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Original Paper

Kynurenine Aminotransferases I, II and III Are Present in Saliva

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Key Words
Saliva • Kynurenine aminotransferase • Human • Sex • cat • KAT inhibition • γ-acetylenic GABA • cerebrolysin • D-cycloserine • pellet • supernatant

Abstract
Background/Aims: Fluids of the human body such as serum, cerebrospinal fluid and saliva contain a wide variety of proteins. Because kynurenic acid (KYNA) has been detected in human saliva, we wondered if KYNA could be produced in saliva by KYNA-synthesising enzymes, namely the kynurenine aminotransferases KAT I, KAT II and KAT III. ***Methods:*** Thirty samples of human saliva from control volunteers were investigated. KAT activity was measured in the presence of 1 mM pyruvate and 2 μM or 100 μM L-kynurenine and KYNA production was assessed by high-performance liquid chromatography. ***Results:*** Saliva dose- and time-dependently produced KYNA. KAT activity ranged between 900 and 1050 pmol/mg protein/h: 900 for KAT I, 950 for KAT III and 1050 for KAT II. KYNA was synthesised in saliva at a physiological concentration of 2 μM L-kynurenine and at a higher concentration of 100 μM. Investigation of the distributions of the enzymes in saliva revealed that KAT I, KAT II and KAT III activity in a centrifuge-obtained pellet ranged from ~100% to 120%; in the supernatant, the percentage was between 0% and 20%. We observed a nonsignificant tendency for lower KAT activity in women's saliva than in men's. KATs present in saliva were sensitive to the GABA-transaminase inhibitor γ-acetylenic GABA, with a concentration of 100 μM γ-acetylenic GABA significantly blocking the formation of KYNA (50% of control, $p < 0.05$). Furthermore, KATs in saliva were sensitive to anti-dementia drugs, such as D-cycloserine and cerebrolysin, in an *in vitro* study. ***Conclusion:*** Our data revealed for the first time the presence of KAT I, KAT II and KAT III proteins in human saliva. KAT activity was found mostly in pelleted cells, suggesting their presence in salivary gland cells. KAT proteins in saliva are sensitive to drugs blocking KYNA formation. Our data indicate the presence of cells in saliva involved in the biochemical machinery of the kynurenine pathway. Their role in the digestive process remains to be clarified. We speculate that modulation of KYNA formation in the mouth by food and/or drugs might affect glutamate neurotransmission and cholinergic activity in the CNS and/or periphery and play a role under physiological as well as pathological conditions.

Berthold Kepplinger died on 31 December 2019.

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Introduction

Saliva is a fluid that is continuously secreted by the buccal glands and the three pairs of salivary glands, the parotid, submandibular and sublingual [1]. Salivation is entirely under nervous control, with parasympathetic stimulation keeping the mucous membranes moist and a feeling of dryness occurring when sympathetic stimulation dominates [1]. Food stimulates the glands to heavily secrete saliva, but the smell, sight, touch and even sound of food increase saliva secretion. These stimuli constitute psychological activation and involve learned behaviour that is laid down in the cerebral cortex [2]. The cortex sends impulses to the nuclei in the brain stem via the extrapyramidal pathway to activate the salivary gland. Interestingly, psychological activation of the gland has some benefits because it allows the mouth to start chemical digestion as soon as food is ingested.

Saliva contains a large variety of constituents and its physicochemical properties are important for the maintenance of oral health [3, 4]. In addition, saliva plays an important role in maintaining a balanced microbiota. Saliva contains 99.5% water and 0.5% solutes. The solutes include salts, dissolved gases, various organic substances, serum albumin and globulin, mucin, bacteriologic enzymes, digestive enzymes and salivary amylase [2-4]. A large number of diseases and medications can affect salivary secretion through different mechanisms, leading to salivary gland dysfunction and associated oral problems, including xerostomia, dental caries and fungal infections [1-4]. Studies by Chiappelli et al. [5, 6] showed that the levels of kynurenic acid (KYNA) in saliva are increased after psychological stress. Central and peripheral tissues are intimately involved in tryptophan metabolism along the kynurenine pathway to form several neuroactive compounds [7, 8]. One such compound, KYNA, is a well-known endogenous antagonist of the glutamate ionotropic excitatory amino acid receptors [9, 10] and of the nicotine cholinergic subtype alpha-7 receptor [11, 12]. Importantly, increased levels of KYNA in an experimental animal model impaired memory capacity [13] whereas decreased KYNA levels improved cognitive function [14]. Similarly, increased KYNA content has been found in various neuropsychiatric and immunological disorders, as well as during the ageing process and in age-related diseases, and a role for KYNA in memory and cognition impairments has been suggested [15-23]. KYNA is present in various human liquids, such as cerebrospinal fluid (CSF), serum and saliva [15, 17, 21, 23, 24].

The concentration of KYNA is regulated by many pyridoxal-5-phosphate–dependent enzymes, named kynurenine aminotransferase(s) (KATs), which catalyse the conversion of L-kynurenine to KYNA in the CNS and periphery of mammals [7, 8, 10]. Various KATs have been identified and characterised in the human brain and peripheral tissues [25-28]. For example, KAT I, KAT II and KAT III show different catalytic characteristics with respect to optimum pH: 9.6 for KAT I, 8.0 for KAT III and 7.4 for KAT II [25, 26, 28].

