Susceptibility to stress plays a crucial role in the development of psychiatric disorders such as unipolar depression and post-traumatic stress disorder. In the present study the chronic mild stress rat model of depression was used to reveal stress-susceptible and stress-resilient rats. Large-scale proteomics was used to map hippocampal protein alterations in different stress states. Membrane proteins were successfully captured by two-phase separation and peptide-based proteomics. Using iTRAQ labeling coupled with mass spectrometry, more than 2000 proteins were quantified and 73 proteins were found to be differentially expressed. Stress susceptibility was associated with increased expression of a sodium-channel protein (SCN9A) currently investigated as a potential antidepressant target.

Differential protein profiling also indicated stress susceptibility to be associated with deficits in synaptic vesicle release involving SNCA, SYN-1, and AP-3. Our results indicate that increased oxidative phosphorylation (COX5A, NDUFB7, NDUFS8, COX5B, and UQCRB) within the hippocampal CA regions is part of a stress-protection mechanism.

Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.016428, 1–12, 2012.

Stress exposure can lead to serious illnesses such as depression and post-traumatic stress disorder and have fatal consequences (1–3). However, some individuals are able to cope with even severe stressors and seem to be stress resilient (4, 5). It is widely accepted that resiliency is not merely a lack of stress susceptibility, but it is an active process involving physiological as well as psychological adaptations. The resilience phenotype is important, both with respect to discovering biomarkers to the pathological state distinctly associated with stress susceptibility and with regard to understanding the biological underpinnings for resiliency.

The hippocampal formation is involved in the stress response as a vital part of the negative feedback system regulating circulating glucocorticoids (6, 7). Moreover, the hippocampus is coupled to stress through fear and emotional processing via its connections to the amygdala (8, 9). The hippocampal formation is believed to be implicated in the etiology of depression, potentially through an important role in stress responses (10–12). Based on the role of the hippocampal formation in stress and stress-related psychiatric disorders, one might expect to find markers of stress-susceptibility/resilience in this region of the brain.

The development of the chronic mild stress (CMS)1 rat model of depression was based on the rationale that stress can induce depression (13, 14). The model fulfills all standard validity criteria and has a face validity including several symptoms of depression, with anhedonia as hallmark symptom of the disease (15). Anhedonia is defined as a lack of interest in otherwise pleasurable events or stimuli. In rodents exposed to the CMS regime this symptom appears as decreased reward sensitivity, measured as a reduced sucrose consumption/preference (16, 17) or increased threshold to intracranial self-stimulation (18).

Using the CMS paradigm we have repeatedly found that the majority of rats exposed to chronic mild stress show a gradual reduction in consumption of sucrose solution, indicating an anhedonic-like state. The remaining rats do not reduce their sucrose intake and appear resilient to the stress-induced effects on sucrose intake (17, 19, 20).

1 The abbreviations used are: CMS, chronic mild stress; CA, cornu ammonis; DG, dentate gyrus; iTRAQ, isobaric tag for relative and absolute quantitation; SRM, selected reaction monitoring; BDNF, brain derived neurotrophic factor; VEGF, vascular endothelial growth factor; SNCA or Na,1.7, sodium channel protein; CADPS2, calcium-dependent activator for secretion protein 2; ENSA, alpha-endosulfine protein; SNAP91 or AP180, clathrin coat assembly protein; SNCA, alpha-synuclein; SYN-1, synaptogyrin 1; AP-3, adaptin-related protein complex 3; GABA, gamma-aminobutyric acid; COX5A,B, cytochrome c oxidase subunit 5A,B; NDUFB7, NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7; UQCRB, ubiquinol-cytochrome c reductase binding protein.
In the present study the aim was to search for proteins linked to the stress-susceptible (termed anhedonic) and stress-resilient phenotype within the hippocampal cornu ammonis (CA) region. The results showed that the anhedonic and resilient phenotypes were associated with distinct protein regulation patterns. In particular, in the resilient group, a large number of proteins were regulated, demonstrating that resilience implies molecular plasticity within the CA region. In stress-susceptible rats a sodium channel, currently investigated as a novel antidepressant target, was up-regulated. Moreover, expression levels of proteins involved in vesicular release/turnover differed significantly between stress susceptible and stress resilient groups, indicating a potential deficit in vesicle turnover in stress-susceptible rats. Finally, a comparison of control and stress-resilient rats revealed mitochondrial oxidative phosphorylation as a potential mechanism underlying resiliency.

**EXPERIMENTAL PROCEDURES**

**Subjects**—Male Wistar rats (Taconic M&B, Ry, Denmark) were used in the study. The initial experimental group contained 300 rats. Twenty-seven rats were used in the main large-scale proteomics study and in the validation study, respectively. Rats were grouped into three categories, based on criteria of behavior during stress experiments. The animals were singly housed, food and water was available ad libitum, and animals were kept on a standard 12-h light/dark cycle except when one of these parameters was changed because of the stress regime. All animal procedures were approved by the Danish National Committee for Ethics in Animal Experimentation (2008/561–447).

