Protein biomarkers in a mouse model of extremes in trait anxiety

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Abbreviations used: CM-M, cross-mated offspring; EP, enolase phosphatase; EPM, elevated plus-maze; Glx1, glyoxalase-I; HAB-M, high anxiety-related behavior mouse; LAB-M, low anxiety-related behavior mouse; NAB-M, normal anxiety behavioral-related phenotype mouse; PVN, paraventricular nucleus
SUMMARY

Brain proteome analysis of mice selectively bred for either high or low anxiety-related behavior revealed quantitative and qualitative protein expression differences. The enzyme glyoxalase-I is consistently expressed to a higher extent in low anxiety as compared to high anxiety mice in several brain areas. The same phenotype-dependent difference was also found in red blood cells with normal and cross-mated animals showing intermediate expression profiles of glyoxalase-I. Another protein that shows a different mobility during twodimensional gel electrophoresis was identified as enolase phosphatase. The presence of both protein markers in red or white blood cells, respectively, creates the opportunity to screen for their expression in clinical blood specimens from patients suffering from anxiety.

Keywords: trait anxiety; biomarker; animal model; affective disorders; diagnostics
INTRODUCTION

Biomarkers are measures of biological parameters of disease that also can predict which new molecular entities will be effective and safe in treating patients (1). We have focused our biomarker discovery efforts on the analysis of mouse models (2) in order to avoid the inter-individual differences of human specimens that often result in a low biomarker signal to background noise ratio.

The manifestation of anxiety in a number of psychiatric disorders such as generalized anxiety disorder, depression, panic attacks, phobias, obsessive-compulsive disorders and post-traumatic stress disorder (3) highlights the importance of gaining a better understanding of associated reliable biomarkers in proper animal models. An animal model to study behavioral, neuroendocrine and genetic concomitants of trait anxiety including psychopathology should represent a good approximation to score symptoms of anxiety disorders and possibly comorbid depression (4, 5, 6). In order to avoid inter-strain comparisons, likely to reveal differences in more than just anxiety-related indices, we have been using intra-strain breeding approaches to focus on particular traits, including anxiety-related behavior (7), depression-like behavior (8), avoidance behavior or receptor functions likely to be associated with differences in anxiety (9, 10, 11, 12). The technique of selective bidirectional breeding enhances the representation of genetic material associated with a particular trait shifting the animals’ phenotype bidirectionally from the strain mean (13).

Genetic approaches currently available in the mouse make this model organism particularly powerful for the functional analysis of candidate genes and in defining gene products underlying trait anxiety and possibly depression (14). For this reason we have generated and validated hyper-anxious (HAB-M) and hypo-anxious (LAB-M) CD1 mouse lines as model of extremes in trait anxiety and have used comparative proteomics to identify anxiety-related protein markers (2). One of the identified markers, glyoxalase-I, shows expression level
differences between HAB-M and LAB-M animals and recently has attracted increasing
attention for its role in psychopathogenic mechanisms (15). The other protein that exhibits a
qualitative difference of unknown nature between the two mouse strains is enolase
phosphatase.

EXPERIMENTAL PROCEDURES

Mice. The animal studies were conducted in accordance with the “Guide for the Care and Use
of Laboratory Animals of the Government of Bavaria”. Mice were housed in groups of three
to five in plastic cages (25 x 20 x 14 cm) with a 12-h light/dark schedule (lights on at 6 a.m.)
at 23°C, 60% humidity with food and tap water available ad libitum. Experiments were
carried out between 8 a.m. and 12 p.m. As previously described (2) we used over 250 animals
from more than 25 litters of the outbred Swiss CD1 strain (Charles River, Sulzfeld, Germany)
as a starting point for selective and bidirectional breeding for anxiety-related behavior on the
EPM at the age of seven weeks. Males and females that spent either the least or most time on
the open arms of the EPM were mated to establish the HAB-M and LAB-M mouse lines,
respectively. The first generation (G1) was weaned and separated by sex at three weeks of age
and tested on the EPM four weeks later. Again, extremes were mated and offspring was
reared in the same manner for all following generations. Outbreeding across families but
within closed lines was practiced in order to minimize random fixation of alleles other than
those selected for, and also to maximize the number of genes influencing anxiety-related
behavior within each line. After nine generations of outbreeding, an inbreeding protocol was
started based on strict sib-mating. We generated 4-6 independent families within the HAB-M
and LAB-M lines, respectively. In the experiments, animals from all inbred HAB-M and
LAB-M families from seven generations (G12-G18) were included to avoid both fortuitous
correlations and fluctuations across time due to genetic drift (8).
CM-M offspring comprised reciprocal crosses between HAB-M and LAB-M mice. They were housed under the same conditions as HAB-M, LAB-M and “normal” CD1 (NAB-M) animals, the latter being selected as controls independent of their performance on the EPM. All behavioral tests were conducted during weeks 7 to 13. Mice were initially tested on the EPM and, at least two days later, in one or more additional tests. The minimum recovery time between the tests was one day.

