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Fluctuations of hippocampal neuronal protein levels over the estrous cycle in the rat

Wei-Fei Diao [a], Wei-Qiang Chen [a], Harald Höger [b], Arnold Pollak [a], Gert Lubec [a,*]

[a] Department of Pediatrics, Medical University of Vienna, Waehringer Guertel 18, A-1090 Vienna, Austria
[b] Institute for Animal Genetics, Medical University of Vienna, Brauhausgasse 34, A-2325 Himberg, Austria

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Abstract

Hippocampal function is known to be estrous-cycle-dependent but information on estrous-cycle-dependent protein expression is limited. It was therefore the aim to study protein levels of the neuronal network over the estrous cycle in the hippocampus of female rats and in males showing protein chemical neuroanatomy in this area. Female and male OFA Sprague–Dawley rats were used and females were grouped to proestrous, estrous, metestrous and diestrous by using vaginal smears. Hippocampal tissue was taken, proteins extracted, run on two-dimensional gel electrophoresis and proteins were identified by mass spectrometry methods (MALDI-TOF-TOF and nano-LC-ESI-MS/MS). Spot volumes were quantified with specific software.

A Synapsin-1 expression form was differentially regulated between proestrous and diestrous, a Synapsin IIa expression form was differentially regulated between proestrous and metestrous, the sum of ERC-2 proteins organizing the cytomatrix at the nerve terminals active zone was showing sex-dependent levels in the proestrous phase and Neurofilament triplet L protein was differentially expressed between the estrous phase and males.

The findings may represent estrous-cycle-dependent hippocampal synaptic function that has been shown already in terms of electrophysiology and neuroanatomy. Neurofilament changes over the estrous cycle may reflect endoskeleton changes over the estrous cycle.

We learn from this study, although increasing complexity of protein knowledge, that the estrous cycle and not only the sex per se has to be taken into account for design of future studies and interpretation of previous work at the protein level.

Keywords: Neuronal; Estrous cycle; Hippocampus; Mass spectrometry; Synapse

Hippocampus biochemistry, morphology and function vary across the female estrous cycle (EC) as well as between sexes. Proestrous presents with increased dendritic spine density and numbers as revealed by histological investigations (Cooke and Woolley, 2005; Woolley and McEwen, 1992). Estrogen has beneficial effects on hippocampal synaptic plasticity in vivo and in vitro (Woolley, 1998), and increases performance in different hippocampus-dependent tests for cognitive function (Korol and Kolo, 2002; Sandstrom and Williams, 2001; Wood et al., 2001), an observation in agreement with human verbal and spatial performance (Halpern and Tan, 2001). However, controversial results were obtained in different behavioral tasks (Berry et al., 1997; Stackman et al., 1997; Warren and Juraska, 1997) and may be the consequence of different molecular mechanisms underlying different types of tasks (Mizuno and Giese, 2005) or due to concomitant changes of estrogen, progesterone and other sex hormones over the EC (Chesler and Juraska, 2000). Effects of gonadal hormones on expression of genes involved in synaptic modeling/remodeling have been reported at the transcriptional level (Devidze et al., 2005) over the estrous cycle (Adams et al., 2001; Crispino et al., 1999).

Synaptic communication requires an efficient synaptic-vesicle cycle, including vesicle biogenesis, transport and interaction with the cytoskeleton, uptake and storage of neurotransmitters, and regulated endocytosis and exocytosis (Jahn, 2004; Sudhof, 2004). These processes are governed by vesicle-associated or integral membrane proteins. Numerous presynaptic proteins have been identified (for reviews: Royle and Lagnado, 2003; Sudhof, 2000) including those involved in vesicle biogenesis, vesicle fusion. Proper functioning of the nervous system requires precise control of neurotransmitter release and since synaptic vesicles are loaded with neuro-

* Corresponding author. Tel.: +43 1 40 400 3215; fax: +43 1 40 400 6065.
E-mail address: gert.lubec@meduniwien.ac.at (G. Lubec).

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transmitters, much endeavor has been made to describe the effect of some individual synaptic proteins on neurotransmission and hence synaptic plasticity. Changes in synaptic interconnections among neurons, such as regulation of number of synaptic boutons and pool size of synaptic vesicles, are established to characterize the mechanism of synaptic plasticity. It has been well documented that expression alterations of various presynaptic proteins involved in synaptic function and homeostasis occur in several neurological and psychiatric disorders accompanied with cognitive deficits (Mirnics et al., 2001; Mirnics et al., 2000; Virmani et al., 2005).

To the best of our knowledge systematic studies on protein profiling over the EC of neuronal network-related proteins have not been published so far thus forming the rationale for the current study. It was therefore the aim of this work to address potential fluctuations of these corresponding hippocampal proteins over the EC and to search for sex-related changes.

1. Experimental procedures

1.1. Materials

Immobilized pH-gradient (IPG) strips and IPG buffers were purchased from Amersham Biosciences, part of GE Healthcare. Acrylamide/piperazine-diacrylamide (PDA) and the other reagents for the polyacrylamide gel preparation were purchased from Bio-Rad Laboratories (Hercules, CA, USA). CHAPS was obtained from Roche Diagnostics (Mannheim, Germany), urea from AppliChem (Darmstadt, Germany), thiourea from Fluka (Buchs, Switzerland), 1,4-dithioerythritol (DTE) and EDTA from Merck (Darmstadt, Germany), and tributylphosphine (TBP) from Pierce Biotechnology (Rockford, IL, USA).

