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# Expression of *Ext1*, *Ext2*, and Heparanase Genes in Brain of Senescence-Accelerated OXYS Rats in Early Ontogenesis and during Development of Neurodegenerative Changes

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**Abstract**—Heparan sulfate (HS) and heparan sulfate proteoglycans (HSPG) play a significant role in brain development, and their structural and quantitative changes are revealed during aging and in neurodegenerative disorders. The mechanism of these changes is not clear, but is likely to be associated with alteration in the expression and/or activity of enzymes responsible for HSPG biosynthesis and degradation. The contents of mRNAs of the genes *Ext1* and *Ext2* encoding polymerization enzymes and of gene *Hpse* of heparanase degrading HS were determined in the brain of prematurely aging OXYS rats during early postnatal development and during appearance of signs of brain accelerated aging (at age of 1, 7, 14, 30, 60, and 420 days). Wistar rats of the same age were used as controls. Expression levels of the genes *Ext1*, *Ext2*, and *Hpse* in the brain of rats of both strains were maximal during the two first weeks of life, and the contents of mRNAs of all genes in the brain of newborn and 7-day-old OXYS rats were significantly higher than in Wistar rats. By the 14th day of life the differences leveled, but at the age of 30 days on the background of a decrease in the contents of mRNAs of *Ext1*, *Ext2*, and *Hpse* in OXYS rats they became more pronounced (three-, four-, and twofold, respectively). Differences between the strains were absent at the age of 60 days and 14 months, and expression of all the genes was significantly lower than in the newborn animals. A strong positive correlation was found between contents of mRNAs of all the studied genes, and this suggested that heparanase should be involved in HSPG metabolism together with *Ext1* and *Ext2*. Based on these and earlier findings, we conclude that development of the OXYS rat brain occurs on the background of significant alterations in HSPG metabolism that precede the development of neurodegenerative manifestations recently detected by magnetic resonance imaging.

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*Key words*: brain aging, heparan sulfate proteoglycans, expression of *Ext1*, *Ext2*, and *Hpse* genes, senescence-accelerated OXYS rats

The role of glycoconjugates (including proteoglycans, PG) during development, in physiological and pathological processes in the adult organism, and during aging is now actively discussed [1, 2]. Heparan sulfate proteoglycans (HSPG) are integral components of the cell surface and extracellular matrix (ECM) of all animal cells [3]. These complex molecules consist of a protein core and unique sulfated carbohydrate chains of glycosaminoglycans (GAG) covalently bound with it. The GAGs are composed of disaccharide units consisting of glucuronic/iduronic acid and N-acetylglucosamine. Structures of heparan sulfate (HS) chains are different in different organs and cells and change during development and in pathology. There is no matrix for biosynthesis of HS. All HS chains are synthesized *de novo*, and it is still unclear what determines their structure [4]. Biosynthesis of a carbohydrate chain is a complex process involving enzymes responsible for chain polymerization (Ext1 and Ext2) and also enzymes responsible for chain modification.

In nervous system tissues HSPG are expressed mainly as a family of transmembrane syndecans [5] and a family of GPI-anchored glypicans [6]. HSPG have been shown to influence the surrounding cells through interaction with nerve cell adhesive molecules (NCAM), members of the family of fibroblast growth factors (FGF), and

*Abbreviations*: ECM, extracellular matrix; GAG, glycosaminoglycans; HS, heparan sulfates; HSPG, heparan sulfate proteoglycans; PG, proteoglycans.

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of the family of transforming growth factors (TGF) [7, 8]. HSPG are involved in cell migration, neuron elongation, synapse formation, and regulation of intracellular signaling pathways [9, 10]. Heparan sulfates influence formation of the brain structures and its size [11], and disorders in HS metabolism are associated with development of neurodegenerative processes [2, 11]. The crucial importance of HS for embryogenesis of mammals is evidenced by high expressions of *Ext1* and *Ext2* in the developing brain and also by pronounced neurodegenerative changes in *Ext1*-knockout mice (they have abnormally small brain cortex and lack many conductive pathways and olfactory bulbs) resulting in the death of the embryo at the stage of late embryogenesis [12].

