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Kynurenic acid and its analogue can alter the opioid receptor G-protein signaling after acute treatment *via* NMDA receptor in rat cortex and striatum

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ABSTRACT

Previously, we have shown that the *N*-methyl D-aspartate (NMDA)-receptor antagonist kynurenic acid (KYNA) and its analogue KYNA1 do not bind directly to mu, kappa and delta opioid receptors *in vitro*. On the other hand, chronic administration of KYNA and KYNA1 resulted in region (cortex *vs* striatum) and opioid receptor-type specific alterations in G-protein activation of mouse brain homogenates. Here we describe for the first time the acute effect of KYNA and KYNA1 on opioid receptor function with the possible involvement of the NMDA receptor.

The acute 30 minute *in vivo* KYNA1 and KYNA treatments altered opioid receptor G-protein signaling or ligand potency depending on the opioid receptor type and brain region (rat cortex *vs* striatum) using [^{35}S]GTPγS binding assays. Pretreatment with the NMDA receptor antagonist MK-801 impaired or reversed the effects of KYNA1 and KYNA. These results suggest an NMDA receptor mediated effect. After acute 30 minute treatment HPLC measurements revealed a similar KYNA1 and a higher KYNA plasma concentration compared to cerebrospinal fluid concentrations. Finally, KYNA, KYNA1 and MK-801 showed comparable results in opioid receptor G-protein activity and ligand potency with acute *in vivo* treatments when they were administered *in vitro* for 30 min on isolated cortex and striatum slices.

We previously demonstrated that KYNA1 and KYNA acutely altered opioid receptor function *in vivo* and *in vitro* through the NMDA receptor depending on the opioid receptor type and brain region. This study may lead to a new, indirect approach to influence opioid receptor signaling.

1. Introduction

The kynurenine pathway of the tryptophan metabolism has emerged in recent years as a key regulator of the production of both neuroprotective (*e.g.*, kynurenic acid), and neurotoxic metabolites such as 3-hydroxykynurenine [21]. Kynurenic acid (KYNA) (Fig. 1A) is a glutamate receptor antagonist [36] and acts as an agonist on GPR35 receptors [31]. It has been proven that KYNA plays an important role in endogenous protective mechanisms, therefore it is a good target for pathophysiologycal investigation of certain neurodegenerative disorders like Huntington's and Parkinson's disease [43].

Kynurenine and KYNA have been implicated in various pain processes [4,11,28,29,40]. KYNA can also induce antinociception in a dose-dependent manner by intrathecal administration in the von Frey test [39] and enhance the acute antinociceptive effects of morphine when co-administered [26]. The opioid system – where morphine acts - is well known for its crucial role in pain relief. The system is composed of three opioid receptors, namely mu, kappa and delta (MOP, KOP and DOP) and a series of endogenous opioid peptides. Opioid receptors

Abbreviations: 3-NLT, 3-nitro-L-tyrosine; CNS, central nerve system; CSF, cerebrospinal fluid; BBB, blood-brain barrier; DAMGO, Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol; DOPr, δ opioid peptide receptor; 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; KOPr, κ opioid peptide receptor; KYNA, kynurenic acid; MOPr, μ opioid peptide receptor; S.E.M., standard error of means; TEM, Tris-HCl, EGTA, $MgCl_2$; Tris-HCl, tris-(hydroxymethyl)-aminomethane hydrochloride.

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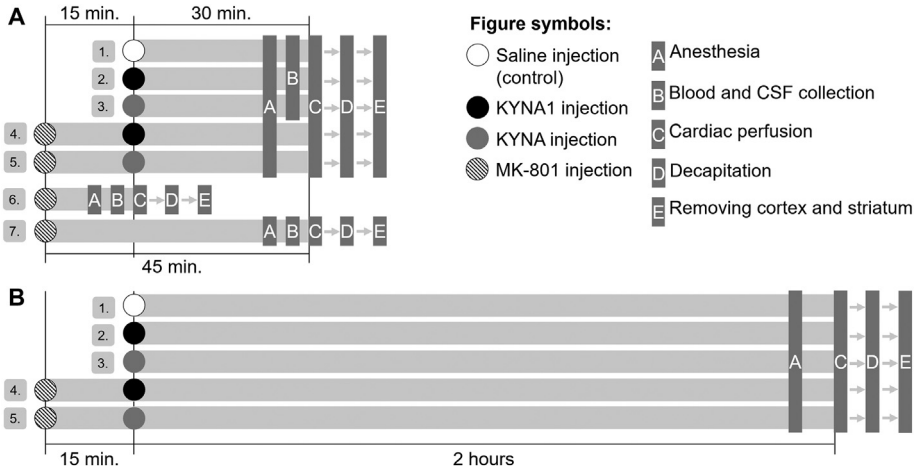

