

REVIEWS

DIVERSIFICATION OF SYNAPTIC STRENGTH: PRESYNAPTIC ELEMENTS

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Synapses are not static; their performance is modified adaptively in response to activity. Presynaptic mechanisms that affect the probability of transmitter release or the amount of transmitter that is released are important in synaptic diversification. Here, we address the diversity of presynaptic performance and its underlying mechanisms: how much of the variation can be accounted for by variation in synaptic morphology and how much by molecular differences? Significant progress has been made in defining presynaptic structural contributions to synaptic strength; by contrast, we know little about how presynaptic proteins produce normally observed functional differentiation, despite abundant information on presynaptic proteins and on the effects of their individual manipulation. Closing the gap between molecular and physiological synaptic diversification still represents a considerable challenge.

SYNAPTIC STRENGTH
The relative amplitude of the postsynaptic response that is generated by the activity of the presynaptic neuron.

Functional studies of synaptic transmission initially developed around relatively few experimentally accessible cases: the vertebrate neuromuscular junction (NMJ), in which the quantal nature of fast transmission was discovered^{1,2}; the giant synapse of the squid, in which many details of the role of Ca²⁺ in triggering transmitter release were elucidated³; and crustacean NMJs, which provided definitive evidence for amino acids as neurotransmitters⁴, and for presynaptic inhibition⁵ and various forms of presynaptic facilitation⁶. With time, many other synapses, in fish⁷, lamprey⁸ and chicken⁹, and even a giant synapse in the mammalian auditory pathway, the calyx of Held¹⁰, have received increasing attention. For the most part, these synapses have attracted experimentalists because they are relatively large and identifiable, but they are also highly specialized.

With the advent of the patch-clamp technique, some of the smaller, more representative synapses in the mammalian central nervous system (CNS) were studied, revealing great diversity of functional performance. Some synapses produce large signals in their postsynaptic followers, whereas others produce small signals. With repeated activation, some responses augment greatly, whereas others decline¹¹. These findings in the mammalian CNS were previewed several decades earlier by similar observations in invertebrate preparations¹²,

underlining the universality of the phenomena. Clearly, differences in response patterns are important for information transfer within the nervous system: the pathways in which the different synapses occur need specialized synaptic performance to carry out their functions.

Confronted with the apparently universal occurrence of synaptic functional diversity, we can ask several general questions. Given that synaptic diversity is functionally important, can one explain the need for particular types of synapse in defined neural pathways? How does diversity arise? Is it developmentally programmed or does it arise from 'experience' of the nerve cells in a pathway? With regard to mechanisms, where and how does synaptic diversity arise? Can it be attributed to pre- or postsynaptic features of the synapse? Can synaptic diversity be explained by the structural organization of the synapse or is it due mainly to specialized molecular differentiation? If the latter is the case, can we explain synaptic differentiation in molecular terms?

Here, we limit our discussion chiefly to the last two questions. We focus mainly on SYNAPTIC STRENGTH, which we define as the response that is produced in a target cell by the synapses of a neuron on initial stimulation. Specifically, we assess presynaptic contributions to synaptic strength; these include the organization and

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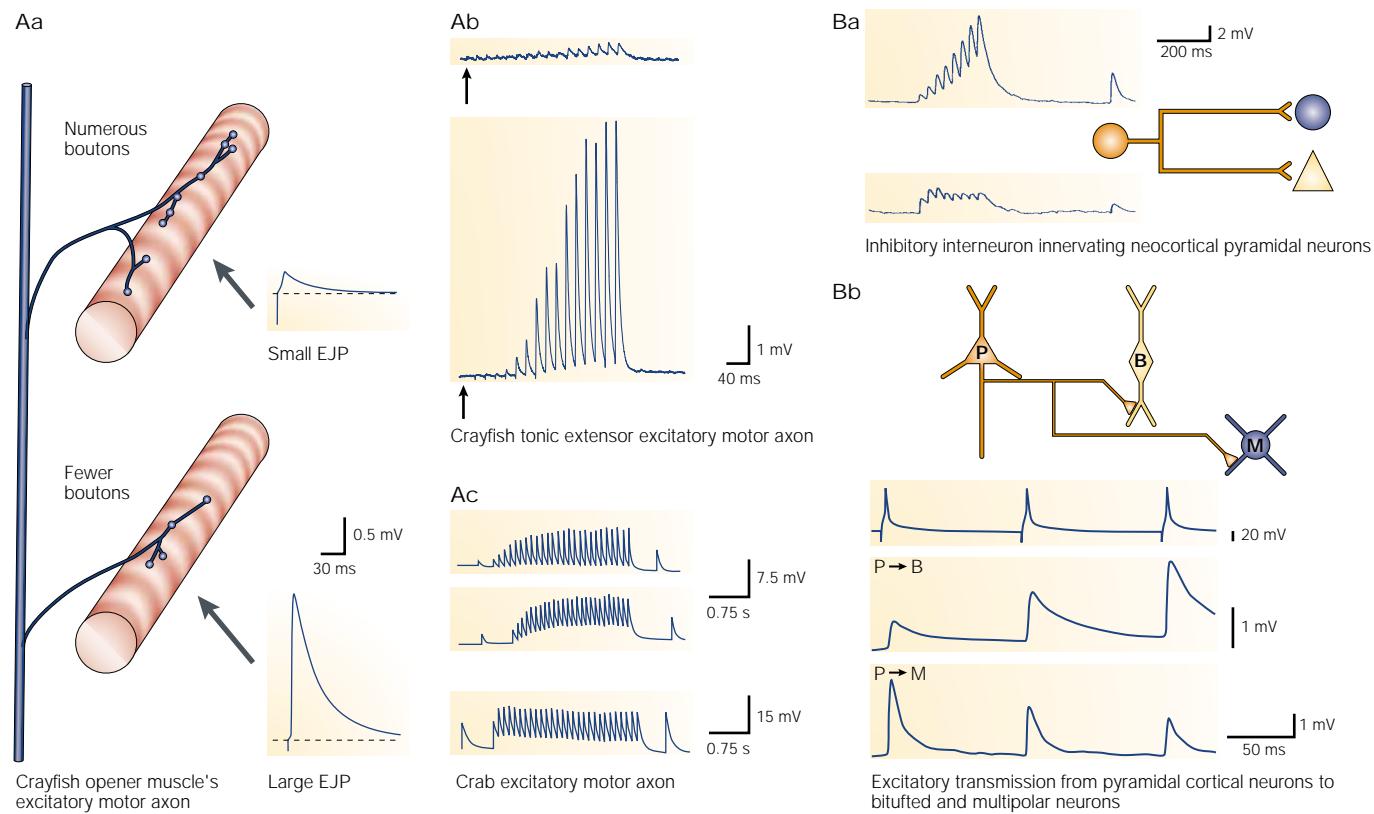


Figure 1 | Synaptic functional differentiation, case I: differences in the responses of a target cell to a single presynaptic neuron. Both crustacean and mammalian examples show large differences in the initial synaptic response and in short-term plasticity (facilitation or depression of the postsynaptic potential during repetitive stimulation). **A** | In crustacean limb muscles, a single motor neuron that innervates different muscle fibres produces synaptic potentials of varying amplitude (**Aa**) and degrees of facilitation (**Ab**) in different postsynaptic followers. Large, poorly facilitating potentials and small, highly facilitating potentials appear in defined muscle fibres (**Ac**). The amount of short-term facilitation in follower cells depends on impulse frequency. EJP, excitatory junction potential. **B** | In the mammalian central nervous system, single inhibitory cortical interneurons produce facilitating and non-facilitating synaptic potentials in different pyramidal neurons (**Ba**). Single cortical pyramidal neurons (P) produce facilitating and non-facilitating (depressing) synaptic potentials (**Bb**) in two different interneuron types (bitufted, B; multipolar, M). In general, target-cell influences are thought to govern case I differentiation. Part **Ab** adapted, with permission, from REF. 157 © 1997 The Company of Biologists Ltd; part **Ac** adapted, with permission, from REF. 19 © 1972 The Rockefeller University Press; part **Ba** adapted, with permission, from REF. 29 © 2000 American Association for the Advancement of Science; part **Bb** adapted, with permission, from REF. 158 © 1998 Macmillan Magazines Ltd.

properties of presynaptic structures, which can account for some, but not all, features of functional differentiation. This fact leads to the conclusion that differences at the molecular level are important for synaptic diversity. Although much has been discovered in the past decade about presynaptic proteins, we argue that a clear case cannot yet be made for their normal roles in functional differentiation.

What is a synapse?

Originally, the term 'synapse' was used to denote the contact between neurons at which transmission occurs. But as explained by Korn¹³, the structural definition of a synapse is most commonly taken to mean the individual, specialized contact that comprises electron-dense pre- and postsynaptic membranes, and the specialized structures of variable appearance in both pre- and postsynaptic compartments. According to this view, the ACTIVE ZONE is a specialization within the presynaptic membrane and is not equivalent to the synapse, whereas the ensemble of synapses between one neuron and another can be termed a synaptic connection.

ACTIVE ZONE
The site of the presynaptic terminal at which synaptic exocytosis occurs.

These distinctions are important for comparisons of synaptic strength, because differences in the properties of a postsynaptic response could arise from the number of synapses formed by the presynaptic cell (a larger or smaller synaptic connection) or from the properties of the individual synapses (such as the probability of vesicle fusion or the number of active zones).

Manifestations of synaptic diversity

The synapses that were initially selected for physiological investigations, such as the frog NMJ and the squid giant synapse, were very large and specialized for rapid, reliable excitation of their postsynaptic followers, indicating a correlation between structure and function. Large synapses have many active zones; consequently, a single presynaptic nerve impulse discharges many synaptic vesicles (hundreds at the frog NMJ, thousands at the squid giant synapse). Most synapses in the CNS are much smaller, often endowed with a single active zone; only one (or no) vesicles are fused by a single nerve impulse¹⁴. Synaptic strength at a single central synapse of this type is less than that at specialized giant synapses,

largely because the physical design of the synapses is hugely different. But how far can we extend the argument of synaptic structure to account for synaptic functional diversity? And are the principles of synaptic transmission that were discovered in giant synapses applicable to the small CNS synapses?

Instead of forming a giant synapse, crustacean motor neurons and molluscan ganglionic neurons provide numerous small synapses to their followers. Synaptic potentials of these cells were found to have a much greater range of short-term plasticity. Short- and long-term facilitation, and different forms of short-term depression, were readily discerned, and proved to be dependent on the pattern of stimulation and on the particular neuron that was activated. Observations on lower vertebrate and mammalian central synapses presented a similar picture, with even more diverse forms of short- and long-term synaptic plasticity^{15,16}. By comparison, the giant synapses seemed rather dull; they readily showed short-term depression (synaptic fatigue) with continuous stimulation, and their limited facilitation processes were revealed only when transmitter output was artificially lowered. We can surmise that the structural and functional specializations that are needed for sledgehammer transmission at giant synapses are elaborated at the expense of more subtle forms of plasticity that are prevalent at smaller synapses.

In addition to obvious neuron-specific synaptic differences, branch-specific differences became evident in the 1950s at crustacean NMJs (FIG. 1A). Intracellular recordings by Hoyle and Wiersma¹⁷ showed that a single neuron could evoke different responses in different follower cells; subsequent studies confirmed and extended this finding¹⁸. Extracellular focal recordings established that differences in synaptic performance were largely presynaptic in origin; however, in addition, the follower cells showed morphological, electrical and chemical differences¹⁹. The concept of target-cell specification of synaptic functional properties was enunciated by Frank²⁰ for crustacean NMJs, and has been echoed in more recent work, in which parallels are drawn between crustacean NMJs and central synapses of insect mechanosensory pathways²¹. The fact that branch-specific synaptic differentiation is a general feature has been borne out by the discovery of more examples in the mammalian CNS (FIG. 1Ba). Although facilitation and depression patterns vary greatly between different branches of a single neuron, a general trend can be discerned: on repeated stimulation, synapses that release less transmitter initially are more likely to show short-term facilitation than depression, whereas the converse applies to synapses that have a large initial transmitter output. As an example, the synaptic connections of a cortical pyramidal cell on multipolar neurons produce a relatively large synaptic potential that depresses, whereas the connection of the same neuron to a bitufted interneuron has a smaller initial amplitude, but facilitates (FIG. 1Bb). Another case of target-cell-specific synaptic differentiation is found between excitatory synapses of hippocampal CA3 pyramidal cells on their followers²².

CLIMBING FIBRES

Cerebellar afferents that arise from the inferior olive nucleus, each of which forms multiple synapses with a single Purkinje cell.

