NEUROSCIENCE
INTERNATIONAL BRAIN IBRO RESEARCH ORGANIZATION

RESEARCH ARTICLE

C. Song et al. / Neuroscience xxx (2017) xxx–xxx

Quantitative Analysis of Kynurenine Aminotransferase II in the Adult Rat Brain Reveals High Expression in Proliferative Zones and Corpus Callosum

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Abstract—Kynurenic acid, a metabolite of the kynurenine pathway of tryptophan degradation, acts as an endogenous antagonist of alpha7 nicotinic and NMDA receptors and is implicated in a number of neurophysiological and neuropathological processes including cognition and neurodegenerative events. Therefore, kynurenine aminotransferase II (KAT II/AADAT), the enzyme responsible for the formation of the majority of neuroactive kynurenic acid in the brain, has prompted significant interest. Using immunohistochemistry, this enzyme was localized primarily in astrocytes throughout the adult rat brain, but detailed neuroanatomical studies are lacking. Here, we employed quantitative *in situ* hybridization to analyze the relative expression of KAT II mRNA in the brain of rats under normal conditions and 6 h after the administration of lipopolysaccharides (LPSs). Specific hybridization signals for KAT II were detected, with the highest expression in the subventricular zone (SVZ), the rostral migratory stream and the floor of the third ventricle followed by the corpus callosum and the hippocampus. This pattern of mRNA expression was paralleled by differential protein expression, determined by serial dilutions of antibodies (up to 1:1 million), and was confirmed to be primarily astrocytic in nature. The mRNA signal in the SVZ and the hippocampus was substantially increased by the LPS treatment without detectable changes elsewhere. These results demonstrate that KAT II is expressed in the rat brain in a region-specific manner and that gene expression is sensitive to inflammatory processes. This suggests an unrecognized role for kynurenic acid in the brain's germinal zones. © 2017 Published by Elsevier Ltd on behalf of IBRO.

Key words: subventricular zone, astrocytes, doublecortin, lipopolysaccharides, *in situ* hybridization, tanycytes.

INTRODUCTION

Kynurenic acid (KYNA), a metabolite of the kynurenine pathway (KP) of tryptophan degradation, acts as an endogenous antagonist of alpha7 nicotinic and NMDA receptors in the brain (Perkins and Stone, 1982; Ganong et al., 1983; Kessler et al., 1989; Hilmas et al., 2001) and may also target additional recognition sites (Stone et al., 2013). Of special interest to neuroscience, and neural immune function, the compound is implicated in a number of neurophysiological and neuropathological processes (see, (Schwarcz and Stone, 2017) for review). For example, KYNA may be neuroprotective in disorders in which excitotoxicity is likely to play a causal role. This includes cerebral ischemia, epilepsy and neurodegenerative diseases such as Alzheimer's, Parkinson's and Huntington's disease (Schwarcz et al., 2012; Stone et al., 2012; Szalardy et al., 2012). On the other hand, elevated brain KYNA levels lead to cognitive impairments and an array of neurotransmitter abnormalities (Schwarcz et al., 2012; Pershing et al., 2016). This may explain the cognitive deficits seen in people with schizophrenia who show increased levels of KYNA in brain and cerebrospinal fluid (Erhardt et al., 2001; Schwarcz et al., 2001; Miller et al., 2008).

Because of these links to brain physiology, the biosynthesis of KYNA in the mammalian brain has been examined in considerable detail. So far, four aminotransferases have been shown to catalyze the irreversible transamination of the pivotal KP metabolite L-kynurenine ("kynurenine") to KYNA (Guidetti et al.,

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Abbreviations: AADAT, α-aminoadipate aminotransferase; cc, corpus callosum; DAB, 3,3′-diaminobenzidine; DCX, doublecortin; GFAP, glial fibrillary acidic protein; IWMNs, interstitial white matter neurons; KAT II/AADAT, kynurenine aminotransferase II; KP, kynurenine pathway; KYNA, Kynurenic acid; LPSs, lipopolysaccharides; ME, median eminence; PBS, phosphate-buffered saline; RMS, rostral migratory stream; SGZ, subgranular zone; SSC, saline citrate buffer; SVZ, subventricular zone; α7nACh, α7 nicotinic acetylcholine receptors.

https://doi.org/10.1016/j.neuroscience.2017.11.001
0306-4522/

1997, 2007; Han et al., 2010). Of these, kynurenine aminotransferase II [KAT II; α-aminoadipate aminotransferase (AADAT); (Tobes and Mason, 1975, 1977; Okuno et al., 1991)] is primarily responsible for the rapid "de novo" synthesis of KYNA in the brain (Amori et al., 2009); see (Schwarcz et al., 2012), for review, with biochemical studies revealing a wide distribution of enzymatic activity across rat brain regions (Guidetti et al., 1997). Of note, genetic elimination of KAT II (Yu et al., 2004; Potter et al., 2010) or selective pharmacological inhibition of KAT II (Wu et al., 2010; Kozak et al., 2014) improves cognitive functions in rodents. Due to this critical role of KAT II in regulating the levels and effects of KYNA in the brain, the structure, function and cellular distribution of this enzyme became a focus in neuroscience research (Guidetti et al., 1997; Goh et al., 2002; Guidetti et al., 2007; Potter et al., 2010).

