ENU MUTAGENESIS AS A TOOL FOR IDENTIFYING NOVEL MOUSE MODELS OF EPILEPSY

E.V. Borisova[*1, 2], *E.A. Turovsky*[3], *M.V. Turovskaya*[3], *A.N. Tomilin*[4], *S.A. Nedospasov*[5, 6, 7], *V.S. Tarabykin*[1, 2]

[1] Institute of Neuroscience, Lobachevsky University of Nizhny Novgorod, Nizhny Novgorod, Russian Federation
[2] Institute of Cell Biology and Neurobiology, Charité-Universitätsmedizin Berlin, corporate member of Freie Universität Berlin, Humboldt-Universität zu Berlin, and Berlin Institute of Health, Berlin, Germany
[3] Institute of Cell Biophysics of the Russian Academy of Sciences, Federal Research Center "Pushchino Scientific Center for Biological Research of the Russian Academy of Sciences", Pushchino, Russia
[4] Institute of Cytology Russian Academy of Science, Saint Petersburg, Russia
[5] Center of Genetics and Life Sciences, Sirius University of Science and Technology, Sochi, Russia
[6] Lomonosov Moscow State University, Moscow, Russia
[7] Engelhardt Institute of Molecular Biology of Russian Academy of Sciences, Moscow, Russia

* Corresponding author: brusnichka_ne@mail.ru

Abstract. Epilepsy affects around 1% of the population in the world. Thus, it is imperative that new more effective and safe treatments be found. In order to understand the nature of epilepsy, new and better animal models are needed in that they offer valuable resources for researchers. Such models provide an opportunity to characterize seizures in the whole organism, to understand the molecular basis of these processes and to test the effectiveness of treatments and therapies. In this study, we have shown that screening after chemical mutagenesis can be used as a tool to identify new genes that may be involved in the mechanism of epilepsy formation.

Keywords: mouse mutagenesis, ENU mutagenesis, phenotype screening, spontaneous mutation, audiogenic epilepsy, SNP mapping.

List of abbreviations

ENU – N-ethyl-N-nitrosourea
SNP – Single nucleotide polymorphism
ARMS-PCR – Amplification-refractory mutation system-polymerase chain reaction
AGS – Audiogenic stimulation

Introduction

Neurological diseases such as epilepsy represent a significant health problem, and the incidence of these diseases increases with life expectancy. Around 60 million people worldwide have epilepsy. In the quest to uncover the molecular underpinnings of neurological diseases mouse genetics play an essential role. The generation of gene-deficient mouse lines is a widely used approach for identifying loss-of-function phenotypes. In the late 1990s, several large-scale mutagenesis programs were established as systematic approaches for creating mouse models of human diseases (Brown & Nolan, 1998, Hrabe de Angelis et al., 2000, Nolan et al., 2000). There are several different strategies, among them the treatment of male mice to intraperitoneal administration of N-ethyl-N-nitrosourea (ENU), a strong chemical mutagen that causes random single nucleotide substitutions in the genome with an average frequency of 1 mutation per 700 loci (Acevedo-Arozena et al., 2008, Cordes, 2005). ENU may cause loss-of-function mutations, antimorphs and gain-of-function mutations. Subsequently, the offspring is analyzed for specific phenotypic changes. In this study, we applied this approach to study the genetic counterparts and molecular basis of epilepsy.

Materials and methods

ENU treatment and breading scheme. ENU treatment was performed as previously described (Borisova et al., 2018). Briefly, 80 μg/kg ENU was intraperitoneally injected to

eight-week-old C3H mice. In the G2 generation C57BL/6 mice were included in the breading scheme to obtain genetic polymorphism. To study autosomal-recessive traits, three-generation screen with backcrossing of G2 females with their fathers was performed to increase the probability of detecting mutations.

Mice were housed in the Institute of Neuroscience animal facility at 25 ± 3°C with a 12-h light/dark cycle and free access to food and water. All animal experimentation met the requirements described in the Rules for the Work using Experimental Animals (Russia, 2010) and the International Guiding Principles for Biomedical Research Involving Animals (CIOMS and ICLAS, 2012); the ethical principles established by the European Convention for the Protection of Vertebrate Animals used for Experimental and Other Scientific Purposes (Strasbourg, 2006) were strictly observed. The Ethical Committee of the Lobachevsky State University of Nizhni Novgorod approved the protocol of these experimental studies on animals.