In human saliva, KYNA occurs in low concentrations (nM) [24]. Because the secretion of proteins from salivary glands is stimulated by parasympathetic and sympathetic stimulation and because cortical regions play a pivotal role in stimulus induction, we were interested in investigating the composition of saliva, particularly the ability of saliva to synthesise KYNA. Accordingly, the aim of this study was to determine if human saliva contains KATs, which synthesise KYNA. Because KATs are mitochondrial enzymes, we examined whether, due to saliva centrifugation, cells separated in the pellet exhibit KAT activity. The activity of KAT I, II and III was measured in the saliva of healthy participants and correlations between KAT activity according to sex were analysed. Subsequently, the effect of γ-acetylenic GABA, a compound that inhibits KAT activity [29], on KYNA formation in human saliva was evaluated. We also analysed the effect of the anti-dementia drugs cerebrolysin [30] and D-cycloserine [31] on KAT activity in human saliva in an *in vitro* study.

Materials and Methods

Chemicals

L-kynurenine, KYNA and pyridoxal-5′-phosphate were purchased from Sigma-Aldrich Handels GmbH Wien, Austria. Cerebrolysin was obtained from EVER Pharma, Unterach, Austria, and diluted to 215.2 mg in 1 ml H_2O. All other chemicals used were of the highest commercially available purity.

Participants

In total, 33 healthy volunteers—27 therapists from the Institute of Therapy, Psychiatric Hospital Mauer-Amstetten, and 6 employees or relatives of employees from the Karl Landsteiner Institute—participated in this study. The participants ranged in age from 4 to 80 years. Saliva was collected in the morning. Participants first washed their mouth with water and waited about 15 min before saliva collection. The saliva was stored in a refrigerator and used immediately for research. Saliva samples were coded and the study was conducted according to Lower Austrian Ethical Regulations.

Animals

Saliva was collected from a male cat, 10 years old, lacking teeth but otherwise in excellent health. The cat produced increased amounts of saliva when its back was massaged or when its food was being prepared; the saliva was collected and immediately frozen and for use in our research. Six saliva samples were collected from the cat in the morning over a 6-week period (i.e., one sample per week) and used in the experiments as a comparison.

Assay of KAT I, KAT II and KAT III activity

KAT I, KAT II and KAT III activity in saliva were measured using an enzymatic assay described by Baran et al. [17] with minor modifications. Briefly, the reaction mixture contained 25, 50 or 75 µl saliva, 2 µM or 100 µM L-kynurenine, 1 mM pyruvate, 70 µM pyridoxal-5′-phosphate and 150 mM 2-amino-2-methyl-l-propranol buffer pH 9.6 for KAT I, 150 mM Tris-acetate buffer pH 7.4 for KAT II, or 150 mM Tris-acetate buffer pH 8.0 for KAT III in a total volume of 200 µl. After incubation at 37°C for 30 min, 1 h or 1.5 h, respectively, the reaction was stopped by the addition of 14 µl of 50% trichloroacetic acid and 1 ml of 0.1 M HCl. Denatured proteins were removed by centrifugation (30 min at 11, 000 rpm) and the synthesised KYNA was quantified by high-performance liquid chromatography (HPLC). Blanks were prepared by the addition of 14 µl of 50% trichloroacetic acid to the reaction mixture before incubation.

KYNA measurement

KYNA was measured according to Swartz et al. [32] with modifications described by Baran et al. [17]. KYNA was quantified by HPLC coupled with fluorescence detection. If necessary, KYNA samples were purified on a Dowex 50-W cation-exchange column as described by Turski et al. [33] before being applied to the HPLC system.

Determination of proteins

Proteins were determined by a method described by Lowry et al. [34] with modifications by Peterson [35].

Determination of the dose-dependency of KAT activity in saliva

To determine the dose-dependency of KAT activity in saliva, different amounts of saliva—25, 50 and 75 µl—were used. The KYNA produced was verified after 1 h of incubation in the presence of the reaction mixture. KYNA formation was analysed as described in the Materials and Methods.

Determination of the time-dependency of KAT activity in saliva

To determine whether the synthesis of KYNA in saliva (50 µl) was time-dependent, various incubation times—0.5 h, 1 h and 1.5 h—were tested and KYNA production was determined as described in the Materials and Methods.

Measurement of KAT activity in human saliva under physiological conditions

To determine if saliva is able to form KYNA in the presence of physiological concentrations of L-kynurenine, KAT activity was examined with concentrations of 2 μM and 100 μM of L-kynurenine in the incubation mixture. KAT activity was determined using the standard assay conditions for KAT I, KAT II and KAT III as described in the Materials and Methods.

Measurement of KAT I, KAT II and KAT III activity in human saliva

In the saliva of the human participants, KAT activity was determined using standard assay conditions for KAT I, KAT II and KAT III, as described in the Materials and Methods. KAT activity was calculated per microlitre of saliva or per milligram of protein. Furthermore, human KAT activity was evaluated according to sex.

