Kynurenic acid and it's amide analogue might be possible drug candidates for controlling the activity of opioid system

Ph.D. thesis

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LIST OF THE PUBLICATIONS

This thesis is based on the following publications:

I. Ferenc Zádor, ***Reza Samavati***, Eszter Szlávicz, Bernadett Tuka, Engin Bojnik, Ferenc Fülöp, József Toldi, László Vécsei, Anna Borsodi. **Inhibition of opioid receptor mediated G-protein activity after chronic administration of kynurenic acid and its derivative without direct binding to opioid receptors.** CNS Neurol Disord Drug Targets. 2014;13(9):1520-9. (Impact factor: 2.628)

II. ***Reza Samavati***, Ferenc Zádor, Edina Szűcs, Bernadett Tuka, Diána Martos, Gábor Veres, Róbert Gáspár, István Mándity, Ferenc Fülöp, László Vécsei, Sándor Benyhe, Anna Borsodi. **Kynurenic acid and its analogue can alter the opioid receptor G-protein signaling after acute treatment via NMDA receptor in rat cortex and striatum.** Journal of the Neurological Sciences. doi: 10.1016/j.jns.2017.02.053 (Impact factor: 2.128)

Other publications unrelated to this thesis:

I. Adriano Mollica, Roberto Costante, Ferenc Zádor, ***Reza Samavati***, Borsodi Anna, Benyhe Sandor, Azzurra Stefanucci, Irina Vetter, Richard J. Lewis, Stefano Pieretti. **Design, characterization and biological evaluation of novel multi-target compounds with opioid agonist and *N*-type voltage-sensitive calcium-channel blocking activity.** Chem Biol Drug Des. 2014 Nov 13. (IF: 2.507)

II. Adriano Mollica, Alfonso Carotenuto, Ettore Novellino, Antonio Limatola, Roberto Costante, Francesco Pinnen, Azzurra Stefanucci, Stefano Pieretti, Ferenc Zádor, ***Reza Samavati***, Anna Borsodi, Engin Bojnik, Sándor Benyhe, Peg Davis, Frank Porreca, Victor J. Hruby **Development of potent opioid peptides: synthesis, biological evaluation and conformational properties of two new cyclic biphalin analogues.** ACS Med Chem Lett. 2014 Jul 14;5(9):1032-6. (IF: 3.703)

III. Zádor F, Lénárt N, Csibrány B, Sántha M, Molnár M, Tuka B, ***Samavati R***, Klivényi P, Vécsei L, Marton A, Vizler C, Nagy GM, Borsodi A, Benyhe S, Páldy E.. **Low dosage of rimonabant leads to anxiolytic-like behavior via inhibiting expression levels and G-protein activity of kappa opioid receptors in a cannabinoid receptor independent manner.** Neuropharmacology. 2014 Oct 16;89C:298-307. (IF: 4.936)

IV. Judit Bóta, Judit Hajagos-Tóth, Eszter Ducza, ***Reza Samavati***, Anna Borsodi, Sándor Benyhe, Róbert Gáspár. **The effects of female sexual hormones on the expression and function of α1A- and α1D-adrenoceptor subtypes in the late-pregnant rat myometrium.** DOI: 10.1016/j.ejphar.2015.11.015. (IF: 2.730)

V. Hajagos-Toth J, Bota J, Ducza E, Csanyi A, Tiszai Z, Borsodi A, ***Samavati R***, Benyhe S, Gaspar R. **The effects of estrogen on the α2-adrenergic receptor subtypes in rat uterine function in late pregnancy in vitro.** Croat Med J. 2016 Apr 23;57(2):100-9. (IF: 1.483)

VI. Monti L, Stefanucci A, Pieretti S, Marzoli F, Fidanza L, Mollica A, Mirzaie S, Carradori S, De Petrocellis L, Schiano Moriello A, Benyhe S, Zádor F, Szűcs E, Ötvös F, Erdei A, ***Samavati R***, Dvorácskó S, Tömböly C, Novellino E. **Evaluation of the analgesic effect of 4-anilidopiperidine scaffold containing ureas and carbamates.** J Enzyme Inhib Med Chem. 2016 Apr 11:1-10. (IF: 3.428)

VII. Hajagos-Tóth J, Bóta J, Ducza E, ***Samavati R***, Borsodi A, Benyhe S, Gáspár R. **The effects of progesterone on the alpha2-adrenergic receptor subtypes in late-pregnant uterine contractions in vitro**. Reprod Biol Endocrinol. 2016 Jun 14;14(1):33. (IF: 2.147)

VIII. Mollica A, Pelliccia S, Famiglini V, Stefanucci A, Macedonio G, Chiavaroli A, Orlando G, Brunetti L, Ferrante C, Pieretti S, Novellino E, Benyhe S, Zador F, Erdei A, Szucs E, ***Samavati R***, Dvrorasko S, Tomboly C, Ragno R, Patsilinakos A, Silvestri R. **Exploring the first Rimonabant analog-opioid peptide hybrid compound, as bivalent ligand for CB1 and opioid receptors**. J Enzyme Inhib Med Chem. 2017 Dec;32(1):444-451. (IF: 3.428)

IX. Hajagos-Tóth J, Ducza E, ***Samavati R***, Vari SG, Gaspar R. **Obesity in pregnancy: a novel concept on the roles of adipokines in uterine contractility**. Croat Med J 58:(2) pp. 96-104. (2017). (IF: 1.483)

X. Szűcs KF, Grosz G, Süle M, Nagy A, Tiszai Z, ***Samavati R***, Gáspár R. **Identification of myoelectric signals of pregnant rat uterus: new method to detect myometrial contraction**. Croat Med J 58:(2) pp. 141-148. (2017). (IF: 1.483)

Total impact factor: 32.084

LIST OF ABREVIATIONS

3-NLT …….....3-nitro-L-tyrosine

AC ……Adenylyl cyclase

BBB …….Blood-brain barrier

CHO ……....Chinese hamster ovary cell line

CHO-DOPr …….....Chinese hamster ovary cell line overexpressed with delta opioid peptide receptor

CHO-KOPr ……Chinese hamster ovary cell line overexpressed with kappa opioid peptide receptor

CHO-MOPr ……...Chinese hamster ovary cell line overexpressed with mu opioid peptide receptor

CNS …….Central nerve system

CSF ……..Cerebrospinal fluid

DAMGO ……..Tyr-D-Ala- Gly-(NMe)Phe-Gly-ol

DOPr ……δ opioid peptide receptor

DRG …….Dorsal root ganglia

EGTA ……..Ethylene glycol tetraacetic acid

GDP ……Guanosine 5'-diphosphate

GPCR ……..G-protein coupled receptor

GTP …….Guanosine 5'-triphosphate

GTPγS …….Guanosine-5'-O-[γ-thio] triphosphate

KMO ……Kynirenine-3-monooxygenase enzyme

KOPr ……κ opioid peptide receptor

KYNA …….Kynurenic acid

KYNA1 ……...*N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride

MOPr ……...μ opioid peptide receptor

NOPr ……Nociceptin peptide receptor

S.E.M. ……..Standard error of means

TEM ……Tris-HCl, EGTA, $MgCl_2$

Tris-HCl……Tris-(hydroxymethyl)-aminomethane hydrochloride

TRP …….Tryptophan

1 REVIEW OF THE LITERATURE

1.1. Opioids and the endogenous system

1.1.1. Opium poppy

Opium is the dried latex extracted from the ripening pods of the poppy (*Papaver somniferum*; Fig. 1) [1]. Opium has been known for millennia to relieve pain and its use for surgical analgesia has been recorded for several centuries. The Sumerian clay tablet (about 2100 BC) is considered to be the world's oldest recorded list of medical prescriptions. A goddess from about 1500 BC shows her hair adorned probably with poppy-capsules and her closed eyes disclose sedation.

Figure 1. The flower and capsule of opium poppy (*papaver somniferum*) after scratch and the latex collection.

The first authentic reference to the milky juice of the poppy is find by Theophrastus at the beginning of the third century BC. In the first century the opium poppy and opium were

known by Dioscorides, Pliny and Celsus and later on by Galen. Celsus suggests the use of opium before surgery and Dioscorides recommended patients should take mandrake (contains scopolamine and atropine) mixed with wine, before limb amputation. The Persian physicians used opium very extensively and about 1000 AD it was recommended by Avicenna especially in the diarrhea and diseases of the eye. Polypharmacy, including a mixture of nonsensical medications were often used. Fortunately for both patients and physicians, many of the preparations contained opium. The goal was a panacea for all diseases. A famous and expensive panacea was theriaca containing up to sixty drugs including opium. Opium latex contain more than 25 natural alkaloids such as morphine (analgesic), codeine (antitussive), thebaine (convulsive, stimulant, chemical precursor for semi-synthetic drugs), papaverine (smooth muscle relaxant) and Noscapine (cough-suppressing), therefore it was known as a gift of God in the medieval ages. Several morphine-like drugs have been synthesized to minimize adverse effects and abuse potential. Combination therapy, innovative delivery systems and long-acting formulations may improve the clinical utility. Naloxone, pure opioid antagonist developed by Sankyo in the 1960s. It is also used for overdose therapy [2].

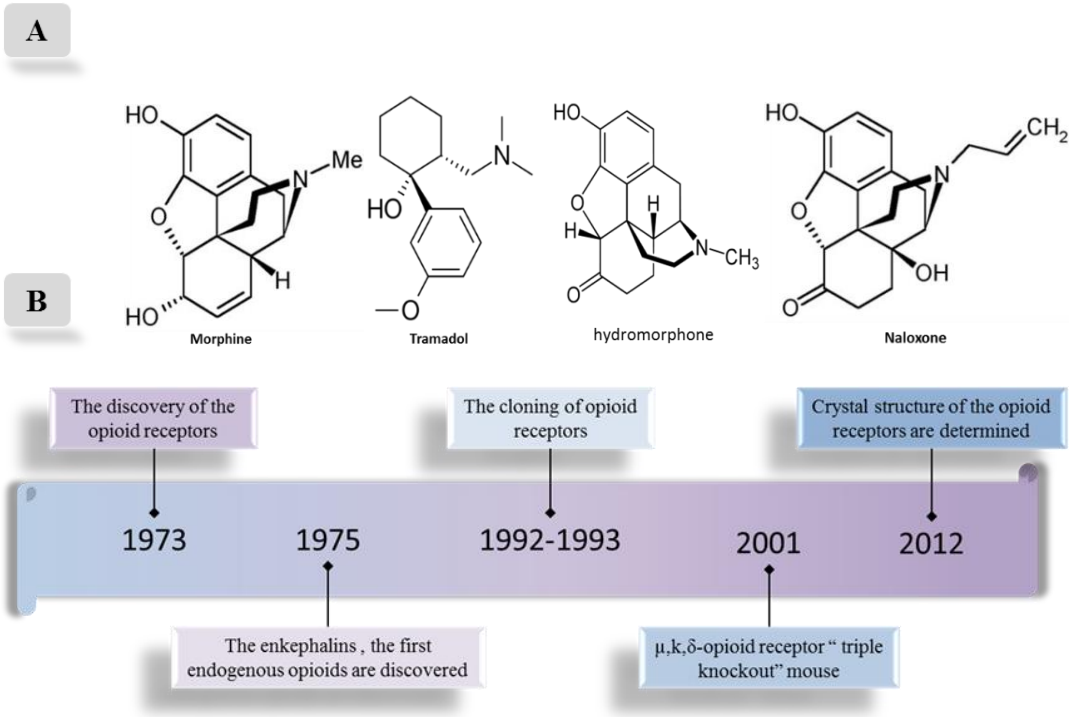


Figure 2. A) Morphine, it's synthetic and semi-synthetic derivatives and opioid antagonist naloxone. B) Short chronology of important opioid research.

1.1.2. Opioid receptors

The opioid ligands express their effects by binding to the specific receptors, called opioid receptors. These receptors belong to the GPCR superfamily and they are mostly coupled to Gi/o type G-proteins [3]. Therefore, they inhibit adenylyl cyclase (AC) activity [4,5], decrease calcium ion influx [6,7] and increase potassium ion efflux [6,8], which leads to inhibit the presynaptic release of different types of neurotransmitters such as acetylcholine, noradrenaline, dopamine or GABA [9,10]. There are three types of classic opioid receptors: the mu, kappa and delta opioid peptide receptor (MOPr, KOPr and DOPr respectively) [11]. Additionally, the nociceptin peptide receptor (NOPr) is also referred to as an opioid receptor [12,13] because the semi-synthetic neutral opioid antagonist, naloxone cannot inhibit nociceptin receptor specific binding, unlike the other three opioid receptors binding [1].

Opioid receptors	Distribution area	Physiological function
μ	Amygdala, cerebral cortex, PAG, thalamus, NAcc, GI tract, SGR	Analgesia, physical dependency, respiratory depression, miosis, euphoria, reduce GI motility
k	Amygdala, SN, PAG, hypothalamus, NAcc, GI tract, SGR	Analgesia, anticonvulsant, dissociative effect, duresis, dysphoria, neuroprotection, sedation
δ	Amygdala, olfactory bulbs, pontine nuclei, crebral cortex	Analgesia, antidepressant, convulsant, physical dependency

Table 1. The distribution and physiological effects of opioid receptors. This table was designed based on the following publications: [1, 10, 14,15].

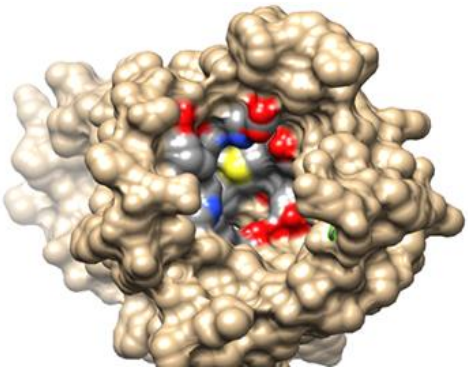
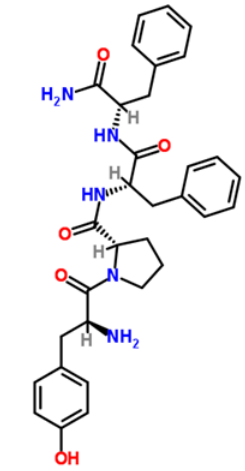


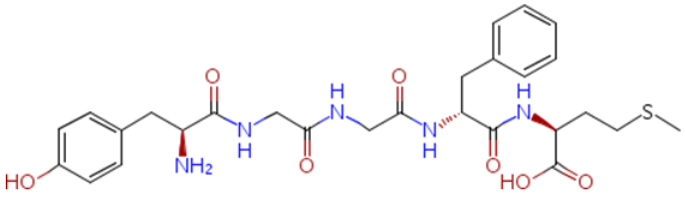
Figure 3. Binding pocket and receptor surface of MOP. Computed and designed by Dr. Ötvös Ferenc.

1.1.3. The endogenous opioid system

In normal physiological condition endogenous opioid receptors, have their specific endogenous opioid ligands as well (Fig. 4). Endogenous opioids are peptide natured neurotransmitters, neuromodulators or even neurohormones [16, 17], and the most notable ones are the enkephalins [18] endorphins [19], dynorphins [20], nociceptin [13] and endomorphins [21]. These endogenous opioids are synthesized from precursor proteins (prepro-opiomelanocortin, preproenkephalin and preprodynorphin) [22,23].



Endomorphin 2



Enkephalin

Figure 4. Endogenous opioid peptides endomorphin 2 and enkephalin

Therefore, this system which includes all the necessary enzymes for synthesizing and degrading endogenous opioids, together with the receptors, called the endogenous opioid system. This system is under strict regulation [16, 17].

1.2. G-protein coupled receptors (GPCR)

1.2.1. The GPCRs structure

More than 75 GPCR crystal structures have been determined from 18 different rhodopsin-like GPCRs [24], comprising notable GPCRs like rhodopsins, β adrenoreceptors, dopamine and opioid receptors as well. All members of the GPCR superfamily have almost similar structure which can be divided into three parts [24,25] (Fig. 5,6A): (1) the extracellular domain, containing the N terminal and three extracellular loops (ECL1-ECL3); (2) the transmembrane (TM) part, consisting seven α-helices (TM1- TM7) and finally (3) the intracellular region, including of three intracellular loops (ICL1-ICL3), an intracellular amphipathic helix (H8), and the C terminal. The extracellular surface modulates ligand access; the structural core of the receptor forms by the TM region, binds to the ligands which then induce conformational changes in the TM region [26]. This structural change is transmitted to the intracellular region which associates with cytosolic signaling proteins, mainly G-proteins (Fig. 7), but GPCR kinases and arrestins can also interact with GPCRs.