Fig. 1. Animal treatments, groups, treatment duration times and main steps following the injections. A: represents groups 1–5 in which the animals were sacrificed 30 min after KYNA/KYNA1 treatment together with groups 6 and 7 where animals received a single MK-801 injection and sacrificed 15 and 45 min after treatment. B: represents groups 1–5 in which the animals were sacrificed 2 h after KYNA/KYNA1 treatment. Circles and grey rectangles marked with letters (A–E) represent the injection time points and the steps following treatments, respectively. For additional information see under Sections 2.2.2 and 2.2.1.

belong to the G-protein coupled receptor (GPCR) superfamily and they are mostly coupled to $G_{i/o}$ type G-proteins. Activation of these receptors leads to the inhibition of adenylyl cyclase which causes hyperpolarisation in the cell and inhibits the release of certain neurotransmitters (for review see [1,2]). Opioid receptors are expressed in the gastrointestinal tract, spinal cord and in high quantity in the cortical and striatal regions of the brain [3].

In our earlier paper, we reported that KYNA and KYNA1 (Fig. 1B) - a structural analogue (also known as SZR72) with considerable blood-brain barrier (BBB) permeability [27] - did not bind directly to any opioid receptor, but after chronic administration they caused significant changes in receptor function in the cortex and striatum of mice [42]. Our hypothesis regarding to these effects was that KYNA and KYNA1 may alter opioid receptor function through the co-localized NMDA receptor.

In this study for the first time we investigated the acute effect of KYNA and KYNA1 on opioid receptor function together with the possible involvement of the NMDA receptor. Rats were acutely treated with a single dose of KYNA or KYNA1 intraperitoneally (i.p.) alone or in combination with the NMDA receptor antagonist MK-801. Additionally, KYNA and KYNA1 plasma and cerebrospinal fluid (CSF) concentration was measured after acute administration. Finally, isolated rat brain slices were treated *in vitro* with KYNA, KYNA1 and MK-801, to exclude the BBB and the peripheral metabolization of KYNA and KYNA1, *etc*. Samples from the cortex and striatum were investigated in opioid receptor mediated G-protein functional studies, similarly to our previous study [42].

2. Materials and methods

2.1. Chemicals

The MOR specific agonist enkephalin analogue Tyr-D-Ala-Gly-(NMe)Phe-Gly-ol (DAMGO) and the KOR agonist peptide dynorphin 1–13 were purchased from Bachem Holding AG (Bubendorf, Switzerland). The structurally modified DOR specific deltorphin II derivative, Ile^{5,6}deltorphin II was synthesized in the Laboratory of Chemical Biology of the Biological Research Center (BRC, Szeged, Hungary). Acetonitrile and perchloric acid was purchased from Scharlau (Barcelona, Spain), acetic acid was purchased from VWR International (Radnar, PA, USA). EGTA, Tris-HCl, $MgCl_2 \times 6H_2O$, NaCl, KCl, $NaHPO_4$, $NaHCO_3$, $CaCl_2$, $MgSO_4$, D-glucose, GDP, the GTP analogue GTPγS, kynurenic acid (KYNA), MK-801 hydrogen maleate (NMDA receptor blocker) 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. The radiolabeled GTP analogue, [^{35}S]GTPγS (specific activity: 3.7×10^{13} Bq/-mmol; 1000 Ci/mmol) was purchased from Hartmann Analytic (Braunschweig, Germany). For dissolving all ligands in receptor assays highly pure distilled water was used and they were stored in 1 mM stock solution at −20 °C.

2.2. Animals and treatments

2.2.1. Animals

For *in vivo* experiments 64 male Sprague-Dawley rats were used and 20 for *in vitro* with average body weights of 300 g. 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), and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§).