Verification of KAT activity in the pellet and supernatant of saliva

KAT I, KAT II and KAT III activity was measured in human saliva and a supernatant and pellet obtained after saliva centrifugation at 5000 rpm for 30 min. The pellet was re-suspended in 5 mM Tris-acetate buffer pH 8.0 to the same volume of the saliva sample used and KATs were determined as described in the Materials and Methods.

Effect of γ-acetylenic GABA on the activity of KAT I, KAT II and KAT III in human saliva

To determine the effect of γ-acetylenic GABA on the activity of KAT I, KAT II and KAT III in human saliva, saliva (50 μl) was incubated in the presence or absence of 100 μM *γ*-acetylenic GABA under standard assay conditions and the amount of KYNA formed was determined. Six independent experiments were performed.

Effect of D-cycloserine on the activity of KAT I and KAT II in human saliva

To determine the effect of D-cycloserine on the activity of KAT I and KAT II in human saliva, saliva (25 μl) was incubated in the presence of 0, 168.3 and 336.6 μM D-cycloserine under standard assay conditions and the amount of KYNA formed was determined. Six independent experiments were performed.

Effect of cerebrolysin on the activity of KAT I and KAT II in human saliva

To determine the effect of cerebrolysin on the activity of KAT I and KAT II in human saliva, saliva (25 μl) was incubated in the presence of 0, 1.25 and 2.5 μl cerebrolysin under standard assay conditions and the amount of KYNA formed was determined, as described in the Materials and Methods. Six independent experiments were performed.

Measurement of KAT I, KAT II and KAT III activity in cat saliva samples

Cat saliva samples were investigated in parallel to the determination of KAT activity in human saliva.

Data analyses

All data are presented as the mean ± the standard error of the mean (SEM). Each sample was examined in duplicate or triplicate. One-way analysis of variance (ANOVA) and the Student's *t*-test were applied to determine statistical significance. The level for statistical significance was set at $p < 0.05$.

Results

Determination of KAT activity using different amounts of saliva

KAT I, KAT II and KAT III activity was determined in an incubation mixture containing different amounts of saliva (25, 50 and 75 μl). KYNA synthesis was found to be dose-dependent under standard assay conditions for KATs. No KYNA formation was seen in deactivated saliva (data not shown).

Determination of KAT activity in saliva using different incubation times

KYNA synthesis in saliva (50 μl) was time-dependent up to 1.5 h using different incubation times (0.5, 1.0 and 1.5 h) under standard assay conditions for KAT I, KAT II and KAT III. Fig. 1 shows the time-dependent formation of KYNA in saliva under standard assay conditions for

KAT III; data for KAT I and KAT II are not shown. Five participants (2 women and 3 men) with a mean age of 56.2 ± 7.3 years were used. No effect on KYNA formation was seen with deactivated saliva.

Measurement of KAT activity in human saliva in the presence of 2 or 100 µM L-kynurenine

We verified that saliva is able to form KYNA in the presence of physiological concentrations of L-kynurenine (2 µM) and at higher concentrations (100 µM). Data on KYNA synthesised due to KAT I, KAT II and KAT III are shown in Fig. 2. ANOVA revealed significant differences in KYNA synthesis between the two L-kynurenine concentrations used: KAT I, F = 7.41182 (p = 0.02615); KAT II, F = 5.63702 (p = 0.04495); and KAT III, F = 5.91125 (p = 0.04112). KAT activity was determined under standard assay conditions for KAT I, KAT II and KAT III as described in the Materials and Methods.

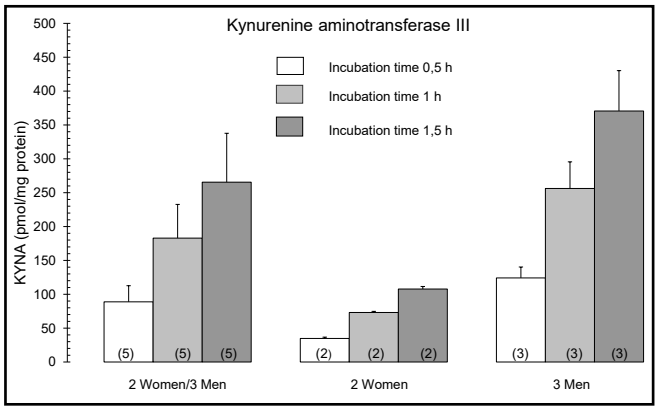

Fig. 1. Time-dependency of KYNA formation in human saliva under standard assay conditions for KAT III. Data represent the mean ± SEM. KAT III activity is expressed as KYNA formed (pmol/mg protein). Five saliva samples were used.

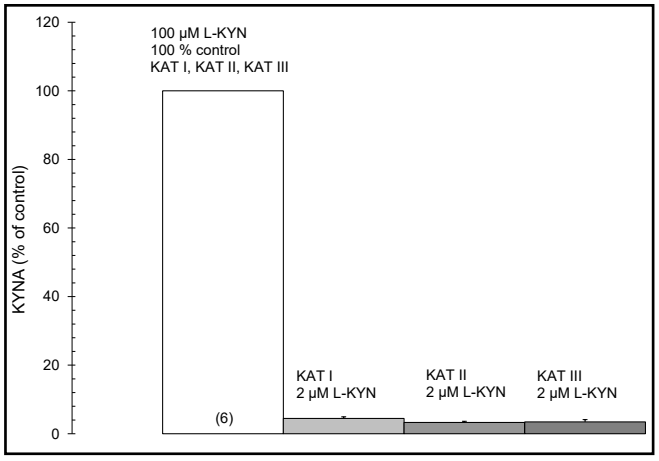

Fig. 2. Comparison of KYNA synthesis in human saliva using 2 µM and 100 µM L-kynurenine (L-KYN) in the incubation mixture under standard assay conditions for KAT I, KAT II and KAT III. Data represent the mean ± SEM. KAT I, II and III activity is expressed as % of control. KAT activity in the presence of 100 µM L-KYN in the incubation medium is expressed as 100%. Six saliva samples were used.

