Neurotransmitters, Drugs and Brain Function.
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4 Neurotransmitter Release

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INTRODUCTION

Release of transmitter from a neuron is triggered by the arrival of a propagated nerve impulse at its terminals. This wave of excitation causes the opening of voltage-gated Ca^{2+}-channels or mobilisation of Ca^{2+} from intracellular stores (e.g. the endoplasmic reticulum or 'calcisomes'). As a result, there is a phasic increase in free intracellular Ca^{2+}, probably to a concentration of about 0.1–1 mM, in regions of the terminal adjacent to the site of transmitter release (the 'active zone'). The subsequent fusion of neurotransmitter storage vesicles with the axolemma, together with the extrusion of their contents into the synapse, is thought to take about 100–200 μs; this cascade is therefore fast enough to effect rapid signalling between neurons.

While this chapter is concerned primarily with the neurochemical mechanisms which bring about and control impulse-evoked release of neurotransmitter, some of the methods used to measure transmitter release are described first. This is because important findings have emerged from studies of the effects of nerve stimulation on gross changes in transmitter release and intraneuronal stores. The actual processes that link neuronal excitation and release of transmitter from nerve terminals have been studied only relatively recently. The neurochemical basis of this stimulus–secretion coupling, which is still not fully understood, is described next. The final sections will deal with evidence that, under certain conditions, appreciable amounts of transmitter can be released through Ca^{2+}-independent mechanisms which do not depend on neuronal activation.

MEASUREMENT OF TRANSMITTER RELEASE

ESTIMATION OF TRANSMITTER TURNOVER *EX VIVO*

Until the development of sensitive assays and sophisticated collection techniques, release studies relied on measuring changes in the concentration of neurotransmitters in whole organs, or dissected brain regions, following nerve stimulation. However, under resting conditions, the transmitter content of any given organ or brain region is remarkably constant. The store of classical transmitters (monoamines and acetylcholine) in nerve terminals is rarely depleted by physiologically relevant rates of neuronal stimulation. This suggests that transmitter synthesis normally keeps pace with release. It follows that the rate at which the store of transmitter is replaced ('turnover rate') can be used to estimate its rate of release. Although this approach is rarely used nowadays, it is outlined here because it uncovered some important findings which are relevant to current studies of the regulation of transmitter release.

The turnover rate of a transmitter can be calculated from measurement of either the rate at which it is synthesised or the rate at which it is lost from the endogenous store. Transmitter synthesis can be monitored by administering [^{3}H]- or [^{14}C]-labelled precursors *in vivo*; these are eventually taken up by neurons and converted into radiolabelled product (the transmitter). The rate of accumulation of the radiolabelled transmitter can be used to estimate its synthesis rate. Obviously, the choice of precursor is determined by the rate-limiting step in the synthetic pathway: for instance, when measuring catecholamine turnover, tyrosine must be used instead of *l*-DOPA which bypasses the rate-limiting enzyme, tyrosine hydroxylase.

One limitation of this method is that the specific activity of the radiolabel is progressively diluted as the radiolabelled transmitter is released from neurons and replaced by that derived from unlabelled substrate. This method also assumes that there is no compartmentalisation of the terminal stores, yet there is ample evidence that newly synthesised acetylcholine and monoamines are preferentially released. An alternative approach is to monitor the rate at which the store of neurotransmitter is depleted after inhibition of its synthesis (Fig. 4.1). However, the rate of release of some neurotransmitters (e.g. 5-HT) is partly governed by their rate of synthesis and blocking synthesis blunts release.

It is already evident that the turnover rate of a transmitter is only a crude measure of its release rate. Further limitations are that there is appreciable intraneuronal metabolism of some neurotransmitters: notably, the monoamines. In such cases, turnover will overestimate release rate. Another problem, again affecting monoamines, is that some of the released neurotransmitter is taken back into the nerve terminals and recycled. This leads to an underestimate of release rate. Despite these drawbacks, studies of turnover rates uncovered some important features of transmitter release. In particular, they provided the first evidence for distinct functional pools of monoamines, acetylcholine and possibly other neurotransmitters: a 'release' pool, which could be rapidly mobilised for release, and a 'storage' or 'reserve' pool which had a slower turnover rate.

TECHNIQUES *IN VITRO*

In situ preparations

Many early studies of transmitter release depended on measuring its concentration in the effluent of a stimulated, perfused nerve/end-organ preparation. This technique is still widely used to study drug-induced changes in noradrenaline release from sympathetic neurons and the adrenal medulla. However, it is important to realise that the concentration of transmitter will represent only that proportion of transmitter which escapes into the perfusate ('overflow') (Fig. 4.2). Monoamines, for instance, are rapidly sequestered by uptake into neuronal and non-neuronal tissue whereas other transmitters, such as acetylcholine, are metabolised extensively within the synapse. Because of these local clearance mechanisms, the amount of transmitter which overflows into the perfusate will depend not only on the frequency of nerve stimulation (i.e. release rate) but also on the dimensions of the synaptic cleft and the density of innervation.

Synaptosomes

Synaptosomes are 'pinched-off' nerve terminals which become severed from the parent axon during gentle homogenisation of brain tissue and then subsequently reseal. They

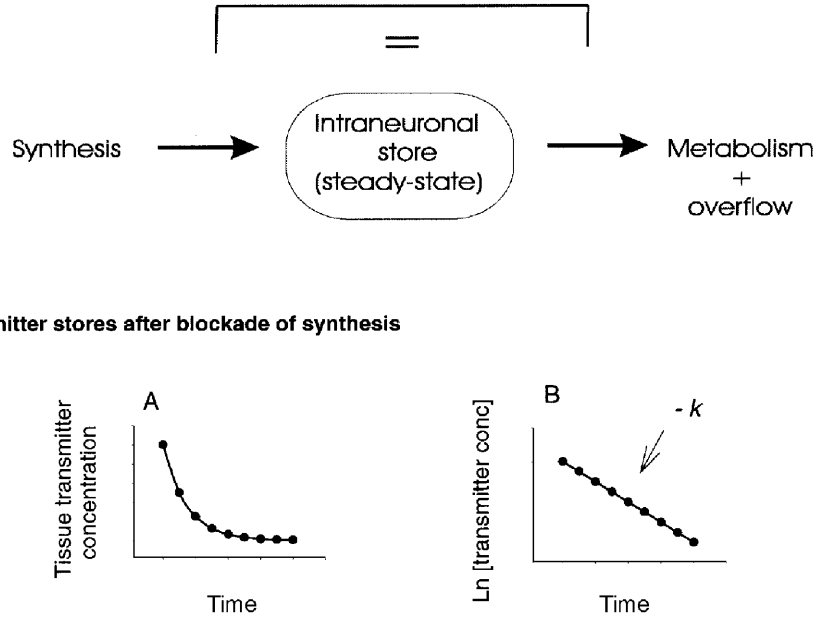

Figure 4.1 Turnover of classical neurotransmitters. At normal rates of neuronal activity, endogenous stores of neurotransmitter are maintained at constant (steady-state) levels, indicating that the supply of new neurotransmitter (through synthesis) meets the demand (determined by release and metabolism). Consequently, the rate of the depletion (A) of the endogenous store of transmitter after inhibition of its synthesis indicates turnover rate and is described by the equation:

$$[\mathrm{T}] = [\mathrm{T}]_0 \mathrm{e}^{-kt}$$

where [T] is the tissue concentration at time t; $[\mathrm{T}]_0$ is the transmitter concentration at time 0; and k is the rate constant for the efflux of transmitter. When plotted semi-logarithmically (B), the exponential decline in tissue stores of transmitter gives a straight line described by the equation:

$$\ln[\mathrm{T}] = \ln[\mathrm{T}]_0 - kt$$

At steady-state there is no net loss of transmitter from the system and so the rate of synthesis of transmitter equals the rate of its efflux. Thus:

$$\text{turnover rate (TOR)} = k \times \text{tissue transmitter concentration at time 0}$$

$$\mathrm{TOR} = k[\mathrm{T}]_0$$

For monoamines, turnover rate is only an approximate measure of release rate because of recycling of released transmitter and spontaneous metabolism of the endogenous store

are separated as a crude synaptosomal pellet from undisrupted tissue by differential centrifugation of the brain homogenates (Fig. 4.3). After resuspension of the synaptosomal pellet, they are perfused with artificial cerebrospinal fluid (aCSF) and the concentration of transmitter in the effluent used as an index of its release.

