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# Maternal methyl-enriched diet in rat reduced the audiogenic seizure proneness in progeny

I.I. Poletaeva [a,*], N.M. Surina [a,1], V.V. Ashapkin [b,2], I.B. Fedotova [a,1], I.B. Merzalov [c,3], O.V. Perepelkina [a,1], G.V. Pavlova [c,3]

[a] Biology Faculty, Moscow State University, Leninskie Gori, 1, build. 12, Moscow 119992, Russia
[b] Belozersky Institute of Physical-Chemical Biology, Moscow State University, Leninskie Gori, 1, build. 40, Moscow 119992, Russia
[c] Institute of Gene Biology, Russian Academy of Sciences, Vavilova str., 34/5, Moscow 119334, Russia

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ABSTRACT

Audiogenic epilepsy proneness was analyzed in the progeny of rats from two strains (audiogenic seizure prone—strain "4"—and audiogenic seizure non-prone, strain "0"). Females were fed by a diet which contained substances enriched with methyl-groups during 1 week before mating (MED), during pregnancy period and 1 week after the delivery. This MED treatment resulted in a decrease of audiogenic seizure fit intensity, which was more evident in rats of strain "0". Control rats of strain "4" displayed intense seizures (tonic seizure, 3.85 arbitrary units). Med "4" rats seizures were less intense (3.23, tonic seizure of lower intensity), control "0" strain rats demonstrated the seizure with mean 3.09 arbitrary units, "0" MED rats only 2.03 arbitrary unit intensity (only clonic seizures, significantly, $p < 0.05$, different from controls). Methyl-enriched diet resulted in the significant changes in methylation status of several genes (Cpne6, Gtf2i, Sctr,1 Sfmbt, Phe2). These genes among others were chosen for analysis as their expression was analyzed in other methylation study. These genes were hypermethylated after "epileptic tolerance". Due to this procedure, the intensity of status epilepticus, produced by kainate in mice, decreased (Miller-Delaney et al., 2012). The modulation of audiogenic seizure intensity as the result of methyl-enriched diet during prenatal and early postnatal ontogeny was demonstrated for the first time.



## 1. Introduction

The genetic predisposition to seizure development was described both in humans (see EPICURE Consortium and EMINet Consortium, 2012; Helbig and Lowenstein, 2013) and in animals (Coppola and Moshé, 2012). As it was demonstrated the processes of brain development are also under epigenetic control, and it is now possible to analyze the role of epigenetic processes and namely the DNA methylation in epileptogenesis (Schatz et al., 1983; Lester et al., 2011; Hwang et al., 2013; Kobow et al., 2009; Kobow and Blümcke, 2011).

The role of DNA methylation in epileptogenesis was investigated in animal experiments (see Sellinger et al., 1986; Tsankova et al., 2004; Miller-Delaney et al., 2012) and in clinic (Kobow and Blümcke, 2012). Gene MBD5 (the product -*methyl-CpG binding domain protein 5*) is the member of methyl-binding group of genes, and it probably participates in brain DNA methylation (Noh and Graham, 2012). The deletion of this gene, found in humans, was accompanied by severe CNS dysfunction, including seizures and developmental delay (Motobayashi et al., 2012). The patterns of gene expression, which are presumably determined (among other factors) by changes in epigenetic processes, vary in animal models of epilepsy (Kobow et al., 2013).

Human brain DNA methylation and epilepsy are shown to be connected. Miller-Delaney et al. (2012) analyzed the effects of seizure preconditioning using status epilepticus (SE) model (seizures induced in C57BL/6 mice by intra-amygdala microinjections of kainic acid). The preconditioning procedure included i.p. injection of kainic acid before SE induction, which promoted seizure tolerance. Based on genome-wide DNA methylation analysis authors discovered changes in methylation status of 288 genes (in hippocampal tissue), and 15 genes from this group were differentially hypermethylated (in comparison to SE group) in the case when pre-conditioning + SE procedures were used. Most of these genes were "novel" and not known previously to be implicated in SE tolerance mechanisms.

The study of epigenetic mechanisms involved in seizure development includes usually the comparison of brain DNA methylation patterns during seizures (or immediately after seizures) vs a normal state. Using this approach changes were found in the methylation level of several genes (Doyle and Sellinger, 1980; Sellinger et al., 1986). At the same time practically no data exist concerning the role of DNA

* Corresponding author at: Biology Faculty, Moscow State University, Leninskie Gori, 1, build. 12, Russia. Tel.: +7 495 9394468; fax: +7 495 9392837.
*E-mail addresses:* ingapoletaeva@mail.ru (I.I. Poletaeva), ashapkin@genebee.msu.su (V.V. Ashapkin).
[1] Tel.: +7 495 9394468; fax: +7 495 9392837.
[2] Tel.: +7 495 939 53 59.
[3] Tel.: +7 499 1352541.

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methylation in determining seizure proneness in genetic animal models of seizure states.

The adequate and easily reproducible generalized seizure fit model is rodent audiogenic epilepsy (Feller et al., 1994; Faingold, 1999; Misawa et al., 2002; Prieto-Martín et al., 2012). The genetic models used are mainly KM strain (Russia), GEPR strains (USA) and WAR strain (Brazil) (Poletaeva et al., 2011). The proneness to these types of seizures has demonstrated genetic components (Poletaeva et al., 2011; McMillan and White, 2004). The physiological mechanisms of audiogenic seizure fit (ASF) are different from those involved in limbic seizure development (Faingold, 1999; Poletaeva et al., 2011).

