ABRIDGEMENT OF THE DOCTORAL DISSERTATION

COMPARATIVE ELECTROPHYSIOLOGICAL STUDY OF KYNURENINES

Éva Rózsa

Supervisor:

Dr. József Toldi

Professor

University of Szeged

Faculty of Science and Informatics

Department of Physiology, Anatomy and Neuroscience

Szeged

2008

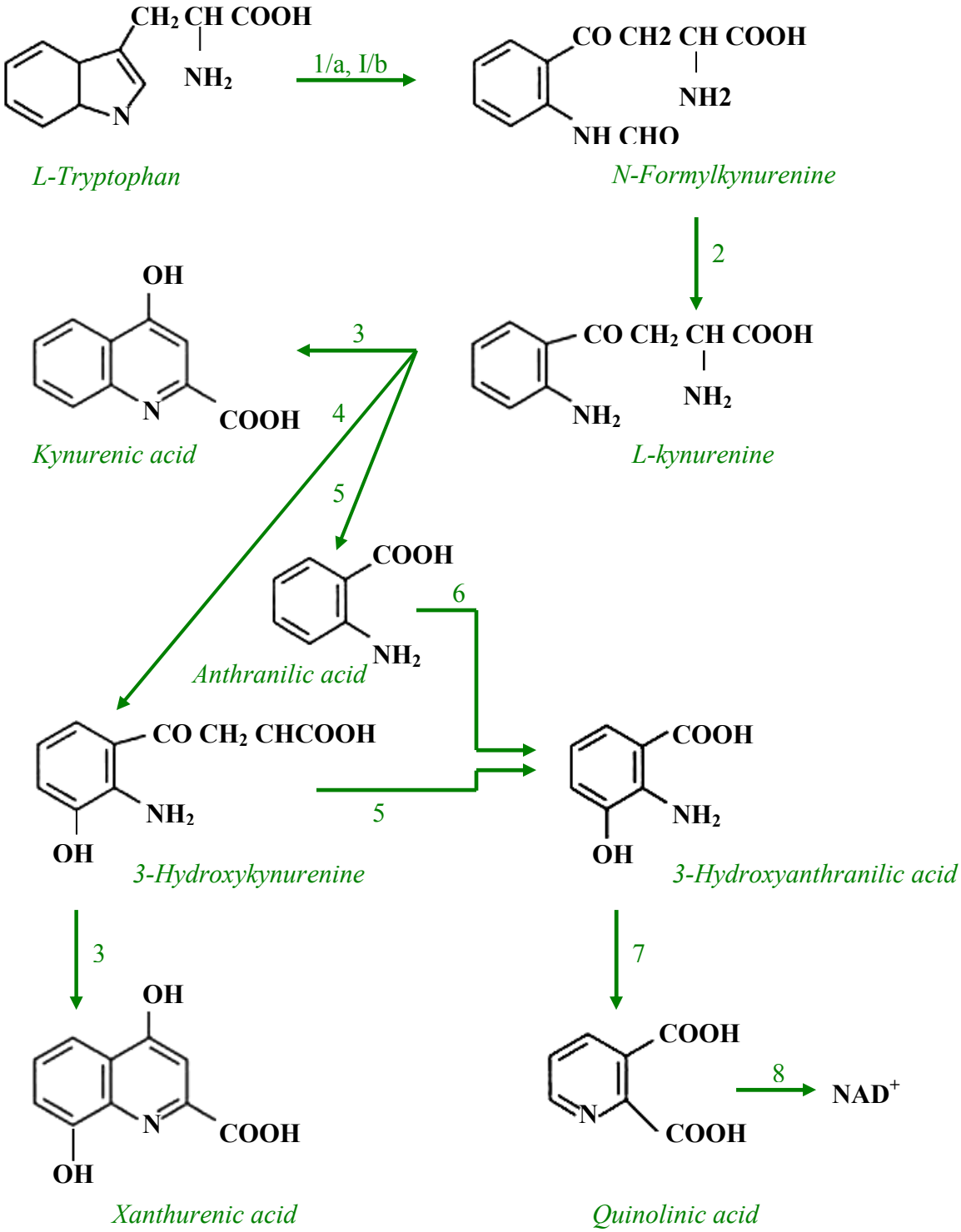
INTRODUCTION

Kynurenic acid was first identified as early as 1853, and half a century later the compound was recognized as a biproduct of the tryptophan metabolism. This route of conversion of tryptophan was in 1947 named the kynurenine pathway. In most tissues, including brain, a major proportion of the tryptophan which is not used for protein synthesis is metabolized along the kynurneine pathway. Kynurenic acid (KYNA) is an endogenous metabolite in the kynurenine pathway of tryptophan degradation and is an antagonist at the glycine site of the N-methyl-D–aspartate (NMDA) as well as at the alpha 7 nicotinic cholinergic receptors. In the brain tissue KYNA is synthesised from L-kynurenine (KYN) by kynurenine aminotransferases (KAT) I and II. KYNA is produced by astrocytes and secreted into the extracellular millieu in the close vicinity of glutamatergic synapses. The KYNA precursor KYN is the key compound of the kynurenine pathway and is present in low concentrations in the blood, brain and peripheral organs and it can easily cross the blood-brain barrier through the large neutral amino acid transporters.

Kynurenic acid has been suggested to be involved in the pathophysiology of several brain disorders, including Parkinson's disease, Huntington's disease, Sclerosis multiplex, Alzheimer's disease and epilepsy. In addition, accumulated data indicate that massively released excitatory amino acids play a major role in mediating the acute ischemic neuronal degeneration. Thus, one of the outstanding debates in the kynurenine field is, whether manipulation of the kynurenine pathway can modify the levels of kynurenic acid sufficiently to antagonize excitatory amino acid receptors. KYNA, the only known endogenous glutamate receptor antagonist, displays a particularly high affinity for the NMDA receptor, however, the its therapeutic use is rather restricted, because KYNA has a very limited abilty to cross the blood-brain barrier.

