced presynaptically, they potentiate the release of transmitter as shown elsewhere (Baux and Tauc 1983, Tauc and Baux 1985, Poulain *et al.* 1986a,b). Accordingly, other cholinergic drugs like curare, hexamethonium, hemicholinium-3, succinylcholine, acetylmethylcholine, acetylthiocholine or pralidoxime, when intracellularly applied in *Aplysia*, decrease or even block synaptic transmission (Tauc and Baux 1985, Baux *et al.* 1986, Baux and Tauc 1987, Fossier *et al.* 1990) without altering ACh quantum size. They can be considered intrasynaptic antagonists. Since decrease in ACh release occurred even in the absence of stimulation, it appears that the pool of releasable ACh is in equilibrium with cytoplasmic ACh; this is not the case with synaptic vesicles.

Apparently, the intracellular pharmacology of ACh release, does not overlap with that of the ACh vesicular uptake blockers: intracellularly applied atropine, which is like curare, a potent antagonist of vesicular ACh uptake (Anderson *et al.* 1983), did not alter the release of ACh (Tauc and Baux 1985, Baux and Tauc 1987).

Because a potent blocker of vesicular uptake, AH-5183 or vesamicol, (Anderson *et al.* 1983, Marshall and Parsons 1987), was shown to block the release of newly synthesized ACh, it was thought that the vesicular theory had been demonstrated (Melega and Howards 1984, Michaelson and Burstein 1985). However, AH-5183 has many other effects such as a blockade of molecular leakage of ACh (Edwards *et al.* 1985). It is not likely that this leakage is related to the insertion of the vesicular ACh transporter into the presynaptic membrane (see Grinnel *et al.* 1989) or to antagonism of postsynaptic receptors (Enomoto 1988). Also, AH-5183 does not block Ca^{2+}-dependent K^+-evoked ACh release from *Torpedo* electric organ synaptosomes (Morot Gaudry-Talarmain *et al.* 1989). Since facilitation of Ca^{2+} entry by aminopyridines reverses transiently the blockade of transmission induced by AH-5183 (Estrella *et al.* 1988), this suggests that AH-5183 probably acts directly on the release mechanism or as an antagonist of intrasynaptic receptors.

Moreover, after blockade of ACh release by AH-5183, further addition of black widow spider venom, a toxin known to induce paroxysmal quantal release of ACh (Fesce *et al.* 1986), can induce the release of newly synthesized ACh (Cabeza and Collier 1988). As AH-5183 blocks the vesicular uptake of ACh, this above observation strongly suggests that ACh is released from a non-vesicular compartment.

The "vesigate hypothesis" as a model of transmitter release mechanism

A satisfactory transmitter release hypothesis should be in complete agreement with the above experimental results and with a minimum of assumptions

to *(i)* explain the formation of quanta, *(ii)* explain the triggering and regulation of release, *(iii)* identify a morphological correlate of release, and *(iv)* provide a mechanism for the translocation of quanta across the presynaptic membrane.

(i) Formation of quanta

Besides the synaptic vesicle (the ACh channel hypothesis being discarded), the other mean to package ACh molecules together to be ejected as a quantum is a membrane-bound structure bearing ACh binding sites which we have discussed above as "presynaptic intracellular ACh receptors". It was proposed (Tauc 1979, 1982) to call this structure a vesigate.

