Kynurenic acid and its amide analogue might be possible drug candidates for controlling the activity of opioid

Ph.D. thesis

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2017

Introduction

Kynurenine pathway (KP), which is presented in varying extents in astrocytes, neurons, micro- and oligodendroglia as well as in macrophages, endothelial- and dendritic cells in the CNS, is the major route of the catabolism of tryptophan (TRP). The tryptophan can convert to L-kynurenine, which can metabolism to neuroprotective kynurenic acid (KYNA). The clinical data shows that KYNA and its metabolites play an important role in the pathogenesis of multiple neurological disorders like Huntingtin disease, Parkinson disease, epilepsy and ischemic stroke. KYNA is a nonselective excitatory amino acid receptor antagonist (like NMDA receptors), participates in the neurotransmission of the glutamate. Since KYNA participates in the endogenous protective mechanism, could be a suitable pharmacological development target in neurological diseases. Furthermore, interactions between G coupled protein receptors (GPCR) and KYNA is known.

The role of the opioid system in analgesia is well known. The classic receptors of this system are called: mu, kappa and delta (μ, κ and δ). The opioid receptors belong to the GPCR superfamily and mostly attach to the $G_{i/o}$ type G-protein. The high number of the opioid receptors is expressed in the gastrointestinal tract, spinal cord and in large quantity in the brain cortex and striatum region. In our earlier study we showed that KYNA and its analog, KYNA1 (the cation centrum is in C-2 side chain, known as SZR72) cannot bind directly to the opioid receptors, but in chronic treatment induces a significant change in the receptor function in mouse cortex and striatum.

On the other hand, we hypothesized that KYNA and KYNA1 might influence the opioid receptors function by interaction with NMDA receptor. We also investigated the acute effect of KYNA on the opioid receptors with the possible involvement of the NMDA receptor, compared to KYNA1. Rats were treated with a single intraperitoneal (i.p.) injection of KYNA, KYNA1 or combined with NMDA receptor antagonist (MK-801). Furthermore we measured the plasma and spinal fluid concentration 30 minutes after i.p. administration. Isolated rat brain tissue was treated in *in vitro* method with KYNA, KYNA1 and MK-801 to exclude the blood brain barrier and the peripheral metabolism of the KYNA and KYNA1. The cortex and striatum samples were used for [^{35}S]GTPγS binding experiments and opioid receptor functional G-protein activity evaluation.

Aim of the study

Several studies revealed that opioid and NMDA blocker co-administration can induce better nociceptive effect even in the lower dose of opiate. Therefore, it must be a relationship between KYNA and opioid system activity. Since it is determined 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. Herein for the first time we characterize the binding properties of KYNA and KYNA1 towards all three opioid receptors in competition binding assays with radiolabeled 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.

Methods

1. Animals

1.1. Chronic treatment

C57/B female mice were used, divided in three groups: control (0,9% saline), KYNA (128 mg/kg/day) and KYNA1 (200 mg/kg/day). They were treated intraperitoneally with the KYNA/KYNA1 for 9 days. On the 9th day and follow by anesthesia the brain cortex and striatum was removed and stored on – 80 °C.

1.2. Acute treatment

Male SPRD rats were used for *in vivo* and *in vitro* experiment.

- ***In vivo* treatment:**

All animals for the in vivo experiments received a single i.p. injection. 7 groups were performed in one setting: 1.) control (saline), 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). These groups were decapitated 30 minutes after treatment. The 6th and 7th groups treated with MK-801 (1mg/kg) were decapitated 15 and 45 minutes after treatment.
The other setting was included five groups: 1.) control (saline), 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). The animals were decapitated 2 hours after treatment.

- ***In vitro* treatment:**

The isolated brain after decapitation was placed in isolated organ bath (36 – 37 °C, artificial cerebrospinal fluid). For treatment we assigned four groups: control, KYNA, KYNA1 and MK-801.
KYNA and KYNA1 in 200 μM, MK-801 in 50 μM concentration were added to the bath for 30 minutes. After treatment the cortex and striatum slices were stored at – 80 °C till they were using for binding assay.