In contrast, the precursor of KYNA, KYN crosses this barrier more readily, and is converted to kynurenic acid in the brain tissue. Furthemore, the level of KYNA can also be controlled by synthetic KYNA derivatives. Some synthetic KYNA analoges penetrate the blood-brain barrier easily and thus can be succesfully used to elevate brain kynurenic acid level. During the past decade, the NMDA receptor has been identified as a primer target of drug development to combat neurological and psychiatric diseases which are thought to be directly or indirectly linked to abnormal glutamatergic neurotransmission.

The kynurenine pathway



1/a: indolamine-2,3-dioxygenase, 1/b: tryptophan-2,3-dioxygenase, 2: kynurenine formamidase, 3: kynurenine aminotransferase I, II, 4: kynurenine-3-hydroxylase, 5: kynureninase, 6: 3-hydroxyanthranilic acid hydroxylase, 7: 3-hydroxyanthranilic acid oxidase, 8: quinolinic acid phosphoribosyl transferase.

PURPOSES

Effects os KYNA

In our *in vitro* experiments, we tested the effects of KYNA on the young rat hippocampus. Our aim was to establish a concentration-response curve for further comparative electrophysiological studies with the KYNA precursor KYN and KYNA derivative SZR-72.

The pentylenetetrazole model

To test the anticonvulsant effects of KYNA, we used an *in vitro* pentylenetetrazole (PTZ) model. PTZ, a chemical convulsant frequently utilized in the study of seizures, exerts its effects by binding to the picrotoxin binding site of the post-synaptic GABA-A receptor.

The KYNA precursor: KYN

Previous studies revealed that rat brain slices have the ability to convert exogenously added KYN to KYNA. Our study was designed to examine the conversion of KYN to KYNA in hippocampal rat brain slices. We applied KYN as a pretreatment in the *in vitro* PTZ model and investigated whether rat hippocampal slices have the enzimatic capacity to convert KYN to KYNA in amount sufficient to provide protection againts PTZ induced hyperexcitation.

Synthetic KYNA analog: SZR-72

SZR-72 is a synthetic KYNA derivative. In our *in vitro* studies, we tested the new KYNA analog in the PTZ model as to compare its anticonvulsive effects with that of the endogenous compound.

Positive neuromodulatory effects

Studies on the mechanism of action of KYNA have demonstrated that KYNA acts as a competitive antagonist at the strychnine-insensitive glycine binding site of the NMDA receptor. In general, the concentration of KYNA required to block glutamate receptors lies in the range of 10-1000 μM. Thus, KYNA exerts it's anticonvulsant properties through the action on excitatory amino acid receptors at non-physiological, high concentrations. Then again, the concentration of KYNA is about 20 nM in the rat brain. The konwledge about the potential physiological significance of the compound is limited.

