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REVIEW

Opioid-Receptor (OR) Signaling Cascades in Rat Cerebral Cortex and Model Cell Lines: the Role of Plasma Membrane Structure

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Summary

Large number of extracellular signals is received by plasma membrane receptors which, upon activation, transduce information into the target cell interior *via* trimeric G-proteins (GPCRs) and induce activation or inhibition of adenylyl cyclase enzyme activity (AC). Receptors for opioid drugs such as morphine (μ-OR, δ-OR and κ-OR) belong to rhodopsin family of GPCRs. Our recent results indicated a specific up-regulation of AC I (8-fold) and AC II (2.5-fold) in plasma membranes (PM) isolated from rat brain cortex exposed to increasing doses of morphine (10-50 mg/kg) for 10 days. Increase of ACI and ACII represented the specific effect as the amount of ACIII-ACIX, prototypical PM marker Na, K-ATPase and trimeric G-protein α and β subunits was unchanged. The up-regulation of ACI and ACII faded away after 20 days since the last dose of morphine. Proteomic analysis of these PM indicated that the brain cortex of morphine-treated animals cannot be regarded as being adapted to this drug because significant up-regulation of proteins functionally related to oxidative stress and alteration of brain energy metabolism occurred. The number of δ-OR was increased 2-fold and their sensitivity to monovalent cations was altered. Characterization of δ-OR-G-protein coupling in model HEK293 cell line indicated high ability of lithium to support affinity of δ-OR response to agonist stimulation. Our studies of PM structure and function in context with desensitization of GPCRs action were extended by data indicating participation of cholesterol-enriched membrane domains in agonist-specific internalization of δ-OR. In HEK293 cells stably expressing δ-OR-$G_i1α$ fusion protein, depletion of PM cholesterol was associated with the decrease in affinity of G-protein response to agonist stimulation, whereas maximum response was unchanged. Hydrophobic interior of isolated PM became more "fluid", chaotically organized and accessible to water molecules. Validity of this conclusion was supported by the analysis of an immediate PM environment of cholesterol molecules in living δ-OR-$G_i1α$-HEK293 cells by fluorescent probes 22- and 25-NBD-cholesterol. The alteration of plasma membrane structure by cholesterol depletion made the membrane more hydrated. Understanding of the positive and negative feedback regulatory loops among different OR-initiated signaling cascades (μ-, δ-, and κ-OR) is crucial for understanding of the long-term mechanisms of drug addiction as the decrease in functional activity of μ-OR may be compensated by increase of δ-OR and/or κ-OR signaling.

Key words

GPCR • Morphine • μ-, δ- and κ-opioid receptors • Rat brain cortex • Adenylyl cyclase I and II • Proteomic analysis • Monovalent cations • Agonist-induced internalization • Plasma membrane structure • Cholesterol • Membrane domains • Fluorescent probes

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Introduction

Hormones, neurotransmitters and growth factors bind to the cell surface membrane receptors, which may be divided into the three main families: i) coupled with guanine nucleotide-binding regulatory proteins (GPCR), ii) ionic channels, and iii) tyrosine-kinases. Binding of hormones or neurotransmitters to the stereo-specific site of receptor molecules located at extracellular side of plasma membrane represents the first step in complicated sequence of molecular events transmitting the signal into the cell interior and initiating the ultimate physiological response. In G-protein-mediated cascades, ligand binding induces conformational change of receptor molecule, which, in the next step, induces dissociation of trimeric G-protein complex (non-active) into the free, active Gα and Gβγ subunits. Subsequently, both Gα and Gβγ subunits activate variety of enzyme activities and/or ionic channels which regulate intracellular concentrations of secondary messengers such as cAMP, cGMP, IP_3, DAG, arachidonic acid, sodium, potassium or calcium cations (Svoboda *et al.* 2004, Drastichova *et al.* 2008).

Receptors for opioid drugs, μ-OR, δ-OR and κ-OR, were classified as members of rhodopsin family of GPCRs. All these receptors are known to inhibit adenylyl cyclase activity in pertussis-toxin-dependent manner by activation of G_i/G_o class of trimeric G-proteins. These proteins (G_i1, G_i2, G_i3, G_o1, G_o2) are present in brain in large quantities and inhibit adenylyl cyclase activity or regulate ionic channels in pertussis-toxin-dependent manner.

Morphine binds to all three types of OR (μ-, δ- and κ-OR) and represents one of the most effective painkillers. Repeated exposure of experimental animals to morphine results in tolerance to this drug, development of physical dependence and a chronic relapsing disorder – drug addiction (Contet *et al.* 2004). Physical dependence contributes to a drug seeking behavior and the continuous drug use with the aim to prevent the onset of unpleasant withdrawal symptoms (Preston *et al.* 1991). Morphine withdrawal generates a set of symptoms like retches, vomiting, blood pressure increase, insomnia, intestines dysfunctions, body shaking and teeth chatter.

Drug addiction to morphine is characterized by a complex etiology including changes in psychology of experimental animals as well as physiology of their brain function. These changes proceed mainly in the brainstem and hippocampus (Connor and Christie 1999, Law *et al.* 2000, 2004, Chen *et al.* 2007). However, some of the long-term behavioral consequences of repeated morphine exposure were related to reorganized patterns of synaptic connectivity in the forebrain (Robinson *et al.* 1999). Morphine-induced changes of brain function were also associated with alterations of neurotransmission, specific signaling cascades, energy metabolism and stability of protein molecules (Miller *et al.* 1972, Kim *et al.* 2005, Li *et al.* 2006, Li *et al.* 2009)

Hyper-sensitization or super-activation of adenylyl cyclase (AC) activity by prolonged exposure of cultured cells or mammalian organism to morphine has been demonstrated in previous studies delineating the mechanism of action of this drug (Preston 1991, Connor and Christie 1999, Law *et al*. 2000, 2004, Contet *et al.* 2004) and considered as biochemical basis for development of opiate tolerance and dependence.

Our previous work on PM fraction isolated from brain cortex in Percoll gradient of rats exposed to morphine for 10 days (10-50 mg/kg) indicated a desensitization of G-protein response to μ-OR (DAMGO) and δ-OR (DADLE) stimulation (Bourova *et al.* 2009, 2010) and specific increase of ACI (8-fold) and ACII (2.5-fold) isoforms (Ujcikova *et al.* 2011). The κ-OR (U-23554)-stimulated [^{35}S]GTPγS binding and expression level of ACIII-IX in PM was unchanged. Behavioral tests of morphine-treated animals indicated that these animals were fully drug-dependent (opiate abstinence syndrome) and developed tolerance to subsequent drug addition (analgesic tolerance detected by hot-plate and hind paw withdrawal tests). The increase of ACI and ACII was interpreted as a specific compensatory response to prolonged stimulation of brain cortex OR by morphine.

