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Modulation of cell surface GABA(B) receptors by desensitization, trafficking and regulated degradation

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EDITORIAL

Modulation of cell surface GABAB receptors by desensitization, trafficking and regulated degradation

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Abstract

Inhibitory neurotransmission ensures normal brain function by counteracting and integrating excitatory activity. γ-Aminobutyric acid (GABA) is the main inhibitory neurotransmitter in the mammalian central nervous system, and mediates its effects *via* two classes of receptors: the GABAA and GABAB receptors. GABAA receptors are heteropentameric GABA-gated chloride channels and responsible for fast inhibitory neurotransmission. GABAB receptors are heterodimeric G protein coupled receptors (GPCR) that mediate slow and prolonged inhibitory transmission. The extent of inhibitory neurotransmission is determined by a variety of factors, such as the degree of transmitter release and changes in receptor activity by posttranslational modifications (e.g., phosphorylation), as well as by the number of receptors present in the plasma membrane available for signal transduction. The level of GABAB receptors at the cell surface critically depends on the residence time at the cell surface and finally the rates of endocytosis and degradation. In this review we focus primarily on recent advances in the understanding of trafficking mechanisms that determine the expression level of GABAB receptors in the plasma membrane, and thereby signaling strength.

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Key words: GABAB receptors; Neuron; Trafficking; Endocytosis; Recycling; Degradation

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FUNCTIONS OF GABAB RECEPTORS

Metabotropic GABAB receptors are widely distributed throughout the central nervous system where they mediate slow, prolonged inhibition to control neuronal excitation, and contribute to synaptic plasticity[1].

GABAB receptors are present at pre- and postsynaptic sites of both inhibitory and excitatory neurons. Electron microscopy revealed that GABAB receptors are located predominantly at areas close to neurotransmitter release sites and at peri- and extrasynaptic areas of spines and dendrites, but only rarely directly at active zones or postsynaptic densities[2-7]. This location of GABAB receptors implies that they are not directly activated by synaptically released GABA. One mechanism to activate GABAB receptors requires intense neuronal activity, resulting in

