

# Multiprotein complex signaling and the plasticity problem

Seth GN Grant\* and Thomas J O'Dell†

Synaptic transmission of distinct patterns of spikes, or 'neural code', leads to plastic changes in synapses and other parts of the neuron, as well as learning in animals. Recent findings indicate that specialized multiprotein structures associated with neurotransmitter receptors and cell-adhesion proteins function as molecular devices that both read the neural code and initiate long-term changes in synaptic structure and function.

## Addresses

\*Department of Neuroscience, University of Edinburgh, Edinburgh EH8 9JZ, UK; e-mail: seth.grant@ed.ac.uk

†Department of Physiology, UCLA School of Medicine, Los Angeles, CA 90095, USA

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## Abbreviations

<b>AMPA</b>	D-2-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid
<b>AMPA</b>	AMPA receptor
<b>GAP</b>	GTPase-activating protein
<b>LTD</b>	long-term depression
<b>LTP</b>	long-term potentiation
<b>MAPK</b>	mitogen-activated protein kinase
<b>MEK</b>	MAPK kinase
<b>mGluR</b>	metabotropic glutamate receptor
<b>NMDA</b>	<i>N</i> -methyl-D-aspartate
<b>NMDAR</b>	NMDA receptor
<b>PKA</b>	protein kinase A

## Introduction

Activity-dependent changes in synaptic strength such as long-term potentiation (LTP) and long-term depression (LTD) have been extensively studied as potential synaptic mechanisms involved in information storage during learning. It is now clear that both LTP and LTD have many forms, which can be distinguished on the basis of their underlying signaling mechanisms, but the best-studied forms of both types of synaptic plasticity remain those induced after patterns of synaptic activity that activate *N*-methyl-D-aspartate (NMDA)-type glutamate receptors (NMDARs) [1]. Not surprisingly, our understanding of the mechanisms responsible for NMDAR-dependent forms of synaptic plasticity initially grew alongside advances in our understanding of NMDAR signaling.

For example, once the central role of NMDAR activation in the induction of LTP was discovered [2], the requirement for coincident presynaptic and postsynaptic activity for LTP induction could be readily explained by the requirement for both ligand binding and membrane depolarization for NMDAR activation. The realization that the NMDAR ion channel has a high permeability to Ca<sup>2+</sup> motivated experiments on Ca<sup>2+</sup>-dependent signaling molecules, and led to the discovery that protein kinases such as protein kinase C and the Ca<sup>2+</sup>/calmodulin-dependent kinase II

need to be activated for LTP induction [3,4]. Although these data led initially to relatively simple signaling models [5], the findings that tyrosine kinase activity is also required for LTP induction [6,7] and that mitogen-activated protein kinase (MAPK) is activated by NMDAR [8] raised new questions over the complexity of kinase pathways.

