INTEGRATION OF BIOCHEMICAL SIGNALLING IN SPINES

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Abstract | Short-term and long-term changes in the strength of synapses in neural networks underlie working memory and long-term memory storage in the brain. These changes are regulated by many biochemical signalling pathways in the postsynaptic spines of excitatory synapses. Recent findings about the roles and regulation of the small GTPases Ras, Rap and Rac in spines provide new insights into the coordination and cooperation of different pathways to effect synaptic plasticity. Here, we present an initial working representation of the interactions of five signalling cascades that are usually studied individually. We discuss their integrated function in the regulation of postsynaptic plasticity.

LONG-TERM POTENTIATION (LTP). An enduring increase in the amplitude of excitatory-postsynaptic potentials as a result of high-frequency (tetanic) stimulation of afferent pathways. It is measured as an increase in the amplitude of excitatory-postsynaptic potentials or in the magnitude of the postsynaptic cell population spike. LTP is most frequently studied in the hippocampus and is often considered to be the cellular basis of learning and memory in vertebrates.

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Memory storage in the brain involves adjustments in the strength of individual synapses to form new neural networks[1]. Changes in strength at excitatory synapses in the CNS are exquisitely controlled by patterns of activity at the synapse and by hormonal influences. Over the past decade, a host of studies have investigated the signalling systems that underlie 'activity-dependent synaptic plasticity'. Two rules have emerged that seem to govern such plasticity. First, the precise timing of transmitter release in relation to the firing of a back-propagating action potential in the postsynaptic neuron determines the direction of change in strength that is induced at a synapse[2,3]. This rule is referred to as 'spike-timing-dependent synaptic plasticity'. When release occurs within 5–15 ms before the firing of a back-propagating action potential, LONG-TERM POTENTIATION (LTP) of the synapse is induced, whereas when release occurs within a similar time window after the firing of a back-propagating potential, LONG-TERM DEPRESSION (LTD) of the synapse is induced. This precise timing controls the NMDA (*N*-methyl-D-aspartate)-type glutamate receptor (NMDAR), which allows influx of Ca^{2+} into the spine when the receptor binds glutamate at the same time that it is subjected to membrane-depolarization[4,5]. Curiously, Ca^{2+} influx through the NMDAR is required to produce both LTP and LTD. This fact has led to the second rule, or 'Ca^{2+} hypothesis', which holds that relatively rapid and large influxes of Ca^{2+} into the spine produce LTP, whereas more prolonged and lower influxes of Ca^{2+} produce LTD[2]. The parameters that govern both of these rules are still being discovered and debated; however, they have raised a new and difficult question: what are the molecular mechanisms through which small changes in Ca^{2+} influx into a spine can produce such distinct functional outcomes?

In the search for molecular mechanisms of LTP and LTD, almost every major signalling pathway has been implicated. The often contradictory studies prompted an influential review by Sanes and Lichtman entitled, 'Can molecules explain LTP?'[6] The authors bemoaned the tendency to confuse indirect effectors and modulators of synaptic plasticity with crucial mediators, and attributed the problem, in part, to the absence of clear cellular definitions of LTP and LTD. We certainly agree that these phenomena are ill-defined at the experimental level. However, Sanes and Lichtman also acknowledged that some cellular phenomena, no matter how well defined, might have no 'core programme', and might, instead, emerge from interactions among many biochemical pathways. We suggest that synaptic plasticity might fall more fully into this category than, say, the action potential, or even transmitter release. To interpret molecular experiments on the mechanisms of postsynaptic regulation more usefully, we need to begin building rigorous quantitative models (BOXES 1 and 2) of the

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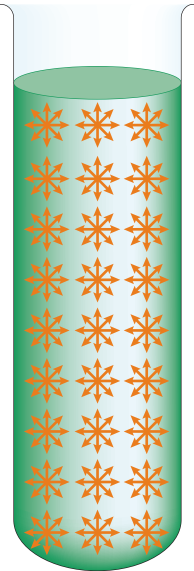
Box 1 | **Quantitative models of interacting signalling events**

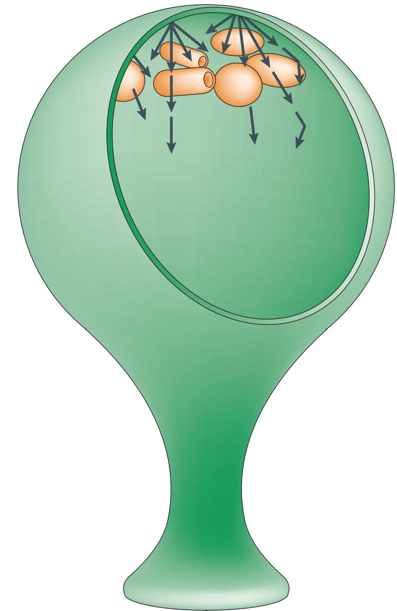
The purpose of 'rigorous quantitative models' of signal transduction pathways is simply to act as an aid for understanding the consequences of our assumptions about the kinetics of the reactions in the pathway and their organization with respect to each other. Most regulatory pathways are too complex to be understood intuitively in all their ramifications. A good model can be used to make quantitative predictions about measurable outputs of the pathway in response to defined inputs. If experiments then show that the predictions are not accurate, we know that the model is missing critical elements or is built with faulty parameters. Models are often most useful to help us to isolate which assumptions are likely to be inaccurate and to indicate additional measurements or experiments that might be useful. A quantitative model can also help to identify 'critical parameters' — those for which a small change in value produces a large change in the output under study.

a Reactions modelled with ordinary differential equations



Reacting molecules well-mixed; reaction rates diffusion-limited

b Calcium signalling in a spine



Spatially-inhomogeneous environment; many reactants immobilized

Compartmental and reaction-diffusion models (for a review, see REF. 9) are based on ordinary differential equations (ODEs) and partial differential equations (PDEs), respectively, and assume that reactions are diffusion-limited and that the reacting elements are well-mixed (panel **a**). In compartmental models, the spatial localization of reactions in a cell is represented by independent 'compartments' that interact only through diffusion of a small subset of reactants across compartmental boundaries. In reaction-diffusion models, localization is represented by spatial variables in the PDEs. Key assumptions of such models are violated in subcellular domains in which the number of molecules is statistically small or in which many of the interacting molecules are immobilized. For example, in spines (panel **b**), Ca^{2+} signalling is initiated by NMDA (*N*-methyl-D-aspartate) receptors when Ca^{2+} flows through the channel and encounters a meshwork of Ca^{2+}-binding effector molecules, most of which are immobilized or diffuse only very slowly. This situation can be simulated by a stochastic model in which binding and reaction rates are represented as probabilities that can be calculated from kinetic rates measured in solution (see BOX 2).

interacting signalling events that can lead to changes in spine functions, and then apply quantitative experimental tests to refine or alter these models. This will be a new endeavor for molecular neurobiologists and will require new tools and new ways of thinking. In this review, we lay out an initial schematic on which such models could be based.