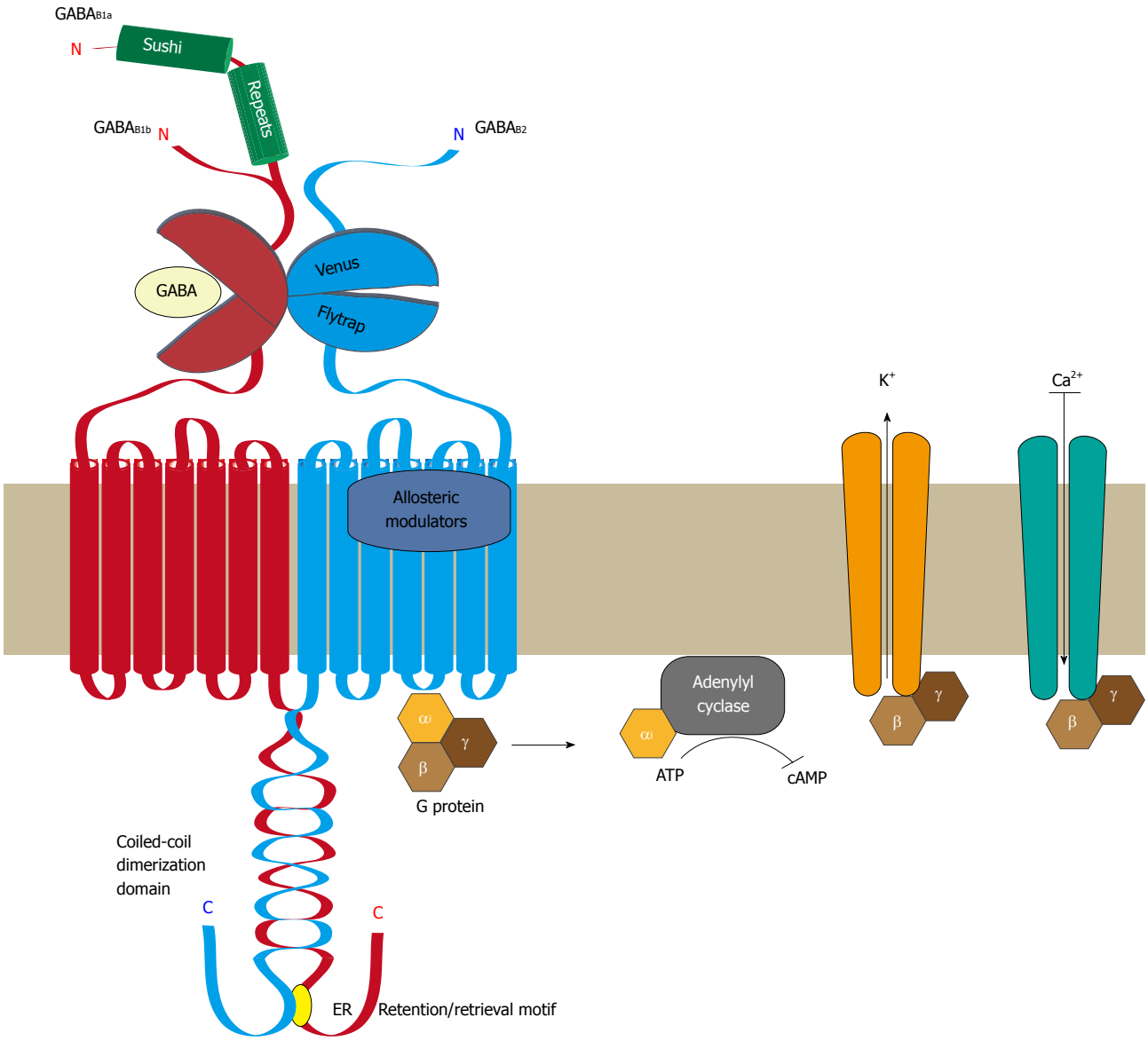

Figure 1 Structural organization of GABA$_B$ receptors. Functional GABA$_B$ receptors are heterodimers composed of the two subunits GABA$_{B1}$ and GABA$_{B2}$. Both subunits are heptahelical membrane proteins with a large extracellular located N-terminal domain containing a "Venus flytrap" module and a large intracellular C-terminal domain containing a coiled-coil protein-protein interaction module. GABA$_{B1}$ and GABA$_{B2}$ heterodimerize *via* their "Venus flytrap" and coiled-coiled domains. An endoplasmic reticulum (ER) retention/retrieval signal is present distal to the coiled-coil domain in GABA$_{B1}$ and prevents ER exit of GABA$_{B1}$ unless it is masked by heterodimerization with GABA$_{B2}$. The "Venus flytrap" module of GABA$_{B1}$ constitutes the GABA binding site, whereas that of GABA$_{B2}$ is inactive and not involved in ligand binding. Instead, the heptahelical domain of GABA$_{B2}$ contains a binding site for allosteric modulators, which affects the affinity of ligands binding to the GABA site. Binding of GABA results in the recruitment and activation of Gαi/o proteins *via* GABA$_{B2}$. The activated Gαi/o subunit inhibits the adenylyl cyclase, resulting in lowered cAMP levels, while the Gβγ dimer activates K$^+$ channels and inhibits Ca^{2+} channels, leading in either case to neuronal inhibition. There exist two isoforms of GABA$_{B1}$, named GABA$_{B1a}$ and GABA$_{B1b}$, which are generated by alternative promoter usage. They only differ by the additional presence of two so-called "sushi repeats" (protein-protein interaction modules) in the N-terminal domain of GABA$_{B1a}$. GABA: γ-Aminobutyric acid; ATP: Adenosine-5'-triphosphate; cAMP: 3'-5'-cyclic adenosine monophosphate.

a spill-over of synaptically released GABA[8]. However, there are also other sources that may increase the ambient level of GABA, such as activity-dependent release of GABA from dendrites and glia cells[9-11]. Recently, it has been shown that basal synaptic activity generates a sufficient concentration of ambient GABA to tonically induce a low level of presynaptic GABA$_B$ receptor activation, which results in the control of transmitter release[12].

Binding of GABA to the GABA$_B$ receptor activates Gi/o-type G proteins[13-18], which in turn modulate three major effector systems: adenylyl cyclases, voltage-sensitive Ca^{2+} channels and inwardly-rectifying K$^+$ channels (Figure 1).

The α subunit of the activated G protein inhibits adenylyl cyclase activity, which decreases cellular 3'-5'-cyclic adenosine monophosphate (cAMP) levels and affects the activity of cAMP-dependent processes. Unfortunately, the contribution of GABA$_B$ receptor-induced lowering of cAMP levels to physiological processes is poorly investigated. So far it has been shown that it retards synaptic vesicle recruitment during sustained activity, which reduces transmitter release[19]. In addition, GABA$_B$ receptor-mediated Gαi/o effects may be important for long-term

adaptations involving regulation of protein kinase activity and gene transcription[20-22].

However, the most well established GABA$_B$ receptor actions are mediated *via* the βγ dimer of the activated G protein. At presynaptic sites, voltage-sensitive P/Q- and N-type Ca^{2+} channels are the predominant effectors of GABA$_B$ receptors[23-27]. GABA$_B$ receptor activated Gβγ inhibits Ca^{2+} channel activity by slowing their current activation kinetics[28], which eventually results in reduced transmitter release. Postsynaptically, GABA$_B$ receptor effects are mainly mediated by the family of G protein-gated inwardly rectifying K^+ channels (GIRK1-4 also called Kir3.1-3.4)[29,30]. Gβγ directly binds to GIRK channels[31,32] and activates them[33,34], resulting in an outward K^+ current. This hyperpolarizes the membrane and consequently inhibits neuronal activity. However, there is no strict mechanistic segregation of pre- (Ca^{2+} channels) and postsynaptic (K^+ channels) effector systems. There is accumulating evidence that GABA$_B$ receptors also activate K^+ channels at presynaptic sites, which assists inhibition of transmitter release[35-37]. Conversely, there is also data for GABA$_B$ receptor mediated inhibition of postsynaptic Ca^{2+} channels[38-41]. This provides an additional mechanism for controlling the excitability of dendrites and spines. Thus, the current data is consistent with a complex pattern of regulating the activity of multiple G protein-gated inwardly rectifying K^+ channels and voltage-sensitive Ca^{2+} channels, both at pre- and postsynaptic sites, resulting in the inhibition of neuronal activity.

To ensure efficient activation of the effector system, GABA$_B$ receptors are localized in close proximity to their effector channels[36,42] and may even constitute signaling complexes by physical interaction[36,43].

