

Investigating KYNA production and kynurenergic manipulation on acute mouse brain slice preparations

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Abstract

Manipulation of kynurenic acid (KYNA) level through kynurenine aminotransferase-2 (KAT-2) inhibition with the aim of therapy in neuro-psychiatric diseases has been the subject of extensive recent research. Although mouse models are of particular importance, neither the basic mechanism of KYNA production and release nor the relevance of KAT-2 in the mouse brain has yet been clarified.

Using acute mouse brain slice preparations, we investigated the basal and L-kynurenine (L-KYN) induced KYNA production and distribution between the extracellular and intracellular compartments. Furthermore, we evaluated the effect of specific KAT-2 inhibition with the irreversible inhibitor PF-04859989. To ascertain that the observed KYNA release is not a simple consequence of general cell degradation, we examined the structural and functional integrity of the brain tissue with biochemical, histological and electrophysiological tools.

We did not find relevant change in the viability of the brain tissue after several hours incubation time. HPLC measurements proved that mouse brain slices intensively produce and liberate KYNA to the extracellular compartment, while only a small proportion retained in the tissue both in the basal and L-KYN supplemented state. Finally, specific KAT-2 inhibition significantly reduced the extracellular KYNA content.

Taken together, these results provide important data about KYNA production and release, and *in vitro* evidence for the first time of the function of KAT-2 in the adult mouse brain. Our study extends investigations of KAT-2 manipulation to mice in a bid to fully understand the function; the final, future aim is to assign therapeutical kynurenergic manipulation strategies to humans.

Keywords: kynurenic acid, kynurenine aminotransferase-2 inhibition; acute slice viability; HPLC; immunohistochemistry; *in vitro* electrophysiology

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20 HPLC; immunohistochemistry; in vitro electrophysiology

21 **1 Introduction**

22 Tryptophan (TRP) is catabolized mostly through the kynurenine pathway (KP) in the
23 mammalian brain (Gal and Sherman 1980). Kynurenic acid (KYNA) is one of the neuroactive
24 products of this metabolic route. KYNA is synthesized with the irreversible transamination of
25 L-kynurenine (L-KYN), a reaction catalyzed by kynurenine aminotransferases (KATs) (Han
26 et al., 2010a). KYNA exerts multiple effects on ligand-gated ion channels (Gal and Sherman,
27 1980, Birch et al., 1988, Prescott et al., 2006, Albuquerque and Schwarcz, 2013) and on the
28 G-protein-coupled receptor 35 in the brain (Berlinguer-Palmini et al., 2013, Alkondon et al.,
29 2015). Through these actions, KYNA can modulate neurotransmission systems (Zmarowski et
30 al., 2009, Alexander et al., 2012, Banerjee et al., 2012a, Olsson et al., 2012). Indeed, the role
31 of KYNA in neurophysiological and neuropathological processes has been the subject of
32 extensive contemporary studies. The causal role of diversion of the KP has been proposed in
33 several neurodegenerative and neuropsychiatric disorders (e.g. Alzheimer's disease,
34 Parkinson's disease, Huntington's disease, cerebral ischemia, depression, and schizophrenia)
35 (Vecsei et al., 2013). Thus, kynurenergic manipulation with the aim of therapy has also been
36 proposed (Amaral et al., 2013).

37 To date, cross-species differences of the relevance and regulation of brain KP function and
38 KYNA production are not fully clarified. The majority of the experimental data concerns the
39 rat kynurenine system, however, mouse models are of particular importance as well
40 (Rosenthal and Brown, 2007).

41 Yet, there is neither detailed study about the mechanism and regulation of KYNA release
42 from cells in the mouse brain parenchyma, nor information about the function of relevant
43 KAT enzymes of the KP. For instance, the importance of kynurenine aminotransferase-2
44 (KAT-2) in the rat and human brain is unequivocal, the relevance of KAT-2 in the mouse
45 brain is, however, controversial (Han et al., 2010a).

46 For describing the kynurenine system in model animals and investigating the effect of
47 kynurenergic manipulation, *in vitro* models are essential and complement *in vivo* studies. To
48 this end, acute brain slice preparations provide many advantages over *in vivo* experiments
49 (Cho et al., 2007, Lein et al., 2011). Previously, KYNA production and release to the
50 extracellular compartment upon L-KYN exposure was described in cortical (Turski et al.,
51 1989, Hodgkins et al., 1999) and in hippocampal rat brain slices (Scharfman et al., 1999,
52 Alkondon et al., 2011). However, there is no data about KYNA production of acute mouse
53 brain slice preparations. Furthermore, these studies did not concern the acute slice viability, a
54 factor, which can strongly influence the KYNA production and release.

