Ph.D. Thesis

NEUROPROTECTIVE STRATEGIES WITH KYNURENINES

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Introduction

Relative to other organs, bulk energy and oxygen consumption of the mammalian brain is extremely high. Most of the consumed energy is focused on stabilization and recovery of ionic homeostasis of the nerve cells. Release of excitatory and inhibitory neurotransmitters partly depends on changes to intracellular ion concentrations; therefore steady-state stability of ionic regulation is the most obvious condition of brain function.

L-glutamate (L-Glu) is the main excitatory amino acid in the brain, reaching a concentration of approximately 10mM across the whole brain tissue. However, the concentration of L-Glu in the extracellular space is only around 0.6 mM. Most of this transmitter is stored in synaptic vesicles in nerve endings. Hippocampal pyramidal cells and neural cultures undergo degeneration if they are exposed to 2-5 mM extracellular Glu concentration.

Short-term insufficiency of the blood supply in the brain tissue is followed by serious "energy debt". The ATP content of the neurons decreases, which results in the ATP-dependent transporters in the neurons and glia malfunctioning. Ionic dysregulation and depolarization of the cell membrane leads to glutamate, the main excitatory amino acid in the brain, to be released without control from its neuronal and glial stores. Toxic extracellular Glu concentration occurs within a short time period.

Mediating the harmful effect of toxic extracellular Glu, NMDA-type glutamate receptors play an essential role in this process. Due to a high Ca^{2+} permeability, activation of the receptors results in the depolarization of the neuron, and indirectly the activation of several Ca^{2+}-dependent intracellular messenger pathways. With a view to neuroprotection after ischemic insult, it would therefore seem obvious to antagonize the excessive NMDA receptor function.

Although neuroprotective strategies based on NMDA receptor antagonism were successful in the animal models of brain ischemia, the pharmacons could not be used in human therapy. Global NMDA receptor antagonism results in several adverse side effects that are unacceptable in human therapy. For instance, phencyclidine and ketamine may produce indications that mimic the negative and positive symptoms observed in schizophrenia, beyond respiratory depression or cardiovascular dysregulation. A further disadvantage of this strategy is that it may antagonize not only the excessive NMDA receptor function, but also the NMDA receptor-dependent endogenous neuroprotective processes that play an essential role in neuronal survival. Indeed, the harmful effect of NMDA receptor antagonists has been observed in several experiments after cerebral ischemia.

Concerning clinical expectations, the blockade of excessive NMDA receptor function, along with the conservation of physiological NMDA receptor function, would be ideal. Endogenous kynurenic acid (KYNA) may be able to meet such expectations.

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 brain parenchyma. KYNA is a competitive antagonist at the glycine/D-serine co-agonist site of the NMDAR. Furthermore it plays a versatile role, influencing dopaminergic and cholinergic synaptic transmission. Diversion of the kynurenin pathway has been described in several chronic neurodegenerative and neuropsychiatric disorders. The effect of KYNA has also been characterized in the animal models of these disorders. However, the practical use of KYNA for this purpose can be excluded because it is barely able to cross the blood-brain barrier. To exploit the neuroprotective effect of KYNA the following methods can be employed: one, diversion of the balance of the kynurenine pathway toward the production of KYNA; and, two, the synthesis of kynurenic acid analogs, which can readily cross the blood brain barrier and protect neurons against ischemic injury. Our research group and colleagues have investigated the latter method over several years.

Aims

i) We aimed to estimate the neuroprotective capability of a newly synthetized KYNA analog (2-(2-N,N-dimethylaminoethylamine-1-carbonyl)-1H-quinolin-4-one hydrochloride; Patent Application No: 104448-1998/Ky/me) in the four-vessel occlusion of global forebrain ischemia, measuring the rate of hippocampal CA1 pyramidal cell loss and the preservation of long-term potentiation at Schaffer collateral-CA1 synapses.

ii) We aimed to estimate the effect of the KYNA analog on spatial orientation and learning ability of rat in the Morris Water Maze.

iii) We aimed to estimate the effect of the KYNA analog on exploratory activity of mice in the open field. Furthermore, we aimed to estimate the effect of the dose of the KYNA analog that does not impair exploratory activity on spatial orientation and learning ability of mice in the radial arm maze.

iv) We aimed to prove the neuromodulatory effect of the KYNA analog with the aid of the scopolamine amnesia model in mice.

v) We aimed to investigate whether the diversion of the kynurenine pathway toward the production of KYNA is neuroprotective in temporary focal ischemia in rats.