2. Functional [^{35}S]GTPγS binding test

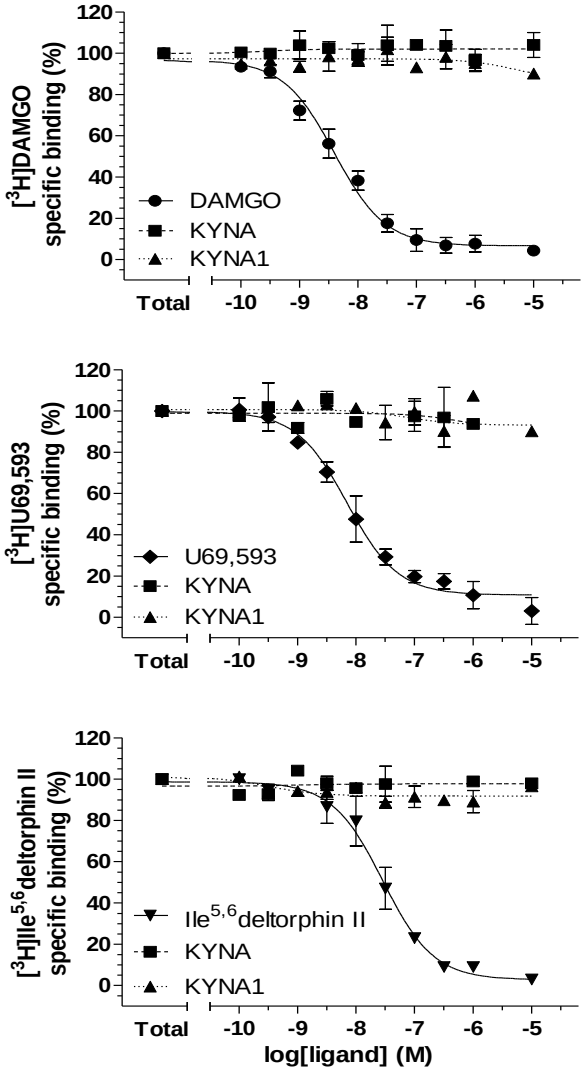
During the G-protein binding assay we monitor the exchange of Gαi/o GDP/GTP with a radioactive indicator, a non hydrolyzing [^{35}S]GTPγS GTP analog.

The amount of the specific attached [^{35}S]GTPγS, binded by the opioid agonist ligands applied in increasing concentrations (10^{-10}-10^{-5} M) can determine the maximum efficacy of the opioid receptors G-protein and ligand potency.