55 The present study was designed to estimate the KYNA production and release of acute mouse
56 brain slices and to assess the effect of the specific KAT-2 inhibitor PF-04859989 on KYNA
57 production *in vitro*.

58 Basing on literature data we presumed that the concentration of KYNA released into the
59 extracellular space falls into the picomolar or low nanomolar range (Schwieler et al., 2006).

60 To ensure a measurable concentration of KYNA for HPLC assessments we incubated our
61 slices in a low bulk volume of artificial cerebrospinal fluid (aCSF) (that is approx. 6 ml of
62 aCSF/100mg wet weight brain tissue) under continuous carbogenation, but without perfusion.

63 In previous studies KYNA increase and release could be measured 2-4hours after L-KYN
64 administration in rat (Turski et al., 1989, Swartz et al., 1990). Results of our pilot experiments
65 are in line with that finding (unpublished data). Therefore, we decided to incubate brain slices
66 for 4-6 hours. Incubation of acute slices inherently initiates progressive damage in the tissue
67 (Fukuda et al., 1995). It is therefore possible that metabolic properties and integrity of cells
68 involved in KYNA production and release might alter during several hours of incubation. In
69 order to characterize the after-incubation viability of the brain tissue we performed
70 histological observations, biochemical and electrophysiological characterization of the slices.

71 To investigate the extra/intracellular distribution of basal KYNA, we compared the KYNA
72 content in the tissue with that liberated into the extracellular space. Then, we wanted to know
73 if we could induce *de novo* KYNA production with the administration of the KYNA precursor
74 L-KYN. Finally, we investigated the effect of the KAT-2 inhibitor on the L-KYN-induced
75 KYNA release.

76 Investigating tissue viability, we found a slight alteration in some of the observed tissue
77 viability measures. However, the slices showed no anatomical or functional abnormalities, so
78 tissue integrity was preserved.

79 Regarding KYNA production, we found that in the course of 4hours incubation slices readily
80 release both basal and *de novo* produced KYNA into the supernatant aCSF, whereas only a
81 negligible fraction of total KYNA is retained in the tissue. The KAT-2 inhibitor, however,
82 conspicuously decreased KYNA release.

83

84 **2 Experimental Procedures**

85 **2.1 Animals**

86 8-12 weeks old male *C57Bl/6* mice obtained from The National Institute of Oncology
87 (Budapest, Hungary) were used (n=30) for the experiments. Animals were kept under
88 controlled laboratory conditions and had free access to food and water. All experiments were
89 in compliance with the guidelines of the European Communities Council Directives
90 (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv.
91 32.§).

92 **2.2 Acute slice preparation**

93 Animals were anesthetized with isoflurane (3%) followed by decapitation (NEMI Guillotine;
94 Braintree Scientific, Inc.). The brain was rapidly removed and submerged in ice-cold aCSF
95 (pH 7.4) composed of (in mM): 234 sucrose, 3.5 KCl, 1 NaH₂PO₄, 24 NaHCO₃, 1 CaCl₂, 3
96 MgSO₄ and 10 D-glucose (Sigma Aldrich) and oxygenated with 95% O₂ and 5% CO₂. 350
97 µm thick sections were obtained with a vibratome (Leica VT1200S, Germany); the two
98 hemispheres were dissected and transferred to a holding chamber. Slices were allowed to
99 recover for 30 min in aCSF (pH 7.4) containing (in mM): 130 NaCl, 3.5 KCl, 1 NaH₂PO₄, 24
100 NaHCO₃, 1.5 CaCl₂, 3 MgSO₄ and 10 D-glucose

101 **2.3 Small volume incubation of brain slices**

102 After 30 min recovering, one hemisphere of the 6 coronal slices were transferred to the
103 incubation chambers containing a low bulk volume of aCSF (appr. 6ml of aCSF/6 half
104 coronal slice), placed on a closed-loop temperature controller pad (TMP-5b, Supertech
105 Instruments UK Ltd.) at 30 °C to reach optimal temperature for KAT function (Banerjee et
106 al., 2012b) (**SV condition hereafter**). The aCSF was continuously bubbled with 95% O₂ and
107 5% CO₂ but was not circulated during the incubation.

