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Intracellular GPCRs play key roles in synaptic plasticity

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Abstract

The trillions of synaptic connections within the human brain are shaped by experience and neuronal activity both of which underlie synaptic plasticity and ultimately learning and memory. G protein-coupled receptors (GPCRs) play key roles in synaptic plasticity by strengthening or weakening synapses and/or shaping dendritic spines. While most studies of synaptic plasticity have focused on cell surface receptors and their downstream signaling partners, emerging data point to a critical new role for the very same receptors to signal from inside the cell. Intracellular receptors have been localized to nuclear, endoplasmic reticulum, lysosomes and mitochondria. From these intracellular positions, such receptors may couple to different signaling systems, display unique desensitization patterns and/or show distinct patterns of subcellular distribution. Intracellular GPCRs can be activated at the cell surface, endocytosed and transported to an intracellular site or simply activated *in situ* by *de novo* ligand synthesis, diffusion of permeable ligands or active transport of nonpermeable ligands. Current findings reinforce the notion that intracellular GPCRs play a dynamic role in synaptic plasticity and learning and memory. As new intracellular GPCR roles are defined, the need to selectively tailor agonists and/or antagonists to both intracellular and cell surface receptors may lead to the development of more effective therapeutic tools.

Graphical Abstract

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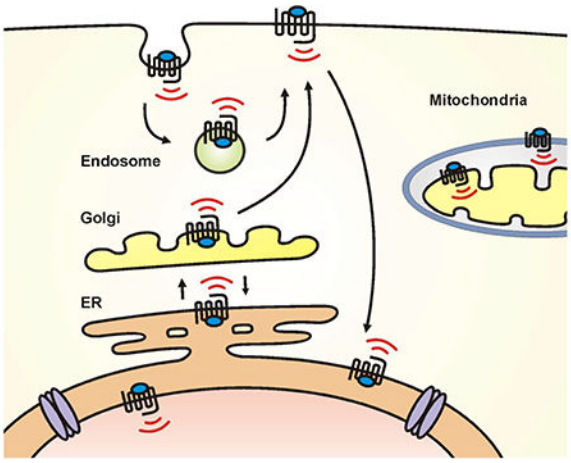

Keywords

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INTRODUCTION

The strengthening or weakening of synapses coupled with the formation or alteration of dendritic spines is thought to underlie synaptic plasticity and ultimately learning and memory. Not surprisingly, GPCRs, which play critical roles in every cellular process, also play key roles shaping how neurons respond to synaptic input which is necessary to learn new skills and generate new behaviors. Just as evidence links GPCRs to normal synaptogenesis, spine morphogenesis, and learning and memory, emerging data have also associated numerous GPCRs to pathophysiological roles in various neurodevelopmental disorders that affect learning and memory (e.g. Fragile X, Autism Spectrum Disorders, schizophrenia, ADHD). Therefore understanding how GPCRs responding to environmental stimuli achieve the necessary changes in synaptic function to learn new tasks and form new memories remains at the cutting edge of this field.

As an important site of learning and memory, the hippocampus has been extensively characterized in terms of the molecules and regulatory cues used in changing synaptic strength. Expression profiling studies (1) indicate that ~300 GPCRs are expressed in the hippocampus and that at least 20 of these receptors are known to play active roles in synaptic plasticity (2). Some like the cannabinoid CB_1 receptor mediate presynaptic plasticity whereas others such as the metabotropic glutamate receptor 5 ($mGlu_5$) primarily modulate postsynaptic processes including increases (potentiation) or decreases (depression) in synaptic strength. Given the importance of these and other GPCRs in the development of normal synaptic plasticity as well as in disorders of synaptogenesis, it's not surprising that their G protein-dependent and independent (e.g. β-arrestin) signaling pathways have been extensively characterized, at least from their classical position on the plasma membrane.

In the last decade however, emerging data show that many GPCRs also signal from inside the cell. For example, GPCRs have been found on the endoplasmic reticulum (ER) where

they are synthesized, folded, modified, and assembled, as well as in sorting vesicles on their way to the cell surface, or on endosomes that have just come off the membrane. Certain intracellular membranes may even serve as alternate destinations or even the preferred location for a number of GPCRs where they may couple to different signaling systems and exhibit distinct patterns of subcellular distribution (3–8). Some of the first examples of intracellular GPCRs include the ocular albinism I (OA1) GPCR, or GPR143 (9, 10) which localizes to melanosomes and late endosomes/lysosomes in pigmented and non-pigmented cells (11); the prostaglandin EP_3 and EP_4 receptors which signal from nuclei in many tissues including the brain (12), and $mGlu_5$ receptors which also signals from neuronal nuclei as well as from ER membranes (13). In addition, GPCRs have been found on vesicles, mitochondria (14), outer and inner nuclear membranes (3, 8, 15, 16), and even within the nucleoplasm on nuclear bodies and/or nuclear invaginations (17–19). Since so many of these receptors are also found in the brain in neurons, astrocytes and microglia, the question becomes do they play a role in processes such as synaptic plasticity and if so, what are the long terms consequences of receptor activation and how might that be different from signaling pathways activated by cell surface receptors? Many of the pioneering studies on intracellular GPCRs have been performed in peripheral systems, thus here we summarize that larger body of data so as to put more recent studies on CNS receptors in context. Where sufficient evidence exists, we have included information on GPCRs functioning on intracellular membranes such as endosomes and mitochondria (7, 20), particularly in the CNS. Finally we highlight several CNS receptors which play a role in synaptic plasticity from inside the cell.

Intracellular GPCRs

Nuclear GPCRs—GPCRs have always been found within the cell, including in the ER where they are synthesized and assembled, or in vesicles on their way to the cell surface, or on endosomes that have just come off the membrane. Previously, GPCRs in these locations were not thought to be functional but rather were considered as receptors on route to the plasma membrane, desensitized, sequestered receptors or receptors on their way for lysosomal destruction. GPCRs were also found on the nucleus. Amongst the first described were peptide receptors which were abundantly expressed both on the plasma membrane and the nucleus (21, 22). For many of these GPCRs, ligand stimulation triggers internalization of the entire receptor and subsequent trafficking to the nucleus (Fig. 1). For example, both coagulation factor II (thrombin) receptor-like 1 (F2rl1) and platelet-activating factor receptor (Ptafr) appear to internalize with their ligands bound to the receptor (16, 23). The oxytocin receptor also moves to the nuclear membrane after ligand binding (24, 25). Alternatively, peptide ligands can directly activate their cognate nuclear receptor via unknown mechanisms; ligand application shows radiolabeled colocalization of nuclear receptors followed by appropriate functional outcomes. For instance, application of radiolabeled gonadotropin-releasing hormone (GnRH) can be found in the nucleus along with the nuclear GnRH receptor where it triggers histone acetylation and phosphorylation within minutes (26).