Structural changes in HSPG during postnatal development of the brain and during development of neurodegenerative states are insufficiently studied. In our work we have compared changes in the expression level of mRNAs of the *Ext1*, *Ext2*, and heparanase genes at different times of postnatal development in the brains of the senescence-accelerated OXYS rats and of Wistar rats. Decreased exploratory activity, impaired ability for associative learning, and increased anxiety are considered to be manifestations of the premature aging of the OXYS rat brain [13-15]. The mechanisms of the accelerated aging of OXYS rats are still unclear, but phenotypic manifestations of premature aging of OXYS rats are known to appear on the background of a significantly decreased total content of PG in the brains of these rats in comparison with the brain of control Wistar rats [16], and this decrease is mainly caused by the low level of HSPG [17].

## MATERIALS AND METHODS

**Animals.** The work was performed on 72 male OXYS and Wistar (control) rats aged 1, 7, 14, 30, 60, 420 days (six animals in each group) from the "Gene Fund of Experimental Animals" of the Institute of Cytology and Genetics, Siberian Division of the Russian Academy of Sciences. All international requirements for experiments with animals were observed (Council of European Communities Directive 86/609/EES). Until the 30th day of age, the animals were kept in the mothers' cages. Beginning from the age of 1 month the rats were placed five animals per cage. The animals were kept under natural light regimen and *ad libitum* access to water and food (the standard granulated food "Chara" for laboratory rodents (Assortment Agro, Russia; GOST PK-120). At the end of the experiment the animals were subjected to light ether anesthesia. The brain was excised and immediately placed into liquid nitrogen and stored at –70°C until examination.

**Isolation of mRNA from rat brain.** The mRNA was isolated from homogenate of the preliminarily weighed whole brain of the animals using Trizol (TRI-REAGENT; GIBCO/Life Technologies, USA) according to the producer's recommendations. During homogenization the protein–ribonucleic particles of a recombinant phage MS2 containing the sequence SNK nonhomologous to any known gene of animals (Vektor-Best, Russia) was added proportionally to the tissue weight as an external standard for further normalization on performing PCR in the real time regimen. Then an additional deproteinization was performed by extraction with phenol–chloroform mixture (1 : 1) and a subsequent removal of phenol admixture by extraction with pure chloroform. Admixtures of genomic DNA were removed by treatment with DNase I (Promega, USA) according to recommendations of the supplier.

**Preparation of cDNA.** The reverse transcription was performed in 50 μl of solution containing 1 μg of the isolated RNA, 0.25 nmol primers ($N_9$ – random nonanucleotide primers from Biosan, Russia), 36 μl of buffer for reverse transcription, 40 units of revertase MoMLV (Vektor-Best), and 0.4 mM dNTP. The cDNA was synthesized at 37°C (1 h), 42°C (30 min), 50°C (10 min). The enzyme was inactivated by heating the mixture at 75°C for 5 min.

**Real-time PCR.** To determine the gene expression levels, real-time PCR was performed in the presence of SYBR Green I dye (Molecular Probes, USA) using an iQ5 amplifier (Bio-Rad Laboratories, USA). As a gene for comparison, an external standard was used that contained the SNK sequence nonhomologous to any known gene of animals. The reaction mixture (20 μl) contained the standard buffer for PCR (67 mM Tris-HCl, pH 8.9, 16 mM $(NH_4)_2SO_4$, 0.01% Tween 20, 10 mM β-mercaptoethanol), 5 mM $MgCl_2$, 0.2 mM dNTP, 1 unit of hot-start Taq-polymerase (Vektor-Best), 0.3 μM forward and reverse primers for rat gene *Ext1* (NM_001130540) (F – 5′-GATAGGGTCAGACACCAGGAA, R – 5′-CAACGAGGAACCAGACAGAA), for the gene *Ext2* (NM_001107751) (F – 5′-CGGCTTCAACCCAAAGAACA, R – 5′-ATCAATGGAGGGGACGAACA), and for rat gene *Hpse* of heparanase (NM_022605) (F – 5′-ATTCCAAAGCGTGAGTCCCT, R – 5′-TCAGAGGTGGGTTCCTTGTT) (Biosan), SYBR Green I in the final dilution of 1 : 20,000 from the stock solution (Molecular Probes). Real-time PCR was performed under the following conditions: heating for 1 min at 94°C; 35-40 main cycles of 15 sec at 94°C, 20 sec at 65°C, 20 sec at 72°C, recording fluorescence of the PCR products at 87°C (*Ext1* and *Ext2*) and at 89°C (*Hpse*) for 10 sec; recording the melting curve from 65 to 93°C. In every experiment the studied cDNA samples with primers for the desired gene were placed on the same plate (four repetitions per cDNA sample); similar samples with primers for the SNK sequence (also four repetitions); "standard" cDNA in dilutions from 1 : 1 to 1 : 27 with the same primers (two repetitions). For each cDNA sample PCR was performed at least twice. Based on the resulting standard calibration curves, the initial contents of the studied cDNAs were determined (relatively to the "standard" cDNA content),

and this value for the desired genes was related to the content of cDNA of the SNK sequence. This resulted in determination of differences in expression levels of the studied genes in the animals of the different strains.

