



Post-translational modification biology of glutamate receptors and drug addiction

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Post-translational covalent modifications of glutamate receptors remain a hot topic. Early studies have established that this family of receptors, including almost all ionotropic and metabotropic glutamate receptor subtypes, undergoes active phosphorylation at serine, threonine, or tyrosine residues in their intracellular domains. Recent evidence identifies several glutamate receptor subtypes to be direct substrates for palmitoylation at cysteine residues. Other modifications such as ubiquitination and sumoylation at lysine residues also occur to certain glutamate receptors. These modifications are dynamic and reversible in nature and are regulatable by changing synaptic inputs. The regulated modifications significantly impact the receptor in many ways, including interrelated changes in biochemistry (synthesis, subunit assembling, and protein-protein interactions), subcellular redistribution (trafficking, endocytosis, synaptic delivery, and clustering), and physiology, usually associated with changes in synaptic plasticity. Glutamate receptors are enriched in the striatum and cooperate closely with dopamine to regulate striatal signaling. Emerging evidence shows that modification processes of striatal glutamate receptors are sensitive to addictive drugs, such as psychostimulants (cocaine and amphetamine). Altered modifications are believed to be directly linked to enduring receptor/synaptic plasticity and drug-seeking. This review summarizes several major types of modifications of glutamate receptors and analyzes the role of these modifications in striatal signaling and in the pathogenesis of psychostimulant addiction.

Keywords: AMPA, NMDA, mGluR, dopamine, phosphorylation, palmitoylation, ubiquitination, sumoylation

INTRODUCTION

The neurotransmitter l-glutamate (glutamate) interacts with specific ionotropic glutamate receptors (iGluR) or metabotropic glutamate receptors (mGluR; Dingledine et al., 1999; Cull-Candy et al., 2001). The former are the ligand-gated ion channel and are classified into N-methyl-D-aspartate receptors (NMDAR), α -amino-3-hydroxy-5-methylisoxazole-4-propionic acid receptors (AMPA), and kainate receptors. The latter are the G protein-coupled receptor. Through various G proteins, they connect to multiple second messenger systems. There are three functional groups of mGluRs (group I–III) classified from eight subtypes (mGluR1–8; Conn and Pin, 1997). Group I mGluRs (mGluR1/5 subtypes) are positively coupled to phospholipase C β 1 through G α q proteins. Activation of mGluR1/5 increases phosphoinositol hydrolysis, resulting in intracellular Ca²⁺ release and protein kinase C (PKC) activation (Conn and Pin, 1997). Both group II (mGluR2/3) and group III (mGluR4/6/7/8) receptors are negatively coupled to adenylyl cyclase through G α i/o proteins. Their activation reduces cAMP formation and inhibits protein kinase A (PKA).

Diverse post-translational modifications occur at intracellular domains of glutamate receptors and represent regulatory mechanisms for controlling many properties of modified receptors. Early extensive studies have established a phosphorylation modification of both iGluRs and mGluRs (Swope et al., 1999; Wang et al., 2006). By targeting specific amino acids (serine, threonine, and tyrosine),

multiple protein kinases and phosphatases control phosphorylation levels of glutamate receptors. Recent work identifies fatty acylation, such as palmitoylation, as another important type of modification at cysteine residues. Lysine residues may also be modified by the enzymatic cascades known as ubiquitination and sumoylation. All these modifications are noticeably physiological events and sensitive to changing synaptic inputs. They are labile, dynamic and reversible in nature, unlike other modifications, such as prenylation, farnesylation, and geranylation, which are semi-permanent and serve to anchor proteins to membranes. Thus, these reversible modifications are believed to activity-dependently regulate and assure normal expression and function of glutamate receptors. As such, malfunction of these modifications is frequently associated with the pathogenesis of various neuropsychiatric disorders.

The striatum, i.e., the dorsal caudate putamen and ventral nucleus accumbens (NAc), is a central structure in reward circuits implicated in drug addiction. This region is enriched with glutamatergic innervation and glutamate receptors. The accumulated data support the role of striatal glutamatergic transmission in drug addiction (Tzschentke and Schmidt, 2003; Hyman et al., 2006). Recently, emerging evidence links post-translational modifications of glutamate receptors to excitatory synaptic plasticity and drug-seeking behavior. Generally, modification processes of striatal glutamate receptors are sensitive to addictive drugs such as the psychostimulants (cocaine and amphetamine). Altered modifications

contribute to enduring receptor plasticity and the addictive properties of drugs. This review will primarily discuss post-translational modifications of glutamate receptor and their associations with drug action, while roles of individual glutamate receptors in drug addiction have been thoroughly reviewed elsewhere (Bird and Lawrence, 2009; Kalivas, 2009; Bowers et al., 2010; Moussawi and Kalivas, 2010; Schmidt and Pierce, 2010). We will focus on several major types of modifications appreciated recently, including phosphorylation, palmitoylation, ubiquitination, and sumoylation, based on the available data linking them to drug addiction. Of note, in addition to addiction, abnormalities in the modifications of glutamate receptors can be observed in many other disease models, such as schizophrenia (Li et al., 2009), anxiety (Delawary et al., 2010), and Parkinson's disease (Ba et al., 2006).