Trafficking: There appear to be diverse mechanisms by which nuclear GPCRs arrive at this destination. For example, certain GPCRs such as the apelin, angiotensin AT_1, α1A and α1B

adenosine and bradykinin B_2 receptors use canonical nuclear localization signals (NLS), i.e. short stretches of basic amino acids, that are subsequently recognized by specific members of the karyopherin superfamily for nuclear import (4). Other GPCRs like the Ptafr traffic to the nucleus via a process involving the small GTPase, Rab11a, and importin-5 (23). Thus there is no one preferred pathway that is involved in this process nor do all nuclear GPCRs contain canonical NLS sequences. Some like $mGlu_5$ receptors contain unidentified targeting sequences which are critical for the receptor's nuclear localization (27). Interestingly, some receptors trafficked from the cell surface are not associated with nuclear membranes but rather appear within the nucleoplasm itself. These include the apelin receptor, chemokine receptor 2 (CCR2), arginine vasopressin receptor1α, sphingosine 1-phosphate receptor 1(S1P1), oxytocin receptor, and Cysteine (C)-X-C receptor 4 (CXCR4) (17, 24–32). For those GPCRs trafficked directly to the nuclear membrane, a simple diffusion-retention model has been proposed since the outer nuclear membrane is contiguous with the ER (33, 34). The diffusion-retention model suggests that proteins synthesized in the ER or retrogradely transported there, rapidly diffuse along the outer nuclear membrane before passing through peripheral channels located between the nuclear pore complex and the pore membrane to become tethered on the inner nuclear membrane via interactions with nuclear lamins or chromatin (35, 36). For example, the $mGlu_5$ nuclear trafficking motif interacts with chromatin via a basic region (pI >9.8) which may promote its nuclear retention (27). Most recently, it was reported that VPAC1, a class B GPCR shared by pituitary adenylate cyclase activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP), was trafficked to the nuclear membrane via palmitoylation of its most N-terminal cysteine (Cys37) in the extracellular domain (37). Thus, nuclear GPCRs arrive at their destination via many different signals and types of processes (Fig. 1).

Ligand Activation of Nuclear Receptors: Intracellular GPCRs can be activated at their subcellular location in a variety of ways (Fig. 2). Ligands can enter cells via diffusion or be made in situ, endocytosed, and/or transported through channels or pores (15, 22, 38). Since ligand binding sites would be within the vesicle or luminal region of the ER or nucleus, extracellular ligands would have to cross both the plasma membrane as well as the intracellular membrane to activate intracellular GPCRs (13). A highly permeable ligand might freely cross such membranes, whereas a less permeable, charged ligand might require an active transport process. Using $mGlu_5$ as an example, at least two uptake systems are responsible for transporting glutamate into a neuron: the sodium-dependent excitatory amino acid transporters and the cystine/glutamate exchanger (39, 40). Conditions that block either type of transporter reduce agonist uptake in cortical, hippocampal and striatal neurons (39, 40, 41). We and others (42) have used microinjection of soluble caged ligands followed by uncaging via restricted photoactivation to directly demonstrate activation of intracellular receptors (42, 43).

In contrast to ligand transport, ligands can also be made in situ via localized biosynthetic machinery. For example, a large number of GPCRs such as the prostaglandin, platelet-activating factor, and lysophosphatidic acid (LPA) receptors, whose ligands are bioactive lipids derived from membrane hydrolysis, are also located on nuclear membranes (44). As ligand-generating enzymes are present on nuclear membranes and because such ligands

readily diffuse through lipid bilayers, Prostaglandin E2 (PGE2), platelet-activating factor, and LPA can easily activate their cognate receptors. Alternatively, many GPCRs exhibit constitutive ligand–independent activity that might allow nuclear receptors to function (22). For example, proteins like Homer1a can lead to agonist-independent $mGlu_5$ receptor activation (45). Agonist-independent activation of the PACAP1 receptor also occurs due to a close association with the insulin-like growth factor 1 receptor and subsequent transactivation by Src (46). Thus as long as a ligand is either made in situ or transported to the site of action, an intracellular receptor can be activated (3, 15, 47; Fig. 2).

Mitochondrial GPCRs—Akin to the novelty of GPCRs being found on nuclear membranes, emerging studies are now pointing to an ever-growing list of GPCRs associated with mitochondria. For example, the angiotensin I and II receptors (AT_1R, and AT_2R) have been reported both in the nucleus and the mitochondria of several cell types. In mitochondria, AT_2Rs have been co-localized with the ligand Ang II on the inner mitochondrial membrane where activation results in nitric oxide formation and suppression of respiration in various cell types including neurons (48). Interestingly, levels of mitochondrial receptor expression can vary depending upon the cell type; in monocytes for example, there is a 40-fold difference in AT_2R in mitochondria versus on the cell surface (48). Moreover, with age AT_2R decreases whereas AT_1R becomes more abundant in mitochondria (48).

Other mitochondrial GPCRs include the purine, P2Y1 and P2Y2, receptors (49), 5hydroxytrptamine ($5\text{-}HT_4$) receptor (50), melatonin MT_1 receptors (51, 52) and cannabinoid CB_1 receptors (53). The latter are thought to play a role in synaptic plasticity described in more detail below. The purine receptors were among the first GPCRs to be localized to mitochondria where they contribute to the regulation of mitochondrial Ca^{2+} uptake. Specifically, activation of P2Y1 stimulates mitochondrial Ca^{2+} uptake whereas activation of P2Y2 inhibits this process in hepatocytes (49). In cardiomyocytes, activation of $5HT_4$ receptors also decreases mitochondrial Ca^{2+} uptake and in turn, respiratory chain activity and ATP production (50). Melatonin, as a small, lipophilic ligand, is found in high levels in mitochondria where it can activate MT_1 receptors. Very recent work shows that mitochondria can synthesize melatonin within the matrix where upon release it activates MT_1 receptors on the outer mitochondrial membrane (52). Mitochondrial MT_1 signal-transduction activates $G\alpha_i$ and blocks adenylate cyclase activity leading to the inhibition of stress-induced cytochrome c release and caspase activation (52). As further evidence of the importance of mitochondrial MT_1, its targeted overexpression inhibited neuronal death resulting from hypoxic/ischemic injury (52). Taken together, there is increasing evidence that mitochondrial GPCRs play important roles in many processes previously thought to be mediated by plasma membrane receptors.