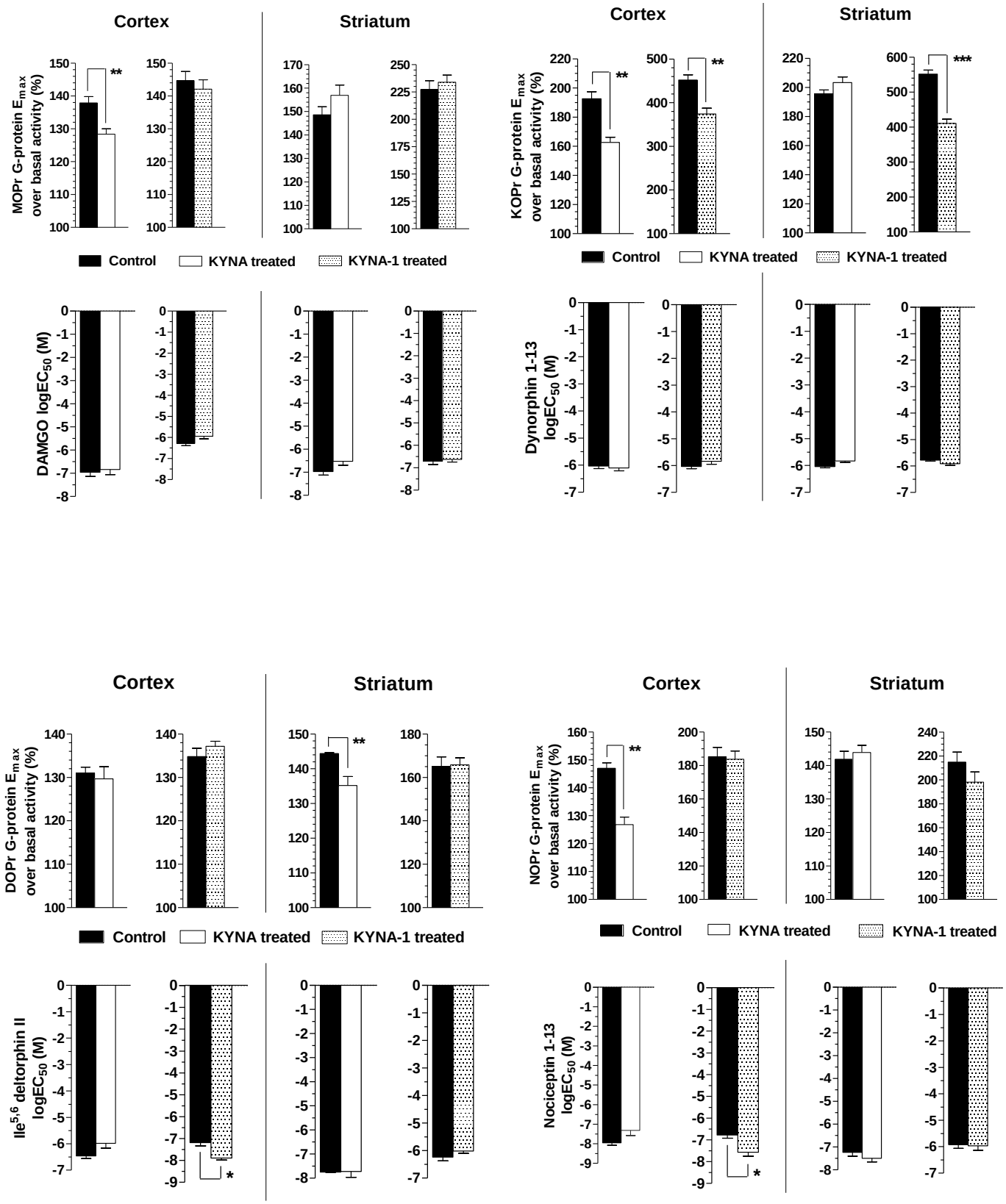
The examination of the three opioid receptor was performed with the belonging selectiv ligand: μ (DAMGO), κ (dinorfin 1 – 13) and δ (Ile5,6-deltoforin II).