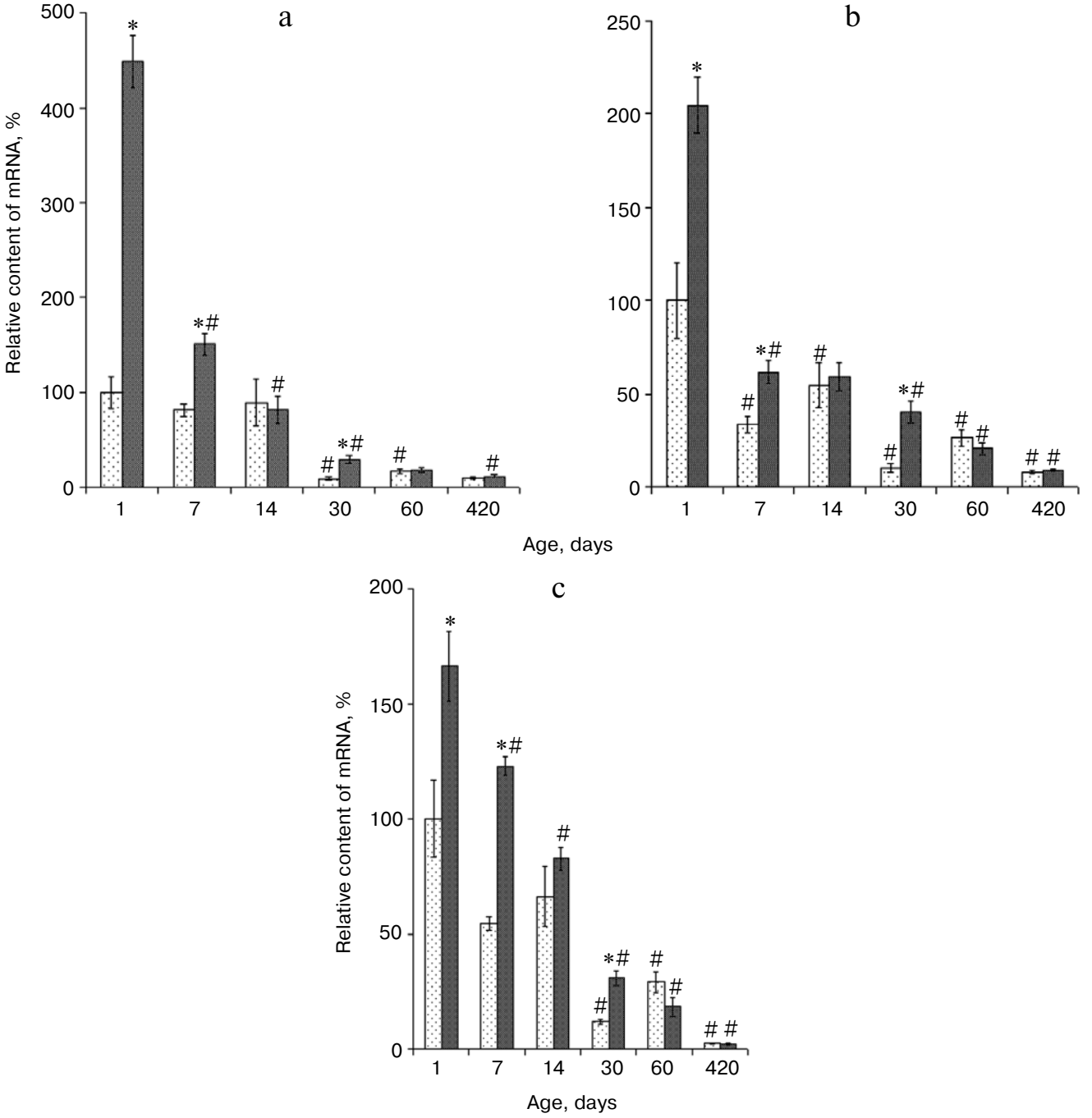
**Statistical processing of data.** The results were processed using ANOVA analysis of variance using the STATISTICA (version 5.5) program with post-hoc comparison of the group average values (Newman–Keuls test). The age and genotype of the animals were considered to be independent factors. Correlations were analyzed using rang correlations on the Spearman scale. The data are presented as arithmetic means and their errors (M ± S. E.).

