Qualitative and quantitative investigation of heterotrimeric G-proteins of brain origin

Ph.D. thesis

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Publications

Full papers:

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II. M. Rottmann, G. Fábián, K. Spicher, S. Offermanns, and M. Szűcs: Receptor-Mediated activation of G-Proteins by kappa opioid agonists in frog (*Rana esculenta*) brain membranes. Brain Res. Bulletin 45/05: 467-474, 1998

III. G. Fábián, C.A. Szabó, B. Bozó, J. Greenwood, P. Adamson, M.A. Deli, F. Joó, I.A. Krizbai, M. Szűcs: Expression of G-protein subtypes in cultured cerebral endothelial cells. Neurochem. Int. 33: 179-185, 1998

IV. M. Szűcs, B. Bozó, E.G. Kicsi, G. Fábián, G. Horváth, M. Szikszay, M. Mácsai, G. Szabó: Distinct receptor regulatory mechanisms are induced by individual opioid agonists in rat brain. Submitted

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X. M. Szűcs, B. Bozó, E. Kicsi, G. Fábián, M. Mácsai, G. Szabó: Ligand-specific regulation of μ-opioid receptors in rat brain. Dolor 14: Suppl. I. 15, 1999

Other papers:

I. Szűcs, M., Fábián, G: G proteinek. Biokémia XVIII/4: 153-160, 1994

CONTENTS

I. INTRODUCTION

1.1 G-PROTEINS

1.1.1 General features of structure and function

The heterotrimeric guanine nucleotide binding proteins - G-proteins - have been discovered about 20 years ago and are such key participants in signal transduction that the discoverers were honored with Nobel prize in medicine in 1993. They function as intermediaries in transmembrane signaling pathways that consist of three proteins: receptors, G-proteins, and effectors[1]. They belong to the superfamily of GTPases that includes factors involved in protein synthesis (for example elongation factor Tu) and small molecular weight (20-25 kDa) monomeric G-proteins, such as p21 ras and its relatives[2-5]. G-proteins consist of three subunits, designated as α, β and γ. Traditionally the type of α subunit is used to define the G-protein oligomer. To date, 23 distinct α subunits encoded by 17 genes have been cloned with molecular mass between 39 and 46 kDa[6]. These can be divided into four subfamilies, namely, G_s, G_i, G_q and G_{12}, based on amino acid sequence homology. Some of them are ubiquitous, like α_s, others are more or less specialized, like α_o for brain tissue or α_{t1} and α_{t2} for retinal rods and cones, respectively. G-protein α subunits are enzymes with inherent GTPase activity. They are also subject to several cotranslational and posttranslational modifications. α_i, α_o and α_z are myristoylated at their N-terminus[7], others are modified by different saturated and non-saturated 12- and 14-carbon fatty acids facilitating membrane attachment of α subunits and increasing their affinity for $\beta\gamma$ dimers[8]. In addition to this irreversible lipid modification, some α subunits, like α_s, are reversibly palmitoylated, what seems to have regulatory function[9]. There are also several possible sites for phosphorylation. However, the most characteristic modification of certain types of G-protein α subunits is the ADP-ribosylation by bacterial toxins. Pertussis toxin catalyzes the covalent binding of ADP-ribose to a cysteine residue located four amino acids from the C-terminus. All α_o and α_i subunits can be modified that way resulting in uncoupling from the receptor by inhibiting the activation of the α subunit. Cholera toxin specifically ADP-ribosylates an arginine residue in α_t, α_s and α_{olf}, leading to inhibited GTPase activity, thus, constitutive activation of those α subunits[10].

Five β (35-37 kDa) and twelve γ (8 kDa) subunits have been described up to now[11-13]. They are tightly associated and form one functional unit. There is evidence, that a degree of

specificity governs βγ dimer assembly and not all possible combinations are formed (reviewed in 6). γ subunits are either farnesilated or geranylgeranylated serving the anchorage to the plasma membrane. It is generally considered that β subunit interacts with the α subunit while γ determines the effector specificity in the action of the dimer.

1.1.2 The role of G-proteins in signal transduction

1.1.2.1 Receptor-G-protein interaction

G-proteins serve as membrane-bound transducers of chemically and physically coded information. That extracellular information is received by receptor (R) molecules that are integrated plasma membrane proteins. Certain classes of those receptors (e.g. ligand-gated ion channels or tyrosine kinase receptors) have themselves effector domains, others, characterized by 7 transmembrane α helical domains (7TM receptors or G-protein-coupled receptors, GPCRs), however, first activate G-proteins, what in turn activate the effector molecules. Steps of that cycle are presented on Fig. 1.
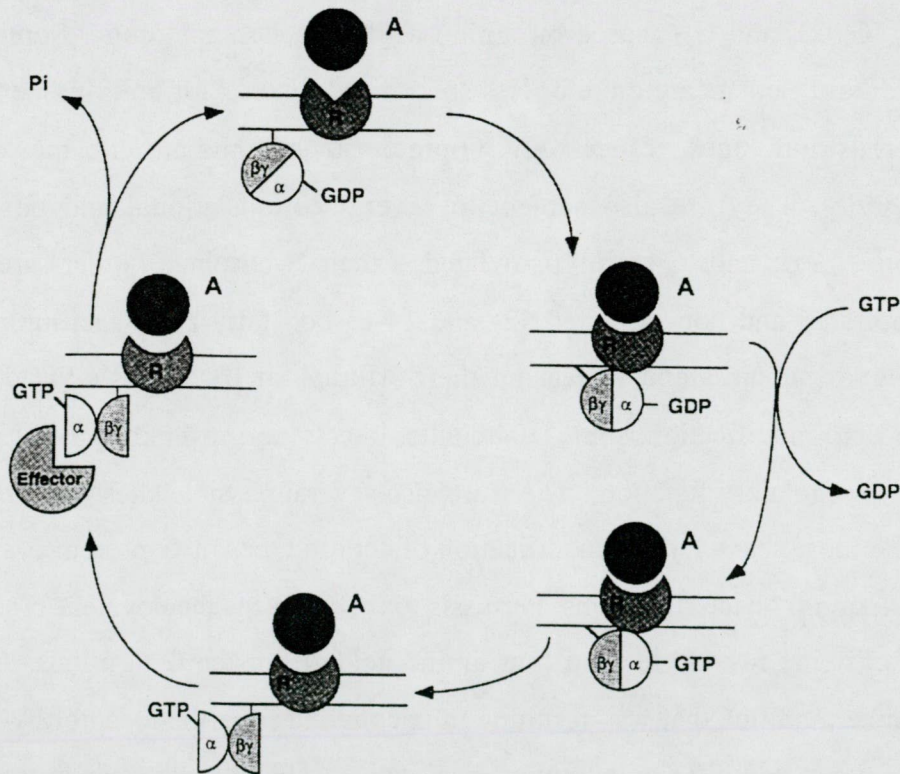
Fig. 1. Ligand activated GTPase cycle of G-proteins. In the resting state heterotrimeric G-proteins bind GDP. Ligand-bound receptor can activate the G-protein resulting in exchange of GDP to GTP and subsequent dissociation of α-GTP and βγ dimer, both of them are capable of activating effectors. The effect is terminated by the inherent GTPase activity of the α subunit and re-association of α-GDP with βγ. R: receptor, A: agonist ligand.

Usually the third intracellular domain and the C-terminal intracellular tail of the receptor molecule determine the R-G-protein interaction. For the activation of G-proteins Mg^{2+} and GTP are essential. Little is known about the regulation of the GTPase cycle, since *in vivo* it goes 10- to 100-fold faster, than *in vitro*, however, recently several proteins were described having GTPase activating properties (GAPs) for $G\alpha$ subunits. They are termed regulators for G-protein signaling (RGS) and definitely the members of this family will increase fast in the future (14 and references cited therein).

G-proteins are also signal amplifiers. It can be achieved at different levels. First, single receptor can activate several G-proteins in turn, second, the dissociation of α and $\beta\gamma$ subunits leads to the bifurcation of the signal, and on the third level G-protein subunits can activate several effector molecules before re-association[174].

1.1.2.2 G-protein-effector interaction

Recent results show that upon activation of a G-protein both α and $\beta\gamma$ subunits are able to interact with different effectors[15] to induce further changes in the state of the cell leading to answer to the extracellular stimulus, or, in a broader sense, to adaptation. The effectors and their activator G-protein subunits are listed in Table 1.

1.1.2.3 Influence of G-proteins on the gene expression

One main pathway for regulation of gene expression by extracellular signals transduced by GPCRs leads via activation of adenylyl cyclase and the subsequent production of cyclic AMP (cAMP). cAMP regulates the transcription of a variety of genes through a distinct DNA sequence termed cAMP response element (CRE) present in their promoter regions. This element is recognized by the cAMP response element binding protein (CREB), a transcription factor of 43 kDa. Activation of CREB is achieved by cAMP-dependent protein kinase (PKA)[17-20].

The other pathway what G-proteins can influence is the signaling route of the receptor tyrosine kinases such as epidermal growth factor, leading to cell differentiation, proliferation and cytoskeletal effects through mitogen-activated protein kinase (MAPK) cascade. There are several convergence points between the two signal transduction pathways, for reviews see 21, 22.

Table 1. Mammalian G-protein subunits and effectors interacting with them

Subtype	Expression	Effectors
α_{sS} (2 forms)[*]	Ubiquitous	Adenylyl cyclase $\uparrow$ (all types)
α_{sL}(2 forms)[*]	Ubiquitous	Ca^{2+} channel $\uparrow$ (L-type)
α_{olf}	Olfact. epithelium	Adenylyl cyclase $\uparrow$ (type V)
α_{gust}	Taste buds, gut	?
α_{t-r}	Retinal rods	cGMP phosphodiesterase $\uparrow$
α_{t-c}	Retinal cones	
α_{i1}	Widely	Adenylyl cyclase $\downarrow$
α_{i2}	Ubiquitous	(types I, III, V, VI)
α_{i3}	Nearly ubiquitous	K^+ channel $\uparrow$
α_{o1}[*]	Neuronal and	Ca^{2+} channels $\downarrow$
α_{o2}[*]	neuroendocrine	(L- and N-types)
α_z	Neuronal, platelets	Adenylyl cyclase $\downarrow$?
α_q	Ubiquitous	Phospholipase-Cβ $\uparrow$
α_{11}	Ubiquitous	($\beta4 \geq \beta1 \geq \beta3 > \beta2$)
α_{14}	Kidney, lung, spleen	
α_{15} (mouse)	Hematopoetic cells	
α_{16} (human)		
α_{12}	Ubiquitous	?
α_{13}	Ubiquitous	?
β_1	Ubiquitous	Adenylyl cyclase $\downarrow$ (type I)
β_2	Ubiquitous	Adenylyl cyclase $\uparrow$ (types II, IV)
β_3	Ubiquitous	Phospholipase-Cβ $\uparrow$
β_4	Ubiquitous	($\beta3 \geq \beta2 \geq \beta1 > \beta4$)
β_{5S}[*]	Mainly brain	K^+ channel $\uparrow$
β_{5L}[*]	Retina	Ca^{2+} channels $\downarrow$
		Receptor kinases (type 2, 3) $\uparrow$
		Phospholipase-A$_2$ $\uparrow$?
γ_1[+]	Retinal rods	Phosphoinositide 3-kinase $\uparrow$?
γ_2	Mainly brain	
γ_3	Mainly brain	
γ_4	Mainly brain	
γ_5	Ubiquitous	
γ_7	Widely	
γ_8[+]	Retinal cones	
γ_{10}	Widely	
γ_{11}[+]	Widely	
γ_{12}	Ubiquitous	

Most of the known G-protein subunits are listed in the table together with the effectors they are interacting with. $\beta\gamma$ combinations apparently not formed are $\beta_2\gamma_1$, $\beta_2\gamma_{11}$; tissue specific combinations are $\beta_1\gamma_1$ for retinal rods and $\beta_3\gamma_8$ for retinal cones. [*] splice variants, [+] these γ subunits are farnesylated, all others are geranyl-geranylated (Table was taken from ref. 16).

1.1.2.4 Role of intracellular G-proteins

Heterotrimeric G-proteins are found not only in the plasma membrane fractions, but also inside the cell, in the cytoplasm or connected to the endo-membrane systems such as the Golgi and the endoplasmic reticulum. They can be found in the non-nervous tissues such as liver[23,24], muscle[25] as well as in the brain[26,27].

