REVIEW

The Organization of the Cholinergic Synapse

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Abstract

In a study of the functional organization of the cholinergic synapse using the electromotor system of the electric ray, *Torpedo marmorata*, two main pools of transmitter have been identified: The cytoplasmic, comprising about 20% of terminal acetylcholine, and the vesicular. The latter is morphologically and metabolically heterogeneous. In resting tissue, about 15% of the vesicles have a relatively low transmitter content and density; they are immature (V_0) vesicles newly arrived from the cell body by axonal transport. Approximately 35% of vesicles belong to the recycling (V_2) pool; these are denser than the main population due to osmotic dehydration attendant upon a reduced osmotic load. The majority (50%) are fully charged 'reserve' (V_1) vesicles. On perfusion, when axonal replenishment and impulse traffic are alike cut off, V_0 and V_2 vesicles take up more transmitter and join the V_1 pool which now comprises over 90% of all vesicles. On stimulation the V_2 population is greatly increased at the expense of V_1 , but in an ensuing period of rest rejoins the V_1 population. Work with isotopically labelled transmitter precursors, false transmitters and endocytotic markers shows that the V_2 population is the origin of quantized release. The cytoplasmic pool, however, is also functionally important since it is the site of transmitter synthesis for vesicular uptake and is subject to 'futile recycling' brought about by the continuous molecular leakage of transmitter into the extracellular space, its hydrolysis by acetylcholinesterase, the uptake of choline and acetate and their resynthesis to acetylcholine. This indicates a control or regulatory function. There is little exchange between this pool and fully charged V_1 vesicles but it supplies the recycling vesicles of the V_2 pool with transmitter. In

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this way, quantal size is kept constant and largely independent of cytoplasmic transmitter concentration. In recent years considerable progress has been made in understanding the organization of the cholinergic synapse, largely by intensive work on one particular model system: the electromotor synapses of the electric rays *Torpedo marmorata* and *T. californica*. Methods have been devised for isolating functional nerve terminals (synaptosomes), presynaptic plasma membranes, synaptic vesicles and vesiculated fragments (microsacs) of the postsynaptic membrane. From the cell bodies of the electromotor neurones, mRNAs have been isolated that code for presynaptic proteins, and from the target cells (the electrocytes), mRNAs coding for polypeptides comprising or accompanying the receptor. From the latter, using the cDNA technique, Numa and colleagues have deduced the structure of the receptor in great detail. The present lecture will concentrate on the functional organization of the presynaptic nerve terminals.

Key words: cholinergic synapse, presynaptic organization, transmitter pools

Electromotor system of the electric ray

The electromotor systems of electric rays of genera *Torpedo* and *Narcine* are now well established models for the study of cholinergic transmission. The electrocytes of the electric organ are derived embryologically from muscle.^{1,2} They are electrically inexcitable but respond to applied acetylcholine.^{1,4} They receive a profuse cholinergic³ innervation from the electric lobes—prominent paired nuclei on the dorsal surface of the brain stem just behind the cerebellum¹ containing the cell bodies of the electromotor neurones. The axons of these cells are heavily myelinated and travel in eight large nerve trunks (four on each side of the neural axis) between the gills into the electric organ. The electromotor cells themselves receive an axo-dendritic, contralateral input from the oval nuclei in the medulla.⁵

A single average-sized specimen of *Torpedo marmorata*, the most readily available species in Europe, provides about 400 g of electric organ containing 500–1000 times more synaptic material than muscle. The tissue is highly collagenous and difficult to homogenize in the conventional way but freezing in liquid nitrogen renders it brittle and it may then be comminuted by crushing.⁶ This process, besides breaking up the tissue, tears open the nerve terminals and by extracting the tissue fragments with iso-osmotic sucrose, saline or sucrose-saline and removing coarse particles by centrifuging, a vesicle-rich cytoplasmic extract is obtained which is a suitable starting material for further purification by isopycnic continuous density-gradient centrifuging in a zonal rotor^{6,7} or exclusion chromatography on porous glass beads^{7,8} or Sephacryl.⁹ In this way synaptic vesicles may be purified to a high and constant concentration of vesicle markers.^{7,10} Such vesicles are extremely rich in acetylcholine—over 6 nmol/mg of

protein corresponding to an internal concentration of 0.9 M or 2×10^5 molecules per vesicle.⁷ In addition vesicles contain a second smaller molecular mass constituent, APT,¹¹ present in about 0.17 M concentration.

