Sex differences after chronic stress in the expression of opioid-, stress- and neuroplasticity-related genes in the rat hippocampus

Matthew Randesi a,b,*, Yan Zhou a, Sanoara Mazid b, Shannon C. Odell b,c, Jason D. Gray e, J. Correa da Rosa d, Bruce S. McEwen e, Teresa A. Milner b,c,e,*, Mary Jeanne Kreek a,1

ABSTRACT

Opioid peptides and their receptors re-organize within hippocampal neurons of female, but not male, rats following chronic immobilization stress (CIS) in a manner that promotes drug-related learning. This study was conducted to determine if there are also sex differences in gene expression in the hippocampus following CIS. Adult female and male rats were subjected to CIS (30 min/day) for 10 days. Twenty-four hours after the last stressor, the rats were euthanized, the brains were harvested and the medial (dentate gyrus/CA1) and lateral (CA2/CA3) dorsal hippocampus were isolated. Following total RNA isolation, cDNA was prepared for gene expression analysis using a RT2 Profiler PCR expression array. This custom designed qPCR expression array contained genes for opioid peptides and receptors, as well as genes involved in stress-responses and candidate genes involved in synaptic plasticity, including those upregulated following oxytocin administration in mice. Few sex differences are seen in hippocampal gene expression in control (unstressed) rats. In response to CIS, gene expression in the hippocampus was altered in males but not females. In males, opioid, stress, plasticity and kinase/signaling genes were all down-regulated following CIS, except for the gene that codes for corticotropin releasing hormone, which was upregulated. Changes in opioid gene expression following chronic stress were limited to the CA2 and CA3 regions (lateral sample). In conclusion, modest sex- and regional-differences are seen in expression of the opioid receptor genes, as well as genes involved in stress and plasticity responses in the hippocampus following CIS.

Keywords: Opioid peptides, stress, plasticity, hippocampus, sex differences

1. Introduction

Over the last two decades opioid use and abuse has risen dramatically (Centers for Disease Control and Prevention, 2013; Centers for Disease Control and Prevention, 2015). Moreover, women are more apt than men to abuse opioids through initial prescription pain reliever use (Jones, 2012; Lee and Ho, 2013). Drug addiction, especially relapse, is often provoked by stress (reviewed by Bruchas et al. (2008); Shalev et al. (2000)). Although stress can have powerful influences on the addictive processes in both sexes (Koob and Kreek, 2007; Koob, 2008; Kreek and Kreek, 1998), females have a heightened sensitivity to stress (Becker et al., 2007). Moreover, unlike male rodents, female rodents have an enhanced cognitive performance following chronic stress (Luine et al., 2007) that may contribute to an accelerated course of addiction and/or reinstatement (Hu et al., 2004; Lynch et al., 2000; Robbins et al., 1999).

Associative learning and motivational incentives (Koob and Volkow, 2010) that critically involve the hippocampus (Luo et al., 2011; Vorel et al., 2001) are importantly involved in addictive processes. Notably, the hippocampus is a target of opioid receptor agonists, such as prescription morphine and methadone, as well as the drug of abuse heroin and various short acting opiates. Notably, the opioid system in the hippocampal CA3 region has been implicated in visual-spatial pattern completion (Kesner and Warthen, 2010), an important element of context associative learning which is essential for associating a drug of abuse with a particular place and set of events (i.e. drug-related

https://doi.org/10.1016/j.ynstr.2018.01.001
Received 31 October 2017; Received in revised form 9 January 2018; Accepted 10 January 2018
Available online 11 January 2018
2352-2895/ © 2018 The Authors. Published by Elsevier Inc. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/BY-NC-ND/4.0/).
learning) (Berke and Hyman, 2000; Kilts et al., 2001; Risinger and Oakes, 1995; Volkow et al., 2006).

Our previous anatomical and physiological studies have demonstrated sex differences in the opioid system in the rat dorsal hippocampus, especially in response to chronic immobilization stress (CIS) (McEwen and Milner, 2017). In particular, females at elevated estrogen states compared to low estrogen states and males, have: (1) elevated levels of enkephalin immunoreactivity in the mossy fibers, (2) greater densities of mu opioid receptor (MOR) immunolabeling on the plasma membrane of GABAergic hilar interneurons, (3) elevated densities of delta opioid receptor (DOR) immunolabeling near the plasma membrane in CA1 and CA3 pyramidal cell dendrites and (4) greater densities of DOR immunolabeling in CA3 dendritic spines contacted by mossy fibers (Harte-Hargrove et al., 2015; Mazid et al., 2016; Torres-Reveron et al., 2008, 2009; Williams et al., 2011b). Such redistribution would enhance excitability and learning processes (McEwen and Milner, 2017). Additionally, female rats at high-estrogen states compared to females in low-estrogen states and males have a lower baseline transmission of the mossy fiber-CA3 pathway that is regulated by MORs and exhibit a DOR-mediated low-frequency form of long-term potentiation (LTP) (Harte-Hargrove et al., 2015). Following CIS, females regardless of estrogen state are “primed” for even greater excitation of CA3 pyramidal cells. Specifically, after CIS, females do not display the dendritic atrophy of CA3 pyramidal cell dendrites and the loss of GABAergic parvalbumin (PARV)-containing interneurons in the dorsal hippocampus observed in males (McEwen, 1999; Milner et al., 2013; Vyas et al., 1999). Rather, in CIS females, enkephalin levels in mossy fibers are elevated and the elevation of MORs and DORs near the plasma membrane in hippocampal pyramidal and interneurons resembles that seen in unstressed females at high estrogen states (Mazid et al., 2016; Milner et al., 2013; Pierce et al., 2014). Moreover, following CIS, DORs are elevated near the plasma membrane of GABAergic hilar interneurons and thus could promote associative learning processes in the hippocampus (Mazid et al., 2016).

