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Research report

Gender- and age-dependent changes in nucleoside levels in the cerebral cortex and white matter of the human brain

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ABSTRACT

Nucleosides are neuromodulators that participate in various neuronal functions in the brain. In previous studies, we described regional differences in the concentrations of nucleosides and their derivatives in the human brain. To better understand the functions of nucleosides in the central nervous system, we investigated gender- and age-dependent changes in the levels of nucleosides and their metabolites. The concentrations of uridine, inosine, guanosine and adenosine as well as uracil, hypoxanthine and xanthine were measured in the frontal cortex and white matter of *post-mortem* brain tissue samples of middle-aged and old men as well as women. The average *in vivo* concentrations calculated from the 40 samples investigated (regardless of anatomical locations, gender or age; mean ± S.E.M.) were as follows (pmol/mg wet tissue weight): 9.7 ± 0.8 adenosine, 85.8 ± 3.9 inosine, 14.3 ± 0.9 guanosine, 37.3 ± 1.8 uridine, 8.9 ± 0.6 uracil, 63.3 ± 2.1 hypoxanthine and 38.7 ± 1.5 xanthine. We conclude that concentration differences between uridine, inosine, guanosine and adenosine in the frontal cortex and cerebral white matter suggest that nucleoside metabolism is altered with aging and regulated differently between men and women.

1. Introduction

The systems biology approach to nervous function includes metabolomic analysis of brain tissue in normal and pathological conditions. Nerve cells have specific and highly controlled intracellular and extracellular metabolite contents that change with physiological and pathological conditions. One of the most important metabolic networks in the cell is the nucleoside metabolic system, which synthesises nucleic acids and plays various regulatory and communicative roles in the nervous system [13,21,27,54,61,65]. In order to understand the operation principles of the nucleoside metabolism network, it is necessary to investigate the distribution of its components in the brain.

Nucleoside metabolic pathways form a complex network with various conversion routes [4,12,70,72,76]. The activities of nucleoside metabolising enzymes, adenosine-receptor density and the distribution of nucleosides are age-dependent, with regulated distribution throughout the brain [11,32,41]. Moreover, gender-dependent changes have been shown for 5′-nucleotidase (5′NT) activity and nucleoside transport in rodents [48,62], suggesting that the levels of nucleosides in distinct brain areas have gender differences.

Previous data collected on nucleosides in the brain revealed that adenosine (Ado) may participate in the regulation of sleep, cognition, and memory function and act to suppress seizure activity [38,47,53,61]. In addition, nucleosides may play a role in neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease [9,39,61,73]. In spite of their neurological side effects, nucleoside derivatives are widely used as anti-tumour and anti-viral agents [10,29,43,52]. In order to better optimise the use of nucleoside-derived drugs, increasing attention must be paid to

Abbreviations: 5′NT, 5′-nucleotidase; A_1 receptors, A_1 adenosine receptors; A_{2A} receptors, A_{2A} adenosine receptors; ADA, adenosine deaminase; Ado, adenosine; Gn, guanine; Guo, guanosine; HGPRT, hypoxanthine-guanine phosphoribosyltransferase; Hyp, hypoxanthine; Ino, inosine; Ura, uracil; Urd, uridine; Xn, xanthine.

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gender and age differences in the human brain. For example, gender differences in the efficiency of treatment of HIV infection by nucleoside analogues have been demonstrated [28]. Recent studies have revealed sex- and age-specific differences in other neurotransmitter systems, including the dopaminergic, serotonergic, cholinergic, GABAergic and opioid systems [20], which could be responsible for the gender-specific differences in the prevalence of depression, Parkinson's disease, and schizophrenia.

We previously described an HPLC technique for measuring nucleosides and their metabolites simultaneously in the same tissue samples [23]. In addition, we developed a back-extrapolation method to estimate the *in vivo* concentrations of nucleosides and their derivatives from *post-mortem* brain tissue samples [45]. In the present study, these methods were applied to investigate gender- and age-related differences in nucleoside and nucleobase levels in the frontal cortex and white matter of the human brain.