We make the assumption that the biochemical pathways that control changes in the strength of synapses in the brain were built and linked together by the logic of evolution. Synapses must respond to environmental stimuli and adjust their strength appropriately so that the information that is stored will be useful for the survival and reproductive fitness of the organism. The signalling pathways must control these responses and, at the same time, institute homeostatic changes that preserve the health and stability of individual neurons and of the brain as a whole[7]. Because we do not have access to the range of influences that shaped the evolution of these pathways, we are probably in for many surprises as we study their organization.

A revolution has been brewing in our view of cellular signalling cascades over the past decade. Two growing realizations have transformed our thinking about how biochemical control pathways work. First, we now know that signalling cascades, once believed to be linear, interact extensively with each other, often starting to interact just a few biochemical steps away from the initiation of a signal by transmembrane receptors[8,9]. Second, the remarkable specificity of regulation that is achieved by relatively few classes of pathway arises from carefully controlled intracellular localization[10–12]. Arrays of scaffold molecules hold receptors near to their appropriate effectors and protein kinases near to their appropriate targets, and might even determine the precise kinetics of individual cascades. Our understanding of the synapse is also being transformed by these revelations[13,14]. Indeed, it is not surprising that interactions among signalling pathways are particularly complex in brain synapses. The subtlety of information encoding in the mammalian brain dictates that synaptic plasticity must be sensitive to many influences.

LONG-TERM DEPRESSION (LTD). An enduring weakening of synaptic strength that is thought to interact with long-term potentiation (LTP) in the cellular mechanisms of learning and memory in structures such as the hippocampus and cerebellum. Unlike LTP, which is produced by brief high-frequency stimulation, LTD can be produced by long-term, low-frequency stimulation.

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Box 2 | **Stochastic models and parameters in MCell**

MCell[117,118] is a publicly-available program designed for building spatially-accurate stochastic models of synaptic signalling. DReAMM (Design, Render and Animate MCell Models) is a visualization tool that can be used to create images that represent MCell output. The program was originally created by Miriam and Edward Salpeter and their students to study the neuromuscular junction[119] and has since been extensively updated. A new version (MCell 3), which is soon to be released, will allow the simulation of pairwise interactions among diffusing molecules in a spatially organized cytosol (T. Bartol, personal communication).

Parameters that are required for an MCell model include: the shape of the compartment to be modelled; the upper and lower boundaries of the number of each molecular species; the diffusion rates of moving molecules; the spatial positions of immobilized proteins to be modelled in the compartment; the relative probabilities of pairs of molecules binding on collision; and the probabilities of state changes of molecules that could occur after a binding event, such as channel opening or activation of enzyme activity.

Because the numbers of signalling proteins in different synapses are likely to vary, it is necessary to know only the range of possible numbers. Different models can then be used to explore the effects of variable numbers on the desired output. Estimates of the numbers of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) and NMDA (*N*-methyl-D-aspartate) receptors in synapses range from tens to a few hundred[120]. The average numbers of many other signalling molecules that have been shown to be present in the majority of spines throughout the forebrain can be estimated by determining their stoichiometries relative to glutamate receptor subunits in synaptosomes or in the postsynaptic density fraction. Such measurements can be made using semi-quantitative mass spectrometry[121] or immunoblotting (A. R. Rosenstein, V. Lucic and M.B.K., unpublished observations). The spatial positioning of proteins can be determined using electron microscopy, as in the elegant studies of Valtschanoff and Weinberg[110]. Finally, the relative probabilities of binding and molecular state changes can be calculated from kinetic rate constants that are measured by electrophysiological or biochemical methods[117].

Cellular processes regulated by spine signalling

The Poster accompanying this review depicts pathways that regulate five postsynaptic physiological processes that have been studied intensively in recent years. Three of these — regulation of AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid)-type glutamate receptors (AMPARs)[15], regulation of the actin cytoskeleton[16–18] and regulation of local protein synthesis[19,20] — are crucial for synaptic plasticity. A fourth process (not shown explicitly in the Poster) that is important for long-term synaptic plasticity and homeostasis is gene expression in the nucleus, which is regulated by signals that arise from several pathways[21] (potential influences on gene expression[22] are indicated by arrows and bars pointing out of the diagram). Finally, neuronal apoptosis[23] has an important role in brain development, and might underlie the pathology of some neurodegenerative diseases[24,25].

The strength of glutamatergic synapses can be regulated postsynaptically by changing the number of AMPARs and/or the current through each AMPAR channel. Phosphorylation of specific serine residues on the glutamate receptor subunit 1 (GluR1) of the AMPAR by Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII), protein kinase A (PKA) or protein kinase C (PKC) increases the current through the activated receptor channel[26,27]. Mutation of these sites leads to defects in both LTP and LTD in mice older than ~4 weeks of age, and interferes with the retention of spatial memory[28]. In mice younger than ~4 weeks of age, regulation of the number of AMPARs is an important mechanism that underlies synaptic plasticity[15]. Insertion of AMPARs into the postsynaptic membrane during LTP is driven by activation of the small GTPase Ras and CaMKII by the NMDAR[29]. Interestingly, in addition to activation of CaMKII and Ras, insertion of AMPARs into the spine membrane also requires the association of GluR1 with a PDZ domain-containing protein[30]. By contrast, activation of Rap drives the endocytic removal of AMPARs that occurs in conjunction with LTD[29]. At present, one challenge is to discern how the rates of insertion and removal of receptors are balanced at each synapse to produce appropriate synaptic strengths.

Together with the regulation of AMPARs, changes in the spine cytoskeleton have a crucial role in synaptic plasticity[16,31,32]. Several mutations in human proteins that regulate the actin cytoskeleton cause mental retardation[18]. The strength of a synapse is correlated with the size of the spine head, which is, in turn, correlated with the size of the postsynaptic density[33,34]. Therefore, it seems that changes in the number of receptors in the postsynaptic membrane, the size of the postsynaptic density and the size of the spine head are coordinated. Several studies have shown that synaptic stimulation alters the spine cytoskeleton[35], and that altering the actin dynamics interferes with synaptic plasticity[36,37]. Actin dynamics in the spine are influenced by Ca^{2+} flux through activated NMDARs[36,38] and by signalling through ephrin receptors, which activate the small GTPase Rac[39,40]. Activation of Rac and other small GTPases of the Rho family initiates the enzymatic reactions that control actin polymerization[18,41]. It will be important for us to understand how regulation of the spine cytoskeleton is coordinated with the other processes that modify synaptic strength.

