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Inhibition of Opioid Receptor Mediated G-Protein Activity After Chronic Administration of Kynurenic Acid and its Derivative without Direct Binding to Opioid Receptors

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Abstract: There is an increasing number of evidence showing analgesic properties of the kynurenic acid (KYNA), and also some studies demonstrate that kynurenine might interact with the opioid system. Therefore in this study, for the first time we investigated the direct binding affinity of KYNA and its structural analog KYNA-1 towards mu, kappa and delta opioid receptor in competition binding experiments applying opioid receptor specific radioligands. The binding affinity measurements were performed in Chinese hamster ovary cell lines overexpressing the corresponding opioid receptor (mu and kappa opioid receptor were rat, delta opioid receptor were mouse sequence). Additionally we also examined the chronic effect of these compounds on mu, kappa and delta opioid receptor and also nociceptin peptide receptor mediated G-protein activity in [^{35}S]GTPγS binding assays performed in mouse cortex and striatum membranes. Our results showed that KYNA and KYNA-1 had no affinity towards any of the three classic opioid receptors. On the other hand the compounds significantly decreased opioid and nociceptin receptor mediated G-protein activity or in some cases enhanced the potency of the activating ligand. Moreover, the alterations were receptor and brain region specific. Accordingly, we conclude that KYNA and KYNA-1 do not interact directly with the opioid receptors, but more likely alter the receptor functions intracellularly.

Keywords: [^{35}S]GTPγS binding, G-protein, kynurenic acid, opioid receptors, radioligand binding.

INTRODUCTION

Kynurenine pathway, 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. Tryptophan may be converted to L-kynurenine, which can be further transformed directly to the characteristically neuroprotective kynurenic acid (4-hydroxyquinoline-2-carboxylic acid; KYNA; Fig. **1A**). Preclinical and clinical data suggest that the KYNA and its metabolites play a major role in the pathogenesis of several neurological disorders such as Huntington's disease, Parkinson's disease, epilepsy and stroke [1-6]. KYNA, as a non-selective excitatory amino acid receptor (such as NMDA receptors) antagonist takes part in glutamatergic neurotransmission and it can block non-competitively the nicotinergic processes in the central nervous system (CNS) [7]. Since KYNA is involved in endogenous protective mechanisms, it would be a good target for pharmaceutical intervention of neurological diseases [8]. Additionally, the G-protein-coupled receptor (GPCR) GPR35, an orphan receptor has been reported to be an interactional partner for KYNA [9, 10].

The combination of the endogenous systems with the enhancement of supplemental exogenous/synthetic drugs has previously been studied in the therapy of pain [11]. However, the kynurenines and their associations with different pain conditions are not fully elaborated [12, 13].

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Since KYNA is barely able to cross the blood-brain barrier [14]), several new KYNA structural analogs, prodrugs or derivatives such KYNA-1, KYNA-2, KYNA-6 and KYNA-11 were designed to overcome this problem [15]. KYNA-1 (*N*-[2-*N*,*N*-dimethylaminoethyl]-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride) may also be considered as a promising neuroprotective agent [2, 16-18] lacking appreciable side-effects [15] (Fig. **1B**).

The opioid system plays a major role in pain relief. Receptors in this system, which are called the opioid receptors belong to the GPCR superfamily and they are mostly coupled to $G_{i/o}$ type G-proteins [19]. They exert their effects through the inhibition of different types of neurotransmitters, such as noradrenaline, dopamine or acetylcholine [20]. Their endogenous ligands, namely the enkephalins, endorphins, dynorphins, nociceptin and endomorphins [21] are called the endogenous opioids, and they are small peptide natured molecules functioning as neurotransmitters, neurohormones or neuromodulators [22, 23]. There are three types of opioid receptors classically: the mu, kappa and delta opioid peptide receptor (MOPr, KOPr and DOPr respectively; [24-26]). Additionally, the nociceptin peptide receptor (NOPr) is also referred to as an opioid receptor [27, 28]. The opioid receptors are expressed widely in the CNS [20, 29] as well as in the peripheral organs, such as the gastrointestinal tract [30]. They have a substantial role in pain regulation, and opioid agonists, such as morphine and the more effective sufentanil [31] were found to have a clear clinical efficacy for the attenuation of certain chronic and acute pain [32].