The main advantage of using synaptosomes is that they are free from any influence of the parent axon. Another is that, since the volume of extracellular space (the incubation medium) is functionally infinite, transmitter will not accumulate near the synaptosomes. This means that reuptake of released transmitter is unlikely to occur and that, under drug-free conditions, transmitter release will not be modified by activation of auto- or heteroceptors (see below).

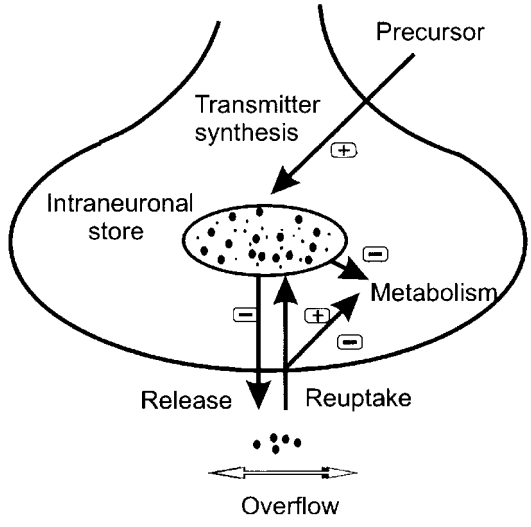

Figure 4.2 The intraneuronal stores of monoamines are maintained by synthesis from precursors taken in with the diet. The pool is depleted by release of transmitter and some spontaneous metabolism of intraneuronal transmitter. Released monoamines are inactivated by reuptake on membrane-bound transporters. Following reuptake, some transmitter might be recycled while the remainder is metabolised. Some transmitter escapes the reuptake process and overflows from the synapse in the extracellular fluid

A disadvantage of using synaptosomes is that they cannot be used to study transmitter release evoked by propagated nerve impulses, but the release, like that from intact neurons, is Ca^{2+}-dependent and K^{+}-sensitive. Pharmacological studies using synaptosomes have also provided evidence that the amount of transmitter that is released following their depolarisation is regulated by the activation of presynaptic receptors.

If the amount of transmitter released into the effluent is too small to be measured by a conventional neurochemical assay, the endogenous store can be 'tagged' by incubating the synaptosomes with radiolabelled transmitter (usually ^{3}H). After washing the synaptosomes, the release of ^{3}H into the perfusate is then measured using (extremely sensitive) liquid scintillation counting. This approach rests on the assumption that the radiolabelled transmitter is taken up only by the 'right' synaptosomes (e.g. that only those derived from noradrenergic neurons will take up [^{3}H]noradrenaline) which is not always the case. Another unjustified assumption is that the radiolabelled transmitter mixes freely with the endogenous (unlabelled) store. Finally, as it is the radiolabel, rather than the actual transmitter itself, which is being measured it must be established that the label remains attached to the neurotransmitter. For instance, [^{3}H]glutamate can be turned into [^{3}H]GABA or [^{3}H]glutamine and so care must be taken to ensure that any findings with ^{3}H refer to release of [^{3}H]glutamate rather than that of its metabolites.

Brain slices

These can be conventional cross-sectional slices up to 0.5 mm thick, or small cubes (0.1–0.5 mm on each plane) or pyramids of brain tissue. The main advantage of using slices is that, either by crudely passing currents across them or, in some cases, stimulating a

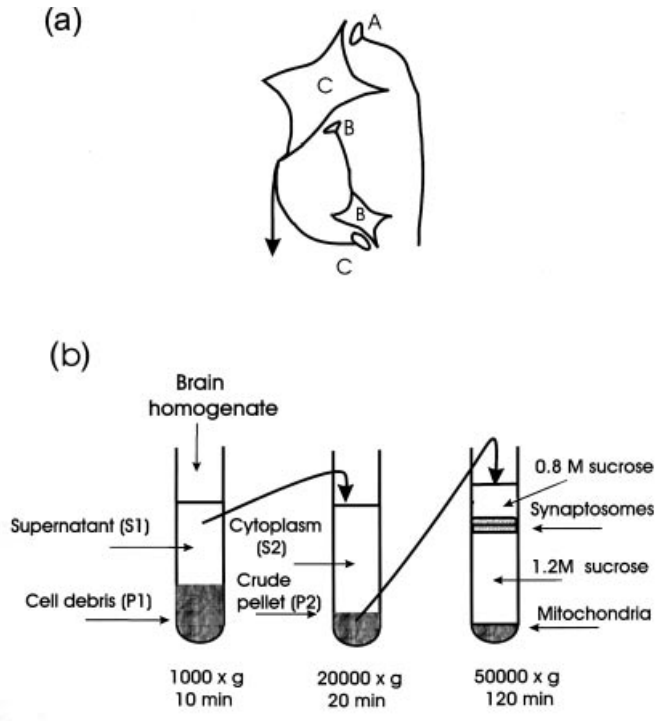

Figure 4.3 The distribution of neurotransmitter in different subcellular fractions. (a) Within the hypothetical brain area shown, where it is assumed that this neuronal arrangement is reproduced many times, the proportion of each neurotransmitter found in the synaptosomes, rather than the cell body (cytoplasmic) fraction, will vary considerably. On the assumption that, although concentrated in nerve terminals, the neurotransmitter will also be found in cell bodies and axons, it is likely that A will be almost entirely in synaptosomes, C mostly in the cytoplasm, while B will be more evenly divided. (b) Procedure for preparation and separation of synaptosomes. Brain tissue is homogenised and spun at $1000 \times g$ for 10 min to remove cell debris. The supernatant (S1) is spun at $20\,000 \times g$ for 10 min and the pellet (P2), containing synaptosomes and mitochondria, is spun through two layers of sucrose for 2 h at $50\,000 \times g$. This is 'sucrose density gradient centrifugation' and is based on the principle that an individual particle will settle in the zone of the sucrose gradient that has the same density. Thus, synaptosomes separate from other elements of the $20\,000 \times g$ pellet and settle at the interface between 0.8 M and 1.2 M sucrose

specific neuronal tract, they can be used to study impulse-evoked release of transmitter. Because the three-dimensional integrity of the tissue is maintained, they can also be used to study modulation of transmitter release by heteroceptors (see below).

One approach, and the first to be adopted, is to study transmitter release from slices which have been preloaded with radiolabelled transmitter. In these experiments, drug-induced changes in the release of transmitter is usually monitored using the 'double-pulse' technique. This involves comparing the effects of a test drug on the amount of transmitter released in response to a reference pulse and a second identical test pulse. If all the radiolabelled transmitter that overflows in the effluent is collected, and the amount which remains in the slice at the end of the experiment is also measured, it is possible to calculate not only how much radiolabelled transmitter was originally contained in the slice but also the effects of drugs on 'fractional release', i.e. the proportion of the store of radiolabelled transmitter which is released by nerve stimulation. As with

synaptosomes, however, it cannot be assumed that incubation of slices in a medium containing radiolabelled transmitter results in its even distribution throughout the slice. Also, the problem of continuous dilution of the radiolabelled store with newly synthesised (unlabelled) transmitter must be borne in mind.

Modern sensitive chromatographic and voltammetric techniques now make it possible to estimate the release of unlabelled endogenous transmitter from slices of brain tissue (commonly the hippocampus and striatum) or spinal cord (Fig. 4.4). However, whatever analytical method is used, the thickness of the slice is paramount. It is important to maintain the balance between preserving the integrity of the tissue (the thicker the slice, the better) against maintaining tissue viability by perfusion with oxygenated aCSF (the thinner the slice, the better).

