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Na^+, K^+-ATPase α3 isoform in frontal cortex GABAergic neurons in psychiatric diseases

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A B S T R A C T

Na^+, K^+-ATPase is an essential membrane transporter. In the brain, the α3 isoform of Na^+, K^+-ATPase is vital for neuronal function. The enzyme and its regulators, endogenous cardiac steroids (ECS), were implicated in neuropsychiatric disorders. GABAergic neurotransmission was also studied extensively in diseases such as schizophrenia and bipolar disorder (BD). Post mortem brain samples from subjects with depression, schizophrenia or BD and non-psychiatric controls were provided by the Stanley Medical Research Institute. ECS levels were determined by ELISA. Expression levels of the three Na^+, K^+-ATPase-α isoforms, α1, α2 and α3, were determined by Western blot analysis. The α3 levels in GABAergic neurons in different regions of the brain were quantified by fluorescence immunohistochemistry. The results show that Na^+, K^+ -ATPase α3 isoform levels were lower in GABAergic neurons in the frontal cortex in BD and schizophrenia as compared with the controls (n = 15 subjects per group). A study on a 'mini-cohort' (n = 3 subjects per group) showed that the α3 isoform levels were also lower in GABAergic neurons in the hippocampus, but not amygdala, of bipolar and schizophrenic subjects. In the temporal cortex, higher Na^+, K^+ -ATPase α3 protein levels were found in the three psychiatric groups. No significant differences in ECS levels were found in this brain area. This is the first report on the distribution of α3 in specific neurons in the human brain in association with mental illness. These results strengthen the hypothesis for the involvement of Na^+, K^+ -ATPase in neuropsychiatric diseases.

1. Introduction

Na^+, K^+-ATPase is a ubiquitous plasma membrane transporter that utilizes the energy from ATP hydrolysis to catalyze the exchange of intracellular Na^+ for extracellular K^+. This enzymatic activity is essential for the regulation of intracellular osmolarity, pH and calcium concentration, maintenance of the plasma membrane electric potential, and co-transport of substances across the membrane (Rossier et al., 2015).

Na^+, K^+-ATPase is a hetero-oligomer composed of two major polypeptides: the α and β-subunits. The α subunit carries the catalytic activity of the enzyme. Three α-subunit isoforms were described in the brain (Clausen et al., 2017): the ubiquitous α1 isoform, the α2 isoform, which is predominantly expressed in glia cells (McGrail et al., 1991), and the α3 isoform, which is mainly localized in neurons (Bottger et al., 2011) and to some extent in dendritic spines (Blom et al., 2011). The isoforms have different kinetic properties and affinities and they exhibit species-, tissue-, and cell-specific patterns of expression, thus allowing for the fine-tuning of Na^+, K^+-ATPase activity (Clausen et al., 2017). For example, the α3 isoform is considered to be optimized for rapid clearance of high intracellular concentrations of Na^+ during rapid neuronal firing (Azarias et al., 2013). Mutations in this isoform were implicated in neurological diseases (Clausen et al., 2017).

Na^+, K^+-ATPase is the only known receptor for cardiac steroids (CS). CS such as ouabain, digoxin and bufalin were originally extracted from plants and amphibians and used for the treatment of congestive heart failure and atrial arrhythmias (Wasserstrom and Aistrup, 2005). The binding of CS to Na^+, K^+-ATPase results in the inhibition of ion transport, and also leads to the activation of several signal transduction cascades, including the Src-kinase, the MAP-kinase and the PKC signaling pathways (Cui and Xie, 2017; Madan et al., 2017). Endogenous cardiac steroids (ECS), compounds resembling the structure of plant-derived CS, have been identified in mammalian tissues, including the brain (Hamlyn et al., 1991; Tymiak et al., 1993), and are considered a hormone family that affects numerous physiological and pathological processes (Bagrov and Fedorova, 2005; Nesher et al., 2007; Schoner and Scheiner-Bobis, 2007). ECS in the brain were shown to be involved in various neuronal functions, suggesting their role as neurosteroids (Hodes et al., 2016; Lichtstein and Rosen, 2001).

Psychiatric disorders are complex and involve dysfunction in multiple brain structures. These include the hippocampus, which is involved in context and focus on tasks, the amygdala, which mediates emotional behavior, and the prefrontal cortex, which modulates activity throughout the limbic system (Grace, 2016). Structural and functional alterations in these brain areas were consistently described in schizophrenia and bipolar disorder (BD). Neuroimaging studies revealed the reduced volume of the hippocampus and amygdala in individuals with BD and schizophrenia which were proportional to the

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severity and duration of the illness, and in some cases counteracted by drug treatment (Otten and Meeter, 2015; Prestia et al., 2015). Frontal and temporal lobe structural and functional changes were reported in schizophrenia and BD (Li et al., 2018; Walton et al., 2018). These were accompanied by an array of genetic and molecular alterations contributing to the neuropsychiatric symptoms (Sellmann et al., 2014).