A few studies have found a relationship between the kynurenine and the opioid systems. Morgan and his co-workers [33] have proved that co-injections of KYNA with morphine have enhanced the acute antinociceptive effect of morphine in rat ventrolateral periaqueductal grey matter (vPAG). Since KYNA is a non-selective NMDA receptor antagonist, it is worth to mention that other non-competitive and competitive NMDA receptor blockers co-administered in low-dose with morphine can inhibit the development of physical addiction on opioids [34]. Also, the co-administration of morphine with certain NMDA receptor antagonists dose-dependently inhibited the full length dynorphin induced characteristic pain responses [35]. Horvath and co-workers demonstrated that in the case of inflammatory pain co-injection of different endogenous ligands in lower doses (EM-1, adenosine, agmantine and KYNA) in triple and quadruple manner, had a more effective impact than the single treatment at spinal level [11]. These results suggest that the interactions and combined therapies of the kynurenine and opioid systems could provide a more effective, well-controlled antinociceptive therapy associated with less undesired side-effects.

The aim of this study was to investigate the effect of KYNA on the opioid system and to compare with its structural analog, KYNA-1. Herein for the first time we characterize the binding properties of KYNA and KYNA-1 towards all three opioid receptors in competition binding assays with radiolabeled receptor specific opioid ligands. The binding experiments were prepared in Chinese hamster ovary cell (CHO) membranes overexpressed with the adequate opioid receptor. Additionally, we investigated the opioid receptor G-protein activity, the initial phase of GPCR signalling, after *in vivo* and *in vitro* KYNA and KYNA-1 administration in functional [^{35}S]GTPγS binding assays. The G-protein activity measurements were performed in mouse striatum and cortex membrane fractions since opioid receptors are widely expressed in these brain regions [20, 29].

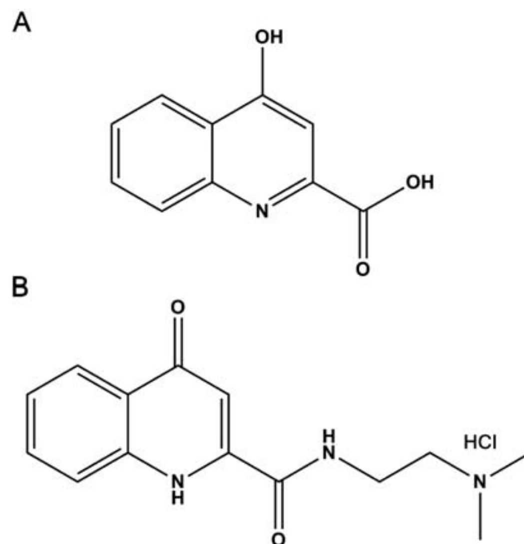

Fig. (1). The chemical structures of KYNA (**A**) and KYNA-1 (**B**). KYNA: kynurenic acid (4-hydroxyquinoline-2-carboxylic acid); KYNA-1: *N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride.

MATERIALS AND METHODS

Chemicals

Tris-HCl, EGTA, NaCl, $MgCl_2$ x $6H_2O$, GDP, the GTP analog GTPγS, the KOPr specific agonist U69,593, and kynurenic acid (KYNA) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The MOPr agonist enkephalin analog Tyr-D-Ala- Gly-(NMe)Phe-Gly-ol (DAMGO), the KOPr and the NOPr agonist peptide dynorphin 1-13 and nociceptin 1-13 respectively were obtained from Bachem Holding AG (Bubendorf, Switzerland). The radiolabeled GTP analog, [^{35}S]GTPγS was purchased from the Isotope Institute Ltd. (Budapest, Hungary). The tritiated U69,593 ([^{3}H]U69,593; [36]) was purchased from PerkinElmer (Boston, USA). The modified DOR specific deltorphin II derivative, Ile^{5,6}deltorphin II was synthesized and tritiated ([^{3}H]Ile^{5,6}deltorphin II; [37]) in our Isotope Laboratory of BRC (Szeged, Hungary) as well as the tritiated DAMGO ([^{3}H]DAMGO; [38]). The opioid antagonist naloxone was kindly provided by the company Endo Laboratories DuPont de Nemours (Wilmington, US). The KYNA structural analog, KYNA-1 was synthetized in the Department of Pharmaceutical Chemistry, University of Szeged [39]. All ligands for receptor assays were dissolved in highly pure distilled water and were stored in 1 mM stock solution at -20°C.

