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# Phosphorylation mediated structural and functional changes in pentameric ligand-gated ion channels: Implications for drug discovery

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Article type: Molecules in Focus
Number of words: main text, 1970; abstract, 147.
No of figures: 2
No of tables: 1
No of references: 33

**Keywords:** Cys-loop receptor, pentameric ligand-gated ion channel, phosphorylation, conformational changes, drug discovery

**Abbreviations:** 5-$HT_3$R: 5-hydroxytryptamine type-3 receptor; CamKII: $Ca^{2+}$/calmodulin-dependent protein kinase; $GABA_A$R: GABA type-A receptor; GlyR: glycine receptor; IPSC: inhibitory postsynaptic current; nAChR: nicotinic acetylcholine receptor; PKA: protein kinase A; PKC: protein kinase C; pLGIC: pentameric ligand-gated ion channel; PTK: protein tyrosine kinase; $PGE_2$: prostaglandin type-$E_2$; VCF, voltage clamp fluorometry.

**Abstract**

Pentameric ligand-gated ion channels (pLGICs) mediate numerous physiological processes, including fast neurotransmission in the brain. They are targeted by a large number of clinically-important drugs and disruptions to their function are associated with many neurological disorders. The phosphorylation of pLGICs can result in a wide range of functional consequences. Indeed, many neurological disorders result from pLGIC phosphorylation. For example, chronic pain is caused by the protein kinase A-mediated phosphorylation of α3 glycine receptors and nicotine addiction is mediated by the phosphorylation of α4- or α7-containing nicotinic receptors. A recent study demonstrated that phosphorylation can induce a global conformational change in a pLGIC that propagates to the neurotransmitter-binding site. Here we present evidence that phosphorylation-induced global conformational changes may be a universal phenomenon in pLGICs. This raises the possibility of designing drugs to specifically treat disease-modified pLGICs. This review summarizes some of the opportunities available in this area.

## 1. Introduction

The pentameric ligand-gated ion channel (pLGIC) family includes nicotinic acetylcholine receptors (nAChRs), $GABA_A$ receptors ($GABA_ARs$), glycine receptors (GlyRs) and 5-$HT_3$ receptors (5-$HT_3$Rs). Although these receptors are involved in many physiological processes, they are probably best known for mediating fast neurotransmission in the nervous system. They have been implicated in numerous channelopathies with neurological manifestations and are pivotal pharmacological targets. For example, nAChRs play a clinical role in nicotine addiction and are therapeutic targets for Alzheimer's disease and schizophrenia (Pollock et al., 2009); $GABA_ARs$ are important clinical targets for epilepsy (Abramian et al., 2010), anxiety, addiction and anaesthesia (Song and Messing, 2005); GlyRs are emerging targets for chronic inflammatory pain (Harvey et al., 2004).

Phosphorylation is well known to influence synaptic function by directly modulating pLGICs (Swope et al., 1999) Indeed, pLGIC phosphorylation has been implicated in various disorders such as nicotine addiction (Wecker et al., 2001), status epilepticus (Terunuma et al., 2008) and chronic pain (Harvey et al., 2004). Phosphorylation of pLGICs can elicit a wide variety of effects, ranging from alterations in the level of surface expression, synaptic targeting and receptor desensitization. Many of these effects are summarized in **Table 1**. Until recently, there have been few attempts to resolve the structural basis of these effects. By doing so, it may be possible to design drugs to specifically treat the consequences of pathological receptor modifications that lead to disease. This review summarizes some of the opportunities available in this area.

## 2. Structure

### *2.1. pLGIC architecture*

Functional pLGICs comprise pentameric assemblies of identical or different subunits **(Fig. 1A)**. The five subunits together form a central water-filled pore that facilitates transmembrane ion flux **(Fig 1B)**. Each subunit can be divided into three domains, an extracellular ligand-binding domain, four transmembrane helices (termed M1-M4) and a large intracellular M3-M4 domain which has a length and primary structure that varies enormously among pLGIC members **(Fig. 1A).** The extracellular domain predominantly comprises a twisted β-sheet sandwich with ligand-binding pockets located at the subunit interfaces. The principal (+) side of the pocket is lined by binding domain loops A, B and C and the complementary (–) side is lined by binding loops D, E and F **(Fig. 1A)**. The transmembrane α-helices form concentric rings around a central ion pore, which is directly lined by five M2 helices **(Fig 1A)**.