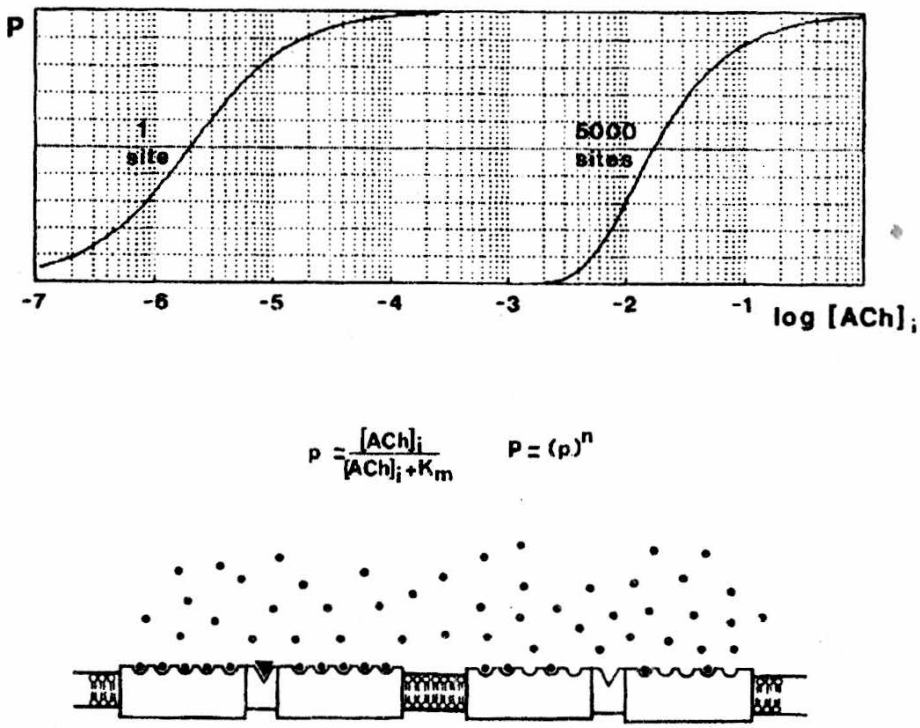

Fig. 2

Schematic representation of vesigates.

Lower panel: vesigates are complex structures inserted in the presynaptic membrane and have acetylcholine binding sites (open half circles) exposed to intracellular molecular acetylcholine (closed circles). According to the existence of subquanta, the release of one acetylcholine quantum results from the synchronous action of several subunits (two are represented in each vesigate). The synchronization site of vesigate subunits is shown as an open triangle. Calcium ions (close triangles) can trigger the release of one quantum only if a vesigate is fully loaded with acetylcholine.

Upper panel: equations show the probabilities of binding of acetylcholine to one binding site or of the saturation of a vesigate having **n** sites (**p** and **P**, respectively). These are represented as a function of intracellular acetylcholine concentration. *Left curve*: probability **(p)** of occupation by acetylcholine of a single binding site, for a $K_m = 2.10^{-6}$ M. *Right curve*: probability **(P)** of simultaneous occupation of a group of n = 5 000 independent binding sites of a vesigate. Each binding site is assumed to have the same K_m.

The vesigate should be apposed to the inner face or be a part of the presynaptic membrane. It should be loaded with ACh from the cytoplasmic compartment; ACh bound to the vesigate would be in dynamic equilibrium with free cytoplasmic ACh and must release ACh extracellularly in a non-electrogenic manner. It should be a structure which binds ACh to saturation before it can operate because quantal size is independent of free ACh concentration. This means that a vesigate will be releasable only when all the binding sites will be occupied by ACh or a releasable agonist (carbachol for instance); occupation of some binding sites by a presynaptic intracellular antagonist (e.g. curare) would lead to incomplete saturation, thereby preventing release.

The process may be described with conventional Michaelis-Menten enzymatic kinetics of receptor/ligand interactions: at a given concentration of cytoplasmic ACh the probability (**P**) that a vesigate of (**n**) binding sites will be fully loaded by ACh (see Fig. 2) is:

$$P = p^n \text{ with } p = [ACh]_i/([ACh]_i + K_m)$$

(**p**) is the probability of binding of one ACh molecule to a binding site; $[ACh]_i$ is the concentration of cytoplasmic ACh; the number of binding sites, (**n**), which must be identical with the number of ACh molecules of a quantum (5 000 to 12 000, Kuffler and Yoshikami 1975, Simonneau *et al.* 1980, Miledi *et al.* 1983); K_m is the dissociation constant for a single receptor. If K_m is considered to be similar to the to the K_{DL} of postsynaptic ACh receptors ($\approx 2.10^{-6}$ M), cytoplasmic ACh concentration required to reach a 50 % probability that 5 000 ACh binding sites of a vesigate would be saturated, would be approximately 20 mM (Fig. 2). This concentration is comparable to experimentally determined values (see above).

The vesigate is more precise than a vesicle in determining the number of releasable ACh molecules in a quantum. The vesigate hypothesis thus predicts that quanta of constant size should be released at a given presynaptic site (i.e. active zone, see below). The appearance of MEPPs of identical amplitude and released at the same site were effectively detected by focal microelectrodes (Kriebel *et al.* 1990). Such an observation cannot be accounted for by the vesicle hypothesis since vesicles of forcibly various quantal content would fuse randomly with the presynaptic membrane during transmitter release.