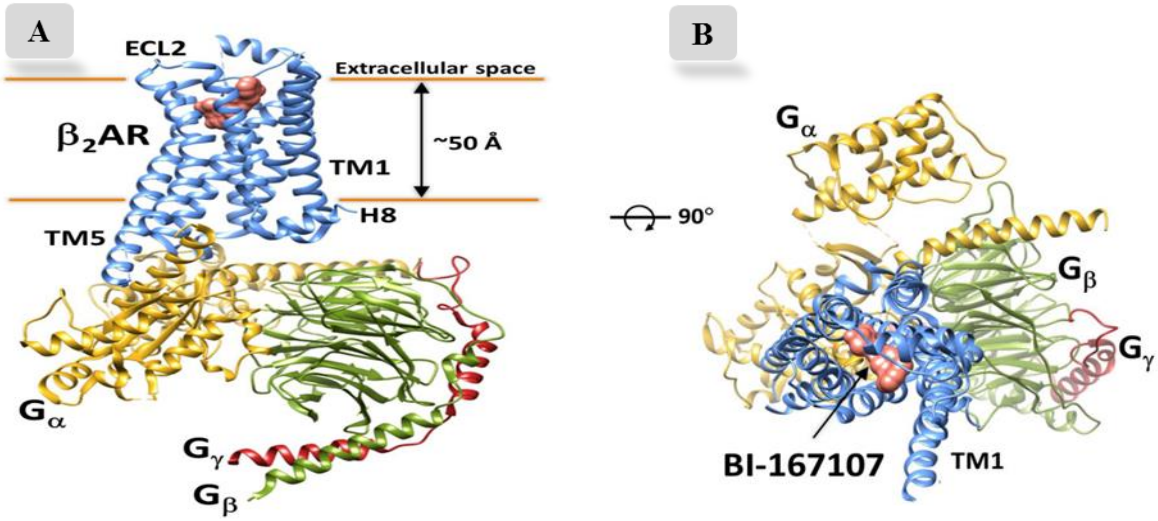


Figure 5. A and B) 3D crystal structure view of the side and extracellular surface of the β2AR-Gs protein complex together with the β2AR agonist BI-1617107 [27].

The GPCRs can have various intermediate structure states, which are stabilized by distinct ligands [28- 29]. The ligands whith agonist property induce the active conformational state of the GPCR [28], which leads the receptor to alter the G-proteins conformation to the active (GTP-bound; see section **1.2.2**) state. Whiles, inverse agonists stabilize the inactive state of the receptor, in which the G-protein remains in the inactive state (GDP bound

state; see section **1.2.2**; [30, 31]). On the other hand neutral antagonists are believed to bind equally to both receptor states, usually with a high affinity, therefore possessing a physiological role by competitively inhibiting agonist and inverse agonist binding [30, 31]. Regarding to these effects, there is a spectrum of efficacies of GPCR ligands initiating from the full and partial inverse agonists, through neutral antagonists to partial and full agonists [30] (Fig. 6B). Therefore agonists increase, inverse agonists decrease, while neutral antagonists do not alter GPCR basal activity. Interestingly, in the absence of basal receptor activity inverse agonists behave as competitive antagonist [32], which explains why numerous of the compounds originally described as neutral antagonists, turned out to be inverse agonists [30]. Inverse agonism phenomenon is discovered not long time ago, and according to increasing numbers of studies, it has a potential therapeutic application in cancer treatment [32] and other human diseases [33-34].

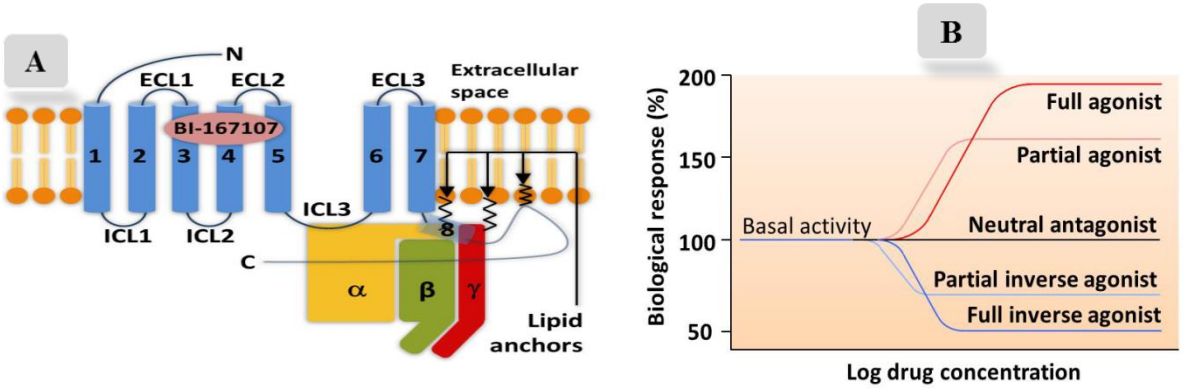


Figure 6. A) The schematic structural view of the β2AR-Gs protein complex viewed within the lipid bilayer. The figure also comprising the lipid anchors of the receptor (palmitate) [35]. B) The spectrum of GPCR ligand efficacy.

1.2.2. GPCR signalling: active / deactive phase regulation

G-proteins subunits are often called molecular switches, since they can initiate and terminate intracellular signaling cascades generated by GPCRs after extracellular stimuli. G-proteins consist of three subunits: α, β and γ (Fig 5), the G_α is the "main switch" and the only G-protein subunit which making direct contact with the receptor, while G_β and G_γ form a functional unit together, and can only be dissociated in denaturing conditions [36]. The main property and role of G_α is its GTP/GDP binding ability and GTPase (GTP hydrolyzing) activity.

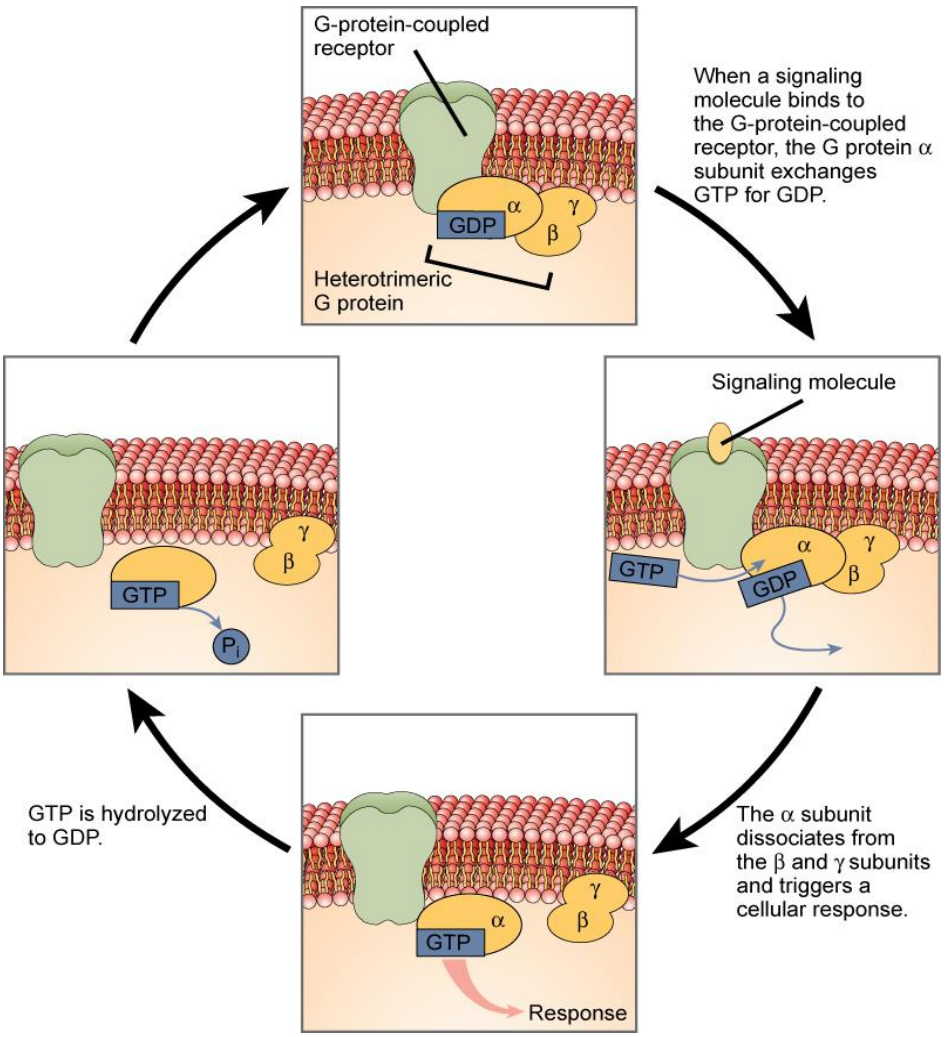


Figure 7. The G-protein activation/deactivation cycle.
Reference: http://cnx.org/content/m44451/latest/Figure_09_01_05.jpg

Stimulation of the $G_{\alpha s}$ subunit activates AC [37], while stimulation of the $G_{\alpha i/o}$ subunit results AC inhibition [38]. Inducing $G_{\alpha q/11}$ leads to activation of phospholipase C (PLC) [39] whiles $G_{\alpha 12/13}$ has a role in the regulation of cell growth [40]. Furthermore, $G_{\alpha i/o}$ and $G_{\alpha s}$ subunits can go through ADP-ribosylation which could be catalyzed by pertussis and cholera toxins respectively [41,42]. The ADP-ribosylation inhibits both G-proteins normal function, resulting altered AC activity levels compared to normal levels.

Approximately few types of G-protein compared to the abundant numbers of the GPCR subfamilies results that G-proteins can interact with many different types of GPCRs, and also several receptors can activate different types of G-protein signaling pathways [41]. The complexity of GPCR signaling is augmented by the large GPCR binding surface of the G-protein [41] which enables it to interact with multiple GPCRs simultaneously. Additionally

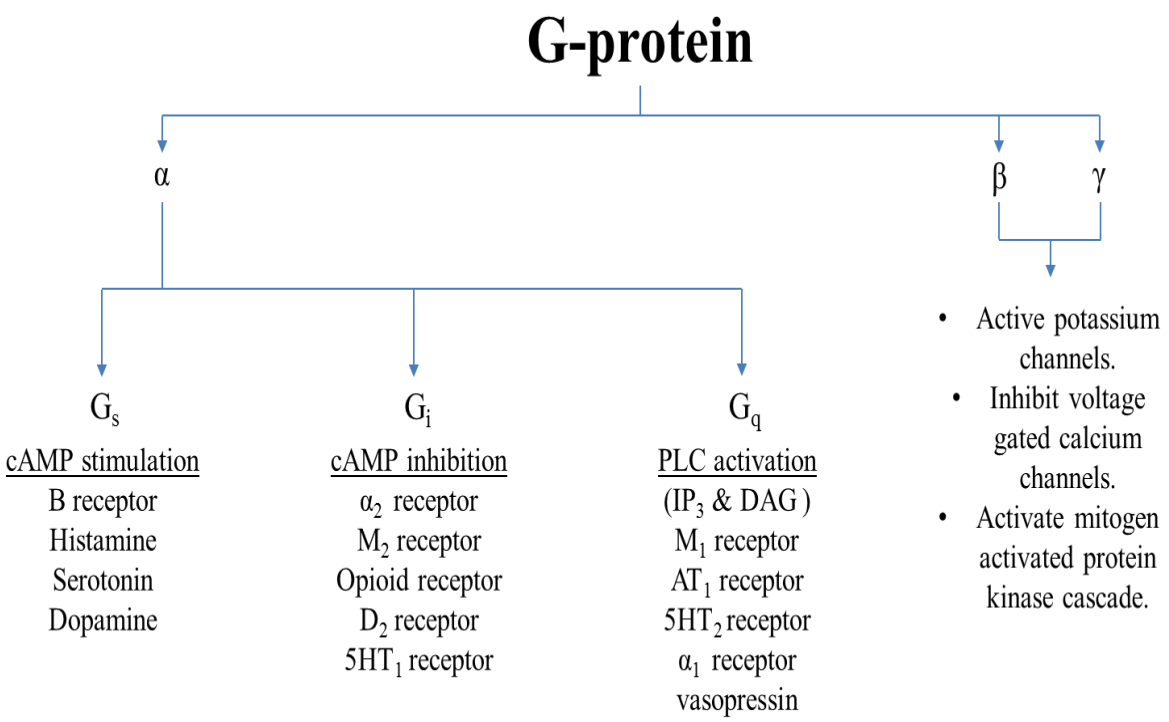


Figure 8. G-protein subunits; their role and effects. PLC = Phospholipase C, cAMP = Cyclic adenosine monophosphate, IP_3 = Inositol trisphosphate, DAG = Diacylglycerol. The figure was designed based on the following publications: [37-39]

receptor homo-, and heterodimerization, or even oligomerization is a usual phenomenon in the GPCR superfamily [43-44]. Therefore, it is not astonishing that GPCRs can cross-regulate each other's signaling pathways, communicate with one and other, altering each other's physiological responses [45-46]. Moreover, there is an emerging concept in GPCR signaling is regarded to "biased agonism" or "ligand-directed signaling", which revealed that distinct agonists can initiate different active receptor conformational states, that leads to initiate distinct signaling pathways [47-48].

1.3 Kynurenines

1.3.1. Tryptophan metabolism

Tryptophan (TRP) is one of the essential amino acids that are required for the appropriate functions of the human body. TRP either binds to albumin around the periphery (90 %) or exists in free form (10 %), but only the free form is capable to cross the blood-brain barrier by specific amino acid transporters. In the central nervous system (CNS), TRP acts as a precursor to the synthesis of different proteins, serotonin, melatonin, tryptamine and especially kynurenine (Fig. 9).

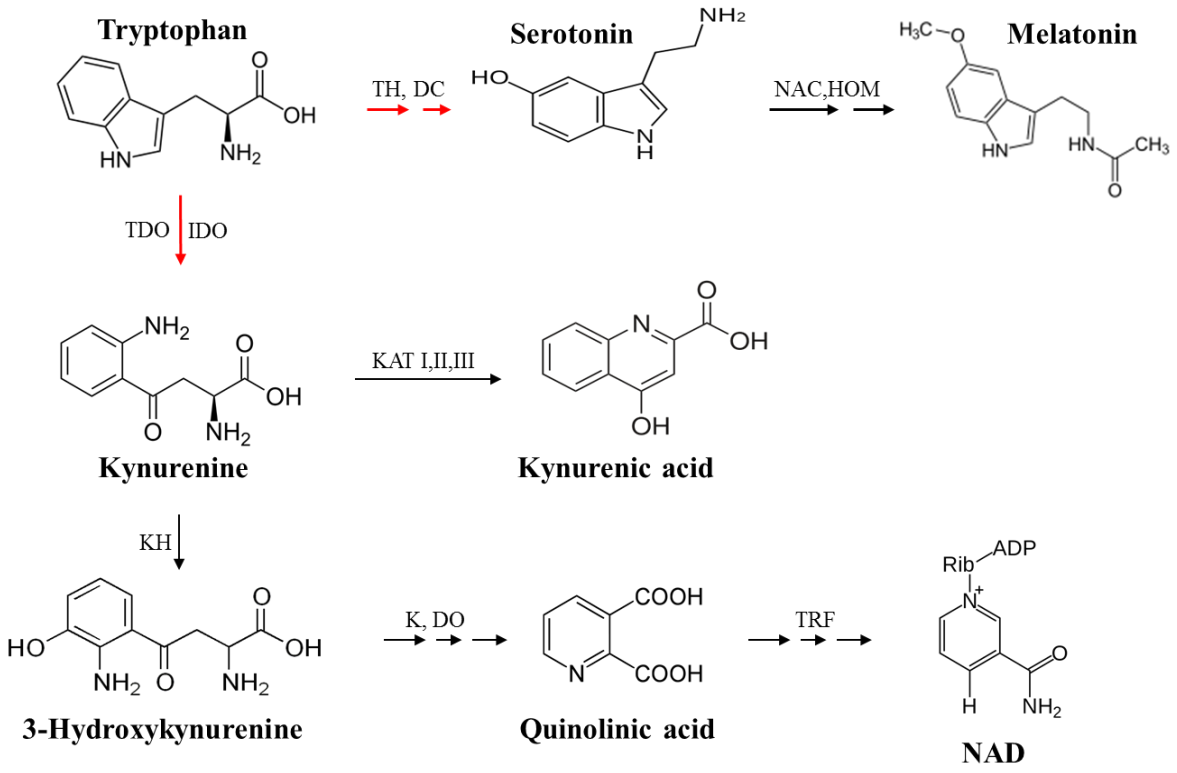


Figure 9. Tryptophan metabolism pathways. TDO (tryptophan 2,3-dioxygenase), IDO (indoleamin 2,3-dioxygenase), TH (tryptophan hydroxylase), DC (L-aromatic amino acid decarboxylase), KH (kynurenine hydroxylase), K (kynureninase), KAT I,II,III, (kynurenine aminotransferases I,II,III), DO (3-hydroxyanthraninate 3,4-dioxygenase), NAC (5HTN-acetylase), HOM (hydroxy indol o-metyltransferase), TRF (Quinolate phosphoribosyl transferase). The figure constructed based on the following publications: [49-50].

Kynurenine pathway (KP), which is presented in varying extents in astrocytes, neurons, micro- and oligodendroglial as well as in macrophages, endothelial- and dendritic cells in the CNS, is the major route of the catabolism of TRP. Approximately, 95% of TRP may convert to kynurenines by the TRP- or indoleamine 2,3-dioxygenase (TDO or IDO-1, IDO-2) enzymes initiated the oxidation of TRP. This conversion results in L-kynurenine (L-KYN), via the formylkynurenine transition product. L-KYN is synthesized in the brain with the rate of 0,29 nmol/g/h and it serves as a key molecule between the neurotoxic and neuroprotective directions of the pathway. Quinolinic acid (QUIN), as a neurotoxic compound is produced from L-KYN, via the additional toxic metabolites (3-hydroxykynurenine and 3-hydroxyanthranillic acid). These agents can generate toxic free radicals, oxidative stress and lipid peroxidation, furthermore in high concentration can cause excitotoxicity due to excitatory amino acid (EAA) receptors. In contrast, the characteristically neuroprotective kynurenic acid (KYNA) is formed directly from L-KYN catalysed by kynurenine aminotransferase (KAT) (Fig. 9) [49].

