Identification of Novel Mutations Controlling Cerebral Cortex Malformations Caused by ENU-Induced Mutagenesis in the Mouse

DOI: 10.17691/stm2018.10.3.8
Received August 2, 2017

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The aim of the study is to identify new gene mutations causing cerebral cortex malformations in mice.

Materials and Methods. To identify genes causing cerebral cortex malformations, chemical mutagenesis was carried out using N-ethyl-N-nitrosourea as a mutagen. A total of 141 male C3H mice aged 8 weeks were injected with the mutagen in order to induce mutations in spermatogonial stem cells. After a period of sterility, the animals were used in three-generation backcross scheme. Satb2-LacZ reporter mice were involved in this strategy to label the neurons forming the corpus callosum.

Results. The animal phenotype displaying primary microcephaly and 6 mutant lines demonstrating audiogenic epilepsy have been described. The phenotypes of these mutants will be further presented and discussed.

Key words: mouse mutagenesis; brain development genetics; phenotype screening; microcephaly; audiogenic epilepsy.

Introduction

In the early stages of development, a formation of the mammalian brain involves coordinated stages of cell division and differentiation. The mammalian telencephalon develops from the most rostral part of the nervous tube, which eventually results in the formation of the neocortex, hippocampus, olfactory bulbs, and basal ganglia. Proper regulation of cellular differentiation (and cell death) contributes to the development of appropriate brain architecture. These events require a wide variety of coordinated cellular biological processes. Today, there

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are studies devoted to investigating genetic regulation of forebrain development, but many things still remain unclear. Further studying the molecular basis of brain formation processes is likely to help explain the causes of many brain diseases [1, 2], including malformations, degenerative diseases developing in adulthood and oncogenesis. Availability of information on the genetic contribution to the development of many diseases provides the possibility to study genetic modifications causing and controlling cerebral cortex malformations.

One of the approaches to studying individual gene changes is based on the use of chemical mutagenesis caused by N-ethyl-N-nitrosourea (ENU). ENU mutagen causes single-nucleotide changes able to produce different types of mutations, including hypo-, hyper-, neo-, zero-, and anti-morphisms [3–5]. Initially, these changes were found difficult to identify. Therefore, after this mutagen was proposed by Russell in 1982 as a method of non-toxic increase in the mutation frequency [6], it was used only by a small number of researchers [7–9].

The tools for genomic analysis and positional cloning were later improved, which led to the increased use of ENU in large-scale screening with a large number of mice [7, 9, 10] and, specifically, in solving particular biological issues [2, 6, 9, 11–13].

The results of these investigations have shown the effectiveness of this approach for creating and studying new mutations with interesting phenotypes. Other studies have demonstrated how this strategy can be used to examine the relationship between a particular gene and a disease [12].

Introduction of ENU to male mice leads to point mutations (single-base changes) in spermatogonia which develop into mature sperm cells with the frequency of about $1.5 \cdot 10^{-3}$, or one mutation per 700 loci. This chemical is an alkylating agent that acts by transferring the ENU ethyl group to nucleotide bases (most often, thymine) in nucleic acids (Figure 1). As a result, O^6-ethylguanine complementary to adenine, not to cytosine, is produced, so there occurs a single-base substitution. This mutagen requires no metabolic activation.