This study was conducted to determine if there also are sex differences in opioid gene expression in the dorsal hippocampus prior to stress (baseline) or following a model of chronic stress. In addition to opioid and stress genes, we assessed synaptic plasticity genes that we previously have shown were up-regulated in the hippocampus of male mice after oxycodone self-administration (Zhang et al., 2015) and/or altered with sex or hormonal levels in the hippocampus (McEwen and Milner, 2007).

2. Materials and methods

2.1. Animals

All procedures were approved by the Rockefeller University Institutional Animal Care and Use Committee 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 (275–325 gm) and female (≈225–250 gm) Sprague-Dawley rats (N = 24; Charles River Laboratories, Wilmington, MA) were approximately 2 months old at the time of arrival. The rats were pair-housed with 12:12 light/dark cycles (Lights on 0600–1800) and had access to food and water ad libitum. All rats were kept in custom-built cabinets especially designed for stress experiments (Phenome Technologies, Inc., Skokie, IL). Each cabinet held two cages (25 cm × 46 cm and 20.5 cm tall). The cabinets are directly attached to the ventilation system. White LEDs are installed in the cabinets.

Estrous cycle stage was determined using vaginal smear cytology (Turner and Bagnara, 1971) at the termination of the experiment. To control for the effects of handling, mock estrous cycling was performed in males at the termination of the experiment. Previous studies in rats showed that CIS had no effect on the length of the estrous cycle or the duration of the individual estrous phases (Milner et al., 2013).

Stress-induced differences in gene expression (RNA levels) are highly sensitive to environmental factors, such as ambient noise, time of day and familiarity of the experimenter, because each can rapidly and significantly alter circulating corticosterone levels, thereby inducing changes in gene expression. Therefore tissues from all groups were collected concurrently to promote the rigor and reproducibility of the results. Importantly, the same individuals conducted the stress experiments and the tissue was harvested at the same time of day, between 9am and 1pm, to control for circadian surges in cortisol levels. Given these attempts to minimize the variation in environmental factors, 24 rats was the maximum number of animals that can be run in a single experiment.

2.2. Chronic immobilization stress

Rats (6 male and 6 female) were transported from their home-room into a procedure room between 9:00 a.m. and 1:00 p.m., and CIS was performed, as previously described (Mazid et al., 2016). Briefly, rats were placed in plastic cone-shaped polyethylene bags with a small breathing hole at the apex and a Kotex mini-pad was placed in the bag underneath them to collect urine. The rats were placed with their nose at the hole and the bag was sealed with tape; they then were left undisturbed on the countertop for 30 min per day for 10 days. Cages containing control rats were brought into the procedure room prior to the stressed rats. The control rats were picked up and then placed back in their cages for 30 min prior to returning them to their homeroom. Animals were euthanized 24 h after the final stress period.

2.3. RNA isolation and cDNA synthesis

RNA was prepared as previously described (Zhang et al., 2014). The rats were anesthetized with CO2, decapitated with a guillotine and their brains were harvested. A coronal section of the dorsal hippocampus was dissected from the brain using a rat brain matrix (ASI Instruments, Warren MI). The medial (dentate gyrus/CA1) and lateral (CA2/CA3) dorsal hippocampus (Fig. 1) were isolated, homogenized in 700 μl of Qiazol (Qiagen Inc., Germantown MD) and immediately frozen and stored at −80°C until the RNA was prepared.

Total RNA was isolated from homogenates using the miRNeasy Kit (Qiagen Inc.), following manufacturer’s recommended protocol. The quality and quantity of RNA from each sample was determined using the Agilent 2100 Bioanalyzer (Agilent Technologies, San Francisco, CA). Starting with 0.5 μg of RNA, complementary DNA (cDNA) was prepared using the RT2 First Strand Kit (Qiagen Inc.), which uses a
proprietary procedure to eliminate contamination of genomic DNA from the RNA samples before reverse transcription (RT). Random hexamers and oligo-dT primers were used in the RT reaction in an unbiased manner. An external RNA control also was included to monitor the RT efficiency and tests for enzyme inhibitors that may contaminate the RNA samples during subsequent gene expression analysis.

