Dynamic Regulation of N-Methyl-D-aspartate (NMDA) and \(\alpha\)-Amino-3-hydroxy-5-methyl-4-isoxazolepropionic Acid (AMPA) Receptors by Posttranslational Modifications*

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Many molecular mechanisms underlie the changes in synaptic glutamate receptor content that are required by neuronal networks to generate cellular correlates of learning and memory. During the last decade, posttranslational modifications have emerged as critical regulators of synaptic transmission and plasticity. Notably, phosphorylation, ubiquitination, and palmitoylation control the stability, trafficking, and synaptic expression of glutamate receptors in the central nervous system. In the current review, we will summarize some of the progress made by the neuroscience community regarding our understanding of phosphorylation, ubiquitination, and palmitoylation of the NMDA and AMPA subtypes of glutamate receptors.

The brain functions efficiently due to accurate communication between neurons. At excitatory synapses, the amino acid glutamate is released from synaptic vesicles present in presynaptic terminals; glutamate diffuses into the synaptic cleft and binds to the extracellular region of glutamate receptor subunits (GluRs).

Glutamate binding to receptors induces structural modification resulting in ion channels opening in the case of ionotropic glutamate receptors (iGluRs) or activation of intracellular signaling cascades upon activation of metabotropic glutamate receptors (mGluRs). Changes in synaptic strength include both potentiation and depression of excitatory neurotransmission, known as long-term potentiation (LTP) and long-term depression (LTD), mechanisms believed to represent cellular correlates of learning and memory (1–3). Over the last three decades, the development of sophisticated biochemical, cellular, and molecular approaches has allowed for in-depth investigation of the detailed mechanisms regulating the content of GluRs at synapses demonstrating that GluRs are dynamic. As shown in Fig. 1, synaptic glutamate receptor localization is regulated by: 1) lateral diffusion to and from synapses; 2) endocytosis and exocytosis at the plasma membrane; and 3) intracellular routing and sorting through endosomal pathways (4–8).

It is clear from a multitude of studies that a variety of posttranslational modifications (PTMs) control GluR trafficking and synaptic expression. For example, these modifications play essential roles in influencing protein activity, signaling cascades, protein turnover, synaptic localization, and interactions with intracellular proteins or lipids. These PTMs include glycosylation, phosphorylation, and palmitoylation, which constitute the addition of a functional group to a substrate, and ubiquitination and sumoylation, which involve the covalent conjugation of the protein ubiquitin or the small ubiquitin-like modifier (SUMO) protein to a substrate. Although each of these PTMs can modify GluRs, the current review is specifically focused on the phosphorylation, palmitoylation, and ubiquitination of two subtypes of iGluRs: \(\alpha\)-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors (AMPARs) and N-methyl-D-aspartate receptors (NMDARs). AMPARs and NMDARs are tetrameric ligand-gated ion channels composed of homologous subunits: AMPARs (GluA1–4) and NMDARs (GluN1; GluN2A-D; GluN3A-B). Each iGluR subunit shares a similar overall topology (Figs. 2 and 3): a long extracellular N-terminal domain (9), a hydrophobic hairpin region forming the pore region that is located between two short intracellular loops (loop1 and loop2), and the first and the second of three membrane-spanning regions. Finally, each subunit has an intracellular C-terminal tail of variable length depending on subtype. The intracellular loops and C-terminal tails have many sites for modifications including palmitoylation, ubiquitination, sumoylation, and phosphorylation, which play critical roles in regulating synaptic expression and intracellular trafficking.

Phosphorylation

Phosphorylation is defined as the reversible addition of a phosphate group (PO_4^{2−}) to a protein, typically to a Ser, Thr, or Tyr residue, although phosphorylation on His, Arg, or Lys has also been reported (10). The presence of this heavily charged group is important for changing the hydrophobicity and electric charge of a protein region and, therefore, it can result in a change in the protein conformation or interactions with other proteins or cell structures. In the particular case of the GluRs, phosphorylation regulates intracellular trafficking and channel...
properties. The best-studied example of the latter is for the AMPAR subunit GluA1 in which the conductance and opening probability are modulated by phosphorylation of Ser-831 (PKC/CaMKII) or Ser-845 (PKA), respectively (11, 12). More recently, the phosphorylation on the NMDAR subunit GluN2B by PKA (Ser-1166) was identified as an important factor affecting Ca²⁺ permeation (13).