Animals and Treatments

79 young adult C57Bl/6J 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 (86/609/EEC) 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 and were approved by the Ethics Committee of the Faculty of Medicine, University of Szeged.

KYNA and KYNA-1 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 KYNA-1 in a dose of 200 mg/kg/day. The dose of KYNA is equimolar with the KYNA-1. 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 preparation.

Cortex and Striatum Membrane Preparations

The membrane fractions of mouse cortex and striatum for [^{35}S]GTPγS binding experiments were prepared according to the method previously described [40]. Briefly, mice were decapitated and the brain was quickly removed. Forebrains were collected and homogenized on ice in 50 mM Tris-HCl buffer (pH 7.4) with a Teflon-glass homogenizer. The homogenate was centrifuged at 40,000 × *g* for 20 min at 4°C and the pellet was resuspended in fresh buffer and incubated for 30 min at 37°C. The centrifugation step was repeated and the final pellet was resuspended in TEM buffer obtaining the appropriate protein content for the assay (~10 μg/ml) and was stored at −80°C until use.

Cell Culture and Cell Membrane Preparations

CHO cells over expressed with rat MOPr (CHO-MOPr) or rat KOPr (CHO-KOPr) or mouse DOPr (CHO-DOPr) were provided by Dr. Zvi Vogel (Rehovot, Israel) and were described earlier [41-43]. 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. Cells were kept in culture at 37°C in a humidified atmosphere consisting of 5% CO_2 and 95% air.

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.

Radioligand Competition Binding Assays

The direct binding affinities of KYNA and KYNA-1 towards opioid receptors were investigated in radioligand competition binding experiments. In this type of receptor assay the inhibition of fix concentrations of a specific radioligand binding in the presence of increasing concentrations of unlabeled competitor ligands is measured.

Firstly the sucrose was removed from the aliquots of frozen CHO-MOPr, CHO-KOPr and CHO-DOPr membranes by centrifugation (40000 × g, 20 min, 4°C). Next the pellets were suspended in 50 mM Tris-HCl buffer (pH 7.4). Membranes were incubated in a final volume of 1 ml containing 10^{-10} - 10^{-5} M concentrations of unlabeled KYNA, or KYNA-1, or for control the appropriate unlabeled opioid receptor specific ligands, together with ~ 1 nM of opioid radioligand. The incubation was carried out at the appropriate temperature and duration depending on the applied radioligand ([^{3}H]DAMGO: 35°C, 45 min; [^{3}H]U69,593: 30°C, 30 min; [^{3}H]Ile^{5,6}deltorphin II: 35°C, 45 min) with gentle shaking. Total and non-specific binding was measured and determined as reported previously, together with the filtration and washing procedures and radioactivity detection [44]. In case of [^{3}H]U69,593 Whatman GF/B, while during the filtration of [^{3}H]DAMGO and [^{3}H]Ile^{5,6}deltorphin II GF/C glass fiber filters were used. The competition binding assays were performed in duplicate and repeated at least three times.

Functional [^{35}S]GTPγS Binding Assays

The G-protein activation of the opioid receptors after chronic KYNA and KYNA-1 treatment was measured in functional [^{35}S]GTPγS binding experiments, in which the nucleotide exchange process is monitored using a non-hydrolysable radioactive GTP analog, [^{35}S]GTPγS in the presence of increasing concentrations of the tested ligand.

The assays were performed according to Sim *et al*. and Traynor and Nahorski, with slight modifications [45, 46]. Membrane fractions of wild type mice 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, Ile^{5,6}deltorphin II or nociceptin 1-13. The determination of total and non-specific binding, together with the filtration, washing procedure and finally the radioactivity detection was accomplished as reported

previously [44]. The [^{35}S]GTPγS binding experiments were performed in triplicates and repeated at least three times.

Data Analysis

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.

RESULTS

Direct Binding Affinity Measurements of KYNA and KYNA-1 on Opioid Receptors

The binding properties of KYNA and its analog KYNA-1 towards all three classic opioid receptors, MOPr, KOPr and DOPr, were analyzed in competition binding experiments using receptor specific tritiated radioactive ligands, namely the MOPr specific [^{3}H]DAMGO, the KOPr specific [^{3}H]U69,593 and the DOPr specific [^{3}H]Ile^{5,6}deltorphin II. The assays were accomplished in CHO cell membranes overexpressing the corresponding opioid receptor.