These intracellular G-proteins can be newly synthesized molecules, which are transported to the cell surface probably in a fully functional state being able to interact with receptors and also with effectors[28,29]. Intracellular G-proteins may also be conveyed from the cell surface as part of the signal transduction process[30-33]. However, recent results show that G-proteins are not only transported as passive molecules, but they also have important functions intracellularly. They have been suggested to regulate various membrane trafficking processes including exocytotic and endocytotic membrane fusion[34-37]. Role of G-proteins in the maintenance of the highly specialized structure of the blood-brain barrier was also suggested[38-40].

1.2 THE OPIOID RECEPTORS

1.2.1 Opioid receptor types and function

Opioid receptors also belong to the family of GPCRs, and so, are characterized by 7 hydrophobic transmembrane segments and the ability to interact with different G-proteins[41-43]. Opioid receptors were identified in pharmacological studies by using peptide and alkaloid ligands and were classified into three main classes, μ, δ and κ[44]. Cloning of the receptors has verified this model[45-48], but failed to prove the existence of opioid receptor subtypes what have been proposed for all three classes on the basis of pharmacological studies. This suggests that the pharmacological subtypes may result from posttranslational, splicing modifications[49] or differential protein-protein interactions between receptors or with associated proteins[50,51].

Pharmacological effects of the opioid receptors are shown on Table 2.

Table 2. Opioid receptor pharmacology

Receptor	Biochemical	Physiological
μ	cAMP inhibition stimulation of IP_3 formation Ca^{2+} channel inhibition K^+ channel stimulation increase intracellular Ca^{2+}	analgesia
$μ_1$		prolactin release acetylcholine turnover feeding
$μ_2$		growth hormon release respiratory depression inhibition of G.I. transit guinea pig ileum bioassay
Morphine-6β-glucuronide		inhibition of G.I. transit analgesia
κ		
$κ_1$	inhibition of cAMP accumulation inhibition of PI hydrolysis Ca^{2+} channel inhibition K^+ channel stimulation	diuresis, sedation rabbit *vas deferens* bioassay feeding
$κ_2$	pharmacology unknown	pharmacology unknown
$κ_3$	inhibition of cAMP accumulation	feeding
KOR-3/ORL-1	K^+ channel stimulation inhibition of cAMP accumulation	hyperalgesia (early) analgesia (later)
δ	inhibition of cAMP accumulation K^+ channel stimulation increase intracellular Ca^{2+}	analgesia mouse *vas deferens* bioassay dopamine turnover
$δ_1$		G.I. motility
$δ_2$		G.I. motility

Table was taken from ref. 52. with minor modifications

1.2.2 Ligand binding to the opioid receptor

Radioligand binding studies combined with site directed mutagenesis of the receptor molecules have provided a great deal of information on the interaction of opioid ligands with their receptors (for review see 53). It is thought that only agonist binding leads to activation of the receptor followed by conformational changes and information transfer. Antagonist binding in contrast would not elicit a biological response. It was shown that certain charged amino acids in the transmembrane regions TM II (Asp114), III (Asp147) and VI (His297) are important for ligand binding and subsequent activation of effectors[54]. It was also shown that opioid peptides

and alkaloids, as well as agonists and antagonists bind to different parts of the receptor molecule[54,55]. In case of δ receptors the third extracellular loop is also important for ligand selectivity[56]. The identification of the specific residues in the κ receptor involved in agonist and antagonist binding may facilitate the further development of therapeutically useful opioids. This will be particularly important in the case of κ receptor since kappa agonists have minimal abuse potential and do not cause respiratory depression, two major side effects of the use of mu receptor selective agonists. In contrast, kappa agonists are effective analgesics and useful diuretic agents. Previous results showed that frog (*Rana esculenta*) brain membranes are remarkably useful for the investigation of this opioid receptor type, since they contain high proportion of κ receptors compared to μ and δ[98]. Frog brain membranes also contain κ receptor subtypes, namely κ₁ and κ₂[134, 152]. Detailed characterization of those binding sites in ligand binding studies indicated that they might couple to G-proteins[147,153,156].

Another way of investigation of ligand-receptor interaction considers energetic aspects. Thermodynamic analysis provides means of determining the underlying driving forces of binding and intermolecular interactions which information can not be easily obtained by other techniques. Thus conformational changes or protein-protein associations should provoke characteristic thermodynamic behavior. Using this approach it is shown that opioid agonist binding is mainly entropy driven, while opioid antagonist binding is exothermic thus enthalpy driven[57-60].

Opioid binding is modulated by a number of reagents. Na⁺ and GTP decrease agonist binding and increase antagonist binding. Divalent cations also differentiate agonist and antagonist binding (61, 62 and references therein). These three agents are also known to be required for functional coupling of opioid receptors to inhibitory G-proteins[63-65]. Thus, thermodynamic analysis of ligand binding might provide key information on the signal transduction function.

1.2.3 Consequences of repeated ligand administration

Chronic use of opiates results in drug addiction, including tolerance to and dependence on the drug, which phenomenon, besides its scientific importance, has a great social impact. Despite intense research on this field the precise molecular mechanism accounts for that is largely unknown.

In biochemical terms long-term presence of the agonist generally leads first to desensitization which means that the receptor is unable to activate effector molecules due to the uncoupling of the receptor from the transducer G-protein. The reason for that is the

phosphorilation of the receptor by specific kinases[66-68]. This occurs on a minute time scale. Desensitization is usually followed by sequestration and internalization of the receptor into endosomal vesicles. This is still a minute to hour-long procedure. Proteins in the endosomal vesicles can be recycled to the cell surface or degraded in lysosomes. On a longer time scale down-regulation of the receptor can occur meaning reduction of the total (surface and intracellular) receptor number. This certainly involves much complicated regulatory steps in the gene expression, translation and/or degradation rate of the certain protein. The above mentioned steps might give rise to the pharmacological phenomenon of tolerance meaning that the same dose of the drug is ineffective to evoke the same response at repeated administration, or conversely, to achieve the same magnitude of effect larger and larger dose of drug is necessary. Dependence refers more to physiological (or somatic) and psychological aspects of addiction, the former characterized by withdrawal symptoms at cessation of drug administration, while the latter by drug seeking behaviour. The different anatomical correlates and molecular mechanisms responsible for the opiate dependence are reviewed in 69-71.

Recent results showed that opioid receptors are regulated by ligand-specific manner[72-76]. However, in the manifestation of tolerance and dependence not only receptors take part but also other elements of the signal transduction pathway. Alteration was detected in the amounts and function of G-proteins[77-82]. Exposure of cells to agonists of receptors linked to G-proteins can result in up- or downregulation of cellular levels or redistribution of G-proteins from membranes to the cytosol. Agonist-induced reductions in G-protein levels have been observed for members of each of the Gs, Gi and Gq families of G-proteins, are likely to be dependent upon the level of receptor expression, and are generally restricted to the G-protein(s) with which the receptor interacts. The mechanisms responsible vary with cell type and include both second messenger-dependent and -independent enhanced protein degradation. Agonist-induced reduction in cellular G-protein levels can provide one mechanism for the development of sustained heterologous desensitization (for review see 126). Various elements of the signal transduction pathway, such as adenylyl cyclase[83,84], protein kinase-C (PKC)[85], G-protein coupled receptor kinase[86], and protein phosphatases[87] are also affected.

II. AIMS

In the present work, we studied the G-proteins in three brain tissues, namely rat brain subcellular fractions, frog brain homogenates and brain endothelial cells. It was of interest to

see if G-proteins of different origin share similar structural features. Also, we raised the question whether certain G-protein types or their absence can be associated with certain tissues. Finally, receptor-G-protein interactions were analyzed in order to gain more insight into the molecular mechanisms involved in opioid signal transduction in opioid naive as well as in morphine-tolerant states. In order to achieve these goals, a spectrum of state-of-the-art techniques were established. The following specific aims were set:

1. Investigation of the ionic regulation and temperature dependence of μ and δ opioid receptor binding in rat brain membranes, and calculation of thermodynamic parameters from the binding data. Investigation of guanine-nucleotide sensitivity of the μ receptor binding, what might reflect receptor-G-protein interaction.

2. Investigation of regulation of μ opioid receptor signal transduction. Detection of effects of *in vivo* chronic morphine treatment on rat brain G-proteins. I want to check whether the treatment causes alteration in the amount, state, function as well as localization of G-proteins. This part of the project also involves investigation of exposure level, affinity, subcellular distribution and signaling properties (desensitization, down-regulation) of μ opioid receptors.

3. Determine the time-course of changes on μ opioid receptors and G-proteins by applying morphine treatments of different length and dose (3, 5 and 10 days) and associate those with the development of analgesic tolerance.

4. Application of protein synthesis inhibitors during the *in vivo* chronic morphine treatments would help to reveal molecular mechanisms underlying tolerance and dependence.

5. *In vivo* treatments of rats with the antagonist naltrexone will also be done to compare resulting changes with that of chronic agonist treatment.

6. Identification of G-protein types in a non-mammalian tissue (frog brain) that is a rich source of κ opioid receptors, and comparison with those in rat brain.

7. Detection of G-proteins in endothelial cell lines and primary cultures of rat brain endothelial cells with highly specialized function, namely, the formation of blood-brain barrier.

III. MATERIALS AND METHODS

3.1 CHEMICALS

For the thermodynamic analysis [³H]naloxone (([³H]NX, 72 Ci/mmol) was synthesized by Dr. G. Tóth and associates[88]. [³H]-DAMGO (Tyr-D-Ala-Gly-MePhe-Gly-ol, 60 Ci/mmol) was purchased from Amersham.

For other experiments urea was purchased form Merck (Darmstadt, Germany), ion-exchange resin for urea purification (AG-501 X8) was from Bio-Rad (Richmond, CA, USA), Low Molecular Weight markers were from Pharmacia (USA), nitrocellulose (Hybond) and ECL were from Amersham (Buckinghamshire, England). Anti-G-protein antibodies AS/7 (anti-$G_{i1,2}\alpha$; 1:500), RM/1 (anti-$G_s\alpha$; 1:500) and GC/2 (anti-$G_o\alpha$; 1:500) were from Du Pont-NEN (Boston, MA, USA). AS 11 (anti-Gβ-common; 1:300)[89], AS 369 (anti-$G_{q/11}\alpha$; 1:1000)[90], AS 269 (anti-$G_{i2}\alpha$; 1:150)[43] and AS 86 (preferentially anti-$G_{i3}\alpha$; 1:500)[91] were characterized and kindly donated by Prof. G. Schultz and Dr. K. Spicher (Freie Universität Berlin, Germany). [α-^{32}P]nicotinamide adenine dinucleotide (([α-^{32}P]NAD; specific activity 800 Ci/mmol) was purchased from New England Nuclear (Boston, MA, USA). Sources of other materials for the photoaffinity labeling have been cited[92,93]. [^{35}S]GTPγS (37-42 TBq/mmol) was obtained from Isotope Institute Ltd., (Budapest, Hungary). For chronic morphine treatment experiments DAMGO and [³H]DAMGO were obtained from Multiple Peptide System (San Diego, CA, USA) via the Drug Supply Program of NIDA (Rockville, USA). EKC and bremazocine was provided by Sterling Winthrop Research Institute, U-50,488 was from RBI, sucrose was from Boehringer (Mannheim, Germany); trasylol (Gordox, aprotinin) was purchased from Gedeon Richter Pharmaceutical Company, (Budapest, Hungary), norbinaltorphimine was from Alkaloida Chemical Company Ltd (Tiszavasvári, Hungary). All other chemicals were purchased from Sigma Chemicals (St. Louis, MO, USA).

3.2 METHODS

3.2.1 Cell cultures, animals

3.2.1.1 Primary cultures of rat CECs and cell lines

Cultures were prepared as described in details earlier[94], from 2-week-old rat brains [III. from Publications, page 2]. All CFY rats used for the primary cultures of CECs were acquired

from the local animal house and were cared for in accordance with international standards and guides. Cultures were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 20% plasma derived bovine serum (PDS), 2.0 mM glutamine and antibiotics at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. To further purify cultures of CECs, a selective cytolysis of contaminating cells by specific anti-Thy 1.1 antibody and complement was performed[94]. After 7 days in vitro, endothelial cells developed continuous monolayers, which were used for the experiments. The cultures were regularly tested for Factor VIII-related antigen, and over 98% of the cells expressed positivity.

GP8 cells[95] were kept in DMEM supplemented with 20% PDS, 1 ng/ml basic fibroblast growth factor (bFGF) and 300 μg/ml geneticine and used between passages 15-20. RBE4 cells[96] were cultured in DMEM plus 10% fetal calf serum, 1ng/ml bFGF and 300 μg/ml geneticine and used between passages 32-47. Both cell lines expressed general endothelial as well as specific cerebral endothelial features as published[95,96].

