Anatomical and functional characterization of kynurenic acid production in the mouse brain

Summary of Ph.D. Thesis

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Szeged, 2018

1.Introduction

Kynurenine pathway (KP) is the main metabolic route of tryptophan (TRP) degradation in the mammalian brain which leading to the production of several neuroactive metabolites. One of the KP end products, kynurenic acid (KYNA) is widely studied as a neuromodulator and neuroprotective agent in the central nervous system (CNS).

KYNA has a broad-spectrum receptor modulatory effect both on ionotropic and metabotropic receptors. The main targets of KYNA in the brain are the N-methyl-D-aspartate (NMDA) and the α7 nicotinic acetylcholine (α7nACh) receptors, through which KYNA can modulate the level of glutamate, acetylcholine, GABA and also dopamine in the CNS.

Along with other metabolites, KYNA has been implicated in several brain disorders, so manipulation of the KP has high therapeutic potential. Decreased KYNA level has been observed in neurodegenerative disorders such as Huntington's and Parkinson's disease, while in disorders where cognitive functions are impaired -e.g. schizophrenia- KYNA is abnormally increased. Therefore both up and down regulation of KYNA production could serve as a potential therapeutical strategy.

KYNA is produced from L-kynurenine (L-KYN) by the action of kynurenine aminotransferases (KAT). So far, four KAT isoforms (KAT-1-4) have been identified from which KAT-2 is considered to be the major biosynthetic enzyme of KYNA in the human brain. Therefore studies investigating KYNA production are focusing on KAT-2 in rodents.The prevailing view about KAT-2 presence and KYNA production based on immunohistochemical studies carried out on rats, that it is localized in astrocytes and KYNA is released mainly by this cell type in the brain. Neuronal expression of the enzyme was only sporadic through the rat brain.

The pharmacological and genetic manipulation of KYNA production has been widely studied in rodents. Although the effect of genetic manipulations of the KYN pathway has been described in mice, the kat-2 knock-out model suffers from the general limitations of the null-mutant models in which peripheral KYNA function is also influenced. **To investigate tissue- and cell-type-specific function of KAT-2, one needs spatio-temporally selective kynurenergic manipulation, which is possible only if the expression profile of KAT-2 is well described in the wild-type animal. However, we have little information regarding the expression and function of KAT-2 in mice.**

While L-KYN readily pass through the blood brain barrier (BBB) kynurenic acid has a very limited ability to penetrate the BBB. So the easiest pharmacological tool to increase brain KYNA level is the administration of L-KYN, which resulted in a significant elevation of KYNA both *in vitro* and *in vivo.* The advantage of using acute brain slice preparations is that kynurenergic manipulation can be studied in a tissue specific manner without affecting the peripheral KYNA function. **Previously KYNA production and release to the extracellular compartment upon L-KYN exposure was described in acute rat brain slices. However, there is no data about the basal and L-KYN induced KYNA production of acute mouse brain slice preparations.**

Inhibiting KP enzymes is another promising tool to decrease or increase KYNA production with the aim of therapy. Shifting the KP toward KYNA synthesis with kynurenine 3-monooxygenase (KMO) inhibition studied in brain disease models like Alzheimer's or Huntington's disease, while lowering KYNA level with selective KAT-2 inhibitors improves cognitive function under conditions considered relevant for schizophrenia. **No pharmacological experiment has yet targeted the mouse KAT-2 function, probably because of its proposed irrelevance in the mouse species.**

Complicating therapeutical KP manipulation that there are prominent differences among mammalian species regarding the kynurenine system. For example, the KYNA synthesizing enzyme isoforms have a different role in different species. In the human and rat brain KAT-2 plays the major role in KYNA production, whereas in mice KAT-2 function is questionable. **Therefore comparative characterization of the kynurenine catabolism in different model species is essential before assigning therapeutical kynurenergic manipulation strategies in humans.**

2. Aims

During our experiments we investigated the kynurenic acid production in the mouse brain. The main aims of the present study were to:

Aim 1: Clarify the presence of kat-2 mRNA and protein in adult mouse brain tissue.

Aim 2: Develop an *in vitro* incubation system, where KYNA production can be investigated and manipulated in acute mouse brain slices.Test the after-incubation state of the brain slices in our system.

Aim 3: Investigate the basal and L-KYN induced KYNA production in acute mouse brain slices.

Aim 4: Evaluate the effect of KAT-2 inhibition in the mouse brain tissue with a selective KAT-2 inhibitor.