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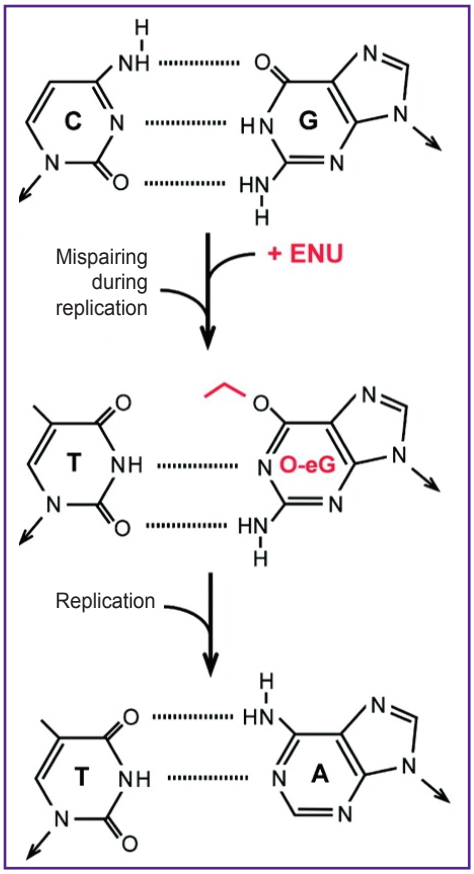

Figure 1. The mechanism of action of ENU mutagen [14]: A — adenine, C — cytosine, G — guanine, T — thymine, O-eG — O^6-ethylguanine

Materials and Methods

This project involved using C3H/HeJ (C3H) and C57BL/6 mice widely used in various fields of research, including investigation of cancer as well as infectious, sensorineural and cardiovascular diseases. The genome sequence was determined for these lines.

A total of 230 C3H male mice were given four series of mutagen injections at the dose of 60–100 μg/kg during the screening aimed to identify new gene mutations responsible for the formation and functioning of the cerebral cortex.

Care and management of experimental animals complied with the Guidelines for Works Involving Experimental Animals (Russia, 2010) and the International Guiding Principles for Biomedical Research Involving Animals (CIOMS and ICLAS, 2012). The work was performed in accordance with ethical principles established by European Convention for the Protection of Vertebrate Animals used for Experimental and other Scientific Purposes (Strasbourg, 2006) and approved by Ethics Committee of Lobachevsky State University of Nizhni Novgorod.

Manufacturing ENU preparation and performing injections. ENU mutagen is sensitive to humidity, light, and acidity. Dilution was made in a new ENU bottle (N3385, Sigma-Aldrich, Germany) before each weekly injection. The bottle was protected from light by foil paper. The solution was prepared by adding 10 ml of 95% ethyl alcohol, stirring until completely dissolved and adding 90 ml of phosphate-citrate buffer (100 mM sodium phosphate, 50 mM sodium citrate, pH 5.0). After dissolving, the solution had the form of a transparent yellow liquid.

The required ENU amount for an injection of a single animal was calculated by the following formula:

$$0.001 \cdot W + \frac{Y}{X} = Z,$$

where W — animal weight in grams; Y — the required ENU dose in micrograms per gram of body weight; X — stock solution concentration in milligrams per milliliter; Z — injection volume in milliliters.

Injection volume was 0.2–1 ml as smaller volumes are difficult to inject, while larger ones can lead to ethanol intoxication [13].

Injections were given to 8-week-old C3H mice intraperitoneally in accordance with the calculated amount. The injection site was chosen along the ventral line of the abdomen, caudal to the chest, but rostral to the bladder.

The period of sterility in the animals. According to the literature [15], the average period of sterility in mice after threefold ENU injection is 10–15 weeks. At this time, spermatogonial cells repopulate the testes. In some cases, males never restore fertility. For example, in case of threefold introduction of mutagen at a dose of 100 mg/kg to C57BL/6J males, 25–50% of them never restored their fertility [15].

Mouse crossing scheme. Various mouse crossing schemes can be used for screening in the studies using ENU mutagen (Figure 2). First-generation mice screening can be used for studying the animals demonstrating both autosomal and, predominantly, X-chromosome-linked inherited traits. Second-generation mice screening can be applied for studying mutations affecting a particular gene, isolating allelic series or identifying interacting genes. This approach is also effective in combination with deletion mutations, for distinguishing mutations pre-localized before the deletion interval and for obtaining animals that demonstrate X-linked recessive traits. Third-generation screening is used for animal generation demonstrating autosomal-recessive traits. Also, this approach often involves backcrossing of G2 females with their fathers to increase the probability of detecting mutations.