Neither KYNA, nor KYNA-1 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. **2**). For comparison the unlabeled opioid ligands decreased the binding of their radiolabeled analogs with an EC_{50} of 3.95 nM (DAMGO; Fig. **2A**), 7.06 nM (U69,593; Fig. **2B**) and 28.67 nM (Ile^{5,6}deltorphin II; Fig. **2C**). Thus according to our competition binding experiments KYNA and KYNA-1 did not interact directly with MOPr, KOPr or DOPr under these conditions.

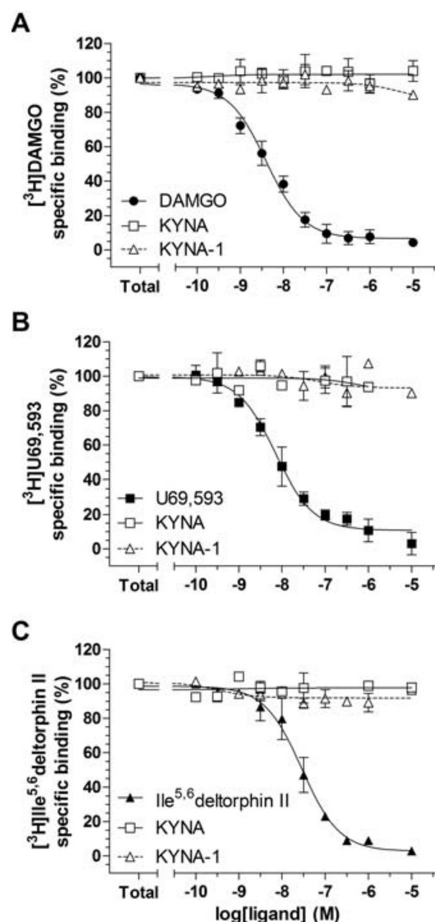

Fig. (2). Direct binding affinity measurements of KYNA and its analog, KYNA-1 towards MOPr (**A**), KOPr (**B**) and DOPr (**C**) in competition binding experiments in CHO cell membrane fractions overexpressed with the appropriate opioid receptor. Data are presented as the percentage of specific binding 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, KYNA-1 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.

Opioid and Nociceptin Receptor G-Protein Activity Measurements After Chronic KYNA and KYNA-1 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 KYNA-1 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 KYNA-1 chronically (9 days) at 128 and 200 mg/kg/day (equimolar to each other) respectively to the animals, i.p.

The chronic, KYNA treatment in mouse cortex significantly inhibited the maximal G-protein activity of MOPr (Fig. **3A**), without altering the potency of the stimulator ligand DAMGO (Fig. **3B**). In contrast chronic KYNA-1 treatment had no significant influence on MOPr G-protein activity (Fig. **3A**) or stimulation in mouse cortex (Fig. **3B**). In mouse striatum, treatment did not have significant impact on MOPr signaling (Fig. **3C**, **D**).

In mouse cortex the KOPr G-protein maximal activity (E_{max}) was significantly attenuated during dynorphin 1-13 stimulation after both KYNA and KYNA-1 chronic administration (Fig. **4A**), but the potency of the stimulator ligand was again unaltered (Fig. **4B**). In contrast, only KYNA-1 attenuated KOPr G-protein maximal efficacy in the striatum (Fig. **4C**), but the potency of the activator ligand did not change (Fig. **4D**).

The DOPr G-protein maximal stimulation was unaffected by either KYNA or KYNA-1 chronic administration in the cortex (Fig. **5A**), however unlike KYNA treatment, KYNA-1 enhanced the potency of the $Ile^{5,6}$deltorphin II stimulator ligand, (Fig. **5B**). In the striatum only KYNA treatment could reduce the maximal efficacy of DOPr G-protein significantly (Fig. **5C**), while the EC_{50} value of $Ile^{5,6}$deltorphin II was not affected by either chronic treatment (Fig. **5D**).

Unlike KYNA-1, KYNA treatment significantly inhibited the maximal effectiveness of the NOPr G-protein in mouse cortex (Fig. **6A**), but the potency of the activator ligand was not affected by KYNA (Fig. **6B**). However KYNA-1, similarly to $Ile^{5,6}$deltorphin II, enhanced the potency of nociceptin 1-13 (Fig. **6B**). In the striatum, similar to MOPr, no significant alterations were observed in NOPr signaling after KYNA or KYNA-1 chronic treatment (Fig. **6C**, **D**).

