Depression and Heart Failure in Male and Female Rats:

Role of Inflammation and Estrogens

by

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Abstract

Clinical and preclinical studies revealed that heart failure induces depression. Injury of myocardial tissue initiates an inflammation cascade that extends to the CNS and might contribute to depression following myocardial infarction (MI). Sex differences were noticed in the progression of heart failure and depression in clinical studies. We hypothesized that depression-like behavior induced by HF post-MI is influenced by sex through modulation of inflammation pathway.

First, we evaluated sex differences in depression-like behavior in male and female rats at 8-10 weeks post-MI, as well as circulating cytokines, the extent of inflammation in the PFC, PVN, and amygdala, and mBDNF levels in the PFC and amygdala that are affected by neuroinflammation. Then we evaluated the effect of ovariectomy (OVX) and whether estrogen replacement with 17β-estradiol (E_2) prevents post-MI induced depression-like behavior through inhibiting neuroinflammation. Thirdly we evaluated the role of inflammation for cardiac dysfunction and development of depression-like behavior in OVX female rats post-MI by oral treatment with pentoxifylline (PTX). Male rats developed depression like behavior by ten weeks post-MI but not females as assessed by sucrose preference test (SPT) and forced swim test (FST). Both developed similar cardiac dysfunction and a comparable increase in plasma and PVN cytokine levels, but cytokine levels increased and mBDNF levels decreased only in the PFC of male rats post-MI. OVX per se decreased sucrose consumption and induced passive behavior assessed by SPT and FST, respectively.The combination of MI and OVX aggravated depression-like behvaiour. E_2 treatment prevented the development of mild depression-like behavior in OVX and severe symptoms in OVX female rats post-MI. E_2 had no effect on cardiac dysfunction in OVX female rats at 10 weeks post-MI. Despite the similar increase in circulating cytokines in OVX ± E_2 at 10 weeks post-

MI, E_2 decreased the proinflammatory cytokines and increased IL-10 (anti-inflammatory cytokine) in the PFC. Finally, we evaluated the role of neuroinflammation in depression-like behavior in OVX female rats post-MI through inhibiting cytokine production by administering oral PTX. PTX prevented depression-like behavior in OVX female rats post-MI and reduced cytokines levels in plasma, PVN and PFC. However, PTX did not affect the progression of cardiac dysfunction at 10 weeks post-MI. Sex determines the development of depression-like behavior in HF post-MI and neuroinflammation appears to play a critical pathway that is affected by sex and can be inhibited by hormonal replacement or anti-inflammatory agents.

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List of abbreviations

(3-HK)	3-hydroxykynurenine
(3-NT)	3-nitrotyrosine
(5-HIAA)	5-hydroxyindoleacetic acid
(5-HT)	Serotonin
(5-HTTLPR)	Serotonin-transporter-linked polymorphic region
(ACE)	Angiotensin-converting-enzyme
(ACTH)	Adrenocorticotropic hormone
(AKT-1)	Protein kinase B
(AMY)	Amygdala
(Ang II)	Angiotensin II
(AP-1)	activator protein-1
(AT1R)	Angiotensin II type 1 receptor
(ARB)	Angiotensin receptor blocker
(BBB)	Blood-brain barrier
(BDI)	Beck depression inventory
(BDNF)	Brain-derived neurotrophic factor
(BH4)	Tetrahydrobioptrein
(BLA)	Basolateral amygdala
(BNST)	Bed nucleus of stria terminals
(BW)	Body weight
(CABG)	Coronary artery bypass graft
(CI)	Cardiac index
(CMS)	Chronic mild stress
(CNS)	Central nervous system
(cNTS)	Caudal nucleus tractus solitarius
(COX-2)	Cyclooxygenase-2
(CREB)	Calcium/cyclic-AMP responsive-element binding protein
(CRH)	Corticotropin-releasing hormone
(CS)	Conditioned stimulus
(CSF)	Cerebrospinal fluid
(CVD)	Cardiovascular diseases
CX3CL1)	Fractalkine
(CXCR1)	CX3C chemokine receptor 1
(DAMP)	Danger-associated molecular pattern
(DBD)	DNA- binding domain
(DRN)	Dorsal raphe nuclei
(E_2)	17β-estradiol
(EF)	Ejection fraction

(eNOS)	Endothelial nitric oxide synthase
(ER)	Estrogen receptor
(ERK)	Extracellular signal regulated kinase
(ERE)	Estrogen response element
(ES)	Escapable shock
(GABA)	Gamma-aminobutyric acid
(GDS)	Geriatric depression scale
(GPER)	G protein coupled estrogen receptor
(GR)	Glucocotrticosteroid receptor
(GRE)	Glucocorticoid response element
(FCT)	Fear conditioning test
(FST)	Forced swim test
(HAM-D)	Hamilton depression rating scale
(HF)	Heart failure
(HPA)	Hypothalamus-Pituitary-Adrenal
(Iba-1)	Ionized calcium-binding adapter molecule-1
(IDO)	Indoleamine 2,3-dioxygenase
(IGF-1)	Insulin-like growth factor
(IL)	Interleukin
(IL-1R)	Interleukin-1 receptor
(IFN)	Interferon
(iNOS)	Inducible nitric oxide synthase
(IP)	Intraperitoneally
(IS)	Inescapable shock
(ITC)	Amygdaloid intercalated cells
(ICV)	Intracerebroventricular
(JAK3)	Janus associated-kinases-3
(KA)	Kynurenic acid
(LAD)	Left anterior descending coronary artery
(LBD)	Ligand binding domain
(LC)	Locus coeruleus
(LPS)	Lipopolysaccharide
(LV)	Left ventricle
(LV dP/dt (-))	Minimum rate of rise of LV pressure
(LV dP/dt (+))	Maximum rate of rise of LV pressure
(LVEDV)	LV-End diastolic volume
(LVESV)	LV-End systolic volume
(LVEDP)	LV-End diastolic pressure
(LVPSP)	LV-Peak systolic pressure
(M1)	Proinflammatory macrophage
(M2)	Anti-inflammatory macrophage
(MAPK)	Mitogen-activated protein kinase
(MDD)	Major depressive disorder

(ME)	Median eminence
(Met)	Methionine
(MI)	Myocardial infarction
(Mincle)	Macrophage Ca+2 dependent lectin receptor
(MMP)	Matrix metalloproteinase
(MWM)	Morris water maze
(NE)	Norepinephrine
(NF-kB)	Nuclear-factor kappa-light-chain-enhancer of activated B cells
(NMDA)	N-methyl-D-aspartate
(NO)	Nitric oxide
(NORT)	Novel object recognition test
(NOX)	NADPH oxidase
(OVLT)	Organum vasculosum of the laminae terminalis
(OVX)	Ovariectomized
(PAMP)	Pathogen-associated molecular pattern
(PFC)	Prefrontal cortex
(PGA2)	Prostaglandin A2
(PGE2)	Prostaglandin E2
(PIC)	Proinflammatory cytokines
(PI3K)	Phosphatidylinositol-3-kinases
(PKC)	Protein kinase C
(PLC)	Phospholipase C
(PPR-ɣ)	Peroxisome proliferator-activated receptor-ɣ
(PVN)	Paraventricular nucleus
(PTX)	Pentoxifylline
(QA)	Quinolinic acid
(RV)	Right Ventricle
(RVLM)	Rostral ventrolateral medulla
(SFO)	Subfornical organ
(SNP)	Single-nucleotide polymorphism
(SNRI)	Serotonin-norepinephrine reuptake inhibitor
(SP-1)	Stimulating protein-1
(SPT)	Sucrose preference test
(SSRI)	Serotonin reuptake inhibitor
(STAT-5)	Signal transducers and activators of transcriptions-5
(TCA)	Tricyclic antidepressant
(TLRs)	Toll-like receptors
(TNF)	Tumor necrosis factor
(TrkB)	Tropomyosin- receptor kinase B
(US)	Unconditioned stimulus
(Val)	Valine

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Chapter 1:

General Introduction

1.0. Overview

Major depressive disorder (MDD) is associated with significant functional disability, morbidity and mortality. It is diagnosed when an individual experiences loss of interest or pleasure, depressed mood without manic episodes, and is associated with recurrent thoughts of death and suicide, insomnia or hypersomnia, and feeling of worthlessness (Diagnostic and Statistical Manual-IV-text revision) (APA 2000). Depression is initiated by the interaction of environmental and genetic factors that lead to monoamines dysfunction. The prevalence of depression is influenced by several factors such as age, gender, socio-economics, and the presence of other comorbidities such as cardiovascular disease, diabetes mellitus and rheumatoid arthritis (Kessler and Bromet, 2013). The prevalence of depression in patients with heart failure (HF) is higher than in the general population (Rutledge et al., 2006).

Both psychosocial factors and critical biological mechanisms may contribute to depression with HF. Indeed, animals with HF also develop depression-like behavior. The comorbidity of depression and HF may reflect an enhancement of inflammation. In patients with HF, plasma pro-inflammatory cytokines (PICs) such as interleukin (IL)-6 and tumor necrosis factor (TNF)-α are higher than in the general population (Deswal et al., 2001). Patients with depression show an increase in plasma IL-2, IL-6 and TNF-α levels (Liu et al., 2012). Male rats post-MI display depression-like behavior associated with an increase in plasma IL-6, TNF-α levels (Kaloustian et al., 2008; Bah et al., 2011a). Caspase-3 activity and TUNEL staining, which reflects apoptosis, increased significantly in the frontal cortex of male rats post-MI (Kaloustian et al.,2008), whereas there were no changes in neural loss in the dorsal hippocampus (Frey et al.,2014). Cytokines may access the central nervous system (CNS) through active transporters across the Blood-Brain Barrier (BBB),

circumventricular organs, or conversion of its signaling to secondary messenger such as prostaglandins (PGE2), and nitric oxide by endothelial cells (Pasic et al., 2003).

In the general population, women have a 1.7-2.7 higher risk to develop depression compared to men through their lifetime (Ferrari et al., 2013). This increase is prominent in particular periods such as in premenstrual, postpartum and postmenopausal period that are associated with a decrease in estrogen levels (Borrow and Cameron, 2014). Hormonal replacement therapy alleviates depression symptoms related to menopause (Toffol et al., 2015). 17β- Estradiol (E_2) treatment protects against the development of depression-like behavior in adult and aged ovariectomized (OVX) female rats (Walf and Frye, 2010; Kiss et al., 2012; Xu et al., 2015). Older women with cardiovascular disease are at higher risk to develop depression, and women with HF and depression have higher mortality risk and HF events (Gottlieb et al., 2004; Wright et al., 2014). Despite the clear evidence of gender differences in humans, there are no studies that evaluate depression-like behavior induced by HF in female rodents. We hypothesized that inflammation in the periphery and CNS links cardiovascular disease and depression, and estrogens influence this pathway. Our study has 3 aims: 1) determine differences in depression-like behavior in male and female rats with HF post-MI and investigate the changes of PICs in the paraventricular nucleus (PVN) and brain areas involved in depression-like behavior such as prefrontal cortex (PFC) and amygdala. 2) assess the effect of OVX in female rats with HF post-MI on depression-like behavior and PICs in the plasma, PVN and PFC, and effects of E_2 replacement. 3) determine the role of neuroinflammation by using the cytokine synthesis inhibitor, pentoxifylline, in OVX female rats with HF post-MI.

1.1. Heart failure induces depression

A number of clinical studies demonstrated the high co-morbidity of depression and HF. This section will highlight the association between HF and depression, followed by the importance of sex differences in clinical and experimental studies.

1.1.1. Heart failure and depression have a bidirectional association

Several meta-analyses report a significant relationship between cardiovascular diseases (CVD) and depression (Van der Kooy et al., 2007; Vogelzangs et al., 2012; Carney and Freedland, 2016). Depression is considered an independent risk factor for developing CVD, after taking into account the presence of other risk factors (Moraska et al., 2013). Both morbidities tend to be present in older patients (Garfield et al., 2014). Older adults with depression are more likely to develop coronary heart disease and acute MI compared to those non-depressed (Brown et al., 2011). Patients with HF class IV exhibited fourfold higher depression rates compared to those in class I (Rutledge et al., 2006). Patients with HF and depression have a significantly worse quality of life and increased functional decline compared to those without depression (Vaccarino et al., 2001; Gottlieb et al., 2004). The association of the two diseases increases the risk of readmission to hospital and all-cause mortality (Faris et al., 2002; Jünger et al., 2005). The Hamilton depression rating scale (HAM-D) score inversely correlated with the ejection fraction (EF) in 209 patients, and depression scores were significantly higher in 45 non-survivors after 24 months of follow-up compared to 164 survivors (Jünger et al., 2005). In 396 patients with HF, 83 suffered from depression, and among these 58% were in class III-IV of HF, while of the 313 patients with HF without depression 87% were in class I-II. Readmission rate and risk of all-cause mortality increased 3-fold after 4 years of follow up in clinically depressed patients with HF compared to those without depression (Faris et al., 2002). The

dominant population in these studies were men, 77%, and 84% respectively (Faris et al., 2002; Jünger et al., 2005).

The standard treatments for depression (tricyclic antidepressant (TCA) or serotonin reuptake inhibitor (SSRI)) failed to reduce the morbidity or mortality associated with the presence of the two diseases. The SADHART-CHF study (O'Connor et al., 2010) included 469 patients with depression and HF (60 % class III-IV), mean age of 60, with 43-38% of females in the sertraline and placebo arms respectively. Sertraline treatment for 12 weeks did not cause a significant improvement in depression symptoms, cardiovascular status, or all-cause mortality after two years of follow up compared to placebo treatment. The non-significant outcomes may have been related to the small sample size and short duration of treatment. Accordingly, the MOOD-HF RCT study was conducted, with a larger sample size (773), and prolonged treatment up to 12 months. 30% of the population were older than 70 years, and participants had HF (50% class III-IV) and depression. Only 25% of the subjects were females in the escitalopram (SSRI) or placebo arms. Again, there was no significant difference in the primary outcome of decreasing hospitalization and all-cause mortality after 24 months. Despite using a sensitive tool (Montgomery-Åsberg Depression Rating Scale) instead of HAM-D for scoring depression, escitalopram did not provide any benefit compared to placebo in alleviating depression symptoms after 12 months (Angermann et al., 2016). Both the SADHART-CHF and MOOD-HF-RCT study did not include a sub-groups analysis for gender differences. The negative results from these two studies suggest that the pathophysiology of depression associated with HF might be different from major depressive disorder per se.

1.1.2. Importance of sex differences in HF and depression

The inherent sex differences in depression rates raise the question of sex differences in the co-presence of HF and depression. Several studies investigated the prevalence and impact of depression in male vs. female patients with HF. Women with HF had a 33 % depression incidence compared to 26% in men with HF (Rutledge et al., 2006). In a study by Gottlieb et al. (2004), 64 % of women with HF reported depression symptoms according to Beck Depression Inventory (BDI) score compared to 44% in men with HF, while patients with HF and depression revealed 46% lower in quality of life compared to non-depressed patients. In 907 cardiac patients and 260 healthy individuals with mean age of 60, HF patients showed the highest incidence of depression 63% compared to 56 % post-MI, 53% coronary artery bypass graft (CABG) and 33% healthy elderly individuals. In subanalysis, women showed higher depression score compared to men in HF (15 vs. 13), post-MI (14 vs. 12), and CABG (15 vs. 12), all significantly higher in women (Moser et al., 2010).

Faller et al., (2007) examined the role of gender in the prognosis of HF in the presence of depression. In 231 subjects with HF (6% class III-IV), 35% of women exhibited depression symptoms and had lower survival rate by 4.5 times compared to non-depressed women with HF after 2.7 years of follow-up, while 28 % of men had depression symptoms and there was no effect of depression on survival rate. The small sample size was not powered to detect relevant gender differences in depression incidence. In a more extensive study, 48117 subjects with depression and HF were included, determined by medical records and prescriptions. 60 % of the subjects were ≥ 75 years old. In patients with HF, after 1-year follow-up, depression incidence was higher in women vs. men (67 % vs. 32 %). The effect of depression on all-cause mortality was analyzed by Cox regression model, and adjusted for age and gender and

medical comorbidities. Depression increases all-cause mortality by 1.2 fold (Macchia et al., 2008). Older women with HF and depression had 1.6-fold higher hospitalization rate compared to those without depression. This risk was not found in older men with HF (Williams et al., 2002).

1.1.3. Depression-like behavior in rodent models of HF

Depression symptoms such as feeling guilt, suicidal thoughts and sad mood in humans cannot be assessed in laboratory animals, but some symptoms can be identified as depression-like behavior. For instance, anhedonia in humans is defined as loss of pleasure and is used to evaluate depression by HAM-D (Hamilton, 1960). In rodents, pleasure can be assessed by the sucrose preference test (SPT) providing two bottles one with plain water, and another containing sucrose (Rizvi et al., 2016). Rats that are subjected to chronic mild stress (CMS), exhibit low consumption of sucrose (Willner et al., 1987) and antidepressant treatment with TCA or SSRI reversed anhedonia (Willner et al., 1987; Muscat et al., 1992). The forced swim test (FST) is widely used to assess despair or passive behavior. When rodent placed in an inescapable cylinder filled with water, first it shows escape-directed behavior such as swimming and climbing (Slattery and Cryan, 2012), then the rodent disengages from the active form to immobile status (passive behavior) (Harro, 2018). Acute or chronic antidepressant treatment results in a decrease of immobility phase of FST (Cryan et al., 2005). A wide range of cognitive functions such as: attention function, long and short-term memory are affected during the depression (Gonda et al., 2015). Negative affective biases, generated by adverse life events, promote distortion and error in the function of high-order cortical centers such as overgeneralization, arbitrary inferences and emotional reasoning (Roiser et al., 2012). Cognitive function can be assessed by learning a new task or by novelty seeking. The novel object recognition test (NORT)

evaluates the ability of rodent to discriminate between a familiar and new object (Ennaceur and Delacour, 1988), and the diminution in discrimination index reflects an impairment in the cognitive function, that was revealed in CMS rats (Elizalde et al., 2008). Emotional associative memory in rodents is assessed by fear conditioning test, in which the rodent learns to anticipate events related to specific context or cued hint, that can be learned through context or cued conditioning of fear (Anagnostaras et al., 2001; Curzon et al., 2009; Darcet et al., 2016). The Morris water maze (MWM) test evaluates hippocampus-dependent spatial learning and reference memory (Vorhees and Williams, 2006). In CMS rats, spatial learning and memory were impaired (Tagliari et al., 2011).

Several pre-clinical studies showed that male rats at two to four weeks post-MI exhibit an increase to 60% in the immobility phase compared to only 25% in sham group, and decreased sucrose preference by 20% in comparison to sham group, with preserved spatial memory assessed by MWM test (Grippo et al., 2003; Wann et al., 2007, 2009; Bah et al., 2011b). Male mice at six weeks post-MI displayed a decline in discrimination index by 20% in NORT, and a decrease by 10% in the cumulative sucrose consumption for eight weeks, correlated negatively to the MI-size (Frey et al., 2014). Despite the apparent gender differences in depression induced by HF in humans, no studies have examined sex differences for depression-like behavior post-MI. Gouweleeuw et al., (2016) examined sex differences in anxiety-like behavior and cognitive function post-MI. Male and female rats developed similar mild HF post-MI, but there were no differences in interest of the new environment, elevated plus maze or NORT.