2.4. RT² profiler array

Gene expression levels were measured using RT² SYBR® Green qPCR Mastermix and a custom RT² PCR Array (both from Qiagen, Inc.) following the manufacturer’s recommended protocol. The gene expression array contained 22 genes of interest (Table 1) and five reference genes. The genes of interest included; seven opioid peptide and receptor related-genes, six genes involved in stress-responses, four genes involved in synaptic plasticity and five kinase or signaling molecule genes important for opioid, stress and plasticity responses including those upregulated following oxycodone self-administration in mice (Zhang et al., 2014). The qPCR reactions were run on an ABI PRISM™ 7900HT Sequence Detection System (Applied Biosystems, Inc., Foster City, CA). The threshold cycle (Ct) values were used to calculate the mean (±SEM) normalized gene expression levels.

2.5. Statistical analysis and figure preparation

A 2 × 2 between-subjects factorial design, in which the factors were Sex (male/female) and Condition (control/stress), was applied. Ct values were normalized by taking their differences to the most stable gene in the analyzed brain region and used as response variables in the statistical model. A two-way analysis of variance (ANOVA) with Sex and Condition as main effects, as well as their interaction was tested for significance at a 5% level. For genes that produced a significant effect of Sex on the expression levels, a two-way ANOVA was performed with Sex and Condition as main effects and their interaction as a covariate. Post-hoc analysis revealed that CIS males have significantly greater expression than CIS females (P = .025). Post-hoc analysis showed that CIS males tended to have greater expression than CIS females (P = .024). Post-hoc analysis showed CIS males to have greater expression than CIS males (P = .069) and that control males tended to have greater expression than CIS males (P = .060; Fig. 2A).

There was a significant main effect of Stress on expression [F(1,20) = 5.46, P = .040]. Post-hoc analysis showed that control males tended to have greater expression than CIS males (P = .030). There was no significant effect of sex or stress on expression of Oprk1, Pdyn, Penk and Pomc in either hippocampal region.

3. Results

At the termination of the CIS experiment, unstressed (control) female rats were in the following estrous cycle stages: proestrus/estrous (N = 1), estrus (N = 4) and diestrus (N = 1), and all CIS female rats were in estrus (N = 6). Statistical evaluation of gene expression was similar in the female rats when data from the proestrus/estrous and estrus groups were carried out. Post-hoc P-values < 0.05 reported as nominally significant and P-values < 0.1 reported, for exploratory purposes. Data are summarized by their mean (±SEM) normalized gene expression levels.

3.1. Opioid genes

Group differences in opioid gene expression were mainly limited to the lateral (CA2/CA3) hippocampal sample.

Two-way ANOVA demonstrated a significant main effect of Sex on Oprm1 expression in the lateral hippocampus [F(1,20) = 5.91, P = .025]. Post-hoc analysis showed that CIS females had a trend toward significantly more expression than CIS males (P = .09; Fig. 2A).

There was a significant Sex x Condition interaction for Oprd1 expression [F(1,20) = 7.04, P = .015] in the lateral hippocampus. Post-hoc analysis showed that CIS females had a trend toward significantly greater expression than CIS males (P = .069) and that control males tended to have greater expression than CIS males (P = .060; Fig. 2B).

There was a significant main effect of Stress on Oprl1 expression [F(1,20) = 5.71, P = .028]. Post-hoc analysis showed that control males had a trend to significantly greater expression than CIS males (P = .030; Fig. 2C).

Table 1: Genes used on RT Profiler PCR array.

| Group               | Gene symbol | Gene name     |
|---------------------|-------------|---------------|
| Opioid              | Oprm1       | Opioid receptor, mu 1 |
|                     | Oprd1       | Opioid receptor, delta |
|                     | Oprk1       | Opioid receptor, kappa |
|                     | Oprl1       | Nociception receptor |
|                     | Pdyn        | Prodynorphin |
|                     | Penk        | Proenkephalin |
|                     | Pomc        | Proopiomelanocortin |
| Stress              | Crh         | Corticotropin releasing factor |
|                     | Chrh1       | Corticotropin releasing factor receptor 1 |
|                     | Chrh2       | Corticotropin releasing factor receptor 2 |
|                     | Avpr1a      | Arginine vasopressin (AVP) receptor 1a |
|                     | Avpr1b      | AVP receptor 1b |
|                     | Fkbp5       | FK506 binding protein 5 |
| Plasticity          | Arc         | Activated cytoskeletal-associated protein |
|                     | Bdnf        | Brain derived neurotrophin factor |
|                     | Nirk2       | Neurotrophic tyrosine kinase, receptor, type 2 |
|                     | Cdh2        | Calcium dependent adhesion transmembrane protein |
| Kinases and signaling molecules | Akt1       | Protein kinase B |
|                     | Mapk1       | Mitogen-activated protein kinase |
|                     | Pim 1       | Pim-1, proto-oncogene, serine/threonine kinase |
|                     | Arh1        | Beta arrestin 1 |
|                     | Arh2        | Beta arrestin 2 |
Fig. 2. Sex differences in opioid gene expression after CIS. Significant group differences were limited to genes in the lateral hippocampal sample. (a) *Oprm1* expression had a main effect of sex with CIS females showing a trend toward significantly more expression than CIS males (+p = .09). (b) *Oprd1* expression had significant sex and condition interaction with CIS females trending toward greater expression than CIS males (+p = .069) and control males showing a trend to have more expression than CIS males (+p = .060). (c) *Oprl1* expression shows control males having significantly greater expression than CIS males (*p = .030).