DISCUSSION

KYNA is well known for having a neuroprotective role in the body [8] but also there is a growing number of evidence supporting analgesic properties of the compound in periphery as well as in CNS [11, 12, 47]. Moreover according to previous studies the combination of kynurenine, or KYNA and opioid ligands shows higher analgesic efficacy with less undesired side-effects. At the same time the possible direct interactions between the opioid system and KYNA so far was not investigated. Therefore in this study, we have examined the direct effect of KYNA and its analog KYNA-1 on the opioid system. Also foremost we have established the effect of high dose KYNA and KYNA-1 on opioid and nociceptin receptor mediated G-protein signaling after chronic administration. The possible direct interactions were examined in competition radioligand binding experiments, while the receptor mediated G-protein signaling was monitored by $[^{35}S]GTP\gamma S$ binding assays.

According to our experiments, the direct effect of KYNA and KYNA-1 can be excluded since neither compound decreased opioid radioligand binding significantly. These results were confirmed by our $[^{35}S]GTP\gamma S$ binding assays when KYNA and KYNA-1 were administered *in vitro* to untreated mouse cortex and striatum membranes (data not

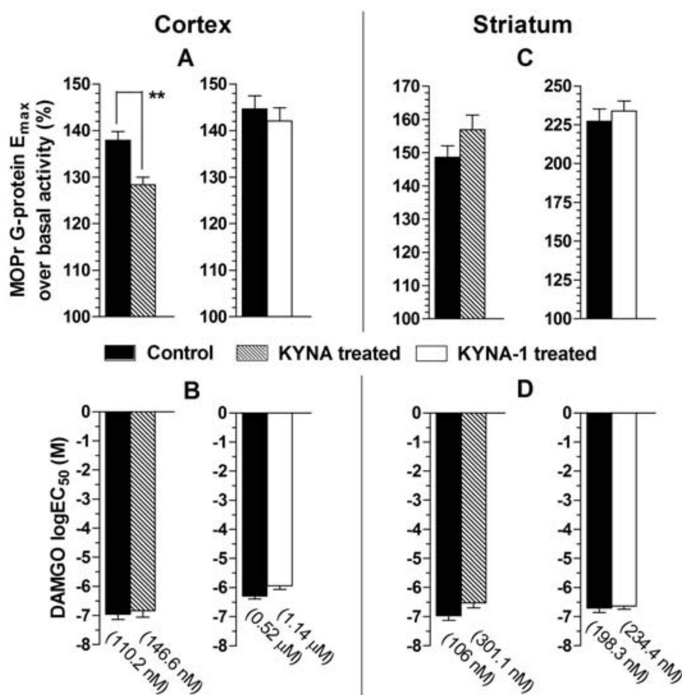

Fig. (3). The effect of chronically administered KYNA and KYNA-1 on the maximal efficacy or E_{max} of MOPr G-protein over basal activity (**A** and **C**) and the potency of DAMGO indicated by the $logEC_{50}$ value (**B** and **D**) in DAMGO-stimulated $[^{35}S]GTP\gamma S$ binding assays in mice cortex (**A** and **B**) and striatum (**C** and **D**) membranes. MOPr 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. The chronic treatment lasted 9 days, i.p. in 128 (KYNA) and 200 mg/kg/day (KYNA-1, equimolar to each other) dosage. 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). **: $P < 0.01$.

