Complex analysis of behavioral, histological and cardiovascular influences of acute L-kynurenine sulfate administration in C57Bl/6j mice

Summary of Ph.D. Thesis

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Introduction

Tryptophan is one of the essential amino acids used for protein biosynthesis. The degradation of tryptophan can lead to the formation of serotonin, a monoamine neurotransmitter known as a regulator in the limbic system. However, the serotonin pathway comprises only a small portion of the tryptophan catabolism, since 99% of the tryptophan is catabolized through the kynurenine pathway. It is a cascade of enzymatic steps generating biologically active compounds.

In this pathway L-Kynurenine (L-KYN) is a central metabolite. The early phase of the catabolic steps takes place mainly in the liver and the kidneys. However, the metabolization of L-KYN can effectively proceeds in the brain. The blood brain barrier strongly limits the penetrability of the kynurenine metabolites from the periphery to the central nervous system, since most of them can only be transferred by passive diffusion with a very low efficacy. One clear exception is the L-KYN, which can enter the brain with the aid of a large neutral amino acid transporter. Thus, the cerebral kynurenine metabolism is very responsive to the peripheral level of the L-KYN. Preclinical studies have shown that growth in the level of systemic L-KYN is particularly associated with a dose-dependent increase of its direct downstream metabolite kynurenic acid (KYNA) in the central nervous system. Evidence suggests that in the physiologically intact brain the most prominent and rapid change after peripheral L-KYN administration is the peak elevation of KYNA.

KYNA is a complex neuromodulator, antioxidant and neuroprotective endogenous molecule. Elevation of brain KYNA content is correlated with

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attenuation in the concentration of extracellular glutamate, dopamine and acetylcholine in distinct cortical and subcortical brain regions. KYNA influences neurotransmission through multiple actions at the pre- and postsynaptic site. KYNA directly attenuates neurotransmitter release, partly by inhibiting α7 nicotinic acetylcholine (α7nACh) receptor located on presynaptic terminals, and partly by stimulating G-protein-coupled receptor 35 (GPR35) localized on neurons and astrocytes. Thus, even the modest fluctuations in endogenous KYNA can bi-directionally control the extracellular levels of glutamate. KYNA hinders postsynaptic N-methyl-D-aspartate (NMDA) receptor currents by competitive antagonism at allosteric glycine binding site of NMDA receptor. Moreover, in the periphery and in the brain during neuroinflammation, KYNA promotes anti-inflammatory responses due to activation of aryl hydrocarbon receptor and GPR35 receptor expressed by immune-cells, as well as it presumably also modulates neuronal survival through extrasynaptic NMDA receptor blockade. Besides its receptor-mediated actions, KYNA itself is a potent antioxidant.

Therefore, elevation of brain KYNA level, either by administration of L-KYN or pharmacological manipulation of the availability of the kynurenine pathway enzymes, has become an attractive strategy to attenuate neuroinflammatory responses and to protect against glutamate induced excitotoxicity associated with ischemic brain injury. Accordingly, we and our collaborators achieved neuroprotection by the administration of L-KYN sulfate (L-KYNs) in experimental models of neurodegenerative diseases and ischemic stroke. Decades after the discovery of the neurotoxic and convulsant properties of glutamate, it has become clear that glutamate

hypofunction is also pathogenic and therefore undesirable. Accordingly, in preclinical studies acute or chronic elevation of brain KYNA content, achieved partly by the peripheral administration of L-KYN, has been suggested to trigger alteration in the behavior of rodents: animals expressed hypoactivity or spatial working memory deficit. Moreover, pre- and postnatal chronic L-KYN exposure provoked long-lasting neurochemical and behavioral abnormalities manifested in adulthood. However, the results assessing the behavioral effects of the kynurenerg manipulations emerged from studies that focused mainly on rats, after various-dose of L-KYNs treatment. Implementing similar experiments in mice is of particular importance, because such data is almost absent from the literature. Additionally, the available information concerning the effects of kynurenerg manipulation beyond neuroprotection is quite incomplete, since study on dose-dependent responses to various L-KYNs treatment is not available.

On a top of these, L-KYN and KYNA were attributed a direct role in the regulation of the systemic circulation. Namely, L-KYN was identified as an endothelium-derived vasodilator, contributing to peripheral arterial relaxation and regulation of blood pressure during systemic inflammation in rats. Furthermore, intravenous administration of low-dose L-KYN (1 mg/kg) has been shown to increase cerebral blood flow (CBF) in conscious rabbits. Therefore, we hypothesized that acute elevation of systemic L-KYN concentration may exert potential effects on mean arterial blood pressure (MABP) and on resting CBF in the adult mouse brain.

