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Abstract

One of the two major endocannabinoids, 2-arachidonoylglycerol (2-AG), serves as a retrograde messenger at various types of synapses throughout the brain. Upon postsynaptic activation, 2-AG is released immediately after *de novo* synthesis, activates presynaptic CB_1 cannabinoid receptors, and transiently suppresses neurotransmitter release. When CB_1 receptor activation is combined with some other factors such as presynaptic activity, the suppression is converted to a long-lasting form. Whereas 2-AG primarily transmits a rapid, transient, point-to-point retrograde signal, the other major endocannabinoid, anandamide, may function as a relatively slow retrograde or non-retrograde signal or as an agonist of the vanilloid receptor. The endocannabinoid system can be up- or down-regulated by a variety of physiological and environmental factors including stress, which might be clinically important.

Introduction

Endocannabinoids retrogradely modulate synaptic transmission widely throughout the central nervous system [1-5]. They are released from postsynaptic neurons, activate presynaptic CB_1 cannabinoid receptors, and suppress transmitter release either transiently (endocannabinoid-mediated short-term depression; eCB-STD) or persistently (endocannabinoid-mediated long-term depression; eCB-LTD). The eCB-STD and eCB-LTD are induced at various types of GABAergic and glutamatergic synapses throughout the brain. The ability of each synapse to express eCB-STD/LTD depends primarily on whether the presynaptic terminal expresses CB_1 receptors. In addition to their well-established functions as retrograde messengers, endocannabinoids might also function in non-retrograde manners [4]. In the last few years evidence has also accumulated to suggest that the endocannabinoid signaling system itself is regulated by various factors [6]. In this article, we review recent advances in the molecular mechanisms of endocannabinoid signaling and its plastic changes induced by neuromodulators and environmental factors.

Standard 2-AG model of eCB-STD

The molecular mechanisms of endocannabinoid release involved in eCB-STD have been studied in a variety of preparations. Here we show the standard 2-AG model (Fig. 1), which can explain most, if not all, results of electrophysiological studies [1-5]. The conditions that induce the production and release of 2-AG are mechanistically classified into three types; increase in intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (Ca^{2+}-driven

endocannabinoid release, CaER), activation of $G_{q/11}$-coupled receptors (basal receptor-driven endocannabinoid release, basal RER), and the combination of these two (Ca^{2+}-assisted RER) [1,2,6]. After released from postsynaptic neurons, 2-AG activates presynaptic CB_1 receptors and suppresses transmitter release. Termination of the retrograde signal depends on degradation of 2-AG by monoacylglycerol lipase (MGL). Although the expression of MGL is highly heterogeneous [7,8], 2-AG is degraded in a synapse non-specific manner by MGL concentrated in particular cell types [8].

The CaER is responsible for the eCB-STD induced by depolarizing a postsynaptic neuron, which is termed DSI (depolarization-induced suppression of inhibition) or DSE (depolarization-induced suppression of excitation) for inhibitory or excitatory synapses, respectively. When depolarization causes a large, transient increase in $[Ca^{2+}]_i$ to micromolar levels through activation of voltage-gated Ca^{2+} channels, diacylglycerol (DG) is produced through some as yet unidentified mechanism (Fig. 1A, red arrows). DG is then converted to 2-AG by diacylglycerol lipase α (DGLα). The basal RER is responsible for the eCB-STD induced by activation of $G_{q/11}$-coupled receptors, such as group I metabotropic glutamate receptors (mGluRs) or M_1/M_3 muscarinic acetylcholine receptors (mAChRs), without need of postsynaptic Ca^{2+} elevation. Many other receptors have also been reported to induce RER, which include 5-HT_2-type serotonin receptors, protease-activated receptor 1 (PAR1), and the receptors for orexin, oxytocin and CCK [3]. When $G_{q/11}$-coupled receptors are activated, DG is produced by PLCβ, the subtype of which depends on brain areas (Fig. 1A, blue arrows). DG is then converted to 2-AG by DGLα. The Ca^{2+}-assisted RER accounts for the eCB-STD induced by the combination of a small increase in $[Ca^{2+}]_i$ and weak receptor activation, both of which can be subthreshold for triggering endocannabinoid release. This synergistic effect can be explained by the Ca^{2+} dependency of receptor-driven PLCβ stimulation [9-11] (Fig. 1A, green arrow).