Endosomal GPCRs—Desensitization and endosomal internalization of GPCRs is a well-known mechanism to regulate receptor number via degradation and/or resensitization. A large body of data has now shown that internalization of receptor/G protein/β-arrestin complexes can lead to stable complexes generating sustained endosomal signals (54). Although initially arrestin-mediated signaling focused on the mitogen-activated protein

kinase/extracellular signal-regulated kinase1/2 (MAPK/ERK1/2) cascade, many other signaling moieties can interact with the receptor/G protein/β-arrestin complex such as protein kinase B (Akt), p38MAPK, c-Jun amino-terminal kinases (JNKs), and activators of transcription (STATs) (55). In turn, these proteins mediate downstream functions such as growth, cell survival, apoptosis, contractility, cell migration and cytoskeletal reorganization (55). Many drug discovery teams are searching for distinct ligands that can modulate β-arrestin, G-protein-independent processes versus G-protein-dependent pathways (56, 57).

Internalized endosomal GPCRs can also trigger G protein-dependent signaling. For example, conformation-specific single-domain antibodies (nanobodies) have been used to directly assess activation of the β2-adrenergic receptor. Using these tools, two activation states of the β2-adrenergic receptor were detected, first at the plasma membrane seconds after ligand application and then a second activation phase on the endosomes. The second, endosomal phase lasted long after the plasma membrane signals had diminished (58). Interestingly, although both the plasma membrane receptor and the endosomal receptor generated cyclic adenosine monophosphate (cAMP), the G protein-dependent response of the endosomal receptor induced unique cAMP-generated transcriptional responses vs those generated by the cell surface receptor (58). In addition to $G\alpha_s$ endosomal signaling (7, 20), Jensen et al. (59) recently demonstrated that internalized neurokinin, NK_1R contributed to sustained endosomal signaling via $G\alpha_q$. Endosomal $NK_1R/G\alpha_q$ signaling but not cell surface NK_1R induced cytosolic cAMP, protein kinase C (PKC) and nuclear ERK resulting in neuronal excitation and nociception; compounds that prevented internalization or blocked endosomal NK_1R were effective in blocking pain transmission. It seems likely that as endosomal signaling pathways are further explored that additional Gα proteins will be discovered which also modulate unique signaling pathways from their position on the endosome.

ER membranes: Given the pleiotropic roles the ER plays within a cell, it can be challenging to isolate a given, receptor-mediated function from interconnected membranes. Ultrastructure studies especially those using immunogold labeling are useful yet may simply reflect synthesis, folding and maturation. A number of GPCRs have clearly been localized on these membranes, however, and various technical strategies have shown functionality. One of the best described ER GPCRs is the G-protein-coupled estrogen receptor-1 (GPER), also known as GPR30, a novel estrogen receptor which in addition to the non-GPCR estrogen receptor mediates signaling in multiple cell types (60, 61). Interestingly, the majority of GPER is localized to the ER and Golgi apparatus in many cancer cells and peripheral cell types (62). In the brain, GPER has a widespread distribution in neurons as well as astrocytes (63). In either cell type, electron micrographs show most GPER is intracellular although in the hippocampus, some GPER is also localized at the cell surface in dendrites and spines (64). Since estrogen is a lipophilic compound it can easily slide through membranes to activate receptors in any membrane throughout the cell. Activated GPER appears to couple to $G\alpha_{i/o}$ and $G\alpha_s$ proteins that together with associated $G\beta\gamma$ subunits regulate many downstream effectors including phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/Akt, ERK1/ 2, adenylyl cyclase, calcium mobilization, nitric oxide synthase (eNOS), and others (2, 65)

GPER is widely expressed in various cancer cell types and tumors where it promotes proliferation, migration, and invasion of cancer cells. However, in some studies, activated GPER inhibited the proliferation of estrogen receptor-negative breast cancer cells, ovarian cancer cells, and prostate cancer cells (65). GPER also influences lipid and glucose metabolism, inflammation and even further estrogen synthesis, actions which further enhance tumor growth and metastasis (65). These opposite and confusing effects may be due to different cell types, different GPER subcellular localization, different subcellular effector molecules and even unique stimuli affecting GPER function.

Another ER membrane GPCR is $mGlu_5$. Although we have primarily characterized $mGlu_5$ receptors on outer and inner nuclear membranes in the striatum, hippocampus and spinal cord dorsal horn neurons (3), ultrastructure studies have also shown large numbers of gold particles on ER membranes (13). Moreover, many earlier ultrastructural studies of various brain regions from the rat, mouse, and monkey have shown large amounts of intracellular receptors in dendrites (66), at the edge of asymmetric postsynaptic specializations and extrasynaptically along the plasma membrane (67–69). We used selective uncaging of glutamate in the presence of cell surface inhibitors to determine whether $mGlu_5$ expressed on dendritic ER was functional. Only the region of the dendrite juxtaposed to the uncaging spot exhibited a change in fluorescence associated with downstream effector formation whereas proximal regions revealed no such fluorescent changes (41). Thus ER $mGlu_5$ is not just undergoing maturation and processing but is also capable of sending signals. As described below, this same study showed that intracellular $mGlu_5$ plays a necessary role in hippocampal long term depression (LTD) (41). It seems likely $mGlu_5$ located on dendritic spine ER is the receptor responsible for the LTD effects (41). Taken together, these two examples of ER GPCR signaling further emphasize that studies investigating molecular mechanisms associated with either GPER or $mGlu_5$'s subcellular distribution and downstream signaling molecules will be critical in the development of effective therapeutic agents targeting these key receptors.

Intracellular GPCRs in synaptic plasticity

GPCRs regulate key aspects of synaptic plasticity both presynaptically and postsynaptically. Presynaptically, various GPCRs affect presynaptic neurotransmitter release either positively or negatively (70, 71). Postsynaptically, GPCR signaling contributes to many processes including long term potentiation (LTP) and LTD, as well as morphogenetic changes associated with dendritic spine alteration and ultimately learning and memory (2, 72). Most of these processes have been explored from the perspective that a GPCR only signals from the cell surface. However, as in more peripheral model systems, it is clear that intracellular GPCRs can affect synaptic plasticity from inside the cell as well. As more intracellular GPCRs are described, this list will surely grow. For now we will highlight those GPCRs for which the best evidence exists for intracellular functions including $mGlu_5$ receptors, M1 muscarinic acetylcholine receptors (mAChRs), CB_1, CB_2 receptors and GPER.