Aims

The experimental work of my thesis can be arranged to three main parts. Our specific research interests focused on the physiological intact C57Bl/6j mice, with the following questions:

1. How will the various L-KYNs treatments influence the locomotion activity, the episodic-like memory formation and the anxiety-like behaviors?

2. Does the L-KYNs treatment interferes with the neuronal activity of brain structures relevant to the behavioral experiments that can be visualized by means of immunohistochemical technique?

3. Does a high-dose L-KYNs treatment influences the systemic blood pressure or the cerebral blood flow anyhow?

Methods

For the experiments $8 - 12$ weeks old adult male C57Bl/6j mice ($n = 216$, body weight 28 ± 3 g) were used, which were housed under controlled laboratory conditions. All housing and experiments were conducted in accordance with the European Communities Council Directives (86/609/ECC) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32.§). Efforts were made to minimize the number of animals used and to reduce pain and discomfort. All of the experiments were approved by the following ethical license: XX/01593/I/2010.

1. Behavioral tests

In the first set of experiments, we wanted to assess the acute effects of L-KYNs treatment on the behavior of the animals, thus the mice were administered a various dose of L-KYNs (25 mg/bwkg, 50 mg/bwkg, 100 mg/bwkg, 200 mg/bwkg, 300 mg/bwkg) intraperitoneally 2 h before the experiments. The mice of control group were injected with the vehicle (0.1M phosphate buffer; pH 7.4). Experiments were video tracked by a coloured CCD camera (Sony, model: SSC-DC378P) connected to a personal computer. Behavioral data were collected and analyzed automatically with the SMART video-tracking system (PanLAB) or scored manually.

The ambulatory activity was assessed in an open field (OF) paradigm. The OF consisted of a square arena (50 × 50 cm) enclosed by continuous, 50-cm-high, light-gray opaque walls made of plexiglass. The animals were allowed to move freely for 8 min, while their horizontal ambulatory activity was tracked with the aid of a video-tracking system (SMART® by Panlab Harvard Apparatus). This allowed us to measure all the required parameters: total distance moved (cm), percentage of time spent immobility (%), average speed (cm/s).

Episodic-like memory performance was tested in a novel object recognition (NOR) paradigm. During this test the animals have to distinguish between a previously seen familiar object and a novel object. In order to analyze the NOR performance of the mice, a discrimination index (DI) was calculated as follows: *novel × 100/(novel + familiar)*, where "novel" is the time spent exploring a novel object and "familiar" is the time spent exploring a familiar one. This shows the object exploration preference, expressed in percentage.

Anxiety-like behavior was evaluated in an elevated plus-maze (EPM) paradigm. The nocturnal rodents instinctively avoid strongly illuminated open spaces. The EPM contains enclosed and open arms illuminated differently. Thus, the risk-bearing capability of the animals can be measured by quantifying the proportion of time spent in the open arms.

2. *Histology*

In order to study possible alterations in neural activity caused by an acute high-dose of L-KYNs administration (300 mg/bwkg) we measured changes of basal c-Fos expression in brain structures relevant to the behavioral experiments. Thus we performed c-Fos immunostaining in the CA1 area of the hippocampus and in the dorsal part of the striatum in the mice. First, we obtained 20-µm-thick coronal sections from the paraformaldehyde (4%) fixed mice brain (+0.54 mm and –2 mm from the bregma). In order to detect c-Fos-positive neurons in the striatum and in the hippocampus, sections were labeled by fluorescent immunohistochemical method. Then, fluorescent photomicrographs were obtained with an Olympus BX51microscope fitted with a DP70 digital imaging system. In both areas a subregion was delineated manually for the analyzation. Then c-Fos+ cells were automatically counted with custom-written software in MATLAB 7.1 (Mathworks, Natick, Massachusetts, USA). After automated threshold adjustment and noise reduction, fluorescent objects in the range 25–400 µm2 were accepted as cells and counted in binary images.