3.2.2 In vivo experiments

After approval had been obtained from the Animal Care Committee of Albert Szent-Györgyi Medical University, female Wistar rats weighing 250-350 g were studied. All experiments were performed in freely moving animals during the same period of the day (8:00-13:00 h) to exclude diurnal variations in pharmacological effects. The animals were randomly assigned to treatment groups (n=6-16 per group) and the observer was blind to the treatment administered.

3.2.2.1 Chronic administration of morphine

The animals were made dependent on morphine by a series of subcutaneous injections of morphine hydrochloride administered twice daily at 8 a.m. and 6 p.m. for 3 (M3), 5 (M5) and 10 (M10) days. The initial dose, 10 mg/kg was increased gradually (see the paradigm in Table). Control animals were handled simultaneously by saline injections [IV., V.]. In another set of experiments rats were treated for 5 days with the antagonist naltrexone (N5) according to the schedule of the morphine treatment [V.].

Table 3. The paradigm of chronic morphine treatment

Dose of morphine (mg/kg)										
Day	1	2	3	4	5	6	7	8	9	10
Morning	10	20	40	40	40	40	50	50	60	60
Evening	20	40	40	40	40	40	50	50	60	60

3.2.2.2. Hot-plate and tail-flick tests

The nociceptive sensitivity after the different treatments (3, 5 or 10 days morphine administration) was assessed by using hot-plate and tail-flick techniques [IV., V.]. The latency of licking one of the hind paws or jumping was measured on the hot-plate (52.5 °C, cut-off time: 60 s). The reaction time in the tail-flick test was determined by immersing the lower 5 cm portion of the tail in the hot water until the typical tail-withdrawal response was observed (51.5 °C water, cut-off time: 20 s). Baseline latencies were obtained immediately before, then 30, 60, 90 and 120 min after the drug injection (saline or 10 mg/kg morphine). Analgesic latencies in acute pain tests were converted to percentage maximum possible effect (%MPE) by using the formula:

%MPE=[(observed latency - baseline latency)/(cut-off time - baseline latency)]*100

Data are presented as means ± S.E.M. Analysis of variance (ANOVA) of data for repeated measures was used for overall effects, with the Newman-Keuls test for post-hoc comparison for differences between means. A level P<0.05 was considered significant.

3.2.3 Membrane preparation

Cell cultures were washed twice with phosphate buffered saline (PBS); the monolayers were detached from the plastic by tissue scraper and suspended in PBS (pH 7.4). Samples were sedimented for 10 min at 3,000 x g (4 °C) and the pellets then suspended in 10 ml lysis buffer (5 mM Tris-HCl, 50 μM CaCl₂, 0.5 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride, pH 8.1) followed by homogenization in a glass teflon potter at 4 °C. The homogenate was centrifuged for 5 min at 3,000 x g and the resulting supernatant spun at 20,000 x g for 30 min. Membrane pellets obtained were suspended in 50 mM Tris-HCl (pH 7.4) to yield about 0.4-1 mg protein/ml and either freshly used for [^{35}S]GTPγS studies or kept frozen at -70 °C until used for immunoblotting [III.].

For thermodynamical analysis particulate membrane fractions were prepared as published[97], with minor modifications [I.]. Briefly, rats (PVG/C strain) were killed by decapitation; whole brains without cerebella were excised and homogenized in 20 volumes (w/v) of ice-cold 50 mM Tris-HCl, pH 7.4 buffer with a Potter-Elvehjem homogenizer. The homogenate was centrifuged for 20 min at 40,000 x g and the supernatant discarded. Pellets were suspended in 20 volumes of fresh buffer and incubated for 30 min at 37°C to remove endogenous opioids. Centrifugation was then repeated as described above. Finally, pellets were suspended in 5 volumes of 50 mM Tris-HCl pH 7.4 buffer containing 320 mM sucrose,

and stored at -70°C. Membranes were thawed before use, diluted with buffer and spun at 40,000 x g to remove sucrose. The resulting pellet was suspended in 80 volumes of buffer to give a protein concentration of about 300 μg/ml and was used for binding experiments.

Frog brain membranes were prepared as described[98]. Adequate measures were taken to minimize pain or discomfort of the experimental animals. Briefly, whole brains were homogenized in 50 mM Tris-HCl buffer (pH 7.4), containing 1 mM EDTA, 0.1 mM PMSF, 10 mg/ml bacitracin, and 40 kIU trasylol. The homogenate was centrifuged (20 min., 25,000 x g, 4 °C). The resulting pellet was resuspended in the same buffer containing 0.32 M sucrose and stored at -70 °C [II.].

After chronic morphine treatment subcellular fractionation of rat brains were done according to Roth et al.[99] and Szücs et al.[33]. Saline treated control (C) and morphine-treated (M3, M5 or M10) brain homogenates were simultaneously assessed in every experiment. Briefly, rat brains were gently homogenized and after repeated centrifugation of the homogenates the combined supernatants are centrifuged at 12,000 x g for 20 min. The pellet is resuspended in 10% sucrose, and consecutive centrifugations at 20,000 x g for 25 min and 14,000 x g for 20 min (twice) result in crude SPM. Crude microsomes are obtained from the 12,000 x g supernatant by consecutive 20,000 x g for 25 min and 165,000 x g for 1 h centrifugations. Purified SPM fractions were finally resolved on a 10%, 28.5% and 34% sucrose density step gradient centrifuged at 100,000 x g for 2 h, whereas microsomes (MI) were obtained from a 10% and 28.5% gradient centrifuged at the same speed. Both SPM and MI from gradient were diluted threefold with TRIS-HCl pH 7.4, pelleted at 100,000 x g for 1 h and resuspended in 50 mM TRIS-HCl pH 7.4 [IV., V.].

In all cases protein content was determined according to Bradford[100].

3.2.4 G-protein methods

3.2.4.1 [35S]GTPγS binding assay

Membranes (≈10 μg of protein) were incubated in Tris-EGTA pH 7.4 buffer containing [35S]GTPγS (0.05 nM) and increasing concentrations (10^{-7} -10^{-3} M) of stimulating ligands in the presence of 100 μM GDP in a total volume of 1 ml for 60 min at 30 °C, according to Sim et al.[101], and Traynor et al.[102]. Nonspecific binding was determined with 10 μM GTPγS and subtracted. Bound and free [35S]GTPγS were separated by vacuum filtration through Whatman GF/B filters with a Millipore manifold. Filters were washed with 3 x 5 ml ice-cold

buffer, and radioactivity was detected after drying in a toluene-based scintillation cocktail in a Searle liquid scintillation counter [II. - V.].

3.2.4.2 ADP-Ribosylation

The protocol for ADP-ribosylation of membrane proteins was developed by Ribeiro-Neto *et al.*[103]. Pertussis toxin was preactivated by incubation with 62.5 mM DTT at 37 °C. Per sample, approximately 180 μg membrane protein was subjected to ADP-ribosylation with $1x10^7$ dpm [α-^{32}P]NAD and 60 μM unlabeled NAD. Samples were spun down, the pellet was resuspended in sample buffer and was electrophoresed [V.].

3.2.4.3 Photolabeling of G-protein α-subunits

[α-^{32}P]GTP azidoanilide was synthesized as described[92]. Membranes were suspended in ice-cold incubation buffer containing 100 mM Hepes, pH 7.4, 10 mM MgCl₂, 2 nM benzamidine, 100 μM GDP, 150 mM NaCl and 0.2 mM EDTA to yield 25 μg protein/30 μl buffer. Sample tubes were preincubated for 3 min at 30 °C with 10 μl of 1 μM opioid ligand (DAMGO or DPDPE for rat brain membranes). Thereafter 20 μl of [α-^{32}P]GTP azidoanilide, diluted in water, were added (2 millions cpm/tube), and the reaction was stopped after 5 min by putting the samples on ice. All the subsequent procedures were performed at 4 °C. The samples were centrifuged at 12,000 x g for 5 min, and the pellets were resuspended in 60 μl of photolysis buffer, containing 50 mM Hepes pH 7.4, 5 mM MgCl₂, 1 nM benzamidine, 2 mM DTT, 150 mM NaCl, and 0.1 mM EDTA. The samples were irradiated for 10 sec at 4 °C with an UV lamp (254 nm, 150 W), from a distance of 3 cm. After irradiation samples were centrifuged again, and the pellets resolved in Laemmli sample buffer[104] for gel electrophoresis.

3.2.4.4 Gel electrophoresis and immunoblotting

SDS-PAGE was performed according to Laemmli[104] with modifications [II., III., V.]. Unlabeled membrane samples of RBE4 and GP8 cells or rat and frog brains were delipidated in methanol : chloroform : water mixture (4:1:3), spun down in an Eppendorf centrifuge, washed with methanol and the pellet resuspended in sample buffer. Cells from the primary culture were lysed in sample buffer, sonicated for 1 min and boiled for 5 min. PTX- or photolabeled samples were resolved in sample buffer. Equivalent amounts of proteins (30-80 μg) were loaded onto a 16 cm long 10% slab gel containing high purity, deionised urea (6 M), which improved the resolution of Gα and Gβ subunits with molecular weights close to each other[89,105]. In case of membranes from saline- and morphine treated rats, different amounts of

protein were loaded onto the same gel between 7.5-60 µg to make the comparison of the matched samples more authentic. Electrophoresis was performed in room temperature at a constant voltage of 150 V for 6 h. After electrophoresis, the separating gels were either, in case of radiolabeled samples, stained with 1 % Coomassie blue G-250, dried and exposed to X-ray films (Kodak X-OMAT AR), or immunoblotted [II., III., V.]. Proteins resolved by SDS-PAGE were transferred onto nitrocellulose membranes at a constant current of 200 mA for 1 h in a three buffer component semi-dry system[106]. Transfer buffer 1 containing 0.3 M TRIS-HCl, 20% methanol; transfer buffer 2 and 3 consisting of 25 mM TRIS-HCl, 20% methanol; and 25 mM TRIS-HCl, 20% methanol, 40 mM ε-amino-caproic acid, respectively . Nitrocellulose membranes were amido-black stained for evaluation of the protein loading and transfer, blocked for 1 h in 3 % ovalbumin in TRIS-buffered saline (TBS), pH 7.4. Thereafter, filters were cut into stripes and incubated for 1 h with various anti-G-protein antisera, which were diluted in TBS (pH 7.4) supplemented with 1% (w/v) BSA (fraction V., protease free) and sodium azide. After washing with TBS supplemented with 0.05 % (v/v) Tween-20 (TBS/Tween) for 15 min 4 times, filters were blocked again as above and subsequently incubated with the second antibody (goat anti-rabbit IgG, peroxidase conjugated, diluted 1:1000) in TBS/BSA for 1 h. The extensive washing was repeated and labeled protein bands were visualized by ECL reagent on Kodak X-OMAT AR film. Films were analyzed by an LKB Ultroscan XL Enhanced Laser Densitometer and GelScan XL Laser Densitometer Program computer software, or were scanned and data files were evaluated with ImageQuaNT software (version 4.1, Molecular Dynamics). In addition to that, photolabeled proteins were identified in the dry gel by careful position of the films onto the gel, and area containing the labeled bands were excised from the gel, solved in 30% H_2O_2 and radioactivity was measured by liquid scintillation counting.

3.2.5 Receptor binding assay

Routinely, the binding assay was performed in a total volume of 1 ml containing 7-800 µl membrane suspension ($\approx$ 250 µg protein), [^{3}H]-labeled ligands at 1 nM concentration with or without appropriate concentrations (10^{-5}-10^{-11} M) of displacers, in case of thermodynamical analysis ions (2 mM $MgCl_2$, 100 mM or 25 mM NaCl for the antagonist and agonists, respectively) and nucleotide (5×10^{-5} M Gpp(NH)p). Incubation was started by the addition of the membrane protein and continued until equilibrium was achieved (1 h at 0 °C, 1 h at 24°C, 30 min at 33°C in the case of [^{3}H]NX, [^{3}H]DHM or [^{3}H]DAMGO; and 90 min at 0°C, 40 min at 24 °C and 33 °C for [^{3}H]DT-B, respectively). In case of the heterologous displacements

additional temperatures were also assayed, 14°C and 18°C for 60 min and 28°C for 40 min. The reaction was stopped by filtration through Whatman GF/B ([^{3}H]NX) or GF/C filters ([^{3}H]DHM, [^{3}H]DT-B and [^{3}H]DAMGO) with Brandel M 24-R cell harvester. Filters were rapidly washed twice with 10 ml ice-cold 50 mM Tris-HCl pH 7.4 buffer, dried and counted in a toluene-based scintillation cocktail in a Beckman LS 5000TD counter.