**Sucrose Consumption Test**—Initially, the animals were trained to consume a palatable sucrose solution (1.5%). The training continued for 5 weeks, with sucrose test conducted twice a week during the first 3 weeks and once a week during the last 2 weeks. Animals were food and water deprived 14 h before the test, which consisted of 1-hour free access to a bottle with sucrose solution. Sucrose consumption was measured by weighing the bottles at the beginning and end of the test. During the stress period, the sucrose consumption test was performed once a week. Independent of the sucrose consumption test, water intake was monitored once a week by weighing the water bottles before and after a 24 h period. Rats with a mean sucrose intake below 8.5 g were designated as innate low-drinkers and were not used for further experiments. A mean baseline standard deviation (S.D.) was calculated from the total population. Rats with a sucrose-intake S.D. more than twofold above the mean S.D. were excluded from the experiment, to avoid subjects with unstable sucrose intake. Baseline sucrose consumption was defined as the mean sucrose consumption during three sucrose tests conducted before stress initiation. Rats were allocated into experimental groups based on their baseline sucrose intake. Following exposure to stress, rats were characterized as anhedonic (defined as a >30% within-subject decrease in sucrose intake) or resilient (defined as a < 10% within subject decrease in sucrose intake). Rats not corresponding to either criterion were excluded from the experiment (see Fig. 1, showing study design).

**Chronic Mild Stress**—On the basis of sucrose intakes in the three final baseline tests, animals were divided into two groups and placed in separate rooms. One group was exposed to 8 weeks of chronic mild stressors and the other was left unchallenged. The stress protocol consisted of seven different stress conditions each lasting 10 to 14 h. One period of intermittent illumination, stroboscopic light, grouping, food or water deprivation, and two periods of soiled cage and no stress, and three periods of 45° cage-tilting.

**Tissue Processing**—Animals were decapitated and the brains were immediately dissected. The hippocampi were removed and subdivided to yield the CA1 and CA3 subregion according to a method reported in detail elsewhere (21). These two regions were pooled and were immediately snap-frozen before used in the proteome analysis. Proteome analysis was conducted on three sample pools corresponding to three rats in each treatment group, respectively.

**Immunohistochemistry**—After an intraperitoneal overdose of sodium pentobarbital (60 mg/ml, 100 mg/kg) the rats were transcardially perfused with 100 ml 0.9% saline, followed by 200 ml of ice cold 4% paraformaldehyde (pH 7.2–7.4). The brains were removed from the skull and the right hemisphere was postfixed overnight in the same solution at 4 °C. The brains were transferred to a solution consisting of 30% sucrose (diluted in phosphate buffer, pH 7.0–7.4) supplemented with 10% sodium azide and stored at 4 °C until they sank.
The brains were sectioned sagittally on a cryostat (Leica, 3050 CS). The 40 μm sections were collected in series of every 10 (−120 sections per brain) and were stored in cryoprotectant antifreeze solution (25% ethylene glycol and 25% glycerin in a 0.05 M phosphate-buffered saline (PBS)) at −20 °C.

The sections were rinsed in cold 0.1% PBS (3 × 10 min.). To detect antigens, the sections were incubated 1 h at 95 °C in 1 mM Tris, pH 9, supplemented with 0.5 mM EGTA. Non specific binding of immunoglobulin was quenched by incubating the sections in 50 μM NH4Cl (30 min.) and were blocked in PBS supplemented with 1% bovine serum albumin (BSA), 0.01% Triton X 100, and 0.2% gelatin (3 × 20 min.). Incubation with the primary antibody (Nav1.7, ab65167, Abcam (Cambridge, UK), 1:500) diluted in PBS and supplemented with 0.1% BSA and 0.01% Triton X100 was done overnight at +4 °C. To control for nonspecific staining, some of the sections were incubated without primary antibody.

The sections were rinsed in 0.1% BSA, 0.2% gelatin, 0.1% Triton X100 in PBS (3 × 10 min) and were incubated with the secondary antibody (Goat, anti-rabbit IgG coupled to Alexa 488, 1:2000) diluted in 0.1% BSA, 0.3% Triton X 100 in PBS for 1 h at room temperature followed by rinsing in PBS (2 × 5 min.). The sections were then mounted with a coverslip in Glycergel antifade medium (Dako) and were inspected on Zeiss Axiosvert 200 M fluorescence microscope using Neofluar 10 × objective.

Protopote Fractionation—Rat brain tissue was homogenized with a Potter-Elvehjem in cold lysis buffer (10 mM Heps, pH 7.2, with complete protease inhibitors (Roche Diagnostics)), sonicated three times for 10 s, and briefly centrifuged (400 × g, 5 min, 4 °C) to remove tissue particles and cell debris. The supernatant from homogenization containing membrane proteins (M) and the S sample were purified by precipitation and further analyzed by proteomics as previously described (25).

Peptide Labeling and Fractionation—The labeling procedure was performed three times corresponding to biological triplicates of S and M fraction. For each triplicate samples from three animals were separated in two phases as previously described (25). Briefly, pellets of membrane proteins were dissolved on ice in −1% Triton X-114. After 15 min incubation at 30 °C a bottom phase of Triton-X114 micelles was formed and collected after centrifugation (13000 × g, 10 min). The Triton-X114 detergent phase, mostly containing membrane proteins (M) and the S sample were purified by precipitation and further analyzed by proteomics as previously described (25).

iTRAQ quantitation was performed in Mascot where normalization to summed intensities was applied to compensate for possible variation in starting material. For details, see http://www.matrixscience.com/help/quant_config_help.html. When identification of a protein yielded several possible protein isoforms, all of them were considered for quantification. In the quantitative calculations, only protein isoforms with iTRAQ values in at least two peptides, each with an expectation value of 0.02 or below were reported for proteins with five or more measured iTRAQ scan values. The significance level of protein identifications was set to 0.001, which resulted in a false discovery rate of less than 0.003 when searched in Mascot against the decoy database of random sequences. Throughout the manuscript, the HGNC symbol (http://www.genenames.org/) obtained from the IPI-database was used to refer to protein hits. iTRAQ values were reported for proteins with five or more measured iTRAQ scan values from at least two peptides, each with an expectation value of 0.02 or below.