2.2.2. In vivo animal treatments

All animals for the *in vivo* experiments received a single intraperitoneal (i.p.) injection. The animal treatments are summarized in Fig. 1, together with the main steps following treatments. One set of animals was 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 sets of animals were decapitated 30 min after the treatments (Fig. 1A). 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 groups of animals was decapitated 15 min, while the other groups of animals were decapitated 45 min 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 min) and right after reaching the peak plasma concentration of KYNA/KYNA1 (15 + 30 min, see the next paragraph) (Fig. 1A). The other set of animals was 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 sets of animals were sacrificed

2 h after treatments (Fig. 1B). In the case of combined treatments MK-801 was administered to the animals 15 min 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 min and 2 h) were based on previously measured plasma concentration (yet unpublished data) of KYNA and KYNA1 after their i.p. administration: 30 min after injection the plasma concentrations of KYNA/KYNA1 was the highest, while after 2 h it was the lowest.

After the treatments, animals were anesthetized at the appropriate time points by chloral hydrate (Fig. 1). Those sets of animals which were anesthetized 30 min after saline, KYNA1 or KYNA alone treatments, the cerebrospinal fluid (CSF) and blood samples were collected immediately afterwards for KYNA and KYNA1 concentration analysis (see later Section 2.3) (Fig. 1A). This was followed by cardiac perfusion of the animals using 0.1 M PBS and finally the animals were decapitated (Fig. 1). After decapitation the perfused brains were removed, and the entire striatum together with the overlying cortex were excised (Fig. 1). Samples then were stored at −80 °C until membrane homogenate preparation.

2.2.3. 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 <30 s and placed 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 10 ml, 37 °C ACSF with oxygen supply. Cortex and striatum slices were incubated for 30 min. After the incubation period KYNA, KYNA1 or MK-801 were added to the bath for 30 min. The concentrations for KYNA and KYNA1 were 200 μM and for MK-801 it was 50 μM [7]. Samples then were stored at −80 °C until for the cell membrane preparation (see under Section 2.4.1).

2.3. Detection of KYNA and KYNA1 concentration in plasma and CSF with HPLC

2.3.1. Sample preparation

Animals that were sacrificed 30 min after the *in vivo* treatments (see Section 2.2.2) the CSF was taken quickly from the suboccipital cistern of rats with 23G needle to Eppendorf tubes (rats were anesthetized and they were taken to the stereotaxic setup in order to fix their head). After collection the CSF samples were stored at −80 °C 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 for KYNA1 concentration detection were collected and stored at −80 °C until use.

The serum samples for KYNA concentration detection were thawed and, after a brief vortex, the serum sample was 'shot' to 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. Additionally, control animals (saline treated) were also used to detect endogenous KYNA levels in the plasma.

2.3.2. Chromatographic conditions for KYNA and KYNA1

For the determination of KYNA1 from plasma and CNS and KYNA from CNS, a Thermo LCQFleet ion trap mass spectrometer was used equipped with an electrospray ionization (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: 4 kV, 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 SecurityGuard pre-column C18, 4 × 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.

The KYNA concentrations of the serum samples were quantified on the basis of the method of Herve et al. [44]. 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 × 4.6 mm I.D., 5 μm particle size (Phenomenex Inc., Torrance, CA, USA) after passage through a SecurityGuard pre-column C18, 4 × 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. The LOD and LLOQ for KYNA in the plasma samples were 1 nM and 3.75 nM, the relative standard deviation was ≤2.2% for the peak area response and ≤0.1% for the retention time for KYNA and the recoveries ranged from 103 to 108% for KYNA.

2.4. G-protein activity assay

2.4.1. 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 the 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.

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

The assays were performed according to previous reports, with slight modifications [34,38]. 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 [42]. The [^{35}S]GTPγS binding experiments were performed in triplicates and repeated at least three times.

2.5. 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 (R [30]).

Experimental data of [^{35}S]GTPγS binding assays were presented and analysed as previously described [42]. Stimulation was given as

percentage of the specific [^{35}S]GTPγS binding observed over the basal activity, which was settled as 100%. In the figures dose-response curves, E_{max} and/or $logEC_{50}$ values have been shown. The significance level was determined by using One-way ANOVA with Bonferroni's Multiple Comparison statistical analysis in GraphPad Prism 5.0. Significance was accepted at the $P < 0.05$ level.

