Signal Transduction: Pathways, Mechanisms and Diseases

Bearbeitet von
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1. Auflage 2009. Buch. XI, 437 S. Hardcover
ISBN 978 3 642 02111 4
Format (B x L): 0 x 0 cm
Gewicht: 918 g

Chapter 2
Regulation of Intraneuronal Trafficking of G-Protein-Coupled Receptors by Neurotransmitters In Vivo

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2.1 Introduction

Most neurotransmitters and related drugs modulate neuronal activity through G-protein-coupled receptors (GPCRs), which are synthesized in the endoplasmic reticulum and mature in the Golgi complex. Immunohistochemical studies at the electron microscopic level demonstrate that GPCRs are targeted to the plasma membrane to interact with neurotransmitters. This membrane targeting of GPCRs leads to their presynaptic or postsynaptic distribution and their synaptic or extrasynaptic localization. This determines the sites of neurotransmitter action and thus the putative role of GPCRs in regulation of neuronal activities.

Activation of these receptors triggers a cascade of intracellular events that involves a wide variety of effector systems and leads to the modulation of neuronal postsynaptic activity (Koenig and Edwardson 1997; Krueger et al. 1997; Yoburn et al. 2004). In vitro studies have widely demonstrated that the abundance and availability of these receptors at the cell surface is regulated by the neuronal environment and is the result of complex intraneuronal trafficking. The amplitude of neuronal response to the neurochemical environment variations depends on, besides the quantity of released neurotransmitter, the number of postsynaptic receptors (Oakley et al. 1999; Anborgh et al. 2000). This control of the abundance and availability of GPCRs at the neuronal membrane probably contributes to modulation of how neurons respond to their endogenous or exogenous ligands under physiological, pathological or therapeutic conditions. It is possible that the modulation of receptor availability at the plasma membrane is a key event of

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A. Sitaramayya (ed.), *Signal Transduction: Pathways, Mechanisms and Diseases*,
DOI 10.1007/978-3-642-02112-1_2,

neuronal activity regulation in vivo, other facets of which include neurotransmitter release and neuronal excitability. However, this regulation is still poorly understood in vivo. Because the mechanisms of GPCR trafficking might differ in vivo and in vitro, as has been shown for the muscarinic ACh receptor M_2, specific analyses are required in vivo (Roseberry and Hosey 2001; van Koppen 2001; Bernard et al. 2003). In vivo analyses of the mechanisms that regulate the availability of GPCRs are thus important for improving our understanding of drug effects in pathology and therapy.

We discuss the role of neurochemical environment in the intraneuronal trafficking of GPCRs in vivo and we present data demonstrating that the abundance of GPCRs at the cell membrane and their intracellular trafficking are modulated by levels of neurotransmitters. We also show that subcellular distribution of GPCRs is determined by different criteria, such as the type of stimulation (acute vs. chronic) and the neuronal compartment (somatodendritic vs. axonal).

2.2 Experimental Approaches used to Study Trafficking of G-Protein-Coupled Receptors In Vivo

Different strategies in which the neurochemical environment is impaired have been used to study GPCR trafficking in animals in vivo. Two such approaches are pharmacological treatment (using direct or indirect agonists or, more rarely, antagonists), and knockout mice for molecules involved in neurotransmitter level. GPCRs are detected in brain sections using antibodies or fluorescent ligands (Yoburn et al. 2004). Alternatively, viral-mediated gene transfer and epitope-tagged GPCRs can be used (Haberstock-Debic et al. 2003). Receptors are usually visualized at the light-microscopic level; immunohistochemistry at this level enables the distinction between membrane and intracytoplasmic localization of the receptors to be made efficiently. Identification of cytoplasmic organelles or compartments containing GPCRs is necessary for understanding the dynamic of trafficking in neurons and for identifying events such as endocytosis, synthesis and degradation. Some organelles can be identified easily on the basis of their ultrastructure: these include the endoplasmic reticulum, Golgi complex and multivesicular bodies. Cytoplasmic trafficking also involves vesicular compartments such as endosomes; the endosomal compartment is identified by its vesicular aspect at the light-microscopic level (Mantyh et al. 1995) or its ultrastructural characteristics (Bernard et al. 1998; Dumartin et al. 1998; Bernard et al. 1999, 2003; Csaba et al. 2001; Liste et al. 2002). Subcellular compartments can be identified at the light-microscopic level by co-detection of GPCRs with molecular markers of cytoplasmic compartments, such as transferrin or the transferrin receptor (Faure et al. 1995; Keith et al. 1998; Csaba et al. 2001; Bernard et al. 2003). Counting immunoparticles at the ultrastructural level is also important for comparing the abundance of receptor in each compartment in basal and

experimental conditions (Bernard et al. 1998, 1999, 2003; Dumartin et al. 1998; Csaba et al. 2001; Liste et al. 2002).

