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Differential regulation of synaptic and extrasynaptic α4 GABA(A) receptor populations by protein kinase A and protein kinase C in cultured cortical neurons

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Abstract

The $GABA_A$ α4 subunit exists in two distinct populations of $GABA_A$ receptors. Synaptic $GABA_A$ α4 receptors are localized at the synapse and mediate phasic inhibitory neurotransmission, while extrasynaptic $GABA_A$ receptors are located outside of the synapse and mediate tonic inhibitory transmission. These receptors have distinct pharmacological and biophysical properties that contribute to interest in how these different subtypes are regulated under physiological and pathological states. We utilized subcellular fractionation procedures to separate these populations of receptors in order to investigate their regulation by protein kinases in cortical cultured neurons. Protein kinase A (PKA) activation decreases synaptic α4 expression while protein kinase C (PKC) activation increases α4 subunit expression, and these effects are associated with increased β3 S408/409 or γ2 S327 phosphorylation respectively. In contrast, PKA activation increases extrasynaptic α4 and δ subunit expression, while PKC activation has no effect. Our findings suggest synaptic and extrasynaptic $GABA_A$ α4 subunit expression can be modulated by PKA to inform the development of more specific therapeutics for neurological diseases that involve deficits in GABAergic transmission.

INTRODUCTION

$GABA_A$-Rs are ligand-gated ion channels that mediate the majority of inhibitory neurotransmission in the CNS. $GABA_A$-Rs are normally heteropentamers that are composed of two α(1–6), two β(1–3), and either a γ (1–3) or δ subunit. The presence of either the γ or δ

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subunit in the assembled receptor influences receptor localization and consequentially the type of $GABA_A$ergic neurotransmission. $GABA_A$-Rs containing the γ subunit, are located synaptically and mediate phasic inhibition[1,2]. Conversely, the δ-containing $GABA_A$-Rs are located exclusively extrasynaptically and mediate tonic inhibition[2,3]. Both synaptic and extrasynaptic $GABA_A$-Rs are crucial to maintaining overall neuronal excitability[3].

The α4 subunit is present in both synaptic and extrasynaptic $GABA_A$-Rs in the mammalian cerebral cortex. The α4γ and α4δ-containing $GABA_A$-Rs have unique physiological and pharmacological properties. α4γ2-containing $GABA_A$-Rs have a lower affinity for GABA but faster desensitization than α4δ-containing $GABA_A$-Rs[4–6]. In addition, other endogenous modulators such as $GABA_A$ergic neuroactive steroids, exhibit higher potency at α4δ-containing $GABA_A$-Rs than α4γ2-containing $GABA_A$-Rs[4]. Both α4-containing $GABA_A$-Rs assemblies are insensitive to classic benzodiazepine agonists such as diazepam[4] although the structurally related benzodiazepine derivatives, imidazobenzodiazepines, such as Ro15-4513 display activity at both receptor subtypes[7]. α4δ $GABA_A$-Rs are also potentiated by low millimolar concentrations of ethanol while α4γ2 $GABA_A$-Rs require a higher concentration[7–9], although this data is controversial, as not all labs have found that δ-containing $GABA_A$-Rs are sensitive to low concentrations of ethanol[10]. Thus, differences in the pharmacological and physiological characteristics of these $GABA_A$-Rs have generated considerable interest in the contributions of these receptors to both physiological and pathological disease states.

Both α4-containing $GABA_A$-R populations have been implicated in multiple disease states, such as alcohol dependence, fragile X syndrome, epilepsy, schizophrenia, and depression[11]. In some disease states, such as alcohol dependence[12], epilepsy[13,14], and schizophrenia[15], downregulation of the δ subunit is accompanied by upregulation of α4γ2-containing $GABA_A$-Rs, suggesting that this change in overall $GABA_A$-R population may be important to the pathogenesis of these diseases. In non-pathological states, genetic ablation of the δ subunit also resulted in an increase in γ2 subunit expression in the cerebellar granule cells[16]. Despite these observations, the intracellular mechanisms that regulate changes in expression of the α4δ and α4γ2 receptors are still largely unknown.

PKA and PKC have long been known to regulate $GABA_A$-R expression either through direct phosphorylation of $GABA_A$-R subunits or through proteins associated with $GABA_A$-Rs[17–19]. PKA is known to modulate expression and function of $GABA_A$-Rs through direct phosphorylation on the β3 subunit serine site S408/409[20]. PKC has been shown to phosphorylate sites on the $GABA_A$ subunits at α4 S443[19], β2 S410, β3 S408/409[20], and γ2 S327[21]. Phosphorylation on these sites contributes to different trafficking[22], stabilization[23], internalization[24], or expression[25], depending on both the phosphorylation site and the composition of the $GABA_A$-R[26]. In addition to direct regulation by protein kinases, indirect regulation of signal transduction by G-coupled protein receptors has also been shown to effect $GABA_A$-R expression and function[27–29].

However, it is still unknown whether PKA and PKC regulate both synaptic and extrasynaptic populations of α4-containing $GABA_A$-Rs. Therefore, we were interested in the role of these two kinases in $GABA_A$-R subunit expression. Previous work in our lab has suggested that

PKA and PKC have opposing effects on $GABA_A$ α4 subunit expression in the presence of ethanol in cortical neurons[25,30]. Thus, the present study sought to determine if PKC and PKA were involved in regulation of both the synaptic and extrasynaptic populations of α4 $GABA_A$-Rs in the absence of ethanol.

MATERIALS AND METHODS

Primary cortical neuron cell culture and treatments

All experiments were conducted in accordance with guidelines from the National Institutes of Health and Institutional Animal Care and Use Committee at the University of North Carolina. Postnatal day 0–1 Sprague Dawley rat pups of both sexes were decapitated and cortices were isolated and cultured as previously described[31]. Neurons were maintained *in vitro* for 18 days in DMEM, B27 (1%, Invitrogen), and penicillin/streptomycin (15 days, 50 U, Invitrogen). On day 18, drugs were diluted in ddH_2O or DMSO. PKA was activated with Sp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (Sp-cAMPs, 50 μM, Sigma Aldrich) and inhibited with Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt (Rp-cAMPS, 50 μM, Sigma Aldrich). PKC was activated with Phorbol 12,13-dibutyrate (PDBu, 100 nM, Sigma Aldrich) and inhibited with Calphostin C (CalC, 300 nM, Sigma Aldrich, St. Louis) All control experiments were exposed to equal volume ddH_2O. All drug exposures were for one hour since previous experiments showed PKA and PKC both alter $GABA_A$ receptors at this time point[25,30].