TECHNIQUES *IN VIVO*

The cortical cup

The cortical cup has been used for many years to monitor changes in transmitter release induced by physiological and pharmacological stimuli (Fig. 4.5). In the past, it was used most commonly to study release of amino acids and acetylcholine. More recently, it has

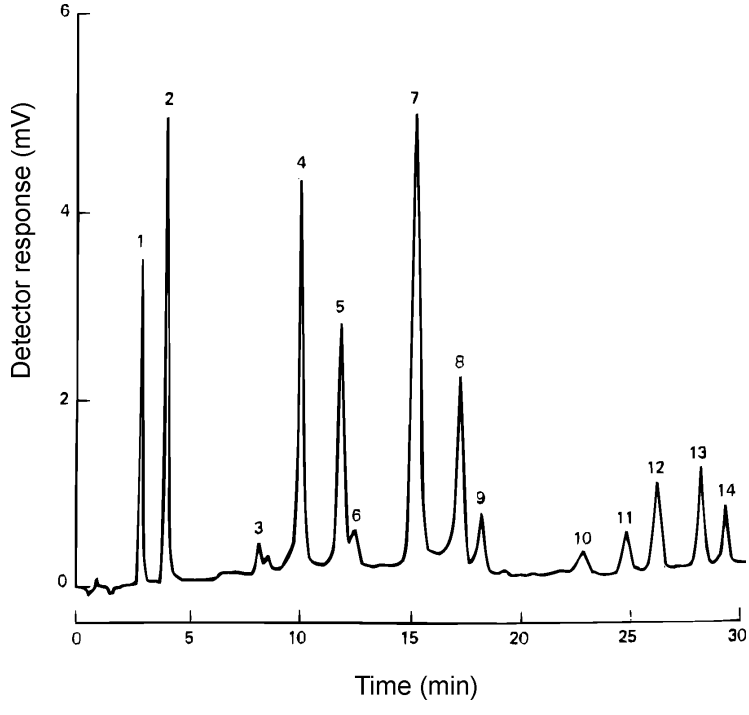

Figure 4.4 Release of amino acids from cortical slices exposed to 50 mM K^+. Measurements by HPLC and fluorescence detection after reaction of amino acids with *o*-phthalaldehyde: 1, aspartate; 2, glutamate; 3, asparagine; 4, serine; 5, glutamine; 6, histidine; 7, homoserine (internal standard); 8, glycine; 9, threonine; 10, arginine; 11, taurine; 12, alanine; 13, GABA; 14, tyrosine. Glutamate concentration is almost 1 pmol/μl which represents a release rate of 30 pmol/min/mg tissue

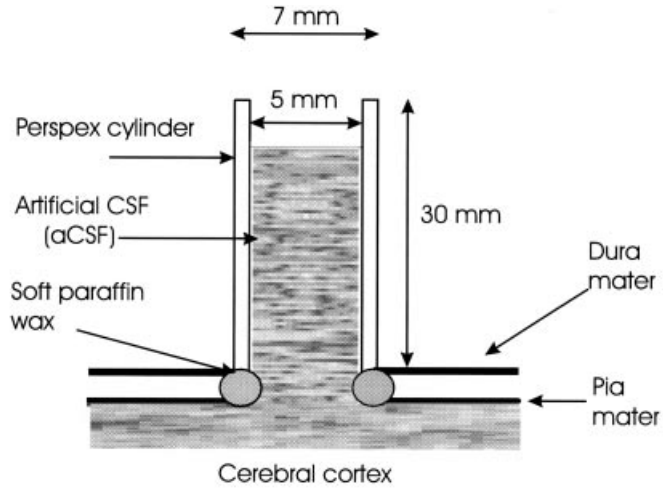

Figure 4.5 The cortical cup. The cup size obviously varies with the animal on which it is used. For the rat, the overall diameter is 6–7 mm and a typical flow rate is 50 µl/min

been adopted in studies of peptide and purine release as well (e.g. Phyllis, Smith-Barbour and O'Regan 1996). The cup comprises a 'well', constructed above a small hole drilled in the skull, which contains aCSF. Transmitters released from neurons near the surface of the brain accumulate in the cup and their concentration in the aCSF can be used as a crude index of release rate. The importance of this technique is that it enabled, for the first time, the monitoring of transmitter release in freely moving animals.

Microdialysis

This is a modification of the earlier push–pull cannula which could be used in anaesthetised animals only. The microdialysis probe which has an outside diameter of about 250 µm (Fig. 4.6) is implanted into the brain under anaesthesia and then subsequently perfused with aCSF. Solutes (including neurotransmitters) in the extracellular fluid of the brain diffuse down their concentration gradient into the probe. By taking samples of the effluent dialysate at regular intervals it is possible to monitor changes in transmitter release. This technique has been used for several years to study release of monoamines (e.g. Sharp, Umbers and Gartside 1997) but is now used to harvest acetylcholine and amino acids as well. Since the molecular cut-off of the dialysis membrane is in the region of 6–20 kDa (depending on the type of membrane used), this technique can also be used to measure release of some small neuropeptides (e.g. oxytocin and vasopressin).

One advantage of microdialysis is that it enables the study of transmitter release in specific brain areas or nuclei. To ensure its correct placement, the probe is implanted, under anaesthesia, by sterotaxic surgery. Another advantage is that the probe can be anchored in place with dental cement and experiments carried out later, in conscious freely moving animals once they have recovered from the anaesthetic. Indeed, comparison of results from studies carried out on both anaesthetised and freely moving subjects has revealed drug interactions with anaesthetics that can affect transmitter release: anaesthetic-induced changes in the regulation of noradrenaline release by α_2-adrenoceptors is a case in point. It is also possible to carry out long-term

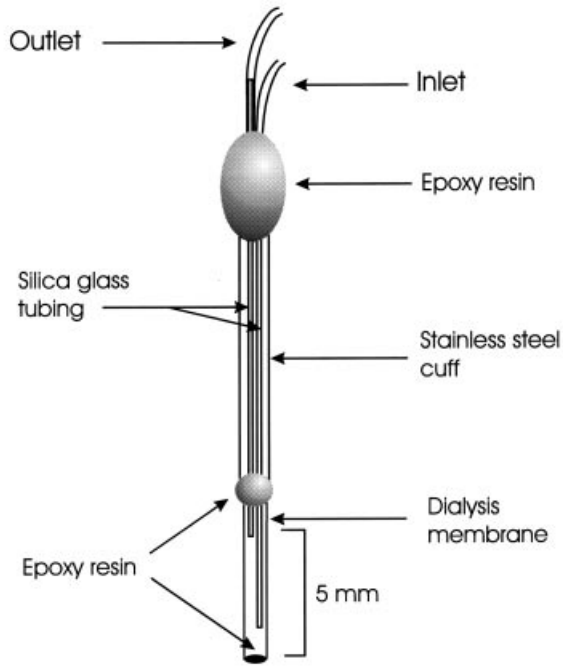

Figure 4.6 The tip of a microdialysis probe, expanded to show dialysis tubing around a steel cannula through the base of which fluid can flow out and then up and over the membrane. The length of membrane below the probe support can be altered (1–10 mm) to suit the size of the animal and the brain area being studied. Flow rates are normally below 2 µl/min

or repeated studies on the same animals but this requires a slight modification of the technique. In this case, a 'guide' cannula is first implanted, under anaesthesia, through which the microdialysis probe is inserted. Unfortunately, for a variety of reasons, each microdialysis probe can be used for only a few hours and so it has to be replaced each day. However, the presence of the guide cannula makes this a relatively straightforward process that requires only light sedation of the animal.

A further advantage of microdialysis is that, unlike the push–pull cannula or the cortical cup, the perfusion medium does not come into direct contact with the tissue being studied. This reduces damage caused by turbulence as well as enzymic degradation of the transmitter. For instance, acetylcholine, but not cholinesterase, will penetrate the probe membrane. Finally, because solutes will pass out of the probe, as well as into it, the probe can also be used for infusing ions (Fig. 4.7) or drugs into specific brain regions ('reverse' or 'retro-' dialysis) so that their local effects on transmitter release can be studied. This avoids many of the problems that arise when trying to determine the synaptic actions of drugs when these are administered systemically.