108 For comparison of tissue viability of slices incubated in SV condition or in standard
109 condition, we performed the same battery of experiments on the corresponding 6 half coronal
110 slices kept under standard conditions (high bulk volume of aCSF; ≈200ml/6 half coronal
111 slice) at 20 °C (**LV condition hereafter**). Through this LV slice incubation routine we could
112 perform stable field excitatory postsynaptic potentials (fEPSP) recordings after 8h incubation,
113 therefore, this condition was used as an adequate control for assessing tissue viability under
114 SV condition.

115 After evaluating tissue viability, we studied KYNA production and kynurenergic
116 manipulation in the SV condition. 10 μ M L-KYN (Sigma Aldrich) and 5 μ M KAT-2 inhibitor
117 PF-04859989 were dissolved in aCSF. The scheme of the protocol used in this study is
118 illustrated in **Supp. Fig. 1**.

119 At the end of the incubations, the supernatants were collected and stored at -80 °C for further
120 biochemical analysis. One portion of the slices were placed in 4% paraformaldehyde (PFA)
121 and fixed overnight for histological studies, whereas the remaining slices were immediately
122 frozen in liquid nitrogen and stored at -80 °C for Western blotting (see in the supplement).

123 **2.4 Electrophysiology**

124 After 4h incubation in the LV or SV condition, the slices were transferred to an interface
125 recording chamber and superfused with aCSF containing (in mM): 130 NaCl, 3.5 KCl, 1
126 NaH_2PO_4 , 24 NaHCO_3 , 3 CaCl_2 , 1.5 MgSO_4 and 10 D-glucose. The temperature (32 °C) and
127 flow rate (2 ml/min) of the aCSF were continuously controlled. Baseline synaptic function of
128 the tissue was tested with input-output (I/O) curve recordings on the CA3-CA1 cell synapses
129 in the hippocampus. Schaffer collaterals were stimulated at 0,05 Hz using a concentric bipolar
130 stainless steel electrode (Neuronelektrod Ltd, Hungary). fEPSPs were recorded from the
131 stratum radiatum of CA1 region with 1,5–3 mOhm resistance glass microelectrodes.

132 Potentials were amplified and filtered with WPI AMP-04 amplifier and digitalized with Axon
133 Digidata 1320A. Recordings were monitored and saved with Axoscope 10.0 (Molecular
134 Devices Corporation, USA). Data were analyzed with Clampfit 10.6 (Molecular Devices,
135 USA) and OriginPro 8.6 (OriginLab Corporation, USA) softwares. In the course of I/O curve
136 recordings to define the minimal stimulus we decreased the stimulus intensity until minimal
137 but unequivocal fEPSP was evoked. The stimulus was then increased in 5 μ A increments. We
138 collected five responses and averaged at each increment. We continued to increase the
139 stimulus intensity until fEPSP response had saturated. fEPSP slope values were normalized
140 and averaged across all slices in each group and plotted as a function of normalized stimulus
141 intensity to construct I/O curves.

142 **2.5 Histology**

143 Fixed brain slices were cryoprotected with sucrose solution and 30 μ m thick sections were cut
144 with a freezing microtome (Reichert-Jung 1206).

145 *Immunohistochemistry*: Free-floating sections were washed in PBT, incubated in 1% NDS
146 and exposed to the primary antibody (mouse anti-NeuN, 1:4000, Millipore) overnight at 4 °C.

147 Next day, they were incubated in the appropriate secondary antibody (1:500; Jackson
148 ImmunoResearch) at room temperature. Primary and secondary antibody were diluted in 0.1
149 M PBT containing 1% NDS. Negative control was prepared from sections incubated without
150 the primary antibody. The sections were coverslipped with antifade mounting medium
151 (ProLong® Gold, Life Technologies).

152 *Cresyl violet staining:* We performed cresyl violet staining for morphological observations
153 after different incubation conditions and time. Sections were rehydrated with descending
154 grades of alcohol and stained with cresyl violet staining solution for 5 min. After staining,
155 samples were passed through ascending alcohol solutions and immersed in xylene for 5 min.
156 Sections were coverslipped with Entellan®.

157 All photomicrographs were obtained with an Olympus BX51 microscope fitted with a DP70
158 digital imaging system.

159 **2.6 Lactate dehydrogenase (LDH) assay**

160 For evaluating tissue viability we performed LDH activity assay on supernatant samples
161 collected after 30 min, 4h and 6 h incubation time. As positive control for LDH release we
162 permeabilized the cell membrane with Triton X-100™ (1%). LDH activity was measured at
163 340 nm and 37 °C using an LDH activity assay kit (Diagnosticum Ltd, Hungary, 46461) on a
164 BioLis 24i Premium system (Siemens). LDH activity was expressed as U/l/100 mg tissue.