1.3.2. Kynurenic acid receptors

According to the several studies, KYNA can act primarily at the strychnine-insensitive glycine-binding site of the N-methyl-D-aspartate (NMDA) receptors and at the α7-nicotinic acetylcholine (nACh) receptors. The most results of the neuroprotective and antinociceptive effects of KYNA are explained by inhibition of the EAA receptors [51].

1.3.2.1. NMDA-receptor

In a comparative study the antagonists of different subunits of the NMDA receptor were investigated in the facial formalin rat model. The ion channel-, the glycine-, the non-selective NMDA site, as well as the NR2A subunit with a competitive drug and the NR2B subunit with a selective antagonist were scanned. The formalin-induced nociceptive behavioral response was effectively suppressed by the inhibition of each binding site, but it seems that the central NR2 subunits have more specific importance in such inflammatory pain diseases [52].

The inhibition of NMDA receptor by 7-chlorokynurenine is proving that it can have a preventive role in the case of Gly-induced persistent anxiogenic nociception model [53].

1.3.2.2. α7-nACh-receptor

Non-competitive antagonist effects of KYNA on the α7-nACh-receptors were observed in neuronal cultures and hippocampal interneurons depending on the experimental conditions and synaptic locations [54, 55]. The effects of the KYNA on this type of receptor could be involved in the pathomechanisms of Alzheimer's disease (AD) and schizophrenia by modulation of kynirenine-3-monooxygenase enzyme (KMO) and dopaminergic system. Inhibition of KMO can lead to a decreased glutamate level in an AD mouse model experiment, which resulted a reduced loss of synapses in the brain, with improved learning and cognitive functions [56]. It is also revealed that levels of kynurenines in the CSF of schizophrenic patients are much higher than in healthy cases. Furthermore, administration of KYNA can develop a similar state than the prefrontal schizophrenia by reduction of the extracellular dopamine concentration [57, 58].

1.3.2.3. GPR35-receptor

Recently, the G-protein-coupled receptor GPR35, an orphan receptor is reported as a target for KYNA. GPR35 protein is expressed in both neurons and glial cells in the brain, but especially, it is present in an abundant number in the small-to medium-diameter neurons at the dorsal root ganglia (DRG) [59], in macrophages and monocytes in the immune system. The hypothesized interaction between the agonist KYNA and the new target is that the KYNA activates the GPR35, which lead to decrease the level of cAMP and the intracellular Ca^{2+} concentration. These alterations induce diminish in neurotransmitters, like glutamate and releasing of the pro-inflammatory mediators from the glial and immune cells. It could be another explanation for the analgesic effects of KYNA in inflammatory models.

The antinociceptive-effects of the GPR35 agonists KYNA and zaprinast were investigated in another study. These drugs could inhibit the adenylate cyclase activity of DRG neurons in a GPR35-specific manner, probably with G-protein-dependent mechanisms [59].

Both compounds mentioned above were examined in an inflammatory pain model as well. The number of the acetic acid-induced writhe behavior was inhibited after KYNA or zaprinast administration. The concentrations of KYNA in the plasma and spinal cord were raised by subcutaneously administered KYN in a dose dependent manner. These

effects were further amplified by probenecid injection. The zaprinast was more effective in lower doses compare with KYN, which is attributable to its more affinity to the GPR35 receptor. These all suggest that the GPR35 could be a promising and innovative pharmacological target for reduces the inflammatory pain [60].

1.3.2.4. Aryl hydrocarbon receptor (AHR)-receptor

New possible targets of the KYNA have been revealed in connection with immunosuppression and carcinogenesis. KYNA may act as an agonist of aryl hydrocarbon receptor (AHR). The AHR protein is involved in the regulation of gene transcription and this is the primary binding site for a potent cellular toxin, dioxin. The AHR affects in the metabolism of xenobiotics, hereby it is important in the detection of foreign substances. KYNA may be an endogenous ligand at the AHR, promoting protective mechanisms against organisms and chemicals. In addition the AHR is essential in the fundamental cell biology, the embryonic development, as well as in the maturation of cells in the immune and nervous system. Through complex immunological processes the binding of KYNA can reduce the maturation of the major antigen presenting cells, so it inhibits the immune surveillance. The KYNA-induced AHR activation precludes the tumor recognition and promotes the growth and migration of the cancer cells. Overall, suppression of the natural anti-tumor mechanisms by the KYNA-AHR binding is due to the activation of IDO/TDO, which can incapacitate the dendritic cells and stop the preventive T cell attack [61,62]. This approach could be useful in the modulation of the tumor-associated pain disorders.

1.3.2.5. Opioid receptors

The investigation of opioid receptors is fundamental in pain research, because it is known that the opioids have analgesic effects, but chronic use can produce physiological dependence. Thus, it appears that using the opioids in combination with other drugs could enhance their efficiency and avoid the overdose by opioids or inducing their adverse effects. Therefore, according to these studies, it would be crucial to investigate the interactions between the kynurenines and opioids [63].

1.3.3. Association of KYNA and opioids

1.3.3.1. Association with morphine

Previously it has been reported that the NMDA receptor blockers alter the pharmacological and behavior effects of acute and chronic opioid administration. However, minimal interactions were observed between these receptors and the discriminative stimulus properties of morphine via concurrent administration of different NMDA antagonists and subcutaneous morphine [64]. Later, the interaction between chronic morphine treatment and NMDA receptors was investigated in isolated medium spiny neurons from nucleus accumbens, *in vitro*. These results have shown that morphine administration did not alter the affinity for certain NMDA receptor agonists, but it can decrease the affinity of the allosteric co-agonist, glycine and the non-competitive NMDA receptor antagonists, 7-chloro-KYNA and ifenprodil. It suggests that beside the NR2B and NR2C, the NR2A subunit expression or function have increased after chronic morphine exposure as well [65].

The intracerebroventricular (i.c.v.) administered morphine is able to induce both synchronous oscillatory discharges and decrease the firing rate of neurons in the locus coeruleus (LC). These effects could be reversed by i.c.v. injection of different specific selective/non-selective NMDA or non-NMDA receptor antagonists, separately. It seems that the EAA input may be involved in the neurotransmitter release and synaptic plasticity caused by morphine-induced synchrony in the LC [66].

Morgan and his colleagues investigated the effect of co-administered glutamate receptor antagonists and morphine on the ventrolateral periaqueductal gray matter (vPAG) of the rat. this area has an important role in morphine-modulated pain relief. Microinjection of KYNA or MK-801 to the vPAG did not affect the nociception, however, their co-injections with morphine have enhanced the acute antinociceptive effect of morphine. This study, was also shown that the reserved tonic glutamate release does not improve the effects of repeated morphine injection [67].

It is possible that these processes could change the excitability of cells, the integrative and neuroadaptative properties of neurons, which may underlie the morphine dependence. There are some data which show, the low-dose administration of non-competitive and competitive NMDA receptor blockers with morphine can inhibit the development of physical addiction by opioids. Additionally, it was found that the antagonism of NMDA/GlyB site with e.g. the 5,7-dichlorokynurenic acid, suppressed the expression of

morphine withdrawal syndrome. The dependency can be attenuated by multiple dose of the GlyB site antagonist, which generates a more pronounced effect than the non-competitive NMDA receptor antagonists [68].

There were trials to answer that question whether what kind of mechanisms lead to the development of opioid withdrawal-induced hyperalgesia. The withdrawal syndrome is demonstrated by either interruption of drug administration or opioid antagonist, for example naloxone. The microinjections of lidocaine, KYNA and a cholecystokinin receptor-2 antagonist to the pain-linked descending facilitatory pathway (rostral ventromedial medulla, RVM) could inhibit both the withdrawal-induced hyperalgesia and its accessory symptoms (e.g: anxiety, diarrhoea). It is evidence that the RVM has a crucial role in the opioid mediated dependency, which can also be modulated by EAA antagonist [69].

1.3.3.2. Association with dynorphin

In an interesting study the functions of opioid peptides (dynorphin A, -B) investigated regarding to their influences for nociceptive behaviors, since the dynorphins may effect by two-ways: inhibition and facilitation of nociceptive transmission. The big dynorphin, a prodynorphin-derived peptide, induced characteristic pain responses, were dose dependently inhibited by i.p. injection of morphine and by i.t. its co-administration with certain NMDA receptor antagonists as well. The mechanisms are mediated through the activation of the NR2B subunit and/or by acting the polyamine recognition site. The glycine binding site, the opioid, the non-NMDA glutamate- and the tachykinin receptor mechanisms were not proved to be effective in the mouse spinal cord [70].

The κ-opioid receptor is the primary target of dynorphin A, but the NMDA receptors may also be influenced by this molecule, thus the role of these receptors was investigated in dynorphin A and its metabolite-induced neurotoxicity. In the physiological conditions dynorphin has antinociceptive effect, however its level can rise after spinal cord injury. It promotes the tissue damage, neurological deficits/degeneration and accidental mortality, which may be attributable to consecutive or accompanying NMDA receptor activation. It is evidenced by that the Gly potentiation site, as well as using the competitive and non-competitive antagonists of NMDA receptor prevent the dynorphin-affected harmful effects [71]. Furthermore the effect of dynorphin A-metabolite on increasing intracellular

Ca^{2+} concentration and reducing cell survival were examined in embryonic mouse spinal cord neurons containing both opioid and NMDA receptors. The different sub-targeting NMDA receptor antagonists prevented the significant loss of neurons, but the co-administration with different active opioid receptor antagonists exacerbated the excitotoxic effects. The opioid receptor antagonists applied alone did not induce neuronal losses. These results indicate that dynorphin can act directly or indirectly through both receptors. In addition, the κ-receptor activation may be moderately protective, since the inhibition of opioid system in the presence of amount of excitotoxic dynorphin causes augmented toxic effects [72].

These processes involve complex interactions with multiple receptors and signalling pathways between the opioid and NMDA systems and may provide a possible treatment of the excitotoxin mediated tissue injury.

1.3.3.3. Association with endomorphines

The relationship between the endomorphine-1 (EM-1) endogenous μ opioid receptor agonist peptide, and KYNA was investigated at the peripheral level in the carrageenan-induced inflammation in rat's joint model. The continuously administered high dose KYNA and the EM-1 has significant antinociceptive potency on the thermal hyperalgesia, moreover their combined treatment can highly potentiate the inhibitory effect on the inflamed joint [73]. The same effects were reported for both compounds when separately applied, as well as in the combination form in a mechanical induced pain model. The elevation of nociceptive threshold and the prolongation of analgesic effect were determined by von Frey filaments. These results indicate an additive interaction between these systems [74].

The co-injection of different endogen ligands (EM-1, adenosine, agmantine and KYNA) in triple and quadruple manner, but lower doses, can be more effective compare with the single treatment at spinal level in the case of inflammatory pain [75].

These results suggest that the interactions and combined therapies of the kynurenine- and opioid systems could provide a more effective, well-controlled and less undesired side-effects associated with pain therapy by opioids.

1.3.4. Kynurenic acid and pain

Kynurenic acid, as a non-selective EAA receptor antagonist takes part in glutamatergic neurotransmission and it can non-competitively block the nicotinergic processes in the CNS [51]. Therefore KYNA is strongly involved in endogenous protective mechanisms, so it could be a good target for pharmaceutical intervention in neurological diseases therapy [76]. As a therapeutic strategy, shifting the neuroprotective/neurotoxic balance towards the formation of KYNA or reduction of the levels of QUIN may come into consideration. Although the features benefit of endogenous molecules is confirmed (specific enzymes in the body; short half-life; lower toxicity) [77], also it is essential to investigate their interactions with other drugs and their possible relationships (specificity, affinity) with different receptors [78]. It may occur that the effectiveness of the endogenous ligand is too low, but this can be ameliorated by the combination of endogenous systems or it can be enhanced by supplemental exogenous/synthetic drugs [79-80].

This theory has been studied in the case of pain therapy [75]. On the other hand functions of endogenous ligands, such as the kynurenines and their associations with different pain conditions are not fully elaborated [81, 82].

The analgesic properties of kynurenines is investigated in different pain conditions. In a study the effects of L-KYN and its combination with probenecid (PROB) or a novel KYNA-analogue pretreatments were investigated in a headache-like animal model (nitroglycerine (NTG)-induced trigeminovascular activation in the rat). The expressions of the neuronal nitric oxide synthase, the Ca^{2+}/calmodulin-dependent protein kinase and the calcitonin gene related peptide (CGRP) - which are proper indicators of the sensitized trigeminal system in this model - have decreased in the caudal trigeminal nucleus of the brainstem by each compound [83, 84].

In the other study the KYN and the KYN+PROB combination (inhibition of the KYN elimination from the brain) are examined in a cortical spreading depression (CSD) rat model of migraine with aura. These pretreatments have significantly suppressed the frequency of CSD, especially in female rats, which suggests the modulating effect of sex hormones referring to the migraine. Following the administration of kynurenines elevated KYNA levels were proven by HPLC analysing in the cortex [85].

The intraperitoneal (i.p.) administration of L-KYN-PROB combination has revealed to be an effective treatment in the lumbar spinal nerve ligation-induced rat model of neuropathic pain. The allodynia was reversed in the combined treatments and even it was

more significant in the case of intrathecal (i.t.) administration of KYNA. The PROB has enhanced the maximal antiallodynic effect of KYNA, additionally increasing the KYNA level was showed in the cerebrospinal fluid (CSF). Impairments of the motor activity was not observed during the experiments, the expression of the KAT II enzyme was unchanged as well [86].

These results propose that L-KYN produced protective effects are attributable to the increased KYNA level in the brain, which can alter the trigeminal nociception by the inhibition of the EAA receptors.

Certain non-steroid anti-inflammatory drugs can increase the KYNA level in the brain, which will further improve with TRP pretreatment [87]. On the other hand the cyclooxygenase enzyme-1 inhibitors can enhance the KYNA level in the brain [88]. These data suggest that some compounds can indirectly modulate each other's impact and potentiate their antihyperalgesic effects, which may give a novel opportunity in the therapy of pain.

There is a controversial study regarded to the pain and kynurenines, which has revealed that the activated IDO1 enzyme and the consecutively elevated KYN level can provoke co-morbidity of pain and depression [89].

1.4. KYNA1

1.4.1. Synthesis and investigated effects

KYNA1 is one of the amide analogue of KYNA which has a cationic centre in the C-2 side chain which is designed and synthetised by Prof. Ferenc Fülöp and his colleagues in the Institute of Pharmaceutical Chemistry, University of Szeged, Hungary [90]. For the first step, the effect of KYNA-1 was investigated in an in vitro electrophysiological experiment together with the other 9 newly KYNA amide analogues and with KYNA itself [90]. KYNA1 and KYNA2 have the similar molecular structure (differing in a $–CH_2$-group), but the others are structurally quite different.

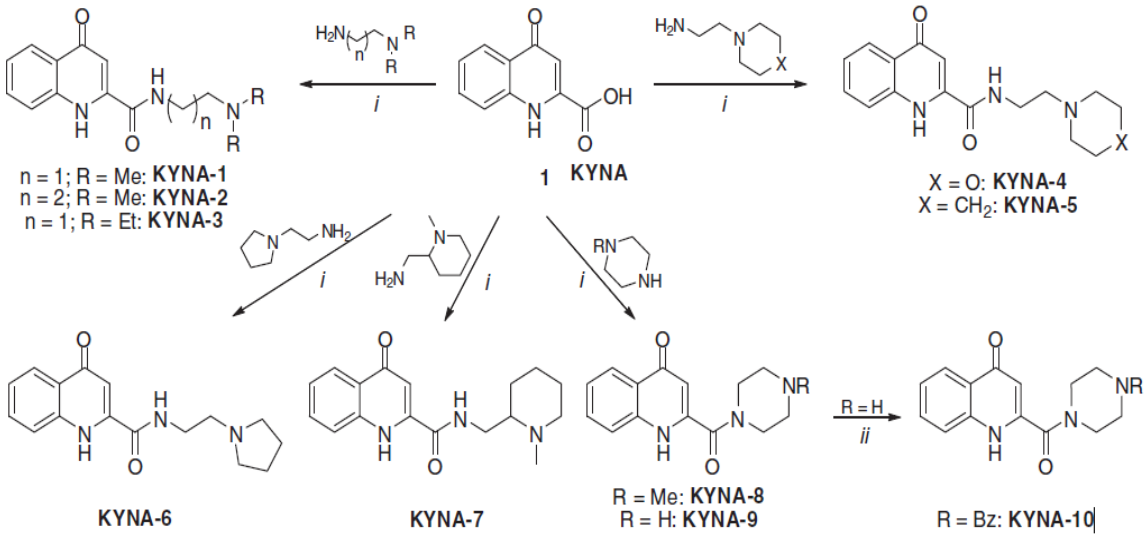


Figure 10. KYNA and its analogues synthesis pathways. Reagents and conditions: (i) 1-Hydroxybenztriazole-hydrate, N,N0-di-isopropylcarbodiimide, DMF, reaction time, 48 h; (ii) Bz–Br, Na2CO3, H2O/toluene, reaction time, 10 h.[90]

KYNA1 was the only KYNA analogue which reduced the amplitude of the fEPSPs, this suggests that KYNA1 exerts an inhibitory effect on the synaptic transmission mediated by NMDA receptors. In the *ex vivo* studies, KYNA1 (200 mg/kg ip) displayed inhibition of the population spike amplitude without any delay and without any transient facilitation as well. Its inhibitory effect attained its maximum within 1 h. Interestingly, even by having similar structure to the KYNA1, KYNA2 did not exhibit any significant effect on the population spike amplitudes. It was also important that KYNA1, which had proved neuroprotective effect in several models, and partially inhibited NMDA-mediated synaptical transmission in the hippocampus, did not induce significant changes in the behavior of the tested animals. This was true for both rats and mice [90].