Proteomic analysis of membrane proteins in rat brain cortex: changes induced by the long-term exposure to increasing doses to morphine

The aim of the next step of our work was the description of an overall change of membrane protein composition and recognition of proteins exhibiting the largest changes induced by morphine. This was performed by proteomic analysis of post-nuclear supernatant (PNS) and plasma membrane-enriched fraction isolated in Percoll gradient. PNS was analyzed because it contains proteins of mitochondrial, endoplasmic reticulum, plasma membrane and cytoplasmic origin. Rats were adapted to morphine for

10 days [10 mg/kg (day 1 and 2), 15 mg/kg (day 3 and 4), 20 mg/kg (day 4 and 5), 30 mg/kg (day 6 and 7), 40 mg/kg (day 9) and 50 mg/kg (day 10)] and sacrificed 24 h after the last dose (group +M10). Control animals were sacrificed in parallel with morphine-treated (group −M10). Post-nuclear supernatant fraction was prepared from brain cortex of both groups and resolved by 2D-ELFO. The gels were stained by Coomassie brilliant blue (CBB) and the altered proteins detected by PDQuest software analysis.

The 10 up-regulated or down-regulated proteins exhibiting the largest morphine-induced change were selected, excised manually from 2D-gel and identified by MALDI-TOF MS/MS. The identified proteins were: 1) (gi|148747414, Guanine deaminase), up 2.5-fold; 2) (gi|17105370, Vacuolar-type proton ATP subunit B, brain isoform), up 2.6-fold; 3) (gi|1352384, Protein disulfide-isomerase A3), up 3.4-fold; 4) (gi|40254595, Dihydropyrimidinase-related protein 2), up 3.6-fold; 5) (gi|149054470, N-ethylmaleimide sensitive fusion protein, isoform CRAa), up 2.0-fold; 6) (gi|42476181, Malate dehydrogenase, mitochondrial precursor), up 1.4-fold; 7) (gi|62653546, Glyceraldehyde-3-phosphate dehydrogenase), up 1.6-fold; 8) (gi|202837, Aldolase A), up 1.3-fold; 9) (gi|31542401, Creatine kinase B-type), down 0.86-fold; 10) (gi|40538860, Aconitate hydratase, mitochondrial precursor), up 1.3-fold. Thus, the ten most highly altered proteins in PNS were of cytoplasmic (proteins no. 1, 4, 5, 7, 9), cell membrane (protein no. 2), endoplasmic reticulum (protein no. 3) and mitochondrial (proteins no. 6, 8, 10) origin and nine of them were significantly increased by morphine (1.3 to 3.6-fold). Correlation with functional properties of these proteins indicated up-regulation of proteins related to guanine degradation (protein no. 1), vacuolar acidification (protein no. 2), apoptotic cell death (protein no. 3), oxidative stress (proteins no. 4, 6, 7, 10), membrane traffic (protein no. 5) and glycolysis (protein no. 8). The role in apoptosis has been also described for glyceraldehyde-3-phosphate dehydrogenase (protein no. 7), already mentioned as major target protein in oxidative stress (Hwang *et al.* 2009). All together, the spectrum of altered proteins suggests a major change of energy metabolism of brain cortex tissue when exposed to increasing doses of morphine. Judged from functional point of view, the most significant change was the up-regulation of proteins related to oxidative stress (proteins no. 4, 6, 7, 10) and apoptotic cell death (proteins no. 3, 7).

We could therefore conclude that the brain cortex of rats exposed to increasing doses of morphine (10-50 mg/kg) for 10 days cannot be regarded as being adapted to this drug. Significant up-regulation of proteins functionally related to oxidative stress and apoptosis indicates the state of severe "discomfort" of brain cells or even damage.

Identification of an active, minority pool of trimeric Gβ subunits responding to chronic morphine in rat brain cortex: proteomic analysis of Percoll-purified membranes

In PM, the altered proteins were of plasma membrane origin [BASP1, Brain acid soluble protein, down-regulated 2.1-fold; GBB, Guanine nucleotide-binding protein subunit beta-1, down 2.0-fold], myelin membrane [MBP, Myelin basic protein S, down 2.5-fold], cytoplasmic [KCRB, Creatine kinase B-type (EC 2.7.3.2), down 2.6-fold; AINX, alpha-internexin, up-regulated 5.2-fold; DPYL2, Dihydropyrimidinase-related protein 2, up 4.9-fold; SIRT2, NAD-dependent deacetylase sirtuin-2, up 2.5-fold; SYUA, Alpha-synuclein, up 2.0-fold; PRDX2, Peroxiredoxin-2, up 2.2-fold; TERA, Transitional endoplasmic reticulum ATPase, up 2.1-fold; UCHL1, Ubiquitin carboxyl-terminal hydrolase L1 down 2.0-fold; COR1A, Coronin-1A, down 5.4-fold; SEP11, Septin-11, up 2.2-fold; RL12, 60S ribosomal protein L12, up 2.7-fold] and mitochondrial [DHE3, Glutamate dehydrogenase 1, up 2.7-fold; SCOT1, Succinyl-CoA:3-ketoacid-coenzyme A, up 2.2-fold; AATM, Aspartate aminotransferase, down 2.2-fold; PHB, Prohibitin, up 2.2-fold].

The only member of GPCR-initiated signaling cascades identified by LC-MS/MS in PM was trimeric Gβ subunit (2-GBB) which was decreased 2-fold in samples from morphine-adapted rats. Similarly, proteomic analysis of protein alterations induced by long-term stimulation of HEK293 cells stably expressing TRH-receptor and $G_{11}\alpha$ protein by TRH, indicated the change of 42 proteins, but none of these proteins represented the plasma membrane protein functionally related to G-protein-mediated signaling cascades (Drastichova *et al.* 2010).

The immunoblot analysis of the same PM resolved by 2D-ELFO indicated that the "active" pool of Gβ subunits affected by morphine, which was decreased 2x, represented just a minor fraction of the total signal of Gβ subunits in 2D-gels (Fig. 1). The total signal of Gβ was