Na^+, K^+-ATPase and ECS were implicated in psychiatric disorders such as BD (Lichtstein et al., 2018): an allelic association between BD and *ATP1A3* has been reported (Mynett-Johnson et al., 1998), followed by the finding of a significant association of BD with six single SNPs in the three genes encoding the α isoforms (Goldstein et al., 2006). Meta-analysis of erythrocyte Na^+, K^+-ATPase activity in BD showed a significant mood-state-related decrease in the enzyme's activity in patients (Looney and el-Mallakh, 1997). Furthermore, Na^+, K^+-ATPase density was significantly lower in BD than in major depressed and schizophrenic subjects (Goldstein et al., 2006). The plasma levels of ECS were significantly reduced in manic individuals, compared with those in non-psychiatric controls (El-Mallakh et al., 2010; Grider et al., 1999). The levels of these compounds were increased in post-mortem samples of the parietal cortex from BD patients (Goldstein et al., 2006).

GABAergic interneurons are present throughout the central nervous system and provide inhibitory control of the projection neurons (Tremblay et al., 2016). Dysfunction of GABAergic neurotransmission is believed to play a role in various neuropsychiatric disorders (Taylor and Tso, 2015). A loss of GABAergic interneurons (Benes et al., 1998) and GABA receptor dysregulation (Fatemi et al., 2017) were reported in post mortem brain samples of BD and schizophrenia patients, as well as a reduction of molecular markers of GABA signaling (Ramaker et al., 2017). The synthesis of GABA is catalyzed by glutamic acid decarboxylase (GAD). GAD67, the isoform located in the somata and dendrites of GABAergic cells and which accounts for 80–90% of overall brain GABA (Kaufman et al., 1991), was consistently implicated in schizophrenia and BD (Akbarian and Huang, 2006).

In view of the central role of GABAergic neurotransmission in brain function and its link to BD and schizophrenia on the one hand, and the potential involvement of Na^+, K^+-ATPase in psychiatric disorders on the other hand, we hypothesized the presence of alterations in α subunit density in GABAergic neurons in pathological states. In the present study, the levels of ECS and of the three Na^+, K^+-ATPase isoforms in post mortem temporal cortex samples of subjects with different psychiatric disorders were compared with those in non-psychiatric controls. A fluorescence immunohistochemical approach was then used to investigate the distribution of the Na^+, K^+-ATPase α3 isoform in post-mortem brain samples of bipolar, schizophrenic and depressed subjects or controls. The results showed that Na^+, K^+-ATPase α3 expression is reduced in GABAergic neurons in the frontal cortex of bipolar and schizophrenic individuals, implicating this isoform in the etiology of psychiatric diseases.

2. Materials and Methods

2.1. Human brain samples

All human brain tissue samples used in this study were obtained from the neuropathology consortium of the Stanley brain collection (Stanley Medical Research Institute, Bethesda, MD, USA). The demographic and clinical characteristics of the population, as well as methods of tissue harvest, preparation, and storage have been previously described in detail (Torrey et al., 2000). Briefly, the subjects were diagnosed as follows: no psychiatric illness, schizophrenia, bipolar disorder, or depression ($n = 15$ per group) and matched for age, sex, race and interval between death and the freezing of brain tissue (postmortem interval), see Supplement Table 1). Cause of death, family history of severe psychiatric disorders, history of substance abuse, medication at time of death and an estimate of total lifetime antipsychotic medication were detailed for all cases (Torrey et al., 2000).

Frozen blocks of temporal cortex tissue from control, schizophrenic, bipolar, or depressed subjects ($n = 15$ per group) were used for ELISA and Western blot analyses. Freshly frozen, 14 μm thick sections on 1.5″ x 3.0″ glass slides were used for fluorescent immunohistochemical analyses. Sections of the hippocampus, amygdala and frontal cortex from control, schizophrenic, and bipolar subjects ($n = 3$ per group; "mini-cohort") were stained for the initial analysis. Frontal cortex sections from control, schizophrenic, bipolar, or depressed subjects ($n = 15$ per group) were used in the subsequent analysis.

2.2. Determination of ECS by ELISA

ECS levels were assessed by the determination of ouabain-like immunoreactivity in brain extracts. Frozen blocks of temporal cortex were kept at −80 °C until analyzed. Extraction of ECS from the tissue was performed as previously described (Hodes et al., 2016; Lichtstein et al., 1998). The assay was performed with a sensitive, competitive inhibition ELISA designed for the quantification of ECS. In this assay, samples were tested for their ability to inhibit the specific binding of anti-ouabain antibodies to solid phase-bound ouabain. The procedure was applied as previously described (Lichtstein et al., 1998), with no modifications.

Samples were randomly processed for ECS levels. ELISA was carried out by a person blinded to the subjects' diagnosis and their identity was revealed by the Stanley Institute after the results were analyzed.

2.3. Quantification of Na^+, K^+-ATPase isoforms by Western blot

Frozen blocks of temporal cortex were kept at −80 °C until analyzed. Samples were homogenized in radioimmunoprecipitation assay buffer supplemented with 1 mM NaVO4 and Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO, USA) and centrifuged (14,000 × *g*). The protein content of the supernatants was determined with the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions.

The samples were subjected to Western blot analysis, as previously described (Goldstein et al., 2006). The following primary antibodies were used: mouse monoclonal anti-Na^+, K^+-ATPase-α1 subunit antibody (Merck, Kenilworth, NJ, USA); rabbit polyclonal anti-Na^+, K^+-ATPase-α2 subunit antibody was kindly provided by Thomas Pressley (Texas Tech University, Lubbock, TX, USA); mouse monoclonal anti-Na^+, K^+-ATPase-α3 subunit antibody and mouse monoclonal anti-α-Tubulin antibody were purchased from Sigma-Aldrich.