Structure of the unperturbed synaptic vesicles

The results of the work of my colleagues and myself on the structure of synaptic vesicles, isolated from resting electromotor nerve terminals are summarized in Fig.^{12,13} The vesicles are larger than those in ordinary motor nerve terminals or autonomic or central terminals—90 versus 50 nm in diameter. All the lipid and almost all of the protein is assigned to the membrane.⁷ The vesicle contains a vesicle specific proteoglycan whose hydrophilic sulphonated carbohydrate residues are directed towards the core.¹⁴ The membrane proteins are about five in number and are of molecular mass 160, 146, 42, 32 and 25 KDa.¹⁰ At least four are functional membrane proteins and comprise a proton-translocating ATPase,¹⁵ a Ca^{2+} , Mg^{2+} -stimulated ATPase,¹⁶ an ADP-ATP carrier^{17,18} and an acetylcholine carrier.¹⁹ The fifth, of molecular mass 42KDa, is a nervous-system specific form of action.²⁰

The internal pH of the vesicle is about 5.5²¹ and the pH gradient probably plays an important part in the uptake of acetylcholine and the second small-molecular-mass component, ATP. Besides these, the vesicle contains appreciable amounts of Ca^{2+} and Mg^{2+} .²² The nuclear magnetic resonance ^{31}P and ^1H spectra show that acetylcholine and ATP are essentially free in solution in the vesicle core^{21,23} and osmotic pressure studies show that they exert an osmotic pressure.²⁴ Water-space measurements²⁵ show that there are three main water compartments, the osmotically active water (65% of total vesicle volume), the water bound to solutes (7%) and the membrane water (%). The non-solvated and hydrophobic components of the membrane account for 17% and the non-solvated solutes for 3% of vesicle volume.

Transmitter pools in resting terminals

The enzyme synthesizing acetylcholine, choline acetyltransferase, has a molecular weight of about 68% KDa and is present in the cytosol.²⁶ It utilizes choline and acetyl-coenzyme A. The result of its activity is a small cytoplasmic pool of acetylcholine. This pool is continuously being replaced even in the resting terminal, since acetylcholine is being continuously lost from the terminal; extracellular transmitter is then rapidly hydrolysed by acetylcholinesterase present in the synaptic cleft and the products by hydrolysis are taken up again by the terminal. As a result of this 'futile recycling', the cytosolic pool of acetylcholine is easily labelled, using radioactive or deuterated choline or acetate. Interestingly, vesicles isolated from blocks of resting tissue exposed to labelled acetylcholine precursors, do not themselves incorporate label, either in the

Fig. 1 Structure of the synaptic vesicle.

form of the precursors or acetylcholine.^{27,28} The proportion of tissue acetylcholine in the cytosolic pool can therefore be quite simply measured non-invasively²⁹ in experiments in which tissue blocks have been exposed to labelled choline by comparing the isotopic ratio $r\text{ACh}$ of tissue acetylcholine with that of its precursor choline $r\text{Ch}$. The ratio of these ratios $r\text{ACh}/r\text{Ch}$ is the proportion by which tissue acetylcholine synthesized from tissue choline has been diluted with non-exchangeable *i.e.* vesicular acetylcholine, *i.e.* the proportion of acetylcholine in the cytosolic pool. This is $22 \pm 3\%$.

Effect of stimulation

Electrophysiologically, electromotor synapses closely resemble other motor synapses *e.g.* in frog muscle. Release of transmitter is quantized and stimulation synchronizes quantized release with the production of an excitatory postsynaptic potential (EPSP).³⁰ It is the summation in series and parallel of these normal-sized EPSPs occurring simultaneously in some 360,000 electrocytes stacked in some 500 columns on each side of the fish,¹ that generates the electric organ discharge (about 40 V measured in air).