MOLECULAR ORGANIZATION OF GABA$_B$ RECEPTORS

Although the GABA$_B$ receptor was discovered in 1980[44], its molecular identity and characterization was delayed for almost 20 years until the first constituent of the receptor was cloned. This delay was due to the fact that all biochemical attempts to purify the receptor failed and expression cloning proved unsuccessful. The development of high-affinity antagonists eventually permitted the successful screening of expression libraries yielding two cDNAs derived from a single gene, GABA$_{B1a}$ and GABA$_{B1b}$[45]. GABA$_{B1a}$ and GABA$_{B1b}$ are generated by differential promoter usage[46] and differ solely by the presence of an additional N-terminal sequence in GABA$_{B1a}$ coding for two protein-protein interaction domains, so-called "sushi domains". GABA$_{B1a}$ and GABA$_{B1b}$ show all the characteristics of class Ⅲ G protein-coupled receptors (e.g., a very large extracellular domain, seven transmembrane-spanning (heptahelical) sequences and a large intracellular located C-terminal domain) (Figure 1). So far, no functional differences among GABA$_B$ receptors containing GABA$_{B1a}$ and GABA$_{B1b}$ have been detected. The cloning of these first GABA$_B$ receptor constituents provided the basis for numerous research efforts analyzing the molecular characterization and function of GABA$_B$ receptors. It soon became clear that functional GABA$_B$ receptors are obligatory heterodimers composed of GABA$_{B1}$ (either GABA$_{B1a}$ or GABA$_{B1b}$) and a second heptahelical membrane protein named GABA$_{B2}$, sharing about 35% sequence identity with GABA$_{B1}$[47-51]. Both subunits serve distinct functions within the heterodimeric receptor complex. GABA$_{B1}$ contains the agonist and antagonist binding site in the large N-terminal extracellular domain, which is most likely arranged in a Venus flytrap-like structure[52-54]. Association with GABA$_{B2}$ is necessary to keep the GABA binding site in a high affinity state[55,56]. On the other hand, GABA$_{B2}$ contains a binding site for allosteric modulators, which is not however associated with the N-terminal Venus flytrap domain, but is located in the heptahelical domain[57]. Binding of ligands to this site does not directly activate the GABA$_B$ receptor but instead affects the affinity of orthosteric agonists and antagonists to GABA$_{B1}$[58]. Finally, GABA$_{B2}$ is responsible for G protein activation[56,59-63] and plays an important role in cell surface trafficking of the heterodimerized receptor complex by masking an arginine-based endoplasmic reticulum (ER) retention/retrieval (RXR) signal present in the C-terminal domain of GABA$_{B1}$[64-68].

THE ROLE OF DESENSITIZATION AND PHOSPHORYLATION ON THE AVAILABILITY OF FUNCTIONAL GABA$_B$ RECEPTORS

Prolonged exposure of G protein coupled receptors (GPCR) to agonists generally leads to a complex series of events in order to attenuate or terminate signal transduction, protecting the cell from overstimulation. Signal transduction is often attenuated by desensitization of the receptors (i.e., abrogating signaling), although the agonist is still present[69,70]. Desensitization of many GPCRs involves phosphorylation-dependent uncoupling of the receptor from the G proteins, followed by internalization of the receptor. Activated GPCRs are usually phosphorylated by G protein-coupled receptor kinase (GRKs) at serine and/or threonine residues residing in the carboxyl-terminal tail- or intracellular loop regions, which rapidly attenuates receptor responses. Phosphorylation leads to the recruitment of arrestins, which is thought to sterically prohibit signaling to G proteins and induces internalization of the receptor by linking it to components (clathrin, AP2 complex) of the endocytosis machinery[69,70]. Internalized receptors are then either degraded in lysosomes or are dephosphorylated and subsequently recycled to the plasma membrane, where they are again available for signaling.

It is well known that prolonged activation of GABA$_B$ receptors commonly leads to their desensitization. Recent studies suggest that there might be more than one mechanism for desensitization of GABA$_B$ receptors[71-75],