Western blot analysis was performed after receiving the patients' code from the Stanley Institute, however the experiments were performed by an individual blinded to the subjects' identity.

2.4. Fluorescence immunohistochemistry

Fluorescence immunohistochemistry was performed as previously described (Bottger et al., 2011), with the following primary antibodies: rabbit polyclonal anti-Na^+, K^+-ATPase-α3 subunit antibody (Novus Biologicals, Littleton, CO, USA); mouse monoclonal anti-GAD67 antibody (clone 1G10.2) and mouse monoclonal anti-tyrosine hydroxylase (TH) antibody (Merck, Kenilworth, NJ, USA). Frozen sections on slides were kept at −80 °C until stained. Slides were fixed for 20 min in 4% paraformaldehyde and washed 3 times × 5 min in phosphate buffered saline (PBS). Nonspecific binding was blocked for 1 h with blocking solution: CAS block (Life Technologies, Frederick, MD, USA) containing 0.3% Triton and 2% bovine serum albumin (BSA, Sigma-Aldrich). Sections were incubated for 2 h in room temperature with primary antibodies (anti-Na^+, K^+-ATPase-α3 and anti-GAD67, or anti-Na^+, K^+-ATPase-α3 and anti-TH), diluted 1:200 in blocking solution, and washed 3 × 5 min in PBS. The sections were then incubated for 45 min with secondary antibodies (Alexa Fluor 568 and Alexa Fluor 647, Abcam, Cambridge, UK) diluted 1:200 in blocking solution without

Triton, then washed 3 × 5 min in PBS and mounted with Fluoroshield mounting medium with DAPI (Abcam) and covered with a 0.15 mm cover glass (Ted Pella, Redding, CA, USA).

GABAergic neurons were stained with an antibody against GAD67 which recognized a single band of 67 kDa in a Western blot experiment on human brain samples (data not shown). Control experiments were performed in parallel with the double staining: sections were incubated with a single primary antibody, following incubation with the non-complementary fluorescent secondary antibody. Imaging of the control slides showed no significant non-specific binding of the secondary antibody (data not shown).

Fluorescence microscopy was performed with a Zeiss LSM 710 laser scanning confocal microscope attached to a Zeiss Axio Observer Z1 inverted microscope with a LCI Plan-Apochromat x25 objective with oil immersion. Alexa Fluor 647 was excited with a 633 nm HeNe laser, Alexa Fluor 568 was excited with a 561 nm diode pumped solid state laser and DAPI was excited with a 405 nm diode laser. For image acquisition, Zen pro software was used. (Zeiss, Oberkochen, Germany).

Images were analyzed with ImageJ software. A minimum 10 fields × 10 cells per field were analyzed in each slide. GAD67 positive cells were identified. For each cell, the α3 fluorescence intensity was calculated by measuring the integrated density of the soma (the cell area excluding the nucleus area) and subtracting the mean fluorescence of the background.

Samples were randomly processed. Analysis was carried out blinded to the subjects' diagnosis and their identity was revealed by the Stanley Institute after the results were analyzed.

2.5. Statistical analysis

All the data are expressed as the mean ± standard error. Statistical analysis was performed by one-way analysis of variance (ANOVA), followed by the Bonferroni test for post-hoc comparisons, or by two-way ANOVA with ECS levels and sex as variables. A value of $p < 0.05$ was considered significant.

3. Results

The present study describes experiments in three human brain collections: 1. Temporal cortex samples of schizophrenic, bipolar or depressed subjects and non-psychiatric controls (n = 15 per group) were used for ELISA and Western blot analysis. 2. Hippocampus, amygdala and frontal cortex samples of schizophrenic, bipolar and control individuals (n = 3 per group, "mini-cohort") and, 3. Frontal cortex samples from schizophrenic, bipolar, depressed and control individuals (n = 15 per group) were used for fluorescence immunohistochemistry.

3.1. ECS levels in temporal cortex of subjects with various psychiatric conditions

The determination of ECS in the brain tissue of psychiatric patients may shed a light on the possible involvement of these steroids in pathological states. In a previous study (Goldstein et al., 2006) it was shown that ECS levels were elevated in the parietal cortex of bipolar subjects, compared with those in depressed, schizophrenic and non-psychiatric controls. To determine whether this is a general phenomenon or a brain-region specific change, ECS levels in post mortem samples from the temporal cortex were now determined. Samples from four groups were analyzed: subjects suffering from BD, major depression and schizophrenia were compared with subjects without known mental illness, as control. The ECS levels were significantly different between men and women, but not between the psychiatric groups. As shown in Fig. 1, ECS levels were higher in women than in men. The ECS levels were lower in depressed and schizophrenic women, as well as in depressed men, vs the respective Control groups. However, these differences did not reach statistical significance, probably due to the large variation in ECS levels in the population.