Materials and methods

Ischemic models

The global forebrain ischemia was induced with the aid of 4VO procedure. Briefly, on the first day, the animals were anesthetized with chloral hydrate (4%, 1 ml/100 g, ip.), and the atlas bone was exposed. Both vertebral arteries were cauterized promptly through the alar foramens located on the lateral surface of the atlas. Thereafter, both common carotid arteries were exposed through a cervical midline incision and, without tightening, ligatures were placed upon them. Both wounds were sutured. 24 h later, under ether anesthesia, both carotid arteries were re-exposed and pulled out by the ligatures, and the blood flow was interrupted with aneurysmal clips.

Inducing temporary focal ischemia operations were carried out under Nembutal anesthesia (60mg/bwkg). 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.

Histological process

For the histological studies, 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 at 4 °C. Coronal, serial sections (20 or30 μm) were obtained with a vibratome (Leica VT1000 S), and were mounted on slides coated with 2% gelatin or were processed free-floating.

Fluoro Jade C staining

To reveal the necrotized neurons, Fluoro Jade C staining and DAPI counterstaining was applied (Sigma, St. Louis MO). After drying, slices were rehydrated through a graduated alcohol series, then were transferred to the 0.06% potassium permanganate solution for 10 min. Thereafter slices were rinsed in Fluoro-Jade C solution for 20 min and air dehydrated, xylene cleared and cover-slipped with Fluoromount (Serva) and subsequently protected from direct light.

Fluorescent immunohistochemistry

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.

Quantitative comparisons

FJ-C-positive cells in the CA1 hippocampal subfield were counted in one field of view, with a magnification of 200x, at the level of inversion of the upper convex curve into the upper concave curve. The number of necrotized neorons was assessed by an investigator who was unaware of the identity of the slices.

FJ-C positive cells on the somatosensory cortices were counted at 40x 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 accepeted as cells and counted in binary images.

Behavioral studies

Morris Water Maze

For Morris Water Maze, male Wistar rats (n=10) weighing 250-300 g were used. Animals were habituated to the inverse dark/light cycle and to the water maze for two weeks. Spatial learning was assessed in a circular tank (diameter 1.6m; depth 0.6m) containing 0.35m deep

water at room temperature. Rats were expected to locate an escape platform (diameter 8 cm) submerged 1.5–2 cm below of surface of the water. Platform was located always in the middle of the same goal quadrant during the whole experiment. After habituation period the acquisition period was performed on seven consecutive days. During acquisition period on rats two probe were performed. Pre-training probe was performed as first swim without platform immediately before the five-trials block of training. Another post-training probe was performed immediately after the five-trials block of training without platform.

Escape latency was considered as the time elapsed to locate the platform from arbitrarily allocated starting points in the first training trial. Summarized escape latency was considered as time elapsed to locate the platform (mean of five-trial training with platform). Preference for goal quadrant was considered as time spent at the goal quadrant related to adjacent and cross quadrants (pre-and post-trial probes without platform).

To facilitate spatial orientation prominent visual cues were located on the room walls (e.g. wall posters, electrical fittings). Two incandescent lamps suspended above the apparatus, and shielded by a white Plexiglas sheet supplied dim light. A low intensity white noise was emitted from an airing ventilator.

Open field

The exploratory activity of 9-16 week old male CFLP mice (n=10 per groups) were measured in the open field. The open field was a square arena in a ground-space of 50X50 cm. Walls were opaque (light grey, so that animals cannot see the room) and 50 cm high. Animals were allowed to move freely in the arena for five minutes. In the Open Field test animals received vehicle, 1 and 2 mmol/bwkg of the KYNA analog, respectively, (dissolved in PB, pH 7.4) i.p. 1 hour before the trial. During the test, we measured the total distance, maximal speed and immobility time.