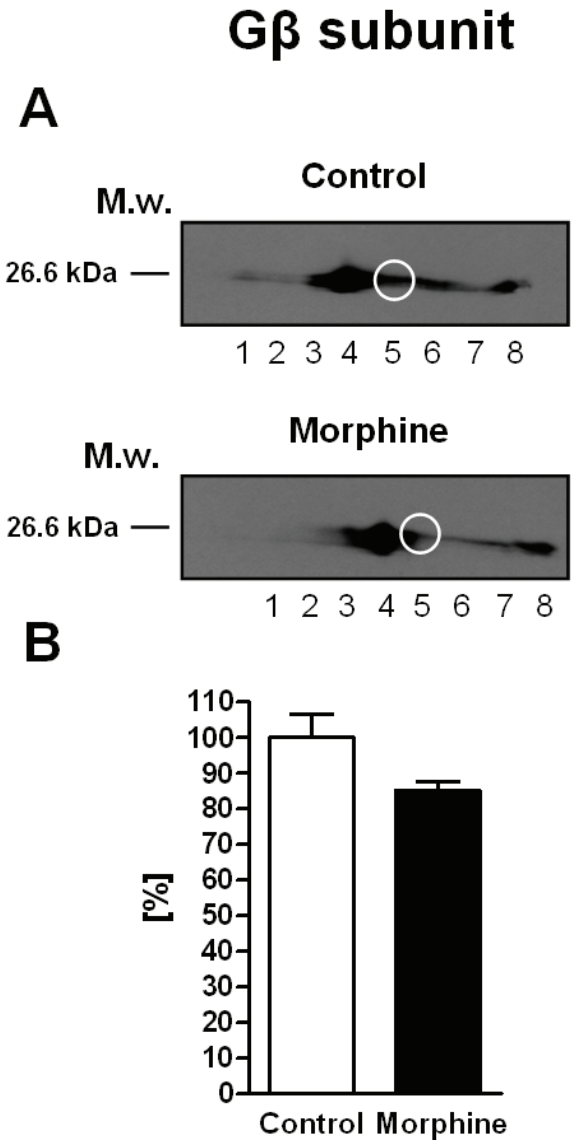

Fig. 1. Morphine-induced decrease of trimeric Gβ subunits in plasma-membrane-enriched fraction: immunoblot analysis of 2D-electrophoresis. **(A)** Two-dimensional resolution of Gβ protein content in PM isolated from control and morphine-adapted rats. PM protein (400 μg) was resolved by 2D electrophoresis using the pI range 3-11 for isoelectric focusing in the first dimension. The white small circle shows the small fraction of the total signal of Gβ which was taken into consideration when analyzed by LC-MS/MS. The second dimension was performed by SDS-PAGE in 10 % w/v acrylamide/0.26 % bis-acrylamide gels (Hoefer SE 600). Gβ was identified by immunoblotting with specific antibody oriented against C-terminal peptide of Gβ. Numbers 1-8 represent spots of Gβ subunits which were subsequently analyzed by LC-MS/MS. **(B)** The average of three immunoblots ± SEM. Difference between (-M10) and (+M10) was analyzed by Student´s *t*-test using GraphPad*Prizm4* and found not significant, NS ($p>0.05$).

decreased 1.2-fold only but dominant/major part of the total signal was unchanged. Accordingly, the immunoblot analysis of Gβ after resolution by 1D-SDS-PAGE in 10 % w/v acrylamide/0.26 % w/v bis-acrylamide or 4-12 % (InVitroGene) gradient gels indicated no change of this protein. We could therefore conclude that proteomic analysis represents a valuable tool for identification of membrane proteins. However, the analysis of low-abundance proteins of OR-initiated signaling cascades in plasma membranes has to be accompanied by specific immublot analysis. Identification of an "active", minority pool of Gβ subunits down-regulated by morphine represents an original finding which has not been described in current literature dealing with drug addiction and morphine effect on mammalian brain.

The effect of lithium and other monovalent cations on ligand binding and efficiency of δ-opioid receptor-G-protein coupling

Lithium is still one of the most effective therapies for depression. Comparison of the effect of lithium, sodium and potassium on δ-opioid receptors was studied in HEK293 cells stably expressing PTX-insensitive δ-OR-$G_i1\alpha$ (Cys^{351}-Ile^{351}) fusion protein. δ-OR-$G_i1\alpha$ (C^{351}-I^{351}) cells represent useful experimental tool as the covalent bond between δ-OR and $G_i1\alpha$ (C^{351}-I^{351}) provides the permanent and fixed 1:1 stoichiometry and C^{351}-I^{351} mutation provides resistance to PTX together with extraordinary high efficacy of coupling between δ-OR and $G_i1\alpha$ (C^{351}-I^{351}) protein (Bourova *et al.* 2003, Brejchova *et al.* 2011).

Agonist [^{3}H]DADLE binding was decreased with the order: $Na^+ \gg Li^+ > K^+ > NMDG^+$. When plotted as a function of increasing NaCl concentrations, binding was best-fitted with a two phase exponential decay considering the two Na^+-responsive sites ($r^2=0.99$). High-affinity Na^+-sites were characterized by $K_d=7.9$ mM and represented 25 % of the basal level determined in the absence of Na^+ ions. Remaining 75 % represented the low-affinity sites ($K_d=463$ mM). Inhibition of [^{3}H]DADLE binding by lithium, potassium and $NMDG^+$ proceeded in low-affinity manner only. Preferential sensitivity of δ-OR-$G_i1\alpha$ to sodium was thus clearly manifested.

Surprisingly, the affinity/potency of DADLE-stimulated [^{35}S]GTPγS binding, quantitatively characterized by comparison of dose-response curves in different ion media (EC_{50} values), was increased in reverse order: $Na^+ < K^+ < Li^+$. This result was demonstrated in PTX-treated as well as PTX-untreated cells (Table 1). Therefore, this finding is not restricted to $G_i1\alpha$ present in fusion protein, but is also valid for stimulation of endogenous G-proteins of G_i/G_o family.

Table 1. DADLE-stimulated [^{35}S]GTPγS binding in membranes prepared from PTX-treated and PTX-untreated δ-OR-$G_i1\alpha$ - HEK293 cells.

A PTX-treated

	EC_{50}		%		B_{basal}		B_{max}		Δ_{max}	
NaCl	5.1×10^{-8} M	**	350	**	0.143	**	0.499	*	0.356	**
KCl	9.6×10^{-9} M	* **	216	NS **	0.241	NS **	0.520	** NS	0.279	NS **
LiCl	5.4×10^{-9} M		231		0.209		0.481		0.272	

B PTX-untreated

	EC_{50}		%		B_{basal}		B_{max}		Δ_{max}	
NaCl	6.5×10^{-8} M	**	327	*	0.178	**	0.582	*	0.404	**
KCl	2.0×10^{-8} M	* **	237	NS *	0.222	NS **	0.526	NS *	0.304	NS **
LiCl	8.4×10^{-9} M		248		0.211		0.523		0.312	

[^{35}S]GTPγS binding was measured in P2 membrane fraction isolated from PTX-treated **(A)** or PTX-untreated cells **(B)** as described in methods. Binding assays were performed in 200 mM NaCl, KCl or LiCl. EC_{50} (M) and B_{max} (pmol x mg^{-1}) values were calculated by GraphPad*Prizm4*. B_{max} values were also expressed as the ratio (%) between maximum DADLE-stimulated (B_{max}) and the basal level (B_{basal}) of binding. Net-increment of agonist stimulation (Δ_{max}) was calculated as the difference between B_{max} and B_{basal} values. Numbers represent the means ± SEM of 3 binding assays, each performed in triplicates. Data were analyzed by one-way ANOVA followed by Neuman-Keuls post test (* $p<0.05$, ** $p<0.01$, NS non-significant). **(A)** In PTX-treated membranes, [^{35}S]GTPγS binding in the absence of ions was 0.622 pmol × mg^{-1} and this level was decreased to 0.143 (NaCl), 0.241 (KCl) and 0.209 (LiCl) pmol × mg^{-1} by addition of 200 mM NaCl, KCl or LiCl, respectively. **(B)** In PTX-untreated membranes, [^{35}S]GTPγS binding in the absence of ions was 0.809 pmol × mg^{-1} and this level was decreased to 0.178 (NaCl), 0.222 (KCl) and 0.211 (LiCl) pmol × mg^{-1} by addition of 200 mM NaCl, KCl or LiCl, respectively.

