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N-Acetylcysteine Inhibits Kynurenine Aminotransferase II

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Abstract

The tryptophan metabolite kynurenic acid (KYNA) may play an important role in normal and abnormal cognitive processes, most likely by interfering with α7 nicotinic and NMDA receptor function. KYNA is formed from its immediate precursor kynurenine either by non-enzymatic oxidation or through irreversible transamination by kynurenine aminotransferases. In the mammalian brain, kynurenine aminotransferase II (KAT II) is the principal enzyme responsible for the neosynthesis of rapidly mobilizable KYNA, and therefore constitutes an attractive target for pro-cognitive interventions. N-acetylcysteine (NAC), a brain-penetrant drug with pro-cognitive efficacy in humans, has been proposed to exert its actions by increasing the levels of the anti-oxidant glutathione (GSH) in the brain. We report here that NAC, but not GSH, inhibits KAT II activity in brain tissue homogenates from rats and humans with IC_{50} values in the high micromolar to low millimolar range. With similar potency, the drug interfered with the *de novo* formation of KYNA in rat brain slices. NAC was a competitive inhibitor of recombinant human KAT II (Ki: 450 μM). Furthermore, GSH failed to S-glutathionylate recombinant human KAT II treated with the dithiocarbamate drug disulfiram. Shown by microdialysis in the prefrontal cortex of rats treated with kynurenine (50 mg/kg, i.p.), peripheral administration of NAC (500 mg/kg, i.p., 120 and 60 min before the application of kynurenine) reduced KYNA neosynthesis by ~50%. Together, these results suggest that NAC exerts its neurobiological effects at least in part by reducing cerebral KYNA formation via KAT II inhibition.

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Keywords

Cognition; Glutathione; Kynurenic acid; Schizophrenia

Introduction

Kynurenic acid (KYNA), a metabolite of the kynurenine pathway (KP) of tryptophan degradation, is increasingly understood to play a significant role in the mechanism(s) that control normal and abnormal cognition. This effect of endogenous KYNA is most likely related to its ability to influence the function of α7 nicotinic acetylcholine (α7nACh) and NMDA receptors, both of which are critically involved in cognitive processes (Timofeeva & Levin 2011; Schwarcz et al. 2012; Yang et al. 2013). In rodents, elevated brain KYNA levels produce cognitive deficits, including impairments in working memory, sensorimotor gating, and attentional processing (Shepard et al. 2003; Erhardt et al. 2004; Chess & Bucci 2006; DeAngeli et al. 2014; Pershing et al. 2015). Conversely, genetic or pharmacological manipulations resulting in a reduction in brain KYNA have pro-cognitive effects in experimental animals (Potter et al. 2010; Pocivavsek et al. 2011; Kozak et al. 2014).

Interestingly, cognitive impairments similar to those caused by KYNA in experimental animals are a core feature of schizophrenia (SZ), leading to poor quality of life in patients (Green 1996; Lewis et al. 2004; de Bartolomeis et al. 2013). As increased levels of KYNA's immediate bioprecursor kynurenine, as well as of KYNA itself, are seen in the brain and cerebrospinal fluid of people with SZ (Erhardt et al. 2001; Schwarcz et al. 2001; Miller et al. 2006; Sathyasaikumar et al. 2011; Linderholm et al. 2012), the behavioral observations made in animals may therefore have translational relevance. Notably, a possible role of cerebral KP metabolism in SZ is also indicated by studies showing increased expression of tryptophan-2,3-dioxygenase (Miller et al. 2006) and reduced kynurenine 3-monooxygenase (KMO) expression and activity (Wonodi et al. 2011; Sathyasaikumar et al. 2011) in the brain of SZ patients. Alone or together, these enzymatic changes may account for increased KYNA formation, further supporting a possible causal role of this metabolite in pathophysiology (Schwarcz et al. 2012). Based on these findings and considerations, kynurenine aminotransferase II (KAT II), the principal enzyme responsible for the synthesis of rapidly mobilizable KYNA in the mammalian brain (Guidetti et al. 2007), is viewed as an attractive target for pro-cognitive interventions in SZ and other major brain diseases (Oxenkrug, 2013; Plitman et al. 2017).

N-acetylcysteine (NAC), an acetylated derivative of the amino acid L-cysteine, has multiple pharmacological characteristics, including antioxidant, neurotropic, and antiinflammatory properties (Samuni et al. 2013; Bavarsad Shahripour et al. 2014; Ooi et al. 2018). Presumably related to one or more of these mechanisms, NAC influences glutamatergic neurotransmission (Olive et al. 2012) and provides cognitive benefits in experimental animals (Otte et al. 2011; Cao et al. 2012).

Because of its oral bioavailability and excellent safety profile (Sheffner et al. 1966; Samuni et al. 2013; Deepmala et al. 2015), NAC has been widely used in humans, especially in efforts to ameliorate cognitive deficits in various psychiatric and neurological diseases

(Skvarc et al. 2017). Specifically, treatment of persons with SZ with NAC for weeks, months or even longer has beneficial effects on working memory (Conus et al. 2018; Sepehrmanesh et al. 2018), processing speed (Rapado-Castro et al. 2017), auditory processing (Retsa et al. 2018) and mismatch negativity (Lavoie et al. 2008). In most of these studies, the improvement of neurocognitive impairments was attributed to the fact that its deacetylation product L-cysteine serves as a bioprecursor of the antioxidant glutathione (GSH), in turn leading to increased glutamate release (Rushworth & Megson 2014; Steullet et al. 2016; Klauser et al. 2018; McQueen et al.2018). Additional or alternative mechanisms of action have been considered as well, however, but have not been assessed experimentally so far (Samuni et al. 2013). Based on the rationale outlined above, we now designed *in vitro* and *in vivo* experiments to explore the possibility that the pharmacological effects of NAC may also involve effects on KYNA biosynthesis.