Radial Arm Maze

Spatial orientation and learning ability of 9-16 week old male CFLP mice were measured in the radial arm maze. The maze consisted of a central platform 24.5 cm in diameter, with eight enclosed arms radiating outwards. Each arm was 38 cm in length, 9 cm in width and 20 cm in height with transparent plastic side walls. During the habituation period, food pellets were scattered throughout the maze. Thereafter food pellets were moved systematically toward the food cups. At last only the food cups were baited. Animals were habituated to the maze for two weeks, as described above. At the end of this period we introduced the four of eight arms baited paradigm. From that time every second arm was baited. The animal was placed into

the central platform and was allowed to move freely in the maze and to find the pellets. The session ended when the animal found the food in all arms or after 4 minutes. Investigating the chronic effect of the KYNA analog, animals were treated and tested eight consecutive days ($n_{control}$=10 and $n_{KYNA\ analog}$=12; 1 mmol/bwkg; dissolved in PB, pH 7,4). The effect of scopolamine (0.2mg/bwkg) and the KYNA analog (1mmol/bwkg) was assessed in a single trial. The preformance of the animals was assessed as follows: Reference memory error was considered as first entry into the non-baited. Total error was considered as entry into the non-baited arms. Working memory error was considered as re-entry into a baited arm. Total Time was considered as time elapsed until finding the food in all four arms.

In the behavioral experiments moving path of the animals was registered with SMART video tracking system.

Electrophysiological measurements

In vitro recordings

The animals were decapitated and the brains were rapidly removed. Coronal brain slices containing the hippocampus (350 μm) were cut with a vibratome (Campden Instruments) in ice-cold artificial cerebrospinal fluid (ACSF) containing the following ingredients (in mM): 130 NaCl, 3.5 KCl, 1 NaH_2HPO_4, 24 $NaHCO_3$, 1 $CaCl_2$, 3 $MgSO_4$ and 10 D-glucose, saturated with 95% O_2 + 5% CO_2. Freshly cut slices were placed in a Haas recording chamber with ACSF (differing only in that it contained 3 mM $CaCl_2$ and 1.5 mM Mg SO_4) for at least 1 h. Experiments were carried out at 34 °C with a stabilized ACSF flow rate.

Field excitatory post-synaptic potential registration

The stimulating electrode (a bipolar glass electrode pulled from a theta capillary) was placed in the stratum radiatum near the border between the CA1 and CA2 regions, in order to allow orthodromic stimulation of the Schaffer collateral/commissural pathway (constant current, 0.2-ms pulses delivered at 0.05 Hz). Field excitatory postsynaptic potentials (fEPSPs) were recorded from the stratum radiatum and stratum pyramidale with a 1–2-mOhm resistance glass microelectrode filled with ACSF and connected to a neutralized, high input-impedance preamplifier (100x gain) with a high-pass filter set at 5 kHz. The test stimulus intensity was adjusted to between 30 and 60 μA so as to induce ~50% of the minimum stimulus intensity that evoked a saturated EPSP (maximum fEPSP response) in the control rats. The fEPSPs were digitized, saved with a PC equipped with a Digidata 1200 interface and an

Axoscope10.0 recording system (Molecular Devices Corporation, Sunnyvale, CA, USA), and analyzed offline with Origin6.0 software (OriginLab Corporation, Northampton, MA, USA). Basal glutamergic synaptic properties were tested by means of fEPSP recording, expressed against gradually increased stimulating intensity (0-100 μA) in certain groups.

LTP of the Schaffer collateral-CA1 synaptic response was induced by high-frequency stimulation (0.2-ms pulses delivered at 100 Hz for 5 s) at 100% intensity of the test stimulus. After the high-frequency stimulation, the fEPSPs were evoked and recorded at low frequency for at least a further 60 min. The fEPSPs were monitored for 40–60 min before conditioning, until the amplitudes were generally stable.