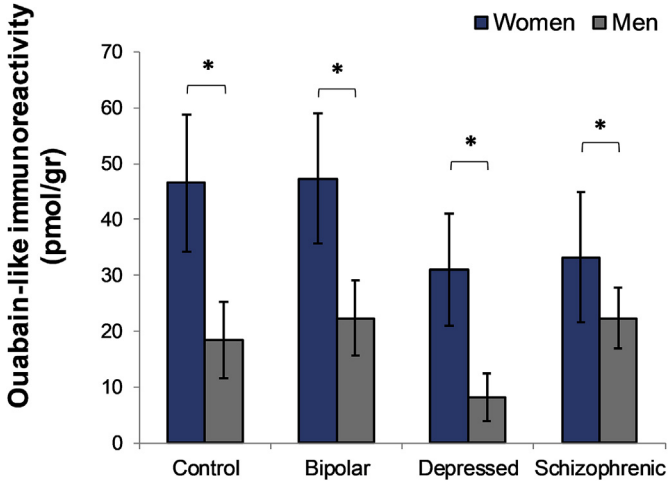

Fig. 1. *ECS levels in temporal cortex.* Ouabain-like immunoreactivity levels in post-mortem temporal cortex samples of control, bipolar, depressed or schizophrenic subjects (n = 15 per group) were determined by ELISA as described in Materials and Methods. Two-way ANOVA: for sex $p = 0.0297$; for group: $p = 0.3477$; for interactions $p = 0.7902$. Bars represent means; error bars represent the standard error of the means; *different between women and men ($p < 0.05$).

3.2. Na^+, K^+-ATPase α isoforms expression in temporal cortex of subjects with various psychiatric conditions

To study the involvement of Na^+, K^+-ATPase in mental illness, and assess the possible contribution of the different isoforms to the pathophysiology of these diseases, the expression levels of the three Na^+, K^+-ATPase α isoforms found in the brain: the ubiquitous α1 subunit, α2 and α3 (predominantly expressed in glia and neurons, respectively), were tested. As shown in Fig. 2A–B, no significant differences in the housekeeping protein α1 levels were detected. The levels of α2 in BD patients was higher than in the controls (159.87% of Control), and no differences were found between controls and depressed or schizophrenic subjects (Fig. 2C–D). The α3 expression was significantly different between the groups: as can be seen in Fig. 2E–F, the α3 level was dramatically elevated by 194.62%, 154.85% and 213.55% in temporal cortex samples of bipolar, depressed and schizophrenic patients, respectively, vs the Control group.

As the most prominent differences between the groups were found in the α3 isoform, subsequent experiments focused on this isoform.

3.3. Na^+, K^+-ATPase α3 isoform levels in GABAergic and dopaminergic neurons in different brain regions of subjects with various psychiatric conditions

To further characterize the changes in the expression of the Na^+, K^+-ATPase α3 isoform it was necessary to determine whether this was a global or region-specific effect. For this purpose, the α3 isoform expression level was examined in GABAergic neurons in different regions of the human brain implicated in psychiatric disorders in afflicted and control subjects. Fluorescence immunohistochemistry was performed with antibodies against Na^+, K^+-ATPase α3, together with GAD67, a marker for GABAergic neurons, in the hippocampus, frontal cortex and amygdala. In this initial analysis, samples from a "mini-cohort" of bipolar, schizophrenic and control subjects ($n = 3$ per group), provided by the Stanley Institute, were used. Representative images of staining of slides from a control subject are shown in Fig. 3A, C, E and G.

Significant differences were found between the groups in the hippocampus and in the frontal cortex, but not in the amygdala. In the