165 **2.7 Hexokinase (HK) assay**

166 Glucose content was measured by HK activity assay on supernatant samples collected after 30
167 min, 4h and 6h incubation time. HK activity was measured at 340 nm and 37 °C using a two-
168 step Glu HK activity assay kit (Diagnosticum Ltd, Hungary, 47361) on a BioLis 24i Premium
169 system (Siemens).

170 **2.8 High performance liquid chromatography (HPLC)**

171 The brain slices were weighed and then homogenized for 30s in 250 ml ice-cold solution,
172 containing trifluoroacetic acid (0.1% v/v) and 2 µM 3-nitro-L-tyrosine (3-NLT, as internal
173 standard). The homogenate was centrifuged at 12000 RPM for 10 min at 4°C. The
174 supernatants were stored at -80°C until further analysis. Similar procedure was applied for the
175 aCSF samples, briefly, 250 µL aCSF was treated with 50 µl of above-mentioned solution, and
176 then centrifuged under the same circumstances as the brain tissues. The resulting supernatants
177 were measured with an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA,
178 USA) combined with a fluorescence (FLD) and a UV detector. For the determination of

179 KYNA, the excitation and emission wavelengths of FLD were set at 344 nm and 398 nm,
180 whereas the UV detector was set at 365 nm for the determination of 3-NLT. Chromatographic
181 separations were performed on an a Kinetex C18 column, 150 x 4.6 mm I.D., 5 µm particle
182 size (Phenomenex Inc., Torrance, CA, USA) preceded by Security Guard pre-column C18, 4
183 x 3.0 mmI.D., 5 µm particle size (Phenomenex Inc., Torrance, CA, USA) with a mobile phase
184 composition of 0.2 M zinc acetate/ACN = 95/5 v/v%, in which pH was adjusted at 6.2 with
185 acetic acid, applying isocratic elution. The flow rate was 1.2 ml/min and the injection volume
186 was 50 µL. As for the method validation, following parameters are reported, briefly,
187 regarding KYNA in tissue and aCSF. The LOD and LOQ in tissue and aCSF samples were 1
188 and 3.75 nM, respectively. With regard to precision, the relative standard deviation was ≤
189 2.2%. The recoveries in tissue and aCSF samples ranged from 103% to 108% and 81% to
190 91%, respectively.

191 **2.9 Data analysis and Statistics**

192 KYNA content was calculated from the concentration values measured by HPLC and data
193 were normalized to the tissue weight. In the case of the aCSF, values represented KYNA
194 content released by 100 mg tissue. LDH release and glucose consumption data were similarly
195 normalized to 100 mg tissue.

196 All statistical computations were carried out with the IBM SPSS Statistics software (version
197 20.). Homogeneity of variance across groups was tested with the Levene's Test of Equality of
198 Error Variances. Distribution of the data was tested with the-Shapiro-Wilk test of normality.
199 Statistical significance was calculated with the GLM univariate model. If assumption of
200 variance homogeneity was violated, Kruskal-Wallis Test was used. For repeated measures of
201 the I/O curve recordings, General Linear Model Repeated Measures method was applied.

202

203 **3 Results**

204 **3.1 Glucose consumption**

205 To our best knowledge, there is no literature data about the minimal bulk volume of aCSF in
206 which acute mouse brain slices can function quasi normally for hours. Therefore, first, we
207 asked if the glucose availability in the aCSF under SV condition exceeds the nutrition demand
208 of the slices.

209 Glucose consumption measurement proved that glucose availability gradually decreased
210 during the incubation. Glucose concentration was already dropped after 30 min, however, the
211 decrease was not statistically significant ($F=4,281$; $p=0,072$; Partial Eta Squared: 0,349). 4h
212 incubation, however, resulted in a significant decrease of aCSF glucose content ($F=20,304$;
213 $p=0,002$; Partial Eta Squared: 0,717) compared to original level (**Fig. 1**). It was further
214 declined during the 6h incubation time (data not depicted).

