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Perspective

Searching for Engrams

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Recent advances in cellular imaging technologies together with novel genetic tools have enabled the observation of minute anatomical changes in the intact brain. This has elevated the search for physical correlates of memory, one of the longstanding questions in modern neurobiology, to a new level. Utilizing these new tools, several studies have recently been published pointing to subcellular structural changes occurring when the brain stores information about the environment. While most of these studies still fall short of investigating memory as commonly defined in neuropsychological terms, they are paving the way to more refined experiments, which come closer to the identification of true "memory traces." In the not too distant future we will be able to observe physical changes that occur during learning in the intact animal in real time, leading the way to understanding these processes in unprecedented detail.

Ever since ancient times, philosophers and scientists have wondered where in the human body past experiences, facts, and motor skills are stored. Initially, thoughts, internal representations, and memories were attributed to organs such as the heart and even the kidney. It took until medieval times to appreciate that mental processes, including memory, must be represented in the brain (see e.g., Descartes, 1649). In more recent times, it was Franz (1912) and later in particular Lashley (1929, 1950) who adapted the word "engram" (coined by Semon, 1904) to describe the place and mechanism of information storage in the central nervous system. While some of Lashley's ideas, such as mass-action, were later disproved experimentally, researchers after him have adopted the concept of the engram and tried to identify the location and physical basis of "memory traces" in the brain.

At the cellular level, Cajal was the first to propose that sites of contact between neurons, later termed synapses by Sherrington (Foster and Sherrington, 1897), could play an important role for memory storage (Ramón y Cajal, 1893). Donald Hebb's specific proposal of a synaptic mechanism for how information could be stored in the brain (Hebb, 1949) was a further specification of how to envisage an engram in the context of memory storage. At the same time, Hebb also explicitly proposed that his "Hebb synapse" would lie at the basis of a distributed memory system, a cell assembly, which stored memories not in one, but in many places of a neural network and as such posed a great challenge to the attempt to find specific engrams for specific memories.

Bearing this concept in mind, it is worthwhile to briefly reflect on how a final proof for a specific memory trace or engram could be made. Clearly, at least for a widely distributed system, data obtained with classical approaches such as lesioning parts of the brain are not always easy to interpret, as a lesion will rarely encompass the entire engram. But even in more realistic cases where engrams are distributed within a couple of relatively circumscribed areas (consider for instance the storage and recognition of faces), lesion studies have their limits: when parts of the brain are experimentally compromised and little or no effect on memory is seen, it cannot be concluded that part of the engram does not reside in the damaged area. In fact, it is the essence of a distributed memory system that redundant storage can compensate for the damaged part of the engram. Also the opposite outcome, that a memory is completely and specifically abolished by inactivating or damaging a well-delineated part of the brain, does not necessarily mean that the respective information is stored in this brain region; one might have just lesioned a functionally essential area, important for the readout of the memory but not related to its storage. Therefore, in systems where memory is distributed, it is difficult to provide definitive proof of the causal relationship between an inactivated brain area harboring the putative engram and the respective memories. As we will discuss later, modern methods of imaging structural changes at a subcellular resolution in the intact brain have a number of advantages in this respect, but even with them it will be difficult to provide definitive proof of an engram.

At this point it seems necessary to discuss how we—at least for the purpose of the present discussion—define an engram or a memory trace. Would a physiological change count? Or does it have to be an anatomical change? Or could it also be exclusively molecular? Ample evidence has been obtained for all of these, but in this review, we will largely focus on the anatomical basis of engrams. The reasons for this somewhat limited perspective are that anatomical changes have been shown to accompany long-term memory formation in a variety of systems and organisms and that several recent studies in mammals have been able to directly follow structural changes associated with the formation of new memories or the adaptation to an altered environment. Notwithstanding the importance of molecular and physiological changes for memory, we will therefore discuss changes in the brain that occur when memories are stored and that can be visualized under the microscope; in other words, true anatomical changes, where a neuronal connection may be made or disassembled or where the strengthening or weakening of a synapse is reflected in a visible anatomical change.