2. Materials and methods

2.1. Materials

Standards for peak identification (uracil/Ura, hypoxanthine/Hyp, xanthine/Xn, uridine/Urd, inosine/Ino, guanosine/Guo and Ado) and other chemicals were obtained from Sigma Chemical Co. (St. Louis, Missouri, USA) and Merck Co. (Darmstadt, Germany) in 99% purity, analytical or HPLC grade.

2.2. HPLC technique

Our HPLC method using a diode array detector and a cooled column system provides sufficient sensitivity and selectivity for the measurement of Ura, Hyp, Xn, Urd, Ino, Guo, Ado, thymidine (Thd) and deoxynucleosides in milligrams of brain tissue [23]. Nucleosides were measured with an HP 1100 series gradient chromatograph. The separation was performed on an HP Hypersil ODS C18, 2.1 mm × 200 mm analytical column and a 2.1 mm × 20 mm guard column. The flow rate was 300 μl/min. Eluent A was 0.02 M formiate buffer containing 0.55% (vol/vol) acetonitrile, pH 4.45 and eluent B was 0.02 M formiate buffer containing 40% (vol/vol) acetonitrile, pH 4.45. The gradient profile was the following: 0% B at 0–10 min, 10% B at 22 min, and 100% B at 30 min. The column temperature was 10 °C. The injection volume was 10 μl. The diode array detector was adjusted to measure at 254 nm (reference wave 360 nm) and 280 nm (reference wave 450 nm). Chromatograms were evaluated using the automatic integrator function of the HP ChemStation software.

2.3. Preparation of human brain samples for chromatography

Human brain samples were collected by the Human Brain Tissue Bank, Budapest, in agreement with the Ethical Rules for Using Human Tissues for Medical Research in Hungary (HM 34/1999). Brains from traffic accident and sudden death victims were removed from the skull 2, 4 or 6 h after death in the Department of Forensic Medicine, Faculty of Medicine of the Semmelweis University. The brains were sliced as 1–1.5 mm thick coronal sections followed by microdissection of different brain areas by the micropunch technique [55]. In total, 20–20 brain samples from middle-aged and old persons were analysed in our study. 50–50% of all samples were obtained from the frontal cortex as well as the white matter from men and women from which we formed experimental groups (Table 1).

We applied a previously tested sample preparation method for the measurement of nucleoside levels in brain tissue samples [45]. Briefly, about 1 mg from each microdissected tissue pellet and 20 μl of chromatographic eluent A were placed into Beckman Airfuge tubes. The tissue samples were homogenised with a Teflon potter for 10 s and were treated immediately with a 1000 W microwave beam for 10 s. Then, the samples were centrifuged at 13,000 rpm for 20 s and 10 μl of the 60 μl supernatants was injected into the chromatograph. The concentrations were calculated based on 1 mg wet tissue weight (w.w.).

2.4. Estimating nucleoside content of post-mortem samples

We used a previously elaborated reverse extrapolation method to estimate nucleoside levels in the living human brain [45]. It is based on the analysis of neuro-surgically dissected human cerebral cortex samples at different *post-mortem* times. Briefly, concentrations of nucleosides and their metabolites were measured at 30 s and at 2, 4, 6 and 24 h *post-mortem* [45]. Back-extrapolation coefficients were calculated by dividing the 30-s sample data with the concentration data of the 2-, 4-, 6-, 24-h samples. The estimation of concentration allowed higher accuracy comparisons of nucleoside concentrations measured from brains at different *post-mortem* times. In this paper, back-extrapolation coefficients used to calculate *in vivo* level of Ura, Hyp, Xn, Urd, Ino, Guo and Ado from 2, 4 and 6 h *post-mortem* brain samples (Table 1) were the following: (2-/4-/6-h): 0.966/0.559/0.375, 0.564/0.505/0.353, 0.679/0.625/0.425, 0.704/0.762/0.635, 1.214/1.352/1.755, 1.089/1.027/1.102, 0.886/1.333/1.234.

The differences in concentrations of nucleosides obtained from the cortex and white matter for middle-aged and old persons, as well as for men and women were compared by ANOVA for correlated samples.