PARALLEL FIBRES

Axons of cerebellar granule cells. Parallel fibres emerge from the molecular layer of the cerebellar cortex towards the periphery, where they extend branches perpendicular to the main axis of the Purkinje neurons and form the so-called *en passant* synapses with this cell type.

These observations lead to the definition of two general cases of synaptic functional differentiation, which we shall term 'case I' and 'case II'.

In case I (FIG. 1), different branches of the same neuron evoke synaptic responses of diverse character in various follower cells. The prevailing view of the origin of these properties is that of Frank²⁰: differences in the follower cells determine specific synaptic properties, most likely by a retrograde trophic influence. Lnenicka and Mellon²³ provided particularly compelling support for this idea by showing that experimental alteration of identified crustacean muscle fibres led to adaptive modification of presynaptic transmitter release. Synaptic homeostasis at the *Drosophila melanogaster* NMJ also supports this view²⁴. The molecular events that underlie case I differentiation have not been fully worked out, but the existence of retrograde effects has been documented through genetic manipulation of *D. melanogaster* NMJs²⁵ and observations of synapse formation of molluscan giant neurons²⁶.

In case II (FIG. 2), different presynaptic neurons that supply the same follower cell evoke different postsynaptic responses. In this case, it cannot be argued that the synaptic functional differences arise entirely from differential retrograde influence of the follower cell. Instead, upstream determination of the presynaptic properties seems to be more significant. Once again, crustacean NMJs provide the clearest and earliest-studied examples: starting with observations by Wiersma and van Harreveld¹² in the 1930s, a general dichotomy between 'fast' (phasic) and 'slow' (tonic) motor axons was recognized and then elaborated by others²⁷. In several limb muscles, one of each type of motor neuron provides the entire excitatory innervation of all muscle fibres (FIG. 2Aa). The synaptic responses of follower cells to the two neurons are enormously different: a single impulse in the phasic axon produces a synaptic potential that is many times larger than its tonic counterpart, even after the latter has facilitated with repetitive stimulation. Among mammalian CNS circuits, an excellent example is the cerebellar Purkinje cell, in which excitatory postsynaptic currents that are evoked by CLIMBING FIBRE stimulation depress, whereas those evoked by PARALLEL FIBRE stimulation facilitate (FIG. 2Ba). The generality of this case is highlighted by its common occurrence elsewhere in the CNS (FIG. 2Bb). A more complete account of examples from the mammalian CNS can be found in reviews by Thomson¹⁶ and by Markram and co-workers^{28,29}. The latter group has described synaptic differentiation between mammalian CNS neurons in great detail; indeed, they claim that "frequency dependence of transmission is potentially unique for each synaptic connection made by a single axon"²⁸. The idea of synaptic uniqueness emerged from earlier invertebrate studies, but until recently, it was not thought to be important in the mammalian CNS.

Phenomenological features of synaptic functional differentiation tell us little about the underlying mechanisms. Elucidating the basis of functional differentiation would aid our understanding of synaptic plasticity and long-term changes in the CNS. We can pursue the basis of functional diversity by addressing several questions.

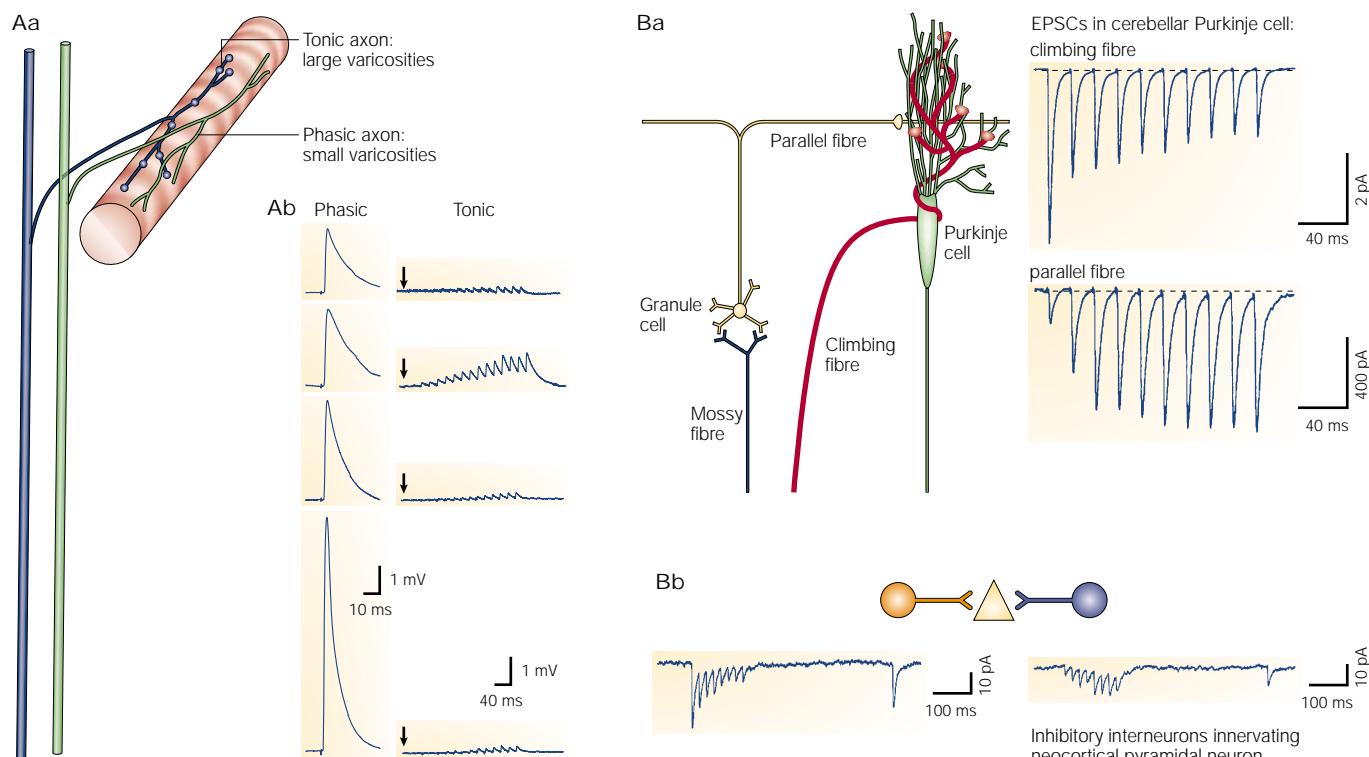


Figure 2 | Synaptic functional differentiation, case II: responses evoked in a single target cell by different presynaptic inputs. Both crustacean and mammalian examples show large differences in synaptic response when different inputs are activated. **A** | In crustacean limb muscles (**Aa**), a single impulse in a phasic motor axon produces large potentials in all of the innervated muscle fibres (**Ab**, left), whereas potentials in the same fibres after stimulation of the tonic motor axon are smaller and become apparent only with repetitive stimulation (**Ab**, right). **B** | Mammalian synapses with a similar dichotomy include the climbing and parallel fibre inputs to cerebellar Purkinje cells (**Ba**), and different interneuronal inputs to cortical pyramidal cells (**Bb**). Presynaptic influences in the regulation of transmitter release are thought to be crucial for case II differentiation. Part **Ab** adapted, with permission, from REF 157 © 1997 The Company of Biologists Ltd; part **Ba** adapted, with permission, from REF 159 © 2000 Society for Neuroscience; part **Bb** adapted, with permission, from REF 29 © 2000 American Association for the Advancement of Science.

First, can we define the extent of pre- or postsynaptic contributions? Second, can we predict function from structure? Third, can we define molecular differences that give rise to functional ones?

Pre- and postsynaptic contributions

The size of the synaptic potential that is produced by a presynaptic neuron in one of its followers depends on several factors that include the number of contributing synapses in the synaptic connection, the amount of neurotransmitter that is released at each synapse and the size of the resulting current at each synapse. In addition, the electrical properties of the postsynaptic cell are important, but will not be considered here.

To simplify the analysis of these factors, we can measure currents at isolated synaptic contacts, ideally contacts that include only one synapse and one active zone. If this can be achieved — as has been in some mammalian preparations, usually from neurons in culture³⁰ — we can observe currents that are produced by single quantal events, and analyse the probability of the occurrence of quantal events per presynaptic impulse. This type of analysis can now be done using optical procedures, by measuring the fluorescence of dyes that are trapped in recycling synaptic vesicles³¹ or analysing synapse-related Ca^{2+} signals at dendritic spines³².

QUANTAL SIZE
The mean amplitude of the postsynaptic response that results from the release of transmitter from a single synaptic vesicle.

The probability of occurrence of quantal events at a synapse is determined primarily by presynaptic factors. Combined structural and physiological observations of crustacean and mammalian CNS synapses have led to the view that probability of transmission is non-uniform across the individual synapses of a synaptic connection³³. Short-term facilitation and depression, which generally reflect changes in probability of quantal occurrence, are also thought to be determined presynaptically, as illustrated by classical work on facilitation at frog and crayfish NMJs. It should be noted, however, that some synapses show short-term depression that arises from postsynaptic receptor desensitization³⁴.

As for QUANTAL SIZE, the postsynaptic membrane provides responsive receptors, and their number and arrangement should determine the amount of current generated by a quantum of transmitter. However, recent observations indicate that quantal size might be determined presynaptically at some synapses, as we discuss later.

Presynaptic scenarios for diversification
Focusing on how differences in presynaptic terminals might affect the probability of release or the number of releasable units, we must take into account the electrical events in the terminal (which depend on its

complement of voltage-gated ion channels), synaptic structural features and their variation, and molecular features and differences.

Changes in the presynaptic action potential affect the release of neurotransmitter by altering Ca^{2+} entry through voltage-gated Ca^{2+} channels. The dynamics of this relationship were investigated extensively in the squid giant synapse³⁵. When the amplitude of the action potential is reduced, transmitter release decreases; this reflects reduced Ca^{2+} entry owing to the incomplete activation of presynaptic Ca^{2+} channels and the steep dependence of transmitter release on intracellular Ca^{2+} . As accurate information about action potential amplitude is not available for many presynaptic terminals, the possibility of differences between terminals has not been fully addressed.

The duration of the presynaptic action potential also affects transmitter release: its prolongation allows a longer-lasting Ca^{2+} entry, which in turn stimulates a larger increase in transmitter release. This effect was initially discovered at the frog NMJ and the squid giant synapse. More recently, mammalian CNS synapses have been found to depend on this feature. At the synapse between granule and Purkinje cells, spike broadening causes more Ca^{2+} entry by prolonging the open time of the presynaptic Ca^{2+} channels, rather than by opening more of them³⁶. Intracellular recordings from HIPPOCAMPAL MOSSY FIBRE boutons showed a similar effect of spike broadening, which occurred

during repetitive activity and could be attributed to the inactivation of presynaptic K^+ channels of the K_{Vi} type³⁷. The calyx of Held does not show such spike broadening³⁸. Variation in presynaptic K^+ channels could turn out to be an important variable that influences synaptic strength.

In the case of synaptic structure and molecular properties, we present in FIG. 3 several possible ways in which presynaptic strength could be modified. The first five of them relate to structural features: the number of active zones per synapse, the number or density of Ca^{2+} channels in or near the active zone, the size of synaptic vesicles, the spacing between READILY RELEASABLE VESICLES and Ca^{2+} channels and the number of readily releasable vesicles. The sixth possibility relates to a possible molecular difference; FIG. 3f presents a difference in Ca^{2+} affinity of the receptor that triggers vesicle fusion. Of course, there are many other molecular differences that we could consider, including differences in VESICLE PRIMING and mobilization.

Presynaptic determinants of quantal size

Synaptic strength depends in part on quantal size: fewer synaptic vesicles would be needed to activate a postsynaptic cell in which quantal size is large. Many studies of LONG-TERM POTENTIATION in the mammalian hippocampus have supported the view that quantal size increases at some synapses, leading to an increase in synaptic strength³⁹.

HIPPOCAMPAL MOSSY FIBRES
Axons of granule cells, which form synapses with CA3 pyramidal neurons. Mossy fibre boutons are among the largest in the central nervous system.

READILY RELEASABLE VESICLES
Synaptic vesicles that are available for rapid fusion with the presynaptic membrane on arrival of a nerve impulse. They are docked to the membrane and have been biochemically primed for release.

VESICLE PRIMING
Primed vesicles are those that have acquired the biochemical attributes for fusion with the presynaptic membrane; they can release their transmitter on the arrival of a nerve impulse.