3. Results

3.1. 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 min significantly decreased the maximum efficacy of DAMGO at the MOP receptor, the reduction was nearly 50% ($P < 0.001$, df: 6; Fig. 2A). 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. 2A). Interestingly 1 mg/kg MK-801 *per se* strongly reduced MOP receptor activity after 15 and 45 min in the cortex compared to control (both $P < 0.01$, df: 6; Fig. 2A) and after 45 min compared to KYNA treatment alone ($P < 0.05$, df: 6; Fig. 2A). The potency of DAMGO was not altered in either treatment condition (data not shown).

Interestingly, 30 minute 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. 2B). This enhancement was reduced by MK-801 in the combined treatments, but the reduction was statistically not significant (Fig. 2B). KYNA did not alter KOP receptor maximum efficacy significantly, neither MK-801 in either treatment condition (Fig. 2B). 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 minute KYNA and KYNA1 or 15 and 45 minute MK-801 acute treatments (data not shown). However, the potency of the DOP receptor agonist Ile^{5,6}-deltorphin II significantly enhanced after 30 minute KYNA1 treatment, which was reversed to control level by MK-801 co-administered with KYNA1 ($P < 0.01$, df: 6; Fig. 2C).

In the striatum in case of MOP and KOP receptor maximum G-protein efficacy was reduced by 30 minute KYNA (MOP receptor: $P < 0.01$, KOP receptor: $P < 0.001$, df: 6; Fig. 2D and E), KYNA1 treatments (both receptors: $P < 0.001$, df: 6, Fig. 2D and E) and also both (15 and 45 min) MK-801 treatments alone (MOP and KOP receptor, 15 and 45 min: $P < 0.001$, df: 6; Fig.2D 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. 2D). In case of KOP receptor co-administering MK-801 resulted a

