Published in final edited form as:

J Chem Neuroanat. 2014 January ; 55: 9–17. doi:10.1016/j.jchemneu.2013.10.004.

HIPPOCAMPAL MOSSY FIBER LEU-ENKEPHALIN IMMUNOREACTIVITY IN FEMALE RATS IS SIGNIFICANTLY ALTERED FOLLOWING BOTH ACUTE AND CHRONIC STRESS

Joseph P. Pierce¹, David T. Kelter¹, Bruce S. McEwen², Elizabeth M. Waters², and Teresa A. Milner^{1,2,*}

¹Brain and Mind Research Institute Weill Cornell Medical College 407 East 61st Street New York, NY 10065

²Harold and Margaret Milliken Hatch Laboratory of Neuroendocrinology The Rockefeller University 1230 York Avenue New York, NY 10065

Abstract

Research indicates that responses to stress are sexually dimorphic, particularly in regard to learning and memory processes: while males display impaired cognitive performance and hippocampal CA3 pyramidal cell dendritic remodeling following chronic stress, females exhibit enhanced performance and no remodeling. Leu-enkephalin, an endogenous opioid peptide found in the hippocampal mossy fiber pathway, plays a critical role in mediating synaptic plasticity at the mossy fiber-CA3 pyramidal cell synapse. Estrogen is known to influence the expression of leu-enkephalin in the mossy fibers of females, with leu-enkephalin levels being highest at proestrus and estrus, when estrogen levels are elevated. Since stress is also known to alter the expression of leu-enkephalin in various brain regions, this study was designed to determine whether acute or chronic stress had an effect on mossy fiber leu-enkephalin levels in females or males, through the application of correlated quantitative light and electron microscopic immunocytochemistry. Both acute and chronic stress eliminated the estrogen-dependence of leu-enkephalin levels across the estrous cycle in females, but had no effect on male levels. However, following acute stress leu-enkephalin levels in females were consistently lowered to values comparable to the lowest control values, while following chronic stress they were consistently elevated to values comparable to the highest control values. Ultrastructural changes in leu-enkephalin labeled dense core vesicles paralleled light microscopic observations, with acute stress inducing a decrease in leu-enkephalin labeled dense core vesicles, and chronic stress inducing an increase in leu-enkephalin labeled dense-core vesicles in females. These findings suggest that alterations in leu-enkephalin levels following stress could play an important role in the sex-

© 2013 Elsevier B.V. All rights reserved.

*Address correspondence to: Dr. Teresa A. Milner Brain and Mind Research Institute Weill Cornell Medical College 407 East 61st Street, RM 307 New York, NY 10065 Phone: (646) 962-8274 FAX: (646) 962-0535 tmlner@med.cornell.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

specific responses that females display in learning processes, including those important in addiction.

Keywords

Stress; hippocampus; female; leu-enkephalin; estrogen; immunohistochemistry; opioids; sex differences; estrogens; chronic stress; acute stress; addiction

1. Introduction

Accumulating evidence indicates that responses to stress are sexually dimorphic, especially in regard to learning and memory processes (reviewed in (Beck and Luine, 2010; Luine et al., 2007; McEwen and Milner, 2007). Chronic stress induces drastically differing, sex-specific effects on both learning and memory tasks, and on the structure of the hippocampal formation (HF), an essential region for such processes. While males display impaired cognitive performance, a pronounced remodeling of CA3 pyramidal cell dendrites, particularly CA3b, and a loss of parvalbumin (PARV)-labeled interneurons (Luine et al., 1998; McEwen, 1999; Sousa et al., 2000), females exhibit enhanced performance on the same memory tasks, no apparent dendritic remodeling or loss of PARV interneurons (Conrad et al., 2003; Galea et al., 1997; Milner et al., 2013). Estrogen probably plays an important role in these distinct stress responses. Ovarian hormones are known to influence hippocampal structure and activity (McEwen and Alves, 1999), and ovariectomized (OVX) female rats perform better on the radial arm maze test following chronic stress after estrogen is added back (Bowman et al., 2002). The effects of acute stress appear more variable. While similar responses from males and females have often been observed following some acute stress paradigms (Park et al., 2008; Uysal et al., 2012), others have yielded sexually dimorphic responses (Beck and Luine, 2010). Sexually dimorphic responses to stress could play an important role in behaviors which are influenced by sex, such as drug addiction: during stressful events, women are more likely to experience craving following exposure to drug-related cues (Robbins et al., 1999), a process that directly involves the HF (Risinger et al., 1995).

Endogenous opioid peptides are critical central mediators of synaptic plasticity within the HF, and could thus play an important role in subserving stress-induced changes. The rat HF contains two known opioid peptide families, the enkephalins and the dynorphins, and mu, delta and kappa opioid receptors [reviewed in (Drake et al., 2007)]. Within the CA3 subregion, both the mossy fiber (MF) pathway (projecting from dentate gyrus granule cells) and the lateral perforant path (LPP; projecting from the entorhinal cortex) contain leu-enkephalin (LENK) (Drake et al., 2007). These pathways converge onto CA3 pyramidal cells, and both some forms of MF-CA3 pyramidal cell synaptic long-term potentiation (LTP), and LPP-CA3 pyramidal cell synaptic LTP are LENK-dependent, requiring the activation of mu opioid receptors (MORs) (Derrick et al., 1992; Do et al., 2002). Additionally, ENKs have a high affinity for delta opioid receptors (DORs) (Corbett et al., 1993). In female rats, estrogen influences LENK expression: levels of LENK within the MF system, particularly CA3b, vary significantly across the estrous cycle, with high levels being

observed in proestrus and estrus, when estrogen levels are highest (Torres-Reveron et al., 2008). These cyclic elevations in LENK levels occur concurrently with changes in the subcellular distributions of MORs and DORs in select neuronal populations (Torres-Reveron et al., 2009; Williams et al., 2011).

Stress is known to alter the levels of opioid peptides in various brain regions (Drolet et al., 2001), and it can induce the release of LENK in the ventral tegmental area (Kalivas and Abhold, 1987) and dynorphins in many brain areas (Bruchas et al., 2008; McLaughlin et al., 2003). However, whether stress, administered either acutely or chronically, can affect LENK levels within the MF pathway has not been explored. The aim of the present study was to determine this, and examine whether any observed changes were sex-specific. To this end, LENK immunoreactive staining patterns in the MF pathway in the CA3b subregion of the HF of cycled female rats and males were first identified and then quantified, by both light microscopy (LM) and electron microscopy (EM).

2. Materials & Methods

2.2 Animals

All procedures were approved by the Rockefeller University and Weill Cornell Medical College Institutional Animal Care and Use Committees and were in accordance with the 2011 Eighth edition of the National Institutes of Health guidelines for the Care and Use of Laboratory Animals. Adult male and female Sprague Dawley rats (~60 d old, N = 90) were obtained from Charles River Laboratories (Wilmington, MA). Same sex rats were housed 3-4 animals per cage with 12:12 light/dark cycles and free access food and water ad libitum. Rats within a single cage were euthanized on the same day so that no rat was housed alone.

2.3 Estrous cycle determination

Only female rats that showed two consecutive, regular 4-5 day estrous cycles prior to initiation of the stressor were used. Estrous cycle stage was determined using vaginal smear cytology (Turner and Bagnara, 1971) daily between 9:00 and 10:00 AM, following one week of acclimation following arrival. To control for the effects of handling, male rats were removed from their cages daily. Blood was collected from the heart immediately before the perfusion fixation and uteri are collected from the fixed rats. Uterine weight and plasma serum estradiol levels via radioimmunoassay were measured as described previously (Torres-Reveron et al., 2008) to confirm estrous cycle stage. Diestrus 2 rats rather than metestrus (diestrus I) were chosen to insure that rats were completely out of the estrus stage. The tissue used in this study was obtained from rats used in our previous studies (Burstein et al., 2013; Gonzales et al., 2011; Milner et al., 2013).