Measurement of KAT I, KAT II and KAT III activity in human saliva

We measured KAT I, KAT II and KAT III activity in the saliva of the human participants (Fig. 3). We expressed KAT activity in femtomole of KYNA produced per microlitre of saliva per hour (Fig. 3A) or in picomole of KYNA produced per milligram of protein per hour (Fig. 3B). The mean total protein values were 2.97 ± 0.55 µg/µl of human saliva and varied between 0.07 and 12.44 µg/µl. A high variability of KAT activity in saliva obtained throughout the day was also observed (data not shown).

Evaluation of KAT activity in human saliva according to sex

Evaluation according to sex revealed lower KAT activity in female participants: KAT I, 52.4%; KAT II, 48.9%; and KAT III, 44.9%, differences were not significant (Fig. 4). KAT activity was determined under standard assay conditions.

Measurement of KAT I, KAT II and KAT III activity in pellet and supernatant of human saliva

After saliva centrifugation, the pellet and supernatant showed significant differences in KAT activity. The highest activity of KAT I, KAT II and KAT III was detected in re-suspended

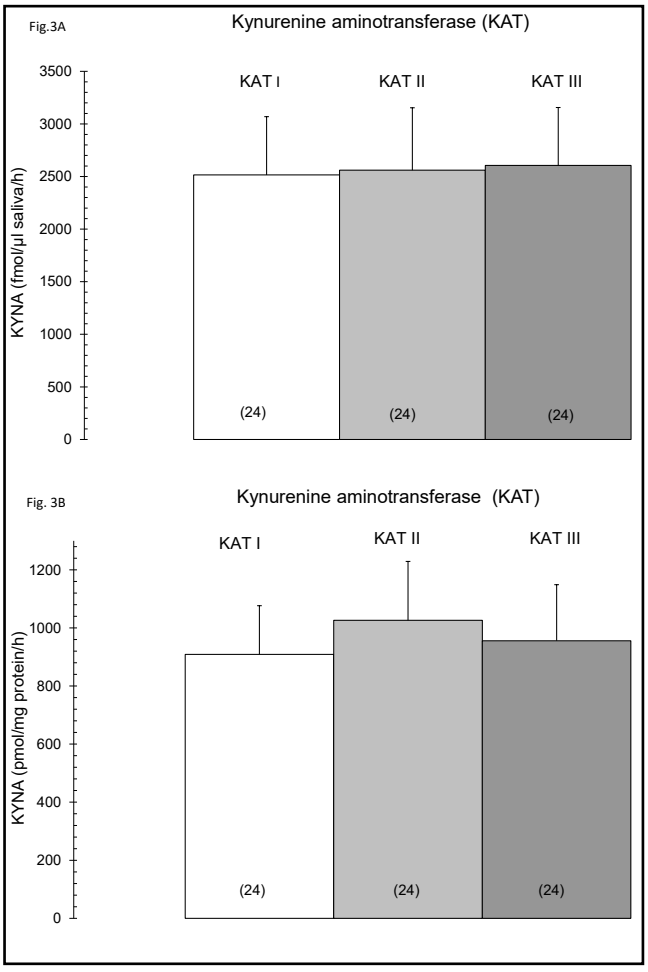

Fig. 3. Determination of KAT I, KAT II and KAT III activity in human saliva. Twenty-four human saliva samples were used. Data represent the mean ± SEM. Data are expressed in femtomole of KYNA formed per microlitre of saliva per hour (Fig. 3A) and in picomole of KYNA formed per milligram of protein per hour (Fig. 3B).

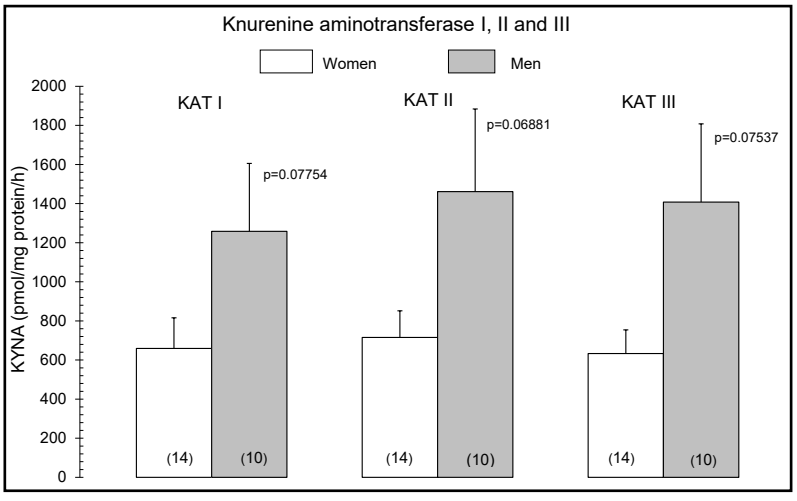

Fig. 4. KAT I, KAT II and KAT III activity in samples of female and male human saliva. Fourteen female saliva samples were used and 10 male samples. Data represent the mean ± SEM. No significant differences were found according to sex: KAT I, p = 0.07754; KAT II, p = 0.06881; and KAT III, p = 0.07537 (Student's t-test).