## RESULTS

The ANOVA analysis revealed that the expression of gene *Ext1* (figure, panel (a)) changes significantly with age ($F_{5,52} = 131$, $p < 0.0001$) and depends on the genotype ($F_{1,52} = 103$, $p < 0.0001$). These factors interacted ($F_{5,52} = 60$, $p < 0.0001$), which suggested a difference in the age-related changes in the gene expression levels between Wistar and OXYS rats. The maximal level of *Ext1* mRNA was observed in the brain of 1-day-old rats of both strains. The difference between the strains was maximal at this age: the content of mRNA of this gene was 4.5-fold higher in the OXYS versus the Wistar rats ($p < 0.0001$). At ages from 1 to 14 days the expression level in Wistar rats did not change significantly, whereas in 7-day-old OXYS rats it became threefold lower than in newborns of this strain ($p < 0.0002$) but remained twofold higher than in Wistar rats of the same age ($p < 0.0001$). By the age of 14 days the level of the *Ext1* mRNA in OXYS rats decreased, and the difference between the strains leveled. At ages from 14 to 30 days the content of the *Ext1* mRNA in OXYS rats decreased threefold ($p < 0.006$) and in Wistar rats – tenfold ($p < 0.01$). As a result, the difference between the strains appeared again: the expression level of this gene in the brain of OXYS rats became threefold higher than in the brain of Wistar rats ($p < 0.005$). At ages from 30 to 60 days the content of *Ext1* mRNA in the brain of Wistar rats increased 1.8-fold ($p < 0.02$), and by the age of 14 months it decreased again and became an order of magnitude lower than in the newborns. In OXYS rats this parameter continued to gradually decrease with age, but beginning from the age of 30 days changes in this parameter were insignificant as compared to the changes in the animals of the preceding age group (figure, panel (a)). Nevertheless, at the age of 14 months the content of *Ext1* mRNA in the brain of OXYS rats was 2.5-fold lower than at the age of 30 days ($p < 0.01$) and 37-fold lower than in the newborns.

The expression of *Ext2* (figure, panel (b)) also changed with age ($F_{5,54} = 71$, $p < 0.0001$) and depended on the genotype ($F_{1,54} = 30.5$, $p < 0.0001$). The factors interacted ($F_{5,54} = 11$, $p < 0.0001$), suggesting the presence of interstrain differences in the age-related changes in expression. As in the case of the *Ext1* gene, the content of *Ext2* mRNA was maximal in the brain of 1-day-old rats of both strains along with the most pronounced difference between them: the content of the *Ext2* mRNA in OXYS rats was twofold higher than in Wistar rats ($p < 0.0001$). By the age of 7 days the expression level of this gene decreased in both Wistar and OXYS rats and became lower than in the newborns by 2.9-fold ($p < 0.010$) and 3.3-fold ($p < 0.0002$), respectively. In the OXYS rats the contents of the *Ext2* mRNA remained 1.8-fold higher than in the Wistar rats ($p < 0.006$), but at the age of 14 days the difference between the strains disappeared. In the Wistar rats at ages from 14 to 30 days the expression decreased 5-fold ($p < 0.004$), and this resulted in reappearance of an interstrain difference: the content of the mRNA of this gene in the brain of OXYS rats became 4-fold higher than in the brain of Wistar rats ($p < 0.001$). At ages from 30 to 60 days in the brain of Wistar rats the content of *Ext2* mRNA increased 2.5-fold ($p < 0.003$), and by the age of 14 months it decreased again to the level observed in 1-month-old animals. In the OXYS rats the content of mRNA decreased with age, and by the age of 14 months it was 22-fold lower than in the newborns ($p < 0.0001$).