2.4 Immobilization stress procedures

Rats that were to receive acute immobilization stress (AIS) were transported from their home room into a procedure room, and AIS was performed as previously described (Lucas et al., 2007; Shansky et al., 2010), between 9:00 AM and 1:00 PM. Rats were placed in plastic cone shaped polyethylene bags with a Kotex mini-pad underneath them to collect urine and small hole at the apex of the cone. Rats were placed with their nose at the hole,

sealed with tape in the bag, and left undisturbed on the countertop for 30 minutes. Immediately after, rats were deeply anesthetized, transported to a neighboring procedure room and their brains fixed via perfusion (described below). Control rats were left in the home-room and anesthetized prior to transfer to the procedure room for perfusion. Rats receiving chronic immobilization stress (CIS) were subjected to AIS as described above for 10 consecutive days. Both CIS and control rats were pair housed in cages. During the stress paradigm, CIS rats were housed in a separate room, and were killed 1 day after the last AIS period. Rats in the CIS group had normal estrous cycles (Milner et al., 2013).

2.5 Tissue preparation

Matched pairs of experimental and control animals were perfused and processed with the same batches of fixatives and buffers. Each animal was first deeply anesthetized with sodium pentobarbital (150 mg/kg, i.p.) and then perfused through the ascending aorta sequentially with solutions of: (1) 10–15 ml of normal saline containing 1,000 U/ml of heparin, (2) 55 ml of 3.75% acrolein (Polysciences, Warrington, PA), and 2% paraformaldehyde in 0.1 M phosphate buffer (PB; pH 7.4), and (3) 200 ml of 2% paraformaldehyde in PB. The portion of the forebrain containing the HF (from –2.0 to –6.65 mm from Bregma) (Swanson, 1992) was then removed as a roughly 5-mm-thick coronal block that was further incubated in the latter fixative for an additional 30 min. Sections (40 μ m thick) through the entire block were cut on a Vibratome (yielding on average 120 sections), collected in 0.1M PB, and transferred serially to tissue culture trays containing a solution (30% sucrose and 10% ethylene glycol in 0.1 M PB) for storage at –25° C. For a detailed description of the tissue preparation, immunocytochemical and analysis procedures used, refer to Milner et al. (Milner et al., 2011).

2.6 Immunocytochemistry

Based on stereological principles, to ensure unbiased sampling, a random systematic series of sections (1:24) through the HF of each animal was selected for LM and EM immunocytochemistry (yielding on average 5 sections). To provide uniform labeling conditions in preparation for making quantitative comparisons (Auchus and Pickel, 1992), sections from control and experimental brains were first punched to allow identification and pooled into single containers prior to processing to immunoperoxidase label LENK. For this purpose, a mouse monoclonal antibody to leu⁵-enkephalin from Sera Labs (Crawley Down, UK), which has been extensively characterized and used previously to immunolabel the MF system (Commons and Milner, 1995; Drake et al., 2002; Milner et al., 1989; Torres-Reveron et al., 2008; Van Kempen et al., 2013) was employed. The immunolabeling procedure first involved incubating pooled tissue in a 1% sodium borohydride solution in PB for 30 min, extensive rinsing, followed by incubation in a 0.5% bovine serum albumin (BSA) solution for 30 min, and further rinsing. Sections were then placed in the primary LENK antibody (diluted 1:1000) in 0.025% Triton-X 100 and 0.1% BSA/Tris saline (TS), for 24 h at RT, followed by 24 h in a cold room. Processes containing LENK then were immunolabeled with the avidin–biotin–peroxidase complex (ABC) method (Hsu et al., 1981), using the following incubations separated by rinses: (a) a 1:400 dilution of horse anti-mouse biotinylated-IgG in 0.1% BSA/TS, 30 min (Jackson ImmunoResearch, West Grove, PA, USA), (b) a 1:100 dilution of ABC in 0.05% BSA/TS (Vectastain Elite Kit, Vector

Laboratories, Burlingame, CA, USA), 30 min, (c) 0.022% 3,3'-diaminobenzidine and 0.003% H₂O₂ in TS, 8 min, and (d) a PB wash, 10 min.

Pooled tissue sections were first sorted by animal, and then subdivided into dorsal and ventral sections based on anatomical landmarks using atlas coordinates as a guide (Swanson, 1992) (yielding 2 to 3 sections per subgroup). Within each subgroup, sections were numbered, and a random number generator was used (to maintain unbiased selection) to select one dorsal and one ventral section for light microscopic analysis, and one dorsal section for electron microscopic analysis (taken from roughly AP -2.45 and AP -3.90 from Bregma). Sections destined for LM analysis were transferred to 0.05 PB and mounted on acid-cleaned slides coated with 1% gelatin. The slides then were air dried, dehydrated through a series of alcohols and xylene, and coverslipped with DPX (Aldrich Chemical, Milwaukee, WI). Sections destined for EM analysis underwent: (a) a PB wash, 5 min, (b) postfixation in 2% osmium, 1 h, (c) dehydration in a series of graded alcohols and propylene oxide, (d) 1:1 Embed 812 (Electron Microscopy Sciences Inc.) and propylene oxide, 12 h, (e) 100% Embed 812, 2 h, (f) flat-embedding between Aclar film (Allied Signal, Pottsville, PA, USA), and (g) polymerization at 60 °C, 72 h.

2.7 LM Analysis

For each dorsal and ventral hippocampal section, a portion of the MF pathway within the CA3b subregion (Lorento de No, 1934) was identified (see Fig. 1A and B, in which the rectangular insets denote the analyzed portion of the CA3b subregion), and the staining pattern and labeling intensity was captured with a 4x objective (the final image being the average of 10 successively captured images) using a Dage MTI CCD-72 camera and control unit (with preset gain and black levels), a Nikon Eclipse 80i microscope and MicroComputer Imaging Device (MCID; Linton, UK) software. Slides were coded for blind analysis. To compensate for uneven illumination across the field, blank fields taken from a slide without tissue were subtracted. For each captured image, average pixel densities (out of 256 gray levels) were then determined using NIH ImageJ software for the MF pathway within CA3b, yielding a dorsal and ventral value per animal. To compensate for background staining levels and to control for variations in the overall illumination level between images, within each captured image, the average pixel density for five small regions that presumably only contained nonspecific staining was determined (such as portions of CA1, which do not contain LENK (Gall et al., 1981)), and this value was subtracted from all density measurements made on that image. The accuracy of the hardware and software was tested by determining pixel densities for captured images of neutral density gelatin filters with defined transmittances ranging from 1 to 80% (Eastman Kodak Co., Rochester, NY), yielding a strong linear correlation between average pixel densities and actual transmittance values ($r = 0.998$). For statistical analysis dorsal and ventral values were pooled. It should be noted that the use of the ABC/peroxidase labeling approach can potentially contribute to a non-linear relationship between antigen and reaction product levels. Correlated quantitative EM analysis of the same fields from the same animals therefore was conducted to provide additional insight into any observed changes.

2.8 EM Analysis

For each flat-embedded dorsal hippocampal section, the same portion of the MF pathway within CA3b analyzed by LM was excised, glued onto a plastic block, trimmed to a trapezoidal pyramid (approximately 1.2×0.8 mm), and thin-sectioned (65 nm) on an RMC ultratome. Sections were collected directly on 400-mesh copper thin bar grids (Electron Microscopy Sciences, Fort Washington, PA). Blocks were coded for blind analysis. Thin sections were collected on thin bar copper grids, counterstained with uranyl acetate and Reynolds' lead citrate prior to examination on a Tecnai electron microscope (FEI Company, Hillsboro, OR, USA) equipped with an Advanced Microscopy Techniques digital camera (Danvers, PA, USA). Captured EM images (at 123x) of selected thin sections were then compared to captured LM images of the blockface before sectioning, allowing one to map the borders of the MF pathway within given thin sections. Since the extent of immunocytochemical labeling decreases with distance from the tissue surface, only grid squares within the MF pathway displaying no obvious tissue damage, but that were adjacent to the Embed812/tissue interface, were selected for analysis (Milner et al., 2011). For each animal/block, two thin sections were selected for analysis, and for each thin section, two to three such complete grid squares ($3025 \mu\text{m}^2$ per square) were selected at low magnification, on the basis of the positional criteria described, randomly, before visual inspection of the grid square field at a higher magnification.