Fig. 3. Sex differences in stress gene expression after CIS. (a) In the medial hippocampal sample, CIS males have significantly greater *Crh* expression than both control males (**p = .0056) and CIS females (p = .0303). (b) CIS males had significantly less *Crhr1* expression than CIS females (*p = .0238) and tended to have less than control males (+p = .068) in the lateral hippocampal sample. (c) In the lateral sample, both CIS females and male controls had significantly more *Avpr1a* expression than CIS males (*p = .0342 and *p = .0328, respectively).
than both control males (P = .006) and CIS females (P = .030; Fig. 3A).

Two-way ANOVA showed a significant main effect of Sex on Crh1 expression [F(1,20) = 7.35, P = .013] in the lateral hippocampus. Post-hoc analysis revealed that CIS males have significantly more Crh1 than CIS females (P = .024), further control males trended toward greater expression than CIS males (P = .068; Fig. 3B).

There was a significant Sex x Condition interaction for Avpr1a expression [F(1,20) = 5.15, P = .034] in the lateral hippocampus. Post-hoc analysis revealed that both CIS females and male controls had significantly more Avpr1a expression than CIS males (P = .034 and P = .033, respectively; Fig. 3C).

There was no significant effect of sex or stress on expression of Avpr1b, Crhr2 and Fkbp5 in either hippocampal region.

### 3.3. Plasticity genes

Group differences in the expression of several plasticity genes were observed in both the lateral and medial hippocampal samples.

There was a significant main effect of Condition on Arc expression in the lateral hippocampus [F(1,20) = 8.70, P = .008]. Post-hoc analysis showed that control males have significantly more Arc expression than CIS males in the lateral (Fig. 4A) hippocampus (P = .004). Further, control females showed a trend toward significantly less Arc expression than control males in the lateral hippocampus (P = .077; Fig. 4A).

There was a significant effect of Sex on the expression of Bdnf [F(1,20) = 5.27, P = .032] in the lateral hippocampal region. Post-hoc analysis showed that female control rats had a trend toward significantly less Bdnf (P = .08) expression than control males (not shown).

Two-way ANOVA showed a significant Sex x Condition interaction for Cdh2 expression [F(1,20) = 5.28, P = .032] in the lateral hippocampus (Fig. 4B). Post-hoc analysis revealed that control males had significantly greater Cdh2 expression than CIS males (P = .027) while CIS females showed a trend to greater Cdh2 expression than CIS males (P = .071).

There was a significant main effect of Condition on Ntrk2 expression in both the lateral hippocampus [F(1,20) = 12.25, P = .002] (Fig. 4C) and in the medial hippocampus [F(1,20) = 5.24, P = .033] (Fig. 4D). Further, the expression of Ntrk2 in the medial hippocampus demonstrated a significant main effect of Sex [F(1,20) = 4.88, P = .039]. Post-hoc analysis showed that males in the control group had significantly more Ntrk2 expression than the CIS males, in both the lateral and medial hippocampus (P = .002 and P = .007, respectively). In addition, CIS females had significantly more Ntrk2 expression than CIS males in the medial hippocampus (P = .008) and trended toward significantly more in the lateral hippocampus (P = .088).

### 3.4. Kinases and signaling molecules

Group differences in the expression of kinase and signaling molecule genes associated with opioids, stress and synaptic plasticity were observed in both the lateral and medial hippocampal samples.

Two-way ANOVA demonstrated significant main effect of Condition on Akt1 gene expression in the lateral hippocampus [F(1,20) = 6.76, P = .017] (Fig. 5A), as well as in the medial hippocampus [F(1,20) = 10.41, P = .004] (Fig. 5B). Post-hoc analysis revealed that control male rats had significantly more Akt1 expression than CIS males in both the lateral and medial hippocampus (P = .011 and P = .001, respectively). In addition, CIS female rats had significantly more Akt1 expression compared to CIS males (P = .018) in the medial hippocampus and trend toward significance in the lateral hippocampus. (P = .071; Fig. 5A and B).

Two-way ANOVA demonstrated a significant main effect of Condition on Arrb1 expression in the lateral hippocampus [F(1,20) = 11.80, P = .003] and in the medial hippocampus [F(1,20) = 5.22, P = .033]. Post-hoc analysis revealed that control males have significantly more Arrb1 expression than CIS males (P = .004) and CIS females show a trend toward significantly more expression than CIS males (P = .069) in the lateral hippocampus (Fig. 5C). While, in the medial hippocampus, Arrb1 expression in the control males had a trend toward significantly higher levels than the CIS males (P = .056; Fig. 5D).

There was no significant effect of sex or stress on expression of Mapk1, Pim1 and Arrb2 in either hippocampal region.