The expression of the heparanase gene (figure, panel (c)) also changed with age ($F_{5,52} = 57$, $p < 0.0001$) and depended on the genotype ($F_{1,52} = 36$, $p < 0.0001$). The factors interacted ($F_{5,52} = 11$, $p < 0.0001$), which was displayed by different aging-related changes in the expression of this enzyme in the Wistar and OXYS rats. The content of the *Hpse* mRNA and also the interstrain difference were maximal in the brain of 1-day-old animals: in the OXYS rats this difference was 2-fold higher than in the Wistar rats ($p < 0.01$). In the Wistar rats the expression of *Hpse* did not change significantly at ages from 1 to 14 days. For the OXYS rats it decreased 1.3-fold by the 7th day of life as compared to its level in the newborns of this strain ($p < 0.003$), but remained 2.6-fold higher than in the Wistar rats of the same age ($p < 0.0002$). By the age of 14 days the content of *Hpse* mRNA in the OXYS rats decreased and the interstrain difference leveled. At ages from 14 to 30 days the content of heparanase mRNA decreased approximately 3-fold in the OXYS rats ($p < 0.0002$) and 5-fold in the Wistar rats ($p < 0.003$). This resulted in reappearance of interstrain difference: the expression of this gene in the brain of OXYS rats became twofold higher than in the brain of Wistar rats ($p < 0.004$). In the brain of Wistar rats the content of *Hpse* mRNA increased 2.7-fold ($p < 0.002$) from the age of 30 to 60 days, and by the age of 14 months it became 4.7-fold lower ($p < 0.0002$) than in the one-month-old animals. For the OXYS rats the content of *Hpse* mRNA continued to decrease with age, and at the age of 14 months it was 13-fold lower than at the age of 30 days ($p < 0.0003$).




Contents of mRNAs of genes *Ext1* (a), *Ext2* (b), and *Hpse* (c) in the brain of OXYS rats (grey columns) and Wistar rats (white columns) of different ages. The 100% values correspond to the content of mRNA in 1-day-old Wistar rats. Data are presented as M ± S. E. ($n$ = 6). * Significant differences between strains in rats of the same age. # Significant differences compared to rats of the same strain of the preceding age

## DISCUSSION

There is now no doubt that molecular interactions mediated by HSPG microdomains play an important role in various signaling pathways in the developing brain and modulate synaptic plasticity [18]. HSPG expression is dynamically regulated by different physiological stimuli, including the neuronal activity. Formation and morphology of postsynaptic dendritic spines considered as a structural basis for memory [12] are associated with changes in the amount and composition of HSPG. Aging and especially neurodegenerative processes are associated with a decrease in the amount of both synapses and dendritic spines, and this decrease correlates with manifestations of behavioral and cognitive disorders [19-21].

Our data show that in the early stages of postnatal development the content of HSPG is high in the brain of rats of both strains [17]. During the first two weeks of life the expression of the *Ext1*, *Ext2*, and heparanase genes were the highest in the brain of both Wistar and OXYS rats. In the brain of newborn and 7-day-old OXYS rats the levels of mRNAs of all three genes were significantly higher than in the brain of Wistar rats at the same age. Neuronal connections are known to be actively formed during this period, and this formation occurs most actively in the brain cortex and cerebellum. In the brain cortex this process is associated with learning and acquisition of conditioned reflexes required for the further life of the animal, whereas in the cerebellum it is associated with development of motility.

The differences leveled by the age of 14 days, but at the age of 30 days on the background of a significant decrease in the expression levels of the *Ext1*, *Ext2*, and *Hpse* genes in rats of both strains, the levels of mRNAs of these genes in the OXYS rats again became higher than in the Wistar rats (respectively, 3-, 4-, and 2-fold). By the age of 30 days the formation of neuronal connections is mainly terminated, which is associated with acquisition of the majority of skills required for the subsequent life of the animal. No interstrain differences were revealed at the age of 60 days and 14 months, and in the rats of both strains the expression of the studied genes stabilized at significantly lower levels than in the newborns. These findings are in correlation with earlier publications that metabolism of proteoglycans in tissues of the adult organism, including the brain, is significantly lower than in the tissues of a developing organism [22].

It is fundamentally important that age-related changes in the expression of the studied genes are coordinated in the rats of both strains. The analysis revealed strong correlations between the expression of the *Ext1* and *Ext2* genes in the brain of Wistar rats ($r = 0.84$, $p < 0.0001$) and OXYS rats ($r = 0.92$, $p < 0.0001$). A direct correlation on the functional level was also observed between the contents of mRNAs of *Ext1* and heparanase ($r = 0.88$, $p < 0.0001$ and $r = 0.94$, $p < 0.0001$ for the Wistar and OXYS rats, respectively), and also between the contents of mRNAs of *Ext2* and *Hpse* ($r = 0.83$, $p < 0.0001$ and $r = 0.91$, $p < 0.0001$ for the Wistar and OXYS rats, respectively).

