RECENT FINDINGS IN KYNURENINE RESEARCH IN THE FIELD OF EXPERIMENTAL EPILEPSY AND STROKE MODELS. ELECTROPHYSIOLOGICAL, BEHAVIOURAL AND HISTOLOGICAL STUDIES

Ph.D. Thesis

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II. **Robotka, H.,** Németh, H., Somlai, C., Vécsei, L. and Toldi, J. (2005) Systemically administered glucosamine-kynurenic acid, but not pure kynurenic acid, is effective in decreasing the evoked activity in area CA1 of the rat hippocampus. *European Journal of Pharmacology*, **513**: 75-80. (IF: 2.477)

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III. Németh, H., **Robotka, H.,** Marosi, M., Kis, Z., Farkas, T., Vécsei, L. and Toldi, J. Kynurenine administered together with probenecid markedly inhibits pentylenetetrazol-induced seizures. An electrophysiological and behavioural study. *11th Annual Meeting of the Hungarian Neuroscience Society, Pécs, 25-29. January, 2005, Clinical Neuroscience* **58**:

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V. **Robotka, H.,** Németh, H., Rózsa, É., Sas, K., Toldi, J., Vécsei, L. Az L-kinurenin neuroprotektív hatásának vizsgálata *in vivo* kísérletekben. *Magyar Élettani Társaság LXX. Vándorgyűlése, Szeged, 2006. június 7-9.*

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Abbreviations

ACSF	artificial cerebrospinal fluid solution
AD	Alzheimer's disease
AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
α7-nACh	α-7-nicotinic acetycholine
BBB	blood-brain barrier
CNS	central nervous system
CSF	cerebrospinal fluid
EAA	excitatory amino acid
EST assembly	Expressed Sequence Tag assembly
fEPSPs	field excitatory postsynaptic potentials
FJ	Fluoro Jade B®
FJ^+	FJ-B positive
GABA	γ-aminobutyric acid
G-KYNA	glucoseamine-KYNA
3-HANA	3-hydroxyanthranilic acid
3-HAO	3- hydroxyanthranilate 3,4-dioxygenase
HD	Huntington's disease
HFS	high-frequency stimulation
3-HK	3-hydroxykynurenine
IDO1	indoleamine 2,3-dioxygenase-1
IDO2	indolamine 2,3-dioxygenase-2
IO	input-output
i.p.	intraperitoneally
KATs	kynurenine aminotransferases
KP	kynurenine pathway
KYNA	kynurenic acid
L-KYN	L-kynurenine
L-KYN+PROB-4VO	L-KYN + PROB pre-treated animals
LTP	long-term potentiation
MCA	middle cerebral artery
mitAAT	mitochondrial aspartate aminotransferase
MRPs	multidrug resistance-associated proteins
MS	Multiple sclerosis
NAD	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NeuN	anti-neuronal nuclei
N-formyl-KYN	N-formyl-kynurenine
NMDA	N-methyl-D-aspartate
PD	Parkinson's disease
PROB	probenecid
PTZ	pentylenetetrazole
QUIN	quinolinic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SC	sham-operated controls
TDO	tryptophan 2,3-dioxygenase
TLE	temporal lobe epilepsy
TRP	L-trypthophan
4VO	4-vessel occlusion
4VO-L-KYN+PROB	L-KYN + PROB post-treated animals

Summary

The kynurenine pathway is one of the metabolic routes by which the essential amino acid tryptophan can be catabolised, producing nicotinamide, a compound that is essential for normal physiological function. Metabolism through the kynurenine pathway yields several neuroactive intermediates, including L-kynurenine, which is formed in the mammalian brain (40%) and is taken up from the periphery (60%), indicating that it can be transported across the blood-brain barrier. It was discovered some 30 years ago that compounds in the kynurenine family have neuroactive properties. L-kynurenine, the central agent of this pathway, can be converted to two other important compounds: the neuroprotective kynurenic acid and the neurotoxic quinolinic acid.

Quinolinic acid is a neurotoxic agent, which can augment the overexcitation of excitatory amino acid receptors and can therefore cause neuronal damage. It has similar neurotoxic effects to those of glutamate in the neocortex, striatum and hippocampus. This glutamate mediated excitotoxic damage can cause abnormalities in cellular Ca^{2+} homeostasis, elevated production of reactive oxygen species, and induce lipid peroxidation. These processes of cell damage may be prevented by using another metabolite of this pathway kynurenic acid.

Kynurenic acid which is one of the few known endogenous broad-spectrum antagonist of excitatory amino acid receptors, especially the N-methyl-D-aspartate receptors behaves as a neuroprotective agent: it can inhibit the overexcitation of these receptors by binding the glycine allosteric site. Moreover, kynurenic acid non-competitively blocks the activity of presynaptic α7-nicotinic acethylcholine receptors.

Kynurenines have been shown to be involved in many diverse physiological and pathological processes. There are a number of neurodegenerative disorders whose pathogenesis has been demonstrated to involve multiple imbalances of the kynurenine pathway metabolism. It is extremely important that the kynurenine pathway abnormality should be suitably prevented or corrected which could attenuate the pathological processes. Therapeutic use of kynurenic acid is difficult enough since it has a very limited ability to cross the blood-brain barrier. The aim is to develop different analogues and pro-drugs of kynurenic acid, which can readily cross the barrier and display similar effectiveness on the affected receptors to that of kynurenic acid.

In our experiments, we examined the effects of a relatively high dose of L-kynurenine (300 mg/kg; i.p.), which leads to an elevated concentration of kynurenic acid in the brain. This effect was augmented by using probenecid (200 mg/kg; i.p.), which prevents the discharge of the organic acids from the cerebrospinal fluid. In our studies, we tested the neuroprotective effects of these treatments in animal models of epilepsy and transient global ischemia. To determine the neuroprotective effects, we investigated these drugs in electrophysiological, histological and behavioural experiments, too.

It is well known that kynurenic acid does not readily cross the blood-brain barrier; hence, its use as a neuroprotective agent is rather difficult. The Department of Physiology, Anatomy and Neuroscience and the Institute of Medical Chemistry at the University of Szeged have developed a new kynurenic acid analogue, glucoseamine conjugate of kynurenic acid, which readily crosses the blood-brain barrier, separate glucoseamine and kynurenic acid in the brain, and increase the kynurenic acid level in the central nervous system. In this study, we examined the effects of peripherally administered glucoseamine-kynurenic acid on hippocampal-evoked activity, in comparison with peripherally administered pure kynurenic acid. These drugs were administered alone, or in combination with probenecid.

Our results and the related literature indicate the importance of the normal function of the kynurenine pathway and the possibilities of neuroprotection with kynurenine derivatives. Elevated levels of kynurenic acid or its analogues in the brain may reduce the overexcitation of excitatory amino acid receptors and may modify or arrest the progression of various neurodegenerative disorders. This can offer a novel therapeutic opportunity where the development of these compounds promises a key for brain neuroprotection.

Introduction

L-tryptophan (TRP), one of the ten essential amino acids, is involved in protein synthesis and acts as a precursor of many biologically active substances. Besides its participation in the process of protein synthesis, in mammals TRP is metabolized in several pathways. The most commonly known is the serotonergic pathway, which is active in platelets and neurons, and yields 5-hydroxy-TRP and then serotonin. TRP is also the precursor of a pineal hormone, melatonin. A less well-known, but actually the main alternative route for the TRP metabolism, is through the kynurenine pathway (KP) (Fig. 1), a cascade of enzymatic steps involving a number of biologically active compounds. It is noticeable that 95% of the TRP is catabolised through the KP within the brain. The metabolites of the KP, collectively termed 'kynurenines', have been shown to take part in many diverse physiological and pathological processes. In particular, fluctuations in the levels of the kynurenines have discrete effects on the nervous and immune systems. Although a century has passed since the kynurenines were first recognized as major catabolic products of TRP, very little attention was paid to their possible involvement in biological processes until the 1980s.

The first KP metabolite of TRP, which was recognized as being neuroactive at the cellular level, was quinolinic acid (QUIN) (Fig.1). This compound depolarises neurons by activating N-methyl-D-aspartate (NMDA) receptors [1]. As a result, it is also able to produce excitotoxicity [2], and this realisation has led to QUIN being implicated in a variety of central nervous system (CNS) disorders (see [3-5]). By definition, excitotoxicity is a result of the overexcitation of glutamate receptors. The concentrations of QUIN causing cell damage do not need to be greatly elevated. The amounts of QUIN in the brain and cerebrospinal fluid (CSF) are normally less than 100nM, but levels only slightly greater than this can cause neurotoxicity when cells are exposed for several hours [6, 7] or weeks [8] with some neurons being damaged after exposure to only 100nM QUIN [9]. QUIN takes part in lipid peroxidation and produces free radicals. It also affects mithochondrial function by decreasing superoxide dismutase activity [10].

The other important metabolite of KP is kynurenic acid (KYNA) (Fig.1), which is one of the few known endogenous broad-spectrum antagonists of excitatory amino acid (EAA) receptors, especially the NMDA receptors.

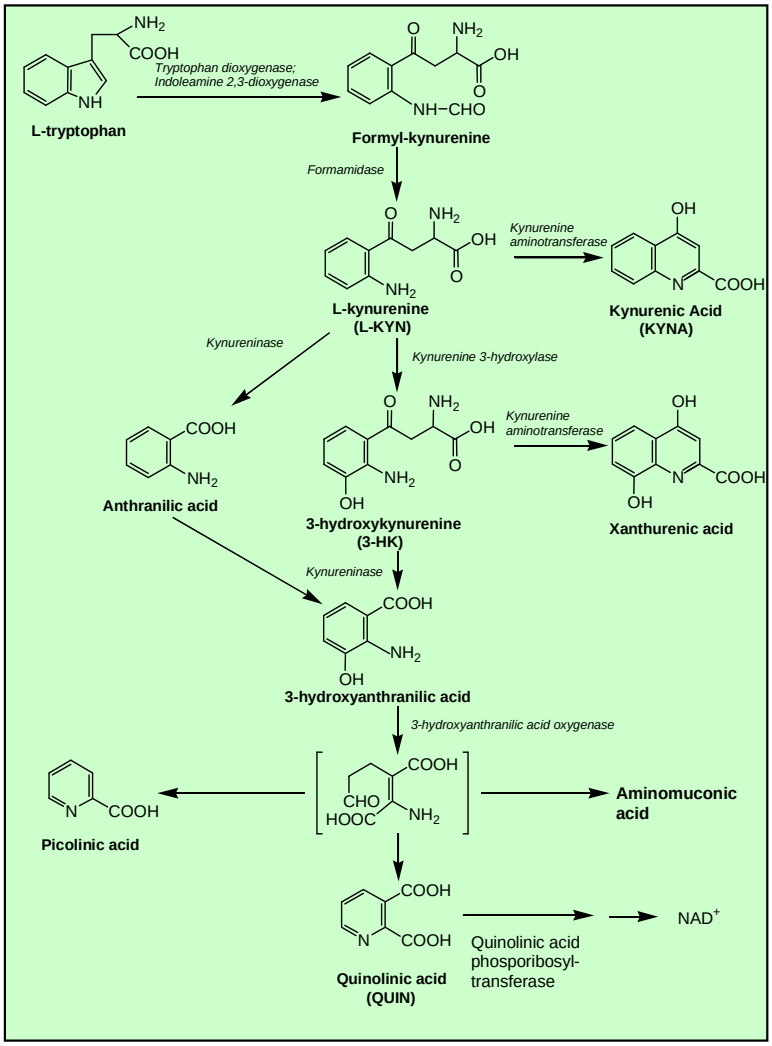

Fig. 1. Kynurenine pathway

KYNA behaves as a neuroprotective agent: it can inhibit the overexcitation of these receptors by binding the glycine allosteric site. It may therefore influence physiological and pathological processes, so it has therapeutic effects in numerous neurological disorders [4, 11, 12]. Furthermore, KYNA displays neuroinhibitory properties in neurophysiological experiments [13] and, like other glutamate receptor antagonists, can cause impairments in learning and behaviour [14]. KYNA can noncompetitively block the activity of presynaptic α-7-nicotinic acetycholine (α7-nACh) receptors [15]. Crosstalk between KYNA and the nicotinic cholinergic system has been presumed to play role in the pathogenesis of numerous brain disorders, including Alzheimer's disease (AD) and schizophrenia, in which brain KYNA levels are elevated and the nicotinic functions are impaired [16].

The enzymatic machinery for the catabolism of TRP exists both in the brain and in the periphery, although it has a much higher capacity in the latter. TRP is converted

by indoleamine 2,3-dioxygenase-1 (IDO1), or in the liver, the more specific enzyme, tryptophan 2,3-dioxygenase (TDO), to N-formyl-kynurenine (N-formyl-KYN), which is further degraded by formamidase to L-kynurenine (L-KYN) (Fig. 1). IDO1 is expressed by a variety of cells and is induced preferentially by the Th1-type cytokine IFN-γ [17-19]. Similarly to TDO, IDO1 catalyzes the first step in TRP degradation, specifically, the formation of N-formyl-KYN, which subsequently deformylates to give L-KYN. IFN- γ is a potent stimulus for IDO1 both *in vitro* and *in vivo*. A third enzyme, indolamine 2,3-dioxygenase-2 (IDO2) recently has been described. The IDO-1 and IDO-2 proteins share significant identity at the amino acid level (43% for human and mouse proteins) but are not related structurally to the TDO protein. The IDO1 and IDO2 proteins are encoded on genes adjacent to each other, on chromosome 8 in humans and mice, suggesting that the genes arose via gene duplication. The discovery of IDO2 suggests that the KP of TRP metabolism is involved in more biological processes than previously thought [20].

L-KYN, a major component of the KP, serves as a source for the synthesis of all the other metabolites of the pathway. L-KYN is present in micromolar concentrations in the blood, brain and peripheral organs, and is transported through the blood-brain barrier (BBB) by the neutral amino acid carrier [21]. L-KYN is further metabolized in three distinct ways. It serves as a substrate for kynureninase, for kynurenine aminotransferases (KATs) and for kynurenine-3-hydroxylase yielding, respectively, anthranilic acid, KYNA and 3-hydroxykynurenine (3-HK).

In the mammalian brain, KYNA is produced irreversibly from L-KYN by the action of the KATs, which are able to catalyse the transamination of L-KYN *in vitro*. Since excitatory amino acid receptor activation takes place in a variety of pathological states, KYNA has been tested as a neuroprotective agent. Two distinct KATs (KAT I and KAT II) have been cloned and extensively studied [22, 23].

KAT I, also known as glutamine transaminase K or cysteine conjugate beta-lyase [24-26], is present in both astrocytes and neurons; its expression increases after birth, reaching a maximum specific enzyme activity in the brain of adult rats [27, 28]. The enzymatic activity of KAT I in the rat brain is optimum at alkaline pH [29]. However, purified human KAT I from Sf9 insect cells/baculovirus expression system exhibits high enzymatic activity under neutral conditions [30], suggesting that KAT I might be an important player in KYNA synthesis under physiological conditions.

KAT II, also known as alpha-aminoadipate aminotransferase, was isolated by RT-PCR from the rat kidney [31] with the EST assembly from mouse and human [32, 33]; it is structurally homologous to aspartate aminotransferase in the pyridoxal 5'-phosphate binding domain. KAT II appears to play a more important role than KAT I in the rat brain in terms of its relative contribution towards the synthesis of KYNA in the cerebral tissue. Lesional and pharmacological studies have confirmed that in most brain regions KYNA originates primarily from KAT II activity [29]. Under physiological conditions, KAT I and KAT II are localized mainly in the astrocytes [34], but they are also present in human brain cells [35–37] and in most of the neurons in the medulla and spinal cord [38].