Amplification-refractory mutation system (ARMS)-PCR and SNP panel. For genetic mapping we developed a single nucleotide polymorphism (SNP) panel of 114 SNPs (6 SNPs for each of 19 autosomes). Primers were designed with primer design web service for tetra-primer ARMS-PCR «Primer1» (Ye et al., 2001). Genomic DNA was extracted from tail biopsies. PCR was performed on a thermal cycler (BIO-RAD Laboratories) in a total volume of 25 µl containing 0,5 µl of 10mM dNTP, 5 µl of 5x buffer (Mg2+ 30mM), 1 unit of Taq DNA polymerase (Evrogen), 100–150 ng of DNA sample, 10 pM of each of Outer Forward, Outer Reverse, Inner Forward and Inner Reverse primers for amplification of allele-specific and non-allele-specific bands. Conditions for amplification were the following:

Step 1: 95º 3 min 1 cycle
Step 2: 95º 30 sec
Step 3: 55º 30 sec
Step 4: 72º 40 sec (35 cycles Step 2 to 4)
Step 5: 72º 5 min

PCR products were subjected to electrophoresis in 2% agarose gel with Serva DNA stain.

Audiogenic epilepsy test. Seizures that can form in response to audiogenic stimulation (AGS) are one of the most adequate and widely applicable models of generalized convulsive epilepsy in humans (Faingold, 1999, Jobe & Browning, 2006). We have chosen this method for testing mice of the G3 generation after ENU mutagenesis.

System for generating AGS in mice was created based on Startle and Fear Conditioning System (PanLab, Spain; Stoelting, USA). The system is a plastic chamber (25x25x25 cm) placed in a soundproof box. The chamber is supplied with a bell and a video camera (Lifecam cinema HD, Microsoft, USA). Testing was carried out on animals of the G3 generation at the age of 20-30 days after birth (P20-P30). The test animal was placed in the chamber, and after 1 minute of the adaptation period, a loud acoustic signal was given. The signal was stopped either when the animal had seizures or 80 seconds after the start of the test (4 intervals of 20 seconds with two-second breaks). The following indicators of the audiogenic response were used for the assessment: seizure intensity (score 0 to 4), the latent period of motor excitation in response to sound stimulation (in seconds), the latent period of seizures (in seconds), and convulsive response character (one- or two-wave).

Mixed cortical neuroglial cell culture. Cell cultures were prepared as previously described (Gaidin et al., 2020). One to three-day-old pups were used. The extracted brains were minced with scissors and then incubated in Versene solution with 0,2 % trypsin for 10 min at 37ºC and 600rpm on a thermocycler. Then, the tissue fragments were washed three times with a cold Neurobasal medium (Gibco). The trypsinized tissue was gently triturated with a pipette and then precipitated (2 min 1000 rpm). The supernatant containing debris was carefully discarded. Further, the cells were resuspended in a Neurobasal medium with Glutamine

(0,5 mM), Supplement B27 (2%), gentamicin (15 mg/μl). Cells were seeded on polyethyleneimine-coated 25-nm glass coverslips (VWR International) and then transferred to the 35 nm Petri dishes (Greiner). All experiments were performed at 9 days in vitro (DIV).

Fluorescence microscopy. The concentration of cytoplasmic calcium ([Ca2+]i) was assessed using a two-wave probe Fura-2 under a well-known method (Gaidin et al., 2020, Turovskaya et al., 2020). The primary cortical cells were stained with the probe Fura-2 AM dissolved in Hanks solution (final concentration 4 μM) containing (in mM): 156 NaCl, 3 KCl, 1 MgSO4, 1.25 KH2PO4, 2 CaCl2, 10 glucose, and 10 HEPES, pH 7.4. Coverslips with cells were incubated with 200 μL of a freshly prepared dye solution for 40 min at 37 °C and then washed twice with Hanks solution.