3. Materials and methods

8-12 week-old male C57Bl/6 mice were used during our experiments.

3.1 Anatomical study of the kynurenine system

3.1.1 Tissue preparation

Animals were deeply anesthetized with an intraperitoneal injection of urethane (1.6 g/bwkg) and were perfused transcardially with ice-cold 0.1 M phosphate buffer (PB, pH 7.4) following 4 % paraformaldehyde (PFA). The brains were removed and post-fixed overnight in 4 % PFA. All solutions were treated with diethylpyrocarbonate (DEPC) to avoid RNAse contamination during RNA in situ hybridization.

3.1.2 RNA in situ hybridization

For detection of kat-2 mRNA, in situ hybridization was performed. Sections were extensively washed in DEPC-treated PB containing 0.2 % Tween-20 (PBT, pH 7.4), digested with proteinase K for and post-fixed with 4 % PFA. After thorough washing in PB, slices were prehybridized for 1 h at 65 ºC in hybridization buffer and then hybridized overnight at 65 ºC in the same solution containing the DIG-labelled sense or antisense probe for kat-2 mRNA (~300 ng/ml). Next day, stringent washes were performed in descending concentrations of the hybridization buffer, then sections were blocked with 1 % NDS and incubated in AP-conjugated anti-DIG antibody overnight on 4 ºC. The following day samples were exposed to the NBT-BCIP substrate. Enzymatic reaction was stopped with PB (pH 7.4) and sections were coverslipped with aqueous mounting medium

3.1.3 Immunohistochemistry

Free-floating sections were washed in PBT and incubated in 1 % NDS. For the detection of KAT-2 and identification of the cells containing the enzyme, sections were exposed to the primary antibodies overnight at 4 ºC. Next day, samples were

incubated in the appropriate secondary antibodies at room temperature. Nuclei were labelled with 4',6-diamidino-2-fenilindol (DAPI).

3.1.4 Transfection and immunocytochemistry

Cells (HeLa ATCC® CCL2 ™) were plated on poly-lysinated dishes and were grown until 80% confluency at 37°C (5% CO_2). Cells were then transfected with 2 µg of the *kat-2* cDNA vector with FuGENE® HD Transfection Reagent prepared as recommended. After 48 hours incubation (37°C, 5% CO_2) cells were washed with PBS, and fixed with 4% PFA for 15 minutes. Cells were permeabilized with 0.1% Triton X-100. Non-specific antibody binding was blocked with 3% bovine serum albumin (BSA)-PBS solution for 1 hour. Cells were incubated in 4µg/ml of the primary antibody diluted in the blocking solution on 4°C, overnight. After thorough washing with PBS cells were incubated in the secondary antibody for 2 hours at RT. Digital photomicrographs were obtained with fluorescence microscope (BZ-X700, Keyence Corp.)

3.1.5 SDS gel electrophoresis (SDS-PAGE) and Western blot

The specificity of KAT-2 primary antibody was tested by SDS-PAGE and Western blot analysis. Protein samples from mouse brain tissue and HeLa cell culture were separated on a 8% gel at 100V for 1 h. Proteins were blotted to a PVDF membrane using a transfer buffer. After blotting, the membrane was washed and blocked with 5% nonfat dried milk. Membranes were probed to the primary antibody at 4˚C overnight. Next day the membranes were washed extensively and incubated with the HRP-conjugated secondary antibody for 1 h. The immunoreactive bands were visualized with a chemiluminescent kit and digital images were captured with Li-Cor C-DIGIT Blot Scanner.

3.2 Functional study of the kynurenine system

3.2.1 Tissue preparation for the *in vitro* studies

350 μm thick sections were obtained with a vibratome (Leica VT1200S, Germany); the two hemispheres were dissected and transferred to a holding chamber. Slices were allowed to recover for 30 min in aCSF. After recovering, one hemisphere of the slices were transferred to the incubation chambers containing a low bulk volume of ACSF (appr. 6ml of ACSF/100mg wet weight brain tissue) at 30 °C to reach optimal temperature for KAT-2 function.

3.2.2 Groups

For testing the after-incubation state of the brain slices in our system we examined two groups:(1) brain slice halves kept under standard conditions (high bulk volume ofACSF; ≈200ml/6half coronal slice) (**LV condition hereafter**) and (2) brain slices transferred to our incubation chambers containing a low bulk volume of ACSF (appr. 6ml of aCSF/100mg wet weight brain tissue) **(SV condition hereafter)**.

