act dorsally to prevent the cell bodies of dorsal root ganglion sensory neurons from migrating along their own axons into the spinal cord.

The second set of questions is triggered by the observation that the effects of boundary cap ablation on establishment of the ventral CNS–PNS interface are only partial. For instance, in the absence of boundary cap cells, Vermeren et al. found no evidence for migration of peripheral cells into the CNS, or for escape of cell bodies other than those of motor neurons. Moreover, motor axons left the spinal cord in apparently normal numbers. This suggests that other cell types (radial glia seem likely candidates), and therefore presumably other signalling molecules, must be involved in setting up these aspects of the boundary. Alternatively, the ability to migrate out of the spinal cord might be programmed by retrograde interactions with the periphery, to which only motor neurons are exposed. The authors also report that grafted neural crest cells show a preferential migration towards presumptive motor exit points, suggesting the existence of a chemoattractive signal that might even prefigure the boundary cap. Thus, a complex interplay of cellular and molecular influences remains to be unravelled; its existence suggests that the importance of establishing a precise boundary during development might well have been underestimated.

Cell body migration along axons: a novel regulated process?
Lastly, the nature of the cellular process in motor neurons controlled by boundary cap cells is worthy of much further investigation. It seems clear that this is not a classical process of cell mixing. By observing individual green-fluorescent-protein (GFP)-labelled motor neurons in electroporated chicken embryos, Vermeren et al. concluded that motor neuron cell bodies migrate along their own axon to leave the spinal cord. This is in some ways reminiscent of the interkinetic movement of nuclei during cell proliferation at earlier stages in the neuroepithelium. It will be interesting to determine whether this influences the position of the leading and trailing processes of motor neurons, and suggests that the definition of axonal versus dendritic properties must be rudimentary at this stage. More generally, these findings suggest that studies using only nuclear markers to follow cell migration in similar situations potentially oversimplify the cellular process under observation, and raise the possibility that correct positioning of the nucleus with respect to even pre-established axonal or dendritic processes might be a regulated event with functional consequences.

Indeed, one could argue that Vermeren et al. potentially underestimate the effects of boundary cap cell ablation on motor axonal guidance, because their observation of axonal outgrowth and targeting was performed at the macroscopic level of neurofilament labelling on sections [1]. Does mispositioning of the cell body in the relatively low percentage of ectopic motor neurons lead to incorrect targeting in the periphery? Recent findings suggest that cell body position and axonal projections could be intimately linked: in mice lacking the motor-pool-specific transcription factor PEA3, the motor neurons that normally express this factor are mispositioned within the spinal cord and fail to innervate their target muscles in a normal manner [6]. Although it is not clear to what extent the axonal phenotype results from the mispositioning, these results raise the possibility that, in maintaining the integrity of motor columns, boundary cap cells also contribute to correct projection of motor neurons to their muscle targets.

The paper by Vermeren et al. [1] therefore offers a striking example of the importance of studying in detail processes involved in laying down the ground plan of the developing nervous system, and provides new confirmation that, in spite of intensive investigation, many surprises await us.

References

Does the fusion pore contribute to synaptic plasticity?

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How synapses change their strength in response to impinging neural activity is a fundamental issue for understanding how the brain moulds its circuitry in response to behavioral experience. Although a growing number of studies reveal the involvement of postsynaptic changes contributing to synaptic plasticity in

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brain circuits, the involvement of presynaptic factors has been implied by several studies. Most recently, several works point to the mechanism of vesicle fusion as a new possible locus for the modification of presynaptic synaptic strength. However, it is not yet clear to what extent such changes affect the simplest form of information transfer in the brain—the transmission of a single action potential.

How synapses change their properties during synaptic plasticity has fascinated scientists for over a decade. The central question has been what aspects of presynaptic and postsynaptic function are modified during synaptic change. Modifications to the postsynaptic side have been demonstrated extensively [1] but presynaptic changes have been more difficult to demonstrate clearly.

