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Protein kinases: which one is the memory molecule?

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Abstract. Encoding of new experiences is likely to induce activity-dependent modifications in the brain. Studies in organisms far apart on the phylogenetic scale have shown that similar, sometimes identical, signal transduction pathways subserve plasticity in neuronal systems, and they may play pivotal roles in the formation of long-term memories. It has become evident that phosphorylation/dephosphorylation reactions are critical for the initiation of cellular mechanisms that embody, retain and modify information in neural circuits. Although physiological investigations on synaptic plasticity have had a major impact, we have concentrated our review on behavioural studies that provide direct or indirect evidence for a role of kinases in mechanisms underlying memory formation. From these, it appears that the learning event induces activation of a variety of kinases with specific time courses. For instance, the calcium/calmodulin-dependent protein kinase II seems to participate in an early phase of memory formation. Apparently, activation of both protein tyrosine kinases and mitogen-activated protein kinases is required for much longer and may thus have a particular function during transformation from short-term into long-term memory. Quite different time courses appear for protein kinase C (PKC) and protein kinase A (PKA), which may function at two different time points, shortly after training and again much later. This suggests that PKC and PKA might play a role at early and late stages of memory formation. However, we have considered some examples showing that these signalling pathways do not function in isolation but rather interact in an intricate intracellular network. This is indicative of a more complex contribution of each kinase to the fine tuning of encoding and information processing. To decipher this complexity, pharmacological, biochemical and genetic investigations are more than ever necessary to unravel the role of each kinase in the syntax of learning and memory formation.

Key words. Memory cellular mechanisms; signalling pathways; protein kinases; PKC; PKA; CaMKII; MAPK and PTK.

How to store information

Information storage in the brain is likely to involve persistent, use-dependent alterations in the efficiency of transmission at synapses. An ever growing number of intracellular signalling pathways have been identified as participating in the translation of signals into the 'syntax' of the neural representation of memory [1]. As pointed out by Dudai [2], these signalling pathways are intricate in extensive cross-talk within the intracellular network whose spatiotemporal activity appears to determine the structural and functional status of the cell at any given time.

Looking at the basic cellular and molecular mechanisms involved in the processing of incoming signals shows surprisingly that neurones follow relatively elementary rules to translate the signal into sophisticated and extremely complex biochemical processes. Intracellular signal processing may start by calcium influx that, apart from many other functions [3], triggers phosphorylation/dephosphorylation reactions [4]. The balance between these two enzymatic systems is supposed to be related to the size of the calcium signal [5–7]. High levels of calcium entry would promote phosphorylation,

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whereas relatively low levels would stimulate phosphatase activity [see Riedel, this issue, 5, 6].

Phosphorylation is a covalent chemical modification used by cells for the control of properties of a wide variety of proteins such as enzymes, receptors, channels and so on. The value of phosphorylation lies in its reversibility but is physiologically limited by the cellular availability of the right catalyst (kinases or phosphatases) and their substrates. Protein kinases are phosphotransferases that catalyse transfer of the γ-phosphoryl group of adenosine trisphosphate (ATP) to the alcohol groups of serine and threonine, or the phenol group of tyrosine in side chains of the peptide. While activating/inactivating their substrates, most protein kinases are themselves subject to regulation so that inhibition by the regulatory domain is relieved due to binding to a second messenger, thereby unfolding the catalytic domain with its active fragment.

However, to be effective for learning and memory, enzyme activity or enzyme-dependent activity of substrates needs to be lasting, sometimes for minutes or hours, and this clearly is beyond the rather short calcium fluxes across membranes. Although stimulated by calcium change, enzyme activity has to become calcium-independent. To achieve such calcium independence, kinases possess two main mechanisms: (i) limited proteolysis that separates the regulatory subunit from the catalytic one, and (ii) (auto)phosphorylation that gives the protein a new conformation. These mechanisms allow the kinase to be switched into an 'on' state that retains its activity even after the calcium signal has ceased. In the case of calcium calmodulin-dependent kinase II (CaMKII), autophosphorylation keeps the enzyme in the 'on' state for extended periods [8, 9] and thereby prolongs biochemical events that are likely to participate in the formation of long-term memories.

Kinase cross-talk

Over the last decade the increased sophistication of biochemical methods, partly inspired by work on long-term potentiation (LTP) and long-term depression (LTD), has provided evidence for a great number of kinases taking part in long-term memory storage. Kandel and colleagues, for instance, have stressed the role of the cyclic adenosine monophosphate (cAMP) cascade for long-term structural changes following learning [10]. In addition, they also consider the possibility that mitogen-activated protein kinases (MAPKs) contribute to long-term memory, at least in *Aplysia* [11–13]. Others [8, 9, 14] have proposed CaMKII as a candidate for exhibiting persistent changes and serving as a memory molecule. However, as one kinase alone may not achieve memory formation, all or at least several kinases are likely to act in parallel or in sequence (fig. 1).

Because all these enzymes are promoting phosphorylation, and each has a huge number of substrates, the involvement of many different kinases allows a very fine tuning of information processing and encoding. Kinases may thus function as (i) coincidence detectors, each activated by its own 'private' stimulus and fulfilling a specific function; (ii) cellular or even subcellular distributors of information according to their substrates and intracellular localisation; (iii) modulators or regulators of the different signalling pathways engaged in the cross-talk at multiple stages of the network (fig. 2).

Data on signal transduction systems, as provided by in vitro work, suggest that alterations in one pathway can have functional consequences on the activity of other pathways. Thus PKC positively regulates the cAMP-dependent protein kinase cascade via stimulation of some isoforms of adenylate cyclase activity (see [15]). The exact nature of this modulation, however, remains unclear, but it appears to be very complex and is still a matter of debate. While Sugita and colleagues [16] found that prolonged activation of PKC increases levels of cAMP in sensory neurones of *Aplysia*, they also showed a PKC-induced inhibition of the cAMP/PKA (protein kinase A) cascades. Furthermore, the MAPK cascade is triggered by tyrosine kinase and is under the positive and negative control of both PKC and PKA (see [17–20]). Interestingly, the activation processes of the protein tyrosine kinase (PTK) demonstrate that kinases and phosphatases do not always interact in an antagonistic way. $pp60^{c\text{-}src}$, a member of nonreceptor PTK that is inactive when phosphorylated in Tyr-527, is activated via a calcium-dependent tyrosine phosphatase. This activity is further increased by PKC-dependent Ser-12 phosphorylation, illustrating some of the synergistic actions between kinases and phosphatases (see [21]).