Materials and Methods

Chemicals and materials

KYNA, L-kynurenine ("kynurenine"), 3-hydroxykynurenine (3-HK), aminooxyacetic acid (AOAA), NAC, GSH, MS grade porcine trypsin and Millipore C18 ZipTips were purchased from Sigma (St. Louis, MO, USA). Endoproteinase Glu-C, LC-MS grade water, acetonitrile, and Optima grade formic acid were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Other chemicals were obtained from a variety of suppliers, as specified below, and were of the highest commercially available purity. All chemicals used in enzyme assays, including kynurenine, NAC and GSH, were dissolved in saline, and the solutions were adjusted to pH 6.8 before experimental use.

Recombinant human KAT II

The expression plasmid (Genscript, Piscataway, NJ, USA) containing human KAT II (hKAT II) as C-terminal intein (chitin binding domain) fusion, in pTXB1 vector, was transformed into BL21 (DE3) cells (Thermo Fisher Scientific). Cells were grown in 2YT media (Thermo Fisher Scientific) at 37°C until the optical density reached 0.6 (measured at 600 nm), and induced with 300 μM isopropyl 2-D-1-thiogalactopyranoside for 18-20 h at 18°C. The cells were then harvested by centrifugation (2,700 x g, 20 min), and the pellet was 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.

Analysis of KAT II by mass spectrometry

Gel-separated hKAT II was de-stained and sequentially digested with trypsin and Glu-C. The resulting peptides were extracted, desalted with C18 ZipTips, and dried. The peptides were then solubilized in 2% acetonitrile and 0.2% formic acid, and pressure-loaded on a 75 μm (i.d.) x 25 cm C18 Acclaim PepMap RSLC analytical column (2 μm, 100 A; Thermo Fisher Scientific). The peptides were separated using a 180 min gradient of 5-40% solvent B (Solvent A: 0.1% formic acid; Solvent B: 80% acetonitrile, 0.1% formic acid) at a flow of 200 nl/min with an EASY-nano 1200 UHPLC in line with an Orbitrap Fusion Lumos ETD UVPD mass spectrometer (Thermo Fisher Scientific). For data-dependent acquisition, a survey FT-MS scan in the orbitrap (mass range 375-1500 Da, 60,000 resolution) was utilized, followed by fragmentation with alternating high energy collision dissociation (HCD) and electron transfer dissociation (ETD) or ETD with 15% supplemental activation energy. HCD MS/MS spectra were acquired in the orbitrap with 30% collision energy, 7,500 resolution, 22 msec maximum injection time (MIT), isolation width of 1.6 Da, and automatic gain control (AGC) at 5.0e4. ETD scans were acquired in the orbitrap at 7,500 resolution, 100 msec MIT, isolation width of 1.6 Da, and AGC at 5.0e4. Additional analysis was performed using collision-induced dissociation MS/MS with fragments detected in the ion trap with a collision energy of 35%, an activation time of 10 msec, an isolation window of 1.6 Da, and an AGC of 1.0e4. MIT was set at 35 msec. Advanced peak determination and monoisotopic precursor selection were enabled. Ions with a +1 charge were excluded from selection. Dynamic exclusion was enabled with a repeat count of 2, a duration of 30 sec, an exclusion duration of 30 sec, and an exclusion mass tolerance of ± 10 ppm for all runs.

Database search

The raw files were searched using Mascot (version 2.4.1) within the Proteome Discoverer 1.4 platform (Thermo Fisher Scientific) against KAT II and digestion enzymes. Parameters for peptide identification were as follows: precursor mass tolerance of 20 ppm, fragment mass tolerance of 0.01 Da for orbitrap MS/MS or 0.5 Da for ion trap MS/MS, 2 missed cleavages, and the following variable modifications on cysteines: S-glutathionylation, conversion to dehydroalanine (Cys -> Dha), and sulfide addition. Oxidation of methionine was included as a variable modification. The raw data were further searched using MaxQuant (v. 1.6.3.3) (Max Planck Institute, Martinsried, Germany) and BioPharma Finder 3.0 (Thermo Fisher Scientific) using the same variable modifications for disulfide bonds. Tandem mass spectra of interest were inspected manually.

Human brain tissue

Brain specimens from the ventral anterior cingulate cortex (Brodmann area 24) were obtained from 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. Tissue donors (n = 4) were free of neurological or psychiatric disorders at the time of death.

Animals

Adult male Sprague-Dawley rats (250-300 g; Charles River Laboratories, Kingston, NY, USA) were used in the *in vivo* experiments. 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. All *in vitro* and *in vivo* studies involving rats were designed in a random fashion and performed by an individual who was unaware of the experimental outline.

Enzyme assays

Kynurenine aminotransferase (KAT II)—Pure recombinant human KAT II protein was thawed and diluted 500 times before performing the assay. Five μl of this solution were then incubated at 37°C for 2 h with kynurenine (10 μM final concentration) using Tris-acetate buffer (150 mM, pH 7.4) containing pyruvate (1 mM) and pyridoxal-5'-phosphate (80 μM), in a total volume of 200 μl. To examine the effect NAC or GSH, the test compounds were added to the incubation mixture in 20 μl aliquots (pH 6.8). Blanks were obtained by the addition of AOAA (1 mM final concentration). The reaction was terminated by the addition of 20 μl of 50% (w/v) trichloroacetic acid and 1 ml of 0.1 M HCl, and the precipitated protein was removed by centrifugation (16,000 x g, 10 min). Twenty μl of the supernatant were applied to a 3 μm C_{18} reverse phase column (BDS Hypersil; 100 mm x 4.6 mm; Thermo Fisher Scientific), and high performance liquid chromatography (HPLC) was performed 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) (Shibata 1988). The retention time of KYNA was ~8 min.