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LTP that lasts longer than 1–2 h (late-phase LTP or L-LTP) requires protein synthesis, some of which occurs locally in the dendrite near the activated synapses[19,42]. Although L-LTP also seems to require increased gene expression in the nucleus, current models suggest that the input specificity that is characteristic of LTP depends on local protein synthesis that drives the remodelling of active spines and, perhaps, a small number of neighbouring spines, along a dendritic segment[43,44]. Local protein synthesis can be stimulated by the activation of NMDARs, metabotropic glutamate receptors (mGluRs) or tyrosine receptor kinase B (TrkB)[20]. Therefore, several regulatory pathways in the spine converge on the dendritic translational machinery. The protein kinases CaMKII and aurora activate translation that is regulated by the cytoplasmic polyadenylation element (CPE)[45], whereas a protein kinase cascade that involves the protein kinases mammalian target of rapamycin (mTOR) and S6 kinase (S6K) stimulate the rate of translation initiation for all mRNAs[20]. The number of local synapses that are modified, and the extent and duration of their modification, might all be affected by the combination of different signals that impinge on the protein-synthetic machinery of dendrites.

An important aspect of homeostatic regulation of a neuron is the control of gene expression in the nucleus, such that appropriate RNA messages are sent into the cytosol in the correct amounts to support the level of activity and the specialized functions of the neuron[21,46]. This interesting and complex topic is beyond the scope of this review. However, it has become clear that synaptic activity and the activation or inhibition of signalling pathways in spines contributes to the ongoing regulation of transcription in each neuron. As with the other processes discussed above, the collective effects of individual synapses and groups of synapses on gene expression depend on the integration of signalling across several pathways over time.

Finally, the accompanying Poster indicates pathways that contribute to the regulation of apoptosis in neurons. Although neuronal cell death is unlikely to be important for normal synaptic plasticity, the apoptotic pathways do have a role in maintaining the homeostasis of brain tissue as a whole, and the signalling pathways that regulate apoptosis also influence a host of other synaptic and neuronal functions[23]. One leading hypothesis about the pathology of Alzheimer's disease is that it begins as a synaptic failure[47,48]. Therefore, it is important to understand how even subtle dysregulation of glutamatergic spines might result in the triggering of neuronal apoptosis. This information might lead to the development of therapies for Alzheimer's disease and other neurodegenerative disorders.

In this review, we concentrate on how the spine signalling pathways that control these functions interact with each other to coordinate regulation, paying particular attention to new studies on the small GTPases Ras, Rap and Rac. The pathways depicted in the Poster are loosely grouped into five categories: those controlled by the second messenger Ca^{2+}; by the second messenger cyclic AMP (cAMP); by growth factors and Ras and Rap; by ephrin receptors and Rac; and by metabotropic receptors and G proteins. Except where indicated, each pathway depicted has been implicated in spine function by pharmacological and/or genetic studies. In many cases, the individual proteins have also been localized to spines by structural studies. Excellent reviews cited throughout our discussion summarize recent studies on the individual signalling pathways. We focus on an integrated view of the five categories, and on what we believe might be crucial coordinating interactions among Ras, Rap and Rac.

Ca^{2+} influx through the NMDAR

The principal receptor that controls activity-dependent plasticity in the spine is the NMDAR. Ca^{2+} flowing through the NMDAR channel can bind to several effectors inside the spine, prominent among which is calmodulin (CaM), a ubiquitous Ca^{2+}-binding regulatory protein. As the effective concentration of Ca^{2+} in the spine increases from a baseline of ~70 nM to a maximum of ~10–30 μM, CaM binds four calcium ions in sequence[49–51]. The Ca^{2+} buffering capacity of spines mostly results from proteins with very low mobility, and these buffers, many of which are also effectors, are not saturated even during the large and relatively prolonged Ca^{2+} influx that is produced by the activation of NMDARs[52]. About 95% of incoming Ca^{2+} is bound to buffers at any given time and 5% is free in the cytosol. Ca^{2+} effectors in the spine, including CaM, compete to bind this available Ca^{2+}. Therefore, only a fraction of the CaM molecules in the spine become saturated with Ca^{2+} each time an NMDAR channel opens[53,54].

There is also competition for the binding of Ca^{2+}/CaM to its targets. At least six CaM-dependent enzymes can be activated by Ca^{2+} flowing through the NMDAR: adenylate cyclase[55], CaMKII (REF. 56), Ras-guanine nucleotide-releasing factor 1 (RasGRF1)[57,58], neuronal nitric oxide synthase (nNOS)[59], phosphodiesterase 1 (PDE1) and calcineurin[60]. These targets compete for limited amounts of Ca^{2+}/CaM[61]. Of the six direct Ca^{2+} targets and the six Ca^{2+}/CaM targets depicted in the Poster, eight are enzymes that mediate 'cross-talk' with other signalling pathways. So, extensive branching of spine regulatory pathways begins immediately after the opening of an NMDAR channel. In fact, it can be argued that competition for Ca^{2+} and Ca^{2+}/CaM near the mouth of the NMDAR channel, and in the more distal spine cytosol, orchestrates the divergent biochemical responses to NMDAR activation. Many spines also contain a small number of voltage-dependent Ca^{2+} channels[62]. The timing and location of Ca^{2+} entry through these channels is different from that of Ca^{2+} entering through NMDARs[52]. It will be interesting to determine the influence of such channels on biochemical signalling in spines; however, here we focus on the NMDAR, which seems to be the primary regulator of Ca^{2+}-dependent biochemical signalling in spines.