Invertebrate Studies

Key to the first successful attempts to identify such memory traces and proving their importance for a specific memory task was the realization that simple organisms provide the best

chances to pinpoint engrams in an intact nervous system. The best-known example is probably the sea slug *Aplysia californica*, where understanding the relatively simple circuitry of the gill- and siphon-withdrawal reflex provided the ground for a detailed analysis of the cellular and molecular mechanisms underlying nonassociative as well as associative learning (Kandel, 2001). These experiments have made a clear and resounding case that the changes observed in these learning paradigms largely take effect at synapses, thus proving Cajal's and Hebb's original ideas on the cellular locus of information storage. Moreover, studies at the time showed that this kind of learning was paralleled by anatomical changes at the respective synapses: long-lasting habituation of the gill- and siphon-withdrawal reflex was shown to be paralleled by the elimination of synapses between the sensory and motor neurons that mediate the behavior, while long-lasting sensitization increased the number of synapses (Bailey and Chen, 1988, 1989; Glanzman et al., 1990). Importantly, the time course of the observed structural changes closely matched the duration of the memory (Bailey and Chen, 1989).

In *Drosophila* and honeybees also, searches for engrams have been conducted for decades. Studies by Quinn, Dudai, and others have pointed to a biochemical mechanism for engrams, which is—like in *Aplysia*—primarily based on cAMP-mediated pathways (see e.g., Dudai, 1988). While structural alterations of sensory neurons in cAMP signaling mutants have been described (Corfas and Dudai, 1991), our knowledge of the detailed anatomical location of the "engrams" is limited to date. While it is well documented that learning and storage of information occur in the mushroom bodies and experience has been shown to alter the dendritic branching pattern of neurons in the mushroom body of adult honeybees (Farris et al., 2001), the precise cellular or subcellular (synaptic) location requires further investigation (Gerber et al., 2004; Menzel and Müller, 1996). There are further examples of other invertebrate models, like *C. elegans*, where the physical changes that accompany learning have been studied in quite some detail, but they would lead us away from what we would like to focus on here, namely the evidence for memory traces in vertebrates.

Learning Paradigms in Birds

Before getting to mammals, we briefly want to consider the work on birds, which have been extensively studied in learning and memory paradigms. The classic example is imprinting (Lorenz, 1981), where a freshly hatched chick learns the characteristics of its parents (or any other moving object, for that matter). Imprinting has been a powerful paradigm to explore memory processes, because it causes dramatic changes in the brains of young birds. However, while a number of prominent alterations can be observed, in particular with respect to the generation of new synapses (Horn, 2004), the distributed nature of the system makes it difficult to prove the causality between the observed changes and the imprinting itself. Another well-studied system is auditory localization in the barn owl. Here it was shown, initially by Konishi and later by Knudsen and collaborators, that these birds have an amazing capacity to adapt to environmental changes in a behaviorally relevant fashion. Their auditory and visual space maps in the superior colliculus align according to visual and auditory experience the owls have made (Knudsen, 2002). This system is another example where it is well established that learning is correlated with anatomical rearrangements of neuronal connections (in this case of the projection between the internal and the external nucleus of the inferior colliculus). A further powerful paradigm where substantial progress has been made with respect to the demonstration of engrams, is the song-learning system in birds. Juvenile birds learn songs from conspecific tutors (usually their fathers), and a whole plethora of brain areas is occupied with this task (for a review see Bolhuis and Gahr, 2006). Very recently, it has been shown that learning in this system is again closely associated with structural changes in one of the involved brain regions: a tutor song leads to rapid accumulation and stabilization of dendritic spines on neurons in the HVC (Roberts et al., 2010). These observations make the song-learning system one of the prime candidates where we might be able to actually watch the formation of an engram for a specific memory item.

Memory and Experience-Dependent Plasticity. Two Sides of the Same Coin?

As mentioned above, the demonstration that something is a true engram or memory trace becomes more difficult the more distributed a system is. It is therefore no surprise that none of the examples given above relate to the mammalian neocortex, which is a highly distributed information-processing machine. Nevertheless, it has been shown many times that alterations do occur in the neocortex in response to changes in the outside world. In other words, information about the outside world is stored in the neocortex.