---

**Fig. 4.** Sex differences in plasticity gene expression after CIS. (a) Control females tended (+p = .07) to have less Arc expression than control males in the lateral hippocampus. Control males had significantly higher Arc expression than CIS males in the lateral hippocampus (**p = .0039). (b) In the lateral hippocampus, CIS males had significantly less Cdh2 expression than control (**p = .027) and tended to have less Cdh2 expression than CIS females (+p = .071). (c, d) Control male rats had significantly more Ntrk2 expression than CIS males, in both the lateral and medial hippocampus (**p = .0021 and *p = .0070, respectively). CIS females have significantly more Ntrk2 expression than CIS males (**p = .0079) in the lateral hippocampus and trend toward significance in the lateral hippocampus. (+p = .088).
4. Discussion

These studies demonstrate that sex and CIS impact the expression of opioid, stress, plasticity, as well as kinases and signaling genes associated with these processes in the rat hippocampus (Fig. 6). Few sex differences were seen in hippocampal gene expression in control (unstressed) rats. However, following CIS, gene expression in the hippocampus was altered in males but not females. In males, opioid, stress and plasticity genes were almost exclusively down-regulated following CIS. The one exception was Crh, which was up-regulated. These findings are consistent with our previous anatomical and physiological findings (McEwen and Milner, 2017) that, unlike males, the female rodent hippocampus is resistant to the deleterious effects of chronic stress.

4.1. Methodological considerations

As we could not consistently separate the CA1 region from the dentate gyrus, we choose to process these regions together (i.e., the medial sample). Our rationale for this was that our primary interest was the opioid system. In hippocampus, granule cells produce most of the dynorphin and enkephalin (Drake et al., 2007) and thus changes in expression for these genes in the medial sample would correlate with changes in these cells. Moreover, interneurons containing MORs and DORs are sparsely distributed in the CA1 and dentate gyrus so Oprm1 and Oprd1 mRNA would be easier to detect if the samples were combined. However, as pyramidal and granule cell neurons in rats have low levels of Oprd1 expression, our medial sample would not be able to distinguish between these. Thus, these technical considerations could have contributed to the lack of sex and stress effects on the expression of opioid genes, as well as some stress and plasticity genes in the medial sample. As the CA1 and dentate gyrus have different functions and connections, future studies would explore gene expression in the CA1 and dentate gyrus separately.

In the present study, we chose to study the effect of CIS on gene expression one day after discontinuing the last stressor. We selected this time point because we wanted to correlate gene expression changes with our previous studies examining changes in enkephalin levels and opioid receptor trafficking changes in the hippocampus after CIS (Mazid et al., 2016; Milner et al., 2013; Pierce et al., 2014). One day after CIS, previous studies have demonstrated that corticosterone levels are elevated in male rats (Lakshminarasimhan and Chattarji, 2012).

4.2. Baseline sex differences in hippocampal gene expression

Of the opioid, synaptic and stress genes analyzed in the hippocampal samples, the present study found that Arc expression tended to be less in female rats compared to male rats in the CA3 region (lateral sample). Arc (activity-regulated cytoskeleton protein, also known as Arg3.1) is an immediate early gene that is targeted to synapses and is an important "master regulator" of protein synthesis-dependent forms of synaptic plasticity (reviewed in Bramham et al. (2010)). In particular, Arc is up-regulated during brain-derived neurotrophic factor (BDNF)-
stimulated long-term potentiation (Wibrand et al., 2006) and following enhancement of TrkB signaling by antidepressants (Larsen et al., 2007; Molteni et al., 2008). Notably, hormone levels influence Arc expression (Christensen et al., 2015) as well as BDNF levels in the mossy fiber pathway (Scharfman and MaClusky, 2014) and the number and types of cellular profiles containing phosphorylated TrkB in the hippocampus (Spencer-Segal et al., 2011). The majority of rats in the present study were in estrus (i.e., the phase when estrogen levels are declining and progesterone levels are rising), therefore, it is possible that Arc expression in females may differ in other estrous cycle phases. Additionally, it is also possible that Arc is involved in different synaptic plasticity processes (e.g., LTP vs neurogenesis) in females and males.

Few papers have looked at gene expression differences in the hippocampi of adult male and females rats at basal conditions. In adult male Wistar rats compared to females, genes involved in exocytosis, glutamate signaling and synaptic transmission were elevated in the hippocampus (Biala et al., 2011). Moreover, in this same study genes involved in negative regulation of apoptosis and in programmed cell death were upregulated in the hippocampi of adult female rats compared to male rats (Biala et al., 2011).

