Brain Structure and Function

Astrocytic and neuronal localization of kynurenine-aminotransferase II in the adult mouse brain

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Abstract:	During catabolism of tryptophan through the kynurenine (KYN) pathway, several endogenous metabolites with neuromodulatory properties are produced, of which kynurenic acid (KYNA) is one of the highest significance. The causal role of altered KYNA production has been described in several neurodegenerative and

	neuropsychiatric disorders (e.g. Parkinson's disease, Huntington's disease, schizophrenia) and therefore kynurenergic manipulation with the aim of therapy has recently been proposed. Conventionally, KYNA is produced from its precursor L-KYN with the aid of the astrocytic kynurenine aminotransferase II (KATII) in the murine brain. Although the mouse is a standard therapeutic research organism, the presence of KATII in mice has not been described anatomically. This study demonstrates the presence of KATII mRNA and protein throughout the adult C57Bl6 mouse brain. In addition to the former expression data from the rat, we found prominent KATII expression not only in the astrocyte, but also in neurons in several brain regions (e.g. hippocampus, substantia nigra, striatum and pre-frontal cortex). A significant number of the KATII positive neurons were positive for GAD67; the presence of the KATII enzyme we could also demonstrate in mice brain homogenate and in cells overexpressing recombinant mouse KATII protein. This new finding attributes a new role to interneuron-derived KYNA in neuronal network operation. Furthermore, our results suggest that the thorough investigation of the spatio-temporal expression pattern of the relevant enzymes of the KYN pathway is a prerequisite for developing and understanding the pharmacological and transgenic murine models of kynurenergic manipulation.
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Astrocytic and neuronal localization of kynurenine aminotransferase II in the adult mouse brain

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Abstract

During catabolism of tryptophan through the kynurenine (KYN) pathway, several endogenous metabolites with neuromodulatory properties are produced, of which kynurenic acid (KYNA) is one of the highest significance. The causal role of altered KYNA production has been described in several neurodegenerative and neuropsychiatric disorders (e.g. Parkinson's disease, Huntington's disease, schizophrenia) and therefore kynurenergic manipulation with the aim of therapy has recently been proposed. Conventionally, KYNA is produced from its precursor L-KYN with the aid of the astrocytic kynurenine aminotransferase II (KATII) in the murine brain. Although the mouse is a standard therapeutic research organism, the presence of KATII in mice has not been described in detail.

This study demonstrates the presence of *katII* mRNA and protein throughout the adult *C57Bl6* mouse brain. In addition to the former expression data from the rat, we found prominent KATII expression not only in the astrocyte, but also in neurons in several brain regions (e.g. hippocampus, substantia nigra, striatum and prefrontal cortex). A significant number of the KATII positive neurons were positive for GAD67; the presence of the KATII enzyme we could also demonstrate in mice brain homogenate and in cells overexpressing recombinant mouse KATII protein. This new finding attributes a new role to interneuron-derived KYNA in neuronal network operation. Furthermore, our results suggest that the thorough investigation of the spatio-temporal expression pattern of the relevant enzymes of the KYN pathway is a prerequisite for developing and understanding the pharmacological and transgenic murine models of kynurenergic manipulation.

Keywords: kynurenine aminotransferase II, astrocyte, interneuron, mouse, hippocampus, striatum, prefrontal cortex, substantia nigra, immunohistochemistry, *in situ* hybridization

Introduction

Tryptophan (TRP) is catabolized mostly through the kynurenine pathway (KP) in the mammalian brain (Gal and Sherman, 1980). Kynurenic acid (KYNA) is a neuroactive end-product of this metabolic route, and it exerts multiple effect on ligand-gated ion channels in the brain (Gal and Sherman, 1980, Birch et al., 1988, Prescott et al., 2006, Albuquerque and Schwarcz, 2013) It is also an endogenous, agonistic ligand of the G-protein-coupled receptor 35 (GPR35) primarily in peripheral tissues but also in the central nervous system (Berlinguer-Palmini et al., 2013, Alkondon et al., 2015).

Altered level of brain KYNA is implicated in the pathophysiology of several neuropsychiatric disorders such as Huntington's disease (Stoy et al., 2005), Alzheimer's disease (Gulaj et al., 2010) and schizophrenia (Linderholm et al., 2012).

The manipulation of the kynurenine system with the aim of therapy has been widely studied recently (Vecsei et al., 2013). However, no detailed anatomical study describing the spatio-temporal expression pattern of KYN pathway enzymes has been performed alongside the genetic and pharmacological studies.

KYNA is synthetized with the irreversible transamination of L-kynurenine (L-KYN) and is excreted into blood without further metabolism. KYN-KYNA transformation is catalyzed by kynurenine aminotransferases (KATs), of which KATII is of greatest importance both in the murine and human brain (Guidetti et al., 2007a). Based on the anatomical studies performed in rats (Okuno et al., 1990, Guidetti et al., 2007b), the prevailing view is that KATII is localized in astrocytes and KYNA is produced and released by this cell type (Guillemin et al., 2001). However, it has been reported that KATII or its isoforms localized also in rat and human neuronal cell cultures (Rzeski et al., 2005, Guillemin et al., 2007) and in the Ammon's horn in the rat hippocampus (Okuno et al., 1990, Du et al., 1992). Moreover, neuronal localization of KATs was detected outside the rat brain, specifically in the medulla and spinal cord (Kapoor et al., 1997) and in the inner retina (Rejdak et al., 2001).

Little is known about the mechanism and regulation of KYNA transport in the brain nor is it known how cells liberate KYNA. KYNA uptake by human and rat organic anion transporters (hOAT and rOAT) was shown using *Xenopus laevis* oocytes (Uwai et al., 2013). KYNA release from rat astrocyte and neuron cell culture and from rat brain slices have also been reported (Turski et al., 1989, Rzeski et al., 2005), but the details of these mechanisms are unknown.

Although investigating the role of the KP in neurodegenerative and neuropsychiatric diseases in mice is of great relevance, most of the data regarding the expression profile of KATII and transport mechanism of KYNA are derived from rat and human samples. To our best knowledge, no description about the localization and distribution of KATII in the mouse brain is available in the literature.

The aim of this study was to clarify the presence of *katII* mRNA and protein in adult mouse brain tissue. We examined the expression of *katII* mRNA with *in situ* hybridization and we performed fluorescent immunohistochemistry to identify KATII containing cells in brain structures in which the

role of KYNA related to neuropsychiatric disorders has been proposed formerly. To verify the specificity of the primary anti-KATII antibody we performed Western blot analysis on mouse brain homogenate and on homogenate of cells transfected with mouse *katII* cDNA.