Untransformed binding data were analyzed with the nonlinear least-squares regression computer program LIGAND[107] to obtain K_d (dissociation constant) and B_{max} (number of binding sites) values [I., IV., V.]. For comparing changes in receptor number due to agonist treatments, the significance was determined by a t-test in B_{max} from matched samples of treated vs. control.

3.2.6 Thermodynamical analysis

For thermodynamical analysis, from the calculated affinity constants, K_a (1/K_d) the following thermodynamic parameters were calculated [I.]:

Gibb's free energy: $\Delta G^{o'} = -R\,T\,\ln K_a$

standard free enthalpy: $\Delta H^{o'} = -R\,m$

standard free entropy: $\Delta S^{o'} = (\Delta H^o - \Delta G^o)\,/\,T$

where R is the gas constant (8.31 J mol^{-1} K^{-1}), T is the absolute temperature in degree Kelvin, m is the slope of the van't Hoff plots ($\ln K_a$ vs. 1/T) which was fitted by the computer program 'Microstat' of Ecosoft, 1984).

In the case of curvilinear van't Hoff plots a non-linear regression analysis of the $\Delta G^{o'}$ values as a function of temperature was performed according to the following equation[108]:

$$\Delta G^{o'} = a + bT + cT^2 \qquad (1)$$

The coefficients a, b, c were calculated from the curve which fitted best the experimental results of $\Delta G^{o'}$ versus T. $\Delta H^{o'}$ and $\Delta S^{o'}$ were evaluated from the mathematical derivatives of the above equation using the calculated a, b, c values:

$$\Delta H^{o'} = d(\Delta G^o\,/\,T)\,/\,d(1\,/\,T) = a - cT^2 \qquad (2)$$

$$\Delta S^{o'} = d(\Delta G^o)\,/\,dT = -b - 2\,cT \qquad (3)$$

IV. RESULTS

4.1 THERMODYNAMICAL ANALYSIS OF RECEPTOR – G-PROTEIN COUPLING

A widely used experimental paradigm for measuring GTP-shift of agonist affinity is to measure the displacement of a radiolabeled antagonist binding by unlabeled agonist in the absence or presence of guanine nucleotide. In our experiments where 10^{-5} - 10^{-11} M unlabeled dihydromorphine (DHM) was tested as displacer of 1 nM [^{3}H]naloxone ([^{3}H]NX) at μ opioid binding sites, analysis of the data with LIGAND resulted in a one-site binding either in the absence (control) or in the presence of regulators. The van't Hoff plots resulted in straight lines with negative slopes in the absence of regulators, or in the presence of Na^+ or Mg^{2+} (r>0.9) (Fig. 2. A,B,C)
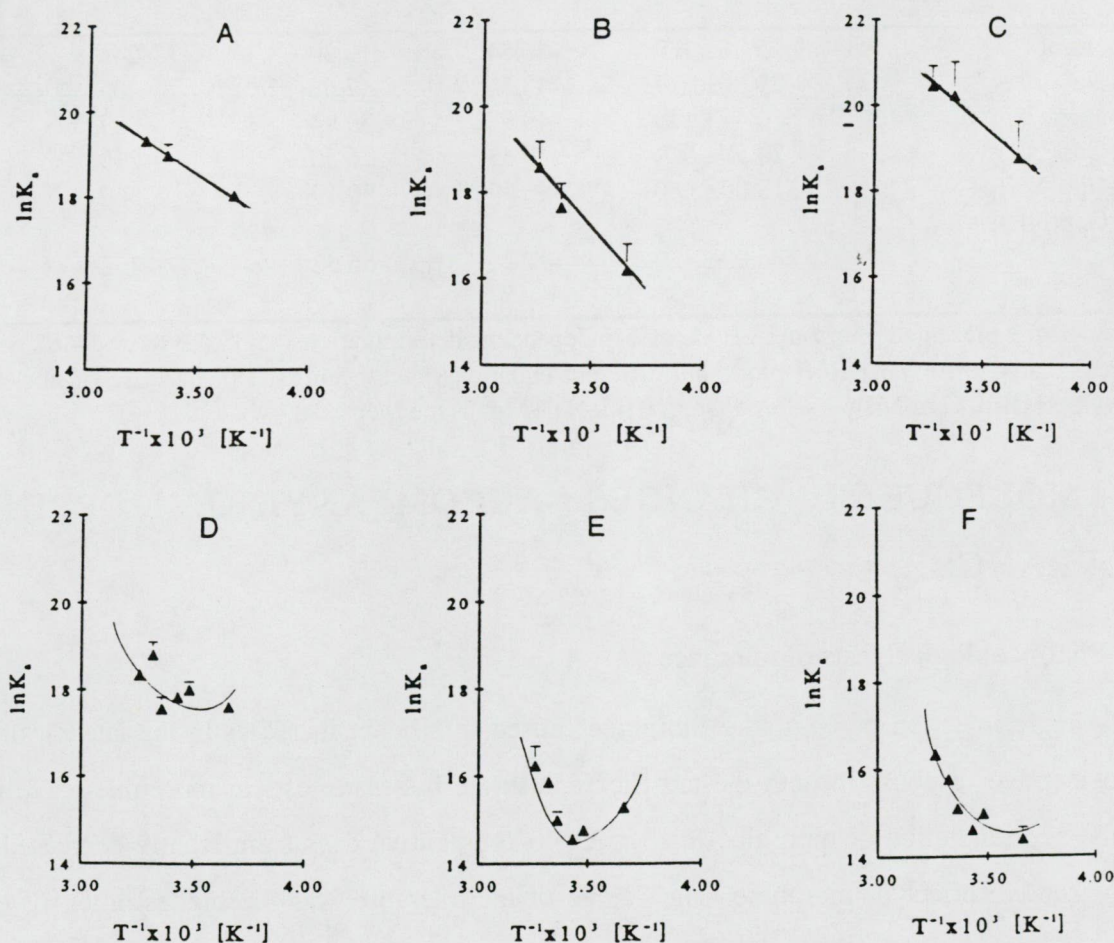

Fig. 2. Van't Hoff plots of unlabeled DHM (10^{-10}-10^{-5} M) competition against 1 nM [^{3}H]NX with no addition (A), in presence of Na^+ (B), Mg^{2+} (C), Gpp(NH)p (D), Gpp(NH)p+Na^+ (E), or Gpp(NH)p+Na^++Mg^{2+} (F). Values shown are the mean±S.E.M. of lnK_a of at least 3 independent determinations at each temperature.

However, the presence of 50 µM Gpp(NH)p (a hydrolysis-resistant GTP analog), the simultaneous presence of Gpp(NH)p + Na$^+$, or Gpp(NH)p + Na$^+$ + Mg^{2+} changed the temperature dependence of the system which was reflected as a break in the plot (r=0.6, 0.5 and 0.85, respectively) (Fig. 2. D,E,F). Such non-linear van't Hoff plots can reflect complex binding interactions involving more than one step, or conformational change. As shown in Table 4. the thermodynamic parameters, especially ΔH$^{o'}$ and ΔS$^{o'}$ undergo major changes when uncoupling of the receptor from G-proteins presumably takes place. Thus increased entropy and enthalpy changes were detected when Gpp(NH)p, Na$^+$ and Mg^{2+} were simultaneously present in the incubation mixture.

Table 4. Thermodynamic parameters of the displacement of 1 nM [^{3}H]NX by 10^{-5}-10^{-11} M unlabeled DHM at 24°C in the absence (control) or in the presence of Na$^+$, Mg^{2+} and/or Gpp(NH)p

	K_D (nM)	ΔG$^{o'}$ (kJxmol^{-1})	ΔH$^{o'}$ (kJxmol^{-1})	ΔS$^{o'}$ (Jxmol^{-1}xK^{-1})
control	5.86± 1.5	-46.84	17.80	246
+Na$^+$	29.80±16.9	-43.56	47.68	307
+Mg^{2+}	2.47± 1.1	-49.97	35.94	287
+Gpp(NH)p	24.50± 6.7	-43.33	26.50	239
+Gpp(NH)p+Na$^+$	313.00±59.0	-36.99	70.19	362
+Gpp(NH)p+ Na$^+$+Mg^{2+}	275.00±10.0	-37.26	66.62	352

K_D values are presented as mean±S.E.M. of 3 independent determinations. ΔG$^{o'}$, ΔH$^{o'}$ and ΔS$^{o'}$ values were calculated from van't Hoff plots, but different equations have been used in the case of non-linear plots (Gpp(NH)p, Gpp(NH)p+Na$^+$ and Gpp(NH)p+Na$^+$+Mg^{2+}) (see Methods).

4.2 MOLECULAR CHANGES ACCOMPANYING MORPHINE TOLERANCE

4.2.1 Pharmacological tests of tolerance

A single injection of 10 mg/kg morphine caused significant increases in the latency of the antinociceptive response in both the tail-flick and in the hot-plate tests in morphine-naive rats. Chronic administration of morphine for 3, 5 or 10 consecutive days significantly decreased the antinociceptive effect of morphine. The degree of the tolerance was the highest after 10 days administration of morphine.

4.2.2 Changes in µ opioid receptor binding due to chronic morphine *in vivo*

Agonist-induced changes in the ligand-binding parameters of µOR were analyzed with

homologous displacement experiments using [³H]DAMGO binding. Brains of control and morphine treated animals were subjected to subcellular fractionation to yield in highly purified synaptic plasma membranes (SPM) and microsomes (MI) as described in Methods. The receptor affinity (K_D) did not change due to chronic morphine treatment in either fractions (data not shown). There were about equal number of μ binding sites in the SPM and MI fractions of control animals. Three days of morphine treatment caused no change compared to those in vehicle-treated brain fractions and the animals showed no signs of tolerance/dependence (data not shown). Upon 5-day morphine treatment, the number of surface μOR did not change, the B_{max} values were 203±26 fmol/mg and 187±70 fmol/mg in control and M5 SPM, respectively. In contrast, a 68% increase in B_{max} values of [³H]DAMGO binding sites from 199±15 fmol/mg to 336±46 fmol/mg was measured in control and M5 MI, respectively. Chronic morphine treatment for 10 days resulted in elevated levels of the total number (surface + intracellular) of μ binding sites. The resulting B_{max} values of [³H]DAMGO binding were 320±71 fmol/mg and 263±3 fmol/mg in M10 SPM and M10 MI (Fig. 3).
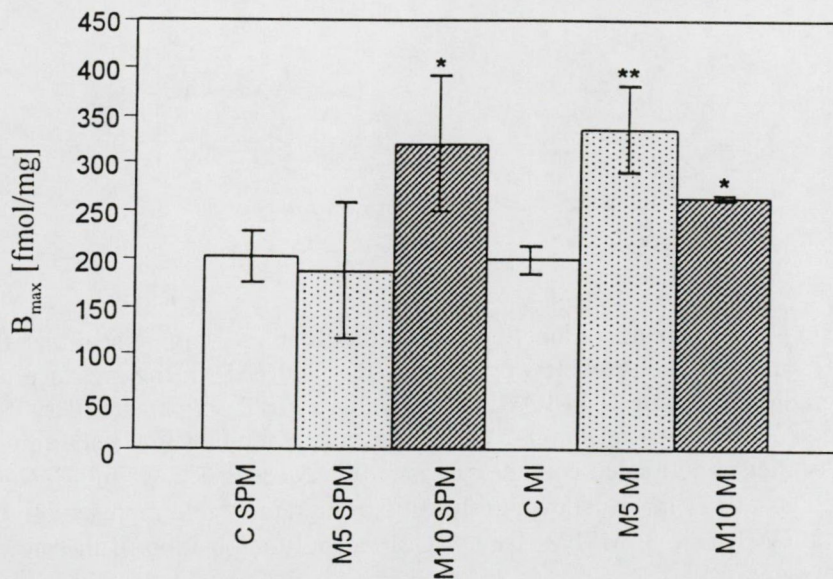

Fig. 3. Changes in the B_{max} of [³H]DAMGO binding due to opioid exposure. Animals were treated with saline (C) or increasing dose of morphine (M) for 3, 5 or 10 days. Subcellular fractionations of brain homogenates were performed to prepare synaptic plasmamembrane (SPM) and microsomal (MI) membranes as described in Methods. Results shown are expressed as fmol/mg protein in each fraction. Mean ± S.E.M., n = 3-8. Significance was determined by t-test, * p< 0.05 , ** p< 0.01