PHOSPHORYLATION OF GLUTAMATE RECEPTORS AT SERINE/THREONINE

α -AMINO-3-HYDROXY-5-METHYLISOXAZOLE-4-PROPIONIC ACID RECEPTORS

Phosphorylation of AMPARs at serine/threonine has been well established (Wang et al., 2006). Most AMPARs become functional upon a heteromeric assembly of four subunits (GluA1–4 or GluR1–4). A tetrameric structure seems to be prototypic and is assembled by dimer-of-dimers of GluA2 and either GluA1, GluA3, or GluA4. As a membrane-bound receptor, four subunit proteins share the same conformation in the plasma membrane: four transmembrane domains (TMD; M1–4) with an extracellular N-terminus and an intracellular C-terminus. C-termini are variable in length among subunits and are intracellular domains for protein–protein interactions and serine/threonine phosphorylation (Carvalho et al., 2000). To date, the GluA1 C-terminus has four identified phosphorylation sites at serine 818 (S818), S831, threonine 840 (T840), and S845 (Roche et al., 1996; Barria et al., 1997; Mammen et al., 1997; **Table 1**). Other subunits are also similarly phosphorylated at their C-terminal serine and/or threonine residues (Chung et al., 2000; Wang et al., 2006). S818 is phosphorylated by PKC (Boehm et al., 2006) and S831 by both PKC and Ca²⁺/calmodulin-dependent protein kinase II (CaMKII; Roche et al., 1996; Barria et al., 1997; Mammen et al., 1997). T840 is not a substrate of the common kinases such as PKA, PKC, or CaMKII, but it appears to be a substrate of p70S6 kinase (Delgado et al., 2007). Finally, S845 is specific for PKA-mediated phosphorylation (Roche et al., 1996).

Phosphorylation is one of the post-translational modifications that are labile and reversible. In fact, AMPAR phosphorylation is subject to the vigorous regulation by synaptic signals. Regulated phosphorylation in turn adjusts expression, distribution (synaptic delivery) and function of the receptor usually in a site-specific fashion. Acute phosphorylation of GluA1 S818 by PKC promoted GluA1 synaptic incorporation and an activity-dependent form of synaptic plasticity, long-term potentiation (LTP; Boehm et al., 2006). PKC/CaMKII-sensitive S831 and PKA-sensitive S845 phosphorylation potentiated AMPAR currents and augmented LTP (Roche et al., 1996; Derkach et al., 1999; Banke et al., 2000). Activity-driven PKA phosphorylation of S845 also drove AMPARs to synapses, which was necessary for LTP (Estaban et al., 2003). T840 is dephosphorylated by NMDAR signals, which is implicated in long-term depression (LTD; Delgado et al., 2007).

AMPA receptors are densely expressed in striatal medium spiny output neurons and certain types of interneurons (Martin et al., 1993; Bernard et al., 1997). These postsynaptic receptors are present in the perikarya, dendrites, and spines with a significant amount of extrasynaptic receptors on these subcellular specializations. The site-specific phosphorylation of striatal GluA1 subunits in response to drug exposure has been extensively studied. It is apparent that dopamine is a strong regulator of their phosphorylation. By stimulating D1 dopamine receptors and associated cAMP/PKA pathways, the D1 agonist and psychostimulants (cocaine and amphetamines) increased GluA1 phosphorylation preferentially at S845 in striatal neurons (Price et al., 1999; Snyder et al., 2000; Chao et al., 2002). This facilitated surface/synaptic delivery of AMPARs and potentiated efficacy and strength of excitatory synapses (Price et al., 1999; Mangiavacchi and Wolf, 2004; Swayze et al., 2004). In contrast to D1 signals, the D2 receptor inhibits S845 phosphorylation (Hakansson et al., 2006).

Plastic changes in AMPAR phosphorylation may constitute an important layer of underlying mechanisms for receptor plasticity and drug addiction. Several studies in chronic drug administration models (passive repeated administration or operant self-administration) have established an association (correlation) between AMPAR phosphorylation and enduring behavioral plasticity (behavioral sensitization and more significantly drug-seeking behavior), although a causal link between them remains to be proven experimentally. For instance, cocaine-sensitized or heroin self-administering rodents were associated with increased phosphorylation at S845 in the NAc (Zhang et al., 2007; Edwards et al., 2009; Chen et al., 2010; but Mattson et al., 2005; Chen and Manev, 2010) and at S831 in the CPU (Kim et al., 2009). Increased accumbens shell S831 phosphorylation was seen in animals showing reinstatement of cocaine-seeking (Anderson et al., 2008). In addition to GluA1, elevated GluA2 S880 phosphorylation in the NAc was related to reinstatement of cocaine-seeking (Famous et al., 2008). These results imply a phosphorylation-dependent mechanism for AMPAR plasticity and drug-seeking. S845/S831 phosphorylation is likely to be upregulated to increase surface AMPAR expression and thereby enhance AMPAR transmission related to behavioral plasticity (Boudreau and Wolf, 2005; Conrad et al., 2008). However, self-administration of cocaine induced lesser S845 phosphorylation in the striatum as compared to acute cocaine injection, establishing a tolerance of S845 phosphorylation in response to chronic cocaine (Edwards et al., 2007). This tolerance may reflect a downregulated GluA1 function in accumbens neurons and may contribute to cocaine sensitization and cocaine-seeking behavior (Sutton et al., 2003; Bachtell et al., 2008). These results underscore the complexity of AMPARs' roles in drug action. Distinct roles of the receptor may derive from differences in subsets of synapses, subpopulations of projection and interneurons, subdivisions of the NAc (core versus shell), early or late withdrawal times and addiction stages, different administration/test regimens, and types of behavior surveyed.