Quantitative PCR

Following treatment, cells were homogenized in Trizol according to manufacturers instructions. RNA was purified using Direct-zol RNA miniprep kits (Zymo) and 260/280 and 260/230 ratios >1.8 were determined using a Nanodrop 1000 (ThermoScientific). RNA was reversed transcribed into cDNA using High Capacity RNA-to-cDNA kit (Applied Biosystems). qPCR was performed using 10 ng of cDNA per reaction, TaqMan Gene Expression Assays (Life Technologies), and TaqMan Gene Expression Master Mix (Life Technologies). Each reaction was run in duplicate and analyzed with the $\Delta\Delta C_t$ method with glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) as a loading control.

Membrane, synaptic and extrasynaptic fractionation

Membrane, synaptic and extrasynaptic fractions were produced as described previously[5,32–35]. Following drug exposures, cells were lysed by brief sonication in PBS containing 0.32M sucrose. The nuclear fraction and cell debris were removed by centrifugation at 1,000×g for 10 minutes at 4 °C. The membrane fraction was produced by centrifugation of the supernatant at 12,000×g for 30 minutes at 4 °C. The resulting pellet was resuspended in 0.32M sucrose PBS buffer containing 0.5% (v/v) Triton-X100 and incubated at 4°C under rotation for 20 minutes. The synaptic fraction was produced by centrifugation at 32,000×g for 20 minutes at 4 °C. The resulting pellet containing the synaptic fraction was resuspended in PBS containing protease and phosphatase inhibitors. The supernatant containing the extrasynaptic fraction was incubated in acetone (1:8 v/v) overnight at −20°C to insolubilize and concentrate the protein. This solution was pelleted by centrifugation at 3000×g for 15 min at 4 °C. The resulting pellet containing the extrasynaptic

fraction was resuspended in PBS containing protease and phosphatase inhibitors (Halt™, ThermoScientific).

Biotinylation for isolation of surface proteins

Isolation of surface proteins with biotinylation was performed using the Cell Surface Protein Isolation Kit (Pierce) according to manufacturers instructions. An aliquot was taken before avidin pulldown in order to analyze expression in the total fraction. The eluted biotinylated fraction was then subjected to western blot analysis. Surface expression was analyzed as the ratio of α4 in the biotinylated fraction over α4 in the total fraction. β-actin was probed in the biotinylated fraction as a control to insure that there were no intracellular proteins in the biotinylated fraction. Results were then normalized to the control for each fraction.

Western blot analysis

Protein concentrations were determined using BCA assay (Pierce). 30–50μg of protein was electrophoresed on 4–16% Tris-Glycine polyacrylamide gels (Biorad) and transferred to iBlot PVDF membranes (Invitrogen), blocked for 1 h in 1–5% BSA and incubated overnight at 4 °C with either anti-$GABA_A$ α4 (Abcam, #ab117080, 1:500), anti-$GABA_A$ β2 (Novus, #NB300-198, 1:1000), anti-$GABA_A$ β3 (Novus, #NBP1-47613, 1:1000), anti-$GABA_A$ δ (Novus, #3002-200, 1:750), anti-$GABA_A$ γ2 (Novus, #NB300-190, 1:1000), anti-$GABA_A$ phospho-γ2 (Ser^{327})(PhosphoSolutions, p1130-327, 1:1000), anti-$GABA_A$ phosphor-β3 (Novus, NBP2-29508, 1:1000) anti-PSD95 (Novus, #NB300-198, 1:2000), anti-Gephyrin (BD Transduction, #610584, 1:1000), anti-neuroligin2 (Alomone Labs, ANR-036, 1:1000) or β-actin (Novus, #NB600-501, 1:3000) Membranes were then incubated with peroxidase-labeled secondary antibodies (Mouse, rabbit, goat, Jackson Laboratories, 1:10000) and signals were developed using ECLPrime (GE) on a LAS 4000 Imager (GE). Bands were quantified using GE ImageQuant software and normalized to β-actin to control for loading.

Statistical analysis

Student's *t* test was used to determine significance for all comparisons between two groups. One-way ANOVA was used to determine significance for more than two groups. Tukey's posthoc test was used to determine significance between groups after one-way ANOVA. All analysis was performed using GraphPad Prism (version 6).

RESULTS

PKA and PKC activation have opposite effects on α4 $GABA_A$-R subunit expression

PKA and PKC activation are known to have opposite effects on $GABA_A$-R function and expression cerebral cortical neurons in the presence of ethanol[25,30]. To determine if PKA and PKC activation cause changes in α4 expression independent of ethanol, we activated PKA with Sp-cAMPs and activated PKC with PBDu then prepared membrane fractions and analyzed α4 expression using western blots (Fig 1A). Our results indicate that PKA activation decreases α4 expression (Fig 1B) while PKC activation increases α4 expression (Fig 1C) in the membrane fraction. We next used quantitative PCR to determine if there were also changes in gene transcription. PKA activation increases *Gabra4* expression (Fig 1D) while PKC activation caused no change in *Gabra4* expression (Fig 1E). We also used

qPCR to analyze *Gabrd* and *Gabrg2* expression after all four drug treatments and found no significant changes in either transcript (*Gabrd*: Sp 1.21±0.19; Rp 0.83±24; PDBu 1.22±0.45; CalC 1.14±0.36. *Gabrg2*: Sp 1.18±0.22; Rp 1.00±0.08; PDBu 1.20±0.41; CalC 1.17±0.11). To determine if PKA and PKC activation caused changes in surface expression we used biotinylation to isolate surface receptors. Our results indicate that PKA activation decreases α4 surface expression (Fig 1F) while PKC activation increases α4 surface expression (Fig 1G). There was no change in total α4 expression levels after either PKA or PKC activation (data not shown).