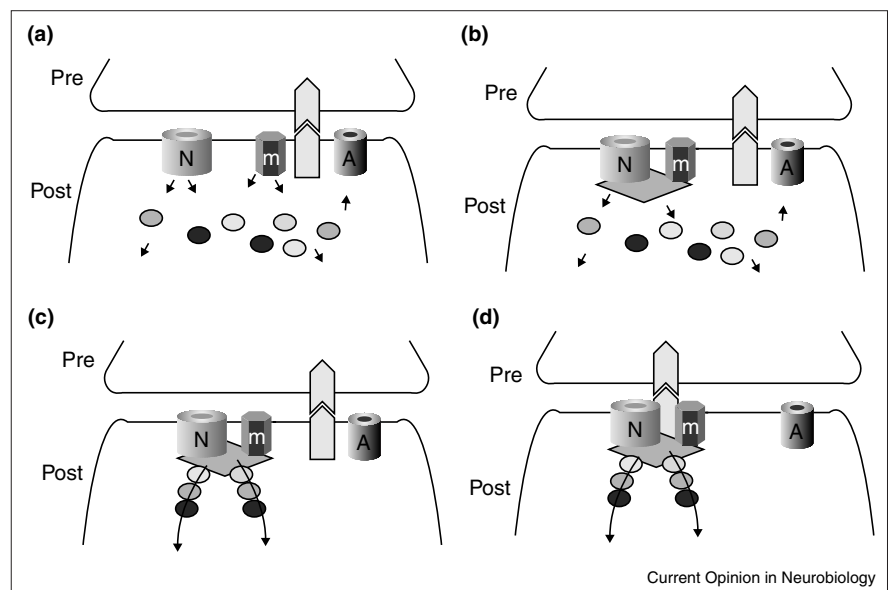
As investigators began to probe the signaling mechanisms responsible for LTP more deeply, the molecular complexity of LTP induction mechanisms rapidly expanded beyond the point where the host of signaling molecules implicated in LTP induction could be incorporated into a simple scheme of NMDAR signaling [9••]. Classifying these molecules reveals roles for numerous neurotransmitter receptors, cell-adhesion proteins, adaptor molecules, signaling proteins, proteases, translation and transcription factors and cytoskeletal proteins. Although some have suggested that the lengthy list of molecules involved in LTP is unnecessarily complicated, and thus most probably wrong [9••], it seems equally likely that this complexity arises from the fact that the induction of LTP results in a diverse set of cellular and synaptic changes. For example, inducing LTP not only leads to changes in the activity and/or number of postsynaptic D-2-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors (AMPA) [10], but also seems to involve formation of new synaptic contacts [11] that depend on cytoskeletal changes, cell-adhesion molecules, transcription and translation, and so on. If this is the case, then an immediate challenge is to understand the composition and relationships of these postsynaptic signaling networks and, more importantly, how they are regulated in a concerted way by NMDAR activation.

Just as the initial progress in our understanding of the mechanisms of LTP benefited from the physiology and pharmacology of NMDAR, new proteomic approaches are beginning to shed light on both the complexity of the signaling processes responsible for LTP and how the activation of postsynaptic NMDAR may lead to coordinated changes in a number of different aspects of neuronal function.

As it is beyond the scope of this review to discuss systematically the large list of molecules implicated in LTP or specific protein–protein interactions [12••], we focus on key elements and emerging themes regarding advances in our understanding of how multiprotein complexes assemble the key pathways and mechanisms, including MAPK and cell-adhesion molecules, that underlie induction of plasticity. Figure 1a illustrates a synapse in which the neurotransmitter receptors, signaling proteins and adhesion proteins are arranged as separate components — a picture similar to that in many textbooks and reviews. As we describe below, new results suggest that this view of

Figure 1

The emerging view of postsynaptic architecture. **(a)** The traditional view of the synapse in which NMDA (N), metabotropic (m) and AMPA (A) receptors are at the postsynaptic synaptic membrane, which contacts the presynaptic terminal through cell-adhesion proteins (light gray boxes). The cytoplasmic signal transduction molecules (shaded spheres) that are activated (arrows indicate signaling events) by  $\text{Ca}^{2+}$  influx through NMDAR or mGluR are shown 'floating' in the spine, and on activation promote changes in AMPARs and other features of the synapse and neurone. **(b)** NMDAR and mGluR are linked to one another by adaptor proteins, and AMPARs are in separate complexes. A set of adaptor proteins (PSD-95, GKAP, Shank, Homer) is shown as a gray diamond linking NMDAR and mGluR. The signaling machinery to downstream pathways and AMPARs is shown distributed in the cytoplasm. **(c)** Multiple signaling pathways are recruited to the complex. The cytoplasmic kinases, phosphatases and other enzymes are physically tethered to NMDAR–PSD95 complexes. The signaling proteins (spheres) are shown attached via the adaptors to the receptors. This assembly paradigm allows these pathways to respond to their linked receptors and confer spatial signaling specificity to otherwise ubiquitous enzymes. The NMDAR–PSD95–SynGAP complex can



integrate several distinct pathways. PSD-95 couples the MAPK pathway to NMDAR through SynGAP, and also couples MAPK-independent pathways to NMDAR. Together, these pathways regulate synaptic plasticity and thus represent intracomplex mechanisms for signal processing. These pathways (and others) might send multiple outputs to AMPARs and other cellular mechanisms.