3. Results

3.1. Concentrations of nucleosides in the human brain

Our analysis was limited to nucleosides and their metabolites, which were reliably detectable in each sample. Thus, Urd, Ino, Guo and Ado as well as Ura, Hyp and Xn were measured in all human cortical and white matter samples. The average concentrations of compounds were calculated from a total of 40 samples regardless of their anatomical locations, gender or age. To calculate concentration values in living brain tissue, back-extrapolation coefficients were applied. The following concentration data were obtained (average ± S.E.M. in pmol/mg wet tissue weight): 9.7 ± 0.8 Ado, 85.8 ± 3.9 Ino, 14.3 ± 0.9 Guo, 37.3 ± 1.8 Urd, 8.9 ± 0.6 Ura, 63.3 ± 2.1 Hyp and 38.7 ± 1.5 Xn.

3.2. Age dependence of brain nucleoside levels

Levels of Ino and Ado increased with age in samples from the frontal cortex of old men and women as compared to their middle-aged counterparts (Fig. 1A and B). The concentration of Ura was significantly higher in the cortex of old men than middle-aged men. The levels of Hyp and Xn were lower and higher, respectively, in cortical samples of old women. Concentrations of Urd and Ino increased significantly with age in male white matter samples while the levels of nucleosides and their metabolites did not change significantly with age in the white matter of women (Fig. 1C and D).

When comparing age-dependent changes in cortical as well as white matter samples in men, we found that Ino levels significantly increased with age (Fig. 1A and C). However, the concentration of Ado and Ura were significantly higher only in cortical samples, while the level of Urd increased in white matter samples with age (Fig. 1A and C). In women, levels of Ado, Ino as well as Xn increased, but Hyp decreased in cortical samples from old individuals. However, the levels of nucleosides did not change significantly with age in white matter (Fig. 1B and D).

Table 1
Specifications of all 8 experimental sample groups were used to compare of nucleoside concentration in man and woman cortical and white matter samples.

Brain area	Sex	Sample groups	Age (years, and average ± S.E.M.)	*Post-mortem* time (hours, and average ± S.E.M.)
Frontal cortex	Man	Middle-aged	31, 32, 41, 42, 47 (38.6 ± 3.1)	6, 6, 2, 2, 2 (3.6 ± 1.0)
		Old	80, 80, 80, 81, 83 (80.8 ± 0.6)	4, 2, 4, 6, 2 (3.6 ± 0.8)
	Woman	Middle-aged	28, 36, 37, 38, 42 (36.2 ± 2.3)	6, 2, 2, 2, 2 (2.8 ± 0.8)
		Old	74, 76, 78, 89, 89 (81.2 ± 3.3)	6, 4, 2, 2, 2 (3.2 ± 0.8)
White matter	Man	Middle-aged	31, 41, 41, 42, 47 (40.4 ± 2.6)	6, 4, 2, 2, 2 (3.2 ± 0.8)
		Old	68, 80, 80, 81, 83 (78.4 ± 2.7)	2, 2, 4, 6, 2 (3.2 ± 0.8)
	Woman	Middle-aged	28, 40, 42, 46, 48 (40.8 ± 3.5)	6, 2, 2, 2, 2 (2.8 ± 0.8)
		Old	74, 76, 78, 89, 89 (81.2 ± 3.3)	6, 2, 2, 2, 2 (2.8 ± 0.8)

3.3. Gender dependence of brain nucleoside levels

As shown in Fig. 1, cortical nucleoside levels depend on gender both in the middle-aged and old groups. The concentration of Guo was higher, but the concentration of Ado was lower in middle-aged as well as in old cortical samples from women compared with samples from men (Fig. 1E and F). However, levels of Urd and Ino were significantly higher only in cortical samples from middle-aged women, but not from old women, while we found significantly lower Hyp levels in cortical samples from old women. We also found that the level of Ino was higher in cortical as well as in white matter samples from middle-aged women and white matter samples from old women. The concentrations of Urd and Guo were higher only in white matter samples from middle-aged women (Fig. 1G and H).