**Elevated Plus-Maze Test.** The EPM was made of black plastic and consisted of two open arms (30 x 5 cm; 300 Lux), and two enclosed arms (30 x 5 x 15 cm; 10 Lux). The arms extended from a central platform (5 x 5 cm; 90 Lux). The EPM was located 120 cm above the floor. Male and female mice were carried into the EPM room 24 h prior to testing and stayed in their home cages until the experiment was carried out. At the beginning of the experiment, each mouse was placed on the central platform facing a closed arm. During the 5-min test, the % time spent on the open arms, the number of entries into the closed and open arms, and the latency to the first open arm entry were scored using the computer software program “plus-maze” (E. Fricke, Munich, Germany). Mice were considered to have entered an open or closed arm when both front paws and front shoulders were on the arm, but also full entries (all four paws) were counted. After each test, the EPM was cleaned with water containing detergent and dried with tissue.

**Forced Swim Test.** Each mouse was placed in a glass cylinder (height: 23.5 cm, diameter: 11 cm) containing 15 cm of water at 22-23°C for 6 min. At the end of each videotaped trial, immobility time (floating) was scored using the computer program Eventlog 1.0 (EMCO Software Ltd., Reykjavik, Iceland). A mouse was judged to be immobile when it stopped any movements except those which were necessary to keep its head above water.

**Protein Sample Preparation.** Three to five days after behavioral testing, HAB-M, NAB-M, CM-M, and LAB-M mice were sacrificed under isoflurane anaesthesia, their trunk blood
collected (see Western blot) and their brains removed. The hypothalamus (containing the PVN and suprachiasmatic nuclei), amygdala (containing the basolateral, central, and medial nuclei), and motor cortex were dissected according to the mouse brain atlas (16). The areas were weighed, immediately frozen in liquid nitrogen and stored at –80°C. Brain specimens were prepared for 2D-PAGE with the PlusOne Sample Preparation Kit (GE Amersham Biosciences, Piscataway, NJ) using IEF rehydration buffer (7 M urea, 2 M thiourea, 0.2% biolytes 3-10, 2% CHAPS, 100 mM DTT supplemented with the protease inhibitor cocktail “Complete”, PMSF and pepstatin). Protein content of the samples was measured using the Bradford Protein Assay (BioRad Laboratories, Hercules, CA). The number of animals analyzed is given in the respective legends to the figures.

**Twodimensional-Polyacrylamide Gel Electrophoresis.** The brain area extracts (12 replicates for each HAB, LAB and NAB group) with 300 µg of protein in IEF sample buffer were applied to a 17 cm, pH 4-7 immobiline strip (BioRad). After active rehydration at 50 V for 12 h paper wicks were used and IEF was carried out at 20°C in a PROTEAN IEF cell (BioRad) using the following conditions: 250 V for 15 min, 10,000 V for 3 h and 10,000 V until 60,000 Vh were reached. For reduction and alkylation of the proteins, the strips were equilibrated in 2% DTT for 30 min and then in 2.5% iodoacetamide for 30 min. For the second dimension SDS polyacrylamide gel electrophoresis, strips were layered on top of a 5% stacking gel and 12% separating gel and were run in batches of 12 gels each at 50-200 V overnight.

