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POST-ISCHEMIC TREATMENT WITH L-KYNURENINE SULFATE EXACERBATES NEURONAL DAMAGE AFTER TRANSIENT MIDDLE CEREBRAL ARTERY OCCLUSION

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Abstract—Since brain ischemia is one of the leading causes of adult disability and death, neuroprotection of the ischemic brain is of particular importance. Acute neuroprotective strategies usually have the aim of suppressing glutamate excitotoxicity and an excessive N-methyl-D-aspartate (NMDA) receptor function. Clinically tolerated antagonists should antagonize an excessive NMDA receptor function without compromising the normal synaptic function. Kynurenic acid (KYNA) an endogenous metabolite of the tryptophan metabolism, may be an attractive neuroprotectant in this regard. The manipulation of brain KYNA levels was earlier found to effectively enhance the histopathological outcome of experimental ischemic/hypoxic states. The present investigation of the neuroprotective capacity of L-kynurenine sulfate (L-KYNs) administered systemically after reperfusion in a novel distal middle cerebral artery occlusion (dMCAO) model of focal ischemia/reperfusion revealed that in contrast with earlier results, treatment with L-KYNs worsened the histopathological outcome of dMCAO. This contradictory result indicates that post-ischemic treatment with L-KYNs may be harmful.

Key words: focal cerebral ischemia, neuroprotection, glycine co-agonist site, NMDAR, MCAO model, kynurenines.

INTRODUCTION

As a result of the high energy demands of the central nervous system (CNS), a deprivation of oxygen and glucose leads in a short time to abnormal glutamatergic transmission. Malfunctioning of the ATP-dependent transporters results in a disturbance of ionic homeostasis, depolarization and the excessive release of glutamate from neural and glial stores in the extrasynaptic space. Acute or prolonged over-activation of N-methyl-D-aspartate receptors (NMDARs) allows the excessive entry of Ca^{2+}, initiating glutamate excitotoxicity, the common core feature of many neuropsychiatric disorders, including stroke, epilepsy, Alzheimer's disease and Huntington's disease (Endres and Dirnagl, 2002; Moskowitz et al., 2010).

Neuroprotective strategies usually have the aim of suppressing an excessive NMDAR function. Indeed, a number of NMDA antagonists have proven to be robust neuroprotectants in animal models of an ischemic/hypoxic (I/H) state, but many failed in clinical trials in consequence of their adverse side-effects (Ikonomidou and Turski, 2002; Muir, 2006).

The destructive effect of NMDAR over-activity is in contrast with the phenomenon that synaptic NMDAR activity mediates the survival of several types of neurons (Hetman and Kharebava, 2006; Hardingham, 2009). It has been reported that neurodegeneration in the basal ganglia is exacerbated by NMDAR antagonists (Ikonomidou et al., 2000), that an NMDAR antagonist enhanced apoptotic cell loss in a head trauma model (Pohl et al., 1999), and that synaptic NMDAR activity boosts intrinsic antioxidant defenses (Papadia et al., 2008). Furthermore, the targeting of ischemic brain areas by global NMDAR antagonism can confuse the functioning of brain areas unaffected by ischemic damage (Gunduz-Bruce, 2009). In this regard, a clinically tolerated neuroprotectant should antagonize the NMDAR function when it is excessive, but not later, without compromising the normal synaptic function.

NMDAR activation requires the definite depolarization of the cell and the presence of both glutamate and the full co-agonists glycine or D-serine (Kussius and Popescu, 2009; Papouin et al., 2012). Furthermore, the glycine co-agonist site is not saturated under physiological conditions, but is in a hyperactive state (Li et al., 2009; Fuchs et al., 2012). Glycine-site antagonists may be attractive neuroprotectants in this respect.

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Abbreviations: 3-HK, 3-hydroxykynurenine; CNS, central nervous system; dMCA, distal middle cerebral artery; dMCAO, distal middle cerebral artery occlusion; EEG, electroencephalography; I/H, ischemic/hypoxic; KYNA, kynurenic acid; L-KYNs, L-kynurenine sulfate; NDS, normal donkey serum; NMDA, N-methyl-D-aspartate; NMDAR, N-methyl-D-aspartate receptors; PB, phosphate buffer; QUIN, quinolinic acid.

