Published in final edited form as:

Neuroscience. 2017 December 26; 367: 85–97. doi:10.1016/j.neuroscience.2017.10.006.

Xanthurenic Acid Formation from 3-Hydroxykynurenine in the Mammalian Brain: Neurochemical Characterization and Physiological Effects

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Abstract

Xanthurenic acid (XA), formed from 3-hydroxykynurenine (3-HK) in the kynurenine pathway of tryptophan degradation, may modulate glutamatergic neurotransmission by inhibiting the vesicular glutamate transporter and/or activating Group II metabotropic glutamate receptors. Here we examined the molecular and cellular mechanisms by which 3-HK controls the neosynthesis of XA in rat, mouse and human brain, and compared the physiological actions of 3-HK and XA in the rat brain. In tissue homogenates, XA formation from 3-HK was observed in all three species and traced to a major role of kynurenine aminotransferase II (KAT II). Transamination of 3-HK to XA was also demonstrated using human recombinant KAT II. Neosynthesis of XA was significantly increased in the quinolinate-lesioned rat striatum, indicating a non-neuronal localization of the process. Studies using rat cortical slices revealed that newly produced XA is rapidly released into the extracellular compartment, and that XA biosynthesis can be manipulated experimentally in the same way as the production of kynurenic acid from kynurenine (omission of Na^+ or glucose, depolarizing conditions, or addition of 2-oxoacids). The synthesis of XA from 3-HK was confirmed *in vivo* by striatal microdialysis. In slices from the rat hippocampus, both 3-HK and XA reduced the slopes of dentate gyrus field EPSPs. The effect of 3-HK was reduced in the presence of the KAT inhibitor aminooxyacetic acid. Finally, both 3-HK and XA reduced the power of *gamma*-oscillatory activity recorded from the hippocampal CA3 region. Endogenous XA, newly formed from 3-HK, may therefore play a physiological role in attentional and cognitive processes.

INTRODUCTION

In mammals, the essential amino acid tryptophan is mainly degraded via the kynurenine pathway (KP), leading to the formation of several bioactive metabolites and, eventually, NAD^+ (Fig. 1). Of special interest to brain research, one branch of the catabolic cascade contains two neurotoxic metabolites, 3-hydoxykynurenine (3-HK) and quinolinic acid

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(QUIN). In excess, 3-HK can cause neuronal death by generating reactive free radicals (Eastman et al., 1992, Okuda et al., 1996, 1998), while QUIN is an excitotoxin acting both as an agonist of the N-methyl-D-aspartate (NMDA) receptor (Stone and Perkins, 1981) and as a free radical generator (Schwarcz et al., 1983, Santamaria et al., 2001). A competing arm of the KP produces the neuroinhibitory compound kynurenic acid (KYNA), which targets NMDA and ±7 nicotinic receptors as well as other sites (Moroni et al., 2012, Stone et al., 2013), and has anticonvulsant and neuroprotective properties (Foster et al., 1984). In mammals, dysregulation of the KP, resulting in hyper- or hypofunction of active metabolites, is associated with a plethora of pathological conditions (Stone et al., 2003). Abnormalities in the brain have been plausibly associated with the occurrence of neurodegenerative and other neurological disorders, as well as with major psychiatric diseases (see (Stone and Darlington, 2002, Schwarcz et al., 2012) for reviews).

Xanthurenic acid (XA), a metabolite of the KP formed from 3-HK, is a structural analog of KYNA (Fig. 1). Although its presence in living organisms has been known for many decades (Musajo, 1935, Lepkovsky et al., 1943), the biological actions of XA have received only sparse attention to date. Its role has so far been primarily studied in invertebrates – for example in mosquitoes where XA is present in the head and mid-gut (Bhattacharyya and Kumar, 2001). In the malaria parasite *Plasmodium*, XA can trigger gametogenesis via a mechanism involving cGMP-dependent protein kinase and a rise of intracellular calcium once the parasite enters the host's blood (Billker et al., 1998, Garcia et al., 1998). Notably, however, recent studies have also begun to indicate a physiological role of the metabolite in mammals. Thus, XA, which exhibits antioxidant properties both *in vitro* and *in vivo* (Christen et al., 1990, Murakami et al., 2001, Lima et al., 2012), has vasorelaxing properties (Fazio et al., 2017a), attenuates tetrahydrobiopterine biosynthesis (Haruki et al., 2016) and may regulate glucose homeostasis (Favennec et al., 2016). Notably, XA induces apoptotic cell death in cultured lens epithelial cells (Malina et al., 2002) and has been repeatedly linked to various pathological events, including type 2 diabetes (Oxenkrug, 2015).

Examination of the localization, transport and release of XA in the rodent brain suggests that the metabolite is involved in synaptic signaling pathways (Gobaille et al., 2008), possibly by targeting G-protein-coupled receptors (Taleb et al., 2012). Specifically, XA may function as an endogenous modulator of glutamatergic neurotransmission, causing a net reduction in extracellular glutamate levels (Fukuyama et al., 2014). This effect may be related to the ability of XA to inhibit the vesicular glutamate transporter (Bartlett et al., 1998, Neale et al., 2013) and/or to interact with Group II (mGlu 2 and mGlu 3) metabotropic glutamate receptors (Copeland et al., 2013, Fazio et al., 2015). As Group II receptors may be implicated in the etiology of schizophrenia and are considered targets for novel antipsychotic drug treatments (see (Li et al., 2015), for review), these properties of XA may be of special relevance in the pathophysiology of psychiatric diseases. Of interest in this context, the levels of XA are reduced in both brain and serum of patients with schizophrenia and their first-degree relatives (Fazio et al., 2015).

Using a radiochemical method (intracerebral infusion of ^{3}H-L-kynurenine), XA (i.e. ^{3}H-XA) has been shown to be rapidly produced in the rat brain *in vivo* (Guidetti et al., 1995, Ceresoli et al., 1997). Although not verified experimentally in these studies, XA was assumed to be

formed by the irreversible transamination of its immediate bioprecursor 3-HK by the same enzyme(s) that convert the pivotal KP metabolite L-kynurenine to kynurenic acid (Guidetti et al., 1997) (Fig. 1). These kynurenine aminotransferases (KATs) have been characterized extensively in the mammalian brain (Okuno et al., 1991b, Guidetti et al., 1997, Guidetti et al., 2007a).

The present study was designed to directly examine the neosynthesis of XA from 3-HK in rat, mouse and human brain, using a variety of biochemical approaches *in vitro* and *in vivo*. In complementary electrophysiological experiments, we used tissue slices from the rat hippocampus to compare the effects of 3-HK and XA on synaptic transmission and circuit function.

MATERIALS AND METHODS

Chemicals

XA was purchased from MP Biomedicals, LLC (Illkirch-Graffenstaden, France). L-Kynurenine sulfate (L-kynurenine; purity: 99.4%) was obtained from Sai Advantium (Hyderabad, India). L-[1-^{14}C] glutamic acid (0.1 mCi/mL) was purchased from Sigma Aldrich (St. Louis, MO, USA). The selective KAT II inhibitor (*S*)-(-)-9-(4-aminopiperazin-1-yl)-8-fluoro-3-methyl-6-oxo-2,3-dihydro-6H-1-oxa-3a-azaphenalene-5-carboxylic acid (BFF-122) was kindly provided by Dr. Y. Kajii (Mitsubishi-Tanabe, Yokohama, Japan). DL-3-hydroxykynurenine (DL-3-HK), quinolinic acid (QUIN) and L-±-aminoadipic acid (L-±AA) were purchased from Sigma Aldrich. Other chemicals used were obtained from various commercial suppliers and were of the highest available purity. Recombinant human KAT II was generously provided by Drs. S. Pidugu and E. Toth (Center for Biomolecular Therapeutics, University of Maryland School of Medicine, Rockville, MD, USA).

Animals

Adult male Sprague-Dawley rats (250–300 g; Charles River Laboratories, Kingston, NY, USA) were used for biochemical experiments. Studies in mice (FVB/N strain) were performed using 2–3-months-old male wild-type and KAT II knockout animals (Yu et al., 2004). Animals were housed in a temperature-controlled, AAALAC-approved animal facility on a 12/12h-light/dark cycle with unlimited access to food and water. The experimental protocol was approved by the Institutional Animal Care and Use Committee of the University of Maryland School of Medicine.

Animals were anesthetized with CO_2 and killed by decapitation, and their brains were quickly removed and placed on ice. For regional distribution studies, the olfactory bulb, frontal cortex, striatum, hippocampus, brainstem, and cerebellum were rapidly dissected on ice and processed as described below.

Electrophysiological experiments were conducted on tissue from adult (>6-weeks old) Sprague-Dawley rats (Harlan, Bicester, UK), housed on a 12/12h-light/dark cycle with unlimited access to food and water. Experimental conditions and procedures were in

accordance with the UK Animals (Scientific Procedures) Act 1986 and associated guidelines.

Human brain tissue

Human brain samples (cortex) were provided by the Maryland Brain Collection, a repository of postmortem tissue maintained in cooperation with the office of the Chief Medical Examiner of the State of Maryland and housed at the Maryland Psychiatric Research Center. The brains used in this study were from six donors (age: 62.1 ± 7.8 years, post-mortem interval: 7.6 ± 1.8 h, M/F: 5/1, 4 Caucasians, 2 African-Americans) who had died without a history of neurological or psychiatric disease. The anterior prefrontal cortex (Brodmann area 10) was dissected out, and the tissue was stored at −80°C prior to analysis.