Stimulation mobilizes synaptic vesicles and causes them to release transmitter by exocytosis. The vesicles reform and refill at the expense of cytoplasmic acetylcholine, which tends to fall during and after stimulation to about a third of its resting value. The reutilization of vesicles is shown by the fact that at low rates of stimulation (0.1 to 4.15 Hz), acetylcholine can be released for a long period without significant diminution of vesicle numbers. However, the recycling vesicles (identified by the uptake of dextran particles into their lumina) became smaller and denser than the reserve population^{31,32} and so can be separated from them by density gradient centrifugation^{31,33} or exclusion chromatography.³⁴ In experiments with labelled precursors, these recycling vesicles incorporate labelled acetylcholine from the cytosol, and on restimulation, the labelled acetylcholine is preferentially released.

Reversibility of the effects of stimulation: All the changes in the morphology and biophysical properties of vesicles just described are reversible.^{32,33,35,36} Fig. 2 shows the result of a recent experiment with perfused blocks of electric tissue. The electric organs were stimulated in anaesthetized fish *in vivo* via the electric lobe at 0.15 Hz for 3.33 h to generate a large pool of recycling vesicles and were then removed for perfusion with deuterated choline and allowed to recover. Cytoplasmic extracts of the blocks were prepared at suitable time intervals and submitted to continuous density gradient centrifuging in a zonal rotor. As will be seen in Fig. 2, at 2 h endogenous acetylcholine was bimodally distributed in the gradient and newly synthesized deuterated acetylcholine had been taken up selectively by the denser, recycled vesicles. During the 16 h period of recovery, the depleted acetylcholine stores of the tissue were replenished and the recycled vesicle gradually recovered the biophysical properties of the reserve population as shown by the diminishing density of the labelled, recycled vesicle population (see insert). By 16 h, the recycled vesicles had regained the characteristics of the reserve population.

Fig. 2 The subpopulation (a) of recycled (VP₂) vesicles present in blocks of stimulated electric organ, labelled with deuterated acetylcholine and smaller (b) than the reserve (VP₁) population recovers (c) the biophysical properties of the reserve population after 16 h rest.³⁶ The physiological integrity of the blocks was preserved by perfusion. (d) Plot of gradient volume (○) separating the peaks of reserve and recycling vesicles as a function of time, also recovery by recycling vesicles of the density (▲) and water content (■) of the reserve pool.³⁵

Basis for the greater density and smaller size of recycled vesicles: The increased density and smaller size of recycling or recently recycled vesicles can be precisely accounted for by a model in which partial reloading of vesicles emptied by exocytosis and reformed in a fully functional state by endocytosis is followed by osmotic dehydration in response to the reduced osmotic load. The lower osmotic load is apparent from the reduced osmotic fragility of recycled vesicles compared to that of reserve vesicles from stimulated or non-stimulated tissue³⁷ and their lower ratio of acetylcholine to a stable vesicle marker such as vesicular proteoglycan^{14,36,38} (Fig. 3). The density changes induced in the two subpopulations of vesicles by changes in the osmotic pressure of the suspension medium are consistent with osmometer-like properties and a lower osmotic loading of the recycled subpopulation. Measurements of water space show a depleted water space in the recycled vesicles.

Table 1 lists the biophysical parameters of the two populations. Since those of

Fig. 3 Isolation of synaptic vesicles on a continuous gradient in a zonal rotor after extraction from a frozen and crushed electric organ 48 h after *in vivo* injection of $^{35}\text{SO}_4^-$ into the lobe. Redrawn from¹⁴. Here ATP ($\triangle \triangle$ - -) is used as a vesicle marker (nmol recovered, $3,350 \pm 70$ (2)). The distribution of ^{35}S in the gradient (total counts recovered, 6.6×10^5) is indicated by the filled squares. Open symbols are the results with the contralateral organ, the nerves to which were cut before the injection of $^{35}\text{SO}_4^-$. It will be noted that ^{35}S comigrates with the vesicle marker, indicating the presence of newly arrived vesicles in the vesicle pool, only when the afferent nerves are intact, though unlabelled vesicles are present (open triangles). Although these vesicles were derived from an unstimulated organ, a small number of recycling vesicles are present, perhaps due to adventitious stimulation, as shown by the shoulder (arrow) on the dense side of the ATP peak. Note that the ATP: ^{35}S ratio is lower here than in the peak fractions, indicating that such vesicles are only partially reloaded with osmotically active small molecules.