More physiological ways of inducing eCB-STD are via synaptic activity (synaptically-driven eCB-STD) [1] (Fig. 1B). If glutamate is released from excitatory presynaptic terminals in a sufficient amount to induce postsynaptic Ca^{2+} elevation and/or mGluR activation, 2-AG is released through the mechanism for CaER, basal RER or Ca^{2+}-assisted RER. Excitatory synaptic activity is therefore potentially effective in inducing 2-AG release. Synaptically-driven eCB-STD can be either homosynaptic (Fig. 1B, red arrow) or heterosynaptic [1] (Fig. 1B, blue arrow).

The standard 2-AG model described above is supported by a considerable number of studies. DSI, DSE, receptor-driven eCB-STD and synaptically-driven eCB-STD are all inhibited by pharmacological blockade of DGL [12] and genetic

deletion of DGLα [13-15]. The mGluR- or mAChR-driven eCB-STD is blocked by genetic deletion of PLCβ1 for hippocampal neurons [9] or PLCβ4 for cerebellar Purkinje cells [10]. The termination of DSI/DSE is prolonged by genetic deletion [8,16] and pharmacological blockade of 2-AG hydrolyzing enzyme (MGL), but not anandamide (another major endocannabinoid) hydrolyzing enzyme (fatty acid amide hydrolase, FAAH) [17].

On-demand vs. pre-formed

It is generally thought that 2-AG is not stored in neurons, but synthesized on demand upon stimulation. However, this "on-demand synthesis model" was challenged by an alternative model that 2-AG is pre-formed by DGLα, pooled within cells, and mobilized from this hypothetical pre-formed 2-AG pools upon stimulation without the contribution of DGLα [18,19]. This model was developed to reconcile the apparent discrepancy in the experimental results between genetic and pharmacological blockade of DGL. DGLα knockout mice were generated independently by three groups, and they all exhibit complete loss of eCB-STD [13-15]. In contrast, the reported effects of acute pharmacological blockade of DGL were highly controversial [19]. A classical DGL inhibitor, tetrahydrolipstatin (THL), inhibited eCB-STD in some studies, but not in others. Inconsistent results were also observed with a novel potent DGL inhibitor, OMDM-188. OMDM-188 failed to inhibit DSI in one study [20], whereas it inhibited DSI but not mGluR-driven STD in another study [21]. More recently, however, effects of OMDM-188 on multiple types of eCB-STD including CaER, basal RER, Ca^{2+}-assisted RER and synaptically-driven eCB release were systematically reexamined in acute slices of the hippocampus, cerebellum and striatum and in cultured hippocampal neurons [12]. The data clearly demonstrate that DSI, DSE, receptor-driven eCB-STD and synaptically-driven eCB-STD in these preparations are consistently blocked by the treatment with OMDM-188 [12]. Importantly, application of OMDM-188 as short as 2 min was sufficient to block DSI in cultured hippocampal neurons [12]. This study has confirmed that there is no discrepancy in the results between genetic and pharmacological blockade of DGL, supporting the "on-demand synthesis model" rather than the "pre-formed pool model".

Given that the "on-demand synthesis model" is correct, why were DGL inhibitors ineffective in blocking eCB-STD in some studies? One possibility is insufficient penetration of the inhibitors. Since THL and OMDM-188 are highly lipophilic, they are difficult to penetrate into brain slices. The effectiveness of these DGL inhibitors therefore depends on whether their targets (e.g., soma or dendrites) are

located near the surface or in the depth of slices. The effectiveness of THL and OMDM-188 also depends on whether or not DGL activity is rate limiting for 2-AG production. If DGL activity is rate limiting for CaER, but not for RER, these two types of eCB-STD can have different sensitivity to the DGL inhibitors, as previously reported [21]. Whether the suppression of synaptic transmission by 2-AG is maximal or submaximal is also a key factor determining the effectiveness of DGL inhibitors. The inconsistent results of pharmacological studies might result from these differences.