1.2. Animals

Eight to 10 weeks old male ($n = 10$) and female ($n = 50$) OFA Sprague–Dawley rats were used. They were bred and maintained in an animal barrier facility in Makrolon type IV cages with woodchip bedding. A standard rodent diet and water from automatic valves were available ad libitum. Lights were on from 5:00 to 19:00. State of the estrous cycle in females was detected by daily vaginal smears, taken between 9:00 and 11:00. A small cotton tip moistened with water was inserted into the vagina and smeared on a microscopic slide. After fixation with methanol and staining with Giemsa, stain cells were examined under a microscope. By the appearance of vaginal smears the female rats were assigned to five phases (Baker, 1979; Maeda et al., 2000), proestrous (PE), estrous (E), early metestrous (ME1), late metestrous (ME2) and diestrous (DE). Only rats showing at least two regular 4–5 days cycles were used for the study. After euthanasia with CO_2 dissection and removal of brain was done between 10:00 and 12:00. Hippocampal tissues were dissected, frozen immediately in liquid nitrogen and stored at −80 °C until use and the freezing chain was never interrupted. Experiments were performed according to the EU guidelines for animal experiments.

1.3. Sample preparation

Individual rat hippocampus was powderised in liquid nitrogen and suspended in 2 ml sample buffer and processed as given previously (Myung and Lubec, 2006). The protein content of the supernatant was determined by the Bradford assay (Bradford, 1976).

1.4. Two-dimensional gel electrophoresis (2-DE)

2-DE was performed essentially as reported (Yang et al., 2004). Samples of 750 μg protein were applied on immobilized pH 3–10 nonlinear gradient strips. Focusing started at 200 V and the voltage was gradually increased to 8000 V at 4 V/min and kept constant for a further 3 h (approximately 150,000 V h totally). The second-dimensional separation was performed on 10–16% gradient SDS-PAGE. After protein fixation for 12 h in 50% methanol and 10% acetic acid, the gels were stained with colloidal Coomassie blue (Novex, San Diego, CA, USA) for 8 h and excess of dye was washed out from the gels with distilled water. Molecular masses were determined by running standard protein markers (Bio-Rad Laboratories, Hercules, CA, USA), covering the range of 10–250 kDa. Isoelectric point values were used as given by the supplier of the immobilized pH gradient strips.

1.5. In-gel digestion

Spots were excised with a spot picker (PROTEINEER sp™, Bruker Daltonics, Leipzig, Germany), placed into 96-well microtiter plates (Bruker Daltonics, Leipzig, Germany) and in-gel digestion and sample preparation for MALDI analysis were performed by an automated procedure (PROTEINEER dp™, Bruker Daltonics) (Gulesserian et al., 2007).

1.6. MALDI-TOF-TOF mass spectrometry and data processing

A target (AnchorChip™, Bruker Daltonics, Bremen, Germany) was wiped using paper tissues in sequence with acetone and *N*-heptane, followed by ultrasonicated in isopropanol and then in HPLC grade water, and dried in air. Four microliter extracted peptides were directly applied onto the target that was loaded with α-cyano-4-hydroxy-cinnamic acid (Bruker Daltonics, Bremen, Germany) matrix thinlayer. The mass spectrometer used in this work was an Ultraflex™ TOF/TOF (Bruker Daltonics, Bremen, Germany) operated in the reflector for MALDI-TOF peptide mass fingerprint (PMF) or LIFT mode for MALDI-TOF-TOF MS/MS with a fully automated mode using the FlexControl™ software. Samples were analyzed by one PMF from MALDI-TOF, followed by additional LIFT-TOF/TOF MS/MS analysis of three peptides. Data were accumulated from 200 consecutive laser shots to produce PMF and MS/MS spectra. Peptide standard was used as external calibration. The *m/z* range is 700–4000 for PMF and 40–2560 for MS/MS. PMF and MS/MS spectra were interpreted primarily with the FlexAnalysis™ software (Bruker Daltonics, Bremen, Germany). Signal-to-noise ratio threshold was set as three. Autoproteolysis products of trypsin were used as internal calibration. Database searches, through the MASCOT 2.1 (Matrix Science, London, UK) against MSDB 20051115 database, using combined one PMF and three MS/MS datasets were performed via BioTools 2.3 software (Bruker Daltonics, Bremen, Germany). Species were limited to rodents. Enzyme was limited to trypsin with one maximum missing cleavage site. A mass tolerance of 25 ppm for PMF, MS/MS tolerance of 0.5 Da, fix modification of carbamidomethyl(C), and variable modification of oxidation of methionine and phosphorylation (Tyr, Thr, and Ser) were considered. A randomized MSDB database (http://www.matrixscience.com/help/decoy_help.html) was searched simultaneously and false positive results were omitted. Protein identification returned from MASCOT were manually examined and filtered to create a confirmed protein identification list. Positive protein identifications were based on a significant MOWSE score.

1.7. Analysis of peptides by nano-LC-ESI-MS/MS and data processing

Spots that were not identified by MALDI-TOF-TOF were analyzed by a second mass spectrometrical approach (Chen et al., 2006). Database search was performed as above.

1.8. Quantification

Protein spots representing neuronal proteins from all gels (10 per group; total $n = 60$) were outlined and matched, first automatically and then manually, and quantified using the Proteomweaver software (Definiens, Munich, Germany). The level of each protein spot volume was represented by its intensity in the 2-DE gel (Frischer et al., 2006).