PROLONGED ALCOHOL CONSUMPTION INFLUENCES microRNA EXPRESSION IN THE NUCLEUS ACCUMBENS OF THE RAT BRAIN

M.I. Airapetov[1,3]*, S.O. Eresko[1,2], S.A. Shamaeva[1], N.M. Matveev[1], E.R. Bychkov[1], A.A. Lebedev[1], P.D. Shabanov[1]

[1]Institute of Experimental Medicine,
12 Akad. Pavlova str., St. Petersburg, 197376 Russia; *e-mail: interleukin1b@gmail.com
[2]North-Western State Medical University named after I.I. Mechnikov,
41 Kirochnaya str., St. Petersburg, 195067 Russia
[3]Military Medical Academy of S.M. Kirov,
6G Akad. Lebedeva str., St. Petersburg, 194044 Russia

The microRNA (miR) species analyzed in this study are involved in molecular mechanisms of TLR4 and TLR7 signaling, mediating the development of neuroinflammation and neurodegeneration. We have investigated the expression levels of miR-let7b, miR-96, miR-182, miR-155, and the mRNA content of HMGB1, TLR3, TLR4 in the *nucleus accumbens* (NAc) of the brain of rats exposed to long-term alcoholization. The long-term alcoholization caused a decrease in miR-let7b, miR-96, miR-182, and TLR7 mRNA levels; this was accompanied by an increase in miR-155, TLR4, and Hmgb1 mRNA levels in the NAc of rat brain. TLR7 is functionally linked to miR-let7b. The data of a simultaneous decrease in miR-let7b and TLR7 mRNA are of interest for further studies; they may indicate on the lack of functionally significant links between Hmgb1 and the miR-let7b-TLR7 system in NAc. The existing evidence of a functional relationship between TLR4 with miR-155 and miR-182 and our observations on their expression changes during chronic alcoholization are very interesting and require further investigation. The suggestion about the development of neuroinflammatory process in NAc under prolonged alcohol exposure are relevant for studying the level of TLR gene expression in NAc, as well as the expression of miR species, which may have a functional relationship with the TLR system.

Key words: *nucleus accumbens*; alcohol; neuroinflammation; TLR; miR

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INTRODUCTION

Chronic alcohol consumption causes biochemical, functional, and structural changes in various parts of the brain [1–4]. Certain evidence exists about occurrence of such changes in the *nucleus accumbens* (NAc) of the brain during prolonged alcohol consumption [5–7]; however, the mechanisms mediating these effects remain poorly understood. At the same time, it is well known that NAc is an important part of the mesolimbic pathway and serves as a key link in the system of internal reinforcement, mediates the effects of psychostimulants, in particular, ethanol [3, 4, 8, 9].

Altered content of microRNA (miR) species has been found in plasma and in brain structures during prolonged exposure to alcohol [10, 11]. MicroRNAs are a class of small non-coding RNAs that can participate in the mechanisms of regulation of protein synthesis in the cell by targeting mRNAs. In addition to this well-known mediated regulatory role, miR species can also act as physiological specific ligands for toll-like receptors (TLRs) and thus initiate immune response signaling cascades [12].

Many miR species can potentially control the key mechanisms determining the development of neuroinflammation and neurodegeneration during long-term alcohol consumption [10, 13–17]. Some TLR subtypes are also associated with the development of neuroinflammatory and neurodegenerative events in the brain during long-term alcohol consumption [14, 15, 18]. Results of some studies suggest existence of possible functional links between miR-let7b, miR-96, miR-182, miR-155, and TLR in the CNS [13–17]. In this regard, the aim of this work was to compare the levels of these miRNAs and the expression of TLR genes in NAc under conditions of prolonged alcoholization in rats.

MATERIALS AND METHODS

Animals

Fourteen male Wistar rats (initial age 2-3 months, body weight 280±30 g) obtained from the Rappolovo nursery (Russia) were used in this study.

Long-Term Alcoholization

Long-term alcoholization of rats (n=7) was modeled by intragastric administration of 20% ethanol solution, which was administered using a gastric tube

for 1 month at a dose of 2 g/kg of ethanol daily from Monday to Friday (20 injections in total). Body weight was measured weekly for accurate administration of the exact dose of ethanol. During the experiment animals of the control group (n=7) received an equivalent volume of water via the gastric tube.