The rate at which the probes are perfused with aCSF is a compromise between the time required for the solutes in the CSF to reach equilibrium with those in the probe (the slower, the better) versus the ideal time-frame for studying changes in transmitter release (the shorter, the better). In general, flow rates of around 1–2 µl/min are used and the time which elapses between taking samples is determined by how much transmitter

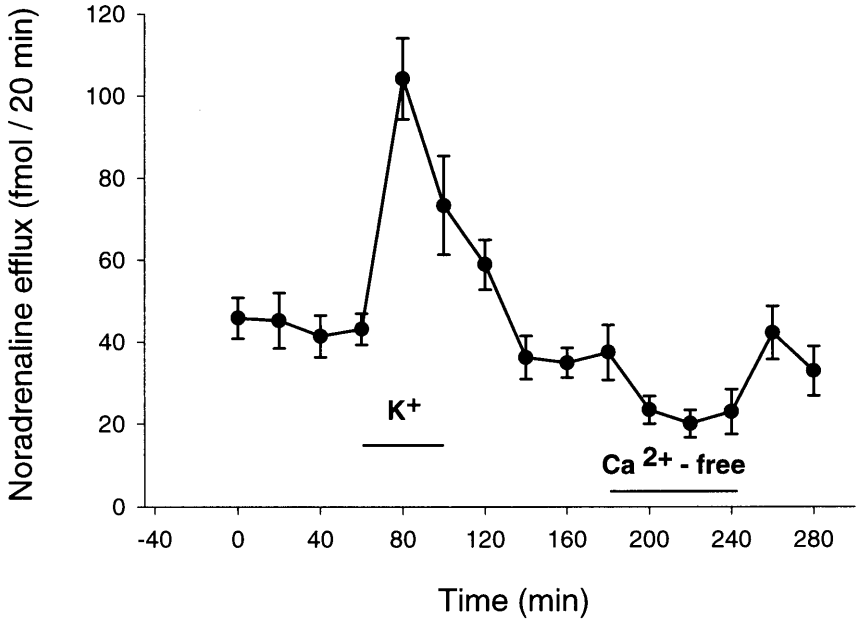

Figure 4.7 The effect of perfusion of the microdialysis probe with a medium containing a depolarising (80 mM) concentration of K^+, or Ca^{2+}-free medium, for the periods indicated by the bars. The graph shows efflux of noradrenaline in the frontal cortex of anaesthetised rats. Increasing the concentration of K^+ in the medium infused via the probe increases noradrenaline efflux whereas removing Ca^{2+} reduces it

is needed for analysis: i.e. by the sensitivity of the assay system. It is acknowledged that the solutes are not in equilibrium with the CSF outside the probe. In any case, the efficiency of the probe membrane limits the net influx (or efflux) of solutes to about 10–20% of the theoretical maximum. It should also be borne in mind that the microdialysis probe is not sampling the transmitter in the synapse: only that transmitter which escapes metabolism in, or reuptake from, synapses will migrate towards the probe. In the drug-free state, any change in the transmitter concentration in the dialysis samples is usually assumed to indicate a change in its rate of release from nerve terminals; this is supported by the spontaneous efflux of transmitters being Ca^{2+}-dependent and K^+-sensitive (Fig. 4.7). However, efflux does not always reflect release rate, especially if experimental interventions (e.g. infusion of monoamine uptake inhibitors) interfere with the clearance of transmitter from the synapse (Fig. 4.8).

Voltammetry

This can be carried out *in vitro* (in brain slices, cultured cell preparations) or *in vivo* and involves penetrating the experimental tissue with a carbon-fibre electrode of 5–30 μm in diameter (Fig. 4.9). This serves as an oxidising electrode and the Faradaic current generated by the oxidation of solutes on the surface of the electrode is proportional to their concentration. Obviously, only neurotransmitters which can be oxidised can be measured in this way so the technique is mainly limited to the study of monoamines and their metabolites. The amplitude of each peak on the ensuing voltammogram is a measure of solute concentration and individual peaks can be identified because different

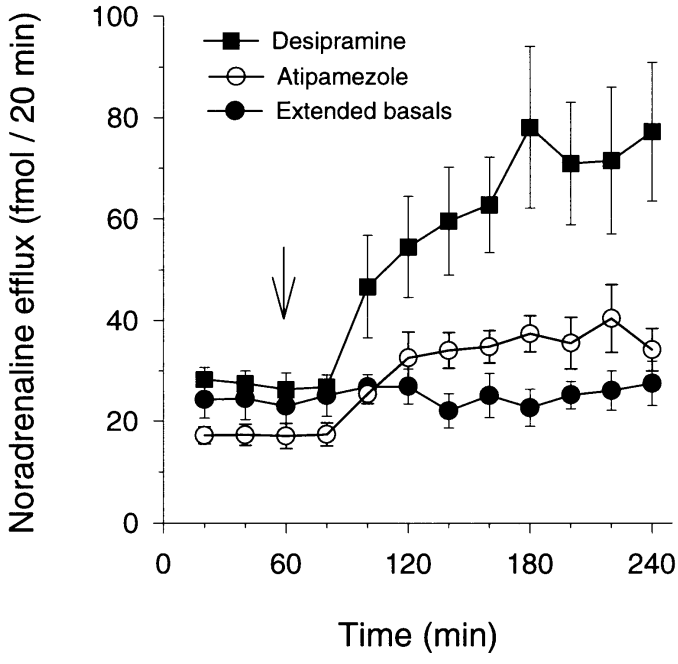

Figure 4.8 Noradrenaline concentration in dialysis samples from probes implanted in the rat frontal cortex. Spontaneous efflux of noradrenaline is stable throughout a 4 h sampling period ('extended basals') but is increased markedly when either the noradrenaline reuptake inhibitor, desipramine (5 μM), or the α_2-adrenoceptor antagonist, atipamezole (0.5 μM), is infused into the extracellular fluid via the microdialysis probe ('retrodialysis')

solutes oxidise at different potentials. Changes in the concentration of transmitters are monitored by rapid cycles of voltage scans (e.g. Palij and Stamford 1994). Since a complete scan takes only about 20 ms, the time resolution with voltammetry is much better than with microdialysis and is suitable for studying rapid, transient changes in transmitter release.

One difficulty with this method is that all oxidisable solutes in the external medium will be incorporated into the voltammogram and interfering peaks can be a problem. In fact, the concentration of monoamine metabolites and oxidisable solutes can be considerably greater than those of the parent amines which can be difficult to distinguish as a result. Ascorbic acid and uric acid are particularly problematic in this respect, although recent work suggests that an increase in the concentration of extracellular ascorbic acid could be a marker for the early phase of cerebral ischaemia. In general, voltammetry is most useful for measuring rapid (subsecond) changes in monoamine release. Under these circumstances, slower changes in the metabolites and other compounds do not interfere. Another problem is that the life of the electrode is limited by progressive 'poisoning' which diminishes its sensitivity. As a rule, voltammetric electrodes are best suited to 'acute' rather than 'chronic' measurements.

Biosensors

As the term suggests, the use of biosensors to measure transmitter release rests on exploiting a biological response which is proportional to the amount of transmitter in

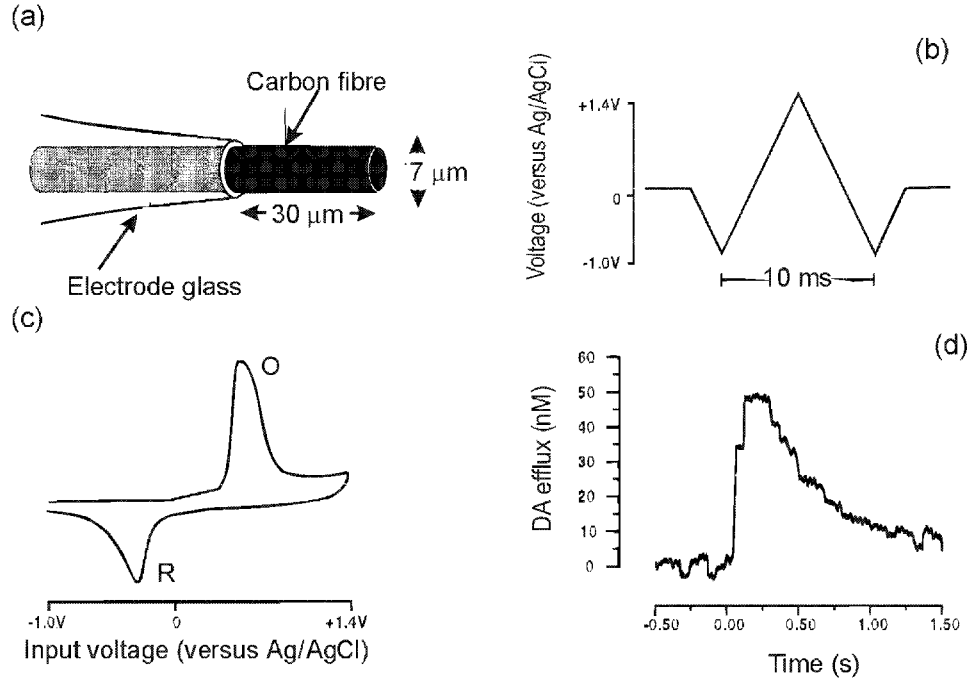

Figure 4.9 Basics of voltammetry. (a) The cut tip of the microelectrode surrounded by the glass insulation. (b) Input voltage waveform to the potentiostat (−1.0 to +1.4 V versus Ag/AgCl, 480 V/s). (c) Background current-corrected cyclic voltammogram of catecholamine obtained by plotting the Faradaic current against the input voltage waveform. The areas marked O and R are the oxidation and reduction currents. (d) Typical dopamine release and reuptake event following local electrical stimulation of a striatal slice. The trace is a plot of the oxidation peak height against time, calibrated for dopamine. (Figure and legend kindly supplied by J. A. Stamford)