CB_1 receptors: In addition to its well-described localization on the presynaptic plasma membrane, CB_1 receptors have also been localized to mitochondrial, endosomal, and lysosomal compartments (14, 73–76). Activation of these mitochondrial CB_1 receptors

suppresses respiration whereas blockade is associated with increased mitochondrial biogenesis, increased β-oxidation, and increased energy production (14). Short term consequences of mitochondrial CB_1 receptor signaling include synaptic depression; long term consequences can include memory loss (77), metabolic defects and apoptosis (78). Current data suggest that mitochondrial CB_1 receptors are coupled to $G\alpha_i$ which appears to inhibit soluble adenylyl cyclase within the mitochondrial matrix (77). Interestingly, cell surface, presynaptic CB_1 receptors remain unchanged (77).

A second cannabinoid receptor, CB_2, traditionally thought to mediate peripheral immune function, is also present in the brain including the prefrontal cortex and hippocampus (79). In both regions, CB_2 receptors appear to be mostly neuronal, influencing excitatory synaptic transmission, plasticity, and long-term potentiation (80–82). Subcellular fractionation techniques, western blotting, binding assays and electrophysiology data from prefrontal cortex slices show that CB_2 receptors are located intracellularly and that 2-arachidonoylglycerol (2-AG) activation results in inositol triphosphate 3 (IP_3) receptor-dependent opening of Ca^{2+}-activated chloride channels and decreased neuronal excitability (74, 83, 84). Thus both CB_1 and CB_2 receptors play a role inside the cell that contributes to synaptic plasticity.

$mGlu_5$: Besides presynaptic GPCRs modulating neurotransmitter release, postsynaptic receptors are also highly linked to learning and memory, including the Group 1 receptor, $mGlu_5$. In the hippocampus, gene knock out studies show that LTP and LTD are impaired in $mGlu_5$ null animals. This is in agreement with data showing $mGlu_5$ plays a key role in the protein synthesisdependent phase of LTP (85–87) as well as LTD (88, 89). $mGlu_5$ receptors are also highly expressed on the cell surface and intracellular membranes of hippocampal CA1 neurons where only intracellular $mGlu_5$ activation triggered sustained Ca^{2+} responses in dendrites. Using an ex vivo slice approach, an important role for intracellular $mGlu_5$ was also seen for electrically- and chemically-induced, protein-synthesis-dependent LTD but not for LTP in acute hippocampal slices (41; Fig. 3).

M1 mAChRs: M1 mAChRs are also linked to LTP and LTD. For example, M1 knock out mice show severe deficits in hippocampal LTP, working memory and memory consolidation (92–93). Using silver-enhanced immunogold staining of both the cortex and the hippocampus, Yamasaki et al. (94) found that M1 mAChRs were primarily expressed in pyramidal cells where a large amount of the receptor was found intracellularly in association with the ER and Golgi. Subsequent studies revealed ER and Golgi-localized M1 receptors in the hippocampus and cortex of rats, mice and humans (95). As with $mGlu_5$ receptors, pharmacological isolation was used to determine that while both cell surface and intracellular M1 mAChRs enhanced phosphoinositide turnover, only intracellular M1 mediated ERK1/2 activation (95). Importantly, carbachol-facilitated LTP was also differentially regulated in that only early (5–15 min) stages of potentiation were blocked by the M1 impermeant antagonist whereas late stages of potentiation (45–60 min; LTP) were blocked by a permeable antagonist as well as a MAPK blocker (95). Thus, just as intracellular $mGlu_5$ is necessary for hippocampal LTD (41), intracellular M1 mAChRs are necessary for LTP (95). M1 mAChRs also induce LTD in hippocampal CA1 in a protein

synthesis-, ERK1/2- and rapamycin (mTOR)-dependent fashion (96). Whether intracellular M1 mAChRs play a role in LTD remains unknown at present.

GPER: Estrogens are known to influence a wide array of behaviors including hippocampal learning and memory in female rodents. Mechanisms underlying some of these behaviors are due to interactions between $mGlu_1$ and the canonical estrogen receptors leading to estrogen E_2induced phosphorylation of ERK1/2, PI3K, and Akt regulating local protein synthesis (97). GPER also plays a key role in promoting hippocampal memory formation since its agonist, G-1, enhances social recognition, object recognition and spatial memory in ovariectomized female mice, whereas G-15, the GPER antagonist, blocks these processes (98, 99). G-1 treatment of GPER also increases dendritic spine density in the CA1 hippocampus further underscoring its role in morphogenetic processes associated with learning and memory (100). Of interest here, ligand activation of GPER enhances memory consolidation by activating JNK, which, in turn, facilitates gene expression via transcription factors such as ATF2 (98). Although these studies have not directly shown whether GPER effects are due to cell surface and/or intracellular receptors, most GPER is found on intracellular membranes especially the ER (61). Thus, it seems likely that at least some percentage of intracellular GPER is regulating hippocampal learning and memory.

Intracellular GPCR signaling in disorders of synaptic plasticity

Fragile X syndrome (FXS): Emerging evidence furthers the notion that GPCR localization also plays an important role in various disease conditions including disorders of synaptic plasticity. In part, this idea grew out of the discovery that the FXS gene product, the Fragile X mental retardation protein (FMRP), can act as a translational repressor of subsets of neuronal mRNAs, including ones involved in synaptic plasticity (101). A prominent hypothesis of FXS is that symptoms arise due to exaggerated signaling of $mGlu_5$ which normally oppose the function of FMRP (102). Work in animal models showed that loss of FMRP enhanced $mGlu_5$ signaling leading to the prediction that $mGlu_5$ antagonists should restore normal synaptic balance and improve behavioral phenotypes (103). Consistent with this notion, several different $mGlu_5$ inhibitors (MPEP, fenobam, CTEP) did indeed improve FXS-like phenotypes in various animal models (102, 104, 105). These findings prompted clinical trials with various $mGlu_5$ negative allosteric modulators (106). Unfortunately, despite early promise, these trials were discontinued due to negative outcome results (107).

