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Differential expression patterns of sodium potassium ATPase alpha and beta subunit isoforms in mouse brain during postnatal development

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A B S T R A C T

The sodium potassium ATPase (Na^+/K^+ ATPase) is essential for the maintenance of a low intracellular Na^+ and a high intracellular K^+ concentration. Loss of function of the Na^+/K^+ ATPase due to mutations in Na^+/K^+ ATPase genes, anoxic conditions, depletion of ATP or inhibition of the Na^+/K^+ ATPase function using cardiac glycosides such as digitalis, causes a depolarization of the resting membrane potential. While in non-excitable cells, the uptake of glucose and amino acids is decreased if the function of the Na^+/K^+ ATPase is compromised, in excitable cells the symptoms range from local hyper-excitability to inactivating depolarization. Although several studies have demonstrated the differential expression of the various Na^+/K^+ ATPase alpha and beta isoforms in the brain tissue of rodents, their expression profile during development has yet to be thoroughly investigated. An immunohistochemical analysis of postnatal day 19 mouse brain showed ubiquitous expression of Na^+/K^+ ATPase isoforms α1, β1 and β2 in both neurons and glial cells, whereas α2 was expressed mostly in glial cells and the α3 and β3 isoforms were expressed in neurons. Furthermore, we examined potential changes in the relative expression of the different Na^+/K^+ ATPase isoforms in different brain areas of postnatal day 6 and in adult 9 months old animals using *immunoblot* analysis. Our results show a significant up-regulation of the α1 isoform in cortex, hippocampus and cerebellum, whereas, the α2 isoform was significantly up-regulated in midbrain. The β3 isoform showed a significant up-regulation in all brain areas investigated. The up-regulation of the α3 isoform matched that of the β2 isoform which were both significantly up-regulated in cortex, hippocampus and midbrain, suggesting that the increased maturation of the neuronal network is accompanied by an increase in expression of α3/β2 complexes in these brain structures.

1. Introduction

The Na^+/K^+ ATPase is a membrane bound protein which adjusts the intracellular Na^+ concentration to approximately 10 mM and elevates the intracellular K^+ concentration to about 100 mM by transporting 3 Na^+ out of the cells and 2 K^+ into the intracellular space (Clausen et al., 2017; Ellis-Davies and Kaplan, 1990; Horisberger and Geering, 2009; Lingrel, 1992; Morth et al., 2007; Ogawa et al., 2009; Skou 1989, 2004). An up-regulation of the expression of Na^+/K^+ ATPase speeds-up neuronal hyperpolarization after enhanced activity and due to an enhanced pump current, it leads to a slight permanent hyperpolarization (Brodie and Sampson, 1989). Na^+/K^+ ATPase consumes approximately 50% of the total brain energy (Attwell and Laughlin, 2001; Azarias et al., 2013; Erecinska et al., 2004). Till date, three alpha isoforms: α1, α2 and α3 (Berrebi-Bertrand et al., 1990; Chen et al., 2013; Cholet et al., 2002; McGrail et al., 1991; Sweadner, 1989) and three beta isoforms: β1, β2 and β3 have been identified in brain tissue (Antonicek et al., 1987; Gloor et al., 1990; Lecuona et al., 1996; Martin-Vasallo et al., 1989; Zlokovic et al., 1993). The α1 isoform is expressed ubiquitously in all cell types of the brain, whereas, the α2

Abbreviations: P, postnatal day; GL, granule layer; MRN, midbrain reticular nucleus; GFAP, glial fibrillary acidic protein; NeuN, neuronal nuclear antigen

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and β2 isoforms are predominantly expressed in glial cells. On the other hand, α3 and β1 isoforms are expressed mostly in neurons in the rat nervous system (McGrail et al., 1991; Watts et al., 1991). The expression of the β3 isoform in mouse brain has not been studied in detail; however, it is known from Malik et al. (1996) that the mRNA of the β3 isoform is highly expressed in testis, whereas kidney, spleen, lung and brain showed a moderate expression (Malik et al., 1996).

Since the Na^+/K^+ ATPase regulates the cellular content of the major electrolytes, a deficiency in Na^+/K^+ ATPase causes severe physiological consequences. As a prominent example, autosomal dominant mutations in *Na^+/K^+ATPase α3*gene result in Rapid-onset Dystonia Parkinsonism (RDP). Patients with RDP show sudden onsets of irreversible dystonia in addition to motor defects and psychiatric symptoms in early adulthood (Brashear et al., 2012; Clausen et al., 2017). These patients do not respond to L-DOPA or any other drug therapy. This observation raises the question, whether Na^+/K^+ATPase α3 is predominantly expressed in midbrain structures. The Na^+/K^+ATPase α3 is expressed in GABAergic neurons in all basal ganglia nuclei; striatum, globus pallidus, subthalamic nucleus and substantia nigra, which are involved in movement control (Bøttger et al., 2011). Yet, potential changes in the expression level of the α3 subunit of the Na^+/K^+ATPase in the mouse midbrain region during development have so far not been investigated.

Previous studies characterized the distribution of the Na^+/K^+ ATPase in fetal and aged rat brain tissues. The aging rat cerebellum shows a down-regulation of the α3 isoform compared with the α1 isoform (Chauhan and Siegel, 1997). Moreover, the aging rat hippocampus shows a selective increase in the α1 isoform compared to the juvenile rat (Chauhan and Siegel, 1996). Furthermore, during development, the expression of the Na^+/K^+ ATPase isoforms depends on the cell type. Na^+/K^+ ATPase α2 is expressed in both neurons and glial cells during the late gestational time period, whereas in adult mouse brain tissues, the α2 isoform becomes localized exclusively to glial cells (Moseley et al., 2003). Knockdown of the α2 isoform in the late gestational period induced death shortly after birth in mice exhibiting lack of movements, defects in gross brain development and reduction of respiratory rhythm activity with irregular and smaller population bursts in the offspring (Moseley et al., 2003).

Since the development of the whole set of isoforms during postnatal development to early adulthood have so far not been studied in detail, we here compared the relative expression level of Na^+/K^+ ATPase isoforms α1, 2, 3 & β1, 2, 3 in postnatal day 6 (neonatal) and 9 months old (adult) mouse brains. These time points correspond to gestational weeks 36–40 (Bockhorst et al., 2008; Lebel and Beaulieu, 2011) and 25–30 years of age in humans (Dobbing and Sands, 1979; Lebel et al., 2012; Semple et al., 2013). First, we investigated the localization of Na^+/K^+ ATPase isoforms α1, α2, α3, β1, β2 and β3 in both neurons and glial cells immunohistochemically in brain sections from various regions. Then we studied the expression of Na^+/K^+ ATPase isoforms quantitatively using immunoblotting in different brain regions at both time points in mouse development. Our findings show a localization of the α2 isoform to glial cells and the α3 isoform to neurons as well as a significant up-regulation of most isoforms during adolescence.

2. Materials and methods

2.1. Mice

Postnatal day 6 (neonatal), postnatal day 19 (young) and 9 months (adult) old *C57BL/6* mice were obtained from the animal facility of the department of Biochemistry II and Faculty of Biology and Biotechnology, Ruhr University, Bochum, Germany. The animals were housed under a 12 h light/dark cycle with free access to food and water. All animal procedures were carried out in accordance with the German legislation.