To register changes in the ([Ca2+]i), we used the Cell Observer image analysis system (Carl Zeiss, Germany) based on an Axiovert 200M inverted microscope with an AxioCam HSm monochrome CCD camera and a Ludl MAC5000 high-speed excitation filter change system. This study used a Plan-Neofluar 10×/0.3 objective, and a UV light source equipped with an HBO 103W/2 mercury lamp was used for fluorescence excitation. To excite and register Fura-2 fluorescence, we used a 21HE filter set (Carl Zeiss, Germany) with excitation filters BP340/30 and BP387/15, FT409 beam splitter, and BP510/90 emission filter. Round cover glass with a cell culture was mounted in a special measuring chamber to measure fluorescence. The volume of the medium in the chamber was 0.5 ml. In neurons, the spontaneous firing was recorded, and epileptiform activity was modeled by removing Mg2+ ions from the medium as described earlier (Turovskaya et al., 2020). The measurements were carried out at 28°C with an interval of 1 frame in 3 seconds. The obtained time series of two-channel images (at exciting light wavelengths of 340 and 380 nm) were processed using the ImageJ software with the Time series analyzer plugin. The amplitude of calcium responses in single cells was calculated as the Fura-2 fluorescence signals ratio at excitation of 340 and 380 nm and the frequency of the ([Ca2+]i) (?) oscillations. The Origin 8.5 software was used for plotting and statistical processing. The results are presented as signals of single cells or as the average signal of cells in the field of view ± standard error (SE).

Results

Five series of ENU mutagen injections were performed in accordance with the protocol for screening aimed to identify new gene mutations responsible for epilepsy. In this work, we used a three-generation backcross mating scheme. In the second generation, C57BL/6 mice were included in the breading to introduce genetic polymorphism used for further mapping of detected mutations (fig. 1a) (Beier & Herron, 2004, Moran et al., 2006). G2 generation females were crossed with their fathers (G1 generation) to obtain recessive mutations in the G3 generation.

G3 generation mice were tested for audiogenic epilepsy at the age of 20–30 days. To date, the total number of animals tested is 2364. Overall, the offspring of 67 males injected with ENU mutagen has been investigated. Animal lines sensitive to the epileptogenic action of sound have been revealed and labeled: S1-3, S2-3, S3-2, S5-1, S8-3, S9-4, S32-1, S34-1, S63-1.

We developed a fixed single nucleotide polymorphism (SNP) panel of 114 SNPs for genetic mapping. We selected the SNPs that differ between the C3H/HeN and C57Bl6 lines. The SNP identification was performed using the ARMS-PCR (Fig. 1b). By using two pairs of primers, two different and specific segments were amplified for each nucleotide. The specificity of each of these two primers was ensured by a mispairing at the 3’ ends of the inner primers. Inner primers were specific to only one allele of the SNP. The first two non-allele-specific (outer) primers amplified the DNA sequence that comprises the SNP. This fragment served as a template for the PCR using second pair allele-specific (inner) primers. The two allele-specific fragments can be distinguished by