1.2. Potential mechanisms involved in depression and HF

Several mechanisms may contribute to the development of depression in patients with HF. The following will provide a summary of the mechanisms that are involved in depression in general and may contribute to depression in HF:

1.2.1. Mechanisms of depression

1.2.1.1. Neuroadaptation to stress:

The limbic-cortical-striatal-pallidal-thalamic circuits play a significant role in depression. There are two major circuits integrated with PFC which perceive and respond to stress events that are either of psychological or somatosensory nature (Hamon and Blier, 2013). First, the PFC interacts with the orbital prefrontal cortex, and visual areas inferior temporal cortex, somatosensory insula, and frontal operculum, as well olfactory, taste areas gustatory cortex to analyze reward, aversion and relative values. The second circuit is PFC, cingulate cortex and entorhinal cortex connected to the limbic system and responsible for emotional behavior (Drevets et al., 2008). Glutamatergic neurons project from the PFC to basolateral amygdala (BLA) and amygdaloid intercalated cells (ITC); and synapse with gamma-aminobutyric acid (GABA) neurons in the BLA and ITC , which in turn projects to central amygdala and inhbit amygdala activity (LeDoux, 2000; Akirav and Maroun, 2007; Marek et al., 2013). In normal rats, the highest activity of the PFC is associated with the least freezing behavior in extinction fear test (Milad and Quirk, 2002), and a lesion in the PFC causes resistance to freezing extinction (Morgan et al., 1993). Restraint stress attenuates GABAergic neurons in the BLA, which enhances freezing behavior (Manzanares, 2005).

Excitatory projection from the PFC to the locus coeruleus (LC) increases the firing rate in the LC during stress events, and from LC to other brain areas (McDevitt et al., 2009). LC projects directly to the PVN and stimulates corticotropin-releasing hormone (CRH) release to initiate stress response (Reyes et al., 2005). Under acute stress, norepinephrine (NE) neurons project from LC to the BLA and central amygdala, and promote freezing behavior by increasing their activity, while chronic stimulation of the BLA via this pathway was found to induce depression-like behavior (McCall et al., 2017). LC also projects to the dorsal raphe nuclei (DRN). An increase of NE release in the DRN reduces serotonin (5-HT) release from the DRN (Hopwood and Stamford, 2001). The DRN is a part of raphe nuclei that is responsible for 5-HT neurocircuit projections to a wide range of cortical and subcortical brain areas (Morrissette and Stahl, 2014).

Under normal conditions, the PFC regulates the bed nucleus of stria terminals (BNST) via glutamatergic projections, which in turn has GABAergic synapses with PVN neurons that inhibit PVN activity (Ziegler, 2002; Radley and Sawchenko, 2011). BNST is considered a key relay connecting limbic forebrain structures to the hypothalamus, that play a role in controlling neuroendocrine and autonomic function (Crestani et al., 2013). Amygdala projects GABAergic neurons to BNST. During stress, amygdala activity increases. LC projects back to the PFC via NE neurons which inhibits PFC activity (Arnsten, 2000; Chandler et al., 2014), and thereby inhibits the negative inputs from BNST to the PVN, leading to increase in PVN activity (Dayas et al., 1999; Radley and Sawchenko, 2011; Reser, 2016).

The DRN is tonically active to support goal-directed motor and cognitive function when awake. Glutamatergic neurons project from PFC to DRN which induce mobile phase in FST (Warden et al., 2012), these glutamatergic neurons either

synapse to 5-HT neurons dirctley or GABA neurons in the DRN which inturn inhibit the serotoninergic neuron activity (Geddes et al., 2016; Soiza-Reilly et al., 2019; Weissbourd et al.,2014). The DRN projects back to the PFC and increases the activity of PFC (Puig et al., 2010). The cognition of PFC to the stressor type modulates the 5-HT neurons that affect the controllability of stress (Robbins, 2005). Under normal conditions, when male rats are having an inescapable shock (IS) (uncontrollable stress), their 5-HT neurons in the DRN are transiently (few minutes) activated, but intensely and induce fear behavior, but not in the escapable shock (ES) (controllable stress). The DRN projects to amygdala through 5-HT neurons and an increase in 5-HT neurons activity increases amygdala activity during IS combined with inhibition in the PFC (Maier and Watkins, 2010). This marked increase in DRN activity sensitizes the autoreceptors in the presynaptic neurons leading to prolonged inhibition in the target regions after IS (Christianson et al., 2009).

During stress, the PFC activates LC to increase the release of NE, and sustained exposure to stress increases the demand for NE synthesis. Tyrosine hydroxylase enzyme levels increase in the LC of CMS rats (Melia et al., 1992; Wang et al., 1998). After repetitive exposure to stress, NE demands outweigh the synthesis, resulting in a decrease in NE levels in the cortex, hippocampus, and LC (Weiss et al., 1980). Patients with depression show a parallel decline in NE and 5-HT concentration in the cerebrospinal fluid (CSF) (Mongeau et al., 1997). 5-HT metabolite 5-hydroxyindoleacetic acid (5-HIAA) was lower in the CSF of patients with depression compared to healthy subjects (Asberg et al., 1976), that was related to a decrease in the rate-limiting enzyme tryptophan hydroxylase for 5-HT synthesis (Jans et al., 2007).

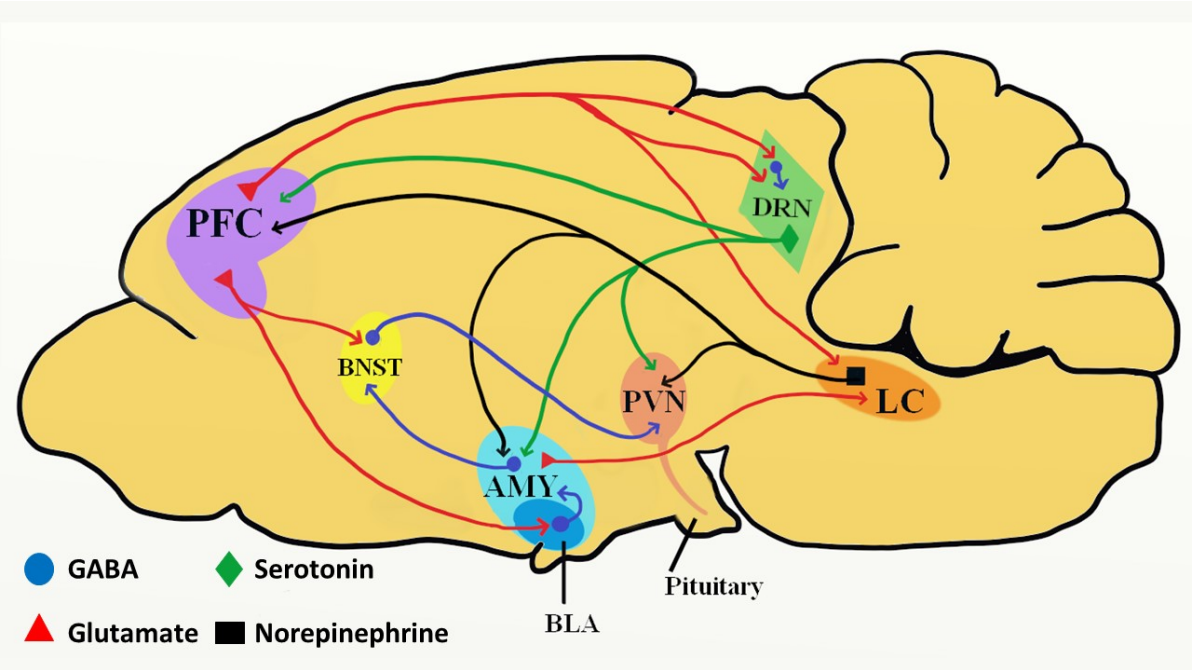

Figure 1.1 Regions involved in stress and neural pathways activated during stress. A stressful event perceived by the prefrontal cortex (PFC), will activate glutamate projections to locus coeruleus (LC) that increases norepinephrine (NE) release from LC, which in turn stimulates amygdala (AMY) (AMY consist of basolateral central amygdala (BLA) and intercalated amygdaloid cells) and paraventricular nucleus (PVN) to respond to stress. PFC also controls DRN via glutamate neurons which synapse with 5-HT or GABAergic neurons in the DRN, leading to inhibition of serotonin (5-HT) release. Chronic stress reduces the indirect PFC negative inputs to AMY and bed nucleus stria terminalis (BNST), which inhibits GABA neurons that produce negative input on PVN, and thereby increases CRH release from PVN which activates HPA axis.

1.2.1.2. Hypothalamus-Pituitary-Adrenal (HPA) -axis dysregulation

In response to stress, the HPA axis is activated by CRH-releasing neurons in the PVN and release of adrenocorticotropic hormone (ACTH) from the pituitary. PVN can be regulated directly via amygdala and LC projections or indirectly by PFC (previously mentioned). An increase of NE in the PVN activates the HPA axis, for example NE microinjection in the PVN increases plasma ACTH and corticosterone levels (Cole and Sawchenko, 2002).

Hyperactivity of the HPA axis caused by overexpression of the CRH1 receptor enhances depression-like behavior (Holsboer and Ising, 2008). Diminished sensitivity of glucocorticosteroid receptors (GR) leads to glucocorticoid feedback disinhibition at pituitary and hypothalamus levels and disinhibits CRH and ACTH release (Pariante and Lightman, 2008). This dysfunction of HPA-axis leads to an increase in corticosterone, hypoactivity in the PFC and hippocampus, but hyperactivity in the amygdala and depression-like behavior (Reser, 2016).

Corticosterone treatment in male mice for 21 days, enhanced passive behavior assessed by FST and caused anhedonia as well decreased discrimination index evaluated by NORT (Gourley et al., 2008; Wu et al., 2013). Glucocorticoids decrease activity of 5-HT neurons that project from DRN to PFC, inhibit tryptophan hydroxylase (Prouty et al., 2019), and increase 5-HT transporters in the DRN and hippocampus (Zhang et al., 2012). Glucocorticosteroids are critical in consolidation and facilitate memory that is associated with negative emotions. High levels of corticosterone increase the excitability of amygdala neurons in response to acute stress (Duvarci and Pare, 2007).

1.2.1.3. Neuromodulation and plasticity

Neural plasticity allows the CNS to adapt to the external and internal environment, and cellular mechanisms that control plasticity are correlated with learning and memory in the brain (Kandel, 2001). Neurotrophins play an essential role in neuronal plasticity and neurogenesis. Brain-derived neurotrophic factor (BDNF) is a neurotrophin that promotes the survival of existing neurons and encourages the growth and differentiation of new synapses and neurons (Numakawa et al., 2010). Downstream signaling of BDNF- tropomyosin-receptor kinase B (TrkB) includes the activation of Ras-mitogen-activated protein kinase (MAPK) and phosphatidylinositol-3-kinases (PI3K) that increases neuronal survival (Duman and Voleti, 2012). Activation of 5-HT receptors 5,6,7 or β- receptors increases cAMP, which in turn increases BDNF gene expression through calcium/cyclic-AMP responsive-element binding protein (CREB) (Duman et al., 1997). Social defeat male rodents exhibited a decrease in the apical dendritic length of the hippocampus compared to the dominant animal. CMS male rats demonstrated a shorter apical length and less complexity in the branching at radial distance compared to their controls (Magariaos et al., 1996; Radley et al., 2004). Similarly, dendritic retraction in proximal and distal of the PFC neurons were found in CMS male rats (Liston et al., 2006; Shansky and Morrison, 2009). Acute or chronic mild stress decreases BDNF mRNA expression in the hippocampus of male rats (Murakami et al., 2005; Nair et al., 2007). BDNF protein expression decreased in the hippocampus and PFC of CMS male rats (Grønli et al., 2006; Filho et al., 2015). BDNF knockdown in the hippocampus decreases sucrose intake by 10% and increases immobility by 30% compared to the wild type mice (Taliaz et al., 2010). BDNF microinjection in the hippocampus of learned

helplessness rats decreases their immobility phase in FST. However, there was no effect on the conditioned avoidance test or open field test (Shirayama et al., 2002).

In clinical studies, plasma BDNF levels were found lower in patients with depression compared to healthy subjects (Brunoni et al., 2008) and correlated negatively with suicidal attempt rate in patients with depression (Kim et al., 2007). A decrease of BDNF was found in the postmortem hippocampus of subjects with depression (Chen et al., 2001). Eight weeks of paroxetine (SSRI) or milnacipran (SNRI) treatment, increased serum BDNF levels 1.8-2.6-fold compared to baseline, which was negatively correlated with (HAM-D) score (Gonul et al., 2005; Yoshimura et al., 2007).

1.2.1.4. Genetics

Serotonin-transporter-linked polymorphic region (5-HTTLPR) is a degenerate repeat in the gene encoding the serotonin transporter (SLC6A4). The s/s genotype of this region is exhibited reduced 5-HT binding site in PET imaging studies compared with l/l individuals (Caspi et al., 2010). The s/s genotype was detected in 19% of 2622 European American but only 6% in 958 African American individuals (Odgerel et al., 2013). The S-genotype increases the vulnerability to depression by 20 % compared to L-genotype (Caspi et al., 2003). The -1019 G/G allele derepresses 5-HT1A autoreceptor expression, by disrupting an inhibitory transcription factor binding site and thereby reducing serotonergic neurotransmission. The expression of the homozygous allele was twice fold higher in patients with depression compared to healthy subjects (Lemonde et al., 2003; Serretti et al., 2004). Patients with depression and G/G-carrier showed a higher amygdala function and a lower PFC function compared to patients with depression and C/G or C/C-holder (Domschke et al., 2006).

The single-nucleotide polymorphism (SNP), with methionine (Met) substituted for Valine (Val) at codon 66 (Val66Met) of the BDNF gene, is associated with a decrease in the hippocampus volume in patients with depression and healthy subjects (Frodl et al., 2006; Hajek et al., 2012; Gatt et al., 2015). Mice with Val66Met exhibit an increase in sensitivity to stress (Yu and Chen, 2011). Expressing Val66Met affects BDNF trafficking and release in neurons, in which vesicles containing BDNF are trafficked to neural dendrites, axons, and terminals, by sortilin and BDNF interaction. This localization is controlled by BDNF pro-domain, the region containing Val66Met, and by expressing Val66Met, BDNF attenuates dendritic trafficking as well as depolarization-induced BDNF secretion (Martinowich et al., 2007). The presence of this gene alteration is associated with a lower response to SSRI anti-depressant treatment (Martinowich and Lu, 2008).

1.2.1.5. Cytokines and depression

Depressive symptoms were noticed in patients receiving immunotherapy such as IL-2 for renal cell carcinoma and interferon (IFN)-α for hepatitis C (Felger and Lotrich, 2013). Chronic diseases such as cardiovascular disease, chronic kidney disease, and Diabetes Mellitus, have a higher incidence of depression (Kessler et al., 1994). These all exhibit low-grade inflammation that might contribute to depression (Smith, 1991). Male mice treated with lipopolysaccharide (LPS), showed an increase in immobility phase in FST by 13%, and sucrose consumption decreased by 30% (Ge et al., 2015). Furthermore, male rats treated with IFN-ɣ for 7 days, showed a 40 % increase in immobility phase in FST (Fischer et al., 2015). Similarly, CMS male mice exhibit depression-like behavior, associated with an increase in IL-1β and TNF-α in plasma and PFC (Couch et al., 2016).

Susceptible social defeat mice showed a significant increase in plasma leukocytes, IL-6, and IL-1β levels compared to control (Leboeuf et al., 2014). Treatment with imipramine (TCA) for 21 days increased anti-inflammatory cytokines (IL-10 and IL-4) in the hippocampus of CMS rats, accompanied with alleviation in the depression-like behavior (Han et al., 2015). In IL-10 knock-out mice, only female mice exhibited depression-like behavior but not males (Mesquita et al., 2008). Human recombinant IL-10 s.c. administered for 21 days, prevented depression-like behavior induced by CMS in female mice (Voorhees et al., 2013).

Although the CNS is protected from peripheral stimuli by the BBB, abnormalities in the peripheral immune system can induce neuroinflammation through several mechanisms. This section will review mechanisms through which the peripheral immune system activates the CNS immune cells (microglia) and modulates monoamine metabolism

1.2.1.5.1. Peripheral immune signals extend to the CNS

The peripheral immune system can induce inflammation in the CNS through several pathways:

1. The expression of Toll-like receptors (TLRs) in circumventricular organs such as the subfornical organ (SFO), and organum vasculosum of the laminae terminalis (OVLT), and the choroid plexus, enables circulating cytokines to activate microglia or macrophages in these regions (Quan et al., 1998), increasing the local production of cytokines (Konsman et al., 2004), which can access the CSF through volume diffusion (Vitkovic et al., 2000).
2. BBB expresses active transporters for circulating cytokines to enter the CNS. However this system is saturable (Banks, 2009).

3. Cerebral vascular endothelial cells express IL-1 receptors (IL-1R), and in response to circulating cytokines increase the expression of cyclooxygenase-2 enzyme (COX-2), and thereby increases prostaglandin-A2 (PGA2) and PGE2 levels (Konsman et al., 2004), leading to increase in permeability of the BBB and activation of local inflammation (Dalvi et al., 2015).
4. Peripheral cytokines activate directly afferent nerves such as vagal nerve and trigeminal nerve, increasing glutamate release in the caudal nucleus tractus solitarius (cNTS) (Maniscalco and Rinaman, 2018), ultimately increasing the release of CRH from PVN by activating NE neurons projects from cNTS-PVN (Rinaman, 2010), and induces avoidance behavior through an increase in NE levels in the amygdala (Dayas et al., 2004).

1.2.1.5.2. Inflammation modulates monoamines

Indoleamine 2,3-dioxygenase (IDO) enzyme: Tryptophan is the precursor of 5-HT. In peripheral tissues IDO enzyme convert tryptophan to kynurenine, that readily crosses the BBB (Sadok et al., 2017). TNF-α and IFN-ɣ stimulate the activity of IDO in macrophages and microglia that increase tryptophan conversion to kynurenine instead of 5-HT in the CNS (Murakami et al., 2016). The three major metabolites from kynurenine are: 1) Quinolinic acid (QA) primarily produced in the microglia, and is an N-methyl-D-aspartate (NMDA) receptor agonist, that stimulates the production of reactive oxygen species (Sahm et al., 2013). 2) 3-hydroxykynurenine (3-HK) generates free radicals that cause oxidative stress (Schwarcz and Pellicciari, 2002). 3)Kynurenic acid (KA) is NMDA receptor antagonist, and an inhibitor of α7-nicotinic acetylcholine receptor (Albuquerque and Schwarcz, 2013) that has anti-inflammatory properties (Ren et al., 2017), also reduces dopamine release in the striatum (Wu et al., 2007). CMS increases the concentration

of IDO enzyme in the cortex of male mice (Zhang et al., 2015). Moreover, in social defeat rats KA and 3-HK increase significantly in the amygdala and ventral hippocampus compared to controls (Fuertig et al., 2016).