*2.2. Structure and importance of the M3-M4 cytoplasmic domain*

Unlike the extracellular and transmembrane domains, the M3-M4 domain is poorly conserved both in terms of length and amino acid sequence. Therefore, it is likely to exhibit structural variation. Low-resolution structural data suggest that the *Torpedo* nAChR M3-M4 domain forms a 'hanging basket' type structure connecting the pore with the cytoplasm (Miyazawa et al., 1999). This structure incorporates a lateral ion permeation pathway (or portal) linking the cytoplasm with the inner vestibule at the base of the pore. Charged residues lining these portals influence the single channel conductance of nAChRs, GlyRs and $5HT_3$Rs (Peters et al., 2010). Interactions between the M3-M4 loop and other proteins or ions are well known to modulate pLGIC activity, assembly and trafficking (e.g., Luscher et al., 2011). These interactions are highly specific to different pLGIC subunits. The M3-M4 domain is also the only pLGIC region known to house phosphorylation sites.

*2.3. Receptor phosphorylation: structural changes induced by kinases?*

Phosphorylation results from the kinase-mediated covalent attachment of the γ-phosphate group of ATP to the hydroxyl group of serine, threonine or tyrosine (**Fig. 1D**). The best-characterized protein kinases include cAMP dependent protein kinase A (PKA), protein kinase C (PKC) and protein tyrosine kinase (PTK). Of these, PKA and PKC phosphorylate both Ser and Thr residues and PTK (including Src family kinase) phosphorylates Tyr residues. Many other kinases also exist such as $Ca^{2+}$/calmodulin-dependent protein kinases (CaMKII) which also phosphorylate both Ser and Thr residues (Swope et al., 1999, Davis et al., 2001). The consensus phosphorylation site for Ser/Thr or Tyr specificity is determined by the structure of the catalytic cleft of the kinase and the local interactions between the kinase cleft and protein phosphorylation site. This binding between the kinase and the receptor protein often provides additional binding interactions, and occasionally, provides allosteric regulation and localization to specific cellular compartments or structures (Ubersax and Ferrell, 2007). Phosphorylation can have many effects on pLGIC function, including alterations in open probability and desensitization, surface expression levels, turnover rate and synaptic targeting (Swope et al., 1999, Davis et al., 2001) (**Fig. 1E**).

## 3. Biological function

*3.1. Chronic inflammatory pain is mediated by phosphorylation of α3 GlyRs*

Perhaps the most direct evidence for phosphorylation-induced structural changes in a pLGIC, and its relationship with a disease, has emerged from studies on the α3 GlyR. This will now be

described in detail. Inflammatory pain is mediated by the production of prostaglandins in the spinal cord. Electrophysiological studies have identified prostaglandin $E_2$ ($PGE_2$) as the most active prostaglandin in the spinal cord, where it specifically inhibits glycinergic inhibitory postsynaptic currents (IPSCs) in lamina I and II nociceptive neurons (Ahmadi et al., 2002). This effect is mediated via activation of the $EP_2$ receptor involves the activation of PKA. Although α3 GlyRs are generally sparsely expressed, they are highly expressed along with α1 subunits at glycinergic synapses in lamina I and II neurons (Harvey et al., 2004). As only α3 GlyRs are inhibited by $PGE_2$ via PKA-dependent phosphorylation at S346 in the M3-M4 domain, it was hypothesised that the α3 subunit was modulated by $PGE_2$ (Harvey et al., 2004). Although GlyR $\alpha3^{-/-}$ mice displayed no overt behavioural phenotype, normal and knockout mice responded similarly to non-painful tactile stimuli and acute inflammatory pain stimuli. However, chronic inflammation produced pain sensitisation in normal animals but not in the GlyR $\alpha3^{-/-}$ animals (Harvey et al., 2004). Thus, $PGE_2$ induces chronic pain by phosphorylating, and inhibiting, α GlyRs. This reduces the magnitude of glycinergic IPSCs in spinal pain sensory neurons, thereby 'disinhibits' them and increasing the likelihood of pain impulses being transmitted to the brain. Thus, drugs that potentiate (i.e., restore) α GlyR IPSCs should be analgesic. Indeed, α GlyR-potentiating agents do exert potent analgesic effects in animal pain models. For example, synthetic cannabinoid derivatives modified to prevent their binding to CB1 or CB2 cannabinoid receptors exert potent analgesia via direct effects on α GlyRs (Xiong et al., 2012).