2.2. Immunocytochemistry

Young mice were transcardially perfused with 4% (w/v) PFA in phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1,8 mM KH_2PO_4) PBS. The brains were carefully isolated and post fixed overnight, followed by a serial incubation in 10%, 20% and 30% sucrose (each 12 h), and frozen in Tissue Freezing Medium (Leica, Solms, Germany). Tissues were sectioned at 40 μm-thickness and stored at −20 °C in antifreeze solution (10% 10x PO_4 buffer (pH 7.0), ethylene glycol 30% (v/v), glycerol 30% (v/v)). Prior to staining, the sections were permeabilized in PBS containing 0.1% Triton-X for 20 min. The sections were then incubated in 3% normal goat serum prepared in PBS containing 0.1% Triton-X for 30 min to saturate non-specific epitopes and thus reduce non-specific binding. Then the sections were incubated with the Na^+/K^+ ATPase isoform antibodies [Anti-Na^+/K^+ATPase α1, Millipore (05–369) dilution 1:1000; Anti-Na^+/K^+ATPase α2, Millipore (07–674) dilution 1:2000; Anti-Na^+/K^+ATPase α3, Millipore (06–172) dilution 1:1000; Anti-Na^+/K^+ATPase β1 Millipore(06–170) dilution 1:1000; Anti-Na^+/K^+ATPase β2 Millipore(06–171) dilution 1:1000, Anti-Na^+/K^+ATPase β3, Novus biologicals (H00000483−B01P) dilution 1:500] for 12 h at 4 °C. The antibodies against the Na^+/K^+ ATPase α2, 3 and β 1, 2 isoforms had been raised in rabbits and the Na^+/K^+ ATPase α1 and β3 antibodies were obtained from mice. Co-stainings with the neuron specific marker, NeuN [Anti-NeuN, Millipore (Mab377) dilution 1:200] and the astrocyte specific marker, GFAP [Anti-GFAP, Sigma (G9269) dilution 1:300] were used to identify the cell type in which the specific Na^+/K^+ ATPase isoforms were expressed. Since the Na^+/K^+ ATPase α1, Na^+/K^+ ATPase β3 and NeuN antibodies were from mice, the co-localization of Na^+/K^+ ATPase α1 and Na^+/K^+ ATPase β3 were studied by co-staining with a GFAP antibody which had been raised in rabbit. After incubation with the primary antibody, the sections were washed three times with PBS for 5 min each. Then the sections (nine sections from 3 animals per isoform) were incubated with the corresponding secondary antibodies [goat anti-rabbit-Alexa flour 594, Invitrogen (A11012) dilution 1:1000, goat anti-mouse-Alexa flour 488, Invitrogen (A11001) dilution 1:1000] for 2 h at room temperature. Hoechst 33342 dye was used for staining the nuclei. Subsequently, the sections were washed with PBS and mounted on commercially available pre-coated microscope glass slides (Thermo Scientific Superfrost plus) and coverslipped using fluoromount (Sigma, F4680). Pictures were captured using a confocal laser-scanning microscope LSM 510 meta (Zeiss) and images were post-processed using Image J-1.46r (NIH, U.S.A). A blinding strategy was followed while imaging the brain sections.

2.3. Sample preparation for immunoblotting

Neonatal and adult mice were sacrificed through cervical dislocation. Cerebral cortex, hippocampus, mid brain and cerebellum were dissected and lysed with buffer containing 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate, 150 mM NaCl, 2 mM EDTA, 50 mM NaF, 10% protease inhibitor cocktail (Sigma P2714-1BTL). Tissue homogenization was performed on ice using an Ultra-Turrax homogenizer followed by collecting the supernatant after centrifugation at 12000 rpm for 20 min at 4 °C. The supernatant was aspirated and the protein concentration was measured using the Bio-Rad DC™ protein assay kit.

2.4. Immunoblotting

Immunoblotting was carried out using standard procedures (Sambrook et al., 1989). Briefly, 15 μg of proteins were resolved with 10% SDS-PAGE and transferred to a nitrocellulose membrane. Blots were blocked with tris-buffered saline containing 5% milk powder for 60 min and incubated with the different antibodies against Na^+/K^+ ATPase isoforms (antibodies used were the same as the ones used for

the immunohistochemical investigations) and loading control β -tubulin (Sigma/T4026, dilution 1:5000) for 12 h at 4 °C. Detection was performed with horseradish peroxidase conjugated secondary antibodies followed by developing the blots using West Femto enhanced chemiluminescent solution (Thermo Scientific). Blots were analyzed using Image J-1.46r (NIH, U.S.A). After background subtraction, the bands were normalized to their corresponding loading control (β-tubulin) and the values were used for calculations of relative changes in band intensities. We followed a semi-blind strategy with the experimenter not knowing the sample details while quantifying the blots.

2.5. Statistical analysis

We normalized the protein expression of the Na^+/K^+ ATPase α and β isoforms using β-tubulin as a loading control. Statistical significance was assessed by using simple analysis of variance (one way-ANOVA) followed by Bonferroni post-hoc analysis. $p \leq 0.05$ was considered to be statistically significant. At least four animals were taken into this study for each experiment.

3. Results

3.1. Expression of Na^+/K^+ ATPase isoforms in the mouse brain

In order to investigate whether the different α1, 2, 3 and β1, 2, 3 Na^+/K^+ ATPase isoforms are expressed in a cell-type specific manner, we performed an immunohistochemical investigation on postnatal day 19 mouse brain sections using antibodies as detailed in section 2.2.

It is known from Schneider et al. (1988) that the Na^+/K^+ ATPase α1 isoform is expressed ubiquitously, showing the strongest expression in epithelium and neural tissues. Our immunohistochemical stainings revealed a consistent expression of the Na^+/K^+ ATPase α1 isoform in neurons as well as glial cells, as indicated by the co-localization of the Na^+/K^+ ATPase α1 staining signal with GFAP positive as well as with GFAP negative cells (Fig. 1a). We further observed that Na^+/K^+ ATPase α2 was expressed mostly in glial cells, but not in neurons, as demonstrated by absence of co-localization of the Na^+/K^+ ATPase α2 isoform signal with the NeuN staining (not shown) and a coexpression with GFAP (Fig. 1b). In contrast, the Na^+/K^+ ATPase α3 staining signal co-localized well with the NeuN positive cells (Fig. 1c). These results indicate that Na^+/K^+ ATPase α1 is expressed in both neurons and glial cells. In cortex Na^+/K^+ ATPase α2 was expressed exclusively in the non-neuronal cell types and Na^+/K^+ ATPase α3 showed its localization mostly in neurons. However, Na^+/K^+ ATPase β1 & 2 isoforms were expressed in most brain cell types (Fig. 2a and b). These findings are in line with a previous report (Banerjee and Chaudhury, 2001). Na^+/K^+ ATPase β3 expressed mostly around the nuclei of the cells. Na^+/K^+ ATPase β3 showed only a weak colocalization with the GFAP signal (Fig. 2c). As both the β3 isoform and NeuN antibodies had been raised in mice, we could not perform a colocalization study for neurons.

3.2. Region-specific expression of Na^+/K^+ ATPase α and β isoforms

To visualize the distribution and expression patterns of Na^+/K^+ ATPase isoforms in cortex, hippocampus, cerebellum and midbrain, we used postnatal day 19 murine brain sections. In the cortex, the α2 isoform of the Na^+/K^+ ATPase showed a more prominent expression compared to α1 and α3 (Fig. 3). In the hippocampus, Na^+/K^+ ATPase α2 isoform expressed in both areas CA2 and CA3, whereas Na^+/K^+ ATPase α1 and α3 isoforms showed less intense staining. In comparison, the Na^+/K^+ ATPase α isoforms showed a moderate expression in cell somata of Purkinje cells, but were not expressed in dendrites except for the α2 isoform (Fig. 3). Na^+/K^+ ATPase α isoforms showed also an adequate expression in the midbrain, in particular midbrain reticular nucleus (MRN) (Fig. 3).

The distribution patterns of Na^+/K^+ ATPase β isoforms showed a similar pattern in the cortex, where all the three Na^+/K^+ ATPase β isoforms showed only a faint expression over all cortical layers (Fig. 4). However, in the hippocampus, the β2 isoform of the Na^+/K^+ ATPase expressed strongly in both areas; CA2 and CA3, whereas Na^+/K^+ ATPase β1 showed much less expression in hippocampus, but the β3 isoform showed less expression in CA3 with no expression in the CA2 region. In the cerebellum, Na^+/K^+ ATPase β1 isoforms showed a much stronger expression in the cell bodies and dendrites of Purkinje cells, whereas Na^+/K^+ ATPase β2 and β3 isoforms showed a weak expression. Most remarkably, the β3 isoform showed a strong expression in structures like blood vessels. Na^+/K^+ ATPase β isoforms showed also an adequate expression in the midbrain (Fig. 4).