Such complex cross-talk should be taken into account when the role of one particular kinase is investigated through interventions such as pharmacological or genetic manipulations. Although we are aware of the cross-talk, this review will deal with kinases individually. On the one hand, most studies focus on one kinase in particular. On the other hand, it appears to be much easier to grasp the summarised information and it is, of course, clearer to present.

Undoubtedly, we have benefited a great deal from the popularity of models of synaptic plasticity, for example LTP and LTD, but in this review, we shall concentrate on behavioural studies that have produced direct or indirect evidence for a role of kinases in mechanisms underlying memory formation. Despite the detailed knowledge about PKC and its involvement in memory formation, particular weight is given to other kinases that may be the centre of attention in years to come. Thus, the main question still needs to be resolved: it is

to decipher the complex network of cellular mechanisms and interactions underlying memory formation.

PKC

Among the kinases that are critically involved in cellular processes of information storage, PKC has attracted the greatest attention in the last 15 years. Because the possible role of PKC in synaptic plasticity and learning has been extensively reviewed in a recent special issue [Prog. Neuro-Psychopharmacol. Biol. Psychiat. 21 (1997)], we will simply highlight a few points that deserve mention.

To date the PKC enzyme family consists of 12 isozymes that can be further categorised into calcium-dependent and -independent isoforms. Although the different iso-

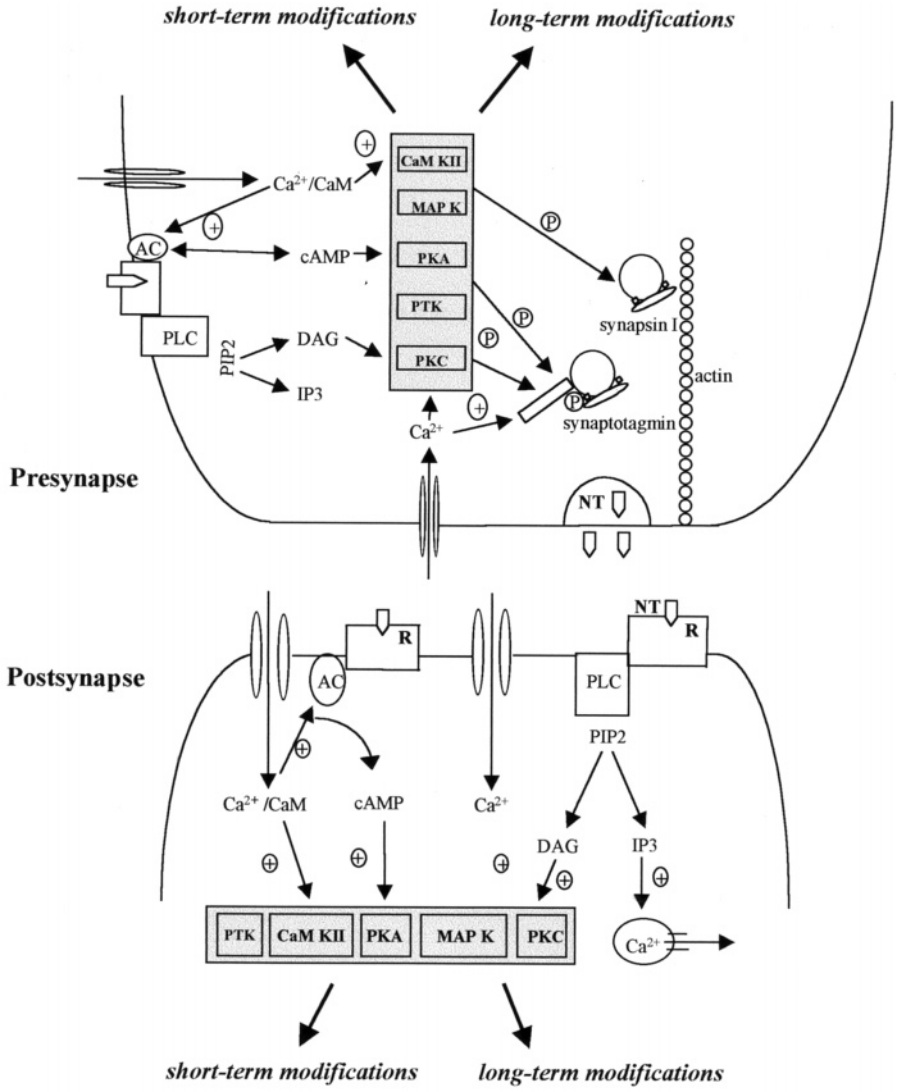

Figure 1. Protein kinases and synaptic interplay. Despite being localised both pre- and post-synaptically, the function of protein kinases might differ. Activation of several second messengers due to extracellular stimulation of voltage-sensitive calcium channels or metabotropic receptors enhances protein kinase activity (grey box). Arrows reflect direct or indirect biochemical relationships between elements with (+) indication stimulation. Enzymes promote phosphorylation of numerous substrates (synapsin, synaptotagmin and many others) which brings about both short-term and long-term changes required for memory formation. Short-term changes include phosphorylation of ion channels, receptors, enzymes or proteins of the cytoskeleton; long-term changes are phosphorylations of transcription factors, activation of early and last effector genes. AC, adenylyl cyclase; CaM, calmodulin; cAMP, cyclic adenosine monophosphate; DAG, diacylglycerol; IP3, *myo*-inositol-D-1,4,5-triphosphate; NT, neurotransmitter; P, phosphate radical; PIP2, phosphatidylinositol 4,5-biphosphate; PLC, phospholipase C; R, receptor.

