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Review

Ras-related and MAPK signalling in neuronal plasticity and memory formation

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Abstract. Ras-related guanosine triphosphatases (GTPases) couple receptor activity to a number of intracellular signalling events culminating in the control of cell morphology and gene transcription. In culture cells, the best-understood Ras-dependent signalling pathway involves the mitogen-activated protein kinase/extracellular-regulated kinase (MAPK/ERK) cascade. A growing body of evidence has recently been accumulating to suggest a crucial role of Ras and MAPK signalling in neuronal functions connected to synaptic plasticity. In the present review article we discuss the experimental basis supporting the notion that the Ras/MAPK pathway interacts with other synaptic mechanisms to regulate invertebrate and vertebrate behavioural responses such as those implicated in learning and memory processes.

Key words. Ras/MAPK signalling; synaptic plasticity; learning and memory; hippocampus.

Introduction

Cognitive functions such as learning and memory formation are believed to require plastic synaptic changes. Increasing evidence suggests that, in specific sets of neurons, both the strengthening of preexisting synapses and the growth and maintenance of new synaptic connections underlie memory consolidation [1, 2]. Activity-dependent synaptic remodelling requires the coordination of a variety of molecular events, both in the cytoplasm and within the nucleus. Whereas short-term memory depends mainly on the modification of preexisting cellular proteins, long-term memory requires alteration in gene expression and synthesis of new proteins [3, 4].

Despite considerable effort, the biochemical processes involved in activity-dependent synaptic changes are still poorly understood. By interfering either pharmacologically or genetically with various signalling molecules, several protein kinase cascades, which involve protein kinase A and C (PKA and PKC), tyrosine kinases of the Src family and the Ca^{2+}/calmodulin-dependent kinases (mainly CamKII and CamKIV), have been implicated in the process of long-term memory formation [5]. Unexpectedly, recent findings point to a pivotal role of the Ras/mitogen-activated protein kinase (MAPK) signalling pathway in modulating synaptic functions. Thus, in addition to the well-described ability to tightly control cell growth, the Ras/MAPK cascade appears to be a critical regulator of memory consolidation and long-term neuronal plasticity.

Ras and MAPKs in neuronal systems

The Ras subfamily of small GTP-binding proteins plays an essential role in a variety of cellular events, including normal and malignant proliferation, differentiation and

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survival [6]. A detailed description of the biochemistry of Ras signalling can be found in recent reviews [7–9]. In neuronal cells, activation of the Ras pathway is mediated by a variety of receptor systems, including receptor tyrosine kinases (RTKs) for peptide factors, G-protein-coupled serpentine receptors (GPCRs) for neurotransmitters, and calcium influx through voltage-gated calcium channels or N-methyl-D-aspartate (NMDA) receptors for glutamate. Ras activation initiates multiple intracellular signalling cascades, eventually leading to gene transcription.

The best-understood effector system downstream of Ras is the MAPK pathway, an evolutionarily conserved signalling cascade. The gene products of ERK1 and ERK2, the two best-characterised MAPKs, are serine/threonine kinases that act as critical transducers of growth factor signalling to the nucleus in mammalian cells. Activation of Ras by extracellular signals leads to sequential activation of Raf (MAPK kinase kinase), MEK (MAPK kinase) and ERKs/MAPKs (fig. 1). Activated MAPKs in turn phosphorylate a large and growing range of substrates, both in the cytosol and in the nucleus. Amongst the major substrates of MAPKs are the Rsks (ribosomal S6 kinases, also known as MAPK-activated kinases, MAPK-APKs). Protein kinases of this class directly phosphorylate the cAMP response element (CRE)-binding factor CREB, which plays an essential role in inducing expression of many immediate-early genes (IEGs) such as Fos. This fact is particularly relevant, since many forms of neuronal plasticity and learning require functional CREB [10]. In addition, MAPKs can directly phosphorylate and activate serum response element (SRE)-binding proteins, such as Elk1, thus contributing to the control of gene transcription [11].

Pharmacological experiments

Several lines of evidence suggested a potential involvement of Ras/MAPK signalling in synaptic functions. First, many components of the signalling pathway, such as Ras itself, ERK1/2 MAPKs and Ras regulators such as RasGRF, SynGAP and NF1, are highly expressed in the adult central nervous system (CNS), in particular in associational areas implicated in learning and memory (hippocampus, neocortex and cerebellum) [8]. Second, synaptic activation in neuronal cultures or in slices, causing elevation of intracellular cAMP and calcium, also potently activates MAPK signalling [12–15].