Table 1 Parameters of Reserve (VP_1) and Recycled (VP_2) Vesicles at 800 mOsm

Symbol	Parameter	How			VP ₂	
		Units ^a	Calculated	VP ₁	No Re- covery	2 h Re- covery
Vesicle						
d	diameter	nm	—	90 ^b	71.1 ^c	72.4 ^c
V	volume	al	$\pi d^3/6$	0.382	0.188	0.199
ρ	density	fg.al ⁻¹	—	1.056 ^d	1.067 ^d	1.006 ^d
Membrane						
d _m	tnickness	nm	(d-d)/2	4.1	8.7	8.7
V _m	volume	al	V-V	0.095	0.107	0.107
ν_m	volume fraction	—	1- ν_c	0.25	0.57	0.54
ρ_m	density	fg.al ⁻¹	—	1.13 ^e	1.11 ^f	1.11 ^f
Core						
d _c	diameter	nm	$3 \sqrt{6V/\pi}$	81.8	53.7	56.0
V _c	volume	al		0.287	0.081	0.092
ν_c	volume fraction	—		0.75	0.43	0.46
ρ_c	density	fg.al ⁻¹	—	1.03	1.003	1.012
ν_G	glycerol space			0.65	0.42	0.44
ν_S	volume fraction of hydrated solutes ^g			0.10	0.01	0.02

^aNone that 1 al (atto litre)= 10^{-18} l= 10^6 nm³, 1 fg (femtogram)= 10^{-15} g, 1 fg.al⁻¹=1 g.ml⁻¹
^bref11 ^cref13 ^diso-osmotic density gradient measurements of intact vesicle, ^eosmotically collapsed ghost [11]; ^fassumes constant membrane mass and 12% increase in volume by imbibition of water; ^gdetermined by measuring the increase in the glycerol space on lysis, proportionally for VP_2 vesicles by the observed extent of reloading.

the recycled vesicles vary with recovery time, two sets of values are given: those relating to zero and 2 h recovery respectively.²⁴ There is no need to assume any change in the mass or composition of the vesicle membrane or in macromolecular constituents of the core which in any case probably consist only of glucosaminoglycan side chains of a proteoglycan firmly anchored in the membrane; indeed any such change would be inconsistent with the recovery, by the recycled vesicles of the density and size of the reserve vesicles during recovery from stimulation.

Labelling of the transmitter pools by analogues: In theory, the preferentially released newly synthesized, labelled acetylcholine could have been released not from recycling vesicles³⁹ but directly from the cytosolic compartment through some kind of gate, pore or carrier.⁴⁰ However, experiments with analogues of choline that are also substrates for choline acetyltransferase and are acetylated to acetylcholine analogues have enabled the cytosolic pool and that in the recycling vesicles to be differentially labelled.⁴¹ The possibility of doing this depends on the fact that the uptake of acetyl-

Fig. 4 Experiments illustrating the incorporation of false transmitters into the fraction (VP_2) of recycling vesicles and their release from this fraction.

Blocks of electric organ were loaded with 3H -homocholine and ^{14}C -choline after having been depleted of endogenous transmitter by stimulation through the nerve at 1 Hz for 30 min. On restimulation (10 Hz for 5 min) 8 h later in the presence of paraoxon (to stabilize released esters) and hemicholinium-3 (to inhibit reuptake of label), both radioactive labels were released, 3H as a mixture of homocholine and acetylhomocholine, ^{14}C as acetylcholine (a).

The label was incorporated exclusively in the fraction of recycling vesicles (b).

The ratio in which the true and false transmitters were released (c, block R) is the same as in the fraction of recycling vesicles (c, block V) and much lower (because of preferential vesicular uptake of acetylcholine relative to acetylhomocholine plus homocholine) than in whole tissue (c, block T). By contrast, the ratio in which the labels were released during the pre-stimulation resting period (c, left) is much higher than even the tissue ratio, reflecting a preponderance of homocholine and acetylhomocholine relative to acetylcholine in the cytoplasm.