Standard 2-AG model of eCB-LTD

eCB-LTD is induced mostly by repetitive afferent stimulation with or without postsynaptic depolarization, but also by postsynaptic firing [1,4,22,23]. Most types of eCB-LTD are mediated by 2-AG. It is generally thought that eCB-LTD and eCB-STD share the same molecular mechanisms for 2-AG release, that is, CaER, basal RER and Ca^{2+}-assisted RER. Typically, afferent stimulation causes glutamate release from excitatory presynaptic terminals, activates AMPA receptors and mGluRs on the postsynaptic membrane, and induces 2-AG release. 2-AG then activates CB_1 receptors on the same presynaptic terminals releasing glutamate (homosynaptic) (Fig. 2, red arrow) or neighboring presynaptic terminals (heterosynaptic) (Fig. 2, blue arrow).

Although presynaptic mechanisms of eCB-LTD have not been fully elucidated, inhibition of the cAMP/PKA pathway seems to be crucial [4]. Involvement of calcineurin, RIM1α and Rab3 has also been suggested [4]. In most if not all cases, activation of CB_1 receptors is not enough to induce LTD, and an additional presynaptic mechanism(s) is required [24], such as presynaptic electrical activity [25,26], Ca^{2+} elevation [25,26], NMDA receptor activation [27], M_2 receptor activation [28] and/or D_2 receptor activation [29,30] (Fig. 2). This associative feature may ensure the selective induction of eCB-LTD at the active synapses.

Contribution of anandamide to endocannabinoid signaling

The other major endocannabinoid, anandamide, also contributes to the modulation of synaptic transmission (Fig. 3). Several recent studies reported anandamide-mediated LTD [31-35] (Fig. 3A). Lerner and Kreitzer reported two types of eCB-LTD in medium spiny neurons (MSNs) driving the indirect pathway in the dorsolateral striatum [31]. Low frequency stimulation induces 2-AG-mediated LTD, whereas high frequency stimulation induces CB_1-dependent, anandamide-mediated LTD [31] (Fig. 3A, eCB-LTD). Anandamide may contribute to LTD not only as a cannabinoid agonist but also as an agonist for transient receptor potential vanilloid receptor type 1 (TRPV1). In

indirect pathway MSNs of the nucleus accumbens, Grueter et al. suggested that low frequency stimulation induces anandamide-mediated LTD, partly by activating presynaptic CB_1 receptors (Fig. 3A, eCB-LTD) and partly by activating postsynaptic TRPV1 channels (Fig. 3A, TRPV1-LTD) [32]. In the dentate gyrus, Chavez et al. reported that pairing stimulation induces CB_1-independent, TRPV1-dependent LTD, which is expressed postsynaptically and mediated by anandamide [33]. On the other hand, Puente et al. suggested that 2-AG mediates both eCB-STD and eCB-LTD in the striatum, whereas 2-AG and anandamide mediate eCB-STD and TRPV1-LTD, respectively, in the extended amygdala [34].

Anandamide may also generate the endocannabinoid tone (Fig. 3B) which contributes to homeostatic plasticity in the hippocampus [36] and the regulation of hypothalamic-pituitary-adrenal (HPA) axis activity in the amygdala [37] (Fig. 4A). Of note, chronic MGL blockade (continuous elevation of 2-AG) causes down-regulation of CB_1 receptors and functional antagonism of the endocannabinoid system, whereas blockade of FAAH (continuous elevation of anandamide) causes sustained agonism without down-regulation of CB_1 [38]. This finding implies that anandamide has an advantage over 2-AG for functioning as a tonic signal. Taking all these findings into account, it is conceivable that the two major endocannabinoids, 2-AG and anandamide, mediate different types of signals for synaptic modulation [5,38]. 2-AG primarily transmits a rapid, transient, point-to-point retrograde signal, whereas anandamide may function as a relatively slow, retrograde or non-retrograde signal or as a vanilloid agonist.

