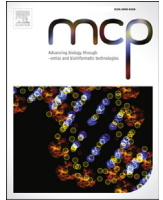




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Short communication

Copy-number variation of housekeeping gene *rpl13a* in rat strains selected for nervous system excitabilityRuslan Kalendar^{a,*}, Alexander Belyayev^b, Tatiana Zachepilo^c, Alexander Vaido^c, Dmitry Maidanyuk^d, Alan H. Schulman^{a,e}, Natalia Dyuzhikova^c^a LUKE/BI Plant Genome Dynamics, Institute of Biotechnology and Viikki Plant Science Centre, University of Helsinki, P.O. Box 65, FI-00014 Helsinki, Finland^b Laboratory of Molecular Cytogenetics and Karyology, Institute of Botany, Czech Academy of Sciences, Zámek 1, CZ-25243 Průhonice, Czech Republic^c Laboratory of Genetics of Higher Nervous Activity, Pavlov Institute of Physiology of the Russian Academy of Science, Nab. Makarova, 6, 199034 Saint Petersburg, Russia^d Laboratory of Genomic and Proteomic Research, Immanuel Kant Baltic Federal University, 236041 Kaliningrad, Russia^e Green Technology, Natural Resources Institute Finland (Luke), Latokartanonkaari 9, FI-00790 Helsinki, Finland

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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.

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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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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- γ -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 μ g 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 T_m 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 T_m (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- T_m 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

Table 1
Gene primers used in real-time PCR.

Primer ID	Sequence ^a	Primer location	Tm (°C)	Fluorescent label ^b	Gene source; primer combination; PCR band size
3881	GTGAGTGCATCTGCGTCTCAGGA	32->54	61.0		<i>rpl13a</i> (60S ribosomal protein L13a); 3881–3884, 3881–3885; 345bp
3884	CTTGCTGCGACCAAGGCGCTTTAGCCGTACAAGG	355<-376	59.4	5'-Cy5	
3885	CTTGCTGCGACCAAGGCGCTTTAGCCGTACAAGG	355<-376	59.4	5'-FAM	<i>ywhaz</i> (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide); 3877–3883; 363bp
3883	CTTGCTGCGACCAACATCGGATACCCAAGGAGACG	813->834 (D17615)	58.5	5'-Cy5	
3877	GAAGCAGTTTGAACACTGGCCCT	1163<-1185 (D17615)	60.5		<i>grin1</i> (glutamate receptor ionotropic, N-methyl-D-aspartate (NMDA)); 3858–3865; 386 bp
3858	AGGCCCCATAACACAGCTGGAC	22069<-22090	61.9		antiprimer (universal quencher)
3865	CTTGCTGCGACCAAGGCTGTGGCTTACTAGTGCCT	21705->21726	59.8	5'-FAM	
3871	TGGGTCGAGCAAG		52.8	3'-Eclipse [®] Dark Quencher	

^a Without tail; oligonucleotide concentration 200 nM.

^b 5'-FAM (6-carboxyfluorescein, emission at 520 nm); 5'-Cy5 (emission at 673 nm).

was performed in a LightCycler[®] 480 System (Roche) in 384-well plates. The 15- μ l reaction volume contained 1X Phire[®], 15 ng DNA, 200 μ M dNTP, and 0.12 U Phire[®] Hot Start II DNA polymerase (Thermo Scientific). Each reaction contained two DNA primers (200 nM) fluor-labeled at the 5'-end and two non-labeled primers (200 nM), as well as the antisense primer (500 nM), labeled with the 3' Eclipse[®] Dark Quencher. The amplification program comprised: 98 °C, 1 min; 15 cycles of 10 s at 98 °C, 10 s at 62 °C, 10 s at 72 °C; 40 cycles of 10 s at 98 °C, 20 s at 67 °C, 5 s at 72 °C, 30 s at 50 °C. Fluorescence was monitored during the last step. The qmPCR was performed to simultaneously detect two genes in a single tube. Each well was examined for the characteristic fluorescent emissions of both fluorescein (FAM channel) and Cy5 (Cy5 channel). Three independent experiments were performed for each gene to generate a mean relative copy number. Relative quantification compares the cycle threshold (Ct) of unknown samples against the standard curve of a sample with known copy numbers. All DNA samples were repeated four times per 384-well plate. The Ct values were automatically selected on a LightCycler[®] 480 System for each assay type and the data were exported into Microsoft Excel for further analysis. Amplification efficiency of the reaction is an important consideration when performing relative quantification. A standard curve with a correlation coefficient of about 0.99 and a slope of about -3.3 on a semi-logarithmic plot was sought. A ten-fold difference in the concentration of the target gene should result in Ct values with a difference of 3.3. In practice, the correlation coefficient was close to ideal (0.97–0.99) for all primer combinations that were used in qPCR. Results were processed using the nonparametric criterion of Mann-Whitney (*U* test) with the program Statgraphics Centurion (version XV). Summary data are shown in Table 2 in groups as ratio of CNV between two genes.