The tissue ratio observed is consistent with a 25% cytoplasmic acetylcholine pool and a 77% cytoplasmic homocholine plus acetylhomocholine pool. In other experiments, it was deduced that the proportion of acetylcholine that is cytoplasmic in resting tissue without any loading stimulus is 22%.

Blocks are mean values of eight experiments; bars are SEMs.

choline into vesicles is carrier mediated and that the carrier has its own, distinctive specificity, different from that of choline acetyltransferase. Homocholine is one of several suitable choline analogues (Fig. 4). Both it and its acetylated product are taken up into recycling vesicles, but the ratio of homocholine to acetylhomocholine in the vesicular pool is much lower than that in the cytosol. At rest the analogues leak out of labelled tissue in the same ratio as they are found in the cytosol. On stimulation, however, the ratio in which the two transmitter analogues are released is that in which they are present in the vesicles and not that of the cytosol. The only way the proponents of the cytosolic release theory can accommodate this result would be to postulate that their hypothetical carrier changes its specificity on stimulation to that of the vesicular storage mechanism (or that new carriers are activated by stimulation). These assumptions seem far-fetched.

Recently the compound AH 5183, which in nM concentrations blocks the uptake of acetylcholine into vesicles⁴² has been used to block vesicle recycling in perfused tissue. Under these circumstances, recycled vesicles are unable to take up labelled cytosolic acetylcholine (*i.e.* recycling is effectively blocked) and the continued release of transmitter under these circumstances depends on the recruitment of the reserve pool.⁴³ The transmitter released is unlabelled; since it is inconceivable that any cytosolic gate could distinguish between labelled and unlabelled transmitter, the released transmitter *must* have been released directly from the reserve pool of vesicles *via* exocytosis.

Original of cholinergic synaptic vesicles

Except perhaps for a limited protein-synthetic activity by terminal mitochondria, it is generally accepted that the nerve terminal is dependent on the neuronal perikaryon for the synthesis of its proteins and organelles. In other systems there is evidence that synaptic vesicles are made in the cell body and conveyed to the terminal by axonal transport. The perikarya of the electromotor neurones—which are among the largest motor neurones found in vertebrates—are packed with rough and smooth endoplasmic reticulum, polysomes and vesicles. Calculations show that each perikaryon has to maintain some 30–50 times its own volume of nerve terminal.

As mentioned earlier, synaptic vesicles contain a specific proteoglycan of the heparan sulphate type. This can be detected immunochemically, immunocytochemically and by labelling *in vivo* with ³⁵S injected into the electric lobe. These techniques have been recently used to follow the synthesis of cholinergic synaptic vesicles in the cell bodies of the electromotor neurones and their export via the axon hillock and the nerve axons to the terminals in the electric organ. Fig. 5a shows punctate immunofluorescence staining of synaptic vesicles in the axon hillock area of an electromotor perikaryon awaiting export down the axon;⁴⁴ Fig. 5b shows the accumulation of stain above

Fig. 5 (a) An electromotor neurone cell body immunocytochemically stained with an antiserum raised against synaptic vesicle proteoglycan showing the distribution of synaptic vesicles in the perikaryon. The cell outline is indicated by the dashed line. Note the high concentration of vesicles in the axon hillock awaiting axonal transport. (b) Accumulation of immunoreactive material in electromotor axons above a ligature (between broad arrows). (c, d) Immunoreactive material is concentrated at the innervated faces of electrocytes but the amount present is reduced by ligation of the axon (c).

ligature and Figs. 5c and d its presence in the nerve terminal (c) before and (d) after ligation; the reduced staining reaction following ligation is apparent.⁴⁵ Fig. 6a shows the distribution after separation on a zonal density gradient of covalently bound particulate ^{35}S extracted from nerve trunks while the wave of rapidly transported ^{35}S was passing down the axon following the injection of $^{35}\text{SO}_4^{2-}$ into the electric lobe.^{46,47} Fig. 6b,c and d shows the corresponding distribution ^{35}S after extraction of vesicles from the electric organ, 18, 32 and 46 h after injection of ^{35}S into the lobe. It will be seen that initially the main peak of the highly sulphonated labelled vesicular proteoglycan does not coincide with that of the classical vesicle marker ATP; after 32 h it does. At the intermediate time points the labelled proteoglycan is bimodally distributed. It can be concluded from this experiment that vesicles arriving at the nerve terminal from

