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Differential gene expression between inbred Roman high- (RHA-I) and low- (RLA-I) avoidance rats

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ABSTRACT

Microarray technology was used to explore differences in brain gene expression under basal conditions in two strains of psychogenetically selected rats which differ in anxiety/stress responses, the inbred Roman High-(RHA-I) and Roman Low-(RLA-I) Avoidance rats. Microarray analysis detected 14 up-regulated and 24 down-regulated genes in RLA-I vs. RHA-I rats functionally related to neurobiological processes. The differentially expressed genes CAMKK2, CRHBP, EPHX2, HOMER3, NDN, PRL and RPL6 were selected for microarray validation using qRT-PCR. EPHX2, CAMKK2 (both up-regulated in RLA-I vs. RHA-I rats) and HOMER3 (down-regulated in RLA-I vs. RHA-I rats) showed a similar tendency and fold-change both in microarray and RT-PCR analyses; PRL (up-regulated in RLA-I vs. RHA-I rats), CRHBP and RPL6 (both down-regulated in RLA-I vs. RHA-I animals) showed a similar tendency but a different order of magnitude of change among experiments; finally, NDN was validated neither in tendency nor in magnitude of change.

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Microarray technology provides an approach that has made possible to analyze genome-wide gene expression in one single experiment [32]. In the present study, microarray technology was used to explore differences in brain gene expression under basal conditions in two strains of rats selected on the basis of their differences in the acquisition of two-way active avoidance: the inbred Roman High-(RHA-I) and Roman Low-(RLA-I) Avoidance rats (derived from the Swiss sublines RHA/Verh and RLA/Verh). This selection procedure led to stable between-strain divergences related to anxiety/fearfulness and coping style [11]. These differences seem to be related to neuroanatomical [6,20,49], neurochemical and molecular divergences [17,22,33] found in brain regions known to modulate anxiety, impulsiveness, and proneness to substance abuse. The present study dealt with the screening of candidate genes whose differential brain expression could underlie the RHA/RLA differences in fear and anxiety [10].

Female 4-month-old RHA-I ($n = 4$) and RLA-I ($n = 4$) rats (weight 250–280 g) were used. Animals were housed in pairs with food and water continuously available. Room temperature was kept at about 22 °C. Animals were maintained under a 12-h light/12-h dark cycle with lights on at 8:00 a.m. The experiment was conducted following European Union (EU) guidelines on the use of animals for research (86/609/EEC).

Rats were decapitated and the brains immersed in liquid nitrogen and stored at −80 °C. For RNA isolation, brain tissue was homogenized in a 10-fold volume (w/v) of ice-cold Trizol reagent (Life Technologies, Gaithersburg, MD). After extraction, total RNA was precipitated by adding isopropanol and centrifuged at 15,000 × *g* for 12 min. RNA quality was assessed in an A2100 Bioanalyser (Agilent Technologies, Waldbronn, Germany).

Samples were hybridized onto the Whole Rat Genome Codelink Bioarray (Amersham Biosciences) encompassing more than 55,000 rats DNA probes. The protocols for sample preparation and

Abbreviations: RT-PCR, real-time reverse transcription-polymerase chain reaction; QTL, quantitative trait loci; RNA, ribonucleic acid; cDNA, complementary deoxyribonucleic acid; RHA-I, inbred roman high avoidance rats; RLA-I, inbred roman low avoidance rats; RHA/Verh, outbred swiss roman high avoidance rats; RLA/Verh, outbred swiss roman low avoidance rats; F2, second filial generation; W/V, weight/volume; DEPC, diethylpyrocarbonate; VS, versus; ML, milliliters; NG, nanogram; MMOL, millimole; MMLV, moloney-murine leukaemia virus; FDR, false discory rate; CaMKs, CaM kinases; HR, High responders; LR, Low responders; HPA, hypothalamic-pituitary-adrenal axis.