Learning in invertebrates

The first evidence of a role for the MAPK cascade in invertebrate neuronal plasticity came from studies of long-term facilitation (LTF) in *Aplysia*, a model for long-term sensitisation of the gill withdrawal reflex. LTF of the connections between sensory and motor neurons contributes to the enhancement of the gill withdrawal reflex and is mediated by multiple spaced pulses of the neurotransmitter serotonin [16, 17]. Kandel and co-workers have shown that MAPK phosphorylation is both associated with and necessary for the establishment of long-term, but not short-term, facilitation in *Aplysia*. Inhibiting MAPK function with neutralising antibodies or with a pharmacological inhibitor of MEK (the direct activator of MAPK) blocked LTF of presynaptic sensory neurons without affecting short-term facilitation. These results suggest that MAPK activation and nuclear translocation induce the transcription and expression of new proteins necessary for LTF [16]. Indeed, apMAPK is able to phosphorylate in vitro at least two major transcriptional regulators, for example apCREB-2 and apC/EBP [18]. In addition, an important cytosolic target of activated MAPK is the transmembrane form of apCAM, a neural cell-adhesion molecule which regulates adhesive interactions at the synapse [16, 18]. Phosphorylation of apCAM leads to its internalisation and degradation, possibly by the ubiquitin proteasome pathway. Selective removal or reduced expression of this molecule results in neurite defasciculation and initiation of synaptic remodelling, thus facilitating the formation of new synapses. Interestingly, repeated exposure of sensory neurons to serotonin induces the expression of a ubiquitin C-terminal hydrolase, which plays a fundamental role in proteolytic events associated with LTF [19]. An intriguing possibility is that MAPK plays a dual role in triggering synaptic reorganisation: MAPK may first phosphorylate apCAM, thus rendering it susceptible to proteolytic cleavage and internalisation; then, following nuclear translocation, it may induce expression of ubiquitin C-terminal hydrolase, which as a component of the ubiquitin proteasome could direct the internalisation and degradation of the tagged apCAM [20].

Additional evidence of a potential role of MAPK signalling in invertebrate learning comes from a recent study in *Hermissenda*. Both in single-trial and multitrial classical conditioning paradigms MAPK activity is rapidly induced and efficiently inhibited by the same MEK inhibitor used in *Aplysia*, PD098059 [21]. Unfortunately, the influence of such inhibition on the behavioural paradigms has not yet been tested.

Plasticity in mammalian models

Importantly, also in mammalian systems increasing evidence implicates MAPK as a critical component of neuronal plasticity. Electrophysiological and biochemical studies of rat hippocampal slices indicated that

MAPK signalling is required for long-term potentiation (LTP), which is a strong candidate as a cellular mechanism for learning and memory [4, 22]. In particular, high-frequency tetanic stimulation that induces LTP in the hippocampus also potently activates $p42^{ERK2}$ MAPK, whereas pharmacological inhibition of MEK partially inhibits LTP formation in this area [15, 23]. The reasons ERK1 does not seem to be activated under these conditions are unknown, but this finding is potentially intriguing since it suggests a differential function for those two kinases.

In addition, it has recently been shown that ERK-dependent signalling is also required for LTP elicited with more physiological stimulation such as θ frequency (5 Hz). Application of both PKA and ERK inhibitors strongly inhibits this kind of LTP, whereas inhibition of protein synthesis has no effect. This scenario is rather different from other types of LTP, such as early LTP induced by single 1-s 100-Hz train (PKA-, ERK- and protein synthesis-independent) or late LTP induced by repeated 100-Hz tetani (which depends on all three factors) [24].

In addition to Ras, at least in some contexts, two intracellular kinases seem to be required for MAPK activation: PKA and PKC. Both stimulation of hippocampal slices with forskolin (PKA activator) and phorbol diacetate (PDA) (PKC activator) causes p42 phosphorylation, which is reverted by addition of MEK inhibitor [25, 26]. At the moment it is not clear what the direct targets of PKA and PKC are: activation of ERK2 in hippocampus by these two kinases might be dependent on small GTPases different from Ras, although the lack of specific pharmacological inhibitors for use in vivo has so far hampered efforts to answer this question. In particular, the Ras-related GTPase Rap1 has been suggested to couple cAMP/PKA signalling to ERK activation via the action of the B-Raf kinase [9, 27]. Interestingly, PKA- and PKC-dependent phosphorylation of CREB is largely inhibited by MEK inhibitor, clearly indicating that MAPKs are major mediators of both signals, at least in the hippocampus.