The CNV in 27 samples of hippocampus and 27 samples of bone marrow in a set of rat strains (intact and subjected to stress) was measured by qmPCR as described above. The copy numbers of genes *grin1*, *gapdh*, *rpl13a*, and *ywhaz* are presented in Table 2. Targeted *grin1* CNV was not found, but a number of effects associated with *rpl13a* were identified. In HT, *rpl13a* copy number was significantly reduced compared with other genes and in comparison with other rat lines. The reduction was seen in both hippocampus and bone marrow. The *rpl13a* values were up to 30-fold reduced compared to *grin1* and *ywhaz* and up to 40-fold reduced compared to *gapdh*. Variability in the other gene copy numbers was not observed in HT, closely matching a 1:1 ratio. No significant differences were observed between copy numbers of the studied genes for the LT line. However, in bone marrow of the LT rats in comparison with the control Wistar line, there was a two-fold

increase in the *rpl13a* gene copy number (versus the reference gene *gapdh*). The influence of Stress 2 (immobilization) on CNV, relative to gene *grin1*, in the bone marrow of HT line rats was manifested by a reduction in the number of copies of the gene compared with the control. In the Wistar line, a moderate, two-fold decrease in the copy number of the same gene, *rpl13a*, for Stress 1 (foot-shock) was detected (but only relative to gene *gapdh*).

Selection on the high excitability of the nervous system was accompanied by a progressive decrease in *rpl13a* copy number compared with the same parameters for the Wistar control. This was manifested both in bone marrow (highly proliferative) and in neural (non-proliferative) tissues. In the opposite selection line (LT), only in one variant and only in bone marrow was a pattern of increase observed. Confirming the association of psycho-emotional stress with CNV for *rpl13a*, a decrease in *rpl13a* copy number also occurred under Stress 2 (immobilization; Table 2) in the HT rat line (bone marrow, *grin1* as reference). Moreover, also Stress 1 was associated with reduced *rpl13a* copies in Wistar rats (*gapdh* as reference). Thus, the stress-associated CNV effect was seen only for *rpl13a* gene, in tissue-specific manner, affecting bone marrow. In line LT, changes under stress were not observed. Full original data are presented in Supplementary Table 3.

It is known that various stresses may be associated with CNV in certain genes. For example, heat shock alters the copy number of *Drosophila* gene *hsp 70* [39] and psychosocial stress both the copy number of the salivary amylase gene *Amy1* [40] and promoter polymorphism in the *Per3* gene [41]. Low copy numbers of some genes (e.g., complement component C4) are regarded as risk factors, but high copy numbers protective, for autoimmune diseases such as *videlicet* systemic lupus erythematosus in humans [42]. Here, there may be a genetic predisposition for PTSD induction under stress in the HT line compared to the LT line. We observed here, for the first time, significant CNV for one presumptive housekeeping gene, *rpl13a*. Moreover, stress leads to a significant reduction in *rpl13a* copy number, particularly in animal lines that initially showed only a minor decrease in the amount of *rpl13a*. The decreased copy-number of this gene appears to be specific for the HT strain and was associated with a high threshold on the excitability of the nervous system. In contrast, in the bone marrow of the LT control group, an increased number of copies of this gene was found compared to the Wistar line. This suggests that even the most carefully maintained inbred strains carry genetic differences that could have widespread effects on gene expression and phenotype.