Within each field, LENK-labeling was invariably associated with dense core vesicles (DCVs) or presynaptic regions immediately adjacent to DCVs, as previously reported (Thureson-Klein and Klein, 1990). In this context, any DCV that displayed distinct labeling over at least the central core was considered labeled (see Fig. 3). Labeled DCVs (IDCVs) were observed almost exclusively in either axonal or terminal profiles (identified by standard criteria (Peters et al., 1991)). Briefly, terminal profiles contain small clear and larger DCVs and form presynaptic specializations with dendritic profiles, and axonal profiles (in this case unmyelinated axonal profiles) are small and thin (usually $0.5 \mu\text{m}$ or less in diameter) and often also contain a few vesicles. MF terminal profiles are distinct in terms of both their large size and the complexity of their form, which often envelopes spinous profiles (Blackstad and Kjaerheim, 1961). For quantification, each grid square field was first completely examined at high magnification to generate a tally of the total number of IDCVs in axonal profiles within the field. IDCVs also were occasionally observed in small, apparent *en passant* terminals, but since these were infrequent, rarely displayed active zones in the plane of section, and contained little morphometric detail, they were included in the axonal tally. Second, images of all MF terminal profiles containing at least 1 IDCV within the field were captured at 23,000x for morphometric analysis. Each labeled MF terminal profile was then traced, examined, and characterized in terms of: 1) cross-sectional area (in μm^2), 2) perimeter length (in μm), 3) the number of LENK IDCVs, 4) the number of unlabeled DCVs (unIDCVs), 5) the number of active zones on the profile membrane and 6) the number of spine profiles apposed to the MF terminal (both those along the outer perimeter and those seen 'within' the MF terminal in the plane of section examined). Active zones and puncta adhaerentia were distinguished based on the criteria of Peters et al. (Peters et al., 1991), although in the identification of puncta adhaerentia, a greater emphasis was placed on the presence of a thick "presynaptic" cytoplasmic density than on the absence of associated

clear vesicles, since many structures that appeared to be active zones also were associated with relatively few clear vesicles. Profile areas and perimeter lengths were determined from the tracings with NIH ImageJ.

Statistical Analysis and Figures Values are presented in the text and figures in the form: mean $\pm$ standard error of the mean. Statistical relationships were examined with t tests, analysis of variance (ANOVA) with Tukey's post hoc tests, and bivariate plots (implemented through JMP software (SAS, Cary, NC)). Figures were generated using Photoshop (Adobe Inc.).

3. Results

3.1 Light and EM Labeling for LENK

As reported in previous LM studies in males (Chavkin et al., 1985; Gall et al., 1981) and females (Torres-Reveron et al., 2008), diffuse LENK immunolabeling was present in the entire MF pathway, both within the hilus and throughout CA3c, with the intensity of the labeling visibly increased at ventral levels of the HF (Fig. 1). Additionally, very light diffuse LENK-labeling was observed within the LPP in the dentate gyrus and CA3, which was also more prominent at ventral levels. Since previous studies in males have shown that stress significantly affects dendrites in CA3b (McEwen, 2000) and that LENK levels in CA3b are sensitive to fluctuating estrogen levels (Torres-Reveron et al., 2008), this region was chosen for EM analysis as well as densitometric analysis after stress (Fig. 1A, inset).

Ultrastructural examination of the CA3b revealed that the LENK-immunoperoxidase labeling was restricted to specific DCVs within presynaptic processes (Fig. 3). The LENK-immunoperoxidase labeling was usually focused over the core of the DCV, with lower levels found both around the core and in surrounding areas of the cytoplasm of the process (presumably due to leakage of LENK from the DCV during tissue processing and/or the diffusability of the peroxidase reaction product). Notably, clearly unlabeled DCVs were frequently observed adjacent to labeled DCVs, allowing the quantitation of both as distinct populations (Fig. 3).

3.2 Stress induces changes in MF LENK levels that are specific to sex and stress duration

Consistent with recent observations (Torres-Reveron et al., 2008), control female rats displayed fluctuations in LENK immunolabeling levels in CA3b across the estrous cycle (Fig. 2A,B), with significantly higher levels being observed at proestrus (10.4 ± 0.7) versus estrus and diestrus (6.6 ± 0.6 and 6.8 ± 0.4 respectively) (ANOVA with Tukey post hoc testing, $p = 0.00001$, $F = 13.46$, $n = 66$, data pooled from acute and chronic stress control groups). Male rats displayed values that were not significantly different from estrus and diestrus females (ANOVA, 6.3 ± 0.6 , $p = 0.8$, $F = 0.17$, $n = 64$).

Following the administration of AIS to female rats, LENK immunolabeling levels in CA3b were selectively altered in just the proestrus phase: values were reduced from an average of 10.0 ± 0.8 in the control group to 6.9 ± 0.8 in the experimental group (t test, $p = 0.01$, $n = 19$, Fig 2A). In contrast, LENK immunolabeling levels were not significantly altered for female rats in the estrus and diestrus phases, or for male rats following AIS. Thus, acute

stress had the net effect of both eliminating the influence of cycle phase on LENK levels (ANOVA, $p = 0.8$, $F = 0.24$, $n = 35$), and shifting overall levels to near the lowest point observed in control animals.

Surprisingly, the effects of CIS appeared to mirror image of those of acute stress: LENK immunolabeling levels were selectively altered in the estrus and diestrus phases (Fig. 2B). Estrus values increased from 7.0 ± 0.8 to 9.3 ± 0.7 (ANOVA, $p = 0.04$, $n = 24$, see Graph 1) and diestrus values increased from 6.6 ± 0.8 to 9.2 ± 0.6 (ANOVA, $p = 0.02$), but neither proestrus nor male levels were significantly altered following CIS. Thus, chronic stress also had the net effect of eliminating the influence of cycle phase on LENK levels (ANOVA, $p = 0.3$, $F = 1.26$, $n = 38$), however in this instance overall levels were shifted to near the highest point observed in control animals.

3.2 Ultrastructural changes in the density of LENK-labeled DCVs parallel light microscopic densitometric changes

To further explore how both AIS and CIS differentially alter the levels of LENK within the CA3b of female rats, tissue from the conditions that had displayed the most significant changes by LM (proestrus animals subjected to AIS, and diestrus animals subjected to CIS) were prepared for ultrastructural analysis. The best measure of the intensity of 'overall labeling' at the EM level is the density of LENK IDCVs within the neuropil (IDCVs/100 μm^2), calculated from the total number of IDCVs in both axonal and terminal processes in an average randomly selected grid square (defining a 3025 μm^2 field of neuropil) (Pierce et al., 1999).