N-METHYL-D-ASPARTATE RECEPTORS

Like AMPARs, NMDARs are tetrameric assemblies of two obligatory GluN1 subunits and two modulatory GluN2 subunits (Dingledine et al., 1999; Cull-Candy et al., 2001). The difference

Table 1 | Post-translational modifications of glutamate receptors.

| Modification | Receptor | Subtype | Site | Enzyme | Physiological/ pathophysiological impact | References | |
|-----------------|--------------------------|------------|---------------------|--|---|---|---|
| Phosphorylation | AMPA | GluA1 | S818 (CT) | PKC | Promote synaptic incorporation and critical for LTP | Boehm et al. (2006) | |
| | | | S831 (CT) | PKC/CaMKII | Increase currents and channel conductance | Roche et al. (1996), Barria et al. (1997), Mammen et al. (1997) | |
| | | | T840 (CT) | p70S6 | Dephosphorylation links to LTD | Delgado et al. (2007) | |
| | | | S845 (CT) | PKA | Increase synaptic delivery/peak currents and critical for LTP | Roche et al. (1996), Estaban et al. (2003) | |
| | | GluA2 | S880 (CT) | PKC | Disrupt GRIP1/2 binding and promote endocytosis and LTD | Chung et al. (2000) | |
| | | | Y876 (CT) | Src | Disrupt GRIP1/2 binding and promote endocytosis and LTD | Hayashi and Huganir (2004), Ahmadian et al. (2004), Fox et al. (2007) | |
| | | NMDAR | GluN1 | S890 (CT) | PKC | Disperse surface clustering | Tingley et al. (1997) |
| | | | | S896 (CT) | PKC | ND | Tingley et al. (1997) |
| | | | | S897 (CT) | PKA | ND | Tingley et al. (1997) |
| | | | GluN2A | Y1325 (CT) | Src | Increase NMDAR activity and contribute to depression | Taniguchi et al. (2009) |
| | GluN2B | | S1303 (CT) | PKC/CaMKII | Enhance NMDAR function | Omkumar et al. (1996), Liao et al. (2001) | |
| | | | S1323 (CT) | PKC | Enhance NMDAR function | Liao et al. (2001) | |
| | | | S1480 (CT) | CK2 | Increase endocytosis | Sanz-Clemente et al. (2010) | |
| | | | Y1336 (CT) | Fyn | Increase calpain cleavage | Wu et al. (2007) | |
| | GluNC | Y1472 (CT) | Fyn | Link to LTP | Nakazawa et al. (2001) | | |
| | | S1244 (CT) | PKA/PKC | Regulate channel kinetics | Chen et al. (2006) | | |
| | mGluR | mGluR1a | S1096 (CT) | PKB/Akt | Increase surface expression | Chen and Roche (2009) | |
| | | | T695 (IL2) | PKC | Desensitization | Medler and Bruch (1999), Francesconi and Duvoisin (2000) | |
| | | | mGluR5 | T840/S839 (CT) | PKC | Generate oscillatory Ca ²⁺ responses | Kawabata et al. (1996), Kim et al. (2005) |
| | | | mGluR5 | T606/S613 (IL1), T665/T681 (IL2), S881/S890 (CT) | PKC | Desensitization | Gereau and Heinemann (1998) |
| mGluR5 | | | T1164/S1167 (CT) | CDK5 | Increase Homer binding | Orlando et al. (2009) | |
| mGluR2 | | | S843 (CT) | PKA | Inhibit function | Schaffhauser et al. (2000) | |
| mGluR3 | | | S845 (CT) | PKA | Inhibit function | Cai et al. (2001) | |
| mGluR4a/7a/8a | | | S859/S862/S855 (CT) | PKA | Inhibit function | Cai et al. (2001) | |
| mGluR7 | | | S862 (CT) | PKC | Inhibit calmodulin binding and increase surface expression | Airas et al. (2001), Suh et al. (2008) | |
| Palmitoylation | | | AMPA | GluA1/2/3/4 | C585/C610/C615/C611 (TMD2) | GODZ/DHHC3 | Golgi retention |
| | C811/C836/C841/C817 (CT) | ND | | | Disrupt 4.1N binding and promote endocytosis | Hayashi et al. (2005) | |

(Continued)

Table 1 | Continued

| Modification | Receptor | Subtype | Site | Enzyme | Physiological/ pathophysiological impact | References |
|----------------|----------|------------|--|----------------|--|--|
| Ubiquitination | NMDAR | GluN2A | C848/C853/ C870 (CT, 1st cluster) | GODZ/ DHHC3 | Increase tyrosine phosphorylation and surface expression | Hayashi et al. (2009) |
| | | GluN2A | C1214/C1217/ C1236/C1239 (CT, 2nd cluster) | GODZ/ DHHC3 | Golgi retention | Hayashi et al. (2009) |
| | | GluN2B | C848/C854/ C871 (CT, 1st cluster) | GODZ/ DHHC3 | Increase tyrosine phosphorylation and surface expression | Hayashi et al. (2009) |
| | | GluN2B | C1215/C1218/ C1239/C1242/ C1245 (CT, 2nd cluster) | GODZ/ DHHC3 | Golgi retention | Hayashi et al. (2009) |
| | Kainate | GluK6 | C827/C840 (CT) | ND | Regulate PKC phosphorylation of GluK6 | Pickering et al. (1995) |
| | mGluR | mGluR4 | ND | ND | ND | Alaluf et al. (1995) |
| | NMDAR | GluN1 | ND | ND | Decrease expression | Ratnam and Teichberg (2005), Kato et al. (2005) |
| | GluN2B | ND | ND | Mind bomb-2 | Decrease NMDAR activity | Jurd et al. (2008) |
| Sumoylation | Kainate | GluK6 | ND (I884 is critical for binding enzyme) | ND | Decrease surface expression | Salinas et al. (2006) |
| | mGluR | mGluR1a/5 | ND | Siah1A | Decrease expression | Moriyoshi et al. (2004) |
| | Kainate | GluK6 | K886 (CT) | PIAS3 | Agonist-induced endocytosis | Martin et al. (2007) |
| | Kainate | GluK7 | ND | SUMO-1 | ND | Wilkinson et al. (2008) |
| | mGluR | mGluR8 | K882 (CT) | PIAS1 | ND | Tang et al. (2005) |
| | mGluR | mGluR4/6/7 | ND | SUMO-1 | ND | Wilkinson et al. (2008) |