For kinetic analysis, the concentration of kynurenine in the assay was varied between 0.5 and 1.5 mM (final concentration), and incubation was performed in the absence (control) or presence of NAC (1 mM final concentration), as detailed above.

Rat and human brain tissues were weighed while frozen and then homogenized (1:5, w/v) by sonication (Branson Ultrasonics; Danbury, CT, USA) in ultrapure water. Tissues were further diluted (1:2, v/v) in 5 mM Tris-acetate buffer (pH 8.0) containing pyridoxal-5'-phosphate (50 μM) and 2-mercaptoethanol (10 mM). Eighty μl of the homogenate were then incubated at 37°C for 2 h with kynurenine (100 μM final concentration) using Tris-acetate buffer (150 mM, pH 7.4) containing pyruvate (1 mM) and pyridoxal-5'-phosphate (80 μM), in a total volume of 200 μl. As in the experiments with human recombinant protein (see above), the effect NAC or GSH was assessed by adding 20 μl aliquots of the test compounds (pH 6.8), and blanks were obtained using AOAA (1 mM final concentration). The reaction was terminated, and KYNA levels were quantified, using the same protocol as described above.

Kynurenine 3-monooxygenase (KMO)—Rat and human brain tissues were weighed while frozen and then homogenized (rat: 1:15, w/v; human: 1:25, w/v) by sonication

(Branson Ultrasonics) in 100 mM Tris–HCl buffer (pH 8.1) containing 10 mM KCl and 1 mM EDTA. Eighty μl of the preparation were incubated for 40 min at 37°C in a solution containing 1 mM NADPH, 3 mM glucose-6-phosphate, 1 U/ml glucose-6 phosphate dehydrogenase, 100 μM kynurenine, 10 mM KCl and 1 mM EDTA, in a total volume of 200 μl. To examine the effect of NAC, 20 μl of the drug solution (pH 7.8) were added to the incubation mixture. Blanks were obtained by adding the KMO inhibitor Ro 61-8048 (final concentration: 100 μM). The reaction was stopped by the addition of 50 μl of 6% perchloric acid. After centrifugation (16,000 x g, 15 min), 20 μl of the supernatant were applied to a 3 μm HPLC column (HR-80; 80 mm x 4.6 mm; ESA, Chelmsford, MA, USA), using a mobile phase consisting of 1.5% acetonitrile, 0.9% triethylamine, 0.59% phosphoric acid, 0.27 mM EDTA and 8.9 mM sodium heptane sulfonic acid, and a flow rate of 0.5 ml/min. In the eluate, the reaction product, 3-HK, was detected electrochemically using a HTEC 500 detector (Eicom Corp., San Diego, CA, USA; oxidation potential: +0.5 V) (Heyes & Quearry 1988). The retention time of 3-HK was ~10 min.

Experiments with tissue slices

Rats were euthanized using a CO_2 chamber and decapitated. The brain was rapidly removed from the skull, and the entire cortex was dissected out on ice. The tissue was placed on plastic discs, and brain slices (1 mm x 1 mm) were prepared with a Mcllwain chopper (Mickle Laboratory Engineering, Gomshall, UK). The slices were immediately immersed in a container filled with freshly oxygenated Krebs-Ringer buffer (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 and 3 mM Na_2HPO_4; pH 7.4) and maintained on ice.

Two cortical slices were placed in each well of a Falcon 48-Well Cell Culture Plate containing 90 μl of Krebs-Ringer buffer. Using tissue from a single rat for each experiment (n=4 animals), the slices were pre-incubated with various concentrations of NAC (0.03,.3 and 3 mM) or 1 mM AOAA for 10 min at 37°C and then incubated further on a shaking water bath for 2 h at 37°C in the presence of 2 μM kynurenine. Following incubation, the culture plate was immediately placed on ice, and the incubation medium was rapidly transferred to 0.5 ml tubes containing 10 μl 1 N HCl/25% perchloric acid. After centrifugation in a microfuge (18,000 x g, 10 min), the supernatant was diluted as needed, and 20 μl were subjected to HPLC for KYNA analysis (see above). The slices were re-suspended in 100 μl of ultrapure water and kept at −80°C for protein determination. Protein was measured according to the method of (Lowry et al. 1951) using bovine serum albumin as a standard.

In vivo microdialysis

Rats were anesthetized in a chamber filled with 5% isoflurane using a vaporizer and were then mounted in a stereotaxic frame (David Kopf, Tujunga, CA, USA). Anesthesia was maintained during the entire surgery period using a nose mask which continuously delivered 2.0-3.0% isoflurane mixed with oxygen. A guide cannula (MAB 2.14.G, SciPro Inc., Sanborn, New York, USA) was then positioned over the medial prefrontal cortex (mPFC; AP: 3.2 mm anterior to bregma, L: ±0.8 mm from the midline, V: 2.0 mm below the dura) and secured to the skull with anchor screws and acrylic dental cement. After surgery, the

animals were allowed to recover and were housed individually in acrylic cages with full access to food and water.

On the next day, a microdialysis probe (MAB 9.14.2, membrane length: 2 mm; SciPro) was inserted through the guide cannula. The probe was then connected to a microinfusion pump set to a speed of 1.1 μl/min, and the freely moving rats were perfused with Ringer solution (144 mM, NaCl; 4.8 mM, KCl; 1.7 mM, $CaCl_2$; 1.2 mM, $MgSO_4$; pH 6.7). Microdialysis samples were collected every 30 min. After the establishment of baseline conditions (2-3 h), animals received two intraperitoneal (i.p.) injections of either NAC (125 or 500 mg/kg, pH 6.8) or vehicle (0.9% saline, pH 6.8) 2 h and 1 h prior to the systemic administration of kynurenine (50 mg/kg, i.p.) (Harvey et al., 2008; Zmarowski et al., 2009; Konradsson-Geuken et al., 2010). Sample collection proceeded for 6 h after the kynurenine injection. Dialysates were diluted 1:2 (v/v) using ultrapure water, and 30 μl were subjected to HPLC for the analysis of KYNA, as described above. Data were not corrected for recovery from the microdialysis probe.