LONG-TERM POTENTIATION
A long-lasting increase in the efficacy of neurotransmission, which can be elicited by diverse patterns of synaptic activation.

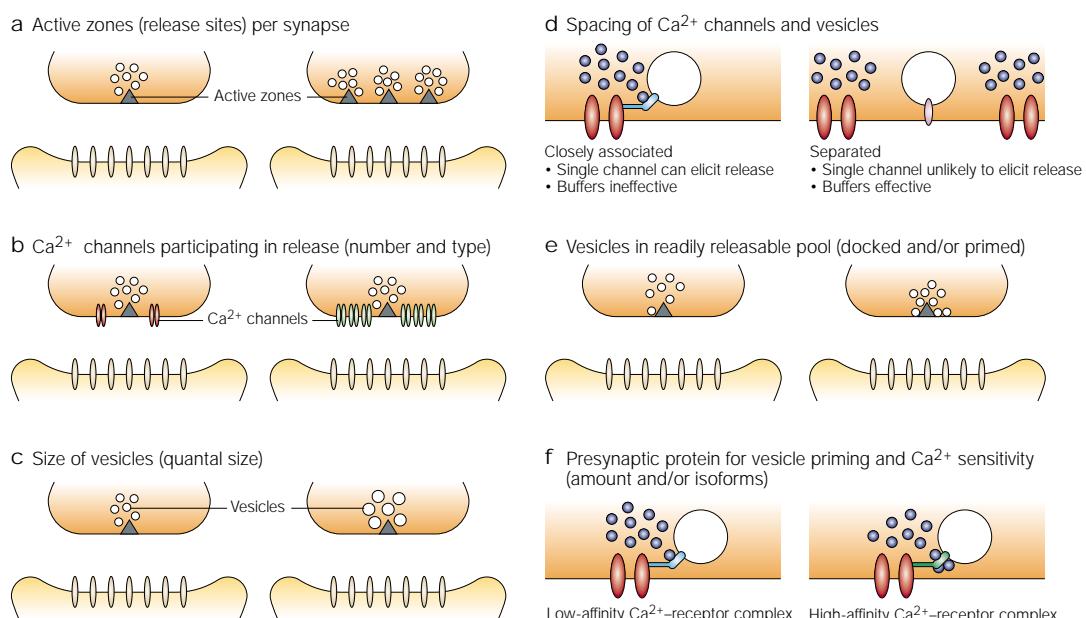


Figure 3 | Presynaptic determinants of synaptic strength. Several hypothetical mechanisms are illustrated. **a** | Individual synapses have different numbers of release sites (active zones). An extreme example is the calyx of Held in the mammalian auditory pathway. **b** | Voltage-dependent Ca^{2+} channels at individual active zones differ in number and/or type, allowing more Ca^{2+} to enter at some active zones after a nerve impulse, eliciting the fusion of more synaptic vesicles. **c** | Synaptic vesicles differ in size, generating correspondingly different quantal units that depend on their transmitter content. **d** | The effectiveness of individual Ca^{2+} channels to cause vesicle fusion depends on channel–vesicle spacing. Intracellular buffers have a more significant influence on transmission when channels and vesicles are more separated. **e** | Synaptic vesicles that are available for release (close to or docked at the synaptic membrane, and appropriately primed) are more numerous at some synapses. **f** | Qualitative and quantitative differences in presynaptic proteins impart different properties to the Ca^{2+} receptors, affecting the probability of vesicular fusion after Ca^{2+} entry.

Box 1 | Presynaptic or postsynaptic determination of quantal size?

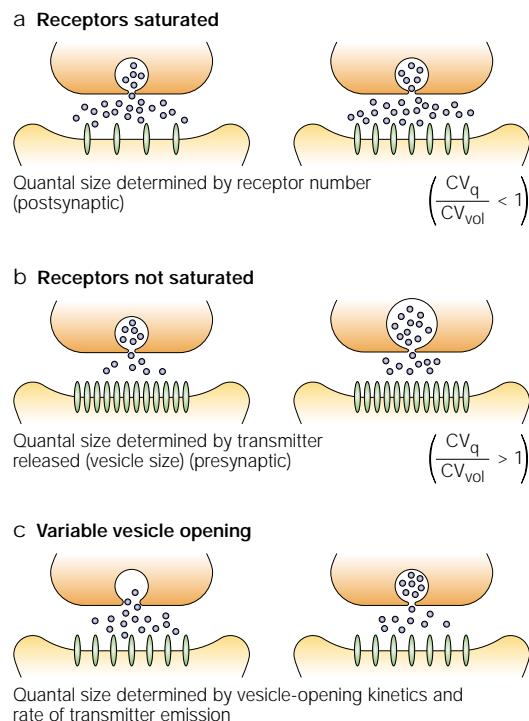
Postsynaptic determination of quantal size (see panel a of the figure) occurs if the number of available postsynaptic receptors differs between synapses. In this case, the same amount of transmitter released from a synaptic vesicle produces quantal events of different sizes, depending on the number of activated receptors. At synapses in which postsynaptic receptors are saturated, quantal variance (CV_q) is small and does not reflect the variation that arises from the vesicular content of transmitter (which could be determined by the variation in vesicle volume, CV_{vol}). The quantal variance reflects intrinsic variability that arises from receptor–transmitter interactions. So, $CV_q < CV_{vol}$ and $CV_q/CV_{vol} < 1$. At unsaturated synapses, if quantal variance results purely from variation in vesicle transmitter content, then $CV_q = CV_{vol}$. However, if we also include intrinsic variability, then $CV_q > CV_{vol}$. Therefore, $CV_q/CV_{vol} > 1$ at synapses that possess unsaturated postsynaptic receptors.

Presynaptic determination of quantal size occurs when the transmitter released from single vesicles is variable, either because vesicles vary in volume and consequently in transmitter content (panel b), or because vesicle opening after fusion is variable in extent and/or rate (panel c). In such cases, $CV_q/CV_{vol} > 1$ because further stochastic variance (FIG. 4a) will contribute to CV_q .

Several mechanisms could underlie changes in quantal size (BOX 1). At single synapses, if the number of postsynaptic receptors is limited, then the transmitter content of a synaptic vesicle could saturate them; postsynaptic factors would therefore limit quantal size, and synapses with more available receptors would generate larger quantal events. However, both pre- and postsynaptic factors could, in principle, contribute to the final outcome. If receptor availability is not a limiting factor, quantal size could be determined by the amount of transmitter that is released from the vesicle, or possibly by the rate at which it leaves the vesicle⁴⁰. There is now evidence for all of these possibilities.

Examination of the variation in synaptic quantal events (QUANTAL VARIANCE) helps to discriminate between the possibilities that are illustrated in BOX 1. A large quantal variance points to a presynaptic locus, whereas small values point to a postsynaptic locus. For example, at some central synapses, the available evidence indicates that the number of postsynaptic receptors, not the amount of released transmitter, dictates quantal size⁴¹. In such a case, quantal variance would be close to zero, as transmitter molecules would occupy all of the postsynaptic receptors, generating a near-maximal response that is characteristic of that synapse⁴². Bekkers *et al.*⁴³ were the first to provide evidence against this view by showing that quantal variance at hippocampal synapses is large. This indicated that receptor number is not the limiting factor; the receptors are not saturated, and quantal size is dictated by the amount of released transmitter.

QUANTAL VARIANCE
A measure of the variability of quantal events normalized to their mean amplitude. From the statistical point of view, quantal variance corresponds to the coefficient of variation of the responses — the standard deviation divided by the mean.



Faber *et al.*⁴⁴ pointed out that quantal variance could not be zero at any synapse because the stochastic nature of receptor–transmitter interactions ensures that postsynaptic receptor binding sites are never fully occupied. Therefore, saturated postsynaptic receptors have a small quantal variance (5–15%) that arises from the intrinsic variability of the binding reaction⁴⁵, whereas unsaturated postsynaptic receptors have, in addition, a larger variance that is attributable to variation in the concentration of transmitter at the cleft. Recent experimental results from cultured mammalian hippocampal neurons⁴⁶ illustrate these principles (FIG. 4a). Glutamate pulses of constant amplitude that were applied to a single synaptic bouton yielded postsynaptic responses with small variance, as would be expected for the transmitter–receptor interaction. Increasing the amount of glutamate in the pulse produced a larger event with the same variance. By contrast, the normally occurring spontaneous quantal events showed much larger variance. These and other experiments strongly indicate a presynaptic determination of quantal amplitude and variance at many central synapses⁴⁷. Such observations point to a ‘vesicle-volume hypothesis’, in which vesicular transmitter concentration is constant but vesicle volume varies, producing in turn the variation in quantal size.

Several presynaptic factors might affect quantal size. Let us first look at vesicle size. If quantal variance reflects the amount of transmitter that is released, a probable determinant of this variation is the size of vesicles, assuming that all of them are filled to the same concentration^{43,48}. Support for this idea comes from several recent studies. In monoaminergic neuroendocrine

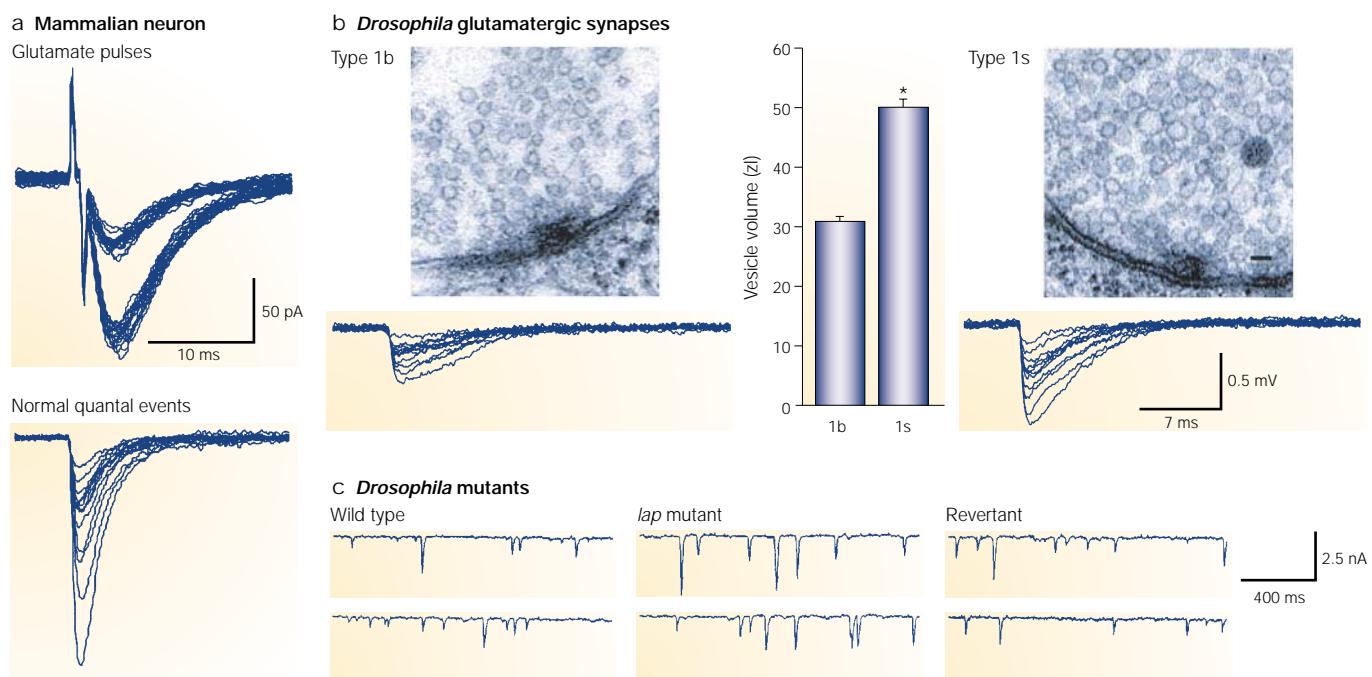


Figure 4 | Quantal size and variance: evidence for presynaptic influence. **a** | When pulsed repeatedly with two different concentrations of glutamate, individual synaptic contacts of cultured mammalian neurons show synaptic currents with small pulse-to-pulse variability (top). By contrast, spontaneously occurring quantal events (bottom) show much larger variability, indicating that the amount of glutamate that is released by individual synaptic vesicles at a single synapse varies considerably. **b** | A link between quantal- and vesicle-size variability is found at *Drosophila* neuromuscular junctions that are formed by two different neurons. One of them provides type 1b terminals (with smaller vesicles), whereas the other one provides type 1s terminals (with larger vesicles). Quantal events at the two terminal types match their respective vesicle sizes, but quantal variance is not significantly different for the two terminals. **c** | *Drosophila* *lap* mutants, which have defects in synaptic vesicle recycling, have larger-than-normal vesicles and correspondingly larger quantal currents after spontaneous exocytosis. Both vesicle size and quantal size are restored to wild-type values when the normal *lap* gene is replaced. Part **a** adapted, with permission, from REF. 46 © 1999 Elsevier Science; part **c** adapted, with permission, from REF. 52 © 1998 Elsevier Science.

cells⁴⁹ and leech serotonergic neurons⁵⁰, AMPEROMETRIC measurements were shown to correlate well with observed vesicle volumes. Similarly in mossy fibre synapses, giant monoquantal events were linked to the presence of exceptionally large synaptic vesicles⁵¹.