2.3 Regulation of the Intraneuronal Distribution of GPCRs Under Physiological Conditions: Constitutive Endocytosis

In vitro, many studies have demonstrated that some GPCRs display constitutive endocytosis, indicating that a proportion of the receptor population spontaneously undergoes internalization in the absence of stimulation with an agonist (Leterrier et al. 2004; Xu et al. 2007).

The neuron is a highly polarized cell that comprises two large domains: the somatodendritic compartment and the axonal compartment. The somatodendritic compartment receives and transduces external signals, and the axon and axonal terminal transmit a relevant response. Regulation of receptor distribution on the cell surface, including axonal polarization may be the result of constitutive internalization under physiological conditions. This polarization is achieved and maintained through a specific sorting signal that selectively targets the neuronal membrane proteins to somatodendritic or to axonal compartments (Higgins et al. 1997; Burack et al. 2000).

A recent in vitro study has shown that constitutive somatodendritic endocytosis is required for the proper axonal targeting of the type 1 cannabinoid receptor (CB1R). Blockade of constitutive somatodendritic endocytosis abolished CB1R targeting to the axonal plasma membrane (Leterrier et al. 2006).

In vivo, constitutive endocytosis may be a means to adapt receptor density to the intensity of stimulation and thus to regulate neuronal activity e.g., neurotransmitter release for axonal receptors, neuronal excitability for somatodendritic receptors. Indeed, there seems to be a correlation between the intensity of the stimulation by the endogenous neurotransmitter and the availability of the receptors at the plasma membrane. For example, two striatal neuronal subpopulations have been identified according to the density of M_4 muscarinic ACh receptors in their plasma membranes (Bernard et al. 1999). These subpopulations are localized in two different striatal territories (striosomes and matrix) that display different ACh levels (Graybiel and Ragsdale 1978; Graybiel 1986; Hirsch et al. 1989; Lowenstein et al. 1989). The higher the ACh-mediated activity, the fewer ACh receptors are present at the membrane. In addition, an inverse relationship exists in rat brain between the density of somatostatin-containing afferents and the density of somatostatin sst2A receptors at the plasma membrane (Dournaud et al. 1998). Similarly, μ-opiate receptor trafficking has been shown to be regulated by afferent inputs in dorsal horn neurons (Morinville et al. 2004).

2.4 Regulation of GPCR Distribution in the Somatodendritic Field After Acute and Chronic Stimulation

2.4.1 Decreased Number of GPCRs at the Plasma Membrane

In vivo data suggest that the mechanisms of regulation and adaptation of GPCR compartmentalization are different after acute and chronic stimulation (Bernard et al. 1998; Dumartin et al. 1998, 2000; Riad et al. 2001; Bernard et al. 2003; Riad et al. 2008). Several processes seem to control the abundance of GPCRs at the plasma membrane, including endocytosis, recycling, degradation and neosynthesis. In both acute and chronic conditions, the stimulation of GPCRs is associated with a decrease in receptor density at the plasma membrane of cell bodies and dendrites (Figs. 2.1 and 2.2). Acetylcholinesterase (AChE) and dopamine-transporter knock-out mice, which are models of constitutive chronic receptor stimulation by ACh and dopamine, display a decreased density or even disappearance from the plasma membrane of M_2 receptors and dopamine D_1 receptors, respectively (Bernard et al. 1998, 2003; Dumartin et al. 1998, 2000). However, although the abundance of these receptors is reduced at the plasma membrane following both acute and chronic stimulation, their intracytoplasmic fate and trafficking are different. Anatomical studies have demonstrated that different GPCRs are differentially redistributed in different intraneuronal compartments.