One of the most common strategies for studying the effects of phosphorylation is the use of mutants in which the phosphorylation is either blocked or mimicked by replacing the phosphorylated residue with a non-polar amino acid (usually alanine) or a negatively charged one (usually aspartate or glutamate), respectively. This approach has proved powerful and provided valuable information, but it is important to recognize the caveats; it may alter some properties of the protein, masking the effect of the phosphorylation. Therefore, it is not uncommon that both phospho-deficient and phospho-mimetic mutations result in a similar phenotype. For this reason, it is preferable to combine a variety of approaches including biochemical characterization of the mutants and the use of complementary techniques (e.g. pharmacological blockade and/or activation of the kinase). Furthermore, a null phenotype with a phospho-mimetic mutation is not uncommon due to a supposed need for the dynamic on and off of true phosphorylation. One could imagine a protein needing phosphorylation for ER egress, but dephosphorylation for stabilization at the synapse, for example, and a surface expression measure could be confounded.

**FIGURE 1.** Cellular mechanisms regulating synaptic expression of GluRs. The synaptic molecular content of iGluRs is controlled by multiple cellular events.

**FIGURE 2.** PTMs decorate GluN2A and GluN2B C-terminal tails. The GluN2A and GluN2B C termini contain several amino acids (aa) modified by phosphorylation (serine (S) or tyrosine (Y)), ubiquitination (lysine (K)), and palmitoylation (cysteine (C)). Kinases targeting a specific residue are illustrated.

**NMDAR Phosphorylation**

Phosphorylation is a key regulatory mechanism controlling the trafficking of NMDARs (see Fig. 2 for a list of phospho-sites in the GluN2A/2B C termini). Strikingly, phosphorylation regulates the surface and synaptic expression of NMDARs in a subunit-specific manner, providing a highly plastic and precise mechanism to accurately control different subunits in response to stimuli. For example, GluN2B is internalized in response to synaptic activity resulting in reduced surface expression (Fig. 4). Internalization from the plasma membrane is mediated by clathrin and tightly controlled by the phosphorylation of GluN2B on Tyr-1472 by Fyn/Src kinases. This residue is part of the YEKL endocytic motif that is recognized by the clathrin adaptor AP-2 as a required step to induce GluN2B internalization. GluN2B Tyr-1472 phosphorylation blocks AP-2 binding, thus preventing the endocytosis of the receptor and, therefore, increasing its surface expression (14–16). Fyn/Src can directly bind to the family of membrane-associated guanylate kinase (MAGUK) proteins, including PSD-95 and SAP102. Therefore, GluN2B phosphorylation on Tyr-1472 is promoted by the interaction of the receptor with these scaffolding proteins and, consistently, there is elevated phosphorylation of GluN2B on Tyr-1472 associated with synaptic GluN2B. The phosphorylation of GluN2B Ser-1480 by casein kinase 2 (CK2) inversely controls the phosphorylation of GluN2B Tyr-1472. GluN2B Ser-1480 phosphorylation occurs within the PDZ ligand and
disrupts binding of the receptor with MAGUK proteins (17). Therefore, phosphorylation of GluN2B on Ser-1480 disrupts anchoring with the postsynaptic density and allows NMDARs to diffuse laterally to extrasynaptic sites corresponding to dephosphorylation of Tyr-1472 by the action of the phosphatase STEP (18). In addition, the disruption of the PDZ ligand “uncouples” the receptor and Fyn/Src kinases, decreasing phosphorylation of Tyr-1472. Therefore, phosphorylation of GluN2B on Ser-1480 results in a decrease in Tyr-1472 phosphorylation, thus promoting internalization (16). A third phosphorylation site on GluN2B is involved in regulating synaptic expression in an activity-dependent manner (22). Unfortu-
GluN2C is analogous to the CaMKII site, Ser-1303, on GluN2B. Although these two analogous residues on different GluN2 subunits are phosphorylated and functionally important, they have divergent consensus sequences resulting in differing kinase specificities.