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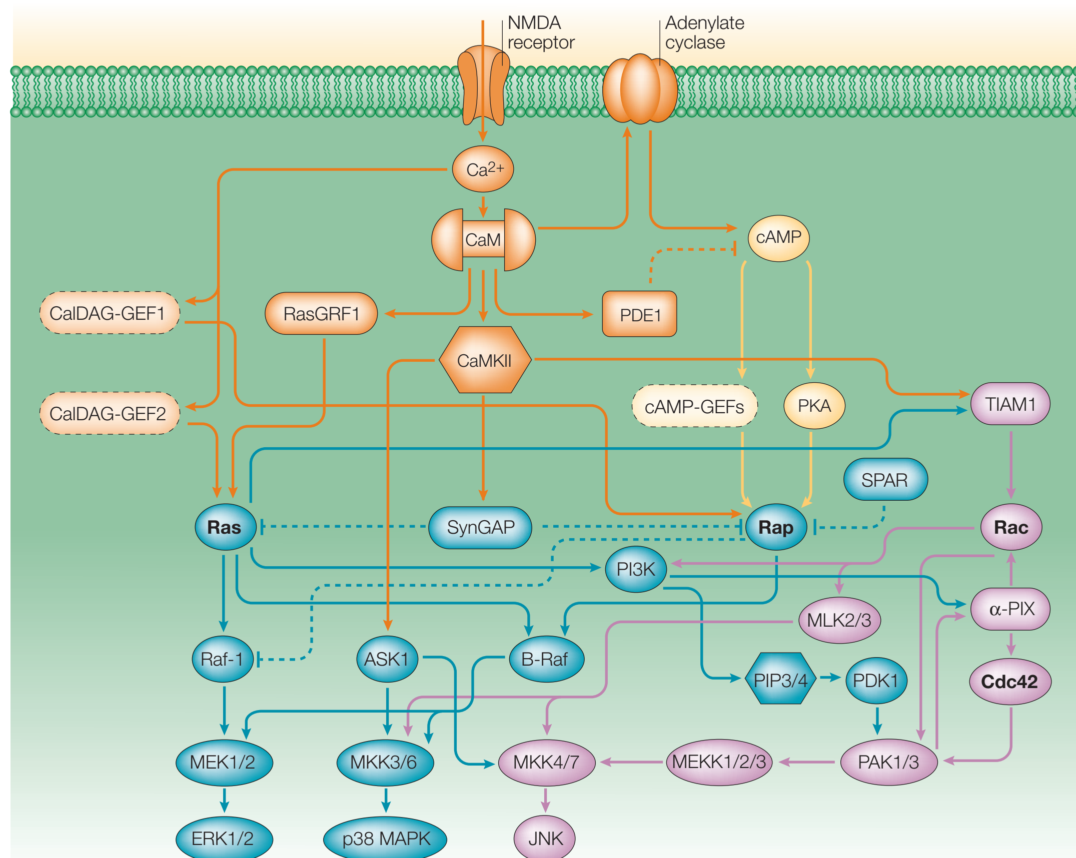

Figure 1 | **Signalling from the NMDA receptor to Ras, Rap, Rac and Cdc42.** As discussed in the text, Ca^{2+} flowing through the NMDA (*N*-methyl-D-aspartate) receptor regulates pathways that influence the ratio of the activated small GTPases Ras, Rap, Rac and Cdc42 (derived from cell division cycle 42) in the spine. Small GTPases are highlighted in bold letters. The activity of the guanine-nucleotide exchange factors (GEFs) RasGRF1 and TIAM1 (from T-cell lymphoma invasion and metastasis 1) and the synaptic GTPase-activating protein SynGAP are influenced by NMDA receptor activity. Cdc42 can be activated by the p21-activated kinases 1 and 3 (PAK1/3) through the GEF α-p21-activated kinase-interacting exchange factor (α-PIX). Four interlocking signalling pathways are depicted: those initiated by the second messengers Ca^{2+} (orange) and cyclic AMP (cAMP; yellow); by growth factors and the small GTPases Ras and Rap (green); and by ephrin receptors and Rac (pink). Proteins depicted by symbols with lighter shading and a dashed outline are highly expressed in specific brain areas, but have not yet been explicitly implicated in postsynaptic regulation or directly localized to spines. Solid lines with arrows indicate activation or stimulation. Dashed lines with a bar indicate inhibition. We have not tried here to distinguish between small and large influences between proteins because their magnitudes will vary under different circumstances. ASK1 (MAP3K5), mitogen-activated protein kinase kinase kinase 5; B-Raf, v-raf murine sarcoma viral oncogene homologue B1; CalDAG-GEF1/2, calcium and DAG-regulated guanine nucleotide exchange factor 1/2; CaM, calmodulin; CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; DAG, diacylglycerol; ERK1/2, extracellular signal-regulated kinase 1/2; JNK, c-Jun amino (N)-terminal kinase; MEK1/2, MAPK/ERK kinase1/2; MEKK1/2/3, mitogen-activated protein kinase kinase kinase 1/2/3; MKK3/4/6/7, mitogen-activated protein kinase kinase 3/4/6/7; MLK2/3, mitogen-activated protein kinase kinase kinase 10/11; p38 MAPK, p38 mitogen-activated protein kinase; PDE1, phosphodiesterase 1; PDK1, phosphoinositide-dependent kinase 1; PI3K, phosphatidylinositol 3-kinase; PIP3/4, phosphatidylinositol-bisphosphate 3/4; PKA, protein kinase A; Raf-1, v-raf-1 leukaemia viral oncogene 1; RasGRF1, Ras-guanine nucleotide-releasing factor 1; SPAR, spine-associated Rap GTPase-activating protein.

Ras and Rap

An influx of Ca^{2+} through NMDARs leads directly or indirectly to the activation of the small GTPases Ras, Rap, Rac and Cdc42 (derived from cell division cycle 42; FIG. 1). Ras and Rap are closely related members of the Ras subfamily[63,64] that have both distinct and shared regulators and effectors (BOX 3). Despite their close relationship, the activation of these two GTPases produces opposite effects on the number of AMPARs in the postsynaptic membrane. As mentioned earlier, Ras activation leads to the insertion of AMPAR subunits, whereas Rap activation leads to their removal[29]. For this reason, there is great interest in understanding the processes that control the differential activation of Ras and Rap. RasGRF1, a Ca^{2+}/CaM-dependent Ras-guanine nucleotide-exchange factor[58] (GEF; see TABLE 1), can be activated directly by Ca^{2+} flowing through NMDARs. RasGRF1 specifically activates several Ras isoforms, but does not activate Rap. It binds to the NR2B subunit of NMDARs and has been shown to mediate 60–70% of the NMDAR-mediated activation of the Ras/Raf/extracellular signal-regulated kinase (ERK) cascade in cultured hippocampal neurons and a significant fraction in the intact brain[57,65].