In this study, third-generation animal screening was carried out using backcross stage to obtain recessive mutations.

LacZ gene promoter. Satb2 is a DNA-binding protein which is involved in the organization of chromatin and regulates gene expression, it is also needed for initiation of a specific genetic program in upper cortical layer neurons. Satb2 also serves as Ctip2 repressor and regulator of cortical-cortical interactions in the cerebral cortex [16]. Beta-galactosidase (β-gal) encoded by the *LacZ* gene is widely used as a reporter enzyme transgene. The ability to visualize *LacZ* expression in living transgenic animals extends the limits of using this reporter. Beta-galactosidase is an enzyme that hydrolyzes beta-galactosides. X-gal segregation (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) results in the appearance of blue color.

Introduction of the Satb2-*LacZ* transgene into the genome of mutant mouse strains after ENU mutagenesis can be used for easier visualization of changes in cerebral cortical cytoarchitecture and connections between cells.

LacZ genotyping. Polymerase chain reaction (PCR) protocol was developed to determine a presence of *LacZ* transgene in mice. For this purpose, mouse tail samples were taken by separating the end fragment with scissors and placing the resulting sample in a sterile tube. Then, DNA was lysed and isolated by precipitation with isopropyl alcohol. The isolated samples were used as a matrix for PCR-based diagnostics. To perform amplification of *LacZ* gene fragment, primer system (SatB2 Fwd, SatB2 Rev, and SatB2 LacZ Rev), the optimal temperature of primer annealing (58ºC) and the number of cycles (35) were chosen.

Polymerase chain reaction was performed on a programmable thermal cycler (BIO-RAD Laboratories, USA) in total volume of 20 μl containing 2 μl of 10-fold buffer (Mg^{2+} — 30 mM); 0.4 μl of 10 mM dNTP; 1 unit of Taq DNA polymerase; 10 pM of each of SatB2 Fwd, SatB2 Rev, and SatB2 LacZ Rev primers for amplification of *LacZ* gene fragment of 250–300 nucleotide pairs in length, 2 μl of DNA sample, in the following mode:

1) 95ºC 3 min, 1 cycle;
2) 95ºC 30 s;
3) 58ºC 30 s;
4) 72ºC 30 s, steps 2–4 to be repeated in 35 cycles;
5) 72ºC 5 min, 1 cycle;
6) storage at 12ºC.

To visualize DNA sample fragments, 2% of an agarose gel containing ethidium bromide (0.5 μg/ml) was introduced into the wells and horizontal electrophoresis was performed at 200 A amperage for 40 min in one-fold Tris-acetate electron (TAE) buffer. Genotypes were identified by qualitative PCR using the Quantum gel

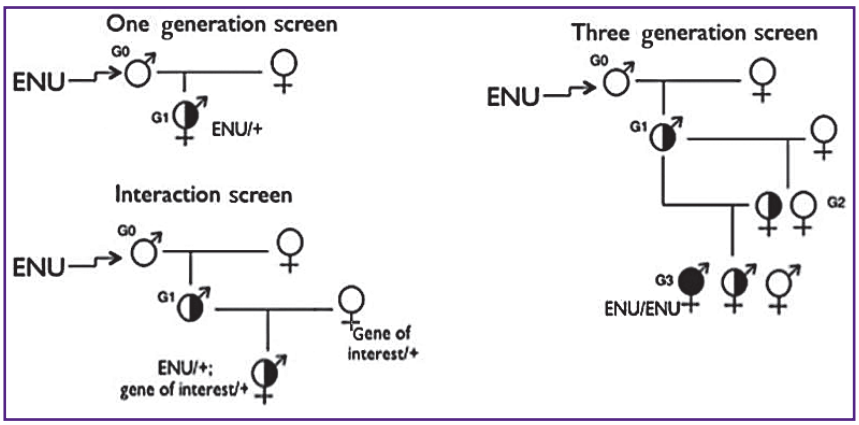

Figure 2. Variants of crossing schemes employed for ENU mutagenesis [13]: G0, G1, G2, G3 — generation numbers

documentation system (Vilber Lourmat, France) and VisionCap software.