Synaptic and extrasynaptic receptors can be separated by detergent Triton X-100

Since the $GABA_A$ α4 subunit is present in both synaptic and extrasynaptic populations of $GABA_A$-Rs, we utilized a biochemical approach previously used for glutamate[32] and $GABA_A$ receptors[5,33] to investigate both synaptic and extrasynaptic receptors. We validated this approach for separation of $GABA_A$ receptors in cortical cultured neurons by probing for synaptic markers neuroligin 2, gephyrin, postsynaptic density protein-95 and for the $GABA_A$ δ subunit that is exclusively localized extrasynaptically (Fig 2)[3]. The GABA specific synaptic markers, neuroligin 2 and gephyrin were highly enriched in the synaptic fraction, along with postsynaptic density protein 95, while the $GABA_A$-R δ subunit was enriched exclusively in the extrasynaptic fraction. Of note, the $GABA_A$-R α4, α1, and γ2 subunits were found in both fractions as expected from previous studies[1–3,5].

PKA and PKC regulation of synaptic α4 $GABA_A$-Rs

Following validation of our strategy to separate synaptic and extrasynaptic populations of $GABA_A$-Rs, we determined if PKA and PKC regulate the two different populations of α4-containing $GABA_A$-Rs. Activation of PKA decreased synaptic α4 expression (Fig 3B) while activation of PKC increased synaptic α4 expression (Fig 3F). We next analyzed γ2 expression, and found that PKA activation caused a decrease in γ 2 expression (Fig 3C) while PKC activation did not alter γ2 expression (Fig 3G). We found that neither PKA activation nor PKC activation caused a change in β3 expression (Fig 3A, Sp-cAMPs 110.4±14.9 % control; PdBU 99.9±23.4 % control).

We next determined if inhibition of PKA or PKC caused changes in synaptic α4 expression. We inhibited PKA with the cAMP derivative Rp-cAMPs and found that there was no change in $GABA_A$ α4 expression (Fig 3A). Inhibition of PKC with CalC also caused no change in expression (Fig 3A).

The β3 subunit has two known PKA and PKC phosphorylation sites on S408 and S409, therefore we were interested if activation of PKA or PKC caused direct phosphorylation of β3 S408/409. Interestingly, we found that PKA activation increased phosphorylation on β3 S408/409 (Fig 3D), but PKC activation did not (Fig 3H). Intrigued by the lack of PKC-induced phosphorylation of β3 S408/409 on the β3 subunit, we next tested to see if there was increased phosphorylation on γ2 S327, another known $GABA_A$-R site phosphorylated by PKC but not PKA. PKC activation increased phosphorylation of γ2 S327 (Fig 3I). As expected, PKA activation did not increase γ2 S327 phosphorylation (Fig 3E).

PKA and PKC regulation of extrasynaptic α4 $GABA_A$-Rs

Regulation of extrasynaptic $GABA_A$-Rs by protein kinases is still poorly understood, and to date, no definitive phosphorylation site has been identified on the δ subunit despite the intracellular loop (316–410) of the $GABA_A$ δ subunit containing putative serine phosphorylation sites for PKA and PKC at δ S305/404 and δ S364/390 respectively (http://www.cbs.dtu.dk/services/NetPhos/, accessed 3/4/15)[36]. In the present study, we found that activation of PKA caused an increase in the expression of α4 (Fig 4B) and δ subunits (Fig 4C) in the extrasynaptic fraction, while activation of PKC did not change in either extrasynaptic α4 (Fig 4C) or δ expression (Fig 4F). We then inhibited PKC with CalC and analyzed the extrasynaptic fraction by western blot. Interestingly, we found that inhibition of PKC resulted in a decrease in δ subunit expression (51.9±10.4 % control, Fig 4A) but no change in α4 expression (109.4±12.34 % control, Fig 4A), suggesting that PKC inhibition may alter other extrasynaptic δ-containing $GABA_A$-Rs. We found that neither PKA nor PKC activation altered β3 S408/409 phosphorylation (Fig 4A, D), or β3 expression (Fig 4A; Sp-cAMPs 111.4±7.2 % control; PDBu 103.4±15.0 % control) in the extrasynaptic fraction.

Effects of PKA and PKC activation can be prevented

Since pharmacological activation of PKA and PKC may have off target or downstream effects, we next wanted to test that the activators were selective for PKA and PKC. Therefore, we simultaneously activated and inhibited PKA and analyzed α4 expression in the synaptic and extrasynaptic fractions. Our results indicate that simultaneous activation and inhibition of PKA prevented changes in α4 expression in the synaptic and extrasynaptic fractions (Fig 5A–C). Simultaneous activation and inhibition of PKC prevented the increase of α4 expression in the synaptic fraction, and had no effect on the extrasynaptic α4 expression (Fig 5B–E).

Since PKC and PKA appear to regulate synaptic $GABA_A$-Rs in opposing directions (Fig 3B, F) we sought to determine if simultaneous activation of both PKA and PKC would negate the effects on synaptic $GABA_A$-Rs. Our results indicate that simultaneous activation of PKA and PKC restores α4 expression to control levels (Fig 5G, H). In contrast, simultaneous activation of PKA and PKC did not prevent PKA-induced up-regulation of α4 expression in the extrasynaptic fraction (Fig 5G, I), consistent with the conclusion that PKC does not regulate extrasynaptic α4 receptors.

DISCUSSION

Dysfunction of GABAergic systems that contribute to the development of neurological diseases likely stems from changes in $GABA_A$-R expression, however little is known about the underlying mechanisms that facilitates these changes. We used a pharmacological and biochemical strategy to investigate the role of PKA and PKC on $GABA_A$-Rs containing the α4 subunit. We focused on the α4 subunit because of its unique physiological properties[37] and due to its dysregulation in many disease states[11,25,38–42]. We focused on PKA and PKC because both kinases have long been known to modulate $GABA_A$-R function[17,20,21,30,43–45] and expression[18,46–48]. We found that the activation of PKA or PKC had opposite effects on α4 expression, with PKA activation decreasing α4 expression and PKC noticeably

increasing α4 expression (Fig 1). Our results further indicate that changes in $GABA_A$-R trafficking are likely responsible for changes in α4 expression, as surface expression changed, but total α4 expression did not (Fig 1). Since $GABA_A$-R expression is thought to occur through either *de novo* insertion or reinsertion following internalization[49] and since we didn't observe increased *Gabra4* mRNA levels following PKC activation we propose that increases in α4 surface expression are due to changes in receptor trafficking as opposed to *de novo* synthesis. Similarly, PKA activation increased *Gabra4* expression but decreased α4 surface expression, again indicating that changes in α4 expression are likely due to changes in $GABA_A$-R trafficking. These results indicate that PKA and PKC activation have opposite effects on α4 membrane and surface expression in cultured cortical neurons, possibly through a trafficking mechanism. Future studies will need to address if changes in surface expression of α4 occur due to stabilization, changes in insertion, or recycling of these receptors.