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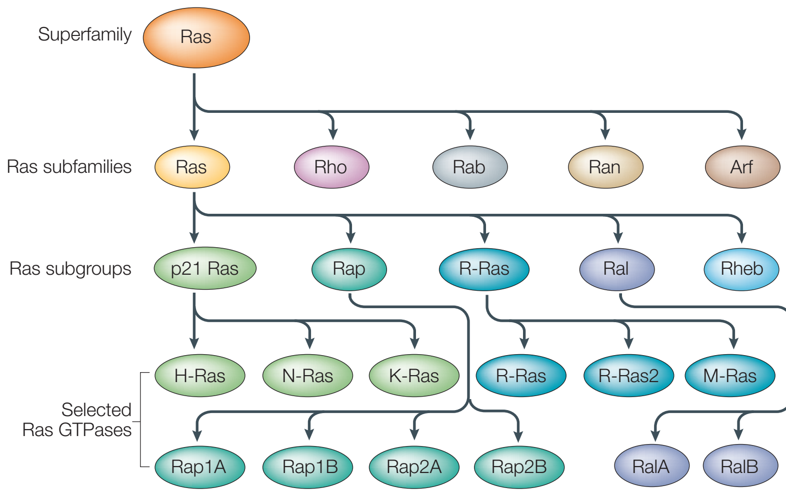
Box 3 | **Achieving signalling specificity: the Ras superfamily**



The Ras-related GTPases that make up the Ras superfamily (figure) have high sequence identity (40–85%)[63,64,122], although the individual proteins have unique functions and preferred targets. For example, Rap1 and p21 Ras seem to have opposing actions on several cellular functions, including AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid) receptor trafficking in the spine[29]. Recent studies have begun to clarify several mechanisms that allow the highly similar and ubiquitous Ras proteins to mediate distinct biological functions.

Ras GTPases act as molecular switches by cycling between an inactive GDP-bound state and an active GTP-bound state. This cycling is facilitated by guanine nucleotide exchange factors (GEFs) and GTPase-activating proteins (GAPs), which can be highly specific for individual Ras family members (see TABLE 1). Therefore, the timing and location of the activation of Ras proteins is controlled by tissue-specific expression, subcellular localization and temporal regulation of specific GEFs and GAPs. For example, Ras-guanine nucleotide-realeasing factor 1 (RasGRF1), which is activated by the binding of Ca^{2+}/calmodulin, binds directly to the NR2B subunit of the NMDA (*N*-methyl-D-aspartate) receptor and is, therefore, responsible for a large fraction of Ras activation by Ca^{2+} influx[57].

Another mechanism through which signalling specificity can be achieved is the targeting of GTPases to distinct membrane subdomains by post-translational modifications. The addition of prenyl groups to a CAAX (cysteine-alanine-alanine-any amino acid) motif in the carboxy (C)-terminal domain anchors Ras proteins in the membrane. Furthermore, individual Ras proteins contain either a polybasic sequence that targets them to relatively disordered domains of the plasma membrane, or a palmitoylation site that targets them to lipid rafts. In this way, for example, H-Ras is sequestered in lipid rafts, whereas K-Ras 4B is targeted to the more disordered plasma membrane[123,124].

Ras family members have significant differences in their affinities for certain effectors. For example, Raf-1 (v-raf-1 leukaemia viral oncogene 1) is preferentially activated by Ras proteins in the following order: K-Ras>N-Ras>H-Ras[125], whereas R-Ras and M-Ras only weakly activate Raf-1 (REF. 126). Therefore, the regulation of GEFs and GAPs, post-translational modification and effector affinity are all mechanisms by which signalling specificity is achieved in the Ras superfamily.

Interestingly, tyrosine phosphorylation by Src (v-src sarcoma viral oncogene homologue) seems to activate latent RacGEF activity in RasGRF1[66].

In many neurons, Rap is activated by the cAMP pathway[67]. Ca^{2+} flowing through NMDARs stimulates the synthesis of cAMP by the Ca^{2+}/CaM-sensitive adenylate cyclases AC1 and AC8 (REFS 55,68). Rap and its target B-Raf (v-raf murine sarcoma viral oncogene homologue B1) can then be activated by a mechanism that depends on cAMP-dependent PKA[69]. The synthesis of cAMP can be potentiated by the coincident activation of β-adrenergic receptors that release the G-protein subunit $G_{s\alpha}$, which then activates adenylate cyclase[70]. The removal of cAMP is also regulated by Ca^{2+}/CaM through the activation of the phosphodiesterases 1A, B and C (PDE1A/B/C), which are expressed in most neurons. Although it has not been shown directly, it is generally assumed that these phosphodiesterases are regulated by the influx of Ca^{2+} through NMDARs. Mice with a targeted disruption of PDE1B show impaired spatial learning[71], which suggests that PDE1B has a crucial role in synaptic plasticity.

Additional families of neuronal Ras and Rap GEFs have recently been characterized. Ca^{2+} and diacylglycerol (DAG)-regulated GEFs 1 and 2 (REF. 72) (CalDAG-GEFs 1 and 2, also known as Ras-GRPs 2 and 1, respectively[73]) are activated synergistically by Ca^{2+} and diacylglycerol. CalDAG-GEF1 principally activates Rap GTPases and CalDAG-GEF2 activates Ras GTPases. CalDAG-GEF2 is highly expressed in neurons throughout the forebrain[74], whereas CalDAG-GEF1 is particularly highly expressed in the medium spiny neurons of the striatum[72,74]. Their location in synapses and modes of activation in neurons have not yet been reported; however, they might represent additional direct targets of Ca^{2+} entering through NMDARs in spines. cAMP-GEFs (also known as EPACs; TABLE 1) are activated by cAMP, catalyse the specific activation of Rap and are widely expressed in the nervous system[75]. One study has shown that in hippocampal neurons, the activation of ERK by the 5-hydroxytryptamine (5-HT, serotonin) receptor 7 (HTR7) requires cAMP-GEFs and does not require PKA[76]. The full range of regulatory roles for CalDAG-GEFs and cAMP-GEFs in neurons remains to be investigated.

Ras and Rap GTPases differ in their influence on the downstream effector Raf-1 (v-raf-1 leukaemia viral oncogene 1). Ras activates Raf-1, whereas Rap inhibits it. Rap and Ras both activate a distinct Raf kinase, B-Raf. Both Raf-1 and B-Raf activate the ERK pathway, but B-Raf also leads to the activation of p38 mitogen-activated protein kinase (MAPK)[77]. Therefore, an increase in the proportion of activated Rap over Ras leads to a relative increase in the level of activation of p38 MAPK in hippocampal neurons[29]. Recent evidence indicates that p38 MAPK can, in turn, activate Rab5, a small GTPase that regulates membrane traffic from the plasma membrane to early endosomes and drives endocytosis of AMPARs[78,79].