ND, not determined; IL1, intracellular loop 1; IL2, intracellular loop 2; CT, C-terminus. See text for other abbreviations.

in GluN2 subunit compositions (GluN2A–D) largely determines the distinct gating and pharmacology of the channel. As a transmembrane receptor, each NMDAR subunit shares an extracellular N-terminus and an intracellular C-terminus. The C-terminal region is large especially for GluN2A and GluN2B, which provides a spacious area for protein kinase interactions and serine/threonine phosphorylation. Indeed, three NMDAR subunits (GluN1, GluN2B, and GluN2C) have been found to undergo active phosphorylation at distinct serine/threonine sites. Within the GluN1 C-terminal domain, PKC phosphorylates S890 and S896 (Tingley et al., 1997). An S896-neighboring site, S897, is a substrate of PKA (Tingley et al., 1997). GluN2B S1303 is subject to phosphorylation by CaMKII (Omikumar et al., 1996). PKC may be another kinase for this site as well as S1323 (Liao et al., 2001). A more recently identified site on the GluN2B C-terminus, S1480, is within a PDZ-binding domain and is sensitive to casein kinase (CK2; Sanz-Clemente et al., 2010). The GluN2C C-terminus is phosphorylated at S1244 by both PKA and PKC (Chen et al., 2006) and at S1096 by protein kinase B (PKB)/Akt (Chen and Roche, 2009). The phosphorylation level at these sites has a significant impact on some key properties of

the receptor. PKC phosphorylation of S890 dispersed the surface clusters of GluN1 (Tingley et al., 1997). PKC-sensitive phosphorylation of GluN1 and GluN2B (S1303 and S1323) enhanced NMDAR function (Hisatsune et al., 1997; Liao et al., 2001). CK2 phosphorylation of GluN2B S1480 drove GluN2B endocytosis and facilitated developmental switch from GluN2B to GluN2A at synapses (Sanz-Clemente et al., 2010). PKB phosphorylation of GluN2C S1096 enhanced surface expression of GluN2C-containing NMDARs and supported neuronal survival (Chen and Roche, 2009).

GluN2A and GluN2B are the predominant GluN2 subunits in the striatum. Major tetrameric subtypes of NMDARs in this region are therefore thought to be 2GluN1/2GluN2A, 2GluN1/2GluN2B, or 2GluN1/1GluN2A/1GluN2B, in addition to some binary subtypes containing one GluN1 and one GluN2A or GluN2B subunit (Dunah et al., 2000; Dunah and Standaert, 2003). Both striatonigral and striatopallidal projection neurons and cholinergic interneurons express a high level of NMDARs (Landwehrmeyer et al., 1995; Chen et al., 1996). Most GluN1/GluN2A receptors are incorporated into synapses, while GluN1/GluN2B receptors are present at both synaptic and extrasynaptic sites (Tovar and Westbrook, 1999).

Enriched expression of NMDARs in the striatum implies their roles in drug action and provides an opportunity to investigate adaptive changes in the receptor phosphorylation in response to psychostimulants. Acute amphetamine increased GluN1 S896 phosphorylation primarily in D1 receptor-bearing striatonigral neurons (Liu et al., 2004). Direct stimulation of D1 receptors with D1 agonists enhanced PKA-sensitive GluN1 S897 phosphorylation through the D1/cAMP/PKA pathway (Dudman et al., 2003). The enhanced S897 phosphorylation seems to lead to a cytosolic Ca^{2+} rise, which synergizes with the cAMP/PKA signals to activate the transcription factor Ca^{2+} /cAMP response element binding protein (CREB) to facilitate gene expression. These results show the ability of psychostimulants to modify NMDAR phosphorylation. Through modifying the receptor, stimulants regulate gene expression and construct transcription-dependent neuroadaptations essential for long-lasting drug action.

In addition to the D1 receptor, the D2 receptor is involved in the regulation of NMDAR phosphorylation (Liu et al., 2006). A single dose of cocaine induced a heteroreceptor complex formation between D2 receptors and GluN2B in D2 receptor-bearing striatopallidal neurons. The interaction of D2 receptors with GluN2B disrupted the association of CaMKII with GluN2B, thereby reducing phosphorylation at the CaMKII-sensitive site S1303 and inhibiting NMDAR currents. Behaviorally, this phosphorylation-involved D2–GluN2B interaction suppressed the inhibitory indirect pathway to promote a full motor response to cocaine.

Chronic cocaine reduced GluN1 S896 phosphorylation in the rat frontal cortex at 24 h, although not 14 days, of withdrawal (Loftis and Janowsky, 2002). However, cocaine self-administration had a minimal influence over S896 phosphorylation in the monkey striatum (Hemby et al., 2005). Acute, repeated, and self administration of cocaine increased GluN1 S897 phosphorylation in the rat striatum (Edwards et al., 2007). These results demonstrate that in the striatum at least S897 is a sensitive site modified by cocaine. Future studies are needed to elucidate how S897 phosphorylation mediates NMDAR plasticity and drug craving.

