Neuroscience 138 (2006) 149–158

STRAIN-DEPENDENT EXPRESSION OF SIGNALING PROTEINS IN THE MOUSE HIPPOCAMPUS

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Abstract—Individual mouse strains may differ significantly in terms of behavior and cognitive function. Hippocampal gene expression profiling on several mouse strains has been carried out and points toward substantial strain-specific variation of more than 200 genes including components of major signaling pathways involved in neuronal information storage.

Strain-specific hippocampal protein expression, however, has not been investigated yet. A proteomic approach based on two-dimensional gel electrophoresis coupled with mass spectrometry has been chosen to address this question by determining strain-dependent expression of signaling proteins in hippocampi of four inbred and one outbred mouse strain. Forty-six spots corresponding to 37 different signaling proteins have been analyzed and quantified. Statistical analysis revealed strain-dependent expression of *serine/threonine protein phosphatase 1*, *serine/threonine protein phosphatase 2A*, *large GTP binding protein OPA1*, *guanine nucleotide-binding protein beta*, *putative GTP-binding protein Ran*, *receptor of activated protein kinase C1*, *WASP-family protein member 1*, *voltage-dependent anion channel 2* and *14-3-3 protein gamma*.

Differential expression of signaling proteins in the hippocampus may contribute to the molecular understanding of strain-dependent behavioral and cognitive performance. Moreover, these data highlight the importance of the genetic background for the analysis of signaling pathways in the hippocampus in wild-type mice as well as in gene-targeting experiments. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: protein expression, brain, inbred mouse strain, proteomics, synaptic plasticity.

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Abbreviations: Balb, Balb/c; C57, C57BL/6J; CaMKII, Ca^{2+}/calmodulin kinase II; DTT, 1,4-dithioerythritol; EDTA, ethylenediaminetraacetic acid; FVB, FVB/N; GNB1, guanine nucleotide-binding protein beta subunit 1; LTP, long-term potentiation; MALDI-TOF, matrix-assisted laser desorption/ionization-time of flight; MAPK, mitogen-activated protein kinase; NIS, neuronal information storage; NMDA, *N*-methyl-D-aspartate; NMDAR, NMDA receptor; OF, OF1; OGP, β-D-glucopyranoside; PMF, peptide mass fingerprint; PP, protein phosphatase; PP1, protein phosphatase 1; PP1-beta, serine/threonine protein phosphatase 1, beta catalytic subunit; PP2A, protein phosphatase 2A; RACK1, receptor of activated protein kinase C 1; VDAC, voltage-dependent anion channel; WAVE-1 protein, WASP-family protein member 1; 129Sv, 129S2/Sv; 2-DE, two-dimensional gel electrophoresis.

0306-4522/06$30.00+0.00 © 2005 Published by Elsevier Ltd on behalf of IBRO.
doi:10.1016/j.neuroscience.2005.11.004

Individual inbred mouse strains differ from each other in behavior, learning and memory (Crawley et al., 1997; Upchurch and Wehner, 1989), long-term potentiation (LTP), a type of synaptic plasticity that may underlie some forms of learning and memory (Nguyen et al., 2000; Gerlai, 2002) and hippocampal gene expression (Fernandes et al., 2004). Therefore, results obtained from cognitive and behavioral studies using one specific strain cannot be simply extrapolated or compared. The actual situation is even more complex: the use of genetically engineered mice for such studies may be hampered by their genetic background, harboring genomes from several mouse strains and problems with interpretation of results may arise if the background is not corrected by appropriate controls. In addition, knowledge on the "protein chemical background" tentatively underlying synaptic plasticity and neuronal information storage (NIS), in the individual strains is limited (e.g. Fordyce et al., 1994; Bowers et al., 1995). The importance for addressing strain distribution at the proteome level is further underscored by the difficulty in predicting protein characteristics from genomic sequence data alone. These characteristics include posttranslational modifications, subcellular distribution, stability, biomolecular interactions and function. Notably, several diverse mechanisms can result in many protein variants from the same locus in one species: single nucleotide polymorphisms, gene splicing, alternative splicing of pre-mRNA, RNA editing, translational frame shifts and posttranslational modifications.