In addition to KATII containing astrocytes, we found prominent neuronal enzyme localization in the substantia nigra, while neuronal expression of KATII was sporadic in the hippocampus, striatum and prefrontal cortex. Interestingly, the vast majority of the KATII containing neurons were interneuron in all brain areas. Taken together, our data indicate that KYNA synthesis and release may also be relevant in neurons in mice. This new finding attributes a new role to brain KYNA in network operation.

Materials and Methods

Animals: For the histological and molecular studies, 8 weeks old male *C57Bl/6* mice obtained from The National Institute of Oncology (Budapest, Hungary) were used (n=6). Animals were kept under controlled laboratory conditions and had free access to food and water. All experiments were in compliance with the guidelines of the European Communities Council Directives (2010/63/EU) and the Hungarian Act for the Protection of Animals in Research (XXVIII.tv. 32. §), and were approved by the ethical license: I-74-16/2015.

Tissue preparation: Animals were deeply anesthetized with an intraperitoneal injection of urethane (1.6 g/bwkg) and were perfused transcardially with ice-cold 0.1 M phosphate buffer (PB, pH 7.4) following 4% paraformaldehyde (dissolved in 0.1 M PB, pH 7.4) for the immunohistochemical experiments. The brains were removed and post-fixed overnight in 4% paraformaldehyde. 20-µm sagittal (for immunohistochemistry) and 30-µm coronal (for *in situ* hybridization) sections were obtained with a vibratome (Leica VT1000S). For the Western blot analysis, animals were perfused with cold 0.1 M PB (pH 7.4) and the brains were processed immediately on ice, as described below.

All solutions were treated with diethylpyrocarbonate (DEPC) to avoid RNAse contamination during RNA *in situ* hybridization.

Labelled RNA Probe design: *katII* cDNA clone (Aadat NM 011834 Mouse cDNA clone, OriGene Technologies) was removed from its vector with KpuI and HindIII digestion. The resulted 1600-bp fragment was amplified with *katII* specific primers carrying T7 or SP6 RNA polymerase promoter sequences. The primer sequences were as follows: 5'-GGA TCC TAA TAC GAC TCA CTA TAG GGA GAA CCA GAG GGA TTC CAT A-3' (KATII forward) and 5'- GGA TCC ATT TAG GTG ACA CTA TAG AAG AGG AAA GCT ATT TGG CAA TGT G -3' (KATII reverse). Sense and antisense probes were synthesized with the aid of *in vitro* transcription using the amplified DNA templates, the T7 or SP6 RNA polymerase (Sigma-Aldrich) and digoxigenin (DIG) labelled nucleotides (DIG RNA Labelling mix, Sigma-Aldrich).

RNA *in situ* hybridization: For detection of *katII* mRNA, *in situ* hybridization was performed. Sections were extensively washed in DEPC treated PB containing 0,2% Tween-20 (PBT, pH 7.4), digested with proteinase K (2µg/ml, Sigma-Aldrich) for 5 min and post-fixed with 4% PFA for 20 min. After thorough washing in PB, slices were prehybridized for 1 h at 65 °C in hybridization buffer containing 50% formamide, 5X saline-sodium citrate (SSC), 0.1 mg/ml sonicated chicken DNA, 0.1% Tween-20 and 0.1 M citrate (pH 4.5) and then hybridized overnight at 65 °C in the same solution containing the DIG-labelled sense or antisense probe for KATII mRNA (~ 300 ng/ml). Next day stringent washes were performed in descending concentrations of the hybridization buffer, then sections were blocked with 1% normal donkey serum (NDS) and incubated in AP-conjugated anti-DIG antibody (sheep anti-DIG-AP, 1:1000, Sigma-Aldrich) overnight on 4°C. The following day

samples were exposed to the NBT-BCIP substrate (1:50, pH 9.5, Sigma-Aldrich). Enzymatic reaction was stopped with PB (pH 7.4) and sections were coverslipped with aqueous mounting medium (Sigma-Aldrich).

Immunohistochemistry: Free-floating sections were washed in PBT and incubated in 1% NDS. For the detection of KATII and identification of the cells containing the enzyme, sections were exposed to the primary antibodies (rabbit anti KATII, 1:1000, Proteintech; rat anti-GFAP, 1:4000, Sigma; mouse anti-NeuN, 1:4000, Millipore and mouse anti-GAD67, 1:1000, Millipore) overnight at 4 °C. Next day, samples were incubated in the appropriate secondary antibodies (1:500; Jackson ImmunoResearch) at room temperature (RT). Primary and secondary antibodies were diluted in 0.1 M PBT containing 1% NDS. Negative control was prepared from sections incubated without the primary antibodies. The sections were coverslipped with antifade mounting medium (ProLong® Gold, Life Technologies). Fluorescent photomicrographs were obtained with an Olympus BX51 microscope fitted with a DP70 digital imaging system.

Results

***katII* mRNA expression:**

In order to study *katII* mRNA localization in the mouse brain, we performed RNA *in situ* hybridization. *katII* mRNA expression was prominent throughout the brain both in the white and gray matter. We detected significant labelling in several brain areas, including the CA1 subfield of hippocampus **(Fig. 1.)**. A large fraction of the labelled cells showed small and dense astrocytic-like cell bodies in the CA1 and also in the corpus callosum. Another cell population expressing *katII*, pronouncedly in the pyramidal cell layer, had large and pale neuron-like somata **(Fig.1 b and c)**. No signal was detected when brain sections were hybridized with *katII* sense probes **(Fig. 1a)**.

KATII protein expression:

The presence of KATII was studied with double fluorescent immunolabelling. KATII was widely distributed in the whole mouse brain.

Similar to previous observations achieved in rats (Guidetti et al., 2007b), we found pronounced KATII immunopositivity in astrocytes in several brain areas in mice. As illustrated in **Fig. 2**, KATII was co-localized with the astrocytic marker GFAP in all layers of the CA1 subregion of the hippocampus and was expressed mostly in the somatic region and in the primary branches of the astrocytes. Additionally, there were a large number of $KATII^+$, but $GFAP^-$ star-shaped cells with highly-branched processes e.g. in the mouse striatum and prefrontal cortex (unpublished data). These cells are presumably astrocytes with low levels of GFAP. KATII positivity in astrocytes was also prominent in the white matter of the mouse brain **(see in Fig. 3a and b)**, which coincides with the results of *in situ* hybridization. Furthermore, KATII was detected in glial end-feet surrounding blood vessels. This phenomenon supports the astrocytic expression of the enzyme **(Supp. Fig.1)**.