Recombinant human KAT II

The expression plasmid containing human KAT II (hKAT II) as C-terminal intein (chitin binding domain) fusion, in pTXB1 vector, was transformed into BL21 (DE3) cells. Cells were grown in 2YT media at 37°C to an OD_{600} of 0.6, and induced with 300 μM isopropyl ^{2}-D-1-thiogalactopyranoside for 18–20 h at 18°C. The cells were harvested by centrifugation at 2,700 × g for 20 min. The pellets were re-suspended in chitin column buffer, pH 8.5, containing 20 mM Tris, 500 mM NaCl and 1 mM EDTA in 10% glycerol and 5 mM ^{2}-mercaptoethanol. hKAT II was purified by affinity chromatography using a chitin column (New England Biolabs, Ipswich, MA, USA). Intein was cleaved on the column by washing with the chitin column buffer containing 50 mM dithiothreitol. This was followed by ion exchange chromatography using a DEAE column (GE Healthcare Life Sciences, Marlborough, MA, USA). Pure hKAT II was obtained after a final round of purification by size exclusion chromatography, using a S200 column (GE Healthcare Life Sciences). Protein (in 20 mM Tris, 50 mM NaCl and 40 μM pyridoxal-5′-phosphate, pH 8.5) was concentrated to 10.2 mg/ml, flash-frozen in liquid nitrogen and stored at −80°C.

Striatal quinolinic acid lesions in rats

Rats were placed in a stereotaxic frame (David Kopf, Tujunga, CA, USA) while under chloral hydrate anesthesia (360 mg/kg, i.p). One μl of a solution containing 300 nmoles of QUIN, prepared in phosphate-buffered saline (PBS) (pH 7.4), and 1 μl PBS, respectively, were then infused into the two striata (Schwarcz et al., 1983). Seven days later, animals were euthanized, and the striata were rapidly dissected and frozen on dry ice. The success of the lesion was determined by measuring glutamate decarboxylase (GAD) activity (Schwarcz et al., 1983).

Aminotransferase assays

Tissue samples (rat, mouse and human) were weighed while frozen and then homogenized (brain: 1:5, wt/vol; liver: 1:50, wt/vol) by sonication (Branson Ultrasonics Corp., Danbury, CT, USA) in ultrapure water. Based on the study of Okuno et al. (Okuno et al., 1991b), 60 μL of the original tissue homogenate were routinely incubated for 2 h at 37°C in a solution containing 150 mM Tris-acetate buffer, pH 7.4 (i.e. the optimal pH for KAT II but not for KAT I/KAT III; (Guidetti et al., 1997, Pinto et al., 2014), 1 mM pyruvate, 80 μM pyridoxal-

5′-phosphate and 500 μM ascorbic acid in a total volume of 200 μL Ten μM (rat) or 100 μM (human) of either L-kynurenine or DL-3-HK were used for measuring the synthesis of KYNA and XA, respectively. Blanks were obtained by adding 20 μL of the non-specific aminotransferase inhibitor aminooxyacetic acid (AOAA) to the incubation solution (final concentration: 1 mM). KAT inhibitors [final concentrations: 10 mM glutamine (inhibitor of KAT I/KAT III) (Han et al., 2010, Pinto et al., 2014) or 100 μM BFF-122 (KAT II inhibitor; (Amori et al., 2009)] were added to the incubation mixture in 20 μL aliquots where indicated. The reaction was terminated by the addition of 20 μL of 50% (w/v) trichloroacetic acid and 200 μL of 0.1 N HCl, and the precipitated proteins were removed by centrifugation (16,000 × *g*, 10 min).

The same protocol was followed for measuring KAT activity in mouse brain and liver, albeit using 400 μM DL-3-HK as a substrate.

Using recombinant hKAT II, 5 μL of the pure enzyme preparation were incubated with 10 μM DL-3-HK, and KAT activity was measured under routine incubation conditions as described above.

KYNA measurement

After suitable dilutions, 20 μL of the acidified supernatant (see above) were applied to a 3 μm C_{18} reverse-phase column (HR-80; 80 mm × 4.6 mm; ESA, Chelmsford, MA, USA), and KYNA was isocratically eluted using a mobile phase containing 250 mM zinc acetate, 50 mM sodium acetate and 3% acetonitrile (pH 6.2) at a flow rate of 1 mL/min. In the eluate, KYNA was detected fluorimetrically (excitation wavelength: 344 nm; emission wavelength: 398 nm; S200 fluorescence detector; Perkin-Elmer, Waltham, MA, USA). The retention time of KYNA under these conditions was <7 min.

XA measurement

Twenty μL of the same supernatant used for KYNA analysis were applied to a 5 μm C_{18} reverse-phase HPLC column (Adsorbosil; 150 mm × 4.6 mm; Grace, Deerfield, IL, USA), and XA was isocratically eluted using a mobile phase containing citric acid buffer (pH 3.0), 7–8% acetonitrile at a flow rate of 600 μL/min (Okech et al., 2006). In the eluate, XA was detected electrochemically using a HTEC 500 detector (Eicom Corp., San Diego, CA, USA; oxidation potential: +0.85 V). The retention time of XA was ~9 min.

In pilot experiments, we also used UV detection to measure XA. To this end, 20 μl of the supernatant were applied to a 5 pm C_{18} reverse-phase HPLC column (150 mm × 4.6 mm; Adsorbosil; Grace, Deerfield, IL, USA), and XA was isocratically eluted using a mobile phase containing 0.1 mM citric acid buffer (pH 3.0) and 8% acetonitrile at a flow rate of 800 μL/min. In the eluate, XA was detected by UV absorbance at 340 nm (SPD-10A VP series; Shimadzu Corp., Columbia, MD, USA). The retention time of XA was ~8 min.

Studies using rat brain tissue slices

Tissue slices from rat brain (1 mm × 1 mm base) were prepared using a McIlwain chopper (Mickle Laboratory Engineering, Gomshall, UK) and immediately placed in culture wells

(two slices per well; total volume: 100 μL) containing ice-cold Krebs-Ringer buffer (KRB) (118.5 mM NaCl, 4.75 mM KCl, 1.77 mM $CaCl_2$, 1.18 mM $MgSO_4$, 5 mM glucose, 12.9 mM NaH_2PO_4, 3 mM Na_2HPO_4). Before use, KRB was oxygenated for 20–30 min, and the pH was adjusted to 7.4. After pre-incubation (10 min at 37°C), the slices were incubated for 2 h at 37°C in the presence of DL-3-HK (100 μM) on a shaking water bath. Ascorbic acid (final concentration: 500 μM) was added to the wells to minimize the effects of spontaneous oxidation of DL-3-HK (Cornelio et al., 2006).

To terminate the reaction, the wells were placed on ice, and the medium was rapidly separated from the tissue and acidified with 10 μL of 1 M HCl. Twenty μL of the medium were subjected to HPLC analysis, and XA was measured as described above. The tissue slices were homogenized in 100 μL of ultrapure water and frozen at −80°C for protein determination. In all experiments, assays were performed in triplicate.

To test the role of Na^+, choline chloride and Tris-phosphate buffer were substituted for NaCl and NaH_2PO_4. In experiments using 50 mM K^+ buffer, the NaCl concentration was reduced to 73 mM to maintain ionic strength. The effects of AOAA (final concentration: 1 mM), BFF-122 (100 μM), veratridine (50 μM), L-±-AA (1 mM), pyruvate (5 mM), and ±-ketoglutarate (5 mM) were determined by adding 10 μL of each solution to regular KRB immediately prior to the preincubation period.

In vivo microdialysis in the rat striatum

Rats were anesthetized with chloral hydrate (360 mg/kg, i.p.) and mounted in a David Kopf stereotaxic frame (Tujunga, CA, USA). A guide cannula (outer diameter: 0.65 mm) was positioned over the striatum (AP: + 1.1 mm from bregma, L: ± 2.5 mm from the midline, V: 3.0 mm below the dura) and secured to the skull with an anchor screw and acrylic dental cement. A concentric microdialysis probe (membrane length: 2 mm; SciPro, NY, USA) was then inserted through the guide cannula. The probe was connected to a microinfusion pump set to a speed of 1 μL/min and perfused with Ringer solution containing 144 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4$, and 1.7 mM $CaCl_2$ (pH 6.7). Samples were collected every 30 min for a total of 8 h. XA was determined in aliquots of the microdialysate as described above. The reported concentrations are not corrected for recovery from the microdialysis probe.

Protein measurement

Where indicated, protein was determined according to the Lowry method (Lowry et al., 1951), using bovine serum albumin as a standard.

Electrophysiological experiments

Rats were killed by decapitation, and the brains were removed and placed into ice-cold oxygenated sucrose Krebs medium containing (mM): sucrose 202, KCl 2, KH_2PO_4 1.25, $MgSO_4$ 10, $CaCl_2$ 0.5, $NaHCO_3$ 26, ascorbic acid 0.5, glucose 10. The brain was hemisected along the midline, and either 300 μm parasagittal slices (for synaptic studies) or 400 μm horizontal slices (for studies of *gamma* oscillation) were prepared with an oscillating microtome (Integraslice, Campden Instruments, UK). Slices were then transferred to a

recovery chamber kept at room temperature and containing oxygenated Krebs solution (mM): NaCl 124, KCl 2, KH_2PO_4 1.25, $MgSO_4$ 1, $CaCl_2$ 2, $NaHCO_3$ 26, ascorbic acid 0.5, glucose 10. Following at least 1 h of recovery, individual slices were transferred to an interface recording chamber where they were perfused with Krebs solution at either 30–32°C (synaptic studies) or 33–33.5°C (*gamma* oscillation studies). Extracellular field potential recordings were made using Krebs solution-filled glass micropipettes (resistance 3–7 M&).