For investigating the L-KYN induced KYNA production we use 10 μM L-KYN, while the effect of KAT-2 inhibition was tested with PF-04859989 dissolved in ACSF in a final concentration of 5μM.

3.2.3 HPLC

KYNA level in the supernatants derived from the tissue and ACSF were measured with an Agilent 1100 HPLC system combined with a fluorescence and a UV detector. For the determination of KYNA, the excitation and emission wavelengths of FLD were set at 344 nm and 398 nm, whereas the UV detector was set at 365 nm for the determination of 3-NLT (internal standard). Chromatographic separations were performed on a Kinetex C18 column preceded by Security Guard pre-column C18.

3.2.4 Cresyl violet staining

We performed cresyl violet staining for morphological observations after different incubation conditions. Sections were rehydrated with descending grades of alcohol and stained with cresyl violet staining solution for 5 min. After staining, samples were passed through ascending alcohol solutions and immersed in xylene for 5 min. Sections were coverslipped with Entallan®.

3.2.5 Immunohistochemistry

Fixed brain slices were cryoprotected with sucrose solution and 30 µm thick sections were cut with a freezing microtome (Reichert-Jung 1206). For staining protocol see section 3.1.3.

3.2.6 Electrophysiology

Baseline synaptic function of the tissue was tested with input-output (I/O) curve recordings, while short term plasticity with paired-pulse paradigm on the CA3-CA1 cell synapses in the hippocampus. After 4 h incubation in the LV or SV condition, the slices were transferred to an interface recording chamber and superfused with aCSF. Schaffer collaterals were stimulated using a concentric bipolar stainless steel electrode (Neuronelektrod Ltd, Hungary). fEPSPs were recorded from the stratum radiatum of CA1 region with 1,5–3 mOhm resistance glass microelectrodes. Potentials were amplified and filtered with WPI AMP-04 amplifier and digitalized with Axon Digidata 1320A. Recordings were monitored and saved with Axoscope 10.0.

3.2.7 Lactate dehydrogenase (LDH) assay

For evaluating tissue viability we performed LDH activity assay on supernatant samples collected after 30 min, 4 h and 6 h incubation time. As positive control for LDH release we permeabilized the cell membrane with Triton X-100™ (1%). LDH activity was measured at 340 nm and 37 ˚C using an LDH activity assay kit on a BioLis 24i Premium system (Siemens). LDH activity was expressed as U/l/100 mg tissue.

3.2.8 Hexokinase (HK) assay

Glucose content was measured by HK activity assay on supernatant samples collected after 30 min, 4h and 6h incubation time. HK activity was measured at 340 nm and 37 ˚C using a two-step Glu HK activity assay kit on a BioLis 24i Premium system (Siemens).

4.Results

4.1 Anatomical study of the kynurenine system

4.1.1 Localization of kat-2 mRNA and KAT-2 protein

First we examined the kat-2 mRNA and KAT-2 protein localization in the mouse brain tissue with RNA *in situ* hybridization and immunohistochemistry. Similar to previous observations achieved in rats, glial presence of KAT-2 was significant both at the mRNA and protein level in several brain areas.

However, neuronal expression of KAT-2 was also pronounced throughout the mouse brain. Neurons expressing KAT-2distributed sporadically in the CA1 area of the hippocampus, dorsal striatum, and all layers of the medial prefrontal cortex , whereas there was a significant number of KAT-2^{+}/NeuN^{+}cells in the substantia nigra. The vast majority of these neurons were identified as GABAergic inhibitory cells in the examined brain areas.

We also examined the presence of KAT-2 in adult male Wistar rats. We found similar astrocytic and neuronal expression pattern of KAT-2 in the rat brain, which indicates that it is phylogenetically conserved in rodents.

These results support the growing body of evidence about neuronal KYNA synthesis and contradicts the previous common view that astrocyte is the only relevant KYNA producing cell type. Astrocytes and neurons may have a distinctive role in KYNA production in health and disease, which should be investigated in the future.

4.2. Functional study of the kynurenine system

4.2.1 The structural and functional integrity of the acute brain slices

During the functional studies we used a custom made *in vitro* incubation system, where the brain slices were incubated in a small volume of ACSF. Therefore we first tested the structural and functional integrity of brain slices in our system after 4 hour incubation time. We performed anatomical, biochemical and electrophysiological experiments.