Although there can be many explanations for this conclusion (species differences; erroneous and/or inadequate disease modeling, paucity of clinically relevant outcome markers, tolerance, etc.), another possibility is differential inhibition of $mGlu_5$ receptors on cell surface or intracellular membranes. For example, not only do drug candidates have unique chemical properties but different populations of neurons also have distinctive membrane constituents and lipophilic characteristics which might underlie differential receptor efficacy (108). One of the most dramatic examples of differential lipid composition in biological membranes is that of cholesterol which is heterogeneously distributed between cellular membranes and among different cell types (109). Recent studies show that the level of cholesterol in a membrane significantly increases or decreases the stability, ligand-binding properties and/or function of many different GPCRs including mGlu receptors (108). At

least three different cholesterol binding motifs have been described in various GPCRs (110), suggesting a potential allosteric role for cholesterol in modulating GPCR functions. Inasmuch as cholesterol is enriched in the plasma membrane (20–25% of lipid molecules; 109) but is only 1% of ER membrane lipids (109), it is conceivable that membrane-specific cholesterol levels might differentially affect GPCR function at the cell surface versus ER or nuclear membranes. A drug that can readily access the brain parenchyma and easily modulate a cell surface GPCR may not have the same effect on an intracellular receptor due to membrane phospholipid composition. In addition to the other reasons the FXS clinical trials might have failed, we speculate that the drugs used for the Fragile X clinical trials may not have blocked all the $mGlu_5$ necessary to achieve remediation of the disorder

Cognition: Intracellular GPCRs also play key roles in responding to environmental stimuli and individual experiences that underlie synaptic change and normal brain function. As one of the most abundant GPCRs in the brain (111), CB_1 receptors are especially important in mediating either physiological and/or pathological stimuli presynaptically, postsynaptically and from within the cell (112–114). As described above, pharmacological and genetic isolation of mitochondrial CB_1 receptors have underscored their role in excitatory synaptic transmission as well as in memory performance (77). Specifically, activation of mitochondrial CB_1 receptors induces memory impairment (amnesia) after training in a novel object recognition task whereas the same experiment done in animals that are unable to traffick CB_1 receptors to the mitochondria shows no effect (77). The CB_1-mediated memory impairment appears to be due to decreased protein kinase A (PKA)-dependent phosphorylation of complex I proteins, leading to decreased ATP and decreased mitochondrial respiration. Thus there appears to be a direct link between mitochondrial CB_1 receptors, bioenergetics and higher brain function (114). The exact mechanisms underlying this process are still unclear, but dissecting the specific proteins/pathways involved in this process may generate new therapeutic tools for brain disorders leading to memory loss.

GPCR regulation of ATP production and respiration may be a more generalized phenomenon. For example, the mitochondrial GPCRs (AT_1R, AT_2R, P2Y1, P2Y2, 5-HT_4, MT_1) might also contribute to higher brain function via modulation of ATP levels in critical tissues at critical time points. Determining if and how mitochondrial receptors modulate respiration and other mitochondrial functions such as oxidative stress, fission/fusion, intracellular motility, apoptosis, and Ca^{2+} buffering, etc. may be critical in understanding how these receptors affect higher brain functions such as learning and memory.

Social Learning: Estrogens are known to influence a wide variety of behaviors including social preferences, aggression, dominance, social recognition and social learning (62, 98, 115). Traditionally, these behaviors are thought to involve the canonical estrogen receptors, ERα and ERβ. However, not all such behaviors can be ascribed to ERα and ERβ. The discovery and characterization of GPER has led to the recognition that its wide expression in both the central and peripheral nervous system may mediate many of estrogen's physiological and pathological functions. In support of this notion, social learning involves GPER, not ERα or ERβ (98). For example, studies investigating GPER-specific effects in the dorsal hippocampus showed that infusion of the GPER specific agonist, G-1, led to a

dose and time-dependent improvement in social recognition and object recognition but not object placement learning in female rats (116). Using a similar paradigm, Kim et al., (117) also found that activation of GPER enhanced object recognition whereas GPER inhibition impaired memory. Interestingly, estrogen effects via ERα and ERβ resulted in ERK1/2 activation whereas Kim et al., found that GPER activation led to phosphorylation of JNK. Infusion of a JNK inhibitor blocked G-1 enhanced object recognition whereas ERK inhibition did not (117). Taken together these experiments underscore the diversity of signaling mechanisms associated with hippocampal memory formation and further emphasize that a single ligand, in this case estrogen, can affect many pathways independently to regulate learning and memory.

Other neuronal functions in which activation of GPER has been linked to positive outcomes include depression, pain, metabolic regulation (body weight, energy balance), and post ischemic stroke (62). Since most of the neurological studies have focused on targeting GPER per se, and because GPER-specific agonists and antagonists are permeable, it is unclear whether the observed neuronal effects are due to cell surface and/or intracellular GPER. Based on its widespread distribution and large body of data showing that the majority of GPER is within the cell, it seems likely that intracellular GPER participates in at least some of these behaviors. Improved pharmacological or genetic tools will help determine which pool of receptors is responsible for which behavioral outcome.

Summary

The wealth of new data demonstrating a plethora of receptors on almost every type of intracellular membrane (nuclei, ER, mitochondria, lysosomes, endosomes) represents a paradigm shift in GPCR research and opens the door for a host of new translational applications. Although for some GPCRs, receptor activation and/or inhibition may occur at the cell surface; for others, whether a ligand gets across a given cellular membrane may change its functional response. Although pharmacological isolation provides evidence of a given receptor's in vivo physiological role, the development of genetically isolated animals in which receptors are targeted or excluded from a given intracellular membrane, would reinforce the role of an intracellular receptor. For example, Hebert-Chatelain et al. (77) discovered that removal of the first 22 amino acids at the N-terminus of CB_1 receptors (DN22-CB_1) prevents the receptor from going to or affecting mitochondrial processes such as respiration or mobility. Genetic isolation in vivo via viral re-expression of CB_1 and DN22-CB_1 in CB_1 knock out animals revealed that only mitochondrial CB_1 receptors mediated hippocampal synaptic transmission and memory formation. Joyal et al. used a similar approach to distinguish plasma-membrane from nuclear F2rl1 functions in vivo (5, 16). In these studies (5, 16) intravitreally injected viral constructs were targeted to either the cell surface or nuclei of retinal ganglion neurons of F2rl knock out mice. Plasma membrane-localized F2rl1 retinas exhibited increased Ang1, an angiogenic factor associated with vascular remodeling and maturation. In contrast, nuclear-localized Frl1showed increased Vegfa expression which is associated with neovascularization (5, 16). Injection of the native F2rl1 increased the expression of both angiogenic factors (5,16). Animals such as these can potentially serve as model systems for the development of drugs optimized for a desirable cell surface and/or intracellular response. In the latter case, the same key parameters