METABOTROPIC GLUTAMATE RECEPTORS

The mGluR is an equally sensitive substrate for phosphorylation and PKC's roles in mGluR phosphorylation have been the most extensively studied (Kim et al., 2008; Mao et al., 2008). The long form group I mGluRs (1a, 5a, and 5b) have a characteristically large C-terminal tail. A threonine residue (T840) or an adjacent S839 in the proximal region of mGluR5a C-terminus undergoes PKC-mediated phosphorylation (Kawabata et al., 1996; Kim et al., 2005). This single site phosphorylation determines the pattern of Ca^{2+} responses to mGluR5 stimulation (Kawabata et al., 1996; Uchino et al., 2004). PKC also possibly phosphorylates other serine/threonine sites (T606, S613, T665, T681, S881, and S890 for mGluR5a; T695 for mGluR1a). Phosphorylation at these sites is involved in rapid desensitization of the receptor (Gereau and Heinemann, 1998; Medler and Bruch, 1999; Francesconi and Duvoisin, 2000). In addition to PKC, cyclin-dependent kinase 5 (CDK5) phosphorylates two residues (T1164/S1167) within the domain of mGluR1/5 C-termini that interacts with the scaffold protein Homer (Orlando et al., 2009). This phosphorylation increased the binding of the receptor to Homer.

Group II/III mGluRs are also phosphorylated at serine/threonine. PKA phosphorylates mGluR2 S843, mGluR3 S845, mGluR4a S859, mGluR7a S862, and mGluR8a S855 on the C-terminal tail to inhibit their function as presynaptic receptors (Schaffhauser et al., 2000; Cai et al., 2001). PKC phosphorylates a conserved site (S862) on mGluR7 C-termini to inhibit calmodulin binding *in vitro* (Airas et al., 2001). In addition, mGluR7 S862 phosphorylation, in combination with the binding of the PDZ domain-containing protein PICK1, stabilized surface mGluR7 expression *in vivo* (Suh et al., 2008). At present, studies on the regulation of mGluR phosphorylation by addictive drugs are limited, partially due to the lack of phospho- and site-specific antibodies. One study revealed that relative serine phosphorylation of the mGluR2/3 monomer was elevated in both the NAc and prefrontal cortex after repeated cocaine, which was associated with an enduring reduction of mGluR2/3 function in inhibiting glutamate release (Xi et al., 2002). How phosphorylation modifications of mGluRs precisely contribute to drug-induced mGluR plasticity and drug-seeking behavior remains an interesting topic in future studies.

PHOSPHORYLATION OF GLUTAMATE RECEPTORS AT TYROSINE

In addition to serine and threonine, tyrosine is another phosphorylation site on glutamate receptors. GluA1 and GluA2 intracellular domains are tyrosine-phosphorylated (Hayashi and Haganir, 2004; Wu et al., 2004). The distal region of the GluA2 C-terminus possesses multiple tyrosine residues. Non-receptor Src family tyrosine kinases phosphorylate tyrosine 876 (Y876; Hayashi and Haganir, 2004), the last tyrosine residue near the end of C-terminus that lies within the PDZ ligand motif. Y876 phosphorylation disrupted the association of GluA2 with the PDZ domain-containing proteins, such as glutamate receptor interacting proteins 1 and 2 (GRIP1/2), and thereby promoted endocytosis of GluA2 (Hayashi and Haganir, 2004) and LTD (Ahmadian et al., 2004; Fox et al., 2007). GluN2, although not GluN1, subunits are also tyrosine-phosphorylated in their C-termini (Lau and Haganir, 1995; Menegoz et al., 1995; Dunah et al., 2000). Both Src-family tyrosine kinases (Src and Fyn) and Src-independent tyrosine kinases can carry out the phosphorylation (Suzuki and Okumura-Noji, 1995; Zheng et al., 1998; Xu et al., 2009). Multiple tyrosine residues (7 of 25, including 1252, 1336, and 1472) within the GluN2B C-terminus are responsive to phosphorylation. Y1472 seems to be a major site (Nakazawa et al., 2001) comparable to a major site (Y1325) on GluN2A (Taniguchi et al., 2009). GluN2 phosphorylation usually enhances NMDAR-mediated currents and NMDAR-dependent LTP via mechanisms involving the regulation of trafficking and protein–protein interactions (Wang and Salter, 1994; Lau and Haganir, 1995; Rostas et al., 1996; Dunah et al., 2004). Moreover, the influence of tyrosine phosphorylation can be site-selective. Y1472 and Y1336 phosphorylation was associated with enrichment of synaptic and extrasynaptic NMDARs, respectively (Goebel-Goody et al., 2009). Fyn-mediated Y1336 phosphorylation site-dependently controlled GluN2B cleavage by calpain (Wu et al., 2007). Regarding mGluRs, mGluR5 was abundantly tyrosine-phosphorylated in striatal neurons *in vivo*, based on pharmacological studies with tyrosine kinase or phosphatase

inhibitors and immunoprecipitation experiments with anti-phosphotyrosine antibodies (Orlando et al., 2002), although accurate site(s) among a few tyrosine residues on the mGluR5 C-terminus have not been determined.