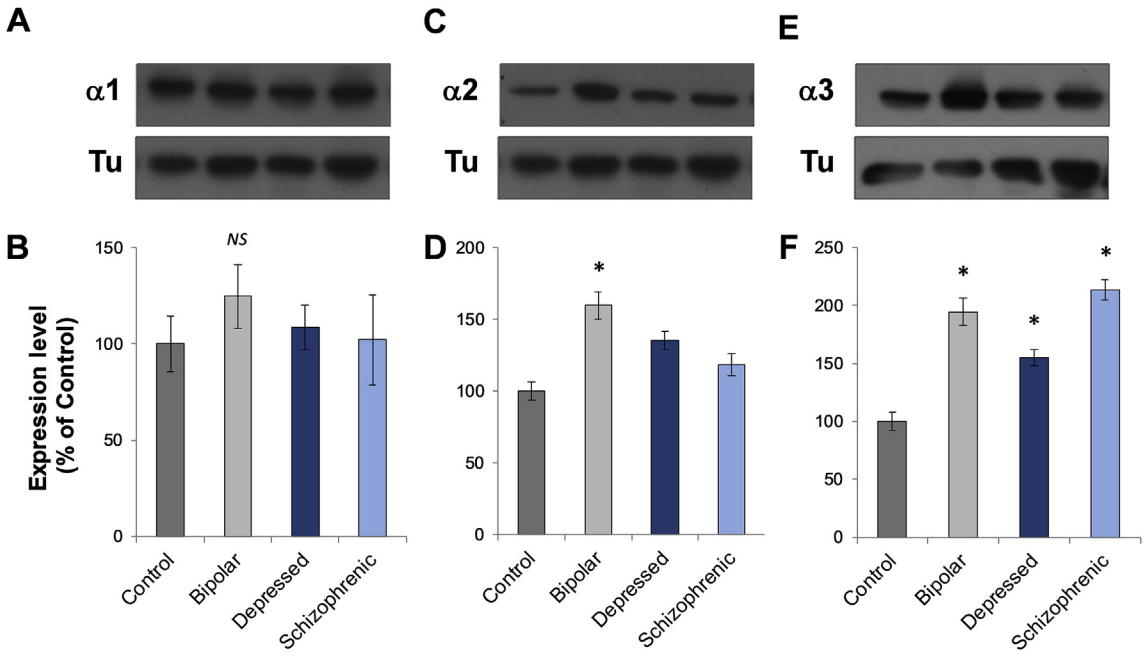

Fig. 2. *Na^+, K^+ -ATPase α isoforms expression in temporal cortex.* Na^+, K^+-ATPase α subunit isoforms expression in post-mortem temporal cortex samples of control, bipolar, depressed or schizophrenic subjects (n = 15 per group) were determined by Western blot analysis as described in Materials and Methods. (A–B): α1 isoform expression. (C–D): α2 isoform expression. (E–F): α3 isoform expression. The values were normalized to α-tubulin (Tu) and expressed as the percentage of the level in the Control group. (A, C, E): representative Western blot bands; (B, D, F): graphical representation of quantification analysis. One-way ANOVA for α1: $F_{3,56} = 1.24$, $p = 0.31$; One-way ANOVA for α2: $F_{3,56} = 3.38$, $p = 0.024$; One-way ANOVA for α3: $F_{3,56} = 15.55$, $p < 0.0001$; Bars represent means; error bars represent the standard error of the means; *different from the Control group (Bonferroni test post hoc; $p < 0.05$); *NS* non-significant.

hippocampus (Fig. 3A–B), the bipolar and schizophrenic groups were significantly different from the Control group. As can be seen in Fig. 3B, the α3 levels in the GAD67 positive neurons in the hippocampus were lower in bipolar individuals, i.e. 65.74% of Control. An even lower level was observed in the schizophrenic group - 50.92% of Control. In the frontal cortex (Fig. 3E–F) significant difference were found in the bipolar and schizophrenic groups compared with the Controls. As shown in Fig. 3F, in the frontal cortex, like in the hippocampus, α3 levels in GAD67 positive neurons were significantly lower in the bipolar (67.88% of Control) and schizophrenic (78.24% of Control) groups. To test for neuronal cell specificity in the expression of the Na^+, K^+-ATPase α3 isoform, the expression level of this isoform in frontal cortex dopaminergic neurons was also examined. There were no significant differences in α3 levels in TH positive neurons between the groups (Fig. 3G–H). These results suggest that the reduction in α3 is a region- and cell-specific phenomenon rather than a global change in brain tissue.

3.4. Na^+, K^+-ATPase α3 isoform levels in GABAergic neurons in the frontal cortex of subjects with various psychiatric conditions

In view of the above described results, the next examinations focused on changes in α3 levels in GABAergic neurons in the frontal cortex, where the most prominent changes were observed in bipolar and schizophrenic subjects. Fluorescence immunohistochemistry with antibodies against Na^+, K^+-ATPase α3 together with anti-GAD67 antibodies was performed in frontal cortex samples from depressed, bipolar, schizophrenic and control subjects in a large cohort ($n = 15$ per group) received from the Stanley Institute. Sections from all 60 cases were successfully stained and analyzed except for one case that had very little tissue on the slide, resulting in 14 cases for the BD group. Representative images of the staining are depicted in Fig. 4A.

Significant differences were found between the groups of subjects. As shown in Fig. 4 and in agreement with the “mini-cohort" experiments (Fig. 3), lower levels of α3 expression were found in GABAergic neurons in bipolar (70.26% of Control) and schizophrenic (69.52% of Control) but not in depressed subject. Interestingly, this is different from the results of total α3 levels which were elevated in temporal cortex in all psychiatric groups (Fig. 2). This demonstrates that Na^+, K^+-ATPase isoforms levels are tissue-, cell- and disease-specific.

4. Discussion

In this study possible changes in α isoforms of Na^+, K^+ -ATPase in human post-mortem brain samples of psychiatric patients were evaluated. To the best of our knowledge, this is the first report on the distribution of α3 in human brain and of altered levels of this protein in specific neurons in association with disease states. The main finding of this study is that the Na^+, K^+ -ATPase α3 isoform levels, determined by fluorescence immunohistochemistry, are lower in GABAergic neurons in the frontal cortex of bipolar and schizophrenic individuals. Western blot analysis revealed a higher level of this protein in the temporal cortex of psychiatric patients. In addition, a fluorescence immunohistochemistry analysis in a “mini-cohort” of patients showed that the α3 isoform level was also lower in GABAergic neurons in the hippocampus, but not amygdala, of bipolar and schizophrenic patients. No change in α3 was found in dopaminergic neurons in the frontal cortex. Furthermore, the levels of the endogenous regulators of Na^+, K^+ -ATPase, ECS, were not altered in the temporal cortex of bipolar, schizophrenic and depressed patients, as compared with healthy individuals.