Comparing the gender dependence of nucleoside levels in middle-aged individuals, we found that the levels of Urd, Ino and Guo were significantly higher in cortical as well as in white matter samples from females compared to samples from males. Moreover, the level of Ado was lower only in the cortex of middle-aged women (Fig. 1E and G). Changes in nucleoside concentrations relative to gender were somewhat different in old individuals (Fig. 1F and H). Levels of Ado and Hyp were lower but the Guo concentration was higher in cortical samples of old women as compared with cortical samples from old men. However, we found that only the concentration of Ino was higher in white matter samples from old women

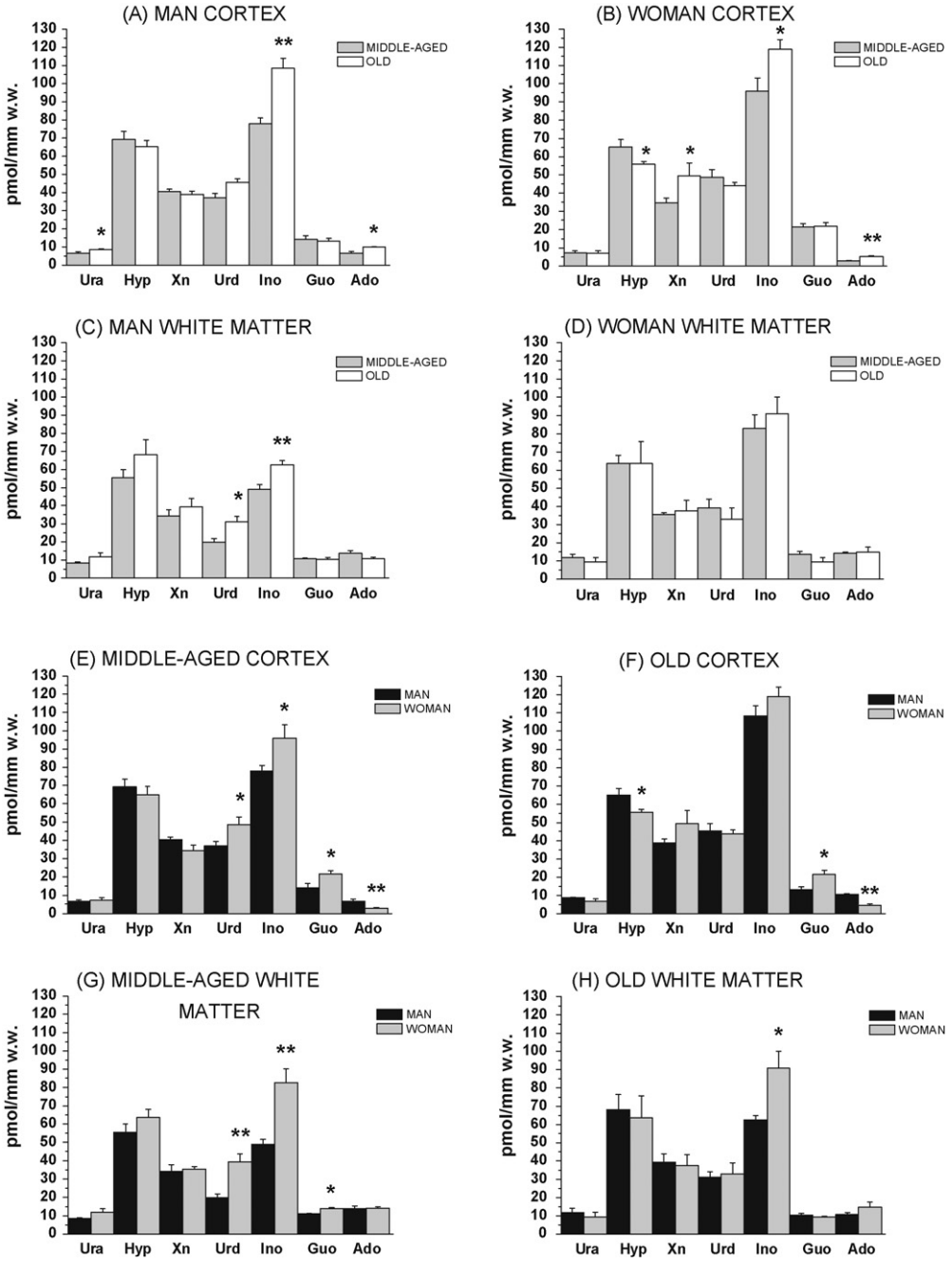

Fig. 1. Concentration of nucleosides and their metabolites in the cortical and white matter samples from old and middle-aged men and women. Abbreviations: w.w.: wet tissue weight; Ura: uracil; Hyp: hypoxanthine; Xn: xanthine; Urd: uridine; Ino: inosine; Guo: guanosine; Ado: adenosine; * labels $p < 0.05$ and ** labels $p < 0.005$ level of significance.

than in those from old men, while the concentrations of other nucleosides and their metabolites did not change significantly in the white matter of old individuals.