Another line of evidence in support of the vesicle-volume hypothesis comes from recent observations of *Drosophila* synapses. Here, neuron-specific differences in vesicle size occur and are associated with corresponding differences in quantal size (FIG. 4b). Quantal size was shown to be augmented in *lap*⁵² (FIG. 4c) and *stoned*⁵³ mutants, in which synaptic vesicles are larger than normal. These results support the view that quantal size depends on vesicle volume.

A second possibility is that intravesicular transmitter concentration might vary. Overexpression of transporter activity on synaptic vesicles produced an increase in quantal size, and inhibition of transporter activity reduced quantal size at developing cholinergic synapses⁵⁴. Altering GABA (γ -aminobutyric acid) metabolism in inhibitory hippocampal interneurons seems to change transmitter concentration in synaptic vesicles in concert with quantal size; whether vesicle size was altered is unknown⁵⁵. Although the means by which vesicles acquire different transmitter contents can vary, the generality of presynaptic influence on quantal size and variance (BOX 1) has been sustained in different neuronal types.

A third possibility is that quantal amplitude and time course are influenced by the vesicular fusion event (BOX 1). Evidence for partial or delayed emptying of transmitter was provided by studies of mammalian central synapses during long-term potentiation and developmental maturation⁴⁰. A transient fusion pore, resulting from rapid fusion and retrieval of a synaptic vesicle ('kiss and run' exocytosis)⁵⁶, might restrict the full discharge of transmitter from synaptic vesicles, producing quantal events that are smaller than normal. However, available data indicate that transmitter could still be fully discharged in kiss-and-run exocytosis without affecting quantal size⁵⁷. Although transmitter discharge through a fusion pore might be a non-instantaneous process⁴⁰, the influence of this factor on quantal size and its variation remains to be established.

Despite the wealth of presynaptic mechanisms, functional differentiation of synaptic strength owing to a difference in quantal size has not been carefully investigated; the contribution of this factor to synaptic diversity under normal circumstances is not known. Most studies produce effects on quantal size through severe experimental interventions. The example of paired glutamatergic *Drosophila* neurons (FIG. 4b) is clear evidence that such differentiation can normally occur. How might it arise?

AMPEROMETRY
An electroanalytical technique that is based on the measurement of current flow through an electrochemical cell. In the case of exocytosis, the release of oxidizable substances (such as catecholamines) from single vesicles can be studied by amperometry using a carbon fibre electrode. The electrode is held at a voltage that causes the oxidation of the transmitter, and the resulting redox currents are measured with a patch-clamp amplifier.

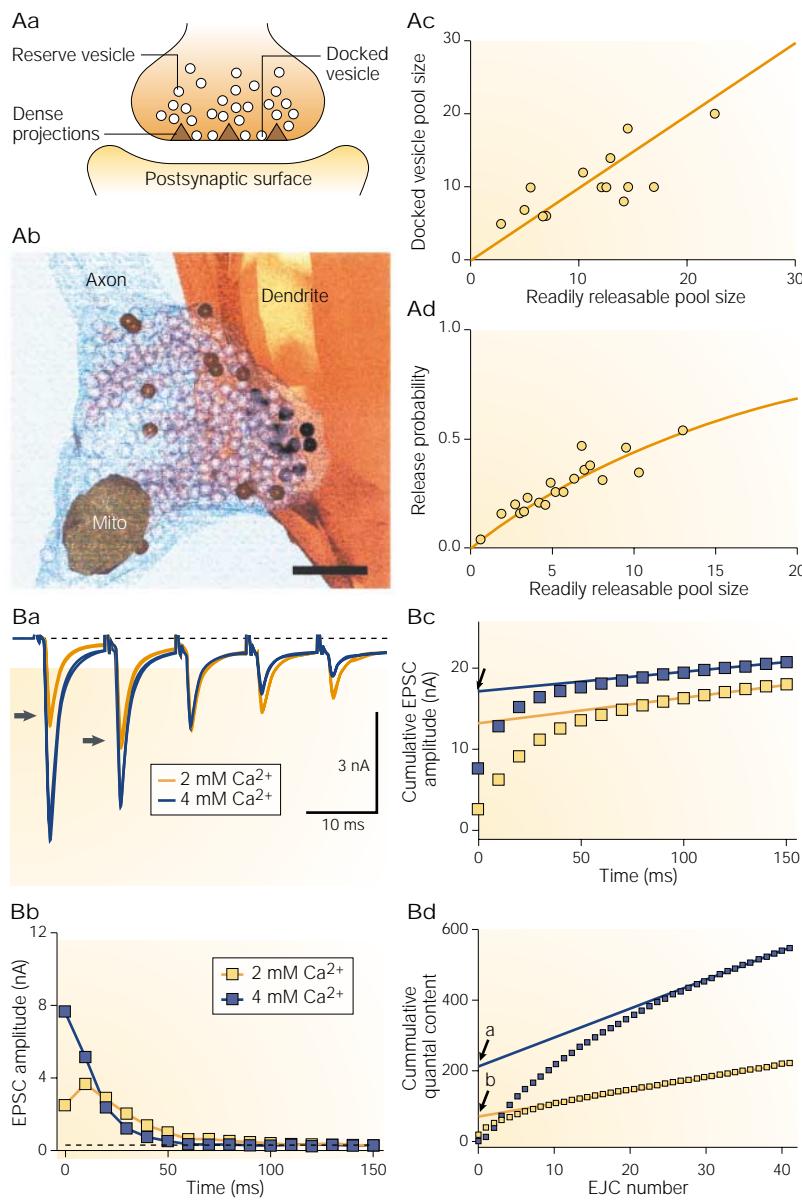


Figure 5 | Synaptic vesicle pools and the putative contribution of readily releasable vesicles to synaptic strength. **A** | The structure of a typical mammalian central synapse (**Aa,b**) allows the docking of a limited vesicle number among presynaptic ‘dense projections’. After fusion with the presynaptic membrane, recycled vesicles can be visualized (**Ab**) and used to estimate the readily releasable vesicle pool. Synapses from cultured mammalian neurons show a strong correlation between docked and readily releasable pool sizes (**Ac**), and between readily releasable pool size and release probability (**Ad**). So, release probability is determined, in large measure, by the docked vesicle pool size, which is often related to the contact area of the synapse. **B** | Physiological estimation of readily releasable vesicles at a mammalian synapse (**Ba–c**) and at two crustacean synapses (**Bd**). The calyx of Held depresses rapidly during a train of closely spaced stimuli (**Ba**): elevating Ca^{2+} levels enhances this effect. A plot of excitatory postsynaptic current (EPSC) amplitude to individual stimuli is shown in **Bb**. Plotting the cumulative EPSC amplitude (**Bc**) provides a measure of the steady-state value by extrapolation of the line (which represents the equilibrium between vesicle loss and vesicle recruitment) back to time 0 (arrow). When the amplitude of a single quantal event is known, the number of rapidly depleted quantal units can be estimated. It is assumed to equal the number of synaptic vesicles in the readily releasable pool. In **Bd**, this procedure was used to estimate the readily releasable vesicles in boutons of phasic and tonic crayfish motor neurons. The estimate for the tonic neuron (blue trace, a) was about three times larger than that for the phasic neuron (yellow trace, b). Readily releasable vesicles are more redundant in the tonic neuron, despite its much lower vesicle release probability (see FIG. 2). EJC, excitatory junction current. Part **Ab** adapted, with permission, from REF. 69 © 2001 Macmillan Magazines Ltd; parts **Ac–d** adapted, with permission, from REF. 71 © 2001 Elsevier Science; parts **Ba–c** adapted, with permission, from REF. 74 © 1999 Elsevier Science; part **Bd** adapted from REF. 75.

One possibility is that differences in quantal and vesicle size are linked to the ongoing level of neuron activity. Activity-dependent regulation of synaptic vesicle size has been observed in electroreceptor afferents in gymnotid electric fish⁶⁸, lamprey reticulospinal axons⁵⁹ and in the electric organ of *Torpedo*⁶⁰. In all three cases, increased activity produced smaller vesicles.

Collectively, these studies indicate that activity affects quantal size by altering the amount of transmitter that is released from synaptic vesicles. Either vesicle filling or size can respond to neural activity. The first effect is rapidly produced by intense stimulation and is linked in some cases to synaptic depression⁶¹. The second effect can be more persistent and might be an adaptive response: larger vesicles (and larger quantal events) appear in less active nerve terminals, enabling them to produce a larger postsynaptic response when activity is resumed. The difference in vesicle volume that is found in *Drosophila* neurons (FIG. 4b) agrees with this hypothesis: the neuron with smaller vesicles is normally much more active than its partner during fictive locomotion. In this and other cases, presynaptic quantal size might respond on an intermediate time scale, providing adaptation to maintained changes in neuronal activity.

Readily releasable vesicles

Synaptic vesicle replenishment and mobility are essential for ongoing synaptic transmission: metabolic poisons⁶² and mutations in genes that affect vesicle replenishment⁶³ cause rapid disappearance of vesicles and concurrent transmission failure. Synaptic strength depends on the presence of a readily releasable pool of vesicles and on their finite probability of release by an action potential. In addition to the readily releasable pool, two other populations of vesicles have been distinguished: recycling vesicles and reserve vesicles. After fusion with the presynaptic membrane, vesicles are retrieved and recycled (recycling pool). Several modes of vesicle recycling are known. At the frog NMJ, which has a large vesicle population, many vesicles are retrieved by endocytosis, and they coalesce with internal compartments before being reformed and refilled with transmitter⁶⁴. By contrast, small mammalian CNS synapses in culture have a small number of vesicles; recycling vesicles retain their identity, are not reformed and are rapidly reused^{65,66}. The recycling pool is a small fraction of the total, exchanging slowly with the reserve pool (FIG. 5A).

Estimation of vesicle pools was originally made on the basis of electrophysiological measurements of synaptic responses during rapid stimulation ('tetanic rundown'), as illustrated in FIG. 5B. Electron microscopy offered a structural view: vesicles that were close enough to the synaptic membrane to be released by an action potential could be counted. However, not all of these DOCKED VESICLES could be assumed to be rapidly released, because biochemical priming is also needed. More satisfactory ways of observing vesicular release have evolved with the advent of fluorescent lipophilic dyes (such as FM1-43) and fluorescent lipids⁶⁷, which are taken up