2 AIMS OF THE STUDY

All these studies which mentioned above (see section 1.3.) shows that it must be a relationship between KYNA and opioid system activity. Since it is revealed that both KYNA and opioid system have neuroprotective role in pathopysiological conditions, it would be very important to investigate the possible reactions and effect of KYNA on opioid system. But till now there is no coherent and focused study about this relationship. For the first time we were investigate the effect of KYNA on the opioid system and to compare with its analogue, KYNA1, which has a cationic centre in the C-2 side chain. Herein for the first time we characterize the binding properties of KYNA and KYNA1 towards all three opioid receptors in competition binding assays with radiolabelled receptor specific opioid ligands.

The aims of the study presented in this thesis were the following:

- To measure the binding affinity of KYNA and KYNA1 towards opioid receptors in competition binding assays with opioid receptors specific radioligands performed in Chinese hamster ovary cell (CHO) membranes overexpressed with the adequate opioid receptor.
- Investigation of the opioid receptor G-protein activity, the initial phase of GPCR signalling, after *in vivo* and *in vitro* KYNA and KYNA1 administration in functional [^{35}S]GTPγS binding assays.
- Investigation of the opioid receptor G-protein activity after chronic treatment of the mice with KYNA and KYNA1 by functional [^{35}S]GTPγS binding assays.
- To examine the effect of acute treatment with KYNA and KYNA1 on the opioid receptor G-protein activity in functional [^{35}S]GTPγS binding assays carried out in rat's brain membranes.
- Try to uncover the mechanism of action of KYNA and KYNA1 on opioid system activity through *in vivo* and *in vitro* experiments.
- Investigation of possible interaction between opioid receptors and NMDA receptors.

3 MATERIALS AND METHODS

3.1. Chemicals

3.1.1. Radiochemichals

The tritiated U69,593 ([^{3}H]U69,593) [91] was purchased from PerkinElmer (Boston, USA). The modified DOP receptor specific deltorphin II derivative, Ile^{5,6}deltorphin II was synthesized and tritiated ([^{3}H]Ile^{5,6}deltorphin II) [92] in our Laboratory of Chemical Biology at the Biological Research Centre (BRC, Szeged, Hungary) as well as the tritiated DAMGO ([^{3}H]DAMGO) [93]. The radiolabeled GTP analogue, [^{35}S]GTPγS (specific activity: 3.7 x 1013 Bq/mmol; 1000 Ci/mmol) was purchased from Hartmann Analytic (Braunschweig, Germany) [94].

3.1.2. Receptor ligands and the fine chemicals

The MOP receptor specific agonist enkephalin analogue Tyr-D-Ala- Gly-(NMe)Phe-Gly-ol (DAMGO) and the KOP receptor agonist peptide dynorphin 1-13 were purchased from Bachem Holding AG (Bubendorf, Switzerland). The structurally modified DOP receptor specific deltorphin II derivative, Ile^{5,6}deltorphin II was synthesized in our Isotope Laboratory of BRC (Szeged, Hungary). We were purchased acetonitrile and perchloric acid (PCA) from Scharlau (Barcelona, Spain), acetic acid was purchased from VWR International (Radnar, PA, USA). EGTA, Tris-HCl, $MgCl_2$ x $6H_2O$, NaCl, KCl, $NaHPO_4$, $NaHCO_3$, CaCl, $MgSO_4$, D-glucose, GDP, the GTP analogue GTPγS, kynurenic acid (KYNA), MK-801 hydrogen maleate and 3-nitro-L-tyrosine (3-NLT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). The KYNA analogue, KYNA1 was synthesized in the Department of Pharmaceutical Chemistry, University of Szeged.

3.2. Animals

3.2.1. Animals and chronic treatment

79 young adult C57/B female mice (17-20 weeks old, 25-30 g body weight) were used in this study. The animals were bred and maintained under laboratory conditions on a 12-h dark 12-h light cycle at 22-24 °C and ~65% relative humidity in the Laboratory Animal House of the Department of Neurology in Szeged. Standard mouse chow and tap water were available *ad libitum*. All experimental procedures were carried out in

accordance with the European Communities Council Directive (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§). The investigations were in harmony with the Ethical Codex of Animal Experiments and in the eighth edition of Guide for the Care and Use of Laboratory Animals.

KYNA and KYNA1 were dissolved in saline and the pH of the solutions was adjusted to ~7,4 with NaOH and HCl. The mice were divided into three groups. In the control group, 43 animals received vehicle, which was 0.9 % saline. One group of 8 mice was treated with KYNA in a dose of 128 mg/kg/day. The third group of 28 mice received KYNA1 in a dose of 200 mg/kg/day. The dose of KYNA is equimolar with the KYNA1. Single intraperitoneal (ip.) injections of the drugs were administered in a volume of 0.1 ml to each animal, once a day, at the same time for 9 days. The treatments were well tolerated by the animals.

After 4 h of the injections of the ninth day, mice were deeply anesthetized with isoflurane (Forane®; Abott Laboratories Hungary Ltd., Budapest, Hungary) and perfused transcardially with artificial cerebrospinal fluid (ACSF) solution (NaCl 122 mM, KCl 3 mM, Na_2SO_4 1 mM, KH_2PO_4 1,25 mM, D-glucose10 mM, $MgCl_2$ x 6 H_2O 1 mM, $CaCl_2$ x 2 H_2O 2 mM, $NaHCO_3$ 26 mM, pH=7,5). The perfused brains were removed, and both entire striatum and the overlying cortex were excised. Samples were stored in Eppendorf tubes at -80 °C until the membrane homogenate preparation [63].

3.2.2. Animals and acute treatments

For *in vivo* experiments 64 male SPRD rats were used and 20 for *in vitro* with average weights of 300g. The animals were bred and maintained under laboratory conditions on a 12-h dark 12-h light cycle at 22-24 °C and ~65% relative humidity in the animal house of the Department of Neurology, Faculty of Medicine, University of Szeged (Szeged, Hungary). All experimental procedures were carried out in accordance with the European Communities Council Directive (2010/63/EU), the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§) and the ethics committee of the Biochemistry Institute, Biological Research Centre (Szeged, Hungary) [94].

3.2.2.1. In vivo animal treatments

All animals for the *in vivo* experiments received a single intraperitoneal (i.p.) injection. The animal treatments are summarized in Figure 11, together with the main steps following treatments. One set of animals were divided into 7 groups as follows: (1) control (saline), (2) KYNA1 (296 mg/kg), and (3) KYNA (189 mg/kg) (4) MK-801 (1 mg/kg) + KYNA1 (296 mg/kg), (5) MK-801 (1 mg/kg) + KYNA (189 mg/kg), that each group was included 5 animals (KYNA1 and KYNA doses were equimolar, 1 mmol). These set of animals were decapitated 30 minutes after the treatments (Fig. 11A). Additionally, two further groups containing 3 animals per group were set up receiving only a single i.p. MK-801 (1 mg/kg) injection. One of the group of animals were decapitated 15 minutes, while the other group of animals were decapitated 45 minutes after the treatment. These two groups were representing a control for the combined treatments to examine the effect of MK-801 *per se* on the opioid system prior to the administration (15 minutes) and right after reaching the peak plasma concentration of KYNA/KYNA1 (15+30 minutes, see the next paragraph) (Fig. 11A). The other set of animals were divided into 5 groups: (1) control (saline), (2) KYNA (189 mg/kg) and (3) KYNA1 (296 mg/kg), (4) MK-801 (1 mg/kg) + KYNA (189 mg/kg), (5) MK-801 (1 mg/kg) + KYNA1 (296 mg/kg). Groups (1)-(3) contained 7, while groups (4) and (5) contained 6 animals per group. These set of animals were sacrificed 2 hours after treatments (Fig. 11B). In the case of combined treatments MK-801 was administered to the animals 15 minutes prior to KYNA and KYNA1 treatment, in this way NMDA receptors will be already blocked by MK-801 when KYNA and KYNA1 are administered.

The chosen time phases for decapitation (30 minutes and 2 hours) were based on previously measured plasma concentration (yet unpublished data) of KYNA and KYNA1 after their i.p. administration: 30 minutes after injection the plasma concentrations of KYNA/KYNA1 was the highest, while after 2 hours it was the lowest.

After the treatments animals were anesthetized at the appropriate time points by chloral hydrate (Fig. 11). Those set of animals which were anesthized 30 minutes after saline, KYNA1 or KYNA alone treatments, the cerebrospinal fluid (CSF) and blood samples were collected immediately afterwards for KYNA and KYNA1 concentration analysis (Fig. 11A). This was followed by cardiac perfusion of the animals using 0.1M PBS and finally the animals were decapitated (Fig. 11). After decapitation the perfused brains were removed, and the entire striatum together with the overlying cortex were excised (Fig. 11). Samples then were stored at -80 °C until membrane homogenate preparation.

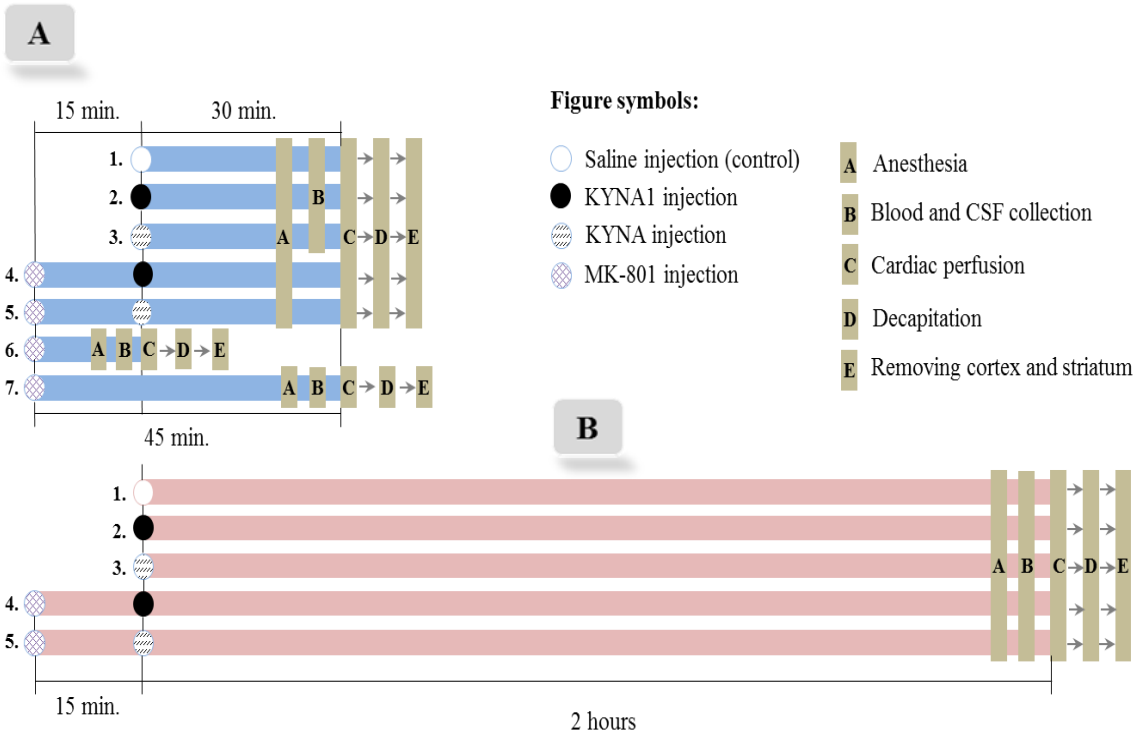


Figure 11. Animal treatments, groups, treatment duration times and main steps following the injections. **A**) Represents group 1-5 in which the animals were sacrificed 30 minutes after KYNA/KYNA1 treatment together with group 6 and 7 where animals received a single MK-801 injection and sacrificed 15 and 45 minutes after treatment. **B**) Represents group 1-5 in which the animals were sacrificed 2 hours after KYNA/KYNA1 treatment. Circles and khaki rectangles marked with letters (A-E) represent the injection timepoints and the steps following treatmens, respectively. For additional information see under sections 3.2.2. and 3.2.2.1.

3.2.2.2. In vitro isolated rat cortex and striatum treatments in isolated organ baths

The *in vitro* studies were performed in isolated organ baths using 3 animals for each group (control, KYNA, KYNA1 and MK-801 treatment groups). After decapitation, brains were removed immediately in less than 30 seconds and replaced it at 36-37 °C (physiological rat body temperature) artificial cerebrospinal fluid (ACSF, consisted of 130 mM NaCl, 3.5 mM KCl, 1 mM NaH_2PO_4, 24 mM $NaHCO_3$, 3 mM $CaCl_2$, 1.5 mM $MgSO_4$ and 10 mM D-glucose) supplied with oxygen. Afterwards the striatum and cortex were quickly separated and transferred to the isolated organ bath chambers containing 36-37 °C ACSF with oxygen supply. Cortex and striatum slices were adapted for 30 minutes to the new condition. After adaptation KYNA, KYNA1 or MK-801 were added to the bath for 30 minutes. The concentrations for KYNA and KYNA1 was 200 µM and for MK-801 it was 50 µM [95]. Samples then were stored at -80 °C until for preparation (see under 3.4.2).

3.3. Cell lines and cell culture

CHO cells over expressed with rat MOP receptor (CHO-rMOPr) or rat KOP receptor (CHO-rKOPr) or mouse DOP receptor (CHO-mDOPr) were provided by Dr. Zvi Vogel (Rehovot, Israel) and were described earlier [96–98]. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) and in α-minimum essential medium (αMEM, Gibco), respectively. Both media were supplemented with 10% fetal calf serum, 2 mM glutamine, 100 IU/ml penicillin, 100 mg/ml streptomycin, 25 mg/ml fungizone and 0.5 mg/ml geneticin. The Parental CHO cells (pCHO) were cultured in F-12 medium with L-glutamine which contained 10% fetal bovine serum. Both CHO cell lines were kept in culture at 37˚C in a humidified atmosphere consisting of 5% CO2 and 95% air.

3.4. Membrane preparations

3.4.1. Cell line membrane preparations

Cell membranes were prepared from subconfluent cultures for competition binding experiments. Cells were washed three times with 10 ml PBS and homogenized in 50 mM Tris–HCl buffer (pH 7.4) with a glass homogenizer in ice-bath. Homogenates were centrifuged two times at 18.000 g for 20 minutes. The final pellet was resuspended in 50 mM Tris buffer (pH 7.4) and stored in aliquots at -80 ˚C until use.

3.4.2. Mouse, rat brain cortex and striatum membrane preparations

The membrane fractions of rat cortex and striatum for [^{35}S]GTPγS binding experiments were prepared after cardiac perfusion and decapitation of the animals. Briefly, the cortex and striatum were collected and homogenized on ice in 50 mM Tris-HCl, 1 mM EGTA and 5 mM $MgCl_2$ buffer (TEM, pH 7.4) with a teflon-glass homogenizer (potter homogenizer). Protein content for the assay was 10 µg/ml and stored at −80 °C until use.

3.5. HPLC analysing, KYNA and KYNA1 concentration detection in the plasma and CSF

3.5.1. Sample preparation

In *in vivo* experiments after treatment of animals the CSF was taken quickly from the suboccipital cistern of rats with 23G needle to Eppendorf tubes (rats were anaesthetized and they were taken to the stereotaxical setup in order to fix their head). After collection the CSF samples were stored at -80C until use for concentration detection of KYNA or KYNA1. After collecting the CSF, Blood samples were collected immediately from the left ventricle into the cool, EDTA containing tubes and centrifuged at 12,000 rpm for 10 min at 4 °C. The serum samples were collected and stored at - 80 °C until use. Before analysis, the serum samples were thawed and, after a brief vortex, the serum sample was 'shot' onto a precipitation solvent (containing PCA with 3-NLT as internal standard, with resulting concentrations of 2.5 w/w% and 2 µM, respectively). The samples were subsequently centrifuged at 12,000 rpm for 10 min at 4 °C, and the supernatants were collected for measurement.

3.5.2. KYNA and KYNA1 concentration detection in the plasma and CSF

The KYNA concentrations of the serum samples were quantified on the basis of a slight modification of a literature method [99]. Briefly, we used an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) equipped with fluorescence and a UV detector; the former was applied for the determination of KYNA and the latter for the determination of the internal standard (3-NLT). Chromatographic separations were performed on a Kinetex C18 column, 150 mm x 4.6 mm I.D., 5 µm particle size (Phenomenex Inc., Torrance, CA, USA) after passage through a Security Guard pre-column C18, 4 x 3.0 mm I.D., 5 µm particle size (Phenomenex Inc., Torrance, CA, USA) with a mobile phase composition of 0.2 M zinc acetate/ACN = 95/5 (v/v), the pH of which was adjusted to 6.2 with acetic acid, applying isocratic elution. The flow rate was 1 ml/min and the injection volume was 20 µl. The fluorescence detector was set at excitation and emission wavelengths of 344 and 398 nm. The UV detector was set at a wavelength of 365 nm.