In the course of *ex vivo* experiments on anaesthetized animals, especially when KYNA or glucosaminekynurenic acid was administered in low dosage, on rare occasions it was observed that KYNA not only did not decrease the evoked responses, but in fact increased their amplitudes. These unexpected observations suggest that in these cases KYNA exhibits a reversal effect. Accordingly, we set out to test the hypothesis that KYNA at low dosage (nM) acts not as a neuroinhibitor, but as a neuroexcitatory agent. Our hypothesis was tested on young rat hippocampal slices, under well-controlled *in vitro* circumstances.

METHODS

Slice preparation

Animals were killed by decapitation and sagittal slices (400 μm) were prepared from their hippocampi with a vibratome (Campden Instruments), in a solution composed of (in mM): 130 NaCl, 3.5 KCl, 1 NaH_2PO_4, 24 $NaHCO_3$, 1 $CaCl_2$, 3 $MgSO_4$ and 10 D-glucose (all from Sigma, Germany), saturated with 95% O_2 + 5% CO_2. The slices were then transferred to a Haas recording chamber and incubated at room temperature for 1 h in the solution used for recording (differing only in that it contained 3 mM $CaCl_2$ and 1.5 mM $MgSO_4$).

Electrophysiology

To stimulate the Schaffer collaterals (constant current, 0.2 ms pulses delivered at 0.033 Hz), a bipolar stainless-steel microelectrode (FHC, USA) was used. The stimulus intensity was adjusted to between 25 and 40 μA to evoke the half-maximum response. The glass micropipettes (~1.5 MΩ) used for recording were filled with artificial cerebrospinal fluid (ACSF). The recordings were carried out in the CA1 region of the hippocampus at 34 ^{o}C. The recorded data were amplified (SEC-LX05, npi, Germany), filtered (1 Hz–3 kHz), acquired at a sampling rate of 10 kHz on a pClamp8 system (Axon Instruments, USA, Digidata 1320 A/D board), and analysed off line with Origin software (Microcal Software, USA).

Animals

The young (postnatal days 20-30) Wistar rats used in all experiments were kept under constant conditions of temperature and humidity and with free access to food and water. The principles of animal care (NIH publication No. 85-23), and the protocol for animal care approved by the Hungarian Health Committee (1998) and the European Communities Council Directive (86/609/EEC) were followed. All efforts were made to minimize animal suffering and to reduce the number of animals used.

Statistical analysis

All statistical analyses were carried out with SPSS Version 9.0. Results are expressed as means ± S.D. Statistical analysis was performed with one-way ANOVA followed by the post hoc Bonferroni test. $p < 0.001$ was considered statistically significant. Data for the statistical analysis were taken from time points where the changes in amplitude had reached their plateau (approximately 20-40 min after the application of each drug and 5 min prior to drug application in the controls).

DISCUSSION

To test the effects of KYNA, we used an *in vitro* PTZ model. PTZ, a chemical convulsant frequently utilized in the study of seizures, exerts its effects by binding to the picrotoxinbinding site of the post-synaptic GABA-A receptor. PTZ is known to suppress the inhibitory effects of certain neurotransmitters, and especially GABA, thereby leading to an easier depolarization of the neurones. In our experiments, PTZ administered *in vitro* at 1 mM induced a considerable increase in the amplitude of the fEPSPs recorded from the hippocampal CA1 region. When applied locally in an extremely high concentration (20 mM), PTZ resulted in characteristic wavelets. However, KYNA administration not only decreased the amplitude of the hippocampal CA1 responses evoked by Schaffer collateral stimulation, but also afforded protection from the PTZ-induced response enhancement. The KYNA precursor KYN also blocked the development of the 1 mM PTZ-induced high increase in amplitude. To prove that the KYN→KYNA conversion did take place in our experiments and that it was KYNA which afforded the protection against the effects of PTZ, we applied *N*-omega-nitro-l-arginine, an inhibitor of KAT I and II. After pretreatment with both KYN and L-NNA, the application of PTZ resulted in the marked increase in the fEPSP amplitudes, which indicates that the enzime inhibitor disabled KYNA production. SZR-72, a synthetic kynurenic acid derivative, applied *in vitro*, prooved to be also effective in preventing the high increase in fEPSP amplitudes, generated by PTZ.