3.3. Changes in the expression levels of Na^+/K^+ ATPase isoforms during development

In order to find out, whether the expression of the different isoforms of the Na^+/K^+ ATPase changes during postnatal development, we performed immunoblottings of Na^+/K^+ ATPase α and β isoforms in neonatal and adult mouse brain using the same antibodies. To elucidate potential changes during postnatal maturation in different regions of the mouse brain, we compared the relative levels of protein expression of the different isoforms in cell lysates from cerebral cortex, hippocampus, midbrain and cerebellum from postnatal day 6 and 9 months old animals.

3.3.1. Changes in expression of Na^+/K^+ ATPase α1-isoform in neonatal and adult mouse brain tissues

Although the immunohistochemical stainings had revealed considerable differences of Na^+/K^+ ATPase α1 expression between individual cells in different layers (Figs. 1 and 3) the average protein expression level did not differ significantly between the different brain regions at a given time point investigated (Fig. 5b). However, the immunoblots revealed a consistently increased expression of Na^+/K^+ ATPase α1 in all four regions of adult mouse brain as compared with P6. In the cerebral cortex, hippocampus and cerebellum of adult mouse brain, the levels of the α1 isoform of the Na^+/K^+ ATPase were expressed significantly higher as compared to neonatal mouse brain (Fig. 5a and b). The midbrain region showed a slightly higher expression of Na^+/K^+ ATPase α1 in adult mouse brain than in neonatal mouse tissue, however, this was not significant ($p \geq 0.05$).

3.3.2. Differential expression of Na^+/K^+ ATPase α2 and α3-isoforms in neonatal and adult mouse brain tissues

Concerning the protein expression levels of the α2 isoform of the Na^+/K^+ ATPase an expression pattern complementary to that of the α1 isoform emerged: like the α1 isoform, the overall expression was smaller in the postnatal compared with the adult tissue and showed a slightly, but insignificantly higher expression in postnatal cerebellum, compared with the other three brain regions investigated. Overall, the protein expression level of the Na^+/K^+ ATPase α2 isoform showed an increase in adult mice as compared with neonatal mice (Fig. 5a and c). In contrast to the change in expression of the α1 isoform, the α2 isoform of the Na^+/K^+ ATPase showed only an insignificantly ($p \geq 0.05$) increased expression in cerebral cortex, hippocampus and cerebellum. In the midbrain, however, its protein level increased significantly ($p \leq 0.01$). Thus, the α1 isoform shows a stronger age-related increase in cortex, hippocampus and cerebellum, whereas the α2 isoform of the Na^+/K^+ ATPase shows a stronger increase in the midbrain during brain development.

Similar to the expression levels of the two other α isoforms, the expression of the Na^+/K^+ ATPase α3 isoform did not differ significantly between the four brain regions within the same time point of investigation and in all brain areas investigated, the expression level increased with development (Fig. 5aand d). The adult mouse brain tissue showed an increased protein expression of Na^+/K^+ ATPase α3 in

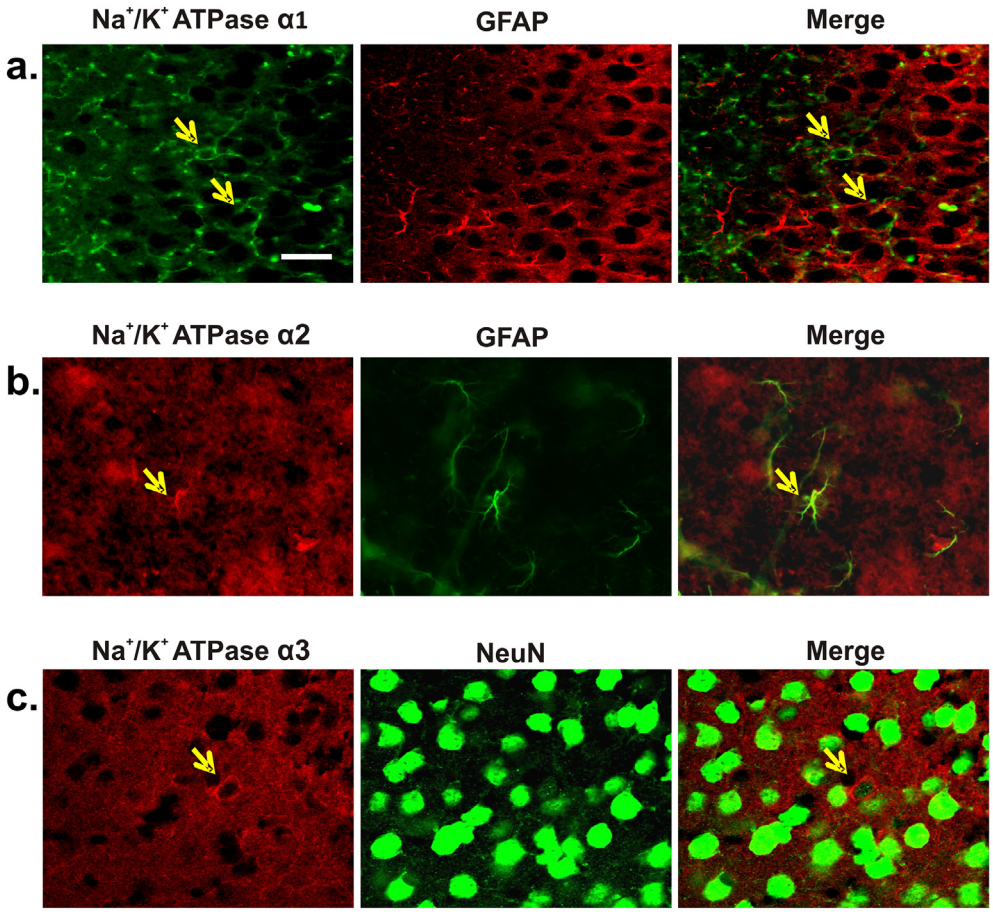

Fig. 1. Cell type specific expression of Na^+/K^+ ATPase α isoforms in mouse brain cortex. (a) Representative images of somatosensory cortex stained with antibodies against the Na^+/K^+ ATPase α1 isoform (left) and the astrocyte marker GFAP (middle). The yellow arrows indicate Na^+/K^+ ATPase α1 isoform stained cells (left) and the co-localization of the Na^+/K^+ ATPase α1 and GFAP positive cells in the right panel. Note that in the left panel the upper yellow arrow points to a Na +/K + ATPase α1 positive neuron and the lower yellow arrow points to a stained astrocyte (left). (b) Representative images of cortical slices stained with antibodies against Na^+/K^+ ATPase α2 (left) and the astrocyte marker GFAP (middle). The yellow arrows point to a Na^+/K^+ ATPase α2 isoform stained cell (left) and the colocalization of Na^+/K^+ ATPase α2 and a GFAP positive cell in the right panel. (c) Representative images of cortical slices stained with antibodies against the Na^+/K^+ ATPase α3 (left) and the neuronal marker NeuN (middle). The merged image (right) displays the co-localization of the two markers in mouse brain cortex The yellow arrows indicate a cell stained against the α3 isoform of the Na^+/K^+ ATPase (left) and the colocalization of Na^+/K^+ ATPase α3 and a NeuN positive cell in the right panel (C). Scale bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cortex, hippocampus and midbrain. The cerebellar tissue showed only an insignificant increase in the expression of the Na^+/K^+ ATPase α3 isoform between the neonatal and adult time point of investigation.

Taken together, all Na^+/K^+ ATPase α isoforms showed an increased expression between P6 and 9 months of age. This increase was insignificant for α1 in midbrain, in which α2 and α3 increased significantly. On the other hand, in the cerebellum only α1 increased significantly, potentially due to an already higher expression level of the other two isoforms at P6. In cortex and hippocampus, Na^+/K^+ ATPase α1 as well as α3 increased significantly.