What kinds of stimuli can induce MAPK activation in hippocampal neurons? Early evidence showed that depolarising stimuli such as KCl and electroconvulsive shock potently activate MAPKs in the hippocampus [28, 29]. More recently, several receptor types have been shown to activate MAPKs, including NMDA and metabotropic glutamate receptors, dopamine receptors, muscarinic acetylcholine receptors and β-adrenergic receptors [24, 26, 30]. At least in the case of dopamine and β-adrenergic receptors, activation of MAPKs requires PKA [26]. In addition, depolarisation of hippocampal neurons in cultures, which causes Ca^{2+} influx, also leads to CREB activation via PKA, ERKs and Rsk2. In particular, PKA signalling seems to be required for nuclear translocation of the two latter kinases [25].

The evidence of involvement of MAPKs in long-term plasticity is strong in the hippocampus; however, this finding seems to be extendible also to other brain structures. In vivo electrical stimulation of the glutamatergic cortico-striatal pathway leads to induction of IEGs such as c-fos, zif-68 and MKP-1 [31]. Such activation depends on both Elk-1 and CREB. Intrastriatal injection of the MEK inhibitor PD098059 completely blocks IEG induction. A similar result can also be obtained with striatal slices stimulated in vitro with glutamate: PD treatment blocks Elk-1 and CREB phosphorylation, emphasising the critical role of MAPKs in the modulation of IEGs in striatal neurons [32]. Interestingly, inhibition of CamKII causes a reduction in ERK and CREB activation, suggesting cross-talk between these pathways. Finally, long-term depression (LTD) in cultured Purkinje cells, another model of plasticity particularly important for cerebellar-dependent learning, is blocked by MEK inhibitor treatment along with MAPK inhibition [33].

Recent results have also provided direct evidence of the involvement of MAPK signalling not only in neuronal plasticity but also in behaviour. Several recent publications have addressed the role of MAPKs in vivo by using various MEK inhibitors. In a first report the drug was given systemically; therefore, it is not known precisely which structures were involved in the observed phenotype. Rats undertaking associative learning tasks, such as contextual fear conditioning, had a peak of P42 MAPK phosphorylation in the hippocampus within the first hour post-training, whereas the signal was already back to baseline levels by 2 h. Pretraining treatment with the MEK inhibitor SL327 caused a clear deficit in memory retention, demonstrating the importance of MAPKs in this type of test [34]. The same compound was also found in mice to inhibit acquisition in the Morris navigation test, a typical measure of spatial memory [35]. Additional results with both contextual and auditory fear-conditioning tests were obtained by intraventricular injection of a different MEK inhibitor, PD098059 [36]. Three additional papers have demonstrated the role of ERK signalling in other types of learning, namely conditioned taste aversion (CTA), spatial learning and inhibitory avoidance. Bilateral infusion of the PD098059 inhibitor directly into the insular cortex attenuated long-term taste aversion without affecting short-term memory. Interestingly, in addition to ERK1/2 kinases, Jun N-terminal kinases 1 and 2 (JNKs), but not Akt or p38MAPK, were found to be activated upon behavioural training, albeit with slower kinetics [37]. It would be interesting to know whether inhibition of JNKs also resulted in behavioural impairment. In addition, bilateral infusion of PD098059 in the

Table 1. Summary of experimental evidence for a role of Ras and MAPKs in cognitive functions.