The ratio of activation of Ras and Rap in synapses can also be controlled by the differential activation of GTPase-activating proteins (GAPs) that bind the activated GTPases and stimulate the hydrolysis of bound GTP to GDP, leading to their inactivation. Two GAPs (TABLE 1) that are both highly enriched in spines contribute to this regulation. The synaptic GTPase-activating protein SynGAP is an abundant component of the NMDAR signalling complex[80], and binds to the NMDAR scaffold protein PSD-95 (from postsynaptic

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Table 1 | **Selected regulators of small GTPases in brain neurons**

	Abbreviation	Alternative name	Specificity	Activators	Expression	References
Guanine nucleotide exchange factors						
Ras-guanine nucleotide-releasing factor 1	RasGRF1		H-Ras, N-Ras, K-Ras, R-Ras (Rac*)	Ca^{2+}/calmodulin (Src*)	Brain neurons; binds NMDA receptors	57,58,65,66
Ras-guanine nucleotide-releasing factor 2	RasGRF2		H-Ras, N-Ras, K-Ras, Rac	Ca^{2+}/ calmodulin	Widely expressed	58,65
Kalirin 7		Duo	Rac	EphB	Brain specific; enriched in spines	39,91
TIAM1	Name derived from 'tumour invasion and metastasis 1'		Rac	H-Ras-GTP, CaMKII	Widespread; highly expressed in brain and synapses	38,92,93
Calcium and diacylglycerol-regulated guanine nucleotide exchange factor 1	CalDAG-GEF1	RasGRP2	Rap1, Rap2	DAG, Ca^{2+}	Highly expressed in medium spiny neurons of the striatum	72–74
Calcium and diacylglycerol-regulated guanine nucleotide exchange factor 2	CalDAG-GEF2	RasGRP1	H-Ras, K-Ras, M-Ras, R-Ras2, R-Ras	DAG, Ca^{2+}	Highly expressed in neurons in the forebrain and cerebellum	73,74
Cyclic AMP-regulated guanine nucleotide exchange factor I	cAMP-GEF I	EPAC 1	Rap1A, Rap2B	Cyclic AMP	Expressed in most tissues including the brain	75,76
Cyclic AMP-regulated guanine nucleotide exchange factor II	cAMP-GEF II	EPAC 2	Rap 1A	Cyclic AMP	Highly expressed in neurons in the forebrain and cerebellum	75,76
GTPase-activating proteins						
SynGAP	Name derived from 'synaptic GTPase activating protein'		Rap1, Rap2, Ras	CaMKII	Brain neurons; highly enriched in spines	80,81
SPAR	Name derived from 'spine-associated Rap GTPase-activating protein'		Rap1A, Rap2A	(Downregulated after expression of serum-induced kinase (SNK))	Enriched in spines	88,89

*Phosphorylation of RasGRF1 by Src family kinases activates its Rac-guanine nucleotide exchange factor (GEF) activity. CaMKII, Ca^{2+}/calmodulin-dependent protein kinase II; DAG, diacylglycerol; EphB, ephrin B receptor; Kalirin 7, RhoGEF kinase; NMDA, *N*-methyl-D-aspartate; Rac, Ras, Rap, small GTPases.

density-95)[80,81]. SynGAP stimulates the GTPase activity of both Ras and Rap, thereby stimulating their rate of inactivation[80–82]. Phosphorylation of SynGAP by CaMKII increases SynGAP's stimulation of the GTPase activity of Ras[83]. This would increase the rate of inactivation of Ras, which, consequently, could reduce the rate of AMPAR insertion into the postsynaptic membrane. It has not yet been determined whether SynGAP phosphorylation by CaMKII similarly alters SynGAP's stimulation of the GTPase activity of Rap. Because NMDAR activation results in the phosphorylation of SynGAP by CaMKII, it is likely that NMDARs modulate the steady-state levels of Ras and, perhaps, Rap, through the activation of both GEFs and GAPs in the spine[83].

The importance of SynGAP in the regulation of several synaptic and neuronal functions is evident from the various phenotypes shown by mice with SynGAP deletions. Mice with a heterozygous deletion are deficient in spatial learning[84]. Hippocampal slices from these mice have reduced LTP[84,85]. Mice with a conditional deletion of SynGAP that causes the level of SynGAP to begin to fall in forebrain pyramidal neurons about one week after birth show enhanced neuronal apoptosis in the hippocampus and cortex[86]. Homozygous mutant mice die a few days after birth, and hippocampal neurons cultured from these mice show precocious spine and synapse formation, and have larger spine heads[87].

In an important recent study, Krapivinsky *et al.*[82] showed that SynGAP and CaMKII also bind to the scaffold protein MUPP1 (from multiple PDZ domain protein 1). They found that activation of synaptic NMDARs for 5 min causes dissociation of SynGAP from MUPP1, which is accompanied by dephosphorylation of SynGAP and decreased activation of p38 MAPK. When they artificially dissociated SynGAP

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from MUPP1 in neurons by introducing inhibiting TAT PEPTIDES, they found an increase in the insertion of AMPARs into the postsynaptic membrane. At present, the biochemical events that underlie these observations are not fully understood. However, it is clear that SynGAP can influence the levels of active Ras and Rap at synapses, and thereby influence the steady-state level of synaptic AMPARs.

Homeostatic regulation of spines over a period of many hours occurs through a completely different mechanism that involves the synaptic RapGAP known as SPAR (from spine-associated RapGAP). Enhancement of excitatory synaptic activity in hippocampal neurons for 18–24 h induces the expression of serum-activated kinase (SNK), which binds to and phosphorylates SPAR in spines, leading to the degradation of SPAR by the proteosome[88,89]. Overexpression of recombinant SNK in neurons has the same effect, and results in the loss of mature spines and enhanced formation of dendritic filopodia. The precise role of the RapGAP activity of SPAR in these events has not yet been determined; however, it is possible that loss of SPAR leads to upregulation of active Rap and p38 MAPK, which would drive endocytosis of AMPARs and contribute to the loss of mature synapses.

The insertion and removal of AMPARs is just one of many neuronal functions that might be influenced by the balance of activated Ras and Rap. FIGURE 1 illustrates how these two GTPases participate in the regulation of the three main MAPK families: ERK, p38 MAPK and c-jun amino (N)-terminal kinase (JNK). Each of these protein kinases regulates a distinct set of transcriptional regulators, and, depending on their location, participates in the regulation of various cellular processes[90]. So, we still have much to learn about the consequences of biochemical events that are initiated just a few steps downstream of Ca^{2+} influx through NMDARs.