3.3.3. Expression of Na^+/K^+ ATPase β isoforms in neonatal and adult mouse brain tissues

Like the Na^+/K^+ ATPase α isoforms, the β isoforms showed an upregulated protein expression in adult animals compared with P6 and there were no significant differences between the protein levels within the different areas at both time points investigated. Although all brain regions showed a tendency for an up-regulation of β1 levels, they were only significantly increased in hippocampus and cerebellum of adult mice (Fig. 6a and b). The Na^+/K^+ ATPase β2 isoform displayed a slightly, but insignificantly increased protein level in cerebellar tissue compared with the other three brain regions in both adult and neonatal mice. During development, the pattern of significant increase resembled that of the α3 isoform: cerebral cortex, hippocampus and midbrain showed a significant ($p \leq 0.005$, $p \leq 0.001$) increase in the Na^+/K^+ ATPase β2 expression in the adult mouse brain compared with P6 mouse brain (Fig. 6a and c). Interestingly, all four brain regions of adult mouse brain showed an increased expression level of Na^+/K^+ ATPase β3 isoform compared with the respective neonatal brain regions. During development, the increased expression pattern of the β3 isoform partially resembled α1 which showed a significant ($p \leq 0.001$) increase in cerebral cortex, hippocampus and cerebellum of the brain and α3 showed a significant increase in cerebral cortex, hippocampus and midbrain of the brain (Fig. 6a, b and d).

4. Discussion

This study set out to investigate whether the level of expression of the different isoforms of the Na^+/K^+ ATPase change during postnatal development in different regions of the mouse brain (cortex, hippocampus, cerebellum and midbrain).

As a first step, we analyzed a potential cell-type specific expression pattern of different Na^+/K^+ ATPase isoforms in the cortex of young adult mice using immunohistochemistry. The α1 isoform was observed in all brain cell types, whereas, the α2 isoform was detected only in the glial cells and α3 was found only in neurons. These observations support previous findings showing mRNA expression of the α1 isoform in all cell types of the rat central nervous system, while α3 was localized specifically to neurons (Banerjee and Chaudhury, 2001).

In the mouse brain, Na^+/K^+ ATPase β isoforms are expressed in both neurons as well as glial cells. However, the less studied β3 isoform showed a weak expression in GFAP positive cells. It has previously been demonstrated that the β3 isoform is predominantly expressed in one of the glial cell types, oligodendrocytes in the cortical white matter, hypothalamic fornix, corpus callosum and optic tract of rat brain tissue (Martin-Vasallo et al., 2000). Since oligodendrocytes produce myelin, an insulating sheath required for the saltatory conduction of electrical impulses along axons, presumably, the β3 isoform plays an important role in regulation of neuronal signal conduction. In addition, the β3 isoform is also expressed in early passages of the rat C6-Glioma line, an experimental model system for glioblastoma multiforme (GBM) (Arystarkhova and Sweadner, 1997). Consistently, in a recent finding,

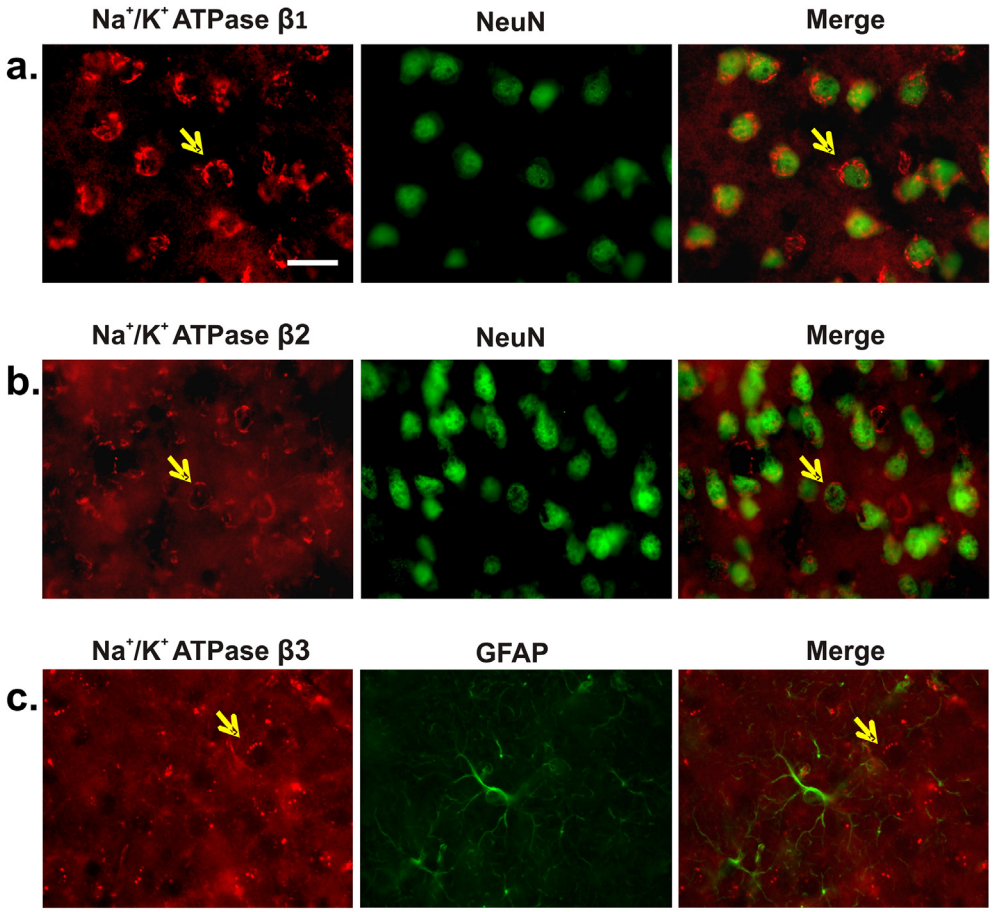

Fig. 2. Cell type specific expression of Na^+/K^+ ATPase β isoforms in mouse brain cortex. (a) Representative images of cortical slices stained with antibodies against Na^+/K^+ ATPase β1 (left) and the neuronal marker NeuN (middle). The yellow arrows indicate the peri-nuclear localization of β1 subunits in neurons in the merged image (right), showing nuclei of neurons in green and the β1 isoform in red. (b). Representative images of cortical slices stained with antibodies against Na^+/K^+ ATPase β2 (left) and the neuronal marker NeuN (middle). The merged image shows the β2 isoform in red and the neuronal nuclei in green. The yellow arrow highlights a β2 positive cell co-expressing NeuN. (c) Representative images of cortical slices stained with antibodies against the Na^+/K^+ ATPase β3 (left) and the astrocyte marker GFAP (middle). The yellow arrow highlights a β3 positive cell. Scale bar = 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

through a colocalization study of the β3 isoform with different brain cell type markers in tissues of GBM patients, it has been shown that the β3 isoform is expressed ubiquitously in astrocytes, endothelial cells, and pericytes (Rotoli et al., 2017). Our immunohistochemical data showed a prominent expression of the β3 isoform in blood vessel like structures. Although we did not perform any double staining of antibodies against the β3 isoform with endothelial cell specific markers (e.g. CD31), the staining pattern is highly consistent with the β3 positive, CD31 expressing endothelial cells of GBM patients' tissues (Rotoli et al., 2017). In particular, we observed a puncta like staining of the β3 isoform around in the nuclei of the cells and weak expression in astrocytes (Fig. 2c). The β3 puncta like staining signals which we observed in the postnatal day 19 mouse brain sections may resemble the β2 isoform staining pattern, synaptic button like signals on mesencephalon level brain tissues of the lizard *Gallotia galloti*, where it was proposed that the β2 isoform might play an important role in the synaptic setup (Arteaga et al., 2003). In the same line of thought, findings from our study showed dots or punctate staining for the Na^+/K^+ ATPase β3 isoform (Fig. 4). Thus we hypothesize that the β3 isoform might play a critical role in creating an adequate ionic environment for neuronal signal conduction and/or signal contacts. Since the β3 antibody and most of the existing neuronal markers had been raised in mouse we could not perform a colocalization study to confirm the expression of the β3 isoform in neurons. Thus, a future immunohistochemical colocalization study demonstrating the expression of the β3 isoform with neuronal markers, in addition to pre- and postsynaptic markers, would provide further information about the functional properties of the β3 isoform in the brain.