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Kynurenic acid (KYNA) is an endogenous metabolite of the tryptophan metabolism. It is produced from its precursor L-kynurenine (KYN) by the enzyme kynurenine-aminotransferase II (KATII), and discharged from the astrocytes in the CNS (Swartz et al., 1990). KYNA is a competitive antagonist at the glycine/D-serine co-agonist site of the NMDAR. Furthermore, it plays a versatile role in pathological states, including inflammatory (Moroni et al., 2012), vascular (Sas et al., 2003) and antioxidant (Lugo-Huitron et al., 2011) processes. Acting on the α7 nicotinic acetylcholine receptor, KYNA also influences the excitability of neurons (Banerjee et al., 2012). A huge body of evidence indicates that manipulation of the brain KYNA levels can effectively ameliorate the histopathological outcome of experimental I/H state (Stone, 2000; Wu et al., 2000; Schwarcz and Pellicciari, 2002; Stone and Addae, 2002; Vamos et al., 2009; Zadori et al., 2009). The neuromodulatory properties of KYNA are now well-established (Vecsei et al., 2013).

In the present study, we investigated whether L-kynurenine sulfate (L-KYNs) administered after reperfusion (in a dose, formerly proved to be neuroprotective) diminishes the neuronal damage triggered by short-term occlusion of the distal middle cerebral artery (dMCA) in the rat cerebral somatosensory cortex. This novel dMCA occlusion (dMCAO) model was recently developed and characterized from histological and electrophysiological aspects in our research group (L. Knapp, manuscript under review).

EXPERIMENTAL PROCEDURES

Animals

Male Wistar rats ($n = 23$) weighing 200–250 g were used. The animals were kept under controlled laboratory conditions with free access to food and water. The experiments were carried out in accordance with the protocol for animal care approved by both the Hungarian Health Committee (1998) and the European Communities Council Directive (86/609/EEC).

Surgical procedure

Experiments were carried out under Nembutal anesthesia. The body temperature was maintained at 37 ± 0.5 °C with a self-regulating heating pad and rectal probe (Supertech TMP-5a). The animals were fixed in a stereotaxic headholder (David Kopf Instr.) and the left masticatory muscle was removed. The surface of the temporal skull was cleaned and the brain was exposed with a high-speed microdrill. The exposed cortical surface involved the trunk and main branches of the MCA. To induce ischemia, the MCA was carefully lifted through 1200 μm with a Fisher microsurgery hook with the aid of a micromanipulator, and occluded for 30 min. To terminate the occlusion, the hook was carefully removed, and restoration of the blood flow was confirmed under an operating microscope. Finally, the dura and the temporal muscle were replaced, the skin was closed with a silk suture and the wound was cleaned with iodine solution. All interventions were strictly synchronized in time, to make the effect of Nembutal on the experiment uniform.

Electrophysiology

60 s of electroencephalography (EEG) was recorded on the surface of the skull with a silver electrode (2 mm lateral to the sutura sagittalis and 3 mm behind the bregma), promptly before and in the 29th–30th min after dMCAO (sampling rate: 1024 Hz; gain: 1000×) with Experimetria NeuroSys software (Experimetria Ltd., Hungary).

EEG power analysis was performed with the EEGLab toolbox (Delorme and Makeig, 2004) and custom-written MATLAB 7.1 (Mathworks, Natick, Massachusetts, USA) software.

The range of frequency of interest was assigned to 2–20 Hz and further analysis was performed within this range.

Histology

Tissue processing. For the histological study, 5 days after dMCAO, animals were anesthetized with an overdose of urethane and perfused transcardially with ice-cold phosphate buffer (PB, 0.1 M, pH 7.4) and 4% paraformaldehyde (dissolved in 0.1 M PB, pH 7.4). The brains were removed and postfixed overnight in paraformaldehyde. On the next day, 20-μm coronal sections were obtained with a vibratome (Leica VT1000 S) between 0.5 and 4 mm behind the bregma (Paxinos et al., 1980). Two adjacent slices were collected in 500-μm steps, one for double immunostaining and the other for Fluoro Jade-C staining. Fluorescent photomicrographs were obtained with an Olympus BX51 microscope fitted with a DP70 digital imaging system.