Additionally we have shown, that KYNA in submicromolar concentration range has a positive neuromodulatory effect. In nM concentrations, kynurenic acid does not give rise to inhibition, but in fact facilitates the field excitatory postsynaptic potentials, recorded from the hippocampal CA1 region. The administration of KYNA at below 500 nM increased the amplitudes of the fEPSPs. The maximum facilitatory effect of KYNA was found at 250 nM. This effect was reversible and could also be washed out. KYNA at 250 nM resulted in an average increase of 20% in the amplitudes of the fEPSPs. Similar to KYNA, the synthetic derivative SZR-72 also displayed a positive neuromodulatory effect in nM concentrations.

CONCLUSIONS

According to our findings, a summary of following conclusions can be made:

KYNA, applied *in vitro* in μM concentrations decreases the fEPSP amplitudes in the young rat hippocampus. KYNA administration is effective in affording protection from the PTZ-induced hyperexcitability. Treatment with KYNA precursor KYN, or the synthetic kynurenic acid derivative, SZR-72, even at very low μM concentration, has also an effect on enhanced neural excitability and thus support the hypothesis that manipulations of the kynurenine pathway might be a rewarding target in different neuronal disorders affected by neuronal hyperexcitation. Besides its neuroprotective and anticonvulsant effects, KYNA in submicromolar concentration range has a positive neuromodulatory effect. The fact that KYNA displays neuromodulatory effects in low nanomolar concentrations in the young rat hippocampus leaves the question open as to whether KYNA has a significant role in the developing rat brain. At early developmental stages non-functional AMPA receptors have been found in both CA3 and CA1 hippocampal regions. The mechanism by which these silent synapses are converted to functional ones is still unknown and KYNA might affords an answer. The *in vivo* significance of KYNA remains to be determined and is of peculiar interest.

PUBLICATIONS BASED ON THE DISSERTATION

Rozsa E, Robotka H, Nagy D, Farkas T, Sas K, Vecsei L, Toldi J: **The pentylenetetrazole-induced activity in the hippocampus can be inhibited by the conversion of l-kynurenine to kynurenic acid: An in vitro study.** *Brain Res Bull* 2008, 30;76(5):474-9
IF: 1.684

Rózsa E, Robotka H, Vécsei L, Toldi J: **The Janus-face kynurenic acid.** *J. Neural Transm.* 2008, DOI 10.1007/s00702-008-0052-5
IF: 2.938

PUBLICATIONS RELATED TO THE DISSERTATION

Nemeth H, Robotka H, Kis Z, **Rozsa E**, Janaky T, Somlai C, Marosi M, Farkas T, Toldi J, Vecsei L: **Kynurenine administered together with probenecid markedly inhibits pentylenetetrazol-induced seizures. An electrophysiological and behavioural study**. *Neuropharmacology* 2004, **47**(6):916-925.
IF: 3.734, Független idéző: 2

Robotka H, Sas K, Agoston M, **Rózsa E**, Szénási G, Gigler G, Vécsei L, Toldi J.: **Neuroprotection achieved in the ischaemic rat cortex with L-kynurenine sulphate** Life Sci 2008, 82(17-18):915-9.
IF: 2.257

Katalin Sas, Hermina Robotka, **Éva Rózsa**, Márta Ágoston, Gábor Szénási, Gábor Gigler, Máté Marosi, Zsolt Kis, Tamás Farkas, László Vécsei and József Toldi: **Kynurenine diminishes the ischemia-induced histological and electrophysiological deficits in the rat hippocampus.** *Neurobiol of disease* 2008, (Article in press).
IF: 4,377

FURTHER PUBLICATIONS

Szegedi V, Fulop L, Farkas T, **Rozsa E**, Robotka H, Kis Z, Penke Z, Horvath S, Molnar Z, Datki Z: **Pentapeptides derived from Abeta 1-42 protect neurons from the modulatory effect of Abeta fibrils - an in vitro and in vivo electrophysiological study**. *Neurobiol Dis* 2005, **18**(3):499-508.
IF: 4.048, Független idéző: 3