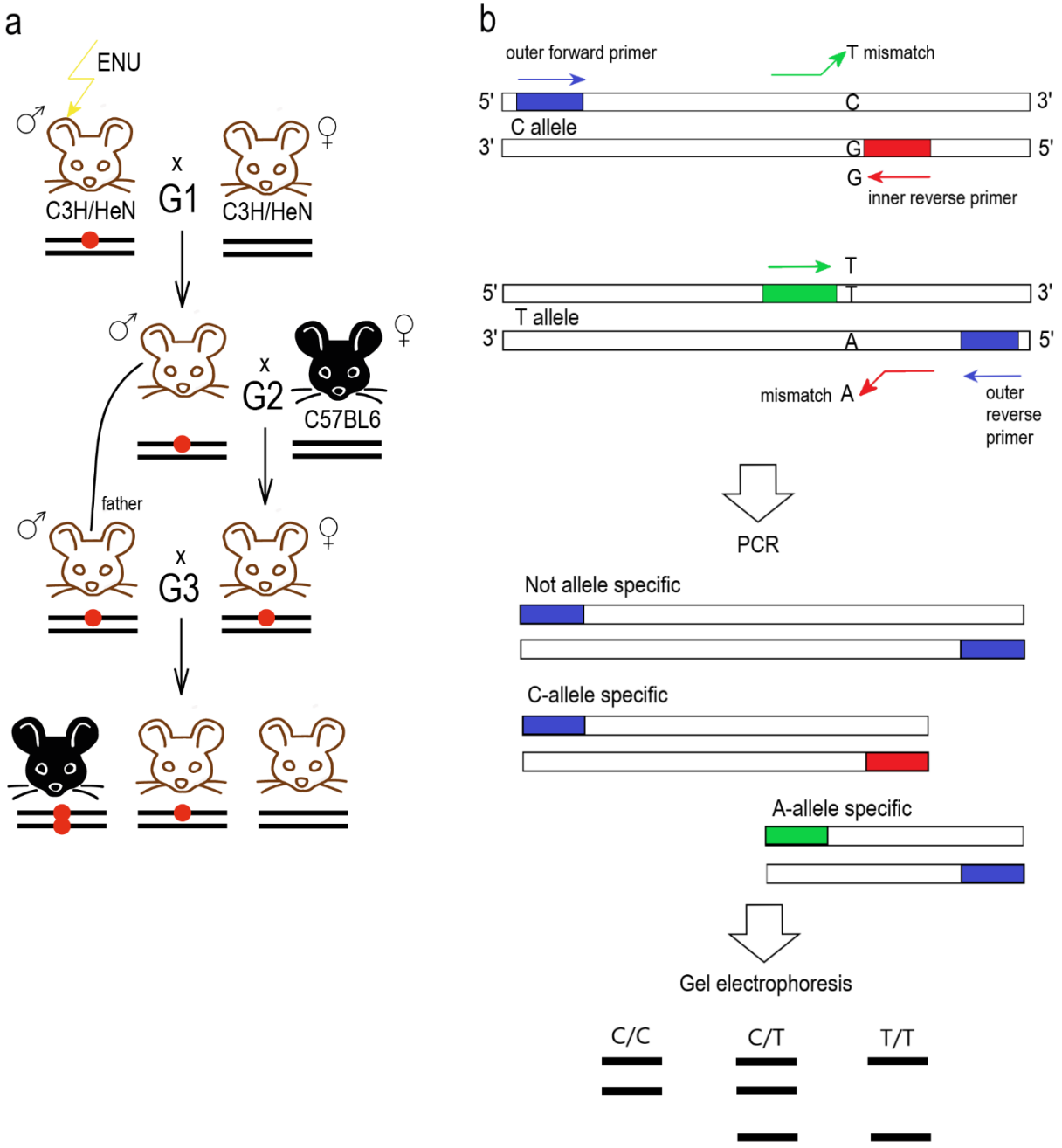

Fig. 1. Breeding strategy and overview of primer design:
a. Three-generation crossing scheme with backcross stage employed in this study; b. Schematic drawing of tetra-primer ARMS-PCR method

their different sizes in an agarose gel because the outer primers are placed at different distances from the SNP.

Using the SNP panel method outlined above (Fig. 1), we identified the exact chromosomal location of the mutation on the 8th chromosome for the S8-3 mutation (8:28057473-32291828).

To further analyze the physiological consequences associated with this mutation, we prepared primary cortical neuroglial cell cultures using the S8-3 line. Cell cultures were loaded with Ca2+ sensitive probe Fura-2 to study the activity of neuronal networks in vitro at rest (Fig. 2a, b), and Ca2+ dynamics were recorded