As described above, there are many studies of the functional roles of anandamide. It should be noted, however, that the evidence for the involvement of anandamide in synaptic plasticity is largely indirect, because enzymatic pathways for anandamide production are complex and not fully understood. In some studies, the contribution of anandamide was suggested principally by the ineffectiveness of DGL inhibitors. Considering the fact that pharmacological results with DGL inhibitors have been highly controversial and are not as reliable as genetic manipulations, experimental data should be interpreted with caution. More direct, convincing evidence will be required in future studies.

Other possible mechanisms

Several additional mechanisms have been suggested to contribute to endocannabinoid signaling, including (1) *N*-arachidonoyldopamine (NADA) [39], (2) glial [40-42] and (3) mitochondrial [43] CB_1 receptors, (4) FAAH-like anandamide transporter (FLAT)

[44] and (5) PLA_2 [45,46]. (1) NADA, like anandamide, is an agonist at both cannabinoid receptors and TRPV1 channels. Recently, it was reported that NADA is tonically released from dopamine neurons, but not neighboring GABAergic neurons, to suppress their inhibitory inputs in the Substantia Nigra pars compacta [39]. (2) Timing-dependent LTD at cortical excitatory synapses has been shown to require the activation of CB_1 receptors, which is believed to be located on presynaptic terminals (Fig. 2). This hypothesis was challenged by a recent study, which suggested that the CB_1 receptors mediating LTD are not located on presynaptic terminals, but on perisynaptic astrocyte processes [41]. (3) Using mitochondrial fractions and anti-CB_1 polyclonal antibodies, it was shown that CB_1 receptors are expressed on mitochondrial membranes and are involved in the regulation of neuronal energy metabolism [43]. However, these findings were questioned by a more recent study [47], which has demonstrated that the previously used anti-CB_1 antibodies can bind to the mitochondrial protein stomatin-like protein 2, and the previously reported effects of cannabinoid agonist on mitochondrial respiration can be replicated in synaptosome-enriched, but not pure mitochondrial preparations. (4) FLAT was reported as a critical molecular component of anandamide transport in neural cells [44]. The study showed that FLAT is a catalytically silent splice variant of FAAH, is expressed robustly throughout the brain, and facilitates the translocation of anandamide into cells. A more recent study, however, suggested that FLAT does not serve as a global intracellular anandamide carrier [48]. The study showed that the expression of FLAT is very low in the brain, and that overexpressed FLAT displays residual catalytic activity, suggesting that its capability to potentiate anandamide uptake may stem from its enzymatic rather than transport activity. (5) A recent study using PLA_2-knockout mice proposed that Ca^{2+} elevation activates PLA_2 to liberate arachidonic acid which is then converted to 2-AG and induces DSE in cerebellar Purkinje cells [45]. However, since this result cannot be reconciled with the standard 2-AG model (Fig. 1A) that is supported by a large number of previous studies, further careful studies are required to judge the validity of the result.

Regulation and plasticity of the endocannabinoid system

The endocannabinoid system depends on many molecular elements, including Ca^{2+} channels, various types of $G_{q/11}$-coupled receptors, PLCβ, DGLα, MGL and CB_1 receptor. Thus, any changes in their functional activities or expression levels influence the endocannabinoid system. Evidence has accumulated to suggest that the endocannabinoid system is regulated by various postsynaptic and presynaptic receptors, and also influenced by environmental and pathological factors [6].

Postsynaptic and presynaptic receptors can regulate endocannabinoid release and CB_1 signaling, respectively. In the striatum, postsynaptic D_2 and A_{2A} receptors regulate endocannabinoid release and eCB-LTD positively and negatively, respectively, by modulating mGluR signaling through regulator of G protein signaling 4 (RGS4) [31]. Enzymatic activity of DGLα is reduced after phosphorylation by CaMKII [49], suggesting the possibility that DGLα might be regulated by CaMKII-involving signals under physiological conditions. Presynaptic CB_1 receptor signaling is influenced by the activity of colocalized presynaptic receptors. In the hippocampus, tonic activation of A_1 receptors by endogenous adenosine inhibits presynaptic CB_1 signaling [50]. Because CB_1 and A_1 receptors are both $G_{i/o}$-coupled, it is conceivable that they use overlapping sets of Gα subunits and interact with each other.