Yu *et al*. [39] recently discovered 'KAT III' as a novel member of the KAT family. Genomic structure analysis indicates that *kat3* and *kat1* share the same exon-intron boundary, indicating that they belong in the same subfamily. 'KAT III' shares several features with KAT I, including a similar genomic structure, and a similar mRNA expression in multiple tissues, including the kidney, liver, heart, lung and neuroendocrine organs, but no enzyme activity has so far been associated with 'KAT III'.

The results of Guidetti *et al.* [40] suggested the existence of a third, quisqualate-sensitive KAT ('KAT IV') in the brain and at the same time provided further support for the notion that KAT I plays only a minor role in cerebral KYNA formation in the rodent brain under physiological conditions. The enzyme was purified, characterized, and found to be identical with mitochondrial aspartate aminotransferase (mitAAT). Mitochondrial aspartate aminotransferase is clearly distinct from the above mentioned 'KAT III', which was recently isolated from mouse and human brain cDNA libraries and suggested to be a heretofore unrecognized KYNA-forming enzyme. In contrast, mitochondrial aspartate aminotransferase is coded by a known gene, and the enzyme's biochemical characteristics as well as its role in cell biology are reasonably well understood. By catalyzing the formation of KYNA, mitochondrial aspartate aminotransferase may be involved in a range of physiological and pathological processes associated with glutamatergic and nicotinergic function.

L-KYN is further metabolized to 3-hydroxykynurenine (3-HK) by the kynurenine 3-hydroxylase present in the liver, placenta, spleen, kidney and brain [41], this conversion requiring nicotinamide adenine dinucleotide phosphate (NADPH) and molecular oxygen (Fig. 1). No important physiological function in the brain has so far

been assigned to 3-HK, but in primate lenses it acts as a major UV filter together with its glucoside derivative, L-KYN and 4-(2-amino-3-hydroxyphenyl)-4-oxobutanoic acid O-β-$_D$-glucoside, and may be useful in protecting the retina from UV radiation [42].

Furthermore, it should be mentioned the synergetic effects of 3-HK and QUIN. Guidetti and Schwarcz [43] examined possible synergistic interactions between 3-HK and QUIN in the rat brain *in vivo*. Intrastriatal coinjection of these agents in low doses which caused no or minimal neurodegeneration on their own, resulted in substantial neuronal loss, determined both behaviourally and histologically. Their data indicate that an elevation of 3-HK levels constitutes a significant hazard in situations of excitotoxic injury.

3-HK is further metabolized by kynureninase, leading to 3-hydroxyanthranilic acid (3-HANA) formation. This is then converted by 3- hydroxyanthranilate 3,4-dioxygenase (3-HAO) to 2-amino-3-carboxymuconate-semialdehyde, an unstable compound, which is transformed non-enzymatically into QUIN. Examinations during recent years have revealed that the neurotoxic properties of QUIN involve at least four mechanisms. Guillemin *et al.* described them in a review [44]. 1) QUIN can activate the NMDA receptor in pathophysiological concentrations. 2) QUIN can inhibit glutamate uptake into the synaptic vesicle, leading to excessive microenvironment glutamate concentrations and neurotoxicity [45, 46]. 3) More recently, it has become clear that a major mechanism of QUIN neurotoxicity is taking place through lipid peroxidation [47, 48]. 4) Finally, QUIN can potentiate its own toxicity and that of other excitotoxins (*e.g.* NMDA and glutamate) in the context of energy depletion [49, 50]. These findings have led to the suggestion that there may be a link between endogenous excitotoxins and human neurodegenerative disorders, and the application of QUIN has been proposed as a model for certain neurodegenerative disorders [51-53]. 3-HAO has been shown to be present in the brain [54]; its activity is increased in various pathological states, *e.g.* in the striatum of Huntington's disease (HD) patients [55], in the gerbil hippocampus after global ischemia [56], and in epileptic rats [57].

Finally, QUIN is degraded by quinolinate phosphoribosyltransferase to nicotinic acid mononucleotid and then nicotinamide adenine dinucleotide (NAD), the end-products of the pathway. One of the main functions of the KP is to metabolise L-TRP into NAD, which is essential for cell survival. NAD participates in many biological processes such as the control of energy metabolism [58], DNA repair [59, 60] and transcription [61, 62].

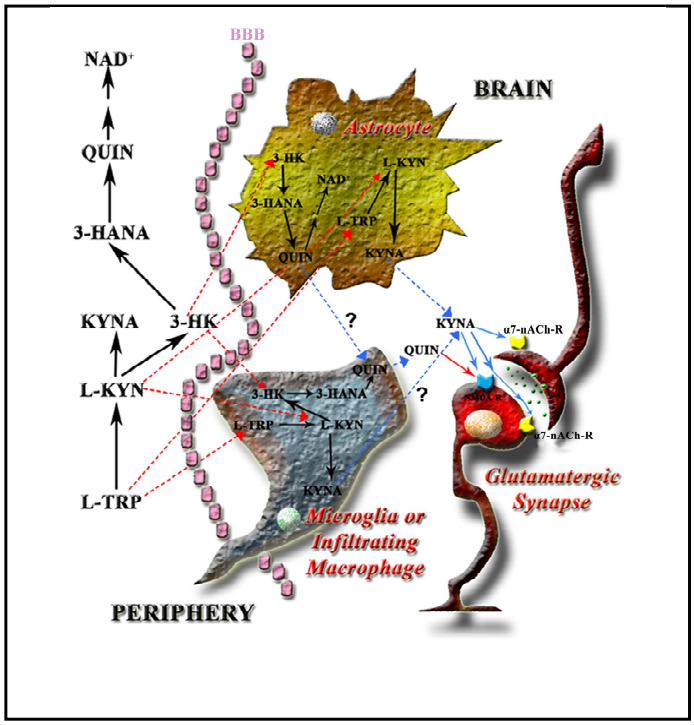

Fig. 2. Dynamics of the cerebral & extracerebral KP metabolism. Broken arrows: brain entry/cellular uptake (red), release (blue). Solid arrows: enzymatic conversion (black), receptor agonist (red), receptor antagonist (blue). α7-nACh-R: α7-nicotinic acetylcholine receptor; 3-HANA: 3-hydroxyanthranilic acid; 3-HK: 3-hydroxykynurenine; L-KYN: L-kynurenine; L-TRP: L-tryptophan; NMDA-R: NMDA-receptor; QUIN: Ouinolinic acid [189].

In the brain, TRP catabolism occurs in the astrocytes and microglia [63-65], and also happens in neurons [36], though 60% of the brain L-KYN is contributed from the periphery [66]. The astrocytes produce mainly KYNA, and the microglia and infiltrating macrophages mainly QUIN [35, 67, 68]. The astrocytes have been demonstrated to metabolize the QUIN produced by the neighbouring microglia [35] (Fig. 2).

It should be noticed that TRP catabolism plays a key role in the regulation of the immune response. The KP is one of the major regulatory mechanisms of the immune response [69]. The immune system continuously modulates the balance between responsiveness to pathogens and tolerance to non-harmful antigens. The mechanism that mediate tolerance, are not well understood but recent findings have implicated TRP catabolism as one of many mechanisms involved. Two theories have been proposed to explain how TRP catabolism facilitates tolerance. One theory posits that TRP breakdown suppresses T cell proliferation by dramatically reducing the supply of this critical amino acid. The other theory postulates that the downstream metabolites of TRP catabolism act to suppress certain immune cells, probably by pro-apoptotic mechanisms. In this issue, there are a number of fine pieces of studies and reviews [for example: [69-72].

Changes in the absolute and relative concentrations of the compounds involved in the KYN pathway (in particular KYNA and QUIN) in the brain have been implicated in a great number of neurodegenerative disorders, for example, stroke, epilepsy, ischemia, AD, HD, Parkinson's disease (PD), multiple sclerosis (MS) and encephalopathies. Both agents are able to act on the NMDA-receptor complex and influence the glutamatergic transmission. Elucidation of the importance of the KP in the brain function has facilitated research relating to the alterations in the KP in various neurological disorders.

A number of extensive studies have been published on disorders associated with alterations in the KP [4, 11, 12, 73, 74] featuring recent results concerning various pathological states.

Hereinafter, we focused on epilepsy and ischemia from the above mentioned neurological disorders, because the accepted models of these two diseases were used in our works.

Epilepsy is one of the most common neurological disorders affecting 0.5-2% of the world's population. It varies widely in type and severity of seizures and should not be considered as a single disorder. It is currently defined as 'a tendency to have unprovoked recurrent seizures'. Epilepsy can result from brain injury caused by head trauma, stroke or infection, but in 6 out of 10 people seizures have no known cause. Seizures are the result of excessive neuronal firing temporarily disrupting neuronal signalling. This aberrant brain activity is the result of a shift in the balance between excitation and inhibition created by ion channels. In this context, it has been hypothesized that seizures and epileptogenesis may be due to either over-activation of excitatory pathways, utilizing glutamate or other excitatory amino acids, and/or a lesser activity of inhibitory pathways utilizing γ-aminobutyric acid (GABA) and other inhibitory neurotransmitters [75-77]. Recent investigations in this field have focused on changes in the function of excitatory and inhibitory systems and morphological adaptive processes, including changes in the synaptic plasticity and growth of new inter-neuronal connections [78-80]. There is some evidence that brain amino acid concentrations may be altered in epilepsy [81, 82]. However, the relationship between changes in the local concentration of amino acids in different brain structures and seizures is not well recognized.

Oxidative stress in the CNS has been shown in several rodent models of experimental epilepsy, such as the amygdala kindling model [83], the kainic acid model

[84], the pentylenetetrazole (PTZ)-induced kindling model [84], and in acute PTZ-induced seizures [85, 86]. Free radical generation can induce seizure activity by direct inactivation of glutamine synthase and glutamate decarboxylase, thereby permitting an abnormal build-up of excitatory (glutamate) and inhibitory GABA neurotransmitters [87, 88].

One of the most frequent and devastating forms of epilepsy involves the development of an epileptic focus in temporal lobe structures. Prolonged seizures (status epilepticus), induced in experimental models by kainic acid or pilocarpine are known to activate neuronal cell death mechanisms in temporal lobe structures similar to other neurodegenerative disorders. This neuronal cell death is also observed in human temporal lobe epilepsy (TLE) and is one of the most important aspects of epileptogenesis. Specifically in the hippocampus, the loss of CA1 and CA3 pyramidal neurons, with relative sparing of the granular neurons of the dentate gyrus and some types of interneurons, is the histopathological hallmark of Ammon's horn sclerosis. Probably the most important factor, preceding neuronal cell death after status epilepticus, is the increased level of reactive oxygen species (ROS) observed in various models of experimental epilepsy, such as after kainate-induced hippocampal damage, after pilocarpine treatment or in low Mg^{2+}-induced epileptiform activity of brain slices and slice cultures. Mitochondrial respiratory chain complex I is very likely to be the most important source of production of these ROS [89].

PTZ, a chemical convulsant frequently utilized in the study of seizures [90, 91], exerts its effects by binding to the picrotoxin-binding site of the post-synaptic GABA-A receptor [92]. PTZ is known to suppress the inhibitory effects of certain neurotransmitters, and especially GABA, thereby leading to an easier depolarization of the neurons [93, 94]. PTZ may trigger a variety of biochemical processes including the activation of membrane phospholipases, proteases and nucleases. Marked alterations in membrane phospholipid metabolism result in the liberation of lipid peroxides and free radicals [95]. Similarly, Basra Deniz Obay *et. al* [96] found that acute PTZ-induced epileptic seizures lead to an increase in oxidative stress, an indicator of lipid peroxidation in erythrocytes, and liver and brain tissues.

In searching for new treatments for epilepsy, a key component is the identification of cellular mechanisms that will reduce neuronal hyperexcitability, ideally in a way that is specific to abnormal cells. Most drugs currently used in the treatment of epilepsy act through a limited number of molecular targets and mechanisms such as the

enhancement of GABA-ergic function or voltage-gated potassium channels, or the inhibition of voltage-gated sodium channels or calcium T-channels [97, 98]. Although the currently used drugs are effective in many individuals, the continued occurrence of seizures in a large proportion of patients suggests that agents with other mechanisms of action should be considered. Increasing information suggests that the pharmacology of epileptic systems may be quite different from that of normal brains, observations that suggests that the identification of new therapies should involve models that better represent the human condition.

Glutamate receptors, especially NMDA receptors, are logical targets for new antiepileptic compounds. Unfortunately, early attempts at attenuating the function of these receptors by using conventional NMDA-receptor antagonists revealed serious side effects [99]. For reasons that are not fully understood, these harmful consequences of direct NMDA-receptor blockade can be avoided by targeting the glycine co-agonist ($glycine_B$) site on the receptor for seizure suppression [100, 101]. Since glutamatergic neurotransmission plays a pivotal role in the pathogenesis of epilepsy, therefore antagonists of glutamate receptors are powerful anticonvulsants. In line with this, L-KYN, KYNA and their synthetic analogues are generally efficacious anticonvulsants in a variety of models of experimental epilepsy [102-104]. Moreover, reduction of KYNA levels increases vulnerability to excitotoxic insults, whereas elevation of KYNA content has an opposite effect [105].

Stroke is the third major cause of death in the major industrialized countries after cardiovascular disease and cancer. The overall incidence of stroke is predicted to increase over the next decade by 12% but by around 20% in low-income families. More than 30% of the stroke survivors will have severe disability, and calculations suggest that over 50 million healthy life-years will be lost by 2015 [106]. Saver [107] undertook the task of calculating the impact a stroke has on nervous tissue. His calculations are sobering. Patients experiencing a typical large-vessel acute ischemic stroke will lose 120 million neurons, 830 billion synapses and 714 km of myelinated fibres each hour. Compared with the normal rate of neuron loss during aging, the ischemic brain will age 3.6 years for every hour the stroke goes untreated. The large majority (85%) of strokes in the western world are ischemic, that is, a stroke resulting from an occlusion of a major cerebral artery, commonly the middle cerebral artery (MCA) by a thrombus or embolism. The other strokes are hemorrhagic, where a blood vessel bursts either in the brain or on its surface. Ischemic brain injury results from a complex sequence of

pathophysiological events that evolve over time and space. The major pathogenetic mechanisms of this cascade include excitotoxicity, peri-infarct depolarization and inflammation leading to cell death by apoptosis and necrosis [108]. The main therapeutic approaches in patients with acute cerebral ischemia are thrombolysis and neuroprotection but none of them is really satisfying. Thrombolysis, which is aimed at restoring the cerebral blood flow, is restricted to few patients because of a narrow therapeutic window and a high hemorrhagic risk [109]. Over the last 25 years, neurochemists have provided a great deal of information on the chain of events that follows an ischemic episode (the so-called 'ischemic cascade'). Many compounds that act on one or more of these mechanisms have been developed and these compounds do indeed prevent ischemia-induced cell death in the brain of experimental animals. Because a pathological increase in glutamate was one of the early changes to be shown to occur after an episode of cerebral ischemia, many compounds interfering with glutamate function were developed during the late 1980s and early 1990s. These were predominantly NMDA receptor subtype antagonists, but some α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor antagonists were investigated and also a glycine modulator site antagonist. A major problem with glutamate antagonism as a mechanism suitable for therapeutic intervention target is that pathological glutamate release is an event occurring very early in the ischemic cascade. This presumably makes it necessary to give such drugs very quickly after the ischemic insult, and rapid admission is often a major clinical problem [108, 110, 111]. However, other problems that also arose with some of these drugs included poor pharmacokinetics, poor brain penetration and a failure to protect subcortical structures.