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hybridization were adapted from the CodeLink Technical Manual. Total RNAs were prepared from a biological sample and a set of bacterial mRNAs of known concentrations (which are provided by the manufacturers and have complementary sequences to the positive control probes on the Bioarrays). One microgram of total RNA was reverse transcribed into cDNAs and amplified into cRNAs, using in vitro transcription. The cRNAs are labelled with avidin, and the fluorescent dye (Cy-5) was bound with streptavidin to the labelled cRNA. Then the labelled cRNAs were hybridized to a CodeLink Bioarray presynthesized. The hybridized microarrays were scanned in a Axon 4100A scanner (Molecular Devices, Sunnyvale, CA, USA) and data extracted with GenePix Pro 6.0 software (Molecular Devices, Sunnyvale, CA, USA).

Total RNA (1 μg) was reverse-transcribed using an Advantage RT-for-PCR kit (Clontech, Palo Alto, CA, USA). Each sample was diluted to a final volume of 12.5 ml in diethylpyrocarbonate (DEPC)-treated water, and 1 ml oligo (18-dT) was added, heated at 70 °C for 2 min, and kept on ice until 6.5 ml mastermix–4 ml 5′ reaction buffer, 1 ml dNTP mix of 10 mmol each, 0.5 ml recombinant RNase Inhibitor, 1 ul MMLV (Moloney-Murine Leukaemia Virus) reverse transcriptase was added. The reaction was incubated at 42 °C for 1 h, and heated at 94 °C for 5 min to stop cDNA synthesis and destroy DNase activity. The products were diluted to a final volume of 100 ml in DEPC-treated water. Real-time PCR was performed using a LightCycler FastStart DNA Master SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer's instructions on a Roche LightCycler platform (Roche, Göttingen, Germany). We employed 200 ng of cDNA for each analyzed sample. A calibration curve was included in each experiment (five serial dilutions). The final products were analyzed by using the provided software (Roche Molecular Biochemicals LightCycler Software v3.5). Data are presented as the ratio between the expression of the gene vs. that of the human GAPDH gene. Melting curves were analyzed to confirm amplification specificity. The genes CAMKK2, CRHBP, EPHX2, HOMER3, NDN, PRL and RPL6 were used for microarray validation. Forward and reverse primer pair sequences for each gene were (5′–3′): CAMKK2, Fw–GCCCTTTCATGGATGAACGAA, Rv - TCCTCTTCGGTCACCTCGA; EPHX2, Fw–GGCCCTCTAAACTGGTATCGA, Rv - AGTCTTCAGCCACTTGATGAGA; HOMER3, Fw–CGCTACGAAGAGGAACTGGA, Rv - TGTTCCGAGCCAAAGCCTA; NDN, Fw–GTACAAGAGATGTGCTGTGCTA, Rv - CAGTCCAGTTCAAATCAGTCCA; PRL, Fw-CCGGAAGTTCTTTTGAACCTGA, Rv - GCAGGGATGGGAGTTGTGA; RPL6, Fw - GAGTTCCTGTGCGTAGGACA. Rv - GCAAAATCTGCGAGTCCACA; CHRBP, Fw - CGGGTCCATGAACCAGGAA Rv - TCCACAAAGTCTCCAGTTCCA.

Statistical analysis was carried out using the R software [35] and the appropriate Bioconductor packages [3] run under R. Densitometry values between arrays were normalized using the quantiles normalization function implemented in the Bioconductor limma package. Statistically significant differences were identified using the multitest function implemented in the Bioconductor package (FDR < 0.05). Those genes showing both an absolute fold change (RLA/RHA) value >1.5, and FDR < 0.05 were considered as significant. Gene symbols, fold-changes and functional annotations were uploaded from Ingenuity Pathways Analysis platform [24].