(ii) Triggering and regulation of the release

The vesigate hypothesis fully agrees with the role of Ca^{2+} influx and instantaneous Ca^{2+} concentrations at critical zones as a trigger for transmitter release (Fig. 2) (see review by Augustine *et al.* 1987). One putative source of regulation may now have to be considered: the control of the number of quanta released by the free ACh concentration (Baux and Tauc 1983, Poulain *et al.* 1986a,b). Contrary to the vesicular hypothesis in which releasable neurotransmitter is prepackaged into a vesicle serving as a stable store, the vesigate hypothesis does not assume the formation of stable quanta in the terminal. Rather, continuous exchange of the transmitter occurs between the vesigate ACh binding sites and the cytoplasmic free ACh pool. As a critical number of binding sites may or may not be

occupied by ACh at any moment, the status of a vesigate will continually change from releasable to non-releasable. Statistically, however, the number of releasable vesigates for a given cytoplasmic concentration of the transmitter would remain constant.

(iii) Identification of vesigates

It is tempting to speculate that there is a relationship between the vesigate and the active zone or even that active zones are vesigates. This concept is supported by the expectation that a complex macromolecular structure like a vesigate would be visible ultrastructurally. In elecron micrographs of central synapses, the prominent feature facing the postsynaptic receptors is indeed the presynaptic densification or active zone which Gray and Akert (Gray 1976) identified in some terminals and termed "presynaptic grid". The repeated pattern of this structure provides morphological support for the existence of vesigate subunits. These subunits must be postulated to explain the subminiature postsynaptic potentials observed at neuromuscular junctions and the *Torpedo* electric organ that reflect the release of subquanta (Kriebel *et al.* 1990, Müller and Dunant 1985). The "mediatophore" protein isolated by Israel's group may function as a part of the vesigate. Clearly such a system requires a triggering and synchronizing molecular structure (Tauc 1982, Dunant 1986) which would permit simultaneous activation of all vesigate subunits. Such a role may even be assumed by the mediatophore molecules themselves.

The identification of the vesigate as the active zone implies that an active zone is able to release only one quantum at a time. This point was most clearly demonstrated at inhibitory synapses on the Mauthner cell of the goldfish where synaptic contacts consist in only few boutons each having a single active zone. In this preparation, the number of quanta released at a time is never more and usually less than the total number of boutons (Faber and Korn 1985). Because the vesigate must be recycled in order to be "reset", one could expect the existence of a refractory period during which an active zone is unable to release the transmitter.

(iv) Translocation of acetylcholine – role of the synaptic vesicles

The vesigate hypothesis excludes vesicular exocytosis and simple ACh channels, but gives no indication of how the transmitter is translocated across the presynaptic membrane. The protein or "mediatophore" isolated from the membrane of *Torpedo* synaptosomes (Birman *et al.* 1986) or from the rat brain (Israel *et al.* 1988) may assume this function. Another candidate is a protein which, when blocked by an antibody, leads to blockade of ACh release (Eder-Colli *et al.* 1989).

The control of synaptic transmission requires that the release of transmitter not only starts but also stops as quickly as possible. Therefore, calcium must be removed rapidly from the proximity of the release sites. Synaptic vesicles are strategically located near the active zones in great numbers and can take up Ca^{2+} very efficiently (Michaelson *et al.* 1980, Israel *et al.* 1980); their exocytosis will then extrude Ca^{2+} from the terminal. This may explain why under nonphysiological conditions, that potentiate considerably the entry of Ca^{2+}, a correlation was observed between the number of quanta released and the number of exocytosis as

seen by electronmicroscopy (Heuser 1989). This, naturally, does not preclude the possibility that vesicles serve as a storage site for the transmitter and have some other additional functions.

Conclusions

In conclusion we propose that, under normal conditions, neither vesicles nor acetylcholine channels translocate ACh across the presynaptic membrane. Rather we believe that quantal release of ACh is performed by a complex macromolecular membrane structure or "vesigate" composed of several subunits that possess receptors for cytoplasmic ACh. The vesigate receptors are thought to be in dynamic equilibrium with cytoplasmic ACh; in order to be releasable, all or most of the receptors have to be occupied by ACh. Synchronized activation of a given number of subunits forms a full quantum. Release of subquanta may result from a nonsynchronous activation of subunits. The translocation mechanism is unknown but it is triggered by Ca^{2+} and may involve a membrane protein already identified as "mediatophore". We suggest further that morphologically identified presynaptic active zones may, in fact, be vesigates. Synaptic vesicles have multiple functions; they probably serve as reserves for ACh, but their immediate function may be the buffering of Ca^{2+} that enters the terminal during stimulation.

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