Mitogen-activated protein kinase (MAPK): Cytokines induce GR resistance through activation of p38 MAPK. IL-1α activates the p38 MAPK pathway, leading to decreased glucocorticoid response element (GRE) GR binding (Wang et al., 2004). It also decreases the GR function and translocation of GR from the cytoplasm to the nucleus, decreasing DNA-GR binding (Pariante et al., 1999). TNF-α disrupts the GR-DNA binding and prevents the up-regulation of GR by activating the MAPK/ERK pathway that prevents the transactivation of anti-inflammatory genes (Onda et al., 2006). IL-2 activates Janus associated-kinases-3 (JAK3) /signal transducers and activators of transcriptions-5 (STAT-5) pathway that forms complexes with GR, disrupting the GR-DNA binding, and also inhibits translocation of GR (Goleva et al., 2002). In social defeat mice, the GR translocation from cytoplasm to the nucleus was decreased compared to their controls (Quan et al., 2003). GR has 2 isoforms α and β with α isoform having 12 α helices whereas in β isoform, the 12th helix is absent and the 11th modified, that inhibits the ligand to activate GR pathway and limit the activity of α-isoform (Lewis-Tuffin and Cidlowski, 2006). TNF-α and IL-1 activate nuclear-factor kappa-B (NF-*k*B), that increases the expression of β-GR, and thereby decrease the effect of glucocorticoids (Webster et al., 2001). Collectively, these mechanisms contribute to HPA- axis dysregulation.

Tetrahydrobiopterin (BH4): is a cofactor of the rate-limiting enzymes of 5-HT, NE, dopamine, and nitric oxide (NO) synthesis. BH4 is highly sensitive to non-enzymatic oxidation and can be converted irreversibly to dihydroxanthopterin (Haroon et al., 2012).IL-6 and IFN-α increased inducible nitric oxide synthase (iNOS)

(Ogłodek and Just, 2018) which increases the oxidation status and leads to decrease BH4 levels in the raphe area and amygdala (Kitagami et al., 2003). The utilization of BH4 in non-enzymatic oxidation decreases monoamine synthesis. IFN-α decreased CSF dopamine and BH4 levels, and BH4 levels were correlated negatively with CSF IL-6 (Felger and Lotrich, 2013).

1.2.1.5.3. Microglia

Microglia is the brain resident macrophage. Microglia have a distinctive morphology that can change according to the microenvironment. In healthy CNS, microglia have small soma and fine, ramified cellular processes. When the CNS is affected by detrimental factors such as infection, ischemia, injury, the morphology of microglia changes to hypertrophic soma and retracted processes that can migrate to the source of injury or infection following the chemotactic gradient (Kettenmann et al., 2011). In CMS male rats the activity of microglia was increased in the PFC, hippocampus, and infralimbic cortex (Bian et al., 2012; Hinwood et al., 2012; Kopp et al., 2013). Microglia activity is regulated by several soluble factors, such as pathogen-associated molecular patterns (PAMPs), endogenous danger-associated molecular patterns (DAMPs), angiotensin II (Ang II), Fractalkine (CX3CL1). Under normal physiology angiotensin II type 1 receptors (AT1R) are localized on astrocytes and neurons. In the presence of neuroinflammation AT1R are also expressed on microglia(Wright and Harding, 2013). Peripheral LPS administration increases microglia activity in the PVN and induces cytokines release in the PFC, hippocampus and PVN, as well as induces depression-like behavior. These effects were prevented by peripheral candesartan treatment (Benicky et al., 2011). Losartan decreases IL-1β release and NF-*k*B transcription in microglia activated by LPS (Miyoshi et al., 2008). Intracerebroventricular (ICV) infusion of Ang II in healthy rats causes activation of

microglia in the PVN (Shi et al., 2010; Jun et al., 2012). Ex-vivo Ang II stimulation upregulates the expression of AT1R on the microglia in the PVN (Biancardi et al., 2008). In vitro, Ang II increases release of TNF-α by microglia through NF-*k*B translocation (Rodriguez-Pallares et al., 2008; Borrajo et al., 2014). Activated microglia increase local cytokine production, which in turn stimulates IDO enzyme in microglia that consumes tryptophan and thereby decreases 5-HT production in neurons. The increase of kynurenine metbolites such as QA and 3-HK, and prostaglandins synthesis in microglia leads to increase ROS levels. Cytokines directly activate NADPH oxidase and increase ROS and RNS (reactive nitrogen species) levels in neurons. The high levels of free radicals in neurons convert BH4 cofactor irrevesibly that is needed for monoamine synthesis. The increase of kynurenine metabolites and oxaditive stress ultimately decrease glutamate reuptake in neurons which decreases BDNF production (Parrott et al.,2016; Kitagami et al., 2003)

Fractalkine (CX3CL1) is released from neurons that binds to microglia CX3CR1 and maintains microglia in rest phase (Biber et al., 2007). CX3CL1 knock-out mouse exhibited an increase in IL-1β in the hippocampus, an increase in microglia activation, and a deficit in spatial memory assessed by MWM test (Rogers et al., 2011). In social stress male mice, CX3CR1 mRNA expression decreases in the hypothalamus and rostral cortex (Wohleb et al., 2013). Stress or cytokine challenge cause dysregulation in CX3CL1-CX3CR1 signaling, decrease neuron-microglia cross talk and enhance neuroinflammation (Wohleb et al., 2016). The release of cytokines serves cell-cell communication via auto or para signaling cell-cell interaction. Activation of microglia increases the expression of several cytokines receptors such as IL-IR, IL-2R, and IL-6R, and TNF-αR leading to increase microglia phagocytic activity (Fu et al., 2014), and release local cytokines and chemokines that produce

positive autocrine loop (Frucht et al., 2001; Carson, 2002; Kettenmann et al., 2011). Cytokines stimulate microglia through several mechanisms for instance: IL-1 upregulates NF-*k*B transcription pathway in microglia and neurons (Koo et al., 2010). The release of IL-6 activates other microglia cells, acts on astrocytes that have tissue repair attempts (Hanisch, 2002). TNF-α and IL-6 increase AMPA receptors and promote excitation, leading to glutamate excitotoxicity (Qiu et al., 1998; Stellwagen, 2005).

1.3. Cytokines and Heart failure

Acute MI induces local inflammation cascade in response to heart tissue injury, which stimulates the immune system (Grippo et al., 2002; Grippo, 2009). IL-1β and TNF-α expression were increased, and IL-10 decreased significantly in the myocardium up to 4 weeks post-MI, followed by a decline in their levels but not returning to control values (Irwin et al., 1999a; Hwang et al., 2001; Berry et al., 2004; Kaur et al., 2006). This increase is not limited to the myocardium tissue. Plasma TNF-α and IL-1β and IL-6 levels increase significantly, as early as 2 hours up to 6 week post-MI (Kang et al., 2006, 2008, 2010; Guggilam et al., 2007, 2008; Bah et al., 2011b; Chen et al., 2014). Patients with HF also show a significant increase in plasma TNF-α and IL-6 (Munger et al., 1996; Torre-Amione et al., 1996, 1999; Kubota et al., 2000). Besides the local production of cytokines, there is persistent recruitment of monocytes from bone marrow and spleen. Proinflammatory macrophages (M1) increased significantly in the non-infarct region after 10 days post-MI, in contrast anti-inflammatory macrophages (M2) increased modestly in the infarct region compared to the sham group. However, all types of macrophages increased significantly up to 12 weeks post-MI (Rafatian et al., 2014). Macrophages in the

myocardium tissue were increased 5 fold in 8 weeks post-MI, specifically, M1 showed a significant increase but not the M2 (Ismahil et al., 2014).

1.3.1. Cytokines extend to the brain

In rodents, post-MI cytokine levels showed an increase in the CNS. At 4 weeks post-MI, IL-1β, and TNF-α mRNA and protein levels were increased in the PVN of male rats (Kang et al., 2006, 2008, 2010, 2011; Guggilam et al., 2007, 2008; Wei et al., 2014b; Yu et al., 2018a). An increase was also found in the SFO of male rats at 4 weeks post-MI, associated with an increase of TNF and IL-1 receptors in neurons in the SFO (Yu et al., 2018). The increase in cytokines levels was associated with an increase in inflammatory mediators such as COX-2, PGE2, and NF-*k*B (Kang et al., 2008, 2011), and increases oxidative stress, via an increase in NADPH oxidase (NOX) 1, NOX2, and 3-nitrotyrosine (3-NT) (Guggilam et al., 2007, 2008). Ter Horst, (1999) One study only showed that TNF-α increased in the PFC, cingulate cortex, peri limbic area, and hippocampus in male rats 72 hours post-MI.

1.3.2. Heart Failure activates microglia

24-hours after MI, microglia showed an increase in ionized calcium-binding adapter molecule-1 (Iba-1) and macrophage inducible Ca+2-dependent lectin receptor (Mincle) that are upregulated during activation of microglia, in the PVN of male rats (Wang et al., 2018). Activation of microglia was persistent in male rats up to 16 weeks post-MI (Rana et al., 2010; Dworak et al., 2012). Microglia were also activated in the PFC and hippocampus of male rats 2 days post-MI (Rinaldi et al., 2015) that induces depression-like behavior in male rats 8 weeks post-MI (Wang et al.,2019) and might contributed to decrease monoamine synthesis and BDNF expression. ATR1 upregulation was found in the PVN and SFO in male rats post-MI (Wei et al., 2013, 2014a; Yu et al., 2018b). ICV infusion of minocycline, that has anti-inflammatory

properties through binding to peroxisome proliferator-activated receptor ɣ (PPAR-ɣ), attenuates microglia activation and decreases IL-1β, IL-6 and TNF-α levels in the PVN, rostral ventrolateral medulla (RVLM) and NTS at 12 weeks post-MI (Hamblin et al., 2009; Dworak et al., 2014; Wang et al., 2019). In male mice at 8 weeks post-MI, genes that stimulate microglia were found upregulated in the PFC and hippocampus (Frey et al., 2014) and oral minocycline treatment reduces depression-like behavior and cytokine levels in the PFC and PVN of male rats 8 weeks post-MI (Wang et al., 2019) . Sex differences for microglia activity in HF, have not yet been studied.

1.3.3. Heart Failure may induce depression-like behavior via neuroinflammation

Depression-like behavior was associated with an increase in Bax/Bcl2 ratio (apoptosis) in the PFC but not amygdala or hippocampus at 2 weeks post-MI (Wann et al., 2006, 2009) as well a decrease in the BDNF levels in the PFC and an increase in the medial amygdala in male rats at 7 days post-MI (Kaloustian et al., 2008). Pentoxifylline (PTX) is a non-specific phosphodiesterase inhibitor that increases cAMP intracellularly, blocks NF-*k*B and thereby inhibits the production of IL-1α, IL-1β, TNF-α, and IFNɣ. It also inhibits the phagocytic activity of macrophages and lysosomal enzyme released by polymorphonuclear cells (D'Hellencourt et al., 1996). Oral PTX treatment attenuates cardiac dysfunction in patients with HF, but not other anti-inflammatory agents such as: etanercept (TNF-α inhibitor), infliximab (TNF-α AB), and Vesnarinone (TNF-α inhibitor) (Chung et al., 2003; Cohn et al., 1998; Deswal et al., 1999; Sliwa et al., 1998). There are no studies so far that assessed effects of anti-inflammatory agents on depression in patients with HF. Peripheral or ICV infusion or bilateral microinjection of PTX prevented the increase of IL-1β and TNF-α in the plasma and PVN and reduced the expression of AT1R in the PVN in

male rats at 4 weeks post-MI (Kang et al., 2008, 2010). PTX 10 mg/kg/day intraperitoneally (IP) for 2 weeks initiated 15 min before MI induction, decrease IL-1β plasma levels and prevented development of despair behavior assessed by FST and anhedonia assessed by SPT in male rats at 2 weeks post-MI (Bah et al., 2011b).

1.4. Effect of Estrogens on depression and depression-like behavior:

Estrogens have a strong connection with depression through women lifetime. This section will review estrogens properties, cellular action, and the behavioral changes associated with changes in endogenous estrogens.

1.4.1. Estrogens and estrous-proestrus cycle

Estrogens are synthesized mainly in the endoplasmic reticulum of ovarian granulosa cells during premenopause. Estrogen plasma levels fluctuate 50-250 ng/L according to the proestrus-estrus, while in post menopause phase, stomal adipose cells produce estrogens but to a lower extent and estrogens plasma levels drop to 20 ng/L (Halbreich and Kahn, 2001; Nilsson et al., 2001). The primary estrogens in females are 17β-estradiol, estrone, 16α-estriol, and 17β-estriol (Schmidt et al., 2002). 17β-estradiol is the principal circulating product, and the main effects of estrogens on different organs are derived from the circulating levels. In pre-clinical studies, serum 17β-estradiol levels of adult female rats vary 5-140 pg/mL depending on the estrous cycle (Ström et al., 2012). Local production of estrogens occurs by aromatase converting enzyme that converts androgen precursors to estradiol in the CNS (Luine, 2014). It is localized in different brain areas such as the PFC, hippocampus, and amygdala (Barker and Galea, 2009; Wei et al., 2014a). Females show a higher expression of aromatase compared to males (Wei et al., 2014a). The local production of E_2 in OVX females provides a comparable concentration of E_2 compared to sham females in the PFC and amygdala but not in the hippocampus 15 days after

ovariectomy (Barker and Galea, 2009). Several studies showed the effect of circulating estrogen deficiency on cognitive function (below will be illustrated), and the importance of timing of administration of estrogen. There are no studies that assessed to what extent local production may affect behavior.

1.4.2. Estrogen receptors

Estrogen receptors (ER) have α and β isoforms that share a high degree of sequence homology in the DNA-binding domain (DBD) and Ligand binding domain (LBD), except in their NH2 terminal domain. However, ER-α and ER-β are products of separate genes located on different chromosomes (Heldring et al., 2007; Kumar et al., 2011). α and β are distributed differently in the brain. β isoform is more abundant in the PVN and RVLM than α (McEwen et al., 2012) while α is predominant in the prefrontal cortex and amygdala (McEwen and Alves, 1999; Pérez et al., 2003) and the hippocampus showed equal distribution (McEwen and Alves, 1999; Dahlman-Wright et al., 2006). The presence of nuclear and extranuclear ER suggests that ER has genomic and non-genomic effects with different time of onset: genomic mechanism has a latency of 20 min or more for the onset of action, and shorter (acute) in case of non-genomic mechanism (McEwen and Alves, 1999).

Genomic pathway

The DBD binds to the estrogen response element that directly promotes DNA transcription regions or indirectly enhances regions located distant from the BDB: Estrogen response element (ERE) transcription site (Kumar et al., 2011)

Direct association to DNA: activation of ERE promotes or inhibits 38 genes. The majority are related to sex hormone genes such as oxytocin, prolactin, and luteinizing hormone genes, as well as estrogen and progesterone receptors gene (Klinge, 2001).

Indirect association to DNA: ER interacts with stimulating protein-1 (SP-1) that enhance the SP-1 to bind to its site and induce endothelial nitric oxide synthase (eNOS), and c-*fos*, while interaction with c-*rel* subunit inhibits NF-*k*B which reduce IL-6 gene expression (Kalaitzidis and Gilmore, 2005). ER interact with Fos and Jun at activator protein-1 (AP-1), which induce expression of insulin-like growth factor (IGF-1), cyclin D1 genes that involved in cell proliferation (Marino et al., 2006).

Non-genomic pathway

ERs are also localized on the cell membrane that enable estrogens to have an excitatory effect on postsynaptic neurons in the hippocampus and suppress GABA receptors in the hypothalamic arcuate (Wong and Moss, 1991; McEwen and Alves, 1999). Another rapid action of estrogen is through G protein-coupled estrogen receptors (GPER), that activates four classes which are phospholipase C/ protein kinase C (PLC/PKCs), Ras/Raf/ MAPK, PI3K/ protein kinase B known by (AKT), and cAMP/PKA depending on the type of tissue (Marino et al., 2006; Bean et al., 2014; Lu and Herndon, 2017). Estrogens can upregulate BDNF expression, via the genomic pathway, in which ERE directly binds to the BNDF gene and promotes transcription or indirectly through activating the second messenger of Raf/ extracellular signal regulated kinase (ERK) or PI3K/AKT, thereby activating CREB (Luine and Frankfurt, 2013).

1.4.3. Estrogen effects on behavior

Menopause and post-menopause are associated with an increased incidence of depression (Grigoriadis and Kennedy, 2002). Accumulating evidence suggests that hormonal deficiency is not only responsible for somatic symptoms but also for depressive symptoms and possibly for an impairment in cognitive function (Schmidt et al., 2002; Harlow et al., 2003; Freeman et al., 2006). Hormonal replacement

alleviates most of these symptoms (Halbreich and Kahn, 2001; Stoppe and Dören, 2002; Miller, 2003; Studd, 2011; Toffol et al., 2015). Most studies also showed an improvement in the spatial and working memory that depends on the hippocampus and PFC (Keenan et al., 2001; Krug et al., 2006) as well cognition (Maki et al., 2001; Bagger et al., 2005). Others showed no benefit (Craig et al., 2005).

In animal studies, ovariectomy resulted in depression-like behavior in mice after 12 weeks, as assessed by SPT and FST as well impaired in cognitive memory by the novel object recognition (Bastos et al., 2015). Young and adult (3-8 months) female rats showed at 14 days of ovariectomy an increase in immobility phase by ~10% in FST but not middle aged 12-13 months (Kiss et al., 2012). E_2 replacement immediately after ovariectomy alleviates depression-like behavior or in 24 months aged female mice (Bernardi et al., 1989; Walf and Frye, 2010; Kiss et al., 2012; Bastos et al., 2015). The timing of initiating E_2 replacement appears critical. Administering E_2 after five months of estrogen deficiency did not improve depression-like behavior (Walf et al., 2009).

1.4.4. Anti-inflammatory actions of estrogen

Ovariectomy activates microglia (Vegeto et al., 2006; Benedusi et al., 2012; Villa et al., 2016). At 4 weeks after ovariectomy, female rats exhibited up-regulation of microglia-associated genes in the PFC (Sárvári et al., 2012). Microglia activation was also found in the hippocampus of female mice at 5 months after ovariectomy (Benedusi et al., 2012). Microglia activation in response to LPS in the hippocampus and cortex was attenuated by E_2 replacement initiated 3 weeks after ovariectomy in 6 weeks old female mice compared to the vehicle group (Vegeto et al., 2006). In vitro, E_2 suppresses PICs and iNOS expression in microglia induced by LPS (Bruce-Keller et al., 2000; Vegeto et al., 2001). ER-α and β are localized on microglia cells, and E_2

treatment inhibits IL-1β and TNF-α release from microglia activated by LPS (Ishihara et al., 2015). In vitro, propylpyrazoletriol (ER-α agonist) causes a greater inhibition of COX-2, IL-1β, and TNF-α production in microglia activated by LPS, compared to diarylpropionitirle (ER-β agonist) (Smith et al., 2011). Estrogens block PAMPs and NF-*k*B that up-regulate TLRs and increase cytokines synthesis (Paimela et al., 2007; Cunningham, 2014). E_2 also accelerates the resolution of inflammation through STAT-3 signaling leading to blockade of PICs (IL-1β) synthesis and increase anti-inflammatory cytokine (IL-10) production (Villa et al., 2016).

1.5. Rationale for the study

Several clinical studies showed an increased incidence of depression in patients with HF. Peripheral and CNS inflammation are present in both depression and HF. Male rats also display depression-like behavior post-MI (Wann et al., 2007; Bah et al., 2011b; Frey et al., 2014). Inflammation that originates from MI, extends to different parts in the brain such as PVN, RVLM, PFC, cingulate cortex, and hippocampus (Ter Horst, 1999; Kang et al., 2006, 2008). In clinical studies, gender modulates depression in patients with HF (Rutledge et al., 2006). To the best of our knowledge there are no studies that assessed whether sex affects depression-like behavior in rodents with HF post-MI.