The transcript for KAT II was initially identified in liver and kidney and later confirmed to be identical to the gene coding for the protein KAT II (Buchli et al., 1995; Yu et al., 1999; Han et al., 2010). Analyses of the mRNA sequence by our laboratory revealed an 88% homology between rats and mice and 76% conserved sequences between human and rat mRNA. Both rodent and human mRNAs contain mitochondrial leader cleavage signals and a conserved pyridoxal phosphate binding site, highlighting the evolutionary relevance of this protein. Using immunohistochemistry, KAT II was localized primarily in astrocytes throughout the adult rat brain (Guidetti et al., 2007), and KYNA formation via KAT II was confirmed using cultured human astrocytes (Kiss et al., 2003). However, studies with human cell cultures, as well as a recent study in mice (Heredi et al., 2017), have also indicated that KAT II is expressed in other cells, including neurons (Rzeski et al., 2005; Wejksza et al., 2005).

Due to regional differences in the production of KYNA in the brain (Turski et al., 1989), the present study was designed to fill a void by mapping the cellular distribution of KAT II/AADAT gene expression in the adult rat brain. To this end, we performed *in situ* hybridization histochemistry with radioactive riboprobes both under normal conditions and in animals receiving lipopolysaccharides (LPSs), an established experimental approach to stimulate an immune response as well as KP metabolism in the mammalian brain (Walker et al., 2013; Larsson et al., 2016). In complementary experiments, we examined the cellular expression of KAT II by immunohistochemistry. Results of these studies converged to demonstrate high levels of KAT II in the brain's neurogenic and gliogenic niches, pointing to a potential novel role of KYNA in adult brain cellular homeostasis.

EXPERIMENTAL PROCEDURES

Animals and treatments

Two-month-old male and female Wistar rats were obtained from Charles River Laboratories (Cambridge, MA, USA) and kept at the animal facility of the University of Maryland School of Medicine. Animals were housed in groups of three in Plexiglas cages with standard food pellets and water available ad libitum. All animals were maintained on a 12:12-h light:dark cycle (lights on at 07:00 AM) at a constant temperature of 23 °C. The rats were left undisturbed for one week and then handled daily for an additional week before starting the experimental procedures. Male rats were injected intraperitoneally (i.p) with 2 mg/kg LPS (Sigma–Aldrich, St. Louis, MO, USA; serotype 055:B5) (n = 17) or 0.9% saline (n = 17) between 09:00 and 10:00 AM and monitored 3 and 6 h later for sickness behavior and temperature. Female rats (n = 6) were injected with saline only. At the later time point, all animals were killed and the tissue was processed for *in situ* hybridization or immunohistochemistry. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Maryland, Baltimore.

Riboprobe generation

Liver tissue was processed for mRNA extraction using TRIzol (Invitrogen, Carlsband, CA, USA) as described previously (Tonelli et al., 2004; Gunsolly et al., 2010). Total RNA was treated with DNAse I (Invitrogen) for 15 min at room temperature according to the manufacturer's instruction. Specific sequences were produced to target regions 411–1151 or 69–1145 (Fig. 1A) corresponding to the rat KAT II mRNA (aminoadipate aminotransferase, Aadat, Genebank accession number NM_017193.1). The specific fragments were produced by PCR amplification using the PCR SuperMix High Fidelity enzyme mixture (ThermoFisher Scientific, USA). Both sequences produced a single band of expected size of 741 bp (Fig. 1B) or 1077 bp (Fig. 1C). These fragments were cloned into the TA Dual Promoter pCR™ II vector (ThermoFisher Scientific) and sequenced to confirm the length, identity and orientation of the template sequence. Antisense and sense (control) riboprobes were labeled by *in vitro* transcription in the presence of 10 μM [^{35}S]-UTPαS (PerkinElmer, Waltham, MA, USA; > 1000 Ci/mmol), 1 μg of linearized plasmid, and 20 units of T3 or T7 RNA polymerase using the RNA labeling kit (ThermoFisher Scientific) according to the manufacturer's protocol. After transcription, the template DNA was digested with DNAse I for 15 min at 37 °C. Unincorporated [^{35}S] UTPαS was removed by centrifugation through ProbeQuant G-50 micro columns (ThermoFisher Scientific).

In situ hybridization histochemistry

The *in situ* hybridization procedure was carried out as described previously (Tonelli et al., 2004; Gunsolly et al., 2010). Briefly, fresh frozen serial consecutive coronal brain sections (n = 6 male, 6 female saline, 6 male LPS) were cut in a cryostat at 20 μm of thickness through the entire rat brain and collected onto silanated slides and stored at −80 °C until processed. Sections were fixed for 10 min with a 4% paraformaldehyde solution in phosphate-buffered saline (PBS), rinsed twice in PBS, acetylated with 0.25% acetic anhydride in 0.1 M triethanolamine-HCl (pH 8.0) for 15 min and dehydrated through graded ethanols. Each slide was treated with 150 μl of hybridization buffer containing 40,000 cpm/μl of

Please cite this article in press as: Song C et al. Quantitative Analysis of Kynurenine Aminotransferase II in the Adult Rat Brain Reveals High Expression in Proliferative Zones and Corpus Callosum. Neuroscience (2017), https://doi.org/10.1016/j.neuroscience.2017.11.001