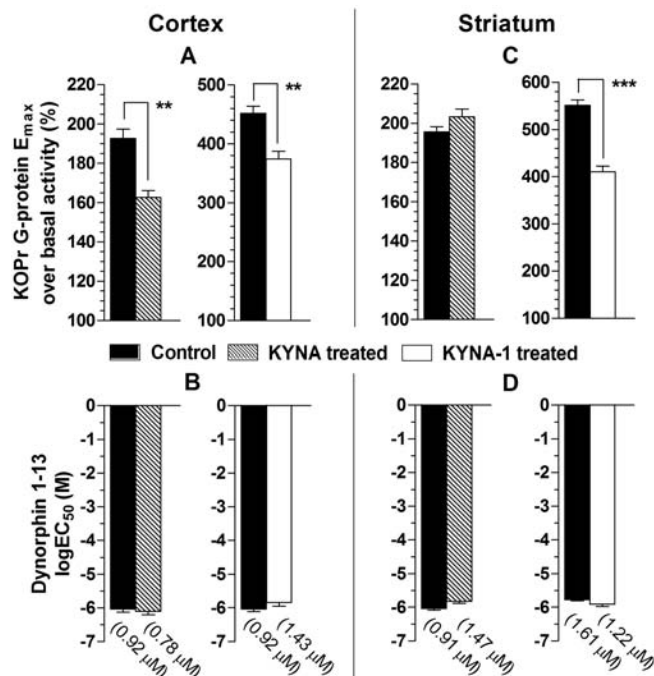

Fig. (4). The effect of chronically administered KYNA and KYNA-1 on the maximal efficacy or E_{max} of KOPr G-protein over basal activity (**A** and **C**) and the potency of dynorphin 1-13 indicated by the $logEC_{50}$ value (**B** and **D**) in dynorphin 1-13-stimulated [^{35}S]GTPγS binding assays in mice cortex (**A** and **B**) and striatum (**C** and **D**) membranes. KOPr 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. The chronic treatment lasted 9 days, i.p. in 128 (KYNA) and 200 mg/kg/day (KYNA-1, equimolar to each other) dosage. 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). ***: $P < 0.001$; **: $P < 0.01$.

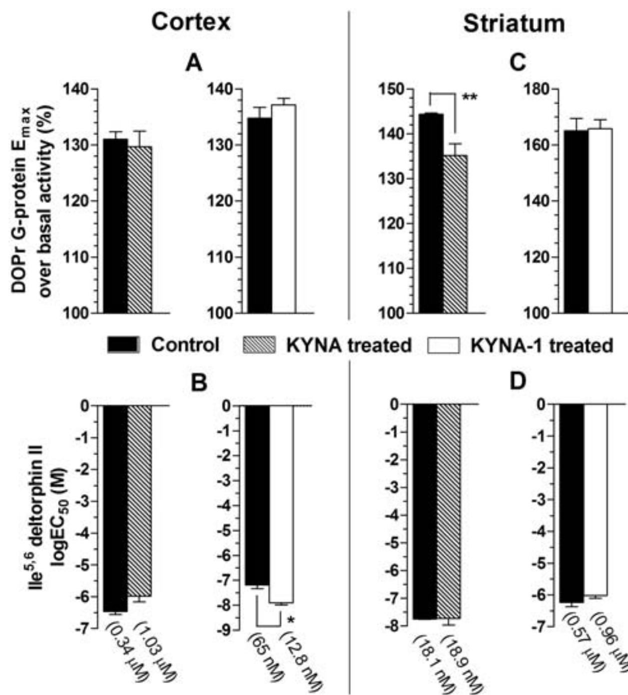

Fig. (5). The effect of chronically administered KYNA and KYNA-1 on the maximal efficacy or E_{max} of DOPr G-protein over basal activity (**A** and **C**) and the potency of $Ile^{5,6}$deltorphine II indicated by the $logEC_{50}$ value (**B** and **D**) in $Ile^{5,6}$deltorphine II-stimulated [^{35}S]GTPγS binding assays in mice cortex (**A** and **B**) and striatum (**C** and **D**) membranes. DOPr 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. The chronic treatment lasted 9 days, i.p. in 128 (KYNA) and 200 mg/kg/day (KYNA-1, equimolar to each other) dosage. 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). **: $P < 0.01$; *: $P < 0.05$.