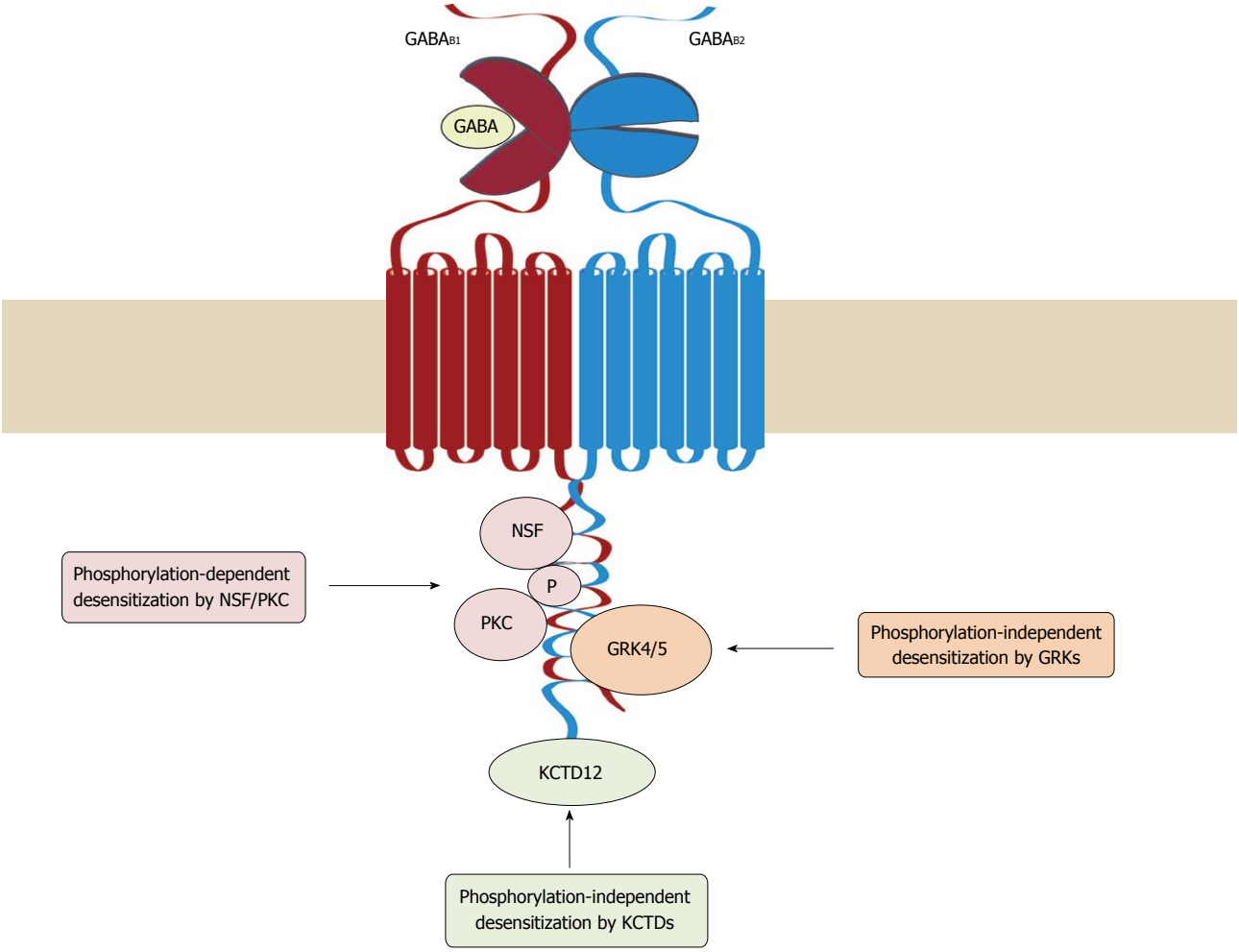

Figure 2 Mechanisms of GABA$_B$ receptor desensitization. Three distinct mechanisms have been so far implicated in the desensitization of GABA$_B$ receptors. In cerebellar granule cells, G protein receptor kinase (GRK) 4 and 5 associate with GABA$_B$ receptors and induce desensitization of the receptors in a phosphorylation-independent manner. In cortical and hippocampal neurons, desensitization of the receptors involves the interaction of NEM-sensitive fusion protein (NSF) with GABA$_{B1}$ and GABA$_{B2}$, which is thought to prime the receptor for phosphorylation by protein kinase C (PKC). Association of potassium channel tetramerization domain-containing (KCTD) proteins 12 and 12b with the C-terminus of GABA$_{B2}$ appears to render the receptor complex competent for desensitization. GABA: γ-Aminobutyric acid.

which does not follow the classical desensitization pattern of GPCRs described above (Figure 2). Distinct desensitization mechanisms for GABA$_B$ receptors may be operative in different neuronal populations.

A study conducted in mouse cerebellar granule cells showed that GRK4, which is mainly expressed in testes and cerebellum[69,76,77], promotes agonist-induced desensitization of GABA$_B$ receptors *via* direct association, but does not involve GABA$_B$ receptor phosphorylation[78]. These findings were confirmed by Kainaide *et al*[75], who demonstrated that the association of GABA$_{B2}$ with GRK4 or GRK5, but not GRK2, -3 or -6, leads to agonist-induced receptor desensitization in *Xenopus* oocytes and baby hamster kidney cells. Interestingly, GRK4 and -5-mediated desensitization was partially suppressed by application of S(+)-ketamine, which leads to inhibition of the GABA$_B$ receptors/GRK complex formation by an as yet unidentified mechanism[79]. It is currently not understood how GRK4 and -5 mediate desensitization of GABA$_B$ receptors. However, it might well be that the binding of GRK4 and -5 disrupts GABA$_B$ receptor/G-protein interaction.

On the other hand, for cortical and hippocampal neurons, a phosphorylation-dependent desensitization mechanism of GABA$_B$ receptors was reported[74]. This mechanism is based on the direct interaction of NEM-sensitive fusion (NSF) protein with the C-terminal domains of GABA$_{B1}$ and GABA$_{B2}$, which primes the receptor for recruitment of protein kinase C (PKC). The data indicate that the association of GABA$_B$ receptors with NSF is a prerequisite for recruiting PKC to the receptor upon agonist activation. PKC phosphorylates the receptor leading to its desensitization, and induces dissociation of NSF from the receptors. The precise roles of NSF and PKC in this complex process remain to be determined. NSF might be required for unmasking phosphorylation sites of the receptor or involved in PKC activation. In addition, it is unclear whether NSF dissociates from the receptor before desensitization occurs or whether releasing NSF initiates recovery of the receptor from desensitization.

Another factor determining desensitization of GABA$_B$ receptors was recently discovered by functional proteomics[80]. Members of the potassium channel tetramer-

ization domain-containing (KCTD) protein family were found to interact as tetramers with the C-terminus of GABA$_{B2}$, generating high-molecular mass protein complexes. Depending on the co-expressed KCTD subtype, distinct parameters of GABA$_B$ receptor function were affected, such as agonist potency, signaling onset or desensitization. Interestingly, only when GABA$_B$ receptors were co-expressed with KCTD-12 or -12b desensitization of GABA$_B$ receptors was observed, whereas in the presence of KCTD-8 or -16 the receptors displayed no desensitization[80]. This finding may explain the observation that GABA$_B$ receptor desensitization varies among different neuronal populations. One striking example is the ventral tegmental area (VTA). GABA$_B$ receptors expressed in GABAergic neurons of the VTA display baclofen-induced, largely non-desensitizing, currents, whereas in dopaminergic neurons of the VTA baclofen elicited desensitizing currents[81]. These findings suggest that the general ability of GABA$_B$ receptors to desensitize may be determined by the associated KCTD subtype, which may then recruit distinct desensitization mechanisms depending on the neuronal population.