4.2.3 Effect of chronic morphine treatment on G-proteins in rat brain

Islet-activating protein (pertussis toxin, PTX), which catalyzed ADP-ribosylation of the α-subunits of G_i/G_o proteins was used to assess the G-protein distribution in subcellular fractions of

rat brain. The proteins incubated with $[\alpha\text{-}^{32}P]NAD$ were analyzed by SDS-PAGE and autoradiography. PTX catalyzed the labeling of proteins of 39-42 kDa that were present in all fractions (Fig. 4.). There was a 20% increase in density of the main labeled band of 42 kDa in the SPM fraction, while the density of the same band increased by 89% in the MI compared to their saline treated counterparts after 5-day chronic morphine treatment (Fig. 4.).
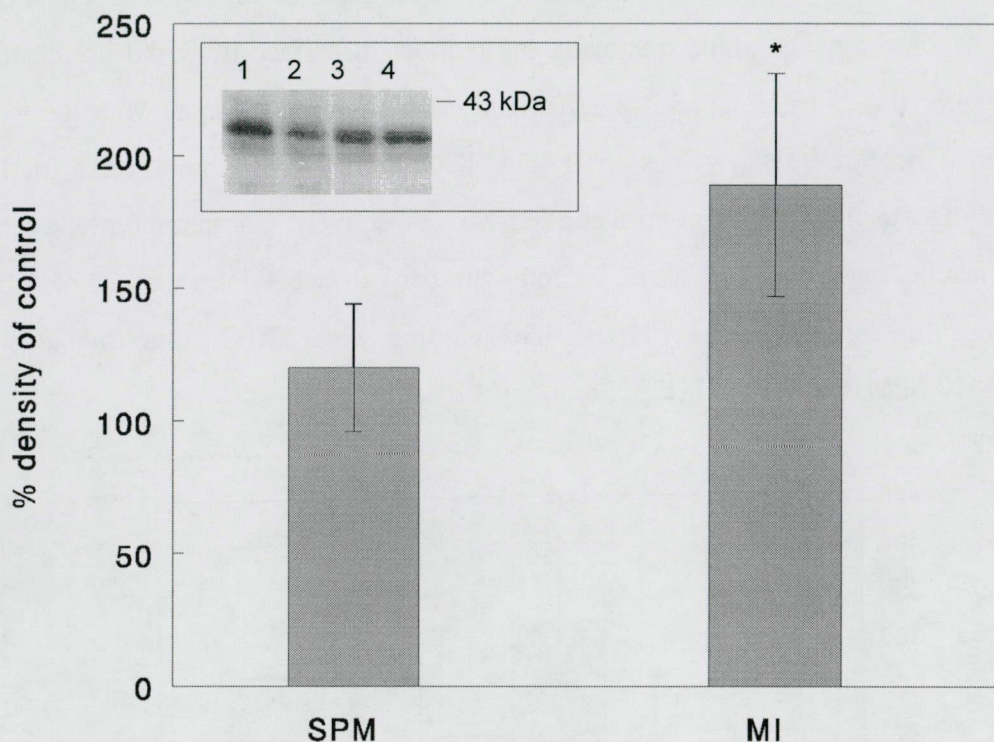

Fig. 4. Pertussis toxin (PTX) dependent labeling of G-proteins in rat brain subcellular fractions after chronic morphine treatment (M5). Proteins were labeled by $[P^{32}]NAD$ in the presence of PTX. Main labeled band of autoradiograms after SDS-PAGE were evaluated by laser densitometry. Shown are the % changes in the morphine treated rat brain synaptic plasmamembrane (SPM) and microsomal (MI) fractions compared to the saline treated counterpart, as 100%. Mean ± S.E.M., n = 3, significance was determined by t-test, * p< 0.05. Insert: shown is the autoradiogram of one representative experiment. Lane 1: MMI, lane 2: CMI, lane 3: MSPM, lane 4: CSPM, and the position of the molecular weight marker is on the right side.

Morphine induced changes in the level and subcellular distribution of various G-proteins was also studied by Western-blotting α-subunits of G_s, G_{i1}, G_{i2}, G_o and $G_{q/11}$. It was shown that the density of the labeling was proportional to the protein amount loaded onto the gel (not shown). The results of the analysis are expressed as percentage of the densities of appropriate antibody staining in morphine-treated vs. the saline treated (control) fractions (Fig. 5.). We detected a rapid and transient decrease of $G_{i1}\alpha$ in SPM ($73\pm13\%$, $p<0.05$) and that of G_s ($77\pm8\%$, $p<0.05$) in MI after 3-day morphine treatment (Fig. 5.A). These alterations were not

observed after 5-day morphine treatment. Significant decrease of G_s (57±3.5%, p<0.01) in M5 SPM and increase of G_{i1}, G_{i2} and G_o in M5 MI were measured (125±8.6%, 121±2.7% 152±22%, resp., p<0.05 and p<0.001 for G_{i2}) (Fig. 5.B). This tendency of increase in the MI fraction was even more pronounced after 10-day morphine treatment. In the latter, besides G_{i1}, G_{i2} and G_o (150±24%, 130±9% and 165±21%, resp., p<0.01 except G_{i1}, where p<0.05), $G_{q/11}$ levels were also elevated (175±17%, p<0.001). In parallel, the exposure level of G_o and $G_{q/11}$ was significantly decreased by 68±10% and 65±7%, (p<0.01) in M10 SPM, respectively (Fig. 5.C). Membranes from 5-day-naltrexone treated rat brains were also subjected to Western-blotting. No significant changes were detected in N5 SPM. An overall elevation, reaching statistically significant levels for G_{i1} and G_o, was found in N5 MI (Fig. 5.D).
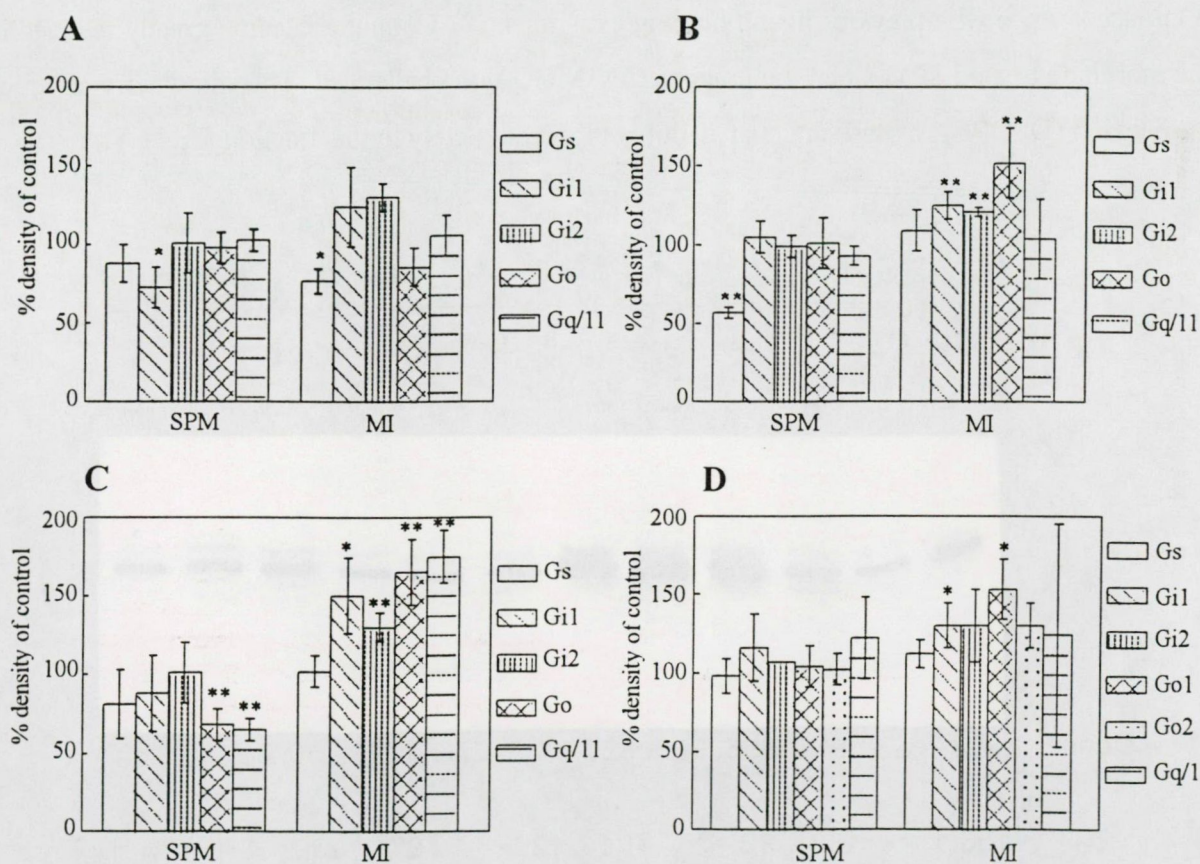

Fig. 5. Western-blotting of different G-protein α subunits by subtype specific antibodies. Samples from saline and chronic morphine or naltrexone treated rat brain synaptic plasmamembrane (SPM) and microsomal (MI) fractions were run on SDS-PAGE, blotted onto nitocellulose and incubated with the appropriate antibody. Immuno-labelled proteins were visualized by ECL chemiluminescent detection system onto Kodak X-OMAT AR film. Films were scanned and data were evaluated by ImageQuant software (Molecular Dynamics). Results are presented as percent densities of saline-treated control as 100%. A) 3 days morphine (M3); B) 5 days morphine (M5); C) 10 days morphine (M10); D) 5 days naltrexone (NX) treated membranes. Mean ± S.E.M., n = 3, significance was determined by t-test, * p< 0.05, ** p<0.01.

4.2.4 Morphine-induced changes in receptor – G-protein coupling

Possible changes in the functional coupling between opioid receptors and G-proteins were investigated by measuring opioid stimulation of photoaffinity labeling of G-proteins with [α-^{32}P]GTP azidoanilide ([α-^{32}P] AA-GTP) in subcellular fractions of saline as well as morphine treated M5 brain homogenates. Preliminary experiments were run to optimize the concentration of Na$^+$ and GDP in the reaction mixture to obtain the best ratio of basal and stimulated labeling in our system. It was found that the presence of 100 μM GDP, 150 mM NaCl gives the best signal to noise ratio, thus they were included in further experiments. It was found that both μ and δ opioid agonists can stimulate the photoincorporation of [α-^{32}P] AA-GTP into the G_α subunits in the untreated SPM, which indicates a functional coupling between the receptor and the transducer (Fig. 6.). Highly attenuated functional coupling of opioid receptors was detected in control MI (Fig. 6.) in accordance with previous ligand binding experiments[33]. Coupling became greatly reduced in the morphine treated SPM when the μ agonist DAMGO was challenged. The stimulatory effect of the δ ligand (DPDPE) was also attenuated, but not lost completely in this fraction (Fig. 6.)

Fig. 6. Photoaffinity labeling of Gα with [α-^{32}P]GTP azidoanilide in subcellular fractions of control (C) and morphine-treated (M) rat brains. DPDPE: δ peptide agonist; or DAMGO: μ peptide agonist were added prior to photolysis to check functional coupling. Autoradiograms after SDS-PAGE were evaluated by laser-densitometry, or labeled bands were excised from the dry gel and the total incorporated radioactivity was counted by liquid scintillation spectrometry. cpm: total incorporated radioactivity; %: total incorporated

radioactivity in percent compared to the basal activity (100 %) of each protein sample. Shown is a representative experiment which was repeated with similar results.

4.3 STUDIES ON G-PROTEINS AND THEIR COUPLING TO κ OPIOID RECEPTORS IN FROG BRAIN

The G-protein composition of frog brain membranes was studied by Western blotting experiments using highly subtype specific antibodies that were raised against known sequences of mammalian G-protein types that were also applied in our studies with rat brain subcellular fractions (Chapter 4.2.3). This was necessary since the G-proteins of frog brain were not previously identified, thus, no antibodies against the proteins of this species were available. Preliminary experiments resulted in the labeling of proteins with appropriate molecular weights in frog brain membranes, similar to those detected in rat brain as outlined below. The α_{common} antibody strongly reacted with proteins of about 39-41 and 44-45 kDa molecular weights. The α_i common antibody stained weakly a band at ≈44 kDa, but strongly a band at about 39 kDa, the latter also being labeled by the α_{i2} antibody. This band disappeared when the blot was incubated with the α_{i2} antiserum that was pre-incubated with an excess of the peptide, which was used to generate the antibody, thus demonstrating the specificity of the labeling. The α_s antibody gave a strong signal at 43 kDa that disappeared when the antibody was pre-incubated with α_s peptide. The affinity-purified α_o common and α_{o1} antibodies both strongly reacted with a protein of ≈40 kDa. Immunoblots were also tested for β subunits of G-proteins. The β_{common} antibody immunoreacted with a doublet of bands of about 35 kDa which resembled very much those found with the β_1 antibody (Fig. 7.).