Considering that the endocannabinoid system contributes to many brain functions including learning, memory, stress regulation and analgesia, its plastic changes induced by environmental factors are clinically important. One such factor is stress. There is growing evidence for functional interaction between the endocannabinoid system and activity along the HPA axis. The endocannabinoid system plays a key role in regulating the HPA axis under both basal and stressful conditions. It was proposed that a tonic level of anandamide within the basolateral amygdala provides a steady-state inhibition of HPA axis activity, and that glucocorticoid-induced 2-AG release from paraventricular nucleus neurons mediates rapid negative feedback effects of glucocorticoids on the HPA axis [37]. Conversely, stress-induced HPA axis activity alters the endocannabinoid system, either positively or negatively depending on the brain region, age and conditions of stress, in several brain regions including the hippocampus, amygdala, hypothalamus, nucleus accumbens and prefrontal cortex [6,37,51]. For example, repetitive immobilization stress impaired DSI/DSE by down-regulating CB_1 receptors in the paraventricular nucleus of the hypothalamus [52], whereas chronic stress enhanced DSI partially by down-regulating MGL in the basolateral amygdala [53]. Interestingly, in vivo recordings of medial prefrontal cortex (mPFC)-evoked spike probability from the bed nucleus of the stria terminalis showed that acute restraint stress switched CB_1-dependent plasticity from LTD to LTP, and that the stress-elicited shift in plasticity was controlled by CB_1 receptors on excitatory terminals [54]. Mechanisms of these stress-induced plastic changes in the endocannabinoid system remain to be elucidated.

A possible relationship between the endocannabinoid system and psychiatric disorders has also been suggested, by exploring genetic morphisms in the human genes for the CB_1 receptor and FAAH [55], and by examining eCB-STD/LTD in animal

models of psychiatric disorders including autism, schizophrenia and Fragile X syndrome [56-60]. In these animal models, alteration in DSI, mGluR-driven eCB-STD, eCB-LTD or the endocannabinoid tone has been reported. Further studies are required to understand the mechanisms that cause these alterations.

Conclusions

The endocannabinoid 2-AG plays an important role as a retrograde messenger at both excitatory and inhibitory synapses. It is synthesized and released from postsynaptic neurons in an activity-dependent manner, and suppresses transmitter release transiently (eCB-STD) or persistently (eCB-LTD). Although considerable progress has been made in recent years with regard to the molecular mechanisms of eCB-STD and eCB-LTD, several questions remain unsolved. First, the enzyme that produces DG upon Ca^{2+} elevation has not been identified. Second, it is not determined whether 2-AG can move across postsynaptic and presynaptic membranes, as well as the synaptic cleft, freely or requires some special machinery. Third, presynaptic mechanisms of eCB-LTD are not completely clear. Fourth, anandamide synthetic pathways are not fully understood. Although accumulating evidence suggests that the endocannabinoid system plays a role in many brain functions, and can be up- or down-regulated by a variety of factors, the precise mechanisms are poorly understood. Of note, the endocannabinoid system contributes to the regulation of the HPA axis and stress response and, conversely, undergoes plastic changes in response to stress condition. Understanding these features of the endocannabinoid system may be clinically important for the development of the methods of treating patients with stress-related disorders.

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Figure legends

Figure 1.

Molecular mechanisms of endocannabinoid-mediated short-term depression (eCB-STD). **A**: The production of diacylglycerol (DG) is induced by either a large Ca^{2+} elevation, which is caused by depolarization-induced activation of voltage-gated Ca^{2+} channels (VGCC), through unidentified mechanisms (red arrows), or strong activation of $G_{q/11}$-coupled receptors such as mGluRs and mAChRs through PLCβ (blue arrows). If weak activation of $G_{q/11}$-coupled receptors is combined with a small Ca^{2+} elevation, both of which are subthreshold for DG production when given alone, receptor-driven PLCβ stimulation is enhanced by Ca^{2+} to produce DG (green arrow). DG is then converted to 2-AG by diacylglycerol lipase α (DGLα). 2-AG is released from the postsynaptic neuron, and activates presynaptic CB_1 receptors to suppress transmitter release (STD). 2-AG is hydrolysed mostly by presynaptic monoacylglycerol lipase (MGL) and arachidonic acid (AA) is produced. **B**: Excitatory synaptic activity releases glutamate, which activates postsynaptic AMPA-type glutamate receptors (AMPARs) and mGluRs. Activation of AMPARs causes Ca^{2+} elevation through activating VGCCs. The resulting postsynaptic Ca^{2+} elevation and/or mGluR activation causes the release of 2-AG through the mechanisms illustrated in **A**. 2-AG activates CB_1 receptors on the same presynaptic terminals releasing glutamate (red arrow, homosynaptic) or neighboring terminals (blue arrow, heterosynaptic), and suppresses transmitter release (STD).