After AIS, the density of LENK IDCVs was significantly altered in proestrus tissue, decreasing to 53% of control values (from 1.5 ± 0.1 to 0.8 ± 0.1 IDCVs/100 μm^2 , ANOVA, $p = 0.002$, $n = 13$, see Table 1), closely paralleling LM observations. Interestingly, this decrease could be completely accounted for by changes that occurred within just the MF axonal profiles. Their contribution to the average neuropil density also decreased to roughly half of control values (from 1.02 ± 0.05 to 0.49 ± 0.05 IDCVs/100 μm^2 , $p = 0.0001$). In contrast, the remaining contribution from MF terminal profiles, did not change significantly (from 0.44 ± 0.08 to 0.35 ± 0.08 IDCVs/100 μm^2 , $p = 0.4$). Similarly, no significant changes were found in the number of either IDCVs or unIDCVs per mossy terminal profile, the percentage of DCVs within mossy terminal profiles that were labeled, or the density of IDCVs along the inner surface of the mossy terminal membrane (IDCVs/ μm ; since DCVs are almost invariably distributed in close association with the inner surface of mossy terminal membranes, IDCVs/ μm of perimeter, and *not* IDCVs/ μm^2 of cross-sectional area, is the appropriate measure of the density of DCVs within mossy terminal profiles (Pierce et al., 1999) (see Table 1). Some significant alterations in the structure of MF terminals were observed: their circularity was increased slightly (from 0.22 ± 0.02 to 0.28 ± 0.02 , ANOVA, $p = 0.04$, $n = 41$) and the number of spinous profiles they were apposed to decreased (from 5.1 ± 0.4 to 3.5 ± 0.4 , $p = 0.005$). Other structural features, such as average cross-sectional area, perimeter length, and the number of active zones, were not significantly altered (Table 1).

The ultrastructural effects observed following CIS also mirrored what had been at the LM level. The density of LENK IDCVs within the neuropil of diestrus animals was significantly elevated, increasing to 250% of control values (from 0.8 ± 0.2 to 2.0 ± 0.2 IDCVs/ $100 \mu\text{m}^2$, ANOVA, $p = 0.004$, $n = 12$, see Table 1). In this case, the strong change in IDCV neuropil density was the result of significant increases in the numbers of IDCVs associated with both axonal (from 0.54 ± 0.07 to 1.03 ± 0.07 IDCVs/ $100 \mu\text{m}^2$, $p = 0.0004$) and terminal (from 0.3 ± 0.2 to 1.0 ± 0.2 IDCVs/ $100 \mu\text{m}^2$, $p = 0.02$) profiles. The number of IDCVs associated with individual MF terminal profiles also increased significantly after CIS (from 2.5 ± 0.5 to 3.9 ± 0.3 , ANOVA, $p = 0.03$, $n = 64$), as did the density of IDCVs within the profiles (from 0.2 ± 0.04 to 0.33 ± 0.3 IDCVs/ μm , $p = 0.007$) and the percentage of DCVs within mossy terminal profiles that were labeled (from $36 \pm 4\%$ to $53\% \pm 3\%$, $p = 0.001$). The last measure also resulted from a significant decrease in the number of unlabeled DCVs per profile (from 5.9 ± 0.6 to 3.3 ± 0.4 , $p = 0.002$). No significant changes in the structure of MF terminal profiles were observed after CIS (Table 1).

4. Discussion

This study demonstrates for the first time that both acute and chronic stress can eliminate the influence that estrous cycle stage exerts on LENK levels in the MF system of female rats. However, the effect was achieved through distinctly different processes: AIS reduced proestrus MF LENK levels to those observed during estrus and diestrus, while CIS elevated estrus and diestrus MF LENK levels to those observed during proestrus. Ultrastructural analysis revealed that the AIS-induced reductions were the apparent result of a loss of a 'backup' population of LENK DCVs, since the loss was only seen in MF axonal profiles, where DCVs are being transported to terminals. In contrast, CIS-induced elevations reflected the apparent accumulation of LENK DCVs in both MF axons and terminals. These changes occur in association with known changes in MOR and DOR populations, parallel observed physiological and behavioral findings, and could have important implications for the understanding of sex-specific learning, including that relevant to addiction.

4.1 Estrous cycle LENK fluctuations are eliminated by stress

The role that estrous cycle fluctuations LENK levels play in the normal functioning of the female HF remains to be fully elucidated. LENK levels in the CA3 subfield are highest at proestrus and estrus (Torres-Reveron et al., 2008), corresponding to the phases during which estrogen and progesterone levels first peak, and then decline, suggesting that the fluctuations in LENK levels directly follow the fluctuations of these ovarian hormones. Further support for the influence of estrogen comes from the fact that its administration to OVX females results in a significant increase in LENK levels in CA3 24 hours later (Torres-Reveron et al., 2008).

LENK is thought to be critically involved in mediating learning and memory processes within CA3. As a neuropeptide stored in DCVs, its release into the extracellular environment requires high frequency stimulation (Thureson-Klein and Klein, 1990), which is often associated with the modification of synaptic function. In male rats, some forms of LTP at the MF-CA3 pyramidal cell synapse, and LPP-CA3 pyramidal cell synapse LTP, are

LENK-dependent, requiring the activation of MORs (Derrick et al., 1992; Do et al., 2002). The MOR-dependence of LPP-CA3 pyramidal cell LTP could arise through disinhibition (Drake et al., 2007): naloxone-blockage of LTP at the LPP-granule cell synapse, which displays similar characteristics, can be prevented from the co-application of a GABA_A receptor antagonist (Bramham and Sarvey, 1996). In CA3, MORs are frequently detected on PARV-containing basket cells (Drake et al., 2007), which innervate the somata of CA3 pyramidal cells, where they can exert a strong influence on activity (Paulsen and Moser, 1998). Recent findings indicate that PARV-containing basket cells are involved in generating gamma and theta field potential oscillations, and endogenously released LENK could set the level of synchrony and thus the mode of hippocampal information processing (Gulyas et al., 2010). The loci for the MOR-dependence of MF-CA3 pyramidal cell LTP is much less clear. Not only are MORs and phosphorylated MORs present on interneurons (Drake et al., 2007) but phosphorylated MORs, and the MOR1D splice variant are located on MF terminals (Abbadie et al., 2000) (Gonzales et al., 2011). Determining such mechanisms is complicated by the fact that, unlike classical transmitters, neuropeptides are thought to frequently act at relatively distant sites from their point of release within the neuropil (Nassel, 2009).

As suggested by these findings, both physiological and behavioral parameters vary across the estrous cycle in female rats, paralleling changes in LENK levels. CA1 pyramidal cell LTP alters across the estrous cycle, with the greatest degree of potentiation being observed in proestrus (Bi et al., 2001; Warren et al., 1995). A form of opioid-dependent LTP at the MF-CA3 pyramidal cell synapse that is only present during proestrus has also been reported (Varga-Wesson et al., 2011). Behaviorally, anxiety-related task performance (using elevated plus maze, open field and social interaction tests) alters across the estrous cycle, with the anxiolytic, anti-anxiety behaviors highest at proestrus (Frye et al., 2000). The contention that estrogen acts as an upstream modulator of these changes is reinforced by studies conducted on OVX female rats. Following estrogen administration, awake OVX rats display significantly greater levels of LTP in CA1 than controls (Cordoba Montoya and Carrer, 1997), and enhanced performance in hippocampal dependent spatial memory tasks (Fader et al., 1998; Korol and Kolo, 2002; Luine et al., 1998). Of course, it should be recognized that LENK is just one mediator in a complex array of hippocampal effects that appear to be initiated by estrogen. Most notably, estrogen induces dramatic fluctuations in the density of CA1 spines and synapses across the estrous cycle (reviewed in (McEwen et al., 2001)).