For synaptic studies, field excitatory postsynaptic potential (fEPSP) responses were evoked (0.1 ms pulses applied every 20 s; 3.2–4.5 V adjusted to approximately 80% of the maximal spike-free response) by a bipolar stimulating electrode positioned in the lower to middle portion of the hippocampal molecular layer. Responses recorded from dentate gyrus (DG) of the hippocampus were digitized (5 kHz) via a CED1401 interface and stored on a computer with Spike2 software (Cambridge Electronic Design, UK).

For *gamma* oscillation studies, extracellular field potential signals were recorded from the lower to middle portion of the hippocampal CA3 region, digitized (10 kHz) via a CED1401 interface, and stored on a computer with CED Spike2 software. *Gamma* oscillatory activity could be elicited by the addition of carbachol (5 μM) to the recording medium. This activity typically stabilized after a period of 30 min, and the carbachol application was subsequently maintained at 5 μM throughout the remainder of the recording. Signal power in the 0.1-100 Hz frequency range was computed using CED Spike 2 (version 7) software, and stable *gamma* oscillations with peak power in the 32–42 Hz region were quantified by computing RMS power using a Fast Fourier Transform (CED Spike2, version 7). Once stable *gamma* oscillatory activity was achieved, test compounds (DL-3-HK or XA) were added to the carbachol-containing perfusate for 15 min periods and then washed out. Effects of the two KP metabolites were expressed as percentage reductions in *gamma* frequency range RMS power compared to control.

Data analysis

The statistical tests used are indicated in the Figure legends. A p value of <0.05 was considered significant in all cases.

RESULTS

XA production from DL-3-HK in rat and mouse forebrain *in vitro*

In preliminary experiments, we examined the production of XA from DL-3-HK in rat cortical tissue homogenate *in vitro* using optimal assay conditions for the study of the formation of KYNA from kynurenine under physiological conditions (Okuno et al., 1991b). In addition, the incubation media always contained 500 μM ascorbic acid (see Materials and Methods). Using 10 μM DL-3-HK as a substrate, *de novo* formation of XA was observed after a 2-h incubation period (Fig. 2A and B), and the identity of newly formed XA was validated further by spiking the samples with a known amount of XA (Fig. 2C).

More detailed characterization of the conversion of DL-3-HK to XA revealed linearity up to 4 h of incubation (data not shown) and over a wide range of substrate concentrations (3 μM

to 1 mM; Fig. 3A), and substantially less neosynthesis of XA when the incubation was performed at 20°C (Fig. 3B). Using standard assay conditions for rat cortical tissue homogenate (10 μM DL-3-HK, 2-h incubation), we also confirmed the production of XA in mouse forebrain tissue homogenate (7.8 ± 0.3 pmoles/h/mg tissue; n = 3).

XA production in rat brain: regional differences

Neosynthesis of XA from DL-3-HK was distributed unevenly in the rat brain (Fig. 3C). The highest enzyme activity was observed in the cortex, followed by the hippocampus, olfactory bulb, striatum, and brainstem. Homogenate from the cerebellum exhibited substantially lower enzyme activity than all other brain areas examined.

XA production in the quinolinate-lesioned rat striatum

The cellular localization of XA neosynthesis was examined in the QUIN-lesioned rat striatum. Confirming the success of the neuronal lesion, GAD activity in the QUIN-treated striatum was reduced by 74% compared to contralateral, PBS-injected controls (26.1 ± 4.7 nmoles/h/mg protein vs. 101.4 ± 6.0 nmoles/h/mg protein; Fig. 3D). Incubation of the same striatal tissue homogenates with 10 μM DL-3-HK revealed significantly greater XA production in the lesioned compared to the contralateral striatum (8.0 ± 0.6 vs. 3.9 ± 0.1 pmoles/h/mg protein).

Role of KAT in XA synthesis in rat and mouse tissue homogenate

The next set of experiments used rat cortical tissue homogenates to evaluate the roles of KAT I/KAT III and KAT II, respectively, in XA synthesis. To this end, we compared the ability of glutamine, which inhibits KAT I and KAT III, and BFF-122, a specific inhibitor of KAT II (Guidetti et al., 2007a, Amori et al., 2009), to prevent the *de novo* formation of XA and KYNA from DL-3-HK and L-kynurenine, respectively, under physiological conditions (i.e. pH 7.4;(Guidetti et al., 1997). As shown in Table 1, a majority of both XA and KYNA production from their respective substrates was found to be catalyzed by KAT II.

As KAT II accounts for ~95% of hepatic KAT (Guidetti et al., 2007a), we next incubated liver tissue homogenates from KAT II knockout and wild-type mice (Yu et al., 2004) with DL-3-HK. XA formation was essentially non-existent in tissue obtained from the mutant animals (Table 1).

De novo synthesis of XA by KAT in human brain tissue

The next experiments were designed to examine and characterize the conversion of XA from 3-HK in human cortical tissue homogenate (Brodmann area 10). As in the studies with rat brain homogenate, the formation of XA and KYNA from DL-3-HK and L-kynurenine, respectively, were compared in parallel using the enzyme inhibitors glutamine and BFF-122. Similar to the results obtained with rat cortex, addition of 100 μM BFF-122 to the incubation medium revealed that KAT II accounted for a large proportion of XA and KYNA production in human brain (~74% and 50% inhibition, respectively). In contrast, 10 mM glutamine decreased the production of XA and KYNA only by ~22% and ~18%, respectively (Table 1).

Finally, we used recombinant human KAT II to directly examine its ability to convert DL-3-HK to XA. The pure enzyme catalyzed the reaction very effectively, and no neosynthesis of XA was observed when the KAT II inhibitor BFF-122 was added to the incubation medium (Table 1).

XA production in rat cortical slices: effects of KAT inhibitors, ionic milieu and 2-oxoacids

We next investigated the release of XA from rat cortical tissue slices after a 2-h exposure to 100 μM DL-3-HK. After pilot studies confirmed that newly produced XA can be liberated into the incubation medium (data not shown), we tested the effects of various experimental manipulations in this paradigm. The presence of AOAA (1 mM final concentration) in the incubation blocked the appearance of XA in the extracellular medium almost completely (Fig. 4A). Addition of BFF-122 (100 μM final) decreased XA formation by 78%, and L-±AA (1 mM final), a competitive substrate of KAT II (Tobes and Mason, 1977), reduced the extracellular presence of XA by 67% (Fig. 4A). Taken together, these data support the results from our studies in cell-free preparations, indicating that KAT II is mainly responsible for the synthesis of rapidly mobilizable XA in the rat brain.

In the next series of experiments, we varied the composition of the incubation medium in order to investigate possible influences of physiologically relevant modifications on the synthesis and release of XA in cortical slices. Depolarizing conditions, induced either by raising the K^+ concentration to 50 mM or by adding veratridine (final concentration: 50 μM) to the incubation medium, inhibited the formation of XA by 43% and 72%, respectively (Fig. 4B). Elimination of glucose from the incubation medium also decreased the *de novo* production of XA from DL-3-HK by 40% (Fig. 4B). To elucidate the role of Na^+, we either removed Na^+ from the Krebs-Ringer buffer or added tetrodotoxin (final concentration: 1 and 2 μM), a blocker of voltage-gated Na^+ channels, to the incubation medium. While neither concentrations of tetrodotoxin had an effect (data not shown; n = 3 per dose), deletion of Na^+ *increased* XA production by 61% (Fig. 4B).

The roles of pyruvate and ±-ketoglutarate (±-KG), two effective co-substrates of KATs (Guidetti et al., 1997), were examined using a final concentration of 5 mM. Both oxo-acids augmented XA neosynthesis compared to controls (by 205% and 109%, respectively; Fig. 4C).

Effect of DL-3-HK on extracellular XA in the rat striatum *in vivo*

Microdialysis was performed to examine the conversion of DL-3-HK to XA in the rat striatum *in vivo*. After collecting four 30-min fractions for baseline measurements, DL-3-HK (10 μM) was applied for 2 h by reverse dialysis, and microdialysis then continued for an additional 4 h. Basal extracellular XA levels were barely detectable with our methodology (<4 nM), but XA could be reliably measured after its synthesis was stimulated. Thus, XA levels increased rapidly following the introduction of DL-3-HK, reached a maximal concentration of approximately 350 nM, and dropped precipitously soon after the removal of the bioprecursor (Fig. 5).