For the determination of KYNA1, a Thermo LCQFleet ion trap mass spectrometer was used equipped with an ESI ion source combined with a Dionex Ultimate3000 HPLC system. The ionization parameters were as follows: heater temperature: 500°C, sheath gas flow rate: 60, auxiliary gas flow rate: 20, spray voltage: 4kV, capillary temperature: 400°C. Chromatographic separations were performed on an Kinetex C18 column, 100 mm × 4.6 mm 2.6 µm particle size (Phenomenex Inc., Torrance, CA, USA) after passage through a Security Guard pre-column C18, 4 x 3.0 mm I.D., 5 µm particle size (Phenomenex Inc., Torrance, CA, USA) with a mobile phase composition of 0.05% aqueous CH_3COOH/ACN = 90/10 (v/v), applying isocratic elution. The flow rate and the injection volume were 1 ml/min and 50 µl, respectively.

3.5.3. Calibration curve and linearity

The calibrants were prepared at 6 different concentration levels, from 1 to 100 nM and 0.5 to 5 µM for KYNA, KYNA1 and 3-NLT, respectively. Three parallel injections of each solution were made under the chromatographic conditions described above. The peak area responses were plotted against the corresponding concentration, and the linear regression computations were carried out by the least square method with the freely available R software. Very good linearity was observed throughout the investigated

concentration ranges for KYNA, KYNA1 and 3-NLT when either fluorescence or UV detection was applied.

3.5.4. Selectivity

The selectivity of the method was checked by comparing the chromatograms of KYNA and KYNA1 and the internal standard for a blank serum and CSF samples and those for a spiked sample. All compounds could be detected in their own selected chromatograms without any significant interference.

3.6. G-protein activity assay

3.6.1. Functional [^{35}S]GTPγS binding assays

The assays were performed according to previous reports, with slight modifications [15,100]. Membrane fractions of rat cortex and striatum were incubated in a final volume of 1 ml at 30 °C for 60 min in Tris-EGTA buffer (pH 7.4) composed of 50 mM Tris-HCl, 1 mM EGTA, 3 mM $MgCl_2$, 100 mM NaCl, containing 20 MBq/0.05 cm^3 [^{35}S]GTPγS (0.05 nM) together with increasing concentrations (10^{-10}-10^{-5} M) of DAMGO, dynorphin 1-13 or Ile^{5,6}-deltorphin II. Total binding (T) was measured in the absence of the opioid ligands, non-specific binding (NS) was determined in the presence of 10 μM unlabeled GTPγS and subtracted from total binding. The difference (T-NS) represents basal activity. Bound and free [^{35}S]GTPγS were separated by vacuum filtration through Whatman GF/B filters with Brandel M24R Cell harvester. The filtration and washing procedure together with radioactivity detection was performed as reported previously [101]. The [^{35}S]GTPγS binding experiments were performed in triplicates and repeated at least three times.

3.7. Data analysis

In the HPLC analysis, the peak area responses were plotted against the corresponding concentration, and the linear regression computations were carried out by the least square method with the freely available R software [102].

Experimental data were presented as means ± S.E.M. in the presence of the applied concentration points in logarithm form. Points were fitted with the professional curve fitting program, GraphPad Prism 5.0 (GraphPad Prism Software Inc., San Diego, CA), using non-linear regression. During the competition binding assays the 'One-site competition' fitting equation was applied to determine the concentration of the competitor ligands that displaced 50% of the radioligand (IC_{50}). The specifically bound opioid radioligands were given in percentage, the total specific binding and the non-specific binding was defined as 100% and 0% respectively. In the [^{35}S]GTPγS binding assays the 'Sigmoid dose-response' fitting equation was used to establish the maximal stimulation or efficacy (E_{max}) of the receptors G-protein, and the potency (EC_{50}) of the stimulator ligand. For better understanding only the E_{max} and logEC_{50} values have been presented in the [^{35}S]GTPγS binding assay result figures, the binding curves were not indicated. Stimulation was given as percent of the specific [^{35}S]GTPγS binding observed over the basal activity, which was settled as 100%. The significance level determined by using unpaired t-test with two-tailed P value statistical analysis in GraphPad Prism 5.0. Since the stimulator ligands were presented in the logarithm form, the curve fitting program could only calculate S.E.M. for the logarithm form of EC_{50} (logEC50) values. At the same time their antilogarithm form has also been indicated on the figures for better understanding. Significance was accepted at the $P < 0.05$ level.

4 RESULTS

4.1. Direct binding affinity measurements of KYNA and KYNA1 on opioid receptors

The binding properties of KYNA and its analogue KYNA1 towards all three classic opioid receptors, MOP, KOP and DOP, were investigated in competition binding experiments using receptor specific tritiated radioactive ligands, namely the MOP receptor specific [^{3}H]DAMGO, the KOP receptor specific [^{3}H]U69,593 and the DOP receptor specific [^{3}H]Ile^{5,6}deltorphin II. The assays were accomplished in CHO cell membranes overexpressing the corresponding opioid receptor.

Neither KYNA, nor KYNA1 had any effect on either opioid receptors specific ligand binding: they both failed to inhibit the total specific binding in case of all three specific opioid radioligand significantly, even at the highest concentrations (Fig. 12). For comparison the unlabeled opioid ligands decreased the binding of their radiolabeled analogues with an EC_{50} of 3.95 nM (DAMGO; Fig. 12A), 7.06 nM (U69,593; Fig. 12B) and 28.67 nM (Ile^{5,6}deltorphin II; Fig. 12C). Thus, according to our competition binding experiments KYNA and KYNA1 did not interact directly with MOP, KOP or DOP receptor.

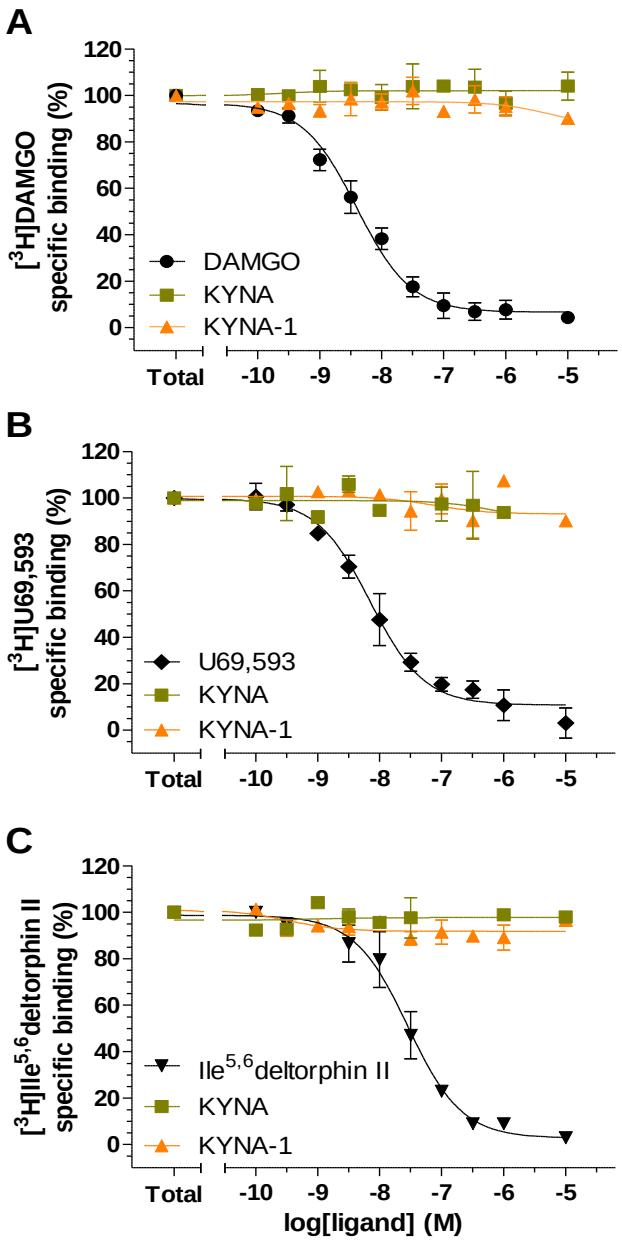


Figure 12. Direct binding affinity measurements of KYNA and its analogue, KYNA1 towards MOP receptor ***(A)***, KOP receptor ***(B)*** and DOP receptor ***(C)*** in competition binding experiments in CHO cell membrane fractions overexpressed with the appropriate opioid receptor. Data are presented as the percentage of specific binding of the appropriate specific opioid radioligand in fixed concentrations (~1 nM) observed in the presence of increasing (10^{-10}-10^{-5} M) concentrations of unlabeled KYNA, KYNA1 and the corresponding opioid receptor specific ligand. "Total" on the X axis refers to the points which did not contain competitor ligands. Points represent means ± S.E.M. for at least three experiments performed in duplicate.

4.2. Opioid and nociceptin receptor G-protein activity measurements after chronic KYNA and KYNA1 treatment in agonist-stimulated [^{35}S]GTPγS binding assays in mice cortex and striatum membranes

Further on we investigated the effect of both compounds on opioid receptor and also nociceptin receptor mediated signaling, namely the G-protein activation of the receptors during agonist stimulation. This was measured in functional [^{35}S]GTPγS binding assays in mice cortex and striatum membranes. Since KYNA and KYNA1 did not affect either of the opioid receptors or nociceptin receptors G-protein activation under *in vitro* (data not shown) conditions, we applied KYNA and KYNA1 chronically at 128 and 200 mg/kg/day (equimolar to each other) respectively to the animals, i.p.

The chronic KYNA treatment (9 days) in mouse cortex significantly inhibited the maximal G-protein activity of MOP receptor (Fig. 13A), without altering the potency of the stimulator ligand DAMGO (Fig. 13B). In contrast chronic KYNA1 treatment had no significant influence on MOP receptor G-protein activity (Fig. 13A) or stimulation in mouse cortex (Fig. 13B). In mouse striatum neither treatment had significant impact on MOP receptor signaling (Fig. 13C and 13D).

In mouse cortex the KOP receptor G-protein maximal activity (E_{max}) was significantly attenuated during dynorphin 1-13 stimulation after both KYNA and KYNA1 chronic administration (Fig. 14A), but the potency of the stimulator ligand was again unaltered (Fig. 14B). In contrast only KYNA1 attenuated KOP receptor G-protein maximal efficacy in the striatum (Fig. 14C), but the potency of the activator ligand did not changed (Fig. 14D).

The DOP receptor G-protein maximal stimulation was unaffected by either KYNA or KYNA1 chronic administration in the cortex (Fig 15A), however unlike KYNA treatment, KYNA1 enhanced the potency of the Ile^{5,6}deltorphin II stimulator ligand, (Fig 15B). In the striatum only KYNA treatment could reduce the maximal efficacy of DOP receptor G-protein significantly (Fig. 15C), while the EC_{50} value of Ile^{5,6}deltorphin II was not affected by either chronic treatment (Fig. 15D).

Unlike KYNA1, KYNA treatment significantly inhibited the maximal effectiveness of the NOP receptor G-protein in mouse cortex (Fig. 16A), but the potency of the activator ligand was not affected by KYNA (Fig. 16B). However KYNA1, similarly to Ile^{5,6}deltorphin II, enhanced the potency of nociceptin 1-13 (Fig. 16B). In the striatum,

similar to MOP receptor, no significant alterations were observed in NOP receptor signaling after KYNA or KYNA1 chronic treatment (Fig. 1C and 16D).

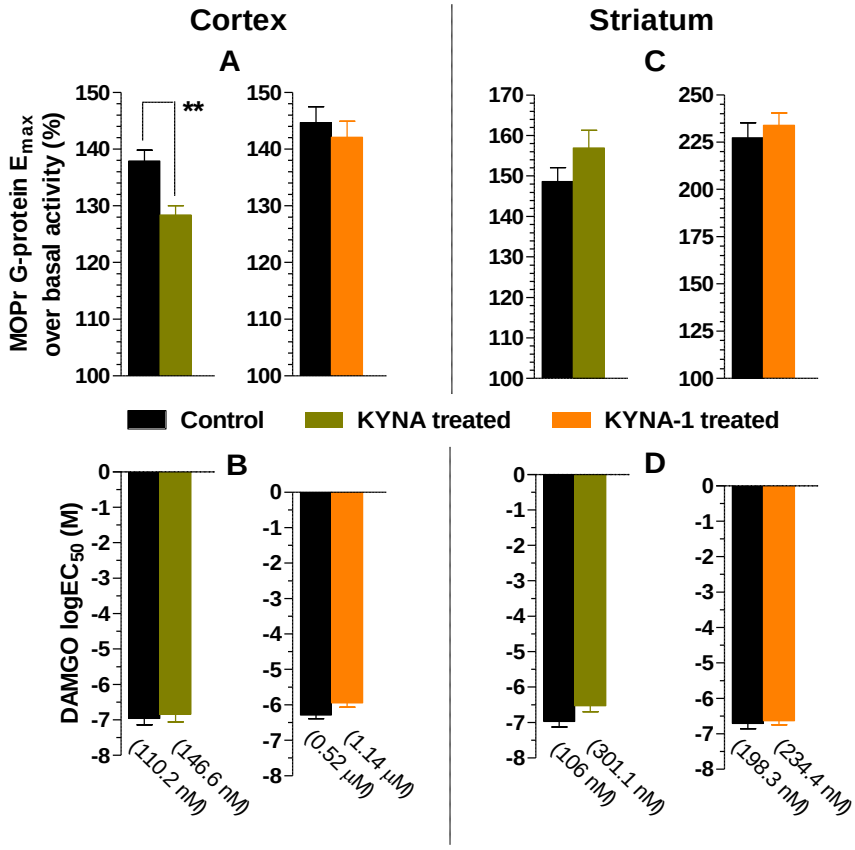


Figure 13. The effect of chronically administered KYNA and KYNA1 on the maximal efficacy or E_{max} of MOPr G-protein over basal activity ***(A and C)*** and the potency of DAMGO indicated by the $logEC_{50}$ value ***(B and D)*** in DAMGO-stimulated [^{35}S]GTPγS binding assays in mice cortex ***(A and B)*** and striatum ***(C and D)*** membranes. MOP receptor was activated by increasing (10^{-10}-10^{-5} M) concentrations of DAMGO, points were fitted as described under the *Data analysis* section, afterwards based on the binding curves the E_{max} and $logEC_{50}$ values were calculated. Columns represent means ± S.E.M. for at least three experiments performed in triplicate. In brackets the antilogarithm form of $logEC_{50}$ (EC_{50}) values are presented. The significance level of $logEC_{50}$ values are indicated by asterisks (unpaired t-test, two-tailed P value). **: $P < 0.01$.

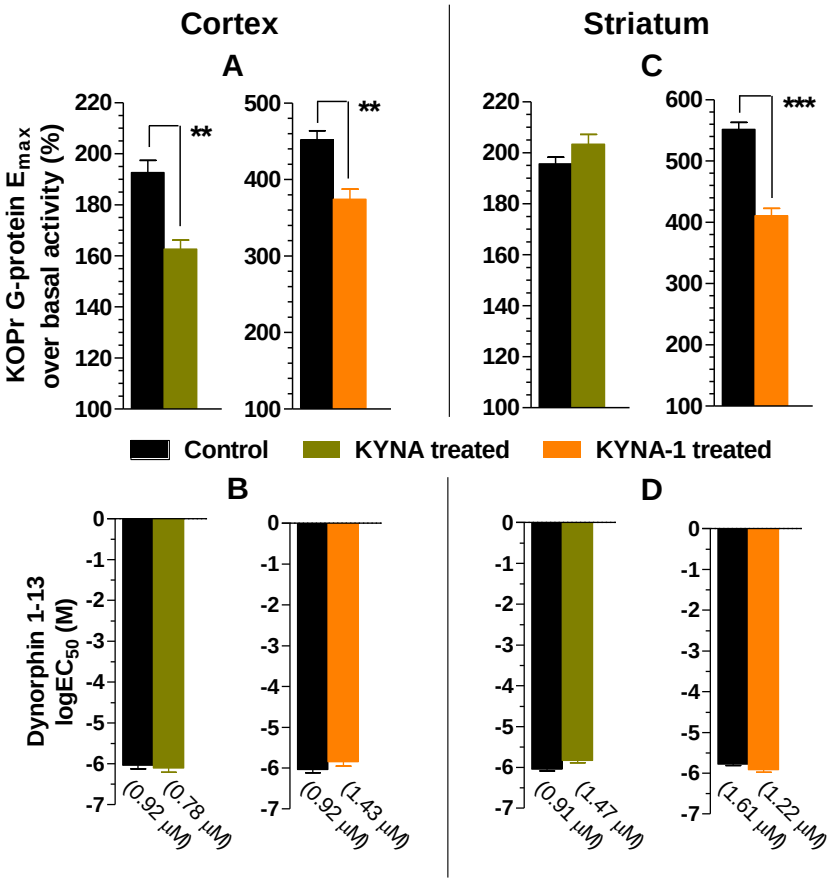


Figure 14. The effect of chronically administered KYNA and KYNA1 on the maximal efficacy or E_{max} of KOPr G-protein over basal activity **(A** and **C)** and the potency of dynorphin 1-13 indicated by the $logEC_{50}$ value **(B** and **D)** in dynorphin 1-13-stimulated [^{35}S]GTPγS binding assays in mice cortex **(A** and **B)** and striatum **(C** and **D)** membranes. KOP receptor was activated by increasing (10^{-10}-10^{-5} M) concentrations of dynorphin 1-13, points were fitted as described under the *Data analysis* section, afterwards based on the binding curves the E_{max} and $logEC_{50}$ values were calculated. Columns represent means ± S.E.M. for at least three experiments performed in triplicate. In brackets the antilogarithm form of $logEC_{50}$ (EC_{50}) values are presented. The significance level of E_{max} values are indicated by asterisks (unpaired t-test, two-tailed P value). ***: $P < 0.001$; **: $P < 0.01$.