Interestingly, PKA-dependent phosphorylation appears to counteract desensitization of GABA$_B$ receptors. Couve *et al*[71] showed that PKA exclusively phosphorylates serine 892 (S892) in the C-terminal domain of GABA$_{B2}$, resulting in reduced receptor desensitization. This effect on desensitization can be overcome by activation of the receptors, which results in inhibition of adenylyl cyclases, reduced cAMP levels and consequently diminished PKA activity and GABA$_{B2}$-S892 phosphorylation. The precise mechanism as to how PKA phosphorylation of GABA$_{B2}$-S892 affects desensitization of GABA$_B$ receptors remains unclear. There is an indication that it stabilizes cell surface GABA$_B$ receptors and thereby increases effector coupling[71,82]. This is, however, unlikely because it is now well accepted that prolonged agonist exposure does not trigger increased internalization of cell surface receptors[78,82-85]. However, GABA$_{B2}$-S892 phosphorylation provides a mechanism for regulating the extent of GABA$_B$ receptor desensitization by the activity of Gα_s-coupled GCPRs that enhance PKA activity.

Another kinase that is involved in regulating GABA$_B$ receptor activity is the 5'AMP-dependent protein kinase (AMPK). AMPK directly binds to the C-terminus of GABA$_{B1}$ and phosphorylates S917 and S783 in the C-terminal domains of GABA$_{B1}$ and GABA$_{B2}$, respectively[86]. Functional analysis revealed that phosphorylation of S783 resulted in a stabilization of baclofen-induced K^+ currents[86]. This effect has been shown to be of particular relevance in limiting neuronal cell death in experimental ischemia. Anoxic or ischemic conditions are associated with neuronal over-excitation, a decline in cellular adenosine-5'-triphosphate (ATP) and a rise in Ca^{2+} and AMP levels, which are all factors activating AMPK[87,88]. Under such conditions, increased phosphorylation of GABA$_{B2}$-S783 was detected along with an over-expression of a GABA$_{B2}$ mutant that cannot be phosphorylated at this site associated with increased neuronal death[86]. These findings support a mechanism in which AMPK functions as a metabolic sensor that detects severe cellular stress and phosphorylates, amongst others, GABA$_B$ receptors. This is thought to result in enhanced GABA$_B$ receptor signaling that counteracts over-excitation of the neuron and limits neuronal death.

REGULATION OF GABA$_B$ RECEPTORS BY TRAFFICKING

The lifecycle of a plasma membrane protein like the GABA$_B$ receptor starts with its synthesis at the rough ER where the nascent protein is co-translationally incorporated into the ER membrane. After folding, initial posttranslational processing, and assembly, the receptor is exported to the Golgi apparatus where it is further processed and finally transported *via* the trans-Golgi network to the plasma membrane. After a certain time span of function, the receptor is internalized and recycled back into the plasma membrane for another cycle of function, or is eventually degraded into lysosomes. To ensure a constant number of receptors in the plasma membrane for signaling, these trafficking events need to be precisely coordinated. On the other hand, regulation of each of the different trafficking steps permits adjusting the number of cell surface receptors, and thus signaling strength, according to the physiological requirements.

ER export of GABA$_B$ receptors

Little is known about the early stages in the lifecycle of GABA$_B$ receptors. So far it is clear that exit of heterodimeric GABA$_B$ receptors from the ER is controlled by an arginine-based ER retention/retrieval signal (RXR) present in the C-terminal domain of GABA$_{B1}$[64-66]. The mechanism that prevents cell surface trafficking of GABA$_{B1}$ appears to involve the coat protein complex I (COP I), which plays a central role in the retrograde transport of proteins from the Golgi apparatus back to the ER[89]. COP I binds to the ER retention/retrieval signal of GABA$_{B1}$ and shuttles monomeric GABA$_{B1}$ that reached the cis-Golgi apparatus back to the ER. Heterodimerization with GABA$_{B2}$ masks the ER retention/retrieval signal and permits forward transport[64-68]. In contrast to GABA$_{B1}$, monomeric GABA$_{B2}$ can leave the ER and reach the cell surface. However, it is assumed that the GABA$_{B2}$ expression level in the ER is a limiting factor for ER exit of the heterodimeric GABA$_B$ receptors. This mechanism is thought to ensure that only correctly folded and assembled (i.e., functional) receptors are exported to the cell surface.

Endocytosis of GABA$_B$ receptors

There are two principal mechanisms by which GPCRs are internalized from the plasma membrane, constitutive endocytosis and agonist-induced endocytosis. Constitutive endocytosis constantly removes receptors from the

cell surface, whereas agonist-induced endocytosis initiates removal of receptors from the plasma membrane upon activation of the receptors and ensures fast termination of signaling. It is now well established that GABA$_B$ receptors undergo constitutive endocytosis, whereas the presence of agonist-induced internalization of the receptors is less clear.

Heterologously expressed, as well as neuronal, GABA$_B$ receptors display fast constitutive internalization, as evidenced by distinct experimental approaches including immunofluorescence staining and microscopy, live cell imaging and cell surface biotinylation methods[83,84,90-93]. Constitutive internalization of GABA$_B$ receptors is a fast process, as shown by the rapid loss of labeled receptors from the cell surface, which reaches a plateau after 10-30 min (40% of labeled receptors remain at the cell surface), with rates of internalization of ranging from 2-10 min[92-94]. GABA$_B$ receptors internalize as heterodimers and are not dissociated into its subunits prior to endocytosis[84,90,92,95]. The rate of internalization appears to be determined by GABA$_{B2}$. GABA$_{B1}$, which contains an inactivated ER retention signal so that it is exported to the plasma membrane, displays a considerably faster rate of internalization than the GABA$_{B1,2}$ heterodimer[92]. This is due to a dileucine motif within the coiled-coil domain of GABA$_{B1}$, which gets masked upon assembly with GABA$_{B2}$.