This surprising but fully reproducible result may be considered in connection with clinical use of lithium in the treatment of manic depression. In electrically active cells, Li^+ enters the intracellular compartment *via* "fast" sodium channel (Richelson, 1977) and also *via* ouabain-sensitive K^+-influx catalyzed by Na,K-ATPase. However, the efflux of Li^+ *via* Na,K-ATPase is limited because ATP+Mg+Na-dependent phosphorylation proceeding at inner side of the plasma membrane and outward oriented efflux of Na^+ cations *via* Na+-pump are strictly specific for sodium. Thus, if available in extracellular space, the intracellular Li^+ concentration will be slowly increased. It is reasonable to assume that such conditions may arise in neuronal or glial cells of depressive patients as the effective range of plasma Li^+ concentrations under clinical conditions is 0.6-1.0 mM. The 2 mM LiCl is regarded as toxic. This is exactly the concentration range in which the first significant inhibition of the basal level of [^{35}S]GTPγS binding was detected in our experiments. The first significant decrease of the basal level of [^{35}S]GTPγS binding measured in the absence of cations was noticed at 1-2 mM NaCl, KCl and LiCl; the 50 % inhibition was reached at 62 mM NaCl, 88 mM LiCl and 92 mM KCl, respectively (Vosahlikova and Svoboda 2011). Thus, in the treatment of acute depression, competitive effect of Li^+ on inverse agonist-like effect of Na^+ on δ-OR and, in parallel, on G_i/G_o class of G-proteins, might be considered as one of plausible mechanisms of Li^+ action besides its numerous other effects on overall cell metabolism (Young 2009).

The role of cholesterol, cholesterol depletion and membrane domains/rafts in structural organization of plasma membrane and transmembrane signaling through G-protein-coupled receptors

Cholesterol constitutes a major component of mammalian plasma (cell) membrane. Its correct distribution among plasma membrane and intracellular membrane compartments is essential for the homeostasis of mammalian cells and intracellular membrane traffic plays a major role in the correct disposition of internalized cholesterol and in the regulation of cholesterol efflux (Maxfield and Wüstner 2002, Scheidt *et al.* 2003). Furthermore, lateral and transbilayer organization of cholesterol molecules in the plasma membrane determines plasma membrane structure and dynamics. However, neither its intracellular pathways of trafficking nor its precise lateral organization in cholesterol-enriched microdomains such as membrane rafts and caveolae is fully understood. The same applies to the transbilayer distribution between the two leaflets of biological membranes (Simons and Ikonen 1997, Brown and London 1998, Anderson and Jacobson 2002).

Cholesterol- and sphingolipid-enriched membrane domains, characterized by high content of cholesterol, saturated phospholipids, glycolipids and sphingomyelin, have been described as lipid platforms capable to harbor and confine trimeric G-proteins in high amounts (Simons and Ikonen 1997, Brown and London 1998, Anderson 1998, Moffett *et al.* 2000, Oh and Schnitzer 2001, Anderson and Jacobson 2002, Pike 2004, Quinton *et al.* 2005). Considering the function of trimeric G-proteins in membrane domains containing caveolin, heterologous desensitization of GPCR signaling was described as specific binding of G-proteins to caveolin (Murthy and Makhlouf 2000).

These structures were also reported to play an important role in both positive and negative regulation of transmembrane signaling through G-protein-coupled receptors (Gimpl *et al.* 1995, Klein *et al.* 1995, Feron *et al.* 1997, De Weerd and Leeb-Lundberg 1997, Schwencke *et al.* 1999, De Luca *et al.* 2000, Dessy *et al.* 2000, Lasley *et al.* 2000, Igarashi and Michel 2000, Ostrom *et al.* 2000, 2001, Rybin *et al.* 2000, 2003, Ushio-Fukai *et al.* 2001, Gimpl and Farenholz 2002, Sabourin *et al.* 2002, Ostrom and Insel 2004, Pucadyil and Chattopadhyay 2004, 2007, Monastyrskaya *et al.* 2005, Savi *et al.* 2006, Xu *et al.* 2006, Allen *et al.* 2007, Ostasov *et al.* 2007, 2008, Chini and Parenti 2009). More specifically, the functional significance of OR presence in membrane domains is far from being understood as cholesterol reduction by methyl-β-cyclodextrin attenuated δ-OR-mediated signaling in neuronal cells but enhanced it in non-neuronal cells (Huang *et al.* 2007).

In HEK293 cells stably expressing δ-OR-$G_i1α$ fusion protein, depletion of PM cholesterol was associated with a decrease (by one order of magnitude) in affinity/potency of G-protein response to agonist stimulation. The maximum response was unchanged (Brejchova *et al.* 2011). Hydrophobic interior of isolated PM became more "fluid", chaotically organized and more accessible to water molecules. The analysis of PM environment by fluorescent derivatives of cholesterol (22- and 25-NBD-cholesterol) in living δ-OR-$G_i1α$-HEK293 cells confirmed these results because the alteration of plasma membrane structure by cholesterol depletion made the membrane more hydrated (Ostasov *et al.* 2013). Our data also indicated that small perturbation of PM structure by low, non-ionic detergent concentrations increased GPCR-G-protein coupling, while the high concentrations were strictly inhibitory (Sykora *et al.* 2009). The close-to-zero level of basal and agonist-stimulated G-protein activity is the typical feature of detergent-resistant membrane domains (DRMs) prepared at high detergent concentrations, 0.5-1 % Triton X-100 (Bourova *et al.* 2003).

Agonist-induced internalization of δ-opioid receptors

The first evidence for agonist-induced internalization of GPCRs was brought by subcellular fractionation studies of cell homogenate using differential or sucrose density gradient centrifugation. The internalized, endosomal pool of receptor molecules was separated from the major pool of receptor molecules in plasma membranes and was found to be increased by agonist stimulation (Stadel *et al.* 1983, Waldo *et al.* 1983, Clark *et al.* 1985, Hertel *et al.* 1985, Sibley *et al.* 1987). In intact cells, the specific, agonist-induced sequestration and internalization of GPCRs was detected by immunofluorescence microscopy of cells expressing $β_2$-adrenergic receptors. $β_2$-AR were transferred from clathrine-coated pitts (in the plasma membrane) to clathrine-coated vesicles, rab5-containing early endosomes and back to the plasma membrane (von Zastrow and Kobilka 1992, 1994, Moore *et al.* 1995,

Pippig *et al.* 1995). Cellular and molecular mechanisms of GPCR internalization are in focus of OR studies as one the leading theories of drug addiction is directly based on atypical parameters of μ-OR internalization (Whistler and von Zastrow 1998, Whistler *et al.* 1999). When exposed to morphine, μ-OR remain at PM and in this way elude desensitization by β-arrestin.

