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GENERAL BIOLOGY

Increased Expression of the Multimerin-1 Gene in α-Synuclein Knokout Mice

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Abstract—Multimerin-1 (Mmrn-1) is a soluble protein, also known as elastin microfibril interfacer 4 (EMILIN-4), found in platelets and in the endothelium of blood vessels. Its function and role in pathology are still not fully understood. Genetic modifications in alpha-synuclein gene (Snca) locus that mapped 160 Kb apart from Mmrn-1 in mouse genome, could weigh with regulatory elements of Mmrn-1 gene. We have studied the Mmrn-1 expression in brain cortex of three mouse lines with *Snca* knock-out: B6(Cg)-Snca$^{tm1.2Vlb}$/J, B6;129-Snca^{tm1Sud}/J, and B6;129X1-Snca^{tm1Rosl}/J. The 35-fold increase for Mmrn-1 mRNA level have been found in B6;129X1-Snca^{tm1Rosl}/J mice that carry in their genome foreign sequences including bacterial gene neo and a strong promoter of a mouse phosphoglycerate kinase (Pgk1) oriented towards *Mmrn-1* gene. This effect on regulatory elements of *Mmrn-1* gene as a result of modifications in *Snca* locus should be taken into consideration when using B6;129X1-Snca^{tm1Rosl}/J line, that is widely applied for study of neurodegeneration mechanisms.

Keywords: multimerin-1, knok-out mice, α-synuclein

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Physiological functions of the high-molecular weight soluble protein multmerin-1 (Mmrn-1) also known as elastin microfibril interfacer 4 (EMILIN-4) are not completely known. Spontaneous deletion of the gene locus containing the gene encoding Mmrn-1 does not result in any specific phenotype in C57BL/6j-OlaHsd mice. This allows the conclusion that the absence of the *Mmrn-1* gene can be compensated during embryogenesis [1]. However, detailed analysis of bone tissue from C57BL/6J-OlaHsd mice performed by the microcomputer tomography (μCT) method revealed the reduced weight of spongy substance of the epiphyses of tubular bones, and histological study on the cellular composition of bones showed an increased content of osteoblasts with decreased mineralization [2]. This observation together with relatively high expression of the *Mmrn-1* gene in bone tissue made it possible to study the involvement of Mmrn-1 in metabolic processes in osteoblasts and during bone formation. High content of Mmrn-1 is also present in blood vessels endothelium and α-granules of platelets. It is synthesized at a fairly high level in megakaryocytes and was detected on the surface of activated platelets in combination with von Willebrand factor [3].

In mice with deletion of the *Mmrn-1* locus, the changes in the hemostasis system were observed including impaired platelets aggregation and clot formation and addition of multimerin in experiments in vitro prevented these impairments [4]. In blood plasma, Mmrn-1 cannot be detected using routine methods; however, the increased level of this protein in the cellular fraction of blood from acute myelogenous leukemia (AML) patients is correlated with low probability of remission, higher risk of disease recurrence, and low survival of patients [5].

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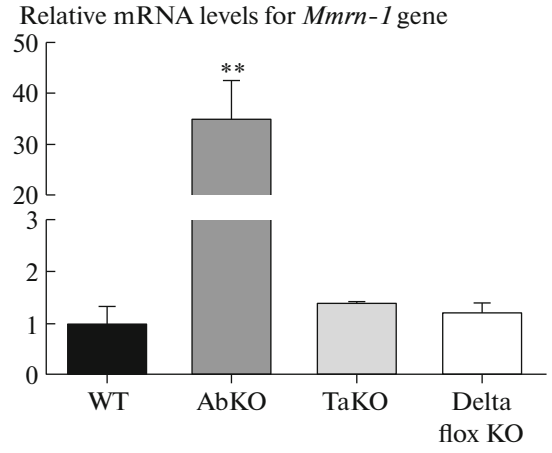

Fig. 1. Comparative analysis of the expression levels of the *Mmrn-1* gene in three mouse strains with α-synuclein gene knockout. Content of Mmrn-1 mRNA was measured using quantitative real-time RT-PCR in the cerebral cortex of B6;129X1-Snca^{tm1Rosl}/J (AbKO), B6;129-Snca^{tm1Sud}/J (TaKO), and B6(Cg)-Snca$^{tm1.2Vlb}$/J (delta flox KO) mice and control wild type (WT) mice. Data are presented as M ± SEM; $n = 6$ for each group; ** $p < 0.01$ for AbKO group versus other groups according to one-way ANOVA followed by Tukey's post hoc test.