It has become clear that LTP and long-term depression (LTD) have many forms which can be distinguished on the basis of their underlying signaling mechanisms (Grant and O'Dell, 2001).

Synaptic plasticity induced following *N*-methyl-D-aspartate (NMDA)-type glutamate receptor activation and signaling cascades involved therein is well-studied. Recognition that NMDAR channels are highly permeable to Ca^{2+} led to discovery that Ca^{2+} dependent signaling molecules, such as protein kinase C and Ca^{2+}/calmodulin kinase II (CaMKII) may play an essential role for synaptic plasticity and NIS. The findings that tyrosine kinase activity is also required for LTP induction (Grant and O'Dell, 2001) and that mitogen-activated protein kinase (MAPK) is activated by NMDAR raise new questions over the complexity of signaling pathways involved in NIS. Since more than 100 molecules have been implicated in LTP-induced synaptic plasticity (Sanes and Lichtman, 1999) it is challenging to understand the relationships and composition of these signaling networks (Grant and O'Dell, 2001). Recently proteomics has been proven a valuable tool for the analysis of signaling proteins specifically with regard to their proposed

role in mediating activity-dependent synaptic plasticity (Yamauchi, 2002; Collins et al., 2005). Although a vast variety of signaling proteins and their relevance for NIS have been studied so far, strain-specific expression of these structures has not yet been systematically evaluated and we therefore decided to carry out the present study.

The aim of this study was to complement the currently available gene expression data at the protein level by focusing on proteins representing candidate molecules involved in neuronal processes underlying basic brain functions. A systematic comparison of wild-type strains should provide some insight into the regulation of basic physiological brain processes as well as the mechanisms potentially contributing to strain-specific phenotypes. Four inbred strains (FVB/N, C57Bl/6J, 129S2/Sv and Balb/c), commonly used for generating genetically modified mice and for conventional experiments in pharmacology and toxicology and one outbred strain (OF1) were selected for studying the expression of signaling proteins in the hippocampus, a region know to be essential for NIS and storage. We have taken this approach further by carefully constructing a data set of hippocampal protein expression profiles specific for a defined brain function, providing a comprehensive tool for candidate protein identification in behavior. Genetically driven expression differences detected from the study can then be related to phenotypic differences among these inbred strains of mice, thus nominating candidate genes that are functional in the sense that they yield expression differences between the strains (Fernandes et al., 2004), although much larger panels of inbred strains and a greater set of proteins will be needed to definitely relate variability in brain protein expression to function.

We herein aimed to determine a "*signaling protein phenotype*" of the individual strains, thus contributing to information available at the behavioral, electrophysiological and mRNA level. Additionally, we attempted generation of a first signaling proteins reference database from hippocampi of individual mouse strains using an analytical tool for the concomitant determination of a large series of signaling structures by a protein-chemical method independent of antibody availability and specificity.

EXPERIMENTAL PROCEDURES

Animals

The subjects were FVB/NHim (FVB), C57BL/6JHim (C57), 129S2/SvHim (129Sv), Balb/cHim (Balb) and Him:OF1 (OF) ($n=10$ for each strain) male, adult mice, approx. 20 weeks old. Animals were housed in standard transparent laboratory cages in a temperature-controlled colony room (22±1 °C). They were maintained on a 12 h artificial light/dark cycle (with lights on at 6:00 A.M.) and provided with food and water *ad libitum*. Mice were killed by neck dislocation and hippocampi were dissected. Tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C until used for analysis. All animal experiments were carried out in accordance with the European Community Council Directive (96/609/EEC) on animal welfare and approved by the local animal committee (confirmation number: LF1-TVG-17/002-2004). All efforts were made to minimize animal suffering and the number of animals used.