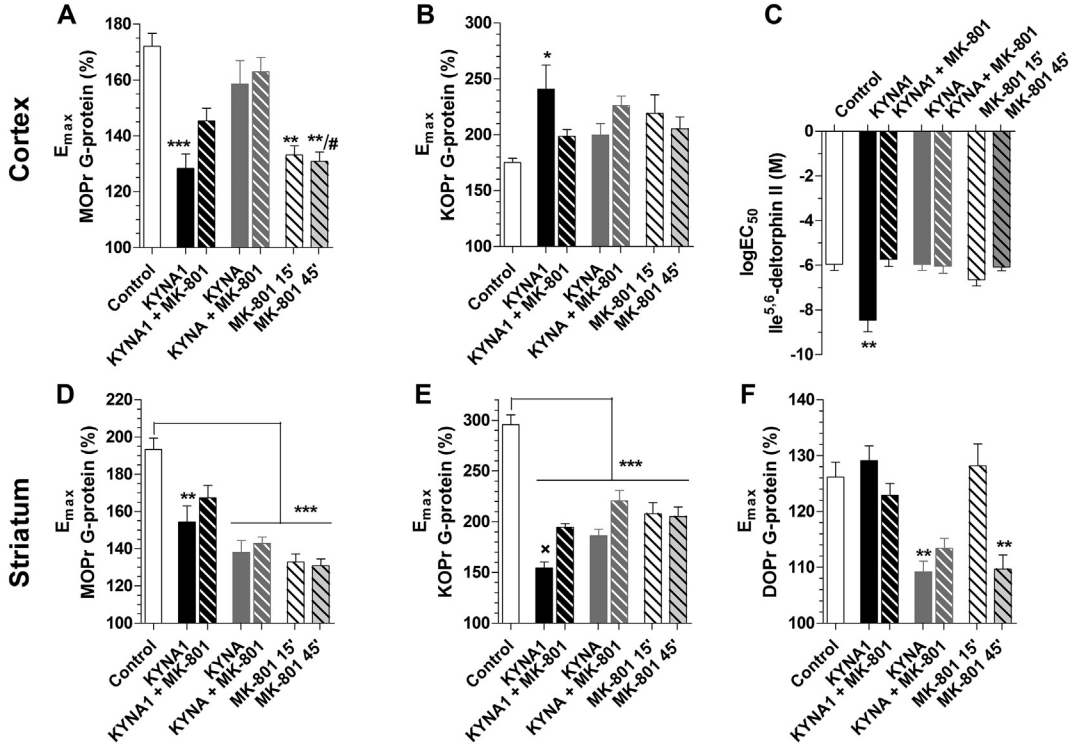

Fig. 2. The acute (30 min, *in vivo*) effect of KYNA, KYNA1 alone and in combination with MK-801 on agonist-stimulated opioid receptor G-protein activity in [^{35}S]GTPγS binding assays performed in rat cortex (A–C) and striatum (D–F). Each animal received a single i.p. injection of 296 mg/kg KYNA1 or 189 mg/kg KYNA dosage, while MK-801 was administered in 1 mg/kg i.p., as described in Section 2.2.2. A, B and D–F represent 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 2.5. *: 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 minute treatment alone. One-way ANOVA with Bonferroni's Multiple Comparison was used for statistical analysis. ***: $P < 0.001$, **: $P < 0.01$, */#/×: $P < 0.05$.

noticeable increase in the E_{max} values compared to KYNA1 and KYNA alone, although the difference was not significant (Fig. 2E). Interesting to note, that KYNA1 treatment alone reduced KOP receptor G-protein activity more effectively than the 15 and 45 minute MK-801 treatment (15 and 45 min: $P < 0.05$, df: 6; Fig. 2E). In the DOP receptor only 30 minute KYNA and 45 minute MK-801 treatments caused a significant reduction with a similar degree in the receptors G-protein activity ($P < 0.01$, df: 6; Fig. 2F). 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 hour 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). Thus, in summary, KYNA1 and KYNA was only effective 30 min after the injection, and the alterations were opposite depending on the brain area and opioid receptor (Table 1).

3.2. KYNA and KYNA1 plasma and CSF concentrations after 30 minute administration

Since the opioid receptor G-protein activity in the cortex and striatum was only altered after 30 min of KYNA1 and KYNA treatment, we measured the concentration of these two compounds in the cerebrospinal fluid (CSF) and compared it to their concentration in the blood plasma. The treatment conditions were the same as described in Section 2.2.2. Additionally, the endogenous KYNA plasma concentration was also determined for control. KYNA1 and KYNA CSF and plasma concentrations were measured by HPLC.

30 min following KYNA treatment the concentration of KYNA dramatically increased compared to endogenous KYNA plasma concentrations as expected ($P < 0.001$, df: 4; Fig. 3). 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. 3). KYNA1 displayed a significantly lower, 124 μM concentration in the plasma compared to KYNA ($P < 0.001$, df: 4; Fig. 3), and 65 μM in the CSF.

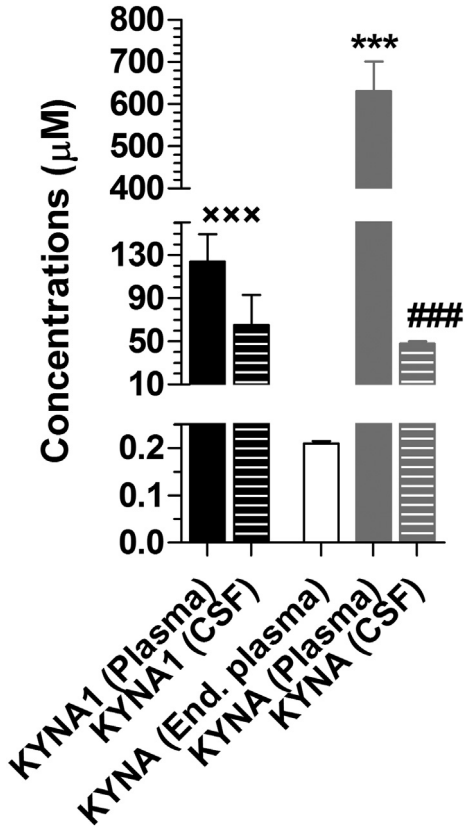

Fig. 3. The blood plasma and CSF concentration levels of KYNA1 and KYNA after 30 min of 296 mg/kg (KYNA1) and 189 mg/kg (KYNA) acute administration of the compounds. Additionally, the endogenous KYNA plasma concentrations (end. plasma) are also indicated for the control. For further information regarding to animal treatments and HPLC analysis see under Sections 2.2.2 and 2.3, 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. ***/###: $P < 0.001$.