In an animal model of transient global ischemia, the level of endogenous KYNA and the activity of its biosynthetic enzymes, KATI and KAT II, are not changed 24 and 72 h after the insult in CA1 hippocampal area [112]. It is in agreement with the results showing no change in KYNA content in the whole hippocampal structure following ischemic insult [56, 113]. The local production of KYNA might be relatively too low in order to prevent pathological changes. On the other hand, KYNA attenuates indeed the ischemic neuronal loss both *in vitro* and *in vivo*. Peripherally administered KYNA protects hippocampal CA1 pyramidal neurons in a transient forebrain ischemia model in gerbils [114]. A recent study of Bari *et al.* [115] on newborn piglets demonstrated that: (1) when applied exogenously at superphysiological concentrations, neither KYNA nor QUIN is vasoactive in the pial vasculature; (2) NMDA- but not hypercapnia-induced

arteriolar dilation can be inhibited by exogenously applied KYNA; (3) KYNA attenuates kainate-dependent vasodilation in normoxic as well as in post-ischemic conditions; and (4) in vulnerable brain regions, they could not detect changes in tissue levels of KYNA and QUIN during the first few hours of reperfusion following global cerebral ischemia. However QUIN levels rose 50-fold over the course of 7 days following the induction of cerebral ischemia in gerbils, mimicking an ischemic stroke [116].

For the last two decades, many neuroprotective drugs gave interesting and promising results in animal models of pre-clinical studies, but the clinical trials performed with these drugs were disappointing and showed no beneficial effects in stroke patients (reviewed in [117, 118]). The possible reasons for the clinical trial failures include the morphological and functional differences between the brains of rodents and humans and the different designs of the preclinical studies and the clinical trials (therapeutic time window, drug-dosing schedule, efficacy assessment). Rodent brains and human brains have many anatomical and histological differences such as e.g. their proportion of white matter [119]. One of the hypotheses, which could explain the failure of neuroprotective trials in stroke patients is the anatomical and histological differences between human and rodent brains. In order to test this hypothesis, Dronne *et al.* [120] built a mathematical model representing the main early pathophysiological mechanisms involved in an ischemic stroke.

Another criticism of the current animal models is that they nearly utilize young healthy animals. In contrast, stroke patients are usually elderly, with a variety of other clinical problems such as hypertension, myocardial infarction and diabetes. Both Davis *et al.* [121] and Schaller [122] have examined older animals and data suggest that neuroprotective efficacy is reduced in these animals. The future of the research on the field of neuroprotection may, therefore depend on the development of better animal models.

The results of a human study [123] strongly support the view that an inflammatory response with increased signs of oxidative stress develops rapidly after a stroke. S100B is one of the most useful markers of brain injury following stroke and its levels rise immediately after stroke and correlate well with the volume of stroke infarct. The significance of this is unclear as, although S100B can protect neurons against acute insults including glutamate receptor activation and hypoglycaemia, it also has the ability to trigger the inflammatory response by activating microglia and macrophages. Thus,

S100B could be a significant factor in generating the inflammatory response to stroke. The results of Darlington *et. al* [123] raise the possibility of introducing some form of early anti-inflammatory intervention to modify the production of a factor such as S100B or the KYN metabolites to reduce the inflammatory response and limit delayed neurodegeneration. Inhibitors of the KP, already shown to reduce ischemic damage in experimental models of stroke and brain inflammation in cerebral malaria, could be useful for such intervention.

Transient global ischemia, which may arise during cardiac arrest and surgery in humans or be induced experimentally in animals, elicits selective, delayed neuronal death. If the ischemia is short, neuronal damage occurs only in vulnerable areas. The pyramidal neurons in the hippocampal CA1 region are particularly vulnerable [124-127]. Animal models of transient global ischemia, including bilateral carotid artery occlusion in Mongolian gerbils [128] and 4-vessel occlusion (4VO) in rats, have demonstrated significant neuronal necrosis in the CA1 region of the hippocampus and impairments in a variety of learning and memory tasks [128, 129] . Other neurons, such as the hippocampal CA3 neurons, are less ischemia-vulnerable [130, 131]. It is widely accepted that activation of the EAA receptors plays an important role in neuronal death in stroke [132]. It has recently been reported that glutamate-induced excitotoxicity and cellular calcium overload are among the key factors of cell death in brain ischemia, especially in the grey matter [133]. By definition, excitotoxicity is a result of overexcitation of the glutamate receptors. In turn, neuroprotective strategies have utilized antagonists of the glutamate receptors to prevent excitotoxic neuronal loss [134].

Long-term potentiation (LTP), also mediated by glutamate receptors is a model of neuronal plasticity [135]. Accordingly, ischemia may likewise impair physiological forms of synaptic plasticity, such as activity-dependent LTP [136]. What is more, global ischemia may induce a special form of plasticity, anoxic LTP [137, 138].

It is currently widely accepted that the neuronal damage which occurs as a result of a stroke is largely attributable not to the immediate hypoxia or ischemia itself, but to the massive release of glutamate from the neurons and glia [139]. Neuronal activity leads to the production of glutamate, which is then taken up, via by Na^+-dependent glutamate transporters, and recycled by astrocytes [140]. Astrocytes convert glutamate either into glutamine or oxidize it via the tricarboxylic acid cycle [141], therefore, they are responsible for maintaining extracellular glutamate concentration. However, QUIN

has been shown to inhibit the uptake of glutamate by astrocytes, thus disrupting the extracellular glutamate balance [46]. Moreover, QUIN has been reported to be able to selectively inhibit the uptake of glutamate into synaptic vesicles in the rat brain without interfering with the uptake of neurotransmitters GABA and glycine [45]. Glutamate activates at least three types of ionotropic receptors, which are sensitive, respectively, to NMDA, kainate and AMPA [142]. These can all increase the intracellular levels of Ca^{2+} [143, 144], and lead ultimately to the generation of NO, ROS and hence to cell death [145]. A therapeutic objective in the pharmaceutical industry, therefore, is to develop agents, which block the activation of glutamate receptors.

Impairments of the KP are strongly involved in neuronal death in various disorders. Prevention or correction of the abnormality, which results from changes in the levels of L-KYN derivatives, could attenuate the pathological processes.

At the moment, there appear to be three different possibilities for the development of therapeutic agents with the aim of modulation of the KP (see recent reviews by [73, 74]). One strategy is to use L-KYN as a precursor of neuroprotective KYNA. L-KYN can cross the BBB and increase the level of KYNA in the CNS. The second approach is to develop different pro-drugs and analogues of KYNA that can easily cross the BBB and act on the glycine-binding site of the NMDA receptors. The third method is manipulation of the KP by administration compounds that block the metabolism of L-KYN → QUIN conversion.

The following aims were set during our work on this topic:

To investigate

- whether L-KYN, which is able to cross easily the BBB by a neutral amino acid carrier and can be transformed into neuroprotective KYNA in the brain, can act against the acute PTZ-induced epileptic seizures?
- whether L-KYN administered intraperitoneally (i.p.) together with probenecid (PROB), (PROB is known to inhibit several transporters; among those are the multidrug resistance-associated proteins (MRPs), which act as organic anion transporters), can be more effective than L-KYN administered alone and whether they are able to protect against the neurotoxic effect of PTZ?

- whether systemic pre- or post-treatment with L-KYN (administered together with PROB; i.p.) decreases the delayed cortical and hippocampal neuronal damage in rats subjected to transient global ischemia (4VO model)?
- whether our newly synthetized KYNA analogue glucoseamine-KYNA (G-KYNA) can readily pass into the brain after systemic administration (alone or together with PROB; i.p.) and can be more effective than KYNA?

Materials and methods

1. Animals

Our studies were performed on adult male Wistar rats (n=235, 250-300g) maintained under controlled environmental conditions at a temperature of 22±2 ˚C and 12-h light/dark cycle. Food and water were available *ad libitum*. Every effort was made to minimize animal suffering. The principles of laboratory animal care (NIH publication No. 85-23), and the protocol for animal care approved by the European Communities Council Directive of 24 November 1986 (86/609/EEC) were followed. In the *in vivo* electrophysiological experiments the summarized number of animals was 107, while 18 rats were used in *in vitro* electrophysiology. The number of animals starting the behavioural tests was n = 50. In the histological evaluation the summarized number of rats was 50, while 10 animals were used in the HPLC-MS/MS analysis.

2. Drugs

L-KYN (300 mg/kg; i.p.), PROB (200 mg/kg; i.p.), PTZ (60 mg/kg i.p.) and KYNA (17 μmol/kg) were obtained from Sigma (Steinheim, Germany), while our new compound G-KYNA (17 μmol/kg) (Fig. 3) was synthetized in the Institute of Medical Chemistry, University of Szeged. L-KYN, KYNA, G-KYNA and PROB was dissolved in 0.1 M sodium hydroxide (1ml) and adjusted with 1 M sodium hydroxide to pH 7.4.

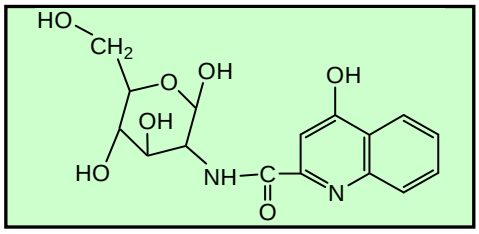

Fig. 3. The chemical structure of G-KYNA

3. Animal models and methods

3.1. Acute PTZ-induced seizures

PTZ is often used experimentally to induce seizures which mimic generalized clonic seizures in humans [146]. The dose of PTZ was chosen on the basis of a previous study of Jiang *et al.* [147]. The PTZ treated animals received a single PTZ injection at a convulsive dose of 60 mg/kg. The drug was given i.p. in a volume of 1ml saline. There is a five point scale in the literature to investigate the intensity of convulsions: 0, no response; 1, ear and facial twitching; 2, myoclonic jerks without rearing; 3, myoclonic jerks with rearing; 4, turn over into side position, clonic-tonic seizures; 5, turn over into back position, generalized tonic-clonic convulsions [148].

3.1.1. In vivo electrophysiology

Surgical procedure: Animals (n=56) were anaesthetized with urethane (1.3 g/kg, i.p.). In some cases, the tail vein of the animals was catheterized. In most of the experiments, the drugs were administered i.p., through a syringe implanted at the beginning of the experiments. For recordings in area CA1, a 2-3 mm diameter hole was drilled over the dorsal hippocampus (3.0-3.8 mm posterior and 1.8-2.3 mm lateral to the sagittal suture) and the recording electrode was lowered 3.3 mm from the cortical surface. Contralaterally, a 1-2 mm hole was drilled for the CA3 stimulating electrode (3.7 mm posterior to the bregma, and 3.3 mm lateral to the sagittal suture: final electrode depth 3.8 mm below the dura). Electrodes were lowered and final positions were adjusted so that the maximum CA1 population spike was obtained in response to contralateral CA3 stimulation (Fig. 4). The sites in area CA1 and CA3 were confirmed histologically. Responses to a range of stimulus intensities were recorded under control conditions to produce an input-output curve by changing the duration (10-100 μs), using current (up to 200 μA) square pulses. Stimuli were triggered at low frequency (0.05 Hz). Response stability was monitored for 30 min prior to drug administration. The electrophysilological recording continued during the following 3h recording period after drug administration.

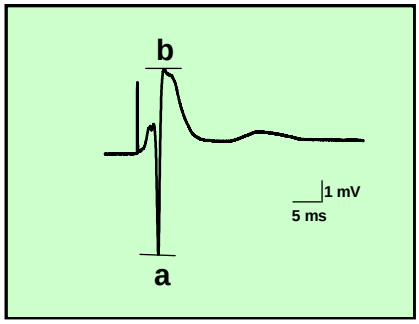

Fig. 4. Population spike recorded in the CA1 area of the rat hippocampus. **a**: minimum point, **b**: maximum point of the response. **b**-**a**= amplitude of the population spike.

Drug administration: The doses of L-KYN and PROB that were chosen were based on earlier work [149, 150]. In pilot experiments, to examine the effects of pure L-KYN or PTZ or PROB, the animals (n=31) were treated with varying doses of these compounds, e.g. L-KYN was administered either i.v. or i.p. in doses of 5, 10, 50, 100, 200, 300 or 400 mg/kg. Since i.p. and i.v. administration resulted in similar effects, i.p. administration was chosen in further studies, because it was more appropriate in the behavioural experiments. On the basis of the literature data (cited above) and our pilot experiments on the dose-dependent effects of these compounds, throughout the main

studies L-KYN was administered in a dose of 300 mg/kg, PROB in a dose of 200 mg/kg, and PTZ in a dose of 60 mg/kg. After the pilot experiments in which the effects of the pure drugs were observed, we studied whether L-KYN + PROB inhibited the effects of PTZ. In these electrophysiological studies, the responses of area CA1 pyramidal cells to contralateral CA3 stimulation were tested for a 30-min control period. L-KYN and PROB administration followed. During the next 2 h, the low-frequency stimulation continued without recording, and PTZ was then administered. The electrophysiological effect was followed during a 3-h recording period (Fig. 5A.)

3.1.2. Behavioural studies

3.1.2.1. Water-maze task

The rats were trained in a large circular swimming pool (160 cm in diameter, 60 cm high) filled with water to a depth of 35 cm. The water was at room temperature and was made opaque by the addition of 2 l milk. The pool was situated in a small rectangular room. The walls were equipped with a variety of spatial cues (pictures and a lamp emitting diffuse light), which remained unchanged during the experiment. The pool was divided into four quadrants, and a removable platform (8 cm diameter) was hidden at one of four positions in the pool, exactly 25 cm from the sidewall. The platform was 1.0 cm below the water surface and not visible to the swimming rat (Fig. 6A,B). The animals performed a block of four consecutive trials, all beginning at a fixed starting point (N, W, S or E). Trials ended either when the platform was found or when 60 s had elapsed. If the rats did not find the platform within 60 s, they were guided to it and left there for 15 s. They were then removed from the pool and either placed back after 15 s (intertrial interval) for the subsequent trial or returned to their home cages after being dried with a towel. The 5-day training consisted of one block of four trials per day, and for each trial the starting point varied in a random order such that no location recurred for a given day. Each trial was recorded and analyzed by using a computer video tracking system. The video camera of the Ethovision System (Noldus) was mounted above the centre of the pool. The animal movements were monitored, timed and processed by the software (Etho Vision 2.0) (Fig. 6C). The sequence of drug administration in the behavioural experiments was similar to the *in vivo* electrophysiology: L-KYN (300 mg/kg i.p.) and PROB (200 mg/kg i.p.) administration preceded the PTZ injection (60 mg/kg i.p.) by 2 h. The water-maze studies were carried out 20 min after the PTZ injection (Fig. 5B).

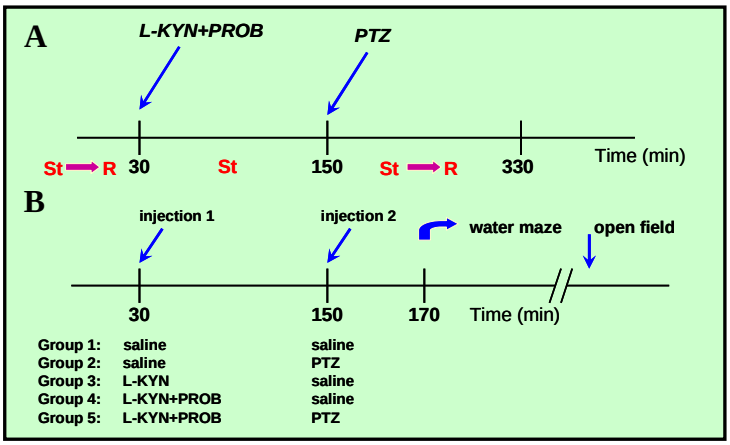

Fig. 5. Schedule of the experiments. (**A**) Time scale for the electrophysiological experiments. Control population spikes were evoked throughout 30 min. **St**, stimulation; **R**, recording. L-KYN and PROB were injected i.p. PTZ injection followed 2 h later. Evoked spike activity was recorded for 3 h following the second injection. (**B**) Time scale for the behavioural observations. The animals in the five groups were injected twice, with a 2-h period between the injections. The water-maze task followed 20 min after the second injection. Before and after the injections, general observations were carried out. The observations in the open-field arena were carried out on another day.