First, we asked if the glucose availability in the ACSF under SV condition exceeds the nutrition demand of the slices. Glucose consumption measurement proved that glucose availability gradually decreased during the incubation. Glucose concentration was already dropped after 30 min, which was further declined after 4h incubation.

Alteration in LDH release to the extracellular space is a sensitive measure of cell viability. Under SV condition LDH release was continuously increasing in the ACSF during 30 min and 4 h. However, Tx-100 treatment resulted in a ≈14 fold increase of LDH in the ACSF after 4h incubation. This indicates that the cell membrane integrity is largely preserved in the course of 4h incubation.

For the histological examination of tissue state we performed NeuN immunolabelling and cresyl violet staining. There was no visible tissue damage in the vulnerable CA1 subregion of the dorsal hippocampus. CA1 pyramidal cell shape and size appeared normal after 4h incubation. However, the structural integrity of pyramidal cells in CA3 was not completely preserved. Cytosolic NeuN immunopositivity decreased and pyramidal cells deformed in the CA3.

To examine whether the function of astrocytes and neurons- the main sources of KYNA- had changed, we performed c-Fos immunolabelling on LV and SV brain sections. c-Fos positivity markedly reduced in neurons of the hippocampal CA1 subregion and strongly increased in astrocytes in other hippocampal areas (and also in the cortex) in our SV condition, indicating functional changes to these cells.

We also tested the baseline synaptic function and the plastic properties of the slices in the dorsal hippocampal area with I/O curve recordings and paired pulse paradigm. There was no significant difference in the I/O curve and paired-pulse ratio between the slices incubated under LV or SV condition, which indicates that the functional integrity of the brain tissue was preserved after 4 h SV incubation.
Although, we detected mild alteration in glial and neuronal function, brain slices remained metabolically active and brain tissue integrity were mainly preserved for further pharmacological experiments.

4.2.2 KYNA production in mouse brain slices

To examine whether mouse brain slices liberate endogenous and *de novo* produced KYNA upon L-KYN administration during 4 h long incubation period we performed HPLC measurements from brain tissue homogenate and from incubating ACSF. Both basal and L-KYN induced KYNA production could be measured in our system. As a result of 10 μM L-KYN administration we found a 6.3 fold increase in the ACSF and a 3.8 fold increase in the tissue KYNA content. Furthermore, ≈97% of the total KYNA content was released to the extracellular compartment (ACSF), whereas only ≈3% remained in the tissue under both condition.
Finally, we investigated the effect of the KAT-2 inhibitor on the L-KYN-induced KYNA release. Similar to former results, high KYNA content could be measured in the L-KYN group. Addition of the inhibitor resulted in a significant decrease of the released KYNA in the ACSF by almost 40%.
Summarize the functional experiments, brain tissue integrity remained sufficient to exclude that the observed KYNA release is a simple consequence of general cell degradation in our incubation system. For the first time in the literature we proved that the mouse brain tissue intensively produces and liberates KYNA into the extracellular milieu. Furthermore, KAT-2 inhibition significantly attenuates KYNA production, which indicates an important role of KAT-2 isoform in the

mouse brain tissue as well. Further *in vivo* and *in vitro* experiments are needed to clarify the precise role of KAT-2 and the mechanism of KYNA production and release in the mouse brain.

Summary

During our experiments we investigated KYNA production in the mouse brain tissue. We got the following main results:

1) We demonstrated for the first time that KAT-2 is localized both in astrocytes and neurons in the adult mouse brain. Furthermore we identified the GABAergic nature of KAT-2 containing neurons.

2) We developed a custom made *in vitro* incubation system. We tested the structural and functional integrity of brain slices in our system after 4 hour incubation time. Although, we detected mild alteration in glial and neuronal function, brain slices remained metabolically active and brain tissue integrity were mainly preserved for further pharmacological experiments.

3) We proved that mouse brain slices intensively produce and liberate KYNA to the extracellular compartment, while only a small proportion retained in the tissue both in the basal and L-KYN supplemented state.

4) Finally, we evaluated the effect of specific KAT-2 inhibition with the irreversible inhibitor PF-04859989. The inhibitor reduced extracellular KYNA content by almost 40% in the ACSF.

Taken together we can conclude that our anatomical and functional results regarding the KYNA production in the mouse brain are filling a research gap in the literature and support future pharmacological and genetic kynurenergic manipulation studies in mice.