Ovarian hormones protect females from development of depression-like behavior. After ovariectomy, female rats exhibit depression-like behavior (Bekku and Yoshimura, 2005; Bastos et al., 2015) associated with a decrease in 5-HT turnover, mBDNF levels in the hippocampus (Kiss et al., 2012; Kramár et al., 2012; Xu et al., 2015), and increase in cytokine levels, TLR-4 and NF-*k*B expression and activated microglia in the hippocampus (Vegeto et al., 2006; Benedusi et al., 2012; Xu et al., 2015, 2016; Villa et al., 2016). Estrogen replacement prevents neuroinflammation,

promotes neurogenesis, and prevents development of depression-like behavior (Benedusi et al., 2012; Kramár et al., 2012; Xu et al., 2015, 2016).

Standard treatment (SSRI) showed no significant improvement in depression symptoms in patients with HF (O'Connor et al., 2010; Angermann et al., 2016). On the other hand, anti-inflammatory agents such as PTX displayed antidepressant effects in male rats at 2 weeks post-MI (Bah et al., 2011b). Several pre-clinical studies showed that anti-inflammatory treatment either peripherally or centrally, inhibits neuroinflammation in the PVN and SFO of male rodents post-MI (Kang et al., 2006, 2008, 2010, 2011; Guggilam et al., 2007, 2008; Wei et al., 2014b; Yu et al., 2018a).

Collectively, neuroinflammation induced by MI may contribute to the development of depression-like behavior, ovarian hormones might have protective effects against development of depression-like behavior induced by HF post-MI, but the mechanisms by which sex hormones affect depression-like behavior in HF have not yet been established.

1.6. Hypothesis

1. Sex modulates neuroinflammation in the PFC, amygdala and PVN of male and female rats with HF post-MI and thereby depression-like behavior induced by HF post-MI.

2. Estradiol replacement inhibits neuroinflammation in the PFC, amygdala and PVN to ameliorate depression-like behavior and HF progression in OVX females with HF post-MI.

3. PICs in the neuroinflammatory pathway are link between HF and depression-like behavior.

1.7. Objectives

To determine modulation by estradiol:

1. Effect of male vs female sex on depression-like behavior in HF post-MI.
2. Effect of estrogen on depression-like behavior in OVX female rats with HF post-MI.
3. Interaction between BDNF and estrogen in the PFC, and amygdala

The impact of inhibition of PIC on depression and HF:

1. Effect of PIC on cardiac remodeling and dysfunction post-MI
2. Effect of PIC on BDNF levels in the PFC and amygdala
3. Role of PIC in depression-like behavior in OVX-female rats post-MI

1.8. Outline of the approach to problem

Chapter 2: Assessment of neuroinflammation and depression-like behavior in male and female rats post-MI, and role of estrogens in depression-like behavior in OVX female rats post-MI

Chapter 3: Role of neuroinflammation in depression-like behavior in OVX female rats post-MI, assessed by oral PTX treatment initiated before inducing MI in OVX female rats.

Chapter 2:

Sex differences in depression-like behavior and neuroinflammation in rats post-MI: role of estrogens.

Sex differences in depression-like behavior and neuroinflammation in rats post-MI: role of estrogens.

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Short Title: Sex and depression in rats with HF post MI

Abstract

Patients with heart failure (HF) have a high prevalence of depression associated with a worse prognosis, particularly in older women. The present study evaluated whether sex and estrogens affect depression-like behavior and associated neuroinflammation induced by myocardial infarction (MI) in rats. MI was induced by occlusion of the left anterior descending artery in young adult male and female Wistar rats or in ovariectomized (OVX) female rats without and with estrogen [17β-estradiol (E_2)] replacement. MI groups showed a comparable degree of cardiac dysfunction. Eight weeks post-MI, male rats with HF exhibited depression-like behaviors, including anhedonia and higher immobility in the sucrose preference and forced swim tests, which were not observed in female rats with HF. In the cued fear conditioning test, male but not female rats with HF froze more than sham rats. After OVX, female sham rats developed mild depression-like behaviors that were pronounced in OVX female rats post-MI and were largely prevented by E_2 replacement. Cytokine levels in the plasma and paraventricular nucleus increased in both sexes with HF, but only male rats with HF showed an increase in cytokine levels in the prefrontal cortex. OVX alone did not affect cytokine levels, but OVX-MI caused significant increases in the prefrontal cortex, which were shifted to an anti-inflammatory pattern by E_2 replacement. These results suggest that estrogens prevent depression-like behavior induced by HF post-MI in young adult female rats by inhibiting proinflammatory cytokine production and actions in the prefrontal cortex.

New & Noteworthy

In contrast to male rats, female rats with heart failure after myocardial infarction do not develop depression-like behavior or increases in prefrontal cortex cytokines. However, after ovariectomy, female rats exhibit similar changes, which are prevented by 17 β -estradiol replacement. Neuroinflammation in the prefrontal cortex in male subjects may contribute to depression-like behavior, whereas its estrogen-dependent absence in female subjects may protect against depression.

Introduction

Patients with heart failure (HF) have a high prevalence of depression, with a meta-analysis reporting an overall point estimate for the prevalence rate of 22%, two- to threefold higher than the rate in the general population (Rutledge et al., 2006). Moreover, patients with HF and depression, or patients with depression after myocardial infarction (MI), have a poorer quality of life and a greater than twofold increased risk of further cardiac events and mortality than those not depressed (Rutledge et al., 2006; Huffman, 2013; Moraska et al., 2013). The comorbidity of depression and HF may reflect an enhancement of inflammation. Patients with HF and major depressive disorder have high plasma proinflammatory cytokines (PIC) levels, specifically IL-2, IL-6, and TNF-α (Deswal et al., 2001; Liu et al., 2012). There are also sex differences in the rate of comorbidity and inflammation. Older women with heart disease, including HF, are at a greater risk of developing depression and have a worse prognosis than men (Gottlieb et al., 2004; Rutledge et al., 2006; Wright et al., 2014). On the other hand, women at an age of 50 yr or less with HF have lower plasma TNF- α levels than men, possibly reflecting an inhibitory action of estrogens (Deswal et al., 2001). Despite these clinical sex differences in the interaction of depression and HF, preclinical studies have used only male rodents. These studies have shown that male rodents with HF induced by MI exhibit depression-like behavior and an increase in plasma PICs, such as TNF-α, IL-1β, and IL-6 (Grippo and Johnson, 2002; Grippo et al., 2003; Francis et al., 2004; Wann et al., 2009; Bah et al., 2011b). This inflammatory state extends to the brain. In rats with HF post-MI, microglia activation and increased IL-1β and TNF- α levels are found in the paraventricular nucleus (PVN) of the hypothalamus (Kang et al., 2006; Rana et al., 2010). The PVN is the major hypothalamus nucleus for cardiovascular regulation in

HF (Pyner, 2014). We postulate that this neuroinflammation may extend to other brain areas, such as the prefrontal cortex (PFC), hippocampus, and amygdala, and contribute to depression-like behavior (Willner et al., 2013). PICs decrease serotonin concentration by activating indoleamine 2,3-dioxygenase (IDO) in microglia and shift tryptophan metabolism from serotonin to quinolinic acid (Miller and Raison, 2016). Quinolinic acid and its metabolites not only stimulate N-methyl-D-aspartate (NMDA) receptors but also induce oxidative damage (Parrott et al., 2016), whereas activated microglia increase the release of PICs and glutamate (Wohleb et al., 2016). As a result, there is increased glutamatergic activation and decreased expression of brain-derived neurotrophic factor (BDNF), which may thereby contribute to depression (Duman, 2004).

The increased rates of depression in older women with HF are correlated with clinical and preclinical work that supports that estrogens protect against depression. For example, postmenopausal women have a higher incidence of depression compared with premenopausal women (Wise et al., 2008). Ovariectomized (OVX) rats exhibit depression-like behavior with a decrease of serotonin in the amygdala, hippocampus, and PFC (Borrow and Cameron, 2014). Hormonal replacement therapy reduces depression in menopause and postmenopausal women (Toffol et al., 2015) and alleviates depression-like behavior in adult and aged OVX rats (Walf et al., 2009; Kiss et al., 2012; Xu et al., 2015). Estrogens may protect against depression through several mechanisms, including promoting microglia resting phenotype (Habib and Beyer, 2015) and increasing serotonin concentration by inhibiting IDO enzyme (Xu et al., 2015) and the expression of BDNF by genomic pathways (Sohrabji and Lewis, 2006). Considering the impact of estrogens, we hypothesized that young adult female rats would exhibit less depression-like behavior and have less neuroinflammation in

the PVN, PFC, and amygdala than male rats with HF post-MI. To test this hypothesis, the present study first assessed the extent of depression-like behavior and neuroinflammation by assessing PIC levels in plasma, PVN, PFC, and amygdala in female and male rats with HF post-MI. These experiments showed clear sex differences in the extent of neuroinflammation in the PFC and in depression-like behavior. We then assessed the role of the ovaries and estrogens for protection against depression-like behavior by inducing HF post-MI in female rats after OVX without or with estrogen [17β-estradiol (E_2)] replacement.

Material and Methods

Experimental Animals

Male and female Wistar rats (9–13 wk old, Charles River, Montreal, QC, Canada) weighing 200–250 g were housed in pairs at 21 ± 2°C and humidity of 46 ± 2% on a 12:12-h light-dark cycle, with the dark cycle starting at 3:00 AM. Rats were provided with standard chow and tap water ad libitum. All experimental procedures conformed with the National Institutes of Health *Guidelines for the Care and Use of Laboratory Animals* and were approved by the University of Ottawa Animal Care Committee.

Experimental Groups and Design

Protocol 1: male versus female rats with HF post-MI. After acclimatization for 5–7 days, rats in both sex groups were randomized into the following surgical groups (surgery to induce MI or sham surgery): male MI (n = 20), male sham (n = 8), female MI (n = 20), and female sham (n = 8). Eleven rats from the male MI group and fifteen rats from the female MI group survived. Rats from the male MI group (n = 3) and rats from the female MI group (n = 5) with MI size < 20% of the left ventricle (LV) were excluded from the study.

Protocol 2: HF post-MI in OVX female rats. Female rats were randomized into the following groups: 1) sham OVX-sham pellet sham MI (sham OVX-sham MI; n = 9), 2) OVX-sham pellet-sham MI (OVX-sham MI; n = 12), 3) OVX- E_2 pellet-sham MI (OVX- E_2-sham MI; n = 10), 4) OVX-sham pellet-MI (OVX-MI; n = 20), and 5) OVX- E_2 pellet-MI (OVX- E_2-MI; n = 20). Estrogen pellets containing 1.5 mg E_2 releasing 25 µg/day (Innovative Research of America, Sarasota, FL) were implanted

subcutaneously in the dorsal neck area. This dose of E_2 results in plasma E_2 levels within the normal range during the estrous cycle of the rat (Nickenig et al., 1998; Hügel et al., 1999). Two weeks after OVX and E_2 pellet implantation, rats underwent MI or sham MI surgery. Eight rats from the OVX-MI group and fifteen rats from the OVX- E_2-MI group survived.

Because of the unusual high mortality in the OVX-MI group, another 20 rats underwent OVX and MI surgery, of which 11 rats survived. Rats from the OVX-MI group (n = 8) and rats from the OVX- E_2-MI group (n = 5) with MI size < 20% of the LV were excluded from the study.

Behavioral tests were carried out from 7 to 9 wk after MI or sham surgery between 7:00 AM and 2:00 PM. At the end, echocardiographic and hemodynamic parameters were obtained, and MI size was measured by planimetry. The final numbers of rats for each of the groups are shown in Tables 1 and 2.

Surgical Procedures

Rats were premedicated with slow-release buprenorphine (1 mg/kg sc, Chiron Compounding Pharmacy, Guelph, ON, Canada) and anesthetized with 2% isoflurane. After intubation, the pericardium was opened via thoracotomy, and the left anterior descending coronary artery was ligated permanently. Sham rats underwent the same surgery but without ligation of the left anterior descending coronary artery.

Bilateral OVXs were performed through a longitudinal incision on each flank. Sham OVX rats underwent the same procedure except that the ovaries were exteriorized but not removed. For sham pellet implantation, a small incision was made but no pellet was implanted. Postsurgery rats were housed individually for 3–5 days and then returned to paired housing.

Behavior Tests

All behavioral tests except the sucrose preference test (SPT) were carried out in the dark cycle and were recorded by a digital camera linked to an EthoVision 11.5 xT video tracking system (Noldus Information Technology, Leesburg, VA). A dark holding room was used to minimize the duration of exposure to dim light in the Morris water maze (MWM) for the rats to see the visual cues and in the forced swim test (FST) and fear conditioning test (FCT) for accurate detection of immobility and freezing. The FST and FCT trials were scored both by hand and Ethovision software (v11.5). Hand score and software results were correlated >90%, and the presented results are calculated by the software. Behavior tests were performed in the following sequence for all rats with at least 1 day in between: SPT, MWM, FST, and FCT.

Tests to Assess Depression-Like Behavior

The SPT is used to evaluate anhedonia. Rats were habituated to the presence of two bottles, one bottle consisting of tap water and the other bottle consisting of 2% sucrose, for 3 days (Taliaz and Stall, 2010). Afterward, daily 1% sucrose and water consumption were measured for 7 days. The bottle location was switched on a daily basis to avoid location preference. Sucrose preference was calculated as the percent of sucrose intake over total daily fluid intake and is presented as the mean for the last 3 days.

The FST is performed to assess despair behavior. Each rat was placed in a 10-liter transparent plastic cylinder (height: 45 cm and diameter: 20 cm) containing 30 cm of tap water at 25 ± 2°C for 10 min. Immobility was defined as the lack of movements except those necessary to prevent the animal from drowning (Slattery and

Cryan, 2012). Percent immobility was calculated as the percent of immobile behavior over the test duration.

Tests to Assess Learning and Memory

The MWM test is performed to evaluate spatial learning and memory. The water maze consists of a circular pool (diameter: 1.84 m) with a clear acrylic glass platform (diameter: 0.10 m) submerged in the center of the southwest quadrant of the pool 1 cm below the surface of water at 25°C. The maze was located in a dimly lit room with different black spatial cues mounted on the white wall in the south, west, and east quadrants of the room. The test consisted of 4 training days, with 2 sessions/day. On the first day, each session had four trials/session, whereas the following training days had 3 trials/session (Vorhees and Williams, 2006). For each trial during training, rats were randomly placed into one of the three quadrants that do not contain the platform. Each rat was allowed to swim for 60 s to find the hidden platform using extra maze visual cues. Probe trials were carried out 24 h after the last training session on days 3 and 5. The platform was removed, and rats were released on the opposite side of the platform location and allowed to swim for 60 s to evaluate the duration spent in the target quadrant where the platform was located. The learning curves for the training sessions and the times spent in the platform quadrant during the two probe sessions are presented.

The FCT is used to assess learning and associative memory. This test was performed using a PhenoTyper cage that is square in shape, has an electrifiable grid floor, a calibrated shock generator, and a sound source as well as a camera mounted in the top unit to allow for continuous video recording (Pham et al., 2009). During the acquisition day, rats were placed in the PhenoTyper chamber for 2 min for habituation and then heard a tone (conditioned stimulus) that coterminated with a shock

(unconditioned stimulus) two times separated by a 1-min interval. The tone-shock pairing consisted of playing of a tone (~2,300 Hz, ~70 dB) for 30 s that ended concurrently with a 2-s foot shock (0.45 mA). Two minutes after receiving the last shock, the acquisition ended, and rats were placed back in their home cages. The second day, contextual fear conditioning was measured as the amount of freezing when the rat was in the same acquisition chamber for 6 min without shock or tone. On the third day, cued fear conditioning was measured in a different PhenoTyper cage with a different color, odor, flooring texture, and triangle shape instead of square to expose the rat to a novel context. To test that the rat did not recognize the context, freezing was measured for the first 3 min in the absence of the tone followed by 3 min in the presence of the tone. Freezing was defined as the absence of movement except those associated with breathing, and it was monitored across the whole trial length.

Echocardiography and Hemodynamic Measurements

An echocardiogram was obtained under 2% isoflurane anesthesia to measure cardiac diameters and ejection fraction (EF) using a Visual Sonics Vevo 770 System (Visual Sonics, Toronto, ON, Canada). A Millar catheter was then placed in the LV to measure LV peak systolic and end-diastolic pressures and dP/dtmax and dP/dtmin for 30 – 60 s.

Tissue Collection

After hemodynamics, trunk blood was collected into prechilled 50-ml tubes containing EDTA and centrifuged at 3,000 rpm for 30 min at 4°C to obtain plasma for cytokine levels. Hearts were removed and kept in cold saline followed by separation of the right ventricle (RV), whereas the LV was longitudinally dissected from the septum, and two to three cuts were performed on the apex to flatten the LV. The area of LV infarction was measured by planimetry, and infarct size was expressed as a

percentage of the LV area. The brain was removed and snap frozen in methylbutane at -20°C. All tissues and plasma were kept at -80°C until further analysis.

Plasma and Brain Cytokines

Plasma cytokine levels were measured by a multiplexed bead-based immunoassay kit (no. 171K1002M, Bio-Rad, Mississauga, ON,Canada). In *protocol 1*, IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, interferon-ɣ, and TNF-α were measured; in *protocol 2*, IL-1α, IL-1β, IL-2, IL-6, IL-10, and TNF-α were measured. Plasma samples were centrifuged at 13,000 *g* for 15 min at 4°C and diluted 1:4. The premixed conjugated beads interacted with targeted cytokines in plasma samples for 1.5 h at room temperature followed by incubation with biotinylated antibodies for 1 h at room temperature, which will interact with the cytokine of interest. The mixture was incubated with streptavidin-phycoerythrin for 10 min at room temperature. Finally, the Bioplex Protein Array System and related software (Bio-Plex Manager version 5.6 were used to determine the levels of cytokines.

Brain punches were collected from the following 50-µm sections: the PFC from (2.8 to 1.8 mm), PVN (-0.8 to -2.3 mm), and amygdala from (-2.5 to -3.1 mm) and then homogenized in tissue lysis buffer [10 mM Tris (pH 7.4), 100 mM NaCl, 1% Triton X-100, 1 mM EDTA (pH 8.0), 1 mM EGTA, 10% glycerol, 0.1% SDS, and 0.5% deoxycholate containing 2 mM protease inhibitor cocktail, 2 mM Na_3VO_4, and 5 mM NaF]. The homogenate was centrifuged at 10,000 *g* for 20 min at 4°C, and the supernatant was collected. Protein concentration was determined by a Micro BCA protein assay kit (no.113225, ThermoFisher Scientific, Rockford, IL). Two PVNs of each rat were pooled, providing ~100–130 µg total protein. Individual tissue samples with 0.5 mg/ml protein were assayed for IL-1α, IL-1β, IL-2, IL-6, IL-10, and TNF-α

using a multiplexed bead-based immunoassay set with a cytokine reagent kit (no. 171304070M, Bio-Rad). Cytokine levels are expressed as picograms per milligram.