Whether there is a behavioral or evolutionary benefit provided to female rats by adjusting the performance capacity of these hippocampal-dependent skills during the estrous cycle remains to be established. The effect of estrogen on CA1 spine formation is known to be preserved in primates (Hao et al., 2003; Leraneth et al., 2002). It has also been suggested, based on both rodent and human studies, that the female HF might be optimized to perform distinct behavioral/cognitive functions during the portion of the estrous cycle when ovarian steroid levels are high and the female is sexually receptive (Desmond and Levy, 1997). In this context, one might consider stress as an overriding environmental factor, that eliminates estrogen-related fluctuations, resetting MF LENK levels either to the lowest control levels observed (rapidly, in response to acute stress) or to the highest control levels observed (over time, in response to chronic stress). These interactions should not be surprising. Stress-

elevated levels of corticosterone act at mineralocorticoid and glucocorticoid receptors, which are highly concentrated in the HF (De Kloet et al., 1990) and are known to modulate learning and memory processes (reviewed in (De Kloet et al., 2005)). Additionally, recent findings indicate that loss of the mineralocorticoid receptor interferes with spatial performance, especially at proestrus, suggesting a strong interaction between the mechanisms induced by stress and ovarian hormones (Ter Horst et al., 2013).

4.2 Acute stress lowers MF LENK levels and chronic stress elevates MF LENK levels in females

Following AIS, MF LENK levels at all stages of the estrous cycle were statistically indistinguishable from the lowest values observed in control animals. Specifically, this meant that proestrus females were selectively affected: diestrus and estrus female, as well as male, MF LENK levels remained statistically unaltered. Acute stress is known to dramatically increase neural activity within the HF. Acute immobilization, as used in this study, produces pronounced hippocampal c-fos labeling (Chowdhury et al., 2000). Such heightened activity levels would presumably result in the rapid release of DCVs from the MF system, over the 30 min. of immobilization. It is thus unexpected that significant reductions in MF LENK immunolabeling were only observed in proestrus females. One possible explanation is that the elevated level of LENK observed at this point is associated with a greater probability of release, such that there is 'readily releasable' pool of LENK DCVs that has accumulated, and is selectively sensitive to the activity induced by acute stress. Tissue from these animals was processed for analysis immediately after the termination of immobilization, so the findings should reflect the direct result of acute stress, before longer term consequences develop.

Ultrastructural field analysis of the CA3b MF field after AIS confirmed the decreases observed by LM analysis, showing that the density of LENK IDCVs in the neuropil was reduced by nearly a half. Surprisingly, subdividing this value into the portions observed in MF axonal processes versus MF terminals revealed that the observed reduction could be almost completely accounted for by a significant reduction in IDCVs associated with axonal processes, again by nearly a half. The numbers associated with MF terminals were reduced, but not significantly. Since DCVs are generated in the cell body and transported to terminals, where they are released (Pierce et al., 1999; Zupanc, 1996), then these findings would seem to reflect a two stage process, where IDCVs were first released from MF terminals during stress, and subsequently replaced by IDCVs that were being transported down nearby axons. The synthesis and transport of new LENK IDCVs in response to this stress-induced loss would take significantly longer than the time frame examined, so the result would be a depletion of IDCVs in axonal processes. Interestingly, ultrastructural analysis also revealed minor but significant changes in the structure of MF terminals. The circularity (a measure of the relative volume to surface area) of the terminals increased, possibly indicating a certain degree of bloating associated with prolonged activity, and the average number of spine profiles associated with MF terminals decreased, possibly related to the apparent relative decrease in surface area.

In contrast, the effects of CIS appeared to be the mirror image of those observed in response to AIS. MF LENK levels across the estrous cycle were statistically indistinguishable from the highest values of control animals, a change that was specifically reflected in selective, significant elevations in diestrus and estrus females, to control proestrus levels. Male MF LENK levels remained unchanged. The chronic stress paradigm used, consisting of ten consecutive days of acute stress, allowed changes to occur progressively over time, and the striking differences observed presumably reflect the development of a response to the repeated effects of stress. Repeated periods of protracted neural activity and DCV release seem to have resulted in an increase in LENK expression, such that elevated levels within the MF system can be maintained one day after the last application of acute stress, when the tissue was analyzed. It is interesting that these apparent compensatory changes are not observed in males, or in proestrus females. One could posit that, if estrogen is a necessary mediator of the compensatory change, it would not be observed in males, and in proestrus females, where estrogen levels are already high, a 'ceiling effect' might already be present in control animals.

Ultrastructural field analysis of tissue from diestrus females after CIS confirmed the LM findings, demonstrating that the density of LENK IDCVs in the neuropil was increased to 250% of control values. In this instance, this change was reflected in significant increases in the portions associated with MF terminal and axonal profiles, indicating that there was both an increase in the number being transported through axons to terminals, and a maintenance of the number of IDCVs in terminals. This also was apparent in individual MF terminals: the average number and the density of IDCVs was significantly increased. Interestingly, the number of unlabeled DCVs per terminal was reduced, and the percentage of DCVs that were labeled was increased, indicating that LENK DCVs were also supplanting other types of DCVs. No significant MF structural or synaptic changes were observed, including no change in MF terminal circularity, as was observed following AIS. Thus, CIS could potentially induce compensatory mechanisms that would help preserve structure, by maintaining things such as water balance, to prevent bloating. This lack of structural change also is consistent with previous findings indicating that, in contrast to males, females do not display either MF terminal clear vesicle clumping (Magarinos et al., 1997) or CA3 pyramidal cell dendritic remodeling (Galea et al., 1997; McEwen, 1999) following chronic stress.

4.3 Functional Implications and Conclusions

In conclusion, these findings demonstrate that stress acts as a primary environmental determinant, eliminating MF LENK fluctuations across the estrous cycle, and resetting MF LENK levels either to the lowest control levels observed (rapidly, in response to AIS) or to the highest control levels observed (over time, in response to CIS). These alterations in peptidergic transmitter levels occur in parallel with known stress-induced changes in their associated receptors, MORs and DORs. As discussed, MORs are found most frequently on PARV-containing basket cells, while DORs are located both on granule and pyramidal cell dendrites, and on interneurons that contain somatostatin or neuropeptide Y (Drake et al., 2007; Williams et al., 2011). AIS significantly reduces MOR levels within dentate gyrus PARV-containing interneurons in females, while comparable values in males were

unaffected (Milner et al., 2013). AIS also reduces DOR levels in CA3 in proestrus females (Hall, Van Kempen and Milner, unpublished data). Thus, females uniquely exhibit concurrent reductions of both MOR and DOR levels at a time point that MF LENK levels are also reset to low values, suggesting that the capacity for LENK to disinhibit pyramidal cell activity immediately after AIS could be greatly diminished. Following CIS, dentate gyrus PARV-containing interneuron numbers in females are selectively preserved (males display a significant loss (Milner et al., 2013), and the density of MOR labeling within these cells is significantly elevated. Comparable changes occur with DORs within CA3 pyramidal cells (Hall, Van Kempen and Milner, unpublished data). Thus, the elevation of MF LENK levels following CIS parallels increased potential receptor availability, suggesting an increase in the capacity for disinhibition, which could support enhanced synaptic plasticity (reviewed in (Simmons and Chavkin, 1996).

Additionally, the effects of acute versus chronic stress on levels of MF LENK, a critical mediator of synaptic plasticity within the HF (Derrick et al., 1992; Do et al., 2002), appear to generally parallel observed effects on performance in learning and memory tasks, particularly in regard to sex. Following acute stress, MF LENK levels in females are comparable to those observed in males. Conversely, following chronic stress elevated MF LENK levels in females strongly contrasting with those observed in males. In this instance, there appears to be a consensus that males display impaired cognitive performance (reviewed in (Luine et al., 2007)) while females exhibit enhanced performance on the same memory tasks (Conrad et al., 2003; Kitraki et al., 2004). This suggests that alterations in LENK levels following chronic stress could be involved in the sex-specific responses that females display in stress-associated behaviors, like drug use relapse and a greater propensity to addiction (Elman et al., 2001; Hu et al., 2004), that involve the HF. Importantly, opioid receptor blockage in CA3, particularly CA3b, in male rats disrupts visual-spatial pattern completion which is important in learning relevant to drug abuse (Kesner and Warthen, 2010). Further research will be necessary to elucidate the role that stress induced changes in the endogenous HF opioid peptide systems play in sex-differences in these processes.