pellet (the pellet was re-suspended up to the volume of the saliva used for the centrifugation); the supernatant showed only marginal activity. KAT II activity was 117.99% that of the saliva in the re-suspended pellet and 9.075% that of the saliva in the supernatant (Fig. 5). ANOVA revealed significant differences between KAT II activity in the supernatant and pellet (F = 37.16249, p = 2.76083E-5). The data for KAT I and KAT III were similar and are not shown.

Effect of γ-acetylenic GABA on the activity of KAT I, KAT II and KAT III

γ-Acetylenic GABA (100 μM) significantly lowered (to approximately 50% of the control) KAT I ($p = 0.03977$), KAT II ($p = 0.04953$) and KAT III ($p = 0.03525$) activity in human saliva under standard assay conditions ($n = 5$; Student's *t*-test; Fig. 6). No effect on KYNA formation was seen using deactivated saliva.

Effect of D-cycloserine on the activity of KAT I and KAT II in human saliva

D-cycloserine significantly and dose-dependently (168.3 and 336.6 μM) lowered the activity of KAT I and KAT II in human saliva: KAT I to 75.9% ($p = 0.0024$) and 65.87% ($p = 7.38998E-4$) of control, respectively, and KAT II to 61.4% ($p = 0.00224$) and 50.7% ($p = 1.9168E-4$) of control, respectively (Fig. 7). One-way ANOVA revealed a significant effect of D-cycloserine (168.3 and 336.6 μM) on KAT I activity ($F = 12.66588$, $p= 6.00249E-4$) and KAT II activity ($F = 18.36759$, $p = 9.27448E-5$).

Effect of cerebrolysin on the activity of KAT I and KAT II in human saliva

Cerebrolysin significantly and dose-dependently (1.25 and 2.5 μl) lowered the activity of human brain KAT I and KAT II: KAT I to 66.09% ($p = 0.00193$) and 54.8% ($p = 1.72252E-4$) of control, respectively, and KAT II to 53.72% ($p = 8.14424E-4$) and 38.8% ($p=5.03103E-5$) of control, respectively (Fig. 7). One-way ANOVA revealed a significant effect of cerebrolysin (168.3 and 336.6 μM) on the activity of KAT I ($F = 14.70106$, $p = 2.91848E-4$) and KAT II ($F = 24.60632$, $p = 1.83455E-5$) in human saliva.

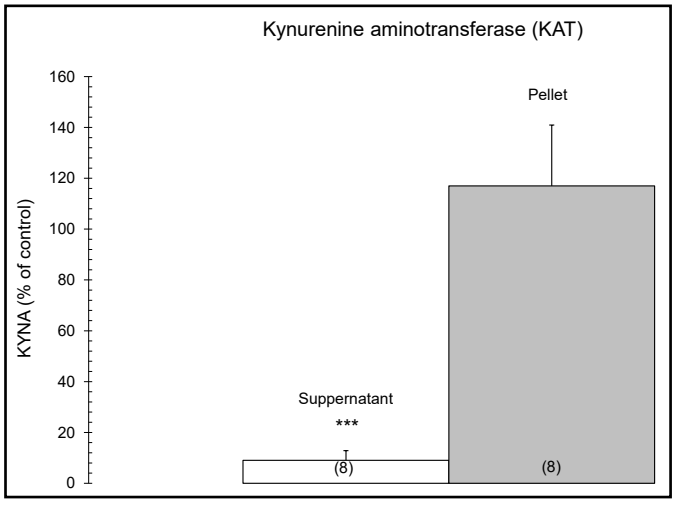

Fig. 5. Activity of KAT I, KAT II and KAT III in saliva and in supernatant and pellet obtained after saliva centrifugation. Six saliva samples were used. KAT activity of saliva is expressed as 100%. Data represent the mean ± SEM. Significant difference between the supernatant and pellet: ***p<0.001 (Student's t-test).