In vivo recordings

In vivo EEG measurements were performed to characterize cortical electrical activity during the dMACAO. EEG was recorded on the surface of skull with a silver electrode (2 mm lateral to the sutura sagittalis and 3 mm behind the bregma; sampling rate: 1024 Hz; gain: 1000x) with Experimetria NeuroSys software (Experimetria Ltd. Hungary). EEG power analysis was performed with the EEGLab toolbox 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.

Results and discussion

The neuroprotective effect of the KYNA analog in global forebrain ischemia

In the first phase of our experiments we investigated the neuroprotective capacity of a newly synthetized KYNA analog with morphological and functional assessments in the four-vessel occlusion model of global forebrain ischemia in rats. The target of our histological and electrophysiological investigations was the hippocampus, which is particularly and selectively vulnerable to global ischemic insult. Morphological and functional changes of the pyramidal cells in the CA1 sub region of Ammon's horn can be easily estimated. Systemic treatment with the KYNA analog significantly decreased the number of FJ-C+ degenerated CA1 pyramidal cells, if administered before and then repeatedly after the ischemic insult ($p=0.001$). The neuroprotective effect also emerged if a single dose of the KYNA analog was administered promptly after the ischemic insult ($p=0.016$).

The protective effect has also been proved with electrophysiological measurements. The synaptic transmission was assessed with the aid of input-output curve registration. Relative to the control group the fEPSP amplitudes evoked by gradually increased stimulus intensity were significantly lower in the 4VO group (p=0,045). However, fEPSP amplitudes recorded in the animals treated with the KYNA analog repeatedly were not significantly different from the amplitudes registered in the control animals (p=0,997). Investigating synaptic potentiation, an increase of 30% of fEPSP amplitudes emerged after high-frequency stimulation in control animals. However, high-frequency stimulation in 4VO animals was not followed by the increase of fEPSP amplitudes. Relative to control group fEPSP amplitudes were significantly lower in the 4VO group (p=0.0001), synaptic potentiation could not be evoked. fEPSP amplitudes recorded in animals treated repeatedly with the KYNA analog were not significantly different from the control level (p=0.99).

The effects of the KYNA analog on spatial orientation and learning

We also investigated the effects of the KYNA analog on spatial orientation and learning in intact rats and mice.

Morris Water Maze

Significant preference for the goal quadrant could not be observed in the pre-training probe on days 5 and 6. Surprisingly, on the 7th day the preference for the goal quadrant increased significantly compared to all other quadrants ($p<0.02$ to all quadrants).

By the 5th day significant decrease of escape latency occurred (p=0.01). Neither vehicle nor analog treatment worsened the performance on days 6 and 7**.** Reference memory was also considered as the summarized mean escape latency of training trials. In this sense escape latency was decreased gradually, best performance could be observed on the 6th and 7th day (p=0,001). Neither vehicle nor analog treatment worsened the performance on days 6 and 7. Working memory performance was considered as daily post-training probe. Preference for goal quadrant was significantly higher on every day (p=0.008 to all quadrants), working memory was built-up successfully. In conclusion, treatment with either vehicle or 1mmol/bwkg of the analog did not worsen the performance of the animals.

Open Field

Treatment with 1 mmol/bwkg of the analog resulted in a marginal decrease in the ambulation of the animals. However 2 mmol/bwkg resulted in significant decrease of this parameter

(p=0.001). Both 1 and 2 mmol/bwkg of the analog decreased the maximal speed of the animals to the same level, however the difference was not significant**.** Interestingly, 1 mmol/bwkg of the analog decreased marginally the resting time of the animals, however treatment with 2 mmol resulted in a significant increase in this parameter (p=0.035). Summarizing our data, we can conclude that the dose of 1 mmol/bwkg did not influence the exploratory activity in the open field. However, the higher dose of the KYNA analog (2 mmol/bwkg) significantly decreased the travelled distance, and significantly increased the immobility time.