Phosphorylation-induced conformational changes in the α3 GlyR have recently been probed using voltage clamp fluorometry (VCF) (Han et al., 2013). VCF takes advantage of the fact that changes in the quantum efficiency of small molecule fluorophores occur in response to changes in their chemical microenvironment. It involves introducing a cysteine into the domain of interest and covalently tagging it with a sulfhdryl-labeled fluorophore, often a rhodamine derivative. This technique enables one to probe conformational changes in protein domains of interest in real time. Using this approach, forskolin-induced PKA phosphorylation of Ser346 in the α3 GlyR cytoplasmic domain elicited a change in the glycine-induced fluorescence response of a label attached to R19'C in the M2-M3 linker or to N203C in loop C of the glycine-binding site (**Fig. 2A**). This effect was not observed if Ser346 was mutated to prevent its phosphorylation (**Fig. 2B and 2C**). Thus, phosphorylation exerts a global conformational change that propagates from S346 in the internal M3-M4 domain to the e glycine-binding site, a distance of >80 **Å**. The experiments shown in Fig. 2 were performed in *Xenopus* oocytes where phosphorylation does not reduce α3 GlyR current. However, when the same receptors are expressed in HEK293 cells, phosphorylation reduced current magnitude and produced a conformational change in the glycine-binding site (Han

et al., 2013). This conformational change appears to be unique because it is accompanied by an increase in both glycine sensitivity and the potency of the competitive antagonist, tropisetron (Han et al., 2013). These results raise the possibility of developing analgesic drugs to specifically target disease-affected receptors.

*3.2 Phosphorylation-mediated conformational and functional changes in other pLGICs*

Phosphorylation also appears to induce conformational changes in $GABA_A$Rs. For example, PKC activators altered the efficacy of benzodiazepines and barbiturates in neuronal $GABA_A$Rs (Gao and Greenfield, 2005). Another study reported that PKC activation reduced the amplitude of GABAergic IPSCs in a manner that was completely inhibited by pre-treatment with neurosteroids (Brussaard et al., 2000). Other studies suggest different PKC isoforms differentially affect $GABA_A$R alcohol sensitivity (Song and Messing, 2005, Qi et al., 2007). Importantly, these later effects have been implicated in alcoholism. Several other examples of $GABA_A$R subunit phosphorylation sites that have been linked to receptor functional changes and neurological disease are summarized in **Table 1**.

Many neuronal nAChRs are also regulated by phosphorylation. For example, dephosphorylation of α7 nAChRs caused a rapid increase in acetylcholine-evoked current and alterations in α-bungarotoxin sites with no change in receptor surface expression (Wiesner and Fuhrer, 2006).

Widely expressed in the brain, the α4β2 nAChRs are proposed to play a major role in the mechanisms that cause nicotine addiction. Another study reported that PKA phosphorylation at Ser467 in the M3-M4 domain increases the relative abundance of α4 available for assembly, which favoured the functional expression of low affinity α4β2 receptors (Bermudez and Moroni, 2006). In addition, previous studies had shown the possible functional significance of phosphorylation/dephosphorylation on α4β2 receptor desensitization at the Ser368 residue. Phosphorylation-mediated time course recovery from desensitization implicated different receptor conformations with continuous nicotine exposure leading to an irreversible desensitized conformation (reviewed in (Quick and Lester, 2002). Recently, a study by Pollock and colleagues reported a dual effect of PKC at multiple sites of α4 subunits associated with mature and immature receptors. Nicotine has been implicated as a chaperone to promote subunit assembly and stabilize the α4β2 receptors at the cell surface (Pollock et al., 2009). Other examples of neuronal nAChR subunit phosphorylation sites that have been linked to receptor functional changes and neurological disease (notably nicotine addiction and cognitive impairment) are summarized in **Table 1**.