The α4 subunit readily assembles into two distinct receptor populations in the cortex, the primarily synaptic $\alpha4_2\beta x_2\gamma2$ and exclusively extrasynaptic $\alpha4_2\beta x_2\delta$ $GABA_A$-Rs. We utilized and validated a biochemical strategy that had previously been utilized for separation of synaptic and extrasynaptic NMDA receptors[32] and for $GABA_A$-Rs *in vivo*[5] and *in vitro*[33] to determine if there was differential regulation of these two populations by protein kinases in cultured cortical neurons (Fig 2). The enrichment of synaptic marker PSD-95 and GABAergic synaptic markers neuroligin 2[50] and gephyrin[51,52] in our synaptic preparation and the presence of the δ exclusively in our extrasynaptic fraction provide ample evidence that our protocol can be used to interrogate the expression of synaptic vs. extrasynaptic populations of $GABA_A$-Rs in cultured neurons. The presence of gephyrin outside the synaptic fraction, was surprising, but is consistent with gephyrin's role as a synaptic organizer[52], but not located exclusively at the synapse[53]. We discovered that there was significant expression of γ2 in our extrasynaptic preparation suggesting that some γ2-containing receptors are localized outside of the synapse consistent with the previous studies[52,54,55]. Differentiation of α4-containing subtypes is important, as in epileptic and alcohol dependence models, there is a downregulation of δ-containing α4 $GABA_A$-Rs and an upregulation of γ2-containing α4 $GABA_A$-Rs[11,12,56]. These receptors mediate different forms of GABAergic inhibition[3] and therefore changes in expression will have important consequences in mediating overall neuronal excitability and neurotransmission. Our methodology provides a relatively simple procedure that could be used to further investigation of endogenous populations of synaptic and extrasynaptic $GABA_A$-Rs.

PKA has been shown to modulate $GABA_A$-R responses and expression in recombinant systems and in cortical neurons[17,20,30]. Consistent with previous findings in the hippocampus[57], PKA activation caused a decrease in synaptic α4 expression and provides further evidence that PKA is a modulator of α4-containing $GABA_A$-Rs (Fig 3). Interestingly, decreased synaptic α4 expression is accompanied by an increase in phosphorylation of the known PKA phosphorylation sites and positive modulator of $GABA_A$-R function β3 S408/409[17]. Phosphorylation of this site has been shown to inhibit binding of $GABA_A$ receptors to the AP2 complex, preventing internalization[58], which appears to be at odds with our current results. However, previous studies in our lab demonstrate that PKA activation increases α1 expression and zolpidem evoked-currents[30],

suggesting that PKA simultaneously down-regulates α4-containing $GABA_A$-Rs and up-regulates α1-containing $GABA_A$-Rs. Future studies are needed to determine whether changes in β3 S408/409 phosphorylation are associated with receptors containing both the α1 or α4 subunits following PKA activation.

Like PKA, PKC modulates $GABA_A$-R function and expression in recombinant and neuronal systems[18,21,22,59]. We found that PKC activation increases synaptic α4 expression in direct opposition to our finding with PKA activation (Fig 3) but consistent with previous results that PKC increases overall α4 surface expression in COS7 cells[19] and cortical neurons[25]. These effects are likely specific to PKC as this effect was blocked by the co-exposure with the PKC inhibitor, CalC (Fig 5). We also observed an increase in γ2 S327 phosphorylation that may account for the increase of synaptic α4 expression following PKC activation. Phosphorylation of γ2 S327 has been shown to stabilize $GABA_A$-Rs at synaptic sites[60]. Further studies are needed to demonstrate that phosphorylation of γ2 S327 is required for the effects of PKC on synaptic α4 receptors.

PKA and PKC appear to be working in opposition on synaptic α4 expression as simultaneous activation of both kinases blocked changes in synaptic α4 expression (Fig 5). PKA and PKC have previously been shown to work in opposition[61] and have opposite effects on $GABA_A$-R function in a cell-type specific manner[5729]. This is also consistent with previous work showing that ethanol exposure for one hour increases both PKA and PKC membrane activity and consequently there is no change in α4 expression[30] suggesting that these kinases have opposite effects on synaptic α4 expression. PKA and PKC may compete for regulation of these receptors, as previous reports have found that PKA phosphorylation of β3 S408/409 is only increased when PKC is inhibited[18].

In contrast to findings in the synaptic fraction, we found that PKC did not regulate extrasynaptic α4 expression (Fig 4). Previous studies examining the effects of PKC on extrasynaptic $GABA_A$-Rs have demonstrated that PKC regulation of extrasynaptic $GABA_A$-Rs is complex and varies depending on receptor composition, cell type, drug exposure, and experimental temperature[19,23,24]. In recombinant systems, pharmacological activation of PKC for twenty minutes decreased α4β2δ surface expression in HEK293 cells while other studies have shown that 10 minute pharmacological activation of PKC increases α4β3δ $GABA_A$-Rs in COS7 cells[19]. PKC activation in dentate gyrus granule cells and thalamic relay neurons caused a decrease in $GABA_A$-R tonic current[24] possibly due to down-regulation of δ-containing $GABA_A$-Rs. In the hippocampus, treatment with PDBu increased α4 surface expression[19]. Other studies have found that neither activating nor inhibiting PKC had any effect on $GABA_A$-R tonic current in cerebellar granule cells[62]. Conflicting reports regarding PKC regulation of extrasynaptic $GABA_A$-Rs may be due to the presence of different PKC isoforms, $GABA_A$-R assemblies, or experimental conditions.