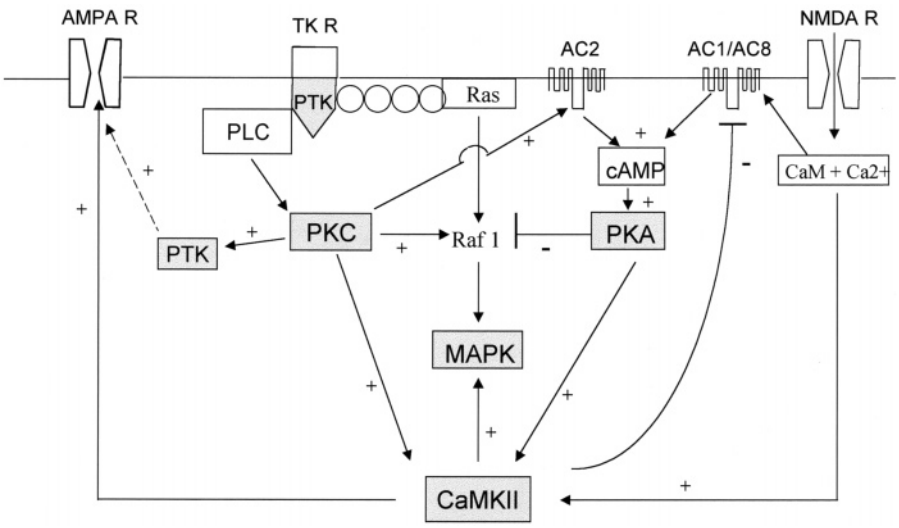

Figure 2. Sketch of some interactions between the different protein kinases described here. Activation and inhibition are both possible and are indicated by (+) and (−). One important end-product is the phosphorylation of AMPA receptors. For abbreviations see figure 1 and: AMPA R, α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid receptor; NMDA R, *N*-methyl-D-aspartate receptor; TKR, receptor associated with PTK.

forms are widely distributed in the brain, higher concentrations were found in the cerebral structures, and these areas are proven to participate in memory processes (see [22, 23]). Most studies so far have examined behaviourally induced PKC modifications limited to the hippocampal formation. However, changes in PKC have also been observed in other parts of the cortex after various learning tasks (see [22]), arguing for a more general role of PKC in the biochemical cascade accompanying learning and memory processes.

Despite the large amount of information available on PKC function in memory processes, it remains unclear whether there is a specific contribution to encoding, storage and/or consolidation. Furthermore, it appears that PKC is universally activated in a wide range of behavioural situations, and there seems to be no specific involvement in one particular form of memory. This may be not surprising given the fact that many PKC isoforms exist, each of which may have its own specific function. In line with this idea, the calcium-dependent isoforms identified in the hippocampus, and more specifically the γ-isozyme, appear to be involved in spatial learning (see [24]). In parallel, changes of γ-PKC or its messenger have been revealed in the hippocampus after LTP induction (see [25]). This functional specificity of PKC subspecies may be accounted for by the cellular or subcellular localisation of the PKC isoforms as well as the particular substrates associated with the enzymes [26, 27].

As for LTP [28], behavioural studies have indicated PKC in memory consolidation processes in mice [29], in some intermediate stages of memory formation in chicks [30, 31], or in long-term memory in both birds and rodents [32, 33]. Collectively, PKC activation seems to be a crucial step in the transformation of short-term into long-term memory. This is further corroborated in ageing studies, which show alterations in calcium-dependent and -independent PKC activities [34–36]. These age-related alterations have been correlated with reduced performance in spatial learning [36–38]. Gene knockout technology has not provided clear-cut results as to which isoform(s) is(are) crucial for hippocampal LTP and memory processes. Mutant mice deficient for γ-PKC with no conventional LTP in the CA1 area have exhibited only mild impairment in spatial learning [39, 40]. Thus, while there is no doubt that PKC plays a prominent role in synaptic plasticity and information storage, its precise nature warrants further investigation.

PKA and cGMP-dependent protein kinase (PKG)

It is widely accepted that PKA plays a major role in various forms of synaptic plasticity and may thus be an essential factor in processes of memory formation. Mammalian PKA includes four regulatory (RIα, RIβ, RIIα, RIIβ) and two catalytic (Cα, Cβ) subunits, each

encoded by a unique gene. Subunit assembly in the tetrameric holoenzyme is likely to differ because each subunit shows a distinct expression pattern across brain regions.

Probably the first insight into the role of PKA in memory formation came from investigations on the cellular mechanisms of the gill-withdrawal reflex in *Aplysia*. Stimulation of the snails' siphon coupled to a noxious tail shock causes the animal to withdraw its gill; this form of conditioning leads to sensitisation so that further siphon stimulation results in gill withdrawal. The number of noxious stimuli determines the strength of the memory with a single shock lasting for minutes and multiple shocks lasting for days. The underlying anatomical substrates of the response and cellular mechanisms involved in memory formation have been established in recent years (for a comprehensive review, see [41]). A serotonergic sensory neurone reporting stimulation of the siphon skin directly activates motor neurones inducing the withdrawal response, and the tail shock is transmitted onto the same synapses via several interneurones (fig. 3).

It has been determined that events leading to both short-term and long-term memory primarily take place in the presynapse of the sensory neurone of the siphon, and the cellular cascades involved have been elucidated. Within these cascades, both PKA and PKC activation can enhance transmitter release by closing potassium channels and increasing calcium influx and thus contribute to short-term sensitisation. But what is the distinct function of PKA in long-term memory formation in *Aplysia*? PKA is heterotetramer composed of two regulatory and catalytic subunits [42]. The catalytic subunit can be traced using fluorescent ratio imaging and translocates to the nucleus of the sensory neurone in response to strong stimulation [43]. PKA then remains active for up to 12 h [44] even in the absence of any further stimulation. This persistent PKA activity is critical for long-term memory formation, and may be due to degradation of regulatory compared with catalytic subunits. Via several steps, including cAMP-response element binding protein (CREB) activation (see Lamprecht, this issue), MAPK and immediate early genes (see Tischmeyer and Grimm, this issue), PKA is further involved in signal translation into long-term morphological modifications of synapses (see Moser, this issue).