3.1.2.2. Open-field observations

This behavioural test was carried out in a circular arena (80 cm in diameter); the height of the wall was 40 cm. Following administration of the compounds, the animals were observed for 5 min (Fig. 6D). The stereotyped behaviour was characterized by the total time spent in grooming and washing. Several parameters were monitored: rearing, stereotyped washing, defecation, time (s) to the onset of grandmal seizures, and the time (min) of death. In the course of the behavioural experiments, with daily PTZ administration, the cumulative number of dead animals was also registered.

Fig. 6. (A,B) Photographs of the circular swimming pool in water-maze task. (C) Single subject tracking (water-maze task) with the Ethovision System (Noldus). (D) Photograph of the open-field arena.

3.2. Four-vessel occlusion (4VO) model of transient global ischemia

4VO was carried out as described previously [151]. In brief, the rats were anaesthetized with Nembutal (CEVA-PHYLAXIA; 60 mg/kg, i.p.). Both vertebral arteries were occluded by cauterization during careful cooling with iced washing. The

wound were then closed, and the animals were allowed to recover for 24 h. On the following day, they were subjected to a 10-min forebrain ischemia by bilateral occlusion of the carotid arteries with atraumatic clips under ether anaesthesia (Fig. 7). The body temperature was monitored, and maintained at 37 ˚C during the surgical procedures, using a thermostatically controlled heating pad. Both vertebral arteries were cauterised, and both common carotid arteries were exposed, but not occluded in the sham-operated animals.

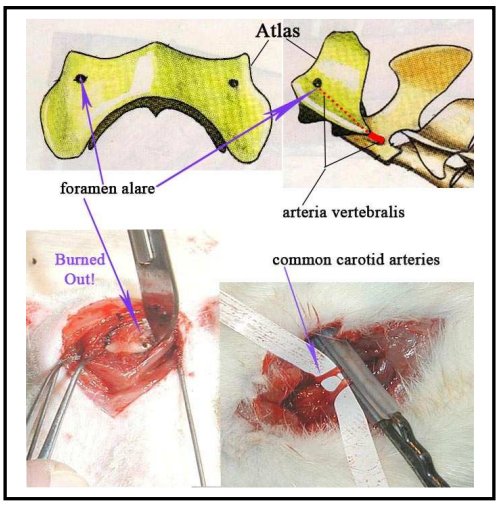

Fig. 7. Surgical procedure of 4VO model of transient global ischemia.

The rats used for cortical histology were divided into 5 groups: the group of control animals (n=5), which contain intact controls (IC) and sham-operated controls (SC); the 4VO group (4VO, n=7), the L-KYN + PROB pre-treated animals (L-KYN+PROB-4VO, n=6) and the L-KYN + PROB post-treated animals (4VO-L-KYN+PROB, n=7).

The rats used for hippocampal histology were divided into 4 groups: SC group (n=5), 4VO group (n=7), L-KYN+PROB-4VO group (n=6) and 4VO-L-KYN+PROB goup (n=7).

The rats used for *in vitro* electrophysiology were divided into 3 groups: SC group (n=6), 4VO group (n=6) and L-KYN+PROB-4VO group (n=6).

Drug administration: L-KYN (300 mg/kg, i.p.) and PROB (200 mg/kg, i.p.) were administered daily for 5 days: in the pre-treated group, the first L-KYN+PROB administration preceded the 10-min carotid occlusion by 2 h; and the animals were treated at the same time on the next 4 days. In the post-treated group, the animals received the first L-KYN+PROB injection at the start of reperfusion. The remaining 4 injections were given at the same time on the next 4 days.

3.2.1 Histological evaluation

Ten days after 4VO, the rats were anaesthetized with urethane (1.3 g/kg, i.p.), and perfused transcardially with 0.1 M phosphate-buffered saline, and then with 4% paraformaldehyde. The literature reports a considerable range of reperfusion times on transient global ischemia [152-154]; our procedure was selected on the basis of these data. The brains were removed from the cranium, post-fixed in formalin, cryopreserved in 20% sucrose (containing 0.05% Na-azide), and sectioned at 32 μm with a cryostat microtome. Serial sections were collected in a cryopreservative solution for storage.

The series of sections were stained for degenerating neurons with Fluoro Jade B® (FJ, Chemicon Int.). Six sections were mounted from all animals in each group. A standard protocol was followed in our experiments. For the detailed steps of FJ staining, see [155].

NeuN (anti-neuronal nuclei), a neuronal specific nuclear protein was used for immunohistochemical detection of surviving pyramidal neurons in the hippocampus. NeuN antibody labels nuclei and cytoplasm of most neuronal cell types in all regions of the adult brain including cerebral cortex, hippocampus, cerebellum, etc. For the detailed steps of NeuN immunohistochemistry, see [156].

The locations of FJ-B positive (FJ^+) cells were observed with a fluorescence microscope (Olympus BX-51, Tokyo, Japan) with an excitation wavelength of 470-490 nm and an emission wavelength of 520 nm. The NeuN labelling was observed under a fluorescence microscope at an excitation wavelength of 530-550 nm, and an emission wavelength of 590 nm. Fluorescence photomicrographs were obtained with an Olympus DP70 (Tokyo, Japan) digital imaging system.

The global ischemia induced damage in both hemispheres, but in each case, the hemisphere with the greater extent of injury was evaluated. In case of cortical evaluation, the number of FJ^+ neurons/mm^2 was determined in each slide of each animal in the respective group. A section was viewed and the injured neurons were counted at 4x magnification. In case of hippocampal evaluation, a section was viewed at 4x magnification and the most dorsal part of CA1 was chosen. Then, at 20x magnification, the image was captured with a 12-megapixel Olympus (DP-70) digital camera. With the aid of home-made software, the damaged regions were encountered and these areas were determined. The labelled cells were then counted manually by a person not involved in the experiments, and who knew nothing about the experimental groups. The

labelled cells were calculated for 1 mm^2. The numbers of FJ^+ and NeuN-immunopositive neurons/mm^2 were determined in each slide of all animals.

3.2.2. In vitro electrophysiology

The electrophysiological recordings were conducted 10 days after 4VO. The rats (n=3x6) were decapitated, and coronal slices (400 μm) were prepared from the middle part of their hippocampi with a vibratome (Campden Instruments, Serial No: 752-903, UK) in an ice-cold artificial cerebrospinal fluid solution (ACSF) 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, saturated with 95% O_2 and 5% CO_2. The slices were then transferred into a Haas-type recording chamber and incubated at room temperature for 1 h to allow the slices to recover in the solution used for recording (differing only in that it contained 3 mM $CaCl_2$ and 1.5 mM $MgSO_4$). The flow rate was 1.5-2 ml/min and the experiments were performed at 34 ˚C. 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 to perform orthodromic stimulation of the Schaffer collateral/commissural pathway (Accupulser A310 [WPI Inc., USA]; constant current, 0.2-ms pulses delivered at 0.033 Hz).

Field excitatory postsynaptic potentials (fEPSPs) were recorded in parallel from the stratum radiatum and stratum pyramidale with two 1-2-MOhm resistance glass microelectrodes that were filled with ACSF and connected to a neutralized, high-input-impedance pre-amplifier (100x gain) with a high-pass filter set at 5 kHz. A homemade amplifier was used; the sampling rate was 10 kHz. The test stimulus intensity was adjusted to between 30 and 60 μA to evoke approximately 50% of a minimal stimulus intensity that evoked a saturated fEPSP (maximal fEPSP response) in SC rats. The fEPSPs were digitized, saved via a PC equipped with a Digidata 1200 interface and an Axoscope 10.0 recording system (Molecular Devices Corporation, Sunnyvale, CA, USA), and analyzed off-line with Origin 6.0 (OriginLab Corporation, Northampton, MA, USA).

LTP of the Schaffer collateral-CA1 synaptic response was induced by high-frequency stimulation (HFS; 0.2-ms pulses delivered at 100 Hz for 6 s) at 100% intensity of the test stimulus, and after HFS the fEPSPs were recorded for further period of at least 60 min. Input-output (IO) curves were created to measure the basal glutamatergic synaptic function. Slices from the same animal were generally used for

both tests, including LTP and IO curves. Two slices were tested from each rat, and each slice was subjected to only one particular test.

3.2.3. HPLC-MS/MS analysis of L-KYN and KYNA levels in the plasma and brain tissue

The vertebral arteries were occluded 24 h before treatments with vehicle or L-KYN + PROB. Two hours later the rats were anaesthetized with pentobarbital (60 mg/kg, i.p.). No bilateral carotid artery occlusion was performed. Blood was taken from the aorta, and centrifuged by a Heraeus Megafuge 1.0 R centrifuge (Kendro Laboratory Products GmbH, Osterode, Germany) for 10 min (RCF: 1625 x g). After decapitation tissue samples were obtained from the hippocampus and cerebral cortex, and weighed. The plasma and brain tissue was stored at about -70 ˚C until analysis. For the detailed steps of HPLC-MS/MS analysis, see [156].

3.3. Comparative in vivo electrophysiological examination of KYNA and G-KYNA

In these experiments, we studied the effects of peripherally administered G-KYNA on the hippocampal evoked activity, in comparison with peripherally administered pure KYNA. These drugs were administered alone, or in combination with PROB (200 mg/kg, i.p.). Two times 15 animals were used to study the effects of KYNA and G-KYNA. The effects of PROB were studied on 3 animals while two times 6 animals were used in the experiments with KYNA+PROB and G-KYNA+PROB. Studies on the changes in blood pressure were carried out on 6 animals. The doses of KYNA and G-KYNA that were chosen were based on earlier pilot experiments, in which both KYNA and G-KYNA were administered i.v. or i.p., in doses of 17, 34, 68 or 136 μmol/kg. Since i.p. and i.v. administration resulted in similar effects, i.p. administration was chosen in further studies. We endeavoured to find the minimum doses that were still effective; accordingly, on the basis of these pilot experiments, both KYNA and G-KYNA were administered in a dose of 17 μmol/kg throughout the main study.

4. Statistical analysis

Antidromic population spikes evoked by CA3 stimulation were measured from peak to peak (see [157], and Fig. 4). Differences between the amplitudes were determined statistically (paired *t*-test, *p* value set at 0.05 for significance). The results of behavioural experiments are expressed as means ± standard deviation (S.D.). Statistical analysis was performed by using the unpaired Student *t*-test or one-way analysis of variance (ANOVA) followed by LSD post-hoc test. Neuronal cell counts are presented

as means ± standard error of mean (S.E.M.), and were analyzed by using one-way ANOVA followed by the Bonferroni test for multiple comparisons with SPSS version 9.0 for Windows software (SPSS Inc., Chicago, IL, USA). A p value of ≤ 0.05 was considered significant. A nonparametric test on two independent samples was chosen for *in vitro* electrophysiological data (Mann-Whitney *U*-test).

Results

1. Acute PTZ-induced seizures

1.1. In vivo electrophysiology

In these experiments we examined whether pharmacological manipulations that lead to increased brain concentrations of KYNA could have any effects on the hippocampal electrical activity, and whether they have any antiepileptic effects. We decided to focus on the hippocampus, and on the question of whether co-administration of L-KYN together with PROB enhances the effects of L-KYN. Accordingly, recordings were made *in vivo* and both behaviour and seizure susceptibility were examined, resulting in a more thorough study of the hippocampal function than if either of these features was studied alone. The evoked responses of hippocampal neurons were chosen as an end-point for these electrophysiological experiments because of the high concentrations of glutamate receptors on the dendrites of these neurons, and because they receive glutamatergic afferents that can be stimulated preferentially *in vivo*.

1.1.1. Effects of L-KYN

The responses of the area CA1 pyramidal cells to contralateral CA3 stimulation were tested before and after the injection of L-KYN. In pilot experiments, L-KYN was administered in doses of 5, 10, 50, 100, 200, 300 or 400 mg/kg, either i.v. or i.p. In >10 mg/kg doses, L-KYN induced some changes in the population spike amplitudes in all cases. This occurred regardless of the mode of application, i.e. after both i.v. and i.p. injection. However, the effects were controversial: in some experiments the systemically administered L-KYN resulted in a transient facilitation of the population spike amplitudes, while in other cases the same dose of L-KYN, administered in the same way, transiently decreased the amplitudes of the population spikes. However, these changes in amplitude very seldom reached the level of significance (Fig. 8A). Similarly controversial results were observed in all experiments when L-KYN was injected in higher doses, either i.v. or i.p. These results were case-dependent. To summarize the data of all those experiments in which 300 mg/kg L-KYN was administered, it can be stated that L-KYN did not significantly change the amplitude of the evoked responses of the area CA1 pyramidal cells (Table 1).

1.1.2. Effects of PROB

PROB administered in a dose of 200 mg/kg did not cause an immediate significant change in the amplitude of the evoked population spike activity. In most cases, it resulted in a decrease in amplitude, but it needed a longer latency to develop

(Fig. 8B). The overall data for all the animals in this group indicate a moderate and not significant inhibitory effect of PROB on the CA1 pyramidal cell responses, with a long delay (Table 1).

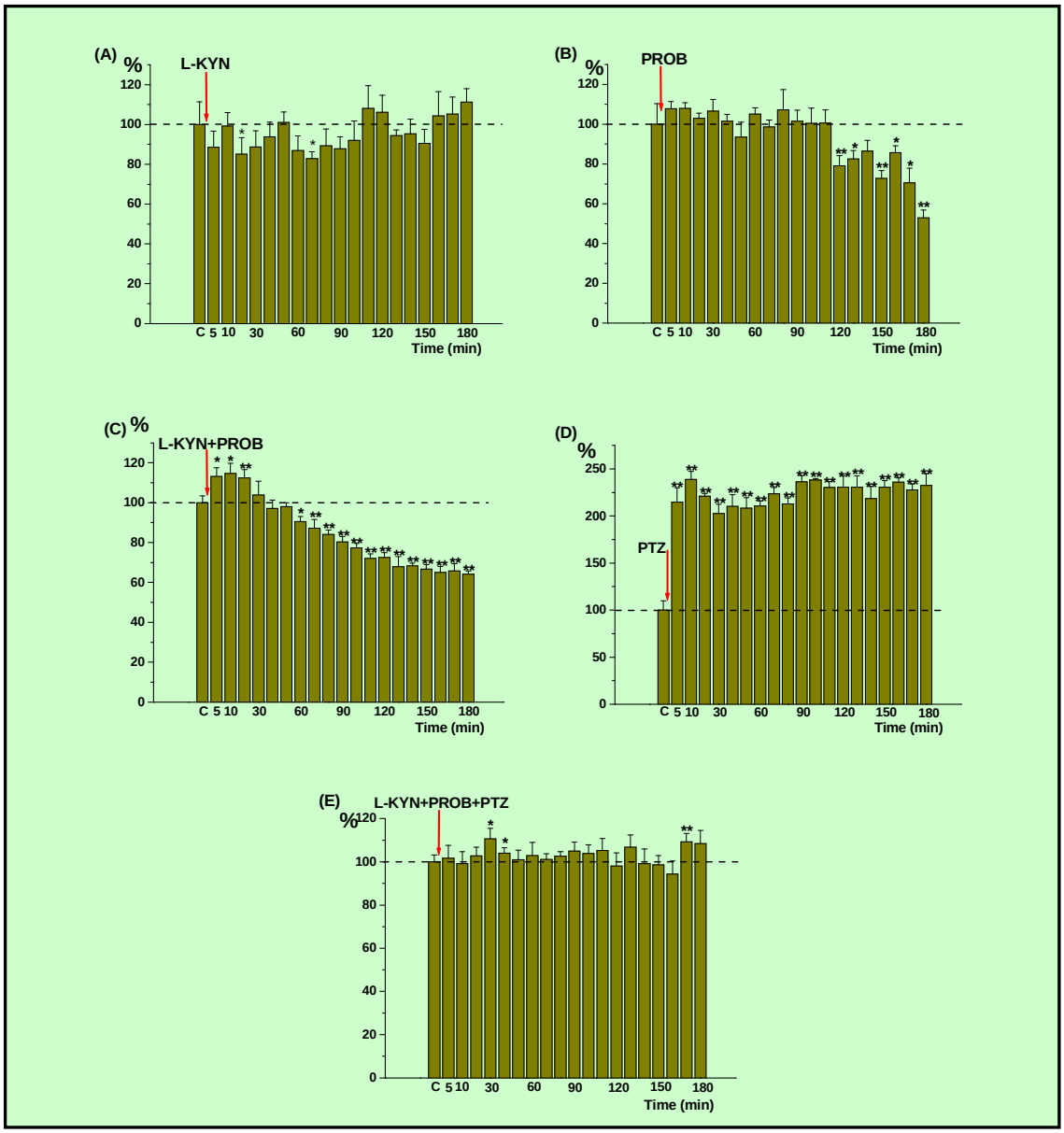

Fig. 8. Examples of the effects of the administered compounds on the population spike amplitudes recorded in CA1. (A) After L-KYN injection, hardly any marked, significant change in amplitude was observed. (B) PROB resulted in decreased CA1 population spikes with 2-h delay. (C) After transient increase, L-KYN+PROB i.p. injection resulted in a rapid and significant decrease in amplitude. Examples of the effects of PTZ (D) and L-KYN+PROB+PTZ (E) on population spikes recorded in the pyramidal layer of area CA1 of rats. Ordinate: Spike amplitudes as percentages of the controls (C). Abscissa: Time in min after drug injection. Each column represents the mean±S.D. for 5 potentials. ***** $p<0.05$, ****** $p<0.01$.