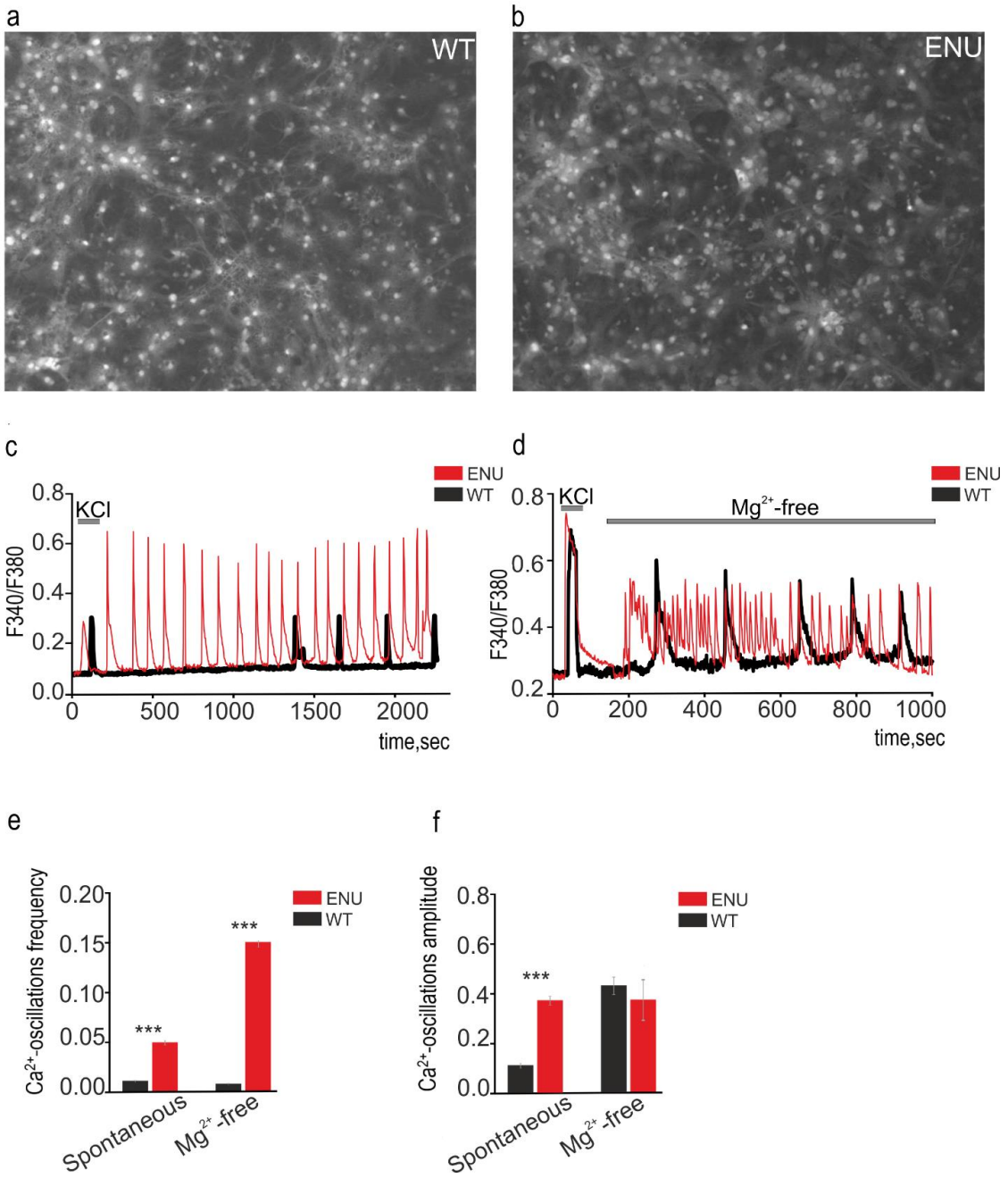

Fig. 2. Ca^{2+} signals of cortical neurons obtained from wild-type (WT) and S8-3 mouse line (ENU):
a, b – images of a neuronal cell culture obtained from wild-type (WT) (a) and S8-3 (ENU) mice (b) on day 9 of cultivation (DIV9), loaded with a Ca^{2+}-sensitive probe Fura-2.
c – spontaneous Ca^{2+} signals in neurons obtained from wild-type (WT, black curve) and S8-3 line (ENU, red curve).
d – Ca^{2+}-signals evoked by a magnesium-free environment (Mg^{2+}-free) in neurons obtained from wild-type (WT, black curve) and S8-3 line (ENU, red curve).
e, f – average frequency (e) and amplitude (f) of spontaneous and Mg^{2+}-free-induced Ca^{2+}-oscillations in neurons obtained from wild type (WT) and S8-3 (ENU) mice.
Panels c, d show Ca^{2+} signals typical for most neurons in the network. Panels e and f show the averaged data obtained from several hundred cells in three independent experiments. *** – differences are statistically significant, $p \leq 0.001$. n/a – differences are statistically not significant, $p > 0.05$. Short-term applications of 35 mM KCl were used to identify neurons