4.3. Sex differences in opioid gene expression after CIS

Together with our previous studies (reviewed in McEwen and Milner (2017)), the present study reveals opposing effects of CIS on hippocampal opioid system in female and male rats. Notably, expression of three of the seven opioid genes examined (Oprm1, Oprd1 and Oprl1) were down-regulated in the CIS males compared to control males and/or females consistent with the idea that CIS essentially shuts down the opioid system in males. Changes in opioid gene expression following CIS were limited to the CA2/3 region (lateral sample) which harbors DOR-containing pyramidal cells (Mazid et al., 2016), and scattered DOR-, MOR- and enkephalin-containing interneurons (reviewed in Drake et al. (2007)). Previous studies have shown that the CA3 opioid system is important in visual-spatial pattern completion, a component of context learning (Kesner and Warthen, 2010). We have shown that DORs are important for low frequency opioid-dependent LTP at the mossy fiber-CA3 pyramidal cell synapse that is unique to females (Harte-Hargrove et al., 2015). At the cellular level, females at high estrogen states compared to males have about three times more DORs in CA3 dendritic spines contacted by mossy fibers (Harte-Hargrove et al., 2015) and about twice as many DORs near the plasma membrane of CA3 pyramidal cell dendrites (Mazid et al., 2016). Moreover, high estrogen-state females compared to males contain about twice as many enkephalin-containing mossy fibers (Pierce et al., 2014) and enkephalin expressing interneurons (Bryson, Milner, Gray unpublished). Thus, down regulation of hippocampal opioid genes in CA2/3 in males following CIS would severely hamper the already limited opioid mediated synaptic plasticity in this region.

4.4. Sex differences in stress gene expression after CIS

The only gene to be up-regulated following CIS was Crh and this occurred exclusively in the males. This finding is consistent with other studies demonstrating an increase in hippocampal Crh mRNA and protein levels in adult rats (sex unspecified) following maternal separation stress (Wang et al., 2014). Cshr1 and Avpr1a expression were down-regulated in males, but not females, after CIS. A similar decrease in hippocampal Avpr1a mRNA has been reported in adult male mice subjected to forced-swim stress (Lesse et al., 2017). Moreover, in humans specific variants in CRH1R, AVPR1A and FKBP5 are associated with heroin addiction (Levran et al., 2014).

Our anatomical studies in hippocampus have shown that unstructured male and female rats contain similar levels of CRF1 receptor-immunoreactivity in CA1 dendrites and that CRF1 receptor often co-localizes with DORs in these dendrites (Williams et al., 2011a). Moreover, DOR agonists can inhibit CRF-induced cAMP accumulation in NG108-15 cells (Williams et al., 2011a) suggesting that DORs regulate CRF receptor signaling. We have recently found that CIS decreases the levels of cytoplasmic CRF1 receptor in CA3 pyramidal cell dendrites in males but not females (McAlin et al., 2016) and that these cellular changes are the opposite as those seen for DORs following CIS (Mazid et al., 2016). Thus, the observed decrease in Crh1 expression in males following CIS is consistent with these findings.

Arginine vasopressin (AVP) containing neurons in the hypothalamus innervate the hippocampal CA2 region (Zhang and Hernandez, 2013) which also contains high levels of phosphorylated DORs (Burstein et al., 2013). Activation of AVP receptors has been shown to facilitate hippocampal LTP (Chepkova et al., 2001; Dubrovsky et al., 2003). Thus, reduction of Avpr1a expression would likely negatively impact hippocampal LTP and thus learning processes. However, as AVP elicits aniogenic and depressive responses (Engelmann et al., 2004; Neumann and Landgraf, 2012), down-regulation of Avpr1a in males following CIS could help reduce these behaviors.

4.5. Sex differences in plasticity gene expression after CIS

Of the plasticity genes examined, Arc, Cdhl2 and Ntk2 were significantly reduced in the hippocampi of CIS males compared to control males and/or females. Moreover, Cdhl2 in CIS females tended to be reduced compared to control females. As discussed above, Arc is important for BDNF-induced LTP (reviewed in Bramham et al. (2010)). Thus, down-regulation of both Arc and Ntk2 following CIS may compromise BDNF-mediated neuroplasticity in males. This may be especially important for antidepressant treatment in which TrkB signaling is thought to promote or restore plasticity through gene expression regulation (Castren et al., 2007; Degenstadt et al., 2006). The finding that Arc expression is disrupted by stress has been shown in other studies. For instance, male rats whose anxiety-like behaviors are minimally disrupted by predator odors have up-regulated Arc expression in the hippocampus whereas rats who exhibit anxiety-like behaviors after predator odors show no up-regulation in Arc (Kozlovsky et al., 2008). Moreover, following prenatal stress, both adult male and female rats exhibit a down-regulation of Arc in the hippocampus (Biala et al., 2011).

Cdhl2 is part of a class of genes encoding synaptic adhesion molecules that not only play roles in adhering presynaptic and postsynaptic membranes together but also neuron-neuron recognition and in generating scaffolds onto which additional proteins can bind (reviewed in Rudenko (2017), Seong et al. (2015)). In line with the present findings in males, chronic restraint stress reduces N-Cadherin protein levels in the hippocampus of male rats (Castaneda et al., 2015). The findings that genes involved in synaptic plasticity are down-regulated in males following CIS supports previous studies that chronic stress negatively impact synaptic structure and function (for reviews see (McEwen, 2016; McEwen and Chattarji, 2007)). In particular, chronic stress results in reduced spine density as well as synaptic turnover in the hippocampus. Moreover, numerous studies in male hippocampi have shown disruption of LTP and calcium currents as well as diminished responses to neurotransmitters following chronic stress (Alfarez et al., 2002; Kamal et al., 2014; Kim et al., 1996; Krugers et al., 2010).