18% using Benjamini and Hochberg’s statistics with extra stringency from the fold change criterion (27, 28). Differences in sucrose consumption were tested
The peptides were trapped on a 2 cm 100 μm inner diameter, 360 μm outer diameter, ReproSil - Pur C18 AQ 5 μm (Dr. Maisch, Ammerbuch-Entringen, Germany) and separated using a 15 cm 75 μm inner diameter, 360 μm outer diameter, ReproSil - Pur C18 AQ 3 μm (Dr. Maisch) reversed phase capillary column. The peptides were eluted using a gradient from 0% to 34% phase B (0.1% formic acid, 90% ACN) over 34 min at 250 nL/min directly into a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific, San Jose, CA). The TSQ Vantage was operated in a nano-electrospray mode. For ionization, 2300 V of spray voltage and 200 °C capillary temperature were used. The selectivity for Q1 was set to 0.7 and Q3 temperature were used. The selectivity for Q1 was set to 0.7 and Q3 (Dr. Maisch, Ammerbuch-Entringen, Germany) and separated using a 15 cm 75 μm inner diameter, 360 μm outer diameter, ReproSil - Pur C18 AQ 3 μm (Dr. Maisch) reversed phase capillary column. The peptides were eluted using a gradient from 0% to 34% phase B (0.1% formic acid, 90% ACN) over 34 min at 250 nL/min directly into a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific, San Jose, CA). The TSQ Vantage was operated in a nano-electrospray mode. For ionization, 2300 V of spray voltage and 200 °C capillary temperature were used. The selectivity for Q1 was set to 0.7 and Q3 (Dr. Maisch, Ammerbuch-Entringen, Germany) and separated using a 15 cm 75 μm inner diameter, 360 μm outer diameter, ReproSil - Pur C18 AQ 3 μm (Dr. Maisch) reversed phase capillary column. The peptides were eluted using a gradient from 0% to 34% phase B (0.1% formic acid, 90% ACN) over 34 min at 250 nL/min directly into a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific, San Jose, CA). The TSQ Vantage was operated in a nano-electrospray mode. For ionization, 2300 V of spray voltage and 200 °C capillary temperature were used. The selectivity for Q1 was set to 0.7 and Q3 temperature were used. The selectivity for Q1 was set to 0.7 and Q3.

Protein Classification and Pathway Analysis—Proteins that passed significance criteria were arranged according to function and process. Protein function and process was inferred from the IPI database (http://www.ebi.ac.uk/IPI/IPIhelp.html) and literature searches (www.ingenuity.com).

Cluster Analysis—Cluster analysis was used to separate the three groups based on between-group similarities in global protein expression profiles. The clustering approach orders objects in a treelike structure and provides information about relations among groups.

The cluster analysis was performed with Cluster software version 2.11 (29). Hierarchical clustering was performed by clustering genes and arrays. Subsequently, the cluster analysis was visualized by Tree View software version 1.60 (http://rana.lbl.gov/EisenSoftware.htm).

RESULTS

Sucrose Consumption—Sucrose consumption was applied to assess stress-induced anhedonic-like and resilient behavior of rats. The data of the groups was normally distributed (as tested by Q-Q-plots). Moreover, no statistically significant difference between the groups indicated that they were well-matched (data not shown). Three-weeks exposure to CMS (n = 164) resulted in significantly decreased sucrose consumption when compared with unchallenged controls (n = 72) (p < 0.00001). Fig. 2A shows the mean group sucrose intake indexed to baseline values.

Forty-three percent of the stressed rats showed a more than 30% decrease in sucrose intake and were defined as anhedonic-like. On the other hand, 23% of the rats exposed to stress did not decrease their sucrose intake and were designated stress resilient. Unchallenged rats did not decrease their sucrose intake. A subset of the unchallenged controls (C), anhedonic-like (A) and resilient (R) rats (nine in each group) were used for the proteome analysis. Fig. 2B shows the sucrose intake (indexed to baseline) of the rats used for proteome analysis.

To confirm that the decrease in sucrose consumption was linked to palatable sucrose and not to general physiology, a 24-hour water consumption was measured once weekly during the experiment in a subset of the control and stress group (n = 20 for each group). No differences were found in water consumption, with both the control and stress group having a 24-hour mean water consumption of 39 ml (data not shown).

Proteome and Cluster Analysis—Large scale protein analysis was performed on hippocampi from the control, anhedonic-like, and stress resilient rats to identify proteins differentially expressed in the three groups. Quantitative data were obtained for 1199 and 1054 proteins from the membrane and soluble sample, respectively, with 242 proteins detected in

with Student’s t-test or One-Way ANOVA followed by bonferroni’s correction when three groups were being compared.