Sample preparation

Hippocampal tissue was powdered and resuspended in 1.0 ml of sample buffer consisting of 7 M urea (Merck, Darmstadt, Germany), 2 M thiourea (Sigma, St. Louis, MO, USA), 4% CHAPS (3-[(3-cholamidopropyl) dimethylammonio]-1-propane-sulfonate) (Sigma), 65 mM DTT (1,4-dithioerythritol; Merck), 1 mM EDTA (Merck), protease inhibitors completeâ (Roche, Basel, Switzerland) and 1 mM phenylmethylsulfonyl chloride. The suspension was sonicated for approximately 15 s. After homogenization samples were left at room temperature for 1 h and centrifuged at 14,000 r.p.m. for 1 h. The supernatant was transferred into Ultrafree-4 centrifugal filter unit (Millipore, Bedford, MA, USA), for desalting and concentrating proteins. Protein content of the supernatant was aimed at determining by Bradford protein assay system (Bradford, 1976). The standard curve was generated using bovine serum albumin and absorbance was measured at 595 nm.

Two-dimensional gel electrophoresis (2-DE)

Samples prepared from each mouse individual mouse ($n=10$ per strain) were subjected to 2-DE as described elsewhere (Weitzdoerfer et al., 2002). Seven hundred micrograms protein was applied on immobilized pH 3–10 nonlinear gradient strips at their basic and acidic ends. Focusing was started at 200 V and voltage was gradually increased to 8000 V over 31 h and then kept constant for a further 3 h (approximately 150,000 Vh totally). After the first dimension, strips (18 cm) were equilibrated for 15 min in the buffer containing 6 M urea, 20% glycerol, 2% SDS, 2% DTT and then for 15 min in the same buffer containing 2.5% iodoacetamide instead of DDT. After equilibration, strips were loaded on 9–16% gradient sodium dodecylsulfate polyacrylamide gels for second-dimensional separation. Gels (180×200×1.5 mm) were run at 40 mA per gel. Immediately after the second dimension run gels were fixed for 18 h in 50% methanol, containing 10% acetic acid, the gels were then stained with Colloidal Coomassie Blue (Novex, San Diego, CA, USA) for 12 h on a rocking shaker. Molecular masses were determined by running standard protein markers (Biorad Laboratories, Hercules, CA, USA) covering the range 10–250 kDa. pI values 3–10 were used as given by the supplier of the immobilized pH gradient strips (Amersham Bioscience, Uppsala, Sweden). Excess dye was washed out from the gels with distilled water and the gels were scanned with ImageScanner (Amersham Bioscience).

Electronic images of the gels were recorded using Adobe Photoshop (Adobe Systems Inc., San Jose, CA, USA) and Microsoft PowerPoint (Microsoft Corp., Redmond, WA, USA) software.

Matrix-assisted laser desorption ionization mass spectrometry

Spots (3×384) each were randomly picked and previous work on hippocampal signaling proteins protein expression was respected (Shin et al., 2004, 2005; Fountoulakis et al., 2005). Spots were excised with a spot picker (PROTEINEER sp™, Bruker Daltonics, Bremen, Germany), placed into 96-well microtiter plates and in-gel digestion and sample preparation for MALDI analysis were performed by an automated procedure (PROTEINEER dp™, Bruker Daltonics) (Suckau et al., 2003; Yang et al., 2004). Briefly, spots were excised and washed with 10 mM ammonium bicarbonate and 50% acetonitrile in 10 mM ammonium bicarbonate. After washing, gel plugs were shrunk by addition of acetonitrile and dried by blowing out the liquid through the pierced well bottom. The dried gel pieces were reswollen with 40 ng/μl trypsin (Promega, Madison, WI, USA) in enzyme buffer (consisting of 5 mM octyl β-D-glucopyranoside (OGP) and 10 mM ammonium bicarbonate) and incubated for 4 h at 30 °C. Peptide extraction was performed with 10 μl of 1% TFA in 5 mM OGP. Extracted peptides were directly applied onto a target (AnchorChip™, Bruker Dalton-