3.3. The effect of in vitro administered KYNA1, KYNA and MK-801 on agonist-mediated opioid receptor G-protein activation in the 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 homogenization for G-protein activity studies as described earlier (Section 2.4.1). MOP, KOP and DOP G-protein activity was measured the same way as in *in vivo* studies (see Section 2.4.2).

In the *in vitro* studies of isolated cortex slices, the maximum G-protein activity 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. 4A). 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. 4A). 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 activity 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. 4B). Interestingly, KYNA did not alter the maximum activity of KOP receptor in the cortex (Fig. 4B) 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 significantly after KYNA, KYNA1 and MK-801 treatments as well ($P < 0.001$, df: 3; Fig. 4C), moreover the ligand potency of $Ile^{5,6}$-deltorphin II was increased after KYNA1 treatment ($P < 0.05$, df: 3; Fig. 4D).

Table 1
Summary and comparison of acute *in vivo* effect KYNA, KYNA1 and MK-801 treatment on opioid receptors mediated G-protein activity and ligand potency.

		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
MOR	Ctx.	∅	∅	↓	∅	↓	∅	↓	∅
	Str.	∅	∅	↓	∅	↓	∅	↓	∅
KOR	Ctx.	∅	∅	↑	∅	∅	∅	↓	∅
	Str.	↓	∅	↓	∅	↓	∅	↓	∅
DOR	Ctx.	∅	∅	∅	↑	∅	∅	∅	∅
	Str.	↓	∅	∅	∅	∅	∅	↓	∅

Ctx.: cortex, Str.: striatum, ↑: significant enhancement, ↓: significant inhibition, ∅: no significant effect.