3. Mean arterial blood pressure (MABP) and cerebral blood flow (CBF) measurements

In the last set of experiment, we set out to obtain comparable information about the potential vascular effects of high-dose L-KYNs administration (300 mg/bwkg). On the day of experiments, the animals were anesthetized with isoflurane (1.5% induction, 1% maintenance in N2O:O2, 70:30%) and allowed to breath spontaneously through a head mask. The left femoral artery was cannulated for the continuous acquisition of MABP. While CBF was assessed through the intact parietal bone with the aid of laser speckle contrast imaging (PeriCamPSI HR System®, Perimed, Järfälla, Sweden). All variables were simultaneously acquired and stored using a personal computer with dedicated softwares (PeriSoft 2.5.5 for BP and PimSoft 1.5.4.8 for CBF Perimed, Järfälla, Sweden). Data analysis was conducted by custom written scripts in a MATlab environment (MathWorks Inc., MA, USA). After taking a baseline of 20 min registration, a single injection of L-KYNs or vehicle of the same volume (0.2ml) was administered intraperitoneally. Subsequently, MABP and CBF were continuously monitored for at least 2.5 h. To determine the changes in CBF, identical regions of interest were positioned over each hemisphere, over the somatosensory cortical area. The size of this area was 2.5 ± 0.3 mm^2. Raw CBF changes were expressed relative to baseline by using the average CBF value of the first 5 min of registration (100%) and the recorded biological zero obtained after terminating the animal (0%) as reference points. In order to discriminate significant CBF variations reliably, a range of normal CBF variation was determined according to a threshold level (± 1.5 stdev) for each individual measurement. Consequently, when CBF dropped below the determined threshold range, a

cerebral hypoperfusion transient, when exceeded that, a cerebral hyperemic transient was noted and the events were taken for further quantitative analysis. The following elements of the CBF transients were characterized: peak amplitude (%), duration (s), and magnitude expressed as area under the curve (% * s).

Results and discussion

1. Behavioral effects

Locomotion activity (OF)

The average speed of the control animals was 9.58 ± 1.1 cm/s, and they spent $28 \pm 10\%$ of the time without horizontal moving activity (immobility). Consequently, their total distance passed was 3390 ± 630 cm. Since the measured parameters of the various control groups were differed slightly from each other, every L-KYNs treated group was compared to a control group assigned on the same day (n = 9 – 10/group). The analyzation of the groups treated with lower-dose of L-KYNs (25 mg/bwkg, 50 mg/bwkg) confirms no statistical difference in any of the measured parameters. A One-Way MANOVA revealed a significant multivariate main effect for the treatment of 100 mg/bwkg L-KYNs (Pillai's trace = 0.419, $F_{(3, 14)} = 3.372$, p = 0.049). The animals moved slower, spent more time in immobility, so the total distance moved was decreased. Interestingly an opposite behavioral pattern emerged as a result of a higher dose of treatment. The administration of 200 mg/bwkg or 300 mg/bwkg L-KYNs significantly elevated movement velocity of the mice as compared with the vehicle-treated groups. Activation was observed mainly in their average speed, while their immobility time did

not decrease. In summary the mice expressed hypoactivity owing to a lower-dose of L-KYNs treatment, whereas the animals become hyperactive as the dose was raised over 100 mg/bwkg. The most prominent locomotor disturbances were seen in the 300 mg/bwkg treated group. Our results fit in the literature. It was shown previously that moderate dose of L-KYNs treatment (100 mg/bwkg) hinders the voluntary ambulatory activity of rats. The effects of a high dose of L-KYNs on the movement velocity was not investigated previously, so our findings are completely new. However, similar behavioral observations were reported earlier following systemic administration of the non-competitive NMDA receptor antagonist MK-801 to C57Bl/6 mice; the injection of a relatively low dose of MK-801 disrupted the movement velocity controls inducing an abnormal hyperactive condition. On the one hand we could verify that moderate L-KYNs treatment hinders the locomotor activity of mice just as it was reported in rats. On the other hand, our data revealed that a high dose of L-KYNs treatment impairs further the controls of voluntary movement by inducing an irregular hyperactive state, which can be correlated at least partly with the blockade of NMDA receptors. Therefore, we uncovered a non-linear dose-dependent characteristic of the L-KYNs treatment on the locomotion activity of mice.