Our analysis of HEK293 cells transiently expressing Flag-epitope tagged version of δ-OR indicated that cholesterol depletion alone induced transfer of receptor molecules into the cell interior (Fig. 2A, upper right and left panels). Incubation of cells with 10 mM β-cyclodextrin (β-CDX, 30 min) caused significant increase of intracellular fluorescence ($p<0.05$), while in control, β-CDX-untreated cells, the small intracellular signal distributed among numerous faint fluorescent patches was unchanged in the course of 30 min incubation in serum-free medium alone (Fig. 2B). Massive transfer of receptor molecules from the cell surface (plasma membrane) into the intracellular compartments was noticed after agonist stimulation (100 nM DADLE). This transfer was decreased in β-CDX-treated cells (Fig. 2A, lower right and left panels). Difference between β-CDX-treated and β-CDX-plus DADLE-treated samples was highly significant ($p<0.01$) (Fig. 2B).

We could therefore conclude that the treatment of HEK293 cells with β-CDX alone, i.e. degradation of membrane domains, induced destabilization of HEK293 plasma membrane structure manifested as spontaneous transfer of a portion of δ-OR molecules into the cell interior. Massive internalization of δ-OR proceeding in the presence of specific agonist was suppressed by β-CDX. This part of internalized receptor molecules may be regarded as functionally related to membrane domains.

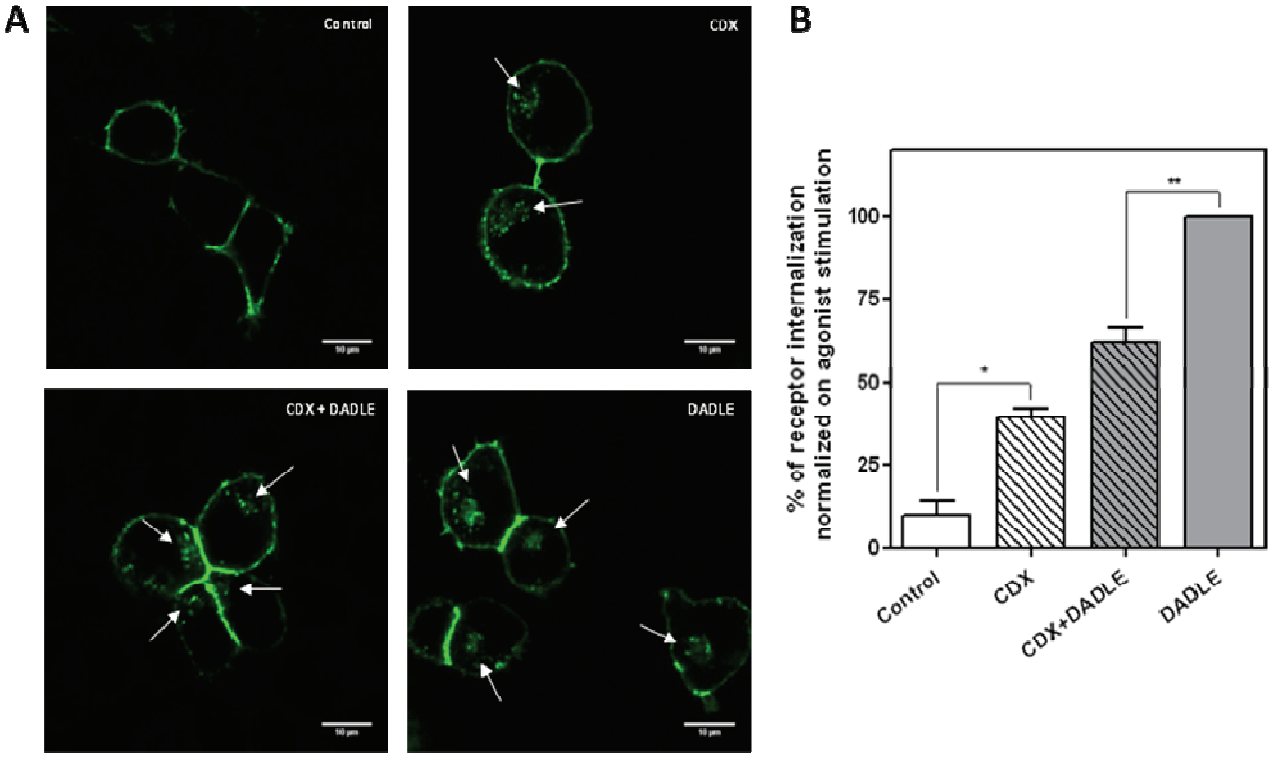

Fig. 2. Agonist (DADLE)-induced internalization of δ-OR is attenuated by cholesterol depletion. HEK293T cells transiently transfected with FLAG-tagged δ-OR were *in vivo* labeled with the corresponding anti-tag antibodies, exposed to serum-free DMEM (Control), 10 mM β-CDX in serum-free DMEM (CDX), 100 nM DADLE (DADLE), or 10 mM β-CDX plus 100 nM DADLE in serum-free DMEM (CDX+DADLE) for 30 min, and fixed. After fixation the cells were subjected to indirect immunofluorescence with Alexa Fluor 488-conjugated secondary antibodies and imaged with laser scanning confocal microscopy. Left panels **(A)** show representative micrographs of cells expressing FLAG-tagged δ-OR and treated as described above. Right panel **(B)** displays results from quantification of micrographs performed by ImageJ software. Fraction of internalized receptors was calculated as a ratio of intracellular to total signal determined in 8 cells per each condition, averaged and normalized to values obtained by agonist (DADLE) stimulation. Data represent the average of three experiments, i.e. three independent transfections, ± SEM. Statistical analysis was performed using one-way ANOVA repeated measurements with Bonferroni *post-hoc* test. *, **, represent the significant difference, $p<0.05$, $p<0.01$.

Conclusions and future perspectives

Understanding of the positive and negative feedback regulatory loops among different OR-initiated signaling cascades (μ-, δ-, and κ-OR) is crucial for understanding of the long-term mechanisms of drug addiction as the decrease in functional activity of μ-OR may be compensated by increase of δ-OR and/or κ-OR signaling. In our experiments using increasing doses of morphine (10-50 mg/kg) for 10 days, the decrease of functional activity of μ-OR in brain cortex PM was measured together with decrease of δ-OR signaling (agonist-stimulated, high-affinity [^{35}S]GTPγS binding). In parallel PM samples, membrane density of adenylyl cyclase I and II was markedly increased; the other AC isoforms (III-IX) were unchanged. The highly positive and "optimistic" result (for drug addicts) was that the up-regulation of ACI and ACII faded away 20 days since the last dose of morphine.