the sample and which can be quantified. One of the earliest biosensors was the dorsal wall muscle of the leech which contracts in the presence of nM concentrations of acetylcholine. Others are the bioluminescent proteins, such as aequorin, which fluoresce in the presence of Ca^{2+}. Within a reasonable range, the fluorescence intensity is proportional to the cation concentration and so it can be used to monitor the increase in the intracellular concentration of Ca^{2+} during excitation of nerve terminals. More recently, biosensors have been developed which comprise electrodes coated with glucose oxidase or lactate oxidase. The activity of these enzymes generates a current that can be used to quantify the concentration of glucose and lactate on the surface of the electrode. This work is playing an important part in research on brain metabolism during neuronal activity. So far, these electrodes are used in 'on-line' assays of samples collected by microdialysis but might be adapted for measurements *in vivo* in the future.

WHERE DOES THE RELEASED TRANSMITTER COME FROM?

Two separate lines of research led to the proposal that transmitter released in response to neuronal excitation is derived from a vesicle-bound pool rather than from the neuronal cytoplasm. One piece of evidence came from electron microscopy which

showed that nerve terminals were packed with vesicle-like organelles (Fig. 4.10). Using differential centrifugation, these vesicles were soon identified as the major storage sites for neurotransmitters. The second was electrophysiological evidence that the effect of neuronal release of acetylcholine on the postsynaptic membrane potential at the neuromuscular junction was quantal in nature, suggesting that this transmitter, at least, was released in discrete packets.

Early neurochemical investigations of the source of released transmitter measured noradrenaline release from chromaffin granules in the adrenal medulla. Chromaffin granules are considerably larger (250 nm diameter) than the storage vesicles in noradrenergic nerve terminals (40–100 nm) and so their experimental use avoided the constraint imposed by the low sensitivity of early assay techniques (see Winkler 1993). Yet, like noradrenergic neurons, the adrenal medulla is derived from the developing neural crest and noradrenaline release is activated by stimulation of preganglionic cholinergic neurons. Chromaffin granules therefore provide a useful model for processes involved in the storage and release of noradrenaline from neurons. Subsequent refinements of assays for noradrenaline enabled studies of noradrenaline release to be extended to stimulated sympathetic nerve/end-organ preparations. These experiments confirmed that noradrenaline was released from vesicle-bound packets of transmitter contained within the terminal vesicles. This is because its release was paralleled by the

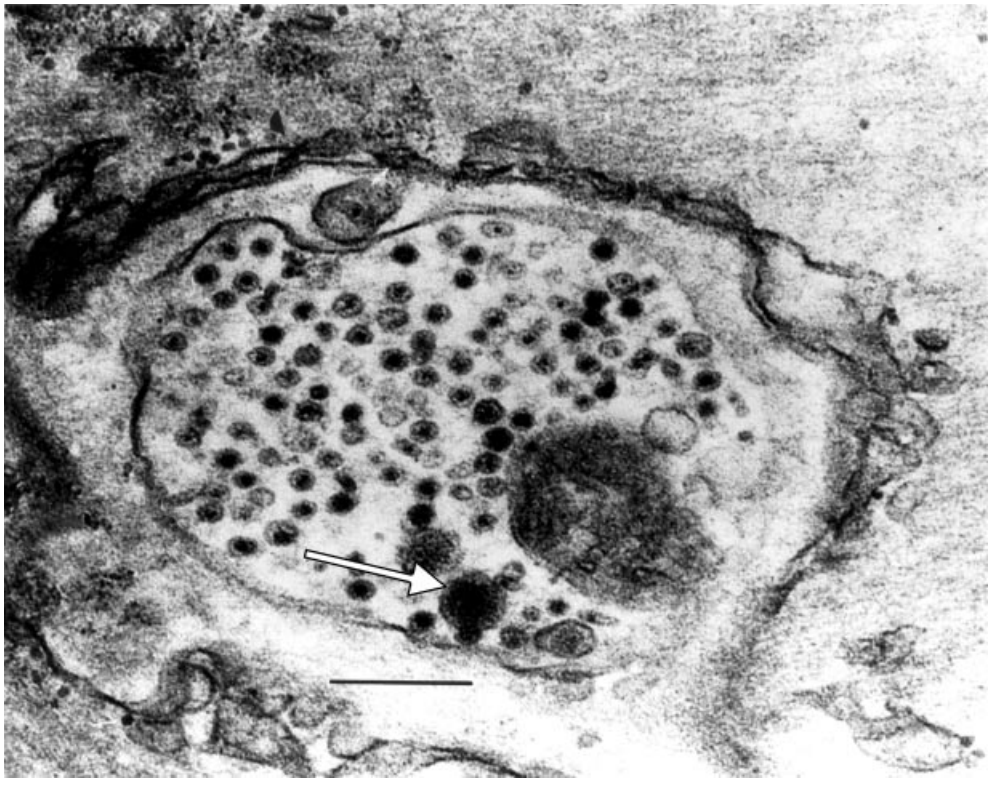

Figure 4.10 An electron micrograph of a terminal varicosity containing a large dense-core vesicle (LDCV), indicated by the arrow and many small synaptic vesicles (SSVs), some of which contain an electron dense core. Calibration mark: 250 nM. (Figure kindly supplied by M. Fillenz)

appearance of the proteins, dopamine-β-hydroxylase and chromogranins, which are found only in noradrenaline storage vesicles, whereas the cytoplasmic enzyme, lactate dehydrogenase, was not found in the perfusate.

Experiments of this kind have provided a great deal of evidence in favour of exocytotic release of vesicular noradrenaline. For example, by administering reserpine (which causes noradrenaline to leak out of the vesicles into the cytoplasm) together with an inhibitor of the enzyme monoamine oxidase (which will prevent metabolism of cytoplasmic noradrenaline), it is possible to redistribute the noradrenaline stored within nerve terminals because it leaks from the vesicles but is preserved within the neuronal cytoplasm. Under these conditions, the total amount of transmitter in the terminals is unchanged but impulse-evoked release rapidly diminishes.

Different evidence, mainly based on histological studies, suggested that acetylcholine is also released by vesicular exocytosis. Landmark experiments used a technique known as 'freeze–fracture' in which tissues are frozen rapidly during periods of intense transmitter release. It is then possible to fracture axolemma membranes in a way that separates their lipid bilayer. Electron microscopy reveals numerous pits in the membranes which are thought to reflect the vesicle/axolemma fusion pore of vesicles in the process of exocytosis. Subsequent studies, combining immunocytochemistry with electron microscopy, showed that proteins in the membranes of vesicles become incorporated into the axolemma during transmitter release. Furthermore, when neurons are stimulated in a medium containing an electron-dense marker, that does not penetrate the neuronal membrane, the marker later appears in vesicles inside the nerve terminals (Basbaum and Heuser 1979). This suggests that such markers are incorporated into the vesicles when they come into contact with the extracellular fluid during exocytosis. There is also some pharmacological evidence for exocytosis of acetylcholine. For instance, impulse-evoked release of this transmitter is prevented by the drug, vesamicol, which blocks uptake of acetylcholine from the cytoplasm into the terminal vesicles (Searl, Prior and Marshall 1991).