**(d)** Adhesion proteins link the NMDAR–PSD-95 complexes. Adhesion proteins are now shown as part of the larger complex. Note that AMPARs remain as separate entities. The assembly of adhesion proteins may allow trans-synaptic signaling via two mechanisms or may help tether the receptors to the appropriate place in the synapse.

NMDAR signaling at the synapse is a gross oversimplification. Instead of floating in a sea of cytoplasm within the postsynaptic dendritic spine as depicted in Figure 1a, many of the downstream signaling molecules involved in NMDAR signaling are actually physically coupled to NMDAR through a variety of protein–protein interactions and adaptor proteins.

## NMDAR is part of a large multiprotein signaling machine

### Identifying interactions in the complex

Using the yeast two-hybrid approach, which detects protein–protein interactions between pairs of proteins, many laboratories have fished out cDNAs encoding proteins that bind to ion channels and neurotransmitter receptors [13,14]. Taking these proteins as bait for another round of fishing has led to the identification of more binding proteins. In this way it is possible to predict that individual or indeed networks of interactions might exist *in vivo*.

The power of this approach has been demonstrated by its prediction that NMDAR might be in the same physical complex as the metabotropic glutamate receptor (mGluR) by links through several intermediate adaptor proteins [15,16•]. Sequential use of the two-hybrid system proposed the following chain of binary adaptor protein

interactions NMDAR–PSD-95–GKAP–Shank–Homer–mGluR. This and other models require protein biochemistry from the intact brain to confirm their predictions. As unfashionable as biochemistry has become, it remains the only way to address some issues related to the problems of LTP and learning.

The biochemical purification of NMDAR and its associated proteins from brain has met with very little success because of the technical difficulty of isolating the complexes. In the absence of large-scale isolation methods, small-scale immunoprecipitations combined with immunoblotting with specific antibodies have been used to test whether 'known' proteins are in the complex; but this reveals nothing about unknown proteins or the size of the complexes. Recently, Husi *et al.* [12••] have systematically compared techniques for isolating NMDAR in complex with PSD-95 [17•] and other proteins from mouse brain, and have shown the first large-scale isolation and characterization of this neurotransmitter receptor complex.

### The NMDAR–PSD-95 complex

The size of the NMDAR–PSD-95 complex isolated was approximately 2000 kDa (2 MDa) [17•]. The size of (tetrameric) NMDAR channel complexes comprising channel subunits alone is roughly estimated at one-third of

that of NMDAR–PSD-95, consistent with the view that many other proteins reside in the complex. Indeed, the high complexity of the complexes was evident by gel staining and subsequent proteomic analysis of specific proteins. Husi and Grant [17•] combined a mass spectrometry analysis of protein bands and immunoblotting with hundreds of antibodies to document the identity of about 75 proteins. In these 75 proteins were many surprises.

Although it was no surprise to find that many of the proteins previously identified by two-hybrid screening interact with NMDAR, some of the predicted interactions, such as mGluRs being part of a complex with NMDAR, were confirmed [16••]. This particular observation, along with the finding that AMPARs are not part of these complexes, leads to a new model of the relationship between postsynaptic glutamate receptors and their involvement in the induction and expression of LTP and LTD. The AMPARs, which mediate fast synaptic transmission and are thought to comprise the expression of LTP, are distinct to a regulatory complex between NMDAR and mGluRs. We can now redraw and update the traditional postsynaptic diagram to reflect this organization (Figure 1b).

#### Signaling modules with the complex

Perhaps the most surprising aspect of the proteomic analysis was the diversity and number of signal transduction proteins found. Many second-messenger enzymes, including kinases and phosphatases, were organized into the complexes. This plethora of signaling proteins seems to have a simple logic that helps resolve a conundrum in LTP — how are all the signaling proteins implicated in LTP organized? More than a dozen signaling proteins previously reported to be required for the induction of LTP and LTD were found in the complex.