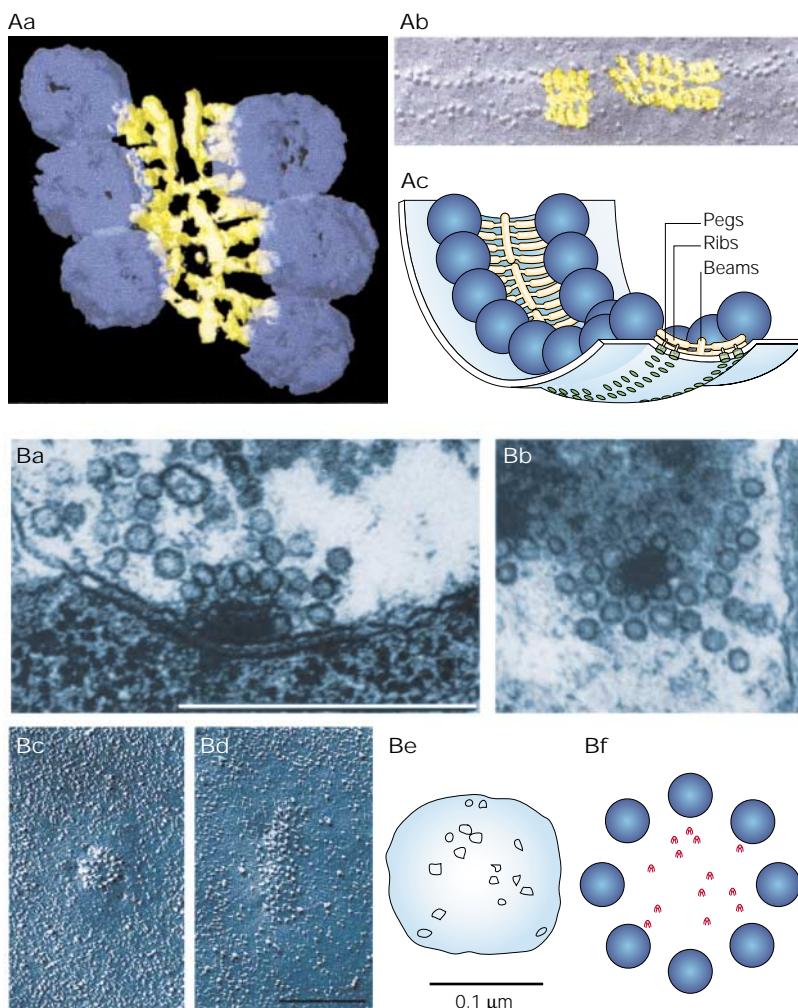


Figure 6 | Active zone structure. **A** | The frog neuromuscular active zone, reconstructed with the aid of electron microscope tomography, exhibits a regular subcellular arrangement in which docked synaptic vesicles are tethered at a uniform distance from large intramembranous particles (putative Ca^{2+} channels). Individual synaptic vesicles are joined by 'ribs' to a central 'beam' that runs the length of the active zone. Small 'pegs' connect the ribs to large active zone particles. This arrangement provides a fixed spatial relationship between docked vesicles and Ca^{2+} channels. **B** | A presynaptic 'dense bar' (seen transversely in **Ba** and tangentially in **Bb**) characterizes the crustacean active zone. Around the edges of the dense bar, there are several docked synaptic vesicles (seven vesicles in **Bb**). Freeze-fracture views of the presynaptic membrane (**Bc**) reveal an array of large transmembrane particles (putative Ca^{2+} channels), usually with images of vesicular fusion around the edge of the particle collection. Individual active zones differ in size and, therefore, in the number of particles and docked vesicles at their edges (**Bc,d**). Mapping the individual active zone particles (**Be**) shows their variable separation from docked vesicles (**Bf**). In this type of active zone, the spatial relationship between Ca^{2+} channels and synaptic vesicles is variable. Scale bars: 1 μm (**Ba,b**); 0.5 μm (**Bc,d**); 0.1 μm (**Be,f**). Part **A** adapted, with permission, from *Nature* (REF. 160) © 2001 Macmillan Magazines Ltd; part **Ba** adapted, with permission, from REF. 161 © 1994 Wiley-Liss, Inc.; parts **Bb,e,f** adapted, with permission, from REF. 162 © Sheffield Academic Press; parts **Bc,d** adapted, with permission, from REF. 163 © 1995 Wiley-Liss, Inc.

DOCKED VESICLES
Synaptic vesicles that are tethered to the presynaptic membrane or the active zone structure. According to current views, not all docked vesicles are fully primed for fusion and release of transmitter.

during endocytosis. With these tools, individual vesicles can be tracked during their fusion cycle, confirming the existence of different pools⁶⁸. The sizes of recycling vesicle pools of cultured mammalian CNS synapses can be measured by counting vesicles that are labelled with FM1-43 that is taken up after exocytosis, which can then be visualized by electron microscopy after photoconversion of the dye (FIG. 5A).

Optical- and electron-microscopic work on cultured mammalian neurons provides a clear picture of the relationship between docked and readily releasable vesicles, and between readily releasable pool size and the probability of vesicular release. As illustrated in FIG. 5A, the size of the readily releasable pool varies by more than tenfold between individual synapses, and there is a linear relationship between docked and readily releasable vesicles; most of the docked vesicles are apparently primed for release⁶⁹. The readily releasable pool size is tightly linked to release probability (FIG. 5A) and in turn to synapse size^{70,71}. These relationships, which have been best established for synapses in culture, have been postulated to apply also to synapses *in vivo*⁴².

The hypothesis that probability of release is proportional to the number of docked vesicles has been examined in several *in situ* studies. The number of readily releasable vesicles and their probability of release could explain the heterogeneity of initial release from SCHAFER COLLATERAL synapses (which have a single release site). Initially, release probability varies considerably between synapses, probably owing to differences in vesicle priming. Short-term plasticity after initial release is also heterogeneous and reflects vesicle recruitment to the releasable state⁷². The situation for synapses *in situ* (even for neurons of the same type) seems to be more complex than for cultured synapses.

The hypothesis has also been tested for case II differentiation in several mammalian and crustacean synapses, but it has not been supported by several investigations. An example comes from the ultrastructural analysis of depressing climbing fibre synapses and facilitating parallel fibre synapses on cerebellar Purkinje cells (see FIG. 2). These synapses are similar in size and in the average number of docked vesicles⁷³. Differences in probability of release cannot be related to numbers of docked vesicles.

This hypothesis can be tested by measuring rapid depletion of readily releasable vesicles. For the calyx of Held, Schneggenburger *et al.*⁷⁴ have estimated the initial readily releasable pool size from plots of cumulative response amplitudes (FIG. 5B). This method works well for crustacean synapses and allows the comparison of readily releasable pool sizes for single boutons⁷⁵. The paired phasic and tonic limb motor neurons (FIG. 2) provide a good test case. Surprisingly, the pool size of tonic boutons is larger than that of phasic boutons (FIG. 5B), despite the much lower probability of transmission for tonic boutons. Electron microscopy has previously shown lower total and docked vesicle counts for crustacean phasic boutons⁷⁶, in support of the electrophysiological measurements. Release probability, and not the number of docked vesicles, determines synaptic strength in these neurons.

The lesson to be drawn from these examples is that neurons of different types (case II) probably differ in their molecular regulation of release probability, and that these differences are superimposed on varying synaptic morphology. So, in the relatively simple situation in culture, the rules that govern vesicle fusion are

probably less complex than in the CNS. The risks of generalizing too broadly from a simplified system to the intact CNS should be recognized.

Active zones: tight or loose organization?

The concept of the active zone emerged from ultrastructural studies, initially at the frog NMJ⁷⁷ and subsequently at other synapses. The frog NMJ provides particularly favourable circumstances for structural studies, because the linear active zones cross the presynaptic membrane at regular intervals of about 1 μm . FREEZE FRACTURE studies⁶⁴ showed paired rows of prominent membrane particles and, beside them, images of vesicular fusion that corresponded to ultrastructurally confirmed locations of docked synaptic vesicles (FIG. 6A). Other vertebrate NMJs have relatively similar arrangements: lizard and mammalian phasic NMJs have closely spaced double rows of the paired-particle arrays, between which docked vesicles are tightly sandwiched; lizard tonic muscles have single rows of paired particles that are fewer in total number⁷⁸. The interpretation of the particle rows is that most of them are voltage-gated Ca^{2+} channels. On theoretical grounds, they have to be in such locations because the time that is available for Ca^{2+} to activate vesicular fusion (about 0.2 ms) requires close vesicle-channel spacing⁷⁹. Other lines of evidence support this interpretation. First, Ca^{2+} channel labelling showed that they are located at the active zone⁸⁰. Second, the immunolabelling of Ca^{2+} channels with antibodies that bear gold particles, followed by their localization on the surface of the presynaptic membrane with an ATOMIC FORCE MICROSCOPE, revealed the expected row-like arrangement in the large calyiform synapse of the chick ciliary ganglion, although their geometry was much less regular than at the frog NMJ⁸¹.

ELECTRON MICROSCOPE TOMOGRAPHY has provided a striking extension of the structural work at the frog NMJ. Reconstructions have shown a repeating array of 'beams' and 'ribs' that connect docked synaptic vesicles to the putative Ca^{2+} channels (FIG. 6A). This active zone is highly organized: docked vesicles are aligned with the Ca^{2+} channels in a preferred orientation and with constant separation.

Is such a tightly organized structure characteristic of other synapses? Although comparable tomographic work has not appeared yet, available electron microscopic work often indicates a less regular organization. Freeze-fracture views of presynaptic membranes of insect and crustacean NMJs do not show the regular particle arrays of their vertebrate counterparts. In crustaceans, the active zones are usually small, discrete patches of variable size, with particles scattered over a region that underlies a presynaptic 'dense bar' or 'dense projection'; vesicles are docked around the edges of this structure, and are therefore at varying distances from the putative Ca^{2+} channels (FIG. 6B).

For central synapses, less correlative information is available. Some have active zones in which dense projections separate docked vesicles in a regular pattern (FIG. 5A). The PRESYNAPTIC GRID can be isolated as a structural unit, a clear indication of a regular array of presynaptic

structural proteins⁸²; it might even be transported to the terminal in preassembled form⁸³. The large calyx of Held and the endbulbs of Held in the cochlear nucleus have variable numbers of docked vesicles at active zones, the general appearance of which is similar to that seen in crustaceans⁸⁴. Precise locations of Ca^{2+} channels are not known for these synapses, but modelling studies point to a variable arrangement⁸⁵. Until ultrastructural details of the relationship between Ca^{2+} channels and docked vesicles are available, especially for CNS synapses, we must surmise that the separation is often variable (FIG. 6B). For those who advocate a close link between synaptic vesicles and presynaptic Ca^{2+} channels on the basis of protein-interaction studies⁸⁶, variable spacing might be disappointing, because it implies that Ca^{2+} channels can function without an obligatory link to a synaptic vesicle and that channels at various distances contribute to transmitter release. On the other hand, synaptic diversity can be better explained in this scenario.

Attempts to correlate differences in active zone structure with synaptic strength have achieved partial success. At frog NMJs, active zone size correlates with synaptic strength⁸⁷. At crustacean NMJs, different endings of the same neuron (FIG. 1, case I) have synapses with different numbers and sizes of active zones, correlating with Ca^{2+} entry and release probability^{88,89}. But the correlation fails for crustacean phasic and tonic endings on the same follower cell (FIG. 2, case II). Here, synapses and active zones are similar in size, and freeze-fracture images show similar numbers of putative Ca^{2+} channels⁸⁰. So, quantitative differences in active zone structure can partially explain case I, but not case II, differentiation. The same conclusion might apply to the mammalian CNS.

Differences in presynaptic Ca^{2+} channels

Transmitter release can be triggered by Ca^{2+} entry through Ca^{2+} channels of different types⁹¹. P/Q-, R- and N-type Ca^{2+} channels are most often reported on nerve terminals⁹². Channels with larger conductance or with a longer opening time could deliver more Ca^{2+} ions to trigger vesicle fusion. Also, the probability of channel opening after an action potential would affect the probability of transmitter release. What is the likelihood that these features account for differences in synaptic strength?

Heterogeneity in single-channel conductance occurs between different channels and for a single channel type in different neurons. There is considerable overlap in single-channel conductance between P/Q-, R- and N-type channels (~ 9 – 20 pS), whereas for T-type Ca^{2+} channels, which are less common at synapses, conductance is lower (~ 8 pS)⁹³. Adding to this complexity, individual channel subtypes can also operate at multiple conductance levels and show multimodal activation kinetics.

Differences in the inactivation kinetics of Ca^{2+} channels could produce differences in the amount of Ca^{2+} that enters the presynaptic terminal. Variation in the pore-forming α_1 -subunit of Ca^{2+} channels produces differences in inactivation rate. In addition, the accessory β -subunits of Ca^{2+} channels affect the inactivation kinetics. Whereas some β -subunits speed the

SCHAFFER COLLATERALS
Axons of the CA3 pyramidal cells that form synapses with the apical dendrites of hippocampal CA1 neurons.

FREEZE FRACTURE
An electron-microscopic method in which rapidly frozen tissue is cracked to produce a fracture plane through the specimen. The surface of the fracture plane is shadowed by a heavy metal, and the specimen is digested away to leave a replica that can be examined under the electron microscope.

ATOMIC FORCE MICROSCOPY
A form of microscopy in which a probe is mechanically tracked over a surface of interest in a series of x-y scans. The force found at each coordinate is measured with piezoelectric sensors, providing information about the chemical nature of a surface.

ELECTRON MICROSCOPE TOMOGRAPHY
A method for the three-dimensional reconstruction of objects from a series of projection images that are recorded with a transmission electron microscope. It offers the opportunity to obtain spatial information on structural arrangements of cellular components.

PRESYNAPTIC GRID
A dense matrix of proteins that is associated with the active zone, which is thought to have a crucial role in defining the transmitter release sites.

rate of inactivation, others have the opposite effect⁹⁴. Different β -subunits are differentially distributed in the brain and might associate with different α_1 -subunits in a cell-specific manner⁹⁵.