Juhasz-Vedres G, **Rozsa E**, Rakos G, Dobszay MB, Kis Z, Wolfling J, Toldi J, Parducz A, Farkas T: **Dehydroepiandrosterone sulfate is neuroprotective when administered either before or after injury in a focal cortical cold lesion model**. *Endocrinology* 2006, **147**(2):683-686.
IF: 5.236, Független idéző: 5

Szegedi V, Juhasz G, **Rozsa E**, Juhasz-Vedres G, Datki Z, Fulop L, Bozso Z, Lakatos A, Laczko I, Farkas T: **Endomorphin-2, an endogenous tetrapeptide, protects against Abeta1-42 in vitro and in vivo**. *Faseb J* 2006, **20**(8):1191-1193.
IF: 6.721, Független idéző: 2

CONFERENCE ABSTRACTS

Rózsa Éva, Penke Zsuzsa, Farkas Tamás, Kis Zsolt, Horváth Szatmár, Soós Katalin, Penke Botond, Toldi József:
Comparative electrophysiological study of pentapeptides in the neurotoxicity produced by beta-amyloid peptide
IBRO International Workshop, 29-31 January, 2004, Budapest, Hungary

Éva Rózsa, Gabriella Juhász-Vedres, Marton Dobszay, Gabriella Rákos, Zsolt Kis, Tamás Farkas and József Toldi:
Dehydroepiandrosterone sulfate is neuroprotectiv ein a focal cortical cold lesion model
Congress of the Hungarian Neuroscience Society, January 27th, 2005, Pécs, Hungary

Gabriella Juhász-Vedres, **Éva Rózsa**, Zsolt Kis, Katalin Soós, Lívia Fülöp, Marta Zarándi, Tamas Farkas, Botond Penke and József Toldi:
Comparative electrophysiological study of aggregation inhibitors on the neuromodulatory effects produced by beta-amyloid peptide
Congress of the Hungarian Neuroscience Society, January 27th, 2005, Pécs, Hungary

Szegedi Viktor, Juhász Gábor, **Rózsa Éva**, Juhász-Vedres Gabriella, Datki Zsolt, Fülöp Lívia, Farkas Tamás, Kis Zsolt, Soós Katalin, Zarándi Márta, Budai Dénes, Toldi József, Penke Botond
Endomorphin II protects againts Ab1-42 induced neuromodulatory effect on CA1 hippocampal neuron sin vivo an din motor cortical slices in vitro
Congress of the Hungarian Neuroscience Society, January 27th, 2005, Pécs, Hungary

Rózsa Éva, Robotka Hermina, Sas Katalin, Vécsei László, Toldi József: **Az L-kinurenin neuroprotekcióban betöltött szerepe in vitro hippocampális agyszelet preparátumon**
Magyar Élettani Társaság LXX. Vándorgyűlése, Szeged, 2006. június 7-9

Robotka Hermina, Németh Hajnalka, **Rózsa Éva**, Sas Katalin, Toldi József és Vécsei László: **Az L-kinurenin neuroprotektív hatásának vizsgálata in vivo kísérletekben**
Magyar Élettani Társaság LXX. Vándorgyűlése, Szeged, 2006. június 7-9

Farkas Tamás, Juhász-Vedres Gabriella, **Rózsa Éva**, Soós Katalin Datki Zsolt, Fülöp Lívia, Kis Zsolt, Penke Botond és Toldi József: **Különböző pentapeptidek vizsgálata az 1-42 β-amiloid által okozott szinaptotoxicitásban**
Magyar Élettani Társaság LXX. Vándorgyűlése, Szeged, 2006. június 7-9

Robotka Hermina, **Rózsa Éva**, Kopcsányi Tamás, Berkecz Róbert, Péter Antal, Szatmáry István, Fülöp Ferenc, Vécsei László és Toldi József: **Egy új kinurénsav származék vizsgálata in vivo és in vitro módszerekkel**
Magyar Idegtudományi Társaság XI. konferenciája, Szeged, 2007. január 24-27,

Vécsei László, Knyihár Erzsébet, Klivényi Péter, Robotka Hermina, **Rózsa Éva**, Toldi József: **Neurodegeneráció, neuroprotekció és kinureninek**
Magyar Idegtudományi Társaság XI. konferenciája, Szeged, 2007. január 24-27