Figure 2.

Molecular mechanisms of endocannabinoid-mediated long-term depression (eCB-LTD). Afferent stimulation and/or postsynaptic depolarization induces 2-AG release by activating postsynaptic AMPARs, mGluRs and VGCCs. Mechanistically, this process is the same as that for eCB-STD, and includes CaER, basal RER and Ca^{2+}-assisted RER. The released 2-AG then activates CB_1 receptors on the same presynaptic terminals releasing glutamate (homosynaptic, red arrow) or neighboring terminals (blue arrow, heterosynaptic). LTD is induced when the activation of CB_1 receptors is combined with some other factors in the presynaptic terminal, such as presynaptic electrical activity, Ca^{2+} influx through VGCCs or NMDA-type glutamate receptors (NMDARs) and the activation of D_2 or M_2 receptors.

Figure 3.

Functions of anandamide in the regulation of synaptic transmission. **A**: Anandamide-mediated LTD. Glutamate released by afferent stimulation activates postsynaptic mGluRs to induce anandamide production. Anandamide activates presynaptic CB_1 receptors to induce presynaptic LTD (eCB-LTD), and activates postsynaptic TRPV1 channels to induce postsynaptic LTD that is expressed as a change in the function or number of postsynaptic glutamate receptors (TRPV1-LTD). **B**: Endocannabinoid tone generated by anandamide. At basal Ca^{2+} levels, anandamide is continuously produced and released. This anandamide-mediated endocannabinoid tone controls synaptic transmission in a sustained fashion.

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This study demonstrates that eCB-STD is consistently blocked by acute pharmacological inhibition of DGL, supporting the "on-demand synthesis model" rather than the "pre-formed pool model". A novel potent DGL inhibitor, OMDM-188, effectively blocks hippocampal DSI, striatal DSI, cerebellar DSI, cerebellar DSE, hippocampal mGluR-driven eCB-STD and cerebellar synaptically-driven eCB-STD after the treatment of slices or cultured neurons with OMDM-188 for 1 hr (slices) or 2-8 minutes (cultured neurons).