Statistical analysis

Sample sizes were chosen, and calculations were performed, in accordance with our previous studies (Zwarowski et al, 2009; Konradsson-Geuken et al., 2010; Bortz et al., 2017). Data are presented using box plots. All *in vitro* data were analyzed by oneway ANOVA followed by Tukey's test for pairwise multiple comparisons, using Graph Prism 8.02 software (GraphPad, San Diego, CA, USA). P values <0.05 were considered statistically significant. *In vivo* experiments were analyzed by two-way ANOVA, with one factor measured within subjects, i.e. with repeated measures. Post-hoc pairwise multiple comparisons were conducted using Bonferroni's adjusted p-values. Additionally, the area under the curve (AUC) was computed for each subject, and oneway ANOVA was performed followed by pairwise multiple comparisons using Bonferroni's adjusted p-values (*p<0.05 vs. kynurenine). IC_{50} values were calculated from dose-response curves using nonlinear fitting and Graph Prism 8.02 software (GraphPad).

Results

Effect of NAC and GSH on human recombinant KAT II

We first tested the effects of NAC and GSH on the activity of pure recombinant hKAT II. NAC inhibited the enzyme with an IC_{50} of ~500 μM whereas GSH had no effect up to a concentration of 3 mM (Fig. 1A). A separate experiment, assessing the effect of 1 mM NAC while the kynurenine concentration was varied from 0.5 mM to 1.5 mM, revealed that NAC inhibited KAT II activity competitively with a K_i value of 450 μM, calculated using a Lineweaver-Burk plot (Fig. 1B).

Treatment of recombinant hKAT II with GSH and the dithiocarbamate drug disulfiram (Rossi et al. 2006) did not induce S-glutathionylation of the low pK cysteine residue of the tryptic peptide, LCVTSGSQQGLCK (Fig. 2).

Effects on KAT II and KMO activity in rat and human brain homogenates

We next investigated the effects of NAC and GSH on rat and human KAT II activity in brain tissue homogenates. Qualitatively and quantitatively very similar results were obtained in both species. Thus, NAC inhibited the enzymes with IC_{50} values of ~2 mM (Fig. 3), i.e. the potency of the drug in cell-free brain homogenates was approximately 4 times lower than in tests using recombinant human KAT II (cf. Fig. 1). In contrast, 3 mM GSH failed to affect the activity of either rat or human KAT II in tissue homogenates (Fig. 3). As GSH was ineffective in all these experiments, only NAC was used in subsequent studies.

NAC had no effect on the activity of KMO in tissue homogenate of either rat or human brain up to a concentration of 3 mM (data not shown).

Effect of NAC on KYNA neosynthesis in rat brain slices

The next experiments were designed to determine the effect of NAC on KYNA formation in rat cortical slices that were incubated with a physiological concentration of kynurenine (2 μM). The concentration of newly produced KYNA recovered from the medium after the 2-h incubation of control tissue (501 ± 43 fmoles/h/mg protein) was in line with previous data (Turski et al. 1989). Similar to the results obtained using tissue homogenate (Fig. 3), addition of NAC reduced the concentration of newly produced KYNA in the extracellular milieu in a dose-dependent manner, with an IC_{50} of approximately 3 mM (Fig. 4).

Effect of NAC on KYNA neosynthesis in vivo

Finally, we examined the ability of NAC to affect the *de novo* formation of KYNA in the mPFC of freely moving rats *in vivo*. As shown previously (Alexander et al. 2012), systemic administration of kynurenine (50 mg/kg, i.p.) increased extracellular KYNA levels, reaching a maximum (44.5 ± 3.9 nM) 2 h following the injection (n = 10) (Fig. 5).

To assess the effects of a high dose of NAC – to duplicate the effects of the high doses needed to provide benefits in humans (Lavoie et al. 2008) – we administered 125, 500 or 1000 mg/kg of the drug i.p. 1 h before kynurenine (50 mg/kg) in a pilot experiment (n = 3 each). While the two lower doses had no significant effect on the neosynthesis of KYNA (data not shown), 1000 mg/kg NAC resulted in adverse behavioral effects and evident signs of pain (squinting eyes, reluctance to move, piloerection and aggressive behavior). We therefore changed the pre-treatment schedule, administering 500 mg/kg NAC i.p. twice, i.e. 2 h and 1 h prior to the systemic administration of kynurenine (n = 7). This procedure, which did not trigger any obvious behavioral problems, prevented the kynurenine-induced increase in extracellular KYNA levels by ~50% ($p < 0.001$). Using the same paradigm, a lower dose of NAC (2 x 125 mg/kg, i.p.) caused a smaller, ~27% reduction in extracellular KYNA levels ($p < 0.05$; n = 7) (Fig. 5). These results were confirmed when we analyzed the areas under the curve (AUCs) of newly produced KYNA that was recovered during the first 4 h after kynurenine administration (Fig. 5, inset).

Discussion

Using several complementary experimental approaches *in vitro* and *in vivo*, the present study provided evidence that NAC, a drug with pro-cognitive, anti-inflammatory and neurotropic properties (Dean et al. 2011; Breier et al. 2018; Conus et al. 2018; Ooi et al. 2018; Yolland et al. 2019), can inhibit the neosynthesis of the endogenous neuromodulator KYNA. Notably, this effect does *not* appear to be related to NAC's well-established role as a precursor of GSH. Our results therefore suggest that a previously unrecognized effect, namely a reduction of KYNA levels in the brain, may account at least in part for the therapeutic properties of NAC.