D1 receptor signals increased synaptic delivery of tyrosine-phosphorylated GluN2A and GluN2B in striatal neurons (Dunah and Standaert, 2001). This event is tyrosine phosphorylation-dependent because the tyrosine kinase inhibitor genistein blocked it and the phosphatase inhibitor pervanadate mimicked D1 signals to induce the delivery. In cultured neurons, D1 receptor stimulation increased GluN2B phosphorylation at Y1472, which increased surface expression of GluN2B/NMDARs in prefrontal cortical neurons (Guo and Wolf, 2008) and facilitated the clustering of GluN2B along the dendritic spine shaft in striatal neurons (Hallett et al., 2006). In addition to D1 signals, brain-derived neurotrophic factor (BDNF) rapidly increased Fyn-sensitive Y1472 phosphorylation and NMDAR activity (Xu et al., 2006). In studies with addictive drugs, acute cocaine increased Src-dependent tyrosine phosphorylation of GluN2A, leading to elevated NMDAR expression and activity in the ventral tegmental area (Schumann et al., 2009). Repeated administration of alcohol elevated Fyn activity, Y1472 phosphorylation, and GluN2B–NMDAR function in the dorsomedial striatum (Wang et al., 2010). Inhibition of Src family tyrosine kinases decreased self-administration of alcohol (Wang et al., 2010). Thus, plastic changes in Y1472 phosphorylation can be a critical element linking NMDAR plasticity to drug-taking. In general, available data indicate the linkage between tyrosine phosphorylation of NMDAR GluN2 and drug action. As for AMPARs and mGluRs, little is known about how tyrosine phosphorylation of these receptors responds to drugs and the role of tyrosine phosphorylation might play in determining drug-induced receptor and behavioral adaptations.

PALMITOYLATION OF GLUTAMATE RECEPTORS AT CYSTEINE

Like phosphorylation, fatty acylation, such as palmitoylation, is another important type of modification. Palmitoylation is the covalent attachment of a 16 carbon saturated fatty acid, palmitate (palmitic acid), to a cysteine residue most commonly via a thioester bond. This process is an enzymatic event and is catalyzed by a family of palmitoyl acyltransferases (PATs). All PATs contain a signature Asp-His-His-Cys (DHHC) Cys-rich domain conserved from yeast to mammals (Planey and Zacharias, 2009). At least 23 mammalian DHHC-containing PATs (DHHC1–23) have been identified. These PATs have distinct substrate selectivity, although it is unclear yet if there exists a consensus PAT palmitoylation motif (Planey and Zacharias, 2009). As one type of lipidation, palmitoylation increases the lipophilicity or hydrophobicity of modified proteins. As such, it often results in an altered protein affinity for the plasma membrane, leading to protein trafficking from one membrane system to another. Additionally, palmitoylation can alter interactions of modified proteins with their binding partners, thereby regulating their subcellular distribution and function. Like phosphorylation and unlike other forms of lipidation, palmitoylation (the thioester bond) is labile and reversible. This nature situates it well as an activity-dependent regulator of synaptic proteins, including glutamate receptors. All four AMPAR subunits (GluA1–4) are palmitoylated constitutively in transfected

HEK 293T cells, cultured cortical neurons, and striatal neurons *in vivo* (Hayashi et al., 2005; Van Dolah et al., 2011). Two conserved cysteine residues on these subunits undergo reliable palmitoylation: one within C-terminal regions (GluA1-C811, GluA2-C836, GluA3-C841, and GluA4-C817) and another within the TMD 2 (GluA1-C585, GluA2-C610, GluA3-C615, and GluA4-C611; Hayashi et al., 2005). These sites are surrounded by basic and hydrophobic residues. They are adjacent to TMD, consistent with a pattern of palmitoylation for transmembrane proteins, i.e., palmitoylation at intracellular cysteines near TMD (El-Husseini and Brecht, 2002). Regarding potential PAT subtypes catalyzing AMPAR palmitoylation, a Golgi-specific protein with a DHHC zinc finger domain (GODZ, also known as DHHC3) promoted the palmitoylation on the TMD 2 site, while the exact PAT subtype for the C-terminal site remains to be identified (Hayashi et al., 2005). In addition to DHHC3, a dendritically localized DHHC2 activity-dependently translocated to the postsynaptic density (PSD) to induce rapid palmitoylation of PSD-95 in cultured hippocampal neurons (Noritake et al., 2009).

As aforementioned, palmitoylation controls redistribution of modified proteins usually in an activity-sensitive fashion. According to Hayashi et al. (2005), GODZ increases palmitoylation of AMPARs on the TMD 2 site and retains the receptor in the Golgi apparatus. In contrast, depalmitoylation releases the receptor from the Golgi for surface delivery. On the C-terminal site, palmitoylation disrupts the interaction of receptors with 4.1N, a synapse-enriched cytoskeletal protein that stabilizes surface AMPAR expression, and enhances susceptibility to agonist-induced internalization trafficking. Depalmitoylation increases the receptor affinity for 4.1N and stabilizes the receptor on surface membrane. Apparently, the balance of palmitoylation and depalmitoylation is regulated by synaptic activity. Such regulatable balance determines subcellular distribution of the receptor.

N-methyl-D-aspartate receptors GluN2A and GluN2B subunits have two distinct clusters of palmitoylation sites in their C-terminal regions (Hayashi et al., 2009). The first cluster is proximal to membrane (GluN2A: C848, C853, and C870; GluN2B: C849, C854, and C871). Palmitoylation of these sites increased tyrosine phosphorylation, leading to enhanced surface expression of the receptor. The second cluster resides in the middle of C terminus (GluN2A: C1214, C1217, C1236, and C1239; GluN2B: C1215, C1218, C1239, C1242, and C1245). Their palmitoylation caused receptors to accumulate in the Golgi apparatus and reduced receptor surface expression. Recombinant kainate receptor GluK6 subunits expressed in HEK cells are palmitoylated at C-terminal C827 and C840 (Pickering et al., 1995). mGluR palmitoylation has been less extensively studied. Available data show that mGluR4 but not mGluR1 α is palmitoylated in heterologous cells transfected with these subtypes (Alaluf et al., 1995; Pickering et al., 1995). Little is known about the palmitoylation status of endogenous mGluRs in neurons.