Acknowledgments

We thank Drs. Annelyn Torres-Reveron, Tanya Williams and Keith Gonzales and Ms. Andreina Gonzales for help with tissue preparation.

GRANT SUPPORT: NIH grants DA08259, HL098351 & HL096571 (TAM), NS007080 (BSM) and DK07313 & AG059850 (EMW)

Abbreviations

AIS	acute immobilization stress
CIS	chronic immobilization stress
DCV	dense-core vesicle
DOR	delta opioid receptor
EM	electron microscopy

HF	hippocampal formation
IDCV	labeled DCV
LENK	leu-enkephalin
LM	light microscopy
LPP	lateral perforant path
LTP	long term potentiation
MF	mossy fiber
MOR	mu opioid receptor
OVX	ovariectomized
PARV	parvalbumin
unIDCV	unlabeled DCV

Reference List

- Abbadie C, Pan YX, Pasternak GW. Differential distribution in rat brain of mu opioid receptor carboxy terminal splice variants MOR-1C-like and MOR-1-like immunoreactivity: Evidence for region-specific processing. *J Comp Neurol*. 2000; 419:244–256. [PubMed: 10723002]
- Auchus AP, Pickel VM. Quantitative light microscopic demonstration of increased pallidal and striatal met5-enkephalin-like immunoreactivity in rats following chronic treatment with haloperidol but not with clozapine: Implications for the pathogenesis of neuroleptic-induced movement disorders. *Exp Neurol*. 1992; 117:17–27. [PubMed: 1618284]
- Beck KD, Luine VN. Evidence for sex-specific shifting of neural processes underlying learning and memory following stress. *Physiol Behav*. 2010; 99:204–211. [PubMed: 19376144]
- Bi R, Foy MR, Vouimba RM, Thompson RF, Baudry M. Cyclic changes in estradiol regulate synaptic plasticity through the MAP kinase pathway. *Proc Natl Acad Sci U S A*. 2001; 98:13391–13395. [PubMed: 11687663]
- Blackstad TW, Kjaerheim A. Special axo-dendritic synapses in the hippocampal cortex: electron and light microscopic studies on the layer of mossy fibers. *J Comp Neurol*. 1961; 117:133–59. 133–159. [PubMed: 13869693]
- Bowman RE, Ferguson D, Luine VN. Effects of chronic restraint stress and estradiol on open field activity, spatial memory, and monoaminergic neurotransmitters in ovariectomized rats. *Neurosci*. 2002; 113:401–410.
- Bramham CR, Sarvey JM. Endogenous activation of μ and δ -1 opioid receptors is required for long-term potentiation induction in the lateral perforant path: Dependence on GABAergic inhibition. *J Neurosci*. 1996; 16:8123–8131. [PubMed: 8987837]
- Bruchas MR, Xu M, Chavkin C. Repeated swim stress induces kappa opioid-mediated activation of extracellular signal-regulated kinase 1/2. *Neuroreport*. 2008; 19:1417–1422. [PubMed: 18766023]
- Burstein SR, Williams TJ, Lane DA, Knudsen MG, Pickel VM, McEwen BS, Waters EM, Milner TA. The influences of reproductive status and acute stress on the levels of phosphorylated delta opioid receptor immunoreactivity in rat hippocampus. *Brain Res*. 2013; 1518:71–81. [PubMed: 23583481]
- Chavkin C, Shoemaker WJ, McGinty JF, Bayon A, Bloom FE. Characterization of the prodynorphin and proenkephalin neuropeptide systems in rat hippocampus. *J Neurosci*. 1985; 5:808–816. [PubMed: 3838345]
- Chowdhury GM, Fujioka T, Nakamura S. Induction and adaptation of Fos expression in the rat brain by two types of acute restraint stress. *Brain Res Bull*. 2000; 52:171–182. [PubMed: 10822158]

- Commons KG, Milner TA. Ultrastructural heterogeneity of enkephalin-containing neurons in the rat hippocampal formation. *J Comp Neurol.* 1995; 358:324–342. [PubMed: 7560290]
- Conrad CD, Grote KA, Hobbs RJ, Ferayorni A. Sex differences in spatial and non-spatial Y-maze performance after chronic stress. *Neurobiol Learn Mem.* 2003; 79:32–40. [PubMed: 12482677]
- Corbett, AD.; Paterson, SJ.; Kosterlitz, HW. Selectivity of ligands for opioid receptors.. In: Herz, A., editor. *Opioids I.* Springer-Verlag; Berlin: 1993. p. 645-680.
- Cordoba Montoya DA, Carrer HF. Estrogen facilitates induction of long term potentiation in the hippocampus of awake rats. *Brain Res.* 1997; 778:430–438. [PubMed: 9459564]
- De Kloet ER, Joels M, Holsboer F. Stress and the brain: from adaptation to disease. *Nat Rev Neurosci.* 2005; 6:463–475. [PubMed: 15891777]
- De Kloet ER, Reul JM, Sutanto W. Corticosteroids and the brain. *J Steroid Biochem Mol Biol.* 1990; 37:387–394. [PubMed: 2257242]
- Derrick BE, Rodriguez SB, Lieberman DN, Martinez JL Jr. Mu opioid receptors are associated with the induction of hippocampal mossy fiber long-term potentiation. *J Pharmacol Exp Ther.* 1992; 263:725–733. [PubMed: 1359112]
- Desmond NL, Levy WB. Ovarian steroidal control of connectivity in the female hippocampus: An overview of recent experimental findings and speculations on its functional consequences. *Hippocampus.* 1997; 7:239–245. [PubMed: 9136053]
- Do VH, Martinez CO, Martinez JL Jr. Derrick BE. Long-term potentiation in direct perforant path projections to the hippocampal CA3 region in vivo. *J Neurophysiol.* 2002; 87:669–678. [PubMed: 11826036]
- Drake CT, Chang PC, Harris JA, Milner TA. Neurons with mu opioid receptors interact indirectly with enkephalin-containing neurons in the rat dentate gyrus. *Exp Neurol.* 2002; 176:254–261. [PubMed: 12093103]
- Drake CT, Chavkin C, Milner TA. Opioid systems in the dentate gyrus. *Prog Brain Res.* 2007; 163:245–814. [PubMed: 17765723]
- Drolet G, Dumont EC, Gosselin I, Kinkead R, Laforest S, Trottier JF. Role of endogenous opioid system in the regulation of the stress response. *Prog Neuropsychopharmacol Biol Psychiatry.* 2001; 25:729–741. [PubMed: 11383975]
- Fader AJ, Hendricson AW, Dohanich GP. Estrogen improves performance of reinforced T-maze alternation and prevents the amnesic effects of scopolamine administered systemically or intrahippocampally. *Neurobiol Learn Mem.* 1998; 69:225–240. [PubMed: 9707487]
- Frye CA, Petralia SM, Rhodes ME. Estrous cycle and sex differences in performance on anxiety tasks coincide with increases in hippocampal progesterone and 3 α ,5 α -THP. *Pharmacol Biochem Behav.* 2000; 67:587–596. [PubMed: 11164090]
- Galea LAM, McEwen BS, Tanapat P, Deak T, Spencer RL, Dhabhar FS. Sex differences in dendritic atrophy of CA3 pyramidal neurons in response to chronic restraint stress. *Neurosci.* 1997; 81:689–697.
- Gall C, Brecha N, Karten HJ, Chang K-J. Localization of enkephalin-like immunoreactivity to identified axonal and neuronal populations of the rat hippocampus. *J Comp Neurol.* 1981; 198:335–350. [PubMed: 6263955]
- Gonzales KL, Chappelle JD, Pierce JP, Kelter DT, Williams TJ, Torres-Reveron A, McEwen BS, Waters EM, Milner TA. The influences of reproductive status and acute stress on the levels of phosphorylated mu opioid receptor immunoreactivity in rat hippocampus. *Front Neuroendocrinol.* 2011; 2:1–10.
- Gulyas AI, Szabo GG, Ulbert I, Holderith N, Monyer H, Erdelyi F, Szabo G, Freund TF, Hajos N. Parvalbumin-containing fast-spiking basket cells generate the field potential oscillations induced by cholinergic receptor activation in the hippocampus. *J Neurosci.* 2010; 30:15134–15145. [PubMed: 21068319]
- Hao J, Janssen WG, Tang Y, Roberts JA, McKay H, Lasley B, Allen PB, Greengard P, Rapp PR, Kordower JH, Hof PR, Morrison JH. Estrogen increases the number of spinophilin-immunoreactive spines in the hippocampus of young and aged female rhesus monkeys. *J Comp Neurol.* 2003; 465:540–550. [PubMed: 12975814]