Our immunohistochemical data showed signals for the β3 isoform in all six cortical layers, the CA3 region of hippocampus, a mild expression in the cerebellar granule layer and an ubiquitous distribution in MRN apparently in both neurons and glia (Fig. 4). These findings are consistent with those of Malik et al., (1998) who demonstrated an ubiquitous distribution of β3 isoform mRNA in rat brain tissues, including cortex, hippocampus, rostral mesencephalon and cerebellum (Malik et al., 1998). However, Martin-Vasallo et al. (2000) showed an expression of the β3 isoform exclusively in rat and mouse white matter oligodendrocytes (Martin-Vasallo et al., 2000). Though our staining data showed an ubiquitous distribution of the β3 isoform in the mouse brain, we observed a puncta like staining around in the nuclei of the cells. Nonetheless, it is possible that in our study the β3 signal could also come from the oligodendrocytes of the mouse brain tissues. Potentially, the discrepancy might be due to the specificity of the β3 antibodies which have been used in both studies. Therefore, a future study using different β3 antibodies and colocalization with different brain cell type specific markers would provide further insights about the cell and brain region specific expression pattern of the β3 isoform.

Overall, immunoblotting revealed an up-regulation of the expression of most of the isoforms in adult animals compared with postnatal mouse brain tissue (Figs. 5 and 6). Moreover, this is in line with a previous study which demonstrated that adult rat brain abundantly expresses α1, α2 and α3 Na^+/K^+ ATPase isoforms at the mRNA level, whereas there is little expression of the α2 isoform in E18 rat brain tissue (Schneider et al., 1988). Since we found a larger increase in α1 and α3 isoforms (expressed in neurons) than those of the α2 isoforms (predominantly expressed in glial cells), we hypothesize that maturing neurons in cortex, hippocampus and cerebellum may acquire more ATPases along with the development of more neuronal processes and contacts.

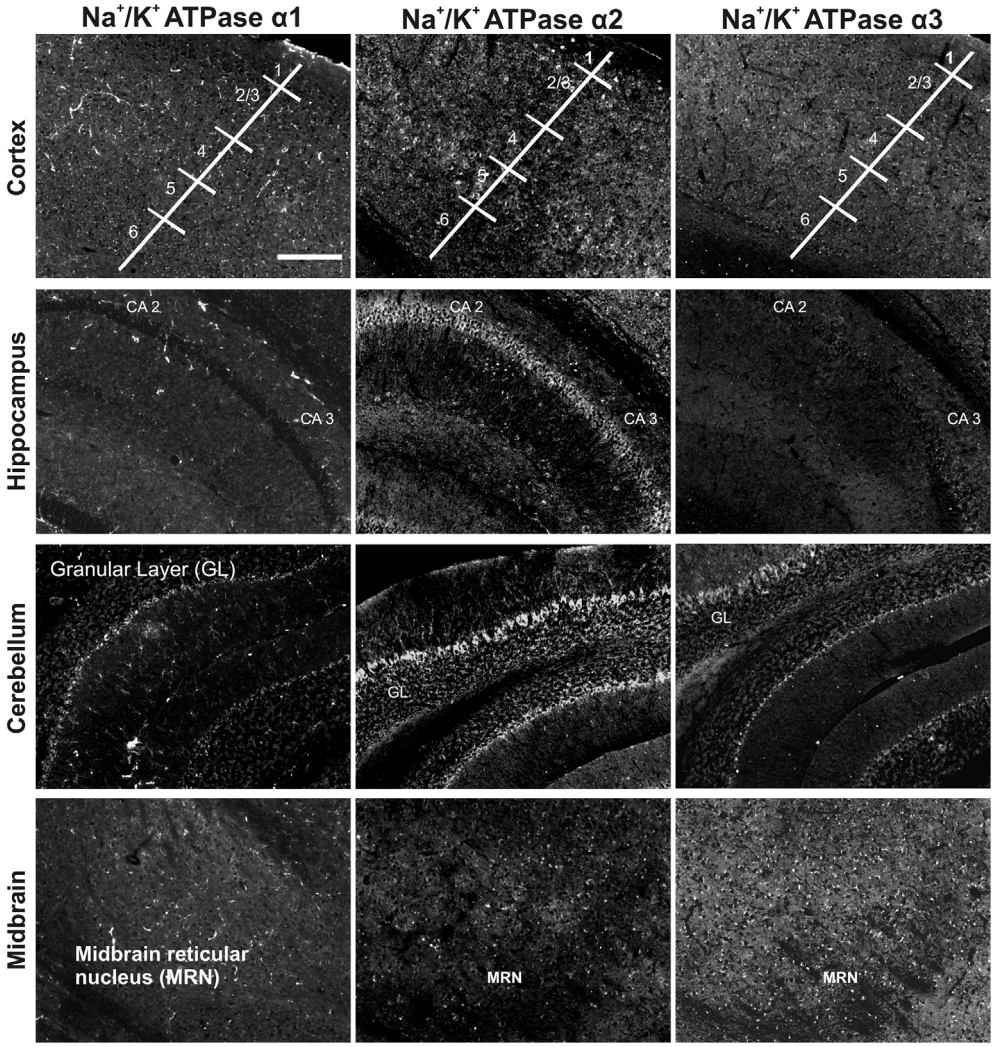

Fig. 3. Detection of Na^+/K^+ ATPase α isoforms in P19 mouse brain. Column one: Na^+/K^+ ATPase α1, Column two: Na^+/K^+ ATPase α2 and, Column three: Na^+/K^+ ATPase α3. Presence of Na^+/K^+ ATPase α isoforms was imaged in somatosensory cortex (cortical layers 1–6 are marked), hippocampus, cerebellum and midbrain region of postnatal day 19 mouse brain sections. Scale bar represents 100 μm.

4.1. Expression pattern of Na^+/K^+ ATPase in cortex and hippocampus

In the mouse brain cortex, the protein expression of the α1, α3, β2 and β3 isoforms was significantly elevated upon development, whereas the expression of the β1 and α2 isoforms was insignificantly elevated (Figs. 5 and 6). Based on the larger increase in the protein level of these isoforms, it is possible that the mouse brain cortices expressed functional binding pairs in an order of α1/β2 = α3/β2 > α1/β1 to maintain the cellular Na^+ and K^+ content (Blanco et al., 1995a,b). Since, using immunoprecipitation experiments in a Madin-Darby Canine Kidney cell line, a previous study showed a potentially high binding affinity of α1/β1 in comparison with α1/β2 (Watts et al., 1991), we expected both α1 and β1 to be upregulated in parallel during mouse development, but the β1 isoform showed only an insignificant elevation at the protein level in the cortex. Presumably, a moderate up-regulation of the β1 isoform is sufficient for the complete binding of most of the upregulated α1 isoforms, whereas the α3 isoform preferentially binds to the β2 isoform (Blanco et al., 1995a,b). However, the apparent affinity of Na^+ to α3/β2 is similar to that of the α1/β1 complex, which is widely expressed in other tissues (e.g. cardiomyocytes) (Blanco et al., 1995a,b; Habeck et al., 2016). The α3/β2 complex displayed a 1.6 fold higher apparent affinity for Na^+ than the α3/β1 complex, suggesting that α3/β1 comes into play when larger Na^+ loads have to be removed out of active cells. Furthermore, our results regarding the β3 isoform raise the possibility that mouse brain cortices may also increase the expression level of the Na^+/K^+ ATPase α1/β3 complex to maintain the cellular Na^+ and K^+ content during development. Since Horisberger et al., 1991, observed that α1 and β3 RNA injected oocytes formed α1/β3 isoenzymes similar to α1/β1 complexes, our findings propose that α1/β3 complexes also contribute to maintain the Na^+ and K^+ content in the developing mouse brain in addition of α1/β2 and α3/β2 (Ackermann and Geering, 1990; Horisberger et al., 1991). However, the binding affinity of α3 towards β3 has not been studied so far. Therefore, we cannot rule out a possible contribution of α3/β3 complexes during mouse brain development. Furthermore, the stainings in Fig. 4 indicate a preferential localization of the β3 subunit to capillaries, suggesting that this subunit might play a role in the function of the blood brain barrier or that this subunit is preferentially expressed in erythrocytes, as some stainings in Fig. 4 suggest. The observation would correspond to the increase in brain blood flow observed with maturation of the brain (Chiron et al., 1992). Although our results do not permit to quantify the exact stoichiometry of the isoform combinations expressed, our data suggests that α1/β2, α3/β2, α1/β3 and α3/β3 isoforms show the largest increase in the cortical maturation of mice.