2.4.2 Redistribution of GPCRs in the Cytoplasm After Acute Stimulation

2.4.2.1 Endocytosis

In vitro, acute stimulation induces endocytosis of GPCRs, which occurs mainly through the formation of clathrin-coated pits (von Zastrow and Kobilka 1992; Koenig and Edwardson 1997; Lamb et al. 2001; Yoburn et al. 2004). After binding of the agonist, the GPCR is phosphorylated and binds β-arrestin, which is responsible for receptor uncoupling from its G protein. Clathrin is then recruited to the plasma membrane to form pits; detachment of these pits from the membrane is induced by dynamin. Alternatively, endocytosis of some GPCRs can involve the formation of caveolae and not of clathrin-coated pits (Lamb et al. 2001; Sabourin et al. 2002). In vivo, light- and electron-microscopic analyses show that acute stimulation reduces the abundance at the plasma membrane of GPCRs, including muscarinic, dopamine, opioid, substance P, serotonin-1A and somatostatin sst2A receptors (Mantyh et al. 1995; Bernard et al. 1998, 1999; Dumartin et al. 1998; Abbadie and Pasternak 2001; Csaba et al. 2001; Riad et al. 2001; He et al. 2002; Liste et al. 2002; Decossas et al. 2003;

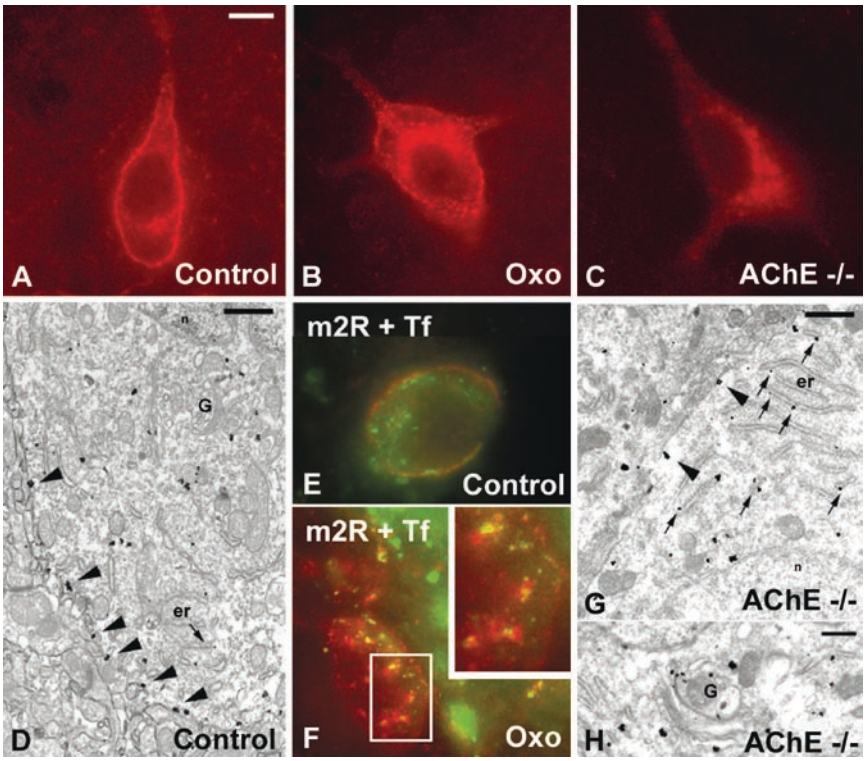

Fig. 2.1 Effect of acute and chronic modifications of ACh levels on the cellular and subcellular distribution of M_2 receptors in neurons of the striatum in vivo. Cellular and subcellular detection of M_2 receptors in striatal neurons. Images were collected under epifluorescence (**a**–**e**) and electron microscopy (**f**–**h**) using fluorescent immunohistochemistry and a pre-embedding immunogold method. (**a**): In control mice, M_2 receptor immunoreactivity is mostly detected at the plasma membrane. (**b**): After acute treatment with oxotremorine ("Oxo"; 0.5 mg/kg subcutaneously for 1 h), M_2 receptor immunoreactivity is seen in the cytoplasm. (**c**): After chronic stimulation of ACh receptors in acetylcholinesterase knockout mice ($AChE^{-/-}$), no staining is observed at the membrane, whereas strong immunoreactivity is detected in the cytoplasm. (**d,e**): Organotypic cultures of striatum were co-incubated for 1 h with NaCl (9 g/L) (**d**), or with oxotremorine (25 μM) and transferrin (Tf; 50 μg/ml) (**e**), a constitutively endocytosed molecule used as a marker of endocytosis. In control animals (**d**), M_2 receptors are localized at the membrane (red) whereas transferrin is endocytosed (green). After oxotremorine treatment (**e**), M_2 receptors (red) are partially co-internalized with transferrin (yellow; the boxed area is enlarged in the inset). This suggests that the stimulation of muscarinic receptors induces the endocytosis of M_2 receptors through clathrin-coated pits. (**f**): In a control mouse, immunoparticles are associated mostly with the internal side of the plasma membrane (arrowheads). Some immunoparticles are associated with the endoplasmic reticulum ("er," small arrow) and the Golgi apparatus (**g**). (**g,h**) In $AChE^{-/-}$ mice, few immunoparticles are detected in association with the plasma membrane (arrowheads). By contrast, numerous particles are seen in the cytoplasm associated with the endoplasmic reticulum and Golgi apparatus. This suggests that when ACh receptors are chronically stimulated, targeting of m M_2 receptors is blocked in the intraneuronal compartments of synthesis and maturation, and thus they are no longer targeted to the membrane. Additional abbreviation: n, nucleus. Scale bars, 10 μm in (**a**–**e**); 500 nm (**f,g**); 50 nm (**h**). Reproduced, with permission, from Bernard et al. 2003