Presynaptic plasticity and the probability of release

The strength of synaptic transmission can be modified by presynaptic factors in two major ways: changes in the probability of vesicle release ($p_r$) following an individual action potential, and regulation of neurotransmitter concentration in the synaptic cleft following a single release event. The initial observation implying a change in $p_r$ during synaptic plasticity has been the decreased failure rate of synaptic transmission following long-term potentiation (LTP) [2–4]. This phenomenon was initially interpreted as crucial evidence for the involvement of presynaptic release machinery in synaptic plasticity. However, the discovery of the 'silent synapse' phenomenon provided an alternative interpretation of these initial observations [5,6]. It was found that NMDA receptors were frequently activated during synaptic failures, suggesting that neurotransmitter was released even during 'failed' synaptic transmission. The simplest interpretation of these results was that such failures originated from synapses that, although presynaptically functional, lacked functional postsynaptic AMPA receptors. Insertion of additional AMPA receptors during LTP was therefore suggested as the primary mechanism for turning silent synapses into functional ones. Since these initial studies, further evidence has accumulated showing that AMPA receptors can indeed be inserted or removed from the synapse during synaptic plasticity [1]. It therefore seemed that this form of synaptic plasticity could be entirely explained by postsynaptic mechanisms, deeming presynaptic factors superfluous.

Mechanism of neurotransmitter release as a possible locus for the regulation of synaptic strength

A renaissance for the presynaptic mechanisms of synaptic plasticity had been triggered by studies showing that the amplitude of quantal synaptic transmission depended on the concentration of glutamate at individual synapses, and that physiological concentrations of transmitter in the synaptic cleft were insufficient to activate all postsynaptic receptors [4,7]. This suggested that glutamate concentration during synaptic transmission might be a possible regulation point of synaptic strength. Subsequent work showed that in young synapses the concentration of glutamate might be low, and therefore fail to activate AMPA receptors but still activate NMDA receptors. An increase in the neurotransmitter concentration following LTP could thus enhance the AMPA receptor response and turn 'silent' synapses into functional ones [8]. In addition, more recent studies showed that, in synapses containing both AMPA and NMDA receptors, NMDA receptors could be activated selectively if the transmitter flux was sufficiently slow [9]. These data together suggested the mechanism of neurotransmitter release as a possible locus for the regulation of synaptic strength and threw the silent synapse controversy back into the presynaptic courtyard, or at least suggested a possible presynaptic contribution. However, it was still unclear to what extent neurotransmitter flux varied during synaptic transmission and plasticity, and to what degree this variation would affect the activation of postsynaptic receptors. Understanding the biophysical processes of transmitter release and its regulation therefore became essential for understanding the presynaptic mechanisms that contribute to synaptic plasticity.

Different modes of transmitter release assayed by FM dyes

Presynaptic release machinery in the central synapses of the brain has been difficult to study, mainly because of the lack of experimental techniques to monitor presynaptic processes directly. However, several recent developments in imaging techniques helped to salvage the situation [10]. In particular, the activity-dependent dye FM1-43 and its variants have provided an interesting tool to measure directly presynaptic vesicle turnover. In cultured hippocampal neurons, Ryan et al. [11] observed an increased rate of FM1-43 de-staining in terminals undergoing LTP, suggesting a higher $p_r$ in these terminals following the induction of LTP. Recent works from the Siegelbaum laboratory [12,13] extended this approach to the hippocampal slice preparation and reported dramatic changes in the kinetics of vesicle turnover after the induction of synaptic plasticity. To understand the reasoning in these studies, the kinetic processes of FM-dye uptake and release must be analyzed (Figure 1). Because of their amphiphilic character, FM dyes partition into the lipid membranes with a fast time constant of $\sim 3$ ms [14]. By contrast, dissociation of FM dyes from the membrane is a much slower process, taking times ranging from 0.6 s to 4.7 s, depending on the length of the terminal hydrocarbon chain of the dye [11,15]. To stain an exocytosed synaptic vesicle, the vesicle lumen must be exposed to the dye-containing extracellular medium for at least 3 ms. By contrast, the time required to de-stain the vesicle depends upon the mode of vesicle release. If the dye-containing vesicle undergoes full fusion and subsequently intermixes with the plasma membrane, the FM dye can diffuse out laterally from the vesicle within the lipid bilayer in $<$1 ms [10]. This process was observed directly by imaging the fusion process of single FM1-43-stained synaptic vesicles using evanescent field fluorescence microscopy [16]. However, if the vesicles use a kiss-and-run mode of exocytosis, the dye must escape the vesicle through a fusion pore and might be released only partially, depending on the duration of the fusion-pore
opening. Specifically, the fraction of the dye that can escape the vesicle after a single cycle of exocytosis depends on the ratio of the fusion-pore open time over the time constant of the FM-dye de-partitioning. However, it must be noted that it is controversial whether partial release of dye from the vesicle is at all possible. Data from goldfish retinae [16] and from leech Retzius neurons [17] argue strongly against the existence of partial neurotransmitter release, although other studies, including two from the Siegelbaum laboratory [12,13] and the most recent study of vesicle cycling in central synapses [18], interpret their data assuming only partial release of neurotransmitter. The discrepancy between these results might be based on the biological systems studied, because all instances of partial neurotransmitter release so far have been reported in the plastic synapses of the hippocampus.