Memory is typically defined as a process that encompasses at least three phases, encoding, storage, and retrieval (Morris, 2006), and it could be validly argued that the response of the visual cortex, e.g., to monocular deprivation, hardly encompasses encoding and as such cannot be called memory. On the other hand, memory can also be seen as one of a broader class of phenomena where the brain is "rewired," i.e., connections are made or abolished to adapt to a changing environment in order to increase—in the Darwinian sense—an organism's fitness. This encompasses learning proper, e.g., memorizing where food is stored or which face is friend and which is foe. But such adaptation to the environment also encompasses rewiring of the brain when the sensory environment changes, for instance because an eye is damaged or some whiskers are removed. In this broader sense, memory or plasticity can be seen as a continuum from adaptations to changes in the sensory environment, through relatively simple "learning" paradigms like fear conditioning, all the way to sophisticated types of learning, such as language acquisition. The above-mentioned example of experience-driven changes in the barn owl's auditory localization system nicely illustrates this idea of a continuum: here, a very simple modification of the sensory input (a shift of the visual field induced by prisms) results in a massive reorganization in the respective circuits in the midbrain, which cause adaptive changes in behavior. Importantly, this adaptation shares important features with learning proper, for example that it can be dramatically enhanced when reward signals (by hunting live prey; Bergan et al., 2005) are present.

But even if the definition of memory given above might be too broad for the taste of some, most people will agree that plasticity phenomena like monocular deprivation and memory as defined by "coding, storage, and retrieval," are most likely based on very similar cellular mechanisms. For example, essential molecular components, like NMDA receptors, the activation of certain protein kinases such as CaM kinase II, as well as further downstream players like BDNF are all shared by learning-induced and sensory-experience-driven plasticity (Berardi et al., 2003; Minichiello, 2009; Morris, 2006; Wayman et al., 2008). In that sense, the investigation of more global phenomena like monocular deprivation is likely to shed light also on the mechanisms underlying learning and memory.

Structural Changes in Neocortex and Hippocampus

The classical studies by Wiesel, Hubel, and their coworkers (Hubel et al., 1977; Shatz and Stryker, 1978; Wiesel and Hubel, 1963) were the first to clearly demonstrate that manipulations of visual input, e.g., monocular deprivation, cause profound changes in cortical circuitry. Experiments of this kind have been immensely helpful in describing the cellular changes following such manipulations, which by now are quite well understood at the phenomenological level. Nevertheless, it has been difficult to pinpoint the exact cellular location of the changes or to reverse these changes and test whether the functional changes are reversed too. While the initial studies of Hubel and Wiesel were carried out in young animals, thus providing clear evidence for a critical period, other authors have demonstrated that similar changes can also occur in the adult mammalian cortex (Merzenich et al., 1984; Knecht et al., 1996; Florence et al., 1998; Kaas et al., 1983, 1990). Again, global changes in connectivity were observed and related to the functional changes, but none of these experiments could point to synapses or other locations on neurons that are causally involved in altered neuronal responses.

A key structure for declarative memory formation in mammals is the hippocampus, and a lot of progress has been made in terms of the cellular basis of memory formation in the hippocampus. Also, recent studies have provided us with a fairly detailed view of the origin of cortical inputs to the hippocampus (Amaral and Lavenex, 2006), but it is much harder to achieve simple and well-controlled manipulations of these inputs than in primary sensory cortex. Nevertheless, a number of studies have attempted to demonstrate structural changes that correlate with the acquisition or storage of information. While some studies have reported such changes to exist (Leuner et al., 2003; Moser et al., 1994), others have not been able to demonstrate them (Rusakov et al., 1997). A potential reason for this discrepancy and why structural changes related to memory formation in the hippocampus have been rather elusive may be that the hippocampus serves as a temporary storage device (Morris, 2006), while the site of long-term memory may be outside of the hippocampus in the neocortex. In fact, a recent study found that the formation of contextual fear memory was correlated with only a temporary increase in dendritic spine density in the hippocampus, while similar changes in the cortex appeared with a delay (Restivo et al., 2009). Another merely practical advantage of the neocortex is that—in contrast to the hippocampus, which is buried deep inside the brain—it lies on the surface of the brain and is therefore much more amenable to approaches studying structural plasticity by optical means.