The DNA methylation level could be modulated using special methyl-enriched diet (Prasolova et al., 2006, 2009; Van den Veyver, 2002; McGowan et al., 2008). In these types of experiments changes in the gene methylation were described as well as some phenotypic changes induced by this treatment (Pliusnina et al., 2007; Gerbek et al., 2010; McGowan et al., 2008; Zhang et al., 2013). These data were the prerequisite for the suggestion that brain DNA methylation level could be analyzed in rats with different proneness for seizures when methylation "load" was induced by means of special diet. In the present study the intensity of ASF intensity, as well as development of audiogenic myoclonic seizures was analyzed in rats of two strains which differed in audiogenic seizure proneness. Special maternal diet should presumably induce changes in brain DNA methylation. The methylation status (using methylation-dependent DNA polymerase chain reaction, PCR) was analyzed in those genes, which were found to be hypermethylated in mice in case of preconditioning + SE experiments, mentioned above (tolerance, by Miller-Delaney et al., 2012).

## 2. Material and methods

### 2.1. Subject and selection procedure

Rats of strains "0" and "4" ($18^{-th}$ selection generation) were used. These strain are selected for the absence of ASF in response to loud sound strain "0" (for "null" reaction to sound) and for its maximal intensity (strain "4", as "4" is the maximal arbitrary unit of this trait intensity, adopted in the laboratory). Selection started using animals of hybrid population between rats of the inbred strain with high audiogenic seizure proneness (Krushinsky–Molodkina or KM strain: see Poletaeva et al., 2011) and Wistar rats. From the group of 60 Wistar rats 4 males and 3 females were chosen that did not develop ASF after 3 successive sound exposures, presented after 1 week interval (Fedotova et al., 2012). Animals from the hybrid population, which did not develop seizures from sound testing, were chosen as parents for strain "0"; animals with maximal ASF intensity were the founders for strain "4". The offspring of these matings were behaviorally tested to sound at 3 months of age using the same protocol. Thus animals used in the present study differed in audiogenic seizure proneness yet to a large extent share a common genetic background. As the selection for "0" degree of ASF slowly proceeded (Fedotova et al., 2012) an absence of ASF was found only in a portion of "0" strain rats (in the control group of "0" rats used in present study there were around 20% of rats without any signs of audiogenic epilepsy), although the ASF intensity of rats from "0" strain was low (see below). Five females from strain "0" and 7 females from strain "4" after mating to males of the respective strains gave birth to a total of 67 pups (see Table 1). Part of them were controls, as their mothers received the normal food (without methyl enrichment, see below), while others were "experimental" (i.e. after maternal methyl enriched diet -MED).

**Table 1**
Numbers of control and experimental (MED) animals of both strains, which participated in the experiment.

| Strain | Group | Male | Female | Total |
|---|---|---|---|---|
| "0" | "Experiment" | 12 | 6 | 18 |
| | "Control" | 5 | 6 | 11 |
| "4" | "Experiment" | 6 | 5 | 11 |
| | "Control" | 10 | 17 | 27 |

### 2.2. Bioethical standards

Animals used in research have been bred in the laboratory and cared for in accordance with the guidelines published in NIH *Guide for the Care and Use of Laboratory Animal* and the principles presented in the "Guidelines for the Use of Animals in Neuroscience Research" by the Society for Neuroscience. The experimental protocol was approved by Moscow State University Ethical Committee and was in accordance to general bioethical principles (namely to EC Convention 2010 rules).

### 2.3. Methyl-enriched diet (MED)

The chemical ingredients, which were added (per 1 kg of food) in order to enrich rat food with methyl-group radicals, were the following: choline—5 g, betain—15 g, folic acid—15 mg, vitamin B12—1.5 mg, L-methionine—7.5 g, and zink (as ZnSO4)—150 mg (by Prasolova et al., 2006). These ingredients were mixed with boiled buckwheat, cottage cheese and fresh eggs. Females received this food for 1 week before mating, during the entire pregnancy period and 1 week after the delivery. Control females received the same food without methyl-group enriching ingredients.

### 2.4. Testing the audiogenic seizure proneness

The testing of audiogenic seizures was performed twice at the age of 3 and 4.5 months. Animal was placed inside the sound attenuating box and the sound (auditory-bell, 120 dB) was switched on. After the development of seizure fit (or after 90 s in cases, when no seizure developed) the sound was switched off. The audiogenic seizure intensity was evaluated according to the arbitrary scale, with "0"—for the absence of fit, "1"—for the stage of "clonic" run (or "wild run stage"), "2"—clonic seizure, "3"— tonic seizure, and "4"— maximal intense tonic seizure of the trunk muscles and extremities and animal falling on the side (Poletaeva et al., 2011). After weaning, at 28 days animals were separated from mother and kept in the female or male groups of 3–4 individuals in A4 cages with water and standard rodent food (Laboratorkorm firm) ad lib. At the age of 3 months all rats were tested for audiogenic proneness for the first time. At the age of 4.5 month a second similar test and the procedure of audiogenic kindling took place. The latter included daily repetitive sound exposure (during 25 days) to test the development of audiogenic myoclonic seizures (Fedotova and Semiokhina, 2002; Galvis-Alonso et al., 2004).

### 2.5. DNA extraction

*DNA extraction* was performed from the whole brain tissue (for technical reasons) by means of standard phenol extraction technique. The tissue samples were taken after three to four weeks after the end of audiogenic seizures and audiogenic kindling testing; thus the previous sound exposure presumably could not affect the methylation status of the DNA.

### 2.6. Methylation level assays

A 200–400 bp segment containing one to five CCGG sequences recognized by restriction endonucleases *Hpa*II and *Msp*I was chosen for each gene in study. These restriction endonucleases are well known isoschizomers having different sensitivity to cytosine methylation. Namely *Hpa*II is inhibited by internal cytosine methylation (Cm5CGG sites) whereas *Msp*I cleaves DNA irrespective of such methylation. Both endonucleases are inhibited by external cytosine methylation