In hippocampus, the changes in protein expression of the Na^+/K^+ ATPase α1, α3 and β3 isoforms were almost similar to that observed in cortical tissue. Interestingly, β1 as well as β2 isoforms were significantly up-regulated in adult mouse hippocampus (Fig. 6b and c).

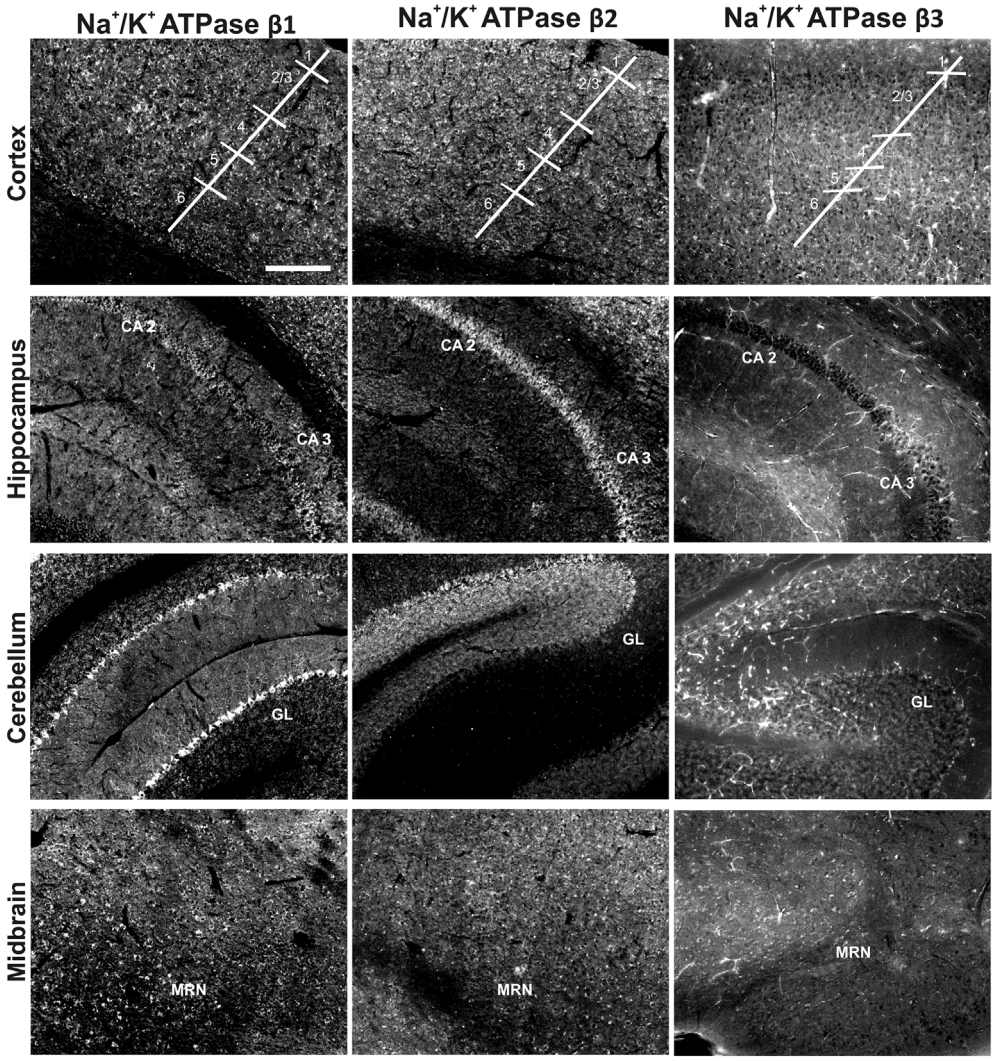

Fig. 4. Detection of Na^+/K^+ ATPase β isoforms in P19 mouse brain. Column one: Na^+/K^+ ATPase β1, Column two: Na^+/K^+ ATPase β2 and, Column three: Na^+/K^+ ATPase β3. Expression of Na^+/K^+ ATPase β isoforms displayed in somatosensory cortex (cortical layers 1–6 are marked), hippocampus, cerebellum and midbrain region of postnatal day 19 mouse brain sections. Scale bar represents 100 μm.

One possible explanation could be that the higher Na^+ load of hippocampal neurons might require more α3/β1 complexes which are additionally activated at higher Na^+ concentrations.

4.2. Expression pattern of Na^+/K^+ ATPase in the cerebellum

In the cerebellum, only α1, β1, β2 and β3 isoforms were significantly up-regulated, whereas the α2 and α3 isoforms showed an insignificant increase, suggesting a vital role of the cerebellar granule layer α1/β1, α1/β2 and α1/β3 complexes in Na^+/K^+ ATPase activity. Since Peng et al., 1997 observed no expression of α1 in Purkinje cells, but an abundant expression of α1 in rat granule cells, the up-regulation of α1 seen in the present study could be attributed to an increased granule cell activity between P6 and adulthood (Peng et al., 1997). This interpretation is additionally favoured by the observation of a strong α1 staining signal in the granule cell layer (Figs. 2 and 3). Inconsistent with the findings of Peng et al. (1997), the presence of β1 isoforms in the soma and processes of Purkinje neurons is more ambiguous (Peng et al., 1997). However, in this study, we have shown a prominent expression of the α2 isoform in Purkinje cell soma and to some extent also in the processes (Fig. 3). This finding is inconsistent with Peng et al. (1997), who observed for the first time an outlining of α2 isoforms in Purkinje cell dendrites using their lab-made antibody. Therefore, our data might indicate that the α2/β1 complex plays a critical role in cerebellar Purkinje cell layer development. However, co-localization staining of Purkinje cell markers and the α2 isoform antibodies would reveal more information about the cell-type specific expression of the α2 isoform in the cerebellum.

4.3. Expression pattern of Na^+/K^+ ATPase in the mid brain tissue

Our immunohistochemical data showed that α1 is expressed uniformly in both neurons and glial cells, but α2 is expressed exclusively in glial cells and α3 only in cortical neurons at P19 which is consistent with previously published reports (Fig. 1a, b, c) (Brines et al., 1991; Cameron et al., 1994). Interestingly, the expression of the α2 isoform in the midbrain region increased in the adolescence stage of the mouse, though our immunohistochemical data showed only a faint staining of the α2 isoform in the midbrain region of P19 mice. One possible reason could be that the glial cells in the mouse midbrain start to express high levels of the α2 isoform later in the adolescence period. Kim et al., (2011) observed that hippocampal glial cells express more GFAP upon development in order to support the functional activity of neurons (Kim et al., 2011). In the same line of thought, we propose that during development midbrain neurons mature, as reflected by their high increase in α3 levels, and their increased activity might challenge the astrocytes