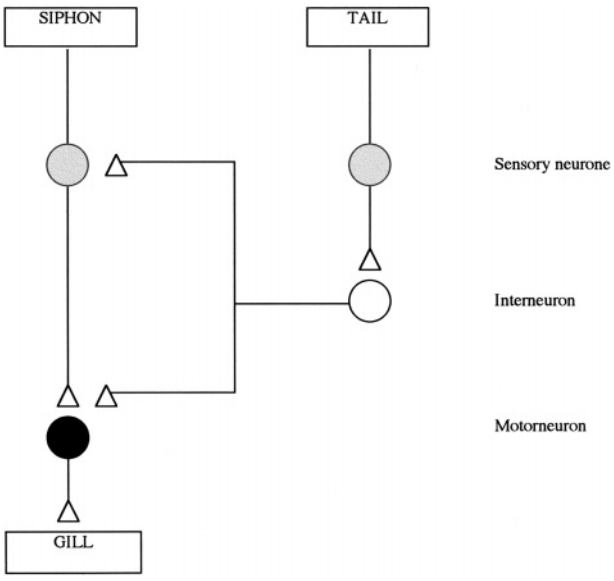

Figure 3. Neuronal circuitry involved in sensitisation in *Aplysia*. Stimulation of the siphon discharges sensory neurons (grey) which directly stimulates motor output to the gill (black). Tail shocks interact with this direct pathway by means of sensory neurone-driven interneurons synapsing on both sensory and motor neurones of the direct siphon withdrawal pathway.

Apart from some studies using pharmacological tools, a more detailed account has become possible, as subunits have been the target of depletion in knockout mice. All of them will be discussed below.

A similar role, yet with a different time course of activation, has been found in vertebrate models of memory formation. In vertebrates, the LTP of synaptic efficacy induced by different stimulation protocols has been extensively studied as a cellular model of memory storage. Drawing the parallel with memory phases, it has been demonstrated that LTP consists of at least two biochemically distinct temporal phases, an early phase lasting 1–2 h and a later, more persistent phase (L-LTP) requiring new protein and RNA synthesis. Numerous studies performed in different synaptic pathways have provided strong evidence that PKA is a critical component of the molecular machinery leading up to the manifestation of the L-LTP [45–48, but see 49]. On the basis of these data, it has been suggested that the cAMP/PKA cascade mediates molecular mechanisms for converting short-term memory into long-term memory. This assumption has received pharmacological support in chicks trained in a one-trial peck-avoidance task. Briefly, this task consists in presenting the chick with a bead dipped in an aversive substance that provokes a typical disgust response. During retention test, chicks were exposed to a similar but dry bead for 10 s. Chicks that pecked during the test trial were scored as amnesic. The pretraining intracerebral injection of PKA inhibitors impaired long-term memory formation, as amnesia occurred 60 min, but not 15–30 min, post-training [50, 51]. The delayed onset of PKA involvement in memory formation has been confirmed recently in rats trained in an inhibitory avoidance task. Intrahippocampal, but not intraamyg-

dala injection of drugs acting on the cAMP/PKA cascade modulates memory consolidation when given 3–6 h after training [52, 53]. Moreover, rats submitted to the inhibitory avoidance learning showed increases in hippocampal cAMP levels and PKA enzymatic activity at the same time window observed in the behavioural experiment [53]. Finally, an increase of phosphorylated CREB immunoreactivity was measured in the CA1 hippocampal field 3 and 6 h after training. This may suggest that the cAMP/PKA signalling pathway in the hippocampus is involved in the late phase of memory consolidation at least of stepdown inhibitory avoidance. However, the neuropsychological significance of these results should be questioned in the sense that hippocampal lesions do not affect or facilitate such conditioning [54].

Gene-targeting technology has been extensively used to investigate the role of specific PKA isoforms in synaptic plasticity and memory processes. In a comprehensive review Brandon and colleagues [55] have discussed the current understanding of behavioural characteristics of mice carrying null mutations in PKA subunits. Mutant mice, deficient in either RIβ or Cβ1, did not show any deficit in learning, including exploration of a novel environment, spatial learning of the Barnes or open field water maze, and contextual or cued fear conditioning [56–58]. Despite these findings, however, RIβ knockout mice expressed significant deficits in mossy fibre CA3 LTP, Schaffer collateral CA1 LTD, and a defect of both LTP and LTD in visual cortex [55]. Although not yet tested for plasticity, RIIβ knockouts appeared normal in behavioural paradigms [55]. The absence of mnemonic effects in these null mutants may be explained by the fact that no detectable change in total PKA activity was observed in the brains of these animals, suggesting that compensatory regulation mechanisms have taken place [55].

Probably the most elegant study used transgenic mice expressing a dominant negative form of the RIα regulatory subunit of PKA termed R(AB) [59]. Expression was limited to the forebrain by means of the CaMKIIα promotor, resulting in a 50% reduction of PKA activity. Although the forebrain of transgenics did not differ anatomically from wild types, long-term, but not short-term, synaptic plasticity as assessed via various tetanization protocols was greatly impaired [59]. In line with an important role of PKA in memory formation, mice did learn normally about space when trained in the open field water maze, but were amnesic when tested for long-term memory days later. A more precise time course of the impairment emerged when animals were trained in a shock-reinforced context versus cue conditioning paradigm and freezing was monitored as an index of learning. R(AB) animals tested for contextual memory 24 h later were significantly impaired, but normal recall was found 1 h after training [59], suggesting normal learning and short-term memory.

This genetic dissection clearly shows that certain PKA subunits may be more critical in the initial cascade of events involved in memory consolidation. Despite these findings, however, the exact activation profile as well as phosphorylation substrates of PKA await further examination.

It is worth mentioning here that a new avenue to be explored in the future is the role of the cGMP-dependent protein kinase (PKG). Activation of PKG is greatly facilitated by the generation of radicals such as NO and CO, blockade of which may cause learning deficits [60]. Elevation of cGMP directly activates PKG, and there is now preliminary evidence from Medina's group showing that blockade of PKG fully blocks, and activation facilitates, memory formation of inhibitory avoidance [61]. This is in agreement with findings in *Drosophila* which aimed at determination of a naturally occurring polymorphism [62]. It turned out that the *foraging* gene has two naturally occurring variants—*sitter* and *rover*—which are responsible for food-search behaviour. *Rover* types move greater distances while searching for food, and also show higher levels of PKG. Genetic overexpression of the *foraging* gene in *sitter* individuals transformed them into *rover*-type flies, providing direct evidence for a correlation between the amount of PKG and the food-search behaviour. These exciting findings warrant further studies on the role of PKG in mechanisms underlying learning and memory in other species.