In contrast, we found that PKA activation increases the expression of extrasynaptic α4 $GABA_A$-Rs (Fig 4), and this effect can be blocked by simultaneous exposure with a PKA inhibitor (Fig 5) but not a PKC activator. This suggests that in contrast to synaptic α4 $GABA_A$-Rs, PKA and PKC do not work in opposition on extrasynaptic α4 $GABA_A$-Rs in cultured cortical neurons. This result agrees with functional studies conducted in our

laboratory showing increases in tonic current in cultured cortical neurons after exposure to a PKA activator but no change in tonic current after PKC activation[33] as well as other studies conducted in the visual cortex finding that PKA activation increases tonic current[63]. However, other studies have shown PKA activation decreases tonic conductance in thalamocortical neurons, while activation of metabotropic G_i/o $GABA_B$ receptors or use of PKA inhibitors increases tonic conductance[27] suggesting that PKA modulation of $GABA_A$-Rs may be brain region or cell type specific. The present results suggest that PKA has differential effects on $\alpha4_2\beta x_2\delta$ and $\alpha_2\beta x_2\gamma2$ $GABA_A$-Rs which backs work in recombinant systems showing that PKA activation increases $\alpha4_2\beta3_2\delta$ spontaneous currents, but not $\alpha4_2\beta3_2\gamma2$ spontaneous currents[59]. Future studies will need to determine the precise mechanism of how PKA activation increases $\alpha4_2\beta x_2\delta$ expression.

The current study demonstrates that PKA and PKC are regulators of α4 expression and provides insight into how activation of these kinases may facilitate changes in α4 expression. Additional studies will be required in order to determine if changes in synaptic and extrasynaptic α4 expression are due to receptor trafficking, surface stabilization, or degradation. Unfortunately, biotinylation interferes with TritonX-100 fractionation and prevents adequate separation of the synaptic and extrasynaptic fractions. Future studies using alternative methods will be needed to determine the mechanism of changes in expression of these two populations of α4 containing $GABA_A$-Rs. Future studies will also need to determine if changes in phosphorylation observed in the current study occur only on the α4-containing $GABA_A$-Rs or other $GABA_A$-R complexes assembled with a different α subunits. These studies could use the methodology that we present in the present study order to further interrogate whether these changes occur on synaptic or extrasynaptic $GABA_A$-Rs.

Overall, our present work suggests that PKA and PKC have opposing effects on synaptic α4 expression while only PKA has effects on extrasynaptic α4 expression in cortical neurons (Fig 6). The results of this study demonstrate one regulatory mechanism for the expression of extrasynaptic $GABA_A$-Rs in the cortex. This could inform diagnostic and therapeutic interventions for alcoholism, depression, epilepsy, stroke, and schizophrenia as well as broaden the knowledge and understanding of the regulation of extrasynaptic $GABA_A$-Rs and inhibitory tonic current in the cortex.

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Abbreviations

PKA	protein kinase A
PKC	protein kinase C
Sp-cAMPS	Sp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt
Rp-cAMPS	Rp-Adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt
PDBu	Phorbol 12,13-dibutyrate

CalC	Calphostin C
PSD-95	postsynaptic density protein-95

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HIGHLIGHTS

- PKA regulates the expression of synaptic and extrasynaptic α4 $GABA_A$-Rs.
- PKC regulates the expression of synaptic but not extrasynaptic α4 $GABA_A$-Rs.
- PKA and PKC have opposing effects on expression of synaptic α4-containing $GABA_A$-Rs.
- Biochemical separation of synaptic and extrasynaptic α4-containing $GABA_A$-Rs shown.

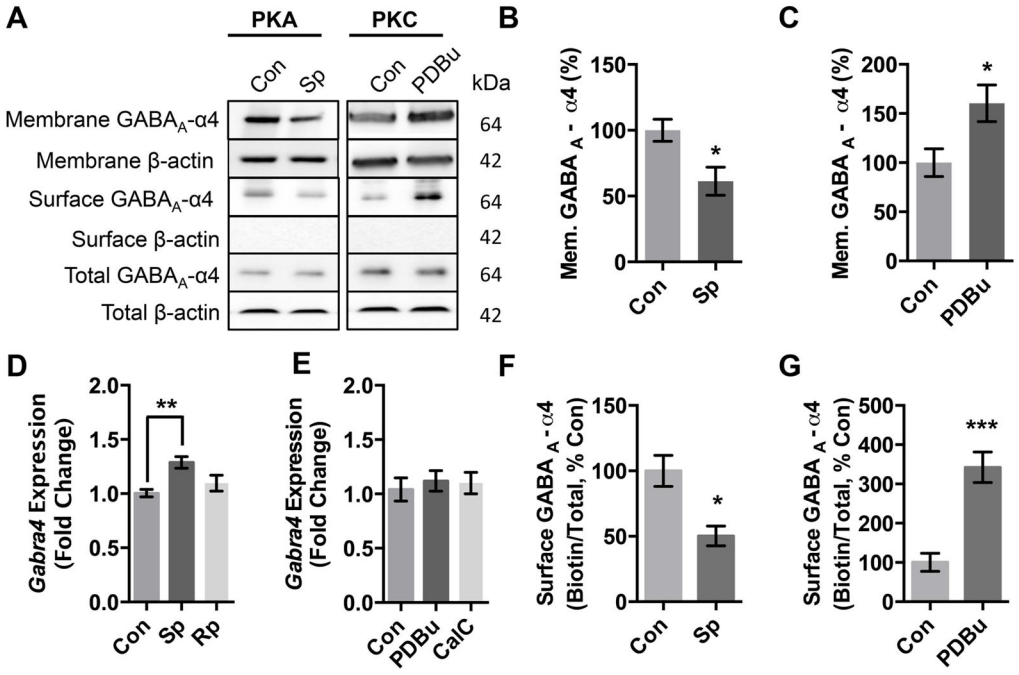

Figure 1. PKA and PKC activation have opposite effects on GABA$_A$ α4 subunit expression

(A) Cortical neurons (DIV 18) were treated 1 hr with either ddH_2O(Con), PKA activator Sp-cAMPs (Sp, 50μM) or PKC activator PDBu (PDBu, 100 nM), followed by membrane fractionation or surface biotinylation and western blot.

(B) Quantification of membrane expression of GABA$_A$ α4 subunit following PKA activation.