Considering the possible translational implications of our hypothesis, and in view of the fact that KAT II is most relevant for the enzymatic formation of KYNA in the mammalian brain (Guidetti et al. 1997) we first examined the effect of NAC on human recombinant KAT II. NAC inhibited the enzyme with an IC_{50} of ~500 μM while GSH did not reduce KAT II activity up to a concentration of 3 mM. This was a relevant distinction since the beneficial clinical effects of NAC are commonly ascribed to the antioxidant effect of its metabolic product GSH, which can modulate the function of various proteins by binding directly to redox-sensitive sites (Chan et al. 2001). Of note in this context, GSH uses NAC-derived cysteine for biosynthesis (Rushworth & Megson 2014; Yolland et al. 2020).

To study the possible relationship of GSH and KAT II in greater depth, we next attempted to S-glutathionylate the pure human protein with GSH and the dithiocarbamate drug disulfiram (Rossi et al. 2006). The highly reactive sulfhydryl group of disulfiram undergoes thiol-disulfide exchange with select protein sulfhydryl groups on low pK cysteine residues and in the presence of GSH, thereby forming GSH-protein adducts, i.e. causing S-glutathionylation (Rossi et al. 2006; Xiong et al. 2011; Hedges et al. 2018). We and others have, in fact, shown S-glutathionylation of proteins with disulfiram and GSH both in vitro and ex vivo (Rossi et al. 2006; Hedges et al. 2018). The fact that we were unable to demonstrate that S-glutathionylation had taken place under these experimental conditions therefore provided direct evidence that GSH does not affect KAT II directly. Finally, given that other cysteine redox posttranslational changes also require low pK cysteine residues (Uys et al., 2011), one would expect a similar lack of effect of other modifications, such as S-nitrosylation. We therefore posit that the competitive inhibition of KAT II by NAC shown here is unrelated to a modification of cysteine residues in the protein.

Experiments using cortical tissue homogenates revealed quantitatively very similar efficacies of NAC to inhibit KAT II activity in rat and human brain, whereas GSH had no effects in either species. These data are in line with the results obtained using the recombinant human enzyme and further support the conclusion that the inhibition of KAT II by NAC does not involve its conversion to GSH.

We next confirmed that NAC also interferes with the production of KYNA from kynurenine in freshly dissected rat brain tissue slices and then proceeded to test the effectiveness of NAC, which readily crosses the blood-brain barrier after systemic administration (Sheffner et al. 1966; Farr et al. 2003; Deepmala et al. 2015), using *in vivo* microdialysis. As in the *in*

vitro studies, the experiments were designed to investigate the ability of NAC to interfere with the neosynthesis of KYNA from its immediate bioprecursor kynurenine, which reliably causes rapid increases in extracellular KYNA levels in the mPFC and other brain areas following systemic administration in rats (Swartz et al. 1990; Zmarowski et al. 2009). After performing pilot experiments to optimize the experimental paradigm, pre-treatment with NAC was indeed found to dose-dependently reduce the kynurenine-induced acute elevation of KYNA *in vivo*.

Because of its ability to inhibit the function of both alpha7 nicotinic and NMDA receptors, i.e. two receptors which are thought to act synergistically to control cognitive processes (Timofeeva & Levin 2011; Nikiforuk et al. 2016), elevated levels of KYNA have been proposed to be causally involved in the cognitive impairments seen in people with SZ (Erhardt et al. 2009; Wonodi & Schwarcz 2010). As cognitive dysfunctions are a core domain of the pathophysiology of SZ and a major determinant of disability and poor functional outcomes in the disease (Green 1996), and as treatment with antipsychotic drugs and other pharmacological approaches provide only very limited benefits in this regard (Keefe et al. 2015), the idea of attaining pro-cognitive effects by reducing brain KYNA levels has recently attracted considerable interest (see Plitman et al. 2017, for review). As detailed earlier (cf. Introduction), the theoretical construct of this hypothesis is supported by an impressive number of animal experiments, and both pharmacological and genetic studies have pinpointed KAT II as an optimal target for KYNA synthesis inhibition in the brain (Potter et al. 2010; Pocivavsek et al. 2011; Kozak et al. 2014). The ability of NAC to inhibit KAT II activity *in vitro* and to interfere with the *de novo* formation of KYNA *in vivo* may therefore indicate a significant role of this effect in the established pro-cognitive effects of the drug (Berk et al. 2013; Breier et al. 2018; Conus et al. 2018; McQueen et al. 2018).

Notably, our new findings do not suggest or imply that the inhibition of brain KYNA production should be considered the lone mechanism by which NAC counteracts cognitive impairments. There is ample evidence that the conversion of NAC to GSH, which is decreased in SZ (Altuntas et al. 2000; Do et al. 2000; Dodd et al. 2008; Gawryluk et al. 2011), the NAC-induced prevention of cytokine production (Tsai et al. 2009; Csontos et al. 2012), and the reduction in oxidative stress associated with these phenomena, are also of substantial importance. Interestingly, all these effects, as well as a reduction in cerebral KYNA, result in an increase in extracellular glutamate levels (see (Schwarcz 2016) for review), which are known to promote cognitive functions in health and disease (Goff & Coyle 2001; Robbins & Murphy 2006; Bridges et al. 2012). Enhanced glutamatergic neurotransmission, the common outcome of multiple modes of action of NAC, may therefore account for the pro-cognitive properties of the drug.