CaMKII

Anatomical localisation and activation profile

Based on in situ hybridisation histochemistry, the distribution pattern of CaMKII messenger RNAs (mRNAs) has been determined throughout the central nervous system. While a detailed anatomical account is beyond the scope of this contribution, some striking observations deserve mention. In the cortex, CaMKII expression is not uniform but rather concentrated in layers II/III and less dense in deeper layers [63]. This has been confirmed in immunohistochemical studies that applied specific antibodies directed against CaMKIIα and β [64]. In the hippocampus, where the highest concentration of CaMKII has been detected, in situ hybridisation and immunocytochemistry revealed dense labelling in the stratum pyramidale of both CA1 and CA2 [63, 64]. Expression was less pronounced in the stratum oriens, stratum radiatum, and in all parts of the dentate gyrus. It is further noteworthy that CaMKII expression is already high in CA3 at postnatal day 1 followed by expression in the external blade of the dentate and CA1

on postnatal day 5 [65]. During further development, CaMKII expression essentially follows the timetable of afferent lamination in the hippocampus, and a temporal overexpression in CA1 and CA3 2 weeks after birth coincides with the emergence of NMDA receptor binding. Immunoreactivity in basal ganglia and diencephalon is dominated by CaMKIIβ expression [64].

CaMKII constitutes up to 2% of protein in certain brain areas such as the hippocampus and is localised in postsynaptic densities of excitatory synapses as the most abundant protein [66]. It constitutes an oligomeric multifunctional isozyme family. The adult enzyme comprises 10–12 subunits consisting of N-terminal kinase motifs, a central regulatory domain and a carboxy-terminal tail for assembly and subcellular localisation. Activation of CaMKII consists of several steps (for review and citations, see [67]): (i) Calcium/calmodulin binding neutralises the inhibitory action of the regulatory domain and, presumably through a conformational change, frees the catalytic domain. (ii) Once activated, the kinase binds as a first substrate Mg^{2+}/ATP. (iii) Finally, a rapid autophosphorylation takes place at threonine 286, constituting a partially calcium-independent form of CaMKII. Calcium-independent CaMKII has been proposed to function as a molecular sensor for synaptic activity [68] or calcium-spike frequency detection [69]. Once activated, the enzyme promotes phosphorylation of numerous substrates such as synapsin I, microtubule-associated protein 2 (MAP-2), tubulin, as well as various glutamate receptors. Thus, CaMKII appears to be appropriately positioned to control synaptic strength by (i) sensing calcium and (ii) phosphorylating synaptic channels.

In accumulating current knowledge about CaMKII, John Lisman [7, 68] has advanced a detailed hypothesis of CaMKII function in processes of synaptic plasticity, such as LTP [70–72] and LTD [73, 74]. The general assumption is that because CaMKII can sense calcium levels, it allows the neurone to differentiate between low and high levels of activity. Moreover, its capability for autophosphorylation enables long-term activation of the enzyme and thus direct storage of information. Since the model could also account for mechanisms of memory formation, it was tested in behaving animals.

CaMKII is involved in early memory processes

In contrast to the great number of reports on the biochemical cascades in which CaMKII takes part, only a very small number of reports have dealt with its role in behaviour directly. Pharmacological blockade of CaMKII by means of trifluoperazine [TFP: 75] or KN62 [76, 77] consistently produced retrograde amnesia. However, TFP is not a specific CaMKII inhibitor [78], questioning the general validity of the results; KN62, on the other hand, appears to be highly selective [79]. When rats trained in a stepdown inhibitory avoidance paradigm were injected with KN62 immediately post-training into either hippocampus or amygdala, full retrograde amnesia was observed in the retention test 24 h later [76, 80]. A reduction or no effect was obtained in animals intrahippocampally infused 30 or 120 min post-training, respectively, suggesting a time-limited activation of CaMKII starting immediately after or during acquisition training. This is indeed the case, because when CaMKII activity was assayed post-training, a pronounced and lasting (more than 30 min) increase was obtained [81]. Naïve controls, or animals receiving shocks alone, showed no change, proving learning specificity of the effect. As the CaMKII activity returned to baseline, phosphorylation of one target protein, AMPA glutamate receptor, increased, suggesting a sequential transformation of the behaviourally relevant signal from CaMKII to AMPA receptors. One prediction based on these results would be that pretraining application of KN62 should prevent both effects. Recent work from Tan and Liang [82] supports this notion. Rats were given a step-through avoidance procedure, in which they receive a footshock upon entering a dark compartment. Intraamygdala administration of KN62 prevented both learning and the learning-induced increase found in controls.

In essence, similar results have been reported for rats trained in the open field water maze [83] and day-old chicks subjected to inhibitory avoidance training [77]. In spatially trained rats, the increase in calcium-independent CaMKII correlated well with the behavioural performance measured shortly before sacrifice [83]. However, the duration of this increase was not determined, and the obtained increase may not purely reflect memory, simply because the training that has been given before decapitation may confound it. Finally, not all forms of learning are paralleled by CaMKII activation. When KN62 was infused after mating into the accessory olfactory bulbs (in mice [84]), olfactory recognition memory exhibited as a pregnancy block was not affected.

First-generation homozygote mice carrying the CaMKII subunit mutation have been tested in a host of behavioural paradigms including spatial, nonspatial and emotional tests. Apart from their deficiency in expressing LTP [85], which may be a secondary consequence of the epileptogenic phenotype [86], a severe deficit in both spatial learning and retention in the water maze were found [87]. This result has been interpreted as being due to hippocampal CaMKII deficiency, because visually guided learning tasks were only mildly impaired. However, a battery of fear conditioning paradigms employed by Cheng and colleagues [88] revealed significant reductions in fear, pain and aggression in homozygote, and

to a lesser degree in heterozygote, CaMKII mutants. It may thus be questioned whether the learning deficits in the aversive water maze task are genuine learning deficits [87], or whether they are by-products of altered fear and emotion.