Organism	Protein/ gene	Test	Technique	Neuronal structure	Effect	Ref
Invertebrate						
Aplysia	MAPK	in vitro stimulation of gill withdrawal reflex	MAPK activation in absence or in presence of MEK inhibitors	presynaptic sensory neurons	block of LTF	16
Hermissenda	MAPK	one- and multi-trial classical conditioning	MAPK activation in absence or in presence of MEK inhibitors	ND	ND	21
Vertebrate						
Rat	MAPK	in vitro electrical and chemical stimulation of neurons	MAPK activation in absence or in presence of MEK inhibitors	hippocampus	block of LTP	23-25
Rat	MAPK	in vivo electrophysiological stimulation of neurons	intrastriatal injection of MEK inhibitors	cortico-striatal pathway	block of CREB and Elk-1 dependent IEG induction	31
Rat	MAPK	in vitro electrical stimulation of neurons	administration of MEK inhibitors	cerebellar Purkinje cells	block of LTD	33
Rat, mouse	MAPK	contextual fear conditioning	in vivo systemic administration of MEK inhibitors	ND	deficit in memory retention	34, 35
Rat	MAPK	contextual and auditory fear conditioning	intraventricular injection of MEK inhibitors	ND	deficit in memory retention	36
Rat	MAPK	conditioned taste aversion	in vivo bilateral infusion of MEK inhibitors	insular cortex	attenuation of long-term taste aversion	37
Mouse	MAPK	Morris water maze	in vivo administration of MEK inhibitors	ND	reduced spatial LTM formation	35
Rat	MAPK	Morris water maze	in vivo bilateral infusion of MEK inhibitors	hippocampus	reduced spatial LTM formation	38
Rat	MAPK	inhibitory avoidance	in vivo bilateral infusion of MEK inhibitors	amygdala, hippocampus, entorhinal cortex	reduced memory retention	38
Mouse	NF1	Morris water maze	NF1 gene targeting	hippocampus	reduced memory of spatial information	52
Mouse	RasGRF	contextual and cued fear conditioning	RasGRF gene targeting	amygdala	compromised LTM consolidation	53
Human	RSK 2		gene linkage analysis	ND	mental retardation	55

Abbreviations: MAPK, mitogen-activated protein kinase; ERK, extracellular regulated kinase; MEK, MAPK/ERK kinase; ND, not detected; LTP, long term potentiation; LTD, long term depression; IEG, immediate early gene; LTM, long term memory; NF1, neurofibromatosis type 1; RasGRF, Ras guanine releasing factor; RSK2, ribosomal S6 kinase 2; CREB, cAMP responsive element-binding protein.

dorsal hippocampus caused a reduction in long-term memory formation without affecting knowledge acquisition in the Morris water maze task [38]. Finally, post-training infusion of PD in the amygdala, CA1 region of hippocampus or entorhinal cortex caused a clear inhibition in inhibitory avoidance task [39, 40].
Taken together, all these lines of evidence clearly support a major role for ERK signalling in plastic changes in different brain areas and in long-term memory formation.

Genetic experiments

So far, direct genetic evidence for the role of Ras-dependent signalling in behaviour is confined to a few reports. Neurofibromin is a major GTPase activating protein (GAP) for Ras proteins, particularly expressed in neuronal tissues. Its malfunctioning causes a common and severe human disease, neurofibromatosis type 1 (NF1) [41–44]. Among various symptoms, NF1 patients often manifest severe cognitive impairments [45–49]. Recently, NF1 mutant mice have been generated [50, 51]. The general assumption is that in these mice the basal activation level of Ras signalling is increased because of the loss of the negative regulatory role normally exerted by neurofibromin. Although homozygous mutants die in utero, heterozygous animals are viable but manifest certain behavioural deficits. In the Morris water maze, acquisition seemed to be normal. However, the probe trial after 10 days (20 trials) of training revealed a reduced retention of spatial information, strikingly similar to what was observed in the same test in rats treated with the MEK inhibitor [52]. Extensive training (14 days, 28 trials) completely rescued the phenotype, as previously observed for other hypomorphic mutants. Surprisingly, the 'rescuing effect' of extensive training is dramatically abolished if NF1 $+/-$ mice are tested in an NMDAR $+/-$ background, suggesting a genetic interaction between Ras and glutamate signalling and confirming the requirement for fine tuning of Ras activity for cognitive functions. Notably, hippocampal functions seem to be particularly sensitive to NF1 dosage since tests for amygdala function, such as cued fear conditioning, did not reveal any deficits. It would certainly be of great interest to have more data on the electrophysiology of the hippocampal formation of NF1 $+/-$ mice as well as a brain-specific NF1 KO mouse strain which presumably would circumvent the embryonic lethality of the NF1 $-/-$ genotype.
Additional support of the involvement of Ras-dependent signalling came from another strain lacking the neuronal specific Ras exchange factor RasGRF. In contrast with NF1 mice, RasGRF $-/-$ mice are viable and fertile and do not manifest gross morphological alterations in overall brain organisation [53]. Yet RasGRF KO mice are severely impaired in amygdala-dependent behavioural tasks, such as operant and classical conditioning tests. While learning and short-term memory are basically intact, long-term memory consolidation is dramatically affected, suggesting a deficit in the Ras-dependent signal transduction mechanism dependent on de novo protein synthesis. The lack of effect in Morris water maze and similar tests in RasGRF KO mice is consistent with intact hippocampal LTP. The apparent discrepancy between the phenotype in NF1 and RasGRF mice could be explained by a difference in importance of NF1 and RasGRF in hippocampal and amygdalar physiology, respectively.
Is anything known about genetic alterations in downstream effectors of Ras, such as the MAPK cascade? At the moment, mouse models are not yet available to test the contribution of various MAPKs (ERK1 and 2, JNK1 and 2, p38, Rsks) to memory formation, but interesting observations have recently been made in humans. Progress in mapping candidate genes for human diseases has led to the identification of several loci involved in neurological dysfunction and, in particular, in mental retardation [54]. Two genes responsible for two different forms of X-linked mental retardation (MRX) code for two kinases associated with small GTPase signalling. Loss of function of Rsk2, one of the CREB kinases downstream to ERKs, causes the Coffin-Lowry syndrome, which is characterised by severe mental handicap. Finally the nonsyndromic MRX30 form is linked to the PAK3 gene, a kinase known to link Rac/Cdc42 GTPases to JNK and p38 SAPKs [55, 56].