Selected Reaction Monitoring (SRM)—Nine proteins were selected for validation by SRM analysis of material from new rat specimens (nine rats from each treatment group) prepared with the same procedures as in the main large scale proteomics study. At least three proteotypic peptides for each protein were SRM analyzed. Isotopically labeled, heavy versions of all the selected peptides were synthesized (JPT Peptide Technologies GmbH), and added to each sample. The digested peptides were resuspended in 0.1% formic acid. Approximately 1 μg peptides were used for each SRM run. The peptides were loaded onto an EASY-nano LC system (Thermo Fisher Scientific). The peptides were trapped on a 2 cm 100 μm inner diameter, 360 μm outer diameter, ReproSil - Pur C18 AQ 5 μm (Dr. Maisch, Ammerbuch-Entringen, Germany) and separated using a 15 cm 75 μm inner diameter, 360 μm outer diameter, ReproSil - Pur C18 AQ 3 μm (Dr. Maisch) reversed phase capillary column. The peptides were eluted using a gradient from 0% to 34% phase B (0.1% formic acid, 90% ACN) over 34 min at 250 nL/min directly into a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific, San Jose, CA). The TSQ Vantage was operated in a nano-electrospray mode. For ionization, 2300 V of spray voltage and 200 °C capillary temperature were used. The selectivity for Q1 was set to 0.7 and Q3 (Dr. Maisch, Ammerbuch-Entringen, Germany) and separated using a 15 cm 75 μm inner diameter, 360 μm outer diameter, ReproSil - Pur C18 AQ 3 μm (Dr. Maisch) reversed phase capillary column. The peptides were eluted using a gradient from 0% to 34% phase B (0.1% formic acid, 90% ACN) over 34 min at 250 nL/min directly into a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific, San Jose, CA). The TSQ Vantage was operated in a nano-electrospray mode. For ionization, 2300 V of spray voltage and 200 °C capillary temperature were used. The selectivity for Q1 was set to 0.7 and Q3 (Dr. Maisch, Ammerbuch-Entringen, Germany) and separated using a 15 cm 75 μm inner diameter, 360 μm outer diameter, ReproSil - Pur C18 AQ 3 μm (Dr. Maisch) reversed phase capillary column. The peptides were eluted using a gradient from 0% to 34% phase B (0.1% formic acid, 90% ACN) over 34 min at 250 nL/min directly into a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific, San Jose, CA). The TSQ Vantage was operated in a nano-electrospray mode. For ionization, 2300 V of spray voltage and 200 °C capillary temperature were used. The selectivity for Q1 was set to 0.7 and Q3 (Dr. Maisch, Ammerbuch-Entringen, Germany) and separated using a 15 cm 75 μm inner diameter, 360 μm outer diameter, ReproSil - Pur C18 AQ 3 μm (Dr. Maisch) reversed phase capillary column. The peptides were eluted using a gradient from 0% to 34% phase B (0.1% formic acid, 90% ACN) over 34 min at 250 nL/min directly into a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific, San Jose, CA). The TSQ Vantage was operated in a nano-electrospray mode. For ionization, 2300 V of spray voltage and 200 °C capillary temperature were used. The selectivity for Q1 was set to 0.7 and Q3 (Dr. Maisch, Ammerbuch-Entringen, Germany) and separated using a 15 cm 75 μm inner diameter, 360 μm outer diameter, ReproSil - Pur C18 AQ 3 μm (Dr. Maisch) reversed phase capillary column. The peptides were eluted using a gradient from 0% to 34% phase B (0.1% formic acid, 90% ACN) over 34 min at 250 nL/min directly into a triple quadrupole mass spectrometer (TSQ Vantage, Thermo Scientific, San Jose, CA). The TSQ Vantage was operated in a nano-electrospray mode. For ionization, 2300 V of spray voltage and 200 °C capillary temperature were used. The selectivity for Q1 was set to 0.7 and Q3.
both fractions (Fig. 3). Extensive lists of proteins with quantitative data are in Supplemental Tables S1 to S3, and detailed identification data are in Supplemental Table S4 to S5.

Out of the 2011 unique protein hits, the main focus was set on those proteins that passed statistical tests. Three experimental groups of rats were analyzed (with triplicates from each, and each triplicate was a mixture from three independent rats): Unchallenged Control (C), Anhedonic-like (A), and CMS-Resilient (R). Table I shows the number of significant protein hits (either down- or up-regulated) for the respective sample comparisons. Seventy-three proteins were significantly altered between the groups (Table II).

Two separate cluster analyses were performed. One was performed on data from all 2011 proteins and another was performed on the 73 proteins that passed selection criteria. According to both cluster profiles, the resilient group diverged from the anhedonic and control group, demonstrating that the protein expression profile of the resilient group differed from the other two groups investigated (Figs. 4A and 4B).

**SRM**—We conducted a validation experiment using subgroups identical to the original experiment and the SRM method to validate the results obtained using iTRAQ labeling. Nine proteins were selected for validation. Two proteins from the soluble sample and seven from the membrane sample. Only one peptide was detected for the Gng-2 protein and therefore this protein was excluded from further analysis. The two proteins from the soluble sample, Pgl-6 and Ap3d1 did not show differential expression levels comparable to the results from the iTRAQ experiment. Differential regulation in line with iTRAQ data was confirmed in five of six proteins, namely Scn9a, Slc1a4, Snca, Syngr1, and Tmpo. Regulation of Cacna1b was not confirmed. A study comparison is shown in Fig. 5.