associated with drug development for cell surface receptors such as efficacy, potency and specificity are still essential for intracellular GPCR drug design. However, strategies that can get a drug or a biomolecule into the cell and even to the appropriate organelle in the cytoplasm without perturbing its cell surface counterpart would be required (118). For example, to prevent NK1 receptor endosomal signaling, Jensen *et al.* (59) synthesized tripartite compounds composed of cholestanol to promote membrane insertion, a polyethylene linker for flexibility and a membrane impermeable NK1 receptor antagonist. This strategy successfully blocked further NK1 receptor endosomal signaling and promoted the desired behavioral response, in this case antinociception (59). Other new techniques include polymer-based nanocarriers, which can be tailored to display a given charge or combined with other biomolecules such as drugs, antibodies, proteins and oligonucleotides to deliver a particular compound to a particular intracellular location (119–121). Taken together these new tools and new strategies will allow an unprecedented ability to deliver therapeutics to every part of the cell.

As highlighted here, intracellular GPCRs have been linked to synaptogenesis, spine formation, learning and memory, cognition and behavior, as well as to pathophysiological roles in disorders such as FXS, Autism Spectrum Disorders, and depression. Therefore understanding how GPCRs responding to environmental stimuli achieve the necessary changes in synaptic function to learn new tasks and form new memories remains at the cutting edge of this field. Thus studies that investigate the molecular mechanisms that determine the subcellular distribution and signaling properties of a given GPCR are critical for developing effective pharmacological agents that target the chosen receptor.

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Abbreviations

ADHD	attention deficit hyperactivity disorder
Akt	protein kinase B
Arc	activity-regulated cytoskeletal-associated protein
AT_1R and AT_2R	angiotensin I and II receptors
ATF2	activating transcription factor 2
ATP	adenosine triphosphate
cAMP	cyclic adenosine monophosphate
CB_1	cannabinoid 1 receptors
CCR2	chemokine receptor 2
CXCR4	cysteine (C)-X-C Receptor 4

egr-1	early growth response protein 1
Elk-1	ETS domain-containing protein
ER	endoplasmic reticulum
ERα and ERβ	estrogen α and β receptors
ERK	extracellular signal-regulated kinase
F2rl1	coagulation factor II receptor-like 1
FMRP	Fragile X mental retardation protein
FXS	Fragile X syndrome
GPER (GPR30)	G-protein-coupled estrogen receptor-1
GPCRs	G protein-coupled receptors
GnRH	gonadotropin-releasing hormone
5-HT$_4$	5-hydroxytrptamine receptor 4
IP$_3$	Inositol trisphosphate
LPA	lysophosphatidic acid
LTD	long term depression
LTP	long term potentiation
JNKs	c-Jun amino-terminal kinases
mAChRs	Muscarinic acetylcholine receptors
MAPK	mitogen-activated protein kinase
mGlu$_5$	metabotropic glutamate receptor 5
MT$_1$	melatonin 1 receptors
mTOR	mammalian target of rapamycin
NK$_1$R	neurokinin 1 receptor
NLS	nuclear localization signals
eNOS	nitric oxide synthase
OA-1	ocular albinism I
P2Y1, P2Y2	purine receptors
PACAP	pituitary adenylate cyclase activating polypeptide
PGE2	Prostaglandin E2

PI3K	phosphatidylinositol-4,5-bisphosphate 3-kinase
PKA	protein kinase A
PKC	protein kinase C
Ptafr	platelet-activating factor receptor
S1P1	sphingosine 1-phosphate receptor 1
STATs	activators of transcription
VIP	vasoactive intestinal peptide
VPAC1	vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide receptor

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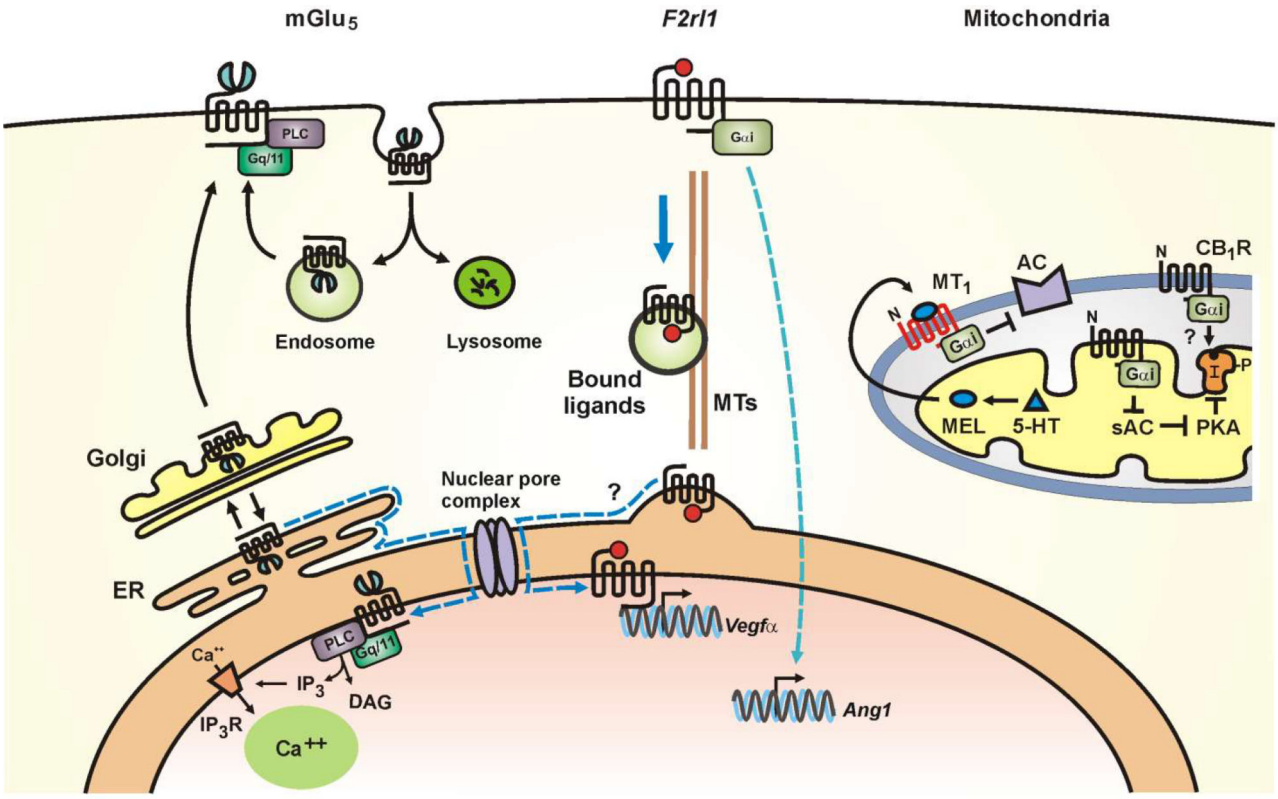

Fig. 1.