The *rpl13a* gene is a ribosomal gene encoding the protein L13a, which performs an extra-ribosomal function in higher eukaryotes,

Table 2
Relative amount of copy number variation between two genes in the hippocampus (H) and bone marrow (B) between rat strains selected for nervous system excitability: HT, LT, and Wistar.

Rat strain, group (N=3)	<i>grin1: ywhaz</i>		<i>grin1: rpl13a</i>		<i>gapdh: grin1</i>		<i>gapdh: rpl13a</i>		<i>ywhaz: rpl13a</i>	
	H	B	H ^a	B ^b	H	B	H	B	H	B
1.HT control	1.8 : 1	2.1 : 1	23.0 : 1	23.7 : 1^a	1.6 : 1	1.3 : 1	24.6 : 1	34.9 : 1	17.1 : 1	24.7 : 1
2.HT Stress1	1.5 : 1	1.9 : 1	24.1 : 1	24.3 : 1	1.6 : 1	1.3 : 1	37.5 : 1	41.2 : 1	16.0 : 1	16.2 : 1
3.HT Stress2	1.6 : 1	2.1 : 1	18.9 : 1	28.7 : 1	1.9 : 1	1.4 : 1	27.6 : 1	41.0 : 1	13.8 : 1	26.2 : 1
4.LT control	1.4 : 1	2.5 : 1	2.8 : 1	1.7 : 1	1.7 : 1	1.1 : 1	2.9 : 1	1.4 : 1	1.8 : 1	2.1 : 1
5.LT Stress1	1.3 : 1	2.6 : 1	2.3 : 1	1.8 : 1	1.6 : 1	1.2 : 1	2.8 : 1	1.9 : 1	1.9 : 1	2.3 : 1
6.LT Stress2	1.1 : 1	2.9 : 1	2.3 : 1	1.7 : 1	1.7 : 1	1.1 : 1	3.2 : 1	1.5 : 1	2.3 : 1	2.1 : 1
7.Wistar control	1.3 : 1	1.9 : 1	2.4 : 1	2.0 : 1	1.9 : 1	1.2 : 1	3.7 : 1	2.4 : 1	2.3 : 1	2.2 : 1
8.Wistar Stress1	1.3 : 1	1.7 : 1	4.0 : 1	3.1 : 1	1.8 : 1	1.4 : 1	5.8 : 1	4.0 : 1	2.3 : 1	3.9 : 1
9.WistarStress2	1.5 : 1	1.8 : 1	2.4 : 1	2.0 : 1	1.7 : 1	1.2 : 1	3.5 : 1	2.7 : 1	2.3 : 1	2.7 : 1

^aBold font – differences are statistically significant from LT and Wistar groups, Mann-Whitney (U- test), p <0.05, M - CNV median values of test gene relative to reference gene.

^bVertical lines - differences in the values are statistically significant between themselves.

including a role in mRNA-specific translational silencing [43]. The gene also has a role in lipid metabolism and regulating oxidative stress. An excessive accumulation of fatty acids activates *rpl13a*, stimulates the snRNA pathway, and triggers apoptosis [44]. The comparative analysis of HT and LT rat lines show that they differ considerably in lipid metabolism parameters. Interesting to note, the synaptic membranes of the rat cerebral cortex of the HT line had a significantly higher amount of sphingomyelin and phosphatidylcholine compared with that of LT animals [45]. Taking these strands together, it is possible to propose that selection on the high threshold of excitability of the nervous system may be manifested as selection for CNV in *rpl13a*, associated with metabolic differences in the HT strain and possibly specific aspects of the stress response. Confirmation will require additional analyses. The observed copy number variation and its link to phenotype may be associated with microevolutionary processes, and reflects the effect of selection on the excitability of the nervous system and stress on the CNV of ribosomal gene *rpl13a*. However, the dynamics of cause and effect during the selection, even given a clear etiology, are non-trivial. Our results can be exploited to model CNVs apparent in the rat and in the human genome [46].

Conflict of interest

The authors declare no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.mcp.2017.02.004>

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