1.1.3. Effects of L-KYN+PROB

The results were quite different when L-KYN was administered together with PROB. In all those cases when L-KYN (300 mg/kg) and PROB (200 mg/kg) were administered together, a significant change in amplitude of the population spikes

occurred within 5-10 min. In some cases, a short, slight and transient increase in amplitude was observed after application of the drugs, but this was followed by a significant decrease in amplitude in all cases (Fig. 8C). In most cases, only the characteristic decrease in amplitude could be detected, as shown by the summarizing data for all the animals in this group (Table 1).

1.1.4. Effects of PTZ

PTZ in a dose of 60 mg/kg induced a significant increase in amplitude of the contralateral CA3 stimulation-induced CA1 responses in all experiments. The increase in amplitude started just after the drug administration, reached its plateau within 10 min, and remained at the elevated level during the 3-h registration period (Fig. 8D. and Table 1). In one of five PTZ-treated animals, the amplitude increased to over 200% of the control (Fig. 8D), while in the other four animals, the increment changed by 15-50% of the control amplitude. Besides the electrophysiological observation, in most of PTZ-treated anaesthetized animals, temporal trembling of the whiskers could also be observed. The administration of PTZ in a dose of 60 mg/kg never resulted in the death of the anaesthetized animals.

1.1.5. Effects of L-KYN+PROB+PTZ

It was reported above that PTZ in a dose of 60 mg/kg induced an immediate and significant increase in amplitude of the CA1 responses in all of the experiments. This PTZ-induced increase in amplitude was completely blocked in those animals in which L-KYN+PROB administration preceded the injection of PTZ (Fig. 8E). The data obtained from those five PTZ-treated animals, which received L-KYN+PROB prior to the PTZ administration clearly demonstrate that L-KYN+PROB did not allow development of the amplitude increase following PTZ administration (Table 1).

	C	Minutes						
		10	30	60	90	120	150	180
KYN	100	95.70 ± 6.1	98.63 ± 12.6	85.73 ± 16.5	87.91 ± 15.5	89.17 ± 19.2	82.76 ± 13.9	90.74 ± 19.6
PROB	100	86.72 ± 22.1	99.71 ± 11.8	98.24 ± 5.9	98.43 ± 10.9	84.79 ± 16.5	74.90 ± 22.9	68.56 ± 23.2
KYN + PROB	100	95.59 ± 17.1	88.67 ± 5.2**	82.82 ± 7.9**	72.98 ± 9.1**	72.26 ± 10.0**	72.85 ± 19.4*	65.22 ± 15.9**
PTZ	100	125.9 ± 10.4*	124.59 ± 13.7*	112.23 ± 4.1**	116.69 ± 1.4**	112.55 ± 6.3*	116.23 ± 1.9**	116.5 ± 7.8*
KYN + PROB +PTZ	100	93.11 ± 15.7	90.42 ± 16.9	82.74 ± 18.7	87.46 ± 17.3	83.81 ± 15.7	80.20 ± 12.5**	88.43 ± 11.9

Table 1. The summarizing data on the effects of L-KYN, PROB, L-KYN+PROB, PTZ, and L-KYN+PROB+PTZ pre-treatment with a PTZ injection 2 h later on the amplitudes of the population spikes recorded in the pyramidal layer of the CA1 region of the hippocampus. **C:** Normalized amplitudes of responses before the drug injection (100%). Amplitudes (means±S.D.) 10, 30, 60, 90, 120, 150 and 180 min after the second injection. L-KYN+PROB injections resulted in a significant decrease in spikes amplitude. This reduction in amplitude (inhibition) after L-KYN+PROB pre-treatment was strong enough to hold the amplitudes below the control level (100%) even after PTZ injection, which itself resulted in a significant increase in amplitude when it was injected alone.

1.2. Behavioural experiments

In parallel with the electrophysiological experiments, behavioural studies were carried out on five other groups of animals: *group 1* (controls, injected with saline), *group 2* (injected with PTZ), *group 3* (injected with L-KYN), *group 4* (injected with L-KYN+PROB) and *group 5* (injected with L-KYN+PROB, and 2 h later with PTZ). The behavioural experiments were carried out on five successive days.

1.2.1. General observations

The animals in group 1 did not display any unusual behaviour. Initially, they were afraid of the saline i.p. injection (two injections, with a 2-h delay), but later they became used to it. The animals in group 3, 4 and 5 (L-KYN, L-KYN+PROB, and L-KYN+PROB and 2 h later PTZ) became calm and slowed down after the first injection. On receiving the second injection of PTZ in group 5, however, these animals did not exhibit the development of any seizures in the course of the 5-day experiment. Instead of seizures, they mimicked hiccup after the PTZ injection. The animals in group 2 were injected with PTZ on each day. After PTZ treatment, the rats exhibited brief myoclonic jerks of their fore-and hind-limbs with a very rapid onset (60-70 s). The animals repeatedly fell down on their side or back, with generalized clonic seizures, which lasted for about 8-10 s. Such myoclonic jerks with the subsequent development of clonic seizures occurred several times. The daily repeated injection of the convulsant resulted in seizures with increasing intensity: 4-5 on the 6-point scale [158]. Further, the animals died in the course of the 5-day experiment, whereas mortality was very limited in group 5, where the animals received L-KYN+PROB prior to the PTZ injection (Fig. 9).

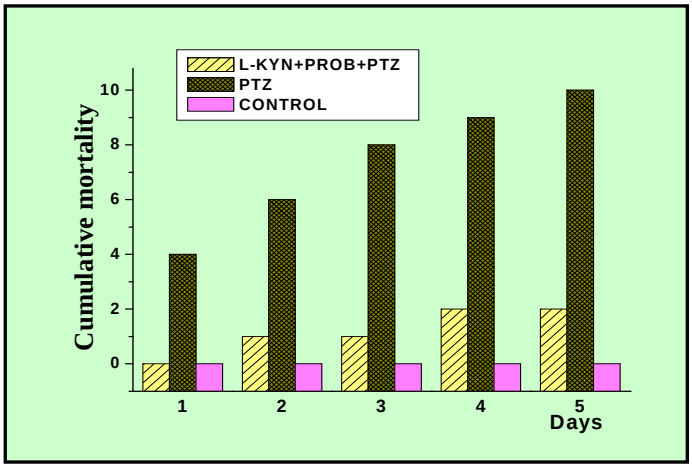

Fig. 9. Cumulative number of dead animals in the groups of controls, PTZ-treated and L-KYN+PROB-pre-treated, then PTZ-injected animals, during the 5-day experiments. Chronically administered PTZ resulted in the death of all the animals in the PTZ-treated group during 5 days. Except for two animals, L-KYN+PROB pre-treatment protected the rats from death induced by the repeated PTZ injections.

1.2.2. Water-maze performance

All five groups of animals started to take part in the water-maze experiments, which began 20 min after the second injection. From the very beginning, the performance throughout the 5 days revealed that the L-KYN+PROB+PTZ-treated animals needed more time to reach the platform than did the controls. This tendency became marked from the sessions on day 2 and the difference then remained significant up to the end of the experiment. This tendency became marked from the sessions on day 2 and the difference then remained significant up to the end of the experiment (F_2=27.337, p_2<0.01; F_3=26.768, p_3<0.01; F_4=28.180, p_4<0.01; F_5=34.652, p_5<0.01). The swimming strategies of the L-KYN+PROB+PTZ-treated animals and those in the other groups were not the same: the rats in group 5 spent more time swimming round the pool, near the wall, and demonstrated a poor performance throughout the 5-day trial. In contrast, the control animals displayed good progress in finding the hidden platform. Similarly, the animals in groups 3 and 4 exhibited a good performance in the water-maze study (Fig. 10). The PTZ-treated animals in group 2 also started to reveal a good performance in the water-maze experiments; however, because of the seizures and their high mortality rate, their testing was interrupted on day 3 (p_2<0.01; p_3<0.05).

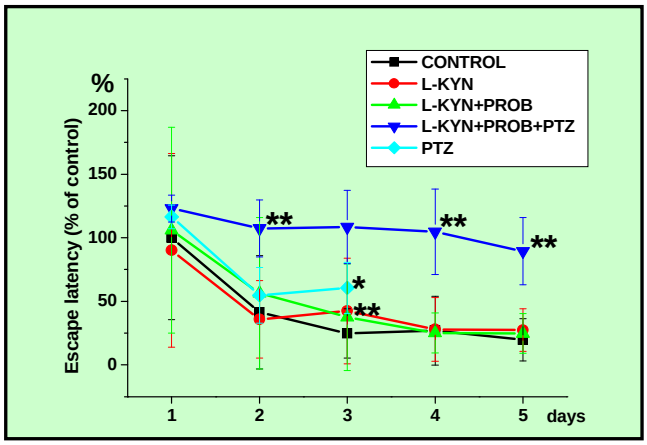

Fig. 10. Performance of five groups of animals in the water-maze test during the 5-day period (4 trials/day). The escape latency at 100% (=mean of the controls) is 47s. The number of PTZ-treated animals fell to 2 by day 4; the evaluation of this group was interrupted. * p<0.05, ** p<0.01. n=10 in controls, in L-KYN and L-KYN+PROB groups; n=10 on days 1-4, and n=9 on day 5 in the L-KYN+PROB+PTZ-treated animals. n=6 on day 1, n=4 on day 2, and n=3 on day 3 in the PTZ-treated group. Remark: on day 3, p<0.01 (control vs. L-KYN+PROB+PTZ) and p<0.05 (control vs. PTZ).

1.2.3. Open-field observations

As detailed above, the animals which received a pure PTZ injection developed seizures, culminating in generalized tonic-clonic activity. Therefore, these animals (group 2) did not take part in the open-field experiment in the arena. The animals in groups 1, 3, 4 and 5 did not display any significant difference between staying in the

centre or at the periphery of the arena. The animals in all groups preferred to stay at the periphery of the arena (Fig. 11A). Differences were observed, however, in rearing, in stereotyped washing (Fig. 11B,C) and in defecation activity between the controls (group 1) and the treated animals in groups 3, 4 and 5, but the differences in defecation activity were not significant (Fig 11D).

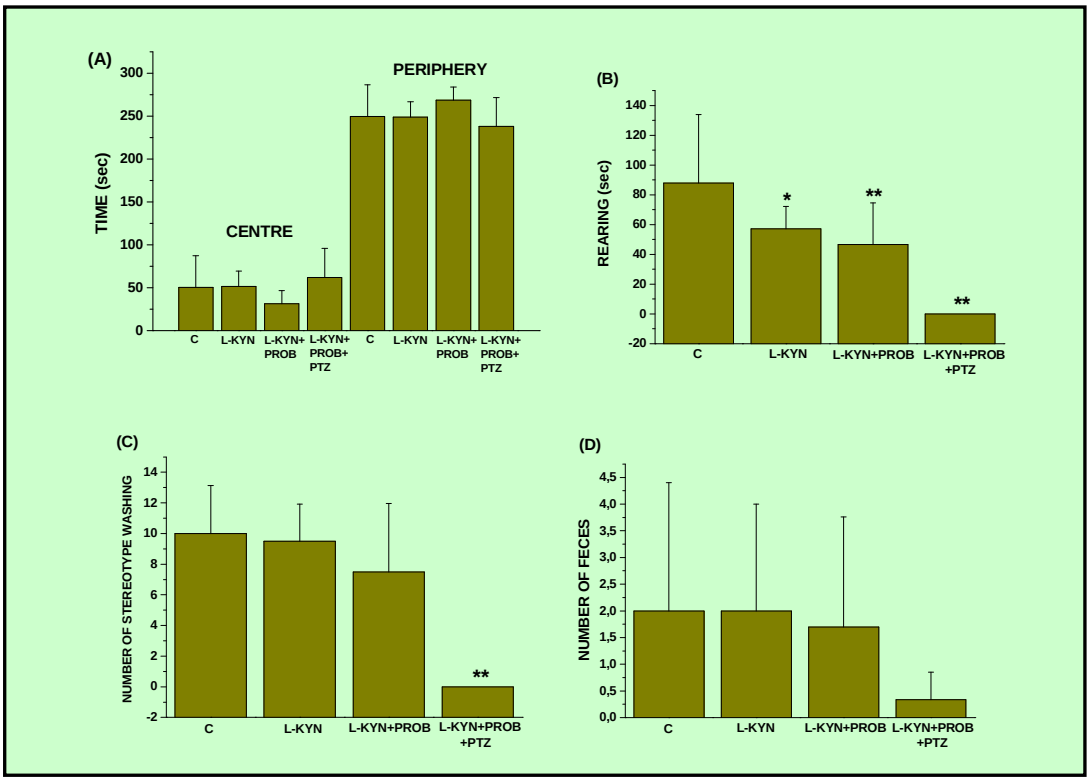

Fig. 11. Observations in an open-field arena. (A) Time (s) spent in the centre and at the periphery of the arena. No difference was found between the control and treated groups. The rats in all four groups preferred to stay at the periphery of the arena. (B) Time spent in rearing during the 5-min observation period. The animals after L-KYN or L-KYN+PROB injection became calm, and slowed down. These changes were significant. (C) The number of washings during the 5-min observation decreased significantly only in group 5 (L-KYN+PROB+PTZ). (D) Number of feces produced during the 5-min period. Data are shown as means±S.D. (n=10 animals), ****** p<0.01.

2. 4VO model of transient global ischemia

2.1. Neuroprotective effects of L-KYN+PROB-treatments on cortical rat brain slices

One day after bilateral vertebral artery cauterization, both carotid arteries were occluded for 10 min. This intervention induced severe neuronal damage in large parts of the neocortex in both hemispheres. The neuronal damage was localized to the temporo-lateral part of the cortex, starting at -3.14, -3.30 mm from the bregma, and reached its maximum at -3.8, -4.30 mm (Fig. 12). The cortical areas with the maximum number of injured neurons (-3.8 mm to the bregma) involved the primary and secondary auditory cortices, the ectorhinal cortex, the perirhinal cortex and the lateral entorhinal cortex, but the maximum was observed in the temporal association cortex. In this region, the FJ^+

neurons were found in a 500-700-µm-wide band involving cortical layers III-V (Fig. 13A,B).

L-KYN (300 mg/kg, i.p.) administered together with PROB (200 mg/kg, i.p.) caused a marked reduction in the number of damaged neurons. In the L-KYN-pre-treated animals, only a few groups of injured neurons could be observed in the cortical areas, whereas a great number of FJ^+ (damaged) neurons were present in the cortical area of the 4VO animals treated with vehicle (Fig. 13C,D vs. Fig. 13A,B).