Western Blot Analysis

Immunoblot analysis was performed using 20 µg total protein from the PFC and amygdala. Proteins were separated by SDS-PAGE (12% gel) for mature BDNF (mBDNF) and transferred onto 0.2 µm PVDF membranes (Bio-Rad). Blots were blocked for 1 h at room temperature in 5% milk in Tris-buffered saline with 0.1% Tween 20 and then probed with rabbit monoclonal anti-BDNF (1:1,000, Ab 108319, Abcam, Toronto, ON, Canada) at 4°C overnight. Blots were then incubated with goat anti-rabbit IgG- horseradish peroxidase (1:5,000, no. 111-035-144, Jackson ImmunoResearch, West Grove, PA) for 1 hr at room temperature. After a wash, the blot was developed with Luminata ECL reagent (MilliporeSigma, Etobicoke, ON, Canada) and visualized with the ChemiDoc XRS_ imager (Bio-Rad). Membranes were reprobed with monoclonal mouse anti-β-actin (1:10,000, A5316, Sigma, Oakville, ON, Canada) or GAPDH (1:10,000, MAB374, Millipore, Toronto, ON, Canada) as a protein loading control. Band densities were quantified using Image Laboratory software (Bio-Rad). The expression of mBDNF protein was calculated as the ratio of its band density relative to the density of β-actin in the same sample.

Statistical Analysis

To assess for sex X MI, two-way ANOVA was performed followed by the Bonferroni post hoc test. To determine differences between the five OVX female groups, one-way ANOVA was conducted, and when significant, the OVX X MI interaction was assessed using two-way ANOVA followed by the Bonferroni post hoc test.

To compare freezing in context and cued test of FCT and spatial learning during the training days in the MWM, two-way repeated measures ANOVA was performed with the Bonferroni post hoc test. *P* values of <0.05 were considered statistically significant. A priori power analyses suggested that *n* = 9/group would be sufficient to

detect a 20% difference in the FST and *n* = 13/group to detect a 10% difference in the SPT.

Results

Cardiac Function

Male versus female rats. Cardiac function of male versus female rats is shown in (Table 2.1). Final body weights showed the expected sex differences but no differences in MI versus sham groups. Male and female rats had similar MI sizes and developed a similar degree of cardiac dysfunction, with clear increases in end-systolic volume and end-diastolic volume compared with sham groups, and a significant decrease in EF. LV peak systolic pressure, LV dP/dtmax, and LV dP/dtmin significantly decreased, whereas LV end-diastolic pressure significantly increased. LV and RV weights significantly increased in female rats post-MI, whereas in male rats post-MI, only RV weight significantly increased.

OVX female rats. Cardiac function of OVX female rats is shown in (Table 2.2) Final body weights were higher in OVX rats but not in OVX-E_2 rats compared with sham OVX rats. Uterus weights were markedly decreased in OVX rats but maintained in OVX-E_2 rats. OVX without or with E_2 replacement did not affect cardiac function in sham MI rats. OVX-MI and OVX-E_2- MI rats showed similar MI sizes and a similar degree of cardiac dysfunction, as assessed by echocardiographic and hemodynamic parameters. LV and RV weights corrected for body weight were lower in OVX rats and showed modest increases post-MI.

Table 2.1 Anatomical, echocardiographic and hemodynamic measurements in male and female rats at 10 wk after MI

Variables	Male sham group	Male MI group	Female sham group	Female MI group
Number of rats/group	8	8	8	10
Body weight, g	605 ± 19.9	593 ± 13.2	368 ± 10.6‡	368 ± 11.2‡
MI size, %	-------	26 ± 1.5	-------	29 ± 1.5
LV/body wt, mg/100g	170 ± 5.0	184 ± 4.5	200 ± 5.2	235 ± 11.2†
Right ventricle /body wt mg/100g	42 ± 2.6	61 ± 12.9*	42 ± 1.7	57 ± 9.4†
Echocardiographic parameters				
LV end-systolic volume, µL/100g body wt	9 ± 1.7	46 ± 4.3*	5 ± 1.2	50 ± 3.8†
LV end-diastolic volume, µL/100g body wt	63 ± 7.5	99 ± 5.4*	59 ± 4.5	102 ± 6.5†
Ejection fraction, %	86 ± 1.6	49 ± 1.9*	91 ± 1.6	52 ± 1.9†
Hemodynamic parameters				
LV peak systolic pressure, mmHg	118 ± 1.9	107 ± 3.9*	125 ± 2.0	112 ± 3.5†
LV end-diastolic pressure, mmHg	3.6 ± 0.3	14.9 ± 1.6*	3.5 ± 0.3	17.1 ± 2.1†
LV dP/dt(+), mmHg/s	7499 ± 176	5864 ± 245*	8108 ± 105	6027 ± 260†
LV dP/dt(-), mmHg/s	6466 ± 130	4474 ± 245*	7092 ± 160	4899 ± 315†

Values are means ± SE. Two-way ANOVA was done for the sex and myocardial infarction (MI) interaction followed by the Bonferroni post hoc test. There was no significant sex X MI interaction for any variable. Body weight sex effect: $F = 266.7$, $P < 0.001$. Right ventricle/body weight MI effect: $F = 10.2$, $P < 0.003$. Left ventricle (LV)/body weight sex effect: $F = 16.1$, $P < 0.0001$. MI effect: $F = 5.8$, $P < 0.022$. LV end-systolic volume MI effect: $F = 135.9$, $P < 0.0001$. LV end-diastolic volume MI effect: $F = 38.1$, $P < 0.0001$. Ejection fraction sex effect: $F = 4.4$, $P < 0.04$. MI effect: $F = 442.7$, $P < 0.0001$. LV peak systolic pressure MI effect: $F = 13.9$, $P < 0.001$. LV end-diastolic pressure MI effect: $F = 74.5$, $P < 0.0001$. LV dP/dt(+) MI effect: $F = 73.9$, $P < 0.0001$. LV dP/d*t*(-) sex effect: $F = 4.8$, $P < 0.036$. MI effect: $F = 76.5$, $P < 0.0001$. *$P < 0.05$ vs. the male sham group; †$P < 0.05$ vs. the female sham group; ‡$P < 0.05$ vs. male groups.

Table 2.2 Anatomical, echocardiographic and hemodynamic measurements in ovariectomized female rats without/with estrogen replacement at 10 weeks post MI

Variables	sham OVX-sham MI group	OVX-sham MI group	OVX-E2 sham MI group	OVX-MI group	OVX-E2-MI group
Number of rats/group	8	12	10	11	9
Body weight, g	362 ± 7.2	466 ± 14.4*†	381 ± 12.7	437 ± 10.0*†	365 ± 10.1
Uterus/body weight, mg/100g	164 ± 13.1	38 ± 3.4*†	162 ± 14.1	37 ± 1.1*†	184 ± 14.6
MI size, %	-------	-------	-------	27 ± 1.4	27 ± 1.7
LV/body wt, mg/100g	204 ± 3.9	163 ± 3.7*	204 ± 9.1	183 ± 5.8‡	230 ± 7.2‡
Right ventricle /body wt mg/100g	46 ± 1.7	37 ± 1.4 †	42 ± 2.3	40 ± 3.5†	50 ± 2.4
	Echocardiographic parameters				
LV end-systolic volume, µL/100g body wt	7 ± 0.7	8 ± 0.7	6 ± 0.4	44 ± 4.1*‡	47 ± 3.4*‡
LV end-diastolic volume, µL/100g body wt	68 ± 5.4	59 ± 1.9	59 ± 2.8	81 ± 6.0‡	103 ± 7.0*‡
Ejection fraction, %	89 ± 0.9	87 ± 0.9	90 ± 0.5	46 ± 2.5*‡	54 ± 2.4*‡
	Hemodynamic parameters				
LV peak systolic pressure, mmHg	128 ± 1.5	127 ± 1.5	130 ± 3.2	121 ± 3.9‡	115 ± 3.9*
LV end-diastolic pressure, mmHg	2.7 ± 0.4	4.1 ± 0.5	3.5 ± 0.3	10.1 ± 0.7*‡	13.3 ± 0.8 *‡
LV dP/dt(+), mmHg/s	8646 ± 261	8773 ± 181	8716 ± 181	6445 ± 257 *‡	6064 ± 282*‡
LV dP/dt(-), mmHg/s	7322 ± 280	7366 ± 141	7572 ± 145	5425 ± 220*‡	5390 ± 271*‡

Values are means ± SE. One-way ANOVA was done to compare all groups. Two-way ANOVA was performed for the ovariectomy (OVX) and myocardial infarction (MI) interaction followed by a Bonferroni post hoc test. Body weight OVX effect: F = 40.8, $P < 0.0001$. Uterus/body weight OVX effect: $F = 222.5$, $P < 0.0001$. Right ventricle/body weight OVX effect: F =15.3, $P < 0.0001$. Left ventricle (LV)/body weight MI effect: F= 12.8, $P < 0.001$. OVX effect: $F = 45.8$, $P < 0.0001$. LV end-systolic volume MI effect: $F = 198.7$, $P < 0.0001$. LV end-diastolic volume MI X OVX effect: $F = 4.6$, $P < 0.031$. Ejection fraction MI effect: $F = 458.5$, $P < 0.0001$. LV peak systolic pressure MI effect: $F = 9.8$, $P < 0.001$. LV end-diastolic pressure MI X OVX effect: $F = 11.7$, $P < 0.001$. LV dP/dt(+) MI effect: $F = 131.1$, $P < 0.0001$. LV dP/dt(-) MI effect: $F = 104.3$, $P < 0.0001$. *$P < 0.05$ vs. the sham OVX-sham MI group; †$P < 0.05$ vs. OVX-17β-estradiol (E_2) groups; ‡$P < 0.05$ vs. the related sham MI group.

Depression-Like Behavior

Male versus female rats. THE SPT. The sex and MI interaction was significant (F=2.0, $P < 0.0001$), with male rats with HF post-MI having a significant decrease in preference for sucrose by 27% compared with sham male rats (Fig. 2.1 *A*). No change in sucrose preference was observed in female MI versus sham rats (Fig. 2.1 *A*), and there was no difference in total liquid between the four groups (Fig. 2.1 *B*).

THE FST. Two-way ANOVA showed a significant MI effect (F=4.7, $P< 0.05$), with male rats with HF post-MI exhibiting a significant increase in immobility compared with sham males but not female rats with HF post-MI (Fig. 2.2 *A*). Independent of MI, female rats were more mobile compared with male rats (sex effect: $F = 26.7$, $P<0.0001$).

OVX female rats. THE SPT. OVX decreased preference for sucrose by 8% in sham MI rats and by ~30% in MI rats (OVX x MI effect: F=5.7, $P< 0.02$). E_2 replacement

prevented these decreases in both OVX-sham MI and OVX-MI rats (Fig. 2.1 *C*). Total liquid intake per 100 g body wt was decreased in OVX groups (Fig. 2.1 *D*).

THE FST. OVX alone caused a modest increase in immobility, whereas OVX-MI caused a marked increase (OVX x MI effect: $F = 4.4$, $P < 0.04$). E_2 replacement prevented this increase (Fig. 2.2 *B*).

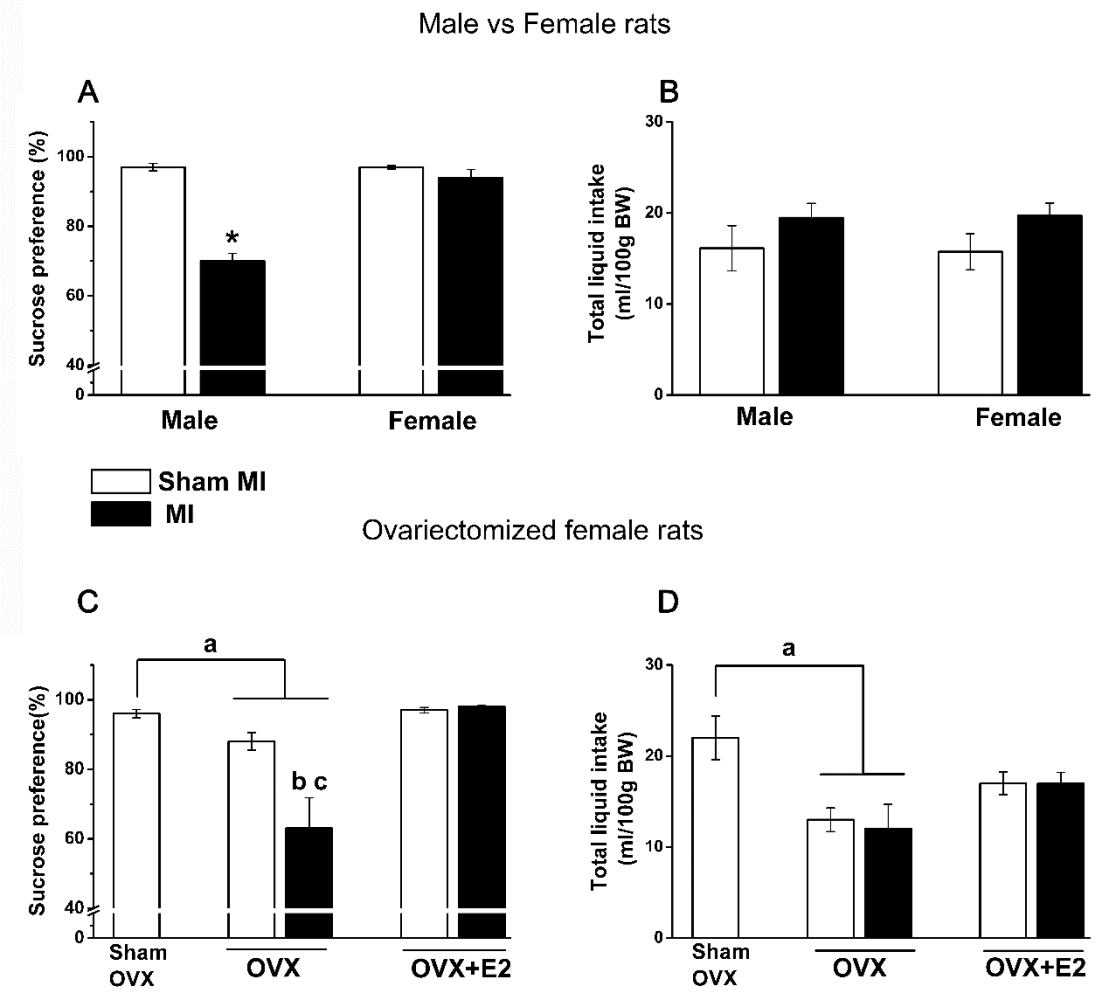
Male vs Female rats
A
Sucrose preference (%)
100
80
60
40
0
*
Male
Female
B
Total liquid intake (ml/100g BW)
30
20
10
0
Male
Female
Sham MI
MI
Ovariectomized female rats
C
Sucrose preference(%)
a
b c
Sham OVX
OVX
OVX+E2
D
Total liquid intake (ml/100g BW)
a
Sham OVX
OVX
OVX+E2

Fig. 2.1. Sex x MI and OVX x MI interactions for the percent of sucrose intake and total liquid intake during the last three days of the test. Values are means ±SE; n = 8–11 rats/group. One-way ANOVA was done to compare all OVX groups with the sham OVX sham-MI group, and two-way ANOVA was done for sex X MI and OVX X MI interactions followed by a Bonferroni post hoc test. *A*: effects of sex and MI on sucrose intake as a percentage of total liquid intake. MI X sex effect: $F = 42.4$, $P < 0.0001$. *B*: total liquid intake of male and female rats. There was no MI or sex effect on liquid intake. *C*: effects of OVX and MI on sucrose preference in OVX female rats with or without 17β-estradiol (E_2) replacement. OVX X MI effect: $F = 5.7$, $P < 0.02$. *D*: total liquid intake. OVX groups showed lower total liquid intake but not OVX-E_2 groups compared with the sham OVX-sham MI group ($F = 3.8$, $P < 0.009$). **P*<0.0001 vs. male sham, [a] *P*<0.05 vs. sham OVX-sham MI, [b] *P*< 0.05 vs. OVX-Sham MI, [c] P<0.05 vs. OVX-E_2-MI group.

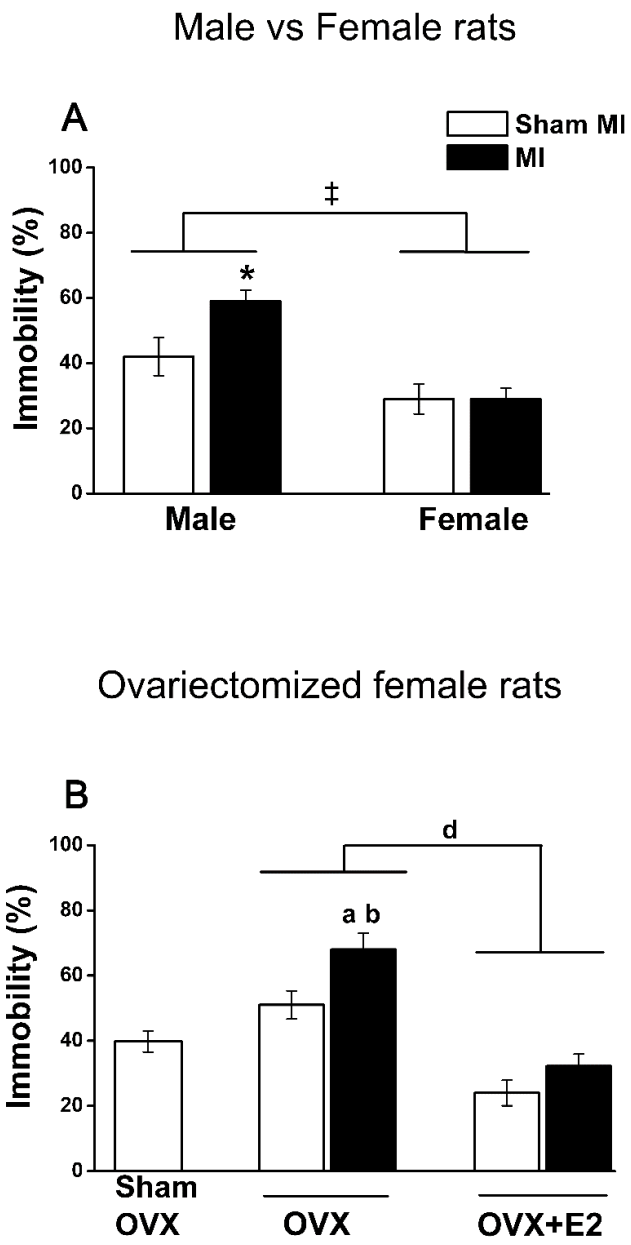

Fig. 2.2. Sex x myocardial infarction (MI) and ovariectomy (OVX) x MI interactions for the percent immobility in the forced swim test. Values are means ± SE; *n* =8–11 rats/group. One-way ANOVA was done to compare all OVX groups with the sham OVX-sham MI group, and two-way ANOVA was done for sex x MI and OVX x MI interactions followed by a Bonferroni post hoc test. *A*: effects of sex and MI. MI effect: $F = 4.7$, $P < 0.039$; sex effect: $F = 26.7$, $P < 0.0001$. *B*: effects of OVX and MI. MI x OVX interaction: $F = 4.4$, $P < 0.04$. OVX effect: $F < 50.5$, $P < 0.0001$. *P <0.05 vs. the male sham group; ‡$P < 0.0001$ vs. male groups; ^{a}P <0.05 vs. the sham OVX-sham MI group; $^{b}P < 0.05$ vs. the OVX-sham MI group; $^{d}P < 0.05$ vs. OVX-17β-estradiol (E_2) groups.