Unexpectedly, we detected large number of KATII containing neurons in the mouse brain tissue. We confirmed the neuronal expression of the enzyme with KATII - NeuN double labelling. Neurons expressing KATII distributed sporadically in the CA1 area of the hippocampus, dorsal striatum and all layers of the medial prefrontal cortex **(Fig. 3a, b and d)**, whereas there was a significant number of $KATII^+/NeuN^+$ cells in the substantia nigra pars compacta and reticulata **(Fig. 3c)**. KATII is expressed predominantly in the cell bodies of the neurons.

To identify the neuron type, we performed GAD67 immunolabelling. KATII and GAD67 were co-localized almost completely in the hippocampus, dorsal striatum and medial prefrontal cortex **(Fig. 4 a, b and d)**. $GAD67^+$ and $KATII^+$ cells were also completely overlapping in the substantia nigra pars reticulata, while none of the $KATII^+$ neurons expressed GAD67 in the pars compacta subregion **(Fig.4c)**. These findings indicate that most of the $KATII^+$ neurons are interneuron in the studied brain regions, suggesting an important role of interneuronal KYNA synthesis.

Finally, we tested whether the neuronal localization of KATII is specific for mice or if it is phylogenetically conserved in rodents. Therefore, we examined the presence of KATII in adult male *Wistar* rats (Charles-River). We found similar astrocytic and neuronal expression pattern of KATII in the rat brain **(Supp. Fig. 2)**. No staining was detected when primary antibodies were omitted.

Antibody validation:

In addition to negative controls in immunohistochemistry, we tested the primary antibody specificity with Western blot analysis both on HeLa cell culture transfected with mouse *katII* cDNA and on mouse brain tissue homogenate. The antibody recognized a single band in the transfected HeLa cell culture preparation at ~ 47 kDa, which is the estimated size of KATII **(Supp. Fig. 3a)**. We found similar clear positivity in mouse brain tissue homogenate **(Supp. Fig. 3d)**

Moreover, we performed immunocytochemistry on the HeLa cell culture. Large number of $KATII^{+}$ cells was seen in the transfected cell culture, while no positivity was observed in the control culture **(Supp. Fig.3b and c)**. These data indicate that the primary antibody binds specifically to KATII in the mouse brain tissue.

Discussion

KATII is expressed by astrocytes and also by neurons in the mouse brain

In the present study we demonstrated for the first time that KATII is localized both in astrocytes and neurons in the adult mouse brain. The vast majority of these neurons were identified as GABAergic cells in the examined brain areas.

The antibody used against KATII was validated on mouse brain tissue homogenate and also on HeLa cells transfected with mouse *katII* cDNA and it showed appropriate specificity in both experiments.

Our observations are partially similar to previous data obtained from rats (Guidetti et al., 2007b) and we found significant astrocytic expression of KATII in the mouse hippocampus, striatum and cerebral cortex and faint positivity in the substantia nigra. We could demonstrate the co-localization of KATII and the astrocytic marker GFAP mostly in the hippocampus, while e.g. in the striatum and prefrontal cortex a large number of $KATII^+$, but $GFAP^-$ cells were seen with astrocytic morphological features. Since GFAP expression in astrocytes of intact mouse cortex and striatum is very low (Kindy et al., 1992, Ben-Gigi et al., 2015), we propose that these $KATII^+$ cells are astrocytes with undetectable levels of GFAP. Co-localization of the S100 astrocytic marker and KATII in the striatum and cerebral cortex (unpublished data) supports this hypothesis. Furthermore, similarly to previous reports from rats (Guidetti et al., 2007b), we detected KATII in astrocytic end feet around brain capillaries. This feature of KATII expression indicates the involvement of astrocytes in L-KYN uptake from blood similarly, as reported in rat (Speciale et al., 1989).

Similar to that described in rats (Du et al., 1992, Roberts et al., 1992), sporadic neuronal KATII immunoreactivity was seen in the mouse hippocampus, striatum and prefrontal cortex. In other brain areas not investigated in former studies (e.g. in substantia nigra pars compacta and reticulata), we found more prominent neuronal KATII expression. Neuronal presence of the enzyme was also significant in other parts of the brain not detailed in this study (e.g. cerebellum, thalamus). Surprisingly, most of the $KATII^+$ neurons were positive for GAD67 interneuron marker in the examined brain areas. This characteristic feature of $KATII^+$ neurons has not been described in previous studies. Considering, that neurons synthesize KYNA at a rate about 2.3 times higher than astrocytes *in vitro* (Rzeski et al., 2005), this cell population may have a significant role in KYNA synthesis in the brain.

The possible mechanism of KYNA release from interneurons and its significance in regulation of GABAergic cells

Previous studies indicate that KYNA is liberated from rat brain slices (Turski et al., 1989) and from neuronal and astrocytic cell cultures (Rzeski et al., 2005). Our own observations also provide evidence that KYNA is released from mouse brain slices to the incubation medium (unpublished HPLC data). However, the exact mechanism of KYNA release from cells and the regulation of release are unknown. There is also no information on whether KYNA has a specific release site on these cells. The prominent interneuronal staining of KATII raises the question of how this cell type liberates KYNA to the extracellular milieu. We detected KATII in the somatic region of the interneurons, so one possible mechanism is that KYNA is released extrasynaptically from the neuronal cell bodies and it exerts a diffuse effect on the neighbouring cell population.

Conventionally, the main ionotropic receptor targets of KYNA are the NMDA and the α7 nACh receptors, respectively. However, the interaction between KYNA and α7nACh receptors is controversial (Albuquerque and Schwarcz, 2013).

α7 nAChRs are widely distributed in the somato-dendritic compartments of interneurons, and also in the presynaptic terminals innervating interneurons in the hippocampus (Zarei et al., 1999, Kawai et al., 2002, Alkondon and Albuquerque, 2004). Moreover, GABAergic cells have tonically active NMDARs diffusely over the neuron, often at extrasynaptic locations (Povysheva and Johnson, 2012, Hsieh et al., 2014). According to Riebe et al., tonically active extrasynaptic NMDARs contribute more prominently to the intrinsic excitation in GABAergic interneurons than in pyramidal cells. Authors showed that low concentrations of the NMDAR blockers memantine and phencyclidine preferentially inhibited tonically active extrasynaptic NMDAR on GABAergic cells (2016).

The role of extrasynaptic NMDA receptors in transmitter release modulation has also been reviewed recently (Petralia, 2012). Considering these data, self-regulation of GABAergic cells and the concomitant alteration of GABAergic and glutamatergic neurotransmission (Sik et al., 1995) are also feasible through extrasynaptic KYNA release.