Effect of DL-3-HK and XA on synaptic transmission in the hippocampal dentate gyrus

Stimulation in the hippocampal molecular layer resulted in field EPSPs (fEPSPs) that could be recorded in the dentate gyrus (DG). Addition of 0.3 mM DL-3-HK to the perfusion medium had little effect on the fEPSP slope, whereas higher concentrations caused significant reductions in fEPSP slopes at 1 mM (14.1 ± 2.0 % reduction, n = 7) and 3 mM (41.2 ± 4.2 % reduction, n = 7) (Fig. 6). Similarly, XA also reduced the slope of the fEPSP (3 mM: 8.4 ± 1.4 % reduction, n = 5). These results are qualitatively similar to those previously obtained with XA in this experimental preparation in the mouse (Neale et al., 2013). As the inhibitor AOAA blocks the formation of XA from DL-3-HK (see above), we next investigated whether AOAA could prevent the effects of DL-3-HK on synaptic transmission. Pre-application of 1 mM AOAA to the slices indeed resulted in a lesser reduction (15.1 ± 3.7 % reduction, n = 5) of the fEPSPs caused by DL-3-HK (3 mM) (Fig. 6).

Effect of DL-3-HK and XA on *gamma* oscillations in the CA3 region of the hippocampus

Incubation of hippocampal slices in 5 μM carbachol for 45 min or longer resulted in stable expression of 30–45 Hz oscillations in the hippocampal CA3 area, similar to the *gamma* oscillations described previously (Colgin et al., 2009, Maier et al., 2013, Chan et al., 2016) (Fig. 7). Addition of DL-3-HK (1 mM) to the bathing medium caused a reduction in the peak power in the *gamma* region by 56 ± 8.9% (n = 7). Similarly, addition of XA (either 1 mM or 3 mM) caused reductions in the *gamma* peak power by 29 ± 3.8% (n = 12) and 91 ± 2.6% (n = 6), respectively, whereas 0.3 mM XA had no significant effect (Figs. 7 and 8).

DISCUSSION

The present study was designed to characterize in detail the mechanisms that control XA production from 3-HK in rat, mouse and human brain. In light of the close similarity of the two biosynthetic processes, most experiments were guided by previous studies which had investigated the cerebral *de novo* synthesis of KYNA from L-kynurenine (Okuno et al., 1991a, Wu et al., 1992, Gramsbergen et al., 1997, Guidetti et al., 2007a). In addition, we conducted electrophysiological studies, describing and comparing the effects of DL-3-HK and XA in hippocampal tissue slices *in vitro*. Jointly, our results showed that XA is produced from 3-HK in the mammalian brain in close analogy to the neosynthesis of KYNA from kynurenine, that 3-HK affects synaptic function and oscillatory activity in the hippocampus, and that at least some of these physiological effects could be attributable to the formation of XA.

In vitro experiments using tissue homogenates from the rat cerebral cortex revealed the expected time- and temperature-dependency of XA neosynthesis from 3-HK. Tissue incubation with up to 1 mM DL-3-HK resulted in proportional elevations in newly produced XA, indicating that the process was not saturated even at high concentrations of the precursor. Although limits in the solubility of DL-3-HK did not permit full kinetic assessment of XA formation, these results indicated that XA production in the rat brain was likely catalyzed by KATs, which have K_m values in the low millimolar range (Han et al., 2004, Guidetti et al., 2007a, Han et al., 2008). This conclusion, which is in agreement with

common assumptions (Guidetti et al., 1997) (cf. Fig. 1), was further supported by the fact that regional differences in XA production in the rat brain showed a similar pattern as previously reported for KAT-catalyzed KYNA synthesis from kynurenine (Okuno et al., 1991b). Taken together, these results imply that the synthesis of KYNA and XA in the brain will depend on the relative availability of the two KAT substrates, kynurenine and 3-HK, respectively. Notably, XA formation from DL-3-HK could also be readily demonstrated in tissue homogenates from mouse and human brain under the same experimental conditions.

The cellular localization of XA production was examined using tissue homogenate obtained from the rat striatum seven days after a focal injection of QUIN, which causes the degeneration of intrinsic neurons and pronounced reactive astrogliosis (Björklund et al., 1986). XA neosynthesis from DL-3-HK was substantially increased in lesioned compared to normal control tissue, paralleling the enhanced production of KYNA from kynurenine in the same paradigm (Guidetti et al., 1997) and suggesting that the transamination of 3-HK takes place mainly in non-neuronal cells. This question needs to be assessed further in healthy tissue, however, and species differences may exist. Thus, a recent immunocytochemical study with antibodies directed against XA itself described an exclusive neuronal localization of the metabolite in the *mouse* brain (Roussel et al., 2016).

Using three complementary experimental approaches, we sought to identify the KAT enzymes that were responsible for the *de novo* synthesis of XA. As KAT II (= L-±AA aminotransferase) is the predominant enzyme catalyzing the rapid transamination of L-kynurenine to KYNA in the adult rat and human brain under physiological conditions (Guidetti et al., 2007a), we first examined the effect of BFF-122, a selective inhibitor of KAT II (Amori et al., 2009). Incubation of DL-3-HK in the presence of this compound dramatically reduced the production of XA, whereas addition of glutamine, which inhibits KAT I (= glutamine aminotransferase) (Cooper, 2004) caused only a modest decrease in XA formation. The efficacy of KAT II was further substantiated using a partially purified preparation of the rat enzyme (data not shown) as well as human recombinant KAT II protein. Finally, as KAT II normally accounts for ~95% of hepatic KAT (Guidetti et al., 2007a), the ability of KAT II to form XA from DL-3-HK was tested using liver tissue homogenate from KAT II knockout mice (Yu et al., 2004). Genetic elimination of KAT II essentially abolished the neosynthesis of XA.

Studies using freshly dissected tissue slices from rat cerebral cortex confirmed that the majority of XA formation from 3-HK is catalyzed by KAT II. Thus, incubation in the presence of BFF-122 decreased the extracellular presence of newly produced XA by 78%. In analogy to experiments designed to investigate KYNA production from kynurenine in brain tissue slices (Gramsbergen et al., 1997), a quantitatively similar reduction of XA neosynthesis was also observed when the competitive KAT II substrate L-±AA was added to the incubation medium. Notably, as KAT II in the rat brain is almost exclusively contained in astrocytes (Guidetti et al., 2007b), these studies, taken together, support the notion that glial cells, rather than neurons, are responsible for the majority of XA neosynthesis (cf. above).

Studies with tissue slices also demonstrated that the mechanisms controlling the cerebral formation of XA from 3-HK are identical to those regulating the conversion of kynurenine to

KYNA in the brain (Gramsbergen et al., 1997). Thus, the levels of newly produced XA were substantially lower when glucose was removed from the incubation mixture or when the tissue was exposed to depolarizing concentrations of K^+ or veratridine. In contrast, pyruvate and ±-KG, two 2-oxoacids which can function as co-substrates of the enzymatic transamination reaction, or removal of Na^+ from the incubation buffer, caused significant increases in *de novo* XA synthesis. As discussed previously in the context of the regulation of KYNA neosynthesis, these phenomena indicate that XA production in the brain is controlled by fluctuations in cerebral energy metabolism and likely involves neuron-glia interactions (Gramsbergen et al., 1997). Of importance in this context, the present electrophysiological experiments using hippocampal slices revealed that pharmacological inhibition of the transamination process greatly reduces the effect of 3-HK on synaptic transmission, demonstrating the functional significance of down-regulated XA production.

In line with our report revealing the cerebral synthesis of ^{3}H-XA from ^{3}H-kynurenine in awake rats (Guidetti et al., 1995), our study also showed that DL-3-HK is promptly converted to XA in the brain *in vivo*. In these experiments, DL-3-HK was applied by reverse dialysis to the rat striatum, and newly synthesized XA was recovered from the perfusate, providing further evidence that exogenously supplied 3-HK enters cells for transamination to XA, which is in turn rapidly liberated into the extracellular compartment. This newly produced XA may, in fact, be released very close to its site of action, providing a possible explanation for our observation that 3-HK was more potent than exogenously applied XA in our electrophysiological experiments. Probably using the same two transporters – one Na^+-dependent and one Na^+-independent – as kynurenine (Speciale and Schwarcz, 1990), brain cells then actively accumulate 3-HK (Eastman et al., 1992), though the mechanism responsible for the release of newly formed XA has not been elaborated so far. Interestingly, and again in analogy to kynurenic acid (Uwai et al., 2012), XA can be removed from the extracellular space by the organic anion transporters OAT1 and OAT3 (Uwai and Honjo, 2013).

The present results, as well as a series of studies demonstrating the ability of XA to influence glutamatergic neurotransmission by inhibiting vesicular glutamate transport (Neale et al., 2013, Neale et al., 2014) and possibly by modulating Group II metabotropic glutamate receptors (Copeland et al., 2013, Fazio et al., 2015, Fazio et al., 2017b), provide support for the idea that endogenous XA, synthesized from 3-HK, plays a role in brain physiology. In addition to the reduction in 3-HK- or XA-induced synaptic transmission and *gamma* oscillations in the hippocampus shown here, previous studies in rodents had demonstrated that local perfusion of XA reduces glutamate release in the substantia nigra (Fukuyama et al., 2014), affects sensory transmission in the thalamus (Copeland et al., 2013), and decreases the amplitude of field excitatory postsynaptic potentials in the hippocampus and cerebral cortex (Neale et al., 2014). Notably, these molecular targets and functional effects of XA, which may also involve specific G-protein-coupled receptors and related increases in intracellular Ca^{2+} concentrations (Taleb et al., 2012), also suggest a possible role of XA in the pathophysiology of schizophrenia – especially with regard to the impairment of the attentional and cognitive processes seen in the disease (Green et al., 2004, Eastwood and Harrison, 2005, Oni-Orisan et al., 2008, Uhlhaas and Singer, 2010, Sodhi et al., 2011) – XA levels are indeed abnormal in both brain and serum of patients with schizophrenia and their

first-degree relatives (Fazio et al., 2015). More generally, dysfunctional KP metabolism has been consistently observed in persons with schizophrenia, and pharmacological interventions targeting specific KP enzymes have been proposed to provide therapeutic benefits to patients (see (de Bie et al., 2016) and (Plitman et al., 2017) for recent reviews).