Gels were stained with colloidal Coomassie Blue and scanned. The scanned images were analyzed and compared to each other with the help of PDQUEST software (BioRad). Spots were detected using the automated spot detection and matching function. After normalization according to total density spot intensities were quantitated and compared between the replicate groups (HAB, LAB, NAB). Group differences were evaluated
statistically applying the student t-test with a significance level of 98 % and the criteria of a minimum of twofold difference (increase or decrease) in spot intensity. The automated analysis was completed by manual matching and quantitation of spots of interest.

**Mass Spectrometry.** Gel spots that represented differences in protein expression levels based on the results from the image analysis were subjected to an in-gel tryptic digest. For this purpose, 50 µl 20 mM ammonium bicarbonate/acetonitrile (1:1) was used for gel cleanup and drying. After the gel pieces were dry, 30 µl 20 mM ammonium bicarbonate containing 0.5 µg trypsin (Promega, Madison, WI) was added and incubated at 37°C overnight. Peptides were extracted twice with 25 µl 5% formic acid by incubation at 37°C for 30 min. The extracts were concentrated in a speedvac and re-dissolved in 12 µl 0.1% formic acid. For mass spectrometry analysis, the tryptic digests were injected onto a C18 cartridge using a FAMOS Autosampler (Dionex, Sunnyvale, CA). After washing the cartridge with 0.1% formic acid (solvent A) for 15 min, the peptides were eluted onto a PicoFrit column (New Objectivce, Woburn, MA) by applying a linear gradient of acetonitrile:water:formic acid (90:10:0.1) over 60 min. The PicoFrit eluate was directly infused into an LCQ DECA XP PLUS iontrap mass spectrometer (Thermo Electron, San Jose, CA). Each full scan was followed by a zoom scan and an MS/MS scan of the most intense signal. The resulting MS/MS data were used to search a non-redundant protein database, release date July 25, 2003, (NCBI, Bethesda, MD) using TURBOSEQUEST (J. Yates, III and J. Eng, University of Washington, Seattle, WA). Mass spectrometry identifications were considered positive when a minimum of three peptides came up with the same protein hit.

**Western Blot.** Red and white blood cells were isolated from 0.8 ml of mouse (HAB-M, LAB-M, NAB-M, CM-M, C57BL/6J and BALB/C) or patient blood that had been collected in EDTA tubes supplemented with trasylol. The cell pellets were frozen at -80°C until used. For red cell lysis, the pellets were thawed on ice, cold water containing 1 mM PMSF was added,
and the suspension was mixed. Proteins from white blood cells were extracted with either IEF or SDS sample buffers. After the removal of cellular debris, the supernatant was stored in aliquots at -80°C. For Western blot, 100 µg of total protein from each red or white cell lysate were run on either a 15% SDS mini gel (BioRad) or a 2D gel (BioRad) and the proteins transferred to an Immobilon PVDF membrane (Millipore, Bedford, MA) at 100V for 1 h with cooling. The membrane was treated with 5% Carnation instant non fat dry milk in TBST overnight and rinsed in TBST. The membrane was then incubated with either Glx1 antiserum (kindly provided by Dr. Kenneth Tew, Fox Chase Cancer Center, Philadelphia, PA) or EP antibody (MorphoSys, Martinsried, Germany) at a 1:2000 dilution in TBST for 2h at RT and washed with water and TBST for 15 min. Incubations with either Protein A horseradish peroxidase (for Glx1) or an anti-c-myc-peroxidase antibody (for EP) (GE Amersham Biosciences, Roche Diagnostics) were carried out for 40 min at RT after which the membrane was washed with water and TBST. Finally, the membrane was incubated with ECL mixture (GE Amersham Biosciences) for 1 min and exposed to ECL film (GE Amersham Biosciences). The membrane was scanned and the band signal strength (optical density) assessed with the help of the QuantityOne software (BioRad).