4. Discussion

We report differences in the concentrations of nucleosides and nucleoside metabolites in the white matter and frontal cortex of the human brain in relation to age and gender. *Post-mortem* human brain samples have been successfully applied in different types of studies using for example proteomic analysis, autoradiography, immunohistochemistry or liquid chromatography/mass spectrometry to measure different compounds of human brain tissues and to reveal pathomechanisms of several diseases in the human central nervous system [34,40,41,49,56,68]. For the concentration measurements, we used an HPLC method, which has been proven successful in previous animal and human brain tissue studies [23,41,44,45]. In addition, we applied back-extrapolation coefficients, which allowed the calculation of *in vivo* concentrations of nucleosides and their metabolites from brain samples with different *post-mortem* intervals [45]. The Human Brain Tissue Bank, Budapest provided us a unique opportunity to select reliable groups of human brain tissue samples in terms of age, sex and cause of death. Sample dissection accuracy and reproducibility are also critically important parts of the study, as previously described [45]. For instance, using samples with different densities of brain capillaries could modify the concentration of nucleosides and their metabolites by affecting hypoxia-induced changes in the brain after death [26].

Fig. 1 shows that the levels of Ino and Ado in the frontal cortex from men and women as well as the levels of Urd and Ino in the white matter collected from men are age-dependent (Fig. 1A–C). Adenosine monophosphate (AMP) can be degraded to Ado and Ado can be anabolised via Ino and Hyp to Xn by 5′NT, adenosine deaminase (ADA), purine nucleoside phosphorylase (PNP) and xanthine oxidase (XO), respectively. Guanosine monophosphate (GMP) can be metabolized to Guo, which can be anabolised via guanine (Gn) to Xn by 5′NT, PNP and guanase, respectively [4,5,72]. Hypoxanthine-guanine phosphoribosyltransferase (HGPRT) is a purine salvage enzyme, which can catalyze Hyp and Gn conversion to IMP and GMP, respectively [4,72]. Urd can be converted to Ura by uridine phosphorylase (UP) [3]. There is evidence of age-dependent changes in the activity of some nucleoside metabolism enzymes, which could result in different nucleoside levels. 5′NT and ADA activity increases and decreases by age, respectively [30,57]. In addition, one of the salvage enzymes of nucleosides adenosine kinase (AK) is saturated in older people [2]. Therefore, Ado content of brain tissues may increase in cortical samples from old subjects. Higher level of Ino in elderly man and women may coincide with increased activity of 5′NT in the old brain [30]. HGPRT activity catalyzing nucleoside salvage as well as the activity of guanase is increased by age [77] and may be in correlation with lower Hyp and higher Xn level in old than middle-aged woman cortex.

Ado is an inhibitory neuromodulator [21,61]. The age differences in Ado metabolism could result in an alteration in the sensitivity of aged human brains to excitatory insults. The number of neurons and the neuron/glia ratio are decreased by age [6,66], which could also contribute to the altered Ado effects in elderly people result from different glial and neuronal nucleoside metabolism [16,51,77]. Thus, our data suggest the need for new investigations regarding the age-dependent effects of Ado, since Ado is a major inhibitory factor in epilepsy [53,61] and is involved in the pathomechanisms of other diseases [9,36,58,73].

The uneven distribution of purinergic receptors in the brain [8,33,61,63] suggests that the intensity of the physiological effects of nucleosides varies with brain areas [27,61,65]. Adenosine receptors play a role in the mechanisms of Alzheimer's, Huntington's and Parkinson's diseases, seizures, cognition, and memory functions [35,36,53,61]. Interestingly, the density of A_1 adenosine receptors (A_1 receptors) decrease while the density of A_{2A} adenosine receptors (A_{2A} receptors) increase with age in the cerebral cortex [25,32]. The decrease in the density of inhibitory A_1 receptors and the increase in the density of excitatory A_{2A} receptors could shift the excitatory/inhibitory balance in aging cells toward excitation. The increased Ado levels in frontal cortical samples of males and females (Fig. 1A and B) may act to further enhance the excitatory state of old neurons. However, an elevated risk of Ado-induced excitotoxicity in aging brain must to be considered. The age-dependent alteration in receptor density and Ado concentration suggests that changes in nucleoside levels may participate in the pathophysiology of learning and memory and also in the sleep-wake cycle.