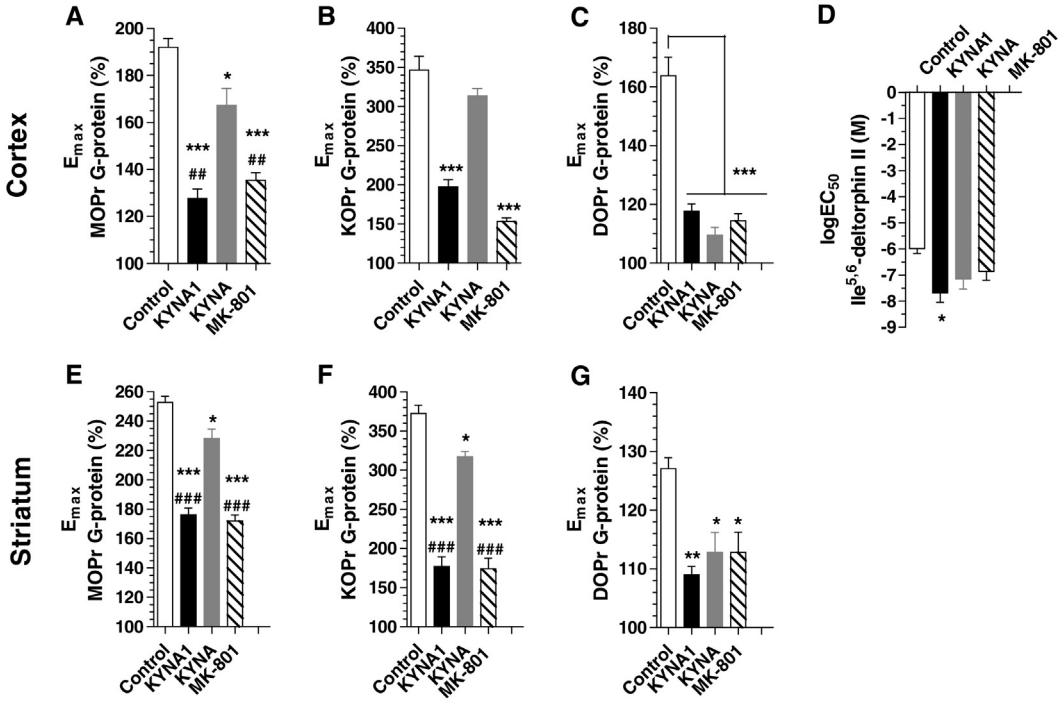

Fig. 4. Agonist-stimulated opioid receptor G-protein activity in [^{35}S]GTPγS binding assays performed in rat cortex (A–D) and striatum (E–G) homogenized brain slices after acute *in vitro* treatment of KYNA1, KYNA and MK 801. KYNA1 and KYNA were administered on cortex and striatum brain slices in 200 μM, while MK 801 in 50 μM concentrations. Samples were treated for 30 min in isolated organ baths as described in Section 2.2.3. A–C and E–G represent 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 2.5. *: 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. ***: $P < 0.001$, **: $P < 0.01$, *: $P < 0.05$.

In the striatum the *in vitro* treatments showed very similar results compared to cortex. For 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. 4E and F). In case of DOP receptors the reduction was less robust than in the cortex region, but KYNA reduced the E_{max} value with comparable extent to KYNA1 and MK-801 similarly to the cortex (KYNA1: $P < 0.01$, KYNA: $P < 0.05$, MK-801: $P < 0.05$, df: 3; Fig. 4G). 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. 4E 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 2). 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.

Table 2
Summary and comparison of acute *in vitro* effect KYNA, KYNA1 and MK-801 treatment on opioid receptors mediated G-protein activity and ligand potency.

		KYNA		KYNA1		MK-801	
		G-protein efficacy	Ligand potency	G-protein efficacy	Ligand potency	G-protein efficacy	Ligand potency
MOR	Ctx.	↓	∅	↓	∅	↓	∅
	Str.	↓	∅	↓	∅	↓	∅
KOR	Ctx.	∅	∅	↓	∅	↓	∅
	Str.	↓	∅	↓	∅	↓	∅
DOR	Ctx.	↓	∅	↓	↑	↓	∅
	Str.	↓	∅	↓	∅	↓	∅

Ctx.: cortex, Str.: striatum, ↑: significant enhancement, ↓: significant inhibition, ∅: no significant effect.

4. Discussion

In this study we have shown for the first time that KYNA1 and KYNA not only alter opioid receptor function after chronic treatment as showed previously [42], 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 [13,17,23,25,26,32].

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 [42]. The NMDA receptor has been demonstrated to bind KYNA - although with low micromolar affinity [35] – also, the interaction of NMDA and opioid receptors have been demonstrated in many levels [22]. Moreover, 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 [24] and anatomical studies [12] and with immunocytochemical labeling [9,10,37]. Thus in order to investigate

the possible role of the NMDA receptor we applied MK-801 (also known as dizocilpine) [41], a highly NMDA receptor selective antagonist [18,19]. MK-801 has also been demonstrated to alter opioid receptor-mediated effects [20,26], however it does not bind directly to opioid receptors [18] similarly to KYNA.

MK-801 alone behaved similarly as KYNA1 or KYNA (Fig. 2A and D). MK-801 in combination with KYNA1 or KYNA displayed a somewhat less pronounced reduction opioid receptor G-protein activity in certain groups (KYNA1 + MK-801: Fig. 2A and D; KYNA + MK-801: Fig. 2F) 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. 2D and E). In case of DOP receptor in the cortex, the enhanced agonist (Ile^{5,6}-deltorphin II) ligand potency followed by 30 minute KYNA1 treatment was reduced to control levels by MK-801 pretreatment (Fig. 2C). Interestingly, MK-801 alone did not cause any alterations in ligand potency following 15 or 45 minute administration (Fig. 2C), 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 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 [15,33], 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 [16]. In fact, a distinctive sodium binding pocket was discovered recently on MOP and DOP receptors [6,14]. 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. 2C).

Since only the 30 minute duration time showed significant results (Table 1), 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. 3). This proves that KYNA1 passes through the blood-brain barrier more easily than KYNA, which is in agreement with previous studies [27]. 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 minute *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 min. 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. 4B *vs* Fig. 2B). 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. 4C *vs* Fig. 2C).

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. 2B). 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 [5,8]. 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.

5. Conclusions

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.

Conflict of interest

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

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