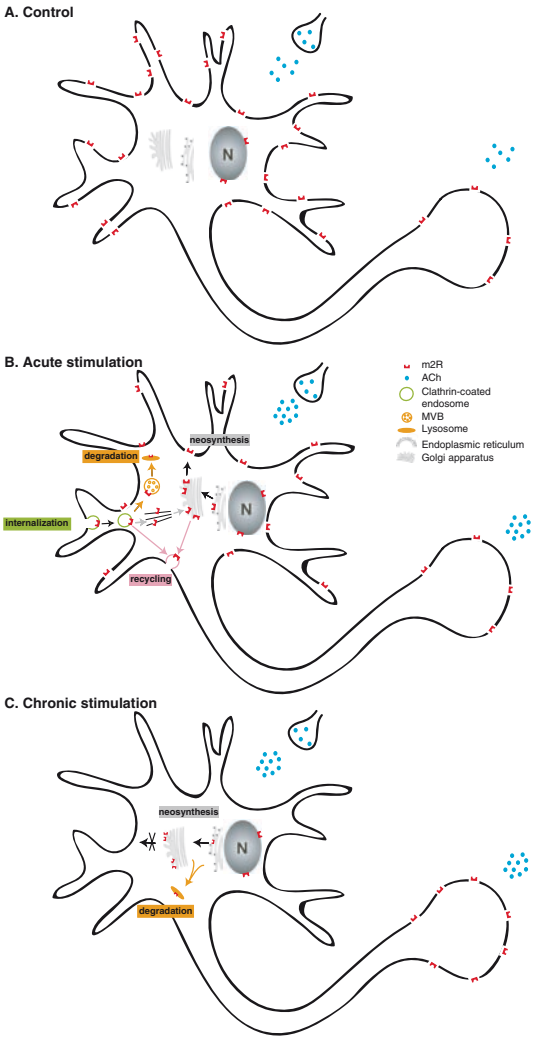

Fig. 2.2 A model of the regulation of the neuronal trafficking of GPCR in vivo by the neurochemical environment based on data obtained for the M_2 receptors in cholinergic neurons of the basalo-cortical or striatal pathways. (**a**): In control mice, most GPCRs are located at the plasma membrane of the somatodendritic compartment and at axonal varicosities. GPCRs are also present in intraneuronal compartments involved in synthesis (endoplamsic reticulum and outer nuclear membrane) and maturation (Golgi apparatus). (**b**): After acute stimulation, GPCRs are internalized from the plasma membrane to the cytoplasm by endocytosis through clathrin-coated pits. After endocytosis, receptors can be either recycled to the plasma membrane or sent to the degradation pathway via multivesicular bodies (MVBs) and lysosomes. Recycling may occur directly after endocytosis or after going through the Golgi apparatus. The receptors continue to be synthesized. (**c**): After chronic stimulation, GPCRs are trapped in the cytoplasm in association with the Golgi apparatus and endoplasmic reticulum, and few of them are targeted to the plasma membrane; mostly to the membrane of the axon terminal. After blockade in compartments of synthesis and maturation, M_2 receptors are degraded in lysosomes

Haberstock-Debic et al. 2003; Morinville et al. 2004; Trafton and Basbaum 2004). GPCRs have also been detected in association with small labeled vesicles in the cytoplasm, without a loss in the total number of receptors (Bernard et al. 1998, 1999, 2003; Dumartin et al. 1998; Csaba et al. 2001; Liste et al. 2002; Decossas et al. 2003). These compartments co-internalize transferrin, a constitutively endocytosed molecule, or co-express transferrin receptors (Csaba et al. 2001; Bernard et al. 2003) (Fig. 2.1). These data demonstrate that acute stimulation induces in vivo, as it does in vitro, internalization of GPCRs with the bound ligand from the cell surface into intraneuronal compartments. This is achieved in the same way as in vitro, by the classical endocytosis of GPCRs through formation of clathrin-coated pits. In vivo, no proof exists for a non-classical clathrin-independent endocytotic pathway involving caveolae (as has been demonstrated for GPCRs in vitro (Feron et al. 1997)).