4.6. Sex differences in kinase and signaling molecule gene expression after CIS

AKT (protein kinase B) is a key signal transduction intermediate that plays a critical role in cell growth and survival and in synaptic protein translation including post-synaptic density (PSD)-95 (Akama and McEwen, 2003; Brunet et al., 2001; Chong et al., 2005). Our previous anatomical studies in rat hippocampus demonstrated that phosphorylated AKT protein levels are significantly decreased in the CA1 pyramidal cell dendrites of males compared to females, regardless of
estrous cycle (Znamensky et al., 2003). The presence of phosphorylated AKT-immunoreactivity in CA1 pyramidal cell synapses fluctuates and follows the estrous cycle with females at high estrogen states having about twice the levels as those seen in males (Yildirim et al., 2011; Znamensky et al., 2003). Additionally, the presence of AKT-immunoreactivity in hippocampal synapses diminishes in aged female rats (Yildirim et al., 2011). The lack of sex differences in the Akt1 expression in the control rats suggests that these changes in phosphorylated AKT protein levels are post-translational. The reduction of Akt1 expression in the CA1 synapses could contribute to the dendritic remodeling and synaptic loss of hippocampal neurons seen in males after chronic stress (McEwen, 2016).

B-arrestins are important regulators of the desensitization and internalization of G-protein-coupled receptors as well as signal transducers (DeWire et al., 2007). B-arrestins are not only important for opioid receptor signal transduction (Al-Hasani and Bruchas, 2011; Fan et al., 2003) but regulating the kinetics and transduction pathway selectivity of CRF1 receptor signaling (Oakley et al., 2007). Thus, the observed down-regulation of Arrb1 in CA1 synapses could impact both opioid and CRF1 receptor signaling in hippocampal neurons.

4.7. Functional considerations

The observed sex differences in hippocampal opioid, stress, plasticity, kinetics and signaling molecules gene expression in response to CRF are consistent with our previous anatomical findings (McEwen and Milner, 2017). Moreover, these studies add to the growing body of literature demonstrating sex differences in hippocampal gene expression in response to stress (Anacker et al., 2016; Bagot et al., 2016; de Azeredo et al., 2017; Labonte et al., 2017; Mychasiuk et al., 2016; Wang et al., 2014). Importantly, the studies to date examining hippocampal gene expression following stress also have shown sexual dimorphisms in the specific genes that are up- or down-regulated depend on the stress model (CIS vs chronic social defeat stress), strain (rats vs. mice) and age of the stress (neonatal vs adult).

The down-regulation of opioid peptide and receptor genes, synaptic plasticity genes and the majority of stress genes in males following CIS would severely impact learning processes. This is consistent with behavioral observations that males, but not females, display impaired cognitive performance after chronic stress (Conrad et al., 2003; Kitraki et al., 2004; Luine et al., 2007; Weiss et al., 2005). In particular, sexual dimorphism in response to stress is important in context learning (Bangasser and Shors, 2010; Beck and Luine, 2010; Becker and Koob, 2016) which is a critical component of drug acquisition and relapse (Crombag et al., 2008; Koob and Volkow, 2010).

Acknowledgements

Study design: MJK, BSM, TAM, JDG; Data collection: SM, SCO, JCR, MR, YZ; Writing of report: MJK, BSM, TAM, MR, JDG. Decision to submit for publication: BSM, TAM, MR, JDG. Supported by NIH grant DA08259 (TAM, MJK, BSM) and Hope for Depression Research Foundation (BSM) and the Dr. Miriam and Sheldon G. Adelson Medical Research Foundation (MJK).

Conflicts of interest

The authors declare no competing financial interests.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.jnstr.2018.01.001.
Lakshminarasimhan, H., Chattarji, S., 2012. Stress leads to contrasting effects in the hippocampus. Neurobiol. Stress. 5, 37.

McEwen, B.S., Chattarji, S., 2007. Neuroendocrinology of stress., Handbook of Biological Psychology. Academic Press, London, pp. 751–811.

Kilts, C.D., Schweitzer, J.B., Quinn, C.K., Gross, R.E., Faber, T.L., Muhammad, F., Ely, E.J., 2017. Sex-specific influences corticotropin-releasing factor receptor colocalization with delta opioid receptors in the hippocampus. Neurobiol. Stress. 5, 37.

Kazarskis, A., Tamminga, C., Mechawar, N., Turecki, G., Zhang, B., Shen, L., Nestler, E.J., 2015. Opioid receptor-dependent sex differences in synaptic plasticity in the hippocampus. Neuron 80, 1159–1171.

Vorel, S.R., Liu, X., Hayes, R.J., Spector, J.A., Gardner, E.L., 2001. Relapse to cocaine-seeking in male and female outpatients. Drug Alcohol Depend. 53, 233–250.

Rudens, G., 2017. Dynamic control of synaptic adhesion and organizing molecules in synaptic plasticity. Neuronal Plasticity, 2017, 65265151.

Scharfman, H.E., MacLusky, N.J., 2014. Differential regulation of BDNF, synaptic plasticity and neurogenesis in the mouse hippocampal pathway of male and female rats. J. Neurosci. 34, 695–708.