Although most evidence supports vesicular exocytosis of acetylcholine (see Ceccarelli and Hurlbut 1980), some researchers contest this view. An alternative suggestion is that an ATPase bound to the axolemma acts as a pore ('mediatophore'). According to this scheme, opening of the pore is triggered by an increase in the concentration of intracellular Ca^{2+} and allows gated release of aliquots of cytoplasmic acetylcholine. The vesicles are thought to serve merely as a reserve pool of transmitter and for sequestration of intracellular Ca^{2+} (Dunant 1994).

TRANSMITTER STORAGE VESICLES

Electron microscopy and biochemical techniques, such as sucrose density-gradient centrifugation (see Fig. 4.3), have enabled the characterisation of at least three types of vesicles in neurons. 'Large dense core vesicles' (LDCV: 75–100 nm), so-called because of their electron-dense core, contain mainly peptides and are thought to derive from the Golgi apparatus in the cell body. They are delivered to the terminals by fast axoplasmic transport and are the only type of vesicle to be found in axons (see Calakos and Scheller 1996). LDCVs are also found in nerve terminals but, in this part of the neuron, it is small synaptic vesicles (SSV: 40–50 nm) which predominate even though the proportion of LDCVs and SSVs varies from organ to organ (see Fillenz 1990). SSVs generally have

an electron translucent core but, when gluteraldehyde is used as a fixative, the majority of SSVs in catecholamine-releasing neurons have an electron dense core and are known as 'small dense-core vesicles' (SDCVs) (Fig. 4.10). SSVs contain the classical transmitters (acetylcholine, monoamines and amino acids) which are mostly loaded in the nerve terminals.

Whether SSVs are derived from LDCVs has been a matter of considerable debate but differences in the protein markers in their membranes, notably synapsin (see below), makes this unlikely (see De Camilli and Jahn 1990). Also, electron microscopic evidence has shown that LDCVs, unlike SSVs, dock at the axolemma at sites remote from the active zone at the synapse. This suggests that they have different functions and regulatory processes which, since they contain peptides, agrees with the finding that their release requires higher frequencies of nerve stimulation than does that of the classical neurotransmitters. Whether this is because LDCVs and SSVs are confined to different regions of the nerve terminal, or have different affinities for, or a topographical relationship with, Ca^{2+} channels is unresolved (Sihra and Nichols 1993).

What happens to vesicles after exocytosis is controversial. Electron microscopy certainly shows that their membranes are recovered after fusion with the axolemma but precisely how this occurs is unresolved. One possibility is that they are retrieved intact from the active zone, immediately after release has taken place. Alternatively, they could become incorporated into, and mix with, the components of the axolemma but are reformed after sorting of the different membrane elements (see Kelly and Grote 1993). Recent studies of exocytosis from retinula cells of the *Drosophila* fly suggest that both these processes for membrane retrieval can be found within individual cells. These studies have shown that there is rapid recovery of vesicular membrane from the active zone. However, a second slower process exists which takes place at sites remote from the active zone and involves the formation of invaginations in the axolemma. This process is thought to precede endocytosis because the formation of these invaginations is followed by the appearance of tubular cisternae within the nerve terminal from which new vesicles bud-off (Koenig and Ikeda 1996). This finding raises the interesting question of whether these different processes lead to the formation of two different populations of synaptic vesicles with different release characteristics.

RELEASE VERSUS STORAGE POOL

Evidence from measurements of transmitter turnover has long suggested that not all transmitter is immediately available for release. This led to the concept of 'release' and 'storage' pools of neurotransmitter but explaining the physiological basis of these different pools has not been straightforward. Some evidence favours the view that the release pool of transmitter comprises those vesicles which are near to the 'active zone' where vesicles fuse with the axolemma and which is richly endowed with Ca^{2+} channels. The reserve pool would then comprise vesicles which are docked, more remotely, on the neuronal cytoskeleton. It is thought that vesicles move from one pool to the other as a result of the actions of protein kinases which effect cycles of phosphorylation/dephosphorylation of proteins, known as synapsins, which are embedded in the vesicle membranes.

SYNAPSINS

Synapsins comprise a family of phosphoproteins that are found only in association with SSVs. Although they account for only about 9% of the total vesicular membrane protein they probably cover a large proportion of their surface. So far, synapsins Ia, Ib, IIa, IIb and III, which are the products of different genes, have been identified.

Recent evidence suggests that, while synapsins might have a role in synaptogenesis, they also regulate the supply of vesicles to the release pool (Hilfiker *et al.* 1999). Experiments *in vitro* have shown that dephosphorylated synapsin I causes growth and bundling of actin filaments which are a major component of neuronal microfilaments. Such findings form the basis of the hypothesis that synapsin I forms a ternary complex with transmitter storage vesicles and the neuronal cytoskeleton, thereby confining vesicles to a reserve pool (Fig. 4.11; see Greengard, Benfenati and Valtorta 1994). All procedures which increase Ca^{2+}-dependent release of transmitter (e.g. electrical stimulation or increasing the concentration of K^+ in the external medium) induce phosphorylation of synapsin I. Phosphorylated synapsin dissociates from the vesicles and *F*-actin, reduces the number of vesicle anchoring sites, and so frees the vesicles to the release pool. Synapsin I is the substrate for several protein kinases but one of these, Ca^{2+}/calmodulin protein kinase II (CAM kinase II), is bound to vesicle membranes to some extent. It is thought that phosphorylation of synapsin I by CAM kinase II is triggered by the influx of Ca^{2+}. This process would enable synapsin to act as a regulator of the balance between the releasable and reserve pools of vesicles.

Much evidence supports this scheme. For example, neuronal depolarisation increases the amount of free synapsin in the cytosol and microinjection of CAM kinase II into the terminals of the squid giant axon or brain synaptosomes increases depolarisation-evoked transmitter release. By contrast, injection of dephosphorylated synapsin I into either the squid giant axon or goldfish Mauthner neurons inhibits transmitter release.

It has also been suggested that synapsin promotes vesicle clustering by a process which is not dependent on phosphorylation. It achieves this by forming cross-bridges between vesicles and by stabilising the membranes of the aggregated vesicles, thereby enabling them to cluster in the active zone without fusing with each other or the axolemma. When synapsin dissociates from the vesicles, as occurs during neuronal excitation, this membrane-stabilising action is lost. This would enable fusion of the membranes of vesicles, clustered near the active zone, with the axolemma. This scheme is supported by evidence that vesicles near the active zone have much lower concentrations of synapsin than those located more remotely (Pieribone *et al.* 1995). Further roles for synapsins are currently being investigated. For instance, it has been suggested that they might also regulate the kinetics of release, downstream of the docking process.

VESICULAR EXOCYTOSIS

Once vesicles detach from the cytoskeleton they are free to participate in the release process but our understanding of precisely how this is brought about is still sketchy, despite the wealth of information which has accumulated over recent years. What is clear is that it involves a complex cascade of regulatory processes focusing on proteins bound to vesicle membranes, the axolemma and some cytoplasmic factors (see Calakos

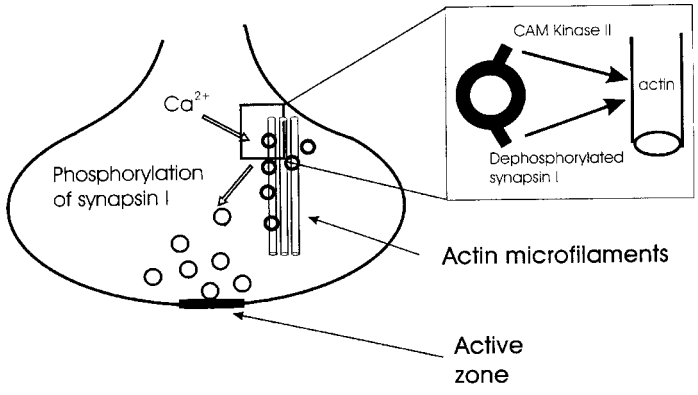

Figure 4.11 Dephosphorylated synapsin, associated with SSVs, is thought to form a heteromeric complex with CAM kinase II (also partially embedded in the vesicular membrane) and actin filaments. An increase in intracellular Ca^{2+} triggers phosphorylation of synapsin I which dissociates from the vesicular membrane. This frees the vesicles from the fibrin microfilaments and makes them available for transmitter release at the active zone of the nerve terminal

and Scheller 1996). The following sections will deal with those factors about which most is known and which are thought to have a prominent role in exocytosis. The extent to which this scheme explains release from large dense-cored vesicles is unclear, not least because these vesicles are not found near the active zone.