Fig. 6 Separation of synaptic vesicles on a continuous density gradient in a zonal rotor (a) 12 h, (b) 18 h, (c) 32 h, after injecting $^{35}\text{SO}_4^-$ into the lobe. Vehicles were extracted from the frozen and crushed nerves (a) or electric organs (b, c, d). Note that 18 h, the main (VP_0) peak of ^{35}S (squares) in the gradient of terminal material has the same density as that of axonal material at 12 h but by 23 h, the ^{35}S -labelled material mainly copurifies with the classical synaptic vesicle marker ATP (triangles). For explanation see text.

the cell body have a low ATP (and acetylcholine) content and—perhaps because of this—a low density. After some hours the vesicles become loaded with acetylcholine and acquire the density of reserve vesicles.^{46,47}

Conclusions concerning vesicle recycling

Our conclusions regarding the transmitter pools and the recycling of synaptic vesicles are summarized in Fig. 7. Vesicles are generated in the cell body and are conveyed by rapid transport to the terminal when they acquire acetylcholine and join the pool of reserve vesicles. On stimulation, some of these are included to undergo exocytosis, a process triggered by the ingress of free Ca^{2+} into the terminal. The molecular mechanisms involved in exocytosis are as yet poorly understood in any system. In the terminal it is a transient event and is followed by the retrieval of the vesicle in a functional state. The empty vesicle rapidly refills, perhaps by a process involving the exchange of inorganic cations acquired during exocytosis for cytosolic

Fig. 7 Scheme showing the organization of the cholinergic nerve terminal as deduced from the experimental work described in this report. As shown by immunocytochemistry and covalent ^{35}S -labelling,^{46,47} synaptic vesicles are formed in the cell body and transported—largely empty—to the terminal (VP_0). Here they fill with cytoplasmic acetylcholine (ACh_c) and enter the pool of *reserve vesicles* (VP_1). On stimulation a proportion of the VP_1 vesicles are recruited into the recycling (VP_2) pool.^{31–35} Such vesicles only partially refill from the cytoplasm and undergo partial osmotic dehydration, becoming smaller and denser.^{24,36–38} At rest, the recycled pool slowly takes up more acetylcholine and reacquires the biophysical properties of the reserve pool.^{33,35,36} By contrast, there is little or no direct exchange between reserve vesicles and the cytoplasm.^{27,28}

Cytoplasmic acetylcholine is subject to 'futile recycling' (black arrows). Transmitter leaking out of the terminal (ACh_0) is rapidly hydrolysed by acetylcholinesterase (ChE) in the cleft and the acetate (Ac_0) and choline (Ch_0) are salvaged; in both cases uptake is facilitated by carriers (AcT , ChT). Cytoplasmic choline (Ch_c) and acetate (Ac_c) are resynthesized to cytoplasmic acetylcholine by the soluble enzyme choline acetyltransferase (ChAT); acetate must however be first converted to acetylcoenzyme A (AcCoA).

The cytoplasmic and VP_2 pools of transmitter can be specifically labelled by means of false transmitters and thus cytoplasmic release and that brought about by vesicle recycling can be readily distinguished (Fig. 4).⁴¹ A similar cycle exists for synaptic vesicle ATP.⁴⁸ There is no evidence yet for antidromic transport of 'worn out' vesicles, though by analogy with other systems this is likely to occur.

acetylcholine, but, since this refilling process is partial and results in a reduced osmotic load, the refilled vesicles lose water to the cytosol, shrink and become denser. They may recycle many times, but, at rest, they take up more acetylcholine and ATP (perhaps by utilizing the proton-gradient known to exist across their membranes²¹), re-acquire water from the cytoplasm and thus rejoin the population of reserve vesicles. Eventually synaptic vesicles must, like other organelles, 'wear out'. Just how such vesicles are disposed of, whether by leaving them unretrieved in the plasma membrane, which must then be adjusted in surface area by some other mechanism, such as membrane intake *via* coated vesicles, or by being passed back to the cell body by retrograde transport, remains obscure.