Fig. 2c, black curve) and line S8-3 (ENU, Fig. 2c, red curve). Cortical neuroglial cell cultures from WT and ENU mice have the same number of Fura-2 positive cells and network density (Fig. 2a, b). However, S8-3 neurons display an increase in primary network activity observed as the generation of spontaneous Ca2+ oscillations in 22±12% of neurons (Fig. 2c, red curve). In contrast, in control (Fig. 2c, black curve), spontaneous Ca2+ oscillations are observed in 11±8% of neurons. Analysis of this spontaneous network activity of S8-3 neurons showed significantly higher frequencies (Fig. 2e) and amplitudes (Fig. 2f) of Ca2+ oscillations compared to WT neurons.

One of the methods for inducing epileptiform activity in vitro is replacing the extracellular medium with a magnesium-free one (Mg2+-free) (Turovskaya et al., 2020). In response to the Mg2+-free medium, the appearance of Ca2+-oscillations is recorded in 38±15% and 17±6% of neurons obtained from the cortex of WT mice and the S8-3 line, respectively (Fig. 2d). In this case, we recorded high-frequency (0.15±0.003 Hz) Ca2+ oscillations in S3-8 neurons (Fig.2d, red curve, Fig.2e) compared to Ca2+ oscillations in WT neurons characterized by a significantly lower frequency, about 0.008±0.001 Hz (Fig. 1d, black curve, Fig. 2e). The oscillation amplitudes induced by a magnesium-free medium were comparable between WT and S3-8 neurons (Fig. 2d, Fig. 2f).

In conclusion, S8-3 primary cortical networks were characterized by increased spontaneous Ca2+ activity, indicative of a propensity of this line to hyperexcitation. This was supported by analysis of the epileptiform activity in vitro since a large percentage of network neurons was characterized by high-frequency Ca2+ oscillations.

Discussion

Recent progress in genome-wide screening after ENU mutagenesis in the mouse has provided scientists with new tools to study mammalian gene function (Potter et al., 2016, Stottmann & Beier, 2014, Weiss et al., 2012). ENU is efficient chemical compound and creates point mutations in coding or splice site regions, and therefore can cause a wide range of effects on the mutated gene. In order for ENU mutagenesis to become an effective tool for identifying new genes involved in the pathology formation and the creation of new animal models, it is necessary to combine mutagenesis with extensive screening and robust and relevant selection for phenotypic traits. Here, we report on the identification of 9 mouse lines with epileptiform activity through screening with AGS assay.

For the primary mapping of the mutation in this study, we created a full genome panel of primers for ARMS-PCR. The candidate locus on chromosome 8 was identified for the S8-3 mouse line.

Alteration in the excitatory/inhibitory balance of neurons is believed to be the underlying mechanism of epileptogenesis (Huberfeld et al., 2011, Reid et al., 2009). In this study, we showed propensity of neurons from S8-3 line to hyperexcitation in vitro. Thus, once again confirming the success of our phenotypic screening after ENU mutagenesis.

Conclusion

In this report, we carried out a genetic screen following genome-wide chemical mutagenesis induced by N-ethyl-N-nitrosourea to identify genes associated with epileptiform activity in mice. Audiogenic seizures were identified in 9 mouse lines. Using a SNP polymorphism analysis and genetic mapping, we identified a homozygosity locus on the 8th chromosome for the line S8-3. Furthermore, we demonstrate that the primary cortical neurons from the S8-3 line were characterized by increased spontaneous Ca2+ activity and hyperexcitability.

Acknowledgements

This work was supported by the Russian Foundation for Basic Research (project 17-00-00327).

In vitro experiments using fluorescence microscopy were carried out with the financial support of the grant from the President of the Russian Federation for M.V. Turovskaya (MK-677.2019.4).

We would like to acknowledge Mateusz Ambrozkiewicz for his comments on the manuscript.

References

ACEVEDO-AROZENA A., WELLS S., POTTER P., KELLY M., COX R.D., BROWN S.D.M. (2008): ENU Mutagenesis, a Way Forward to Understand Gene Function. Annu. Rev. Genomics Hum. Genet., 9, 49–69.