Muscle nAChRs have also been demonstrated as phosphorylation targets. PKA phosphorylates γ and δ subunits, PKC phosphorylates δ and α subunits and PTK phosphorylates β, γ and δ subunits (Swope et al., 1999). Phosphorylation of muscle nAChRs is related mainly to myasthenia gravis (**Table 1**).

## 4. Possible medical applications

Here we demonstrate that neurological disorders can result from conformational changes induced by the 'inappropriate' phosphorylation of pLGICs. For example, PKA-mediated phosphorylation α3 GlyRs (which causes chronic pain) inhibits current and causes a global conformational change that changes the structure of the glycine-binding site (Han et al., 2013), and alcoholism can result from a phosphorylation-induced increase in ethanol sensitivity of γ2-containing $GABA_A$Rs (Qi et al., 2007). We propose that in these and other neurological disorders, phosphorylation induces a global allosteric conformational change that alters the structure of orthosteric or allosteric binding sites.

In classical allosteric theory, receptors are considered to exist in either inactive or active states and drugs that modify their function do so by stabilising one of these states (Nussinov and Tsai, 2014). In reality the situation is much more complex with numerous states existing in unmodified receptors, and even more that can be induced via covalent modifications (such as phosphorylation) or protein-protein interactions. We suggest that an ideal therapeutic drug would bind specifically to a phosphorylated (disease-affected) receptor and promote a conformational change that specifically compensates for the deleterious effect of phosphorylation. As far as we are aware, no such drug has yet been developed for any receptor type. However, there is no reason why this should not be feasible given that phosphorylation is well known to modulate the ability of enzymes to bind specifically to their substrates.

The aim of this brief commentary is to raise awareness of the fact that phosphorylation can cause global allosteric conformational changes in pLGICs and to suggest that these conformations could in principle be targeted by drugs with therapeutic potential.

## Acknowledgements

Research in the author's laboratory is supported by the National Health and Medical Research Council of Australia and the Australian Research Council. S.T. is thankful to Queensland Education and Training International and to The University of Queensland for PhD scholarship support.

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**Figure Legends**

**Fig. 1.** Structure and phosphorylation of pLGICs. **(A)** Side view of pentameric α3 GlyR homology model viewed parallel to the membrane showing five identical subunits, each colored differently. The large M3-M4 cytoplasmic loop is shown as a dashed line as its structure is not known. **(B)** Top down view of the same pentameric assembly showing the central pore and the (+) and (-) subunit interfaces. **(C)** Close-up side view of an extracellular domain subunit interface showing ligand-binding loops A, B and C on the (+) side of the subunit interface and loops D, E and F on the (-) side of the interface. **(D)** Ion channel phosphorylation pathway. (i) Phosphorylation starts with the binding of ATP to the catalytic site of the protein kinase. (ii) Binding of ATP is followed by protein kinase binding to the substrate receptor. (iii) Followed by γ-phosphate transfer from ATP to Ser/Thr or Tyr of the receptor phosphorylation site. (iv) Following phosphorylation, kinase leaves the receptor with ADP bound, following which ADP is released. **(E)** Phosphorylation of pLGICs leads to many possible outcomes, some of which are indicated.