Rac

Rac, along with the other Rho family members Cdc42 and RhoA, has an important role in the regulation of the actin cytoskeleton in spines[18]. During the development of synapses, the activation of NMDARs and ephrin B receptors (EphBs) influences spine morphology by regulating actin remodelling[38]. In mature spines, the activation of NMDARs increases actin polymerization[36], and activation of EphBs is necessary to maintain spine morphology[91]. Tolias *et al.*[38] recently reported that regulation of the cytoskeleton by NMDARs is mediated by TIAM1 (from T-cell lymphoma invasion and metastasis 1), a Rac-specific GEF that is expressed at high levels in the developing and adult brain. Knockdown of TIAM1, or expression of a dominant-negative TIAM1, in hippocampal neurons disrupts the effects of NMDAR activation on the spine cytoskeleton[38]. TIAM1 is present in dendrites and spines[38] where NMDAR stimulation could lead to activation of its RacGEF activity, either by direct binding of activated Ras to TIAM1 (REF. 92) or through phosphorylation by CaMKII (REF. 93). This cascade is an important example of cross-talk bridging three signalling streams in the spine: the Ca^{2+}, Ras and Rac pathways (FIG. 1).

An entirely separate mechanism for activating Rac also has a prominent role in controlling the spine cytoskeleton. Activation of postsynaptic EphB by ephrin B1 induces the rapid formation of mature spines along the dendrites of 10-day old hippocampal neurons in culture[39]. Penzes *et al.* showed that activated EphB recruits the RacGEF kalirin 7 into clusters in the dendritic shaft and spines[39]. The recruitment of kalirin 7 causes the activation of Rac and its targets, which leads to actin polymerization and spine formation. A reduction in kalirin 7 expression in mature cultured neurons by antisense RNA leads to loss of spines and, ultimately, to a reduction in dendritic complexity, which suggests that the activation of kalirin 7 by EphB is important for the maintenance, as well as the formation, of spines[91]. Once Rac is activated, it, in turn, activates the effectors cyclin-dependent kinase 5 (CDK5)[94,95] and p21-activated kinase 1 or 3 (PAK1/3)[96]. PAK then activates the cytoskeletal regulator LIM-domain-containing protein kinase 1 (LIMK1), which facilitates actin polymerization[32]. PAK can also orchestrate the activation of Cdc42, another Rho subfamily member, which drives the nucleation of new actin filaments and the branching of existing filaments[18].

Integration and modulation

Our new understanding of the key roles of Ras family proteins in the regulation of postsynaptic plasticity helps to provide a framework in which we can begin to understand how the many pathways that regulate synapses are integrated and coordinated (see accompanying Poster). Here, we discuss a few examples to illustrate how physiological functions in the spine are subject to combinatorial regulation by many signalling pathways, often including one or more small GTPases.

We described how the balance of active Ras and Rap can be controlled in the spine by GEFs and GAPs that are activated by the second messengers Ca^{2+} and cAMP. This balance can also be shifted by signals from the three other classes of pathway that are depicted in the Poster. TrkB is activated in spines and dendrites by brain-derived neurotrophic factor (BDNF) that is released from neighbouring neurons[97,98]. Depending on the number and availability of cytosolic adaptor proteins, TrkB can then activate Ras, Rap or both. Ras can also be activated by direct recruitment of the adaptors Src homology 2 domain-containing transforming protein C (Shc) and son of sevenless (SOS) to the plasma membrane following phosphorylation of Shc by Src[99]. In spines, Src can be activated in various ways. For example, metabotropic receptors, including mGluR1, activate Src through the intermediate kinase protein tyrosine kinase 2 (PYK2)[100,101]. Activation of EphB leads to phosphorylation of the tyrosine residues in its cytosolic tail that bind to and activate Src[102]. Therefore, the ratio of activated Ras and Rap at a given time will be determined by the sum of signals from several pathways. However, we do not yet know what influence the spatial localization of receptors and targets

TAT PEPTIDES
These are protein domains of interest, fused to the carboxyl terminus of the 11-residue protein transduction domain (PTD) of the human immunodeficiency virus 1 (HIV-1) transcriptional activator Tat protein. The Tat PTD allows the TAT peptide to be taken up into cells by macropinocytosis and then to move across the vesicle membrane into the cytosol.

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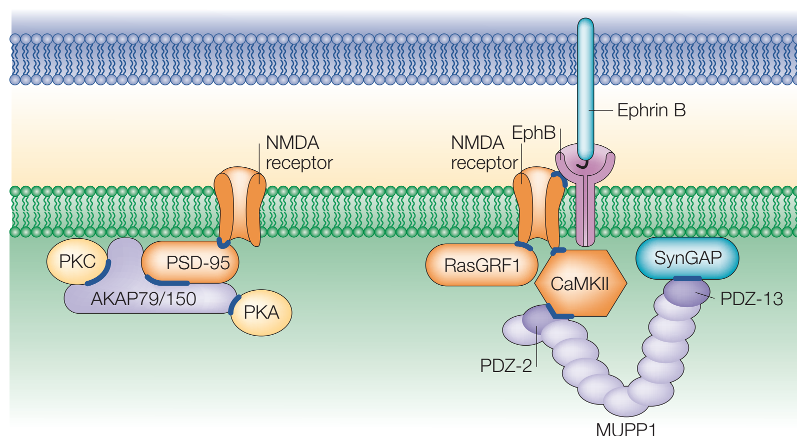

Figure 2 | **New scaffolding interactions in the postsynaptic density.** Three newly described sets of interactions among signalling proteins in the postsynaptic density illustrate how the positioning of signalling enzymes might influence signalling pathways (see text). The scaffold protein AKAP79/150 (from A-kinase-associated protein of molecular weight 79 and 150 kDa) can immobilize protein kinase A (PKA) and protein kinase C (PKC) near the NMDA (*N*-methyl-D-aspartate) receptor complex through its association with PSD-95 (from postsynaptic density-95), a scaffold protein that binds to the NMDA receptor. The interaction of the ephrin B receptor (EphB) with its ligand, ephrin-B, stabilizes an association between the extracellular domains of EphB and the NMDA receptor. Finally, the interaction of MUPP1 (from multiple PDZ domain protein 1) with Ca^{2+}/calmodulin-dependent protein kinase II (CaMKII) at PDZ-2 and with SynGAP (from synaptic GTPase activating protein) at PDZ-13 helps to control the regulation of SynGAP by the NMDA receptor. The interaction might also place SynGAP near Ras proteins that have been activated by Ras-guanine nucleotide-releasing factor 1 (RasGRF1).

has on this summation. Most receptors are positioned in the spine or postsynaptic density by interaction with scaffold proteins[14,103] (see below). So, to fully understand their combinatorial influences, it will be important to directly measure the influence of each receptor on specific targets in different types of synapse.