Rat brain cortex of rats sacrificed 24 h after the last dose of morphine (50 mg/kg) cannot be regarded as "being adapted" as the proteomic analysis suggests a major alteration/reorganization of energy metabolism of brain cortex cells: four out of nine up-regulated proteins were described as functionally related to oxidative stress; the two proteins were related to genesis of apoptotic cell death. This was not an optimistic finding indeed (for drug addicts), however, further research is needed to find out whether these changes are reversible as the up-regulation of ACI and ACII after 20 days of drug withdrawal.

Analysis of model HEK293 cell line expressing the defined type of OR (δ-OR) indicated that degradation of membrane domains/rafts by cholesterol depletion resulted in decrease of affinity of δ-OR-response to agonist stimulation in parallel with increase of "fluidity" and hydration of PM. Agonist binding to δ-OR was unchanged. Perturbation of optimum PM organization by cholesterol depletion deteriorated the functional coupling between δ-OR and G-proteins while receptor ligand binding site was unchanged. Therefore, the biophysical state of hydrophobic PM interior should be regarded as regulatory factor of δ-OR-signaling cascade.

In HEK293 cells expressing δ-OR-$G_i1\alpha$ (Cys^{351}-Ile^{351}) fusion protein, the inverse agonist-like effect of monovalent cations on δ-OR was detected as inhibition of agonist binding. Maximum of G-protein response to agonist stimulation was preferentially oriented to sodium with the order: $Na^+ \gg Li^+ > K^+ > NMDG^+$. Surprisingly, affinity of G-protein response was preferentially supported by lithium.

Modern biophysical and confocal microscopy techniques (fluorescence lifetime imaging, fluorescence resonance energy transfer, raster image correlation spectroscopy) are being introduced at present time for the analysis of agonist-induced change of receptor mobility in HEK293 cell lines transiently expressing δ-OR-CFP, δ-OR-YFP and δ-OR-CFP plus δ-OR-YFP. Stably transfected HEK293 cells expressing TRH-R-eGFP and fluorescence recovery after photobleaching are used as reference standard when testing agonist-specific alteration of receptor mobility in living cells.

Conflict of Interest

There is no conflict of interest.

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References

ALLEN JA, HALVERSON-TAMBOLI RA, RASENICK MM: Lipid raft microdomains and neutrotransmitter signaling. *Nat Rev Neurosci* **8**: 128-140, 2007.

ANDERSON RGW, JACOBSON K: A role for lipid shells in targeting proteins to caveolae, rafts, and other lipid domains. *Science* **296**: 1821-1825, 2002.

ANDERSON RGW: The caveolae membrane system. *Annu Rev Biochem* **67**: 199-225, 1998.

BOUROVA L, KOSTRNOVA A, HEJNOVA L, MORAVCOVA Z, MOON HE, NOVOTNY J, MILLIGAN G, SVOBODA P: delta-Opioid receptors exhibit high efficiency when activating trimeric G proteins in membrane domains. *J Neurochem* **85**: 34-49, 2003.

BOUROVA L, STOHR J, LISY V, RUDAJEV V, NOVOTNY J, SVOBODA P: G-protein activity in Percoll-purified plasma membranes, bulk plasma membranes and low-density plasma membranes isolated from rat cerebral cortex. *Med Sci Monit* **15**: BR111-BR122, 2009.

BOUROVA L, VOSAHLIKOVA M, KAGAN D, DLOUHA K, NOVOTNY J, SVOBODA P: Long-term adaptation to high doses of morphine causes desensitization of μ-OR- and δ-OR-stimulated G-protein response in forebrain cortex but does not decrease the amount of G-protein alpha subunit. *Med Sci Monit* **16**: 260-270, 2010.

BREJCHOVA J, SÝKORA J, DLOUHA K, ROUBALOVA L, OSTASOV P, VOSAHLIKOVA M, HOF M, SVOBODA P: Fluorescence spectroscopy studies of HEK293 cells expressing DOR-Gi1α fusion protein; the effect of cholesterol depletion. *Biochim Biophys Acta* **1808**: 2819-2829, 2011.

BROWN DA, LONDON E: Functions of lipid rafts in biological membranes. *Annu Rev Cell Dev Biol* **14**: 111-136, 1998.

CHEN XL, LU G, GONG YX, ZHAO LC, CHEN J, CHI ZQ, YANG YM, CHEN Z, LI QL, LIU JG: Expression changes of hippocampal energy metabolism enzymes contribute to behavioural abnormalities during chronic morphine treatment. *Cell Res* **17**: 689-700, 2007.

CHINI B, PARENTI M: G-protein-coupled receptors, cholesterol and palmitoylation: facts about fats. *J Mol Endocrinol* **42**: 371-379, 2009.

CLARK RB, FRIEDMAN J, PRASHAD N, RUOHO AE: Epinephrine-induced sequestration of the beta-adrenergic receptor in cultured S49 WT and cyc- lymphoma cells. *J Cyclic Nucleotide Protein Phosphor Res* **10**: 97-119, 1985.

CONNOR M, CHRISTIE MD: Opioid receptor signaling mechanisms. *Clin Exp Pharmacol Physiol* **26**: 493-499, 1999.

CONTET C, KIEFFER BL, BEFORT K: Mu opioid receptor: a gateway to drug addiction. *Curr Opin Neurobiol* **14**: 370-378, 2004.

DE LUCA A, SARGIACOMO M, PUCA A, SGARAMELLA G, DE PAOLIS P, FRATI G, MORISCO C, TRIMARCO B, VOLPE M, CONDORELLI G: Characterization of caveolae from rat heart: localization of postreceptor signal transduction molecules and their rearrangement after norepinephrine stimulation. *J Cell Biochem* **77**: 529-539, 2000.

DE WEERD WFC, LEEB-LUNDBERG LMF: Bradykinin sequesters B2 bradykinin receptors and the receptor-coupled Galpha subunits Galphaq and Galphai in caveolae in DDT1 MF-2 smooth muscle cells. *J Biol Chem* **272**: 17858-17866, 1997.

DESSY C, KELLY RA, BALLIGAND JL, FERON O: Dynamin mediates caveolar sequestration of muscarinic cholinergic receptors and alternation in NO signaling. *EMBO J* **19**: 4272-4280, 2000.

DRASTICHOVA Z, BOUROVA L, LISY V, HEJNOVA L, RUDAJEV V, STOHR, J, DURCHANKOVA D, OSTASOV P, TEISINGER J, SOUKUP T, NOVOTNY J, SVOBODA P: Subcellular redistribution of trimeric G-proteins – potential mechanism of desensitization of hormone response; internalization, solubilisation, down-regulation. *Physiol Res* **57** (Suppl 3): S1-S10, 2008.

DRASTICHOVA Z, BOUROVA L, HEJNOVA L, JEDELSKY P, SVOBODA P, NOVOTNY J: Protein alterations induced by long-term agonist treatment of HEK293 cells expressing thyrotropin-releasing hormone receptor and $G_{11}\alpha$ protein. *J Cell Biochem* **109**: 255-264, 2010.

FERON O, SMITH TW, MICHEL T, KELLY RA: Dynamic targeting of the agonist-stimulated m2 muscarinic acetylcholine receptor to caveolae in cardiac myocytes. *J Biol Chem* **272**: 17744-17748, 1997.