Impaired GABAergic neurotransmission is observed in pathological conditions, e.g. in schizophrenia, where NMDAR hypofunction affects primarily the GABAergic interneurons both in the hippocampus (Grunze et al., 1996) and in the prefrontal cortex (Homayoun and Moghaddam, 2007). This selectivity causes diminished inhibitory control and increased excitation by disinhibition over pyramidal neurons (Homayoun and Moghaddam, 2007). During schizophrenia the level of KYNA is abnormally increased (Linderholm et al., 2012, Kegel et al., 2014). Is it possible that extrasynaptically released KYNA acts on extrasynaptic NMDA and α7 nACh receptors, resulting in the downregulation of interneurons in schizophrenia? To test our hypothesis the exact mechanisms by which the endogenously produced KYNA is released from interneurons and also from astrocytes must be determined, both in physiological and pathological states.

Therapeutical manipulation of KATII expression and KYNA synthesis in model animals of neuropsychiatric disorders

Along with other KP metabolites, altered brain KYNA level is implicated in the pathogenesis of neurodegenerative diseases and mental disorders, like schizophrenia or major depressive disorder (Maddison and Giorgini, 2015, Meier et al., 2016). Therefore, manipulating KYNA synthesis or other branches of the KP holds broad therapeutic perspectives (Dounay et al., 2015).

Recently the genetic (Alkondon et al., 2004, Potter et al., 2010) and pharmacological (Zmarowski et al., 2009, Koshy Cherian et al., 2014) inhibition of KATII has been studied primarily for the treatment of psychiatric and cognitive disorders (Jayawickrama et al., 2015). Kozak et al. showed that lowering KYNA level with selective KATII inhibitor improves cognitive function under conditions considered relevant for schizophrenia in rats (2014). On the other hand, shifting the KP toward KYNA synthesis with kynurenine 3-monooxygenase (KMO) inhibition prevents spatial memory deficits, anxiety-related behavior, and synaptic loss in a mouse model of Alzheimer's disease (Zwilling et al., 2011). Therefore, both up- and down regulation of KYNA production could serve as a potential therapeutical tool.

In the substantia nigra, we detected KATII mostly in interneurons in the pars reticulata subregion, while astrocytes were only faintly positive for KATII. So it is conceivable that the decreased concentration of KYNA observed during e.g. in Parkinson's disease in this brain area (Ogawa et al., 1992) is a consequence of neuronal KATII dysfunction. Taking these results, astrocytes and neurons may have a distinctive role in disorders with abnormal brain KYNA level. Therefore, expression pattern and activity of KATII during neuropsychiatric disorders should be clarified before future therapeutical kynurenergic manipulation strategies are assigned.

Although the effect of genetic manipulations of the KYN pathway has been described in mice, the *katII* knock-out model suffers from the general limitations of the null-mutant models (Alkondon et al., 2004, Yu et al., 2004). To understand tissue- and cell-type specific function of KATII in health and disease, one needs spatio-temporally selective kynurenergic manipulation, which is possible only if the expression profile of KATII is well described in the wild-type animal.

Conclusion

In conclusion, we studied for the first time the expression profile of KATII in the mouse brain. We proved that KATII is expressed not only in astrocytes but also in neurons in brain structures in which the role of KYNA related to neuropsychiatric disorders has been proposed formerly. This result supports the growing body of evidence about neuronal KYNA synthesis and contradicts the previous common view that astrocyte is the only relevant KYNA producing cell type. The majority of KATII containing neurons were interneuron in the examined brain areas. Brain structures composed predominantly of GABAergic neurons (e.g. the substantia nigra) possess the strongest neuronal KATII expression. It is possible in these areas, that interneuronal KATII production is superior to that of astrocyte both in physiological and pathological states. The release mechanism of KYNA is unknown, but the somatic location of the enzyme in the interneurons may indicate an extrasynaptic release mode, through which interneurons possibly control neighbouring cell population including other interneurons and themselves as well.

Taken together, the release mechanisms of KYNA by these two distinct cell populations and the functional significance of the cell type specific expression of KATII during neuropsychiatric disorders should be investigated in the future.

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Figure Captions

Fig. 1 Expression of *katII* mRNA transcripts in the hippocampal CA1 subfield. No signal was detected in the CA1 subregion, when the brain sections were hybridized with the KATII sense probes (negative control) **(a)**. Using antisense *katII* probes, *katII* mRNA expression was prominent in the whole mouse brain, including the CA1 subfield of the hippocampus **(b)**. *katII* mRNA is localized in small and dense astrocyte like cell bodies (arrows) and also in bigger and pale, neuron like somata (arrowheads) through the CA1 subregion. The most significant neuronal KATII positivity was detected in the pyramidal cell layer of the CA1. katII expression in astrocyte like somata was strong also in the *cc* **(c)**. Scale bars are 200 μm in **a** and **b** and 50 μm in **c**. Abbreviations *so:* str.oriens, *sp:* str. pyramidale, *sr:* str. radiatum, *cc: corpus callosum*

Fig. 2 Astrocytic localization of KATII in the hippocampal CA1 subfield. Double labelling of KATII (red) with the astrocyte marker GFAP (green) shows significant co-localization (orange) in all layer of the CA1 **(left panel)**. KATII is expressed mostly in the somatic region and in the primary branches of the astrocytes **(right panels)**. Note the significant number of $KATII^+$ non-astrocytic cells in the *sub* and sporadically in the *so, sp, sr* and *slm* of the CA1. Asterisk indicates the corresponding cell in lower and higher magnification photomicrographs (*). Scale bars are 80 μm (left panel) and 40 μm (right panels). Abbreviations: *sub:* subiculum, *so:* str.oriens, *sp:* str. pyramidale, *sr:* str. radiatum, *slm:* str. lacunosum-moleculare

Fig. 3 Neuronal localization of KATII in the mouse hippocampus, striatum, substantia nigra and prefrontal cortex. KATII (red) is co-localized (orange) with the neuronal marker NeuN (green) in all the four brain areas shown in **a-d**. $KATII^+$ neurons are seen in all layer of the CA1 (*so, sp, sr and slm*) and also in the *sub* **(a)**. In the dorsal part of the striatum KATII is also overlapped with NeuN in large striatal neurons **(b)**. Neuronal KATII expression is strong both in the *SNc* and *SNr* **(c)**, while as in the hippocampus and striatum the enzyme is expressed sporadically in the medial prefrontal cortex **(d)**. Note that KATII is presented predominantly in the cell bodies of the neurons **(right panels)**. Non-neuronal KATII expression is detected in the examined brain areas and also in the cc **(a-b)**. Asterisks indicate the corresponding cells in lower and higher magnification photomicrographs (*). Scale bars are 80 μm (left panels) and 40 μm (right panels). Abbreviations: *sub*: subiculum, *so*: str.oriens, *sp*: str. pyramidale, *sr*: str. radiatum, *slm*: str. lacunosum-moleculare, *cc*: corpus callosum, *SNr*: substantia nigra pars reticulata, *SNc*: substantia nigra pars compacta