(C) Quantification of membrane expression of GABA$_A$ α4 subunit following PKC activation.

(D) qPCR for *Gabra4* in cortical neurons (DIV 18) treated by PKA activators and inhibitors.

(E) qPCR for *Gabra4* in cortical neurons (DIV 18) treated by PKA activators and inhibitors.

(F) Quantification of surface expression of GABA$_A$ α4 subunit following PKA activation.

(G) Quantification of surface expression of GABA$_A$ α4 subunit following PKC activation.

Values are relative to control. *p<0.05, **p<0.01. Error bars indicate ± SEM. n = 3–8 independent experiments.

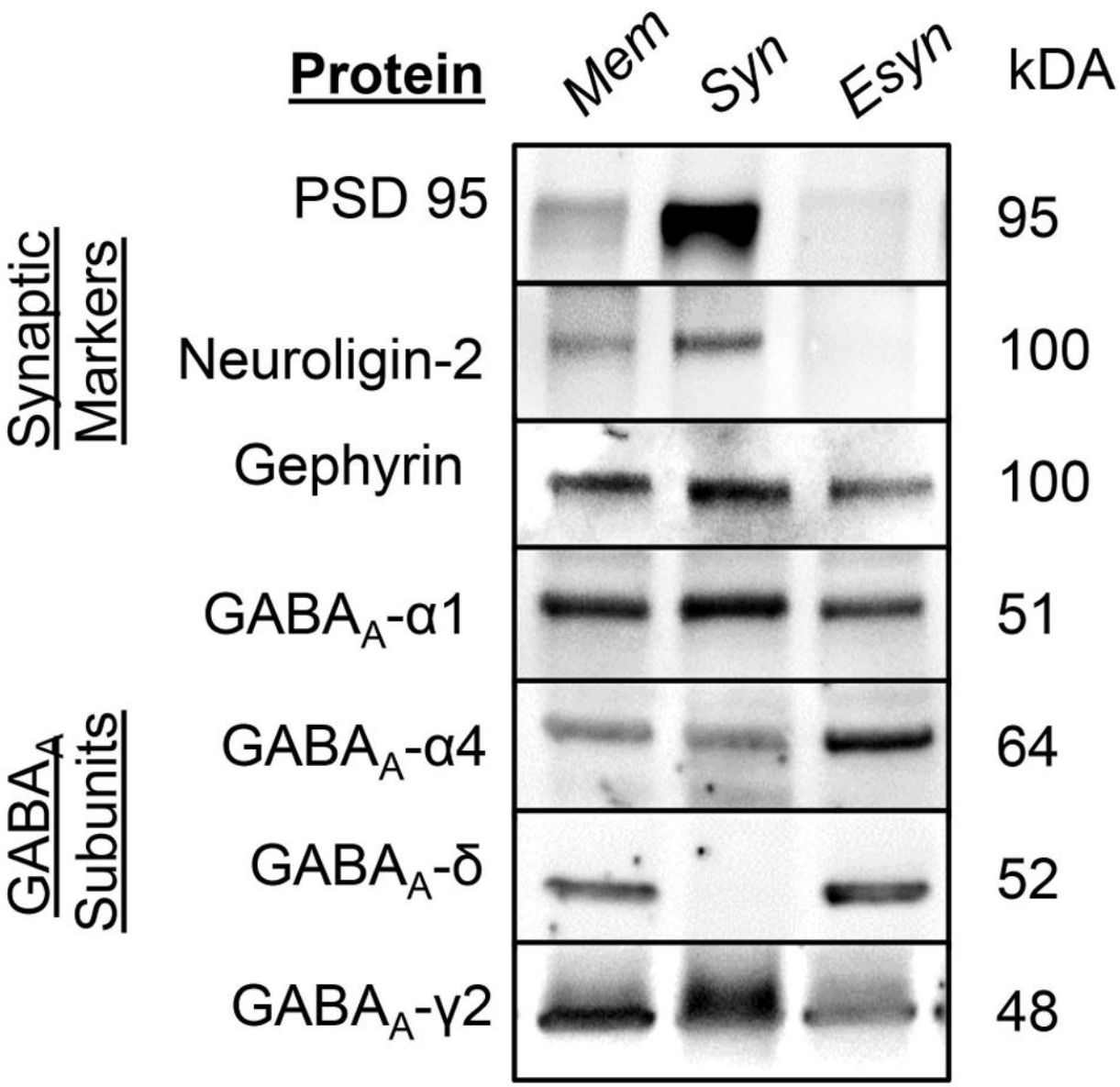

Figure 2. Synaptic and extrasynaptic populations of α4 containing GABA$_A$ receptors can be separated with Triton X-100

Representative blots showing TritonX-100 (0.5%) enriches the synaptic fraction for synaptic markers, neuroligin 2, PSD-95, and gephyrin, while the extrasynaptic fraction is enriched for GABA$_A$ δ subunit.