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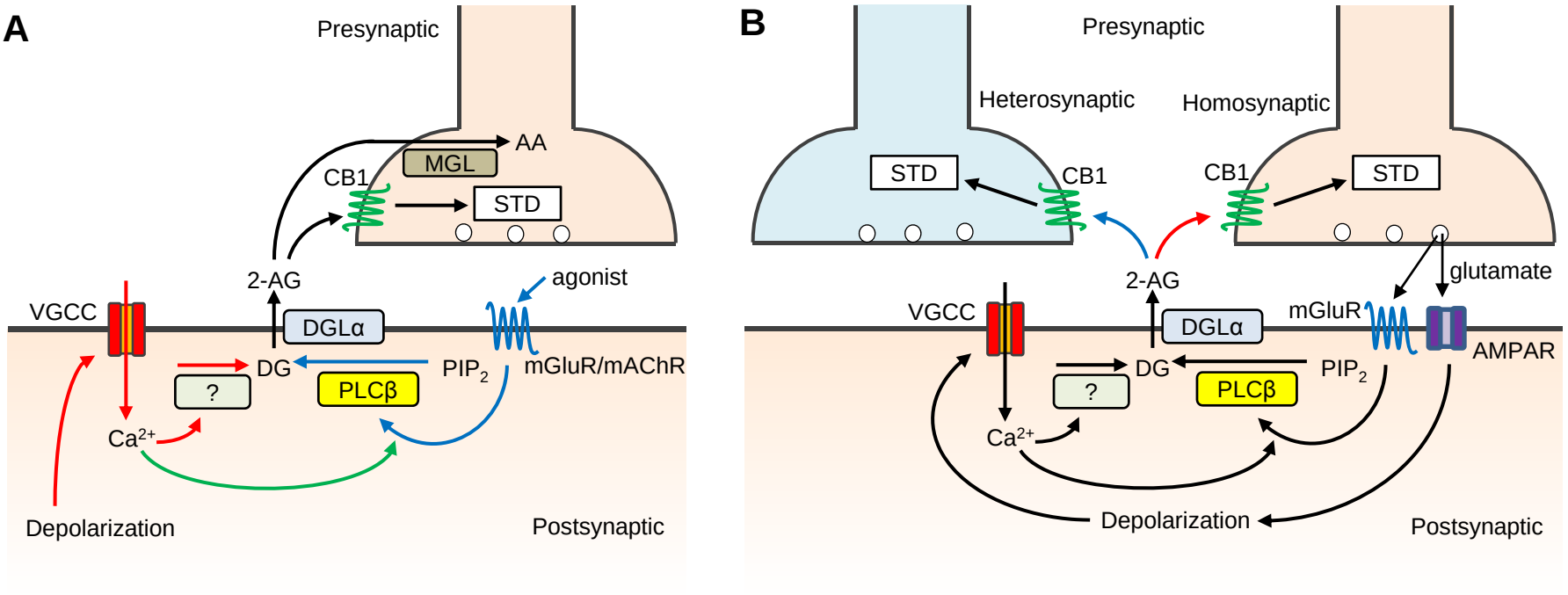
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Presynaptic
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MGL
CB1
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2-AG
agonist
VGCC
DGLα
DG
PIP2
mGluR/mAChR
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PLCβ
Ca2+
Depolarization
Postsynaptic
B
Presynaptic
Heterosynaptic
Homosynaptic
STD
CB1
CB1
STD
glutamate
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VGCC
DGLα
mGluR
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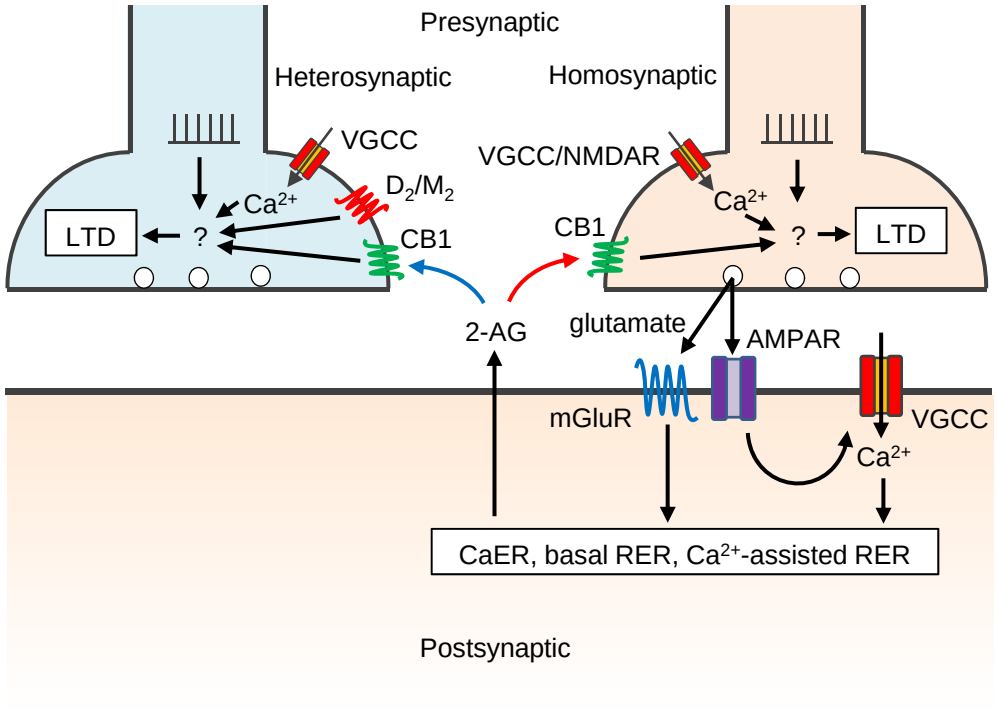
PLCβ
Ca2+
Depolarization
Postsynaptic

Figure 1.