Several important issues concerning XA neurobiology remain to be addressed, importantly including elucidation of the mechanisms which control the cross-talk between peripheral and central 3-HK and XA under physiological and pathological conditions (Fukui et al., 1991, Gobaille et al., 2008). Moreover, in order to better conceptualize the possible participation of XA in brain function and dysfunction, it will not only be necessary to clarify the cellular localization of cerebral XA biosynthesis from 3-HK (see above) but also to unequivocally identify the cells which form 3-HK from kynurenine in the brain (Sathyasaikumar et al., 2016) (Fig. 1). These experiments can be expected to clarify and strengthen arguments for the proposed role of endogenous XA as a functionally significant KP metabolite in the mammalian brain.

Acknowledgments

This work was supported by USPHS grant P50 MH103222 (to R.S.)

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HIGHLIGHTS

- The neosynthesis of xanthurenic acid (XA) from 3-hydroxykynurenine (3-HK) was examined in mice, rats and humans,
- In all 3 species, XA formation from 3-HK was traced to a major role of kynurenine aminotransferase II (KAT II).
- In slices from rat hippocampus, both 3-HK and XA reduced the slopes of dentate gyrus field EPSPs.
- Endogenous XA, newly formed from 3-HK, may play a physiological role in attentional and cognitive processes.

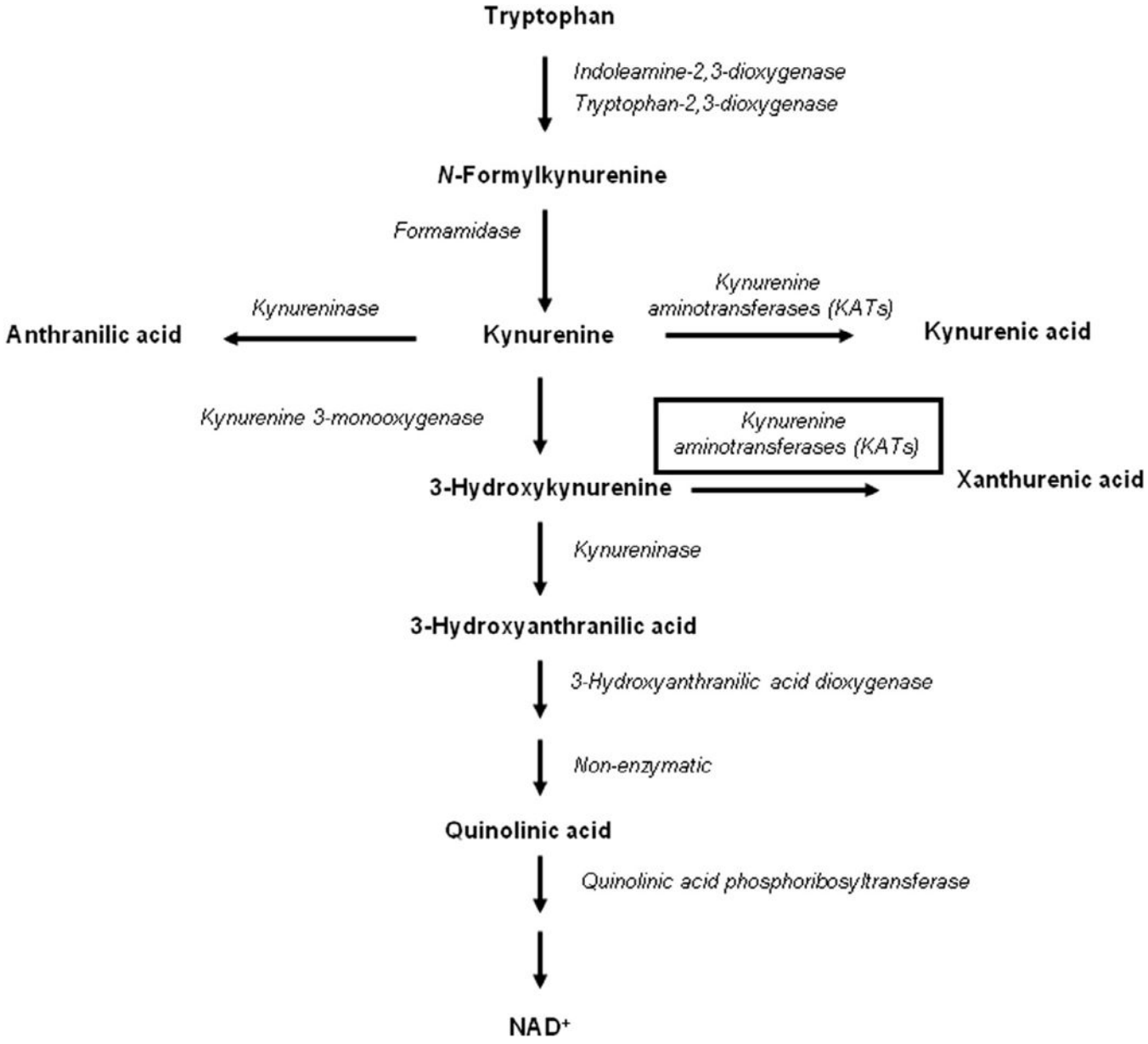

Figure 1.
Schematic diagram of the KP pathway illustrating the enzymatic conversion of 3-HK to XA (box).

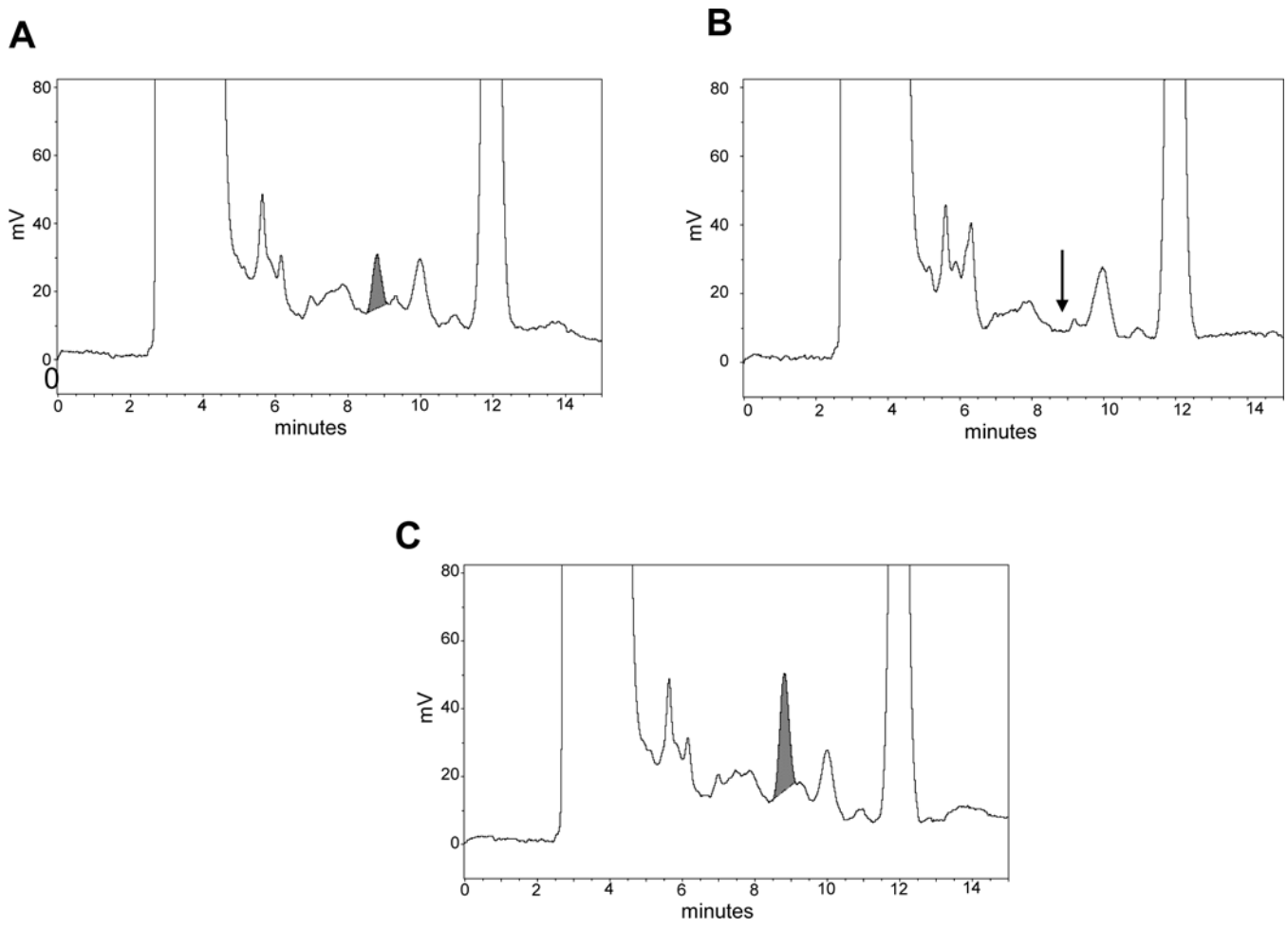

Figure 2.