The fact that NAC interferes with the neosynthesis of KYNA supports the prevailing assumption that astrocytes play a critical role in the effects of NAC in the brain. This was originally concluded from the demonstration that astrocytes readily accumulate NAC from the extracellular milieu (Kranich et al. 1998) and that these cells are essential for the production of GSH from its precursor L-cysteine, the deacetylation product of NAC (Hertz & Zielke 2004; McBean et al. 2017). Notably, the present study suggests an additional mechanism, namely direct interference of NAC with the activity of KAT II, which is

exclusively contained in astrocytes in the rat brain (Guidetti et al. 2007). To elaborate this and other mechanistic options in greater depth, experiments currently in progress in our laboratory are designed to investigate possible effects of L-cysteine and various other molecular processes by which acute or prolonged treatment with NAC may directly or indirectly down-regulate the *de novo* formation of KYNA from kynurenine (Guidetti et al. 1997; Blanco Ayala et al. 2015).

In summary, our new findings showed that KAT II, which is responsible for the formation of rapidly mobilizable KYNA in the mammalian brain, is a *bona fide* target of NAC. As KAT II inhibition has emerged as an attractive novel strategy to overcome cognitive deficits in SZ and other major brain diseases (Oxenkrug, 2013), this mechanism may participate in the beneficial neuropharmacological effects of the drug.

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Frequently used abbreviations:

α7nACh	α7 nicotinic acetylcholine
GSH	Glutathione
3-HK	3-Hydroxykynurenine
KAT	Kynurenine aminotransferase
KMO	Kynurenine 3-monooxygenase
KP	Kynurenine pathway
KYNA	Kynurenic acid
mPFC	medial prefrontal cortex
NAC	N-acetylcysteine
SZ	Schizophrenia

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Highlights:

- N-acetylcysteine (NAC) inhibits kynurenine aminotransferases II (KAT II) in brain homogenates from rats and humans *in vitro*.
- NAC competitively inhibits recombinant human KAT II protein with a K_i of 450 μM, whereas glutathione (GSH) is ~40 times less potent.
- GSH fails to S-glutathionylate recombinant human KAT II after treatment with the dithiocarbamate drug disulfiram.
- NAC affects the de novo formation of kynurenic acid in the medial prefrontal cortex of freely moving rats *in vivo*.

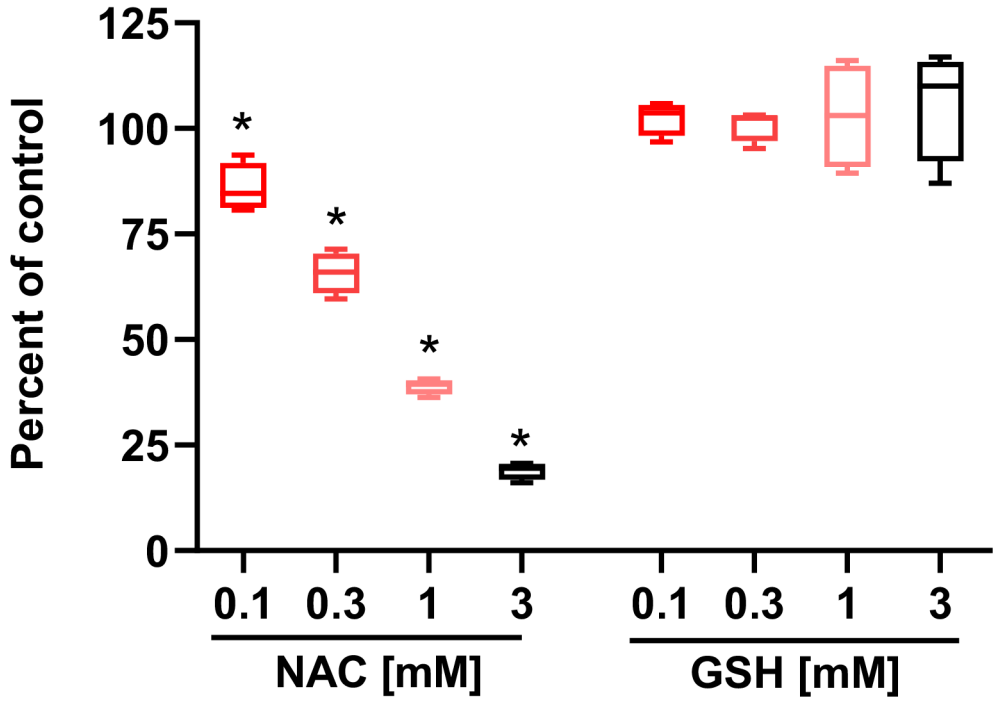

Figure 1A: Effect of NAC and GSH on human recombinant KAT II activity

Four separate experiments were performed as described in the text. Control activity was 0.8 ± 0.2 μmoles KYNA/h/mg protein. The box plots show first quartile, median and third quartile with whiskers from minimum to maximum*$p<0.05$ vs. control.

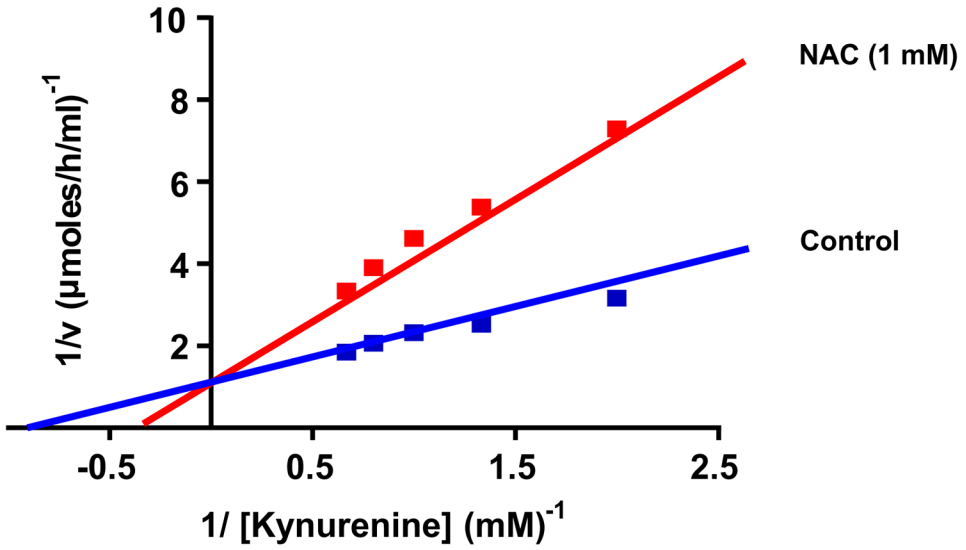

Figure 1B: Effect of NAC on human recombinant KAT II activity with varying substrate concentrations of kynurenine