The molecular mechanisms following ligand binding and endocytosis (phosphorylation of GPCRs, interaction with α-arrestin and uncoupling of the receptor from its G protein) have been described in vitro, but are still poorly understood in vivo. However, phosphorylation of μ-opioid receptors after agonist stimulation and the absence of such phosphorylation in GPCR kinase (GRK3) knockout mice, suggest a role for GPCR phosphorylation in the process of endocytosis of GPCRs in vivo (McLaughlin et al. 2004).

Endocytosis can occur throughout the membrane of the somatodendritic tree or can be compartment specific. Endocytosis of a same receptor may also be brain region specific (Riad, 2001). For example, after acute stimulation, muscarinic, D_1 or sst2A receptors are endocytosed in both the soma and dendrites, whereas μ-opioid receptors are endocytosed only in dendrites (Bernard et al. 1998, 1999, 2003; Dumartin et al. 1998; Csaba et al. 2001; Liste et al. 2002; Decossas et al. 2003; Haberstock-Debic et al. 2003). This might be due to different subneuronal comparmentalization of cytoplasmic regulatory factors involved in the endocytotic pathway (e.g., arrestin and/or dynamin). Such subcellular compartmentalization occurs in retinal photoreceptors for arrestin, which binds to rhodopsin, a GPCR (Elias et al. 2004). Because neuronal functions depend on the integration of neurochemical signals transmitted by different parts of the neuron, these data suggest that endocytosis might occur selectively in different neuronal compartments and thus contribute to the modulation of the membrane receptor availability and, hence, to the neuronal response.

Endocytosis mechanisms in vivo seem to depend on the type of agonist. For example, it has been demonstrated that μ-opioid receptors are internalized after treatment with D-ala2,me-phe4,gly(ol)5-enkephalin (DAMGO), but not after morphine treatment (Abbadie and Pasternak 2001).

2.4.2.2 Fate of GPCRs After Endocytosis

In vitro, degradation and recycling of GPCRs are thought to be important events after endocytosis. They participate in regulation of plasma membrane receptor

abundance and thus modulate the receptivity of neurons to further stimulation (Alvarez et al. 2002). Degradation and recycling have also been shown to occur in vivo. Anatomical data demonstrate that acute stimulation induces an increase in the number of muscarinic receptors associated with multi-vesicular bodies (MVBs) and lysosomes – two subcellular compartments that are involved in the degradation pathway (Bernard et al. 1998, 1999; Liste et al. 2002; Decossas et al. 2003) (Fig. 2.2). MVBs are considered to be intermediary compartments between endosomes and lysosomes where proteins are degraded. This suggests that some of these receptors are degraded after endocytosis. Alternatively, other endocytosed M_2 receptors might be recycled to the plasma membrane hours after acute stimulation (Bernard et al. 1998, 2003). It is possible that some GPCRs follow exclusively one or the other post-endocytotic pathways, as has been shown in vitro. For example, substance P NK1 receptors and μ-opioid receptors are mostly recycled to the plasma membrane, μ-opioid receptors are not, and instead are almost all sent to lysosomes and degraded (Grady et al. 1995; Wang et al. 2003). In vitro, GPCRs might also be degraded in the proteasome, as demonstrated for μ-opioid receptors, but no such data are available in vivo (Li et al. 2000). Ex vivo studies on organotypic cultures of striatum suggest that the re-expression of GPCRs at the membrane might occur without neosynthesis. For example, the blockade of neosynthesis of the M_2 receptor by cycloheximide has no effect on its reappearance at the plasma membrane (organotypic sections incubated first with 1 μM oxotremorine and 100 μM cycloheximide for 20 min, and then with 100 μM cycloheximide for 2 h; V. Bernard, unpublished). Moreover, no increase in levels of M_2 receptor mRNA was observed in neurons of the nucleus basalis magnocellularis (NBM) after acute stimulation, despite the internalization of M_2 receptors (Decossas et al. 2003). This suggests that activation of gene expression might not contribute to the synthesis of new M_2 receptors for recycling to the plasma membrane. It is usually accepted that reclycling occurs directly after endocytosis. Alternatively, it has been recently shown in vivo that after endocytosis, the somatostatin type 2 receptor (sst2) may be retrogradely transported through a microtubule-dependent mechanism to a *trans*-Golgi network, before recycling (Csaba et al. 2007).