There are data suggesting binding of Urd [42] and Guo [69] to a selective receptor in the nervous system. We have only tentative knowledge about the physiological roles and signaling mechanisms of those putative receptors. The uridine receptor is only a putative binding site inducing functional changes in physiological processes but no information is available about the receptor molecule itself. However, accumulating data supporting the participation of Guo, Ino or Urd in physiological as well as pathophysiological processes such as sleep regulation, immunomodulation, and epilepsy [37,42,67,74]. Guo plays a role in psychiatric diseases and modulates memory processes [64], while Urd exhibits antidepressant-like and antiepileptogenic effects [15,75]. Chronic oral administration of nucleoside–nucleotide mixtures containing Ino and Urd, but not Ado, reduces age-related memory and learning deterioration in old senescence-accelerated mice [19]. Nucleosides such as Ino and Urd have been proposed to be neuroprotective agents, which could antagonise cytotoxic effect of high level of Ado [1,7,17,24,37,46,59,64]. These evidences are consistent with the elevation of Ino levels we observed in cortical samples from old men and women (Fig. 1A and B). Urd and Ino level were increased with age in the white matter of men may be relation to their neuroprotective effects. However, nucleoside levels of the white matter were not changed in women (Fig. 1C and D). While our knowledge about gender and age differences in nucleoside metabolism in the human brain remains incomplete, we believe that our results suggest genuine changes in nucleoside metabolism with age in both sexes [2,22,30,41,57,62,77].

Gender differences in brain nucleoside levels are the major findings of our study. The source of gender differences in brain nucleoside content remains difficult to identify because our understanding of the brain nucleoside metabolome is incomplete. There are, however, some relevant sporadic data. It has been described that activities of ectonucleotidases exhibit gender differences in rats [22,62]. Equilibrative nucleoside transporter (ENT1) mRNA levels are significantly higher in male rat brains [48]. It is known that the structure and synaptic morphology of the cortex, the neuron/glia ratio, and hypoxic tolerance all have gender differences [50,60,71]. The lower levels of Ado in cortical samples from the middle-aged and old women compared to men (Fig. 1E and F) may serve as a protective mechanism against the excitation arising from an increase in the density of A_{2A} receptors. However, the levels of neuroprotective Urd, Ino and Guo [14,18,37,64] were higher in samples from middle-aged and old women than in samples from men (Fig. 1E–H). Based on these results we believe that sexually different nucleoside levels might play a part in the reduced vulnerability of female brains to excitotoxic insults.

The available data on gender- and age-dependent differences in nucleoside metabolism are consistent with our findings. However, additional investigations are needed to explain the functional consequences of such gender and age differences. In turn, our data

also suggest that the levels of different nucleosides change differently with age and gender. Nucleoside analogs are widely used as immunosuppressants and anticancer drugs [10,31,38,52]. The effective concentrations of these drugs depend on their metabolism and transport to cells, which show regional differences modulated by age and sex. Further research is needed on gender and age differences in nucleosidergic systems to develop gender- and age-specific treatments for certain diseases (for example neuropsychiatric disorders and viral infections) with nucleoside analogues as well inhibitors of nucleoside metabolic enzymes [28].

In conclusion, we present here the first complex metabolomic analysis of the human brain related to age and gender dependencies of nucleoside levels. We demonstrated that the concentration of metabolically coupled nucleosides is modulated by age and gender in the human brain. Our data are in agreement with formerly disclosed changes in metabolic enzymes in aging and gender difference studies. Our findings suggest that the nucleoside microenvironment in the human brain is an important factor in the aging processes. The results represent one of the first steps towards understanding nucleoside function in the brain using a common metabolomics, proteomics and genomics platform of systems biology.

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