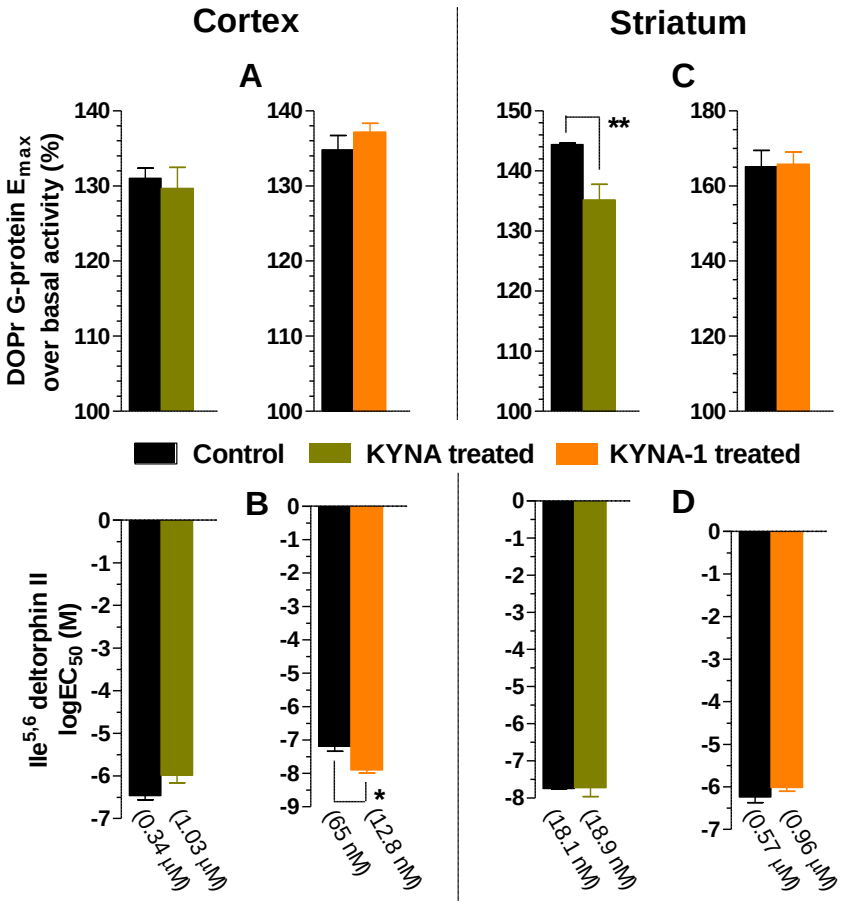


Figure 15. The effect of chronically administered KYNA and KYNA1 on the maximal efficacy or E_{max} of DOPr G-protein over basal activity **(A** and **C)** and the potency of Ile^{5,6}deltorphine II indicated by the logEC_{50} value **(B** and **D)** in Ile^{5,6}deltorphine II-stimulated [^{35}S]GTPγS binding assays in mice cortex **(A** and **B)** and striatum **(C** and **D)** membranes. DOP receptor was activated by increasing (10^{-10}-10^{-5} M) concentrations of Ile^{5,6}deltorphine II, points were fitted as described under the *Data analysis* section, afterwards based on the binding curves the E_{max} and logEC_{50} values were calculated. Columns represent means ± S.E.M. for at least three experiments performed in triplicate. In brackets the antilogarithm form of logEC_{50} (EC_{50}) values are presented. The significance level of E_{max} and logEC_{50} values are indicated by asterisks (unpaired t-test, two-tailed P value). **: $P < 0.01$; *: $P < 0.05$.

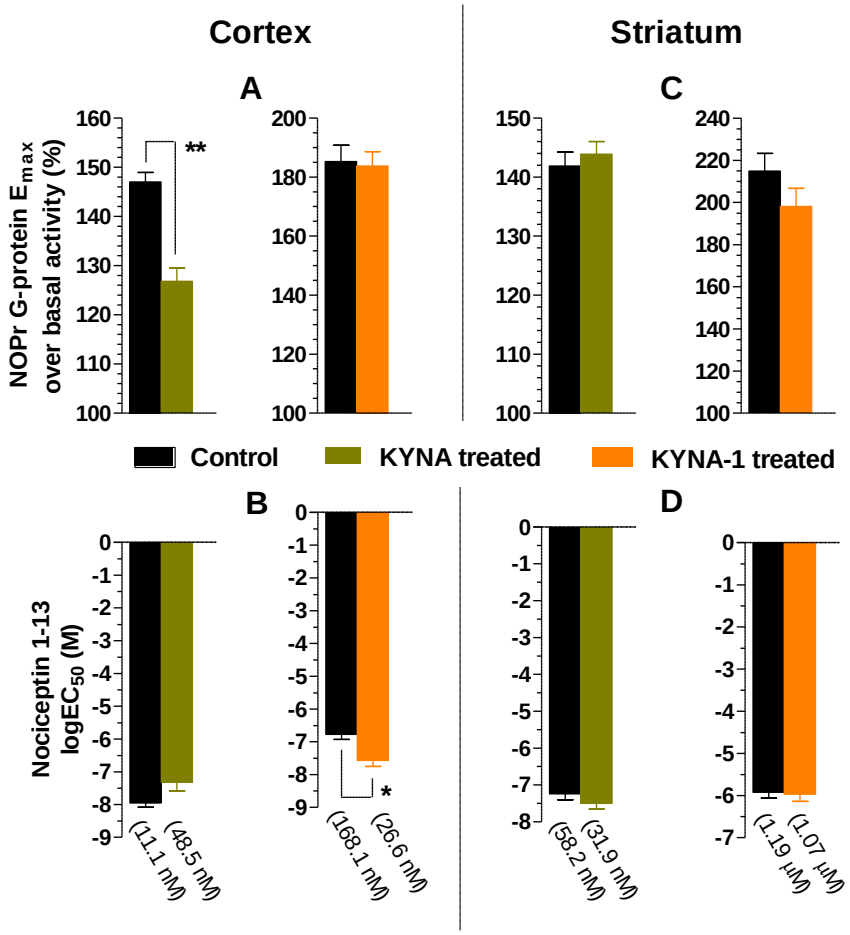


Figure 16. The effect of chronically administered KYNA and KYNA1 on the maximal efficacy or E_{max} of NOPr G-protein over basal activity ***(A and C)*** and the potency of nociception 1-13 indicated by the $logEC_{50}$ value ***(B and D)*** in nociception 1-13-stimulated [^{35}S]GTPγS binding assays in mice cortex ***(A and B)*** and striatum ***(C and D)*** membranes. NOPr was activated by increasing (10^{-10}-10^{-5} M) concentrations of nociceptin 1-13, points were fitted as described under the *Data analysis* section, afterwards based on the binding curves the E_{max} and $logEC_{50}$ values were calculated. Columns represent means ± S.E.M. for at least three experiments performed in triplicate. In brackets the antilogarithm form of $logEC_{50}$ (EC_{50}) values are presented. The significance level of E_{max} and $logEC_{50}$ values are indicated by asterisks (unpaired t-test, two-tailed P value). **: $P < 0.01$; *: $P < 0.05$.

Receptor	Brain area	KYNA		KYNA1	
		G-protein efficacy	Ligand potency	G-protein efficacy	Ligand potency
MOP	Ctx.	↓	∅	∅	∅
	Str.	∅	∅	∅	∅
KOP	Ctx.	↓	∅	↓	∅
	Str.	∅	∅	↓	∅
DOP	Ctx.	∅	∅	∅	↑
	Str.	↓	∅	∅	∅
NOP	Ctx.	↓	∅	∅	↑
	Str.	∅	∅	∅	∅

Table 2. Summarizing the effect of chronic KYNA and KYNA1 treatment on opioid receptor mediated G-protein efficacy and ligand potency values. Ctx.: cortex, str.: striatum, ↑: significant enhancement, ↓: significant inhibition, ∅: no significant effect.

4.3. The effect of acute KYNA and KYNA1 treatment alone and with the combination of MK-801 on agonist-mediated opioid receptor G-protein activation in rat cortex and striatum

Opioid agonist-stimulated G-protein activation was measured in [^{35}S]GTPγS binding assays in cerebral cortex and striatum homogenates of saline (control) or KYNA/KYNA1-treated animals. MOP receptors were activated by the use of DAMGO. KOP receptor activation was measured in the presence of dynorphin (1-13), while DOP receptor stimulation was accomplished with Ile^{5,6}-deltorphin II , a synthetic DOP receptor peptide agonist ligand. All opioid agonist ligands were applied in increasing concentrations to determine the maximum efficacy (E_{max}) of the opioid receptors G-protein and ligand potency (EC_{50}). We also investigated the effect of NMDA receptor specific antagonist MK-801 on opioid receptor activity alone and in the presence of KYNA and KYNA1 (combined treatments).

In the cortex area, 296 mg/kg KYNA1 treatment after 30 minutes significantly decreased the maximum efficacy of the MOP receptor, the reduction was nearly 50% ($P < 0.001$, df: 6; Fig. 17A). This effect was slightly reversed by 1 mg/kg MK-801. 189 mg/kg KYNA treatment alone and in combination with 1 mg/kg MK-801 did not change the MOP receptor mediated G-protein activity (Fig. 17A). Interestingly 1 mg/kg MK-801 *per se*

strongly reduced MOP receptor activity after 15 and 45 minutes in the cortex compared to control (both $P < 0.01$, df: 6; Fig. 17A) and after 45 minutes compared to KYNA treatment alone ($P < 0.05$, df: 6; Fig. 17A). The potency of DAMGO was not altered in either treatment conditions (data not shown).

Interestingly, 30 minutes KYNA1 treatment resulted an opposite effect in KOP receptor G-protein activity in the cortex compared to MOP receptor: the maximum efficacy of the KOP receptor significantly increased compared to control ($P < 0.05$, df: 6; Fig. 17B). This enhancement was reduced by MK-801 in the combined treatments, but the reduction was statistically not significant (Fig. 17B). KYNA did not alter KOP receptor maximum efficacy significantly neither MK-801 in either treatment conditions (Fig. 17B). The potency of the KOP receptor stimulator ligand dynorphin 1-13 was not altered by either KYNA, KYNA1 or MK-801, similarly to DAMGO (data not shown).

In contrast, the DOP receptor G-protein activity did not show any significant difference in the cortex area after 30 minutes KYNA and KYNA1 or 15 and 45 minutes MK-801 acute treatments (data not shown). However, the potency of the DOP receptor agonist $Ile^{5,6}$-deltorphin II significantly enhanced after 30 minutes KYNA1 treatment, which was reversed to control level by MK-801 co-administered with KYNA1 ($P < 0.01$, df: 6; Fig. 17C).

In the striatum in case of MOP and KOP receptor maximum G-protein efficacy was reduced by 30 minutes KYNA (MOP receptor: $P < 0.01$, KOP receptor: $P < 0.001$, df: 6; Fig. 17D and E), KYNA1 treatments (both receptor: $P < 0.001$, df: 6, Fig. 17D and E) and also both (15 and 45 minutes) MK-801 treatments alone (MOP and KOP receptor, 15 and 45 min.: $P < 0.001$, df: 6; Fig.17D and E). MK-801 in combination with KYNA1 and KYNA did not cause any significant alterations in G-protein activity compared to their treatments alone in MOP receptor (Fig. 17D). In case of KOP receptor co-administering MK-801 resulted a noticeable increase in the E_{max} values compared to KYNA1 and KYNA alone, although the difference was not significant (Fig. 17E). Interesting to note, that KYNA1 treatment alone reduced KOP receptor G-protein activity more effectively than the 15 and 45 minutes MK-801 treatment (15 and 45 min.: $P < 0.05$, df: 6; Fig. 17E). In the DOP receptor only 30 minutes KYNA and 45 minutes MK-801 treatments caused a significant reduction with a similar degree in the receptors G-protein activity ($P < 0.01$, df: 6; Fig. 17F). Additionally in the striatum neither of the potency of the opioid receptor selective agonist ligands was altered significantly by KYNA1, KYNA or MK-801 (data not shown).

After 2 hours treatment of KYNA1 and KYNA, no significant changes were observed in either of the opioid receptors G-protein activity or ligand potency regardless of the brain areas (data not shown).

Receptor	**Brain area**	**KYNA (30′)**		**KYNA1 (30′)**		**MK-801 (15′)**		**MK-801 (45′)**	
		G-protein efficacy	Ligand potency	G-protein efficacy	Ligand potency	G-protein efficacy	Ligand potency	G-protein efficacy	Ligand potency
MOP	Ctx.	∅	∅	↓	∅	↓	∅	↓	∅
	Str.	∅	∅	↓	∅	↓	∅	↓	∅
KOP	Ctx.	∅	∅	↑	∅	∅	∅	↓	∅
	Str.	↓	∅	↓	∅	↓	∅	↓	∅
DOP	Ctx.	∅	∅	∅	↑	∅	∅	∅	∅
	Str.	↓	∅	∅	∅	∅	∅	↓	∅

Table 3. Summary and comparison of acute *in vivo* effect KYNA, KYNA1 and MK-801 treatment on opioid receptors mediated G-protein activity and ligand potency. Ctx.: cortex, Str.: striatum, ↑: significant enhancement, ↓: significant inhibition, ∅: no significant effect.

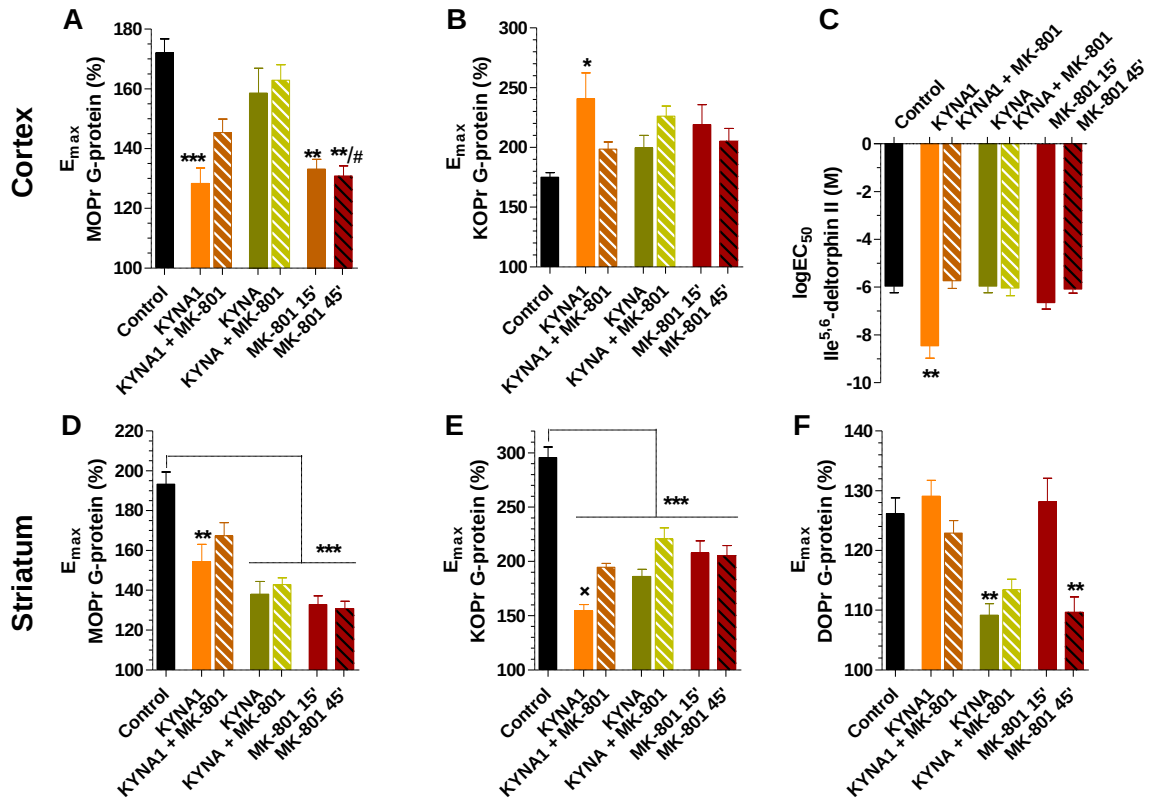


Figure 17. The acute **(30 min, *in vivo*)** effect of KYNA, KYNA1 alone and in combination with MK-801 on agonist-stimulated opioid receptor G-protein activity in [^{35}S]GTPγS binding assays performed in rat cortex (A-C) and striatum (D-F). Each animal received a single i.p. injection of 296 mg/kg KYNA1 or 189 mg/kg KYNA dosage, while MK-801 was administered in 1 mg/kg i.p., as described in section 3.2.2.1. **A**, **B** and **D-F** represents the maximal efficacy (E_{max}) over basal activity (100 %) of MOP and KOP receptors G-protein in cortex (**A** and **B**) and striatum (**D** and **E**) and of DOP receptor G-protein in striatum (**F**), respectively. **C** represents the potency ($logEC_{50}$) of the DOR specific agonist Ile^{5,6}-deltorphin II. Columns represent means ± S.E.M. for at least three experiments performed in triplicate. The calculation of E_{max} and EC_{50} values can be seen under section 3.7. *****: indicates the significance level of E_{max} and $logEC_{50}$ values compared to control. #: indicates the significance level of E_{max} values compared to KYNA treatment alone. ×: indicates the significant reduction of E_{max} value after KYNA1 injection *per se* compared to MK-801 15 and 45 minutes treatment alone. One-way ANOVA with Bonferroni's Multiple Comparison was used for statistical analysis. ***: $P < 0.001$, **: $P < 0.01$, */#/×: $P < 0.05$.