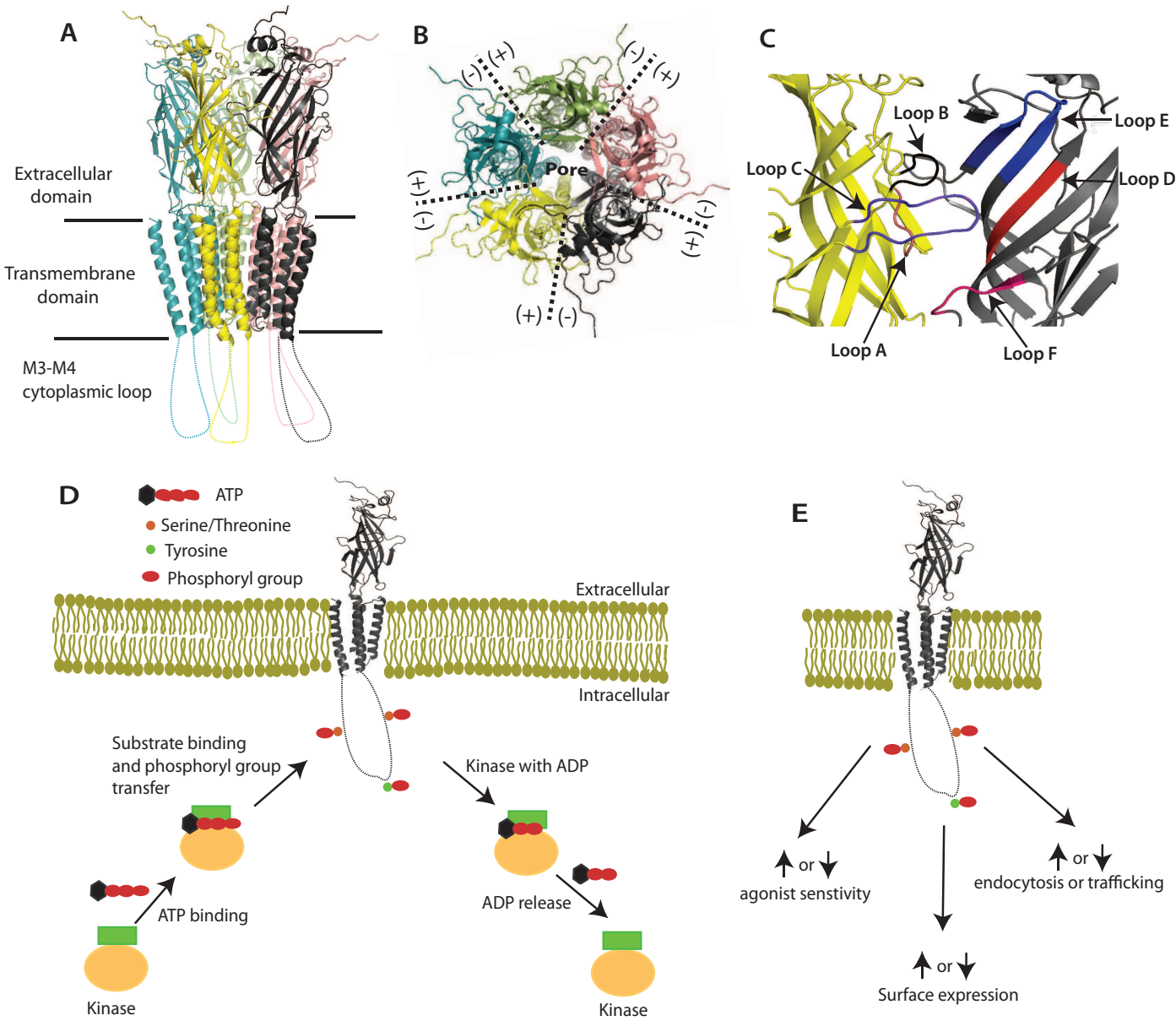
**Fig. 2.** Phosphorylation-mediated conformational changes in the α3 GlyR expressed in *Xenopus laevis* oocytes. **(A)** Cartoon depicting location of labelled residues for VCF in the ligand-binding site (N203C; green), M2-M3 linker (R19'C; blue) and the phosphorylation site (S346G, magenta). **(B)** Example of $EC_{50}$ glycine-induced current (ΔI) and fluorescence (ΔF) responses in fluorescently-labelled α3-R19'C and α3-R19'C-S346G GlyRs before and after a 15 min forskolin (FSK) treatment and after a 15 min wash. Averaged results (right panel) reveal that forskolin inhibits glycine-mediated fluorescence responses only in α3 GlyRs containing a functional S346 phosphorylation site. **(C)** Example and averaged data of saturating glycine- and strychnine-induced ΔI and ΔF responses in fluorescently-labelled α3-N203C and α3-N203C-S346G GlyRs before and after a 15 min forskolin treatment and after 15 min wash. Figure modified from Han et al. (2013).