Closer inspection of the specific proteins shows they form known signaling pathways, of which a striking example is the Ras–MAPK pathway. This pathway, well known to be involved with LTP (see below), can now be considered to be a ‘module’ within the complex of proteins attached to the NMDAR and mGluR receptors. These results build on earlier evidence from PSD-95 mutant mice indicating that NMDAR interactions with PSD-95 and associated signaling proteins control the coupling of pathways regulating induction of plasticity [18]. Together, these data lead to the view that several signaling pathways exist within the complex, and that these pathways control the induction of LTP (Figure 1c).

#### Module organization and adaptor proteins

How are these signaling modules organized, and how do signaling proteins link to the receptors? Important answers to these questions have been provided by studies showing that A kinase anchoring proteins (AKAP) can link protein kinase A (PKA) to NMDAR subunits, as well as to PSD-95 [19•,20•]. One of these AKAPs, known as Yotiao, links PKA to the NR1 subunits of NMDARs, whereas another, AKAP-79, links PKA to PSD-95 (which in turn binds the

NR2 subunits). Moreover, using heterologous expression of the NMDAR with the AKAPs, it has been possible to reconstitute a complex that shows PKA modulation of NMDAR currents in an Yotiao-dependent manner [19•]. Why it is necessary to recruit PKA by different adaptors to the NMDAR remains obscure; however, it may be that Yotiao is more important for modulating the channel, whereas the PSD-95–AKAP-79 linkage is more important for the downstream signaling pathways that involve PSD-95. This physical association of enzymes to the receptors provides a resolution to the puzzling issue of how ‘ubiquitous’ enzymes are involved with specific signaling pathways (Figure 1c).

Why are there so many signaling pathways physically linked to the NMDAR and mGluR receptors? Given that the NMDAR can itself distinguish different patterns of neuronal firing by the depolarization-dependent relief of the  $Mg^{2+}$  block [21], and that the NMDAR is required for LTP and LTD, one possibility is that the multiple pathways may underlie the bi-directionality of synaptic plasticity. This possibility was supported by an earlier study of PSD-95 mutant mice; these mice show a much enhanced form of LTP but no change in synaptic NMDAR currents, indicating the specific uncoupling of an ‘LTP restraining’ or negative-effector pathway [18]. A recent study of mice carrying a mutation in SynGAP, a GTPase-activating protein that binds PSD-95, extends this model and provides evidence that distinct pathways linked to PSD-95 control plasticity and learning (NH Komiyama *et al.*, personal communication).

#### SynGAP links the NMDAR to the MAPK pathway through PSD-95

The Ras to MAPK pathway is well known to be involved with signaling from many receptors in many cells. Although activation of NMDAR stimulates MAPK [8] and pharmacological inhibitors (MEK [MAPK kinase] inhibitors) of this pathway inhibit the induction of some forms of LTP [22–25], neither the upstream signaling events linking NMDAR activation to activation of MAPK nor the downstream substrates involved in LTP are known. Much has been made of the hypothesis that MAPK regulates transcription, as it does in many non-neuronal cells, and how this function could underpin its function in neurons.

The discovery that MAPK is physically tethered in NMDAR–PSD-95 complexes [12••] suggests that it must be regulated in the complex and that it may output to some immediate synaptic substrates rather than transcription factors. Indeed, inhibiting MEK strongly suppresses the early phases of LTP seen shortly after induction, suggesting that inhibiting MAPK activation interferes with the LTP induction machinery, rather than the much later, transcription-dependent, phases of LTP.

Komiyama and colleagues created a knockout mouse for SynGAP [26,27] and have found that this protein is necessary for regulation of the MAPK pathway, which

shows enhanced phosphorylation of MEK and MAPK in the mutant mice (NH Komiyama *et al.*, personal communication). Given that PSD-95 mutants show enhanced LTP, and SynGAP binds PSD-95 and itself restrains MAPK activation, the simplest prediction of the LTP phenotype that might have been observed in these mice would have been enhanced LTP. Surprisingly, it was quite the opposite, in that SynGAP mice show reduced LTP.