**Histology**—We assessed the presence of the SCN9A protein (Nav1.7) in the hippocampus by staining this sodium channel in cryocut brain sections (Fig. 6). In control rats, we found highest immunoreactivity in the CA3, with lower intensities in the CA1, and very little reactivity in the DG. We observed higher immunoreactivity in anhedonic rats compared with controls. Immunoreactivity was confirmed in the dorsal root ganglia, a neural area known to contain a large density of Nav1.7.

### DISCUSSION

By exposing rats to the CMS regime, we obtained rat groups with different stress response phenotypes, using sucrose consumption as readout on hedonic-like status. The hippocampal proteome was analyzed by mass spectrometry-based proteomics and numerous proteins, differentially expressed in the different stress-response phenotypes, were identified. More than 2000 proteins were detected using a two-phase fractionation method designed to facilitate detection of membrane proteins (25). This method proved highly successful with 957 proteins being classified as associated to membranes, a protein group likely to be involved in cell signaling.

We have previously reported that, rats exposed to CMS, show a graded response to stress, using sucrose-intake as behavioral readout (17, 19, 20, 30). This finding was reproduced in the present study, which showed a general effect of 4 weeks of mild stress exposure on sucrose intake with a mean decrease of 23% (Fig. 2A) and no concurrent reduction of water intake. The sucrose consumption data follow a right-skewed normal distribution, thus the definition as anhedonic and resilient rats respectively is based on an operational cutoff chosen by the experimenter. A graded effect of stress exposure is expected and a distinction into stress-susceptible and stress-resilient rats provides a useful approach for identifying potential biomarkers specific for anhedonia, as opposed to general stress effects. Moreover, inclusion of the resilience group improves the translational value of the CMS model, as stress resiliency is a common clinical phenomenon (31). Furthermore, data from the resilient group might shed light on novel treatment opportunities augmenting resiliency. Recently, a resilience phenotype has also been described in studies employing the chronic social defeat model of depression (32, 33) and stress susceptibility/resilience has also been assessed following exposure to cold-swim-stress (34) further arguing for the importance of this subgroup.

The aim of the present study was to determine whether proteome changes in the rat hippocampal CA-subregion following exposure to CMS correlate reliably with stress-sus-
**TABLE II**

| Fraction | IPI number | Abbreviation | Protein description | Function description | Process | R vs A |
|----------|------------|--------------|--------------------|----------------------|---------|-------|
| M        | IPI00369419 | -            | Uncharacterized cofillin-like/19 kDa protein | Cytoskeleton | Actin binding | 0.76   |
| S        | IPI00365286 | Vcl          | Vinculin           | Cytoskeleton         | Protein binding | 0.79   |
| M        | IPI00870837 | Mic1         | Megalencephalic leukemiaophopathy | Ion channel | Membrane ion Channel | 0.76   |
| M        | IPI00480827 | Scn9a        | Sodium channel protein type 9 alpha | Ion channel | Sodium ion transport | 0.77   |
| S        | IPI00870234 | Gstm6I       | Glutathione S-transferase M6-like | Metabolism | Glutathione transferase | 0.80   |
| S        | IPI00214614 | Pabpc3       | Poly(A) binding protein, cytoplasmic | Metabolism | RNA binding | 0.77   |
| S        | IPI00231107 | Ptns         | Parathymosin       | Metabolism | DNA replication | 0.77   |
| S        | IPI00370315 | Elf4h        | Eukaryotic translation initiation factor 4H | Metabolism | Translation initiation factor | 0.82   |

**TABLE II B**

| Fraction | IPI number | Abbreviation | Protein description | Function description | Process | R vs A |
|----------|------------|--------------|--------------------|----------------------|---------|-------|
| M        | IPI00372040 | Arpc4        | Actin related protein 2/3 complex, subunit 4 | Cytoskeleton | Actin binding | 0.78   |
| S        | IPI00421723 | Tbeca        | Tubulin-specific chaperone A | Cytoskeleton | Unfolded protein binding | 0.81   |
| M        | IPI00569279 | Hist2h2ab    | Histone 2A         | Metabolism | Gene regulation | 0.60   |
| M        | IPI00569391 | Tmpo         | Thymopoetin/75 kDa protein | Metabolism | Metabolism | 0.62   |
| M        | IPI00395291 | Slc1a4       | Neutral amino acid transporter | Transmembrane transport | 0.66   |
| M        | IPI00766917 | Ptdrd        | Tyrosine-protein phosphatase delta precursor Rec.-type D | Metabolism | Protein dephosphorylation | 0.74   |
| M        | IPI00421626 | Rps9         | Rps9 40S ribosomal protein S9 | Metabolism | Peptide synthesis | 0.77   |
| M        | IPI00209308 | Nlgn2        | Isoform 1 of Neurologin-2 | Metabolism | Protein binding | 0.78   |
| M        | IPI00767998 | Rab6b        | Member RAS oncogene family | Metabolism | Protein binding | 0.80   |
| M        | IPI00561936 | Acly         | ATP citrate lyase | Metabolism | Lipid metabolism | 0.81   |
| M        | IPI00370527 | Ribosomal S4/22 kDa protein | Metabolism | Peptide synthesis | 0.81   |
| M        | IPI00559107 | -            | Putative uncharacterized protein | Unknown | Unknown | 0.69   |
| S        | IPI0061103 | -            | Putative uncharacterized protein | Unknown | Unknown | 0.82   |
| M        | IPI0188732 | Syngr1       | Synaptogyrin-1 Isoform CRA_C | vesicular | Vesicular function | 0.60   |
| M        | IPI00205493 | Snca         | Isoform Syn1 of Alpha-synuclein | vesicular | Exocytosis | 0.70   |
| S        | IPI00362469 | Pgls         | 6-phosphogluconolactonase | Catabolism | Pentose-phosphate shunt | 1.27   |
| M        | IPI00193333 | Cyb5b        | Cytochrome b5 type B | Catabolism | Cellular respiration | 1.32   |
| S        | IPI00211317 | Dynl2        | Dynine light chain 2, cytoplasmic | Cytoskeleton | Protein transport | 1.26   |