While these mutations were neither region-specific nor temporary, a more advanced generation of mice created by Kandel's group overexpressed CaMKII-Asp^{286} instead of CaMKII-Thr^{286}. This renders the enzyme calcium-insensitive and thus permanently active. Transgenic expression of this dominant mutation results in a systematic shift of CA1 responses, so that low frequency stimulation in θ range (5–10 Hz) results in LTD compared with LTP in wild-type littermates [89]. Although high-frequency stimulation (100 Hz) caused normal LTP in both groups, spatial learning and retention when tested in the Barnes maze were significantly impaired in CaMKII-Asp^{286} mice [90]. A more distinct point mutation of threonine 286 to alanine resulted in reduced LTP over a range of tetanus protocols and impaired spatial learning and retention in the water maze [91]. This memory impairment could be the consequence of abnormal hippocampal spatial representations, as a profound place cell instability has been demonstrated in the CA1 region of the hippocampus in these mutant mice [92]. To achieve temporary inactivation in such mice, the same group generated two strains of mice [93]: one expressing the tetracycline activator (tTA) under the control of the CaMKII promoter, which limits expression to the forebrain, and a second one with the tTA-responsive tet-O promoter linked to the CaMKII-Asp-gene that is sensitive to doxycycline inhibition. When both transgenes are combined into the same mouse, the tet-O gene is activated only in cells expressing tTA. Unfortunately, the location of tTA expression cannot be controlled, but several lines showed characteristic and localised expression. Line B13 carried pronounced overexpression in CA1 and dentate, but was devoid of any significant enzyme in CA3. Recordings from CA1 showed LTD in response to 10 Hz, compared with LTP in wild-type mice. Four weeks of doxycycline treatment in drinking water reversed the effect. In parallel, a severe deficit in acquisition of the Barnes maze was found in B13 mice; no transgenic acquired criterion. Again, doxycycline treatment reversed the deficit and rendered transgenic mice normal. Another line, B21, with CaMKII-Asp^{286} expressed predominantly in striatum, amygdala and olfactory tubercle, was severely impaired in both contextual and cued fear conditioning, and this impairment was also sensitive to doxycycline. Long-term (6 weeks) doxycycline-induced inhibition of CaMKII-Asp^{286} overexpression enabled normal acquisition, consolidation and recall compared with animals exposed to normal water post-training.

What do we take from these data? As demonstrated in genetically engineered mice, activation of CaMKII is required for a number of neuronal processes, and this may include learning. A crucial part of the enzyme constitutes threonine 286, which is sensitive to autophosphorylation, a mechanism required for long-term activation of CaMKII. Especially the pharmacological studies provided compelling evidence for a role of CaMKII in early stages of memory formation. Since block of memory required immediate/early post-training application of antagonists, it can be inferred that the training event leads to fast activation of CaMKII, which, if persistent for 30 min or more, enables additional longer-term processes.

PTKs

Studies on the role of PTKs have revealed important functions in cell proliferation and development. As part of plastic events, PTKs have been implicated in synaptic plasticity and memory formation only in recent years. PTKs exist in two general families: (i) those that are integral parts of the membrane and form receptors for cellular growth factors such as nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF), and (ii) those which are not coupled to receptors (see [21] for a recent review). The former are not well understood with respect to mechanisms of memory formation, since work has predominantly investigated their role in LTP of synaptic transmission. However, there also appears to be a potential role of BDNF in synaptic plasticity [94] and memory formation. A more detailed account, especially with respect to memory formation, used BDNF mutant mice [95]. Homozygotes did not survive, but heterozygote animals showed a significant reduction in BDNF expression. When tested in a spatial version of the open field water maze, young adult BDNF mutants were clearly impaired in acquisition and required twice the number of sessions to meet criterion compared with wild-type animals. No learning was obtained in aged mutants which correlated with reduced BDNF expression in older animals [95].

Considerable insight has been gained recently into the function of nonreceptor PTKs, which include several gene families such as *c-alb*, *c-fps*, *c-src*, and so on [21]. They are widely expressed in postmitotic cells in the brain and some, like *c-fyn*, which is a member of the *c-src* family, show a very high expression in postsynaptic densities [96] of the hippocampus [97]. Compelling evidence has been provided for various receptors being substrates of PTKs; those receptors include nicotinic acetylcholine (nACh) receptors [98], *N*-methyl-D-aspartate (NMDA) receptors [99, 100], γ-amino-butyric acid (GABA) receptors [101], and α-amino-3-hydroxy-5-

methyl-4-isoxazole propionic acid (AMPA) receptors [102, 103]. Although not fully established, the activation of PTKs clearly requires depolarisation of the postsynaptic compartment by way of calcium. This could either arise from activation of metabotropic receptors, through which the breakdown of phospholipids is stimulated resulting in diacylglycerol (DAG), an activator of PKC, and inositol trisphosphate (IP3), which when bound to its receptors releases calcium from internal stores. Alternatively, increases in postsynaptic calcium may be achieved via ligand- or voltage-activated calcium channels. Calcium appears to be the main trigger for tyrosine kinase activation, but activity might be enhanced through PKC-dependent phosphorylation of the enzyme. Once activated, PTKs phosphorylate a great number of substrates. Add to the extensive knowledge of PKC in memory formation the modulation of PTKs, it is plausible to assume a role of PTKs in mechanisms of memory.

A potential role of PTKs in learning and memory formation was strengthened recently when deficits in odour discrimination learning were reported for a mutant *Drosophila* termed *linotte* [104, 105]. The *linotte* gene was found to encode a putative tyrosine kinase, homologous to the human protein RYK. Deficiency in this gene caused a 60% decrease in memory tested 30 min post-training, when animals were presented with the formerly shock-associated odour and a novel odour. This deficit was not due to peripheral impairments, since shock sensitivity and reactivity to octanol, for instance, were normal [104, 105].

One route for alteration of PTK function is the pharmacological approach. Application of PTK antagonists genistein and lavendustin A to hippocampal slices in which extracellular field potentials from area CA1 were recorded in response to Schaffer collateral/commissural fibre stimulation dates back to 1991 [106]. Both compounds blocked the induction of tetanus-induced LTP with the potentiation returning to baseline within 20–25 min. These results have now been verified and extended by Salter's group [103, 107], showing that specific inhibition of *src* PTKs blocks LTP induction. In contrast, selective activation of *src* PTK caused a slow-onset potentiation that occluded tetanus-induced LTP [103]. Unfortunately, both *src* activator peptide [EPQ(pY)EEIPIA] and inhibitor protein (*src* 40–58) have to be applied intracellularly to be effective. Genistein and lavendustin A, on the other hand, would cross the membrane and were thus applied to 1-day-old chicks trained in a single-trial inhibitory avoidance paradigm. A memory deficit emerged in animals tested 90 min or later after training [108]. Because applications were effective only during a narrow time window prior to or immediately after training, PTK activation appears to be an early step in the cascade of memory mechanisms, but does not manifest its behavioural effect before 90 min post-training. Although both studies suggest the requirement of PTKs for longer-lasting processes involved in synaptic plasticity or memory formation, the nonselectivity of both drugs does not allow any firm conclusion as to the specific PTK family involved in these mechanisms.