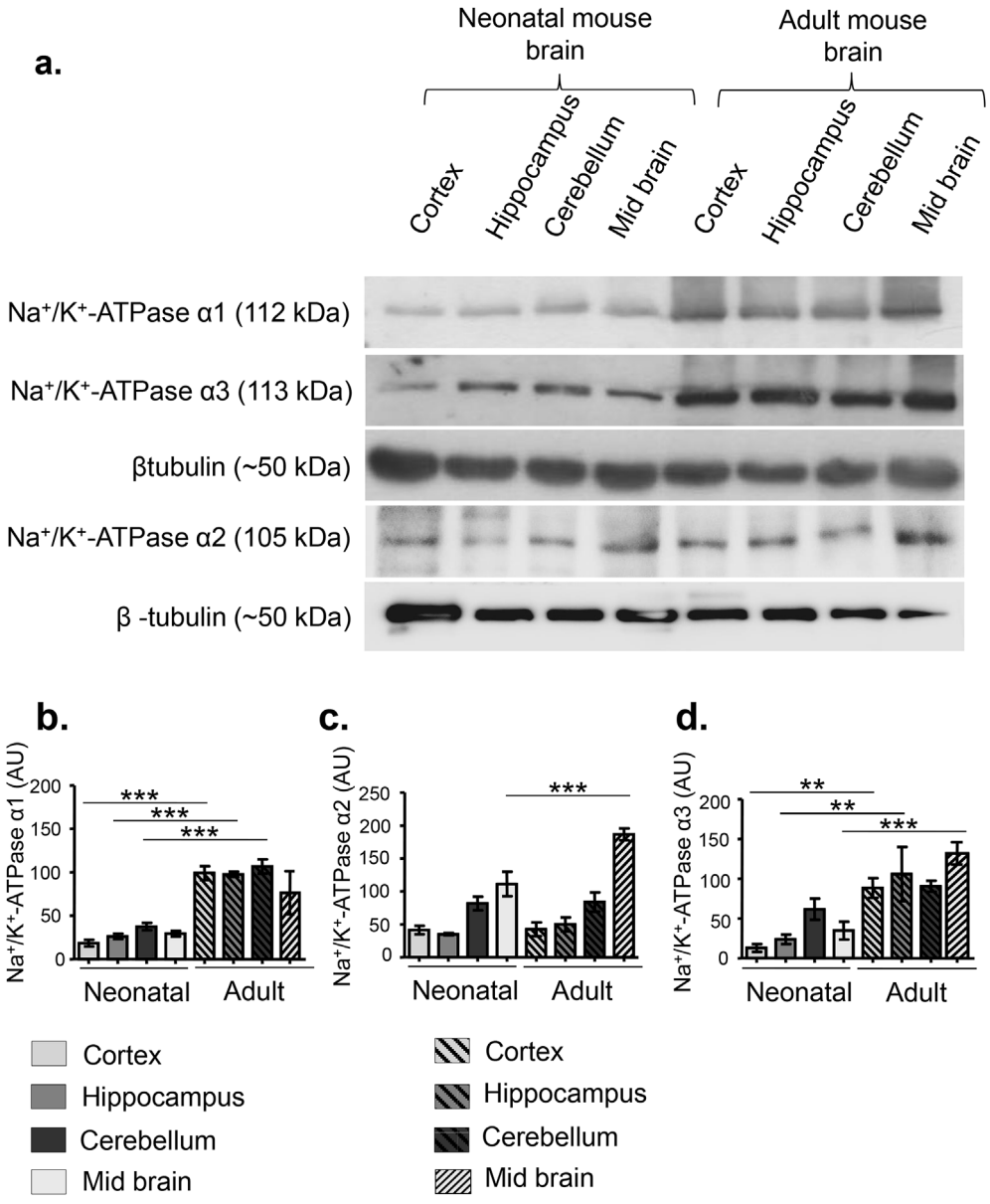

Fig. 5. Immunoblot analyses of Na^+/K^+ ATPase α isoforms in mouse brain. (a) Protein expression of Na^+/K^+ ATPase α isoforms in cortex, hippocampus, cerebellum and midbrain of neonatal and adult mice (b,c,d). Protein expression of Na^+/K^+ ATPase α isoforms normalized to the expression of β-tubulin as an internal control. Bar graphs represent mean ± s.e.m for the different brain regions analyzed in P6 and 9 months old brains. Data were analyzed using one way ANOVA.*** = $p \leq 0.001$, ** = $p \leq 0.01$ and * = $p \leq 0.05$, n = 4.

to clear more glutamate released during neuronal activity via glutamate/Na^+ co-transporters. In order to handle the increased Na^+ load of the cells occurring in the course of enhanced transport activity, astrocytes might express more α2 isoforms in the midbrain at the adult stage. The high level of the α2 isoform in the mouse mid brain could thus reflect an increased functional response of astrocytes to neuronal activity.

Visualizing changes in the expression of various Na^+/K^+ ATPase isoforms in the central nervous system would reveal insights into pathological conditions such as familial hemiplegic migraine type 2 (mutations in the *Na^+/K^+ ATPase α2*) and RDP. Overall, our immunoblotting data suggest that in the course of brain development, from neonatal to adult stage, most of the Na^+/K^+ ATPase isoforms; α1, α3, β1, β2, β3 are up-regulated in the cortex, hippocampus, cerebellum and midbrain regions, but not the α2 isoform. The α2 isoform maintained an equal level of protein expression in cortex, hippocampus and cerebellum at both neonatal and adult stages and was only up-regulated in adult mouse midbrain. Since the α2 isoform showed nearly no changes in most of the mouse brain regions (cortex, hippocampus and cerebellum) upon development, it is possible that after birth the α2 isoform expression was already at its peak in those brain regions. Interestingly, during the gestational period (embryonic day 18.5), the α2 isoform showed a very selective expression in neurons. It was expressed in astrocytes only after birth with a simultaneous α2 isoform gene silencing in neurons (Moseley et al., 2003). The α2 isoform 'gene turn on' mechanism is important for the physiological function of astrocytes, since a direct response of astrocytes is immediately required to maximize K^+ clearance from the extracellular space following neuronal activity (Larsen et al., 2016). The α2 isoform 'gene turn on-turn off' mechanism is not limited to brain cells, but such a mechanism is also observed in rat adipose mesenchymal stem cells (ASCs). Acosta et al. demonstrated that acutely isolated ASCs express all Na^+/K^+ ATPase subunits; α, β, FXYD2 and FXYD7, but after their differentiation into adipocytes and chondrocytes, the α2 gene expression is eventually