Results

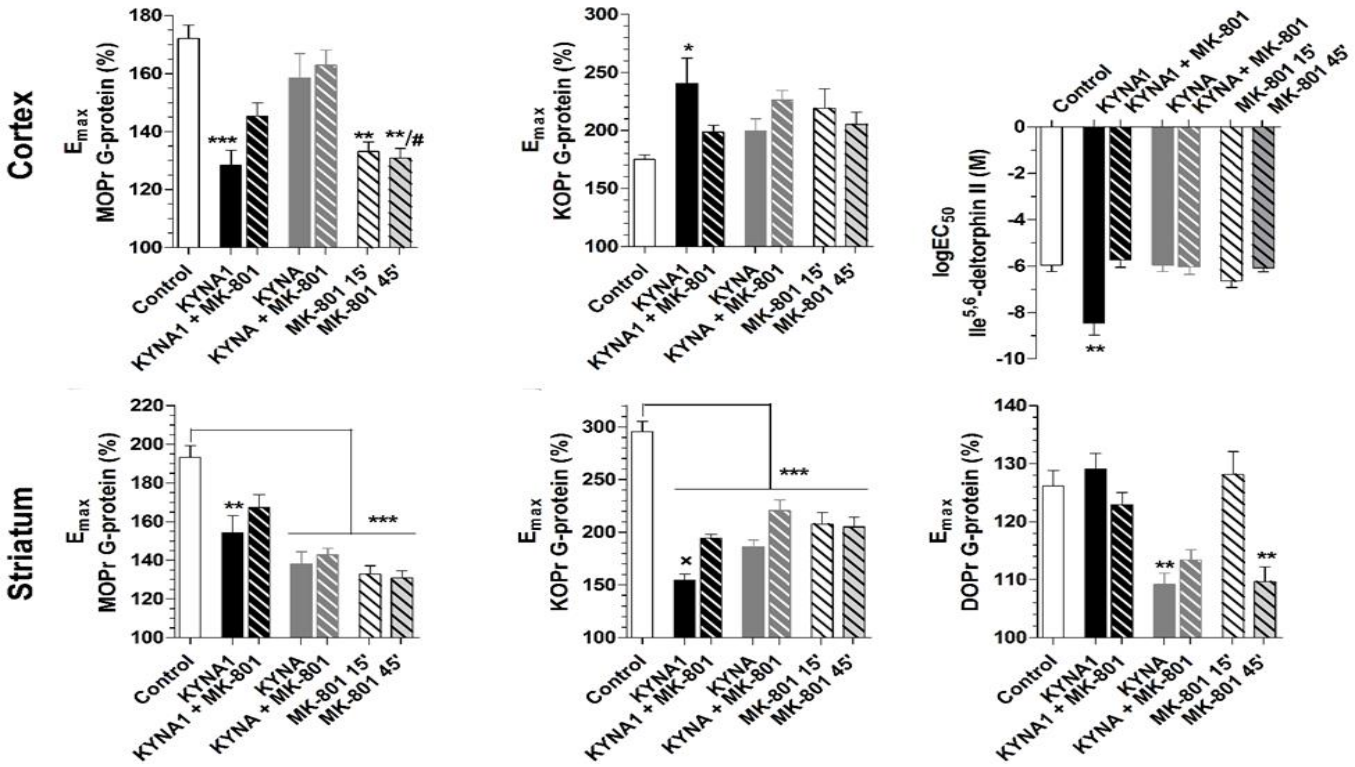
- **Competition binding assay**



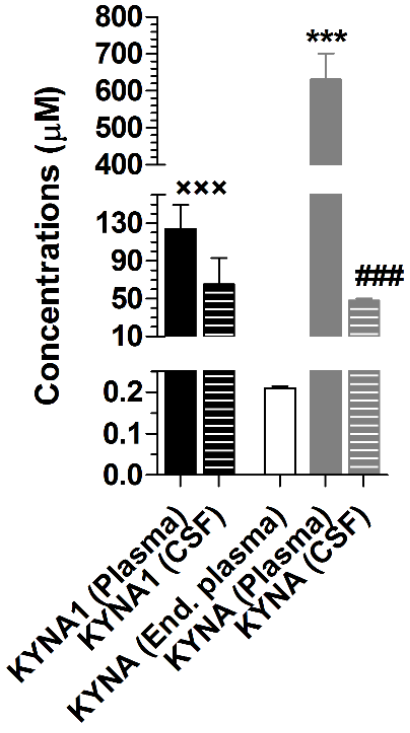
- **Chronic treatment**



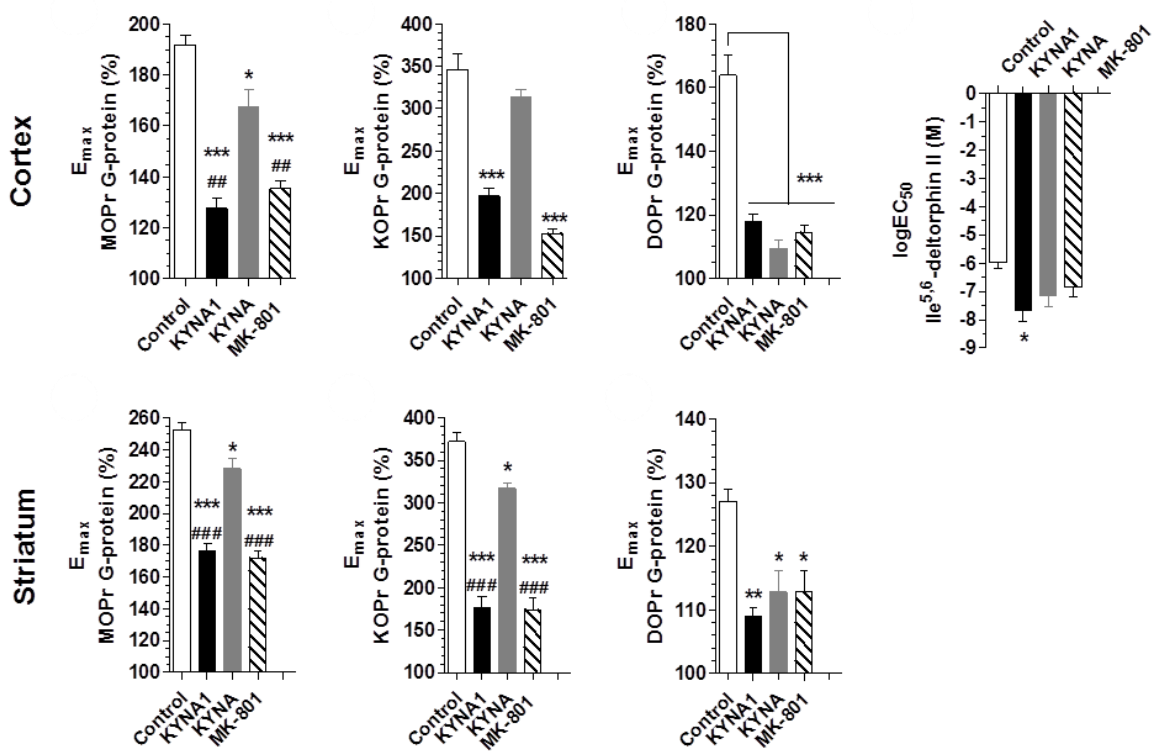
· **Acute treatment**



· **The blood plasma and CSF concentration levels**



· ***In vitro* treatment**



Discussion and summary of results

In this study we have shown for the first time that KYNA1 and KYNA not only alter opioid receptor function after chronic treatment, 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 vitro* experiments in isolated cortex and striatum slices. Our results further support previous findings showing the effect of KYNA on the opioid receptors activity. 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). The NMDA receptor has been demonstrated to bind KYNA - although with low micromolar affinity also, the interaction of NMDA and opioid receptors have been

demonstrated in many levels. Thus in order to investigate the possible role of the NMDA receptor we applied MK-801 (also known as dizocilpine), a highly NMDA receptor selective antagonist. MK-801 has also been demonstrated to alter opioid receptor-mediated effects, however it does not bind directly to opioid receptors similarly to KYNA. MK-801 alone behaved similarly as KYNA1 or KYNA. MK-801 in combination with KYNA1 or KYNA displayed a somewhat less pronunced reduction opioid receptor G-protein activity compared to KNYA1 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. 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. Interestingly, MK-801 alone did not cause any alterations in ligand potency following 15 or 45 minute administration, 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. Since only the 30 minutes duration time showed significant results, 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. This proves that KYNA1 passes through the blood-brain barrier more easily than KYNA, which is in agreement with previous studies. 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. Additionally, in some cases the *in vitro* results showed significant alterations where the *in vivo* setup did not. 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.

The most striking result was the increase of KOP G-protein activity, which was only observed in the cortex following *in vivo* KYNA1 treatment. 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.

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.

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