Chromatographic profiles demonstrating XA production from DL-3-HK. (**A**) XA in rat cortical tissue homogenate incubated in the presence of 10 μM DL-3-HK for 2 h at 37°C; (**B**) Blank obtained using 1 mM of AOAA in the incubation medium; the arrow points to the retention time of XA; (**C**) XA peak following spiking of the supernatant of (**A**) with a known amount of XA (1.25 pmoles) (see text for experimental details).

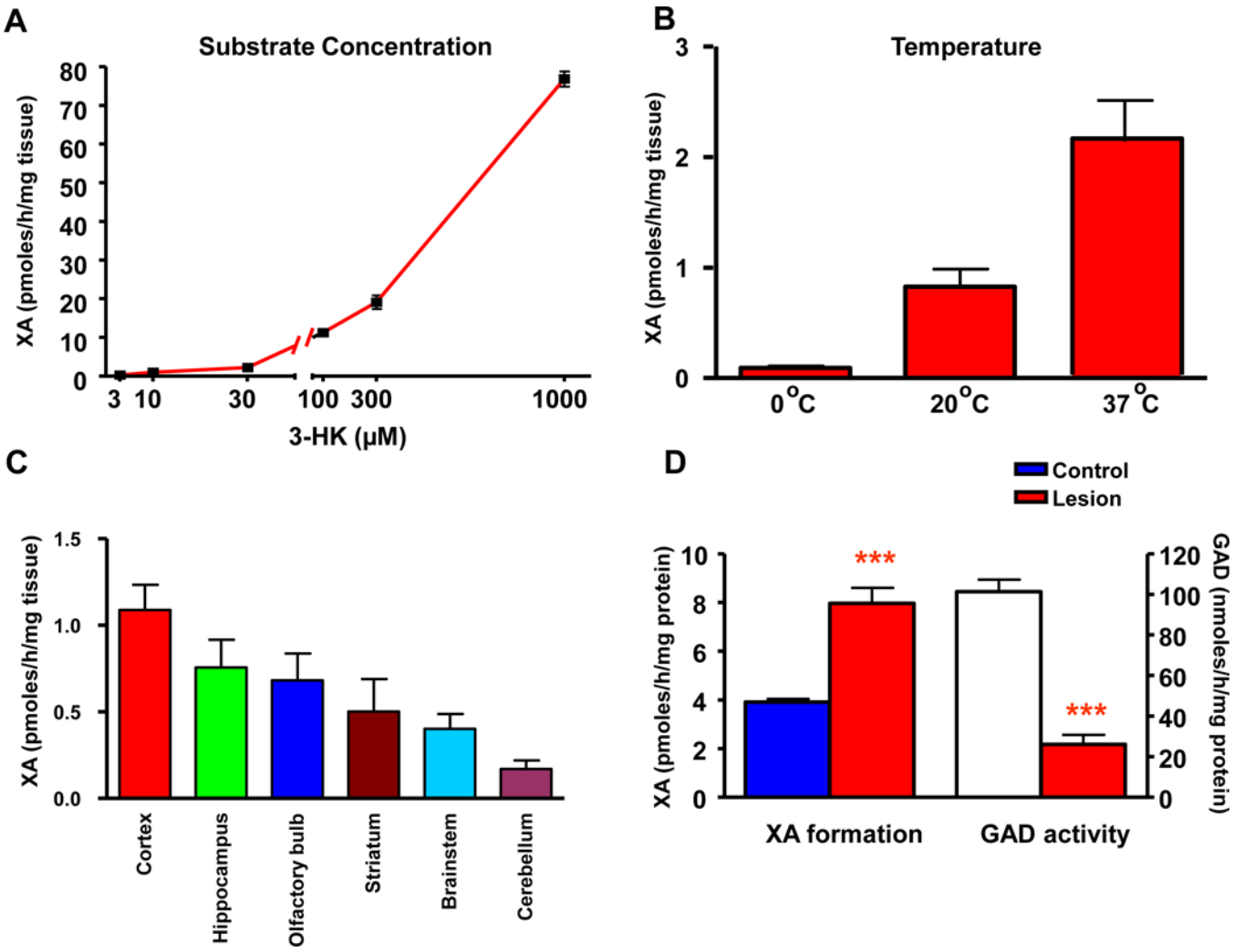

Figure 3.

(A) Concentration-dependency of XA production from DL-3-HK in rat cortical tissue homogenate under standard incubation conditions (see Methods for experimental details); (**B**) Temperature dependency of XA production from DL-3-HK in rat cortical tissue homogenate. Data are the mean ± SEM of 3–5 rats; **(C)** Regional distribution of XA production in rat brain tissue homogenate. Data are the mean ± SEM of 5–7 rats; **(D)** Effect of neuronal depletion on the production of XA from DL-3-HK in the rat striatum. Animals received injections of PBS (1 μl) or QUIN (300 nmoles/1 μl and were euthanized 7 days later. XA production and glutamate decarboxylase (GAD) activity were determined in the same tissue homogenates. Data are the mean ± SEM (6 rats per group). ***p< 0.001 vs. control (Student's t-test).

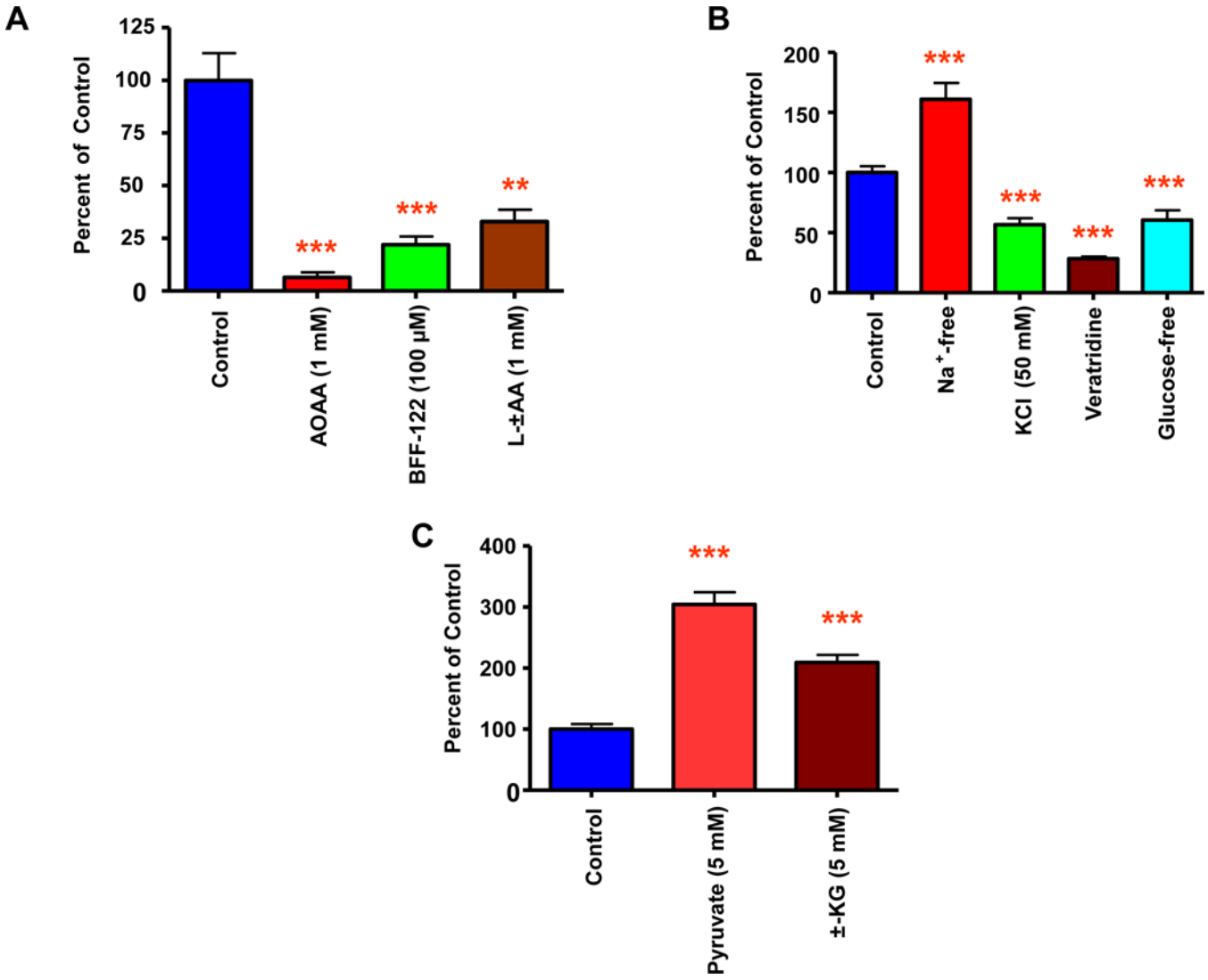

Figure 4.