Fig. 4 Co-localization of KATII (red) with the interneuron marker GAD67 (green) in the mouse hippocampus, striatum, substantia nigra and prefrontal cortex. High proportions of the $KATII^+$ neurons were $GAD67^+$ interneuron (orange) in all the four brain areas shown in **a-d**. The expression pattern of

KAT2$^+$ interneurons is similar as the KATII$^+$/NeuN$^+$ neurons in Fig. 3. Note that in the substantia nigra pars compacta none of the KATII expressing neurons were GAD67$^+$**(c)**, suggesting the non-GABAergic, but dopaminergic characteristic of these cells. The presence of KATII is somatic in the GAD67$^+$ neurons **(right panels).** Non-neuronal KATII expression is detected in all the examined brain areas and also in the cc **(a-b)**. Asterisks indicate the corresponding cells in lower and higher magnification photomicrographs (*). Scale bars are 80 μm (left panels) and 40 μm (right panels). Abbreviations: *sub*: subiculum, *so*: str.oriens, *sp*: str. pyramidale, *sr*: str. radiatum, *slm*: str. lacunosum-moleculare, *cc*: corpus callosum, *SNr*: substantia nigra pars reticulata, *SNc*: substantia nigra pars compacta

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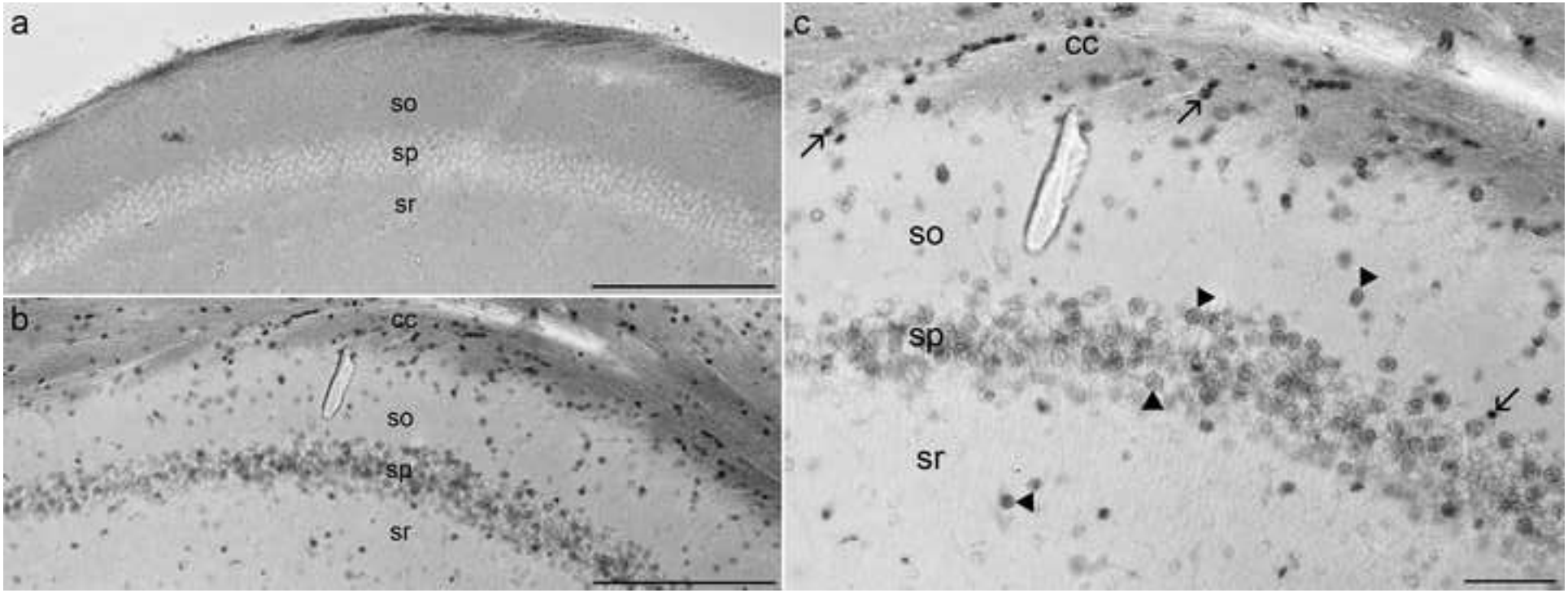

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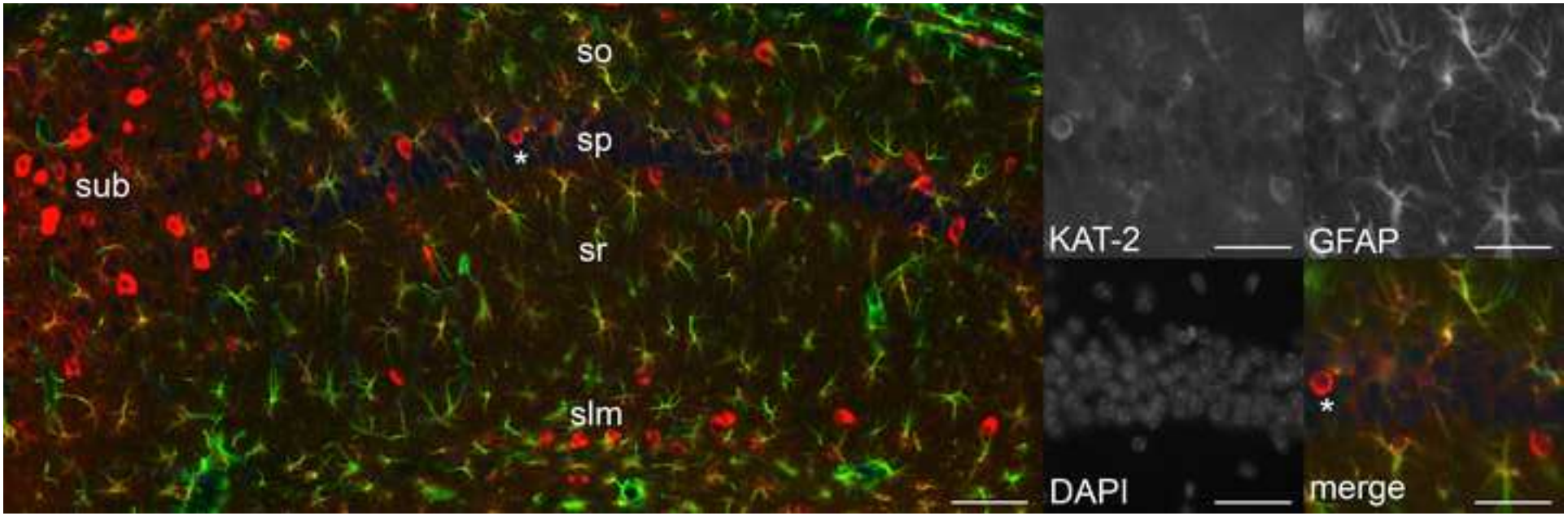