In order to assess the functional coupling of frog brain opioid receptors to G-proteins, the ability of opioid agonists to activate G-proteins was measured with [35S]GTPγS binding assay. EKC, a prototypic κ opiate ligand which however also binds to μ and δ sites at least in rat brain[109], was the most effective among the ligands tested achieving about 60% stimulation over the basal activity at 10 μM concentration (Fig. 8.). Bremazocine, another κ-preferring ligand with cross-reactivity to μ and δ sites at high concentrations, was less efficient in activating G-proteins. The κ_1 selective U-50,488[110] displayed very similar activation to the latter; the maximal stimulation was 20-30% at the highest concentrations tested (Fig. 8.). When the κ antagonist norbinaltorphimine at 10 μM was also included, it completely blocked the stimulating effect of all three ligands tested at 1 μM, implying that their effect is due to activation of κ opioid receptors (data not shown).
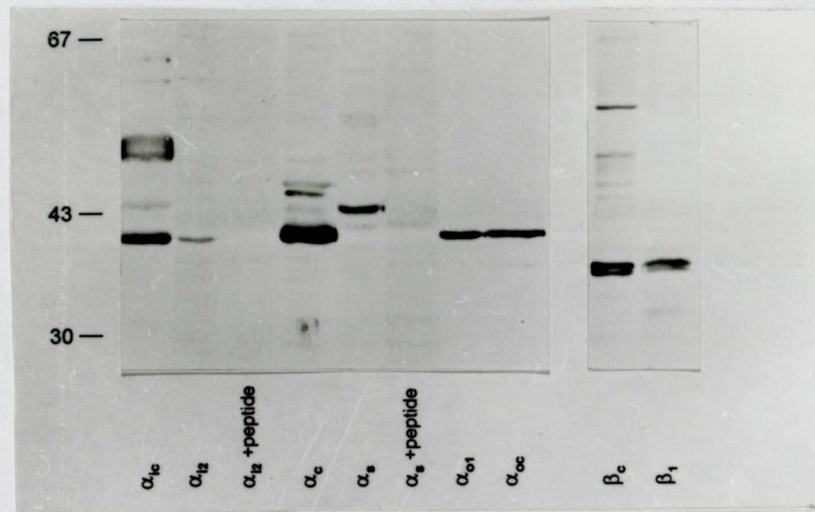
Fig. 7. Immunoblots of G-proteins in frog brain membranes. Proteins were separated on a 10 % acrylamide SDS-gel, then blotted, and subsequently stained using G-protein subunit-specific antibodies, as described in Methods. The G-protein peptide antibodies used were α_c: α_{common}-antibody, α_s: α_s-antibody, $\alpha_{i\,c}$: $\alpha_{i\,common}$-antibody , α_{i2}: α_{i2}-antibody, α_{oc}: $\alpha_{o\,common}$-antibody, α_{o1}: α_{o1}-antibody, β_c: β_{common}-antibody, and β_1: β_1-antibody. "+ peptide" designates the presence of an excess of the peptide to which the particular antibody was raised. Values on the left indicate the migration of molecular weight markers. This is a representative experiment out of 3-5 others giving similar results.
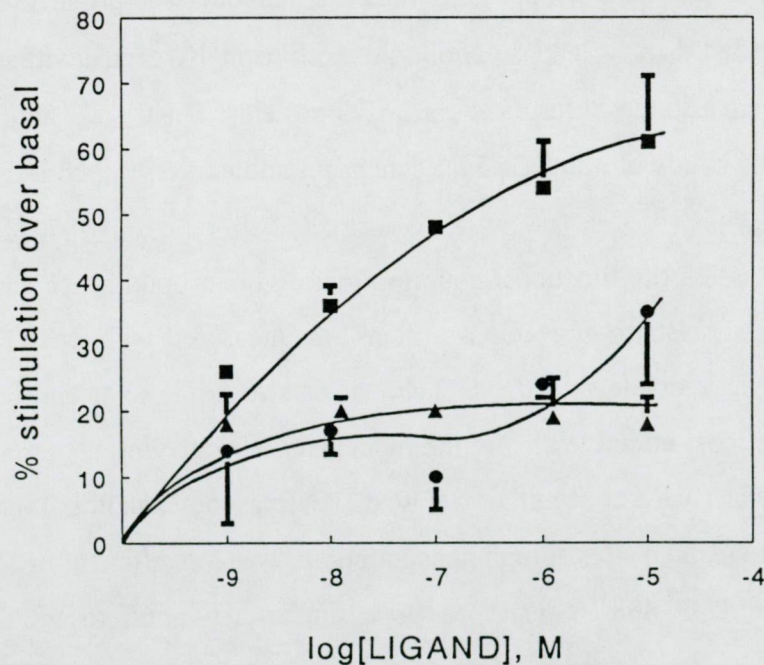

Fig. 8. Effect of opioid agonists on [^{35}S]GTPγS binding in frog brain membranes. Proteins were incubated with 0.05 nM [^{35}S]GTPγS and 10 μM GDP in Tris-EGTA pH 7.4 buffer in the absence (basal activity), or in the presence of various concentrations of EKC (■), U-50, 488 (●) or bremazocine (▲). Basal binding was 84.5 ± 7 fmol/mg protein. Data are mean ± S.E.M. of 2-6 experiments each performed in triplicate.

4.4 G-PROTEINS IN THE RAT BRAIN ENDOTHELIAL CELLS

G-protein composition of primary cultures of rat brain endothelial cell lysates and that of plasma membranes of immortalized rat brain endothelial cell lines, RBE4 and GP8 was studied with immunoblotting. Again, the specific antisera against different mammalian G-protein subtypes were used that were previously characterized in rat and frog brain membranes (Chapter 4.2.3 and 4.3). SDS-PAGE separation of proteins was performed in the presence of urea to improve resolution of the Gα subunits of closely similar molecular weights (39-43 kDa)[89,105]. Basically, all the Gα subunits tested were present in the three types of cerebral endothelial cells (CECs) studied with only slight quantitative differences (Fig. 9.).
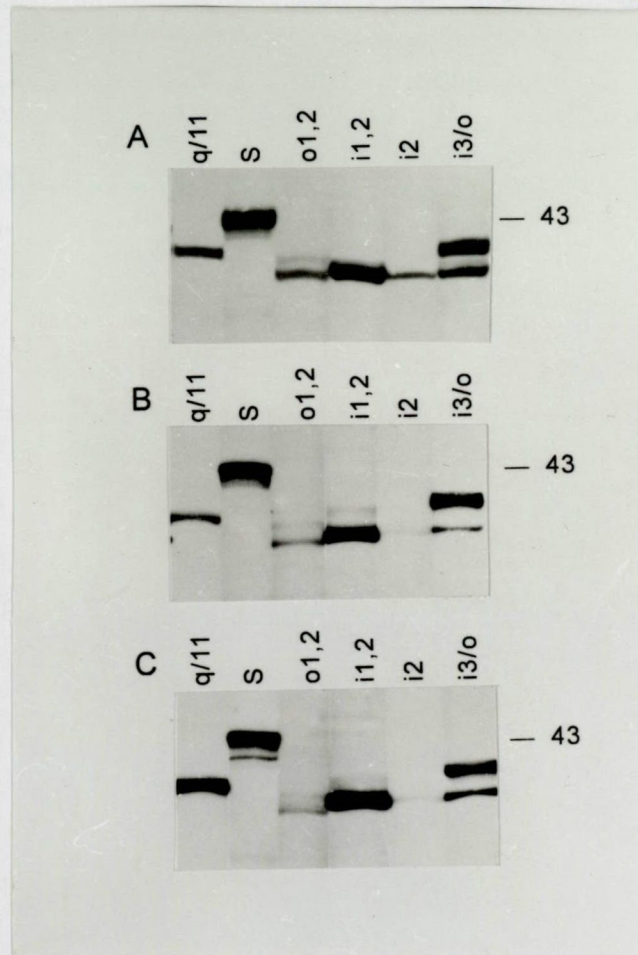

Fig. 9. Immunoblot analysis of G-protein α subunits in primary cultured rat brain endothelial cell lysates (A), or crude membranes of RBE4 (B) and GP8 (C) cell lines. Samples were run on SDS-PAGE containing urea and transferred onto nitrocellulose membranes. Immunoblotting was performed with specific anti-peptide antisera which recognise G-protein α subunits indicated on the top of each lane. Filter-bound antibodies were detected by ECL system. Molecular weights in kDa are indicated on the right.

Antiserum AS 369 (anti-$G_{q11}\alpha$) detected a protein band which, however, was in many experiments resolved into two distinct bands. Even in cases where the resolution was not visible, the densitometric analysis of the films revealed the presence of two peaks very close to each other. Heavy staining of proteins of 43 kDa was seen with antiserum RM/1 (anti-$G_s\alpha$). A minor band of about 42 kDa was also detected mainly in GP8 cell membranes (Fig. 9, panel C). This latter protein was sometimes also seen in the other two CECs tested. Antiserum GC/2 (anti-$G_o\alpha$) detected a faint band at approximately 41 kDa, and a more intense staining at about 39 kDa. Antiserum AS/7 (anti-$G_{i1}\alpha$ and $G_{i2}\alpha$) recognized one bulky band which comigrated with the $G_{i2}\alpha$ labeled band obtained by incubation of the membranes with AS 269 (anti-$G_{i2}\alpha$). A faint band of approx. 42 kDa, what might correspond to $G_{i1}\alpha$ was also detected with AS7 in RBE4 and GP8 cell membranes (Fig. 9.B and C) but was not seen in lysates of primary cell cultures (Fig. 9.A). AS 86 (preferentially anti-$G_{i3}\alpha$) detected 2 bands of which the upper band might be $G_{i3}\alpha$ and the lower one $G_o\alpha$ based on the known cross-reactivity of AS 86 with $G_o\alpha$[91,111], and also the comigration of this band with the lower band of the GC/2 (anti-$G_o\alpha$) detected bands (Fig. 9.).

The Gβ-specific antiserum AS 11 detected a doublet with molecular weights of 40 and 41 kDa in the tissues tested (data not shown) that are somewhat higher than the values obtained in most tissues, i.e. 35 resp.36 kDa[89], and might be due to the presence of urea in the gel.

V. DISCUSSION

In this work we studied G-proteins of brain origin with different techniques to understand some of their roles first of all in the function of the brain opioid receptors.

First we analyzed the opioid receptor – G-protein interaction by analyzing the binding parameters of the receptor. Ligand binding studies performed at different temperatures can be used to get thermodynamical parameters what provide information about the underlying chemical forces of the interaction.

The entropy increase observed when the μ ligand DHM was displacing [^{3}H]NX (Table 4.) might indicate hydrophobic interactions, and since it is present in all cases (also in homologous displacement experiments with subtype specific ligands such as [^{3}H]DAMGO and [^{3}H]DHM for μ and [^{3}H]DT-B for δ opioid receptors as well as a general antagonist, [^{3}H]NX [I.]), it might be one of the first general steps of the ligand binding, when ordered water molecules on the surface of the ligand and the receptors have to be removed[113,114]. Another source of the entropy increase might be a conformational change of the receptor upon ligand binding. Theories based on point mutation experiments of the cloned β2-adrenergic receptor resulting in constitutively active receptor mutants[115,116] propose a model, in which the native receptor is conformationally

constrained in the unliganded, inactive state and hormone binding drives it into the activated, "relaxed" form, in which the third cytoplasmic loop becomes available for G-protein activation. This interaction requires the presence of Na^-, Mg^{2-} and guanine nucleotides[64.65] and results in a conformational change and/or rearrangement of the membrane components.

Na^- has been suggested to affect agonist binding by directly interacting with receptors to cause G-protein uncoupling, thereby converting the receptor into a low affinity state for agonists[117]. Mg^{2-} plays multiple roles in the course of receptor function acting on both the receptor and the G-protein modifying their function and interaction[118]. In the extension of the ternary complex model Samama et al. suggested a direct role for these ions to regulate the receptor ability to isomerize between the active and inactive form[119]. In our experiments monovalent and divalent cations acted similarly to these, i.e. sodium decreased agonist affinity, but increased that of antagonist; and Mg^{2-} increased agonist affinity primarily in case of DT-B at higher temperatures [I.]. Nevertheless, Na^- or Mg^{2-} results only in quantitative changes of $\Delta S^{o\prime}$ and $\Delta H^{o\prime}$ values.