4.4. KYNA and KYNA1 plasma and CSF concentrations after 30 minutes administration

Since the opioid receptor G-protein activity in the cortex and striatum was only altered after 30 minutes of KYNA1 and KYNA treatment, we measured the concentration of these two compounds in the cerebrobrospinal fluid (CSF) and compared it to their concentration in the blood plasma. The treatment conditions were the same as described in section 3.2.2.1 Additionally the endogenous KYNA plasma concentration was also determined for control. KYNA1 and KYNA CSF and plasma concenrations were measured with HPLC.

30 minutes following KYNA treatment the concentration of KYNA dramatically increased compared to endogenous KYNA plasma concentrations as expected ($P < 0.001$, df: 4; Fig. 18). It reached to 630 µM in the plasma (endogenous KYNA plasma concentration: 0.2 µM), while this was reduced to 48 µM in the CSF ($P < 0.001$, df: 4; Fig. 18). KYNA1 displayed a siginificantly lower (124 µM) concentration in the plasma compared to KYNA ($P < 0.001$, df: 4; Fig. 18), and 65 µM in the CSF, which is very close to that of KYNA in the CSF.

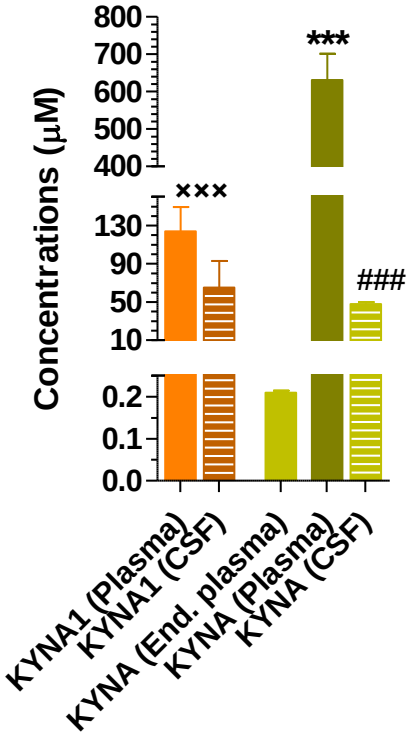


Figure 18. The blood plasma and CSF concentration levels of KYNA1 and KYNA after 30 minutes of 296 mg/kg (KYNA1) and 189 mg/kg (KYNA) acute administration of the compounds**.** Additionally, the endogenous KYNA plasma concentrations (End. plasma) is also indicated for control. For further information regarding to animal treatments and HPLC analysis see under section 3.2.2.1 and 3.5 respectively). Columns represent means ± S.E.M. for at least three experiments. *****: indicates the significant increase in concentrations of KYNA after treatment compared to endogenous KYNA levels in the plasma. #: indicates the significant decrease of KYNA CSF concentration levels compared to plasma after treatment. ×: indicates the significant difference between KYNA and KYNA1 plasma concentration levels. One-way ANOVA with Bonferroni's Multiple Comparison was used for statistical analysis. ***/###: $P < 0.001$.

4.5. The effect of *in vitro* administered KYNA1, KYNA and MK-801 on agonist-mediated opioid receptor G-protein activation in isolated rat cortex and striatum slices

In these studies freshly prepared brain slices of rat striatum and cortex were prepared and treated with KYNA, KYNA1 and MK-801 in an isolated organ bath. Following 30 min incubation tissue specimens were further processed by homogenisation for G-protein activity studies as described earlier (section 3.4.2). MOP, KOP and DOP G-protein activity was measured the same way as in *in vivo* studies (see section 3.6.1).

In the *in vitro* studies of isolated cortex slices, the maximum G-protein efficacy of MOP receptor significantly decreased nearly 60% in the cortex after 200 µM KYNA1 treatment ($P < 0.001$, df: 3), which was significantly lower compared to the reducing effect of 200 µM KYNA ($P < 0.001$, df: 3; Fig. 19A). The results in 50 µM MK-801 treated cortex were very similar to KYNA1 treated samples, the reduction reached 70 % ($P < 0.001$, df: 3; Fig. 19A). The potency of DAMGO on MOP receptor was not altered after either *in vitro* treatments (data not shown). Similar results were observed in KOP receptor G-protein maximum efficacy in the cortex after KYNA1 and MK-801 treatments, but the inhibitory effects were much more prominent in both cases ($P < 0.001$, df: 3; Fig. 19B). Interestingly, KYNA did not alter the maximum efficacy of KOP receptor in the cortex (Fig. 19B) and similarly to MOP receptor agonist, the potency of the KOP receptor agonist ligand (dynorphin 1-13) remained unaltered after in case of all *in vitro* treatments (data not shown). The DOP receptor G-protein activity was reduced signficanlty after KYNA, KYNA1 and MK-801 treatments as well ($P < 0.001$, df: 3; Fig. 19C), moreover the ligand potency of $Ile^{5,6}$-deltorphin II was increased after KYNA1 treatment ($P < 0.05$, df: 3; Fig. 19D).

In the striatum the *in vitro* treatments showed very similar results compared to cortex. All three opioid receptor G-protein activity was reduced by all three compounds compared to control. MOP and KOP receptor G-protein activity was reduced in a similar extent by the compounds and compared to the cortex (KYNA1: $P < 0.001$, KYNA: $P < 0.05$, MK-801: $P < 0.001$, df: 3; Fig. 19E and F). In case of DOP receptor the reduction was less robust than in the cortex region, but KYNA1 reduced the E_{max} value with comparable extent to KYNA and MK-801 similarly to the cortex (KYNA1: $P < 0.01$, KYNA: $P < 0.05$, MK-801: $P < 0.05$, df: 3; Fig. 19G). The main difference in the striatum is that the DOP receptor agonist potency was not altered by either compound (data not shown). Additionally, KYNA1 and MK-801 reduced MOP and KOP receptor maximum G-protein efficacy more effectively than KYNA ($P < 0.001$, df: 3; Fig. 19E and F).

In summary the *in vitro* treatments displayed a reducing effect in receptor G-protein signaling regardless of the opioid receptor type and brain region (except for KYNA in KOP receptor) (Table 4). Also the levels of inhibition were very similar in the two brain regions within the opioid receptor types. Additionally, KYNA1 and MK-801 displayed stronger effects than KYNA in case of MOP and KOP receptor in both cortex and striatum.

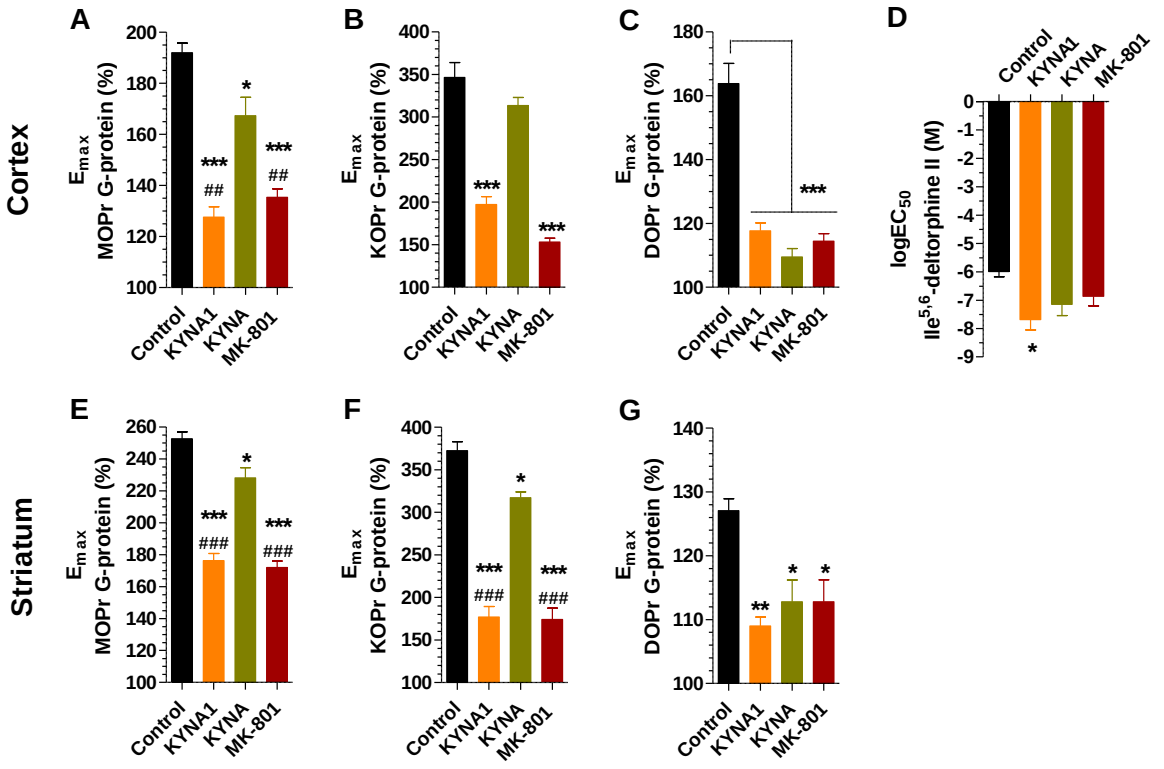


Figure 19. Agonist-stimulated opioid receptor G-protein activity in [^{35}S]GTPγS binding assays performed in rat cortex (A-D) and striatum (E-G) homogenised brain slices after acute *in vitro* treatment of KYNA1, KYNA and MK 801. KYNA1 and KYNA were administered on cortex and striatum brain slices in 200 µM, while MK 801 in 50 µM concentrations. Samples were treated for 30 minutes in isolated organ baths as described in section 3.2.2.2. **A-C** and **E-G** represents the maximal efficacy (E_{max}) over basal activity (100 %) of MOP, KOP and DOP receptors G-protein in cortex (**A-C**) and striatum (**E-G**). **D** represents the potency ($logEC_{50}$) of the DOR specific agonist Ile^{5,6}-deltorphin II. Columns represent means ± S.E.M. for at least three experiments performed in triplicate. The calculation of E_{max} and EC_{50} values is discussed under section 3.7. *: indicates the significance level of E_{max} and $logEC_{50}$ values compared to control. $^{\#}$: indicates the significance level of E_{max} values of KYNA1 and MK-801 compared to KYNA. One-way ANOVA with Bonferroni's Multiple Comparison was used for statistical analysis. ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$.

Receptor	Brain area	KYNA		KYNA1		MK-801	
		G-protein efficacy	Ligand potency	G-protein efficacy	Ligand potency	G-protein efficacy	Ligand potency
MOP	Ctx.	↓	∅	↓	∅	↓	∅
	Str.	↓	∅	↓	∅	↓	∅
KOP	Ctx.	∅	∅	↓	∅	↓	∅
	Str.	↓	∅	↓	∅	↓	∅
DOP	Ctx.	↓	∅	↓	↑	↓	∅
	Str.	↓	∅	↓	∅	↓	∅

Table 4. Summary and comparison of acute *in vitro* effect KYNA, KYNA1 and MK-801 treatment on opioid receptors mediated G-protein activity and ligand potency. Ctx.: cortex, Str.: striatum, ↑: significant enhancement, ↓: significant inhibition, ∅: no significant effect.

5 DISCUSSION

In this study we have shown for the first time that KYNA1 and KYNA not only alters opioid receptor function after chronic treatment [101], but also after acute administration in a tissue and receptor specific way. Moreover, the effects were modified by MK-801, a selective NMDA receptor antagonist, indicating that the changes might be mediated through this receptor. The effects seen *in vivo* were found also in acute *in vitro* experiments in isolated cortex and striatum slices. Our results further support previous findings showing the effect of KYNA on the opioid receptors activity [67, 73- 75, 103 , 104].

When compared the results of chronic (128 and 200 mg/kg/day, i.p., for 9 days) and acute treatments, the data accords just in few cases (DOP receptor in cortex and striatum and KOP receptor in striatum by KYNA treatment). In the other cases, the effects were opposite (KYNA1 treatment on KOP receptor in the striatum), or it did not cause any significant alterations when compared to the appropriate acutely treated group (KYNA1 on MOP receptor). Nevertheless, the molecular mechanisms behind these effects might be due to altered opioid receptor G-protein gene or protein expression or receptor sensitivity, which is most probably mediated through KYNA/KYNA1 specific receptors since they do not bind directly to opioid receptors [101]. The NMDA receptor has been demonstrated to bind KYNA - although with low micromolar affinity [49] also, the interaction of NMDA and opioid receptors have been demonstrated in many levels [105]. Morevoer, a functional interaction and co-localization has been described between the MOP receptor and NMDA receptor in the NAcc shell – which is part of the ventral striatum - with electrophysiological [106] and anatomical studies [107] and with immunocytochemical labeling [108-109]. Thus in order to investigate the possible role of the NMDA receptor we applied MK-801 (also known as dizocilpine) [110], a highly NMDA receptor selective antagonist [111, 112]. MK-801 has also been demonstrated to alter opioid receptor-mediated effects [113, 67], however it does not bind directly to opioid receptors [112] similarly to KYNA.

MK-801 alone behaved similarly as KYNA1 or KYNA (Fig. 17A and D). MK-801 in combination with KYNA1 or KYNA displayed a somewhat less pronunced reduction opioid receptor G-protein activity in certain groups (KYNA1+MK-801: Fig. 17A and D; KYNA+MK-801: Fig. 17F) compared to KYNA1 or MK-801 alone. The results might be explained by the impairment of KYNA1/KYNA and MK-801 individual activity when administered together, since they exerted similar effects alone, indicating an NMDA receptor mediated effect. The same explanation arises in case of MOP and KOP receptors

expressed in the striatum, where KYNA and MK-801 alone or in combination reduced G-protein signaling (Fig. 17D and E). In case of DOP receptor in the cortex, the enhanced agonist (Ile5,6-deltorphin II) ligand potency followed by 30 minutes KYNA1 treatment was reduced to control levels by MK-801 pretreatment (Fig. 17C). Interestingly, MK-801 alone did not cause any alterations in ligand potency following 15 or 45 minute administration (Fig. 17C), which indicates that MK-801 inhibited the effect of KYNA1 through NMDA receptor. Furthermore, it also shows that the enhanced DOP receptor agonist ligand potency is a KYNA1 specific action, whereas the attenuated opioid receptor G-protein activity in the cortex (MOP and DOP receptor) and striatum (all three opioid receptors) are also MK-801 related.

AMPA and kainate receptors are also a direct targets for KYNA and there is evidence that they can also interact with opioid receptors. Their interactions have been studied mainly in terms of opioid addiction and has been described so far in the amygdala and hippocampus [114, 115], which are outside the point of our examined brain regions. To the best of our knowledge there are no current studies so far describing AMPA/kainate-opioid receptor interactions in the striatum or cortex. However, it does not exclude the possibility and may be an alternative mechanism which through KYNA/KYNA1 alters opioid receptor function.

Another possible explanation for our results is the connection between GPCR ion-dependency and glutamate receptors. Sodium ion has been long known to affect allosterically opioid receptor binding [116]. In fact, a distinctive sodium binding pocket was discovered recently on MOP and DOP receptors [117, 118]. Hence, ionotropic glutamate receptors (NMDA, AMPA and kainate receptors etc.) being ligand-gated non-selective cation channels, may alter sodium ion concentrations induced by KYNA/KYNA1, which than may trigger an altered opioid receptor binding. This could be a plausible explanation for the increased DOP receptor agonist potency after KYNA1 treatment in the cortex (Fig. 17C).

Since only the 30 minutes duration time showed significant results (Table 3), we carried out HPLC measurements of KYNA1 and KYNA concentration levels in the CSF following 30 minute treatment. As expected the KYNA CSF concentration levels dramatically reduced compared to plasma levels, while in case of KYNA1 there was only a minor difference (Fig. 18). This proves that KYNA1 passes through the blood-brain barrier more easily than KYNA, which is in agreement with previous studies [90].

Additionally, KYNA1 concentrations were significantly lower in plasma compared to KYNA indicating that KYNA1 might have been metabolized to KYNA.

The observed effect after 30 minutes *in vivo* KYNA1 treatment might be at some part KYNA related. To examine this possibility G-protein activity studies were carried out in cortex and striatum slices treated *in vitro* with KYNA1, KYNA and MK-801 in isolated organ baths for 30 minutes. With this setup we can exclude or at least minimize the peripheral metabolism and elimination of KYNA1 and also exclude the BBB from the system, yet again the possible receptor-receptor interactions can remain intact. Accordingly, KYNA1 displayed the same effect as in *in vivo* experiments (similar to KYNA and MK-801), thus KYNA1 itself does affect opioid receptor G-protein activity. However, in case of KOP receptor in the cortex, the effect was opposite compared to *in vivo* experiments (Fig. 19B vs. 17B). Additionally, in some cases the in vitro results showed significant alterations where the *in vivo* setup did not. For instance, in the *in vivo* experiments KYNA1 did not alter the G-protein activity of DOP receptors expressed in the cortex, whereas *in vitro* this parameter was reduced (Fig. 19C vs. 17C).