Fluoro Jade-C staining. Fluoro Jade-C (FJ-C) staining was performed with the literature protocol (Schmued et al., 2005) with some modification. The slices were mounted on gelatine-coated slides, then coverslipped with Fluoromount. FJ-C-positive (FJ-C+) cells were counted in the ispilateral cortex at 40× magnification. Automated counting of FJ-C+ cells was performed with custom-written software in MATLAB 7.1 (Mathworks, Natick, Massachusetts, USA). After automated threshold adjustment and noise reduction, 25–400-μm^2 fluorescent objects were accepted as cells and counted in binary images.

Immunohistochemistry. Glial reaction was detected with an indirect immunohistochemical method. 20-μm-thick free-floating sections were washed in PB, and then incubated in 10% normal donkey serum (NDS). For the detection of activated microglia (mouse anti-CD11b, clone OX42, 1:1000, Millipore) and reactive astrocytes (rabbit anti-S100, 1:2000, DAKO), sections were exposed to the primary antibodies overnight at 4 °C, and to the appropriate secondary antibodies for 2 h at room

temperature. Primary and secondary antibodies were diluted in 0.1 M PB containing 0.4% Triton-X100, 2% NDS and 0.01 % sodium azide. The sections were coverslipped with an aqueous mounting medium.

Drug administration

The rats were divided into two groups: L-KYNs-treated animals (n = 11) received 300 mg/kg L-KYNs (dissolved in 5% NaOH, pH 7.4) intraperitoneally, immediately after reperfusion, while the control animals (n = 12) were treated with the vehicle.

All chemicals were purchased from VWR Ltd., Hungary, and Sigma, St. Louis, MO, USA.

Statistical analysis

Electrophysiology. EEG power spectra filtered at 2–20 Hz were decomposed at 1-Hz intervals. The EEG power of a given frequency was considered as an individual case. Analysis was performed with General Linear Model/Repeated measures (IBM SPSS Statistics version 20).

Histology. Numbers of FJ-C+ cells were compared with the General Linear Model. The effects of the different rats were used as random effects and the different treatments were used as fixed effects in the mixed effect linear model (IBM SPSS Statistics version 20).

RESULTS

Electrophysiology

The EEG registered for 60 s filtered for 2–20 Hz revealed a marked and characteristic change in EEG during dMCAO (Fig. 1). The power values in each frequency bin were submitted to separate repeated-measures analysis of variance, with period and frequency as within-subject factors. All effects with two or more degrees of freedom were adjusted for violations of sphericity according to the Greenhouse–Geisser correction.

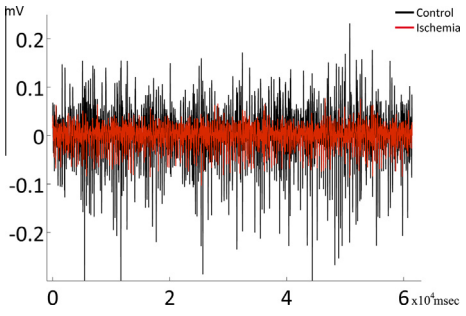

Fig. 1. EEG recordings from a rat somatosensory cortex ipsilateral to the dMCAO. 60-s EEG recordings during control (black) and ischemic (red) periods are superimposed. The EEG filtered for 2–20 Hz revealed a marked change during dMCAO. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The ischemic period significantly reduced the power of the signal as compared with the power of the EEG registered before ischemia (main effect of period: $F(1,21) = 32.989$, $p < 0.0001$, $\eta^2 = 0.61$; Fig. 2, panel A, B). It was earlier observed that somatosensory-evoked responses disappear completely during dMCAO (L. Knapp, manuscript under review). Together, these data indicate, that the dMCAO in our model resulted in a clean-cut decay of activity in the somatosensory cortices, i.e. the animals underwent a 30-min I/H period.