An alternative biochemical approach has been utilised by Dudai's group [109]. Rats were trained in a conditioned taste aversion paradigm in which a novel taste, saccharin, was followed by delayed (50 min) LiCl infusion causing serious malaise. Ten minutes after training, brain tissue—here particular weight was given to the insular or taste cortex—was excised and biochemically analysed for the level of PTK activation by means of polyclonal antibodies. An increase in PTK-induced protein phosphorylation was reported. This increase required both novel taste and malaise (not each component individually), was region-specific (not in olfactory bulb, piriform, occipital or frontal cortex), and lasted for longer than 1 h. A novel, at the time unidentified 180-kD protein, was heavily phosphorylated; it has now been identified as the 2B subunit of the NMDA receptor (Dudai, personal communication), corroborating the important link between PTKs and receptor phosphorylation.

Using a third, genetic approach and creating selective knockout mutants of *fyn*, *src*, *yes* and *abl* (all members of nonreceptor PTKs), their function in both long-term potentiation and learning have been tested more specifically [110]. Surprisingly, only the *fyn* mutants showed severe deficits in the induction of LTP to low-amplitude tetanization, but high-amplitude tetanization produced blunted LTP. All other mutants had no effect on this form of synaptic plasticity, suggesting a potential role of *fyn* in LTP. When tested in the open field water maze task, fyn mutants showed a striking impairment in learning to locate the hidden platform, despite their ability to learn a cued version of this task [110]. This indicates a peculiar role of *fyn*, as opposed to some other PTKs, in mechanisms underlying learning and memory formation. However, because of the importance of PTKs in development, and the lack of *fyn* activity throughout life of the mutants, a careful investigation demonstrated anatomical disorganisation of cell layers in CA3 and dentate gyrus [110]. Interpretation of the data thus requires some caution, since compensatory mechanisms might have been activated during the ontogeny of the mutants. Thus, a more detailed analysis of the behaviour of these *fyn* mutant mice provided clear evidence for sensory-motor deficits which, once overcome, allow them to acquire a spatial water maze task [111]. Although they did not meet the same high and accurate performance that wild-type animals did, a transfer test revealed a significant bias in swimming in

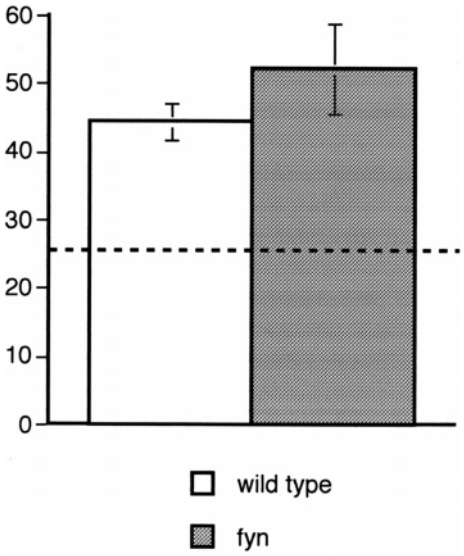

Figure 4. Performance of *fyn* mutant mice in a transfer test in the water maze. Only the trained quadrant is shown. Mean ± SEM. Dotted line denotes chance level. Data modified from [111].

the trained as opposed to all other quadrants (fig. 4). However, this behavioural pattern was achieved with intensified training, lending additional support to the electrophysiological findings of low-amplitude LTP being impaired, but high-amplitude LTP being blunted [110]. Clearly, it is to be expected that multiple training sessions per day may produce stronger neuronal signals compared with low numbers of training sessions.

Furthermore, *fyn* mutants derived from a different source have been tested in a number of learning and control testing paradigms by the group of Niki and colleagues [112, 113]. They reported that spatial learning in a positively reinforced eight-arm radial maze was normal in the knockouts [113], but there was a clear and pronounced increase in fearfulness. When tested in the elevated plus maze, with two open and two enclosed arms, *fyn* mutants spend more time in the enclosed arms [113]. They also spend more time in the dark compared with the illuminated compartment of a novel box and show reduced exploration of a novel open field [112, 113]. Together with the observation of dramatically increased memory in a step-through inhibitory avoidance task [112], these data provide compelling evidence for alterations in emotionality in *fyn* mutant mice, which could be responsible for deficits in the water maze [110], which is a rather stressful test. Once extensive habituation has taken place, however, the impairment may no longer be detectable [111]. Such alterations in emotionality, however, do not affect all forms of learning. When *fyn* mutants were tested for conditioned taste aversion, a paradigm in which a novel taste is coupled with malaise by way of LiCl injection, they performed normally compared with wild-type controls [114].

Collectively, activation of receptor PTKs appears to be an important step during acquisition of new information. By contrast, stimulation of nonreceptor PTKs may be a prerequisite for successful long-term memory formation in the animal kingdom and possibly in humans.

MAPK cascade

A further step in the biochemical intracellular signalling cascades is the stimulation of second messenger-independent protein kinases. One, that has recently become the focus of memory research engages a number of protein kinases to finally activate the mitogen-activated protein kinase (MAPK). Although some details of the MAPK cascade have been elucidated due to its importance in proliferation and differentiation processes of the cell (for review see [115]), determination of an exact role in synaptic plasticity and memory formation awaits further work. The limited number of reports available today, however, allows for some interesting speculations on the function of MAPK in memory formation. There is now considerable evidence that especially long-term memory formation requires activation of transcription factors such as CREB (see Lamprecht, this issue). While direct phosphorylation, and thereby activation, of transcription factors may be achieved via PKA, other transcription factors lack consensus sequences for PKA, suggesting regulation via different enzymes. The direct link between PKA and MAPK is established through PKA regulation of the proto-oncogene Raf-1 or some Ras members of the small G-protein family. Raf-1 itself functions as a direct regulator of MAPK kinase (MEK), which can tyrosine-phosphorylate MAPK for activation. However, the common final step in this cascade appears to be the activation of MAPK (which could be either inactivated or activated by phosphorylation [116]).