10.1074/mcp.M111.016428–6

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ceptible and stress-resilient phenotypes in rats. The CA region was chosen because of its association to cognitive function (35–37) and the profound effects seen in this region in response to stress (38). The macro-dissection method used was designed to obtain the CA-regions (21) and no further distinction of the CA region into CA1 and CA3 was applied.

### TABLE II B—continued

| Fraction | IPI number | Abbreviation | Protein description | Function description | Process | R vs A |
|----------|------------|--------------|---------------------|---------------------|---------|--------|
| M        | IPI00476965 | Cacna1b      | Voltage gated N-type calcium channel Ca(V)2.2 | Ion channel | Ion transport | 1.24 |
| M        | IPI00364599 | Cacna1b      | Pore-forming calcium channel alpha-1B subunit variant a | Ion channel | Ion transport | 1.24 |
| M        | IPI00187596 | Slc6a1       | Sodium- and chloride-dependent GABA transporter 1 | Ion transport | Neurotransmitter transport | 1.28 |
| M        | IPI00829505 | Lap3         | Isoform 2 of Cytosol aminopeptidase CDGSH iron sulfur domain-containing protein 1 | Metabolism | Protein metabolism | 1.22 |
| M        | IPI00870183 | Gis1         | Metabolism | Cellular respiration | 1.22 |
| M        | IPI00200552 | Rpl26        | 60S ribosomal protein L26 | Metabolism | Peptide synthesis | 1.22 |
| S        | IPI00362105 | Psmd6        | Proteasome (prosome, macropain) 26S subunit, ATPase, 6 | Metabolism | Nucleotide binding | 1.28 |
| M        | IPI00205559 | Crtac1       | Cartilaginous protein 1 | Miscellaneous | Ion binding | 1.30 |
| M        | IPI00566814 | Cd200        | OX-2 membrane glycoprotein | Miscellaneous | Protein binding | 1.33 |
| M        | IPI00476554 | Gng2         | Guanine nucleotide binding protein gamma 2 (Fragment) | Metabolism | G-protein signalling | 1.27 |
| M        | IPI00370116 | -            | Putative uncharacterized protein | Unknown | Unknown | 1.20 |
| M        | IPI00212980 | -            | Isoform Long of Brain protein 44 | Unknown | Unknown | 1.21 |
| S        | IPI00198486 | Ap3d1        | Adaptor-related protein complex 3, delta 1 subunit | Miscellaneous | Protein transport | 1.28 |

### TABLE II C

| Fraction | IPI number | Abbreviation | Protein description | Function description | Process | C vs R |
|----------|------------|--------------|---------------------|---------------------|---------|--------|
| M        | IPI00192246 | Cox5a        | Cytochrome c oxidase subunit 5A, mitochondrial | Catabolism | Oxidative phosphorylation | 0.74 |
| M        | IPI00365170 | Ndubf7       | NADH dehydrogenase (ubiquinone) 1 beta subcomplex, 7 | Catabolism | Oxidative phosphorylation | 0.77 |
| M        | IPI00188330 | Ndufs8       | NADH dehydrogenase (ubiquinone) Fe-S protein 8 | Catabolism | Oxidative phosphorylation | 0.78 |
| M        | IPI00193918 | Cox5b        | Cytochrome c oxidase subunit 5B, mitochondrial | Catabolism | Oxidative phosphorylation | 0.79 |
| S        | IPI00194312 | Ola1         | Ola1 Obg-like ATPase 1 | Catabolism | ATP binding | 0.79 |
| M        | IPI00201307 | Uqcrb        | Ubiquinol-cytochrome c reductase binding protein | Catabolism | Oxidative phosphorylation | 0.81 |
| S        | IPI00362469 | Pgl3         | 6-phosphogluconolactonase | Catabolism | Pentose-phosphate shunt | 0.81 |
| M        | IPI00369419 | -            | Uncharacterized coflin-like/19 kDa protein | Cytoskeleton | Actin binding | 0.76 |
| M        | IPI00364599 | Cacna1b      | Pore-forming calcium channel alpha-1B subunit variant a | Ion channel | Calcium ion transport | 0.74 |
| M        | IPI00476965 | Cacna1b      | Voltage gated N-type calcium channel Ca(V)2.2 | Ion channel | Calcium ion transport | 0.76 |
| M        | IPI00208061 | Atp1b3       | Sodium/potassium-transporting ATPase subunit beta-3 | Ion transport | Sodium/potassium transport | 0.81 |
| S        | IPI00214614 | Pabpc3       | Poly(A) binding protein, cytoplasmic 3 | Metabolism | RNA binding | 0.68 |
| S        | IPI00189074 | Pabpc1       | Polyadenylate-binding protein 1 | Metabolism | RNA binding | 0.76 |
| M        | IPI00231771 | S100b        | S100 calcium binding protein B | Miscellaneous | Protein binding | 0.71 |
| M        | IPI00476554 | Gng2         | Guanine nucleotide binding protein gamma 2 (Fragment) | Metabolism | G-protein signalling | 0.82 |
| M        | IPI00362291 | -            | Putative uncharacterized protein/16 kDa protein | Unknown | Unknown | 0.69 |
| M        | IPI00566858 | -            | Putative uncharacterized protein/37 kDa protein | Catabolism | Glycolysis | 1.22 |
| M        | IPI00569391 | Tmop         | Thymopoetin/75 kDa protein | Metabolism | Transcription factor | 1.33 |
| S        | IPI00373214 | Kpna4        | Karyopherin alpha 4 | Miscellaneous | Protein transport | 1.21 |
Cluster analysis on all proteins indicated that, in terms of protein expression levels in the CA-region, resilient rats had a distinct proteome profile (Fig. 4). These findings agree with a microarray study on the nucleus accumbens and the ventral tegmental area in socially-defeated mice (39). Previously, we found by hierarchical clustering analysis that the anhedonic group co-segregates with unchallenged controls in a subcluster diverging from a subcluster including resilient rats and escitalopram-responder rats (20). It seems that the coping mechanisms associated to the resilience phenotype implies a high degree of molecular adaptations across several brain regions.