Using microarray technology and after data normalization and statistical analyses, we detected 14 up-regulated and 24 down-regulated genes in RLA-I vs. RHA-I rats, some of them being related to neurobiological diseases, nervous system development/function and behavior (Table 1). Seven of these genes were selected as candidates for RT-PCR validation. Four of them were those with highest (PRL, EPHX2) and lowest (RPL6, NDN) fold-change, and the other three (CAMKK2, HOMER3, CRHBP) were selected according to their behavior/brain-related functions. The obtained results (Table 2) showed that EPHX2, CAMKK2 and HOMER3 show a similar tendency and fold-change both in microarray and RT-PCR analyses; PRL, CRHBP and RPL6 showed a similar tendency but a different order of magnitude of change among experiments; finally, NDN was neither validated in tendency nor in magnitude of change (see Table 3).

In order to ascertain the possible implication of the differentially expressed genes in common functions and diseases, a gene enrichment functional analysis was further carried out. 18 genes were related to the category "Neurological Diseases", 8 to "Nervous System Development and Function" and 5 to "Behavior", which point to a manifested de-regulation of cerebral related molecules (Table 2).

The five genes validated trough RT-PCR seem to be linked to behavioral/neuroendocrine traits shown to be divergent between Roman rats (i.e. fearfulness, novelty seeking, impulsivity and vulnerability to drug abuse; see also Table 3): EPHX2, PRL, CAMKK2 (up-regulated in RLA-I vs. RHA-I rats), CRHBP and HOMER3 (down-regulated in RLA-I vs. RHA-I animals). EPHX2 gene encodes a member of the epoxide hydrolase protein family [40]. A knockout EPHX2 mouse [29] has shown decreased plasma testosterone and cholesterol levels, reduced novelty-induced exploratory behavior and a decreased hedonic response. This suggests a putative role of EPHX2 gene in the regulation of responses (fearfulness and hedonism) that are divergent in Roman rats ([11], see Table 3).

An important body of evidence indicates that anxiogenic/stressful stimuli activate prolactin-releasing peptide neurons that regulate prolactin levels in several brain areas (see [34], for review), and that prolactin involvement in anxiety and stress seems to vary with the particular type of stress [36,42,48], the amount of control an organism exerts over the stressor [42,44], and the animal's behavioral phenotype [27]. It is therefore all the more remarkable that the Roman rats exhibit large between-line/strain differences in stress-induced prolactin responses, which are particularly exacerbated in the RLA line/strain [41,42], as well as in other strains of rats selectively bred for high anxiety-related behavior [27]. The finding that prolactin gene expression is markedly upregulated in RLA-I, compared to RHA-I rats, seems to be in line with these results and points to prolactin function as one of the most between-line/strain discriminating neuroendocrine traits linked to their psychogenetic profiles ([7], see Table 3).

CaM kinases constitute a family of protein kinases whose activities are modulated by binding Ca^{2+}/calmodulin. CaMKII, in particular, is known to modulate neuronal development, synaptic plasticity (*i.e.* long term potentiation and depression; see [47] for review) and aversive learning/memory [1,12,21,38]. Within this context, Mei et al. [31] found that CaMKII gene expression was downregulated in the hippocampus 6 h after fear conditioning. Kabbaj et al. [25] conducted a study with Sprague-Dawley rats and selected those animals showing high (HR) vs. low rates (LR) of novelty-induced exploratory locomotion, reporting that social defeat increased CAMKII mRNA expression in hippocampus of LR rats only. These results suggest the involvement of CAMKII in both aversive learning/memory and stressful experiences. Interestingly, it is well known that RHA-I and RLA-I exhibit a clearly different pattern of responses when exposed to unconditioned and conditioned anxiety/fear-related stimuli/tasks (*e.g.* [11,28], see Table 3).

The CRHBP gene encodes the corticotropin-releasing factor-binding protein. This protein seems to be related to anxiety, stress and depression [8,45]. In mice, CRHBP expression levels are high in the pituitary, cortex, hippocampus, amygdala and bed nucleus of the stria terminalis, among other areas [39]. CRHBP appears to modulate the neuroendocrine activity of CRH (corticotropin releasing hormone), which has been recognized as one of the major hypophysiotropic (HPA-axis) hormones involved in the regulation of the mammalian behavioral and endocrine/immunological responses to stress [45]. Accordingly, it has been reported that: