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

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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 RT^2 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 oxycodone self-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.

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 Koob, 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

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Abbreviations

BDNF	brain-derived neurotrophic factor
CIS	chronic immobilization stress
CRF	corticotrophin releasing factor
CRFR1	corticotrophin releasing factor receptor 1
Ct	threshold cycle
DOR	delta opioid receptor
FKBP5	FK506 binding protein 5
LTP	long-term potentiation
MOR	mu opioid receptor
PARV	parvalbumin
PSD	post-synaptic density
RIN	RNA integrity number
ROI	region of interest
RT	reverse transcription

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., 2002). 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 CO_2, 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 RT^2 First Strand Kit (Qiagen Inc.), which uses a

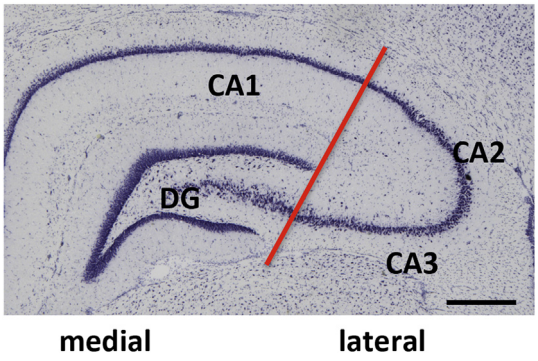


Fig. 1. Schema of hippocampal dissection. A Nissl-stained coronal section of the rat dorsal hippocampus denotes where the medial and lateral samples were dissected (red line). Bar = 0.5 mm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

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. RT^2 profiler array

Gene expression levels were measured using RT^2 SYBR® Green qPCR Mastermix and a custom RT^2 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 up-regulated 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 produce a significant ANOVA results ($P < .05$), either for main effects and/or interaction, Tukey-Kramer HSD post-hoc pairwise comparisons among the four studied 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. Results

At the termination of the CIS experiment, unstressed (control) female rats were in the following estrous cycle stages: proestrus/estrus (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/estrus and diestrus control rats were included and omitted. Thus, the data presented for the control females includes all 6 rats, regardless of estrous cycle stage. A complete listing of the statistical analysis for all genes is in Supplementary Tables 1 and 2.

All RNA samples were of high quality with a RNA Integrity Number (RIN) ranging from 8.0 to 9.0, as determined by the Agilent 2100 Bioanalyzer. Sex and stress affected gene expression in all three categories: opioid, plasticity and stress.

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 *Oprm1* expression than CIS males ($P = .09$; Fig. 2A).

There was a significant Sex x Condition interaction for *Oprd1* expression [$F(1,20) = 5.94$, $P = .024$]. Post-hoc analysis showed CIS females tended to have greater *Oprd1* expression than CIS males ($P = .069$) and that control males tended to have greater *Oprd1* 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 have significantly greater *Oprl1* expression than CIS males ($P = .030$; Fig. 2C).

There was no significant effect of sex or stress on expression of *Oprk1*, *Pdyn*, *Penk* and *Pomc* in either hippocampal region.

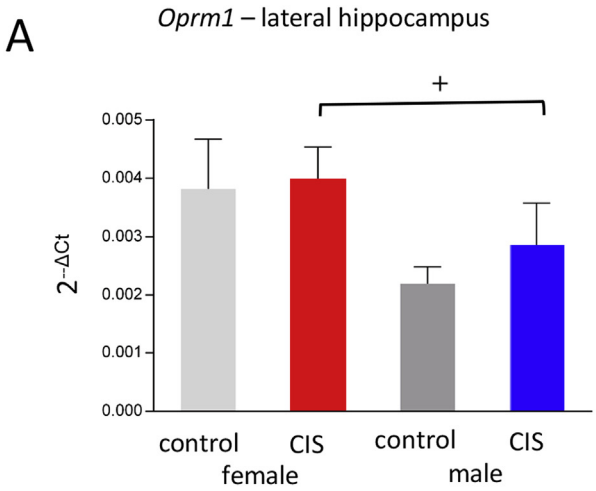
3.2. Stress genes

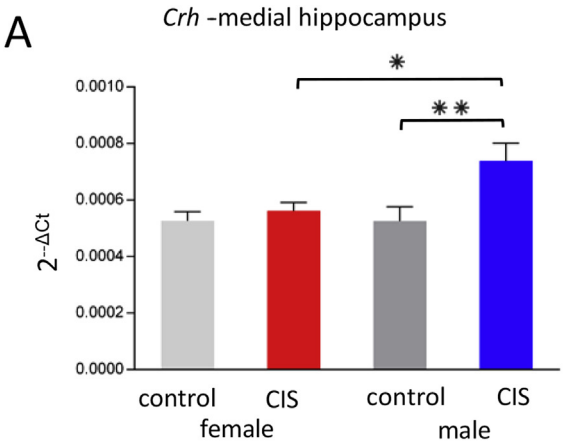
Group differences in the expression of stress-related genes were observed in both the lateral and medial hippocampal samples.