Electrostatic interactions of the ligand with complementary polar residues of the receptor would lead to negative or slightly positive values of $\Delta H^{o\prime}$. While the former is true for [^{3}H]NX binding in homologous displacement experiments, the latter is the case for all the agonists (^{3}H-DHM, ^{3}H-DAMGO, ^{3}H-DT-B) tested in homologous displacement experiments[112]. Importance of charged residues in TM II, III and VI for different functions are being delineated for opioid receptors[54].

The presence of Gpp(NH)p in heterologous displacement experiments (DHM competing with [^{3}H]NX) resulted in curvilinear van't Hoff plots (Fig. 2.). We fitted the data without the lnK_a value obtained at the lowest temperature and this also gave better fit for polynomial than for linear regression. The simultaneous presence of Gpp(NH)p, Na^- and Mg^{2-} increased the enthalpy more than 3 times compared to the control value (66.62 vs. 17.80 kJxmol^{-1}), and at least doubled compared to the presence of a single regulator (Table 4.). Entropy likewise increased from 246 to 352 Jxmol^{-1}xK^{-1}. We suggest that these alterations are the consequences of the molecular rearrangement in the membrane due to uncoupling of the receptor-ligand complex from G-proteins which results in "free" protein molecules. This hypothesis is strengthened by the low affinity of μ-receptor agonist binding measured in this case (275 nM vs. 5.86 nM of the control, Table 4.) indicating the utilization of the binding energy of receptor-ligand to drive G-proteins to the open form, thus weakening the observed binding of the receptor for the ligand.

Physicochemical interpretations of thermodynamic parameters are more or less speculative

for such a complex system, however, some molecular events, such as molecular interactions discussed above can be postulated.

Our further studies on the μ opioid receptor signal transduction aimed to reveal contribution of altered receptor and G-protein function to the manifestation of morphine tolerance and dependence. The availability of membrane fractions originating from the cell surface (SPM) and from 'light vesicles' or microsomes (MI), the latter highly enriched in endoplasmic reticulum and Golgi membranes as well as endosomes, allowed us to study the subcellular distribution of μOR and their cognate G-proteins in opioid naive and morphine-tolerant animals. The fractionation protocol and detailed characterization of the fractions by marker enzymes, electron microscopy and receptor binding experiments was published[33,99,120,121].

Consistent with earlier reports[121], comparable B_{max} values were measured with [^{3}H]DAMGO in control SPM and MI membranes (Fig. 3.). Upon 5-day morphine treatment, the receptor number did not change in the SPM implying the lack of internalization of the surface μOR. In parallel, however, the μ-sites were up-regulated by 68% in MI. When morphine treatment was performed with a higher dose for a longer period (10 days), the number of both surface and intracellular μ binding sites was elevated (Fig. 3.).

Mechanisms of receptor up-regulation include changes in receptor posttranslational modifications, compartmentalization, or turnover. Chronic morphine treatment may enhance the processing of latent or precursor receptors, or it may induce new receptor synthesis, probably from existing pool of mRNA, since literature data reveal no alterations in the mRNA levels of μOR either after opioid agonist or antagonist treatment[122,123]. We do not have direct evidence for such effect of morphine, since our attempt to block the protein synthesis *in vivo* by co-administration of cycloheximid had failed. Although this chemical is widely used in cell culture studies to inhibit mRNA translation on the ribosomes, and as a consequence, prevent protein synthesis, the smallest dose given *in vivo* was toxic for the rats. However, the following observations greatly agree with the possible role of morphine in the regulation of gene expression and protein synthesis. It has been showed before that newly synthesized μOR that are highly enriched in the microsomes of neonatal brain display enhanced coupling to G-proteins compared to their adult counterparts[33]. These data were well supported by autoradiographic studies that showed that while newly synthesized receptors that are in transit from the soma toward the nerve terminals are GTP-sensitive, the internalized opioid receptors undergo retrograde axoplasmic flow and they are GTP-insensitive (29 and references cited therein). Our results found in M5 MI, namely, increased receptor and G-protein density and enhanced coupling between them can be the consequence of protein synthesis. Up-regulation

of intracellular nicotinic acethylcholine receptors (nAChR) was also reported after chronic nicotine (another abusive drug) treatment in primary cultures of fetal rat brain. It was suggested to entail a nicotine-stimulated conversion of the low-affinity reserve pool of nAChR into a high-affinity conformer[124,125].

It is well documented, that not only receptors are involved in the development of drug addiction but G-proteins[77,82,126], as well as of various elements of the signal transduction pathway, such as adenylyl cyclase[83,84], protein kinase-C (PKC)[85], G-protein coupled receptor kinase[86], and protein phosphatases[87] are also affected. Although most emphasis has been placed on analysis of the internalization and redistribution of GPCRs, it has also been recognized that sustained agonist treatment of cells can result in alterations in both the cellular distribution and levels of G-proteins activated by the relevant GPCR[126]. The possible alteration of G_i/G_o proteins, which are selectively labeled by PTX and are known to interact with the opioid receptors[42,43, II.], were assessed after PTX catalyzed ADP-rybosilation in subcellular fractions of rat brain. Most notable was an 89% up-regulation of G_i/G_o proteins noted in the microsomes of morphine-tolerant animals (Fig. 4.).

Results of the immunoblotting experiments also showed increases in the amount of G_i and G_o in M5 and M10 MI (Fig. 5.). A transient decrease of G_{i1} in SPM with simultaneous increase (statistically not significant) of the same protein in MI also occurred after 3 days of morphine treatment. Nonetheless, translocation can not be the single source of G-protein increase in MI, since in M5 membranes only microsomal increase was detected for G_{i1}, G_{i2} and G_o without decrease of the same proteins in SPM. Even in M10 MI the increase was larger than the decrease in the SPM. There was no sign of down-regulation of G_i and G_o in rat brain. Reduced amount of G_o and G_{q11} was detected in M10 SPM, while elevated levels of the same proteins in MI were also noted (although $G_{q/11}\alpha$ is not known to directly interact with opioid receptors). We speculate that it can be explained by internalization of the surface G-proteins. Svoboda et al.[128] detected translocation of $G_{11}\alpha$ subunits from the plasma membrane into the low density membranes and cytosol fractions of transfected HEK293 cells upon chronic exposure to thyrotropin-releasing hormon (TRH). Similarly, redistribution of G_q and G_{11} was induced by stimulation of the muscarinic m1 acetylcholine receptors in Chinese hamster ovary (CHO) cell line[129].

In the case of the stimulatory G-protein G_s, that was shown to play a particular role in morphine tolerance in the peripheral nervous system (130 and references cited therein) not only internalization, but down-regulation might take a place (Fig. 5). The total amount (surface + intracellular) of this subtype tended to be less in M3 and M5 fractions than in membranes from saline treated brains. During longer drug administration that phenomenon seems to be

compensated as in M10 membranes no significant reduction of G_s was measured.

The distinct pattern of changes of G-protein subtypes that were detected after 3, 5 and 10 days of morphine administration (Fig. 5.) might represent different stages of the cellular adaptation to the continuous presence of the drug and might reflect different roles of the G-protein subtypes in this process. Our data fit into the scheme of drug regulation of neuronal gene expression suggested by Nestler[69,70], where one main group of genes targeted by the drug effect is that encoding G-proteins. The altered gene expression of several components of the cell signaling system, resulting in tolerance and addiction, is part of the adaptation processes to compensate the impact of agonist exposure[131].

Functional coupling of μ- and δ-opioid receptors to G-proteins in subcellular fractions of rat brain before and after M5 treatment was also examined by measuring the ability of DAMGO (μ) or DPDPE (δ) ligands added to membrane fractions *in vitro* to stimulate photoaffinity labeling of G-proteins. After chronic morphine treatment DAMGO had completely lost its stimulating effect in M5 SPM indicating functional uncoupling of opioid receptors from G-proteins, while DPDPE had reduced effect compared to control (Fig. 6). The influence of morphine on both μ and δ opioid receptor coupling to G-proteins might be its well-known cross-reactivity between μ and δ opioid receptors, or a cross-talk between the signal transduction pathways[175-177]. In contrast, in M5 MI there was a tendency of increased stimulation by opioid ligands compared to the saline treated MI indicating enhanced interaction between opioid receptors and G-proteins. This might reflect an increased number of μ-binding sites and/or G-proteins which is in a good agreement with the results of the ligand binding (Fig. 3-5). It is worth mentioning that the two different analysis of photoaffinity labeling experiments, namely, densitometry of the autoradiograms and scintillation counting of the incorporated radioactivity into the labeled proteins excised from the gel gave very similar results (Fig. 6).

Since it is well documented that chronic administration of opioid receptor antagonists produces an up-regulation that might be accompanied by increased coupling to G-proteins[121,132], another possibility, namely that morphine behaves like a partial antagonist in our system should also be taken into account. In agreement with this, Sternini et al.[134] showed that morphine partially inhibited the etorphine-induced μOR rapid endocytosis in neurons. However, it does not explain our observation that only the intracellular sites were affected by shorter morphine exposure, but not the surface one (Fig. 3.). Also, there were some distinct patterns of changes when we tested the effect of chronic naltrexone treatment in our system. In agreement with literature data, [³H]DAMGO binding sites on the cell surface were up-regulated by 73% after 5-

day-naltrexone treatment (N5). When membranes were also exposed to PTX, there was no change in the intensity of labeling in N5 SPM, while 58% increase was noted in the N5 MI (data not shown). In photoaffinity labeling experiments N5 treatment caused no change in the stimulating effect of DAMGO (119±2.5 %) and DPDPE (123±12 %) while these ligands had reduced capability to stimulate G-protein labeling after M5 treatment.

The need for novel, strong analgesics free of abuse potential and side effects of morphine led to the intense research of non-μ opioid sites, in particular κ opioid receptors. The difficulty of the investigation of endogenously expressed κ receptors due to their relatively low abundance in mammalian brain was overcame by using frog brain membrane preparations, what is a rich source of κ opioid receptors[98,134]. Next part of this study characterizes the G-proteins present in frog brain membranes, and furthermore their activation by κ opioid agonists. In order to typify the G-proteins we utilized antibodies raised against peptides with either common or subtype-specific sequences of given G-protein subunits deduced from mammalian G-protein sequences as published previously[89,134-139], since no sequence data of G-proteins in *Rana esculenta* have been available. Thus, the reactivity and subtype selectivity of the peptide antibodies had to be examined in frog. The immunolabeling pattern with α_{common} and α_o were compared in rat and frog brain tissues in preliminary experiments. It was concluded that these antisera were able to recognize appropriate G-protein subunits in frog brain with identical or slightly different molecular weights than in rat brain membranes (data not shown). Published data concerning the G-protein composition in amphibian tissues such as *Xenopus laevis* oocytes[140], frog skeletal muscle[25], rod photoreceptors[141], and even in neuronal tissues of different vertebrates including frogs[142-144] have indicated a high degree of homology of the G-proteins of these tissues with their mammalian counterparts. Our results also agree with the above notions as outlined below.

The G-protein composition of frog brain membranes was further elucidated by using more specific antisera (Fig. 7.). Our conclusions concerning the identity of a given immunostained protein band were based on: (i) its comigration with proteins which were reactive to other peptide antibodies in the absence and in the presence of 6 M urea in the separating gels; and that its mobility was in accordance with the molecular range labeled (ii) by [α-^{32}P]GTP azidoanilide; or (iii) pertussis toxin-sensitive proteins. (This latter requirement obviously was not applicable to proteins immunoreactive to α_s or β antibodies.)

On the basis of our results one might speculate that β subunits emerge as two different isoforms of β_1 (Fig. 7.), as the comigration of β_1- and β_{common}-immunoreactive proteins and the absence of β_2-reactive material (not shown) would suggest. The two G_s α forms shown by

peptide-reversible anti-α_s immunostaining after running on a urea-containing gel were also reactive to the α_{common} antibody (not shown) that recognized α_i, α_o, α_s, α_t, and to a lesser extent α_z in mammalian cells[136]. Testing for α_i subunits led to the detection of an α_{i2}-like protein of about 39 kDa which was immunostained with the α_{i2} affinity purified antibody and also coincided with the appropriate anti-α_i common reactive band. The α_i common peptide antibody which recognized the α_{i1}, α_{i2}, and α_{i3} proteins[136] did not however show immunolabeling in the region where α_{i1} would be expected under the experimental conditions in frog brain (Fig. 7.). This result was confirmed with an antibody specific for α_{i1} which also showed no labeling in frog (data not shown). The other, higher molecular weight band stained by the α_i common antibody (Fig. 7.) might correspond to α_{i3} which protein was also detected in frog skeletal muscle[89]. The affinity-purified α_{o1} recognized a protein band at 40 kDa that was also seen with the α_o common antiserum (Fig. 7). An antibody specific for α_{o2} showed no detectable signal (data not shown). These observations suggest that there is only one type of G_o protein in frog brain, and that G_{o1} seems to play a role in κ opioid receptor signaling in frog brain.