215

216 **3.2 Viability of acute brain slices – LDH release**

217 A characteristic sign of the perturbation of normal cellular function is the loss of cell
218 membrane integrity and the concomitant increase of membrane permeability (Cho et al.,
219 2007). Therefore, alteration in LDH release to the extracellular space is a sensitive measure of
220 cell viability. Under SV condition LDH release was continuously increasing in the aCSF
221 during 30 min ($Z= 16,910$; $p=0,959$), 4h ($Z= 16,910$; $p=0,030$) and also 6 h incubation (data
222 not depicted), compared to the initial state. However, Triton X-100 treatment resulted in a ≈ 14
223 fold increase of LDH in the aCSF after 4h incubation ($Z= 16,910$; $p=0,001$) (**Fig. 2**). This
224 indicates that the cell membrane integrity is largely preserved in the course of 4h incubation.
225 The observed KYNA release is not the simple consequence of disrupted membrane integrity
226 (see below).

227 **3.3 Histological observations**

228 For the histological examination of tissue state we performed NeuN immunolabelling and
229 cresyl violet staining. There was no visible tissue damage in the vulnerable CA1 subregion of
230 the dorsal hippocampus. CA1 pyramidal cell shape and size appeared normal after 4h
231 incubation (**Fig. 3**). However, the structural integrity of pyramidal cells in CA1 was not
232 completely preserved after 6h incubation. Compared to the control group, shrunken and
233 deformed pyramidal cells emerged (visual observation).

234

235 **3.4 Synaptic properties of acute brain slices - I/O curves**

236 Basal glutamatergic synaptic properties were tested by means of fEPSP recording, expressed
237 against gradually increased stimulating intensity in certain groups. There was no significant
238 difference between slices recorded immediately after post-slicing recovery period (30 min.),
239 or incubated under LV condition for 4hours and under SV conditions, respectively ($F=0.793$;
240 $p=0,465$; Partial Eta Squared: 0,064). This result indicates that, the baseline synaptic function
241 of the brain tissue was preserved after 4h SV incubation (**Fig. 4**).

242 Considering the decreased glucose availability and increased LDH release, furthermore the
243 visually observed tissue damage after 6 h, the 4h long incubation duration was considered to
244 be the most suitable for KYNA measurement.

245 **3.5 KYNA production in mouse brain slices**

246 To examine whether mouse brain slices liberate endogenous and *de novo* produced KYNA
247 upon L-kynurenine administration during 4h long incubation period we performed HPLC
248 measurements from brain tissue homogenate and from incubating aCSF.

249 Without L-KYN administration $7.01\pm 2.03\text{ng}$ basal KYNA content could be measured from
250 the aCSF and $0.22\pm 0.07\text{ng}$ from the brain tissue homogenate. In contrast, as a result of $10\ \mu\text{M}$
251 L-KYN administration we found a 6.3 fold increase in the aCSF ($44.56\pm 6,99\text{ng}$) ($Z=6,818$;
252 $p=0,009$) and a 3.8 fold increase in the tissue ($0.85\pm 0.21\text{ng}$) KYNA content (**Fig. 5**). No
253 considerable KYNA elevation could be observed in the course of 6h incubation compared to
254 the 4h incubation (data not depicted).

255 In conclusion, $\approx 97\%$ of the total KYNA content was released to the extracellular
256 compartment (aCSF), whereas only $\approx 3\%$ remained in the tissue under both conditions (**Supp.**
257 **Fig. 2**)

258 **3.6 Effect of KAT-2 inhibitor PF-04859989 on *de novo* KYNA release**

259 In the next series of experiments we incubated acute slices in the presence of $10\ \mu\text{M}$ L-KYN,
260 with or without PF-04859989 in a concentration of $5\ \mu\text{M}$. KYNA content of the aCSF was
261 measured after 4h incubation. Similar to former results high KYNA content could be
262 measured in the L-KYN group ($55.19\pm 6.45\text{ng}$). Addition of the inhibitor resulted in a
263 significant decrease of the released KYNA in the aCSF by almost 40% ($34.5\pm 6.93\text{ng}$;
264 $F=23,868$; $p=0,001$; Partial Eta Squared: 0,749) (**Fig. 6**).

265 4 Discussion

266 The implication of kynurenine metabolites in several brain disorders (e.g. schizophrenia,
267 Alzheimer's disease, depression, migraine) shifted much attention to the manipulation of the
268 KP in recent years (Dounay et al., 2015). The KP of tryptophan catabolism is present in many
269 mammalian species (Moroni et al., 1988). Concomitantly, the neuromodulatory metabolites of
270 this route are produced and function, making model animals suitable for extrapolating the
271 effects of kynurenergic manipulation in humans. However, there are prominent differences
272 among mammalian species regarding the kynurenine system (Moroni et al., 1988, Fujigaki et
273 al., 1998), complicating further the understanding of the physiological and pathological role
274 of the KP.