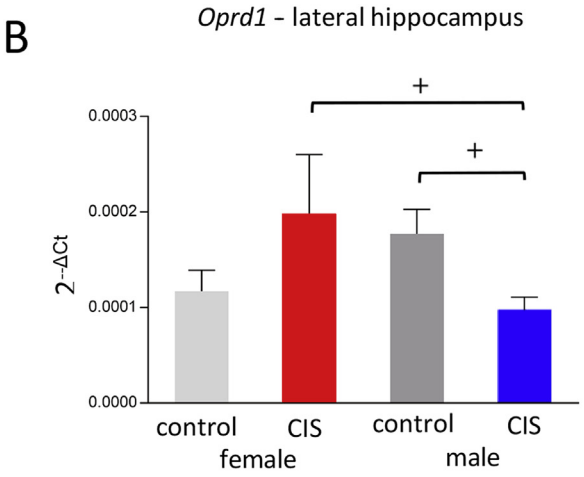
Two-way ANOVA showed a significant main effect of Condition on *Crh* expression [$F(1,20) = 7.04$, $P = .015$] in the medial hippocampus. Post-hoc analysis revealed that CIS males have significantly greater *Crh*

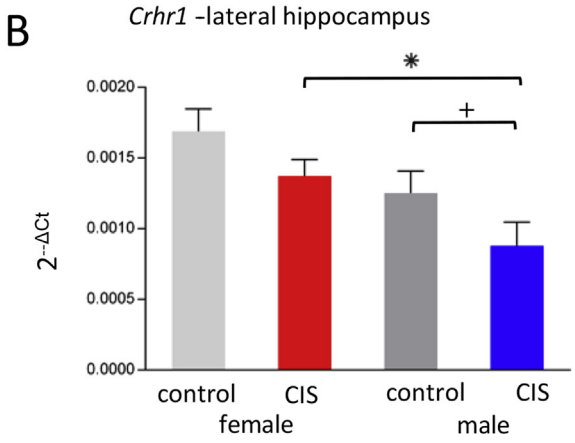
Table 1
Genes used on RT Profiler PCR array.

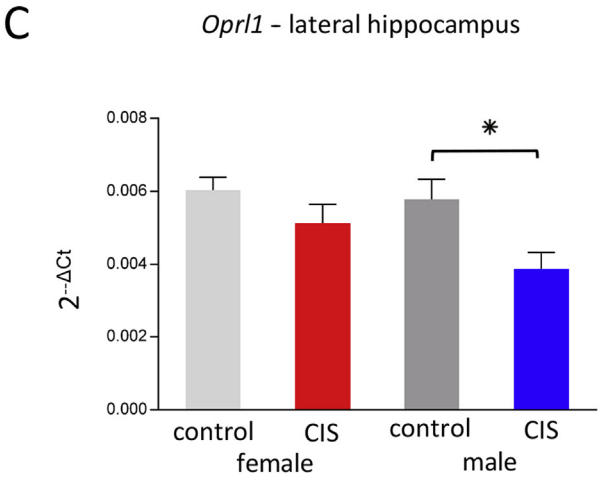
Group	Gene symbol	Gene name
Opioid	*Oprm1*	Opioid receptor, mu 1
	Oprd1	Opioid receptor, delta
	Oprk1	Opioid receptor, kappa
	Oprl1	Nociception receptor
	Pdyn	Prodynorphin
	Penk	Proenkephalin
	Pomc	Proopiomelanocortin
Stress	*Crh*	Corticotrophin releasing factor
	Crhr1	Corticotrophin releasing factor receptor 1
	Crhr2	Corticotrophin releasing factor receptor 2
	Avpr1a	Arginine vasopressin (AVP) receptor 1a
	Avpr1b	AVP receptor 1b
	Fkbp5	FK506 binding protein 5
Plasticity	*Arc*	Activity regulated cytoskeletal-associated protein
	Bdnf	Brain derived neurotrophin factor
	Ntrk2	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
	Arrb1	Beta arrestin 1
	Arrb2	Beta arrestin 2











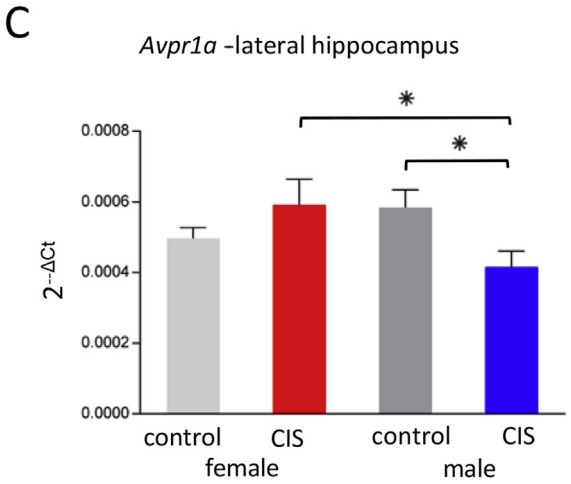


Fig. 2. Sex differences in opioid gene expression after CIS. Significant group differences were limited to genes in the lateral hippocampal sample. (a) *Oprm1* expression had a main effect of sex with CIS females showed a trend toward significantly more expression than CIS males (+p = .09). (b) *Oprd1* expression had significant sex and condition interaction with CIS females trending toward greater expression than CIS males (+p = .069) and control males show a trend to have more expression than CIS males (+p = .060). (c) *Oprl1* expression shows control males have 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 *Crhr1* expression [F(1,20) = 7.35, P = .013] in the lateral hippocampus. Post-hoc analysis revealed that CIS females have significantly more *Crhr1* than CIS males (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 have 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.

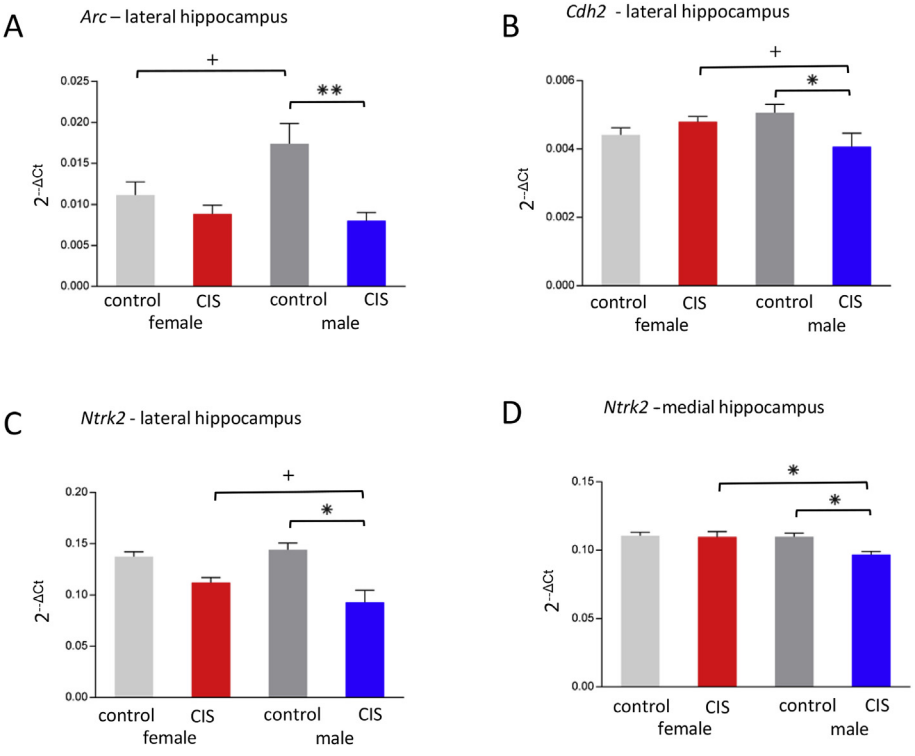


Fig. 4. Sex differences in plasticity gene expression after CIS. (a) Control females tended (+p = .07) to have less *Arc* expression than control males in the lateral hippocampus. Control males had significantly higher *Arc* expression than CIS males in the lateral hippocampus (**p = .0039). (b) In the 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 medial hippocampus and trend toward significance in the lateral hippocampus. (+p = .088).