2.4.2.3 Functional Role of Endocytosis

In vitro, the function of endocytosis is still under debate, but it might be involved in processes of desensitization, resensitization and/or signaling (Ferguson, 2001; Alvarez et al. 2002). Desensitization is a reversible reduction in neuronal response during sustained agonist stimulation. Some authors consider desensitization of GPCRs to be a consequence of endocytosis (Ferguson, 2001). Alternatively, densitization might not be linked to endocytosis, because the blockade of 5-HT2A receptor endocytosis has no effect on agonist-induced desensitization (Gray et al. 2001). In vivo, the links between changes in subcellular compartmentalization of a GPCR and the functions regulated by the same receptor are also unclear. Is densensitization a consequence of endocytosis, or are these processes independent?

Recently, Scherrer et al. (2006) studied the consequences of delta opioid receptor (DOR) sequestration on receptor function in vivo (locomotion) in knockin mice expressing fluorescent DOR. Groups of animals were pretreated with an agonist of DOR. After 2 h, when fluorescent DOR has internalized as a function of agonist concentration , a second dose was injected to all groups. Animals pretreated with either vehicle or the low agonist dose, whose receptors remain on the surface, showed a significant hyperlocomotor response. On the contrary, animals pretreated with doses producing both the locomotor response and receptor redistribution showed no significant increase of locomotor activity in response to the second injection. Mice with endocytosed receptors, therefore, are insensitive to the agonist. This last experiment strongly suggests that DOR internalization prevents further DOR signaling. Thus, that receptor internalization represents a main mechanism for receptor desensitization in vivo.

The relationship between opioid-induced endocytosis and anti-nociceptive tolerance has been investigated but the conclusions were conflicting. In β-arrestin 2 knockout mice, in which endocytosis is blocked, agonist-induced desensitization of μ-opioid receptors is strongly impaired and mice exhibit increased sensitivity to the acute anti-nociceptive effects of morphine (Bohn et al. 1999; von Zastrow 2004). These results suggest that arrestin-mediated endocytosis of opioid receptors is induced by morphine in vivo and contributes directly to the development of physiological tolerance to opioids (Bohn et al. 2000). However, opioid tolerance-related changes in signaling after stimulation of μ-opioid receptors do not correlate with the endocytosis of these receptors in vivo (Trafton and Basbaum 2004).

2.4.3 Redistribution of GPCRs in the Cytoplasm After Chronic Stimulation

2.4.3.1 Downregulation

Downregulation of GPCRs is characterized by a decrease in the total number of receptors in neurons and a decrease in the number of receptors at the membrane. Downregulation can be distinguished from internalization, which is defined by redistribution of receptors from the plasma membrane to the cytoplasm without modification of total receptor number. In vitro, the number of receptors present in cells can be regulated at the level of receptor gene expression and biosynthesis, in addition to the level of receptor degradation (von Zastrow 2001). In the case of β_2 adrenoceptors, proteolysis is believed to be the predominant mechanism of downregulation (Heck and Bylund 1998). In vivo, the decrease in M_2- and M_4-receptor abundance in dendrites (i.e., in the larger compartment of the neuron), and the decrease in the number of membrane-bound M_2 receptors after chronic cholinergic neuron stimulation, show that these receptors are downregulated (Liste et al. 2002; Decossas et al. 2003). Different mechanisms might induce downregulation, including modulation of gene expression. The decrease in receptor M_2 mRNA in NBM or

striatum neurons of mice in which levels of ACh are chronically high (chronic hypercholinergic mice) might partially explain the loss of M_4 receptors in dendrites. A decrease in D_1 receptor mRNA has also been demonstrated in mice with chronically high levels of dopamine (chronic hyperdopaminergic mice) (Giros et al. 1996; Dumartin et al. 2000). Alternatively, downregulation might result from increased proteolysis of GPCRs.

The mechanism by which plasma-membrane abundance of GPCRs decreases after repetitive and/or long-lasting stimulation in vivo seems to involve at least two phenomena: (1) limited delivery of the receptors to the plasma membrane because of their sequestration in protein synthesis and maturation compartments; and (2) degradation in lysosomes.