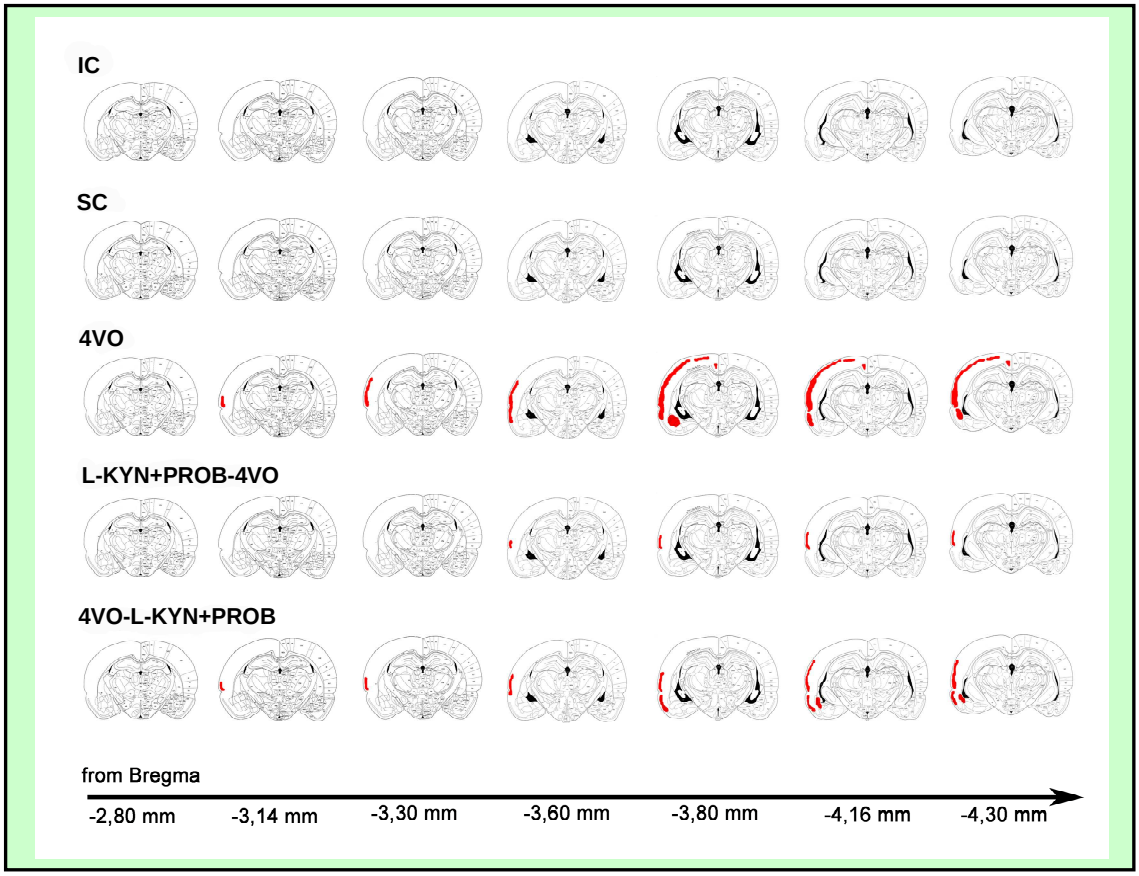

Fig. 12. The localisation of FJ^+ neurons in the cortex of 4VO animals (a representative example in each group). The cortical damage is maximum at 3.8 mm behind the bregma.

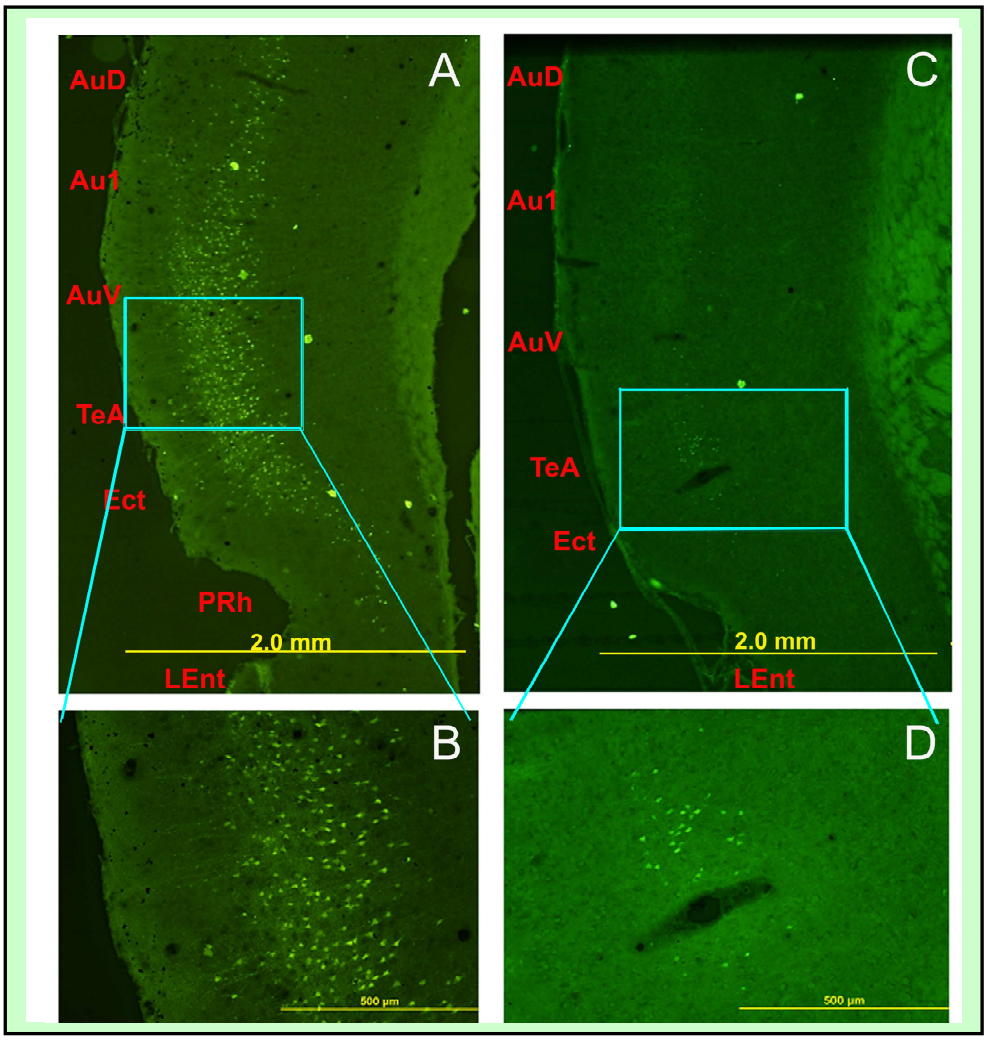

Fig. 13. FJ^+ cells in the cortex. Ten days after 4VO, injured neurons were detected in the extended lateral part of the hemispheres. The injured neurons attained their maximum number at 3.8 mm behind the bregma, in the ventral area of the secondary auditory cortex and in the temporal association cortex (A,B). L-KYN+PROB pre-treatment significantly reduced the number of FJ^+ cells. Only sporadic groups of FJ^+ cells could be found in the cortex of L-KYN+PROB-4VO animals (C,D). **AuD:** secondary auditory cortex dorsal area, **Au1:** primary auditory cortex, **AuV:** secondary auditory cortex ventral area, **TeA:** temporal association cortex, **Ect:** ectorhinal cortex, **PRh:** perirhinal cortex, **Lent:** lateral entorhinal cortex.

The statistical evaluation revealed that L-KYN administration considerably decreased the number of injured neurons. In the 4VO animals, the number of injured neurons/mm^2 was >200, while in the pre-treated group it was only 55-60 neurons/mm^2. The decrease in the number of injured neurons was highly significant not only in the pre-treated group, but also in the group treated with L-KYN after reperfusion (Fig. 14).

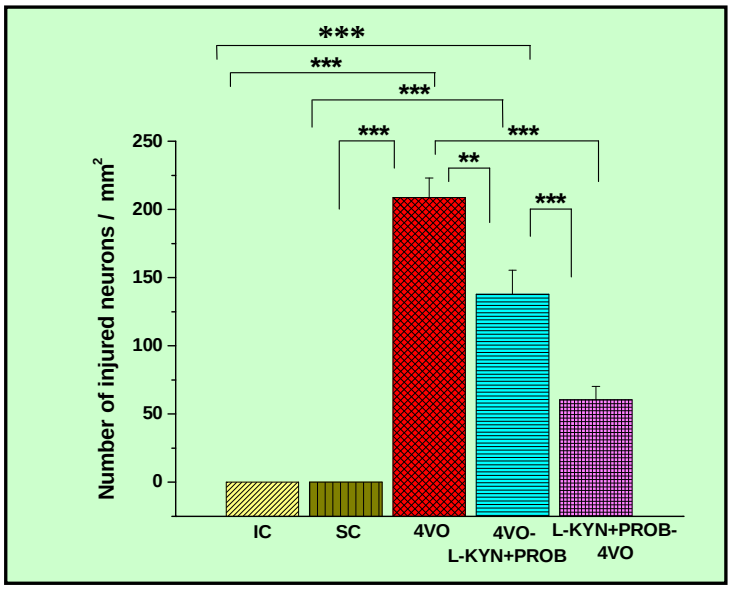

Fig. 14. Numbers of injured neurons per mm^2 in the five groups of animals. The 4VO-induced increase in the number of injured cells was significantly reduced in those animals which received L-KYN+PROB either before (L-KYN+PROB-4VO) or after (4VO-L-KYN+PROB) ischemia induction. The neuroprotection was most effective in the L-KYN+PROB-4VO group; a smaller, but significant reduction was achieved in those animals which received protective L-KYN as post-treatment (4VO-L-KYN+PROB). Values are means±S.E.M. (n=6-7 rats/group). ****** p<0.01; ******* p<0.001 compared to the group indicated in the Figure (one-way ANOVA followed by the Bonferroni test).

The KYNA concentration in the CSF of the rats lay in the range 7-196 nM; in most cases it was 20-30 nM. In accordance with the literature [159] this concentration can be increased 500-800-fold after peripheral L-KYN (300 mg/kg, i.p.) + PROB (200 mg/kg, i.p.) administration.

The importance of this observation is immediately clear: it means that even the post-traumatic administration of L-KYN may be of substantial therapeutic benefit.

2.2. *Neuroprotective effects of L-KYN+PROB-treatments on hippocampal rat brain slices*

In animals subjected to 10-min 4VO, severe neuronal damage was observed in the CA1 area of the hippocampus in both hemispheres 10 days after the intervention. The neuronal damage extended for millimeters in the anterior-posterior direction from -3.14 mm relative to the bregma. In this injured region, numerous of the pyramidal cells in the CA1 region were FJ^+ in each of the coronal sections, while those in the CA3 region and the dentate gyrus were not labelled (Fig. 15A). In accordance with this, the NeuN immunohistochemistry indicated the lack of intact cells in the CA1 region in the 4VO animals, but an intact CA3 region and dentate gyrus (Fig. 15B).

L-KYN administered together with PROB caused a marked reduction in the number of damaged neurons. In the L-KYN-pretreated animals, injured neurons stained with FJ could be observed only sporadically in the CA1 area of the hippocampus (the same was true for the CA3 region and the dentate gyrus; Fig. 15C). Accordingly, NeuN immunohistochemistry gave the impression of a non-injured CA1 region (like the CA3 region and the dentate gyrus) in L-KYN-pre-treated 4VO animals (Fig. 15D).

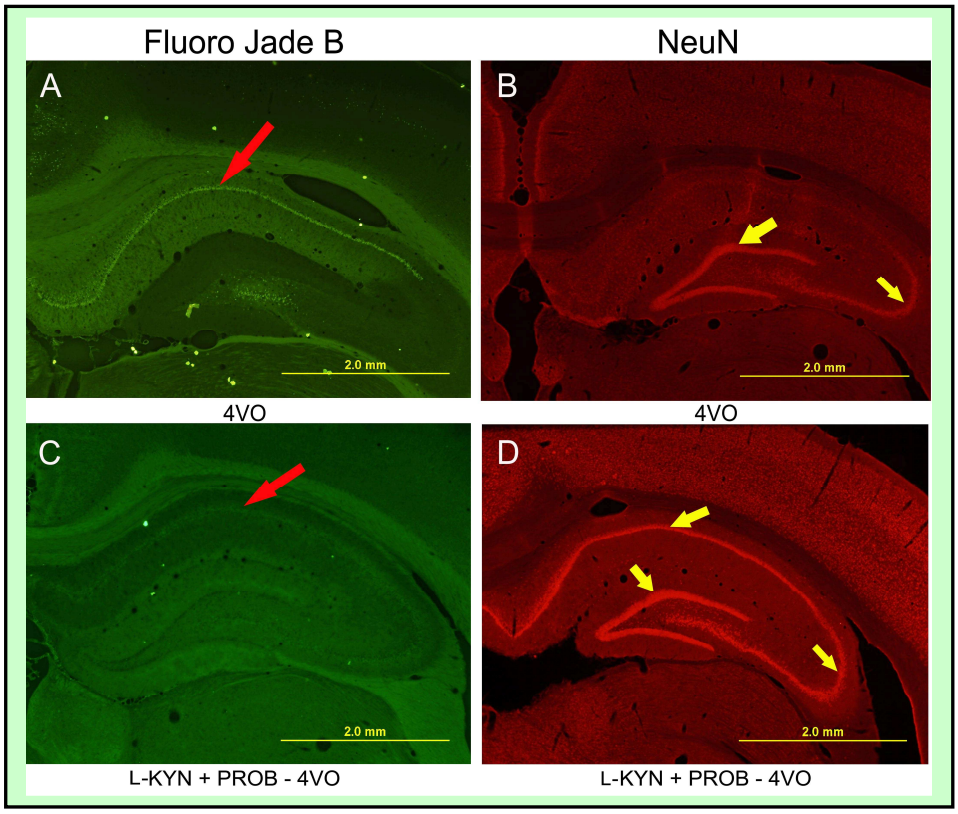

Fig. 15. The effects of pre-treatment with L-KYN on the ischemic hippocampus of the rat. (A) Transient global ischemia (4VO) damaged the CA1 pyramidal cells in the hippocampus (the red arrow shows the injured CA1 region (FJ+ cells) of the hippocampus). (B) The CA3 area and the dentate gyrus are less vulnerable to transient ischemia: the NeuN-immunopositive region of the hippocampus (yellow arrows). (C) Pre-treatment of 4VO animals with L-KYN+PROB (L-KYN+PROB-4VO) protects the hippocampus from the ischemia-induced damage (red arrow). (D) In L-KYN+PROB-4VO-pre-treated rats, NeuN immunohistochemistry reveals intact areas throughout the hippocampus, including the CA1 and CA3 regions and the dentate gyrus (yellow arrows).

L-KYN administration considerably decreased the number of injured neurons in the CA1 region. However, the decrease in the number of injured neurons was highly significant only in the pre-treated group (L-KYN+PROB-4VO). The animals in the post-treated group (4VO-L-KYN+PROB) also exhibited a tendency to a reduction in the number of injured neurons, but this change was not significant (Fig. 16A). The NeuN immunohistochemistry supplemented these results: the number of non-injured cells was highest in the SC group, and lowest in the 4VO animals. Post-treatment with L-KYN

(4VO-L-KYN+PROB) had hardly any effect, while in the 4VO animals which received L-KYN before ischemia (L-KYN+PROB-4VO) the number of intact cells was comparable to the control level (Fig. 16B). In short, the L-KYN+PROB pre-treatment was able to reduce the proportion of damaged cells to 52% relative to the damaged cells induced by 4VO without L-KYN+PROB treatment. The L-KYN+PROB treatment after the 4VO intervention did not prove effective (see Table 2).