Episodic-like memory performance (NOR)

In the vehicle-treated and the 25 mg/bwkg treated group mice spent more time exploring the novel object. In contrast the higher-dose L-KYNs-treated groups (100 mg/bwkg, 300 mg/bwkg) failed to recognize the novel object and spent equal times exploring both objects. As a result, the 100 mg/bwkg and 300 mg/bwkg treated mice object recognition memory performance was significantly hindered, expressed as DIs ($63 \pm 12.6\%$, $61.8 \pm 15.9\%$,

$47.1 \pm 10.7\%$, $48.1 \pm 12.8\%$; vehicle, 25 mg/bwkg, 100 mg/bwkg, 300 mg/bwkg L-KYNs) (n = 10-12/group). Accordingly, we can assume that the elevated brain KYNA content owing to a moderate to high dose of L-KYNs treatment interfered with the formation of recollection of the familiar object (encoding and memory consolidation). Thus it led to the impaired NOR memory performance. Others observed similar results after kynurenergic manipulation. In a radial arm maze paradigm systemic L-KYNs treatment (100 mg/bwkg) impaired the spatial working memory function in rats. Our data reinforce the amnestic property of the kynurenergic manipulation and confirms this effect in mice. Besides we may complete this notion with the following: under moderate dose of L-KYN (100 mg/bwkg) treatment significant memory disruption may not evolve.

Anxiety-like behavior (EPM)

The control animals entered the well-lit open arms of the EPM 12 ± 3 times on average, and spent $15 \pm 7\%$ of the time (300 s) exploring it. The ratio of the open-arm exploration was elevated dose-dependently ($15 \pm 7\%$, $23 \pm 17\%$, $23 \pm 12\%$, $25 \pm 6\%$; vehicle, 25 mg/bwkg, 100 mg/bwkg, 300 mg/bwkg L-KYNs), while the ratio of the enclosed-arm exploration was diminished ($75 \pm 8\%$, $61 \pm 21\%$, $60 \pm 19\%$, $58 \pm 7\%$; vehicle, 25 mg/bwkg, 100 mg/bwkg, 300 mg/bwkg L-KYNs) (n = 10-12/group). However, only the 300 mg/bwkg treated group differed statistically from the control group. Based on these findings we may conclude that the treatment increased the risk-taking behavior of the animals. In a similar set of experiments done by others an acute anxiolytic effect of the systemic L-KYN treatment was challenged. However, significant influence cannot be revealed in rats. Conversely we could demonstrate that the mice spent significantly more time

in the aversive open spaces of the platform. Consequently, the treatment dose-dependent anxiolytic effect was revealed.

2. Histological results

In a separate group of animals we estimated c-Fos expression levels in brain areas corresponding to behavioral paradigm following a systemic administration of a high dose of L-KYNs (300 mg/bwkg) (n = 10/group) administration. The dose was chosen based on a previous set of experiments. In the control group strong c-Fos immunopositivity was observed in the medial part of the dorsal striatum, whereas high number of cells expressing the c-Fos protein in the analyzed subsection (485 ± 245). A similar tendency was observed in the CA1 pyramidal cell layer (56 ± 22). In the L-KYNs-treated group, cells expressing the c-Fos protein were sporadic and their number were lower in the dorsal striatum (220 ± 121) and the hippocampus (32 ± 21) compared to the vehicle-treated group. In rodents the functional integrity of hippocampal CA1 region is essential to the formation of object recognition memory. There is an unequivocal relationship between decreased hippocampal c-Fos expression and episodic-memory impairments. Striatal medium-sized spiny neurons are one of the central components of the sensorimotor cortico-basal ganglia network, which is responsible for controlling voluntary movements and plays a critical role in determining the movement speed. The relationship between the dorsal striatum activity and c-Fos expression is well-described. In our experiment a high-dose of L-KYNs treatment (300 mg/bwkg) decreases the number of c-Fos-immunopositive-cells in both areas. Thus our data revealed that behavioral abnormalities owing to a single exposure of high-dose L-KYNs

may emerges related to the altered basal c-Fos protein expression and the imbalance of the striatal and hippocampal neuronal activity.

3. Circulatory effects

In the control group, MABP and CBF were stable (69 ± 4 mmHg and $100 \pm 5\%$, respectively) throughout the entire data acquisition period. In the L-KYNs-treated group, MABP was similar to that, of control group (73 ± 6 mmHg), while hypoperfusion transients of $22 \pm 6\%$, lasting 7 ± 3 min occurred in the cerebral cortex over the first $60 - 120$ min following drug administration ($n = 8$/group). Here we have shown that the L-KYNs treatment destabilized resting CBF of isoflurane-anesthetized mice by inducing a number of transient hypoperfusion events, with no simultaneous influence on MABP. The present data are at variance with previous observations attributing a vasodilator and blood pressure lowering effect to L-KYN, which may be due to the various L-KYN concentrations used, or the induction or lack of anesthesia, or species difference. While the exact molecular mechanism leading from elevated level of KYNA to vascular diameter changes remain to be clarified. Clearly, a number of possible processes could be operative in our model. One explanation could be that KYNA hinders the tonic activity of cortical astrocytes through non-neuronal, Mg^{2+}-insensitive NMDA receptor and GPR35 receptor. KYNA action on both receptor types leads to attenuated intracellular Ca^{2+} levels, associated with hindered release of vasoactive arachidonic acid substance and impaired control of CBF. Thus, this astrocytic pathway could be implicated in the cerebral hypoperfusion transients in response to high dose L-KYNs administration. The CBF attenuating property of L-KYN or KYNA is

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completely novel finding. Although, up to this point the possibility that this manipulation might interfere with the cerebrovascular regulation was not taken into account.