We have already discussed the different ways that Ras and Rap regulate Raf-1, B-Raf and p38 MAPK. A second crucial difference in their effects is that Ras activates the phosphatidylinositol-3 kinase (PI3K) pathway and Rap does not. PI3K catalyses the addition of phosphate to hydroxyl group 3 on the inositol moiety of phosphatidylinositol-bisphosphate (PIP2)[104]. The resulting phospholipids (PIP3 and PIP4) recruit and activate two potent protein kinases, Akt (v-akt murine thymoma viral oncogene homologue, also known as protein kinase B (PKB)) and phosphoinositide-dependent kinase 1 (PDK1)[105]. Akt is an upstream regulator of mTOR, a protein kinase that activates S6 protein kinase 1 (S6K1). S6K1 phosphorylates ribosomal protein S6 and stimulates the rate of ribosomal translation[20]. Therefore, any pathway that contributes to the activation of Ras in the spine could help to drive local protein synthesis. NMDARs stimulate local protein synthesis through the activation of CaMKII, which phosphorylates cytoplasmic polyadenylation element-binding protein (CPEB), leading to enhanced initiation of translation of a specific class of dendritic mRNA[20,45]. The mTOR–S6K1 and CaMKII–CPEB pathways are the principal mechanisms by which dendritic protein synthesis is activated to stabilize long-term synaptic plasticity[19,20].

A second crucial function of Akt is to phosphorylate and repress proteins that initiate apoptosis[23]. These include the BCL-associated death promoter (BAD), which is a universal inducer of mitochondrial apoptosis; members of the forkhead family of transcription factors, which drive expression of the death ligand FASL; glycogen synthase kinase 3 (GSK3), which can stimulate apoptosis and orchestrate actin depolymerization; and mitogen-activated protein kinase kinase kinase 5 (MAP3K5, also known as ASK1), which stimulates apoptosis through the p38 MAPK and JNK pathways[23]. CaMKII can oppose some of the effects of Akt on apoptosis pathways by phosphorylating and activating ASK1 (REF. 106). So, the same two pathways that are important for regulating AMPARs and dendritic protein synthesis also contribute to the regulation of pathways that can lead to neuronal apoptosis.

In the complex regulatory environment of the spine, the ideas of 'modulation' of a process versus 'mediation' begin to lose their meaning. We suggest that changes in synaptic plasticity, such as LTP and LTD, have, in fact, no single core programme as discussed by Sanes and Lichtman[6], but instead emerge from interactions among many biochemical pathways.

Spatial localization by scaffold proteins

The Poster accompanying this review is based on information about regulatory interactions that influence spine functions. It does not include the wealth of information that has been gathered about the subcellular localization of many of these proteins within spines or the scaffold proteins that are believed to help position them with respect to each other in the postsynaptic density or along the spine cytoskeleton. A complete discussion of this work is outside the scope of this review. However, because scaffold proteins make a crucial contribution to signalling integration, and as information about them will be important for constructing accurate spatial models of signalling pathways in the spine, we discuss a few examples from recent studies.

The core components of the postsynaptic density scaffold have been reviewed many times[14,103,107,108]. Regulatory machinery is anchored to NMDARs and AMPARs at the cytosolic face of the postsynaptic membrane by scaffold proteins of the PSD-95 family. PSD-95 itself is almost exclusively localized in the postsynaptic density and forms a lattice that is located at an average of 12 nm from the inner surface of the membrane[109,110].

In addition to its interactions with specific signalling proteins[14], PSD-95 binds other scaffold proteins. Presumably, these interactions both appropriately position additional signalling proteins and stabilize the entire postsynaptic lattice. One of these scaffold proteins is Shank (from SH3 and multiple ankyrin repeat domains), a sort of 'scaffold of scaffolds', that is centred at an average of 24–26 nm from the cytosolic face of the membrane[110]. Shank links together proteins that interact with NMDARs, AMPARs and mGluRs[111–113].

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Another scaffold protein is AKAP79/150 (A-kinase-associated protein of molecular weight 79 and 150 kDa), which can bind PKCα, PKA and calcineurin[114] (FIG. 2). Interestingly, PKCα and PKA co-precipitate from a postsynaptic density fraction with PSD-95 and AKAP79/150, but calcineurin does not. Instead, PSD-95 competes with calcineurin for binding to AKAP79/150, which indicates that calcineurin is not bound to the portion of AKAP79/150 molecules that bind PSD-95. Specific localization of PKC and PKA near the NMDAR complex will influence the probability with which targets like the AMPAR are phosphorylated when either kinase is activated.

Two newly reported binding interactions (FIG. 2) influence signalling through the Ras and Rac pathways in the NMDAR complex. Krapivinsky *et al.*[82] documented the presence of MUPP1 (REF. 115), a ubiquitous scaffold protein that contains 13 tandem PDZ domains, in the postsynaptic density fraction[82]. MUPP1 associates with SynGAP through PDZ-13 and with CaMKII through PDZ-2. As discussed above, activation of NMDARs dissociates SynGAP from MUPP1 and causes it to become dephosphorylated. This interaction helps to regulate the activation of p38 MAPK by the NMDAR. Dalva *et al.* found that binding of an ephrin ligand to EphB promotes its direct association with NMDARs at synapses[116]. The interaction does not require tyrosine kinase activity of EphB and is mediated by the extracellular domains of the two receptors. It is easy to imagine that this interaction might position CaMKII and RasGRF1, both of which bind the NMDAR, near TIAM1 and Rac, and thereby promote their activation by CaMKII and RasGRF1, respectively (see Poster).

Each of these new scaffolding interactions provides useful information for the formulation of spatially accurate models of spine signalling. It will be interesting to see whether models that incorporate such scaffolding interactions can begin to predict the kinetics of the regulation of pathways through PKA, PKC, SynGAP or EphB. It is likely that postsynaptic scaffolding interactions will comprise an important set of parameters that will need to be understood before we form an accurate picture of the integration of signalling pathways in the spine.

Future directions

We have seen remarkable progress in our understanding of mechanisms of synaptic plasticity and are now at a juncture where we need to begin framing questions about mechanisms differently. Rather than attempting to design experiments to distinguish between mediation and modulation, it will be more useful to ask questions about the quantitative contribution of each pathway to a change in synaptic strength. For example, when we learn that a particular pathway regulates a synaptic process, it will be important to ask what proportion of the total influence comes from that pathway, paying careful attention to how that proportion changes under varying conditions and in different classes of synapse. Stochastic biochemical models that incorporate spatial localization of signalling proteins and measured kinetic parameters will be useful for understanding which parameters determine the contribution of each pathway. It seems that the answers to many questions about the regulation of synaptic plasticity are, like the devil, in the details.

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Competing interests statement

The authors declare no competing financial interests.

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