Presynaptic
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VGCC
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Ca^{2+}
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CB1
CB1
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LTD
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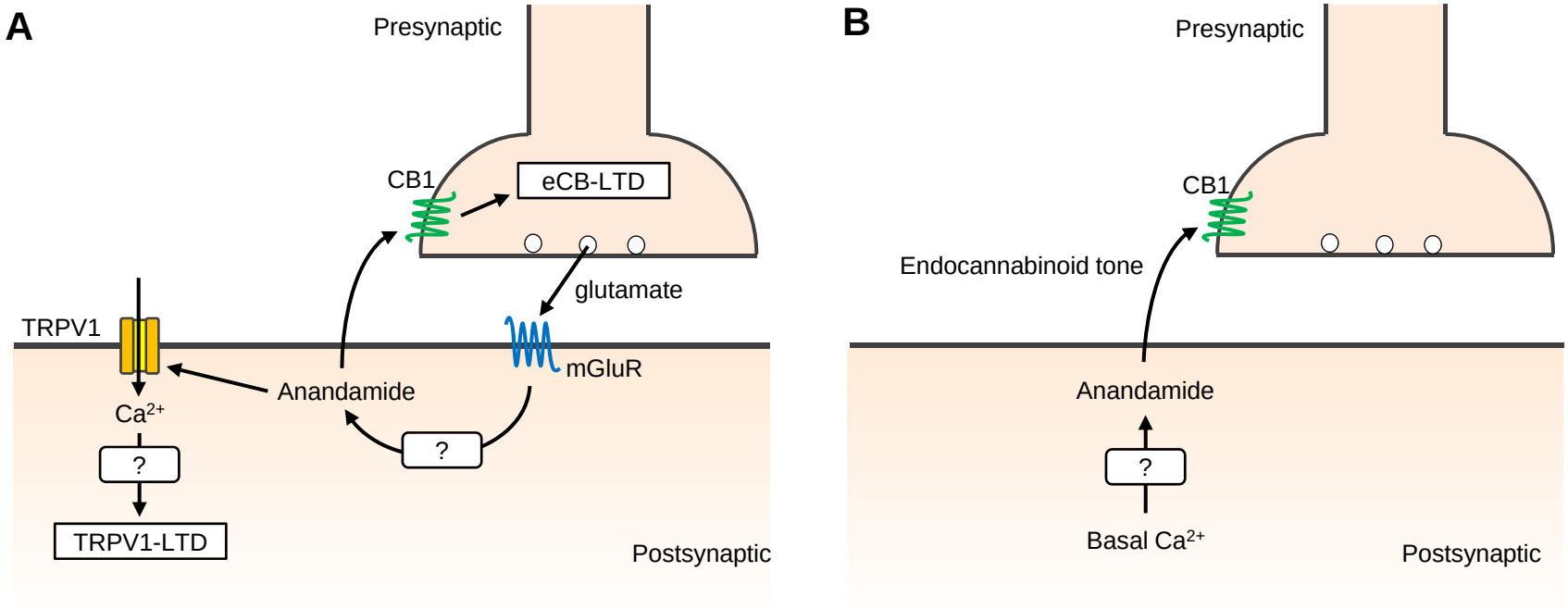
glutamate
AMPAR
mGluR
VGCC
Ca^{2+}
CaER, basal RER, Ca^{2+}-assisted RER
Postsynaptic

Figure 2.


A
Presynaptic
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Ca2+
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TRPV1-LTD
Postsynaptic
B
Presynaptic
CB1
Endocannabinoid tone
Anandamide
?
Basal Ca2+
Postsynaptic

Figure 3.