These differences between *in vivo* and *in vitro* results might be due to rapid peripheral metabolism of the compounds and the presence of the BBB. However, the main effect - which is decreasing the activity of opioid receptors - can be seen in both experimental setups. The different levels of G-proteins or receptors could be another possible explanation. The most striking result was the increase of KOP G-protein activity, which was only observed in the cortex following *in vivo* KYNA1 treatment (Fig 17B). It has been demonstrated that a bolus i.p. injection of a very high dose of KYNA can cause a decrease in the cerebral blood flow, which reduction was more significant in the cortex area (Varga et al., unpublished). KOP receptors are known to contribute to neuroprotection in animal models of cerebral ischemia [119, 120]. The increased KOP receptor activity induced by KYNA1 treatment might be due to a compensatory mechanism of the KOP receptor, representing its neuroprotective effect against reduced cortex blood flow. Furthermore, during this mechanism KYNA1 might be converted to KYNA in the cortex, exerting its vasoconstrictor effect at high dosage. KYNA treatment did not affect KOP receptor activity in the cortex most probably because of its poor BBB penetration. In other words, KYNA did not reach the necessary concentration levels in the cortex to reduce the blood flow in this area, thus it did not trigger the compensatory mechanism of the KOP receptor system.

6 CONCLUSION

The present study for the first time provides evidence for an indirect, NMDA receptor-mediated mechanism regarding the effects of KYNA/KYNA1 on opioid receptor function at the receptor-G-protein level. Thus KYNA and KYNA1 might be possible drug candidates for controlling the activity of the opioid system via the NMDA receptor, for instance, during opioid withdrawing in addiction therapy or pain management.

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Reza Samavati
Szeged, May 2017

APPENDICES

Appendix A: Summary of the Ph.D. thesis in Hungarian

Appendix B: Off-prints of thesis related publications

Appendix A: Summary of the Ph.D. thesis in Hungarian

A kinurénsav és amid analógja, mint lehetséges gyógykészítmények az opioid rendszer aktiváció szabályozására

Ph.D. értekezés összefoglalója

BEVEZETÉS

A kinurenin útvonal, mely jelen van az asztrocitákban, neuronokban, mikro- és oligodendrogliában, makrofágokban, endotél és dendrit sejtekben a központi idegrendszeren belül, fontos útvonala a triptofán katabolizmusának. A triptofán átalakulhat L-kinureninné, mely tovább metabolizálódik neuroprotektív kinurénsavvá (KYNA). Klinikai adatok azt mutatják, hogy a KYNA és annak metabolitjai fontos szerepet játszanak számos neurológiai rendellenesség patogenezisében, úgy mint a Huntington-kór, Parkinson-kór, epilepszia és ischaemiás stroke. A KYNA egy nemszelektív aktiváló aminósav receptor (mint az NMDA receptorok) antagonista, részt vesz a glutamát neurotranszmisszióban. Mivel a KYNA részt vesz az endogén protektív mechanizmusban, megfelelő célpontja lehet a neurológiai betegségek kezelését célzó gyógyszerészeti fejlesztéseknek. Továbbá a G-fehérje kapcsolt receptorok (GPCR) és a KYNA kölcsönhatását már leírták.

Az opioid rendszer fájdalomcsillapításban játszott szerepe jól ismert. A rendszer három opioid receptorból áll: mü, kappa és delta (MOP, KOP és DOP). Az opioid receptorok a GPCR családhoz tartoznak és többnyire a Gi/o típusú G-fehérjéhez kapcsolódnak. Az opioid receptorok a gasztrointesztinális traktusban, a gerincvelőben és nagy mennyiségben az agyi kortexben és a striatum régióiban expresszálódnak. Korábbi tanulmányunkban leírtuk, hogy a KYNA és analógja, a KYNA1 (amelynek kationos centruma van a C-2 oldal láncon, SZR72-ként is ismert) nem kötődik közvetlenül az opioid receptorokhoz, de krónikus kezelés esetén szignifikáns változást okoztak a receptor funkcióban egér kortexben és striatumban.

Másrészről feltételeztük, hogy a KYNA és a KYNA1 befolyásolhatja az opioid receptor funkciót a kolokalizált NMDA receptoron keresztül. Elsőként vizsgáltuk a KYNA akut hatását az opioid receptor funkcióra az NMDA receptor lehetséges bevonásával, összehasonlítva a KYNA1-gyel. Patkányokat kezeltünk akut módon egyszeri dózisú KYNA-val és KYNA1-gyel intraperitoniálisan, önmagukban illetve az MK-801, NMDA receptor antagonistával kombinálva. Továbbá mértük a KYNA és a KYNA1 plazma és gerincvelői folyadékbeli koncentrációit akut kezelés után. Végül, izolált patkány agy szöveteket kezeltünk *in vitro* KYNA-val, KYNA1-gyel és MK-801-gyel, hogy kizárjuk a véragygátat és a KYNA és a KYNA1 perifériás metabolizmusát. A kortex és striatum mintákat opioid receptor vezérelt G-fehérje funkcionális tesztekben vizsgáltuk.

CÉLKITŰZÉSEK

Minden fent említett tanulmány azt mutatja, hogy bizonyára van kapcsolat a KYNA és az opioid rendszer aktivitása között. Mióta felmerült, hogy mind a KYNA mind az opioid rendszer neuroprotektív szerepet játszik kóros körülmények között, fontos lenne a KYNA opioid rendszerre gyakorolt lehetséges reakcióinak és hatásának vizsgálata. De ezidáig nem létezik összefüggő és összpontosított tanulmány erről a kapcsolatról. Jelen dolgozatban jellemezzük a KYNA és KYNA1 kötési tulajdonságait mind a három opioid receptoron kompetitív kötési vizsgálatban radiojelzett receptor specifikus opioid ligandokkal.

Az értekezés tanulmányának céljai a következők:

- Megmérni a KYNA és a KYNA1 kötési affinitását az opioid rendszeren kompetíciós kötési tesztben opioid receptor specifikus radioligandokkal overexpresszáló CHO (Chinese Hamster Ovary) sejt membránokban a megfelelő opioid receptorokkal.
- Megvizsgálni az opioid receptor G-fehérje aktivitását, KYNA és KYNA1 detektálása *in vivo* és *in vitro* a GPCR jelátvitel kezdeti szakaszában [^{35}S]GTPγS funkcionális méréssel.

- Megvizsgálni az opioid receptor G-fehérje aktivitását az egerek KYNA-val és KYNA1-gyel való krónikus kezelése után [^{35}S]GTPγS funkcionális méréssel.
- Megmérni a KYNA-val és KYNA1-gyel történt akut kezelés hatását funkcionális [^{35}S]GTPγS kísérletekben patkány agyi membránokban.
- Feltárni a KYNA és a KYNA1 működési mechanizmusát az opioid rendszer aktivitásának szintjén *in vivo* és *in vitro* kísérletekben.
- Megvizsgálni a lehetséges kölcsönhatásokat az opioiod receptorok és az NMDA receptorok között.

MÓDSZEREK

Állatok

Krónikus kezelés

C57/B nőstény egereket használtunk a kísérletekben. Három csoportra osztottuk őket: kontrol (0,9 %-os szalin), KYNA (128 mg/kg/nap) és KYNA1 csoport (200 mg/kg/nap). A vegyületeket intraperitoneálisan kilenc napon át kapták. A kilencedik napon, altatást követően az agy eltávolításra került és a kortex és a striatum kimetszésre került, majd -80 ^{o}C-on tároltuk.

Akut kezelés

Hím SPRD patkányokat használtunk fel *in vivo* és *in vitro* kísérletekhez.

In vivo kezelések:

Egyik kísérleti beállításban hét csoport i.p. kezelése történt: 1.) kontroll csoport (szalin), 2.) KYNA1 (296 mg/kg), 3.) KYNA (189 mg/kg), 4.) MK-801 (1 mg/kg) + KYNA1 (296 mg/kg), 5.) MK-801 (1 mg/kg) + KYNA (189 mg/kg). Ezek a csoportok a kezelés után 30 perccel dekapitálva lettek. A 6. csoport 15 perccel, a 7. csoport 45 perccel később lett dekapitálva az MK-801-gyel (1 mg/kg) történő kezelés után. A másik kísérleti beállításban 5 csoport szerepelt: 1.) kontroll csoport (fiziológiás sóoldat), 2.) KYNA (189 mg/kg), 3.) KYNA1 (296 mg/kg), 4.) MK-801 (1 mg/kg) + KYNA (189 mg/kg), 5.) MK-

801 (1 mg/kg) + KYNA1 (296 mg/kg). Itt az állatok dekapitálása a kezelések után 2 órával történt meg.

In vitro kezelések:

A dekapitáció után az agyakat azonnal izolált szervi fürdőbe (36-37 ^{o}C, mesterséges gerincvelői folyadék) helyeztük. A kezeléshez négy csoportra osztottuk az állatokat: kontrol, KYNA, KYNA1 és MK-801. A kortex és a striatum leválasztása után a KYNA-t és KYNA1-t 200 µM, az MK-801-t 50 µM koncentrációban adtuk a folyadékhoz 300 percen keresztül, majd -80 ^{o}C tároltuk további felhasználásig.

Funckcionális [^{35}S]GTPγS kötési teszt

A G-fehérje aktivációs kísérletek során a Gαi/o GDP/GTP kicserélődését monitorozzuk egy radioaktívan jelölt, nem hidrolizáló [^{35}S]GTPγS GTP analóggal. Adott liganddal történő, növekvő koncentrációban (10^{-10}-10^{-5} M) aktivált receptor esetén a specifikusan kötött [^{35}S]GTPγS mennyisége információt ad a vizsgált receptor által közvetített G-fehérje maximális aktivitásáról és a receptort aktiváló ligand potenciáljáról.

A három opioid receptor vizsgálata az adott receptorhoz tartozó szelektív ligandokkal történt: MOP – DAMGO, KOP - dinorfin 1-13, DOP - Ile5,6-deltorfin II.

MEGBESZÉLÉS ÉS AZ EREDMÉYNEK ÖSSZEFOGLALÁSA

Először ebben a tanulmányban mutattuk ki, hogy a KYNA és a KYNA1 nem változtatja meg az opioid receptor funkciót krónikus és akut kezelés után sem, szövet és receptor specifikus úton. Ráadásul az NMDA receptor szelektív antagonista, az MK-801 befolyásolta azt, jelezve, hogy a változásokat ez a receptor közvetíti. A hatások láthatóak voltak akut *in vitro* kísérletekben izolált kortex és striatum szövetekben. Kísérleteink alátámasztották korábbi eredményeinket, azaz a KYNA opioid receptor aktivitására gyakorolt hatását.

A krónikus (128 és 200 mg/kg/nap, i.p., 9 napon át) és az akut kezelések összehasonlításakor csak néhány esetben mutattak összhangban (DOP receptor kortexben és striatumban, és KOP receptor striatumban KYNA kezelés esetén). Más esetekben az

ellenkezője történt (KYNA1 kezelés KOP receptoron a striatumban) vagy nem láttunk szignifikáns változást a megfelelő akut kezelt csoport összahasonlításakor (KYNA1 a MOP receptoron). A KYNA NMDA receptorhoz való kötődése igazolt - habár alacsony mikromoláris koncentrációban az NMDA és az opioid receptorok közötti kölcsönhatás is sok szinten. Így annak érdekében, hogy vizsgálni tudjuk az NMDA receptor lehetséges szerepét, MK-801-t (dizocilpin), erős NMDA receptor szelektív antagonistát alkalmaztunk. Az MK-801 opioid receptor közvetítésre gyakorolt hatását igazolták, azonban nem kötődik direkt módon az opioid receptorhoz hasonlóan a KYNA-hoz.

Az MK-801 magában úgy viselkedik, mint a KYNA vagy a KYNA1. Az MK-801 a KYNA-val és a KYNA1-gyel való kombinálása esetén egy enyhe csökkenés jelenik meg az opioid receptor G-fehérje aktivitásában összehasonlítva a KYNA1-gyel vagy az MK-801-gyel önmagukban. Az eredmények magyarázhatóak a KYNA1/KYNA gyengülésével és az MK-801 egyedi aktivitásával az egyidejű detektálásuk esetén, mivel az általuk kifejtett hatások önmagukban hasonlóak, jelezve egy NMDA receptor által közvetített hatást. Ugyanez a magyarázat felmerül, a striatumban expresszálódó MOP és KOP receptoroknál, ahol a KYNA és az MK-801 önmagukban vagy kombinálva csökkentik a G-fehérje jelátvitelt. A DOP receptor a kortexben, a továbbfejlesztett agonista ligand (Ile5,6-deltorfin II) potenciálja 30 perces KYNA1 kezelést követően a kontrol szintig csökkent az MK-801 előkezelés esetén. Érdekes módon az MK-801 magában nem okozott változást a ligand potenciáljában 15 illetve 45 perccel a mérés után, amely azt jelzi, hogy az MK-801 gátolja a KYNA1 hatását az NMDA receptoron keresztül. Továbbá, azt is mutatja, hogy a KYNA1 specifikus hatása potenciálja a továbbfejlesztett DOP receptor agonista ligand affinitását, mivel a legyengült opioid receptor G-fehérje aktivitás a kortexben (MOP és DOP receptor) és a striatumban (mind a három opioid receptor) az MK-801-gyel függnek össze.

Ahogy csak a 30 perces időtartam mutatott szignifikáns különbséget, így a KYNA1 és KYNA koncentrációs szintek HPLC mérését a gerincvelői folyadékban 30 perces kezelést követően hajtottuk végre. Ahogy azt vártuk, a KYNA gerincvelői folyadékbeli koncentráció szintek drámaian csökkentek összehasonlítva a plazma szintekkel, amíg a KYNA1 esetén csak kis különbség volt. Ez bizonyítja, hogy a KYNA1 sokkal könnyebben hatol át a véragygáton, mint a KYNA, amely egybevág az előzetes tanulmányokkal. Továbbá, a KYNA1 koncentrációk szignifikánsan alacsonabbak a plazmában, mint a KYNA-é, jelezve, hogy a KYNA1 esetleg KYNA-vá bomlik.

A megfigyelt hatások *in vivo* 30 perccel a KYNA1 kezelés után kapcsolódhatnak a KYNA-hoz. Ennek lehetőségének tanulmányozására G-fehérje aktivitást mértünk a kortexben és a striatumban KYNA1, KYNA és MK-801-gyel történt kezeléssel izolált szerv fürdőben 30 perces időtartammal. Ezzel a beállítással kizártuk illetve minimalizáltuk a KYNA1 perifériku metabolizmusát és eliminációját és kizártuk a véragygátat a rendszerből, a lehetséges receptor-receptor kölcsönhatás épen tartásával. Eszerint a KYNA1 ugyanazt a hatást mutatja az *in vivo* kísérletekben (hasonlóan a KYNA-hoz és az MK-801-hez), így a KYNA1 önmagában hat az opioid receptor G-fehérje aktivitására. Azonban a KOP receptor esetén a kortexben ellenkező hatást mértünk összehasonlítva az *in vivo* kísérletekkel. Továbbá, néhány esetben az *in vitro* eredmények szignifikáns változást mutattak, ahol az *in vivo* beállítás nem.

Ezek a különbségek az *in vivo* és az *in vitro* eredmények között következhetnek a vegyület gyors perifériku metabolizmusából és a véragygát jelenlétéből. A legmeglepőbb eredmény a KOP G-fehérje aktivitás növekedése volt, amely a KYNA1-gyel történő kezelés után csak a kortexben *in vivo* volt látható. A megnövekedett KOP receptor aktivitást kiválthatja a KYNA1 kezelés a KOP receptor kompenzációs mechanizmusaként, mutatva neuroprotektav hatását a csökkent kortex véráramlás ellenében. Továbbá, a KYNA1 mechanizmusa alatt átalakulhat KYNA-vá a kortexben, kifejtve érszűkítő hatását magas koncentrációban. A KYNA kezelés nem volt hatással a KOP receptor aktivitásra a kortexben, valószínűleg a véragygáton való nehéz átjutása miatt.

KONKLÚZIÓ

Jelen tanulmány először szolgál bizonyítékul egy indirekt, NMDA receptor által közvetített mechanizmusra, tekintettel a KYNA és a KYNA1 az opioid receptor funkciójára gyakorolt hatásaira a receptor G-fehérje szintjén. Így a KYNA és a KYNA1 egy lehetséges gyógyszer-jelölt az opioid rendszer aktivitásának szabályozására az NMDA receptoron keresztül, például opioid megvonás alatt függőség vagy fájdalom kezelés esetén.

Appendix B: Off-prints of thesis related publications

I.

Reza Samavati, Ferenc Zádor, Edina Szűcs, Bernadett Tuka, Diána Martos, Gábor Veres, Róbert Gáspár, István Mándity, Ferenc Fülöp, László Vécsei, Sándor Benyhe, Anna Borsodi. **Kynurenic acid and its analogue can alter the opioid receptor G-protein signaling after acute treatment via NMDA receptor in rat cortex and striatum.** Journal of the Neurological Sciences. doi: 10.1016/j.jns.2017.02.053

Impact factor: 2.126

II.

Ferenc Zádor, ***Reza Samavati***, Eszter Szlávicz, Bernadett Tuka, Engin Bojnik, Ferenc Fülöp, József Toldi, László Vécsei, Anna Borsodi. **Inhibition of opioid receptor mediated G-protein activity after chronic administration of kynurenic acid and its derivative without direct binding to opioid receptors.** CNS Neurol Disord Drug Targets. 2014;13(9):1520-9.

Impact factor: 2.628