The examples above clearly demonstrate that learning indeed alters connectivity in the brain and that these changes in connectivity become visible as modifications in the fine structure of neurons. However, in many of these studies, learning-induced changes in neuronal structure could not be directly observed in individual animals, but had to be indirectly inferred by comparing groups of animals that had been reared under specific conditions or undergone different training regimes. This is a valid and fruitful approach under conditions of massive changes following rather dramatic modifications of neuronal activity patterns or in well-characterized circuits with a small number of neuronal elements known to be involved in the learning process. It often falls short, however, when the changes are subtle or when learning-related changes are distributed over large regions of the brain. As is evident from the examples above, this is typically the case in the cerebral cortex, where many of our memories are thought to eventually be stored.

New Technologies Enable New Insights

A number of relatively recent technical developments have now made it possible to follow the structure of neurons over weeks and months in individual animals. Two-photon microscopy (Denk et al., 1990) now allows visualization of the fine details of neuronal morphology up to several hundreds of micrometers into the cortex of the intact brain. In combination with genetically encoded markers for neuronal structure such as GFP (Chalfie et al., 1994) and a sparse, often "Golgi-like" expression pattern of these markers under the neuronal Thy1.2 promoter (Feng et al., 2000), it has become possible to repeatedly image individual cortical neurons and their processes over extended periods of time (Grutzendler et al., 2002; Trachtenberg et al., 2002). Probably the most important result of these first chronic in vivo imaging studies in mouse neocortex was that the fine structure of excitatory cortical neurons is not stable over time. In particular, their dendritic spines were found to be dynamic structures, which appear and disappear, even under baseline conditions in adult animals. It is important to note that different groups came to somewhat divergent conclusions with respect to the magnitude of the baseline spine turnover rates. This discrepancy might be related to different methodologies and/or cortical areas (Holtmaat et al., 2005, 2009), but it remains undisputed that structural changes on the level of single spines do occur over time.

What makes dendritic spines very attractive candidates for the site of memory traces is the fact that spines are the structural correlates of excitatory synapses. With few exceptions, each dendritic spine of a cortical pyramidal neuron carries an excitatory synapse (Arellano et al., 2007), and, conversely, the vast majority of excitatory inputs to pyramidal neurons terminate on dendritic spines. Importantly, the close correlation between spines and synapses also holds for newly formed spines: in two studies that combined two-photon imaging with post-hoc electron microscopy, it was shown that in the adult cerebral cortex in vivo (Knott et al., 2006) as well as in the hippocampus in vitro (Nägerl et al., 2007) it takes about a day for a new spine to form a synapse. The disappearance of a spine, on the other

hand, has been shown to entail the loss of its synapse (Becker et al., 2008), and the alternative scenario of spine synapses being transformed into dendritic shaft synapse seems unlikely, based on a comparison of the numbers of disappearing spines and existing shaft synapses (Trachtenberg et al., 2002). Thus, observing the appearance and disappearance of dendritic spines is by and large equivalent to watching excitatory synaptic connections being established and abolished.

Plasticity in Dendritic Spines

Once the stage was set for long-term in vivo imaging of synaptic structures, the logical next step was to test the degree to which spine gain and loss were altered by manipulating the level and patterns of neuronal activity in the cortex. This is most easily achieved by partially or completely depriving the cortex of its input. The first experiments of this sort were done in the somatosensory barrel cortex, where it was shown that removal of half of the whiskers on the mystacial pad in a chessboard like fashion resulted in marked changes in spine turnover rates on the apical dendritic tufts of layer 5 neurons (Holtmaat et al., 2006; Trachtenberg et al., 2002). More specifically, while deprivation did not change overall spine density, it stabilized newly formed spines and at the same time destabilized already existing spines. These effects were found to be cell-type specific: in one type of neuron (the ones with a complex apical tuft), the density of persistent spines was enhanced, whereas it was lowered in another type (with a simple tuft; Holtmaat et al., 2006). Using a slightly different deprivation paradigm, complete removal of all whiskers on one side, Zuo et al. (2005) observed that the overall, age-dependent loss of spines was slowed down by this manipulation. This slowdown was caused by a decrease in the rate of spine elimination, while the rate of spine formation remained unchanged.