GIMPL G, KLEIN U, REILÄNDER H, FAHRENHOLZ F: Expression of the human oxytocin receptor in baculovirus-infected insect cells: high-affinity binding is induced by a cholesterol-cyclodextrin complex. *Biochemistry* **34**: 13794-13801, 1995.

GIMPL G, FAHRENHOLZ F: Cholesterol as stabilizer of the oxytocin receptor. *Biochim Biophys Acta* **1564**: 384-392, 2002.

HERTEL C, COULTER SJ, PERKINS JP: A comparison of catecholamine-induced internalization of beta-adrenergic receptors and receptor-mediated endocytosis of epidermal growth factor in human astrocytoma cells. Inhibition by phenylarsine oxide. *J Biol Chem* **260**: 12547-12533, 1985.

HUANG P, XU W, YOON SI, CHEN C, CHONG PLG, LIU-CHEN LY: Cholesterol reduction by methyl-beta-cyclodextrin attenuates the delta opioid receptor-mediated signaling in neuronal cells and enhances it in non-neuronal cells. *Biochem Pharmacol* **73**: 534-549, 2007.

HWANG NR, YIM SH, KIM YM, JEONG J, SONG EJ, LEE Y, CHOI S, LEE KJ: Oxidative modifications of glyceraldehyde-3-phophate dehydrogenase play a key role in its multiple cellular functions. *Biochem J* **423**: 253-264, 2009.

IGARASHI J, MICHEL T: Agonist-modulated targeting of the EDG-1 receptor to plasmalemmal caveolae. eNOS activation by sphingosine 1-phosphate and the role of caveolin-1 in sphingolipid signal transduction. *J Biol Chem* **275**: 32363-32370, 2000.

KIM SY, CHUDAPONGSE N, LEE SM, LEVIN MC, OH JT, PARK HJ, HO IK: Proteomic analysis of phosphotyrosyl proteins in morphine-dependent rat brains. *Brain Res Mol Brain Res* **133**: 58-70, 2005.

KLEIN U, GIMPL G, FAHRENHOLZ F: Alteration of the myometrial plasma membrane cholesterol content with beta-cyclodextrin modulates the binding affinity of the oxytocin receptor. *Biochemistry* **34**: 13784-13793, 1995.

LASLEY RD, NARAYAN P, UITTENBOGAARD A, SMART EJ: Activated cardiac adenosine A(1) receptors translocate out of caveolae. *J Biol Chem* **275**: 4417-4421, 2000.

LAW PY, WONG YH, LOH HH: Molecular mechanisms and regulation of opioid receptor signaling. *Annu Rev Pharmacol Toxicol* **40**: 389-430, 2000.

LAW PY, LOH HH, WEI LN: Insights into the receptor transcription and signaling: implications in opioid tolerance and dependence. *Neuropharmacology* **47**: 300-311, 2004.

LI KW, JIMENEZ CR, VAN DER SCHORS RC, HORNSHAW MP, SCHOFFELMEER ANM, SMIT AB: Intermittent administration of morphine alters protein expression in rat nucleus accumbens. *Proteomics* **6**: 2003-2008, 2006.

LI Q, ZHAO X, ZHONG LJ, YANG HY, WANG Q, PU XP: Effects of chronic morphine treatment on protein expression in rat dorsal root ganglia. *Eur J Pharmacol* **612**: 21-28, 2009.

MAXFIELD FR, WÜSTNER D: Intracellular cholesterol transport. *J Clin Invest* **110**: 891-898, 2002.

MILLER AL, HAWKINS RA, HARRIS RL, VEECH RL: The effects of acute and chronic morphine treatment and of morphine withdrawal on rat brain in vivo. *Biochem J* **129**: 463-469, 1972.

MOFFETT S, BROWN DA, LINDER ME: Lipid-dependent targeting of G proteins into rafts. *J Biol Chem* **275**: 2191-2198, 2000.

MONASTYRSKAYA K, HOSTETTLER A, BUERGI S, DRAEGER A: The NK1 receptor localizes to the plasma membrane microdomains, and its activation is dependent on lipid raft integrity. *J Biol Chem* **280**: 7135-7146, 2005.

MOORE RH, SADOVNIKOFF N, HOFFENBERG S, LIU S, WOODFORD P, ANGELIDES K, TRIAL JA, CARSRUD ND, DICKEY BF, KNOLL BJ: Ligand-stimulated beta 2-adrenergic receptor internalization via the constitutive endocytotic pathway into rab5-containing endosomes. *J Cell Sci* **108**: 2983-2991, 1995.

MURTHY KS, MAKHLOUF GM: Heterologous desensitization mediated by G protein-specific binding to caveolin. *J Biol Chem* **275**: 30211-30219, 2000.

OH P, SCHNITZER JE: Segregation of heterotrimeric G proteins in cell surface microdomains: G(q) binds caveolin to concentrate in caveolae, whereas G(i) and G(s) target lipid rafts by default. *Mol Biol Cell* **12**: 685-698, 2001.

OSTASOV P, BOUROVA L, HEJNOVA L, NOVOTNY J, SVOBODA P: Disruption of the plasma membrane integrity by cholesterol depletion impairs effectiveness of TRH receptor-mediated signal transduction via G(q)/G(11)alpha proteins. *J Recept Signal Transduct Res* **27**: 335-352, 2007.

OSTASOV P, KRUSEK J, DURCHANKOVA D, SVOBODA P, NOVOTNY J: Ca2+ responses to thyrotropin-releasing hormone and angiotensin II: the role of plasma membrane integrity and effect of G11alpha protein overexpression on homologous and heterologous desensitization. *Cell Biochem Funct* **26**: 264-274, 2008.

OSTASOV P, SYKORA J, BREJCHOVA J, OLSZYNSKA A, HOF M, SVOBODA P: FLIM studies of 22- and 25-NBD-cholesterol in living HEK293 cells; plasma membrane change induced by cholesterol depletion. *Chem Phys Lipids* **167-168**: 62-69, 2013.

OSTROM RS, POST SR, INSEL PA: Stoichiometry and compartmentation in G protein-coupled receptor signaling: implications for therapeutic interventions involving G(s). *J Pharmacol Exp Ther* **294**: 407-412, 2000.

OSTROM RS, GREGORIAN C, DRENAN RM, XIANG Y, REGAN JW, INSEL PA: Receptor number and caveolar co-localization determine receptor coupling efficiency to adenylyl cyclase. *J Biol Chem* **276**: 42063-42069, 2001.

OSTROM RS, INSEL PA: The evolving role of lipid rafts and caveolae in G protein-coupled receptor signaling: implications for molecular pharmacology. *Br J Pharmacol* **143**: 235-245, 2004.

PIKE LJ: Lipid rafts: heterogeneity on the high seas. *Biochem J* **378**: 281-292, 2004.