Staining protocol. To visualize changes in the structure of the cerebral cortex, the optimized protocol was used for staining mouse brain preparations with Satb2-LacZ reporter, the composition of the solutions used was selected. Beta-galactosidase encoded by the *LacZ* gene hydrolyzes beta-galactosides, which results in the appearance of blue color. The cleavage product displays long-range red light and fluorescence properties detected by *in vivo* imaging.

At the first stage, mouse brain sections containing *LacZ* transgene were washed in the fresh solution (4 mM $MgCl_2$; 0.01% NaDOC; 0.02% NP40 in phosphate-buffer saline (PBS), pH 7.4). Then the sections were incubated in a staining solution (5 mM $K_3Fe(CN)_6$; 5 mM $K_4Fe(CN)_6$; 0.5 mg/ml X-gal) for 30 min at 37°C. Next, they were washed in PBS and fixed on glass slides for subsequent microscopy.

Testing for audiogenic seizures. Audiogenic seizures (AGS) developing reflexively in rodents in response to sound stimulation are the most popular and adequate experimental models of generalized convulsive epilepsy in humans [17–20].

For the first time, AGS were observed in rats at Wistar Institute (USA) and in mice at I.P. Pavlov Laboratory (Russia) in 1924. Using rodents as experimental models for studying physiological mechanisms of epilepsy was started by Krushinsky and his colleagues in 1948 at the Department of Developmental Biology, Faculty of Biology, Moscow State University [18].

AGS susceptibility in rodents may be genetically inherited or occur as a result of induced mutagenesis [19, 21]. Just like other reflex seizures, AGS is a latent form of epilepsy, therefore, in the absence of specific stimulation, the animal shows no neurological pathology, while epileptic seizures develop only when induced by relevant sensory stimuli [22].

Full-scale AGS comprise several phases which consistently replace each other and reflect the spread of epileptic activity in the brain: the initial motor excitation (paroxysmal running), clonic and tonic seizures. Motor excitation is the characteristic and most common component of AGS in rodents.

Other components such as clonic and tonic convulsions may be absent. Motor excitation may consist of one or two episodes separated by a motionless period ("brake pause").

AGS intensity is determined primarily by the initial level of convulsive sensitivity of the animal and varies from the minimal convulsive response to sound in the form of motor excitation (score 1) to a full-scale seizure with tonic-clonic convulsions of maximum intensity (score 4 according to Krushinsky scale [23] or score 9 according to Jobe scale [20]).

The degree of AGS manifestation in response to sound stimulation can be divided into several universal phases typical for all lines of AGS susceptible animals:

1. The phase of "circus movement" or motor excitation: after sound stimulation, the animals move uncontrollably in the test chamber. This phase may contain 1 or 2 excitation phases: uniphasic running bout (described in the literature as "false start") or bi-phasic running bout.
2. Clonic convulsions are characterized by flexion of the back, neck, fore-, and hindlimbs combined with muscle spasms of the whole body. If the clonus is accompanied by short-term rigidity of the animal body, this type of convulsions will be classified as tonic-clonic.
3. Tonic convulsions can be observed after the running phase or clonus, they are characterized by rigid stretching of the entire animal body. AGS reaching this phase can lead to the death of the animal, which is the most common feature in various mouse lines.
4. Typical post-convulsive period characterized by motionlessness, temporary insensitivity to sound and vocalization can also be observed after clonic, tonic, or tonic-clonic seizures have developed in various rodent lines [24].

Results

Four series of ENU mutagen injections were performed in accordance with the protocol (see the Table) for screening aimed to identify new gene mutations responsible for cerebral cortex formation and functioning.