BEIER D.R. & HERRON B.J. (2004): Genetic Mapping and ENU Mutagenesis. Genetica, 122, 65–69.

BORISOVA E.V., EPIFANOVA E.A., TUTUKOVA S.A., BELOUSOVA I.I., ZHIDKOVA N.M. et al. (2018): Identification of Novel Mutations Controlling Cerebral Cortex Malformations Caused by ENU-Induced Mutagenesis in the Mouse. Sovrem. Tehnol. v Med., 10, 70.

BROWN S.D. & NOLAN P.M. (1998): Mouse mutagenesis-systematic studies of mammalian gene function. Hum. Mol. Genet., 7, 1627–1633.

CORDES S.P. (2005): N-Ethyl-N-Nitrosourea Mutagenesis: Boarding the Mouse Mutant Express. Microbiol. Mol. Biol. Rev., 69, 426–439.

FAINGOLD C. (1999): Neuronal networks in the genetically epilepsy-prone rat. Adv Neurol, 79, 311–321.

GAIDIN S.G., TUROVSKAYA M.V., GAVRISH M.S., BABAEV A.A., MALTSEVA V.N. (2020): The selective BDNF overexpression in neurons protects neuroglial networks against OGD and glutamate-induced excitotoxicity. Int J Neurosci, 130, 363–383.

HRABE DE ANGELIS M.H., FLASWINKEL H., FUCHS H., RATHKOLB B., SOEWARTO D. et al. (2000): Genome-wide, large-scale production of mutant mice by ENU mutagenesis. *Nat. Genet.,* 25, 444–447.

HUBERFELD G., DE LA PRIDE L.M., PALLUD J., COHEN L., LE VAN QUYEN M. et al. (2011): Glutamatergic pre-ictal discharges emerge at the transition to seizure in human epilepsy. *Nat Neurosci.*, 14, 627–634.

JOBE P. C. & BROWNING R. A. (2006): Mammalian models of genetic epilepsy characterized by sensory-evoked seizures and generalized seizure susceptibility. Model. seizures epilepsy, 261–271.

MORAN L. B., DUKE D.C., DEPREZ M., DEXTER D.T., PEARCE R.K.B., GRAEBER M.B. (2006): Whole genome expression profiling of the medial and lateral substantia nigra in Parkinson's disease. Neurogenetics, 7, 1–11.

NOLAN P. M., PETERS J., STRIVENS M., ROGERS D., HAGAN J. et al. (2000): A systematic, genome-wide, phenotype-driven mutagenesis programme for gene function studies in the mouse. Nat. Genet., 25, 440–443.

POTTER P.K., BOWL M.R., JEYARAJAN P., WISBY L., BLEASE A. et al. (2016): Novel gene function revealed by mouse mutagenesis screens for models of age-related disease. *Nature Comm.,* 7, 1–13.

REID C.A., BERKOVIC S.F., PETROU S. (2009): Mechanisms of human inherited epilepsies. *Prog Neurobiol.*, 87, 41–57.

STOTTMANN R. & BEIER D.R. (2014): NEU mutagenesis in the mouse. *Curr Protoc Hum Genet.,* 82, 1–10.

TUROVSKAYA M.V., EPIFANOVA E.A., TARABYKIN V.S., BABAEV A.A. & TUROVSKY E.A. (2020): Interleukin-10 restores glutamate receptor-mediated Ca2+-signaling in brain circuits under loss of Sip1 transcription factor. Int J Neurosci, 1–12.

TUROVSKAYA M.V., GAIDIN S.G., VEDUNOVA M.V., BABAEV A.A. & TUROVSKY E.A. (2020): BDNF Overexpression Enhances the Preconditioning Effect of Brief Episodes of Hypoxia, Promoting Survival of GABAergic Neurons. Neurosci Bull, 36, 733–760.

WEISS J., HURLEY L.A., HARRIS R.M., FINLAYSON C., TONG M. et al. ENU mutagenesis in mice identifies candidate genes for hypogonadism. *Mamm Genome,* 25, 346–355.

YE S., DHILLON S., KE X., COLLINS A. R. & DAY I. M. N. (2001): An efficient procedure for genotyping single nucleotide polymorphisms. Nucleic Acid Res., 29.