Table 1: Effect of phosphorylation on pLGICs and their relevance to diseases.

| Receptor | Subtype | Phosphorylation Site | Protein Kinase | Effect on Receptor Function | Disease relevance | Reference |
|---|---|---|---|---|---|---|
| GlyR | α3 | S346 | PKA | Inhibition of glycinergic currents | Chronic inflammatory pain | (Harvey et al., 2004) |
| $GABA_AR$ | β1 | S409 | PKA, PKC | Enhanced desensitization and prolonged deactivation | Epilepsy | (Hinkle and Macdonald, 2003) |
| | β3 | S408, S409 | PKC | Alterations in cell surface expression. | Status epilepticus | (Terunuma et al., 2008) |
| | | | PKC | BDNF-mediated transient increase in receptor function followed by down regulation | Neuronal Excitotoxicity | (Jovanovic et al., 2004) |
| | | S383 | CamKII | Rapid insertion of extrasynaptic receptors at the cell surface and enhanced tonic currents in hippocampus | Neuropsychiatric disorders | (Saliba et al., 2012) |
| | γ2 | S327 | PKC | Alterations in ethanol and benzodiazepine sensitivity | Alcoholism | (Qi et al., 2007) |
| | | S343 | PKC | Increased amplitude of mIPSCs | Alcoholism | (Song and Messing, 2005) |
| | | Y365, Y367 | Src | Increased postsynaptic receptor expression and enhanced frequency of hippocampal mIPSCs | Spatial memory deficits | (Tretter et al., 2009, Luscher et al., 2011) |
| | α4 | S443 | PKC | Increased cell surface stability and activity | Temporal lobe epilepsy | (Abramian et al., 2010) |
| | α7 | Y386, Y442 | Src | Increased current amplitudes with no change in surface receptor number | Cognition and addiction | (Charpantier et al., 2005) |
| | | Y386, Y442, Y317 | Src | Increased surface receptor numbers with no change in open probability | | (Cho et al., 2005) |
| nAChR (neuronal) | α4 | S368 | PKC | Prolonged nicotine-induced desensitization | Nicotine addiction | (Wecker et al., 2001) |
| | | S365,S472, S491 | PKA | Increased surface expression | | (Guo and Wecker, 2002) |
| | | S467 | PKA | Favoured expression of low-affinity receptors | | (Bermudez and Moroni, 2006, Pollock et al., 2009) |
| | | S550 | PKC | Dual action affecting mature receptors to stabilize the receptors at cell surface and increase transport of immature receptors from endoplasmic reticulum to membrane | | (Pollock et al., 2009) |
| | β | Y390 | Src | Decreased receptor turnover and metabolic stabilisation | Myasthenia gravis | (Rudell and Ferns, 2013) |
| | | Y355 | Src | Enhanced desensitization and immobilization of the receptor | | (Tzartos et al., 1993) |

| | | | | | | |
|---|---|---|---|---|---|---|
| nAChR (muscle) | δ | S361, S362 | PKA | Enhanced desensitization | Myasthenia gravis | (Hoffman et al., 1994) |
| | | Y372 | Src | Stable localisation of the receptor during synaptogenesis | | (Wagner et al., 1991) |
| | γ | S353, S354 | PKA | Enhanced desensitization | | (Hoffman et al., 1994) |
| | | | | | | |