(A) Effect of AOAA (1 mM), BFF-122 (100 μM) and L-±AA (1 mM) on XA production and liberation from rat cortical slices exposed to 100 μM DL-3-HK at 37°C for 2 h. Data (mean ± SEM) are expressed as a percentage of control (9.3 ± 1.2 pmoles XA/h/mg protein) of five separate experiments; **(B)** Effect of Na^+, K^+, veratridine, and glucose deprivation on XA production and liberation from cortical slices exposed to 100 μM DL-3-HK at 37°C for 2 h. Data (mean ± SEM) are presented as a percentage of standard KRB controls (Na^+, glucose: 8.2 ± 0.6; K^+, veratridine: 7.0 ± 1.0 pmoles XA/h/mg protein) of 5–10 separate experiments; **(C)** Effect of co-substrates pyruvate (5 mM) and ±-KG (5 mM) on XA production and liberation from cortical slices exposed to 100 μM DL-3-HK at 37°C for 2 h. Data (mean ± SEM) are expressed as a percentage of control (4.6 ± 0.4 pmoles XA/h/mg protein) of 5 separate experiments. **p< 0.01, ***p< 0.001 vs. control (Student's t-test).

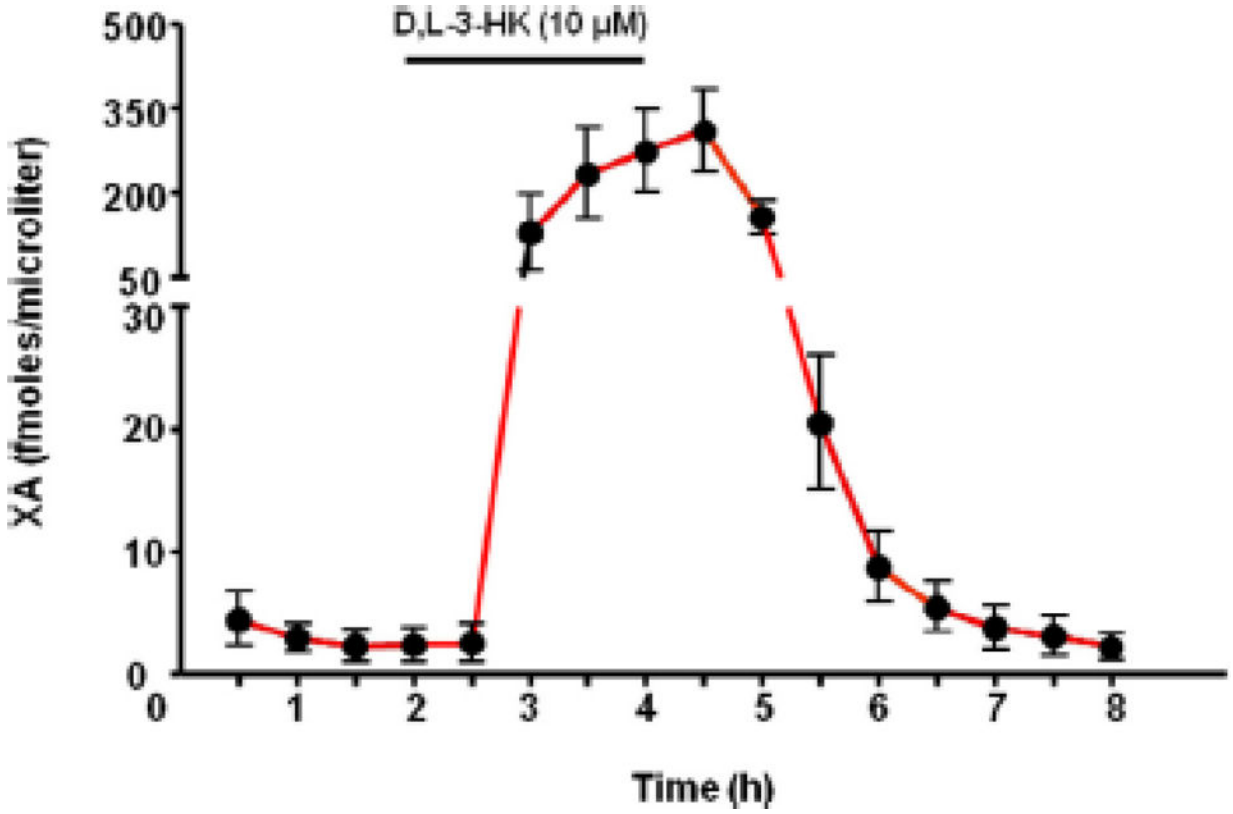

Figure 5.

Effect of reverse dialysis of DL-3-HK (10 µM) (bar) on extracellular XA levels in the rat striatum. Data were obtained from 4 rats and are the mean ± SEM.

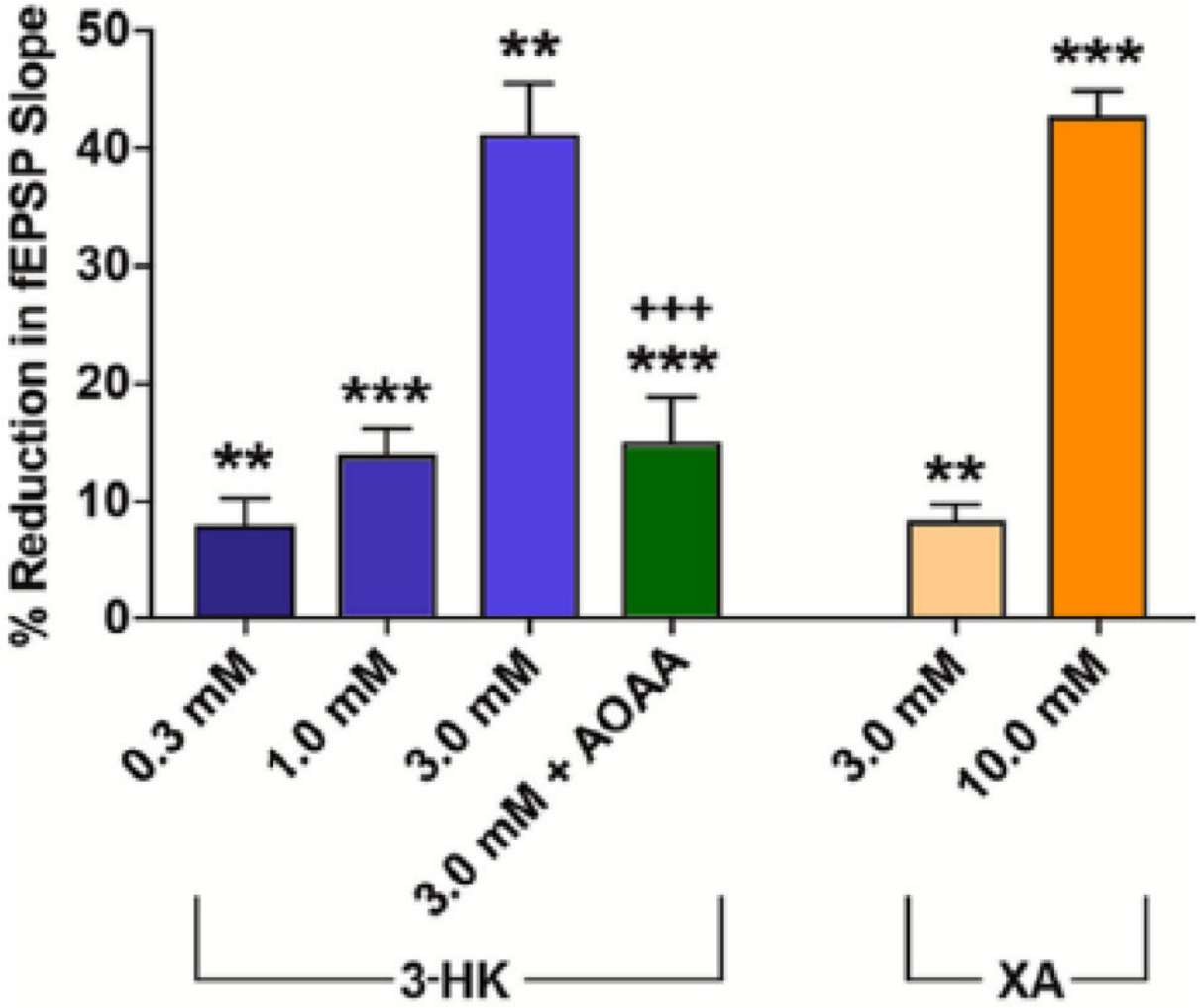

Figure 6.

Effect of DL-3-HK and XA in hippocampal dentate gyrus. Bars show mean percentage reductions (± SEM) from control values of fEPSP slope following bath administration of 3-HK or XA. Note that the effect of 3-HK was substantially attenuated in the presence of AOAA. **p< 0.01, ***p< 0.001 vs. control, $^{+++}$p<0.001 between DL-3-HK and DL-3-HK + AOAA (Tukey's multiple comparison test).