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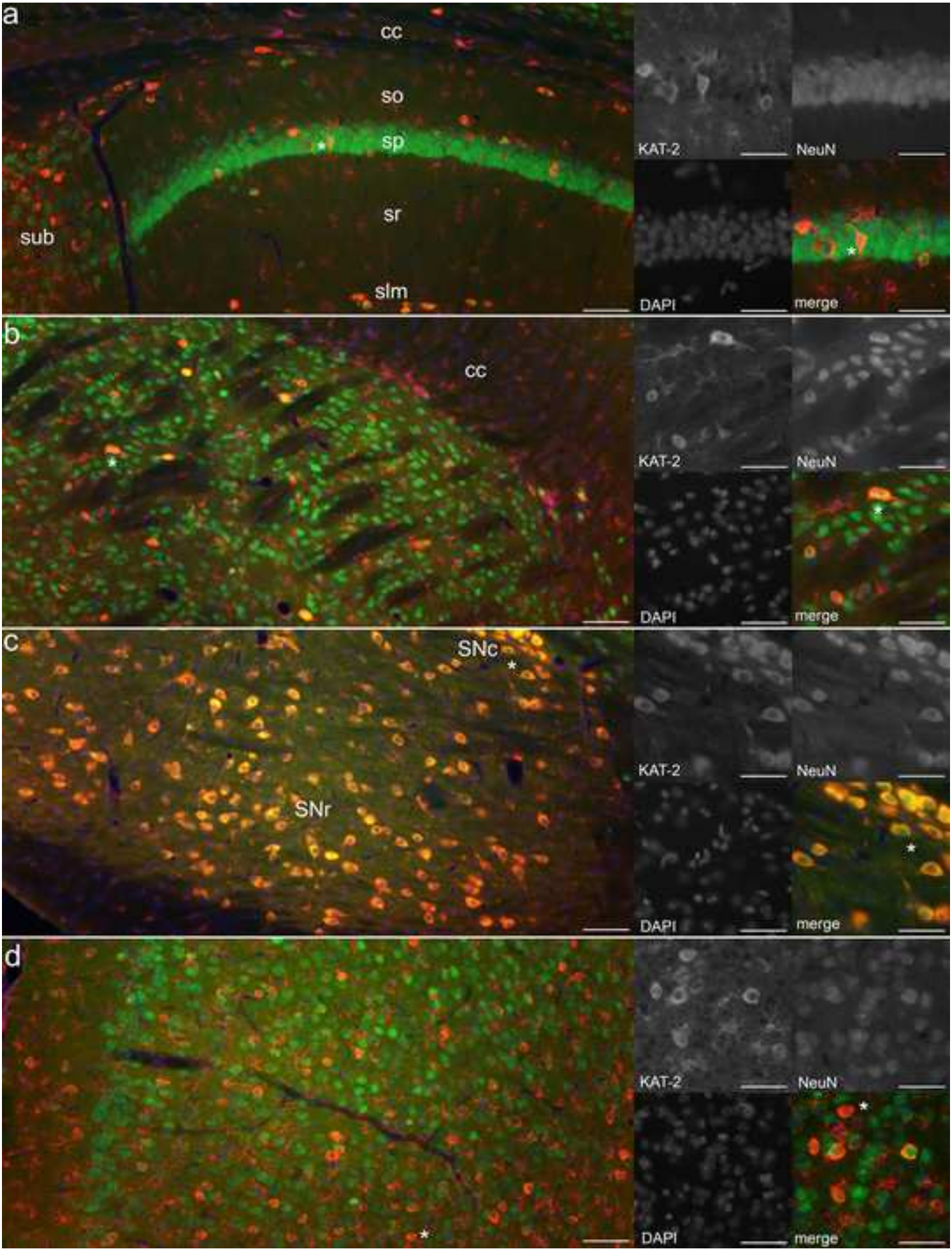

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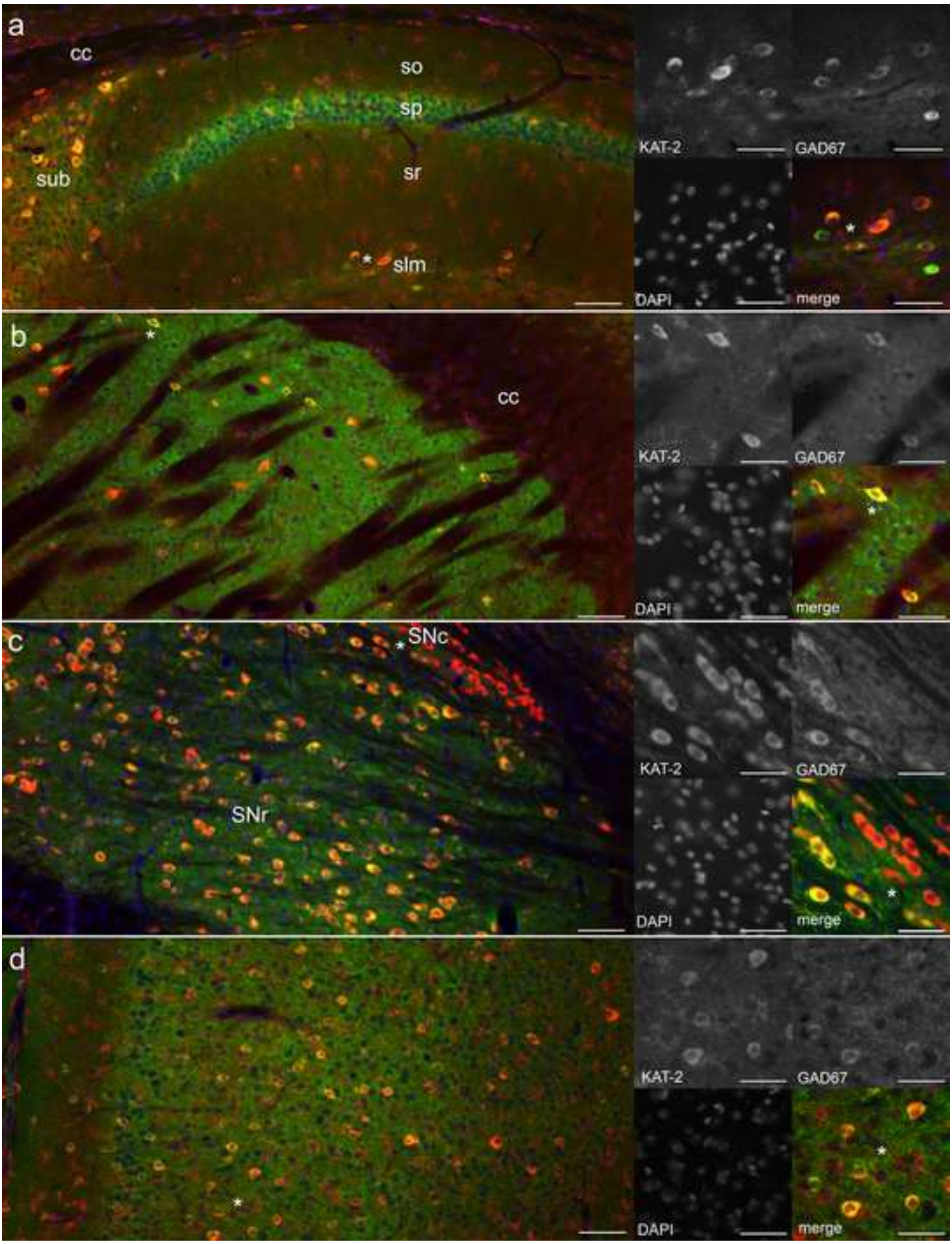