The amount of dye staining and de-staining therefore results from a combination of multiple factors: the mode of vesicle release, $p_r$, and the fusion-pore open time. Measuring the amount of dye staining and de-staining can then provide information about these factors, although the individual contributions could be hard to separate. For instance, the amount of FM dye loaded into the synaptic terminals after a fixed number of action potentials results from a combination of the $p_r$ of individual vesicles and the duration of the fusion-pore open time. If the fusion pore remains open for $> \sim 6\text{ ms}$ (twice the time constant of dye membrane partitioning), the amount of dye loading at single presynaptic terminals is proportional to $p_r$. However, if the fusion pore remains open for $< 6\text{ ms}$, the two factors are not easily separated. In the limit case when no dye enters the terminals, it can be concluded that either these terminals are nonfunctional ($p_r = 0$) or their fusion-pore open time is $< 3\text{ ms}$. Vesicle de-staining results from a combination of all three of the factors mentioned, and is therefore even more difficult to interpret. However, to separate the three possible factors contributing to terminal staining and de-staining, a combination of dyes with different kinetic properties can be used. The ability of the terminal to take up or release these dyes then leads to different interpretations of the fusion-pore open time, as explained in Figure 1.

Zakharenko et al. reported three main observations in their experiments. They reported that long-term depression (LTD) was associated with (i) a reduction in the number of boutons that could be loaded with FM dye, (ii) an increase in the number of boutons that could load but failed to release FM dye, and (iii) a slowing of the rate of de-staining of those boutons that released FM dye. A simple interpretation based on the reduction of $p_r$ would account only for the first and third observation. Instead, taken together, the three observations suggested a
different mechanism – a reduction in the duration of contact between the vesicle lumen and the synaptic space. This might occur by two mechanisms: (i) a shift in the mode of exocytosis of vesicles from full fusion to a kiss-and-run mode [19] and (ii) a shortening of the open time of the fusion pore during the kiss-and-run exocytosis. Both modes of exocytosis have been observed in central synapses previously [11,14,15]. The kiss-and-run mode of vesicle cycling has been recently demonstrated more directly at individual central synapses [18,20]. Therefore, the proposed changes in the mechanism of vesicle fusion during long-term synaptic depression are plausible in the light of recent results. This study provides the most direct demonstration of the changes in presynaptic vesicle cycling after the induction of synaptic plasticity in neuronal slices so far.

Can the fusion pore regulate the strength of synaptic transmission?

Which properties of the fusion pore regulate the strength of synaptic transmission? The amount of neurotransmitter that is discharged into the synaptic cleft during quantal synaptic transmission depends on the product of two factors: the duration of the fusion-pore open time and the rate of neurotransmitter release, which is proportional to the fusion-pore conductance. These two properties of the fusion pore are therefore most important in the presynaptic regulation of synaptic strength. Insights into these properties were initially gained from studies of membrane conductance and capacitance during secretion in mast and chromaffin cells [19,21,22]. The fusion-pore conductance was reported at ~300 pS and the open duration in the range of hundreds of milliseconds. Several groups recently applied the capacitance method to study vesicle fusion in large neuronal synapses. Klyachko and Jackson [23] studied microvesicles located in the posterior pituitary gland and estimated their fusion-pore open times at ~300 ms and the fusion-pore conductance at only 19 pS. Sun et al. [24] reported the time constant required for rapid endocytosis of the vesicles in the calyx of Held synapse as ~50 ms. In small hippocampal synapses, where direct measurements of terminal capacitance and conductance are impossible, reports of the fusion-pore open times range from 6 ms to 6 s, based on estimates from the FM-dye method [14,15]. Most recently, using the pH sensitivity of the synaptophysin fluorescent protein, Gandhi and Stevens [20] reported the duration of the fusion-pore opening in the range 400–860 ms. Fusion-pore conductance in the hippocampal synapses remains unknown.