In the present study, there was a tendency, statistically non-significant, toward lower ECS levels in the temporal cortex in depressed men and in women with depression and schizophrenia (Fig. 1). Due to the large variance of ECS levels in the population (Bagrov et al., 2009; Nesher et al., 2007), a study on a larger cohort of subjects is necessary in order to obtain statistically significant results. The temporal cortex ECS levels were lower in men than in women (Fig. 1). In a previous study, we found increased levels of ouabain-like ECS in the parietal cortex of bipolar individuals, but no sex difference was found

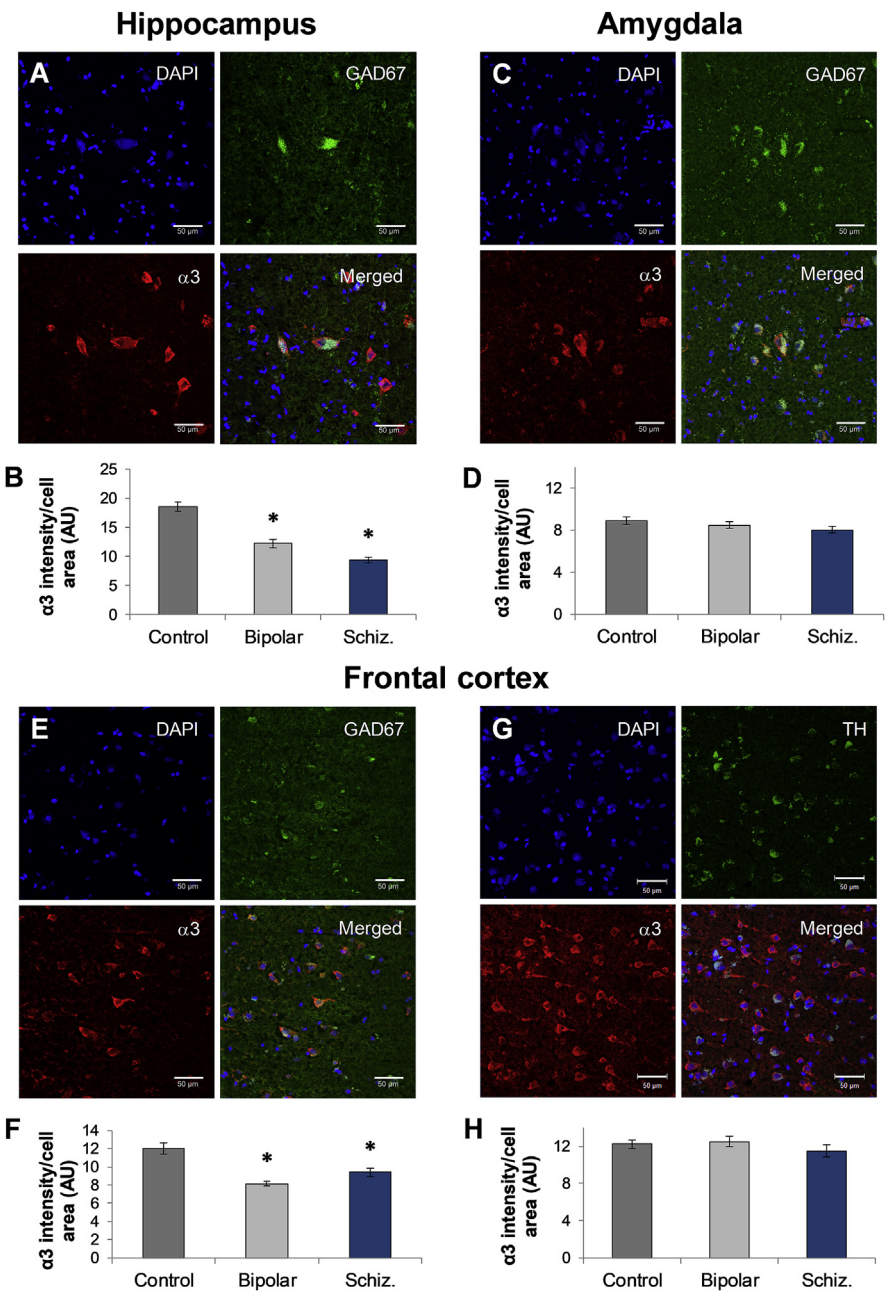

Fig. 3. *Na^+, K^+-ATPase α3 isoform levels in GABAergic and dopaminergic neurons in different brain areas.* Fluorescence immunohistochemistry for Na^+, K^+-ATPase α3 and GABAergic neuronal marker GAD67 (A–F) or dopaminergic neuronal marker TH (G–H) and nuclear marker DAPI in post-mortem brain samples of control, bipolar or schizophrenic subjects (n = 3 per group) from the hippocampus (A–B), amygdala (C–D) of frontal cortex (E–H). Immunostaining was performed as described in Materials and Methods. (A, C, E, G): representative confocal fluorescence images of a control subject; (B, D, F, H): graphical representation of quantification analysis. A minimum 10 fields × 10 cells per field were analyzed in each slide. The fluorescence intensity of α3 was calculated by measuring the integrated density of the soma (the cell area excluding the nucleus area) and subtracting the mean fluorescence of the background for each cell. One-way ANOVA for hippocampus: $F_{2,426} = 35.05$, $p < 0.0001$; One-way ANOVA for amygdala: $F_{2,316} = 1.52$, $p = 0.22$; One-way ANOVA for frontal cortex GAD67 staining: $F_{2,506} = 18.14$, $p < 0.0001$; One-way ANOVA for frontal cortex TH staining: $F_{2,405} = 0.82$, $p = 0.44$. Bars represent means; error bars represent standard error of the means; *different from the Control group (Bonferroni test post hoc; $p < 0.05$). Scale bar = 50 μm.