Astrocytic and neuronal localization of kynurenine-aminotransferase II in the adult mouse brain

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Brain Structure and Function

Supplementary Materials and Methods

SDS gel electrophoresis (SDS-PAGE) and Western blot: The specificity of KATII primary antibody was tested by SDS-PAGE and Western blot analysis. Protein from mouse brain tissue was extracted in tissue protein extraction buffer containing a protease inhibitor cocktail (Boehringer Mannheim) using mechanical homogenization and sonication. Homogenates were centrifuged for 10 min at 12,000 rpm at 4°C. Total protein levels in each sample were quantified by the Bradford assay (Sigma-Aldrich) and 50 µg total protein per lane was loaded onto a 8% gel (Bio-Rad). Homogenate were separated at 100V for 1 h. Proteins were blotted to a nitrocellulose membrane (Millipore Immobilon®- P) using a transfer buffer containing 20% methanol, 25 mM Tris base and 192 mM glycine at 20V for 90 min.

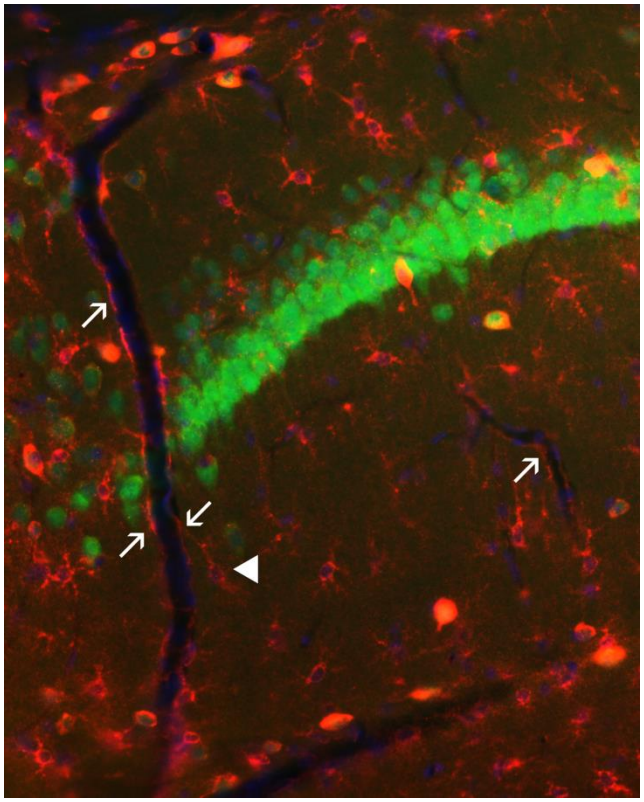
Protein from HeLA cell culture was extracted in extraction buffer (T-PER®, Thermo Scientific) containing a protease inhibitor cocktail (Sigma) using mechanical homogenization and sonication. Homogenates were centrifuged for 10 min at 12,000 rpm at 4°C. Total protein levels in each sample were quantified by the BCA assay (Pierce) and 50 µg total protein per lane was loaded onto a 10% gel (Bio-Rad, TGX FastCast). Homogenate were separated at 100V for 1 h. Proteins were blotted to a PVDF membrane (Millipore Immobilon®- P) using a transfer buffer containing 20% methanol, 25 mM Tris base and 192 mM glycine at 20V for 90 min.

After blotting, the membrane was washed in 1x PBS containing 0.05% Tween-20 (PBST) and blocked with 5% nonfat dried milk (Bio-Rad). Membranes were probed to the primary antibody (rabbit anti-KATII, 1:200, Proteintech) at 4˚C overnight. Next day the membranes were washed extensively and incubated with the HRP-conjugated secondary antibody (HRP-conjugated goat anti-rabbit, 1:10000, Jackson ImmunoResearch) for 1 h at RT. Primary and secondary antibodies were diluted in 1X PBST containing 5% nonfat dried milk. The immunoreactive bands were visualized with a chemiluminescent kit (Immobilon Western, Millipore) and digital images were captured with Li-Cor C-DIGIT Blot Scanner.