2.4.3.2 Intraneuronal Sequestration of GPCRs

Electron-microscopic analyses after immunohistochemistry demonstrate that, in constitutive chronic hyperdopaminergic or hypercholinergic mice, D_1, M_2 and M_4 receptors are trapped in the cytoplasmic compartments of synthesis and maturation (i.e., the endoplasmic reticulum and Golgi apparatus) (Dumartin et al. 2000; Liste et al. 2002; Bernard et al. 2003; Decossas et al. 2003) (Figs. 2.1 and 2.2). In hypercholinergic mice, M_2 receptors are almost absent at the plasma membrane (Fig. 2.1). This suggests that, once synthesized, GPCRs are trapped in endoplasmic reticulum and Golgi apparatus, and not targeted to the plasma membrane of the somatodendritic compartment. The decrease in total M_2 receptor number might also be explained by decrease in receptor neosynthesis, because M_2 receptor mRNA expression is decreased in neurons of AChE knockout mice (Decossas et al. 2003). The molecular mechanisms that prevent the newly synthesized proteins reaching the plasma membrane are still poorly understood. However, a membrane protein associated with the endoplasmic reticulum, dopamine-receptor-interacting protein 78 (DRIP78), has been linked to the transport of GPCRs, including D_1 and M_2, from the endoplasmic reticulum to the cell membrane (Bermak et al. 2001). Neurons from DRIP78 knockout mice do indeed accumulate D_1 and M_2 receptors in the endoplasmic reticulum. We therefore suspect that such a mechanism is impaired during chronic stimulation. Intraneuronal sequestration of GPCRs is a reversible process, because reduction of hyperstimulation enables the receptors to return to the membrane, as has been shown for M_2 and D_1 (Dumartin et al. 2000; Bernard et al. 2003).

2.4.3.3 Fate of Receptors After Sequestration

Under normal conditions, the majority of GPCRs are targeted from the Golgi apparatus to the plasma membrane, and only a few of them are degraded. In vitro, long-lasting stimulation activates degradation of GPCRs in lysosomes, as has been shown for β_2 adrenoceptors (Kallal et al. 1998). In vivo, GPCRs are mainly sent

from the Golgi apparatus to the degradation lysosomal compartment, as observed in the NBM and striatal neurons of hypercholinergic mice (Bernard et al. 2003) (V. Bernard, unpublished).

2.4.3.4 Function of Downregulation

There is probably a functional link between the decrease in number of membrane GPCRs after chronic stimulation and the changes in functions that are regulated by this receptor. For example, in vitro data demonstrate that downregulation of M_3 receptors after chronic stimulation induces desensitization (Detjen et al. 1995). In vivo, AChE knockout mice are resistant to M_2-agonist-induced salivation and hypothermia (Li et al. 2003). This is due to the absence of M_2 receptor stimulation, because the same response has been demonstrated in M_2 receptor knockout mice (Gomeza et al. 1999; Bymaster et al. 2001). Similarly, recycling of the μ-opioid receptor to the plasma membrane correlates with the increase in μ-receptor-mediated anti-nociception (Cahill et al. 2001, 2003).

2.4.4 Relationships Between Endocytosis and Downregulation

The relationship between endocytosis and downregulation of GPCRs is still being debated. In vitro data suggest that they are independent phenomena. Deletion of a part of the third intracytoplasmic loop of the human M_2 receptor inhibits internalization after agonist stimulation, but partially inhibits M_2 receptor downregulation (Tsuga et al. 1998). Alternatively, the mutation of one specific amino acid of the M_2 receptor decreases its ability to display downregulation, without affecting its internalization properties (Goldman and Nathanson 1994). Phosphorylation of some residues of the histamine H_1 receptor is required for receptor transport from endosomes to lysosomes, and thus downregulation has no effect on internalization (Horio et al. 2004). Ex vivo experiments on organotypic cultures of chronic ACh-stimulated striatum (Bernard et al. 2003) suggest that endocytosis does not contribute to the decrease in the abundance of M_2 receptors in the plasma membrane, because M_2 receptors are not co-incorporated with transferrin, which characterizes an endocytotic process. However, other in vivo and in vitro data suggest a link between endocytosis and downregulation (Cahill et al. 2001; Liste et al. 2002). In vivo, internalization and intracytoplasmic sequestration of M_2 receptors might contribute to the decrease in the membrane-bound M_2 receptors. Indeed, subchronic stimulation of muscarinic receptors leads to increased numbers of M_2 receptors in both endosomes and endoplasmic reticulum (Liste et al. 2002).