- Hsu SM, Raine L, Fanger H. Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures. *J Histochem Cytochem.* 1981; 29:557–580.
- Kalivas PW, Abhold R. Enkephalin release into the ventral tegmental area in response to stress: modulation of mesocorticolimbic dopamine. *Brain Res.* 1987; 414:339–348. [PubMed: 3620936]
- Kesner RP, Warthen DK. Implications of CA3 NMDA and opiate receptors for spatial pattern completion in rats. *Hippocampus.* 2010; 20:550–557. [PubMed: 19650123]
- Kitraki E, Kremmyda O, Youlatos D, Alexis MN, Kittas C. Gender-dependent alterations in corticosteroid receptor status and spatial performance following 21 days of restraint stress. *Neuroscience.* 2004; 125:47–55. [PubMed: 15051144]
- Korol DL, Kolo LL. Estrogen-induced changes in place and response learning in young adult female rats. *Behav Neurosci.* 2002; 116:411–420. [PubMed: 12049322]
- Leranth C, Shanabrough M, Redmond DE Jr. Gonadal hormones are responsible for maintaining the integrity of spine synapses in the CA1 hippocampal subfield of female nonhuman primates. *J Comp Neurol.* 2002; 447:34–42. [PubMed: 11967893]
- Lorento de No R. Studies on the Structure of the Cerebral Cortex. 1934:113–177.
- Lucas LR, Wang CJ, McCall TJ, McEwen BS. Effects of immobilization stress on neurochemical markers in the motivational system of the male rat. *Brain Res.* 2007; 1155:108–115. [PubMed: 17511973]
- Luine VN, Beck KD, Bowman RE, Frankfurt M, MacLusky NJ. Chronic stress and neural function: accounting for sex and age. *J Neuroendocrinol.* 2007; 19:743–751. [PubMed: 17850456]
- Luine VN, Richards ST, Wu VY, Beck KD. Estradiol enhances learning and memory in a spatial memory task and effects levels of monoaminergic neurotransmitters. *Horm Behav.* 1998; 34:149–162. [PubMed: 9799625]
- Magarinos AM, Verdugo JM, McEwen BS. Chronic stress alters synaptic terminal structure in hippocampus. *Proc Natl Acad Sci U S A.* 1997; 94:14002–14008. [PubMed: 9391142]
- McEwen B, Akama K, Alves S, Brake WG, Bulloch K, Lee S, Li C, Yuen G, Milner TA. Tracking the estrogen receptor in neurons: Implications for estrogen-induced synapse formation. *Proc Natl Acad Sci U S A.* 2001; 98:7093–7100. [PubMed: 11416193]
- McEwen BS. Stress and hippocampal plasticity. *Annu Rev Neurosci.* 1999; 22:105–122. [PubMed: 10202533]
- McEwen BS. The neurobiology of stress: from serendipity to clinical relevance. *Brain Res.* 2000; 886:172–189. [PubMed: 11119695]
- McEwen BS, Alves SE. Estrogen actions in the central nervous system. *Endo Rev.* 1999; 20:279–307.
- McEwen BS, Milner TA. Hippocampal formation: shedding light on the influence of sex and stress on the brain. *Brain Res Rev.* 2007; 55:343–355. [PubMed: 17395265]
- McLaughlin JP, Marton-Popovici M, Chavkin C. Kappa opioid receptor antagonism and prodynorphin gene disruption block stress-induced behavioral responses. *J Neurosci.* 2003; 23:5674–5683. [PubMed: 12843270]
- Milner TA, Burstein SR, Marrone GF, Khalid S, Gonzalez AD, Williams TJ, Schierberl KC, Torres-Reveron A, Gonzales KL, McEwen BS, Waters EM. Stress differentially alters mu opioid receptor density and trafficking in parvalbumin-containing interneurons in the female and male rat hippocampus. *Synapse.* 2013; 10
- Milner TA, Pickel VM, Reis DJ. Ultrastructural basis for interactions between central opioids and catecholamines: I. Rostral ventrolateral medulla. *J Neurosci.* 1989; 9:2114–2130.
- Milner, TA.; Waters, EM.; Robinson, DC.; Pierce, JP. Degenerating processes identified by electron microscopic immunocytochemical methods.. In: Manfredi, G.; Kawamata, H., editors. *Neurodegeneration, Methods and Protocols.* Springer; New York: 2011. p. 23-59.
- Nassel DR. Neuropeptide signaling near and far: how localized and timed is the action of neuropeptides in brain circuits? *Invert Neurosci.* 2009; 9:57–75. [PubMed: 19756790]
- Park CR, Zoladz PR, Conrad CD, Fleshner M, Diamond DM. Acute predator stress impairs the consolidation and retrieval of hippocampus-dependent memory in male and female rats. *Learn Mem.* 2008; 15:271–280. [PubMed: 18391188]