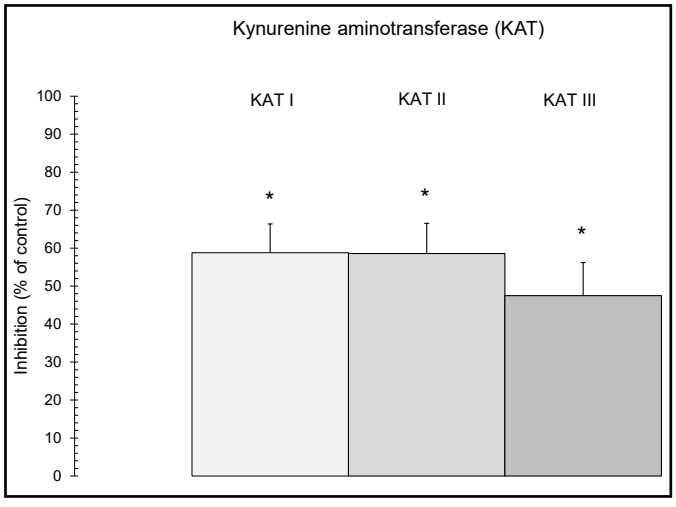

Fig. 6. Influence of γ-acetylenic GABA on KAT I, KAT II and KAT III activity in human saliva. Six saliva samples were used. Data represent the mean ± SEM and the controls, expressed as 100%, are the activity of KAT I, KAT II and KAT III in the absence of γ-acetylenic GABA under standard assay conditions as described in the Materials and Methods. *p<0.05 vs control (Student's t-test).

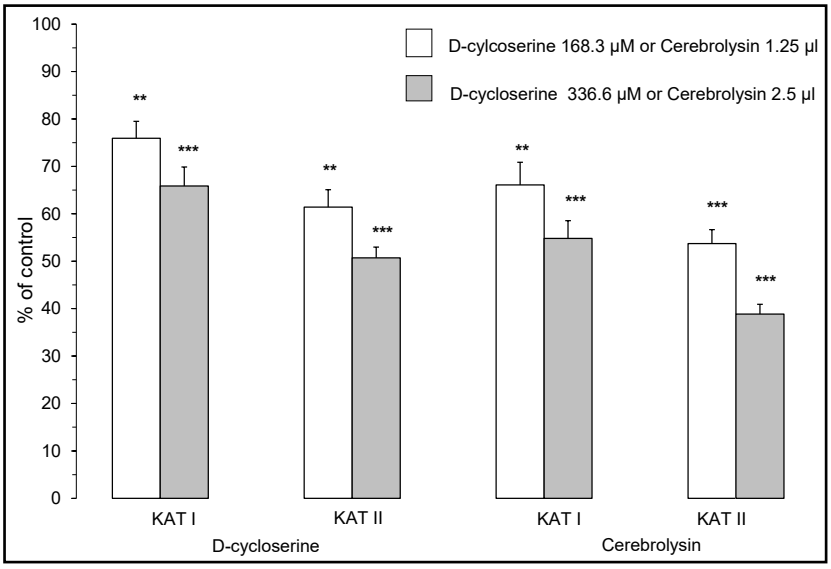

Fig. 7. Influence of D-cycloserine (168.3 µM and 336.6 µM) and cerebrolysin (1.25 µl and 2.5 µl; 1 ml of water contains 215.2 mg cerebrolysin) on KAT I and KAT II activity in human saliva. KATs in saliva without drugs are expressed as 100%. The concentration of cerebrolysin used is described in the Materials and Methods. Six saliva samples were used. **p<0.01 and ***p<0.001 (Student's t-test).

Measurement of KAT activity in cat samples

Measurement of KAT activity in saliva samples (N=6) collected from the cat revealed low values of KYNA produced (fmol/µl of saliva/h): KAT I, 531.5 ± 81.2; KAT II, 668.2 ± 187.1; KAT III, 813.7 ± 290.2.

Discussion

The marginal presence of KAT I, KAT II and KAT III, the enzymes that synthesise KYNA, had previously been described in human fluids such as the CSF and serum [21]. The present study is the first to show the presence of KAT I, KAT II and KAT III in human saliva. We also found KAT activity in cat saliva, with the data suggesting the general presence of KATs in mammalian saliva. The function of these proteins in terms of the digestive process remains to be clarified.

KYNA synthesis in saliva was dose- and time-dependent and was observed at a physiological concentration of 2 µM L-kynurenine, at least in an *in vitro* study. The ability of salivary KATs to synthesise KYNA from a low concentration of L-kynurenine indicates a high affinity of these proteins for L-kynurenine as a substrate, similar to what we observed for KATs from the human brain [25, 26]. Furthermore, the action of transaminase inhibitors on KAT activity in saliva was similar to the action on KATs observed in homogenates of human brain and peripheral tissue. Thus, the transaminase inhibitor γ-acetylenic GABA [29] and the anti-dementia drugs cerebrolysin [36] and D-cycloserine [37], which block KAT activity in human brain homogenate, significantly inhibited KYNA formation in human saliva in an *in vitro* study. In addition, there are significant data showing the presence of KYNA in various vegetables [38, 39]. Interestingly, vegetables are also involved in KYNA formation (observation by H. Baran and B. Kepplinger, paper in presentation) and this might lead to significant changes in KYNA metabolism as well.

The amounts of saliva secreted daily vary considerably and range from 1000 to 1500 ml. Similarly, the numbers of cells in saliva also show considerable time-dependent variations during the day or according to food consumed or health condition [1-4]. The high variability of KAT activity in saliva during the day might reflect the high variability of KYNA levels found in the droppings of various birds [40-41]. Tryptophan metabolites, including KYNA, were investigated in eagle droppings and KYNA was present at high concentrations of 4–12 nmol/mg [40-41]. Interestingly, different concentrations of KYNA levels were found in the digestive system as well [42].