G proteins also mediate inhibition of presynaptic Ca^{2+} channels through their interaction with the α_1 -subunit. The degree of inhibition is dependent on the type of β -subunit of the interacting G protein⁹². G-protein β -subunits are also expressed differentially in several regions of the brain⁹⁶. Together, the multiplicity of protein interactions, and the differential distribution of both Ca^{2+} channel and G-protein subunits, could fine-tune Ca^{2+} entry to create specificity of release efficacy at different synapses.

Given that differences in channel properties — kinetics and opening probability — could contribute to synaptic functional differentiation, are there differences in the occurrence of channel subtypes that can be linked to differences in synaptic strength? It is not yet clear that this is the case. With regard to case I differentiation, Reid *et al.*⁹⁷ reported that, in a population of excitatory cultured hippocampal synapses in which release efficacy was assayed, 45% of them expressed purely P/Q-type Ca^{2+} channels, 45% both P/Q- and N-type channels, and the remaining 10% purely N-type channels. However, the differential distribution of channels did not correlate with differences in release efficacy. The same authors subsequently reported no difference in the degree of cooperativity for transmitter release mediated by either P/Q- or N-type channels⁹⁸. They speculated that differences in release efficacy might instead arise from structural or biochemical differences between synaptic inputs.

Regarding case II differentiation, individual muscle fibres in the closer muscle of the crab *Eriphia spinifrons* are dually innervated by two motor neurons⁹⁹. Both phasic and tonic terminal boutons express P/Q-type Ca^{2+} channels, but N-type channels were found only on phasic boutons and R-type channels on tonic terminals. Differential regulation of transmitter release by peptides was attributed to effects on the different Ca^{2+} channels. However, the large difference in phasic/tonic transmitter release per synapse is probably not due to differences in Ca^{2+} entry at the active zone⁹⁰, and the occurrence of a mix of different channels is probably not the main determinant of synaptic strength in this case.

At the calyx of Held, N- and R-type channels are less effective in triggering transmitter release than P/Q-type channels¹⁰⁰. P/Q-type channels are more closely localized with synaptic vesicle clusters, and this provides an explanation of why N- and R-type channels are less effective in triggering exocytosis. However, this feature is not universal. At the calyiform synapse of the chick ciliary ganglion, N-type Ca^{2+} channels predominate and single-channel openings can trigger vesicle release, indicating a close association of channels with the release apparatus^{31,101}. By contrast, multiple types of Ca^{2+} channel are equally effective at rat MELANOTROPHS in triggering vesicle fusion, indicating that vesicles do not specifically co-localize with or require a specific Ca^{2+} channel for exocytosis¹⁰².

In the case of the mammalian CNS, Westenbroek *et al.*¹⁰³ reported that rat and rabbit isoforms of the Ca^{2+} channel α_{1A} -subunit (which forms P/Q-type channels) are differentially distributed in nerve terminals in the cerebellum and hippocampus. Furthermore, the rabbit isoform can bind the SNARE PROTEINS **syntaxin** and **SNAP-25**, whereas the rat isoform binds only SNAP-25 *in vitro*¹⁰⁴. This example indicates that the specificity of Ca^{2+} channels for SNARE proteins might explain differences in release efficacy between synapses. Numerous other reports indicate interactions between Ca^{2+} channel α_1 -subunits and vesicle-associated or active zone proteins¹⁰⁵. However, experimental data on normally occurring functional synaptic differences that can be linked to these findings have yet to be obtained.

From this survey, we conclude that, although differences in Ca^{2+} channel properties and distribution could, in principle, contribute to differences in synaptic strength, evidence for such a link is, at best, suggestive, as crucial physiological experiments that show a causal relationship *in situ* are still lacking.

Effects of Ca^{2+} channel-vesicle spacing

At each Ca^{2+} channel that is opened by a presynaptic action potential, a local increase in intracellular Ca^{2+} rapidly develops¹⁰⁶, which we shall refer to as a 'microdomain'. The brief lifetime and small size of the microdomain preclude its resolution by light or confocal microscopy with conventional Ca^{2+} indicators. A procedure in which a low-affinity version of the Ca^{2+} -binding photoprotein aequorin was used, together with very rapid imaging, succeeded in showing such events, which might have corresponded to the opening of a few Ca^{2+} channels, at the squid giant synapse¹⁰⁷. Larger events (synaptic 'domains', which are created by an ensemble of Ca^{2+} channels at an active zone) have been described more frequently^{108,109}. The increase in Ca^{2+} that is detected in a nerve terminal generally represents Ca^{2+} distribution throughout the bouton after the dissipation of the individual domains¹¹⁰. Whole-bouton Ca^{2+} concentration is much lower than in local domains; the decline in Ca^{2+} levels with distance from a Ca^{2+} channel is very steep¹¹¹.

When readily releasable vesicles are closely associated with Ca^{2+} channels, the microdomain of a single channel can cause vesicle fusion¹⁰¹. By contrast, when releasable vesicles and Ca^{2+} channels are not closely associated, more than one Ca^{2+} channel must open before the Ca^{2+} concentration at the crucial Ca^{2+} sensor is sufficient to trigger the fusion event. So, the probability of a fusion event depends on the spacing of Ca^{2+} channels and readily releasable vesicles, and on the properties of the Ca^{2+} sensor (such as its affinity for Ca^{2+} , and its binding and unbinding kinetics).

Instead of direct measurements of microdomains, extensive modelling studies have been developed. They incorporate several different channel and vesicle spacings, together with mobile and immobile Ca^{2+} buffers^{112–114} (BOX 2). A general feature of these models is that the outcome of vesicle release depends crucially on the nature of the intracellular Ca^{2+} buffers, including their kinetic properties, their mobility and their concentration.

MELANOTROPHS

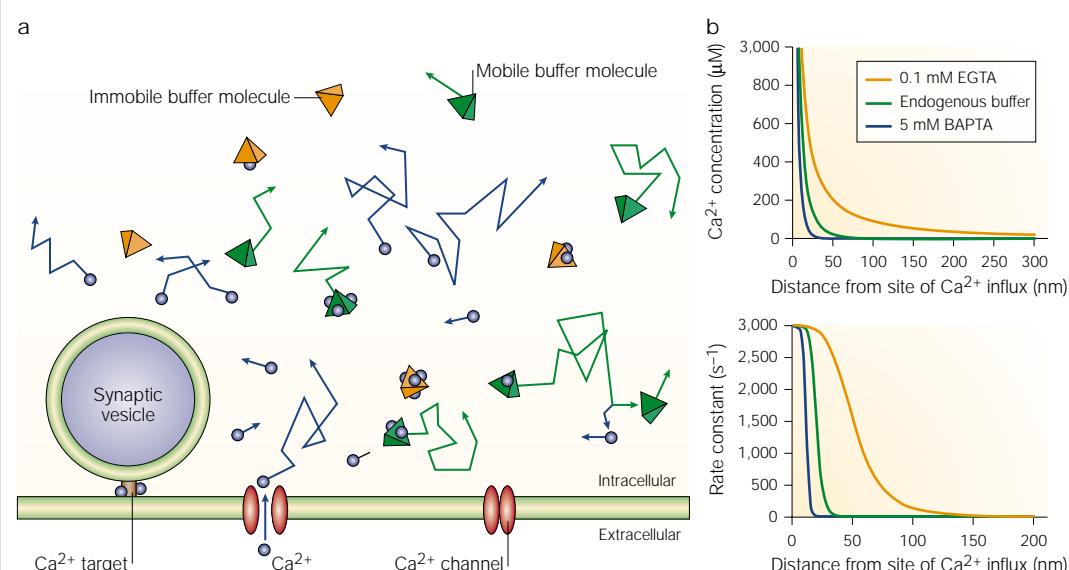
Excitable cells from the pituitary that are specialized in secreting several peptide hormones such as β -endorphin and adrenocorticotropin.

SNARE PROTEINS

A family of membrane-tethered coiled-coil proteins that regulate exocytic reactions and target specificity in vesicular fusion processes. SNARE stands for 'soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptor'.

Box 2 | Effects of presynaptic Ca^{2+} buffers on Ca^{2+} spread and exocytosis

Panel a of the figure shows mobile and immobile Ca^{2+} buffers interacting with Ca^{2+} ions near a docked vesicle at an active zone. Immobile buffers bind entering Ca^{2+} ions, but can also release them in the active zone region. Mobile buffers bind Ca^{2+} ions and transport some of them away from docked vesicles, thereby lowering the local Ca^{2+} concentration and the probability of exocytosis. Buffer effects depend crucially on the on and off rate constants of the Ca^{2+} -buffer interaction. An example from retinal bipolar cells (panel b) shows buffer effects on steady-state free Ca^{2+} near its entry point (top graph), and on the rate of exocytosis as a function of distance from the entry point (bottom graph). The presynaptic terminal behaves as if it normally had a mobile buffer with an efficiency equivalent to that of 1–3 mM BAPTA. Presynaptic Ca^{2+} buffers can limit synaptic strength by intercepting Ca^{2+} before it binds to the Ca^{2+} sensors that regulate exocytosis. This effect is more pronounced with increasing distance between the entry point (the Ca^{2+} channels) and the docked vesicles. Part a adapted from REF. 155; part b adapted, with permission, from REF. 156 © 2002 Elsevier Science.



Furthermore, as intracellular Ca^{2+} buffers can intercept and remove ions before they bind to the Ca^{2+} sensor, they have a greater influence when channels and releasable vesicles are separated (FIG. 3). Indeed, a very close association would severely limit the effects of intracellular buffers on vesicle fusion¹¹⁵. However, information about intracellular buffers is incomplete. Kinetics of the native intracellular buffer have been measured in CHROMAFFIN CELLS¹¹⁶ but not in nerve terminals, although buffer capacities have been established for some of them¹¹⁰.

Our survey of active zone structure (FIG. 6) indicated a highly organized vesicle–channel relationship at some synapses and a less regular organization at others. Physiological experiments aimed at determining the functional importance of channel–vesicle spacing have relied on the introduction of exogenous Ca^{2+} buffers with known properties into the nerve terminal to perturb release. This approach has confirmed the importance of spacing for synaptic diversity.

Experiments with exogenous buffers have given variable results. At the squid giant synapse, a slow buffer (EGTA) was not effective, but a fast buffer (BAPTA) inhibited release¹¹⁷. At the calyx of Held and at other mammalian CNS synapses, both buffers affect release and are differentially effective¹¹⁸. So, differences in channel–vesicle spacing probably exist between synapses.

To illustrate buffer effects, we present in BOX 2 an example from retinal bipolar cells; here, EGTA and BAPTA differentially affect the Ca^{2+} concentration profile and, consequently, the rate of transmitter release near a site of Ca^{2+} entry. Removing buffers by internal perfusion greatly augments transmitter release by transient Ca^{2+} entry. The pool of readily releasable vesicles also increases, probably because vesicles farther from Ca^{2+} channels are recruited for release. At the calyx of Held, modelling similar buffer effects has led to the inference that vesicle–channel distances vary at the different active zones of the calyx, such that releasable vesicles ‘sense’ Ca^{2+} transients that range in amplitude from 0.5 to 40 μM (REF. 85). Such heterogeneity probably determines the fast and slow phases of transmitter release at this synapse.

Perhaps the clearest proof of the importance of channel–vesicle spacing in synaptic functional differentiation comes from the analysis of buffer effects at cortical pyramidal cell synapses (FIGS 1 and 7). Intracellularly applied buffers affected transmitter release differentially at facilitating and depressing synapses (FIG. 7). An interpretation of this difference is that the mean diffusion distance between Ca^{2+} channels and releasable vesicles is longer in facilitating synapses, possibly reflecting a lower channel density¹¹⁸.

CHROMAFFIN CELLS
Cells of the adrenal gland that store and secrete catecholamines. They are known as ‘chromaffin’ cells because of the ability of chromium salts to stain them.