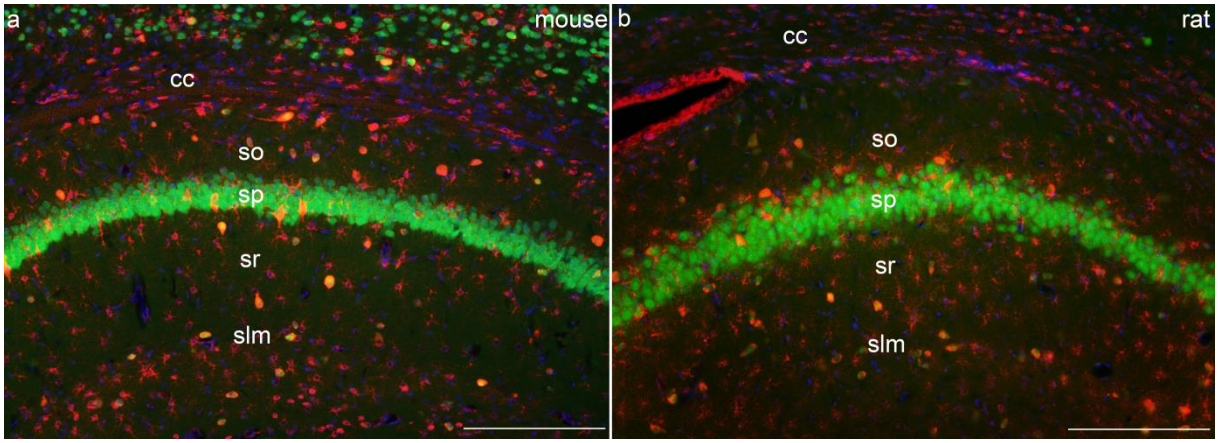
Transfection: Frozen cells (HeLa ATCC® CCL2 ™) were thawed rapidly in 37°C water bath and then resuspended in DMEM complete growth medium supplemented with 10% fetal bovine serum, 4 mM glutamine and 100units/ml penicillin/streptomycin (WAKO). Cells were then plated on poly-lysinated dishes (35mm in diameter; Thermo Fisher Scientific) and were grown until 80% confluency at 37°C (5% CO_2). Cells were then transfected with 2 µg of the *katII* cDNA vector with FuGENE® HD Transfection Reagent prepared as recommended. After 48 hours incubation (37°C, 5% CO_2) cells were washed with PBS, and fixed with 4% paraformaldehyde for 15 minutes. Cells were permeabilized with 0.1% Triton X-100 made in PBS solution for 15 min. Non-specific antibody binding was blocked with 3% bovine serum albumin (BSA)-PBS solution for 1 hour. Cells were

incubated in 4μg/ml of the primary antibody diluted in the blocking solution on 4°C, overnight. After thorough washing with PBS cells were incubated in the secondary antibody diluted in the blocking solution for 2 hours at RT. Digital photomicrographs were obtained with fluorescence microscope (BZ-X700, Keyence Corp.)

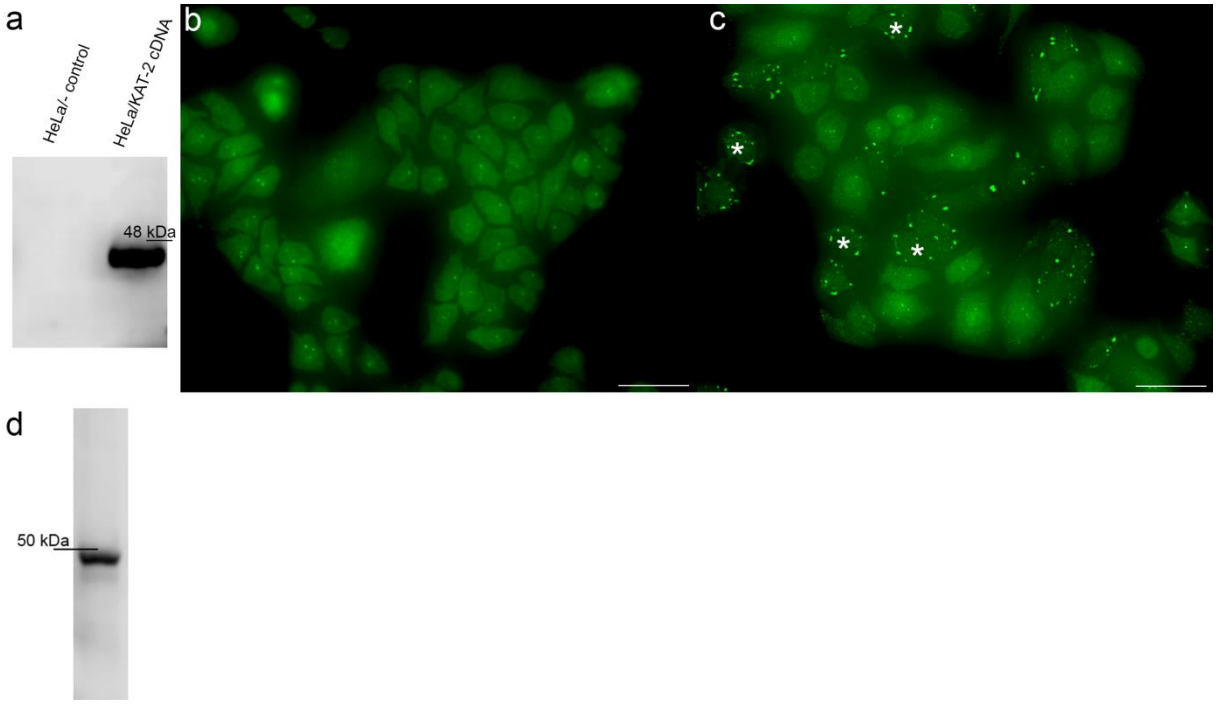
Supplementary Figures and Figure Captions



Supp. Fig. 1 KATII expression around blood vessels in the hippocampus. Arrows indicate the KATII^{+} astrocyte end-feet surrounding blood vessels. Note the long process derives from an astrocyte and ends on the capillary (arrowhead).



Supp. Fig. 2 Neuronal localization of KATII in the mouse and rat hippocampal CA1 subfield. KATII (red) is co-localized (orange) with the neuronal marker NeuN (green) in all layer of the CA1 region *(so, sp, sr and slm)* of the hippocampus both in the mouse **(a)** and rat hippocampus **(b)**. Note the similarity of the expression pattern of KATII in the two species. Scale bars are 200 µm. Abbreviations: so: str.oriens, sp: str. pyramidale, sr: str. radiatum, slm: str. lacunosum-moleculare, cc: corpus callosum



Supp. Fig. 3 Validation of rabbit anti KATII with Western blot analysis and immunocytochemistry. The specificity of the primary antibody was confirmed on HeLa cell culture transfected with mouse KATII cDNA and on mouse brain tissue homogenate. The antibody recognized a single band in the transfected HeLa culture at ~ 47 kDa, while no bands were seen in the control culture **(a)**. With immunocytochemistry, no positivity was observed in the control cell culture **(b),** but prominent KATII immunolabelling (asterisks) was seen in the transfected HeLa cells **(c)**. Western blot with mouse brain tissue homogenate gave the same result, and the primary antibody binds to proteins at ~ 47 kDa **(d)**. Scale bars are 80 µm.