(Goldstein et al., 2006). Hence, presumably, the metabolism of ECS in different brain regions is diverse. Increased levels of ECS were associated with manic-like and depressive-like behaviors in animal models, which were accompanied by biochemical changes in the brain (Goldstein et al., 2006; Hodes et al., 2016). Reduction of ECS in the brain (by administration of anti-ouabain antibodies) showed dramatic anti-depressive – and anti-manic-like behavioral effects and induced a reduction of oxidative stress (Hodes et al., 2018). However, the mechanism by which ECS and their receptor, the α subunit of Na^+, K^+-ATPase, are involved in complex behavior remains unknown. Interestingly, changes in other neurosteroids such as pregnenolone and dehydroepiandrosterone in the brain were reported in schizophrenia and BD (Marx et al., 2006). A post-mortem study revealed that these changes are likely due to altered expression levels of key enzymes in the steroid synthesis pathway (Qi et al., 2018). Although the entire synthetic pathway of ECS was as yet not elucidated, it was established that the first steps of their biosynthesis are similar to those of other neurosteroids (Lichtstein et al., 2012). Hence, it is reasonable to assume that the changes in the enzymatic machinery for neurosteroids are responsible for the alterations in ECS in specific brain regions and in pathological states.

In the present study, the total levels of each of the Na^+, K^+ -ATPase α-subunit isoforms in the temporal cortex of psychiatric patients were compared with those of the controls. In a previous study, no change was found in any of the isoforms in the parietal cortex of subjects with mental illness (Goldstein et al., 2006). Here, substantial changes were observed: the α2 isoform expression was higher in the bipolar group and the levels of α3 were significantly higher in bipolar, depressed and schizophrenic individuals (Fig. 2). The α3 isoform of Na^+, K^+ -ATPase is mainly localized in neurons (Clausen et al., 2017), and it is of particular importance for neuronal function (Pivovarov et al., 2018). Under conditions of extensive neuronal firing, the α3 isoform has an essential role in the rapid clearance of increased intracellular sodium and cell repolarization (Azarias et al., 2013). Furthermore, Na^+, K^+ -ATPase has an essential role in neurotransmitters release and re-uptake (Ikeda et al., 2017; Kurauchi et al., 2018). Given the above, α3