Radial Arm Maze

In this experiment we investigated the effect of chronically administered KYNA analog on spatial orientation and learning ability in the radial arm maze paradigm. During the 8-day experiment, vehicle or 1 mmol/bwkg of the KYNA analog (dissolved in 0.1 M PB, pH 7.4) were administered daily before radial arm maze trials.

By the 6th day of the radial maze imprinting test, the number of reference memory errors decreased significantly (p=0.001). Statistical analysis did not reveal significant difference between the two groups during the eight days (p=0.523).

The number of working memory errors decreased on the 2nd day significantly (p=0.001) and the low error rate stayed steady. Statistical analysis did not reveal significant difference between the two groups during the eight days (p=0.356).

The number of total errors decreased significantly by the 3rd day (p=0.042). This tendency was also observed on the following days. Statistical analysis did not reveal significant difference between the two groups during the eight days (p=0.121).

Mean time elapsed to complete the task decreased significantly by the second day (p=0.001) and continued to do so as the experiment progressed. Oscillation can be observed in the performance of both groups, however both show a tendency to improve. Statistical analysis did not reveal significant difference between the two groups during the eight days (p=0.753).

In conclusion, we were unable to find any significant difference between the improvements of the two groups in any situation. Chronic administration of 1 mmol/bwkg of the KYNA analog did not worsen the performance in the radial maze.

Scopolamin amnesia

In this experiment we investigated the effects of the KYNA analog on scopolamine amnesia. After a training period 0.2 mg/bwkg of scopolamine (dissolved in physiological saline solution) and 1 mmol/bwkg of KYNA analog (dissolved in 0.1 M PB, pH 7.4) were

administered concurrently. Data acquisition and analysis were performed in accordance with previous radial arm maze experiments. Measuring the performance of control, scopolamine treated and scopolamine+KYNA analog treated groups, we found significant differences regarding working memory error ($p=0.029$) and total time ($p=0.003$). Pairwise comparison between groups revealed, that number of working memory errors were not significantly higher relative to the control group, if scopolamine and the KYNA analog were administered concurrently ($p=0.530$). Similarly, time elapsed until completing the task was not significantly higher in the scopolamine+KYNA analog treated group relative to the control group ($p=0.137$). Regarding working memory and total time, the performance of scopolamine+KYNA analog treated group was not significantly lower relative to the control group. Although, in our previous radial arm maze experiment, investigating the chronic effect of the KYNA analog on spatial learning, we found that 1 mmol/bwkg of the analog does not worsen learning ability. With the aid of the scopolamine amnesia paradigm we proved the neuromodulatory effect of this dose.

The effect of L-KYNs administered after brief focal ischemia

In the second phase of our experiments we investigated the effect of L-kynurenin sulphate (L-KYNs) administered systemically after focal permanent brain ischemia. *In vivo* EEG measurements were performed to characterize cortical electrical activity during the dMACAO. The EEG registered for 60 s filtered for 2-20 Hz revealed a marked and characteristic change in EEG during dMCAO. The ischemic period significantly reduced the power of the signal as compared with the power of the EEG registered before ischemia ($p=0,0001$). 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.

Systemic administration of L-KYNs divert the kynurenine pathway toward the production of endogenous KYNA, so we expected a modest neurodegeneration after its administration. However, we experienced unexpected, contradictory results; the extent of reactive gliosis and the number of degenerated FJ-C+ neurons ($p=0,023$) actually increased in the somatosensory cortices of the rat. Our explanation is that the blocking of NMDA receptor-dependent endogenous neuroprotective processes by KYNA is responsible for the extension of neurodegeneration.

Summary

Based on our experiments, we can posit that the development and utilization of KYNA analogs that can readily cross the blood-brain barrier is more advantageous, considering the clinical expectations. These analogs will not be metabolized further, they act rapidly, and can more easily pass through the narrow therapeutic window after brain ischemia. Using analogs we can determine a dose that is neuroprotective but that induces no adverse cardio-respiratory and psychotomimetic side effect, which would be unacceptable in clinical use.

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