Molecular and Cellular Probes 33 (2017) 11–15

Contents lists available at ScienceDirect

Molecular and Cellular Probes

journal homepage: www.elsevier.com/locate/ymcpr

Short communication

Copy-number variation of housekeeping gene *rpl13a* in rat strains selected for nervous system excitability

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ARTICLE INFO

Article history:
Received 2 January 2017
Received in revised form
7 February 2017
Accepted 7 February 2017
Available online 10 February 2017

Keywords:
Copy number variation (CNV)
Quantitative real-time multicolor multiplex PCR (qmPCR)
Nervous system excitability
Hippocampus
Bone marrow
Rat

ABSTRACT

We evaluated copy number variation (CNV) for four genes in rat strains differing in nervous system excitability. *rpl13a* copy number is significantly reduced in hippocampus and bone marrow in rats with a high excitability threshold and stress. The observed phenomenon may be associated with a role for *rpl13a* in lipid metabolism.

Extensive genomic structural variation among individuals is of great importance in mammalian diversity and evolution [1–3]. One manifestation of genomic structural variation is copy number variation (CNV) [4–6]. Gene CNVs have been implicated in a diverse group of human diseases including nervous system disorders [5,7,8] as well as neurological and autoimmune disorders [9,10]. The CNV research on the rat genome emphasizes the ability to model human diseases on this system. Rat strains can be utilized as a model for the microevolution of the nervous system and for modeling diseases such as Post-Traumatic Stress Disorder (PTSD), immune disorders, and autoimmune diseases. Strains selected for specific characteristics of the nervous system, excitability in particular, are of special interest. Excitability includes the functional state of the nervous system, which determines characteristics of normal and pathological behaviors [11,12]. Rat strains that have undergone a long selection for either a low threshold (LT) or high threshold (HT) of the tibial nerve (*n. tibialis*) to electric current have correlated values for direct measurement of excitability of the caudal nerve (*n. caudalis*), midbrain, and hippocampus. The LT and HT strains demonstrate interlinear differences in both in normal behaviors and in response to stress [13,14]. However, the genetic mechanisms for this phenomenon are not clear, and it has been proposed that CNV-specific genes with known functions can play a significant role [15,16].

Central nervous system excitability is closely related to the roles of genes of the glutamate receptors, and, in particular, to *N*-methyl-D-aspartate (NMDA) receptors. These receptors are involved in mechanisms of neural diseases or conditions such as PTSD [17]. Gene *grin1* (glutamate receptor ionotropic, *N*-methyl-D-aspartate) encodes a key subunit (NR1, NMDAR1) of the NMDA receptor, which is important in the regulation of normal and pathological

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http://dx.doi.org/10.1016/j.mcp.2017.02.004
0890-8508/

brain function and plays a crucial role in synaptic plasticity and memory [18–20]. Therefore, we aimed to detect the CNV of *grin1* and of several housekeeping genes with stable expression, including *gapdh*, *rpl13a*, and *ywhaz* [21–28] within LT, HT, and Wistar rat lines under normal conditions and two varieties of stress. These stresses were: 1) emotional painful stress, as a combination of light and electrical current action; 2) emotional hypokinetic stress. The experiments were performed on three-month-old adult males of two rat strains differing in excitability of the peripheral and central nervous systems, which were selected over 60 generations for a high threshold (HT line) and low threshold (LT line) of excitability of the tibial nerve (*n. tibialis*) to electric current. The outbred Wistar population that initially served as the source material for HT and LT strain selection [29] served as the control. Breeding of HT and LT strains was conducted in the Laboratory of Higher Nervous Activity [29] and Wistar rats were maintained in the vivarium of Pavlov Institute of Physiology RAS, St. Petersburg, Russia.

Rats were grown under standard conditions, having free access to food and water. All experiments met the requirements of Directive 86/609/EEC for the use of animals in experimental research. The Commission on Humane Sciences of the Pavlov Institute of Physiology RAS (protocol 113) approved the experimental procedures. Strains differed qualitatively by stress reactivity, dynamics and intervals of saving post-stress behavioral changes, rearrangement of chromatin and heterochromatin in the neurons, as well as by changes in chromosomal aberration frequency in bone marrow and developing hippocampus, by the number of single-stranded breaks and some epigenetic modifications in adult brain, and by morphological parameters of the hippocampus [14, 30–33]. The first generation of randomly mated outbred Wistar albino rats (breeding nursery Rappolovo, Leningrad region), was the source for the HT and LT lines. Initially, individual Wistar rats with high and low thresholds of excitability to electrical stimuli (50 ms duration) were chosen as the parents for the following selection. The first and second generations were obtained by mating of full sibs. After the third generation, due to the negative effects of close inbreeding on genetic variability and increasing inbreeding depression, which reduce the effectiveness of selection, the intra-line breeding in both HT and LT strains was conducted randomly. The effectiveness of selection was evaluated by comparison with randomly mated rats of the initial outbred Wistar strain. After the 10th generation, the differences between HT and LT strains greatly increased, and then the selection reached a plateau [29]. The four-fold difference between the strains was significantly higher than the intra-individual variability.