In the visual cortex, very similar observations have been made in experiments where a small part of the retina was lesioned, thereby permanently depriving a region of the visual cortex from its sensory input (Keck et al., 2008). As had been shown earlier in other species (Gilbert and Wiesel, 1992; Kaas et al., 1990), the initially silenced cortical region became visually responsive again in the weeks and months following induction of the retinal lesion. During this period of functional cortical reorganization, repeated two-photon imaging revealed a substantial increase in the spine turnover rate, which returned to baseline levels about 2 months after the lesion. The prolonged increase in spine turnover led to an almost complete exchange of the initially present set of dendritic spines on the imaged dendrites in the deafferented cortical region. Importantly, this major rearrangement of synaptic structures did not occur when visual input was removed altogether, i.e., when the retina was completely ablated. Thus, the increased spine turnover may well reflect the formation of new cortical circuits that serve the recovery of visual responses. As was the case after checkerboard deprivation in the barrel cortex (Holtmaat et al., 2006), the increased spine dynamics following retinal lesions did not lead to any concomitant changes in spine density. This means that these effects would have gone by undetected, had one simply compared experimental and control groups without having any temporal information from individual animals, again critically highlighting the advantage of the chronic imaging approach.

Searching for Engrams

Monocular deprivation is another well-established model for experience-dependent cortical plasticity, where temporary closure of one eye shifts the balance between the strength of the representation of the two eyes in the visual cortex toward the open eye. In a recent study, inspired by Knudsen's work in the barn owl, our lab has employed this paradigm to demonstrate an engram in the neocortex. These studies were based on an earlier observation that plastic changes occurring in the visual cortex due to monocular deprivation in adulthood were faster and longer lasting if the animal had undergone a similar deprivation (of the same eye) earlier in life (Hofer et al., 2006). In other words, the system "remembered" the earlier experience and adapted more rapidly to a similar challenge later in life. In order to investigate potential structural modifications underlying this memory, we followed the changes that occurred at the level of dendritic spines in the visual cortex during the first and the second deprivation episode. We observed highly specific spine changes, during the first deprivation, which were limited to the binocular part of mouse visual cortex (Hofer et al., 2009). Importantly, many of the spines that had appeared during the first monocular deprivation stayed in place even after reestablishing normal vision and full functional recovery of the system. We could then demonstrate that these new, lasting spines were the basis for the faster and longer-lasting second adaptation, because the second monocular deprivation did not produce a further increase in spine density, indicating that the spines that stayed in place earlier were reutilized to achieve the same physiological response, thus providing a structural basis for faster plasticity. Therefore, the spines generated during the first monocular deprivation show the hallmarks of a structural engram: they encode information about a previous experience that the animal has made, and they allow the animal to adapt more quickly to an environmental challenge that it had experienced earlier. This is very reminiscent of the concept of "savings" put forward more than 100 years ago by Hermann Ebbinghaus (1880), who experimentally demonstrated the everyday experience that relearning is easier than learning. Interestingly, it was apparently realized already early on that synapses that physically persisted could be the basis of faster acquisition times for a second episode of learning (Kandel, 2006).

Thus, already the first chronic in vivo two-photon imaging studies have taught us a great deal about how modified sensory experience leads to structural changes at the level of individual dendritic spines. While classical experiments carried out in fixed tissue had already provided the first evidence for the susceptibility of spines to altered sensory inputs (e.g., Valverde, 1967), the recently developed imaging techniques now allow for a much more detailed and encompassing view on the dynamic nature of these processes.

Very recent experiments using similar techniques have now taken the next step: they were able to demonstrate that behaviorally relevant motor training resulted in the rapid formation of new dendritic spines on pyramidal cells in motor cortex. The newly formed spines induced during learning were preferentially stabilized during subsequent training and persisted over months (Xu et al., 2009). Importantly, a related study in motor cortex suggests that the degree of spine remodeling correlates with

the improvement in behavior seen after learning (Yang et al., 2009). These studies therefore provide a direct link between learning of defined tasks and specific structural changes in the relevant cortical areas.