Figure 1. A schematic representation of Ras/MAPK signalling at the synapse. Several possible receptor mechanisms might activate Ras proteins, including glutamate receptors such as *N*-methyl-D-aspartate receptors (NMDAR), voltage-gated calcium channels (VGCC), G-protein-coupled receptors (GPCRs) or receptor tyrosine kinases (RTKs). All membrane signals converge toward Ras exchange factors such as RAS-GFR, Sos and Ras-GRP, which in turn activate Ras proteins. An additional level of regulation might be achieved by protein kinase C (PKC) inhibition of GTPase-activating proteins such as NF1 and GAP-1. A protein kinase cascade is initiated in the cytoplasm by sequential activation of RAF, MEK and MAPKs of the ERK family. Protein kinase A (PKA) might promote this cascade independently from Ras. Once translocated into the nucleus, MAPKs can phosphorylate transcription factors such as CREB. This effect is believed to be mediated by the Rsk family of protein kinases. Activation of CREB-dependent transcriptional activity leads to immediate early (IE) gene expression. Some of these gene products are themselves transcription factors and might induce late response (LR) genes. These genes are believed to be responsible for establishing long-term memory (LTM).

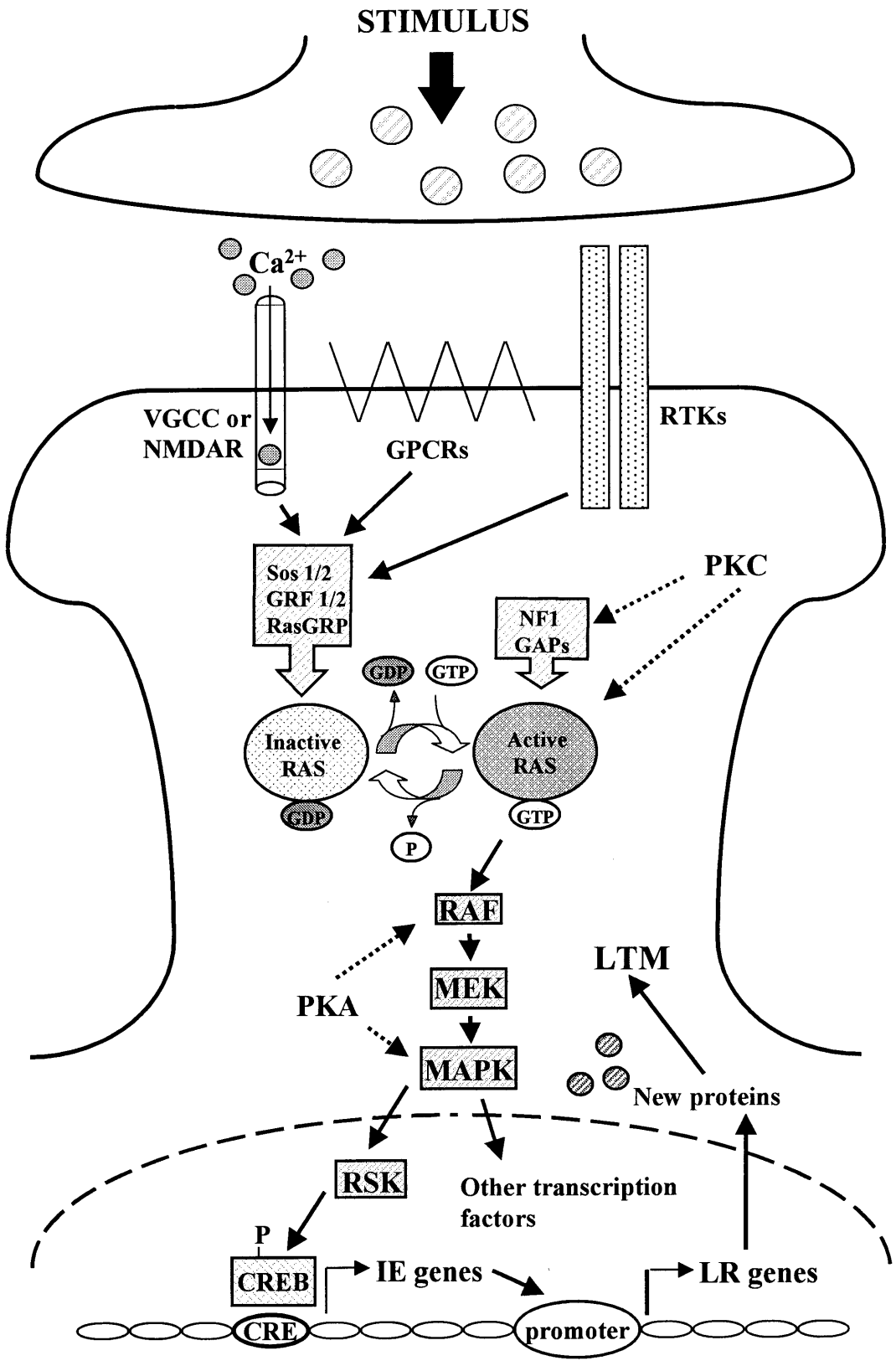
STIMULUS
Ca2+
VGCC or
NMDAR
GPCRs
RTKs
Sos 1/2
GRF 1/2
RasGRP
PKC
NF1
GAPs
GDP
GTP
Inactive
RAS
Active
RAS
GDP
GTP
P
RAF
MEK
MAPK
PKA
LTM
New proteins
RSK
Other transcription
factors
P
CREB
IE genes
LR genes
CRE
promoter

Fig. 1.