Stresses were as follows: Stress 1, Massed painful emotional stress exposure in rats: 15 series of 13 min each (195 min in total) in a stochastic way [34]. A 13-min exposure included 6 non-reinforced (10 s) and 6 light signals backed by current (2.5 mA for 4 s). The combination of signals at each 13-min series was new and unexpected for the animal. Light signals were fed to a transparent chamber with an electrified floor with 1-min intervals between signals. (Experiment 1). Stress 2, Emotional hypokinetic stress - immobilization [34]; within seven days at a specific time in accordance with the scheme, the animals were fixed in special tubes providing immobility. Hypokinesia and free mobility alternated as follows: first day, beginning of immobilization at 9:00; second day, end of immobilization at 9:00; third day, end of immobilization at 9:00, beginning of immobilization at 13:00, end of immobilization at 17:00; fourth day, beginning of immobilization at 8:00, end of immobilization at 17:00; fifth day, beginning of immobilization at 9:00, end of immobilization at 17:00; sixth day, beginning of immobilization at 12:00; seventh day, end of immobilization at 9:00 (experiment 2). The scheme precluded the formation of reflection on time and was designed to maintain the emotional stress. The animals were sacrificed 40 min after the end of exposure. Extraction of the dentate gyrus was carried out by the method of Hagihara [35] modified by us. Extraction of bone marrow was performed by the standard conventional method.

Four genes were investigated. *Grin1* is the glutamate receptor of the ionotropic, *N*-methyl-D-aspartate (NMDA) subtype. The encoded protein is a critical subunit of *N*-methyl-D-aspartate receptors; the subunits play a key role in the plasticity of synapses, which is believed to underlie memory and learning. The second gene was *Gapdh*, glyceraldehyde-3-phosphate dehydrogenase, an enzyme also having nitrosylase activity. *Gapdh* participates in nuclear events including transcription, RNA transport, DNA replication, and apoptosis. The third was *Rpl13a*, encoding the 60S ribosomal protein L13a, which also plays a role in the repression of inflammatory genes as a component of the IFN-gamma-activated inhibitor of translation (GAIT) complex. The fourth was *Ywhaz*, the tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide. Belonging to the 14-3-3 family of proteins, which mediate signal transduction by binding to phosphoserine-containing proteins, it interacts with the IRS1 protein, suggesting a role in regulating insulin sensitivity. For DNV analysis, total DNA was prepared using an extraction buffer (1 M guanidine thiocyanate, 10 mM EDTA, 5% Tween 20, 0.5% Triton X-100, 50 mM HEPES, pH 5.3 with 200 μg of proteinase K) in 2 ml Eppendorf Safe-Lock tubes (http://primerdigital.com/dna.html). The sample was incubated overnight at 37 °C. The aqueous phase was extracted at 65 °C with hot chloroform-isoamyl alcohol, and DNA precipitated by adding an equal volume of isopropanol. The DNA pellet was dissolved in TE, pH 8.0 (with RNAse A) at 55 °C. DNA was thereafter precipitated and dissolved in TE, pH 8.0.

Quantitative real-time multicolor multiplex PCR (qmPCR) primers were designed to match exons (Table 1) using FastPCR software [36]. The tail consists of a 14-base oligonucleotide added to the 5′ end of the forward or reverse gene-specific primer, depending on which placement is less likely to contain secondary (hairpin) structures. The Tm of this oligonucleotide tail and the antiprimer complementary to it is 53 °C, as calculated by FastPCR [36]. The gene-specific portion of the primer is designed to have a higher Tm (58–65 °C). At an annealing-extension temperature of 67–72 °C, the primers can bind the target and induce polymerization without much interference from the lower-Tm antiprimer. When the temperature is subsequently decreased to 50 °C, the antiprimer binds the tail of the free, single-stranded primer, but not the double-stranded PCR product. Because the antiprimer concentration is 2- to 3-fold higher than that of the primer concentration, the majority of the free primer is expected to bind the antiprimer at 50 °C, thus strongly quenching the primer fluorescence. Because the 5′ end of the primer tail is opposite to the 3′ end of the antiprimer, the interaction is mediated via an excitation interaction [21,37], i.e., direct contact-quenching, between the 5′-fluorophore and the 3′-quencher present on the tail and antiprimer, respectively, which for most fluorophores provides stronger quenching than fluorescence resonance energy transfer. The 3′ Eclipse® Dark Quencher was used; having a wide range of absorbance wavelengths (a quenching range of 390–625 nm), it is appropriate for quenching multiple fluorophores simultaneously, including the FAM and Cy5 used for multiplex PCR. By its design, the antiprimer does not participate in primer-dimer formation because the 3′-quenching molecule is also an effective polymerase block. Primers were synthesized at Eurofins MWG Operon (Germany).

The antiprimer-based quantitative real-time multicolor multiplex PCR method (qmPCR) for the analysis of copy number variation is special, not much used, but very sensitive [38]. The qmPCR