Palmitate is the most abundant fatty acid in the brain. Thus, inducible, regulatable, and reversible palmitoylation could be a common mechanism for regulating normal glutamate receptors and excitatory synapses. An attractive speculation is that glutamate receptor palmitoylation could also be plastic in response to drug exposure and is thus involved in enduring synaptic and behavioral plasticity. At present, the study linking palmitoylation to addiction

is limited partially due to the lack of palmito- and site-specific antibodies that are needed to detect site-specific palmitoylation responses to drugs in *in vivo* adult rodent brains. Nevertheless, one recent study shows that palmitoylation of AMPARs is subject to the regulation by a psychostimulant (Van Dolah et al., 2011). Acute cocaine induced a transient and reversible increase in overall GluA1 and GluA3 palmitoylation in the rat NAc, while cocaine did not affect GluA2 and GluA4 palmitoylation. This increase was correlated well with a temporary loss of surface GluA1/3. Pharmacological inhibition of protein palmitoylation reversed the loss of local GluA1/3. Thus, the increased palmitoylation appears to cause reduction of GluA1/3 surface expression, probably via an enhanced internalization rate due to disrupted interactions with the surface-stabilizing protein 4.1N (see above). Noticeably, AMPAR palmitoylation contributes to the regulation of behavioral sensitivity to cocaine. Since blocking AMPAR palmitoylation responses to cocaine augmented motor responses to the drug, the induced palmitoylation may downregulate surface AMPAR expression to prevent motor overstimulation. It will be interesting to explore how palmitoylation of AMPARs responds to chronic drug administration and whether palmitoylation is another modification that needs to be investigated for elucidating mechanisms underlying AMPAR plasticity and drug addiction.

UBIQUITINATION OF GLUTAMATE RECEPTORS AT LYSINE

Ubiquitination is a stepwise enzymatic process carried out by a set of three enzymes. Initially, a small regulatory protein ubiquitin is activated by the ubiquitin-activating enzyme (E1). Active ubiquitin is then transferred to the ubiquitin-conjugating enzyme (E2). Finally, the C-terminal glycine of ubiquitin recognizes and binds the lysine in the modified protein via the ubiquitin-protein ligase (E3). The whole process can be repeated until a short chain of ubiquitin is formed (polyubiquitination) to target the modified protein to degradation by proteasomes. The ubiquitin-proteasome system (UPS) serves as a fundamental mechanism for the regulation of protein expression and function. Increasing evidence supports its role as an important regulator of synaptic plasticity (Yi and Ehlers, 2005). To date, a subset of synaptic scaffolds, including Shank, guanylate kinase-associated protein (GKAP) and A-kinase anchoring protein (AKAP), have been found to be polyubiquitinated in cultured hippocampal or cortical neurons (Ehlers, 2003) or in the rat striatum *in vivo* (Mao et al., 2009). PSD-95 may arguably be ubiquitinated (Colledge et al., 2003; Bingol and Schuman, 2004). Among glutamate receptor subtypes surveyed, there is evidence showing that GluN1 and GluN2B could be the direct targets of ubiquitin in heterologous cells or in cultured neurons, although results are not always consistent (Ehlers, 2003; Kato et al., 2005; Ratnam and Teichberg, 2005; Jurd et al., 2008). GluK6 is ubiquitinated (Salinas et al., 2006). Group I mGluRs (mGluR1 α and mGluR5) can also undergo polyubiquitination in heterologous cells via a specific E3 ligase, seven in absentia homolog 1A (Siah1A; Moriyoshi et al., 2004). Multiple lysine sites at mGluR5 intracellular loops and the C-terminal tail are among the sites ubiquitinated, although the exact sites remain to be identified. Functionally, polyubiquitination determines the degradation rate and expression level of the receptor, while mono-ubiquitination may carry out some non-proteolytic roles, including the regulation of receptor endocytosis.

Given the regulatory role of ubiquitination and degradation in synaptic plasticity, it is intriguing to explore whether ubiquitination is directly involved in the remodeling of excitatory synapses and behavioral plasticity in response to drug exposure. Mao et al. (2009) have recently found that chronic amphetamine administration enhanced ubiquitin conjugation to synaptic proteins in the rat striatum. The specific substrates sensitive to the drug in their ubiquitination include Shank and GKAP. Since these two scaffold proteins are known to anchor PSD-95 and PSD-95-linked GluN2B to the PSD microdomain, the ubiquitination-dependent removal of Shank/GKAP may have a significant impact on the stability of PSD-95/GluN2B, even though PSD-95/GluN2B are not direct ubiquitin targets. In fact, striatal PSD-95 (Yao et al., 2004) and GluN2B proteins were reduced in parallel with reduced levels of Shank and GKAP (Mao et al., 2009). Inhibition of proteasomes reversed the reduction of all four proteins (Shank, GKAP, PSD-95, and GluN2B) in the striatum of amphetamine-treated rats. Destabilization of GluN2B through this mechanism further translates to the inhibitory modulation of synaptic plasticity in the form of LTD at cortico-accumbal glutamatergic synapses and contributes to behavioral sensitization. These data link ubiquitination to glutamate receptor plasticity in relation to drug action and pave the way for further studies to characterize this new layer of mechanisms.