The molecular mechanisms leading to the downregulation are still unclear. However, if internalization and downregulation are linked, we can hypothesize that these two processes share common molecular mechanisms at least in the first step, such as phosphorylation of GPCRs and endocytosis in clathrin-coated pits,

which has been demonstrated for β_2 adrenoceptors in vitro (Gagnon et al. 1998). In addition, downregulation of the μ-opioid receptor in vitro involves molecules activated during endocytosis, such as GPCR kinase (GRK), arrestin 2, dynamin, rab5 and rab7 (Li et al. 2000).

2.5 Regulation of GPCR Distribution in the Axonal Field After Acute and Chronic Stimulation

Regulation of GPCR compartmentalization at neuronal terminals by the neurochemical environment might contribute to modulation of functional responses, including neurotransmitter release. Few studies have addressed this question in vitro or in vivo. In vitro, the metabotropic glutamate mGlu5 receptor, the neurotensin NTS1 receptor and the dopamine D_1 receptor (three GPCRs) display endocytosis in axons and/or terminals after acute stimulation by their respective agonists (Martin-Negrier et al. 2000; Nguyen et al. 2002; Fourgeaud et al. 2003). In vivo, no modification of M_2 receptor density at varicosities was shown after acute stimulation. Conversely, chronic stimulation of ACh receptors induces an increase in M_2 receptor density at cortical cholinergic varicosities (Decossas et al. 2003) (Fig. 2.2). The mechanisms underlying these different effects remain unidentified. However, we hypothesize that different regulation of the sorting signals by chronic stimulation might direct M_2 receptors from the Golgi apparatus to the terminals, and so lead to accumulation of the receptors in varicosities.

2.6 Concluding Remarks

The results obtained for different GPCRs in the brain suggest a model of trafficking of GPCRs in vivo under acute and chronic stimulation conditions (Fig. 2.2). Acute stimulation induces endocytosis of GPCRs through clathrin-coated pits. These receptors might then be either degraded directly in lysosomes or recycled to the plasma membrane. Chronic stimulation inhibits the delivery of receptors to the plasma membrane from synthesis and maturation compartments (the endoplasmic reticulum and Golgi apparatus). The receptors that are no longer targeted to the membrane are thus directly degraded in lysosomes, leading to the downregulation of GPCRs.

Chronic high levels of ACh had opposite effects on the regulation of M_2 receptor density at the plasma membrane in postsynaptic somatodendritic and presynaptic axonal compartments of the same neuron in AChE knockout mice (Decossas et al. 2003) (Fig. 2.2). In addition to the intraneuronal redistribution observed in the somatodendritic field, M_2 receptors were redistributed along the plasma membrane of the soma, dendrites and axon: the M_2 receptor density decreased at the

plasma membrane of the somatodendritic field and increased at the membrane of terminals. This suggests that the mechanism regulating the GPCR membrane targeting by the neurochemical environment differs at the plasma membrane depending on the subcellular compartment. The molecular mechanisms that underlie the targeting of M_2 receptors to varicosities are unclear (Trimmer 1999).The different effects at somatodendritic and axonal membranes might also result from subcellular compartmentalization of cytoplasmic regulatory factors involved in trafficking of GPCRs. The sorting signals that direct M_2 receptors from the Golgi apparatus to the nerve terminals might be regulated, which could lead to accumulation of the receptor in varicosities, as suggested by the increase in the total receptor numbers at the terminals of basalocortical cholinergic neurons. The regional differences might also result from the differences in receptor membrane recycling and degradation efficiencies between the somatodendritic and axonal fields, as has been demonstrated for the neurotensin receptor NT1 (Nguyen et al. 2002). More of this receptor might be recycled, and less of it degraded, in axon terminals than in the soma and dendrites. The opposing regulation of the abundance of receptors at presynaptic and postsynaptic sites suggests differences in the functions transmitted by these GPCRs at these sites. This has been observed for the adenosine A_1 receptor, which differentially desensitizes the neuronal response depending on its presynaptic or postsynaptic localization (Wetherington and Lambert 2002).

We have reviewed the trafficking of GPCRs after stimulation; however, inhibition of receptors by antagonists also induces changes in receptor distribution that shed additional light on multiple mechanisms for trafficking of GPCRs (Gray and Roth 2001). Further investigations will be required for a better understanding of the link between intraneuronal trafficking of GPCRs and neuronal responses induced by GPCR activation. This might enable the development of new strategies for treating neurological diseases associated with altered GPCR signaling, such as Parkinson's and Alzheimer's diseases (Levey 1996; Muriel et al. 1999; von Zastrow 2001).

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