DOCKING AND FUSION

Because exocytosis is so rapid, it is believed that Ca^{2+} must trigger release from vesicles which are already docked at the active zone. The processes leading to docking and fusion of the vesicle with the axolemma membrane are thought to involve the formation of a complex between soluble proteins (in the neuronal cytoplasm) and those bound to vesicular or axolemma membranes. Much of this evidence is based on studies of a wide range of secretory systems (including those in yeast cells) but which are thought to be conserved in mammalian neurons.

From evidence collected to date, a scheme has emerged, known as the SNARE hypothesis (see Söllner and Rothman 1994) (Fig. 4.12). The soluble proteins referred to above include N-ethylmaleimide sensitive factor ('NSF', an ATPase) and SNAPs which comprise a family of 'soluble NSF attachment proteins'. Evidence, largely derived from studies of the Golgi apparatus, suggests that SNAPs have a general role in protein–protein interactions underlying membrane fusion. Proteins thought to act as SNAP receptors ('SNARES') are found both in the axolemma (known as 'target SNARES' or tSNARES) and vesicles (vSNARES). The vesicle protein, synaptobrevin (also known as 'vesicle associated membrane protein' or 'VAMP') is thought to act as a vSNARE and couples with the tSNARE proteins: syntaxin and SNAP-25 (synaptosomal associated protein: 25 KDa). It is envisaged that this complex of the two SNARES enables sequential binding of the soluble SNAPs and NSF. Subsequent hydrolysis of ATP by NSF enables dissociation of the complex and fusion of the membrane so that the vesicle contents can be discharged into the synapse.

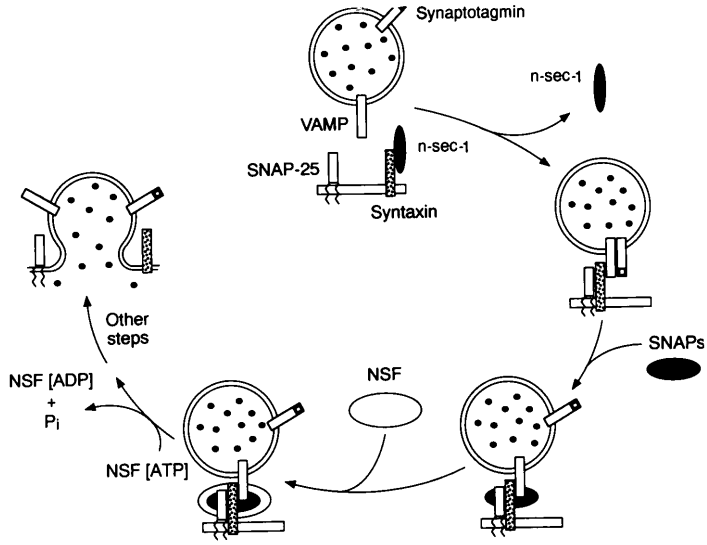

Figure 4.12 Hypothetical model of the action of SNAP receptors (SNARES) during vesicle docking, activation and fusion. It is thought that synaptic vesicle docking to the presynaptic plasma membrane requires the removal of a regulatory cytosolic protein, n-sec-1, from a VAMP (synaptobrevin) binding site on syntaxin. This results in the dissociation of synaptotagmin from the SNARE complex and binding of SNAPs and NSF, thus initiating fusion. The ATP hydrolysed by NSF results in disassembly of the SNARE complex. How the interconversion of these complexes occurs and which components trigger these processes is poorly understood. Proteins such as rab 3A, Ca^{2+} binding proteins and Ca^{2+} channels are likely to be involved. (From Söllner and Rothman 1994, page 346 with permission from Elsevier Science)

Much evidence supports a role for these proteins in exocytosis. For instance, injection of recombinant SNAP into the squid giant axon increases vesicular exocytosis. Also, membrane SNAP-25 and syntaxin are both targets for botulinum toxin while the vesicule protein, synaptobrevin, is a target for tetanus and botulinum toxins; both these toxins are well known for disrupting transmitter release.

How all these processes are influenced by Ca^{2+} is uncertain but another vesicle membrane-bound protein, synaptotagmin, is widely believed to effect this regulatory role (Littleton and Bellen 1995). Synaptotagmin has a single membrane-spanning domain with the NH_2–tail penetrating the vesicle and the COOH–tail extending into the cytoplasm. This tail binds Ca^{2+} and could enable synaptotagmin to act as a Ca^{2+}-sensor but, although it is found in adrenergic and sensory neurons, it appears to be absent from motor neurons.

Another protein, synaptophysin (p38), is the most abundant of the vesicle proteins and is found in the membranes of both SSVs and LDCVs. Its transmembrane structure resembles that of connexins which form gap junctions and has provoked the theory that neuronal excitation might cause synaptophysin to act as a fusion pore. There is no doubt that many other factors are involved in regulating the docking–fusion–extrusion process, including the Rab family of GTP-binding proteins and the Rab3 effectors, Rabphilin and Rim. For a detailed review of the role of all these factors in the exocytotic cycle, see Benfanati, Onofri and Giovedi 1999.

RECEPTOR-MEDIATED MODULATION OF Ca^{2+}-DEPENDENT TRANSMITTER RELEASE

Regulation of transmitter release does not rest solely on the frequency at which nerve impulses reach the terminals. Early experiments using stimulated sympathetic nerve/end-organ preparations *in situ*, or synaptosomes, indicated that release of [^{3}H]noradrenaline was attenuated by exposure to unlabelled, exogenous transmitter. This action was attributed to presynaptic adrenoceptors, designated α_2-adrenoceptors, which were functionally distinct from either α_1- or β-adrenoceptors. Later experiments have confirmed that α_2-adrenoceptors comprise a family of pharmacologically and structurally distinct adrenoceptor subtypes.

It is now generally accepted that there are receptors on nerve terminals which, when activated by released transmitter, attenuate its further release; these presynaptic receptors are known as 'autoreceptors'. However, there is some dissenting evidence. For instance, autoreceptors can only be synthesised in the cell bodies of neurons and are delivered to the terminals by axoplasmic transport. Yet α_2-adrenoceptors have not been found in either the cell bodies or axons of sympathetic nerves. Recently, it has been found that 'autoreceptor'-mediated modulation of transmitter release does not occur in cultured neurons unless they are co-cultured with target cells. Such findings fuel speculation that feedback inhibition of transmitter release might involve a transsynaptic mechanism. Nevertheless, many different types of neurons appear to have autoreceptors, including those that release acetylcholine (M_2), dopamine (D_2/D_3), GABA ($GABA_B$), 5-HT (5-HT_{1B} or 5-HT_{1D}) and histamine (H_3).

Electrophysiological studies in the CNS have exposed the presence of an α_2-autoreceptor with a different function. These are found on the cell bodies of noradrenergic neurons in the nucleus locus coeruleus of the brainstem. When activated, they depress the firing rate of noradrenergic neurons in the nucleus. This means that changes in the concentration of noradrenaline in the medium bathing these somatodendritic α_2-autoreceptors will modify the firing rate of central noradrenergic neurons. Other types of neurons have equivalent autoreceptors. Examples are: 5-HT_{1A} receptors on serotonergic neurons in the Raphé nuclei and $D_{2/3}$-autoreceptors on central dopaminergic neurons in the ventral tegmental area and substantia nigra.

Autoreceptor-mediated feedback control of transmitter release will obviously depend on enough transmitter accumulating in the synapse to activate the receptors. If the trains of stimuli are either too short, or their frequency too low, then transmitter release is not augmented by the administration of autoreceptor antagonists, implying that there is no autoreceptor activation (Palij and Stamford 1993). This is known as 'pseudo-one-pulse' stimulation. Conversely, at higher frequencies and long trains of stimulation, it becomes harder to inactivate the autoreceptors with antagonist drugs, presumably because of competition with increased concentrations of transmitter in the synapse.