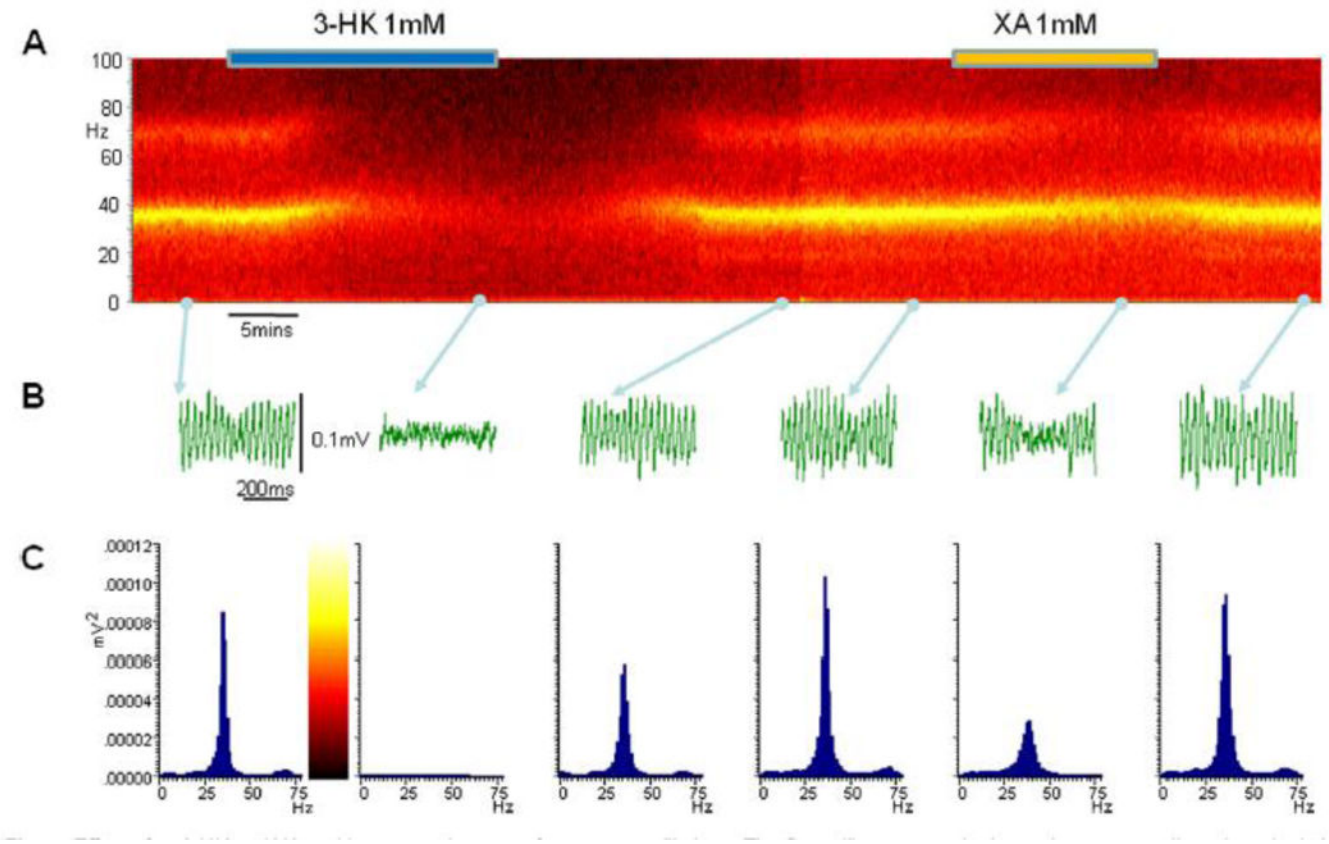

Figure 7.

Effect of DL-3-HK and XA on hippocampal *gamma*-frequency oscillations. The figure illustrates a single continuous recording where both 3-HK and XA were separately applied at different time points. **A:** continuous sonogram of signal power up to 100 Hz, showing the application of either DL-3-HK or XA (bars above the sonogram). The highest power in the sonogram is represented in yellow, the lowest in black (sonogram calibration is seen in **C**); **B:** Voltage recordings taken at different time points (indicated by the arrows); **C:** Power spectra computed from data recorded for 1 min at different times during the recording (indicated by the arrows in A/B). Note that both 3-HK and XA evoked reductions in power are in the gamma-frequency range.

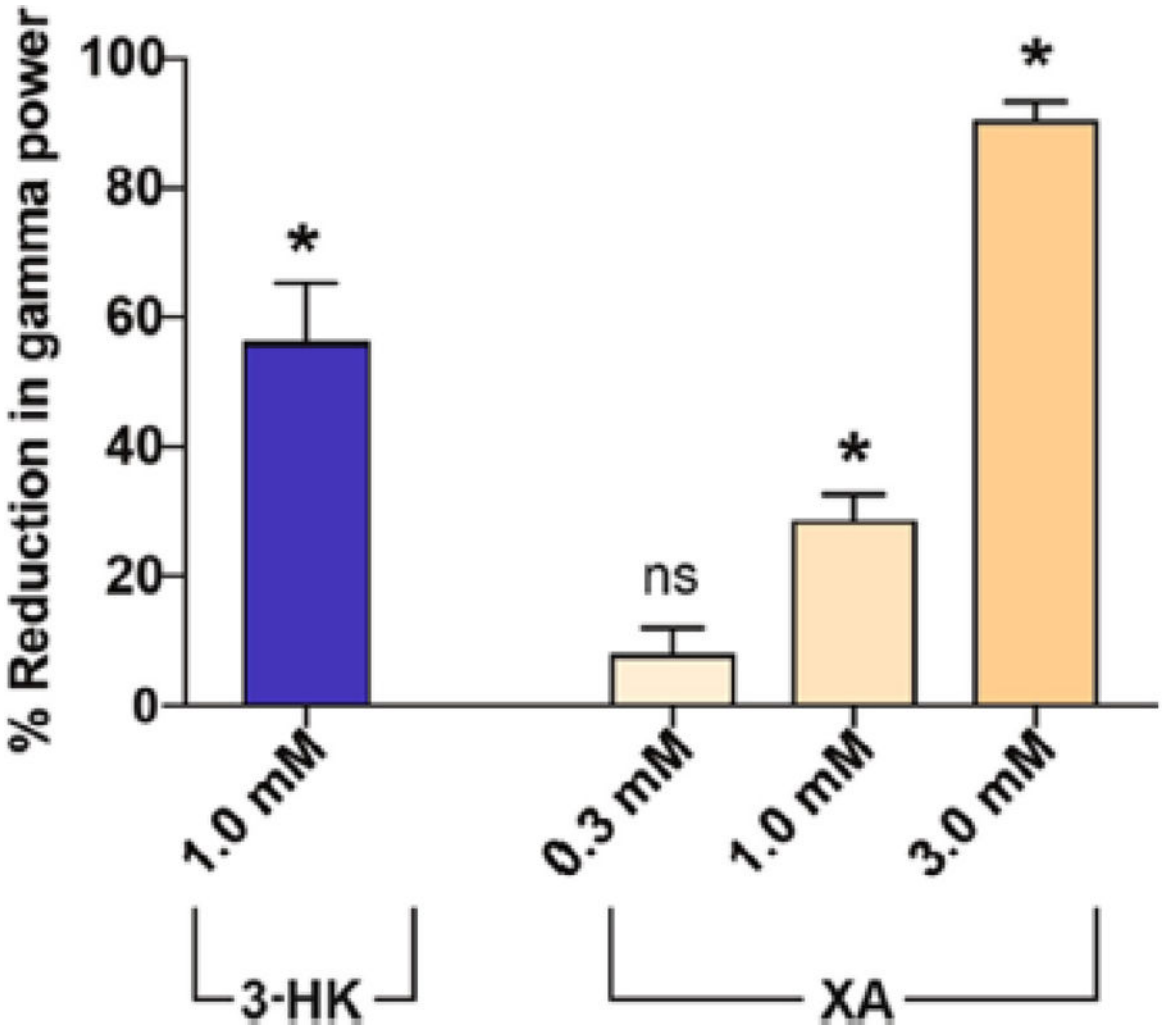

Figure 8.

Effect of DL-3-HK and XA on hippocampal *gamma*-frequency power. Bars show mean percentage reductions (± SEM) from control values of power following bath administration of either DL-3-HK or XA. *p< 0.05, vs. control (Tukey's multiple comparison test).

Table 1

Role of KAT II in the *de novo* formation of XA from 3-HK in rats, mice and humans

Pharmalogical Inhibition	Inhibitors	XA (% Inhibition)	KYNA (% Inhibition)
Rat Brain	Glutamine (10 mM)	25.2 ± 2.0	25.3 ± 1.2
	BFF-122 (100 μM)	84.5 ± 1.8	82.8 ± 0.8
Human Brain	Glutamine (10 mM)	21.9 ± 1.7	17.6 ± 1.9
	BFF-122 (100 μM)	74.2 ± 3.8	50.0 ± 5.2
Human Recombinant KAT II Protein	BFF-122 (100 μM)	94.1 ± 3.0	not done
Genetic Inhibition		**XA (% Elimination)**	
KAT II Knockout Mouse Liver		99.0 ± 0.1	not done

See text for experimental details. Data are the mean ± SEM of 3–6 experiments.

Absolute control values: Rat brain: XA: 1.1 ± 0.05, KYNA: 3.4 ± 0.2 pmoles/h/mg tissue; Human brain: XA: 0.6 ± 0.09, KYNA: 2.0 ± 0.3 pmoles/h/mg tissue; Human recombinant KAT II protein: XA: 86.8 ± 8.7 nmoles/h/mg protein; KAT II mouse liver: XA: 2.6 nmoles/h/mg tissue.