Biomaterial Sampling

At the end of the experiment, the rats were guillotined without anesthesia on the last day of alcoholization, and the brain NAc was sampled using the boundaries defined in the rat brain atlas. Brain samples were immediately frozen and stored at -80°C.

RNA Isolation

Total RNA was isolated using the ExtractRNA reagent (Evrogen, Russia) in full accordance with the manufacturer's instructions. Samples were treated with DNase (Promega, USA). Concentrations of the resultant RNA was measured using an Implen NanoPhotometer P330 spectrophotometer (Implen, Germany). The purity of the isolated product was evaluated by the ratio A_{260}/A_{280} (normal ≤1.8).

RT-PCR

cDNA synthesis was performed by reverse transcription (RT) using the MMLV RT kit (Evrogen) in full accordance with the manufacturer's instructions. Before RT, miR was polyadenylated using *E. coli* poly(A) polymerase (New England Biolabs Inc., USA) according to a previously described method [19]. RT for miR was performed in 10 μl using the MMLV RT kit (Evrogen) and a specific PolyT adapter (5′-GCGAGCACAGAATTAATAC GACTCACTATAGGTTTTTTTTTTTTVN-3′) [19]. Polymerase chain reaction (PCR) with real-time detection (RT-PCR) was carried out in an Mx3005P amplifier (Stratagene, USA) in 10 μl of the reaction mixture containing SYBR Green MIX (Biolabmix, Russia) and a mixture of specific forward and reverse primers (Beagle, Russia) shown in the table. The relative level of mRNA and miR was calculated using the $2^{\Delta\Delta Ct}$ method; the mRNA content was normalized to the expression level of the *Gapdh* gene, the miR level was normalized to the expression level of the *U6* gene.

Statistical Data Processing

Statistical processing of results was performed using the program Graph Pad Prizm v. 6. Groups were compared using the Mann-Whitney U-test for independent small datasets. Differences were considered as statistically significant at $p \leq 0.05$.

RESULTS AND DISCUSSION

It is known that the miRs species analyzed in this study may be functionally linked to the signaling pathways of toll-like receptors (TLR7 and TLR4), which are involved in the initiation of neuroinflammatory events that develop during chronic alcoholism [10, 13–17]. miR-let7b is known to be an endogenous TLR7 agonist [13, 18]. Changes in the miR-let7b expression may have a functional relationship with the TLR7 signaling cascade [13, 15, 18]. It has been shown that activation of TLR4 is accompanied by an increase in miR-155 in brain microglia of wild type mice, but not in Tlr4 gene knockout mice (TLR4-KO) [14]. In TLR4-KO mice, the content of miR-96 in the cerebral cortex remained unchanged, while prolonged alcoholization in wild-type mice decreased the miR-96 level [16, 17].

In our experiment, prolonged alcoholization of rats for 1 month led to a 1.71-fold decrease in the miR-let7b level in brain NAc, a 3.87-fold decrease in the miR-96 level, and a 2.29-fold decrease in the miR-182 level. On the contrary, the content of miR-155 demonstrated a 1.41-fold increase (Fig. 1).

Table. Sequences of primers used in this study

Gene	Primers	
	Forward (5′-3′)	Reversed (5′-3′)
Tlr4	ACTCTGATCATGGCATTGTT	GTCTCAATTTCACACCTGGA
Tlr7	TGAAAATGGTATTTCCAATGTG	TAAGGGTAAGGTTGGTGGTA
Hmgb1	CTCTGATGCAGCTTATACGA	AAAAGACTAGCTTCCCCTTG
miR-182	TTTGGCAATGGTAGAACTCACACCG	GCGAGCACAGAATTAATACGAC
miR-155-5p	TTAATGCTAATTGTGATAGGGGT	GCGAGCACAGAATTAATACGAC
miR-96-5p	TTTGGCACTAGCACATTTTTGCT	GCGAGCACAGAATTAATACGAC
miR-let-7b	GCGGCGGCTATACAACCTACTGC	GCGAGCACAGAATTAATACGAC
U6	TGCTTCGGCAGCACATATAC	AGGGGCCATGCTAATCTTCT
Gapdh	GCCAGCCTCGTCTCATA	GTGGGTAGAGTCATACTGGA

Prolonged alcoholization caused a decrease in the level of both TLR7 mRNA and miR-let7b, in NAc (Fig. 2). Unidirectional changes (increase contents) were also found in the case of TLR4 mRNA (Fig. 2) and miR-155.