A range of both preclinical and clinical studies has shown the hippocampal region to be affected in depressed subjects, without showing direct causal linkage (40, 41). These studies have given rise to investigations on potential molecular pathways and targets that could explain these effects, pointing to neurotrophines such as BDNF and VEGF (42, 43), or proteins involved in the cyclic-AMP pathway (44). However, as with the anatomical effects, no direct causal relation has been established between a given protein, or molecular pathway, and depressive behavior. Given this lack of knowledge on specific proteins and pathways associated to depression, we used a general, hypothesis-generating bioinformatic approach to search for novel molecular targets potentially involved in the pathology of depression. Based on Panther (45), Ingenuity, and Pubmed searches, some of these proteins were selected for more detailed description with respect to function and possible relation to depression.

To validate the group protein regulation patterns a validation study was conducted, using material from new rat specimens (nine rats from each treatment group) prepared with the same procedures as in the main large scale proteomics study. In general there was a good correlation between the iTRAQ data and SRM data, but three proteins did not show comparable group regulation patterns. This could be because of false positives, or a potential regulation could be masked by biological variation. Consequently conclusions based on these proteins should be taken with caution.

Comparing the control and anhedonic rats, 22 proteins passed significance criteria. Ingenuity pathway analysis revealed a number of proteins of potential interest to psychiatric disorders in general, but also specifically with respect to depressive disorder.

The calcium-dependent activator for secretion protein 2 (CADPS2) was significantly up-regulated (24%) in the anhedonic group compared with controls. CADPS is involved in regulating exocytosis at dense core vesicles (49). Moreover, CADPS2 plays a role in sleeping patterns and is a critical protein in the differentiation of hippocampal neurons (50). Although the literature does not provide direct linkage between CADPS2 and depression, a disrupted sleeping pattern is a common symptom of depression (51). Moreover, hippocampal neurogenesis and differentiation may be linked with depression (43), suggesting that stress-induced perturbed CADPS2 levels may occur in depressive disorder. Interestingly, the Cadps2 gene has also been associated with bipolar disorder (52).

The alpha-endosulfine (ENSA) protein was significantly up-regulated (20%) in the anhedonic group compared with controls. The role of ENSA in brain function is not well-established, but a stress-induced up-regulation of ENSA has previously been reported in rats following swim-stress (53). In addition, Dou and colleagues report a neurotoxicity-induced up-regulation of ENSA following streptozocin injection. Based on these findings, one could speculate that ENSA plays a role in stress-induced neuronal atrophy.

Clathrin coat assembly protein (SNAP91 or AP180) was significantly down-regulated (38%) in the anhedonic group compared with control. SNAP91 has been shown to be important for synaptic vesicle recycling (54, 55) and may be implicated in psychiatric disorders such as schizophrenia and bipolar disorder by imposing a deficit in vesicular turnover (56, 57).

Comparing resilient and anhedonic rats, 32 proteins passed significance criteria. Among these proteins, several are involved in psychiatric disorders.
involved in vesicular function thus indicating that alterations in neurotransmission might underlie resiliency. For example, alpha-synuclein (SNCA) was significantly down-regulated (30%) in the resilient group as compared with the anhedonic group. SNCA is expressed throughout the brain, but has been shown to be enriched in presynaptic terminals (58). Moreover, SNCA is involved in synaptic plasticity and neurotransmission, potentially through regulating vesicle density by inhibiting exocytosis (59, 60). In addition, SNCA is involved in Parkinson disease as a major constituent in the development of Lewy bodies (61). Its relation to depression is not well-established, but the comorbidity of Parkinson disease and depression is high (62). Further, a recent study found SNCA mRNA was correlated to scores obtained on the Beck depression inventory (63).