A solution to this paradox was found when it was discovered that LTP in PSD-95 mutants does not require the MAPK pathway (MEK inhibitors have no effect), and indeed the analysis of SynGAP/PSD-95 double mutants showed that the SynGAP–MAPK pathway was uncoupled by the PSD-95 mutation (NH Komiyama *et al.*, personal communication). In other words, there must be another pathway downstream of PSD-95 other than the SynGAP–MAPK pathway. Moreover, these pathways seem to be key regulators of bi-directional plasticity, and under normal conditions work in combination to control the direction and magnitude of change in synaptic strength after trains of stimuli. This genetic dissection of distinct pathways involving interacting proteins in the complex now allows us to redraw the complex in such a way as to represent a ‘signal integration’ device (Figure 1d).

Although it is generally accepted that the activation of NMDAR is responsible for changes in many aspects of neuronal cell biology including synaptic and non-synaptic effects (such as AMPAR phosphorylation, trafficking, cytoskeletal changes, synaptic and dendritic structural changes, signals to the nucleus for transcription, translational activation and synaptic tagging), there has been no clear model of how all these effects might be coordinated. The characterization of multiple signaling modules in the complex provides a model in which the signaling complex — by channeling signals to distinct output pathways — can orchestrate the ensemble of changes that together reflects neuronal plasticity.

AMPA receptors are the most intensively studied of all the downstream effector or expression mechanisms, yet, despite recent advances in our understanding of AMPAR modulation in LTP, it still remains unclear how the large NMDAR–PSD-95 complex regulates AMPAR complexes. An important clue may be that the very same kinases, phosphatases and other proteins (NSF [28–30], Dynamin [31]) that regulate AMPA turnover are found in NMDAR complexes [12\*\*], and may therefore mediate their function by translocating from an NMDAR complex to an AMPAR complex.

#### **Adhesion proteins are linked to the NMDAR complex and tyrosine kinase receptors to PSD-95**

The proteomic study yielded a final surprise: several well-known cell-adhesion proteins are present in NMDAR complexes, including N-cadherin and L1 [12\*\*]. Each of these proteins (and other cell-adhesion proteins including

N-CAM and integrins) have been previously implicated in the induction of LTP [32], but like most implicated proteins [9\*\*] were considered to be structurally independent of NMDAR.

The immediate physical proximity of adhesion proteins with NMDAR and mGluR may allow signaling cross-talk and, as such, couple two forms of trans-synaptic signaling: chemical neurotransmission and contact-adhesion signaling. Although this could be an attractive way of sending anterograde and retrograde trans-synaptic signals, it may be that the adhesion proteins are there simply as some basic structural scaffold holding the synapse and its components together. Tanaka *et al.* [33\*\*] have shed light on these models by showing that N-cadherin distribution can be dynamically regulated by neuronal activity (and this specifically requires NMDAR); they propose that the function of N-cadherin is to transiently change the shape or apposition of the presynaptic and postsynaptic terminals. Although the involvement of N-cadherin in dynamic synaptic changes will require further study, the recent use of fluorescent proteins for synaptic imaging is leading away from the view that synapses are structurally static towards one of substantial dynamism in shape, turnover and structural integrity [10,11,34].

These structural changes must also involve regulation of the intracellular cytoskeleton by signaling proteins. Using the frog optic tectum model, in which NMDAR signaling is known to influence dendritic branching, Li *et al.* [35\*] have shown that small GTPase proteins, including Rac and Rho, are involved in this regulation. This fits well with the observation that members of this class of protein are found in NMDAR complexes, and supports the view that one of the output pathways from this complex is to drive cytoskeletal changes. Further evidence that PSD-95 is involved in regulating spine structure and number has been provided by El-Husseini *et al.* [36\*], who found that overexpressing PSD-95 in cultured hippocampus neurons leads to increases in the number and size of dendritic spines.