**AMPAR Phosphorylation**

Since the mid-1990s, the cytosolic C-tails of AMPAR subunits have been shown to be targets of a variety of kinases (12, 27–31), which regulate AMPARs in many important ways including endocytosis, intracellular trafficking, channel conductance, and synaptic plasticity (11, 12, 31–34). As shown in Fig. 3, all four AMPAR subunits (GluA1–4) are demonstrated substrates of least one of the following kinases: CaMKII, Fyn, JNK, PKA, PKC, and PKG (5, 35). However, most of our knowledge regarding AMPAR phosphorylation is limited to GluA1 and GluA2, which are widely distributed in the brain. Indeed, GluA1/2 heteromers constitute the majority of AMPARs in the hippocampus (36, 37).

GluA1 was the first AMPAR subunit for which the phosphorylation of the C-terminal tail (Fig. 3) was identified at Ser-831 and Ser-845 (12). Subsequent studies showed that CaMKII specifically phosphorylates Ser-831 (31, 32), which leads to an enhanced single channel conductance (11, 32), whereas the phosphorylation of GluA1 Ser-845 by PKA increases the opening probability of homomeric GluA1 (38). Surprisingly, no interacting partners seem to depend on the phosphorylation state of Ser-831, although Ser-831 phosphorylation regulates recycling (39), whereas Ser-845 dephosphorylation correlates with mechanisms associated with LTD (40, 41). Evidence also suggests that the phosphorylation of GluA2 on Tyr-876 and Ser-880 is essential for receptor endocytosis (42–44). Indeed, GRIP1/2 and PICK1 bind to the extreme GluA2 C-terminal region to the PDZ ligand. Phosphorylation provides elegant specificity of binding as phosphorylation of GluA2 on Tyr-876 and Ser-880 disrupts the binding of GRIP1/2, but is still permissive for PICK1 binding to GluA2 to promote internalization and LTD (43, 45).

It is interesting to note that consensus motifs can be misleading. For example, both PKC and CaMKII recognize very similar sequences (46), but the amino acid sequence surrounding GluA1 Ser-831 does not conform well to the prototypic CaMKII/PKC consensus motif as the residue at position –3 is not basic but hydrophobic (a proline). Thus, it is unclear what other molecular determinants dictate the kinase specificity for Ser-831. In addition to sequence specificity, other factors can modulate receptor phosphorylation such as receptor-binding proteins or other PTMs. For example, SAP97, the only PDZ protein known to bind GluA1, could play a role in regulating PTMs (47). Indeed, a model proposes that SAP97 binds to activated α-CaMKII firmly attached to NMDARs, which provide a solid platform for the synaptic anchoring of newly inserted GluA1-containing AMPARs (48). Thus, SAP97 binding to AMPARs and CaMKII could be a critical mechanism underlying LTP and receptor trafficking (4, 49–55).

Recent studies have revealed the important regulation of GluR trafficking dictated by mechanisms targeting the intracellular loops of GluRs, which include ER retention motifs (56) and residues that are targets for a variety of kinases. Indeed, we found that GluA1 is phosphorylated by CaMKII on Ser-567, a residue in the loop1 region of AMPARs. Surprisingly, phosphorylation on this residue inhibits GluA1-containing AMPAR synaptic insertion under basal condition (57). Instead of promoting AMPAR synaptic expression, the phosphorylation of GluA1 on Ser-567 may represent the first example of an LTD-specific CaMKII substrate that is distinctively different from standard CaMKII substrates such as GluA1 Ser-831 and GluN2B Ser-1303 (58).

It is likely that the AMPAR intracellular loop contains even more regulatory sites, and it seemed unlikely that CaMKII was...
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the only kinase targeting this region. Indeed, in subsequent studies, we found that CK2 phosphorylates the loop1 region of both GluA1 and GluA2 (59). This study shows that blocking phosphorylation of the major CK2 phosphorylation site on GluA1, Ser-579, impairs AMPAR surface and synaptic expression. Interestingly, CK2 can also phosphorylate, at least in vitro, the GluA1 Ser-567 residue previously identified as a CaMKII phosphorylation site (57). Furthermore, casein kinase 1 is another potential kinase that could regulate AMPARs by phosphorylating the loop1 of AMPARs (59). Phosphorylation of this region might not only regulate trafficking, but due to the close proximity to the pore region, could potentially have potent effects on channel properties. Thus, the loop1 of AMPARs may represent an overlooked region with great potential for gaining insight into core mechanisms regulating glutamate receptor function.