275 The physiological concentration of KYNA is different in model animals and in humans. The
276 lowest basal KYNA level is found in the mouse brain, whereas it is the highest in the human
277 neocortex (Moroni et al., 1988). The KYNA synthesizing enzyme isoforms have also a
278 different role in different species. In the human and rat brain KAT-2 plays the major role in
279 KYNA production, whereas in mice kynurenine aminotransferase-4 (KAT-4) was supposed to
280 be the main KYNA synthesizing enzyme (Alkondon et al., 2004, Guidetti et al., 2007).
281 However, KAT-2 null mutation resulted in the decrease of extracellular KYNA level in
282 transgenic mice (Potter et al., 2010). Therefore, comparative characterization of the
283 kynurenine catabolism in different model species is essential before assigning therapeutical
284 kynurenergic manipulation strategies in humans.

285 Investigating the KP *in vitro* is of particular importance. In contrast to *in vivo* assays, acute
286 brain slice preparations provide the possibility to exclude the perturbation effect of the
287 peripheral KP function, while replicating or even changing many aspects of the *in vivo*
288 context. A large panel of pharmacological interventions can be efficiently evaluated;
289 furthermore, endogenous factors influencing brain KYNA production might also be easily
290 clarified.

291 4.1 The after-incubation state of mouse brain slice preparations

292 One of the major limitations of acute brain slice models is the viability of nervous tissue,
293 which endures on average for \approx 6-8 hours (Buskila et al., 2014). However, there are specific
294 brain areas (e.g. dorsal hippocampus) and cell populations where this window is even
295 narrower (\approx 4hours) (Fukuda et al., 1995). To ascertain that KYNA release is not an
296 uncontrolled function of a deteriorated brain tissue, we examined the after-incubation

297 viability. After 4h and 6h incubations in the SV aCSF, respectively, we observed slight
298 alterations in tissue integrity and function.

299 The change of cell membrane integrity was evaluated by measuring the cytosolic LDH
300 released into the aCSF. LDH could be measured and was continuously increasing during the
301 incubation time, a phenomenon that verifies the limited and relatively narrow working
302 window with acute brain slices. The considerably higher LDH content of the supernatant
303 aCSF after membrane permeabilization indicates that after 4h incubation membrane integrity
304 of the nervous tissue is largely preserved.

305 Changes in glucose consumption and glucose availability we estimated with a glucose
306 hexokinase assay. We found that the available glucose was gradually decreased in the course
307 of 4h and 6h incubation (not depicted). Although, continuous aCSF LDH level increase
308 suggests a gradual loss of membrane integrity, decrease of glucose availability in the course
309 of 4h and 6h incubation is possible only if, the nervous tissue actively metabolizes glucose. In
310 the nervous tissue both astrocytic and neuronal glucose up-take is possible through facilitative
311 membrane glucose transporters (Chuquet et al., 2010, Lundgaard et al., 2015). A shift toward
312 one of these mechanisms we did not estimate, however we may conclude a generally
313 preserved glucose up-take, consumption-machinery and functioning brain tissue.

314 Although the selective vulnerability of the CA1 region of the hippocampus to a variety of
315 insults (e.g. excitotoxicity, cerebral ischemia) has been reported (Davolio and Greenamyre,
316 1995, Kovalenko et al., 2006) we observed no visible tissue damage in this brain area.

317 Furthermore, Schaffer-collateral CA1 pyramidal cell synapses were equally functional in the
318 compared groups. There was no significant difference between slices recorded immediately
319 after post-slicing recovery period (30 min.), or incubated under LV condition for 4hours and
320 under SV conditions, respectively. This indicates that functional integrity of vulnerable region
321 was largely preserved during a 4h SV incubation.

322 **4.2 Basal and *de novo* KYNA production in mouse brain slices**

323 Our first and basic question was, whether KYNA production can be measured and elevated in
324 mouse brain slice preparations. To increase KYNA production L-KYN concentration (10 μ M)
325 was chosen on the basis of previous studies on slice preparations (Urbanska et al., 2000,
326 Okuno et al., 2011). Similar to rat brain slices (Alkondon et al., 2011), mouse brain slices
327 could produce KYNA upon L-KYN exposure and liberate newly formed KYNA to the
328 extracellular space after 4h incubation (**See Fig. 5.**). In trial experiments we measured KYNA
329 production after 2h incubation, but there was no sufficient KYNA increase in the L-KYN

330 treated group on which the inhibition of KYNA synthesis could reliably be studied (not
331 depicted). Therefore, 4 h long incubation was necessary to examine KYNA production and
332 the effect of KAT-2 inhibition.