RESULTS

In our efforts to identify biomarkers for trait anxiety, a mouse model was established by using a bidirectional inbreeding protocol. Anxiety-like behavior on the elevated plus maze was the key selection criterion (Fig. 1A). The increasing divergence between HAB-M and LAB-M animals is depicted in Fig. 1B, with the former showing significantly heightened anxiety-related behavior compared to their LAB-M counterparts (G4-G21: p<0.001). The time outbred CD1 mice spent on the open arms of the EPM ranged from 0 to 54.6% for males and
from 0 to 65.1% for females. Independent of gender, both selected lines differed significantly from unselected NAB-M controls, as shown in Fig. 1B.

Male and female LAB-M mice also displayed significantly less immobility time than HAB-M, NAB-M and CM-M mice in a depression-related behavioral assay, the forced swim test (males: $F_{3,138}=41.3$, females: $F_{3,115}=61.9$, $p<0.001$; Fig. 2). Individual pairs of data (% time EPM vs. time spent immobile in the forced swim test) revealed a significant correlation (2).

Both behavioral assays, the EPM and the forced swim test, showed a very consistent difference in the phenotype of the two mouse strains, which was a prerequisite for subsequent proteomic analyses.

For biomarker identification, protein extracts from individual brain areas from HAB-M and LAB-M mice were subjected to 2D-PAGE (Fig. 3). The first dimension IEF was run with a pH gradient of 4-7 and the second dimension in a 12% SDS gel. After staining the gels with colloidal Coomassie Blue, the scanned images were compared by computer analysis. Each gel contained approximately 800 detectable protein spots. One of the protein spots differed significantly in its intensity between all the HAB-M and LAB-M animals in all three brain areas analyzed (hypothalamus, amygdala, motor cortex). In order to obtain a good estimate of the relative expression levels, we used Western blot analysis. Based on the signal intensity of the Glx1 protein band, the level of this protein’s expression was approximately tenfold higher in LAB-M compared to HAB-M animals (Fig. 4A,B). Another protein showed a different migration pattern in the gels indicating a qualitative variance that is either due to an altered post-translational modification or an allelic difference. In HAB-M animal tissue extracts, the EP protein exhibits a slower mobility in the second dimension than for LAB-M and NAB-M mice. No significant difference in the spot intensities was observed for EP between the three mouse lines (LAB-M: 0.123, HAB-M: 0.180, NAB-M: 0.133; Fig. 3). The exact nature of the observed difference in migration will be determined in future experiments. Both marker
proteins were identified by cutting out the stained gel spots from HAB-M, LAB-M and NAB-M tissue extract gels, and subjecting the proteins to in-gel digestion with trypsin followed by tandem mass spectrometry. Mass spectrometry data were used to search the non-redundant protein database and resulted in the identification of Glx1 and EP, respectively. The following peptide sequences were identified for the two proteins:

Glx1: SLDFYTR; VLGLTLLQK; RFEELGVK; DFLLQQTMLR

EP: QLQGHMWK; AAFTAGR; VDSESYR; PGNAGLTDDDEK

This represents a 18.48% and 12.84% amino acid coverage for Glx1 and EP, respectively.

Since 2D-PAGE is a rather laborious method for studying protein expression in tissues, we next wished to set up a high throughput method for the quantitative analysis of Glx1 and EP. Since Glx1 is expressed ubiquitously in many cells and tissues (17), we were able to use red blood cells as a source from now on. The protein sequence of Glx1 is highly conserved among higher vertebrates, which allowed us to use a polyclonal antiserum that was generated against human Glx1 for the detection of the mouse enzyme by Western blot analysis (Fig. 4A). In the case of EP we obtained an antibody from a commercial source. Since EP is not expressed in red blood cells, we used HAB-M and LAB-M mouse brain tissue to validate the expression difference that we had observed in 2D-PAGE (Fig. 4C).

In the case of Glx1 the Western blot enabled us to screen a great number of mouse red blood cell specimens. To extend our studies, we now also included samples from “normal” NAB-M and cross-mated CM-M controls, which displayed intermediate scores in the EPM test (Fig. 4A). Independent of their gender, both groups, NAB-M and CM-M animals, also showed intermediate levels of Glx1 expression as compared to LAB-M and HAB-M mice, the former again expressing significantly more Glx1 thus confirming the brain tissue data (Fig. 4A,B).