Notably, the highest synthesis of KYNA in saliva was detected in a pellet obtained after centrifugation, suggesting the presence of KATs in the mitochondrial fraction of cells released from salivary glands. Recently, we proposed the therapeutic efficacy of compounds that block KAT activity in dementia [36, 37, 43, 44] and it is reasonable to believe that the ability of saliva to synthesise KYNA could be a new therapeutic approach with regard to the delivery of selected drugs. It is possible that, during food consumption, compounds affecting KAT activity might also have an impact on KYNA production and the transmission of signals into the CNS and/or periphery.

Measurement of KAT activity beyond the CSF and serum, particularly in the supernatant and/or pellet of saliva, might be important for the diagnosis [45] of various neuropsychiatric diseases or even cancers.

We found that women had approximately 50% lower KAT activity in saliva than men. While this observation is of interest, it must still be confirmed. Studies with larger groups of participants are needed to further explore this observation. The within-group data variability was higher in the male group and this also needs to be evaluated.

We found a large variation in protein levels in the saliva samples and our data are in line with previous findings [46]. The authors described the influence of the ageing process on protein amounts in saliva in participants up to 35–40 years old. Accordingly, information on older people is lacking and needs to be obtained in the near future.

Conclusion

The current data indicate that human saliva is able to form KYNA due to the KATs present in the saliva. Although this property may be of potential benefit for the CNS and for the periphery, the mechanism(s) of KYNA action remain to be investigated. The influence of variations in KAT activity on the digestive process is unclear. The effect of increased or decreased KYNA catabolism due to the impact of different foods and/or medications also must be elucidated. In this regard, it would be of particular interest to determine if the KATs in saliva could be useful in the treatment of dementia. The potential of KAT activity measurement in the saliva [45] as a diagnostic marker for neuropsychiatric disorders and cancer should be evaluated in the near future.

Abbreviations

ANOVA (analysis of variance); CSF (cerebrospinal fluid); HPLC (high-performance liquid chromatography); KAT (kynurenine aminotransferase); KYNA (kynurenic acid); L-KYN (L-kynurenine); SEM (standard error of the mean).

Acknowledgements

We want to express our sincere gratitude and appreciation to all the therapists from the Institute of Therapy, Landesklinikum Mauer (Neuropsychiatric Hospital Mauer) as well as the employees of the Karl Landsteiner Institute and their relatives for generously supporting this study with their participation.

Author contributions: H. Baran designed the study. H. Baran and C. Kronsteiner carried out the analysis and B. Kepplinger contributed to the analysis. H. Baran evaluated the data and wrote the first draft of the manuscript. All authors contributed to and have approved the final manuscript.

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In memory of Primar Berthold Kepplinger MD, MSc

On 31 December 2019, the neurologist and psychiatrist Berthold Kepplinger MD, MSc died at the age of 73. Dr. Kepplinger was a pioneer in both neurorehabilitation and pain treatment and was a renowned educator who taught several generations of Austrian neurologists and anaesthesiologists. He was the head of the acute neurology department and stroke unit at Amstetten General Hospital as well as the neurology and neurorehabilitation department and pain treatment clinic at Neuropsychiatric Hospital Mauer (later renamed Landesklinikum Mauer), where he also served as medical director. In 1988, together with his enthusiastic team comprising Dr. H. Papst, Dr. C. Derfler, Dr. P. Kalina, Dr. H. Schmid, Dr. F. Memelauer, Dr. H. Imb, Dr. M. Winninger, Dr. C. Allen and Dr. G. Rettensteiner, Dr. Kepplinger succeeded in establishing CT diagnostics at Mauer and thereby also started CT-targeted interventions for pain therapy. During 1988, Dr. Kepplinger was one of the first representatives of the 'conservative' discipline in Austria, implanting epidural pain stimulation probes and spinal pain pumps, either by himself or with Dr. A. Kainz, and the work was very successful. Dr. Kepplinger was also instrumental in establishing magnetic resonance imaging for diagnostic and therapy in 1993. Dr. Kepplinger' dream and goal were to establish a pioneering pain clinic, which was ultimately achieved thanks to, as he always said, "the great effort and excellent work of his team of doctors," including Dr. H. Papst, Dr. H. Erhart, Dr. D. Schafelner, Dr. D. Zeiner, Dr. J. Wallner, Dr. A. Kainz, Dr. S. Eigner, Dr. P. Kalina, Dr. A. Barwari, Dr. R. Badawi-Nagy, Dr. K. Nescak, Dr. E. Pallinger and Dr. J. Reuss. Dr. Kepplinger also established great working relationships with other departments and hospital administrators, including Ms. I. Halla, the head of Medical-Technical Services as well as the hospital's 40 therapists who cooperated in his research work.