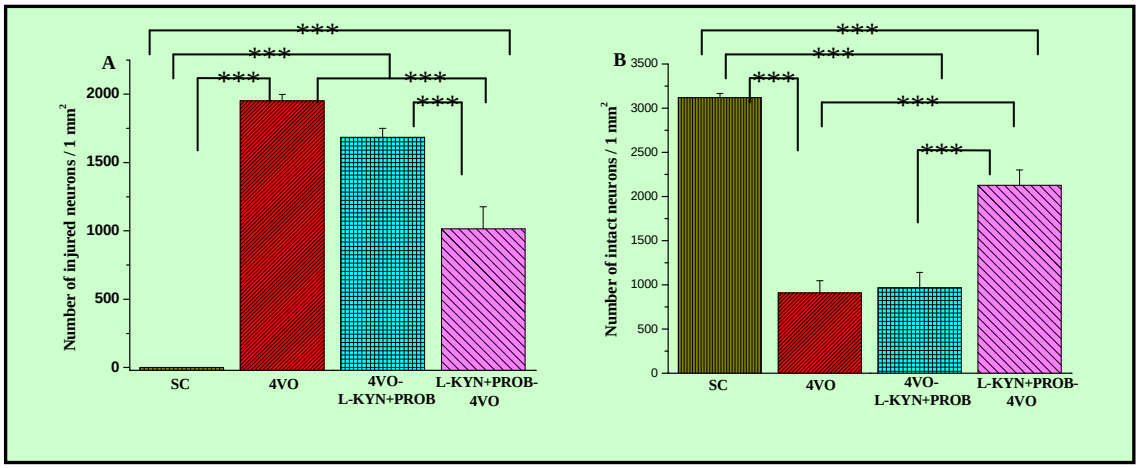

Fig. 16. The effects of L-KYN administration on the cell number/mm^2 in the CA1 region of four groups of animals. (A) The transient global ischemia (4VO)-induced increase in the number of injured cells was reduced in those animals which received L-KYN+PROB. The reduction was significant only in those animals which received L-KYN+PROB before transient global ischemia (L-KYN+PROB-4VO), though the number of FJ$^+$ cells was slightly altered in the post-treated animals (4VO-L-KYN+PROB). (B) NeuN immunohistochemistry supplemented the above results: the number of intact cells/mm^2 in the CA1 region of the hippocampus was appreciably reduced in the 4VO animals. Post-treatment was ineffective, whereas pre-treatment strongly increased the number of intact cells/mm^2 in the CA1 region of the hippocampus. Values are means±S.E.M. (*n*=6-7 rats/group). Differences between the SC and 4VO groups and between the 4VO and L-KYN+PROB-4VO groups were significant ($p<0.001$, one-way ANOVA followed by the Bonferroni test.

Groups:	Fluoro Jade B-positive(%):	NeuN-labeled (%):
SC	0	100
4VO	100	29,19
4VO-L-KYN+PROB	86,29	31,02
L-KYN+PROB-4VO	51,96	68,17

Table 2. The proportions of injured and intact neurons in the CA1 region of the hippocampus. In the case of FJ-B positivity, the result for the 4VO group was taken as 100%, for the animals in this group exhibited the most extreme injuries. On the other hand, all 4VO-intervention groups were compared with the SC group in the case of NeuN labelling.

2.3. In vitro electrophysiology

First of all, we explored the basal synaptic properties of the fEPSPs in order to evaluate the ischemia-induced impairment of the Schaffer collateral-CA1 synaptic transmission (Fig.17).

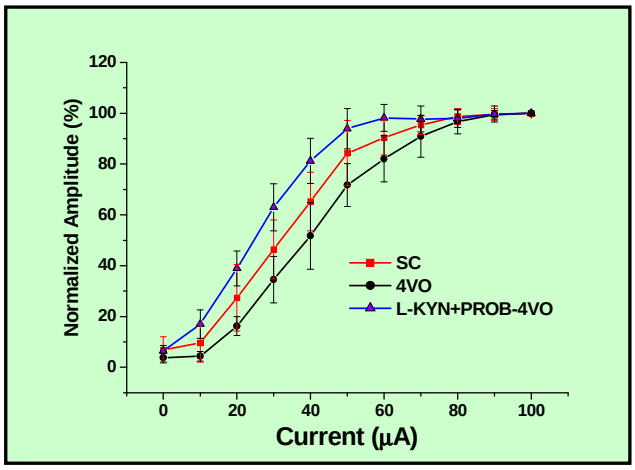

Fig. 17. Input-output (IO) curves of sham-operated controls (SC), 4VO animals, and 4VO animals which received L-KYN+PROB treatment. IO curves were established by plotting the fEPSP amplitudes against various test pulse intensities from 0 to 100 μA. No significant difference was found between the IO curves in the three groups, implying that the basal functions of the pyramidal cells and synapses were not affected by complete ischemia.

For this purpose, the IO curves were established by plotting the fEPSP amplitude against various test pulse intensities from 0 to 100 μA. The IO curve for the 4VO animals was positioned below that for the controls (while the curve for the L-KYN+PROB-4VO animals was positioned above it). However, there was no significant difference between the IO curves in the three groups, implying that the basal functions of the registered pyramidal cells and synapses were not affected by complete ischemia.

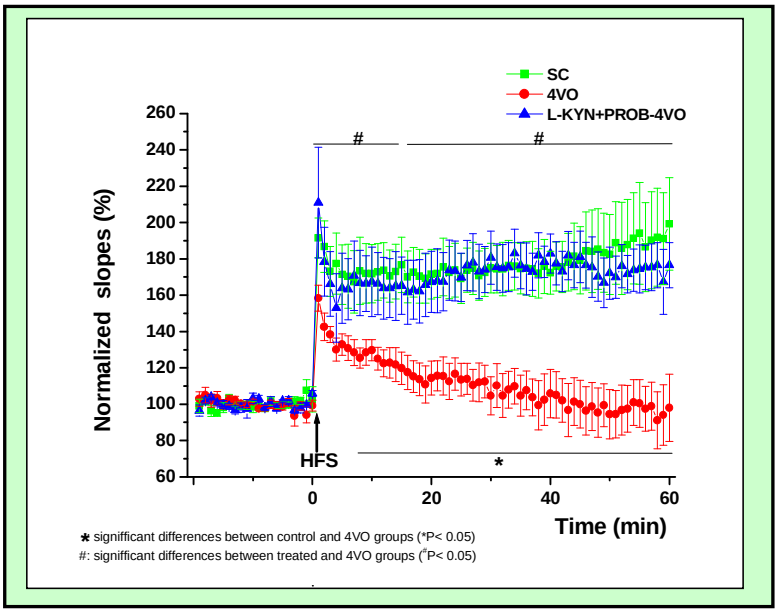

Fig. 18. Administration of L-KYN and PROB prevented the impairment of the long-term potentiation (LTP) response caused by global ischemia. In the SC group (n=5), LTP was induced by high-frequency stimulation of the Schaffer collaterals (HFS; 600 pulses at 100 Hz). LTP after HFS was not present in the 4VO group (n=11) 10 days after 10-min global cerebral ischemia. The L-KYN+PROB-4VO group (n=6) was subjected to the same carotid occlusion that was preceded and followed by i.p. injections of L-KYN and PROB. The course of LTP in this group was identical to that in the controls. Data points are means±S.E.M. of normalized slopes of fEPSPs. Statistically significant differences are denoted: (*****), (#) significant differences between SC and 4VO groups (***** p<0.05), and between treated and 4VO groups ($^{\#}$ p<0.05; nonparametric test on two independent samples was used, the Mann-Whitney U-test).

LTP was induced by HFS of the Schaffer collateral-CA1 synapses. The fEPSPs were monitored for 40-60 min before conditioning stimulation until the amplitudes were generally stable, and their mean value was determined as the 20-min-long baseline before LTP induction. In the SC group, the HFS caused a robust increase (170-180%) in the slope of the fEPSPs (Fig.18, SC) and this increase in slope (and in amplitude) remained at the elevated level during the 1-h registration period. The same conditioning protocol did not induce a significant, lasting increase of the fEPSPs in the majority of the 4VO animals. In this group, the elevation of the amplitudes was only transient; no LTP was observed. At the end of the registration period, the slopes had returned to the control level, or decreased below the baseline (Fig. 18). The administration of L-KYN and PROB protected slices from the 4VO-induced LTP impairment. L-KYN restored the fEPSP slopes to the control level, and these parameters were stable until 60 min after HFS.

2.4. Plasma and brain L-KYN and KYNA concentrations

In the sham-operated rats, L-KYN concentration was approximately 3-fold higher in the plasma than in the cortex and hippocampus. On the other hand, the KYNA concentration was higher in the cortex and hippocampus than in the plasma ($p<0.001$), and higher in the cortex than in the hippocampus. As a consequence, the KYNA concentration in the sham-operated rats reached only 1.5% of the L-KYN concentration in the plasma, but 8% and 12% of the L-KYN concentrations in the hippocampus and cortex, respectively. Treatment with L-KYN+PROB considerably increased both the L-KYN and KYNA concentrations in the plasma and brain, and also altered their proportions within the compartments studied. The L-KYN concentration increased 37-fold in the plasma and approximately 70-fold in the hippocampus and cortex, while the KYNA concentration was elevated roughly 300-fold in the plasma and 50-fold in the hippocampus and cortex. The L-KYN concentration increased more in the brain than in the plasma, and the L-KYN levels were similar in the plasma and brain after L-KYN+PROB administration, but this was probably due to the relatively large scatter of the data. Likewise, since the KYNA concentration increased more in the plasma than in the brain, the KYNA concentrations were similar in the plasma and brain, reaching 7-12% of the L-KYN concentration in the brain (Table 3).

	Plasma concentration, ng/ml		Hippocampal concentration, ng/wet weight		Cortical concentration, ng/g wet weight	
	L-KYN	KYNA	L-KYN	KYNA	L-KYN	KYNA
Vehicle, n=5	602±21	8.8±0.5	212±18***	17.7±2.1***	235±22	28.2±3.2***,#
L-KYN+PROB, n=5	22518±5392	2695±783	14270±3801	996±318	1710±4492	1497±534
Ratio treated/SHAM	37	305	67	56	73	53

Table 3. The concentrations of L-KYN and KYNA in the plasma, brain cortex and hippocampus in rats in which the vertebral arteries were occluded bilaterally on the day before treatment. The animals were treated with vehicle or L-KYN+PROB 2 h before sacrifice, but no bilateral carotid artery occlusion was performed. Statistically significant differences are shown. ******* $p<0.001$ vs. plasma. ***** $p<0.05$ vs. cortex.

3. Comparative in vivo electrophysiological examination of KYNA and G-KYNA

3.1. Effects of KYNA

The responses of the area CA1 pyramidal cells to contralateral CA3 stimulation were tested before and after the injection of KYNA. KYNA was administered in doses of 17, 34, 68 or 136 μmol/kg. Three animals were tested at each dose, with the exception of the 17 μmol/kg dose, at which 6 animals were examined. Marked effects of the i.p. injected drug were not observed in any of the treated animals, apart from some transient, inconsistent deviations from the control level. In the main series of experiments, to facilitate comparability with the results of administration of G-KYNA (see below), 17 μmol/kg (equimolar) KYNA was administered (i.p.); this resulted in only a slight, if any decrease in the amplitude of the CA1 responses (Fig. 19A and Table 4).

3.2. Effects of G-KYNA

In pilot experiments, G-KYNA was administered in doses of 17 (n=6), 34 (n=3), 68 (n=3) or 136 μmol/kg. G-KYNA, administered either i.v. or i.p., in a dose of 136 μmol/kg resulted in 100% mortality within 5 min. Similarly, G-KYNA administered in a dose of 68 μmol/kg i.v. resulted in a stoppage of breath, but the animals could be resuscitated. I.p. injection of this dose (68 μmol/kg) of drug did not stop the breathing of the animals. These findings led us to reduce the dose of the drug to a level as low as possible, which still resulted in a marked and clear-cut decrease in amplitude of the CA1 pyramidal cell responses. We found that, in a dose of ≥ 17 μmol/kg, G-KYNA induced consistent and appreciable decreases in the population spike amplitudes in all cases. This change started 50-60 min after drug administration, regardless of the mode of application. In some cases, a small and transient increase in amplitude was observed shortly after the application of the drug, but this was followed by a significant lasting decrease in amplitude in all cases (Fig. 19B and Table 4).

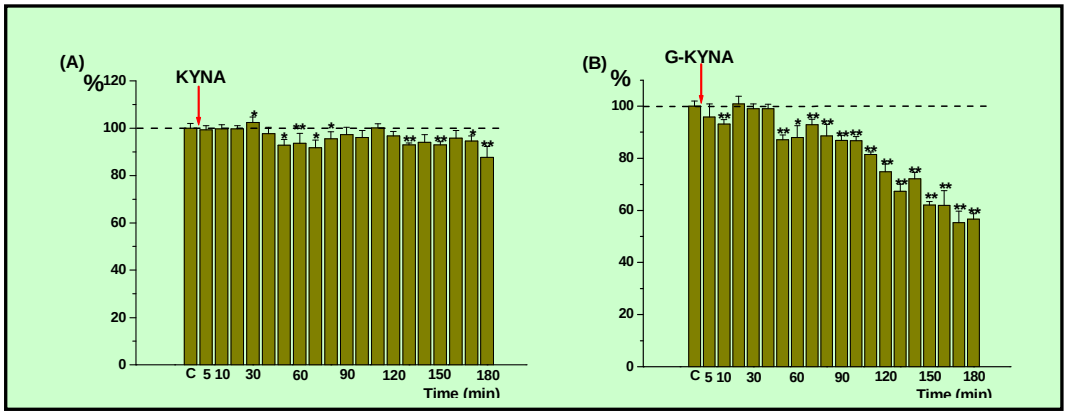

Fig. 19. Examples of the effects of the administered compounds on the population spike amplitudes recorded in CA1. (A) After KYNA injection there were some decreases in amplitude, but these changes were not marked. The observations were similar after the i.p. injection of KYNA in higher doses up to 136 μmol/kg. (B) G-KYNA injection resulted in marked and significant decreases in the CA1 population spike amplitudes with a 50-60 min delay. Ordinate: spike amplitudes as percentages of the controls (C). Abscissa: time in min after drug injection. Each column represents the mean±S.D. for 5 potentials. ***** $p<0.05$, ****** $p<0.01$.

3.3. Effects of PROB

PROB administered in a dose of 200 mg/kg i.p. did not cause an immediate significant change in the amplitude of the evoked population spike activity. In most cases, it resulted in a decrease in amplitude, but this needed a longer period (60-90 min) to develop. This observation is fully consistent with our results in acute PTZ-induced seizures, and is therefore not shown here.

3.4. Effects of KYNA+PROB

The results were similar when KYNA was administered together with PROB. In most cases, when KYNA (17 μmol/kg) and PROB (200 mg/kg) were administered together, there was no change in the amplitudes of the responses during 1 h following the drug application, except for a transient, slight facilitation in a few cases, but with a 1.5-2 h delay, a slight decrease in amplitude was registered in all cases (Fig. 20A and Table 4).

3.5. Effects of G-KYNA+PROB

The results were quite different when G-KYNA was administered together with PROB. G-KYNA (17 μmol/kg) administered together with PROB (200 mg/kg) resulted in a progressive and, by the end of the recording period, a considerable decrease in amplitude of the CA1 population spikes. The decrease in amplitude began and became significant within 10-20 min, and continued over the 3 h registration time (Fig. 20B and Table 4). In some of the experiments, the registration continued over 4.5 h. In these animals, the reduction in amplitude was progressive throughout the whole experiment (Fig. 20C).