Structural Changes in Axons…

Most of the studies described above have focused on dendritic spines when searching for structural correlates of sensory experience or learning. Can we expect to find a similar degree of structural plasticity on the presynaptic side? Again, some older, anatomical experiments using more conventional techniques have provided clear evidence for this. In fact, the shrinkage and expansion of ocular dominance columns seen after monocular deprivation in higher mammals directly reflects the retraction and growth of thalamic fibers in the visual cortex (Antonini and Stryker, 1993; Shatz and Stryker, 1978). Intracortical axons, too, can grow new branches in response to altered activity patterns, as shown for example in the visual cortex following retinal lesions (Darian-Smith and Gilbert, 1994) or in the somatosensory cortex after peripheral injury (Florence et al., 1998). So far, few studies have investigated changes in axons and boutons using chronic structural imaging. In adult mouse (De Paola et al., 2006) as well as macaque (Stettler et al., 2006) cerebral cortex axonal boutons exhibited some degree of structural plasticity under baseline conditions, while overall axon branching patterns remained stable. In a recent series of experiments, Yamahachi and colleagues employed two-photon imaging in macaques to follow the remodeling of GFP-labeled intracortical axons after retinal lesions over extended periods of time (Yamahachi et al., 2009). They observed an immediate increase in the number of axonal boutons as well as the addition of axon collaterals in the affected cortical region. Thus, both, pre- and postsynaptic elements of the cortical circuitry change in parallel following removal of a small part of retinal input (Keck et al., 2008; Yamahachi et al., 2009). The logical next experimental step will now be to express genetically encoded labels of different colors in axons and dendrites, thus allowing for concurrent imaging of structural alterations of both synaptic partners as well as potential trans-synaptic signaling molecules mediating the synchronization of pre-and postsynaptic changes.

…and Inhibitory Neurons

In contrast to excitatory neurons, on which most studies have focused so far, relatively little attention has been paid to structural modifications of inhibitory neurons. Lee and colleagues observed that the branch tips of dendrites of inhibitory neurons in mouse visual cortex undergo constant remodeling, indicating that inhibitory neurons, too, are capable of participating in structural plasticity (Lee et al., 2006, 2008). Two-photon calcium imaging has revealed that inhibitory neurons also undergo functional changes following sensory deprivation (Gandhi et al., 2008; Kameyama et al., 2010), but so far the relationship between functional and structural changes of inhibitory neurons remains unclear.

Functional Correlates

One of the biggest problems in searching for engrams is knowing where exactly to look for them in the brain. In particular, the recent experiments on experience-dependent structural plasticity of dendritic spines described above have made headway in this respect. The observed effects were found to be specific to certain elements of the columnar architecture of the barrel cortex (Holtmaat et al., 2006), regions of the visual cortex that had undergone functional changes (Keck et al., 2008; Hofer et al., 2009), or the relevant regions of motor cortex (Xu et al., 2009; Yang et al., 2009). Clearly, though, this is only a beginning, as one would ideally like to demonstrate correlations between functional and structural changes at the level of individual neurons. So far this has been very difficult: while it is now possible to chronically image subcellular structural changes in living animals over weeks or months, achieving chronic functional imaging has turned out to be very demanding. Electrical recordings allow this to a certain extent, but the crucial drawback is that there is no anatomical information about the recorded cells. This problem can be overcome in principle by in vivo calcium imaging using synthetic indicators (Ohki et al., 2005; Stosiek et al., 2003), yet these do not seem to permit repeated recordings (Dombeck et al., 2007; Mrsic-Flogel et al., 2007). Recently developed genetically encoded calcium indicators solve this problem, allowing the functional properties of individual neurons to be followed over several weeks (Mank et al., 2008; Tian et al., 2009). Granted that some technical problems like continuous high-level expression of the indicators still need to be solved, coexpression of genetically encoded structural and functional markers will soon make it possible to relate the magnitude of experience-dependent modifications of a neuron's response properties with subcellular structural changes, e.g., at the level of single spines. This approach will be immensely helpful in understanding the cell-to-cell variability with respect to the degree of structural changes seen in many studies. For example, neurons in the visual cortex exhibit a large range of changes in spine turnover following monocular deprivation (Hofer et al., 2009). It is tempting to speculate that this variability reflects the different degrees to which individual neurons shift their ocular dominance after closure of one eye (Mrsic-Flogel et al., 2007), but without actually doing the experiment this remains a speculation.