The data so far suggest that for endocytosis, GABA$_B$ receptors are recruited to clathrin-coated pits and internalized in a dynamin-dependent manner[83,90,95]. Clathrin-coated pits are composed of clathrin heavy and light chains that form a polymeric lattice and contain numerous adaptor and endocytic accessory proteins. For endocytosis, the cargo-loaded clathrin-coated pit invaginates and is eventually released from the plasma membrane in a GTP-dependent reaction mediated by dynamin[96]. There is evidence based on colocalization and immunoprecipitation data that GABA$_B$ receptors interact with the AP2 adaptor, which is one of the adaptor complexes that recruit membrane proteins to clathrin-coated pits[83,84,90].

Colocalization studies with marker proteins for various endosomal compartments revealed that endocytosed GABA$_B$ receptors first enter early endosomes and are then either sorted to Rab4 or Rab11-positive recycling endosomes, or to Rab7-positive late endosomes, and finally to lysosomes for degradation[84,90,92,95,97,98].

In addition to the colocalization data, there is also functional evidence that endocytosed GABA$_B$ receptors constitutively recycle back to the cell surface. Using immunofluorescence staining and tagged GABA$_B$ receptors transfected into hippocampal neurons Vargas *et al*[90] showed that a significant fraction of endocytosed receptors recycle back to the cell surface. Quantitative cell surface biotinylation and immunofluorescence-based methods indicate that the vast majority of native GABA$_B$ receptors in cortical neurons are rapidly recycled to the plasma membrane. After 15 min, about half of the internalized receptors have recycled back to the cell surface, and after 30 min this has increased to the majority of the receptors[84,94].

In summary, the current data indicate that GABA$_B$ receptors constitutively internalize at a high rate *via* the classical clathrin-dependent pathway and rapidly recycle back to the cell surface. Since endocytosis and recycling are highly energy-consuming processes, this mechanism is most likely of significant physiological relevance. The most obvious explanation is that a high rate of constitutive internalization and recycling generates a pool of intracellular receptors that can be immediately inserted into the plasma membrane to increase the cell surface number of receptors by increasing the rate of recycling while leaving the rate of internalization constant. In the case of synaptic AMPA receptors, such a mechanism has been proposed to contribute to increasing the level of the receptors during the early phase of long-term potentiation, which is thought to underlie learning and memory formation[99].

Degradation of GABA$_B$ receptors

Most cell surface receptors are eventually degraded in lysosomes, the major catabolic cellular compartment. After endocytosis, the endocytic vesicles carrying the receptors fuse with early endosomes, which then mature to late endosomes containing the material destined for degradation. Mature late endosomes are competent to fuse with lysosomes that contain a variety of hydrolases for the breakdown of all kinds of macromolecules[100].

There is now solid data that, at the end of their lifetime, GABA$_B$ receptors are endocytosed and degraded in lysosomes. This is evidenced by the intracellular accumulation of internalized GABA$_B$ receptors upon inhibition of lysosomal function[83,84,101] and the colocalization of intracellular GABA$_B$ receptors with marker proteins for late endosomes and lysosomes[84,92]. GABA$_B$ receptors are most likely sorted by the ESCRT (endosomal sorting complex required for transport) machinery to lysosomes, because the knockdown of tumor susceptibility gene 101 (TSG101), an integral component of the ESCRT machinery, prevents degradation of the receptors[101]. Three distinct ESCRT complexes sequentially target mono- and K63-linked polyubiquitinated membrane proteins to late endosomes[102]. However, it remains to be shown whether GABA$_B$ receptors are ubiquitinated and whether ubiquitination serves as a lysosomal sorting signal.

Another unresolved issue is how the decision is made as to whether a receptor is sorted to the degradation pathway. As discussed above, the vast majority of endocytosed GABA$_B$ receptors recycle back to the plasma membrane and only few are degraded. However, pharmacological inhibition of recycling leads to rapid lysosomal degradation of the receptors (about 50% of the total receptor population within 30 min)[84]. This indicates that recycling and degradation of GABA$_B$ receptors is tightly controlled, and decreasing the rate of recycling constitutes a mechanism to rapidly reduce the receptor number (discussed below).