Histology

After a 5-day survival period, definite FJ-C staining and astrocyte/microglial activation throughout the somatosensory cortices emerged in approximately half of the animals, ipsilateral to the dMCAO (6/12 of the saline-treated animals; 5/11 of the L-KYNs-treated animals). In the remaining animals, no FJ-C staining and no glial reaction were observed, i.e. complete staining negativity. Ipsilateral to the dMCAO, astrocyte activation was characterized by hypertrophic astrocytes with prominent, thick processes and small vacuoles in the cell bodies as compared with the contralateral cortex (Fig. 3, panel A and insert). The microglia also revealed the activated phenotype ipsilateral to the dMCAO. Enlarged somata and the loss of secondary and tertiary branching were characteristic (Fig 3, panel B and insert). The glial reaction was more prominent in the L-KYNs-treated group (visual observation). The FJ-C staining distribution was similar to that in the activated microglia (compare Fig. 3, panels B, C). The groups were compared quantitatively for FJ-C staining. The number of FJ-C+ neurons was significantly higher in the L-KYNs-treated group (Fig. 4, General Linear Model; $p = 0.023$).

DISCUSSION

Physiological glutamatergic transmission through NMDARs is essential in the brain, playing a key role in development and synaptic plasticity. Due to its high permeability for Ca^{2+}, the NMDAR is linked to several cell-signaling pathways, and to learning and memory (Nakazawa et al., 2004; Zhang et al., 2007). In certain acute and chronic neuropsychiatric disorders, however, Ca^{2+} entry is the key mediator of glutamate excitotoxicity and the NMDAR is the primary source of a toxic Ca^{2+} influx (Stanika et al., 2012). NMDAR antagonism is therefore an obvious neuroprotective approach.

The failure of numerous antagonists in clinical trials is due in part to the different roles of synaptic and extrasynaptic NMDARs during excitotoxic processes. The hypothesis that extrasynaptic NMDARs mediate cell death, while synaptic NMDARs may promote survival was recently discussed (Hardingham and Bading, 2010; Li and Ju, 2012). From this respect, the selective targeting of extrasynaptic receptors without interfering with the normal synaptic function will involve a great advance (Chen and Lipton, 2006).

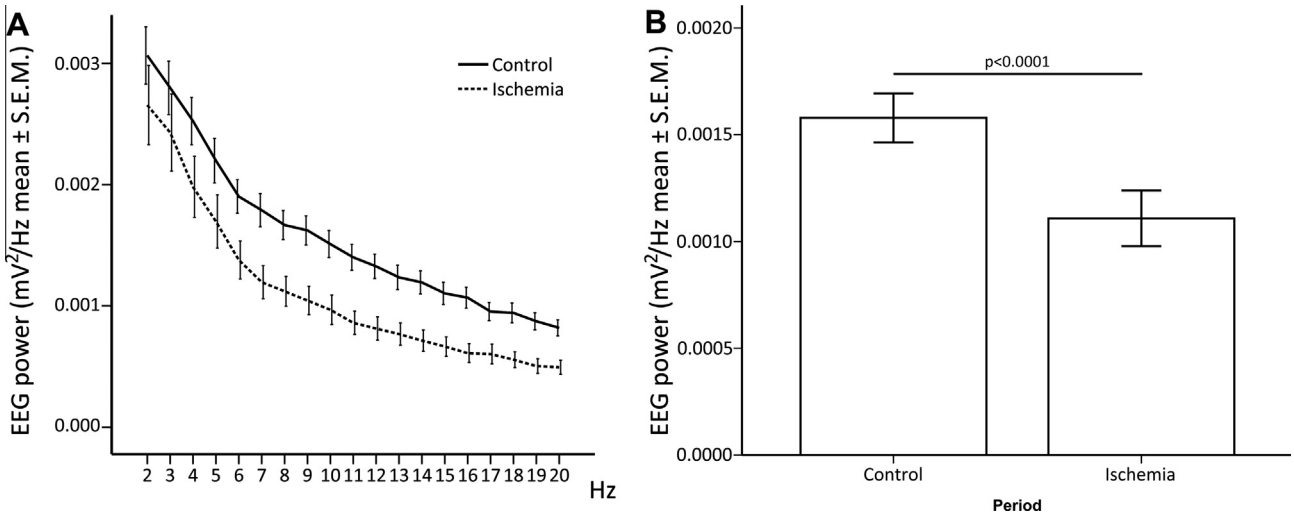

Fig. 2. (Panel A) EEG power decomposed at 1-Hz intervals. Lines demonstrate the EEG power of given frequencies during the control (line) and ischemic (dashed line) period (mean ± S.E.M.). (Panel B) The EEG power decreased significantly during the ischemic period (Repeated measures: $F(1,21) = 32.989$, $p < 0.0001$, $\eta^2 = 0.61$; mean ± S.E.M.).