As an enthusiastic supporter of interdisciplinary access to pain research and pain therapy, starting in 1988 Dr. Kepplinger, together with Dr. H. Schmid, regularly invited nationally and internationally renowned pain experts such as Dr. Albert D. Ray, Prof. Dr. Endre Csanda, Prof. Dr. Guenter Corssen, Prof. Dr. Ahmet E. Oygar, Prof. Dr. Heber Ferraz-Leite, Prof. Dr. Sinerik Ayrapetyan, Dr. Paul Leonard, Prof. Dr. Dieter Klingler, Prof. Dr. Wilfried Ilias, Prof. Dr. Josef Donnerer, Prim. Dr. P. Pauly, Prof. Dr. Rudolf Likar, and Prof. Dr. Leopold Saltuary and many other excellent specialists to interdisciplinary symposia in Lackenhof and Mauer Öhling, Lower Austria, and published the scientific contributions presented there as conference proceedings (Pain - Clinical Aspects and Therapeutical Issues I, II, III and IV, Edition *Selva Verlag).*

Dr. Kepplinger combined many medical meetings with art exhibitions to showcase paintings featuring interesting ways of expressing pain.

In 2005, Dr. Kepplinger and I established the Karl Landsteiner Research Institute for Neurorehabilitation and Pain Treatment Mauer with the Neurochemical Laboratory at the Neuropsychiatric Hospital Mauer. The institute is dedicated to basic research in neuropsychiatry as well as the scientific processing of topics related to neurorehabilitation and pain therapy. Dr. Kepplinger performed stochastic resonance therapy and repetitive transcranial magnetic stimulation at Landesklinikum Mauer. The neurochemical basis for the effects of these treatments on depression, pain and spasms informed the doctoral thesis of Dr. Sedlnitzky-Semler, which was examined in cooperation with Veterinary Medical University. Many other scientists from Veterinary Medical University Vienna, including Dr. M. Draxler, Dr. M. Attam, Dr. M. Bertignol, Dr. L-M Glenk and Dr. B. Semler-Sedlnicky, as well as Julius Maximilians University Würzburg Mag. C. Kronsteiner successfully completed their education while working at the University and at the Karl Landsteiner Research Institute Mauer. Mag. Kronsteiner is now working on tryptophan metabolism in the snail Helix pomatia during aging process and for her doctoral thesis she is investigating the influence

of the tryptophan metabolite kynurenic acid and anti-dementia substances on learning behaviour and memory in Helix pomatia memory model.

The subject of one of our major studies was the role that tryptophan metabolites in cerebrospinal fluid and serum play in various neuropsychiatric disorders and the aging process. We demonstrated a significant age-correlated increase in the concentration of kynurenic acid in the cerebrospinal fluid. This work was published under the title, 'Age-Related Increase of Kynurenic Acid in Human Cerebrospinal Fluid - IgG and ß2-Microglobulin Changes', in *Neurosignals* (2005; 14: 126–135). The authors included Dr. Kepplinger, Dr. Kainz, Dr. Ferraz-Leite, Dr. Newcombe, Dr. Kalina and myself. An extended investigation of tryptophan metabolites in CSF during the aging process revealed that not only the level of kynurenic acid, but also that of L-tryptophan and the biological precursor of kynurenic acid L-kynurenine increase with advancing age. This work, under the title "Increased Levels of Kynurenic Acid in the Cerebrospinal Fluid in Patients with Hydrocephaluswas," was also published in *Neurosignals* (2019, 29:1–11), and was authored by Dr. Kepplinger, Mag. Kronsteiner, Dr. Reuss and myself. Interestingly, we found increased kynurenic acid levels in cerebrospinal fluid in hydrocephalus patients with dementia.

Kynurenic acid has been known for over 40 years as a neuroprotective, anticonvulsant metabolite that influences glutamatergic and cholinergic activity. Research in the field of dementia has identified a deficit in cholinergic activity, particularly in the limbic system and in the frontal lobe of the brain, and in 1999 we showed that metabolism of kynurenic acid is increased in most brain regions in Alzheimer's patients ("Kynurenine metabolism in Alzheimer's disease" by Baran et al., J Neural Transm, 1999: 106: 165-181). The results of this study led to the conclusion that declining memory capacity in old age may be caused by an increase in kynurenic acid in the brain, given that kynurenic acid blocks both glutamatergic and cholinergic activity. Substances capable of curbing excessive kynurenic acid production in aging brains with clinical signs of dementia may be the next candidates' anti-dementia therapy. Indeed, in our work we have shown that certain anti-dementia medications (D-cycloserine or cerebrolysin) as well as natural remedies (Hawthorn berry extract or Jerusalem balsam) lower the production of kynurenic acid.

Berthold Kepplinger not only was an empathic, skilled physician and a visionary scientist, but also was intimately involved in various medical societies such as the Austrian Vegetative State/Wachkoma Society and the Austrian Pain Society. He was a member of the editorial board of the magazine 'Pain News' from 2004 to 2007 and from 2012 to 2016 and secretary of the Austrian Pain Society from 2009 to 2013.

His humanity, his sensitivity and, above all, his sense of responsibility were particularly evident during the incident in Austria known as the "Fritzl case," during which he pleaded with the media and the paparazzi to "finally allow the family to rest, to find themselves."

Dr. Kepplinger's charisma, knowledge, reliability and calm demeanour were always evident to the physicians he trained and the patients he treated.

His beautiful paintings mostly produced as a night-time hobby concerned the facial expressions related to various emotions. He was a wonderful and kind person with a great sense of humour, who was fully committed and devoted to the work, friendships and love he created.

Dr. Kepplinger will be deeply missed by all who knew him.

Professor Halina Baran, PhD

Disclosure Statement

The authors declare no conflict of interest exists.

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