To take the argument on finding the precise location of the individual elements of an engram even further: as pointed out earlier, the ultimate challenge and goal is to demonstrate that the synapse on a newly formed spine is causally involved in altering a neuron's response properties. We can think of two experimental approaches to achieve this goal. (1) One could attempt to observe new spines generated in a plasticity paradigm and functionally characterize these new inputs individually using calcium imaging in single spine heads. Single-spine calcium imaging has successfully been done in vitro (Oertner, 2002) but will be much harder to implement in the intact brain. (2) Ultimate proof for a causal link could be achieved by a highly refined version of a lesion experiment. One might attempt to selectively remove all or at least a substantial fraction of a neuron's newly grown spines and test whether the functional change can be reverted.

Many of the studies aimed at finding structural correlates of plasticity in the neocortex have used modifications of sensory inputs like monocular deprivation or whisker removal to induce changes in neuronal circuitry. The reasons for this approach

are obvious: such manipulations are easy to carry out, and they often lead to dramatic changes in the pattern and level of neuronal activity in the affected neuronal circuits, which in turn result in major structural changes that are relatively easy to detect. There are good reasons to believe that many of the molecular and cellular changes induced by gross manipulations of sensory inputs are similar to those employed when an animal learns a stimulus association, a specific motor task, or navigating in a novel environment. Still one wants to demonstrate rather specific localized changes rather than global ones, to really establish an engram.

Attributing Functional Significance to Structural Changes

The first step in achieving this goal is identifying individual neurons whose responses change in a specific fashion when an animal learns something new. As pointed out above, there are several examples in invertebrates where such neurons have been identified, but only very few examples of this have been reported in the neocortex. In some of these experiments, the presentation of a specific sensory stimulus was paired with direct activation of the neuron, either by electrically stimulating it (Frégnac et al., 1988; Meliza and Dan, 2006), by local application of neuromodulators such as acetylcholine (Shulz et al., 2000), or by activation of cholinergic inputs (Froemke et al., 2007), resulting in altered response properties of the recorded cortical neurons. In a clever variation of this theme, Ahissar and colleagues (Ahissar et al., 1992) changed the functional connectivity between a pair of neurons in monkey auditory cortex, as measured with cross-correlation, by pairing the spiking activity in the two neurons, utilizing one neuron's natural response to an auditory stimulus.

While all these studies were important in showing that synaptic changes can be induced experimentally in the intact brain, none of them demonstrated alterations of neuronal responses related to the learning of a behaviorally relevant task. In a very elegant set of experiments, Miyashita and colleagues (Miyashita, 1988; Miyashita and Chang, 1988; Sakai and Miyashita, 1991) came closer to this: these investigators could show that when monkeys learn visual pair associations, neurons in inferotemporal cortex change their stimulus selectivity such that a cell initially responsive to only one image of the pair will also start firing when the paired associate is presented. A completely different, well-documented example for learning-related changes in receptive field properties are hippocampal place cells, where recordings in awake behaving rats have made it possible to observe directly how a neuron's place field emerges while the rat is exploring (and learning about) a novel environment (Wilson and McNaughton, 1993).