333 It is important to note that, extending the extracellular space of the acute slices with the
334 incubating aCSF (approx. 100 mg wet weight brain tissue/6ml aCSF) results in a considerably
335 smaller intracellular/extracellular volume ratio than found in the intact mouse brain (approx.
336 500mg wet weight brain tissue/0,04ml CSF (Artru, 1993). That difference means a steep
337 concentration gradient and driving force toward the aCSF for any released molecules. After
338 4h incubation the amount of the released KYNA was more than 30 times higher than that
339 retained in the tissue (**See Supp. Fig. 2.**). This release mechanism of KYNA is still unknown
340 and should also be investigated in future work. Nevertheless, a high local KYNA
341 concentration might be reached upon L-KYN treatment in the close apposition of the KYNA
342 release sites.

343 It is important to note that our results regarding KYNA production may not reflect completely
344 the *in vivo* mechanisms. The brain-to-blood elimination of brain KYNA through probenecid-
345 sensitive organic acid transport is continuous *in vivo* (Miller et al., 1992), however, the ratio
346 of elimination in the mice is not described. Furthermore, the composition of aCSF differs in
347 several aspects from the *in vivo* physiological extracellular milieu. For instance, there are no
348 amino acids in the aCSF (e.g. aspartate, tryptophan), which can negatively impact the activity
349 of the KAT isoenzymes (Han et al., 2010b). The applied incubation temperature was lower
350 than physiological, but higher temperature curtails the lifespan of acute slices as a
351 consequence of bacterial growth and cellular metabolism (Buskila et al., 2014). Lower
352 temperature slows down these adverse processes and prolong the time period during which
353 the slices can be kept functional. Beyond tissue viability aspects, and *in vitro*
354 electrophysiological routines we chose 30 °C for incubation basing on the paper of Banerjee
355 et al. (2012), where this temperature was used as an optimal temperature for KAT-2 (Banerjee
356 et al., 2012). These differences may lead to altered KAT activity and to a modified
357 production of KYNA. However, we did not find altered KAT-2 expression level of the
358 samples incubated under SV condition (F = 0.000; p =0.989; Partial Eta Squared: 0.000),
359 therefore a quasi normally KYNA production can be suggested (**Supp. Fig. 3.**)

360 The ≈ 6.3 fold elevation in aCSF KYNA content by this small amount of tissue after L-KYN
361 addition indicates a high KAT capacity. Previously it has been shown on purified KAT
362 preparation from rat liver that KATs have high capacity and Km value (~ 1 mM) for L-KYN

363 (Bender and McCreanor, 1985). Indeed, KYNA production in the rat brain was saturable only
364 at high L-KYN (≈ 1 mM) concentration (Turski et al., 1989), which is concordant with our
365 findings.

366 In conclusion, mouse brain tissue intensively liberates endogenous and *de novo* synthesized
367 KYNA into the extracellular milieu *in vitro*, whereas the retained KYNA in the tissue is
368 negligible.

369 **4.3 Effect of KAT-2 inhibitor PF-04859989 on *de novo* KYNA release**

370 The advent of highly specific KAT-2 inhibitors opened new perspectives in clarifying KP
371 function in rodents (Dounay et al., 2012, Nematollahi et al., 2016). However, no
372 pharmacological experiment has yet targeted the mouse KAT-2 function, probably because of
373 its proposed irrelevance (Guidetti et al., 2007). In our previous anatomical study, we found
374 prominent KAT-2 immunopositivity in astrocytes and in GABAergic cells in the adult mouse
375 brain (Heredi et al., 2017). We therefore hypothesized that KAT-2 has a specific role in
376 mouse brain KYNA function. Because of the low basal KYNA content of the tissue
377 homogenate and that of the aCSF (usually close to the detection limit of HPLC), we estimated
378 the effect of the inhibitor on the KYN induced *de novo* KYNA production.

379 Applying the highly specific KAT-2 inhibitor PF-04859989 in a similar concentration, which
380 was found in the rat CSF after parenteral application (Dounay et al., 2012), resulted in a
381 decrease of *de novo* KYNA production by almost 40%. Although, this is higher than has
382 previously been reported using alternative methods, KYNA production was not completely
383 abolished following KAT-2 inhibition; 60% of total KAT activity remained. This indicates
384 that, in a whole other KAT enzymes make a greater contribution in the mouse brain, however,
385 it does not exclude the important role of mouse KAT-2. Indeed, an incomplete inhibition of
386 KAT activity is more suitable in the experimental models of therapeutical KYNA level
387 reduction. Our results indicate that the investigation of the effects of pharmacological KAT-2
388 inhibition should be extended to mouse models, which was largely neglected in this relation.