In vitro studies with mutant forms of Rsk2 and PAK3 proteins clearly demonstrated their inability to transduce signalling. In particular, a commonly found amino acid substitution in Rsk2, R383W, causes a reduction in kinase activity to 15–20% of the wild type, probably by precluding the essential autophosphorylation event at Ser-386. In addition, R383W mutant is unable to induce phosphorylation at Ser-133 on its nuclear target CREB [57].

Taken together, these genetic alterations strongly support the involvement of Ras-dependent signalling in cognitive functions both in rodents and in humans.

Future directions and open questions

From the body of evidence provided in the previous paragraphs (summarised in table 1), it seems clear that Ras-dependent signalling and in particular MAPK activation is a crucial event in modulating synaptic functions. However, the molecular details of its involvement in the behaviour of living animals and particularly in the formation of long-term memories remain to be elucidated.

Some important questions will hopefully be answered in the next few years:

1) What are the relative contributions and functional relevance of upstream signals such as neurotransmitter receptors and other intracellular kinase cascades such as PKA and PKC in the activation of both Ras and MAPKs?

2) What are the crucial targets of MAPK signalling besides CRE- and SRE-binding proteins?

3) What is the reason for a differential activation of ERK1 and ERK2 in hippocampus and possibly other brain structures?

4) Besides their involvement in long-term memory formation, are MAPKs also necessary for consolidation, storage or information retrieval?

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