Learning and Memory

Male versus female rats. MWM TEST. All groups had a similar significant decrease in their latency to the platform during the four days of training (Fig. 2.3 *B*). Independent of MI, female rats needed more time to reach the platform compared with male rats (sex effect: F=12.0, $P < 0.05$). For the probe trials, all groups increased their time in the target quadrant on *day 5* (*probe 2*) compared with *day 3* (*probe 1*). However, there was no MI or sex effect in either of the two probe trials (Fig. 2.3 *A*). Similarly, there was no significant difference in locomotor activity as assessed by the speed of traveling and total path length (Table 2.3).

THE FCT. On *day 1*, during habituation, before presentation of the tone or shock, there was a significant higher freezing behavior in male rats compared with female rats, independent of MI (Fig. 2.4 *A*). On *day 2*, on the context test, female rats showed a modest, but significant, increase in freezing compared with habituation on *day 1* (F= 58.8, $P < 0.0001$), but there was no MI effect (Fig. 2.4 *A*). On the cued FCT, all groups showed an increase in freezing behavior when hearing the tone. However, male rats with HF post-MI exhibited significantly more freezing behavior during the 3 min with the tone (Fig. 2.4 *B*) compared with male sham rats, whereas there was no difference between the two female groups.

OVX female rats. MWM TEST. All groups showed a similar significant decrease in latency to the platform during the 4 days of training (Fig. 2.3 *D*). OVX alone or with E_2 did not affect any component of the two probe trials (Fig. 2.3 *C*). The OVX-MI group showed less time in the target quadrant in *probe 1* (Fig. 2.3 *C*), which improved in *probe 2*. Locomotor activity did not differ among the groups (Table 2.3).

THE FCT. On *day 1*, freezing during habituation was very low in the sham groups but significantly higher in the OVX-MI group, which was partially prevented by E_2 therapy (Fig. 2.4 *C*). In contrast, OVX alone and OVX-MI caused a similar marked increase in freezing in the context test compared with the sham OVX-sham MI group, which was partially prevented by E2 therapy (OVX effect: F =21.1, P < 0.001; Fig. 2.4 *C*). During the cued conditioning test, all groups showed an increase in freezing during the tone. OVX alone caused a modest further increase in freezing, and OVX-MI caused a marked further increase. These increases were largely prevented in both groups by E_2 treatment (Fig. 2.4 *D*).

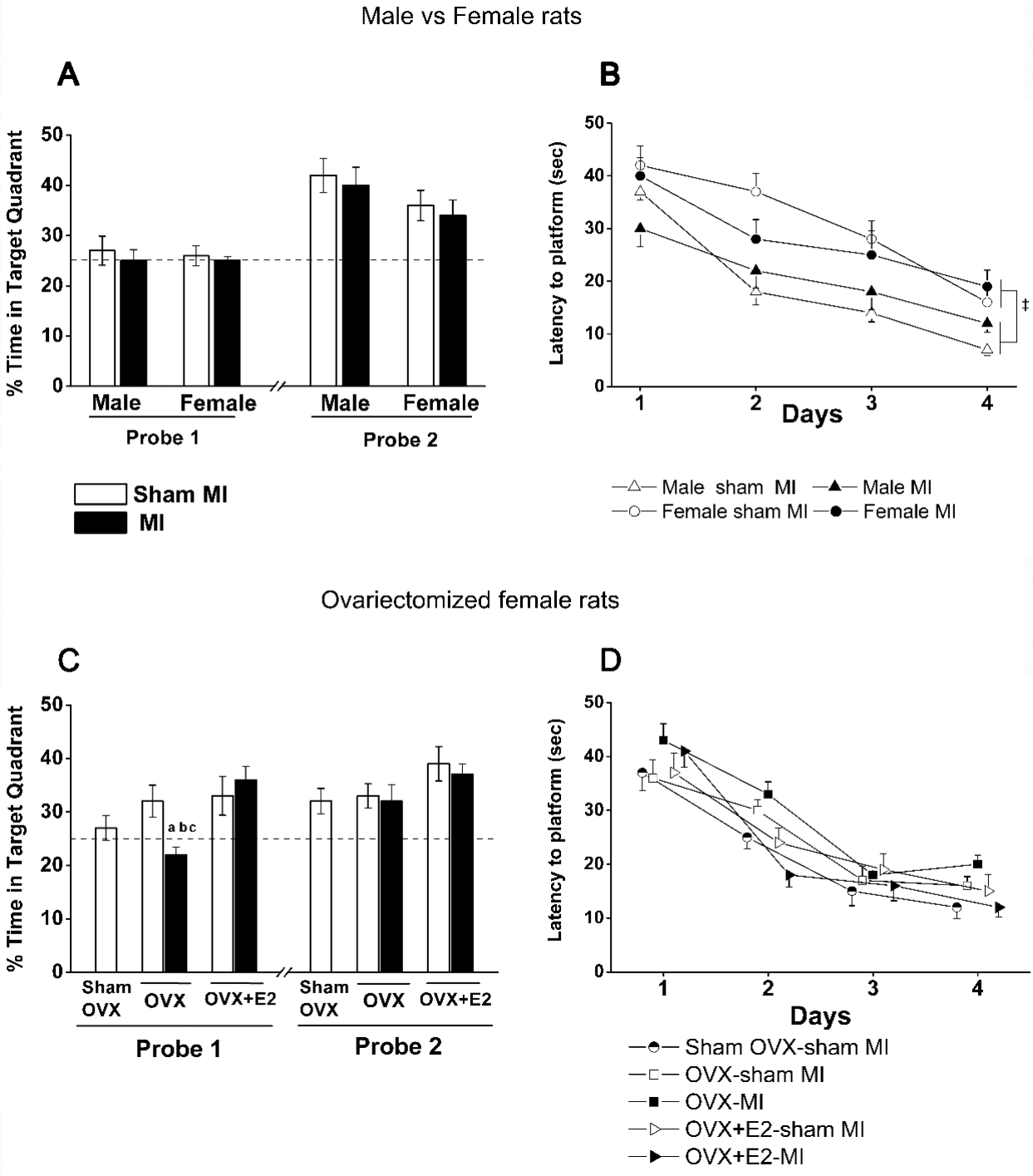
Male vs Female rats
A
% Time in Target Quadrant
50
40
30
20
10
0
Male
Female
Male
Female
Probe 1
Probe 2
Sham MI
MI
B
Latency to platform (sec)
50
40
30
20
10
0
1
2
3
4
Days
‡
Male sham MI
Male MI
Female sham MI
Female MI
Ovariectomized female rats
C
% Time in Target Quadrant
50
40
30
20
10
0
a b c
Sham OVX
OVX
OVX+E2
Sham OVX
OVX
OVX+E2
Probe 1
Probe 2
D
Latency to platform (sec)
50
40
30
20
10
0
1
2
3
4
Days
Sham OVX-sham MI
OVX-sham MI
OVX-MI
OVX+E2-sham MI
OVX+E2-MI

Fig. 2.3. Sex x myocardial infarction (MI) and ovariectomy (OVX) x MI interactions in the Morris water maze test. Values are means ± SE; n = 8–10 rats/group. Repeated-measures ANOVA was performed to compare all groups during training days, and two-way ANOVA was done for sex x MI and OVX x MI interactions followed by a Bonferroni post hoc test. *A*: *probes 1* and *2* were done on the third day and fifth day. Time spent in the target quadrant, where the platform was located in the training sessions, is expressed as a percentage. MI and sex interaction: $F = 0.9$, $P = 0.35$. *B*: latency to the platform on training days. Sex effect: $F = 12.0$, $P < 0.05$. *C*: *probes 1* and *2*. *Probe 1*, MI x OVX interaction: $F = 9.2$, $P < 0.05$. *D*: latency to the platform on training days. ‡P <0.0001 vs. male groups; ^{a}P <0.05 vs. the sham OVX-sham MI group; $^{b}P < 0.05$ vs. the OVX-sham MI group; $^{c}P < 0.05$ vs. the OVX-17β-estradiol (E_2)-MI group.

Table 2.3. Locomotor activity in the first training session of the Morris Water Maze

	Number of rats/ group	Total path length, m	Speed, cm/s
Male vs Female rats			
Male sham group	7	14 ± 2.0	26 ± 1.5
Male MI group	8	15 ± 0.8	26 ± 1.3
Female sham group	8	11 ± 1.8	25 ± 1.8
Female MI group	9	15 ± 0.6	28 ± 0.8
Ovariectomized Female rats			
Sham OVX-Sham MI group	9	15 ± 1.0	28 ± 1.4
OVX-Sham MI group	12	15 ± 1.5	28 ± 1.7
OVX-E_2-Sham MI group	10	15 ± 0.5	31 ± 0.8
OVX-MI group	9	14 ± 0.9	28 ± 0.8
OVX-E2-MI group	9	16 ± 0.7	27± 1.1

Values are means ±SE. Two-way ANOVA was performed for sex x myocardial infarction (MI) and ovariectomy (OVX) x MI interactions. E2, 17β-estradiol. No significant differences in locomotor activity were observed

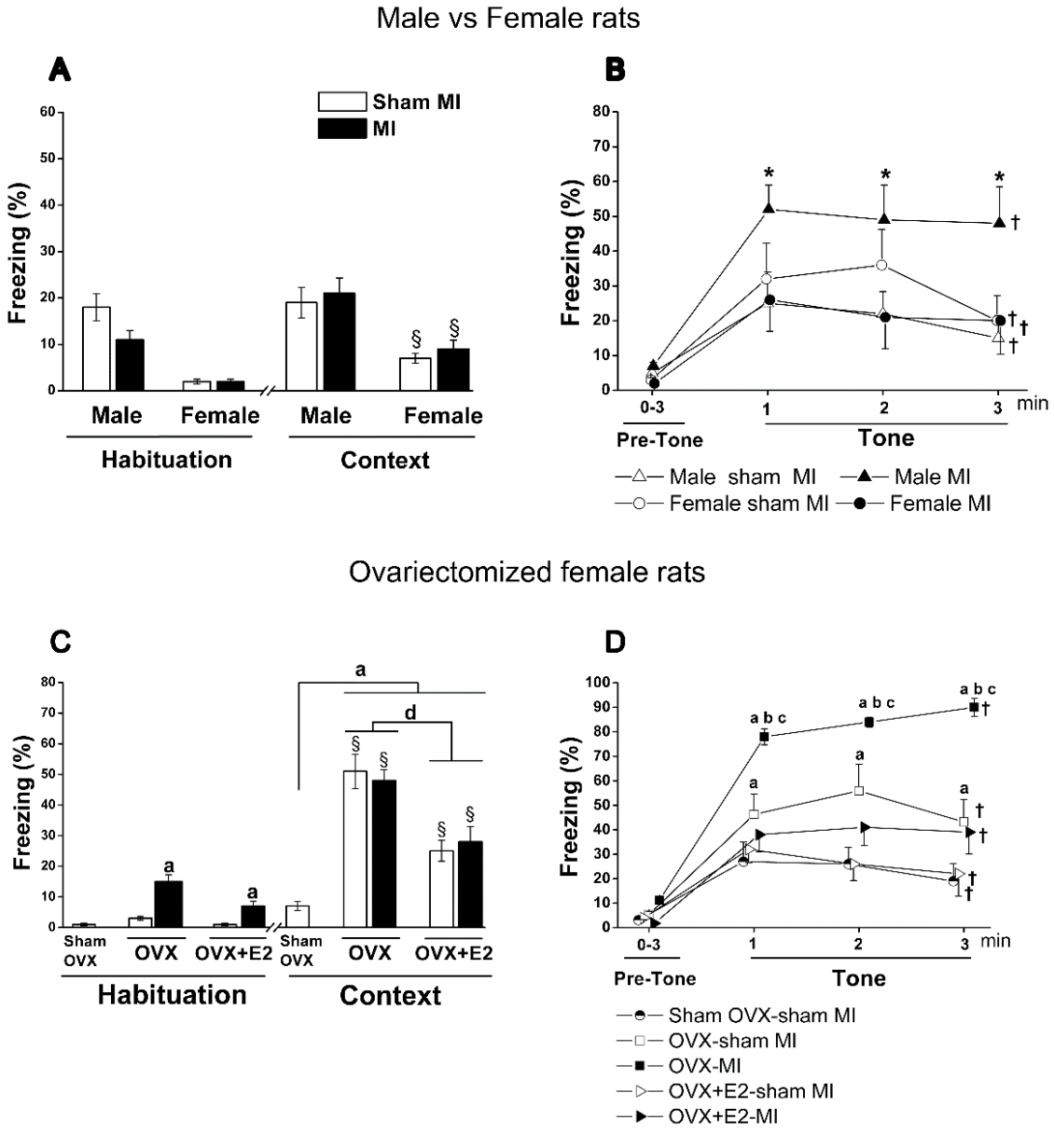
Male vs Female rats
A
Sham MI
MI
Freezing (%)
Male
Female
Male
Female
Habituation
Context
B
Freezing (%)
0-3
1
2
3
min
Pre-Tone
Tone
Male sham MI
Male MI
Female sham MI
Female MI
Ovariectomized female rats
C
Freezing (%)
Sham OVX
OVX
OVX+E2
Sham OVX
OVX
OVX+E2
Habituation
Context
D
Freezing (%)
0-3
1
2
3
min
Pre-Tone
Tone
Sham OVX-sham MI
OVX-sham MI
OVX-MI
OVX+E2-sham MI
OVX+E2-MI

Figure 2.4. Sex x myocardial infarction (MI) and ovariectomy (OVX) x MI interactions in the fear conditioning test. Values are means ± SE; n = 7–11 rats/group. Freezing behavior is expressed as a percentage of time freezing over the specified period. One-way ANOVA was performed to compare all OVX groups with the sham OVX-sham MI group, and two-way ANOVA was performed for sex x MI and OVX x MI interactions. *A*: habituation on *day 1* during the first 2 min of acquisition. Context on *day 2* to assess contextual fear includes all 6 min of the test. Two-way ANOVA revealed a sex effect: $F = 58.8$, $P < 0.0001$. *B*: cued fear conditioning test consisted of 3-min habituation as the pre-tone period followed by the tone period for 3 min. Sex and MI interaction: $F = 4.24$, $P < 0.049$. *C*: habituation on *day 1*. MI effect: $F = 39.2$, $P < 0.001$. Context freezing on *day 2*: OVX effect, $F = 21.1$, $P < 0.0001$. *D*: freezing on cued conditioning. All groups froze significantly more during tone compared with their baseline on *day 3* ($F = 104.2$, $P < 0.0001$). MI effect: $F = 8.0$, $P < 0.008$. OVX effect: $F = 26.1$, $P < 0.0001$. *$P < 0.05$ vs. the male sham group; §$P < 0.05$ vs. first day habituation; †$P < 0.05$ vs. pre-tone; [a]$P < 0.05$ vs. the sham OVX-sham MI group; [b]$P < 0.05$ vs. the OVX-sham MI group; [c]$P < 0.05$ vs. the OVX-17β-estradiol (E_2)-MI group; [d]$P < 0.05$ vs. OVX-E_2 groups.

Cytokine Levels

Male versus female rats. PLASMA. Among the 11 cytokines, IL-1α, IL-4, IL-5, IL-6, IL-12, TNF-α, and interferon-ɣ showed a similar increase in male and female rats with HF post-MI. The anti-inflammatory cytokine IL-10 increased in both HF post-MI groups, but it was only significant in male rats with HF post-MI (Table 2.4). THE PVN, PFC, AND AMYGDALA. In sham rats, cytokine levels in the PVN were similar or somewhat lower in female versus male rats, but in the PFC, they were higher in female rats (Fig. 2.5). The PICs IL-1α, IL-1β, IL-2, IL-6, and TNF-α increased significantly in the PVN of the hypothalamus in both male and female rats with HF post-MI as well the anti-inflammatory cytokine IL-10 (Fig. 2.5). In contrast, PFC cytokines increased significantly in male rats with HF post-MI compared with male sham rats but for IL-2, only up to levels found in female sham rats, and showed no further increase in female rats with HF post-MI (Fig. 2.5). The amygdala showed no changes in any of the cytokines (Table 2.5).

Table 2.4. Plasma cytokine levels increase in both male and female rats at 10 wk post MI

Cytokine, pg/ml	Male sham group n=4	Male MI group n=4	Female sham group n=5	Female MI group n=6
IL-1α	43 ± 5.4	96 ± 23.1*	41 ± 7.4	75 ± 5.4†
IL-1β	220 ± 32.9	223 ± 50.3	188 ± 44.3	145 ± 22.8
IL-2	88 ± 11.1	123 ± 8.8	95 ± 20.9	142 ± 23.8
IL-4	7 ± 0.6	12 ± 1.5*	7 ± 1.1	11 ± 0.6†
IL-5	79 ± 21.7	180 ± 26.0 *	83 ± 19.9	155 ± 7.0†
IL-6	145 ± 17.8	492 ± 184.1*	126 ± 46.3	279 ± 18.5†
IL-10	228 ± 35.8	442 ± 74.2*	255 ± 46.7	370 ± 27.5
IL-12	18 ± 3.4	47 ± 5.8*	21 ± 3.8	42 ± 2.2†
IL-13	28 ± 5.7	49 ± 8.9	35 ± 12.5	41 ± 1.4
TNF-α	127 ± 7.5	290 ± 16.7*	119 ± 15.9	262 ± 6.6†
IFN-ɣ	75 ± 14.8	169 ± 26.2*	62 ± 9.2	141 ± 4.9†

Values are means ± SE; *n*, number of rats/group. Two-way ANOVA was performed for the sex and myocardial infarction (MI) interaction followed by a Bonferroni post hoc test. There was no significant sex x MI interaction for any variable. IL-4 MI effect: $F = 19.1$, $P < 0.001$. IL-5 MI effect: $F = 22.5$, $P < 0.001$. IL-6 MI effect: $F = 9.3$, $P < 0.003$. IL-10 MI effect: $F = 12.7$, $P < 0.003$. IL-12 MI effect: $F = 42.5$, $P < 0.001$. TNF-α MI effect: $F = 158.6$, $P < 0.001$. Interferon-ɣ MI effect: $F = 39.4$, $P < 0.001$. *$P < 0.05$ vs. the male sham group; †$P < 0.05$ vs. the female sham group.