Another way in which PSD-95 might be involved in regulating spine structure is suggested by reports that receptors for the neuregulin growth factors bind to PSD-95 [37,38]. These receptors, ErbB-1 to ErbB-4, are receptor tyrosine kinases and bind to the same PDZ domains of PSD-95 that bind NR2 subunits. Although these studies do not show that NMDAR and ErbB are located in the same complex, Huang *et al.* [38] have shown that activation of ErbB with neuregulins suppresses LTP induction, which suggests that there is some signaling from ErbB to the downstream mechanisms of LTP. The details of this cross-talk are unknown, although it does not seem to be through tyrosine phosphorylation of NMDAR subunits, which would be expected to enhance NMDAR currents and facilitate LTP [38].

The elucidation of these signaling pathways and their precise role in these structural changes will be a fertile area of

research for some time, and it will be particularly important to identify the physiological and behavioral significance of these pathways and events. We can now redraw our synaptic organization to show linkage between adhesion and the receptor signaling complexes (Figure 1d).

### Trafficking, assembly and dynamics of NMDAR complexes

Understanding the rules governing the assembly of large signaling complexes will help in interpreting their function in the developing nervous system, as well as the dynamic changes that must result following their activation. Dynamic protein-protein interactions are the basis for many signaling pathways in non-neuronal cells (e.g. tyrosine phosphorylation and SH2-domain binding). Activation of the NMDAR regulates CaMKII localization [39] and PSD-95 is also modulated by synaptic activity [40]. There has been much less insight into understanding the critical steps in assembly and, to date, no mutant mice have been studied that show altered composition of these complexes, although this is likely to emerge in the future.

By analogy, in *Drosophila* phototransduction in which a calcium channel Trp (transient receptor potential) is assembled with signaling enzymes through the PDZ protein inaD (inactivation no afterpotential), Tsunoda *et al.* [41] have shown that disruptions of the interaction between the channel and inaD indicate that the cytoplasmic components of the complex can pre-assemble. Setou *et al.* [42] have reported the characterization of vesicles carrying NMDA channel subunits being trafficked from the cell body to the synapse through a molecular motor protein (KIF17), which is itself part of a large protein complex. Husi *et al.* [12\*\*] noted that many proteins in the complex are encoded by activity-dependent genes, and Takagi *et al.* [43] have shown that PSD-95 and NMDAR interactions are regulated dynamically by stimulation protocols. It seems likely that the dynamics of the components of the large complexes will result in different patterns of composition, in heterogeneity at synapses and, as suggested by mutations affecting the structure of the complex, in changes in the induction of plasticity.

### Conclusions

Gone are the days of ion-channels as elaborate pores or membrane holes to be studied only by electrophysiological means. The mammalian prototype for a new kind of signaling machine is found in the complex of five classes of proteins: neurotransmitter receptors, cell-adhesion proteins, adaptor molecules, signaling enzymes and cytoskeletal proteins. This complex, which has been referred as the 'hebbosome' because it can provide the molecular functions required of Hebb synapses, can orchestrate the diverse sets of cell biological functions called into play by distinct patterns of synaptic activity. The combination of biochemistry and proteomics with genetics is clearly a powerful approach that can now be used on other receptors and brain complexes. It will be

valuable to place physical measures on these complexes, study their functions as discrete entities and visualize their organization. Perhaps computational models of signaling pathways will now interface with more established models of neuronal plasticity and lead to new models of plasticity and learning.

Maybe the most significant area for the future will be in placing this basic science into the context of human disease. Despite the enormous amount of research on NMDAR basic biology, our understanding of the importance of these receptors in a wide range of neurological and psychiatric disorders is still in its infancy. In this regard, the observations by Husi *et al.* [12\*\*] — that the hebbosome complexes contain several proteins that are known to be defective in human heritable learning impairments — opens a new and exciting approach to the human condition.

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