Enolase phosphatase Western blot analysis of HAB-M and LAB-M mouse brain tissue revealed a complex expression pattern when 2D-PAGE was used for protein separation (Fig.
4C). Despite its complexity an additional spot that migrates slower in the second dimension SDS gel was detected in HAB-M brain tissue extracts and was absent in LAB-M extracts. This isoform pattern is in agreement with the one observed in the original 2D-PAGE analysis of brain tissue from the two mouse strains (Fig. 3). The nature of the different isoform in HAB-M animals is currently under investigation.

In an extension of our studies we have begun to screen human specimens with the long-term goal to correlate the expression of biomarkers that were identified in mouse models with disease phenotypes in humans. Working to our advantage both, Glx1 and EP, are expressed in blood cells which are isolated on a routine basis in clinical laboratories. Whereas Glx1 is found in red blood cells, EP expression can be analyzed in white blood cells as a source. Preliminary screens with a limited number of patient specimens show that both proteins can be detected by Western blot analysis from these cells (Fig. 5). In the case of EP the two immunoreactive bands comigrate with the two spots that were originally detected during 2D-PAGE of the HAB-M and LAB-M amygdala extracts. Due to the small amount of patient blood specimens, we were unable to produce a Coomassie-stained 2D gel of the white blood cell extracts.

DISCUSSION

Selective and bidirectional breeding of CD1 mice for either high or low anxiety-related behavior resulted in two inbred lines that, independent of their gender, differ extremely in a variety of behavioral paradigms (2). The EPM, based on spontaneous, unconditioned behavior, was selected as an initial key evaluation tool. LAB-M mice were not only less anxious compared to HAB-M mice in the EPM test but also showed lower immobility scores in the forced swim test, indicative of a reduced depression-like behavior. Using 2D-PAGE of several brain tissue sections (hypothalamus, amygdala, cortex) and subsequently Western blot
analysis, Glx1 and EP were identified as protein markers, which are consistently expressed at a different level or with an altered pattern, respectively. Other spot differences were not observed in a consistent manner. In our experience the method of 2D-PAGE followed by Coomassie Blue staining is only capable of detecting significant differences of abundant proteins. For a more sensitive analysis of the trait anxiety mouse model proteins, we are planning to use a stable isotope-based proteomics platform in the future.

The significant correlation between anxiety- and depression-related behaviors in HAB-M and LAB-M mice resembles the clinical situation of a high comorbidity between anxiety disorders and depression (18). The correlation in mice, however, is primarily due to the extremely low depression-like behavior of LAB-M, whereas HAB-M mice did not significantly differ from NAB-M and CM-M controls, respectively. Thus, while HAB-M and LAB-M mice selectively bred for a specific anxiety-related phenotype using the EPM show similar differences across a variety of anxiety- and depression-related behavioral paradigms, HAB-M animals show little difference from NAB-M or CM-M controls in the depression-related forced swim test. These findings suggest that test-dependent facets of behavior are involved with at least partially different genetic mechanisms controlling the various anxiety- and depression-related indices.

A major goal in the area of mental disorders is the identification of biomarkers that can categorize subsets of subjects in a more reliable and consistent manner. Our approach demonstrates that proteomic analyses are suited to identify protein markers that are differentially expressed in CD1 mice with different genetic predispositions to anxiety-related behavior. Glx1 represents an enzyme in the cytosolic fraction of cells and tissues of many organisms (17, 19). Although its function is not entirely clear, it has been shown that the enzyme plays a major role in the detoxification of methylglyoxal, which represents a potent cytotoxic metabolite. Glx1 catalyzes the transformation of methylglyoxal and glutathione to S-lactoylglutathione, which is converted to D-lactic acid by glyoxalase-II (19). Due to its
ubiquitous expression, the methylglyoxal pathway is believed to be of fundamental importance for cellular metabolism. Glx1 was identified as the only up-regulated gene in mutant mice sharing common pathogenic mechanisms with Alzheimer patients (15). A possible connection between Glx1 and depression has been found in a linkage study of families with depressive disease. Subgroups of families with unipolar affective disease showed evidence for a linkage or association with the Glx1 locus (20). In a recent study using different inbred strains of mice Hovatta et al. found that Glx1 and glutathione reductase 1 play a causal role in anxiety (21). It remains to be seen if Glx1 represents a risk marker or a risk factor for the anxiety-related phenotype in mice (22).