The mechanism of interaction between the miR-183C miRNA cluster (it includes miR-96 and miR-182) and TLR4 is not fully understood; however, there are indications for such links based on miR-182-5p as an example [16, 17]. In our experiment, an increased content of TLR4 mRNA in NAc corresponded to the reduced expression level of miR-96 and miR-182.

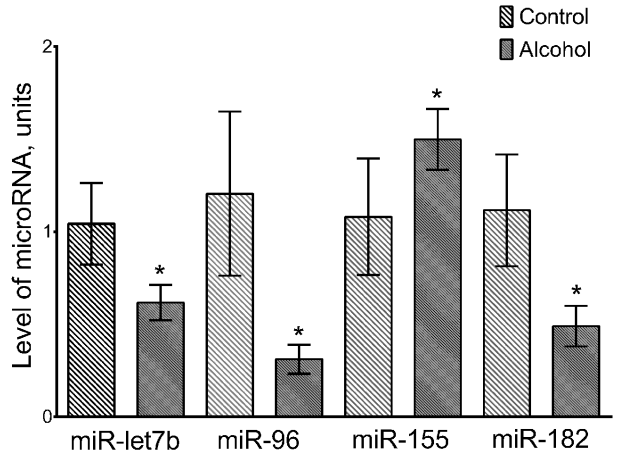

Figure 1. The level of miR in NAc (* – $p \leq 0.05$ versus control).

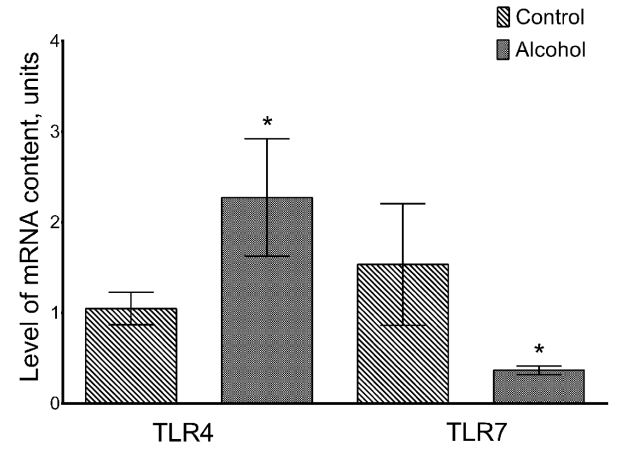

Figure 2. The level of TLR mRNAs in NAc (* – $p \leq 0.05$ versus control).

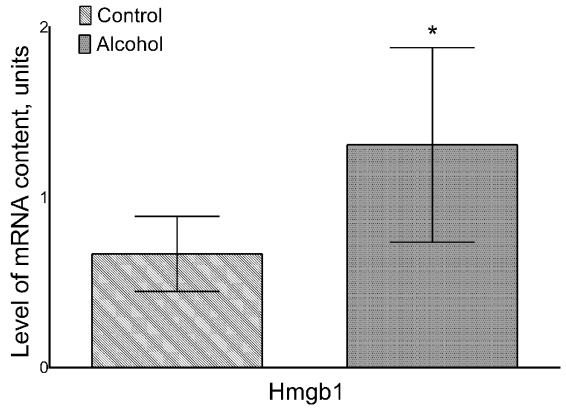

Figure 3. The level of Hmgb1 mRNA in NAc (* – $p \leq 0.05$ versus control).