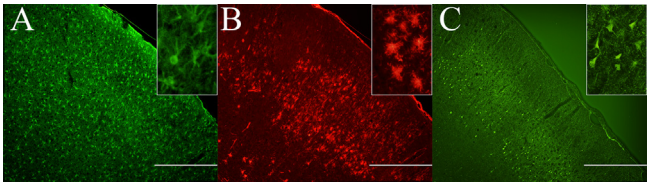

Fig. 3. Representative photomicrograph of the rat somatosensory cortex ipsilateral to the dMCAO after L-KYNs treatment. Double immunostaining of reactive astrocytes and microglia from the same slice; FJ-C staining from the adjacent slice (100× magnification, scale bars = 500 μm). Astrocyte activation was characterized by hypertrophic astrocytes with prominent, thick processes and small vacuoles in the cell bodies (panel A, and insert). The microglia also revealed an activated phenotype, enlarged somata and the loss of secondary and tertiary branching (panel B, and insert). A high number of FJ-C+ neurons were seen throughout the cortex (panel C). The FJ-C staining pattern closely followed the microglia distribution (compare panels B and C).

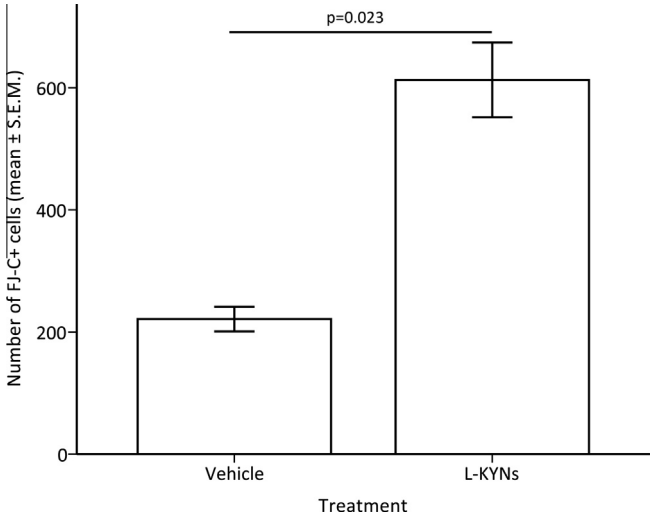

Fig. 4. FJ-C+ cells counted in the rat somatosensory cortex ipsilateral to the dMCAO. The numbers of FJ-C+ cells were compared with the General Linear Model, and plotted in a bar chart. The cell number was significantly higher in the L-KYNs-treated group (General Linear Model; $p = 0.023$; mean ± S.E.M.).

It has been argued that systemically administered L-KYNs is neuroprotective in different I/H states (Gigler et al., 2007; Sas et al., 2008). In such experiments, the I/H model triggered massive excitotoxicity and a high-level, long-lasting glutamate spillover. On the other hand, pre-ischemic treatment was effective, since the

KYN/KYNA transition in the astrocytes is time-consuming (Swartz et al., 1990).

However, in a recent study we showed that a KYNA derivative significantly diminished hippocampal neurodegeneration, even if administered at the time of reperfusion (Gellert et al., 2011).

A relatively brief MCAO evokes clean-cut neurodegeneration in only a fraction of the animals (Memezawa et al., 1992; Aspey et al., 2000; Popp et al., 2009). Similarly, in our experiment only half of the animals exhibited neurodegeneration, irrespectively of whether they received L-KYNs or saline treatment. However, the amplitude of the evoked responses (L. Knapp, manuscript under review) and the EEG power decreased markedly during dMCAO, and it may be therefore postulated, that the somatosensory cortices were subjected to an I/H state. This indicates that endogenous protective processes are able to withstand a short I/H state in this cortical area.

Systemic treatment with L-KYNs in our experiment did not alter the probability of occurrence of neurodegeneration, but extended the damaged area, the glial activation and the number of FJ-C+ cells in the animals, which ignited cell-death pathways.