Experimental studies on impairments of Mmrn-1 functions are mostly performed using the C57BL/6J-OlaHsd mouse strain [6]. In the genome of these mice, spontaneous deletion of a long 350 kbp fragment [1] includes both α-synuclein (*Snca*) and *Mmrn-1* genes located close to each other at a distance of about 160 kbp. A-synuclein (a-syn) is an important constituent of Lewi bodies, which plays a key role in pathogenesis of Parkinson's disease; therefore, several mouse strains with genetic modifications of the *Snca* locus were developed independently [7].

It is possible that the introduced modifications may affect other regions of genome in close vicinity. In the present study, we compared the levels of Mmrn-1 expression in three mouse strains with *Snca* deletion, such as B6;129X1-Snca^{tm1Rosl}/J (AbKO) provided by Rosental's laboratory [8], B6;129-Snca^{tm1Sud}/J (TaKO), provided by Sudhof's laboratory [9], and B6(Cg)-Snca$^{tm1.2Vlb}$/J (delta flox KO) provided by Buchman's laboratory [10, 11].

To find out whether different modifications of the *Snca* locus can affect the expression of the *Mmrn-1* gene located only 160 kbp from the *Snca*, we studied the content of Mmrn-1 mRNA in the cerebral cortex using the real-time RT-PCR method.

Total RNA was extracted from the cortical samples of three-month-old male mice using RNeasy Plus Mini kits (Qiagen, Germany) according to the manufacturer's protocol. Two micrograms of purified RNA were used for the synthesis of a complementary DNA chain in the reverse transcription reaction and subsequent amplification with qPCRmix-HS SYBR reagents (Evrogen, Russia) according to the manufacturer's real-time protocol using a Bio Rad CFX 96 amplifier (Bio Rad, United States). The following primers were used: Mmrn-1 forward (5'-GGACACTGGCTCT-CATTTAC-3') and Mmrn-1 reverse (5'-CAG-CATTGGATGTCTTCTGAG-3'). The glyceraldehydes-3-phosphate dehydrogenase (GAPDH) gene was used as a house-keeping gene. The following primer sequences were used: q_m_GAPDH forward (5'-CACTGAGCATCTCCCTCACA-3') and q_m_GAPDH reverse (5'-GTGGGTGCAGC-GAACTTTAT-3').

We estimated the relative levels of Mmrn-1 expression in three mouse strains with *Snca* gene knockout and found that the expression level was 35-fold elevated in the AbKO mice as compared to the expression level in the cortex of control wild type mice whereas in the delta flox KO and TaKO mice it was similar to that observed in the control mice (Fig. 1). The technology of designing the studied knockout strains included the use of embryonic stem cells at the stage of homologous recombination of the α-synuclein locus with subsequent selection of clones based on the production of the bacterial gene *neo* introduced in the target cassette. At the same time, direct modifications of the sequences in the vicinity to the *Snca* gene were differ (Fig. 2). In the AbKO strain, after deletion of the second and third α-synuclein exons with adjacent sequences (size of deletion is about 3.2 kbp) the modified locus contained a fragment of the plasmid pGT-N39 vector of 1.9 kbp in size, which carried a coding sequence of the *neo* gene under the mouse phosphoglycerate kinase Pgk1 gene promoter (Fig. 2). In the knockout TaKO strain, the second and third *Snca* exons with adjacent sequences were also deleted and the size of deletion was 3.9 kbp. The foreign sequence remained in the modified genomic locus carried a fragment of pTK-Neo3a plasmid, containing the *neo* gene under the control of the mouse subunit A of polymerase II Polr2a gene promoter (Fig. 2).