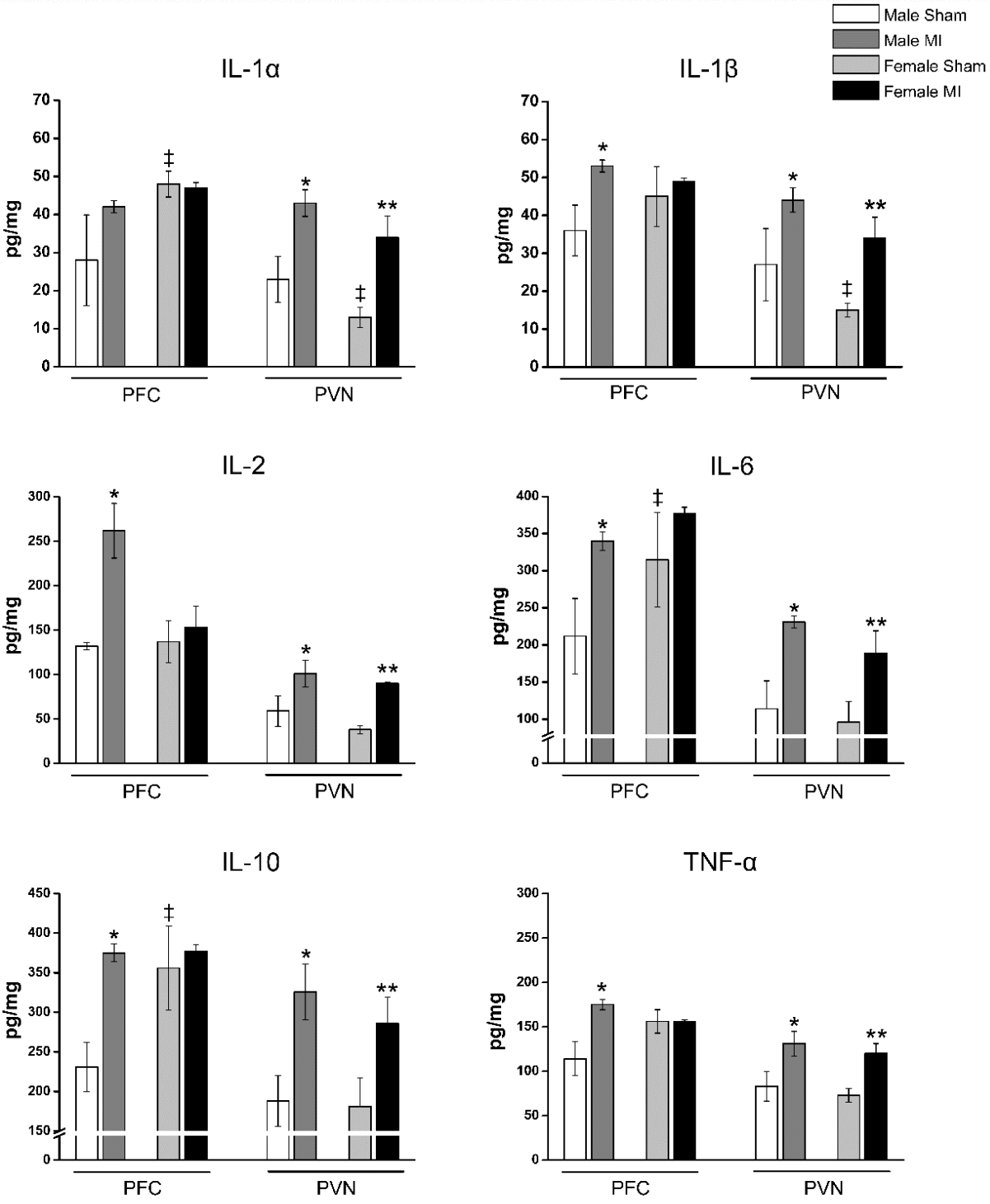
Male Sham
Male MI
Female Sham
Female MI
IL-1α
IL-1β
IL-2
IL-6
IL-10
TNF-α
pg/mg
PFC
PVN

Fig. 2.5. Cytokine levels in the prefrontal cortex (PFC) and paraventricular nucleus (PVN) in male and female rats with heart failure at 10 wk post myocardial infarction (post-MI). Values are means ± SE; n = 4–7 rats/group. Two-way ANOVA was performed for the sex and MI interaction followed by a Bonferroni post hoc test. In the PFC, MI x sex effect: IL-2, $F = 4.7$, $P < 0.03$; IL-10, $F = 25.3$, $P < 0.0001$; and TNF-α, $F = 17.8$, $P < 0.001$. MI effect: IL-6, $F = 6.5$, $P < 0.02$; and IL-1β, $F = 4.6$, $P < 0.05$. Sex effect: IL-1α, $F = 5.6$, $P < 0.03$; IL-6, $F = 11.6$, $P < 0.003$; and IL-10, $F = 36.0$, $P < 0.001$. In the PVN, MI effect: IL-1α, $F = 17.7$, $P < 0.001$; IL-1β, $F = 10.7$, $P < 0.05$; IL-2, $F = 13.9$, $P < 0.002$; IL-6, $F = 13.1$, $P < 0.002$; IL-10, $F = 11.7$, $P = 0.003$; and TNF-α, $F = 14.3$, $P < 0.002$. Sex effect: IL-1α, $F = 4.1$, $P < 0.05$; and IL-1β, $F = 3.9$, $P < 0.05$. *$P < 0.05$ vs. the male sham group; **$P < 0.05$ vs. the female sham group; ‡$P < 0.0001$ vs. the male sham group.

Table 2.5. Cytokine levels in the amygdala of male and female rats are unchanged at 10 weeks post MI

Cytokine, pg/mg	Male sham n=4	Male MI n=5	Female sham n=5	Female MI n=6
IL-1α	24 ± 6.0	29 ± 5.9	31 ± 2.3	29 ± 3.3
IL-1β	28 ± 7.5	36 ± 7.1	29 ± 6.0	33 ± 4.4
IL-2	49 ± 8.4	41 ± 3.8	50 ± 6.3	41 ± 2.0
IL-6	151 ± 45.0	205 ± 43.5	226 ± 16.8	201 ± 34.2
IL-10	217 ± 51.5	259 ± 44.0	267 ± 15.0	250 ± 28.5
TNF-α	124 ± 33.2	150 ± 27.6	226 ± 16.8	201 ± 34.0

Values are means ± SE; *n*, number of rats/group. Two-way ANOVA was performed for the sex and myocardial infarction (MI) interaction. In the amygdala, there was no MI effect for all cytokines. MI effect: IL-1α, $F = 0.02$, $P = 0.9$; IL-1β, $F = 0.5$, $P = 0.5$; IL-2, $F = 2.5$, $P = 0.1$; IL-6, $F = 0.0$, $P = 0.9$; IL-10, $F = 0.03$, $P = 0.9$; TNF-α, $F = 0.2$, $P = 0.7$.

OVX female rats. OVX with or without E_2 with sham MI caused variable nonsignificant changes in plasma cytokines compared with the sham OVX-sham MI group (Table 2.6). Post-MI, plasma IL-2, IL-6, and TNF-α increased significantly in the OVX and OVX-E_2 groups. The anti-inflammatory IL-10 also increased, the most being in the OVX-E_2-MI group. OVX with or without E_2 with sham MI did not affect cytokine levels in the PFC (Fig. 2.6). In the OVX-MI group, IL-2 and IL-6 increased significantly in the PFC. In contrast, in the PFC of the OVX-E2-MI group, IL-2 and anti-inflammatory IL-10 increased significantly, whereas TNF-α decreased markedly (Fig. 2.6).

Table 2.6. Plasma cytokine levels in ovariectomized female rats with or without E_2 replacement at 10 weeks post MI

Cytokine, pg/ml	Sham OVX-sham MI n=6	OVX-sham MI n=7	OVX-E2-sham MI n=7	OVX-MI n=10	OVX-E2-MI n=9
IL-1α	74 ± 9.8	70 ± 9.1	52 ± 11.3	82 ± 9.8	104 ± 19.9
IL-1β	144 ± 46.1	213 ± 27.5	399 ± 86.5	135 ± 20.3	207 ± 60.1
IL-2	123 ± 16.4	131 ± 65.4	82 ± 9.0	193 ± 27.3*	138 ± 9.8*
IL-6	479 ± 85.1	439 ± 109.3	357 ± 82.0	928 ± 173.6*	630± 54.2*
IL-10	107 ± 26.2	82 ± 19.6	33 ± 5.9	131 ± 24.2*	203 ± 34.9*†
TNF-α	213 ± 49.1	149 ± 39.7	76 ± 6.9	288 ± 53.8*	396 ± 64.4*

Values are means ±SE; *n*, number of rats/group. E_2, 17β-estradiol. One-way ANOVA was done to compare all groups, and two-way ANOVA was performed for the ovariectomy (OVX) and myocardial infarction (MI) interaction followed by a Bonferroni post hoc test. OVX x
/0 MI interaction for IL-10: F =5.4, P<0.027. MI effect: TNF-α, F=19.7, P< 0.0001; IL-6, F = 5.0 P <0.023; and IL-2, F=5.1, P< 0.033. There was no MI or OVX effect for IL-1α or IL-1β. *P <0.05 vs. the related sham MI group; †P <0.05 vs. the OVX-MI group.

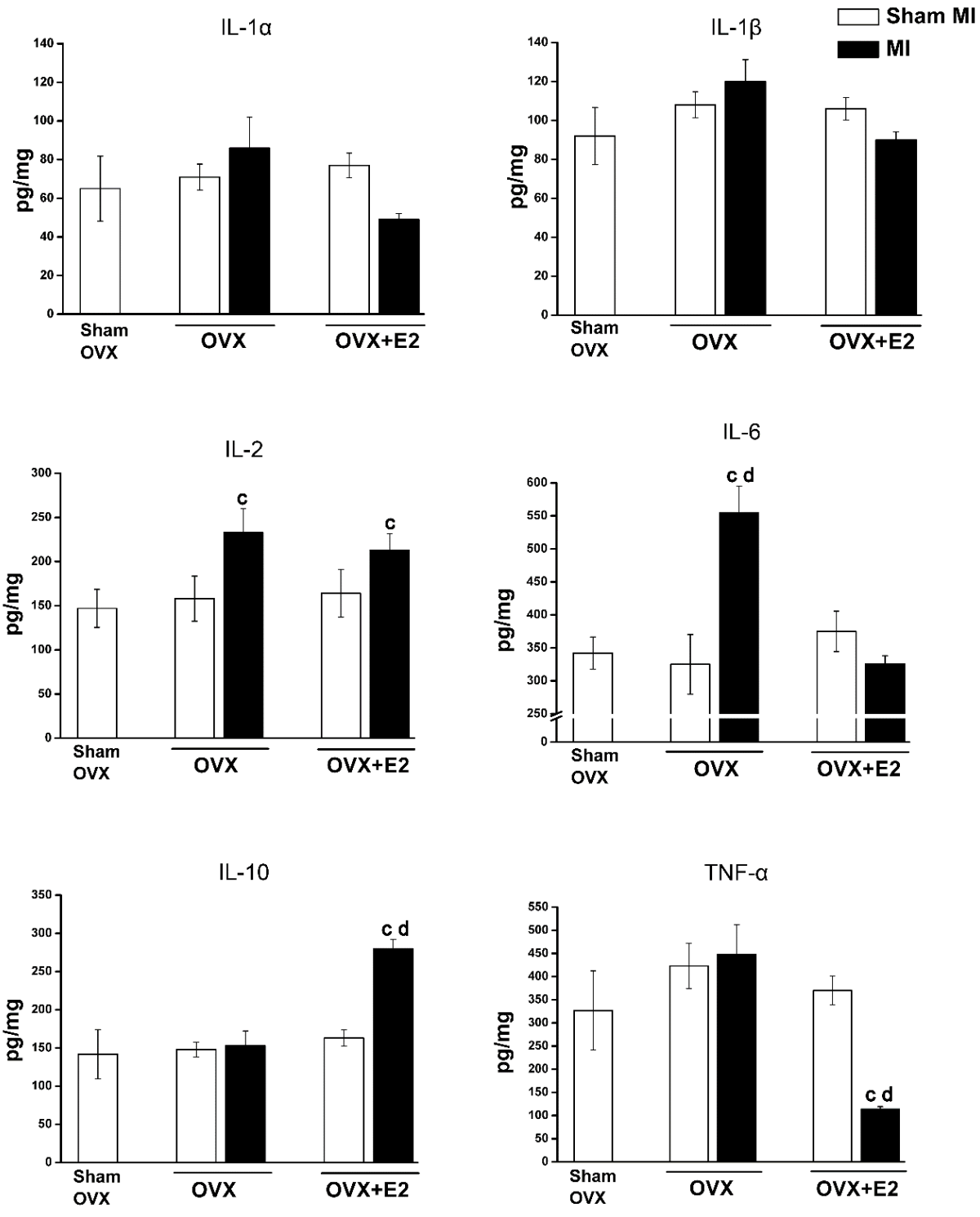
IL-1α
IL-1β
Sham MI
MI
IL-2
IL-6
IL-10
TNF-α
pg/mg
Sham OVX
OVX
OVX+E2
c
c d

Fig. 2.6. Cytokine levels in the prefrontal cortex (PFC) of ovariectomized (OVX) female rats with or without 17β-estradiol (E_2) replacement at 10 wk post myocardial infarction (post-MI). Values are means ± SE; n = 6–11 rats/group. One-way ANOVA was done to compare all groups, and two-way ANOVA was performed for the OVX and MI interaction followed by a Bonferroni post hoc test. OVX x MI interaction: IL-6, $F = 5.9$, $P = 0.024$; IL-10, $F = 12.5$, $P = 0.002$; and TNF-α, $F = 6.2$, $P = 0.02$. MI effect: IL-2, $F = 5.9$, $P = 0.023$. OVX effect: IL-10, $F = 16.7$, $P < 0.0001$; and TNF-α, $F = 12.8$, $P = 0.001$. $^{c}P < 0.05$ vs. the related sham MI group; $^{d}P < 0.05$ vs. OVX-E_2 groups.

BDNF Protein Levels

Male versus female rats. In the PFC, mBDNF protein expression had a nonsignificant trend (F = 2.8, P= 0.071) to decrease in the male post-MI group. The amygdala showed a significant MI and sex interaction (F = 5.4, P = 0.034), with mBDNF levels increasing significantly in male rats with HF post-MI compared with female rats with HF post-MI (Fig. 2.7.).

OVX female rats. OVX with or without E_2 did not affect mBDNF protein expression in the PFC of sham MI groups. In the OVX-MI group, mBDNF protein decreased significantly (F = 4.3, $P < 0.05$), which was prevented by E_2 replacement (Fig. 2.8.). In the amygdala, OVX did not effect mBDNF level, but E_2 replacement increased mBDNF level significantly (F = 5.5, P = 0.024). The OVX-MI group showed a significant increase in mBDNF level compared with their sham group (F = 4.7, P=0.03), but E_2 replacement did not cause a further increase in the mBDNF level.

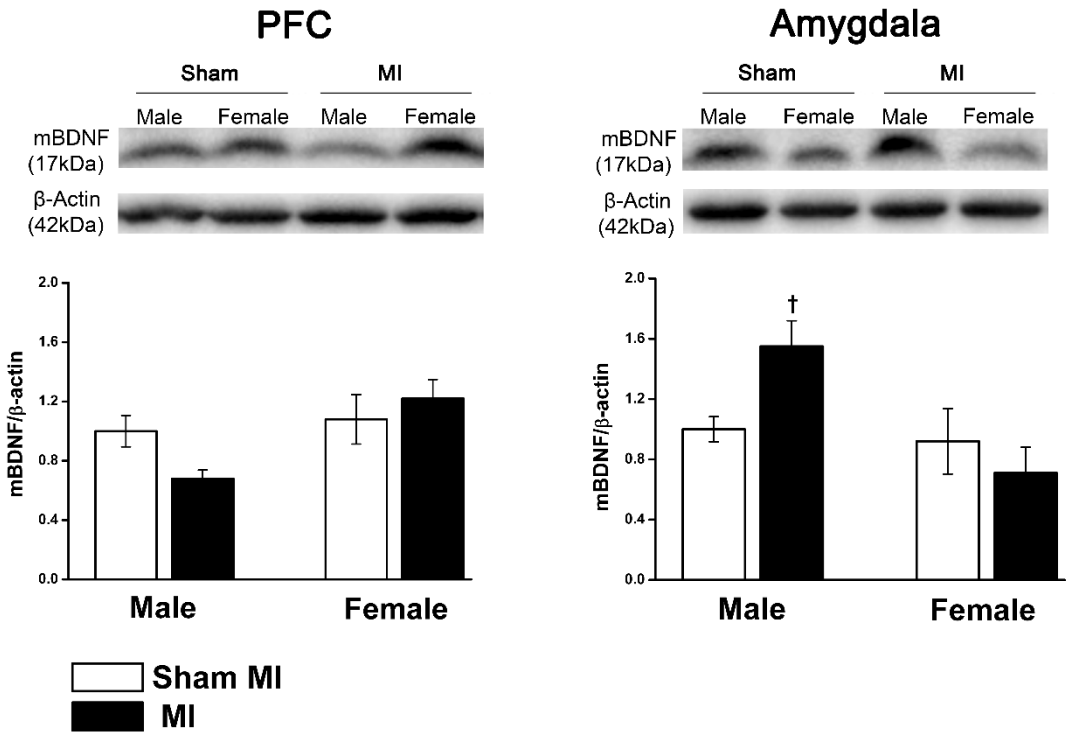

Fig. 2.7. Mature brain-derived neurotrophic factor (mBDNF) protein expression in the prefrontal cortex (PFC) and amygdala of male and female rats with heart failure at 10 wk post myocardial infarction (post-MI). Values are means ± SE; n = 4–7 rats/group. Values for mBDNF/β-actin in the male sham group were normalized to 1. Two-way ANOVA was performed for the MI x sex interaction (F = 5.4, P = 0.034, in the amygdala and F = 2.8, P = 0.071, in the PFC). †P <0.05 vs. the female MI group. Full blots were peer reviewed for validation.

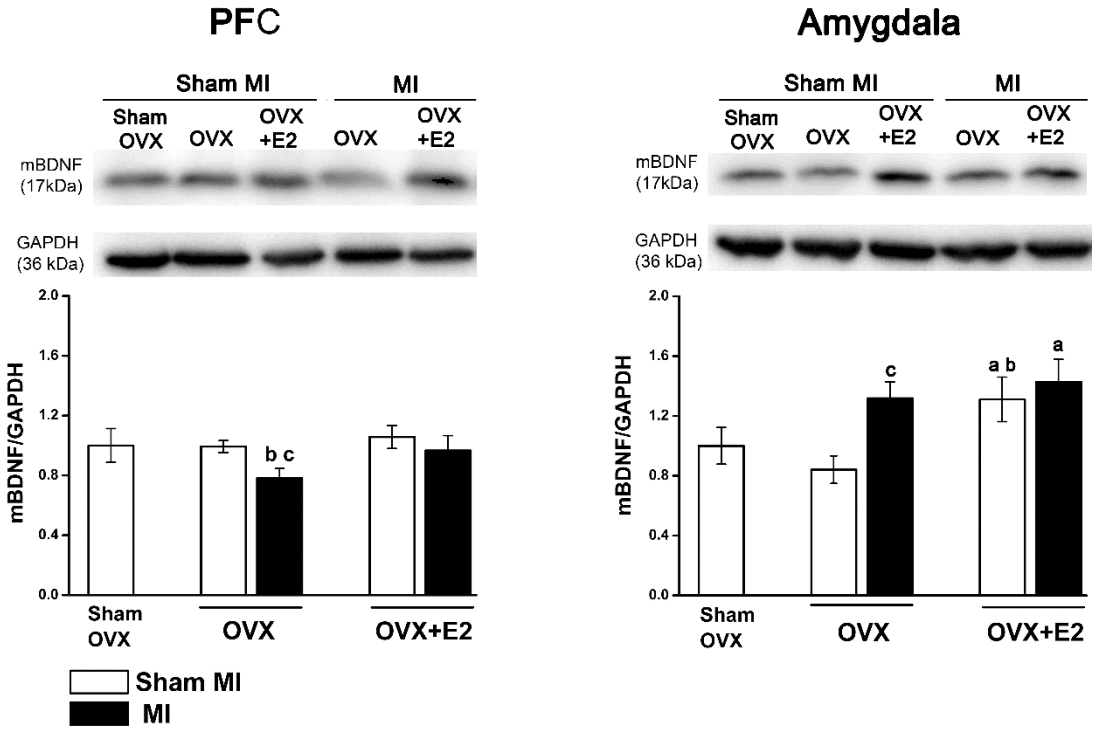

Fig. 2.8. Mature brain-derived neurotrophic factor (mBDNF) protein expression in the prefrontal cortex (PFC) and amygdala of ovariectomized (OVX) female rats with heart failure at 10 wk post myocardial infarction (post-MI). Values are means ± SE; *n*=6–13 rats/group. Values for mBDNF/GAPDH in the sham OVX-sham MI group were normalized to 1. One-way ANOVA was done to compare all groups, and two-way ANOVA was performed for the OVX and MI interaction followed by a Bonferroni post hoc test. In the PFC, MI effect: $F = 4.3$, $P = 0.046$. OVX effect: $F = 6.5$, $P = 0.016$. In the amygdala, MI effect: $F = 4.7$, $P = 0.038$. OVX effect: $F = 5.5$, $P = 0.024$. $^{a}P < 0.05$ vs. the sham OVX-sham MI group; $^{b}P < 0.05$ vs. the OVX-sham MI group; $^{c}P < 0.05$ vs. the related sham MI group. Full blots were peer reviewed for validation.