In our newly developed mouse model of extremes in trait anxiety, Glx1 in brain areas and blood cells has been shown to represent a robust, reliable biomarker for the non-anxious versus anxious phenotype. While expressed at an intermediate level in unselected NAB-M as well as CM-M controls, it showed a bidirectional shift from the strain mean towards reduced (HAB-M) and increased (LAB-M) expression levels, thus mimicking the anxiety phenotype. Very little has been reported on the function of the other marker protein that was identified in our analysis. As its name indicates, EP is an enzyme that has both, enolase and phosphatase activities, and is involved in metabolic pathways of energy production and conversion (23). No association with any disease has been reported to this date. The nature of the different isoforms that were found in HAB-M and LAB-M mice is currently under investigation.

The expression of both marker proteins, Glx1 and EP, can be assessed in human specimens by isolating red and white blood cells, respectively. Future studies with a great number of case and control specimens will tell if the two proteins can be used in the diagnosis of anxiety disorders in patients.
REFERENCES


FIGURE LEGENDS

Figure 1: A, Unlike non-anxious LAB-M mice which explore the aversive open arms of the EPM, the anxious HAB-M mice spend most of the test time in the dim lit closed arms. B, EPM data (% time open arms) of the parental male and female CD1 mice (mean ±SEM, male and female CD1 are displayed as bigger and smaller crosses, respectively) and G1 to G21 generations of male and female HAB-M and LAB-M mice. Unselected CD1 mice (NAB-M) are shown for comparison (horizontal bar). Male animals are shown in solid lines and filled rhombs and circles, and females in dotted lines and empty rhombs and circles, respectively. Independent of gender, HAB-M and LAB-M animals differed significantly in their anxiety-related behavior (G4-G21), and NAB-M differed significantly from both HAB-M and LAB-M animals (n=40-80 per line and generation).

Figure 2: Immobility time of HAB-M, NAB-M, CM-M and LAB-M mice in the forced swim test. **p<0.01, ***p<0.001 vs. HAB-M; ###p<0.001 vs. LAB-M. Results represent data from G14-G15 (adapted from (2) with permission).

Figure 3: Representative 2D-PAGE of amygdala protein extracts from male HAB-M (A), LAB-M (B) and NAB-M (C) animals. The differences in expression of Glx1 and EP protein spots are pointed out with arrows. The basic end of the gel is on the left side and the acidic end on the right side of the gels. Results represent data from G12; a total of 12 animals were analyzed for each group.

Figure 4: Expression analysis by Western blot. A, Red blood cell protein extracts from male HAB-M, NAB-M, CM-M and LAB-M animals representing equal amounts of total protein
(100 µg) were run on a 12 % SDS gel and transferred to a PVDF membrane. Subsequently, the membrane was probed with an anti-Glx1-specific antibody and developed. The immunoreactive Glx1 protein bands are shown (adapted from (2) with permission). B, The signal volumes of the bands obtained from Glx1 Western blot analysis in panel A were quantified with a densitometer. C, EP Western blot of amygdala protein extracts from male HAB-M and LAB-M mice. Extracts were subjected to 2D-PAGE and transferred to a PVDF membrane. The membrane was probed with an anti-EP-specific antibody and developed. The additional EP isoform spot in HAB-M mice that is missing in LAB-M mice is indicated with an arrow.

**Figure 5:** Glx1 (A) and EP (B) Western blot analyses of human red (A) and white (B) blood cell protein extracts from 4 individuals. Immunoreactive bands that represent Glx1 (A) and EP (B) are indicated with arrows.
Figure 1
Figure 2
Figure 3
Figure 4

A  CM-M F1  LAB-M  HAB-M  NAB-M

B

C  HAB-M  LAB-M

Figure 4
Figure 5