What are the physiological consequences of these reports? For the fusion-pore conductance of 300 pS, reported in the fusion of dense-core vesicles [23], the transmitter should escape the vesicles completely within a few hundred microseconds [25]. Although the fusion-pore conductance of the vesicles in the hippocampal terminals is unknown, such fast discharge would be consistent with the observed fast rise times of synaptic AMPA receptor currents [26]. Given the rapid discharge, the fusion-pore open time (of which the shortest reported is 6 ms) is then unlikely to affect the amount of released neurotransmitter in hippocampal synapses. By contrast, for release through fusion pores with lower conductances, such as the 19 pS observed in microvesicles of the posterior pituitary, the neurotransmitter should require as long as ~5 ms for a complete discharge [23]. Such prolonged release would then be more sensitive to the duration of the fusion-pore opening, and might result in an incomplete release of the neurotransmitter [18]. However, because the shortest directly observable fusion-pore open times have been in the range of tens to hundreds of milliseconds [20,23,24], which is longer than the time required for neurotransmitter release even through the narrowest fusion pore (19 pS), it remains an open question to what extent the fusion-pore open times regulate the strength of quantal synaptic transmission. Instead, according to recent studies, the rate of neurotransmitter release has been demonstrated as another factor that modulates receptor activation; it has been shown that slow neurotransmitter flux significantly reduces the activation of the receptors with fast kinetics [9,27]. Rather than the pore open time, the pore conductance, resulting in a more sustained neurotransmitter efflux, might then be a better regulator of synaptic strength. Thus, in addition to the shortening of the fusion-pore open times observed during the induction of LTD recently [13], a decrease in the fusion-pore conductance might be observed as an accompanying phenomenon in the future. Conversely, an increase of the fusion-pore conductance might speed up the rate of glutamate release and increase the levels of AMPA receptor activation during LTP and synaptic maturation [8,9].

Although several studies provide indirect evidence pointing towards the fusion pore as a possible regulator of synaptic strength, direct demonstration of the effects of a changing fusion pore on the activation of postsynaptic receptors is still lacking. This presents a challenge for future research.

References
A common histopathological hallmark of most neurodegenerative diseases is the presence of aberrant proteinaceous inclusions inside affected neurons. Because these protein aggregates are detected using antibodies against components of the ubiquitin–proteasome system (UPS), impairment of this machinery for regulated proteolysis has been suggested to be at the root of neurodegeneration. This hypothesis has been difficult to prove in vivo owing to the lack of appropriate tools. The recent report of transgenic mice with ubiquitinous expression of a UPS-reporter protein should finally make it possible to test in vivo the role of the UPS in neurodegeneration.

Chains of ubiquitin (a small protein of 76 amino acids) conjugated to a substrate protein specifically target that protein for degradation by the proteasome, a huge multimeric protein complex found in all eukaryotic cells [1]. This ubiquitin–proteasome system (UPS) is responsible for the clearance from cells of most soluble proteins and, particularly, of key short-lived regulatory proteins and damaged or misfolded proteins. The first animal model with expression of a reporter protein that allows the detection of UPS impairments has been recently reported [2]. Detecting impairment of the UPS has been a major challenge owing to the complexity of this machinery for regulated proteolysis that requires the coordinated action of many enzymes and protein complexes. These include the E1 ubiquitin-activating enzyme, 20–40 different E2 conjugating enzymes, and hundreds of different E3 ubiquitin ligases. The E2 conjugating enzymes transfer one molecule of activated ubiquitin to a specific E3 ubiquitin ligase that, finally, binds the ubiquitin molecule to a lysine residue in a given substrate protein [3]. Additional ubiquitin molecules are attached to the first one, thus generating the polyubiquitin chain that is recognized by the 19S regulatory complexes located at one or both sides of the 20S proteolytic core of the proteasome [1]. The 19S complexes unfold the polyubiquitinated substrate protein and facilitate its entrance into the 20S proteasome, where it is degraded into small peptides. Polyubiquitin chains are cleaved from the substrate protein and subsequently recycled into monomeric ubiquitin by different deubiquitinating enzymes (DUBs). Alterations in this complex system have been suggested to play a role in different pathological conditions such as cancer, neurodegenerative diseases and muscle-wasting syndromes [4,5].

Impairment of the UPS has been postulated to be at the root of neurodegenerative diseases

The notion that alterations in the UPS might play a role in neurodegenerative diseases arises from the observation that in most of these diseases aberrant proteinaceous deposits can be detected inside the affected neurons using

Testing the ubiquitin–proteasome hypothesis of neurodegeneration in vivo

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