- Paulsen O, Moser EI. A model of hippocampal memory encoding and retrieval: GABAergic control of synaptic plasticity. *Trends Neurosci.* 1998; 21:273–278. [PubMed: 9683315]
- Peters, A.; Palay, SL.; Webster, H.d. The fine structure of the nervous system. 3rd ed.. Oxford University Press; New York: 1991.
- Pierce JP, Kurucz O, Milner TA. The morphometry of a peptidergic transmitter system before and after seizure. I. Dynorphin B-like immunoreactivity in the hippocampal mossy fiber system. *Hippocampus.* 1999; 9:255–276. [PubMed: 10401641]
- Shansky RM, Hamo C, Hof PR, Lou W, McEwen BS, Morrison JH. Estrogen promotes stress sensitivity in a prefrontal cortex-amygdala pathway. *Cereb Cortex.* 2010; 20:2560–2567. [PubMed: 20139149]
- Simmons ML, Chavkin C. Endogenous opioid regulation of hippocampal function. *Int Rev Neurobiol.* 1996; 39:145–96. 145–196. [PubMed: 8894847]
- Sousa N, Lukoyanov NV, Madeira MD, Almeida OFX, Paula-Barbosa MM. Reorganization of the morphology of hippocampal neurites and synapses after stress-induced damage correlates with behavioral improvement. *Neurosci.* 2000; 97:253–266.
- Swanson, LW. *Brain Maps: Structure of the Rat Brain.* Elsevier; Amsterdam: 1992.
- Ter Horst JP, Kentrop J, Arp M, Hubens CJ, De Kloet ER, Oitzl MS. Spatial learning of female mice: a role of the mineralocorticoid receptor during stress and the estrous cycle. *Front Behav Neurosci.* 2013; 7:56. doi: 10.3389/fnbeh.2013.00056.:56. [PubMed: 23754993]
- Thureson-Klein AK, Klein RL. Exocytosis from neuronal large dense-core vesicles. *Int Rev Cytol.* 1990; 121:67–126. [PubMed: 1972143]
- Torres-Reveron A, Khalid S, Williams TJ, Waters EM, Drake CT, McEwen BS, Milner TA. Ovarian steroids modulate leu-enkephalin levels and target leu-enkephalinergic profiles in the female hippocampal mossy fiber pathway. *Brain Res.* 2008; 1232:70–84. [PubMed: 18691558]
- Torres-Reveron A, Williams TJ, Chapleau JD, Waters EM, McEwen BS, Drake CT, Milner TA. Ovarian steroids alter mu opioid receptor trafficking in hippocampal parvalbumin GABAergic interneurons. *Exp Neurol.* 2009; 219:319–327. [PubMed: 19505458]
- Turner, CD.; Bagnara, JT. *General Endocrinology.* W.B. Saunders; Philadelphia: 1971.
- Uysal N, Sisman AR, Dayi A, Ozbil S, Cetin F, Baykara B, Aksu I, Tas A, Cavus SA, Gonenc-Arda S, Buyuk E. Acute footshock-stress increases spatial learning-memory and correlates to increased hippocampal BDNF and VEGF and cell numbers in adolescent male and female rats. *Neurosci Lett.* 2012; 514:141–146. [PubMed: 22426107]
- Van Kempen TA, Kahlid S, Gonzalez AD, Spencer-Segal JL, Tsuda MC, Ogawa S, McEwen BS, Waters EM, Milner TA. Sex and estrogen receptor expression influence opioid peptide levels in the mouse hippocampal mossy fiber pathway. *Neurosci Lett.* 2013; 10
- Varga-Wesson, A.; Harte, LC.; Milner, TA.; MacLusky, NJ.; Scharfman, HE. Differences in mossy fiber plasticity in adult male and female rat hippocampal slices. *Society for Neuroscience*; Washington DC: 2011. Program No. 869.02 2011 Meeting Planner [Online]
- Warren SG, Humphreys AG, Juraska JM, Greenough WT. LTP varies across the estrous cycle: Enhanced synaptic plasticity in proestrus rats. *Brain Res.* 1995; 703:26–30. [PubMed: 8719612]
- Williams TJ, Torres-Reveron A, Chapleau JD, Milner TA. Hormonal regulation of delta opioid receptor immunoreactivity in interneurons and pyramidal cells in the rat hippocampus. *Neurobiol Learn Mem.* 2011; 95:206–220. [PubMed: 21224009]
- Zupanc GKH. Peptidergic transmission: From morphological correlates to functional implications. *Micron.* 1996; 27:35–91. [PubMed: 8756315]

Research Highlights

Stress eliminates the estrous cycle changes in mossy fiber leu-enkephalin levels.

Acute stress resets leu-enkephalin levels in females to the lowest control values.

Chronic stress resets leu-enkephalin levels in females to the highest control values.

Changes in enkephalin levels correlate with immunolabeled dense-core vesicle number.

Hippocampal leu-enkephalin levels in males are unaffected by stress.

Figure 1.

Examples of LENK-immunoreactivity in the dorsal and ventral hippocampus. LENK immunolabeling of the mossy fiber pathway in the dorsal (A) and ventral (B) hippocampus of a proestrus control rat. The rectangular inset denotes the analyzed portion of the CA3b subregion. DG, dentate gyrus; CA3 and CA1, hippocampal subregions. Scale bars: 500 μm , inset- 50 μm .

Figure 2.

The effects of acute and chronic stress on the levels of LENK-immunoreactivity in CA3b vary with estrous cycle phase and sex. (A) LENK immunolabeling of the mossy fiber pathway in the hippocampus of a proestrus control rat. DG, dentate gyrus; CA3 and CA1, hippocampal subregions. The rectangular inset denotes the analyzed portion of the CA3b subregion). (B) Quantitative densitometry showed that LENK-levels are reduced to diestrus levels following AIS. Y-axis data is expressed as pixel density units (out of 256 grey levels). (C) Quantitative densitometry showed that LENK-levels are increased to proestrus levels following CIS. $P < 0.05$. $N = 5-6$ rats per condition. Scale bars: figure- 500 μm , inset- 50 μm .

Figure 3.

Electron micrographs of LENK-labeled mossy fiber terminals. Mossy fiber terminals (MT) from control diestrus rats (**A**) contained significantly fewer LENK-labeled DCVs (red arrows) than mossy fiber terminals from chronic stress diestrus rats (**B**). Unlabeled DCVs are indicated by white arrows. D, dendrite; S, spine. Scale bar: 500 nm

Table 1

1) Acute Stress, Proestrus Animals	Control	Experimental	p	n
Field Analysis (total LDCVs/100 μm^2)	1.5 $\pm$ 0.1	* 0.8 $\pm$ 0.1	0.002	13
Field Analysis (axonal LDCVs/100 μm^2)	1.02 $\pm$ 0.05	* 0.49 $\pm$ 0.05	0.0001	13
Field Analysis (terminal LDCVs/100 μm^2)	0.44 $\pm$ 0.08	0.35 $\pm$ 0.08	0.4	13
LDCVs per Mossy Terminal	3.3 $\pm$ 0.5	3.9 $\pm$ 0.6	0.5	41
UnLDCVs per Mossy Terminal	6.0 $\pm$ 0.7	4.4 $\pm$ 0.8	0.2	41
% LDCVs per Mossy Terminal	38 $\pm$ 5	51 $\pm$ 6	0.1	41
LDCVs/ μm Terminal Perimeter	0.22 $\pm$ 0.03	0.27 $\pm$ 0.04	0.2	41
Mossy Terminal Cross-Sectional Area (μm^2)	4.6 $\pm$ 0.5	4.3 $\pm$ 0.5	0.6	41
Mossy Terminal Perimeter (μm)	16.4 $\pm$ 0.9	14 $\pm$ 1	0.07	41
Mossy Terminal Circularity	0.22 $\pm$ 0.02	* 0.28 $\pm$ 0.02	0.04	41
Synapse # per Mossy Terminal	3.8 $\pm$ 0.5	2.8 $\pm$ 0.6	0.2	41
Spine Profile # per Mossy Terminal	5.1 $\pm$ 0.4	* 3.5 $\pm$ 0.4	0.005	41
2) Chronic Stress, Diestrus Animals				
Field Analysis (total LDCVs/100 μm^2)	0.8 $\pm$ 0.2	* 2.0 $\pm$ 0.2	0.004	12
Field Analysis (axonal LDCVs/100 μm^2)	0.54 $\pm$ 0.07	* 1.03 $\pm$ 0.07	0.0004	12
Field Analysis (terminal LDCVs/100 μm^2)	0.3 $\pm$ 0.2	* 1.0 $\pm$ 0.2	0.02	12
LDCVs per Mossy Terminal	2.5 $\pm$ 0.5	* 3.9 $\pm$ 0.3	0.03	64
UnLDCVs per Mossy Terminal	5.9 $\pm$ 0.6	* 4.4 $\pm$ 0.8	0.002	64
% LDCVs per Mossy Terminal	36 $\pm$ 4	* 53 $\pm$ 3	0.001	64
LDCVs/ μm Terminal Perimeter	0.20 $\pm$ 0.03	* 0.33 $\pm$ 0.03	0.007	64
Mossy Terminal Cross-Sectional Area (μm^2)	4.0 $\pm$ 0.4	3.8 $\pm$ 0.3	0.7	64
Mossy Terminal Perimeter (μm)	13 $\pm$ 1	12.2 $\pm$ 0.8	0.4	64
Mossy Terminal Circularity	0.32 $\pm$ 0.03	0.36 $\pm$ 0.02	0.3	64
Synapse # per Mossy Terminal	4.1 $\pm$ 0.7	3.3 $\pm$ 0.5	0.3	64
Spine Profile # per Mossy Terminal	4.0 $\pm$ 0.6	4.1 $\pm$ 0.4	0.8	64

* significant, $p < 0.05$