PIPPIG S, ANDEXINGER S, LOHSE MJ: Sequestration and recycling of beta 2-adrenergic receptors permit receptor resensitisation. *Mol Pharmacol* **47**: 666-676, 1995.

PRESTON KL: Drug abstinence effects: opioids. *Br J Addict* **86**: 1641-1646, 1991.

PUCADYIL TJ, CHATTOPADHYAY A: Cholesterol modulates ligand binding and G-protein coupling to serotonin(1A) receptors from bovine hippocampus. *Biochim Biophys Acta* **1663**: 188-200, 2004.

PUCADYIL TJ, CHATTOPADHYAY A: Cholesterol depletion induces dynamic confinement of the G-protein coupled serotonin(1A) receptor in the plasma membrane of living cells. *Biochim Biophys Acta* **1768**: 655-668, 2007.

QUINTON TM, KIM S, JIN J, KUNAPULI SP: Lipid rafts are required in Galpha(i) signaling downstream of the P2Y12 receptor during ADP-mediated platelet activation. *J Thromb Haemost* **3**: 1036-1041, 2005.

RICHELSON E: Lithium ion entry through the sodium channel of cultured mouse neuroblastoma cells: a biochemical study. *Science* **196**: 1001-1002, 1977.

ROBINSON TE, KOLB B: Morphine alters the structure of neurons in the nucleus accumbens and neocortex of rats. *Synapse* **33**: 160-162, 1999.

RYBIN VO, XU X, LISANTI MP, STEINBERG SF: Differential targeting of beta-adrenergic receptor subtypes and adenylyl cyclase to cardiomyocyte caveolae. A mechanism to functionally regulate the cAMP signaling pathway. *J Biol Chem* **275**: 41447-41457, 2000.

RYBIN VO, PAK E, ALCOTT S, STEINBERG SF: Developmental changes in beta2-adrenergic receptor signaling in ventricular myocytes: the role of Gi proteins and caveolae microdomains. *Mol Pharmacol* **63**: 1338-1348, 2003.

SABOURIN T, BASTIEN L, BACHVAROV DR, MARCEAU F: Agonist-induced translocation of the kinin B(1) receptor to caveolae-related rafts. *Mol Pharmacol* **61**: 546-553, 2002.

SAVI P, ZACHAYUS JL, DELESQUE-TOUCHARD N, LABOURET C, HERVÉ C, UZABIAGA MF, PEREILLO JM, CULOUSCOU JM, BONO F, FERRARA P, HERBERT JM: The active metabolite of Clopidogrel disrupts P2Y12 receptor oligomers and partitions them out of lipid rafts. *Proc Natl Acad Sci USA* **103**: 11069-11074, 2006.

SCHEIDT HA, MULLER P, HERRMANN A, HUSTER D: The potential of fluorescent and spin-labeled steroid analogs to mimic natural cholesterol. *J Biol Chem* **278**: 45563-45569, 2003.

SCHWENCKE C, OKUMURA S, YAMAMOTO M, GENG YJ, ISHIKAWA Y: Colocalization of beta-adrenergic receptors and caveolin within the plasma membrane. *J Cell Biochem* **75**: 64-72, 1999.

SIBLEY DR, BENOVIC JL, CARON MG, LEFKOWITZ RJ: Molecular mechanisms of beta-adrenergic receptor desensitization. *Adv Exp Med Biol* **221**: 253-273, 1987.

SIMONS K, IKONEN E: Functional rafts in cell membranes. *Nature* **387**: 569-572, 1997.

STADEL JM, STRULOVICI B, NAMBI P, LAVIN TN, BRIGGS MM, CARON MG, LEFKOWITZ RJ: Desensitization of the beta-adrenergic receptor of frog erythrocytes. Recovery and characterization of the down-regulated receptors in sequestered vesicles. *J Biol Chem* **258**: 3032-3038, 1983.

SVOBODA P, TEISINGER J, NOVOTNY J, BOUROVA L, DRMOTA T, HEJNOVA L, MORAVCOVA Z, LISY V, RUDAJEV V, STOHR J, VOKURKOVA A, SVANDOVA I, DUSRCHANKOVA D: Biochemistry of trans-membrane signaling mediated by trimeric G-proteins. *Physiol Res* **53** (Suppl 1): S141-S152, 2004.

SYKORA J, BOUROVA L, HOF M, SVOBODA P: The effect of detergents on trimeric G-protein activity in isolated plasma membranes from rat brain cortex; correlation with studies of DPH and Laurdan fluorescence. *Biochim Biophys Acta* **1788**: 324-332, 2009.

UJCIKOVA H, DLOUHA K, ROUBALOVA L, VOSAHLIKOVA M, KAGAN D, SVOBODA P: Up-regulation of adenylylcyclases I and II induced by long-term adaptation of rats to morphine fades away 20 days after morphine withdrawal. *Biochim Biophys Acta* **1810**: 1220-1229, 2011.

USHIO-FUKAI M, HILENSKI L, SANTANAM N, BECKER PL, MA Y, GRIENDLING KK, ALEXANDER RW: Cholesterol depletion inhibits epidermal growth factor receptor transactivation by angiotensin II in vascular smooth muscle cells: role of cholesterol-rich microdomains and focal adhesions in angiotensin II signaling. *J Biol Chem* **276**: 48269-48275, 2001.

VON ZASTROW M, KOBILKA BK: Ligand-regulated internalization and recycling of human beta2-adrenergic receptors between the plasma membrane and endosomes containing transferrin receptors. *J Biol Chem* **267**: 3530-3538, 1992.

VON ZASTROW M, KOBILKA BK: Antagonist-dependent and -independent steps in the mechanism of adrenergic receptor internalization. *J Biol Chem* **269**: 18448-18452, 1994.

VOSAHLIKOVA M, SVOBODA P: The influence of monovalent cations on trimeric G protein G(i)1α activity in HEK293 cells stably expressing DOR-G(i)1α (Cys(351)-Ile(351)) fusion protein. *Physiol Res* **60**: 541-547, 2011.

WALDO GL, NORTHUP JK, PERKINS JP, HARDEN TK: Characterization of an altered membrane form of the beta-adrenergic receptor produced during agonist-induced desensitization. *J Biol Chem* **258**: 13900-13908, 1983.

WHISTLER JL, VON ZASTROW M: Morphine-activated opioid receptors elude desensitization by beta-arrestin. *Proc Nat Acad Sci USA* **95**: 9914-9919, 1998.

WHISTLER JL, CHUANG HH, CHU P, JAN LY, VON ZASTROW M: Functional dissociation of μ-opioid receptor signaling and receptor endocytosis: implications for the biology of opiate tolerance and addiction. *Neuron* **23**: 737-746, 1999.

XU W, YOON SI, HUANG P, WANG Y, CHEN C, CHONG PL, LIU-CHEN LY: Localization of the kappa opioid receptor in lipid rafts. *J Pharmacol Exp Ther* **317**: 1295-1306, 2006.

YOUNG W: Review of lithium effects on brain and blood. *Cell Transplant* **18**: 951-975, 2009.