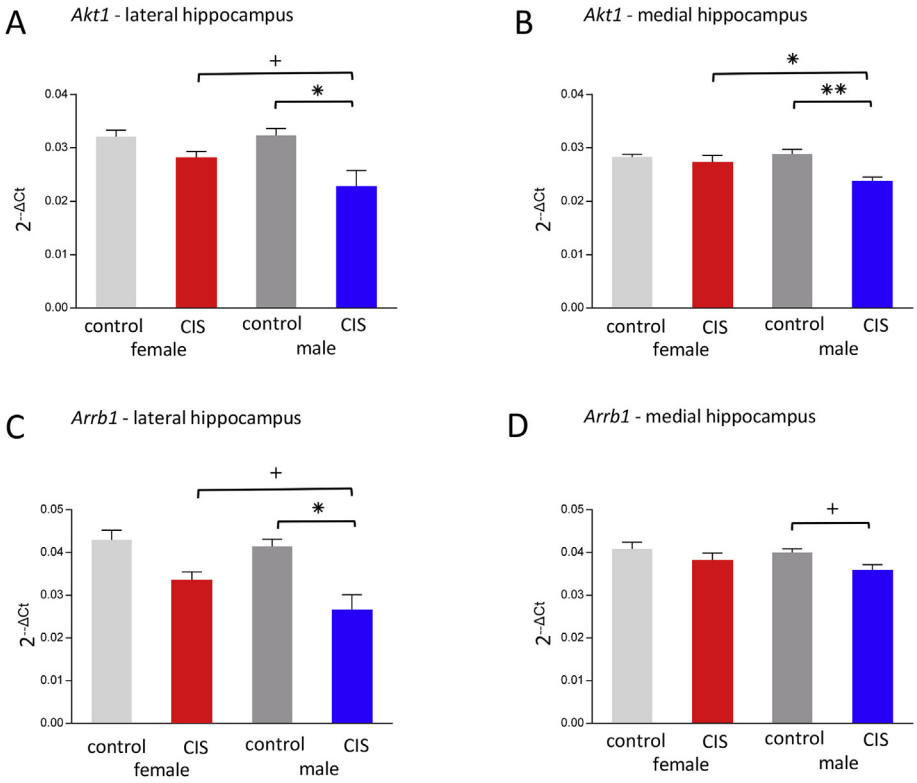


Fig. 5. Sex differences in kinases and signaling molecule gene expression after CIS. (a,b) The male controls had significantly more *Akt1* expression than CIS males in the lateral and medial hippocampus (*p = .0109 and **p = .0013, respectively). CIS females had significantly more *Akt1* expression compared to CIS males in the medial hippocampus (*p = .018) with a trend toward significance in the lateral hippocampus (+p = .071). (c,d) Control males had significantly more *Arrb1* expression than CIS males (*p = .0037) in the lateral hippocampus and trended toward significantly greater than CIS males (+p = .056) in the medial hippocampus. Additionally, CIS females showed a trend toward greater expression of *Arrb1* than CIS males (+p = .069) in the lateral region.

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 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)-

Control			Chronic Stress	
Female	Male		Female	Male
–	–	OPIOID	–	*Oprd1* *Oprl1* ↓
–	–	STRESS	–	*Crh* ↑ *Crhr1* *Avpr1a* ↓
	< *Arc*	PLASTICITY	–	*Arc* *Cdh2* *Ntrk2* ↓
–	–	KINASES & SIGNALING	–	*Akt1* *Arrb1* ↓

Fig. 6. Summary. Schematic diagram of sex differences in the changes in gene expression in the dorsal hippocampus at baseline (control) and following chronic immobilization stress. Arrows indicate direction of change. For simplicity, results from lateral and medial hippocampus are not distinguished.

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). *Crhr1* 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 *CRHR1*, *AVPR1A* and *FKBP5* are associated with heroin addiction (Levran et al., 2014).

Our anatomical studies in hippocampus have shown that unstressed 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 (McAlinin 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 anxiogenic 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, Cdh2* and *Ntk2* were significantly reduced in the hippocampi of CIS males compared to control males and/or females. Moreover, *Cdh2* 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 *Ntrk2* 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; Dagestad 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).

Cdh2 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 over 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 CIS males in both hippocampal regions 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 CIS males could impact both opioid and CRF1 receptor signaling in hippocampal neurons.

4.7. Functional considerations

The observed sex differences in hippocampal opioid, stress, plasticity, kinases and signaling molecules gene expression in response to CIS 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).

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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.ynstr.2018.01.001.

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