Around one-quarter of the extrasynaptic NMDARs in adult hippocampal slices are perisynaptic (within 100 nm of the postsynaptic density). Of the dendritically localized extrasynaptic NMDARs, around one-third is adjacent to glia-like processes (Petralia et al., 2010). KYNA produced in the glia may therefore, antagonize both synaptic and extrasynaptic NMDARs, influencing pro-death or survival mechanisms, respectively.

The emergence of KYNA produced de novo from systemically administered L-KYNs takes time that is considerable from the aspect of an excitotoxic process (Swartz et al., 1990). However, KYNA or KYNA analogs can act quickly after administration. Furthermore, during a brief I/H state the presence of excessive glutamate and concomitant extrasynaptic NMDAR activation can last for minutes (Benveniste et al., 1984; Ikonomidou and Turski, 2002). The phenomenon that the KYNA analog, but not L-KYNs, is neuroprotective when administered after reperfusion may depend on the intensity and duration of the I/H state, the concomitant glutamate spillover, and the duration of the KYN-KYNA turnover.

Another possible explanation would be that L-KYNs administration led to the increased concentrations of quinolinic acid (QUIN) and 3-hydroxykynurenine (3-HK), neurotoxic components of the kynurenine pathway.

Several studies observed that increased brain KYNA levels follow systemic administration of L-KYNs. Swartz et al. found that striatal KYNA level increased gradually as a result of L-KYNs administered systemically in gradually increased doses. The main conclusion of this study was that extracellular levels of KYNA can be dramatically increased by pharmacologic manipulation of precursor levels (Swartz et al., 1990).

In another study concerning the effect of systemically administered L-KYNs on cortical spreading depression, intraperitoneal injections of L-KYNs were found to increase cortical KYNA level about 40-fold in rats (Chauvel et al., 2012).

Investigating the effect of systemically administered L-KYNs on sensory gating, Shepard and associates found that systemic administration of L-KYNs was not followed by an increase of the harmful L-KYN metabolite, QUIN (Shepard et al., 2003).

Astrocytes do not contain kynurenine 3-hydroxylase and therefore cannot produce 3-HK, but are able to produce large amounts of KYN and KYNA, whereas microglial cells preferentially produce intermediates of the quinolinic branch of the KYN pathway. It has also been demonstrated that the other main source of QUIN is the macrophage, infiltrated during inflammatory processes (Guillemin et al., 2001; Wonodi and Schwarcz, 2010).

In the main, the activation of the microglia increases extracellular levels of QUIN or other kynurenines that exacerbate neuronal damage (Schwarcz and Pellicciari, 2002).

In gerbils subjected to a period of cerebral ischemia, 50-fold QUIN level increases were observed 7 days after the onset of ischemia (Heyes and Nowak, 1990).

Finally, increased L-kynurenine influx from the blood exceeds the catabolic capacity of kynurenine 3-hydroxylase in microglia, promoting KYNA production in the astrocytes (Wonodi and Schwarcz, 2010).

Microglia activation and the infiltration of the macrophages follow the ischemic insult with a certain delay. So we might reasonably conclude that the extension of the damaged area in our experiments is not the result of high 3-HK or QUIN levels originated from L-KYNs administered promptly after reperfusion. Extension of the neural damage is attributable to the disturbed NMDAR-mediated survival mechanisms.

These data indicate that kynurenergic manipulation remains a potent strategy against excitotoxic cell death, but the excitotoxic state and treatment pattern should be well-tuned.

CONCLUSION

Suppression of excessive NMDA function has long been the focus of research aimed at neuroprotection after brain ischemia. However, robust NMDA antagonism is not acceptable from the clinical point of view, since normal synaptic NMDA function should not be inhibited, even in the ischemic brain. The endogenous KYNA acting at the glycine/D-serine co-agonist site of the NMDA receptors is a pharmacon that might potentially absolve this contradiction. Indeed, a huge body of evidence indicates that manipulation of the brain KYNA levels can effectively enhance the histopathological outcome of the experimental I/H state. However, the neuroprotective potential of L-KYNs administered after brief focal ischemia has not yet been tested; surprisingly, treatment with L-KYNs worsened the histopathological outcome in our experiments. This contradictory result indicates that post-ischemic treatment with L-KYNs may be harmful.

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