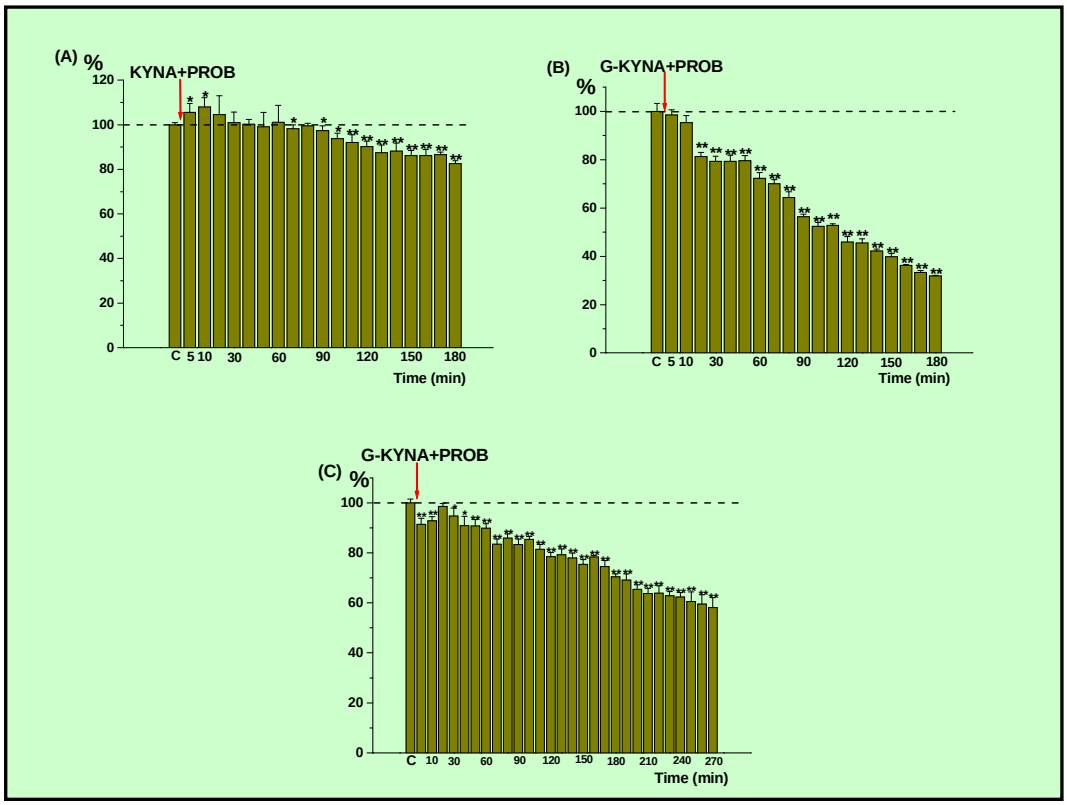

Fig. 20. Examples of the effects of KYNA or G-KYNA administered together with PROB. (A) After a transient increase, KYNA+PROB resulted in decreased CA1 population spikes with a 1.5-2 h delay. (B) G-KYNA+PROB i.p. injection resulted in considerable decreases in amplitude, shortly (10-20 min) after the administration. In some cases (as presented here), the amplitudes fell to 35-40% of the controls, while in most cases, as in panel C below, the amplitudes fell only to 60-70% of the controls, in spite of the relatively long recording period. The ordinate and abscissa are the same as in Fig. 19.

	C	0-30 min	30-60 min	60-90 min	90-120 min	120-150 min	150-180 min
KYNA	100	95,88±3,9	90,55±2,1 *	88,61±1,5 **	90,08±2,8 *	87,20±2,6 *	87,39±2,4 *
G-KYNA	100	100,64±0,5	91,03±4,2	83,96±3,8 *	78,55±4,6 *	63,93±8,2 *	61,65±3,0 **
KYNA+PROB	100	97,79±1,5	105,94±11,9	98,43±4,2	85,75±8,1	84,35±5,7 *	77,76±1,4 **
G-KYNA+PROB	100	93,11±2,5 *	88,43±0,8 **	81,47±2,9 **	74,51±4,2 **	73,50±2,9 **	65,13±6,6 **

Table 4. Changes in population spike amplitudes of CA1 pyramidal cells. Data summarising the effects of KYNA, G-KYNA, KYNA+PROB and G-KYNA+PROB on the amplitudes of the population spikes recorded during 30 min blocks in the pyramidal layer of region CA1 of the hippocampus. C: Normalized amplitudes of responses before drug injections (100%). Amplitudes (means±S.D.). G-KYNA+PROB treatment was the most effective in inducing a persistent and long-lasting decrease in the amplitudes.
***** $p<0.05$ (n=6 animals in each group), ****** $p<0.01$ (n=6 animals in each group).

3.6. Blood pressure

In 6 animals, the basal blood pressure and the effects of KYNA and of G-KYNA on the blood pressure were examined. In all animals, the intra-carotid blood pressure was found to be 100-119 mm Hg. Neither KYNA nor G-KYNA influenced the intra-carotid blood pressure markedly.

Discussion

The neuroinhibitory properties of KYNA were revealed in neurophysiological experiments more than 20 years ago [13]. Later, various experiments proved that KYNA is a broad-spectrum antagonist of EAA receptors; it inhibits the overexcitation of NMDA receptors [160, 161]. At low concentrations, KYNA preferentially blocks the glycine co-agonist site of these receptors [162]. Moreover, KYNA can inhibit α7-nACh receptors presynaptically and hence modify the glutamatergic transmission [15]. Because of its interesting pharmacological characteristics and neuroprotective and anticonvulsant properties [102, 163], KYNA, several of its derivatives and different KP metabolites have become widely used tools in neurobiological research.

The primary finding of our study was that the peripheral administration of L-KYN and PROB eliminated the effects of PTZ; the responses to glutamatergic inputs were decreased in the CA1 region of the hippocampus, and in the behavioural experiments L-KYN and PROB pre-treatment protected the animals from the PTZ-induced kindling of seizures and death.

On the basis of the literature cited in the introduction, we may suppose that, if the KYNA content of the brain is high enough, it has a definite neuroprotective effect. The normal concentration of KYNA in the brain is too low to influence the EAA receptors, and even under pathological conditions the data do not indicate that the concentration elevation will necessarily allow KYNA to influence the co-agonist site of the NMDA receptor.

In our study, we administered a relatively high systemic doses of the precursor, L-KYN (and even higher doses in combination with PROB), which led to a non-physiological concentration of KYNA. This proved effective: L-KYN administered i.p. slightly (not significantly) decreased the population spike amplitude of the CA1 pyramidal cell responses. PROB had a somewhat stronger effect, with a longer delay. The two compounds administered together resulted in a marked and significant decrease in amplitude of the population spikes evoked on the CA1 pyramidal cells. This effect is probably based on the inhibition of the NMDA receptors of the CA1 pyramidal cells by the elevated level of KYNA in the brain tissue, as a consequence of peripheral L-KYN and PROB administration. This seemed to be able to compensate the overexcitation induced by PTZ. Indeed, 60 mg/kg PTZ administered i.p. resulted in a significant increase in amplitude of the CA1 spike activity, and this effect was completely compensated by pre-treatment with L-KYN+PROB. In parallel with the

electrophysiological results, we studied the effects of L-KYN+PROB pre-treatment on the PTZ-induced seizures and death in awake animals. To test the possible protective effect of L-KYN+PROB in behaving animals, we chose the convulsive dose of 60 mg/kg PTZ. The pre-treatment with L-KYN+PROB was effective in protecting the animals against the generalized clonic seizures. Although they exhibited reduced rearing, washing and defecation activity, we did not observe any difference in behaviour between the controls and the treated animals in an open-field arena.

In the water-maze task, the L-KYN+PROB+PTZ-treated animals revealed a significantly impaired performance, while those animals, which received pure L-KYN or L-KYN+PROB did not exhibit a significantly impaired performance as compared with the controls. Until the number of PTZ-treated animals decreased to a critical level, this group also displayed a good performance in the water-maze studies. This is in accordance with the results of Szyndler *et al*. [164], who found that PTZ-kindled rats did not demonstrate any significant differences in short-term and long-term memory in a passive avoidance test.

In accordance with our *in vivo* electrophysiological results, KYNA administered *in vitro* led to a decrease in the amplitude of the evoked fEPSPs in the hippocampal CA1 region [165]. These results have demonstrated that the conversion L-KYN→KYNA produces KYNA in an amount that is not only detectable by analytical methods [166], but also sufficient to prevent the neuroexcitatory effect of PTZ. The L-KYN→KYNA conversion was proved via KAT blocker (L-NNA) administration [165].

It has long been known that a reduction in cerebral blood flow is closely related to different kinds of brain dysfunctions [167, 168]. Understandably, research activity relating to the consequences of cerebral hypoperfusion has recently been high (some recent reviews: [169-171]). The CA1 region of the hippocampus is particularly vulnerable to hypoxic conditions [172, 173]. Our results have shown that 4VO-induced temporal global ischemia results in neuronal damage not only in the hippocampus, but also in the neocortex. Moreover, L-KYN application significantly reduced this cortical neuronal damage after either pre- or post-ischemic administration. Nozaki and Beal [174] first reported that the pre-administration of L-KYN proved neuroprotective in a neonatal model of ischemia. Accordingly, the systemic administration of L-KYN dose-dependently increases the KYNA content of the brain [149]. The extensive literature (see the recent reviews by [74, 175, 176]) suggests that an appropriate elevation of the brain KYNA concentration leads to a neuroprotective effect. The normal concentration

of KYNA that we measured in the CSF by mass spectrometry is 20-30 nM, which is in good accordance with the results of Moroni et al. [177]. A considerable (800-1300-fold) increase in the brain KYNA level following the peripheral administration of L-KYN+PROB was demonstrated more than 15 years ago [159, 174].

Evidence is accumulating which suggests that an ischemia-induced sustained elevation in intracellular calcium concentration $[Ca^{2+}]_I$ contributes to cell death [178-180]. The secondary rise during reperfusion injury is more likely to be responsible for apoptotic than for immediate necrotic damage, which occurs in the CA1 neurons in particular 2-3 days after the induction of global ischemia [181]. These processes of injury of the CA1 neurons are attained via NMDA activation [182, 183].

L-KYN administration has proved to be neuroprotective in histological studies. However, the neuronal degeneration marker used, Fluoro Jade B, does not discriminate between apoptotic and necrotic cell damage. Moreover, recent studies have indicated that Fluoro Jade B can stain neurons degenerating as a result of an acute insult, and it can label activated microglia and astrocytes during a chronic neuronal degenerative process [184, 185]. All these points should be taken into consideration.

The systemic administration of L-KYN together with PROB resulted in concentrations of KYNA in the brain which have proved to be neuroprotective in histological and behavioural studies [128]. However, relatively little is known about its impact on the outcome of synaptic plasticity. A novel finding here is that the administration of L-KYN (+PROB) once before and 4 times after 4VO-induced transient global ischemia proved neuroprotective in histological studies, and also reduced (nearly abolished) the impaired LTP induction in the Schaffer collateral - CA1 pathway in adult rats. Interestingly, the IO curves of the controls, and of ischemic + L-KYN+PROB-treated rats did not display significant changes. This suggests that it is the machinery of LTP that is injured rather than the basal functions of the pyramidal cells and synapses. It should additionally be taken into consideration that, though the FJ-B labelling indicates massive neuronal degeneration in the untreated 4VO group, the NeuN positivity demonstrates that nearly 1/3 of the neurons in this group remained intact, in spite of the complete ischemia.

Although we do not know sufficient about ischemia-induced injuries of the LTP machinery, it is clear that ischemia may impair physiological forms of synaptic plasticity, such as activity-dependent LTP [136]. However, Crepel et al., [186] characterized a pathological form of synaptic plasticity induced by a few minutes of

anoxia and aglycemia *in vitro*. This form of potentiation is referred to as anoxic LTP, which also requires an increase in $[Ca^{2+}]_I$ [187]. In contrast with tetanic LTP, the expression of anoxic LTP is mediated exclusively by NMDA receptors (reviewed by [137]). Anoxic LTP involves the redox modulatory site of the NMDA receptor [188].

In most cases, after transient global ischemia, the surviving neurons displayed normal transmission, except for the reduction in the maximum level of fEPSPs that seems to be a consequence of the cell number reduction due to ischemic cell death. The impaired LTP induction should reflect deficits in the machinery specific to LTP induction in the individual surviving neurons. Although the mechanism is not yet known, we have demonstrated for the first time here that treatment with L-KYN+PROB rescues the Schaffer collateral-CA1 synapses from impaired LTP induction after transient global ischemia.

The results cited above and our observations presented here suggest that L-KYN administration results in the production of the neuroprotective KYNA rather than the neurotoxic QUIN. This assumption is supported by the present complex histological and electrophysiological results, which prove that L-KYN, as a precursor of the glutamate receptor antagonist KYNA, offers advantages over KYNA in the treatment of ischemic brain damage. These results raise the possibility that long-term L-KYN administration may be useful in delaying neuronal loss in certain neurodegenerative disorders.

The primary finding of our comparative *in vivo* electrophysiological examination of KYNA and G-KYNA was that the peripheral administration of G-KYNA in a dose as low as 17 μmol/kg, especially together with PROB, effectively reduced the responses to glutmatergic input in region CA1 of the hippocampus, while pure KYNA injected either equimolarly or in higher doses, did not do so. These results suggests that the manipulation of brain kynurenines, e.g. increase of the KYNA level in the brain, may reduce the overactivation of EAA receptors, and offers novel therapeutic opportunities.

However, the use of KYNA as a neuroprotective agent is rather restricted because it has only a very limited ability to cross the BBB [21]. This is supported by the results we have presented here: systemically administered KYNA in doses of 17, 34, 68 or 136 μmol/kg did not cause any observable change in the anaesthetized animals, either in their breathing, or in the electrophysiological activity of their CA1 region. Against this, G-KYNA in a dose of 136 μmol/kg resulted in the death of the animals in all cases, while 68 μmol/kg (i.v.) G-KYNA induced the stoppage of breathing of the animals, though they could be resuscitated. A dose of G-KYNA as small as 17 μmol/kg was

effective in reducing the CA3 stimulation-evoked activity of the CA1 pyramidal cells in the hippocampus. This effect was augmented when G-KYNA was administered together with PROB, which is known to be an inhibitor of the transport of organic acids from the CSF, and has been demonstrated to increase the brain concentration of KYNA [149]. PROB administered with KYNA was not so effective, probably because i.p. or i.v. injected KYNA does not cross the BBB. A slight decrease in evoked activity was observed with a long (1-1,5 h) delay in these experiments too. This suggests that PROB itself induced an increase in the brain KYNA content, which is probably based only on the endogenous KYNA. These results suggest that G-KYNA passes the BBB much more readily than does KYNA.

The discovery of the importance of the family of kynurenines in the brain function under physiological and pathological conditions has led to the development of powerful new compounds that promise to emerge as some of the first effective treatments for brain neuroprotection and as valuable adjuncts or alternative therapies for other CNS disorders. It is likely that, with continued development, a selection of new agents will soon be suitable for therapeutic intervention in the prevention or treatment of brain damage and neurodegenerative disorders, areas that at present remain almost impossible to treat.

Three potential therapeutic alternatives appear to offer the perspectives for the future (the use of pro-drugs of KYNA, like L-KYN; application of KYNA analogues; and manipulation of the KP by enzyme inhibitors). Nevertheless, it would be especially important to explore the L-KYN metabolism with softer and more sensitive methods in patients with different diseases. A good number of researchers have measured the levels of KYNA and QUIN in the plasma and CSF in certain conditions, but the causes and effects between KYNA and diseases are still not known in detail, and in most cases they are quite unknown. In the future, it would be useful to elicit the relationships between the KP and diseases through the application of sensitive molecular techniques in line with the development of powerful new compounds. In the face of all these challenges, it is clearly necessary to develop and spread the personal therapies of the patients with utilization of the recent research results.

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APPENDIX