Through the years, phosphospecific antibodies, phosphopeptide mapping, mass spectrometry analysis, and genetic approaches generated volumes of data substantiating a critical role for AMPAR phosphorylation in regulating synaptic expression and dynamic AMPAR changes during paradigms of synaptic plasticity. However, a recent study by Hosokawa et al. (60) has attempted to tackle the issue of overall stoichiometry of AMPAR phosphorylation using a different biochemical approach, specifically a Phos-tag SDS-PAGE reagent that resolves molecules by molecular weight, as a reflection of their phosphorylated residues. Thus, the distinction between phosphorylated and not phosphorylated species is possible based on their mobility on SDS-PAGE. Using this technique, 4.3% of all GluA1 found in the hippocampus are phosphorylated at Thr-840, whereas phosphorylated GluA1 at Ser-831 and at Ser-845 represent respectively 0.18% and 0.018% of total GluA1 (60). This estimation is in sharp contrast to other studies that have estimated closer to 15% of surface GluA1 is phosphorylated at Ser-845 under steady-state conditions (61). Furthermore, genetic knock-in approaches have found that GluA1-containing mutations at Ser-831 and Ser-845 display impaired synaptic plasticity (62). Therefore, there are conflicting data, but the study by Hosokawa et al. (60) certainly sheds light on the issue of stoichiometry and how it can be more precisely determined. However, detecting low phosphorylation levels at any given time reflects the transient nature of phosphorylation, and thus studying the stoichiometry of PTMs (i.e., phosphorylation or ubiquitination) on substrates is not necessarily a measure of functional relevance because spatial and temporal resolution is missing.

Ubiquitination

In addition to phosphorylation, other PTMs such as palmitoylation and ubiquitination are gaining attention as well. Indeed, the importance of the ubiquitin (UB) system in regulating virtually all aspects of cell function rivals, and may exceed, the role of protein phosphorylation (63). For example, the UB system preserves cell homeostasis by acting as the primary mechanism of protein quality control, membrane protein trafficking, receptor internalization, and degradation (64, 65).

Ubiquitination is a highly regulated ATP-dependent process that requires the coordinated and sequential action of an E1-activating enzyme, an E2-conjugating enzyme and, finally, an E3 UB ligase. Ultimately, the UB molecule is attached to the substrate via the formation of an isopeptide bond between the C-terminal glycine of UB and an internal lysine within the substrate. The human genome encodes several hundred E3s, and only a few of these have been studied thus far. In mammalian cells, many G protein-coupled receptors and ion channels are ubiquitinated in response to ligand binding (66–74). In addition, the UB-proteasome system influences neuronal activity and glutamatergic neurotransmission. For instance, a study by Ehlers (75) shows that bidirectional homeostatic plasticity triggers activity-dependent ubiquitination and profound modifications of a variety of PSD proteins. This pioneering work, along with studies from other groups, suggests a mechanism for regulating dynamic changes in spine content, morphology, and structure, therefore altering synaptic activity and plasticity (76, 77).

Many studies have identified and characterized the ubiquitination of mammalian iGluRs. For instance, the UB E3 ligases Fbx2 (78) and Mind Bomb-2 (79) ubiquitinate the NMDAR subunits GluN1 and GluN2B in an activity-dependent manner. More recently, GluN2D was shown to be ubiquitinated by Nedd4-1 (80). The ubiquitination of AMPARs, on the other hand, was initially demonstrated in Caenorhabditis elegans (81), and it took another decade before studies demonstrated that mammalian AMPAR subunits were actually ubiquitinated by the UB E3 ligases APC<sup>Cdh1</sup>, Nedd4-1, and RNF167 (68, 69, 72, 73, 82–84). Interestingly, modulation of neuronal activity by repetitive stress induces GluA1 and GluN1 ubiquitination (85). Importantly, two recent proteomic studies performed on rodent brains identified GluN1, GluN2A/2B (Fig. 2), and GluA1–4 (Fig. 3) as being modified by UB (86, 87). Without a doubt, ubiquitination is important for regulating GluRs, but the mechanisms and implications of AMPAR and NMDAR ubiquitination on health and with respect to synaptic dysfunction remain to be investigated in depth.