It was found during the study that males restored their fertility after 21–24 weeks when the mutagen was introduced three times at a dose of 60 mg/kg, after 27–28 weeks when a dose was 80 mg/kg and fertility was never fully restored after a dose of 100 mg/kg (Figure 3).

In this work, we used a three-generation backcross scheme with Satb2-LacZ reporter mice integrated into this scheme to identify recessive mutations (Figure 4). In the second generation, C57BL/6 mice were included in the crossing scheme to obtain genetic polymorphism

A series of mutagen injections given to C3H mice

A series of injections	Dates of injections	Mutagen doses, (μg/kg)/number of animals
1	29.07.2015 05.08.2015 12.08.2015	80/21 90/19 100/20
2	19.10.2015 27.10.2015 03.11.2015	80/18 90/21 100/20
3	19.01.2016 26.01.2016 02.02.2016	60/21 80/17 100/22
4	03.03.2016 10.03.2016 17.03.2016	60/18 80/18 100/15

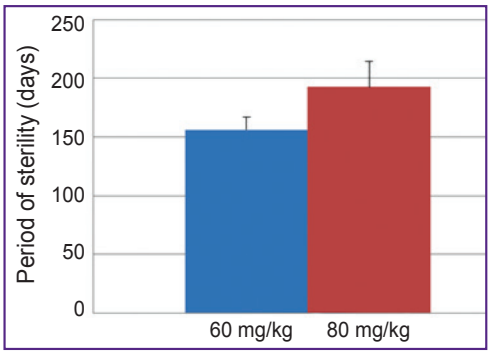

Figure 3. The period of sterility in groups of mice with different ENU mutagen doses

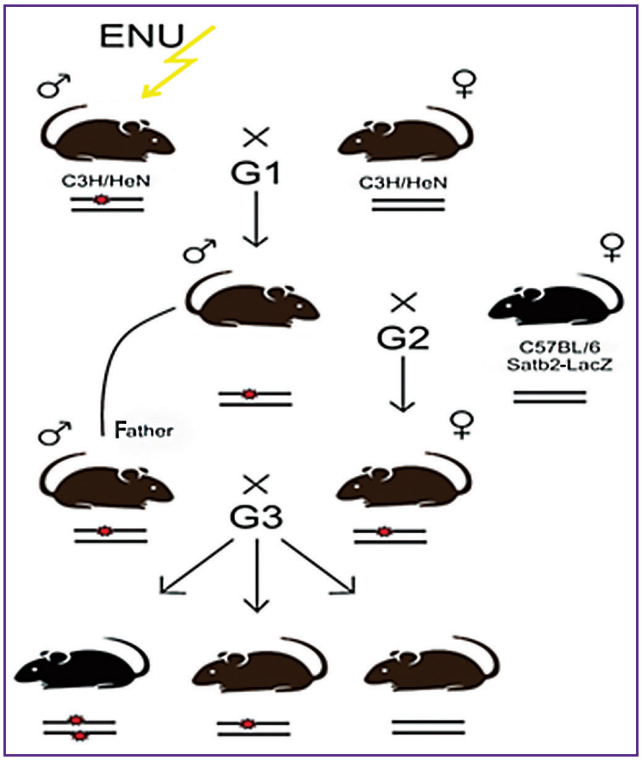

Figure 4. Three-generation crossing scheme with backcross stage employed in this study