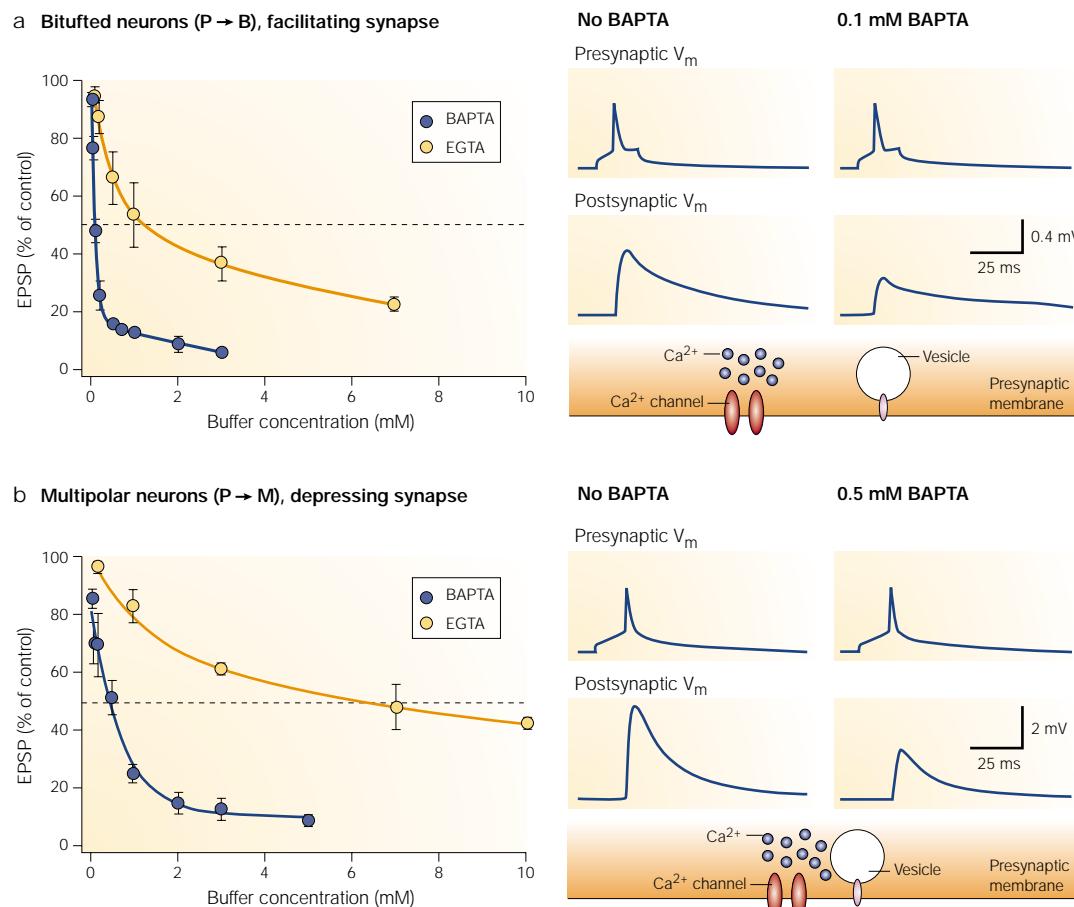


Figure 7 | Differential effects of Ca^{2+} buffers in mammalian central neurons. **a,b** | Excitatory postsynaptic potentials (EPSPs) were measured before and after the infusion of Ca^{2+} buffers into presynaptic cortical pyramidal neurons (P; see FIG. 1Bb), and the dependence of synaptic transmission on buffer concentration was determined. Both slow (EGTA) and fast (BAPTA) Ca^{2+} buffers attenuated the synaptic potential (less transmitter was released), and the fast buffer was more effective. However, the buffer concentrations that were needed to achieve 50% attenuation of the response (dashed horizontal lines) were significantly less for synapses on bitufted neurons (P → B, facilitating synapses; **a**) than for synapses on multipolar neurons (P → M, depressing synapses; **b**). The results indicate that the mean distance between presynaptic Ca^{2+} channels and docked vesicles is greater for facilitating synapses, which produce smaller initial synaptic potentials. V_m , membrane potential. Adapted, with permission, from REF. 118 © 2001 The Physiological Society.

From these examples, we can conclude that differences in vesicle–channel spacing and intracellular Ca^{2+} buffers contribute significantly to differences in synaptic strength. However, ultrastructural information on channel–vesicle spacing is not available for some of the synapses for which physiological information has been obtained.

Ca^{2+} sensitivity of release

One possible mechanism of synaptic functional differentiation is the existence of Ca^{2+} receptors with different affinities (FIG. 3f). Determining the Ca^{2+} concentration that is effective for vesicle fusion requires a method for elevating Ca^{2+} uniformly and rapidly in the presynaptic structure. If this is not done, the effects of channel–vesicle spacing and buffers are likely to create spatial non-uniformities in Ca^{2+} , making it difficult to estimate its concentration at specific locations. The apparent non-uniformity in Ca^{2+} sensitivity between releasable vesicles

of the calyx of Held¹¹⁹ can be adequately explained by variation in channel–vesicle spacing⁸⁵ (BOX 2). Flash photolysis of CAGED Ca^{2+} that is injected into the presynaptic terminal circumvents this problem by rapidly creating a uniform increase in Ca^{2+} throughout the structure¹²⁰.

Estimates of Ca^{2+} levels in or near microdomains close to release sites of the squid giant synapse initially led to the view that 70–300 μM Ca^{2+} is required for rapid release¹²¹. Values in this range were confirmed by flash photolysis in retinal bipolar cells¹²². However, lower values (below 10 μM) were reported for crayfish nerve terminals¹²³. Recently, flash photolysis at the calyx of Held has produced values that are much lower than anticipated — 6–10 μM (REFS 124,125). During a pre-synaptic action potential at this synapse, Ca^{2+} concentration at the release site probably reaches 20–30 μM , which is below saturating values for the Ca^{2+} sensor. In *Aplysia californica*, peptidergic and cholinergic synapses seem to have different Ca^{2+} requirements for release¹²⁶.

CAGED MOLECULE
A labile derivative of a biologically active molecule that will break down after photolysis of the precursor to yield the bioactive compound.

The fact that different values for triggering release have been obtained with flash photolysis lends credibility to the view that functional differentiation can include Ca^{2+} sensitivity as a variable parameter. Manipulation of several presynaptic proteins can produce apparent differences in Ca^{2+} sensitivity^{127,128}. Whether such a mechanism is normally used remains an open question.

Presynaptic proteins and function

Synaptic structural features cannot fully account for differences in synaptic strength. So, underlying molecular differences must make an important contribution to final performance. Features such as Ca^{2+} sensitivity and synchronization of transmitter release ultimately depend on differences in proteins that are associated with synaptic vesicles and the active zone. A further level

of regulation is added by second-messenger systems that modify the functional capabilities of the available proteins, and that might be differentially active between neurons or synaptic terminals. Important differences in mitochondria and ATP supply occur at synapses¹²⁹ and could affect synaptic performance. Other second messengers and enzymes (particularly protein kinases and phosphatases) are likely to vary between neurons. Owing to the complexity of the intracellular milieu, it is not an easy task to expose the identity of individual molecules as prime movers for synaptic functional differentiation. In assessing molecular variables, we must beware of equating the occurrence of a molecular species with its causative role, and we should aim to distinguish the essential agents of a process (mediators) from the more numerous secondary modifiers of the process (modulators)¹³⁰. We must also be aware that combinations of proteins, rather than single molecules, determine performance.

In the case of vesicle fusion and exocytosis, the essential mediators are the SNARE proteins — **synaptobrevin/VAMP** on the synaptic vesicle, and **syntaxin** and **SNAP-25** on the presynaptic membrane. Without them, the fusion process fails; they are universally present at fast synapses and are highly conserved in sequence¹³¹. Most of the other identified presynaptic proteins are not essential for the fusion event. But some are permissive, and others take part in facilitating or inhibiting vesicle movement or other steps. Some are probably important modulators, conferring differences in synaptic plasticity or strength. So, investigations of molecular determinants of synaptic strength are likely to be focused mainly on the roles of modulators of exocytosis, owing to their greater number.

To explain cases I and II of functional differentiation in molecular terms, specific presynaptic proteins or isoforms would have to be differentially localized, as indicated in FIG. 8. This is a prerequisite for a causative role. However, the functional state of the protein must still be ascertained: constitutive and stimulated phosphorylation or dephosphorylation alter protein function, and local conditions that promote these reactions can vary between neurons. So, a one-protein approach to the problem, although necessary as a starting point, is somewhat naive.

How well can the known molecular candidates explain synaptic differentiation? From a survey of those that have been described, we have selected exemplary candidate molecules (TABLE 1) to illustrate the current state of investigations. We shall summarize some general observations, then discuss illustrative examples for case I and case II differentiation.

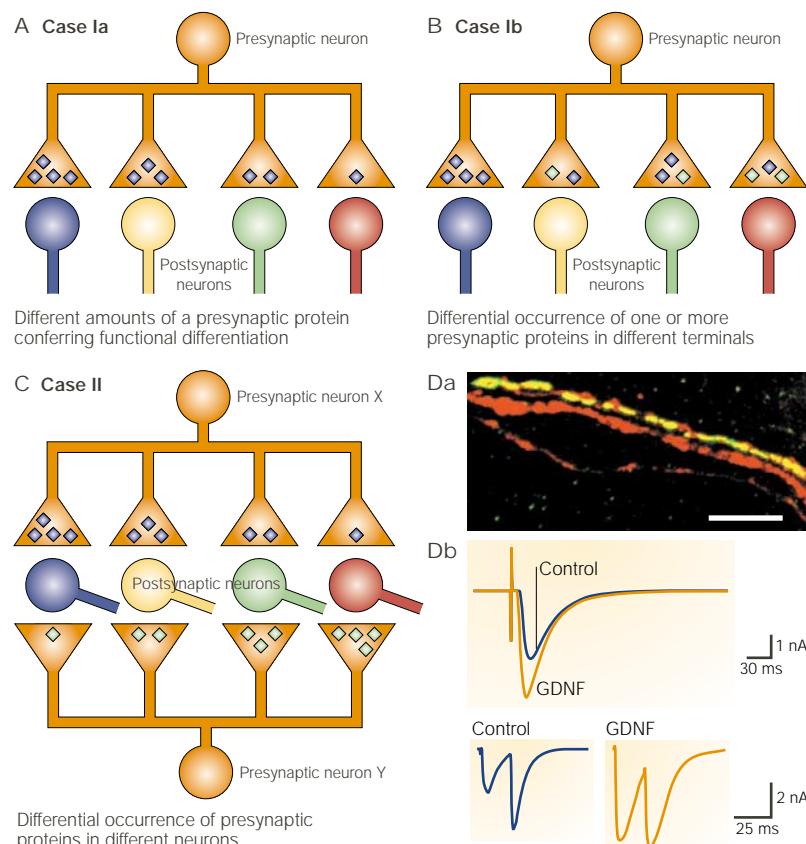


Figure 8 | Possible mechanisms that lead to differential synaptic strength. **A,B** | For case I differentiation, postsynaptic neurons induce or attract different amounts of a strength-regulating presynaptic protein (**A**) or variable combinations of more than one presynaptic protein (**B**). **C** | For case II differentiation, different presynaptic neurons generate their own isoforms or different molecular species of strength-regulating proteins. Induction of such proteins could be regulated by neuronal activity or by specific inputs to the presynaptic neuron. The Ca^{2+} -binding protein **frequenin** provides an example of differential occurrence of a strength-regulating presynaptic protein. **Da** | **Frequenin**, which is known to enhance synaptic transmission, selectively occurs in crustacean phasic, but not in tonic, nerve terminals that innervate the same postsynaptic muscle fibres. An antibody against **synapsin** (red) stained both phasic and tonic terminals, whereas an antibody against **frequenin** (green) stained only the phasic terminal (yellow indicates double staining). Scale bar: 20 μm . **Db** | Synaptic enhancement of *Xenopus laevis* neuromuscular junctions in culture appears soon after treatment with glial-cell-line-derived neurotrophic factor (GDNF), which exerts its effect by increasing the neuronal expression of **frequenin**. Part **Db** adapted, with permission, from REF. 146 © 2001 Elsevier Science.