According to [15], ethanol causes the formation of HMGB1-miR-let-7 complexes in microvesicles; they cause the development of a neurotoxic effect through the activation of TLR7 in neurons. The results of our experiment revealed an increased level of Hmgb1 mRNA in NAc (Fig. 3), while the levels of miR-let7b and TLR7 mRNA were reduced. Such data, apparently, indicate the absence of a functionally significant links between Hmgb1 and the miR-let7b-TLR7 system in NAc, since differently directed responses were obtained regarding the level of expression of Hmgb1 and miR-let7b in this brain structure.

CONCLUSIONS

Prolonged alcoholization influenced the level of microRNA species (miR-let7b, miR-96, miR-182, miR-155), as well as the level of TLR4, TLR7, and Hmgb1 mRNA in rat brain NAc. Based on the literature data, it can be assumed that the observed changes in the miRNA levels may have a functional relationship with the TLR system in NAc under chronic alcohol exposure; however, further studies are needed to confirm these suggestions. It seems interesting to study the content of these miRNAs in animals exposed to pharmacological agents that have a significant impact on the functioning of TLR signaling pathways, such as naloxone, rifampicin, and azithromycin and others.

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COMPLIANCE WITH ETHICAL STANDARDS

The methods used in the work were approved by the Ethical Committee for the Care and Use of Animals of the Institute of Experimental Medicine (protocol No. 21/5 dated February 26, 2015). They fully correspond to the principles of humanity (Directive of the European Community No. 86/609 EC).

CONFLICT OF INTEREST

The authors declare no conflicts of interest.

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ХРОНИЧЕСКАЯ АЛКОГОЛИЗАЦИЯ ИЗМЕНЯЕТ СОДЕРЖАНИЕ МИКРО-РНК В ПРИЛЕЖАЩЕМ ЯДРЕ ГОЛОВНОГО МОЗГА У КРЫС

М.И. Айрапетов[1,3]*, С.О. Ереско[1,2], С.А. Шамаева[1], Н.М. Матвеев[1], Е.Р. Бычков[1], А.А. Лебедев[1], П.Д. Шабанов[1]

[1]Институт экспериментальной медицины,
197376, Санкт-Петербург, ул. Акад. Павлова, 12; *эл. почта: interleukin1b@gmail.com
[2]Северо-Западный государственный медицинский университет имени И.И. Мечникова,
195067, Санкт-Петербург, ул. Кирочная, 41
[3]Военно-медицинская академия имени С.М. Кирова,
194044, Санкт-Петербург, ул. Академика Лебедева, 6, лит. Ж

Исследуемые в работе молекулы микро-РНК (miR) участвуют в молекулярных механизмах реализации TLR4- и TLR7-сигнализации, что опосредует развитие процессов нейровоспаления и нейродегенерации. В работе представлены новые сведения относительно уровня экспрессии miR-let7b, miR-96, miR-182, miR-155 и содержания мРНК HMGB1, TLR3, TLR4 в прилежащем ядре (nucleus accumbens, NAc) головного мозга у длительно алкоголизированных крыс. Длительная алкоголизация вызвала снижение уровня содержания miR-let7b, miR-96, miR-182 и мРНК TLR7, привела к повышению уровня miR-155, мРНК TLR4 и Hmgb1 в NAc головного мозга крыс. TLR7 имеет функциональную связь с miR-let7b. Полученные данные об одновременном снижении miR-let7b и мРНК TLR7 представляют интерес для дальнейших исследований, они могут указывать на отсутствие функционально значимой взаимосвязи между Hmgb1 и системой miR-let7b-TLR7 в NAc. Имеющиеся сведения о наличии функциональной взаимосвязи между TLR4 с miR-155 и miR-182 и полученные нами наблюдения об изменении их экспрессии при длительной алкоголизации также кажутся нам весьма интересным наблюдением, требующим дальнейших исследований. В связи с предположением о развитии нейровоспалительного процесса в NAc при длительном воздействии алкоголя, становится актуальным исследование в NAc уровня экспрессии генов TLR, а также экспрессии молекул miR, которые могут иметь с системой TLR функциональную взаимосвязь.

Полный текст статьи на русском языке доступен на сайте журнала (http://pbmc.ibmc.msk.ru).

Ключевые слова: *nucleus accumbens*; алкоголь; нейровоспаление; TLR; микро-РНК

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