Double-reciprocal representation of KAT II activity measured in the presence and absence of 1 mM NAC. Experiments were performed in duplicate, as described in the text, varying the kynurenine concentration between 0.5 mM and 1.5 mM.

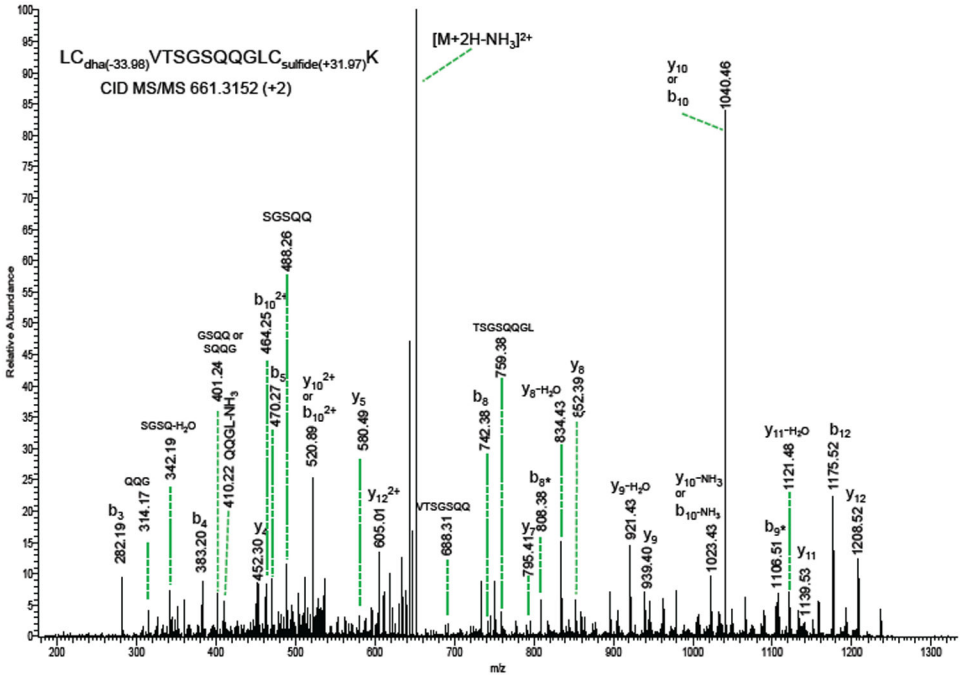

Figure 2: High energy collision dissociation (HCD) tandem mass spectrum of human recombinant KAT II treated with GSH and disulfiram

Experiments were performed as detailed in the text. The tandem mass spectrum of KAT II, residues X-X, is consistent with the presence of an intramolecular disulfide bond between the two cysteine residues. Fragmentation of the disulfide-linked cysteines resulted in the loss or retention of the sulfur yielding a dehydroalanine (−33.98) and sulfide (31.97), respectively. The asterisk indicates fragment ions in which the sulfur remained on the first cysteine.

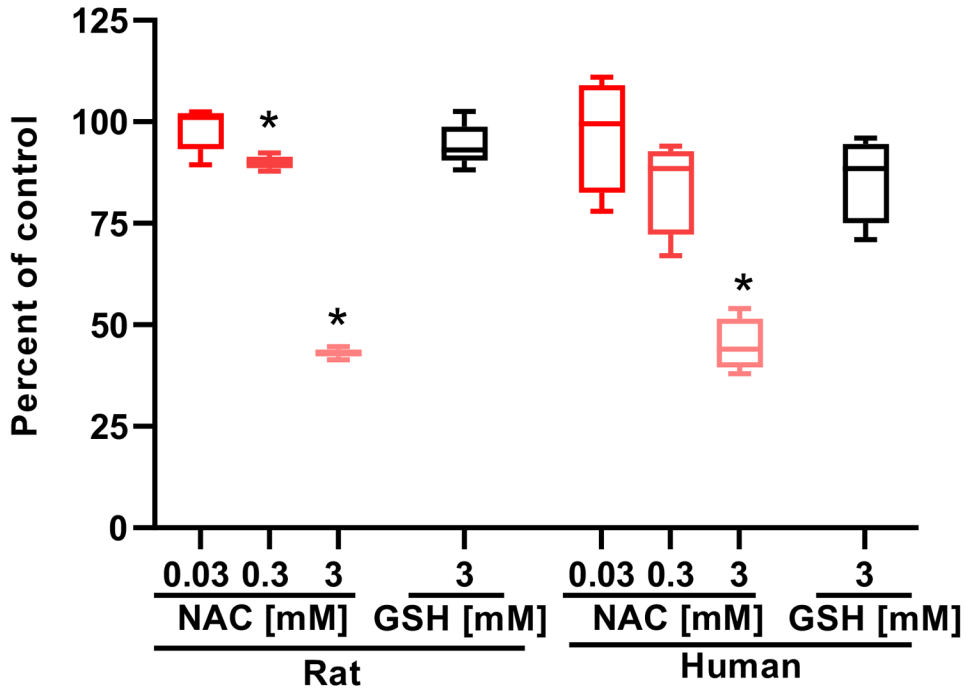

Figure 3: Effect of NAC and GSH on KAT II activity in rat and human brain homogenate

Experiments were performed as described in the text, using tissues from 5 rats and 4 human brains, respectively. The box plots show first quartile, median and third quartile with whiskers from minimum to maximum. Control activities were 443.3 ± 70.8 pmoles/h/mg protein (rat) and 56.7 ± 2.1 pmoles/h/mg protein (human), respectively. *$p<0.05$ vs. control.