Synaptogyrin 1 (SYN-1) is another protein involved in vesicular function and, like SNCA, SYN-1 regulates vesicle density by inhibiting exocytosis (64). The expression level of SYN-1 was also decreased (40%) in resilient rats compared with anhedonic rats. In line with the results regarding SNCA and SYN-1 levels and their potential regulation of exocytosis, a recent study on zinc-deficient mice showed that susceptibility to stress associates with a decrease in exocytosis (65). In addition to SNCA and SYN-1, the adaptor-related protein complex 3 (AP-3) plays a potential role in vesicular function; mice lacking the AP-3 protein have decreased density of synaptic vesicles at inhibitory terminals in the CA1 region (66). The AP-3 protein was significantly up-regulated (28%) in the resilient group compared with the anhedonic group ($p = 0.04$). It has to be noted that the group expression pattern of AP-3 was not confirmed in the validation study. In addition to the above-mentioned proteins several proteins involved in vesicular functions are differentially expressed between the three groups further emphasizing that this biological function is affected by stress.

In support of a theory on CMS-induced alterations of vesicular-related proteins, a recent study on punches of dentate gyrus tissue from CMS-exposed rats also showed alterations in vesicular-related proteins (67). Moreover, an earlier study at our facility likewise identified differentially expressed vesicular-related proteins in rat hippocampus following stress exposure (20).

We speculate that a general hippocampal deficit in vesicular function in anhedonic rats has implications that might explain previously reported results on anhedonic rats, showing a significantly decreased number of miniature inhibitory postsynaptic potentials and a reduced GABA release probability (11). In support of this theory, a study on the role of SNCA showed a significant decrease in the frequency of miniature excitatory postsynaptic potentials in mice overexpressing human SNCA (68). Furthermore, the decrease in hippocampal GABA levels seen in studies on mice exposed to CMS could also be explained by a deficit in vesicular function (69, 70).

In future studies it will be interesting to elucidate whether stress-tolerance-specific alterations of SNCA and other vesicular-related proteins is a general neuromolecular mechanism, or whether this regulation is restricted to the hippocampus.

Comparing resilient and control rats, 19 proteins passed significance criteria. Most cell energy is obtained through oxidative phosphorylation and it is well known that brain tissue has a high energy demand. Studies have shown that chronic stress affects the mitochondrial respiratory chain and several studies have proposed mitochondrial dysfunction as a potential mechanism.

![Expression level comparison between data obtained in original experiment using iTRAQ labeling and validation study using the SRM method.](image-url)
underlying the pathophysiology of psychiatric disorders (71, 72). Five proteins involved in oxidative phosphorylation were significantly up-regulated in the resilient group compared with controls, namely COX5A, NDUFB7, NDUFS8, COX5B, and UQCRB. For all five proteins the expression in stress-susceptible rats was intermediate between control and resilient, thus it could be speculated that the regulation of these proteins provides protection against stress, but in the stress-susceptible rats the level of regulation is insufficient. We speculate that increased oxidative phosphorylation is an adaptive and protective mechanism underlying a stress-resilient phenotype. It could be argued that increased pentose phosphate shunt could prevent oxidative stress, it could be speculated that the increased expression levels of PGLS in the resilient group is a stress protective mechanism. CACNA1b is a voltage dependent calcium channel involved in neurotransmitter release. CACNA1b was (23%) and (24%) up-regulated in the resilient group compared with the anhedonic and control group respectively. Increased levels of CACNA1b could serve to increase hippocampal neurotransmission in the resilient group, as a way to compensate for the decrease in neurotransmission shown following exposure to chronic stress (74). It has to be noted that the group expression pattern of CACNA1b was not confirmed in the validation study. GNG2 does, to our knowledge, not have any previously known association to chronic stress or depression.

In summary, our unbiased proteome-wide approach identified several candidate proteins that may be related to stress-susceptibility and stress-resilience. Regarding stress-susceptibility, we found the sodium channel SCN9A to be up-regulated in stress-susceptible rats. Based on previous studies, we propose that SCN9A may be a potential novel biomarker of depression. Based on the results in the stress-resilient rats, we propose that the conservation of vesicular functioning is central in stress coping strategies. Finally, our results indicate that increased oxidative phosphorylation within the hippocampal CA regions may be important in resistance to stress.

Acknowledgments—We thank Stine Dhiin Heide for her skilled animal handling and care, and Dr Donald Smith for valuable text editing.

* This work was supported by the Lundbeck Foundation, the Augustinus Foundation, and the John and Birthe Meyer Foundation.

This article contains supplemental material.

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Fig. 6. Sagittal SCN9A-stained brain slices of the hippocampus showed increased immunoreactivity in anhedonic rats compared with controls, especially pronounced in the CA1 region. Immunoreactivity was lowest in the dentate gyrus (DG). Immunoreactivity was confirmed in sections of dorsal root ganglion (DRG), known to have a large density of sodium channels.
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In order to cite this article properly, please include all of the following information: Henningsen, K., Palmfeld, J., Christiansen, S., Baiges, I., Bak, S., Jensen, O. N., Gregersen, N., and Wiborg, O. (2012) Candidate Hippocampal Biomarkers of Susceptibility and Resilience to Stress in a Rat Model of Depress- ion. Mol. Cell. Proteomics 11(7):M111.016428. DOI: 10.1074/mcp.M111.016428.