Schematic representation of various intracellular GPCRs. Left, proposed model of $mGlu_5$ receptors trafficking in neurons in which >90% of $mGlu_5$ traffics through the Golgi (27). Subsequently, 15–40% goes to the cell surface where it undergoes a cycle of constitutive endocytosis and recycling (27, 122). Alternatively, 60–85% of $mGlu_5$ is retrogradely trafficked back to the endoplasmic reticulum (ER) and then, via lateral diffusion (dotted blue line), reaches the nuclear membrane (27, 123). Middle, ligand bound F2rll can translocate from the retinal ganglion cell surface to the nucleus along microtubules (MTs); nuclear F2rl1 activates vascular endothelial growth factor (Vegfα) expression. In contrast, signaling from cell surface F2rl1 results in the angiogenic gene, Angl expression (16). Right, a growing number of GPCRs have been localized to the outer (MT_1, CB_1 receptors) and inner mitochondrial membranes (AT_2 receptor). Studies show that serotonin (5-HT) is converted into melatonin (MEL) within the mitochondrial matrix; MEL diffuses freely across membranes to activate MT_1 receptors in the outer mitochondrial membrane (52). CB_1 receptors have also been described in the outer mitochondrial membrane (124) although its downstream signaling machinery is primarily located within the matrix. The exact orientation of mitochondrial GPCRs and their signal transduction pathways is largely unknown. Other abbreviations include AC, adenylyl cyclase; sAC, soluble adenylyl cyclase; “I”, IP_3R, inositol trisphosphate receptors; DAG, diacylglycerol; PLC, phospholipase C; Complex I; “P”, phosphorylation site on Complex I; black arrows indicate enhancement of activity; black bars indicate reduction of activity; αi inhibitory subunit of Gi protein; N, amino terminus; and PKA, protein kinase A.

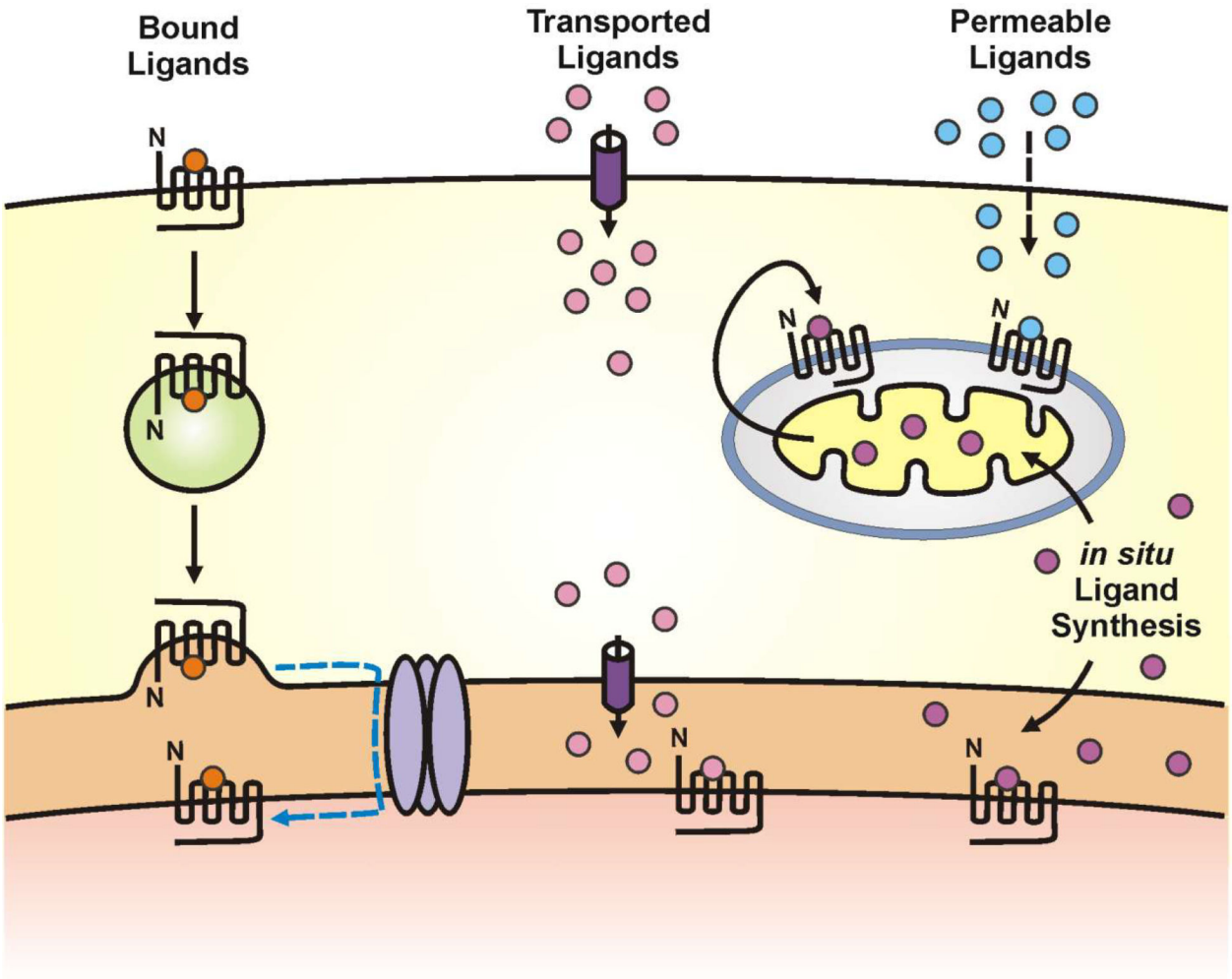

Fig. 2.