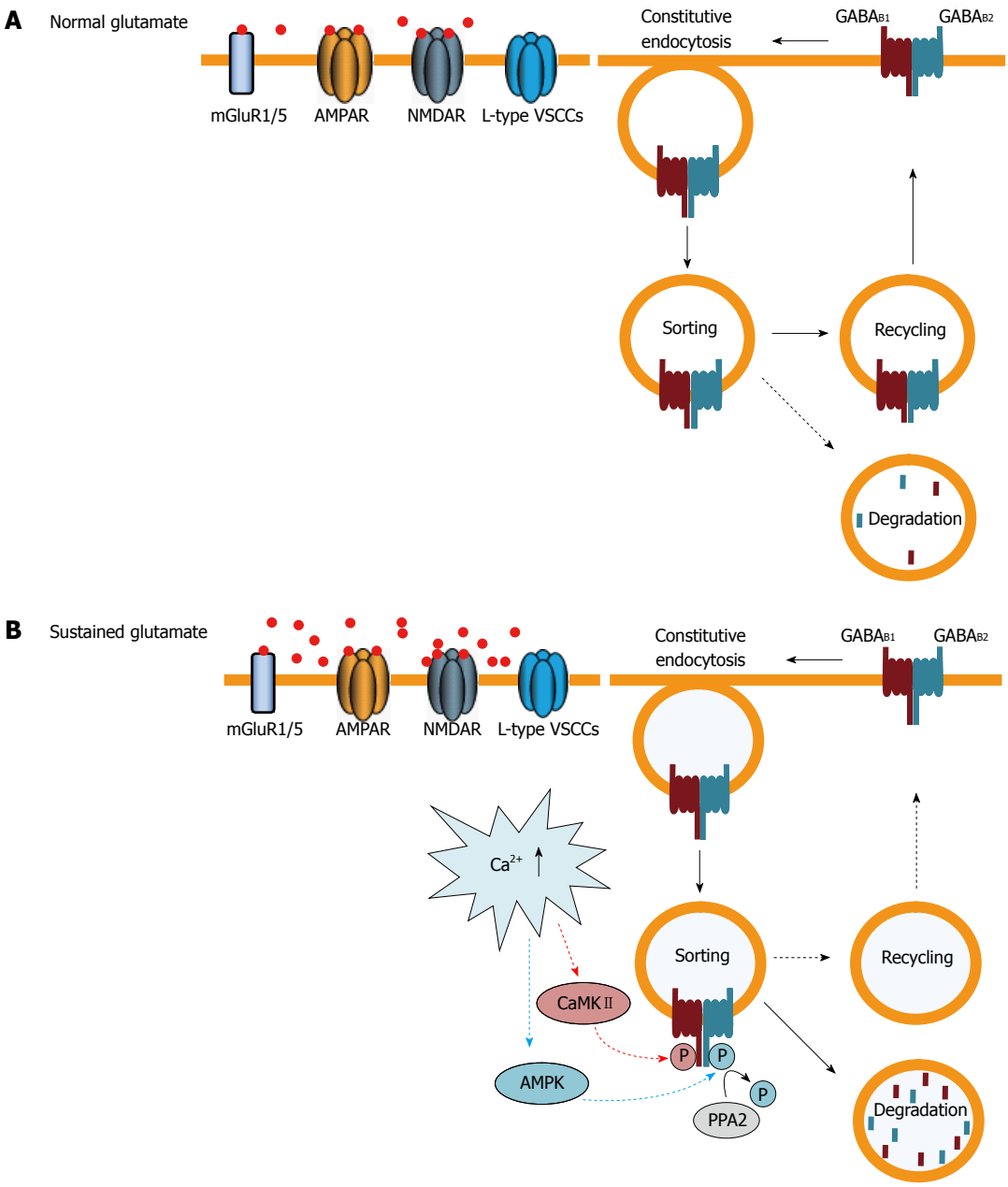

Figure 3 Regulation of cell surface GABA$_B$ receptors by trafficking. A: Under normal conditions GABA$_B$ receptors are constitutively internalized and recycled back to the plasma membrane. Only a small fraction of receptors are sorted to lysosomes for degradation; B: Sustained activation of glutamate receptors [primarily 2-amino-3-(5-methyl-3-oxo-1,2-oxazol-4-yl) propanoic acid (AMPA) and N-methyl-D-aspartic acid (NMDA) receptors] and L-type voltage-gated Ca^{2+} channels raises intracellular Ca^{2+} levels. This induces phosphorylation of GABA$_{B1}$ at serine 867 by calmodulin-dependent protein kinase II (CaMK II) and of GABA$_{B2}$ at serine 783 by adenosine monophosphate (AMP) kinase, followed by slow dephosphorylation, by protein phosphatase 2 (PPA2). These events shift the recycling/degradation equilibrium towards degradation so that the majority of GABA$_B$ receptors are no longer recycled, but instead degraded in lysosomes. Since constitutive endocytosis of the receptors remains unaffected, this mechanism results in a rapid down-regulation of GABA$_B$ receptors. AMPK: 5'AMP-dependent protein kinase; GABA: γ-Aminobutyric acid; VSCCs: Voltage-sensitive calcium channels.

Regulation of cell surface GABA$_B$ receptors by glutamatergic excitatory activity

GABA$_B$ receptors control glutamate signaling *via* presynaptic and postsynaptic mechanisms. They are abundantly expressed at glutamatergic synapses[2-5,103] where they are activated by GABA spillover from adjacent GABAergic terminals and inhibit glutamate release[8,104-106]. This limits activation of postsynaptically located excitatory glutamate receptors (AMPA/kainate and NMDA receptors). Although GABA$_B$ receptors are also located in close proximity to AMPA and NMDA receptors they do not appear to directly modulate AMPA and NMDA receptor excitatory postsynaptic currents (EPSCs)[105]. However, activation of postsynaptic GABA$_B$ receptors seem to limit Ca^{2+}-influx through NMDA receptors by inhibition of the cAMP/PKA signaling pathway, which normally enhance NMDA receptor Ca^{2+} conductance[105].