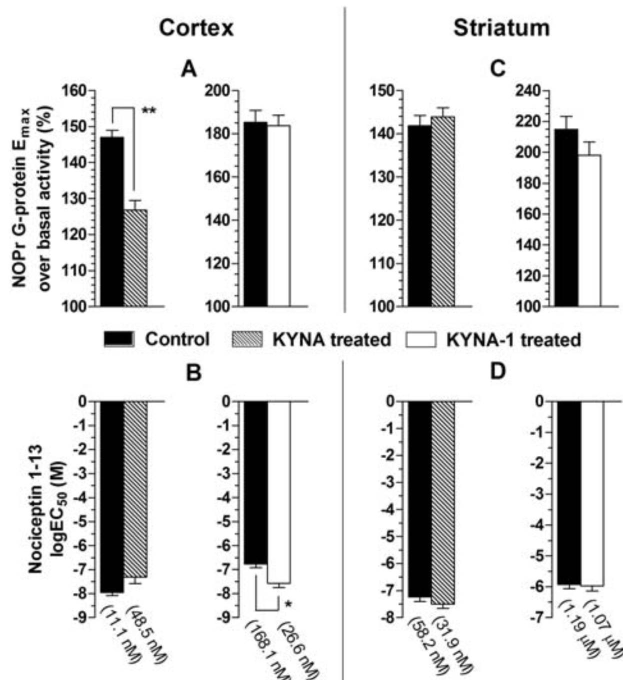

Fig. (6). The effect of chronically administered KYNA and KYNA-1 on the maximal efficacy or E_{max} of NOPr G-protein over basal activity (**A** and **C**) and the potency of nociception 1-13 indicated by the $logEC_{50}$ value (**B** and **D**) in nociception 1-13-stimulated [^{35}S]GTPγS binding assays 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. The chronic treatment lasted 9 days, i.p. in 128 (KYNA) and 200 mg/kg/day (KYNA-1, equimolar to each other) dosage. 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). **: $P < 0.01$; *: $P < 0.05$.

shown). However, administration of high dose KYNA and KYNA-1 chronically did affect G-protein activity and receptor activator ligand potency after the treatment in certain receptors. Interestingly, the alterations were similar in the affected receptors: G-protein activity inhibition (Table **1**) and in case of KYNA-1 stimulator ligand potency enhancement (Table **1**). Another common feature was that the alterations were always distinct from each other: the G-protein activity reduction did not reduce the potency of the activator ligand, while the increasing potency did not imply maximal G-protein activity intensification (Table **1**). This observation also supports the lack of direct interaction between the opioid receptors and KYNA and also KYNA-1.

One possible mechanism which can explain the inhibitory actions in opioid and nociceptin receptor mediated G-protein activation is the reduced G-protein gene or protein expression levels by the chronic treatment with these substances. In this case lower levels of G-proteins would be available for coupling to the receptors resulting a diminished activity. Also both compounds could possibly reduce opioid and nociceptin receptor mediated G-protein activity allosterically. The other interesting observation was that KYNA-1 enhanced the potency of DOPr and NOPr activator ligands which was not observed after KYNA treatment. KYNA-1 treatment possibly increased the expression rate of the receptor proteins or sensitivity, which is an enhanced effect of a drug with chronic use [48].

Table 1. Summarizing the effect of chronic KYNA and KYNA-1 treatment on opioid receptor mediated G-protein efficacy and ligand potency values. Ctx.: cortex, str.: striatum, ↑: significant enhancement, ↓: significant inhibition, ∅: no significant effect.

		KYNA		KYNA-1	
		G-Protein Efficacy	Ligand Potency	G-Protein Efficacy	Ligand Potency
MOPr	Ctx.	↓	∅	∅	∅
	Str.	∅	∅	∅	∅
KOPr	Ctx.	↓	∅	↓	∅
	Str.	∅	∅	↓	∅
DOPr	Ctx.	∅	∅	∅	↑
	Str.	↓	∅	∅	∅
NOPr	Ctx.	↓	∅	∅	↑
	Str.	∅	∅	∅	∅

An interaction between the opioid receptors and KYNA specific receptors can also explain our results (Fig. **7**). There is evidence that the NMDA receptor/glycine site antagonist ACEA-1328 administered chronically into mice decreased the

antinociceptive effect of the KOPr agonist U50,488H [49] and there are further studies reporting the interaction between NMDA and opioid receptors [50]. Additionally NMDA receptors and MOPr are proved to be colocalized not just within a certain brain region but also in the same neuron as well in several parts of the CNS, such as the periaqueductal gray or the caudate putamen [48, 51-53]. The α7 cholinergic nicotine receptor - which KYNA can antagonize - can also interact with opioid receptors, mostly in nicotine addiction [54], thus it may also mediate the chronic effect of KYNA and KYNA-1 on the opioid receptors (Fig. **7**).