Correlation between expression levels of the *Ext1* and *Ext2* genes was described earlier [2, 4, 22, 23]. This correlation is due to formation of heterooligomers of proteins Ext1 and Ext2 in the Golgi apparatus. Changes in expressions of the *Ext1*, *Ext2*, and N-deacetylase/N-sulfotransferase (*Ndst*) genes are coordinated [4], although their colocalization with *Ndst* has not been revealed. However, for other genes encoding synthesis of HSPG, those of 2-O-sulfotransferase and C-5-epimerase, both colocalization and complex formation on HS biosynthesis are shown [24]. A close positive correlation between the expression levels of the *Ext1*, *Ext2*, and *Hpse* genes is shown here for the first time. This correlation suggests a coordinated regulation of expression of these three genes and their joint involvement in the activation of metabolism of HS chains.

Overall, our findings are in agreement with data of work [22], which reported a high level of expression of *Ext1* and *Ext2* in the developing mouse brain during the early postnatal period and especially during embryogenesis, which the authors think to be associated with an active contribution of HSPG to neurogenesis [2]. Expression of heparanase in the brain is also high during early ontogenesis and increases under conditions of hypoxia and on development of tumors [25, 26]. Heparanase acts as a regulator of angiogenesis and is involved in remodeling of damaged blood vessels: the cleavage by heparanase of the heparan sulfate component of the extracellular matrix results in release of some growth factors activating proliferation and angiogenesis. The overexpression of heparanase was recently shown to induce changes in expression of the majority of genes involved in HSPG biosynthesis [27]. Moreover, on the background of overexpressed heparanase the expression of 1042 and 1039 genes in the mouse brain cortex and thalamus, respectively, was changed 2-fold (positively or negatively). Naturally, the expression of heparanase is high during embryogenesis and intensive growth.

In this connection, the interstrain differences in the contents of mRNAs of *Ext1*, *Ext2*, and heparanase found by us are likely to indirectly indicate the difference in the rates of neurogenesis, whereas significantly increased levels of expression of genes encoding synthesis and degradation of PGs in the brain of OXYS rats are likely to be associated with a deceleration of neurogenesis during the late embryogenesis and, as a consequence, its acceleration during the first week of life. However, a high level of heparanase expression can be caused by development of the brain in OXYS rats during early ontogenesis occurring on the background of a delayed formation of the microcirculation and resulting hypoxia. Characteristic signs of adaptation to hypoxia were detected in the brain of 20-day-old OXYS rats when energy metabolism was studied [28].

In fact, based on the data published in [25, 26, 29] the increased expression of the heparanase gene in the brain of OXYS rats during the first week of life can be explained by hypoxia and the associated compensatory reactions. However, we have not found interstrain differences either in the contents of PG [16, 17] or in the contents of mRNAs of *Ext1*, *Ext2*, and *Hpse* in the brain of 14-month-old Wistar and OXYS rats, although magnetic resonance imaging (MRI) revealed in them pronounced neurodegenerative changes on the background of disorders in cerebral blood flow specific for chronic ischemia [30, 31].

The premature aging of the brain of OXYS rats phenotypically manifests itself by disorders in the ability for

associative learning and formation of a passive behavioral stereotype. These signs form at ages from 30 to 90 days along with appearance in the OXYS rat brain of neurodegenerative changes that we have recorded by MRI [15, 28, 31, 32]. The age of 60 days is critical for development of these and other manifestations of the premature aging of OXYS rats.

The association of age-related neurodegenerative diseases with changes in the extracellular matrix is proved and actively discussed. The idea of matrix involvement has evolved from discussion about its contribution to changes in adhesion to acknowledgement of the involvement of PG in complex cell regulations [33]. Note that no interstrain differences in the animals' brain contents of mRNAs of *Ext1*, *Ext2*, and *Hpse* were found by us at the age of either 60 days or 14 months. But it was shown earlier that the content of chondroitin sulfate and heparan sulfate in the brain of 60-day-old OXYS rats was, respectively, 2- and 6-fold lower than in the brain of Wistar rats [17]. In contrast, in the absence of significant interstrain difference in the content of HSPG during the first week of life the expression levels of all genes responsible for HSPG metabolism were significantly increased in the developing brain of the OXYS rats (figure).

At present, we cannot explain in detail the findings, and further studies are required. However, overall the data obtained in this work and that of previous findings [17] indicate that the brain of OXYS rats develops on a background of significant changes in the metabolism of HSPG and of increased expression of key genes responsible for their synthesis and degradation. These changes precede the development of phenotypic manifestations of the premature aging of the brain of OXYS rats, including the development in it of neurodegenerative changes that we recently revealed by MRI [31, 32].

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