389

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392

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399

400 **Conflict of interest**

401 The authors declare no competing financial interests. All co-author agree with the submission
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523

524

525 **Legend to figures**

526 **Fig. 1: Glucose consumption in the course of 4h SV incubation.** Acute slices were
527 incubated as routinely in aCSF having high glucose concentration (10mM). Glucose
528 concentration was already dropped after 30 min ($\approx 8\%$), however, the decrease was not
529 statistically significant ($F=4,281$; $p=0,072$; Partial Eta Squared: 0,349). 4h incubation,
530 however, resulted in a significant decrease of aCSF glucose content ($\approx 20\%$) ($F=20,304$;
531 $p=0,002$; Partial Eta Squared: 0,717). Data are expressed as a percentage of baseline glucose
532 content (glucose content of the aCSF at the starting point of the experiment) and represent the
533 mean \pm SD. $n=5$ animals, 6+6 corresponding brain slices per condition.

534
535 **Fig. 2: LDH release in the course of 4h SV incubation.** At the beginning of the incubation
536 aCSF LDH content is virtually zero (only measuring error is depicted). In comparison,
537 gradual increase of the aCSF LDH content could be measured in the course of 30 min ($Z=$
538 $16,910$; $p=0,959$) and 4h incubation ($Z= 16,910$; $p=0,030$). However Triton X-100 treatment
539 resulted in a ≈ 14 fold increase of LDH in the aCSF after 4h ($Z= 16,910$; $p=0,001$). This
540 indicates that the cell membrane integrity is largely preserved in the case of 4h incubation.
541 Data represent the mean \pm SD. $n=9$ animals, 6+6 corresponding brain slices per condition.

542

543 **Fig. 3: Cresyl violet staining (A-B) and NeuN immunolabelling (C-D) in the dorsal**
544 **hippocampus of mouse acute brain slices.** There was no visible difference between the LV
545 (A-C) and SV (B-D) group in the CA1 area of the hippocampus after 4h incubation. Cells
546 keep their normal appearing in the pyramidal cell layer. Scale bars are 100 μm .

547 **Fig. 4: Basal glutamatergic synaptic properties of acute brain slices.** There is no
548 significant difference between slices recorded immediately after post-slicing recovery period
549 (30 min.), or incubated under LV condition for 4hours and under SV conditions, respectively
550 ($F=0.793$; $p=0,465$; Partial Eta Squared: 0,064). The values represent normalized means \pm SD
551 and were plotted as a function of stimulus strength. $n=17$ animals; 11+9+8 recordings/group.

552 **Fig. 5: Basal and *de novo* KYNA content in the incubating aCSF and in the tissue**
553 **homogenate after 4h incubation.** Without L-KYN administration $7.01\pm 2.03\text{ng}$ basal KYNA
554 content could be measured from the aCSF and $0.22\pm 0.07\text{ng}$ from the brain tissue homogenate.
555 In contrast, as a result of $10\ \mu\text{M}$ L-KYN administration we found a 6.3 fold increase in the
556 aCSF ($44.56 \pm 6,99\text{ng}$) ($Z=6,818$; $p=0,009$) and a 3.8 fold increase in the tissue ($0.85\pm 0.21\text{ng}$)
557 KYNA content. The values represent means \pm SD. $n=5$ animals, 6+6 corresponding brain
558 slices per condition.

559 **Fig. 6: Effect of KAT-2 inhibitor PF-04859989 on *de novo* KYNA release.** Acute slices
560 were incubated in the presence of $10\ \mu\text{M}$ L-KYN with or without PF-04859989 in a
561 concentration of $5\ \mu\text{M}$. KYNA content of the aCSF was measured after 4h incubation. High
562 KYNA content could be measured in the L-KYN group ($55.19\pm 6.45\text{ng}$). Addition of the
563 inhibitor resulted in a significant decrease of the released KYNA in the aCSF by almost 40%
564 ($34.5\pm 6.93\text{ng}$; $F=23,868$; $p=0,001$; Partial Eta Squared: 0,749). The values represent
565 means \pm SD. $n=5$ animals, 6+6 corresponding brain slices per condition.



