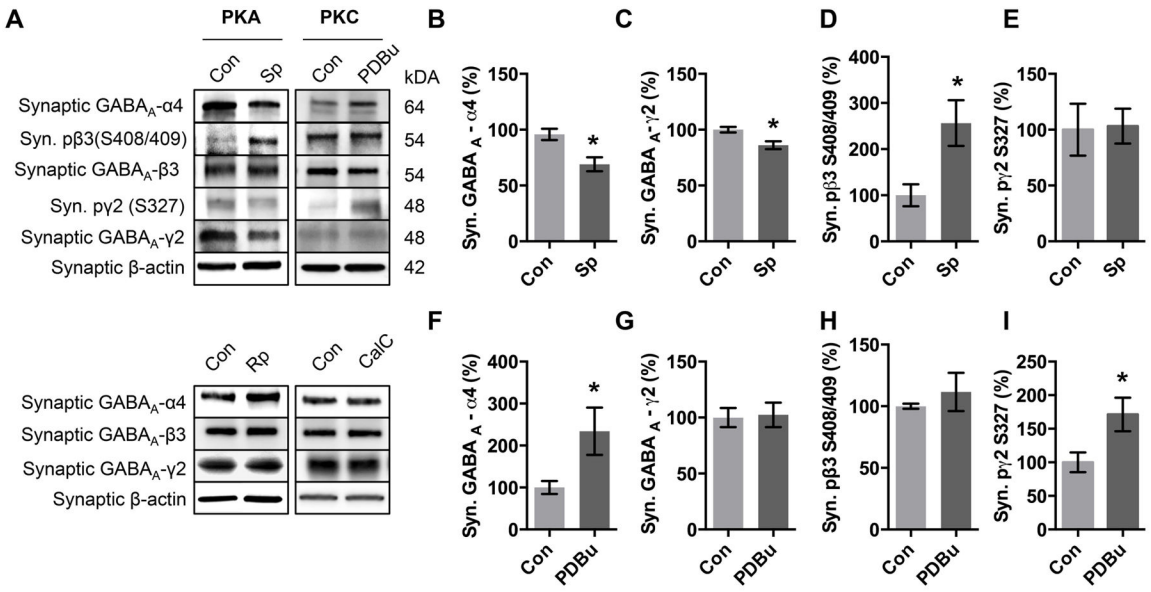

Figure 3. PKA and PKC have opposing effects on synaptic α4 subunit expression

(A) Cortical neurons (DIV 18) were treated 1 hr with either ddH_2O(Con), PKA activator Sp-cAMPs (Sp, 50μM), PKC activator PDBu (PDBu, 100 nM), PKA inhibitor Rp-cAMPs (Rp, 50 μM), or PKC inhibitor Calphostin C (CalC, 300nM) followed by isolation of the synaptic fraction and western blot.

(B) Quantification of synaptic expression of $GABA_A$ α4 subunit following PKA activation.

(C) Quantification of synaptic expression of $GABA_A$γ2 subunit following PKA activation.

(D) Quantification of extrasynaptic expression of $GABA_A$ β3 S408/409 phosphorylation following PKA activation.

(E) Quantification of synaptic expression of $GABA_A$γ2 S327 phosphorylation following PKA activation.

(F) Quantification of synaptic expression of $GABA_A$ α4 subunit following PKC activation.

(G) Quantification of synaptic expression of $GABA_A$γ2 subunit following PKC activation.

(H) Quantification of extrasynaptic expression of $GABA_A$ β3 S408/409 phosphorylation following PKC activation.

(I) Quantification of synaptic expression of $GABA_A$γ2 S327 phosphorylation following PKC activation.

Values are relative to control. *$p<0.05$, Error bars indicate ± SEM. n = 4–9 independent experiments.

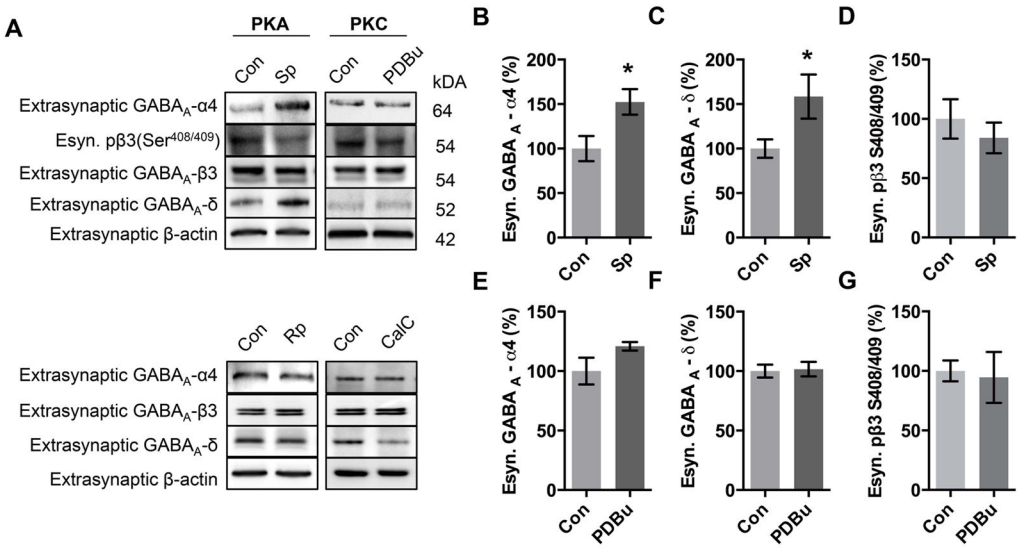

Figure 4. PKA, but not PKC activation, increases extrasynaptic α4 subunit expression

(A) Cortical neurons (DIV 18) were treated 1 hr with either ddH_2O(Con), PKA activator Sp-cAMPs (Sp, 50μM), PKC activator PDBu (PDBu, 100 nM), PKA inhibitor Rp-cAMPs (Rp, 50 μM), or PKC inhibitor Calphostin C (CalC, 300nM) followed by isolation of the extrasynaptic fraction and western blot.

(B) Quantification of extrasynaptic expression of $GABA_A$ α4 subunit following PKA activation.

(C) Quantification of extrasynaptic expression of $GABA_A$ δ subunit following PKA activation.

(D) Quantification of extrasynaptic expression of $GABA_A$ β3 S408/409 phosphorylation following PKA activation.

(E) Quantification of extrasynaptic expression of $GABA_A$ α4 subunit following PKC activation.

(F) Quantification of extrasynaptic expression of $GABA_A$ δ subunit following PKC activation.

(G) Quantification of extrasynaptic expression of $GABA_A$ β3 S408/409 phosphorylation following PKC activation.

Values are relative to control. *$p<0.05$, Error bars indicate ± SEM. n = 4–9 independent experiments.