Palmitoylation

Another common and important PTM that regulates GluR trafficking is palmitoylation. It is defined by the covalent and reversible union of a palmitic acid molecule (saturated 16-carbon lipid) to a cysteine residue in a given protein. The presence of basic and hydrophobic residues surrounding cysteine appears to create a favorable sequence environment for the reaction (88). This likely explains the propensity of palmitoylated cysteines to be identified near the transmembrane-spanning region for membrane proteins (89–92). The addition of the palmitoyl group increases the hydrophobicity of the protein and, therefore, facilitates the interaction with cellular membranes. Palmitoylation can both stabilize proteins in the plasma membrane and control protein shuttling between intracellular compartments (93). Palmitoylation is mediated by a group of enzymes named palmitoyltransferases (PATs), of which there are 23 in humans with each containing an Asp-His-His-Cys (DHHC) Cys-rich domain that confers the molecular signature of PATs (94). Conversely, depalmitoylation is mediated by acyl-protein-thioestersases, of which very few have been identified so far.
The function of many neuronal proteins, including NMDARs and AMPARs, is regulated by palmitoylation. The palmitoylation of GluN2A and GluN2B subunits occurs in “two clusters” (Fig. 2). Cluster I, close to the last transmembrane domain of GluN2A and GluN2B, is associated with an increase in the surface expression of the receptor, whereas palmitoylation of Cluster II, located in the middle of the intracellular C-terminus, plays an opposite role to Cluster I and is associated with receptor accumulation in the Golgi apparatus (95, 96). However, the regulation of NMDARs by palmitoylation is complex and participates in interplay with tyrosine phosphorylation for both GluN2A and GluN2B. In addition, PSD-95 and other synaptic proteins important for controlling NMDARs are also palmitoylated, multiplying the complexity of the regulation of NMDARs by this modification (93). Similarly, all four subunits of AMPARs are palmitoylated at two conserved cysteine residues (Fig. 3): one is located within the C-terminal region, and another is located within the second intracellular loop immediately adjacent to the pore region (89, 97). Although in vitro studies show that AMPAR trafficking and membrane expression are regulated by palmitoylation (95, 97), the study by Van Dolah et al. (98) tackled the function of AMPAR palmitoylation in the brain. In this interesting study, intraperitoneal injection of the psychostimulant cocaine (20 mg/kg) in adult male rats up-regulates the palmitoylation of GluA1 and GluA3 AMPAR subunits in the nucleus accumbens. In fact, cocaine causes the redistribution of AMPARs, increasing the intracellular localization, whereas the surface expression was reduced. Although future studies are required to clarify the function of palmitoylation in AMPAR synaptic plasticity, it is clear that AMPAR palmitoylation can be dynamically controlled by extracellular stimuli in various brain regions (89, 97, 98).

Future Perspectives

Over the last decade, great advances have been made in identifying the specific regulation of AMPARs and NMDARs by PTMs. Several studies demonstrate crosstalk between two or more PTMs to be important mechanisms of synaptic regulation (99). Functionally, crosstalk may occur within the same protein (cis crosstalk) or between PTMs on two different proteins (trans crosstalk). An example of such crosstalk on glutamate receptors is that, after depalmitoylation of GluA1 on Cys-811, the phosphorylation of GluA1 Ser-818 by PKC enhances binding to 4.1N to drive membrane insertion and the expression of LTP (97). As evidenced by this study, future investigations are required for understanding the synergistic/antagonistic effect of PTMs and the directionality of the crosstalk for identifying new mechanisms implicated in spatial and temporal regulation of AMPARs and NMDARs. Interestingly, using mass spectrometry and various enrichment approaches, a recent study suggests the presence of a global crosstalk directionality in which phosphorylation frequently precedes ubiquitination (100). In conclusion, PTMs represent an important set of mechanisms to regulate protein function and cellular signaling, and the importance and complexity of its code remain a major challenge for our complete understanding of brain function.

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