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# Hippocampal mossy fiber leu-enkephalin immunoreactivity in female rats is significantly altered following both acute and chronic stress

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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-specific responses that females display in learning processes, including those important in 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, and 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

*Abbreviations:* AIS, acute immobilization stress; CIS, chronic immobilization stress; DCV, dense-core vesicle; DOR, delta opioid receptor; EM, electron microscopy; HF, hippocampal formation; lDCV, 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; unlDCV, unlabeled DCV.

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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 and Oakes, 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 and methods

### 2.1. 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.

### 2.2. 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 a.m., 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).

### 2.3. 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 a.m. and 1:00 p.m. 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 min. 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 separate 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).

### 2.4. 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 1000 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 μm thick) through the entire block were cut on a Vibratome (yielding on average 120 sections), collected in PB, and transferred serially to tissue culture trays containing a solution (30% sucrose and 10% ethylene glycol in PB) for storage at −25 °C. For a detailed description of the tissue preparation, immunocytochemical and analysis procedures used, refer to Milner et al. (2011).

### 2.5. 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 leu$^5$-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% $H_2O_2$ 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 2–3 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.

### 2.6. 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,