Thus, there are a few examples where also in the mammalian brain individual neurons have been shown to change their stimulus selectivity in a meaningful way during memory formation. This does not necessarily mean that the synaptic changes have occurred on these very neurons (synaptic weight changes elsewhere in the network might be responsible), but these cells provide the reasonable starting point for the search for anatomical changes responsible for their altered response properties. In order to test whether the functional changes are also reflected at the structural level in the very same neurons, it would be necessary to visualize their fine structure during the actual learning process. Until recently, suggesting such an experiment in a behaving animal would have sounded rather daring, but a number of recently introduced techniques now seem to make such an approach feasible, albeit still very demanding. Over the last couple of years, a number of designs have been proposed for head-mounted fiber-optic microscopes (Flusberg et al., 2005; Helmchen et al., 2001; Sawinski et al., 2009), which allow for high-resolution two-photon imaging in freely moving animals. While this is in principle a very important step forward, as it would allow the imaging of animals moving around in what is called a "natural environment," it unfortunately still lacks the resolution required to unequivocally discern structural changes on the level of single spines. Using an alternative approach, Tank and colleagues (Dombeck et al., 2007) have modified a more than 50 year old idea (Hassenstein, 1951) allowing them to image neuronal calcium signals from the cortex of a head-fixed mouse, while the animal is walking on an air-supported styrofoam ball. Once accustomed to walking on the ball, the mice can be trained to navigate in a virtual environment, and they can learn to associate specific locations in this environment with a reward (Harvey et al., 2009). Thus, it seems, we are close to having all the necessary tools to directly watch the formation of a memory trace in a behaving animal.

Sometime in the Future

After having reviewed where we stand experimentally and conceptually, let us for a moment put experimental feasibility aside and consider how we could get closer to proving that a neuron's new connections are actually engaged in mediating the altered behavior following learning. A number of approaches to this question come to mind. (1) One approach would be to selectively remove a large number of the spines that were formed during memory formation and observe whether one thereby abolishes the "memory." This could be done by laser ablation, a technique that has been used at the level of individual neurons in several invertebrate systems (Farrow et al., 2003). More recently, it has also been shown that such an approach can be used in the intact mammalian central nervous system with a precision that allows targeted lesioning of subcellular structures such as individual axons (Ylera et al., 2009). One problem with this approach is that, due to the distributed nature of memory, most likely a large number of spines would have to be ablated, which is experimentally not very practical. A second disadvantage of this approach is that it relies on the permanent removal of the synapses under question. (2) Therefore, an even more elegant experiment would be to temporally inactivate the respective new spines. Over recent years, several techniques for silencing single neurons have been developed, based on the light-triggered activation of ion pumps or channels (Banghart et al., 2006; Zhang et al., 2007). One could imagine that these methods could also be used to silence single spines by way of local shunting of the respective conductances. (3) In a yet more audacious approach, one could think of using "suicide markers" where, at the time of their birth, newly generated spines are tagged with a marker, which later, upon illumination or pharmacological activation, results in self destruction of the respective population of spines. (4) While the above three thought

experiments rely—in one way or another—on the inactivation of newly generated spines, one could also take approaches interfering with the time course of spinogenesis. If there were a way of delaying spine generation for a certain time, better even by a time and to a time point determined by a light flash or some drug application, this could be used to make the interconnection of information storage and spine growth even stronger. One could subject an animal to a learning paradigm but postpone the actual learning to the time of light or drug application. Imagine for a moment that such an experiment could be done. It would provide quite a strong proof that the generation of the spines is indeed an essential step in information storage in the brain.

When considering the feasibility of these approaches, one should keep in mind that the number of neurons that will be reached by light or drugs may be small. On the other hand, a couple of studies have shown that activation of a relatively small set of cortical cells can influence perceptual decisions (Huber et al., 2008; Salzman et al., 1990) and that the firing of single neurons can result in a measurable motor output (Brecht et al., 2004). So one may hope that even a relatively limited number of synapses may have substantial and measurable behavioral effects. Still, despite the conceptual beauty of experiments where one would observe very specific structural changes in small populations or even single cells, they do not completely circumvent the above-mentioned pitfalls of conventional lesion experiments.

While the experiments just suggested might be marked off as outlandish and experimentally unrealistic suggestions, we nevertheless believe that the last couple of years have brought a number of technical improvements and new ideas that together make the "search for engrams" much more likely to succeed than was the case only a few years ago. A number of technical hurdles still have to be overcome, but we are convinced that, in the very near future, substantial progress can be made such that we can start looking for engrams not only in the comparatively simple nervous systems of invertebrates but also in the much more complex network of the mammalian neocortex, which after all is the place where most of our memories are thought to be stored.

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