Kandel's group [117–119] has studied the mechanistic role of MAPK in short-term and long-term sensitisation of the gill-withdrawal reflex in *Aplysia*. As outlined above, plastic events are presynaptic, and alterations are to be expected in the sensory neurone (fig. 3). Specific antibodies labelling MAPK have been constructed, and immunohistochemical analysis of the MAPK distribution revealed a stimulation-dependent translocation of the enzyme into the nucleus of the presynaptic neurone. In line with this observation, blockade of MAPK prevents both long-term sensitisation and translocation [118]. Moreover, several transcription factors are directly modulated by MAPK

[119]. Because short-term facilitation remained normal, these data support the view that activation of the PKA-MAPK cascades permit long-term facilitation in *Aplysia*. In addition, it appears that MAPK phosphorylation of cellular adhesion molecules (CAM) at the membrane causes CAM internalisation and subsequent cell growth [117]. This process is also required for long-term facilitation and is prevented in the presence of MAPK inhibitors.

Confirmation of these findings has been provided for the marine mollusc *Hermissenda* [120] when light paired with rotation thereby elicits foot retraction. Single-trial or multitrial classical conditioning caused MAPK-dependent phosphorylation of extracellular signal-regulated protein kinases ERK1 and 2 with a molecular weight of 44 and 42 kDa, respectively. A similar increase in the 42-kDa, but not 44-kDa, protein had been reported by English and Sweatt [121] after induction of long-term potentiation in hippocampal CA1. Thus it is difficult to reconcile that mutant mice lacking Ras-GRF, a small G-protein family member, did not show abnormalities in hippocampal synaptic plasticity or hippocampus-dependent learning [122]. However, long-term plasticity is abnormal in the amygdala, and long-term memory is reduced for contextual or cued conditioning.

In a very recent report, further evidence was provided that demonstrates that the MAPK cascade plays a prominent role in mammalian associative learning [123]. Fear conditioning in rats was shown to induce an increase in MAPK activation in the hippocampus. Conversely, MK801, an NMDA receptor antagonist, and SL327, a selective inhibitor of MEK that blocked MAPK activation upstream, abolished both cued and contextual fear conditioning. The time course of MAPK activation suggests that MAPK is required for consolidation of associative memories.

In essence, evidence is accumulating for a potential role of the MAPK cascade as a prerequisite for establishing long-term memories.

Conclusions

The aforementioned data do not support the view stressed in the provocative title of this paper. Despite having reviewed a large body of evidence that protein kinases play a critical role in memory formation, there are at present no convincing data that allow us to qualify a particular kinase as a memory molecule [9] or as a kinase to remember [12]. Rather, the neuronal syntax appears to underlie a more complex interaction with multiple kinases (and their counterpart phosphatases—see Riedel, this issue) being directly involved in the early phase of memory formation. Taken together, the different kinases seem to have a time-dependent activation profile with respect to the learning event. It is therefore a cascade, or sequence, of kinases that come into play which as a whole enables formation of memory (fig. 5). Pharmacological, biochemical and transgenic investigations demonstrate that the time window of protein kinase activation appears to be limited, and so far no concrete evidence supports the hypothesis that protein kinases can autonomously sustain a persistent activation over time in vivo (Dudai, personal communication). With respect to the diversity of the substrates (more than 130 have so far been identified for PKC), protein phosphorylation appears to be implicated in many events that could act in concert to form memory either by inducing posttranslational modifications or by promoting more permanent changes such as protein synthesis.

Recent findings have revealed the potential importance of posttranslational processes in long-term plasticity mechanisms. The research of mechanisms that account for input specificity has led Frey and Morris to propose a synaptic tag hypothesis [124–126]. Briefly, this hypothesis, based on hippocampal LTP, assumes that LTP induction sets a short-lasting (around 1 h), protein synthesis-independent synaptic tag at the potentiated synapses, which sequesters de novo synthesised plasticity-related proteins to establish long-term synaptic modifications. The molecular identity of the putative synaptic tag is still speculative. Among the possible candidates, protein kinases or more likely phosphorylated receptors such as NMDA or AMPA receptors, or phosphorylated structural proteins could exhibit potential tagging properties. Strengthening the notion of site specificity that is required by the synaptic tag hypothesis, it is now well established that specific anchoring

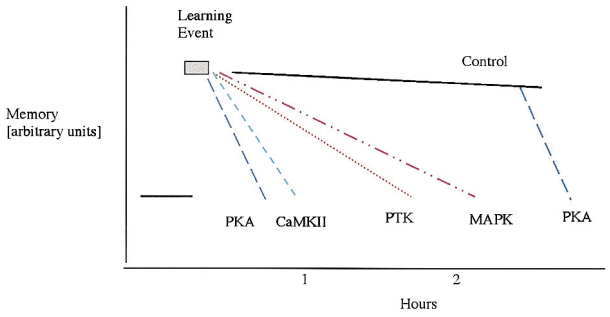

Figure 5. Tentative sequence of protein kinases activated as a consequence of learning. Memory (set in arbitrary units) is inhibited at different time points after application of specific kinase blockers suggesting prolonged activation of these enzymes. This has been supported by biochemical examinations conducted post-learning. For details of the individual kinases, see main text.

proteins located at various sites in the cell compartmentalise the kinases to their sites of action [127, 128]. This subcellular specific targeting enhances the selectivity of kinases by favouring their accessibility to certain substrate proteins. These targeting processes appear to exhibit the same time course as the synaptic tagging [126, 127].

Moreover, the regulatory mechanism of the family of transcription factors such as CREB provides a good example of the cooperativity of the different kinases in the regulating cAMP-induced gene expression. Genes regulated by the cAMP/PKA cascade can be activated by CREB1, but they are concomitantly repressed by CREB2 [41, 10]. Phosphorylation of CREB2 relieves this repression and might thus potentiate the activation process initiated by CREB1 phosphorylation. Whereas CREB1 contains consensus sites for phosphorylation by PKA, CREB2 has only PKC and several MAPK sites [10]. This example clearly demonstrates that the different protein kinases may act in concert to execute positive and negative regulations that mediate long-term memory storage.

These fragmentary considerations emphasise the fundamental role of the protein kinase systems in establishing the syntax of memory formation. They also provide evidence of the necessity of disentangling the complexity of these molecular interactions in a spatiotemporal dimension.

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