Table 1 | Presynaptic proteins that are candidates to participate in functional differentiation

Protein (and proposed role)	Differential expression	Changes to synaptic transmission by altering protein function	References
Munc 13 (vesicle-priming factor)	Munc 13-1 and Munc 13-2 isoforms are found in different synapses	In Munc-13-1-deficient cultured synapses, release cannot be triggered by action potentials, Ca^{2+} ionophores or hypertonic solutions	135,137
Rabphilin (exocytosis and/or vesicle mobilization)	Found in a subset of synapses in hippocampal cultures; in the cerebellum, phosphorylated rabphilin is found only in climbing, but not in parallel, fibres; in the retina, the unphosphorylated form is found only in ribbon synapses	No alteration in synaptic transmission in rabphilin-deficient mice	164,165,166
Synaptotagmin (Ca^{2+} sensor)	Isoform I is found in rostral brain regions; in the rat, it is in sensory and autonomic neurons, but not in motor neurons; isoform II is found in caudal brain regions	Point mutation in synaptotagmin I reduces Ca^{2+} sensitivity to transmitter release in mice; in <i>Drosophila</i> , mutating the C2 Ca^{2+} -binding domain of synaptotagmin I reduced evoked release and increased spontaneous release; isoforms I and IV support different rates of evoked release	138,143,144,167–169
Synapsin (vesicle mobilization)	In cerebellar nuclei, all isoforms are present, but Purkinje cells lack isoform IIa; in the trapezoid body of the brainstem, isoform IIb is present only in a subset of synapses; in the retina, synapsins are absent from ribbon synapses, but are found in conventional synapses	Phenotypes revealed in synapsin-I-deficient mice: (1) delayed recovery of synaptic transmission after a tetanus; (2) increased paired-pulse facilitation; (3) epileptic seizures	170–172
Rab3A/C (regulates exocytosis)	Localized in subpopulations of neurons in the <i>Torpedo</i> central nervous system	In Rab3A-deficient mice, paired-pulse facilitation is enhanced, and long-term potentiation at mossy fibre synapses is abolished	173–175
Synaptophysin (synaptic plasticity)	Synaptophysin I is homogeneously distributed; synaptophysin II is not found in neuron subtypes of the olfactory bulb, hippocampus, striatum, spinal cord or cerebellum	No alteration in synaptic transmission in synaptophysin-I-deficient mice	176–179
SV2 (vesicle fusion)	SV2A is in cell populations of rat hippocampus, diencephalon, superior and inferior colliculus, and cerebellum; SV2B is in cell populations of rat cerebellum and brainstem; SV2C is restricted to a subset of neurons in the basal forebrain	Cultured hippocampal neurons from double mutants lacking SV2A and SV2B showed sustained Ca^{2+} -dependent synaptic transmission in response to two or more action potentials	180–183
VAMP (synaptobrevin) (member of SNARE complex)	In the rat, VAMP1 is more highly expressed in brain nuclei and spinal cord lamellae that are involved in somatomotor function; VAMP2 expression predominates in nuclei that participate in autonomic, motor, sensory and integrative functions	In <i>Drosophila</i> embryos, evoked release is completely abolished, but spontaneous release persists at a diminished rate; in <i>Drosophila</i> larvae, VAMP mutants showed reduced Ca^{2+} cooperativity of synaptic transmission	184–186
SVOP (transporter of unidentified cofactors)	Found in all cortical layers, but strong labelling in pyramidal cells of layers 3 and 5, and low levels in layer 4	Unknown	187
CSPs (regulate transmitter release)	Differentially distributed in many regions; in rat hippocampus, strong expression in CA3 and hilus of the dentate gyrus, less in CA1	<i>Drosophila</i> mutants show reduced transmission and prolonged Ca^{2+} signals in boutons at non-permissive temperatures; CSP enhances Ca^{2+} channel activity in the chick ciliary ganglion calyiform synapse	188–190
Syntaxin (vesicle docking)	Syntaxin IA and IB are differentially distributed in many areas of the rat brain	In <i>Drosophila</i> mutants, synaptic transmission is nearly abolished; they show severely impaired responses to hypertonic solutions	191,192
SNAP-25 (member of SNARE complex)	Isoforms SNAP-25a and SNAP-25b are differentially distributed in regions of the cortex, thalamus, substantia nigra and pineal gland	Mice that lack SNAP-25 display prolonged spontaneous hyperactivity, with region- and neurotransmitter-specific deficiency in release	193,194
Volado (regulates vesicle fusion)	Found in subsets of synapses in the central neuropil and the neuromuscular junction of <i>Drosophila</i> larvae	The neuromuscular junction of Volado mutants shows increased transmission, reduced Ca^{2+} dependence of release, and impaired facilitation, augmentation and post-tetanic potentiation	195
Discs-large (regulates synaptic strength)	Observed in <i>Drosophila</i> type I and III boutons, but not in type II boutons	Mutant <i>Drosophila</i> larvae show increased neurotransmitter release and Ca^{2+} sensitivity	196,197
Frequenin (neuronal Ca^{2+} sensor)	Found in phasic, but not in tonic, boutons at the crayfish neuromuscular junction; in humans, it is highly expressed in the cortex (especially in temporal lobe), and has very low expression in the spinal cord and corpus callosum	Overexpression of frequenin in <i>Drosophila</i> larvae enhanced facilitation; in <i>Xenopus</i> , evoked and spontaneous release were enhanced	147,150,151,198
Piccolo and Bassoon (components of presynaptic cytoskeletal matrix)	Both proteins are found in glutamate and GABA synapses, but not at the neuromuscular junction; Bassoon found in photoreceptors, but not at bipolar ribbon synapses	Unknown	199,200

Munc 13 and rabphilin are candidates to participate in case I differentiation. Rabphilin and the remainder of the molecules are candidates to participate in case II differentiation. CSP, cysteine-string protein; GABA, γ -aminobutyric acid; SV2, synaptic vesicle protein 2; SVOP, SV2-related protein.

difficult, combinations of molecules, rather than individual species, determine the final functional outcome. So, a huge array of molecular possibilities for functional fine-tuning exists^{16,132}. To some extent, co-localization can be best analysed in culture, where function and molecular species can be observed at individual synapses¹³³. However, isolated neurons in culture often differ from their *in vivo* counterparts.

Removal or disturbance. Both in mouse and in *Drosophila*, functional studies of synaptic transmission that are based on mutations and gene deletions have shown that exocytosis can be perturbed or modified by targeting specific proteins (TABLE 1). These studies are the basis for discovering candidate proteins and accepting them as relevant modifiers of synaptic transmission. However, the mutations and deletions that are investigated experimentally do not normally occur in the intact nervous system, and so do not provide, in themselves, a *prima facie* explanation for case I or case II differentiation *in vivo*. When mutations occur under normal circumstances, they sometimes produce a pathological phenotype that can be used to study aspects of function; alternatively, genetic compensation can occur to maintain the normal phenotype.

Normal occurrence. Many molecule–function studies, especially of mammalian central neurons, are based on culture studies, often on isolated neurons that form autapses. This approach provides excellent experimental models, but as the conditions for synapse formation and maintenance are vastly simplified, findings in culture must be verified and extended to a meaningful *in vivo* context. For some species, including molluscs, this has been partially achieved²⁶, whereas for others, such as *Drosophila*, the attempt has been less successful³⁴: mutant phenotypes are observed but the cultured neurons are far from normal, making it difficult to extrapolate to the *in vivo* context.

To illustrate these three general points with specific examples, we first select **Munc 13**, a priming factor for docked synaptic vesicles. This protein is important in preparing vesicles for fusion after they have docked at the active zone, as shown by studies in both mouse¹³⁵ and *Drosophila*¹³⁶. The two mammalian isoforms (Munc 13-1 and Munc 13-2) contribute to synaptic strength and confer different properties of short-term plasticity to their host synapses; Munc 13-1 synapses show depression, whereas Munc 13-2 synapses show facilitation¹³⁷. This provides an attractive possibility for case I differentiation (TABLE 1). In tissue culture, molecule and function co-localize. However, it is not yet known whether this finding extends to normal synapses in the CNS. So, even in this example (which is one of the best), the molecule–function relationship has not been shown to occur *in vivo*.

Synaptotagmin, a vesicle-associated protein, is one of the most thoroughly studied presynaptic proteins. It is thought to be the essential Ca^{2+} sensor that triggers fast exocytosis¹³⁸. Ca^{2+} sensitivity of release¹³⁹ and probably

also fusion pore kinetics¹⁴⁰ depend on this molecule. Phosphorylation and dephosphorylation further modify the Ca^{2+} sensitivity of synaptotagmin¹⁴¹. Among its eight isoforms, some of which are vesicle associated and some membrane associated, a hierarchy of Ca^{2+} affinities prevails, and related functional changes in synaptic transmission occur when different isoforms or mutated forms are expressed in neurons^{142–144}. However, within the normal nervous system, functional attributes of synapses that possess different isoforms have not been shown.

Another example is the Ca^{2+} -binding protein **frequenin**, which was discovered in *Drosophila*¹⁴⁵ and has vertebrate and mammalian counterparts¹⁴⁶. Alteration of its expression in neurons affects synaptic strength¹⁴⁶ and facilitation¹⁴⁷. Increased activation of Ca^{2+} channels¹⁴⁸ and of phosphatidylinositol-4-OH kinase¹⁴⁹ probably contribute to these effects. Within the mammalian CNS, frequenin is heavily localized in the cerebral cortex, but is present at low levels in several other regions¹⁵⁰. The localization pattern has not been correlated with synaptic physiology. However, high-output phasic crustacean neurons express it strongly, whereas low-output tonic neurons do not¹⁵¹ (FIG. 8D). In this case, occurrence is correlated with a functional difference *in situ*, but experimental manipulation of protein level or occurrence while monitoring synaptic function has not been accomplished.

So, we have many possible candidates, but there is no final proof that they are responsible for normally occurring functional differentiation. Zucker and Regehr¹⁵² recently reached a similar conclusion in relation to short-term plasticity.

Conclusions

In the nervous system, individual neurons acquire many of their anatomical and physiological features during development, but continuous adjustment takes place in response to environmental challenges. Developmental programmes impose constraints on physiological plasticity, but within these, considerable modification occurs. The end result is great diversity of synaptic performance. We have concentrated on just one aspect of synaptic physiology — synaptic strength — but when several forms of short-term and long-term plasticity are added to the analysis, the combinations expand to produce many physiological subtypes. Indeed, some authors have concluded that each neuron is unique, both physiologically¹¹ and biochemically¹³².

The problem that we have posed is whether an explanation for differences in synaptic strength can be found in terms of structural and molecular features of synapses. During synapse formation and modification, parallel pathways govern morphology and physiology¹⁵³. To simplify the discussion, we have selected examples that can be classified as case I or case II. In addition to seeking an explanation of why synaptic strength differs in selected neurons, one can search for the upstream controls that are responsible for important features of differentiation.

From our survey, molecular differentiation of neurons *in vivo* — their channel subtypes, their presynaptic vesicle proteins and their second messengers — is exuberant, but seldom matched (*in vivo*) to physiological phenotype. So, in principle, specific combinations of Ca^{2+} channels, G proteins, K^+ channels and various presynaptic proteins, superimposed on variation in metabolic background and combined with structural diversity, provide a rich substrate to match the range of physiological phenotypes. Although we have started to link physiological properties with molecular and structural substrates, our survey indicates that this matching has not progressed very far. An important goal of future research will be to decipher more of the molecular and structural rules that govern the expression of physiological phenotypes.

The structural approach has been the most successful so far, as it has produced some general principles of variations in synaptic strength. For case I differentiation, synaptic strength and non-uniform release probability have been correlated with active zone size and number in crustacean synapses and vertebrate NMJs, with synapse size and docked vesicle number in cultured mammalian CNS synapses, and with Ca^{2+} channel–vesicle spacing at cortical pyramidal cell synapses *in vivo*. On the molecular side, presynaptic molecules have been found to vary in different synaptic boutons of the same neuron. Enough information is available to frame a preliminary hypothesis: case I functional differentiation involves quantitative differences in the synaptic population. The

same molecules might be present in all endings of a parent neuron, but their relative amounts and dispositions vary (FIG. 8A). It is also possible that different molecules occur in separate endings of the same neuron (FIG. 8B), but, at present, this seems less likely.

Case II differentiation presents a greater challenge. Structural explanations are inadequate and the molecular basis for functional differentiation is unknown. Crustacean phasic and tonic synaptic differentiation (FIG. 2) provides an excellent example for heuristic purposes. For a single action potential, individual phasic and tonic boutons differ in quantal output by a factor of 200–1,500. But there are more synapses on the low-output tonic boutons; they are larger on average, have more readily releasable vesicles (FIG. 5), and a larger reservoir of transmitter¹⁵⁴. Freeze-fracture studies of active zone particles and analysis of Ca^{2+} signals indicates no overall superiority of the phasic bouton in either respect⁹⁰. The most likely possibility is a molecular difference that confers much higher fusion probability during the normal Ca^{2+} trigger. In fact, a significant molecular difference in a Ca^{2+} -binding protein that modulates release — frequenin — has been observed (FIG. 8D), but a causative role for this protein in functional differentiation has not yet been shown. In this example, as in others of case II differentiation, closing the loop between molecular and physiological differentiation with unambiguous experiments remains the biggest challenge. So far, this challenge has not been adequately met.

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