**Figure 1**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Extracellular domain
Transmembrane domain
M3-M4 cytoplasmic loop
B
Pore
(-)
(+)
C
Loop B
Loop C
Loop E
Loop D
Loop A
Loop F
D
ATP
Serine/Threonine
Tyrosine
Phosphoryl group
Extracellular
Intracellular
Substrate binding and phosphoryl group transfer
ATP binding
Kinase
Kinase with ADP
ADP release
Kinase
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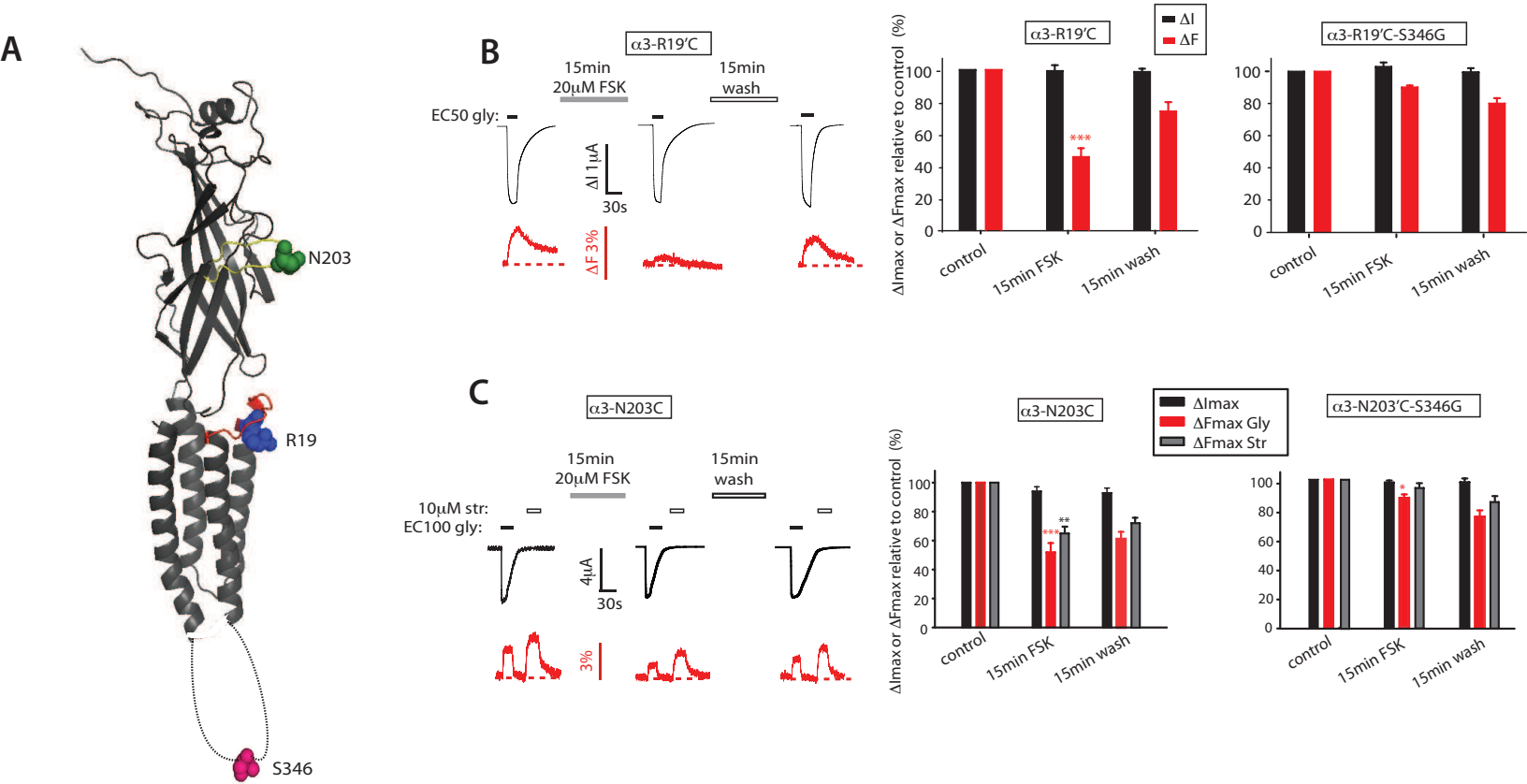
or
agonist senstivity
or
endocytosis or trafficking
or
Surface expression

**Figure 2**


A
N203
R19
S346
B
α3-R19'C
15min
20μM FSK
15min
wash
EC50 gly:
ΔI 1μA
30s
ΔF 3%
ΔI
ΔF
α3-R19'C
α3-R19'C-S346G
ΔImax or ΔFmax relative to control (%)
control
15min FSK
15min wash
C
α3-N203C
10μM str:
EC100 gly:
4μA
3%
ΔImax
ΔFmax Gly
ΔFmax Str
α3-N203'C-S346G