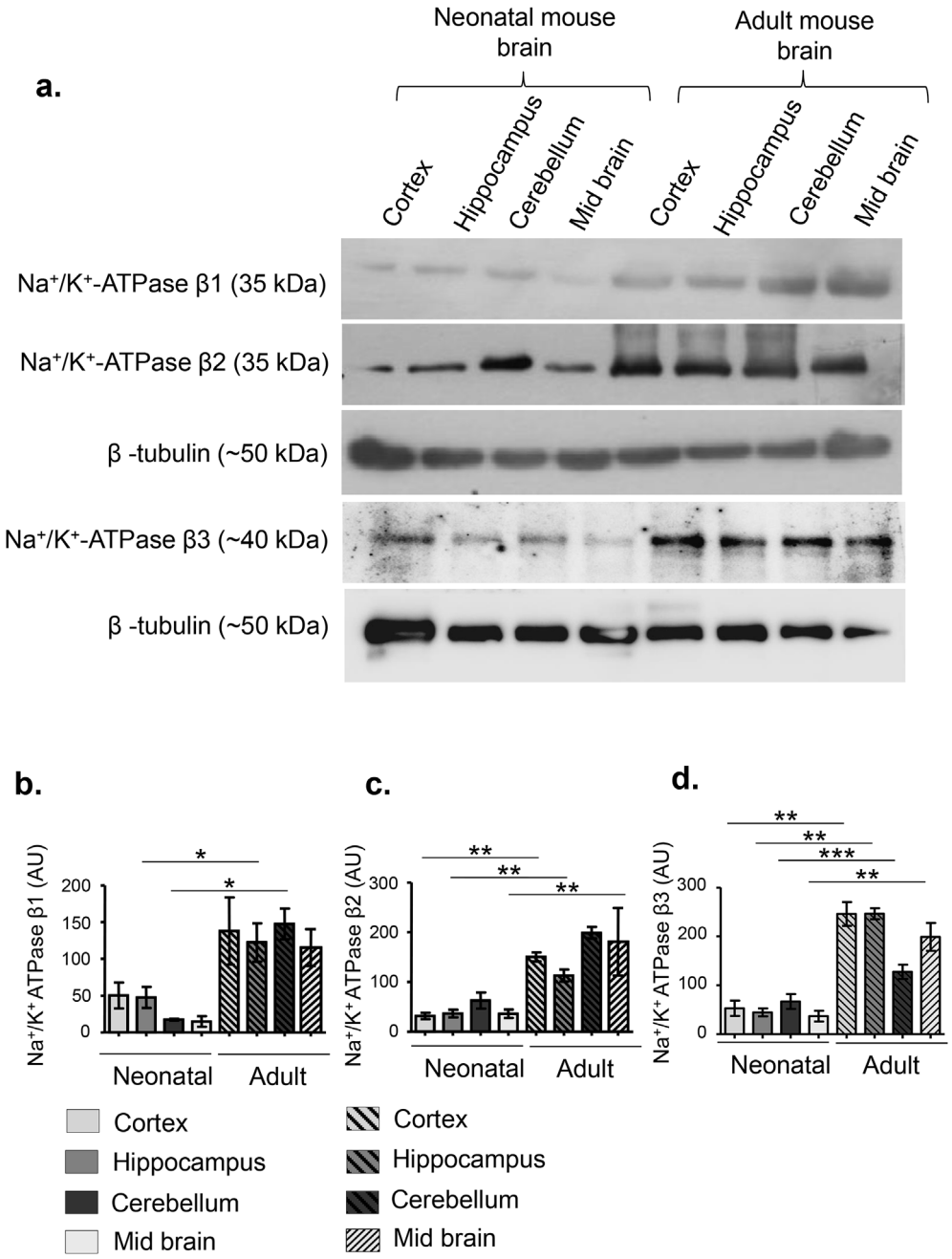

Fig. 6. Immunoblot analysis of Na^+/K^+ ATPase β isoforms in mouse brain. (a) Protein expression of Na^+/K^+ ATPase β isoforms in cortex, hippocampus, cerebellum and midbrain of neonatal and adult mice (b,c,d). Protein expression of Na^+/K^+ ATPase β isoforms normalized to the expression of β-tubulin as an internal control. Internal control β-tubulin blots of Na^+/K^+ ATPase α2 and β3 are from the same set of samples. Bar graphs represent mean ± s.e.m for the different brain regions analyzed in P6 and 9 months old brains. Data were analyzed using one way ANOVA. ** = $p \leq 0.01$ and * = $p \leq 0.05$, n = 4.

turned off (likely from passage 2 of ASCs) (Acosta et al., 2011). Overall, cell type specific Na^+/K^+ ATPase isoform gene turn on or turn off mechanism depends on the physiological functions of different cell types.

Currently, we have little insight into the relationship between the functional significance and the diversity of the different Na^+/K^+ ATPase isoforms in the different regions of the mouse brain. The presence of all three α and three β isoforms suggests a cell-type specific regulation of Na^+/K^+ ATPase isoforms. In particular, α1/β1 and α3/β2 have been shown to be highly regulated isoform-complexes in most mouse brain regions (Blanco et al., 1995a,b). Under basal conditions, both α1 and α3 isoforms contribute to the maintenance of the intracellular Na^+ concentration. Inhibition or dysfunction of the α3 isoform due to mutations causes depolarization of neurons and disruption of network organization (Azarias et al., 2013; Holm et al., 2016).

In addition to confirming the complexity of the expression of the various Na^+/K^+ ATPase isoforms previously noticed by other research groups, here we documented that most of the isoforms increase in their expression during the time period from birth to adulthood. Collectively, these findings are summarized in Table 1. The upregulation of the α1 and α3 isoforms suggests an upregulation in the cells showing most electrical activity. Interestingly, it has been observed, that Na^+ current densities increase as well during postnatal development between p6 and p19 especially in neocortical pyramidal cells (Huguenard et al., 1988), somatosensory cortex (Wang et al., 2009) as well as in Purkinje

Table 1
Distribution and expression pattern of Na^+/K^+ ATPase α and β isoforms in mouse brain.

Na^+/K^+ -ATPase α and β isoforms	Brain cell type	Mouse Brain regional distribution	Protein expression level of Na^+/K^+ ATPase isoforms in adult mouse brain compared to neonatal mouse brain in the present study.			
			Cortex	Hippo-campus	Cerebellum	Midbrain
α1	Neuron, glia (Brines and Robbins, 1993; McGrail et al., 1991; Peng et al., 1997)	Ubiquitous distribution in all cortical layers, hippocampus (mild expression in CA2, CA3), cerebellar granule layer, mild expression in Purkinje cell soma, and ubiquitous cellular distribution in midbrain reticular nucleus (MRN)	Higher	Higher	Higher	Moderately elevated
α2	Glia (Brines and Robbins, 1993; Cameron et al., 1994; Martin-Vasallo et al., 1989; McGrail et al., 1991; Moseley et al., 2003; Peng et al., 1997; Schmalzing et al., 1992)	Cortical layers (prominent expression in layer 5), hippocampusl (CA2 and CA3), cerebellar granule layer, purkinje cells, and ubiquitous cellular distribution in MRN.	Similar	Similar	Similar	Higher
α3	Neuron (Dobretsov et al., 2019; Dobretsov and Stimers, 2005; Peng et al., 1997)	Ubiquitous distribution in all cortical layers (mild expression in layer 6), hippocampus (high distribution in CA3), cerebellar granule layer, Purkinje cell soma, and prominent distribution in MRN.	Higher	Higher	Slightly elevated	Higher
β1	Neuron, glia (Lecuona et al., 1996; Martin-Vasallo et al., 1989; Peng et al., 1997)	Ubiquitous distribution in all cortical layers, hippocampus (CA2 and CA3), cerebellar granule layer, Purkinje cell layer (dendritic tree) and prominent distribution in MRN	Moderately elevated	Higher	Higher	Moderately elevated
β2	Neuron, glia (Antonicek et al., 1987; Gloor et al., 1990; Lecuona et al., 1996)	ubiquitous distribution in all cortical layers, both hippocampal regions (CA2 and CA3), cerebellar granule cells and MRN	Higher	Higher	Slightly elevated	Higher
β3	Possibly in neurons, glia (a weak expression). Oligodendrocytes, astrocyte, pericytes. (Malik et al., 1998, 1996; Martin-Vasallo et al., 2000; Mobasheri et al., 2000; Rotoli et al., 2017)	Ubiquitous distribution in all cortical layers, prominent positive cells in CA3 region, mild expression in cerebellum and ubiquitous distribution in MRN.	Higher	Higher	Higher	Higher

cells (Fry, 2006). The increased Na^+ current density leads to faster action potential upstroke velocities and firing frequencies with maturation, and in addition neuronal bursting activity increases in this developmental period (Franceschetti et al., 1998; Perez Velazquez and Carlen, 1996) suggesting a parallel development of Na^+/K^+ ATPase and Na^+ influx during increased neuronal activity. Furthermore, it has been shown that in skeletal muscle (Harrison and Clausen, 1998) and myotubes (Brodie and Sampson, 1989), the expression of Na^+ channels as tested using saxitoxin binding, preceded the expression of the Na^+/K^+ ATPase as measured by an increase in ouabain binding. Hence, it will be interesting to elucidate in further experiments to what extent the upregulation of the different Na^+/K^+ ATPase isoforms might be a consequence of an enhanced intracellular Na^+ load caused by a larger Na^+ influx into the neurons. Alternatively, a higher relative expression of the different Na^+/K^+ ATPase isoforms could just be a reflection of a more intense fiber network, in which thinner dendrites with a higher surface to volume ratio express more Na^+ channels and Na^+/K^+ ATPases in a given volume of tissue (as reflected by the normalization to β tubulin) at constant membrane densities of both membrane proteins.

4.4. Conclusion

The membrane bound Na^+/K^+ ATPase maintains the concentration difference of Na^+ and K^+ across the plasma membrane using 50% energy of the cell. Although the structure as well as the Na^+/K^+ ATPase transport cycle mechanism are known, the details of regulating factors governing the expression of the various isoforms of the Na^+/K^+ ATPase remain enigmatic. As a first step towards understanding the regulation of the expression of the different isoforms in the brain, we here performed a comparative analysis of Na^+/K^+ ATPase isoform expression in different regions of neonatal and adult mice at postnatal day 6 and 9 month of age. We chose these time points since the postnatal day 6 has been reported to coincide with human neonatal brain and at a time of 9 months the complete neuronal network is mature. We observed that the relative protein level of all Na^+/K^+ ATPase isoforms as normalized to β-tubulin increases during mouse brain development. However, we observed region-specific differences. α3, which is expressed in neurons, was significantly upregulated only in cortex, hippocampus and midbrain. Since this paralleled the significant up-regulation of β2 and β3 isoforms, our results are in line with the assumption that an up-regulation of α3/β2 and α3/β3 complexes reflects the maturation of neuronal networks. The highly specific staining of capillaries for β3 could indicate, that this β subunit is preferentially involved in the function of the epithelial cells and the blood brain barrier or that this subunit is prominently expressed in erythrocytes.

Author contribution

SMS was involved in the most of the experiments and analyzed the results; DS: preparation of mouse brain sections. AE: mouse brain tissue isolation; AF and RH provided valuable reagents and contributed to the manuscript. SMS and ID designed and supervised the study and wrote the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuint.2019.04.009.

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