Summary

The impairment of the kynurenine pathway metabolism is increasingly considered to be involved in the occurrence and the progression of neurophysiological dysfunctions observed in various neurodegenerative diseases. Thus, manipulation of the availability of kynurenine metabolites with the aim of therapy has been extensively investigated recently. **Although, up to this point a detailed dose-response study related to the kynurenine manipulation effects beyond neuroprotection was not done. We have demonstrated that a single moderate L-KYNs administration can provoke numerous behavioral disturbances, whereas these effects become more dominant with the increase of L-KYNs concentration. Therefore, a single high-dose L-KYNs treatment next to the behavioral influences, could also alter genexpression and destabilizes resting CBF, a phenomenon that should be considered by interpreting the effect of kynurenergic manipulation on brain function. By planning clinical trials basing on kynurenergic manipulation possible behavioral and vascular side effects should also be considered.**

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Science metrics

1. Publications: 7
2. Summed impact factors: 18.921
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The thesis is based on the following publications

1. **Varga, D.,** Herédi, J., Kánvási, Z., Ruszka, M., Kis, Z., Ono, E., Iwamori, N., Iwamori, T., Takakuwa, H., Vécsei, L., Toldi, J., Gellért, L. (2015) Systemic L-Kynurenine sulfate administration disrupts object recognition memory, alters open field behavior and decreases c-Fos immunopositivity in C57Bl/6 mice. *Frontiers in Behavioral Neuroscience*, 9, 1-15.
doi: 10.3389/fnbeh.2015.00157.
IF.:3,104

2. Gellért, L., and **Varga, D.** (2016) Locomotion Activity Measurement in an Open Field for Mice. *Bio-protocol*, 6(13): e1857.
doi:10.21769/BioProtoc.1857. **(Online protokoll)**

3. **Varga, D.P.,** Menyhárt, Á., Puskás, T., Bari, F., Farkas, E., Kis, Z., Vécsei, L., Toldi, J., Gellért, L. (2017) Systemic administration of L-kynurenine sulfate induces cerebral hypoperfusion transients in adult C57Bl/6 mice. *Microvascular Research,* 114, 19-25.
doi:10.1016/j.mvr.2017.05.006.
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Other publications

4. Nagy, K., Plangár, I., Tuka, B., Gellért, L., **Varga, D.,** Demeter, I., Farkas, T., Kis, Z., Marosi, M., Zádori, D., Klivényi, P., Fülöp, F., Szatmári, I., Vécsei, L., Toldi, J. (2011) Synthesis and biological effects of some kynurenic acid analogs. *Bioorganic & Medicinal Chemistry*, 19, 7590-6. doi:10.1016/j.bmc.2011.10.029.
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5. Gellért, L., **Varga, D.,** Ruszka, M., Toldi, J., Farkas, T., Szatmári, I., Fülöp, F., Vécsei, L., Kis, Z. (2011) Behavioural studies with a newly developed neuroprotective KYNA-amide. *Journal of Neural Transmission*, 119, 165-72. doi:10.1007/s00702-011-0692-8.
    IF.:2,392

6. Gellért, L., Knapp, L., Németh, K., Herédi, J., **Varga, D.,** Oláh, G., Kocsis, K., Menyhárt, A., Kis, Z., Farkas, T., Vécsei, L., Toldi, J. (2013) Post-ischemic treatment with L-kynurenine sulfate exacerbates neuronal damage after transient middle cerebral artery occlusion. *Neuroscience*, 247, 95-101. doi:10.1016/j.neuroscience.2013.04.063.
    IF.:3,277

7. **Varga, D.P**., Puskás, T., Menyhárt, Á., Hertelendy, P., Zölei-Szénási, D., Tóth, R., Ivánkovits-Kiss, O., Bari, F., Farkas, E. (2016) Contribution of prostanoid signaling to the evolution of spreading depolarization and the associated cerebral blood flow response. *Scientific Reports*, 6, 31402. doi:10.1038/srep31402.
    IF.:4,847