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Behavioral extremes of trait anxiety in mice are characterized by distinct metabolic profiles

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ABSTRACT

No comprehensive metabolic profile of trait anxiety is to date available. To identify metabolic biosignatures for different anxiety states, we compared mice selectively inbred for ~40 generations for high (HAB), normal (NAB) or low (LAB) anxiety-related behavior. Using a mass spectrometry-based targeted metabolomics approach, we quantified the levels of 257 unique metabolites in the cingulate cortex and plasma of HAB, NAB and LAB mice. We then pinpointed affected molecular systems in anxiety-related behavior by an *in silico* pathway and network prediction analysis followed by validation of *in silico* predicted alterations with molecular assays. We found distinct metabolic profiles for different trait anxiety states and detected metabolites with altered levels both in cingulate cortex and plasma. Metabolomics data revealed common candidate biomarkers in cingulate cortex and plasma for anxiety traits and *in silico* pathway analysis implicated amino acid metabolism, pyruvate metabolism, oxidative stress and apoptosis in the regulation of anxiety-related behavior. We report characteristic biosignatures for trait anxiety states and provide a network map of pathways involved in anxiety-related behavior. Pharmacological targeting of these pathways will enable a mechanism-based approach for identifying novel therapeutic targets for anxiety disorders.

1. Introduction

The identification of molecular biosignatures for psychiatric disorders and their subsequent implementation in clinical settings will revolutionize the diagnostic and therapeutic options in the field of neuropsychiatry. Emerging systems biology approaches allow the interrogation of distinct disease/health states in a holistic and hypothesis-free manner. Data derived from omics technologies reveal alterations in pathways, systems and networks, casting light on underlying molecular disease mechanisms and potential therapeutic targets. In this regard, metabolomics captures the status of biochemical pathways at a given time point and is able to define distinct metabolic states in health and disease (Kaddurah-Daouk and Krishnan, 2009; Suhre et al., 2011). Anxiety disorders encompass a wide spectrum of conditions, ranging from panic disorder to agoraphobia (American Psychiatric Association, 2000). Discovering molecular biosignatures for such disorders is of particular importance as they constitute the most common psychiatric disorders with up to one third of the patients not responding to existing treatments (Bystritsky, 2006; Kessler et al., 2005) and no molecular biomarkers for diagnosis/therapeutics available (Filiou et al., 2011b).

To investigate the molecular underpinnings of trait anxiety, a mouse model of high (HAB), normal (NAB) or low (LAB) anxiety-related behavior was generated by selective inbreeding for ~40 generations based on the % time spent on the elevated plus-maze (EPM) open arms (Kromer et al., 2005). Due to the selective enrichment of the genetic risk factors related to anxiety across generations, the HAB/NAB/LAB mouse model is a robust system for studying genetically inherited behavioral extremes of trait anxiety (Landgraf et al., 2007).

We have previously established a multi-omics biomarker discovery platform for animal models of disease (Filiou and Turck, 2012; Frank et al., 2009; Haegler et al., 2009; Zhang et al., 2009). This platform was used to compare cingulate cortex (Filiou et al.,

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Abbreviations

EPM	elevated plus-maze
FDR	false discovery rate
HAB	high anxiety-related behavior
LAB	low anxiety-related behavior
KEGG	Kyoto encyclopedia of genes and genomes
NAB	normal anxiety-related behavior
PLS-DA	partial least square-discriminant analysis
ROS	reactive oxygen species
SAM	significance analysis of microarrays
SEM	standard error of the mean
SRM	selected reaction monitoring
VIP	variable importance in projection

2011a) and plasma (Zhang et al., 2011) proteomes of HAB and LAB mice. We focused on cingulate cortex due to its involvement in the regulation of emotional behavior (Drevets and Savitz, 2008) and on plasma as the specimen of choice for translational applications. HAB mice exhibit energy metabolism and mitochondrial pathway alterations in the cingulate cortex and energy metabolism changes in the plasma compared to LAB mice (Filiou et al., 2011a; Zhang et al., 2011). To further study these differences in energy homeostasis, we analyzed cingulate cortex and plasma specimens of HAB, NAB and LAB mice with a metabolomics platform that is based on selected reaction monitoring (SRM) mass spectrometry and predominantly targets major metabolic pathways (Yuan et al., 2012). In the present study we report distinct metabolic profiles for different trait anxiety states both in cingulate cortex and plasma. We extended our analyses to predict *in silico* affected pathways in trait anxiety and validated selected pathway predictions with molecular methods, providing a map of affected pathways in anxiety-related behavior.