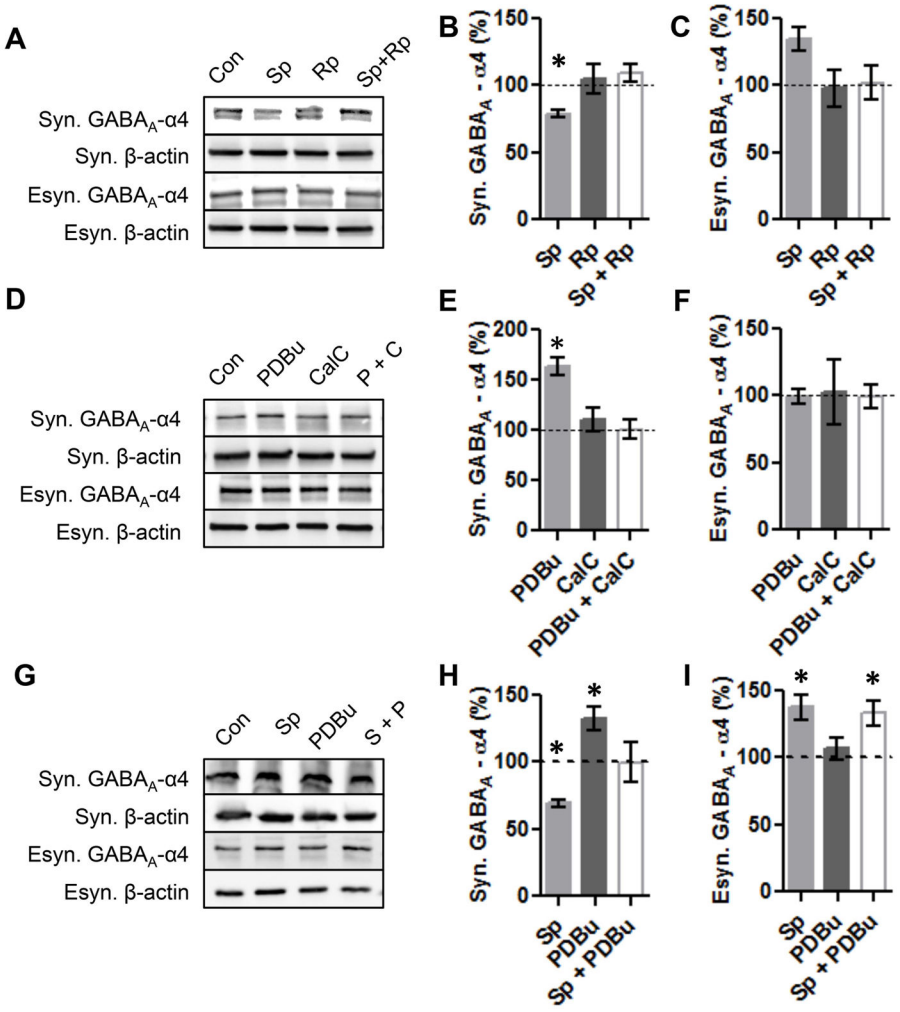

Figure 5. Effects of PKA and PKC activation on synaptic and extrasynaptic α4 expression can be blocked by PKA or PKC inhibitors

(A) Cortical neurons (DIV 18) were treated 1 hr with either ddH_2O(Con), PKA activator Sp-cAMPs (Sp, 50μM), PKA inhibitor Rp-cAMPs (Rp, 50 μM), or both followed by fractionation and western blot.

(B) Quantification of synaptic $GABA_A$ α4 expression following simultaneous PKA activation and inhibition.

(C) Quantification of extrasynaptic $GABA_A$ α4 expression following simultaneous PKA activation and inhibition.

(D) Cortical neurons (DIV 18) were treated 1 hr with either ddH_2O(Con), PKC activator PDBu (PDBu, 100 nM), and PKC inhibitor Calphostin C (CalC, 300nM), or both followed by fractionation and western blot.

(E) Quantification of synaptic $GABA_A$ α4 expression following simultaneous PKC activation and inhibition.

(F) Quantification of extrasynaptic $GABA_A$ α4 expression following simultaneous PKC activation and inhibition.

(G) Cortical neurons (DIV 18) were treated 1 hr with either ddH_2O(Con), PKA activator Sp-cAMPs (Sp, 50μM), PKC activator PDBu (PDBu, 100 nM), or both followed by fractionation and western blot.
(H) Quantification of synaptic $GABA_A$ α4 expression following simultaneous PKA activation and PKC activation.
(I) Quantification of extrasynaptic $GABA_A$ α4 expression following simultaneous PKA activation and PKC activation.
Values are relative to control. $p<0.05$, Error bars indicate ±SEM. n = 4–6 independent experiments.

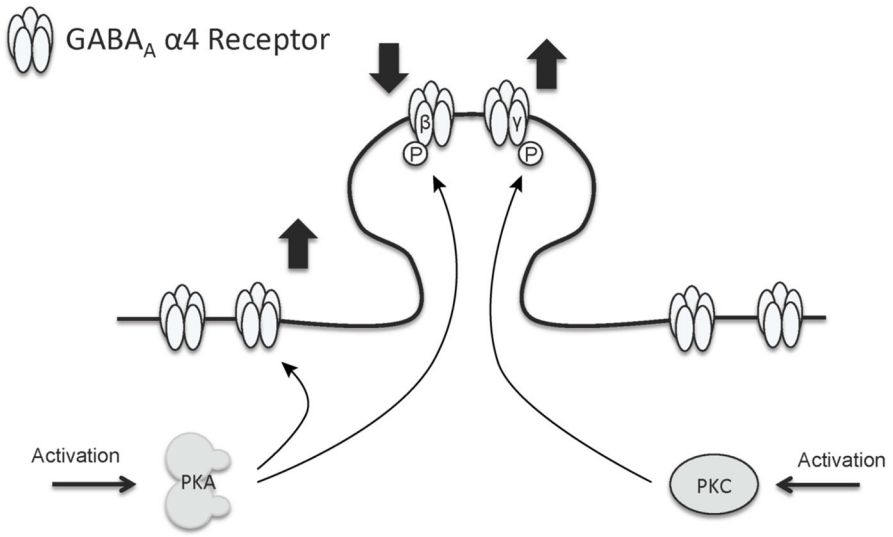

Fig 6. Model of PKA and PKC regulation of subpopulations of α4 GABA$_A$-Rs in cortical neurons

PKA activation decreases synaptic α4 GABA$_A$-R subunit expression in conjunction with increased S408/409 phosphorylation of the β3 subunit, while PKA activation increases extrasynaptic α4/δ GABA$_A$-R expression independent of β3 subunit phosphorylation, suggesting these distinct receptor subtypes are independently regulated in opposite directions by PKA. PKC activation increases synaptic α4 GABA$_A$-R subunit expression in conjunction with increased S327 phosphorylation of the γ2 subunit, while this activation has no effect on extrasynaptic α4/δ GABA$_A$-R expression. PKA and PKC have opposing effects on synaptic α4 GABA$_A$-Rs.