The model advanced in this article for vesicle recycling differs from earlier concepts in stressing the kinetic complexity of the system and the metabolic and functional heterogeneity of a class of organelles whose appearance in electron micrographs is deceptively uniform. However, such a model is more in line with what we know about cell biology in general and goes far to resolve paradoxes of transmitter synthesis, storage and release such as the preferential release of newly formed transmitter once felt to create difficulties for a vesicular model.

Presynaptic plasma membrane

Functions: The plasma membrane surrounding the nerve terminal is an extremely important part of the synapse. It performs, *inter alia*, the following functions:

- it possesses transporters which facilitate the uptake of energy metabolites (*e.g.* glucose) and transmitters (*e.g.* noradrenaline) or their precursors (*e.g.* tyrosine, choline) into the presynaptic nerve terminal;
- it contains receptors for substances regulating transmitter release (*e.g.* muscarinic receptors) and for trophic factors needed for synaptogenesis and synaptic maintenance;
- it contains ionic channels important for stimulus-secretion coupling (*e.g.* the Ca^{2+} channels);
- it interacts with the vesicle membrane during exocytotic (quantized) transmitter release;
- it possesses surface antigens important for the recognition of the target cell and in synaptogenesis.

Presynaptic plasma membranes have not so far been very intensively studied, yet they are easy to prepare from synaptosomes by osmotic lysis and fractionation of the lysate. Our own studies using synaptosomes isolated from electric organ have so far concentrated on two aspects, the high-affinity choline transporter and a cholinergic-specific surface antigen, Chol-1.

High-affinity choline transporter: Fragments of plasma membrane prepared from

electromotor synaptosomes readily reseal to form membrane-bound vesicles (Fig. 8a). When an outwardly directed K^{2+} and inwardly directed Na^{+} gradient is imposed across the membrane, choline is rapidly taken up (Fig. 8b), but leaks out again as the ionic gradient is dissipated.⁴⁹ The initial rate of uptake is a measure of the concentration of the transporter in the membrane. The transporter has been solubilized and reconstituted in an artificial membrane.⁴⁹ The reconstituted system has the same characteristics as the native transporter: the uptake is hemicholinium-sensitive, K^{+} , Na^{+} and Cl^{-} -dependent, and stimulated by valinomycin. Cl^{-} can be partially replaced by Br^{-} and K^{+} by Rb^{+} but the requirement for Na^{+} is absolute.

In more recent work,⁵⁰ the transporter has been labelled with radioactive choline aziridium mustard, an analogue of choline which is a good substrate for the transporter but reacts covalently with it at elevated temperatures. This is possible because of the labile three-member aziridium ring (Fig. 8c) in this analogue. Three proteins were labelled: one, the labelling of which was Na^{+} -dependent and blocked by hemicholinium, is assumed to be the choline transporter; it has an isoelectric point of 5.1 and a molecular mass of 45–50 kDa (Fig. 8d). The two others may be choline acetyltransferase and choline kinase, both of which have choline recognition sites and should therefore readily interact with the analogue. In future work an attempt will be made to sequence the molecule using the cDNA technique.

Cholinergic-specific surface antigens: Antisera have been raised in sheep to electromotor presynaptic plasma membranes.⁵¹ These induce the selective, complement-mediated lysis of the cholinergic subpopulation of mammalian brain synaptosomes,^{52,53,54} thus indicating the presence, on the surfaces of electromotor and central mammalian cholinergic nerve terminals of evolutionarily conserved common epitopes specific for cholinergic neurones (Fig. 9a). The antigens involved are gangliosidic in nature⁵³ and are collectively designated Chol-1; on applying the antiserum to total guinea-pig brain gangliosides after separation on a TLC plate, it recognizes two minor gangliosides,⁵⁵ designated Chol-1 α and β . One of these (β) runs just behind the main GT band, the other (α) below GQ (Fig. 9b). Electric organ contains additional immunoreactive gangliosides. The anti-Chol-1 titre of the antiserum can be raised nearly 10-fold by passing it through a column of immobilized *Torpedo* electric organ gangliosides and eluting the adsorbed anti-Chol-1 antibodies with potassium thiocyanate.⁵⁵ This concentrated anti-Chol-1 antiserum has provided a new immunocytochemical method for studying the distribution of cholinergic neurones in the mammalian central and peripheral nervous systems.⁵⁶