NAC-1 is a transcription factor in brain cells and is localized in the nucleus. Its mRNA levels were increased in the rat NAc 3 weeks after chronic cocaine self-administration (Cha et al., 1997). NAC-1 is deemed to play an important role in modifying long-term behavioral effects of cocaine (Mackler et al., 2000). Interestingly, NAC-1 forms complexes with proteins in the UPS (Shen et al., 2007). NAC-1 and the proteasome were cotranslocated from the nucleus into dendritic spines and the PSD microdomain when synaptic activity was disinhibited by bicuculline (Shen et al., 2007). Thus, NAC-1 has a potential to modify drug-induced synaptic plasticity via recruiting the UPS into synaptic sites.

SUMOYLATION OF GLUTAMATE RECEPTORS AT LYSINE

Analogous to ubiquitination, sumoylation is a sequential enzymatic modification of proteins at lysine residues. Through a defined cascade involving three enzymes (E1–3), small ubiquitin-like modifier or SUMO proteins, a family of small proteins similar to ubiquitin, are covalently attached to and detached from target proteins (Geiss-Friedlander and Melchior, 2007; Heun, 2007). Specifically, at the final step, the C-terminal glycine of SUMO forms an isopeptide bond with an acceptor lysine on the target protein. In contrast to ubiquitin, SUMO is not used to tag proteins for degradation. It instead has diverse functions most frequently linked to protein protection from ubiquitination (stability), protein–protein interactions, cytosol-nuclear transport, and transcriptional regulation (Zhao, 2007). These functions are directly derived from the sumoylation-induced changes in trafficking/subcellular redistribution and binding properties of modified proteins with their interactors. Typically, sumoylation is carried out at a consensus motif Ψ -K-x-D/E where Ψ is a hydrophobic residue, K is the lysine conjugated to SUMO, x is any amino acid, D or E is an acidic residue. Free online engines, such as SUMOplot or SUMOsp (Ren et al., 2009),

can be accessed to predict and score potential sumoylation sites for a given protein of interest. Of note, sumoylation can also be reversibly regulated by desumoylating enzymes.

Early work focused on nuclear proteins. It is now appreciated that proteins in all subcellular compartments are subject to sumoylation, including synaptic proteins such as glutamate receptors (Scheschonka et al., 2007; Sen and Snyder, 2010). GluK6 is a SUMO substrate in rat hippocampal neurons (Martin et al., 2007). GluK6 sumoylation is required for agonist-evoked endocytosis of the receptor. Noticeably, GluK6 is sumoylated at the C-terminal K886 site (Martin et al., 2007), which is adjacent to I884 critical for binding and formation of a complex for GluK6 ubiquitination (Salinas et al., 2006). The proximity of two types of modifications implies possible crosstalk. mGluR8 is sumoylated at its C-terminal K882 site (Tang et al., 2005). Other group III mGluRs (mGluR4/6/7) and GluK7 may also undergo reliable sumoylation (Wilkinson et al., 2008).

A striatum-enriched small G-protein ras homolog enriched in striatum (Rhes) has recently been found to act as a physiological regulator of protein sumoylation (Subramaniam et al., 2010). Rhes-deleted mice exhibited marked and selective reduction of protein sumoylation in the striatum (Subramaniam et al., 2009). Rhes expression is regulated by dopamine signals. Dopamine depletion reduced its mRNA and protein expression in the rat striatum (Harrison and LaHoste, 2006; Harrison et al., 2008). However, dopamine stimulation by acute amphetamine did not alter Rhes mRNA levels in the striatum (Schwendt and McGinty, 2010).

At present, sumoylation in drug action almost represents an uncharted area. It is possible that sumoylation of synaptic proteins including glutamate receptors is regulated by drug exposure. Altered sumoylation may contribute to the regulation of striatal signaling and drug addiction.

CONCLUSION

Glutamate receptors have long been appreciated to be subject to various post-translational modifications. Extensive studies have established glutamate receptors as direct substrates of protein kinases for robust phosphorylation at serine, threonine, or tyrosine

residues. Such phosphorylation is important for regulating trafficking and function of modified receptors. Reversible palmitoylation also occurs to glutamate receptor cysteine residues, which like phosphorylation regulates expression and activity of the receptor. Ubiquitination and sumoylation at lysine residues are other types of glutamate receptor modifications. These modifications control degradation or endocytosis of surface receptors and thus adjust surface expression levels of modified receptors. All these types of modifications are regulated by cellular and synaptic signals. In response to psychostimulants, glutamate receptors in striatal neurons show marked and dynamic changes in phosphorylation and palmitoylation. Even though limited studies have been attempted at present, emerging evidence indicates the linkage between these protein modifications and persistent drug-related plasticity at both receptor and behavioral levels.

In addition to phosphorylation, palmitoylation, ubiquitination and sumoylation summarized in this review, other types of protein modification may be substantial for the regulation of glutamate receptors. These potential modifications include nitrosylation (cysteine), sulfhydration (cysteine), acetylation (lysine), etc. (Sen and Snyder, 2010). Recently, these modifications are appreciated to impact far more proteins than expected and could influence glutamate receptors or their adaptor proteins as prominently and efficiently as phosphorylation. Additionally, different modifications are thought to interact with each other, especially when modifications occur at the same or proximal sites. This could pair two different modifications as a reciprocal process, such as palmitoylation and nitrosylation on cysteines or ubiquitination and sumoylation on lysines. It is anticipated that modification biology will grow rapidly and more modification models on glutamate receptors will be characterized *in vivo*. This will ultimately inform glutamate receptor modifications as to their clinical relevance, and assist in our understanding the pathogenesis of psychiatric illnesses and in developing therapeutic agents for drug addiction.

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