Ligand activation of intracellular GPCRs. Intracellular GPCRs can be activated via receptor-bound ligands (left) that can be internalized with a given GPCR such as F2rl1 receptors (16). Alternatively, channels, transporters or exchangers (middle) can transport specific ligands across the plasma membrane and even intracellular membranes to activate receptors whose ligand binding domain faces the endoplasmic reticulum (ER) or nuclear lumen such as glutamate in the case of $mGlu_5$ or organic cation transporter 3 (OCT3) for norepinephrine activation of α1-adrenergic receptor (α1-AR) (3, 125). Permeable ligands such as endocannabinoids can freely diffuse across cell membranes to activate their corresponding receptor (right). Ligands can also be synthesized within the cell (lower right) and either diffuse or be trafficked to a given cellular compartment.

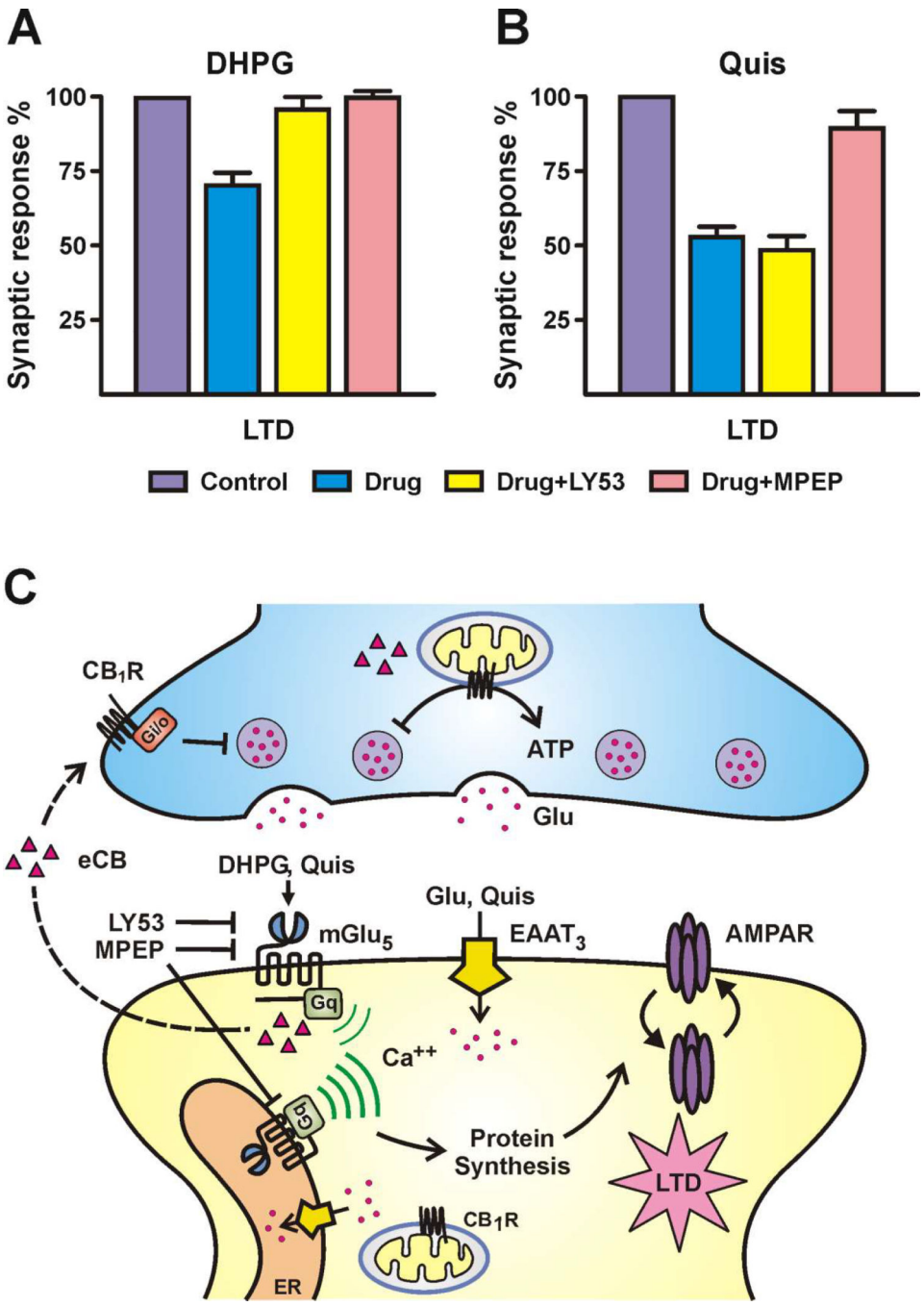

Fig. 3.

Pharmacological isolation demonstrates activation of intracellular $mGlu_5$ receptors can mediate LTD. Information contained in the figure has been extrapolated from (41). Drug addition of DHPG (cell impermeant, non-transported agonist; A) or Quisqualate (Quis; cell impermeant, transported agonist; B) (both 10 μM) induces depressed synaptic responses in hippocampal slices (LTD) shown here at 90 min. DHPG but not Quis LTD is blocked by the impermeant antagonist LY393053 (LY53; 10 μM); whereas both are blocked by MPEP (10 μM) which is permeable. Quis is added in the presence of CNQX (10 μM), CPCCOEt (20

μM), and APV (100 μM) inhibitors to isolate mGlu5 activation; n = 5 for each experiment. C. Conceptual illustration based on information from (3, 41, 77, 112–114). Quis can be transported into the cells via excitatory amino acid transporter 3 (EAAT3). In the hippocampus activation of either cell surface or intracellular $mGlu_5$ receptors leads to activation of $G\alpha_q$, causing formation of IP_3 via PLC and intracellular Ca^{2+} mobilization. $mGlu_5$ activation triggers several signaling pathways ultimately modulating protein synthesis and AMPA receptor (AMPAR) internalization. Studies show that $mGlu_5$ and CB_1 can regulate each other's function since $mGlu_5$ activation generates endocannabinoids (eCBs; triangles) which can freely diffuse across membranes to the presynaptic terminal and/or mitochondrial CB_1Rs decreasing presynaptic glutamate release and mitochondrial ATP production (114, 126). It is worth noting that activation of dendritic $mGlu_5$ receptors leads to a sustained Ca^{2+} response in the hippocampus (41) in contrast to the cell surface response. This might account for the more pronounced Quis-mediated LTD effect (B) and possibly increased production of eCBs.