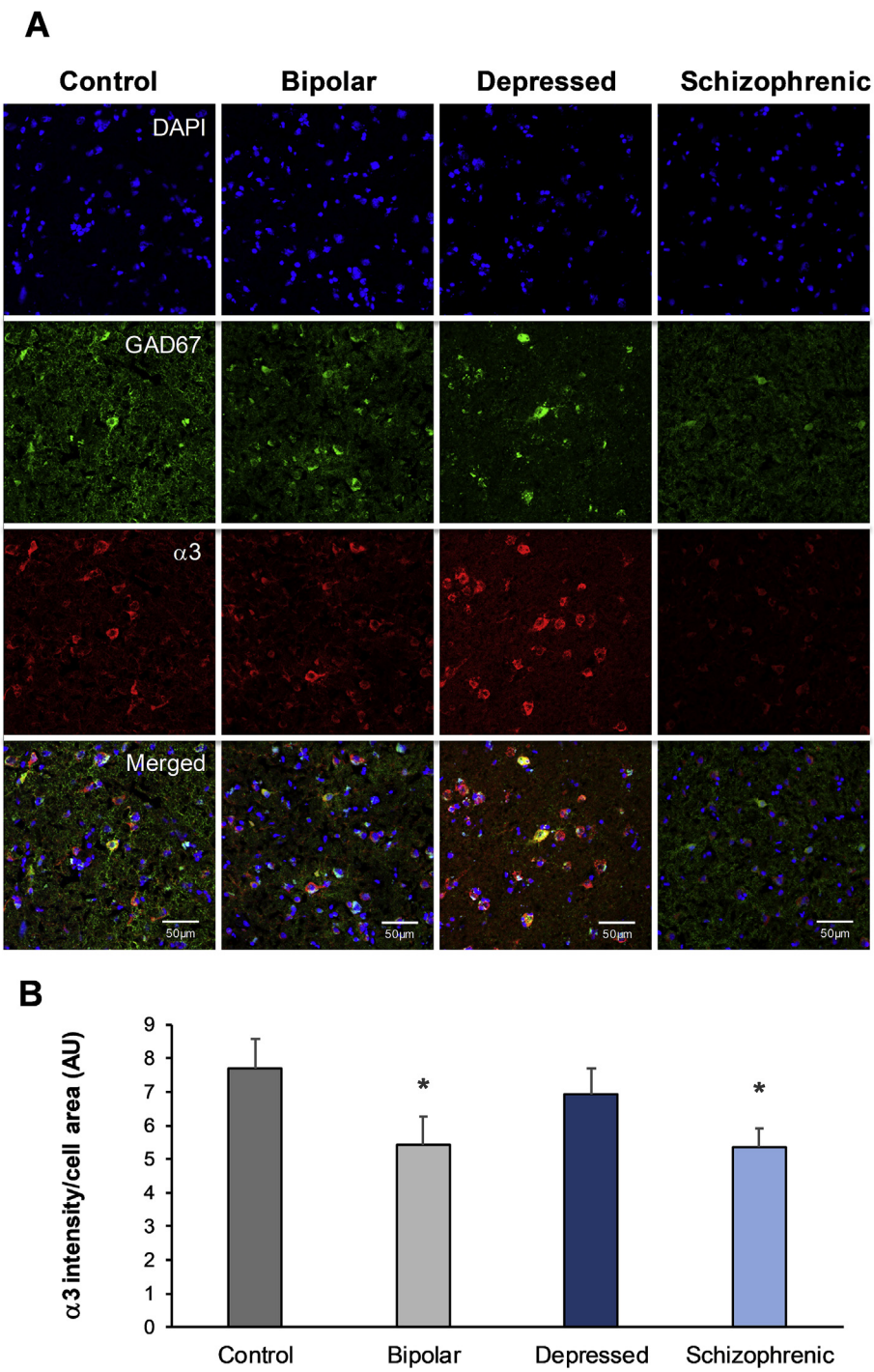

Fig. 4. *Na^+, K^+ -ATPase α3 isoform levels in GABAergic neurons in frontal cortex*. Fluorescence immunohistochemistry for Na^+, K^+-ATPase α3, GABAergic neuronal marker GAD67 and nuclear marker DAPI in post-mortem frontal cortex samples of control, bipolar, depressed or schizophrenic subjects (n = 15 per group). Immunostaining was performed as described in Materials and Methods. (A): representative confocal fluorescence images; (B): graphical representation of quantification analysis. The fluorescence intensity of α3 was calculated as described in the legend to Fig. 3. One-way ANOVA: $F_{3,5483} = 61.23$; $p < 0.0001$. Bars represent means; error bars represent standard error of the means; * different from the Control group (Bonferroni test post hoc; $p < 0.05$).

expression in neurons is a major determinant of these cells' excitability and neurotransmitter metabolism, and alterations in the expression levels of this protein in distinct brain areas may reflect changes in neuronal function in psychiatric disorders (Shrivastava et al., 2018). A number of neurological syndromes are associated with α3 mutations (de Carvalho Aguiar et al., 2004; Demos et al., 2014; Heinzen et al., 2012), and animal models harboring such mutations present with an array of neurological phenotypes (Holm and Lykke-Hartmann, 2016; Kirshenbaum et al., 2011).

In the present investigation, a study of the levels of α3 in GABAergic neurons in brain areas implicated in psychiatric diseases revealed that the α3 isoform level is significantly lower in GABAergic neurons of the frontal cortex of bipolar and schizophrenic patients (Fig. 4). A detailed and elegant study of the mouse brain showed extensive co-localization of this isoform with GABAergic marker GAD67 in multiple brain areas, including the frontal cortex, hippocampus, basal ganglia and more (Bottger et al., 2011). GABAergic neurotransmission in neuropsychiatric diseases has been extensively studied, and was found particularly important in schizophrenia and BD: Dysfunctional GABA activity may cause an imbalance between excitatory and inhibitory cortical activity, which has been hypothesized as one of the molecular mechanisms responsible for psychiatric disorders (Cohen et al., 2015) and an important potential therapeutic target for cognitive defects associated with mental illness (Xu and Wong, 2018). Numerous post mortem studies report lower GAD67 mRNA and protein levels, mainly in the pre-frontal cortex (Curley et al., 2011; Guidotti et al., 2000; Woo et al.,

2008) and hippocampus (Benes et al., 2007; Konradi et al., 2011; Thompson Ray et al., 2011). Given the above, reduced α3 levels in GABAergic neurons would reduce their excitability and neurotransmission, and could contribute to a loss of inhibitory control in the cortex and hippocampus, giving rise to a broad array of disturbances in cognitive and emotional function, such as those seen in schizophrenia and BD.

In conclusion, this study on the post-mortem human brain provides evidence for the involvement of Na^+, K^+ -ATPase, and possibly its' ligand ECS, in neuropsychiatric diseases. Combined with our previously published findings (Goldstein et al., 2006; Hodes et al., 2016), it may be concluded that changes in ECS levels and Na^+, K^+ -ATPase isoform expression are not global changes in brain tissue, but, rather, specific to particular brain areas and cell types. Whereas total levels of Na^+, K^+ -ATPase α3 isoform expression in bipolar and schizophrenic patients are higher in the temporal cortex (Fig. 2), they are reduced in GABAergic neurons (Figs. 3–4). Evidently, such changes will have numerous functional consequences that may underlie the neuropsychiatric symptoms. This is the first report of changes in Na^+, K^+ -ATPase isoform expression in schizophrenia and BD. Further studies are indicated in order to map the Na^+, K^+ -ATPase isoform expression in the human brain in normal and pathological conditions and to understand the mechanism underlying the role of α3 isoform in GABAergic neurons in psychiatric diseases.

Conflicts of interest

None of the authors or funding sources has conflict 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.jpsychires.2019.04.014.

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