The cholinergic subpopulation of mammalian brain synaptosomes has been purified by adsorbing anti-Chol-1 antiserum-treated synaptosomes onto a column of immobilized high-titre anti-sheep-IgG monoclonal antibody.⁵⁴ The adsorbed synaptosomes show a

Fig. 9 (a) Induction of selective complement-mediated lysis of cholinergic synaptosomes. The guinea-pig brain cortical synaptosomes were labelled with a variety of radioactive transmitters (glutamate, Glu; γ -aminobutyrate, GABA; noradrenaline, NA; dopamine, DA; 5-hydroxytryptamine, 5-HT) and a cytoplasmic marker (deoxyglucose, DOG) and treated in the presence of complement with either the anti-Chol-1 antiserum (black blocks) or an anti-cortex antiserum (white block). The anti-Chol-1 serum lyses only 6% of the synaptosomes (DOG release), none of the non-cholinergic transmitters, but 60% of the cholinergic marker choline acetyltransferase (CAT), indicating a selective attack on the cholinergic subpopulation. In other experiments, up to 90% of CAT was released. By contrast, the anti-cortex antiserum (right) induces the lysis of the total synaptosome population (90% release of DOG) in a non-selective way. Results from ref 54.

(b) 1, 2. Separation of total (T) and polysialo (F_p) guinea-pig brain gangliosides by TLC. In 1, the gangliosides are revealed by Ehrlich stain and the main classes identified. In 2, two faintly Ehrlich-positive gangliosides (Chol-1 α and β) are shown to react strongly with anti-Chol-1 serum; the component running behind GQ being enriched in F_p. In 3, 250 µg of T and 10 µg of F_p are shown to inhibit the CAT-releasing action of the anti-Chol-1 antiserum in presence of complement, presumably due to competition between free and membrane-bound, synaptosomal Chol-1 for antiserum. Only membrane-bound antiserum can induce complement-mediated lysis.

close to theoretical enrichment of cholinergic markers and, interestingly, the enrichment of VIP to an extent suggesting that some 75% of brain VIP is localized in cholinergic neurones (Agostone, D. V., *et al.*; submitted for publication)—a figure consistent with the existing immunocytochemical data.

Developmental studies^{57,58} indicate that Chol-1 begins to be expressed at the time that functional synaptogenesis takes place. There is also an increase in Chol-1 in the dentate gyrus of the hippocampus during the 'reactive synaptogenesis' induced in the cholinergic input by section of the non-cholinergic ipsilateral entorhinal input.⁵⁹ Although an earlier expression of Chol-1 in amounts too small to be detected by our current methods cannot be ruled out, we take these results to mean that Chol-1 is less likely to be involved as a receptor for trophic factors operating during development or as a recognition molecule, than as a necessary component for the consolidation of a synaptic contact already provisionally formed. Chol-1 may be needed to provide the correct lipid environment for a presynaptic plasma membrane receptor interacting with elements in, or attached to the postsynaptic membrane. This role for gangliosides has been proposed in the case of cell-cell adhesion.⁶⁰

If indeed cholinergic synaptogenesis can be regulated by the expression of Chol-1, a new and powerful mechanism underlying synaptic plasticity may have been uncovered. Since central cholinergic connexions may well play an important role in learning and memory, would it be too fanciful to suggest that Chol-1 could be a kind of 'memory molecule'?

Chol-1 may also play a role in the aetiology of neuropathies such as Alzheimer's Disease and amyotrophic lateral sclerosis (ALS), a disease marked by a primary degeneration of the cholinergic system. Recent work⁶¹ indicates that in ALS, degeneration of the motor neurones of the ventral horns of the human spinal cord is accompanied by the loss of Chol-1 immunoreactivity. However, it has not been possible so far to detect circulating anti-Chol-1 antibodies in such patients or to establish a primary involvement of Chol-1 in the aetiology of these diseases.

The work that has been described in this report will serve to show how the intensive investigation of a model system can lead to a detailed understanding of the structure and function of the cholinergic synapse at the molecular level and uncover hitherto unsuspected mechanisms of potential importance for human medicine.

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