Seong, E., Yuan, L., Arikat, J., 2015. Catecholamines and catecholamine synapses: A new role for cell adhesion molecules. Curr. Drug Targets. 16, 202–217.

Shea, L.U., Highfield, D., Yap, J., Shaban, Y., 2000. Stress and relapse to drug seeking in rats: studies on the generality of the effect. Psychopharmacology (Berl) 150, 137–146.

Spencer, J.I.L., Waters, E.M., Bath, K.G., Chao, M.V., McEwen, B.S., Milner, T.A., 2011. Distribution of phosphorylated TrkB receptor in the mouse hippocampal formation depends on sex and estrous cycle stage. J. Neurosci. 31, 6780–6790.

Torrance, T.A., Khalid, M., McEwen, B.S., Drake, C.T., CT., McEwen, B.S., Milner, T.A., 2008. Ovarian steroids modulate enkephalin levels and target enkephalins in the female hippocampal mossy fiber pathway. Brain Res. 1222 (70-74), 70–84.

Torrence-Roveron, A., Williams, T.J., Chapleau, J.D., Waters, E.M., McEwen, B.S., Drake, C.T., Milner, T.A., 2009. Ovarian steroids alter mu opioid receptor trafficking in the hippocampal paraventricular GABAergic interneurons. Exp. Neurol. 219, 319–327.

Turner, C.D., Bagnara, J.T., 1971. General Endocrinology. W.B. Saunders, Philadelphia.

Volkow, N.D., Wang, G.J., Telang, F., Fowler, J.S., Logan, J., Childress, A.R., Jayne, A.R., May, M., Wong, C., 2006. Cocaine cues and dopamine in dorsal striatum: mechanism of reward in cocaine addiction. J. Neurosci. 26, 6583–6588. Behav. Brain Res. 51, 457–461.

Vorel, S.R., Liu, X., Hayes, R.J., Spector, J.A., Gardner, E.L., 2001. Relapse to cocaine-seeking after hippocampal theta burst stimulation. Science 292, 1175–1178.

Vyas, A., Mitra, R., Shankaransamy Rao, B.S., Chattarji, S., 2002. Chronic stress increases contractions of dendritic remodeling in hippocampal and amygdaloid neurons. J. Neurosci. 22, 6810–6818.

Wang, A., Nie, W., Li, H., Hou, Y., Yu, Z., Fan, Q., Sun, R., 2014. Epigenetic upregulation of corticotropin-releasing hormone mediates postnatal maternal separation-induced memory deficiency. PLoS One 9, e94394.

Weiss, C., Sametsky, E., Sasse, A., Spiess, J., Disterhoft, J.F., 2005. Acute stress facilitates trace eyeblink conditioning in C57BL/6 male mice and increases the excitability of hippocampal mossy fiber pathway of male and female rats. J. Neurosci. 25, 128–138.

Wilbrand, K., Messaoudi, E., Havik, B., Steenslid, V., Lovlie, R., Steen, V.M., Bramham, D.C., 1999. Comparing levels of corticotrophin-releasing hormone and beta-endorphin in the bitrofrontal cortex, and hippocampus. Neuroscience 322, 489–500.

Williams, T.J., Akama, K.T., Knudsen, M.G., McEwen, B.S., Milner, T.A., 2011a. Ovarian hormones influence corticotropin-releasing factor receptor colonization with delta opioid receptors in CA1 pyramidal cell dendrites. Exp. Neurol. 230, 186–202.

Williams, T.J., Torres-Reveron, A., Chapleau, J.D., Milner, T.A., 2011b. Hormonal regulation of delta opioid receptor immunoreactivity in interneurons and pyramidal cells in the rat hippocampus. Neurobiol. Learn. Mem. 99, 506–202.

Yildirim, M., Jansen, W.G., Lou, W.Y., Akama, K.T., McEwen, B.S., Milner, T.A., Morrison, J.H., 2011. Effects of estrogen and aging on the synaptic distribution of aromatase-expressed Akan immunoactivity in the CA1 region of the female rat hippocampus. Brain Res. 1379, 98–108.

Zhang, L., Hernandez, V.S., 2013. Synthetic innervation to rat hippocampus by vaso-pressin-immuno-positive fibers from the hypothalamic supraoptic and paraventricular nuclei. Neuroscience 228, 139–162.

Zhang, Y., Brownstein, A.J., Buonora, M., Niikura, K., Ho, A., Correa da, R.J., Kreek, M.J., Ott, J., 2015. Self-administration of oxycodone alters synaptic plasticity gene expression in the hippocampus. Neurobiol. Stress. 25, 34–46.

Zhang, Y., Mayer-Blackwell, B., Schlussman, S.D., Randes, M., Butelman, E.R., Ho, A., Kreek, M.J., 2014. Extended access oxycodone self-administration and neuroremitter receptor gene expression in the dorsal striatum of adult C57BL/6J mice. Psychopharmacology (Berl) 231, 1277–1287.

Znamensky, V., Akama, K.T., McEwen, B.S., Milner, T.A., 2003. Estrogen levels regulate the subcellular distribution of pinopodin/Ena/Vasp in hippocampal CA1 dendrites. J. Neurosci. 23, 2340–2347.