Besides the prominent regulation of glutamate signaling by GABA$_B$ receptors, there is now evidence that glutamatergic activity, in return, may affect GABA$_B$ receptor expression to attenuate inhibitory control (Figure 3). Application of glutamate to cultured neurons dra-

matically down-regulates cell surface GABA$_B$ receptors and GABA$_B$ receptor-activated currents[90,94,97,98]. Specific activation of AMPA receptors[94] or NMDA receptors[97,98] was sufficient to induce the down-regulation of GABA$_B$ receptors. Interestingly, the kinetics of AMPA-induced down-regulation of GABA$_B$ receptors was significantly slower than that induced by glutamate and was accelerated upon co-activation of group I metabotropic glutamate receptors[94]. These findings indicate that beside the ionotropic AMPA and NMDA receptors, metabotropic glutamate receptors also contribute to the glutamate-induced down-regulation of GABA$_B$ receptors. The underlying mechanism of this rapid down-regulation of GABA$_B$ receptors is a shift of the recycling/degradation equilibrium towards lysosomal degradation[94,97]. Glutamate application reduced the rate of GABA$_B$ receptor recycling without altering the rate of their internalization and was fully restored after inhibition of lysosomal degradation. The precise intracellular signaling cascade leading to the glutamate-induced shift in sorting the GABA$_B$ receptors preferentially to the degradation pathway is currently not fully resolved. It is clear that the down-regulation of GABA$_B$ receptors depends on the influx of Ca^{2+}[94,98], which is most likely mediated by L-type voltage-gated Ca^{2+} channels[94]. Two downstream effector systems were identified to be involved in the down-regulation of GABA$_B$ receptors (Figure 3). One depends on phosphorylation of serine 867 (S867) in GABA$_{B1}$ by Ca^{2+}/calmodulin-dependent protein kinase Ⅱ (CaMK Ⅱ)[98]. The other involves phosphorylation of serine 783 (S783) in GABA$_{B2}$ by AMP kinase and subsequent dephosphorylation by protein phosphatase 2A (PP2A)[97]. Mutational inactivation of each phosphorylation site prevented glutamate-induced down-regulation of GABA$_B$ receptors. However, while the cell surface expression of the receptors containing the mutant GABA$_{B1}$(S867A) was normal[98], the mutant GABA$_{B2}$(S783A) was expressed to a significantly lesser level in the plasma membrane[97]. This suggests that phosphorylation of S783 in GABA$_{B2}$ is involved in sorting the receptors to the recycling pathway, while phosphorylation of S867 in GABA$_{B1}$ may constitute a direct signal for sorting the receptors to lysosomal degradation. Alternatively, phosphorylation of GABA$_{B1}$(S867) may be required for dephosphorylation of S783 in GABA$_{B2}$, for instance by recruiting PPA2 to the receptor. In this respect it would be very interesting to test whether phosphorylation of GABA$_{B1}$(S867) by CaMK Ⅱ is required for dephosphorylation of GABA$_{B2}$(S783) by PPA2.

What is the physiological relevance of this mechanism? Since glutamate-induced down-regulation of GABA$_B$ receptors has so far only been studied in cultured neurons, the role of this process *in vivo* remains to be shown. However, there are physiological, as well as pathological, conditions involving sustained activity of glutamate receptors where this mechanism might be operative. Under pathological conditions associated with excessive activation of glutamate receptors, such as ischemia, down-regulation of GABA$_B$ receptors results in diminished inhibitory control and may further enhance excitotoxicity and neuronal cell death. This view is supported by an *in vitro* model of ischemia where total GABA$_{B2}$ protein levels were found to be strongly reduced 60 min after the ischemic insult[107]. Likewise, in an *in vivo* model of hypoxia/ischemia, significantly reduced levels of GABA$_B$ receptors were detected[108].

Under normal physiological conditions, glutamate-induced down-regulation of GABA$_B$ receptors may contribute to the process of long-term potentiation, which is thought to be the molecular basis for learning and memory formation, as long-term potentiation is associated with sustained activity of glutamate receptors[109]. In this scenario, enhanced glutamatergic activity would induce the down-regulation of GABA$_B$ receptors and consequently relieve the synapses from inhibition, resulting in a further increase of synaptic excitability.

CONCLUSION

Trafficking events play a pivotal role in the cell surface availability of receptors and largely determine their signaling strength. Currently, we are only beginning to identify and understand the trafficking mechanisms of GABA$_B$ receptors and how cell surface expression of the receptors is regulated. In particular, we almost completely lack knowledge on forward trafficking of GABA$_B$ receptors from the ER *via* the Golgi network to the plasma membrane. In addition, mechanisms on the targeting of the receptors to specific sites in the neuron are unknown. There is an initial indication that GABA$_{B1}$ may be transported independent of GABA$_{B2}$ within the ER into dendrites and are then assembled and exported to the plasma membrane[110]. This finding implies that heterodimerization of GABA$_B$ receptors is a spatially and temporally controlled mechanism, and would provide an additional level to regulate cell surface expression of the receptors. It is now clear that GABA$_B$ receptors are constitutively endocytosed *via* the clathrin and dynamin-dependent pathway, and are predominantly recycled back to the plasma membrane with only a minor fraction being degraded in lysosomes. The equilibrium of sorting the receptors to the recycling and degradation pathway appears to be controlled by phosphorylation/dephosphorylation events and regulated by changes in neuronal activity associated with increased influx of Ca^{2+}. It will be a major future effort to unravel the mechanisms involved in trafficking, sorting and degradation of GABA$_B$ receptors and how they are regulated by physiological and pathological stimuli. It is now well established that receptor trafficking regulates signal transduction and that disturbances in these mechanisms may contribute to disease states[111]. Since GABA$_B$ receptors have been implicated in a variety of neurological disorders-ranging from epilepsy, addiction, schizophrenia, depression, anxiety to chronic pain-it is likely that altered GABA$_B$ receptor trafficking is involved, at least to some extent, in these diseases. We expect that a deeper knowl-

edge of the trafficking mechanisms of GABAB receptors under physiological and pathological conditions will provide the basis for the development of novel and highly selective future therapeutic interventions.

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