Even more sophisticated control of neurotransmitter release is suggested by the possibility of 'heteroceptors'. These receptors are thought to be located on the terminals of, and to modulate transmitter release from, one type of neuron, but are activated by transmitter released from a different type of neuron (Laduron 1985). For example, noradrenaline has been proposed to modulate release of a wide range of transmitters (e.g. dopamine, 5-HT and glutamate) through activation of α_2-heteroceptors on the terminals of each of these different types of neuron. However, one factor that should be borne in mind is that most of the evidence for heteroceptors comes from studies of

tissue slices with [^{3}H]preloaded transmitter stores. It is therefore hard to be certain that heteroceptors are actually located on the terminals of the [^{3}H]labelled neuron and to rule out the possibility that they form part of a polysynaptic loop. To avoid this problem, a few studies have used synaptosomes to test the effects of one transmitter on K^+-evoked release of another. This approach has shown that noradrenaline and histamine, at least, blunt [^{3}H]5-HT release from cortical synaptosomes. Whether the same is true for all the other interactions between noradrenaline and 5-HT release, as well as with other transmitters, remains to be seen.

Evidence suggests that co-transmitters in a terminal have their own autoreceptors and, in some cases, activation of their own presynaptic receptor can influence the release of the co-stored, classical transmitter. For instance, activation of P_{2Y}-autoreceptors by ATP is thought to affect the release of noradrenaline from sympathetic neurons. However, in other cases, feedback modulation of release of classical and their associated co-transmitters seems to have separate control mechanisms. This would suggest that either the two types of transmitter are concentrated in different nerve terminals or that mechanisms for regulating release target different vesicles located in different zones of the terminal (Burnstock 1990).

COUPLING RECEPTORS WITH EXOCYTOSIS

There are several ways in which activation of auto- or heteroceptors on nerve terminals could modify the amount of transmitter released by exocytosis. The fact that this will depend on the influence of second messengers is beyond doubt. What remains to be resolved is whether one mechanism is more important than the others, or whether this varies from tissue to tissue.

Taking α_2-adrenoceptors as an example, several possible mechanisms have been suggested (see Starke 1987). The first rests on evidence that these autoreceptors are coupled to a Gi (like) protein so that binding of an α_2-adrenoceptor agonist to the receptor inhibits the activity of adenylyl cyclase. This leads to a fall in the synthesis of the second messenger, cAMP, which is known to be a vital factor in many processes involved in exocytosis. In this way, activation of presynaptic α_2-adrenoceptors could well affect processes ranging from the docking of vesicles at the active zone to the actual release process itself.

Alternative mechanisms are equally likely. One possibility arises from evidence that activation of α_2-adrenoceptors reduces Ca^{2+} influx; this will have obvious effects on impulse-evoked exocytosis. In fact, the inhibition of release effected by α_2-adrenoceptor agonists can be overcome by raising external Ca^{2+} concentration. Finally, an increase in K^+ conductance has also been implicated: this would hyperpolarise the nerve terminals and render them less likely to release transmitter on the arrival of a nerve impulse. Any, or all, of these processes could contribute to the feedback inhibition of transmitter release. Similar processes could explain the effects of activation of other types of auto- or heteroceptors.

Ca^{2+}-INDEPENDENT RELEASE OF TRANSMITTER

CARRIER-MEDIATED RELEASE

It is now well established that transmitter in the cytoplasm of neurons can be released by a process which is not dependent on Ca^{2+}. For monoamines, this is best illustrated

by the actions of amphetamine and its analogues. Studies of a range of substituted amphetamines, using cultured serotonergic neurons, have confirmed that this release is not prevented by either N-type or L-type Ca^{2+} channel blockers, or removal of Ca^{2+} from the incubation medium, or depletion of the vesicular pool of transmitter. The release is blocked by inhibitors of the axolemma 5-HT transporter on the axolemma that normally carries 5-HT back into the nerve terminals from the synaptic cleft. This suggests that amphetamine-induced release of 5-HT represents a reversed efflux of transmitter on the membrane-bound carrier (Rudnick and Wall 1992). Whether this process of reverse transport accounts for all the 5-HT which is released by amphetamine, or whether this drug has additional actions which affect transmitter release, remains unclear (see also Chapter 9).

Several other conditions can provoke this 'reverse pump' type of release. One is when the transmembrane ionic gradient is reversed. Experimentally this is achieved by reducing extracellular Na^+. Because the neuronal uptake of monoamines from the synapse by the transporter requires co-transport of Na^+ and Cl^-, reversing the ionic gradient (so that the Na^+ concentration is lower outside, than inside, the terminals) will drive the transporter in the 'wrong' direction. Such carrier-mediated release could explain the massive Ca^{2+}-independent release of noradrenaline during ischaemia which increases intracellular Na^+ concentration and reduces intracellular K^+.

Amino acids might also be released in this way (see Attwell, Barbour and Szatkowski 1993). There is evidence that depolarisation of retinal horizontal cells and cultured type 2 astrocytes by glutamate increases intracellular $[Na^+]$ concentration sufficiently to drive the membrane transporter to carry GABA (together with Na^+ and Cl^-) out of the neurons (Fig. 4.13). Glutamate release during ischaemia is also thought to involve such carrier-mediated transport. A similar process might also explain a glutamate-induced increase in glycine release from astrocytes in the hippocampus.

HETEROCARRIER-MEDIATED RELEASE

Finally, there is evidence that transporters for GABA are found on the terminals of neurons releasing other types of transmitters. Moreover, uptake of GABA by this 'heterocarrier' could result in co-transport of sufficient Na^+ to depolarise the terminal and provoke exocytotic release of the resident neurotransmitter. This suggestion arises from findings that exposure of brain synaptosomes to GABA can trigger release of noradrenaline, dopamine and acetylcholine. This release is prevented by inhibitors of GABA uptake but not by GABA receptor antagonists or monoamine uptake blockers. Unlike the carrier-mediated release described above, this form of release is thought to be Ca^{2+}-dependent and to involve vesicular exocytosis. However, the contribution of this process to the physiological control of neurotransmission has not yet been resolved.

CONCLUSION

That impulse-evoked release of neurotransmitters depends on a Ca^{2+}-dependent extrusion from storage vesicles is beyond dispute. However, many details concerning the supply of vesicles that participate in this process, as well as the processes which regulate the docking and fusion of synaptic vesicles with the axolemma, remain uncertain. Nevertheless, it is clear that the amount of transmitter that is released in this

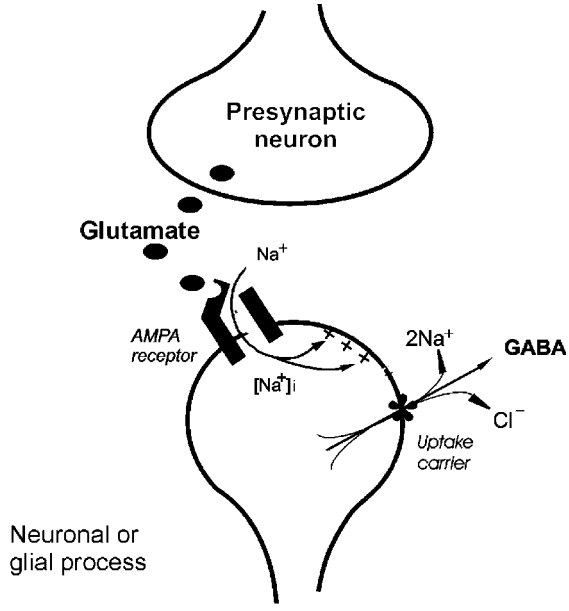

Figure 4.13 GABA release by reversed uptake ('retrotransport'). Depolarization of a neuronal, or glial cell process by glutamate, with a concomitant rise in $[Na^+]i$ reverses the operation of the GABA uptake carrier, raising [GABA]o. (Modified from Attwell, Barbour and Szatkowski 1993, with permission from the publisher Cell Press)

way is influenced by a network of auto- and heteroceptors. Activation of these receptors is coupled to the release process through their respective second messengers. It is also evident that vesicular exocytosis is not the only process which leads to release of transmitter from nerve terminals. Under certain conditions, axolemma-bound transporters can export transmitters from neurons or even evoke exocytosis. It seems that a range of processes contribute to release of neurotransmitters, all of which could have a vital role in the regulation of neurotransmission.

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