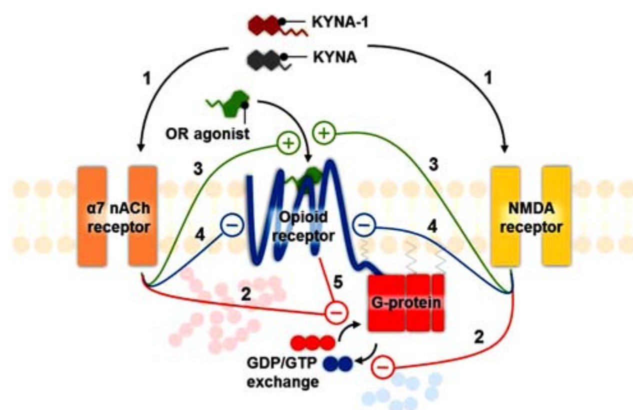

Fig. (7). The possible indirect mechanisms of chronic KYNA and KYNA-1 treatment on agonist stimulated opioid receptor activity. KYNA and KYNA-1 may exert their effect on opioid receptors through their α7nACh or NMDA receptors, which they can both antagonize. KYNA and KYNA-1 by binding to the receptors (**1**) possibly decreases G-protein activity or even expression, which overall results in a reduced nucleotide exchange (**2**). Also KYNA-1 may enhance OR agonist potency through these receptors (**3**). Additionally the chronic treatment may also reduce opioid receptor expression levels (**4**) through α7nACh or NMDA receptors and as a result less G-protein will couple to the receptor, which may lead to reduced G-protein activity (**5**). The black arrows indicate the ligand binding process and the GDP/GTP exchange. The red, blue and green lines indicate the effects on G-protein activity, OR agonist ligand potency and OR expression levels respectively. The positive and negative signs are indicating the direction of the effect. In the GDP/GTP exchange process the three red discs and the two blue discs represents GTP and GDP respectively. OR: opioid receptor; α7nACh receptor: alpha 7 nicotinic type acetylcholine receptor.

Our results also pointed out the brain region specificity of KYNA chronic effect in opioid receptor G-protein efficacy (Table **1**). Accordingly, the observed effects of KYNA reveal itself in the brain regions where the expression rate of the corresponding opioid receptor is known to be higher [55]. In case of KYNA-1 the reduced G-protein efficacy was only observed in KOPr, but both in cortex and striatum (Table **1**). Interestingly we could only observe the same effects of KYNA and KYNA-1 in the KOPr located in the cortex, probably because of the abundance of this opioid receptor type in this brain region [29]. At the same time the different opioid receptor and brain region specific actions of the two compounds are more likely due to their structural differences and the derived properties: KYNA-1 has a water soluble side-chain, a cationic center in the C-2 region, with side-chain substitution compared to KYNA (Fig. **1**). These structural changes allows KYNA-1 to inhibit NR2B subunit of the NMDA receptor more selectively [56], resulting reduced side-effects compared to the complete blockage of the NMDA receptor [57]. Also due to the side-chain substitution KYNA-1 can pass through the blood-brain-barrier more easily [15].

CONCLUSION

In this study we pointed out that KYNA and its structural analog KYNA-1 has no specific binding for either classic opioid receptor. However, after administering the two compounds chronically the function of all three classic opioid receptors and also NOPrs were significantly altered. Moreover, the observed effects were brain region specific. We conclude that KYNA and KYNA-1 are affecting the receptor functions intracellularly. Further studies will focus on the acute effect of KYNA and KYNA-1 on the opioid receptor G-protein activity together with the effect of the compounds on expression levels of the related proteins. Studying the effect of other novel KYNA analogs on the opioid system is also planned. Investigation of the impact of KYNA and its derivatives on the opioid receptor G-protein activity in neurodegenerative animal models seems to be an interesting, feasible approach in the future.

LIST OF ABBREVIATIONS

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 nervous system
DAMGO	=	Tyr-D-Ala- Gly-(NMe)Phe-Gly-ol
DOPr	=	Delta opioid peptide receptor
EGTA	=	Ethylene glycol tetraacetic acid
GDP	=	Guanosine 5'-diphosphate
GPCR	=	G-protein coupled receptor
GTP	=	Guanosin 5'-triphosphate
GTPγS	=	Guanosine-5'-O-(gamma-thio)triphosphate
KOPr	=	Kappa opioid peptide receptor
KYNA	=	Kynurenic acid (4-hydroxyquinoline-2-carboxylic acid)
KYNA-1	=	*N*-(2-*N*,*N*-dimethylaminoethyl)-4-oxo-1*H*-quinoline-2-carboxamide hydrochloride
MOPr	=	Mu 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

CONFLICT OF INTEREST

The authors confirm that there is no conflict of interest in this article.

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