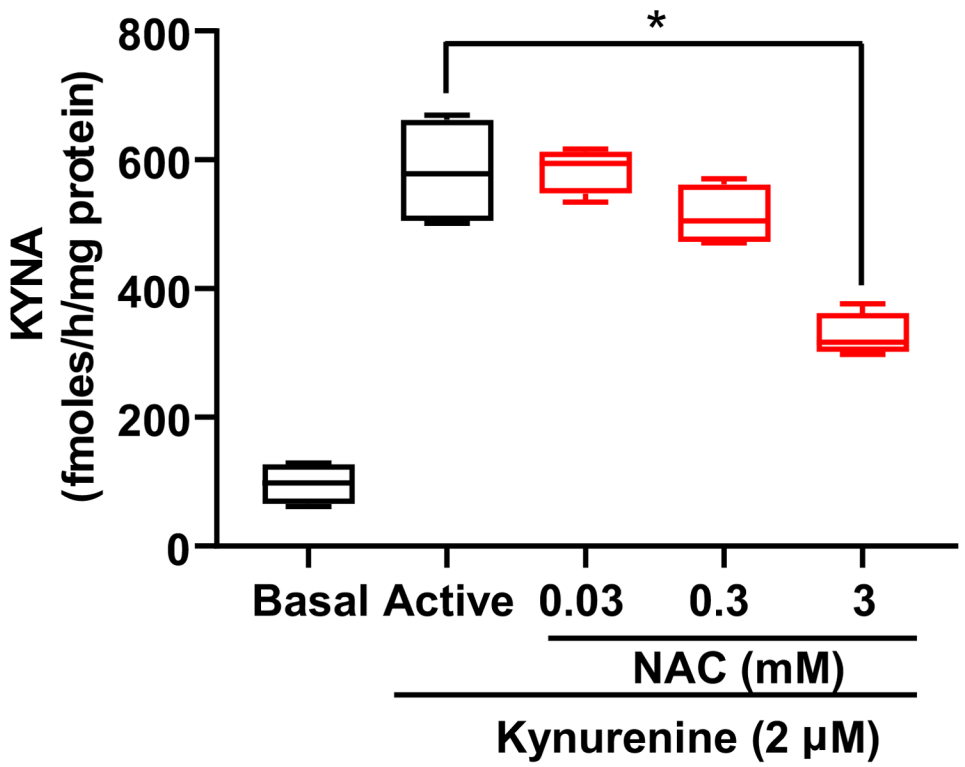

Figure 4: Effect of NAC on KYNA formation in rat cortical tissue slices

KYNA production and liberation was investigated using tissue slices exposed *in vitro* to 2 µM kynurenine for 2 h (see text for experimental details). The box plots show first quartile, median and third quartile with whiskers from minimum to maximum.. *p< 0.05 vs. control.

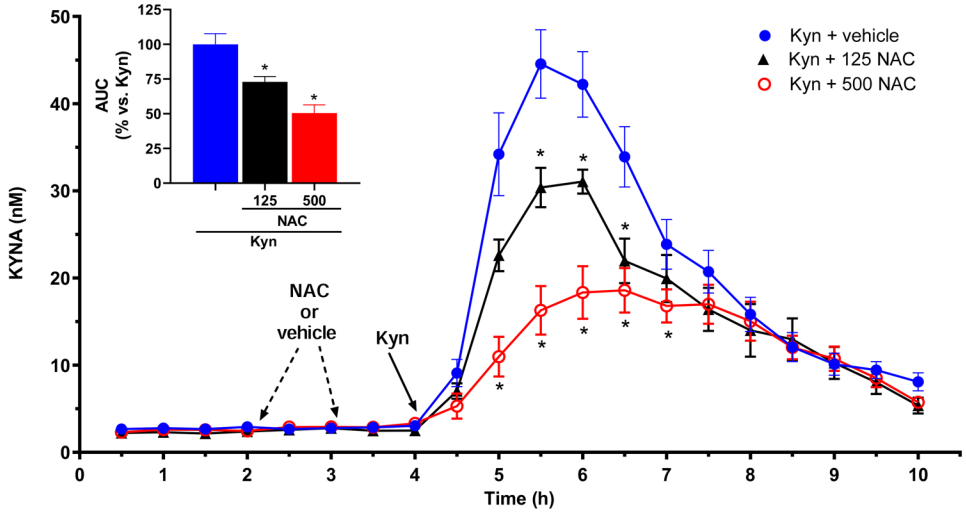

Figure 5: NAC attenuates the neosynthesis of KYNA in the rat mPFC *in vivo*

Microdialysis was performed in awake animals, as described in the text. After 2 h of baseline collection, NAC (125 or 500 mg/kg) or vehicle were administered i.p. twice, i.e. 2 h and 1 h prior to kynurenine (50 mg/kg, i.p.; Kyn) (arrows). Vehicle + Kyn (control): n = 10; NAC (125 mg/kg) + Kyn: n = 7; NAC (500 mg/kg) + Kyn: n = 7, F (2,21)= 9.116 Inset: area under the curve (AUC) of extracellular KYNA levels (mean ± SEM) recovered during 4 h after Kyn administration. *p < 0.05 vs. Kyn, F (2, 21) = 9.338.