2. Materials and methods

2.1. Animals

The animal experiments were approved by local authorities and conducted according to current regulations for animal experimentation in Germany and the European Union (European Communities Council Directive 86/609/EEC). HAB, NAB and LAB male mice from the 38th to the 43rd generations were housed under standard conditions in the animal facility of the *Max Planck Institute of Psychiatry* as previously described (Filiou et al., 2011a). At seven weeks of age anxiety-related behavior was assessed on the EPM. At eight to nine weeks of age animals were sacrificed. Trunk blood was collected and centrifuged for 10 min at 1300 *g*, 4 °C to separate plasma. After perfusion with 0.9% saline, the cingulate cortex was dissected according to the mouse brain atlas (Paxinos and Franklin, 2001). Cingulate cortex and plasma samples were snap-frozen in liquid nitrogen. Separate sets of animals were used for metabolomics analysis and subsequent validation of *in silico* predicted alterations.

2.2. Sample preparation and targeted LC-MS/MS metabolomics

Cingulate cortices were prepared for metabolomics analysis as previously described (Webhofer et al., 2013). Briefly, tissues were homogenized in 30 volumes of ice cold 80% methanol, homogenates were centrifuged and supernatants were incubated on dry ice. The pellets were dissolved in six volumes of ice cold methanol and combined with previous supernatants. Plasma samples were processed according to Bruce et al. (2008) with slight modifications. Ice cold 100% methanol (400 μl) was added to 100 μl plasma and vortexed for 2 min. After a 2 h incubation on ice, samples were centrifuged at 2100 *g* for 10 min at 4 °C. The supernatant was ultrafiltrated and the filtrate was evaporated to dryness for LC-MS/MS analysis. The LC-MS/MS metabolomics analysis was performed at the Beth Israel Deaconess Medical Center Mass Spectrometry Core (Boston, MA) using a 5500 QTRAP triple quadrupole mass spectrometer (AB/SCIEX, Framingham, MA) coupled to a Prominence UFCL HPLC system (Shimadzu, Columbia, MD) by a targeted SRM metabolite analysis workflow (Yuan et al., 2012). Samples were delivered to the mass spectrometer via normal phase chromatography using a 4.6 mm i.d × 10 cm Amide Xbridge HILIC column (Waters Corp., Milford, MA). Several metabolites were targeted in both positive and negative modes by positive/negative polarity switching. All other parameters were as previously described (Webhofer et al., 2013). Five animals per HAB/NAB/LAB line were analyzed for each sample type (cingulate cortex or plasma).

2.3. Metabolomics data analysis

For metabolites measured both in positive and negative mode, only the measurement with the most robust values was considered for further analysis. Metabolites with >10% missing values were excluded. MetaboAnalyst (Xia et al., 2009) was used for statistical analysis of metabolite quantification. After median normalization and Pareto scaling, data were analyzed by a multivariate (partial least square discriminant analysis (PLS-DA)) and a univariate (significance analysis of microarrays (SAM)) method. Groups of metabolites with variable importance in projection (VIP) score > 1.5 and $p < 0.05$ for PLS-DA and important features with false discovery rate (FDR) ≤ 0.05 and $q < 0.05$ for SAM were considered.

2.4. In silico prediction of pathway and network alterations in anxiety-related behavior

Pathway Studio (v 8.0, Ariadne Genomics, Rockville, MD) was used for *in silico* network and pathway prediction. Only metabolites with available connectivity information in the Pathway Studio database were included in the analysis. Connections supported by at least three references in the literature are reported.

2.4.1. Cingulate cortex

To enhance analysis robustness we only considered overlapping metabolites between PLS-DA and SAM for altered pathway prediction in cingulate cortex. These metabolites were searched for downstream common targets. We then investigated literature-based relations between these downstream common targets and altered protein networks between HAB and LAB mice (Filiou et al., 2011a) as well as anxiety. Only downstream common targets with at least three connections with other entities were considered.

2.4.2. Cingulate cortex and plasma

As common metabolites in cingulate cortex and plasma we considered the metabolites that were found in both cingulate cortex and plasma by at least one statistical method (PLS-DA, SAM). These metabolites were searched for downstream common targets and upstream common regulators. The relation of the downstream common targets with selected cell processes was investigated. Furthermore, significantly overrepresented metabolic pathways in downstream common targets and upstream common regulators were calculated by the 'Find Pathways/Groups enriched with Entities' Pathway Studio function (Bonferroni corrected $p < 0.05$). For the latter analysis, only downstream common targets and upstream