It is important that the orientation of the *neo* cassette in the genomes of the AbKO and TaKO strains were differed. In the AbKO mice, the *neo* gene was located more distal relative to the *Snca* gene and oriented in reverse direction relative to the modified gene. In the genome of the TaKO mice, the *neo* cassette promoter is located more proximal relative to the *Snca* promoter and oriented in the same direction (Fig. 2). In the third strain used in the present study, the delta flox KO mice, deletion of the *Snca* gene was associated with minimal locus modifications. From the mouse genome the third exon was deleted containing the START codon and a small fragment of the adjacent intronic sequence of 1.1 kbp in size that is substantially less than in the AbKO or TaKO strains. Furthermore, the *neo* gene and other important foreign sequences were deleted from the modified locus of the knockout mice. The only introduced insertion

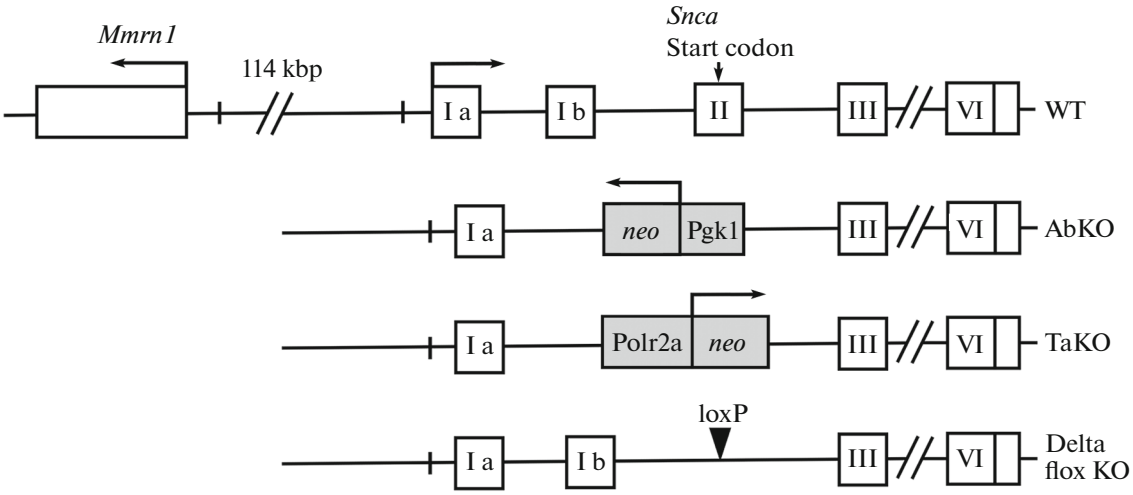

Fig. 2. Scheme of modifications of the *Snca* locus in the knockout mouse strains. The foreign sequences of *neo* gene, LoxP site, and phosphoglycerate kinase (Pgk1) gene and subunit A of polymerase II (Polr2a) gene promoters, introduced into the mouse genome are colored. Arrows indicate the transcription directions.

left in the genome of the delta flox KO mice was the *LoxP* site with a size of 34 bp (Fig. 2).

These data point that the functionally active promoter of the pGT-N39 cassette in the genome of the AbKO mice [8] oriented to *Mmrn-1* could activate the regulatory sequences of this gene. In TaKO mice, the promoter of the pTK-Neo3a vector oriented in the opposite direction from the *Mmrn-1* gene [9] exhibited significantly lower effect on the expression of this gene. In the novel knockout delta flox KO mice [10, 11], the expression of the *Mmrn-1* gene practically did not differ from its expression in the nonmodified genome of the wild type mice on the same genetic background (Fig. 1).

Bioinformatic analysis revealed that potential regulatory sequences are located between the *Snca* and *Mmrn-1* genes, and within the *Mmrn-1* introns there are two enhancers and two binding sites for the CTCF transcription repressor, which is involved in the regulation of chromatin architecture and delimits eu- and heterochromatin [12].

Our data allow us to assume with a high degree of probability that impairments in the functioning of regulatory elements of the genome are the cause of altered expression of Mmrn-1 in the AbKO mice.

Despite the fact that the AbKO strain of knockout mice (B6;129X1-Snca^{tm1Rosl}/J) is widely used in laboratories around the world to study neurodegeneration, the detailed analysis of expression level of Mmrn-1 in these mice was not performed until now.

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COMPLIENCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare no conflict of interest.

Statement of the welfare of animals. Experiments with animals were performed according to the order no. 199n of the Ministry of Healthcare of the Russian federation from April 1, 2016 "On approval of the rules of good laboratory practice."

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