Evaluation of agonists stimulation of [^{35}S]GTPγS binding offers an opportunity to study the direct coupling of a receptor to the activation of G-proteins regardless of the types of G-proteins and effector systems involved. In this respect it is similar to agonist stimulation of photolabeling by [α-^{32}P]AA-GTP or the high K_m GTPase activity measurements. Previous literature data provided evidence for the coupling of kappa (besides μ and δ) opioid receptors to rat brain GTPase[145]. In frog brain membranes, κ opioid ligands resulted in a concentration-dependent stimulation of [^{35}S]GTPγS binding (Fig. 8.) which was fully inhibited by the κ-specific antagonist norbinaltorphimine. The rank order of potency was EKC > U-50,488 $\approx$ bremazocine. The EC$_{50}$ values defined as the concentration of the ligand producing 50% of the maximal response was in the nanomolar range for all three agonists tested (Fig. 8.) which agrees well with the equilibrium dissociation constants of these ligands obtained in receptor binding experiments[134,146-151]. While U-50,488 is considered to be a selective ligand for κ_1 opioid receptors[110] that represent only 20-30% of the kappa receptor pool in frog brain[134,142], the benzomorphan EKC and bremazocine bind to both κ_1 and κ_2 subtypes[152] besides their well-documented cross-reactivity with μ and δ sites[109]. Although the potency of the latter two ligands is similar in most mammalian tissues, this is not necessarily the case in frog brain. Previous experiments revealed unique characteristics of opioid sites of frog brain, among them the antagonistic like binding pattern of these two ligands in vitro[146,153,154], and the ability of EKC to antagonize morphine-induced antinociception in vivo[155]. The observation that EKC was more potent than bremazocine in stimulating [^{35}S]GTPγS binding will require future work,

nevertheless it agrees with recent results of Benyhe et al.[149], who studied the binding of Met-enkephalin-Arg^6-Phe^7, the proposed endogenous ligand of the κ_2 receptors[152]. In their study bremazocine and EKC displaced about 50% and 80% of the specific binding of the radioligand in frog brain membranes, respectively[149]. The potency of Met-enkephalin-Arg^6-Phe^7 has also been evaluated in [^{35}S]GTPγS binding. This full agonist of the κ_2 sites displayed about 120% stimulation over the basal activity at 1 μM concentration[156]. The main conclusion that can be drawn from [^{35}S]GTPγS binding experiments is that kappa receptors including κ_1 and κ_2 subtypes do interact with G-proteins in frog brain. This observation is also supported by previous ligand binding experiments where the binding of [^{3}H]EKC, [^{3}H]dihydromorphine, [^{3}H]etorphine and [^{3}H]U-50,488 was shown to be regulated by guanine nucleotides[142,150,153].

Further experiments by photolabeling of G-protein α subunits by [α-^{32}P]GTP azidoanilide in the absence and presence of opioid agonists identified multiple bands in the molecular weight range of 39-42 kDa. The 39 kDa band which showed very faint labeling comigrated with the protein specifically labeled with the α_{i2} antibody. The 40 kDa band was identified as α_{o1}. The other two bands were also stained with the α_{common} antibody, but were not further identified. Photoincorporation of the label was slightly but significantly stimulated by EKC and U-50,488 into three bands, including proteins identified as α_{o1}, α_{i2}, and the unknown α-subunit with higher molecular weight. Based on the observation that the kappa ligands enhanced photolabeling of multiple proteins by AA-GTP it seems likely that multiple types of G-proteins are able to interact with κ sites in frog brain. Likewise, this was shown to be the case for the cloned rat κ receptors[172,173] and for the δ opioid receptors in NxG 108-15 cells[42], as well as for μ and δ receptors in human neuroblastoma SH-SY5Y cells[43]. Thus multiple types of G-proteins might seem to couple to all three opioid receptors.

The acute and chronic effects of opioid drugs are fundamentally determined by the concentration of the compound in the brain. P-glycoprotein is a transmembrane protein expressed by multiple mammalian cell types, including the endothelial cells that comprise the blood-brain-barrier[178]. P-glycoprotein functions to actively pump a diverse array of xenobiotics out of the cells in which it is expressed. P-glycoprotein acts to limit the entry of some opiates into the brain and that acute administration of P-glycoprotein inhibitors can increase the sensitivity to these opiates, so the BBB can differentially regulate the exchange of related substances between the CNS and blood[179]. In this part of this work we identified the G-proteins that are present in cerebral endothelial cells and might contribute to their specific function.

Highly purified primary cultures of CECs were studied to avoid possible astrocytic and pericytic contamination[157]. By using specific antibodies raised against various G-protein subtypes and immunoblot techniques, we were able to demonstrate the presence of a complex set of G-proteins such as $G_s\alpha$, $G_{i2}\alpha$, $G_{i3}\alpha$, $G_{q/11}\alpha$, $G_o\alpha$ and $G\beta$ in CECs. The same proteins were also present in the two immortalized cerebral endothelial cell lines tested, i.e. RBE4 and GP8 which showed similar morphological and functional characteristics to primary CECs[96,97].

The presence of the principal stimulatory G-protein, $G_s\alpha$ in CECs shown in our study (Fig. 9.) may constitute an important element in coupling different 7TM receptor proteins to intracellular signaling pathways. Previously certain 7TM receptors, known to be coupled to $G_s\alpha$ in other systems, like β-adrenoceptors[158], histamine H-2 receptors[159], and dopamine D-1 receptors[160], have been shown to be expressed by the cerebral endothelium. Activation of these receptors may lead to increased intracellular cAMP levels. Increased endothelial cAMP levels in turn have been shown to regulate BBB permeability *in vivo*[161] and *in vitro*[162,163], and to inhibit endothelial cell proliferation[164].

Inhibitory G-proteins have also been detected on the cerebral endothelium (Fig. 9.). To our knowledge this is the first report describing the presence of $G_o\alpha$ proteins in cells of endothelial origin. This might be characteristic to brain endothelial cells since no detectable amount of $G_o\alpha$ was expressed in peripheral endothelial cells[165], and might support the highly specialized function of brain endothelial cells, namely, the maintenance of BBB. Previous studies have demonstrated the expression of different G_i types ($G_{i2}\alpha$, $G_{i3}\alpha$) in peripheral endothelial cells with the predominance of $G_{i2}\alpha$, and their role in stimulating endothelial cell proliferation and angiogenesis[164,166]. In this respect the CECs have similar characteristics expressing significant amount of $G_{i2}\alpha$ and $G_{i3}\alpha$ (Fig. 9.). Activation of these G-proteins have been shown to decrease intracellular cAMP levels and activate K^+ channels in other systems.

G-proteins possibly coupling membrane receptors to phospholipase-C (PLC) turned out to be present in CECs as well (Fig. 9.). Similarly to its demonstrated role in peripheral endothelium, $G_{q/11}\alpha$ may be involved in mediating the effect of potent vasoactive substances like bradykinin[167], histamine[168], endothelin[169], thrombin[170] and fibrin[171]. Moreover, G-proteins may be involved in the regulation of ion channels as well.

To our knowledge this was the first study to identify the G-protein types in cerebral endothelial cells.

VI. CONCLUSIONS

In this work we presented qualitative and quantitative investigation of heterotrimeric G-proteins of brain origin. Our laboratory was among the first in Hungary introducing ligand stimulated [^{35}S]GTP-γ-S binding and photoaffinity labeling experiments to achieve that. From immunoblotting experiments it can be concluded that the structure of most heterotrimeric G-protein types is well preserved during phylogenesis, since antibodies against mammalian G-proteins could recognize frog, as well as rat G-proteins. G-proteins are expressed ubiquitously in the tissues examined, namely, rat brain subcellular fractions, rat brain endothelial cells and frog brain membranes. Recently strong emphasis was made on functional studies (such as ligand stimulated [^{35}S]GTPγS binding, photoaffinity labeling by [α-^{32}P]AA-GTP, GTPase or adenylyl-cyclase activity assays), because receptor binding experiments only characterize one aspect of the receptors (ligand binding), but not transfer of signal to elicit function. These methods use different assay conditions (GDP and Na$^+$ content, incubation time, which is for example 3 minutes versus 1 hour in photoaffinity labeling and [^{35}S]GTPγS binding, respectively). So, they might have different sensitivity towards G-protein function and photoaffinity labeling due to the short incubation time might detect differences in the fine tuning of the kinetics of G-protein activation, what might be masked during longer incubation time. Also the detection of incorporated radioactivity is different, autoradiography after affinity labeling versus liquid scintillation counting after [^{35}S]GTPγS binding and GTPase assay. However, when these two different analysis were applied for the same experiment, i.e. photoaffinity labeling, densitometry of the autoradiograms and scintillation counting of the incorporated radioactivity into the labeled proteins excised from the gel gave very similar results (Fig. 6.).

The most important findings of this work are summarized below:

1. Thermodynamical analysis of the agonist binding of the μ opioid receptor can reveal receptor-G-protein coupling in the presence of GppNHp, Na$^+$ and Mg^{2+}.

2. *In vivo* chronic morphine treatment increases the density of intracellular μ opioid binding sites and does not cause down-regulation of the receptor.

3. *In vivo* co-administration of cycloheximide, the protein synthesis inhibitor, with morphine was lethal to the animals. So, the source of μ receptor up-regulation (*de novo* synthesis or activation of spare receptors) could not be defined.

4. Density and intracellular localization of G-proteins are also affected by *in vivo* chronic morphine treatment. Alterations in the amount of the different G-protein subtypes are dependent on the length of the treatment (3, 5, or 10 days).

5. Various G-protein subtypes are differently regulated by *in vivo* chronic morphine treatment.

6. The functional coupling of the μ opioid receptor to G-proteins has been changed due to *in vivo* chronic morphine treatment. Increased coupling was detected at intracellular μ sites.

7. *In vivo* chronic naltrexone treatment resulted in different pattern of G-protein alteration than morphine did and it did not alter the receptor-G-protein coupling.

8. κ opioid receptors and G-proteins interact *in situ*, i.e. within the native plasma membrane as revealed by opioid stimulated [^{35}S]GTPγS binding.

9. There are multiple G-proteins activated by κ opioid ligands in frog brain .

10. $G_o\alpha$ proteins are present in cells of endothelial origin. This might be characteristic to brain endothelial cells since no detectable amount of $G_o\alpha$ was expressed in peripheral endothelial cells, and might support the highly specialized function of brain endothelial cells, namely, the maintenance of BBB.

VII. ACKNOWLEDGEMENT

VIII. ABBREVIATIONS

7TM receptors, 7 transmembrane domain containing receptors

$[\alpha^{-32}P]$ AA-GTP, $[\alpha^{-32}P]$GTP azidoanilide

BBB, blood-brain barrier

cAMP, cyclic adenosine monophosphate

CECs, cerebral endothelial cells

CNS, central nervous system

CRE, cAMP response element

CREB, cAMP response element binding protein

$\Delta G°'$, Gibb's free energy

$\Delta H°'$, standard free enthalpy

$\Delta S°'$, standard free enthropy

DAMGO, Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol

DHM, dihydromorphine

DNA, deoxyribonucleic acid

DPDPE, [D-Pen^2,D-Pen^5]enkephalin

DT-B, deltorphin-B (Tyr-D-Ala-Phe-Glu-Val-Val-Gly-NH$_2$)

DTT, DL-dithiothreitol

EKC, ethylketocyclazocine

GAPs, GTPase activating proteins

GDP, guanosine 5'-diphosphate

G-protein, heterotrimeric guanine nucleotide binding regulatory protein

GPCRs, G-protein-coupled receptors

GppNHp, 5'-guanylylimidodiphosphate

GTP, guanosine 5' triphosphate

GTPγS, guanosine-5'-O-(3-thio)triphosphate

MAPK, mitogen-activated protein kinase

$[\alpha^{-32}P]$NAD, $[\alpha^{-32}P]$nicotinamide adenine dinucleotide

NX, naloxone

PKA, cAMP-dependent protein kinase

PLC, phospholipase C

PMSF, phenylmethylsulphonyl fluoride

PTX, pertussis toxin

RGS, regulators for G-protein signaling

SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis

U-50,488, trans-(±)-3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl)benzene-acetamide

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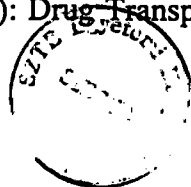
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