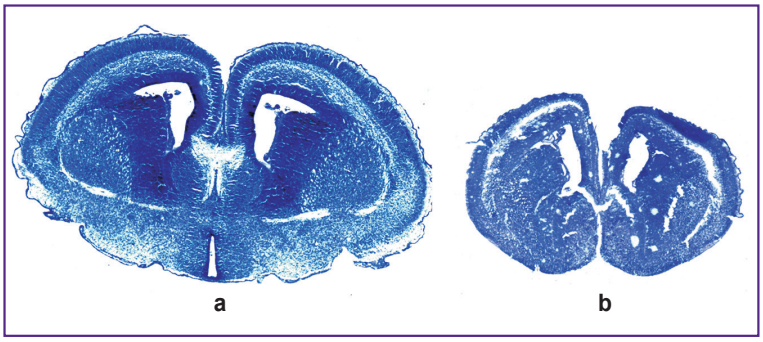

Figure 5. Primary microcephaly:
Mouse brain slices at stage P0, after Nissl staining: (a) control sample; (b) microcephaly, stage E 16.5

used for further mapping of detected mutations [25, 26]. G1 males were obtained by crossing G0 males subjected to mutagen injections with wild-type C3H females. At this stage, Satb2-LacZ line of mice (C57BL/6) was introduced into the crossing scheme for obtaining generation G2. To generate recessive mutants, LacZ-positive G2 females were mated with G1 fathers. The resulting G3 offspring were analyzed for the presence of brain abnormalities on day 4 after birth and for sensitivity to AGS causing epileptic seizures on day 21 after birth.

Cytoarchitecture of the cerebral cortex was studied in 113 lines of mice produced by 20 ENU-induced mutant males (generation G3), using the staining protocol described earlier.

A mutation leading to the development of primary microcephaly phenotype was found. On average, the number of microcephaly in the offspring varied from one sixth to one eighth, which may indicate this mutation recessiveness, on the one hand, and possible partial embryonic lethality, on the other hand. Mice with signs of microcephaly died at birth. It should be noted that embryos with microcephaly were already found at embryonic stage E16.5, but death occurred after birth, as a rule. Microcephaly was characterized by a noticeable decrease in head size, both the skull and the brain, and a small decrease in body size. Brain size reduction was mainly due to a critical reduction in the size of large hemispheres, including the cerebral cortex. At the same time, microcephalic mice were found to have the corpus callosum and hippocampus, which indicates presence of all main brain structures, while having reduced size of its individual parts or the entire brain completely. Microcephaly may be caused by abnormal cell proliferation or increased cell death (Figure 5).

There was created a system for generating AGS in mice on the basis of Startle and Fear Conditioning System (PanLab, Spain; Stoelting, USA), its work was tested. This system (Figure 6) is a plastic camera (25×25×25 cm) placed in a soundproof box. The camera is supplied with a bell and a video camera (Lifecam cinema HD; Microsoft, USA) used to record what is happening inside. The test animal is placed into the experimental chamber and a loud acoustic signal (10 kHz, 100 dB) is given after 1 min of adaptation. The signal stops when the animal starts convulsing or after 80 s (four periods of 20 s, with two-second intervals) from the test start.

To assess the observed effect, the following quantitative indicators of audiogenic response were used: the latent period of motor excitation in response to sound stimulation (in seconds), the latent period of seizure (in seconds), seizure intensity (score 0 to 4) and convulsive response character (one- or two-wave).

G3 generation mice were tested at the age of 20–30 days. To date, the total number

Figure 6. Audiogenic epilepsy test system

of animals tested has been 836. Overall, the offspring of 32 males injected with ENU mutagen has been investigated. Sexual differences were found in the reaction to experimental stimuli, animal lines sensitive to the epileptogenic action of sound have been revealed: S1-3, S2-3, S3-2, S5-1, S8-3, S9-4.

G3 generation mice of these lines will be further tested using various behavioral methods, mapping of these mutations will be performed.

Conclusion

As a result of identifying the genes causing cerebral cortex malformations in mice, which was carried out using chemical mutagenesis with N-ethyl-N-nitrosourea as a mutagen, the phenotype displaying primary microcephaly has been described and 6 lines of animals demonstrating seizures induced by audiogenic stimulation were found.

Study funding. This study was supported by the grant of the Russian Science Foundation No.15-14-10021.

Conflict of interests. The authors have no conflict of interests to disclose.

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