Discussion

Our findings demonstrate that sex and estrogens influence neuroinflammation and depression-like behavior in rats with HF post-MI. In contrast with male rats, female rats with HF did not exhibit depression-like behaviors, even though male and female rats had a similar degree of HF and MI size. OVX female rats displayed mild depression-like behavior that was significantly more pronounced in OVX MI rats and largely prevented by E_2 replacement. Plasma and PVN cytokine levels were likewise elevated in male and female rats with HF. PFC cytokine levels were higher in female versus male sham rats, and post-MI, they only increased in male rats. PFC cytokine levels were not affected by OVX per se, but after OVX, female rats post-MI also exhibited increases in PFC cytokines, which were changed to an anti-inflammatory pattern by E_2 replacement. These findings suggest that the rise of neuroinflammation in the PFC in male subjects post-MI may contribute to the development of depression-like behavior, whereas in female subjects, estrogens may protect against depression by inhibiting cytokine production and actions.

- **HF in Male and Female Rats Post-MI**

Male and female MI groups developed similar MI size (26% vs. 29% of the LV), associated with a similar clear increase in LV volumes, and showed a decrease in EF from ~90% to ~50% at 10 wk post-MI. In addition, LV end-diastolic pressure and RV weight increased and dP/d*t*max decreased similarly in male and female MI groups. Altogether, the magnitude of the cardiac dysfunction was not affected by sex. These findings support previous studies in male and female rats showing similar cardiac dysfunction at 4–6 wk post-MI (Litwin et al., 1999; Chen et al., 2011; Gouweleeuw et al., 2016). On the other hand, male mice from 4 to 12 wk post-MI had worse EF and larger LV dimensions compared with female mice with similar MI size

of ~30% (Cavasin et al., 2004). Two other studies in male and female mice with MI size of 30–40% showed an equivalent decline in cardiac function at 6 and 10 wk post-MI (Bridgman et al., 2005; Shioura et al., 2008). Collectively, these studies suggest that male and female rodents develop a similar degree of cardiac dysfunction for similar MI size. OVX rats with or without E_2 showed the expected pattern of changes in body weight and uterus weight but no significant changes in parameters of cardiac function in sham MI rats (Table 2.2). Consistent with a previous study (Hügel et al., 1999), cardiac function was similarly decreased in OVX-MI and OVX- E_2-MI rats.

- **Plasma and PVN Cytokines in Male and Female Rats Post-MI**

This is the first study to compare the extent of peripheral and central inflammation in male and female rats post-MI. Female rats with HF post-MI had a similar pattern of increase in plasma cytokines compared with male rats with HF post-MI. At 10 wk post-MI, plasma levels of several PICs were increased two- to threefold, associated with an increase in the anti-inflammatory cytokine IL-10. A more variable pattern of changes was noted in OVX-MI-rats, whereas increases in plasma IL-6 and IL-10 were actually larger in OVX- E_2-MI rats. Previous studies have shown that plasma IL-1β, IL-6, and TNF-α levels were significantly increased by two- to threefold in male rats at 4 wk post-MI (Kang et al., 2006, 2008, 2011; Guggilam et al., 2007). Cytokine levels increase in the acute phase after MI as the immune system responds to the cardiac injury (Nian et al., 2004). Male and female mice post-MI showed equivalent trafficking of neutrophils and macrophages to initiate the wound repair process after MI (Cavasin et al., 2004). In the chronic phase, cytokines can further increase (Irwin et al., 1999b) and thus contribute to myocyte apoptosis and the progression of cardiac remodeling and dysfunction. A variety of studies have shown that inhibition of inflammation can inhibit cardiac dysfunction, for example, treatment

of male mice with an IL-2 antibody for 14 days post-MI enhanced macrophage shifts from M1 to M2 and inhibited cardiac dysfunction (Zeng et al., 2016). Treatment of male rats with the TNF-α inhibitor etanercept for 6 wk post-MI inhibited progression in cardiac dysfunction (Berry et al., 2004). Treatment of male rats with the anti-inflammatory cytokine IL-10 for 4 wk post-MI decreased IL-6 and TNF-α levels in the LV and plasma and improved cardiac function (Stumpf et al., 2008). These studies, therefore, highlight the growing recognition about the importance of cytokines in the progression of HF post-MI. This is also the first study to show that female rats with HF post-MI develop inflammation in the PVN with 1.5- to 2-fold increases in the PICs IL-1α, IL-1β, IL-2, and TNF-α as well the anti-inflammatory IL-10, overall similar to the increases in male rats with HF post-MI. Microglia activation was observed by others in the PVN of male rats at 4 wk post-MI (Rana et al., 2010). Increases in plasma cytokines and ANG II may contribute to microglia activation, which then can produce local cytokines (Yu et al., 2018b), which may enhance ANG II-ANG II type 1 receptor signaling with a decrease in GABA levels and an increase in glutamate levels (Kang et al., 2008, 2011; Wei et al., 2014b). Central treatment with the cytokine synthesis inhibitor pentoxifylline decreased IL-1β and TNF-α levels in the PVN and attenuated cardiac decline post-MI (Kang et al., 2008). Similarly, SN50 (which inhibits nuclear translocation of NF-*k*B) administered centrally decreased IL-1β, IL-6, and TNF-α in the PVN and plasma, prevented an increase in plasma catecholamines, and was associated with less increase in LV end-diastolic pressure and less decline in EF in male rats post-MI (Kang et al., 2011). Altogether, the results suggest that in male and female rats, a similar increase of cytokines in the plasma and PVN is consistent with the similar cardiac dysfunction at 10 wk post-MI.

- **Depression-Like Behavior and Neuroinflammation in Male and Female Rats with HF Post-MI**

This is the first study to demonstrate a clear difference in depression-like behavior in male and female rats with HF post-MI. Male rats with HF post-MI displayed clear depression-like behavior, such as anhedonia assessed by sucrose preference and behavioral despair assessed by the FST. This finding is similar to previous studies in male rats with HF post-MI (Armony et al., 1998; Grippo et al., 2003; Wann et al., 2007; Bah et al., 2011a; Frey et al., 2014) and occurred in the absence of any difference between control female and male rats. Spatial memory as assessed by MWM testing was conserved in both male and female rats with HF post-MI, consistent with a study in male rats by Wann et al. (2007). Female rats had a slower learning rate in the MWM, which is consistent with previous MWM studies that compared male and female rats (Kokras and Dalla, 2014). HF post-MI had no effect on acquisition of fear conditioning in either sex group. In contrast, in the cued fear conditioning, male, but not female, rats with HF post-MI froze significantly more, suggesting that male rats with HF post-MI exhibit an enhanced adaptive response to stressful events (Beckers et al., 2013). Taken together, male, but not female, rats with HF post-MI develop depression-like behavior.

Depression-like behavior has been linked to cytokines and neuroinflammation (Miller et al., 2009; Felger and Lotrich, 2013; Xu et al., 2015). Cytokines may contribute to depression-like behavior by several mechanisms, such as a decrease in the expression of BDNF through activating microglia and astrocytes, activating the IDO enzyme, which decreases the concentration of serotonin, and using tetrahydrobiopterin for nitric oxide synthesis, which is essential for dopamine production (Miller et al., 2009; Felger and Lotrich, 2013). Indeed, treatment of male

rats with pentoxifylline for 14 days alleviated anhedonia and immobility in the FST at 14 days post-MI (Bah et al., 2011b). Similarly, treatment of male rats with etanercept for 7 days post-MI prevented anhedonia (Grippo et al., 2003; Johnson, 2006). Consistent with a previous study in male rats (Ter Horst, 1999), in the present study, the PICs IL-1β, IL-2, IL-6, and TNF-α, but also IL-10, increased by 1.5-fold in the PFC of male rats with HF but not in the PFC of female rats with HF. The increase of PICs in the PFC of male rats was associated with a trend for reduction in mBDNF only in male rats with HF, similar to a previous study in male rats (Kaloustian et al., 2008). However, cytokine levels were higher in female versus male sham rats and increased in male rats with HF, largely only to the levels of female control rats. If PICs in the PFC indeed contribute to depression-like behavior, these findings may suggest that female rats are protected against cytokine-induced depression-like behavior by, e.g., estrogens inhibiting downstream responses to cytokines as well as by inhibiting further increases in cytokines post-MI (see *Depression-Like Behavior in OVX Female Rats Post-MI* below). The high densities of estrogen receptor-α in the PFC compared with the PVN (Laflamme et al., 1998; Pérez et al., 2003) may play a role in this regard.

Finally, the amygdala revealed no significant changes in PICs or IL-10 in either sex group at 10 wk post-MI. Only male rats with HF showed an increase in cue-associated freezing, which is dependent on the amygdala (Armony et al., 1998), and an increase in mBDNF in the amygdala. An increase of mBDNF levels in the amygdala was also found in chronic stressed rats (McEwen et al., 2016). Patients with depression showed an increase in the activity of the amygdala (Yu and Chen, 2011). High levels of BDNF in the amygdala might contribute to hyperexcitability of the fear

circuit in the FCT (Rosen and Schulkin, 1998) and enhance the expression of fear (Ou and Gean, 2006).

- **Depression-Like Behavior in OVX Female Rats Post-MI**

Consistent with previous studies (Kiss et al., 2012; Borrow and Cameron, 2014; Xu et al., 2015), after OVX, female rats developed depression-like behavior with a modest decrease in sucrose preference and an increase in immobility in the FST as well as a marked increase in context and cue associated freezing in the FCT, whereas general locomotor activity was not affected. As expected (Xu et al., 2015), these behavior changes were largely prevented by E_2 replacement, indicating that the adverse effects of OVX indeed mainly depend on estrogen deficiency. Enhanced cue-associated freezing was fully prevented by E_2, but context-associated freezing was only partially prevented by E_2, suggesting possible different effects of E_2 in the amygdala versus the hippocampus (Phillips and LeDoux, 1992; Barha et al., 2010). Similar to the findings by Xu et al. (2015), OVX alone or with E_2 replacement did not affect cytokine levels, suggesting that an increase in neuroinflammation per se in the PFC does not contribute to the depression-like behavior by estrogen deficiency. Consistent with this finding, mBDNF levels in the PFC were not affected by OVX alone or with E_2 replacement, similar to a previous study (Luine and Frankfurt, 2013). In contrast, E_2 replacement at physiological levels increased mBDNF levels in the whole amygdala. Altogether, OVX-induced estrogen deficiency may remove a protective effect against cytokine-induced actions on, e.g., IDO enzyme and serotonin production (Xu et al., 2015; Miller and Raison, 2016) or on BDNF levels (Duman, 2004; Sohrabji and Lewis, 2006; Şahin et al., 2015).

The present study is the first study to demonstrate the marked protection provided by the ovaries and estrogens against depression-like behavior in female rats

post-MI. In contrast with intact female rats, OVX rats developed a marked decrease in sucrose preference and an increase in immobility in the FST post-MI, as similarly noted in intact male rats post-MI. Freezing behavior on the cued fear conditioning day of the FCT was markedly increased by MI in OVX rats, reaching ~80% versus ~50% in male rats post-MI. This finding may indicate a further activation of the fear circuit in the amygdala. Estrogen replacement prevented all of the OVX-MI-induced changes in behavior. This finding highlights the potent inhibitory effects of estrogens on mechanisms contributing to depression-like behavior in female rats with HF post-MI. In this regard, we evaluated the interaction of OVX, MI, and E_2 replacement for cytokine levels in the PFC. In contrast with no changes in intact female rats post-MI, after OVX-MI, PICs IL-2 and IL-6 significantly increased, and TNF-α tended to increase in the PFC. E_2 replacement shifted this pattern toward an anti-inflammatory one with a twofold increase in IL-10, possibly preventing an increase in IL-6 and causing a marked decrease in TNF-α (Stumpf et al., 2008). Consistent with the cytokines, mBDNF decreased in the PFC of the OVX-MI group, comparable to the changes in male rats post-MI. E_2 replacement prevents this decrease of mBDNF in the PFC. Further studies with cytokine synthesis inhibitors, such as pentoxifylline (Bah et al., 2011b), or with a specific cytokine inhibitor, such as etanercept (Grippo et al., 2003; Johnson, 2006), are needed to determine the actual role of these cytokine changes for inducing depression-like behavior and the protective actions of estrogens in the setting of HF post-MI. Further studies are also needed to assess whether these same mechanisms play a role in psychological distress-induced depression. In conclusion, in contrast with male rats, female rats with HF post-MI do not develop depression-like behavior; neither exhibit increases in cytokines in the PFC. However, female OVX-MI rats also develop marked depression-like behaviors that are

prevented by E_2 replacement, possibly by inhibiting production and actions of proinflammatory cytokines. Understanding the mechanisms contributing to these sex-specific and estrogen-dependent responses may contribute to new therapeutic strategies that may be sex specific (Regitz-Zagrosek and Kararigas, 2017).

GRANTS

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Chapter 3:

Role of Myocardial infarction – induced Neuroinflammation for Depression-Like Behavior and Heart Failure in Ovariectomized female rats

Role of Myocardial infarction – induced Neuroinflammation for Depression-Like Behavior and Heart Failure in Ovariectomized female rats

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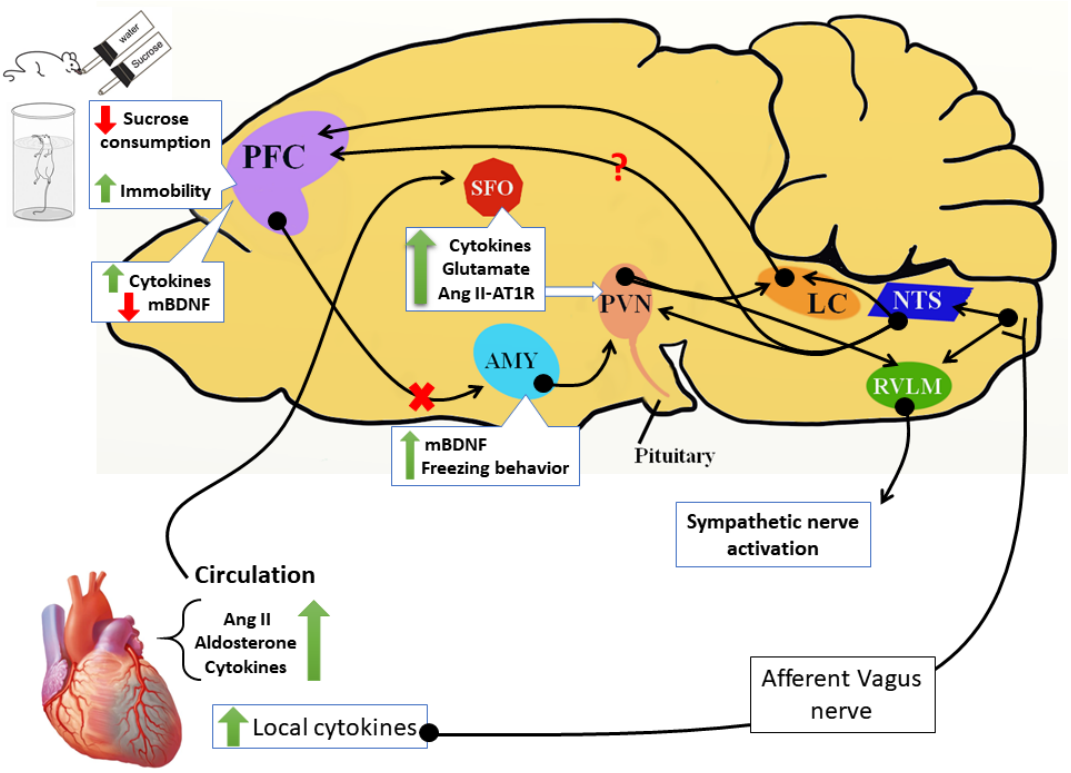
Short Title: Depression in Ovariectomized female rats with HF post-MI

KEY WORDS: Depression, Heart Failure, Neuroinflammation, Cytokines, Ovariectomy

ABSTRACT

After myocardial infarction (MI), ovariectomized (OVX) female rats develop depression-like behaviors and an increase of pro-inflammatory cytokine (PIC) levels in the prefrontal cortex (PFC). We hypothesized that inhibition of neuroinflammation by the PIC synthesis inhibitor, pentoxifylline (PTX) would prevent depression-like behaviors induced by heart failure (HF) post-MI in OVX female rats. PTX treatment was initiated in female Wistar rats, one week after ovariectomy, and one week before MI by occlusion of the left anterior descending artery. Eight weeks post-MI, OVX female rats treated with vehicle or PTX exhibited a similar MI size and degree of cardiac dysfunction. OVX female rats post-MI developed depression-like behaviors consisting of anhedonia, despair behavior and enhanced freezing behavior in the cued conditioning test. PTX prevented the depression-like behavior symptoms and enhanced freezing. Cytokine levels were elevated in plasma and both paraventricular nucleus (PVN) and PFC, and the mature brain-derived neurotrophic factor (mBDNF) was decreased in the PFC of OVX female rats post-MI. PTX treatment limited the decrease of mBDNF, and decreased cytokine levels in plasma, PVN and PFC to (below) sham levels. These findings show that OVX female rats post-MI exhibit an increase in both peripheral and central inflammation. PTX treatment prevents increases in PIC levels in plasma and PVN but does not attenuate the progression of cardiac dysfunction. In contrast, PTX prevents enhanced PIC production in the PFC, as well as limits depression-like behaviors induced by MI in OVX female rats.

GRAPHICAL ABSTRACT



The increase in plasma cytokines, Ang II and aldosterone post-MI can induce neuroinflammation in CVOs such as the SFO that in turn increases local production of cytokines, reactive oxygen species, and Ang II-AT1R signalling in the PVN leading through the RVLM to increase of sympathetic nerve activity. PVN, NTS and RVLM projections increases LC activity which increases norepinephrine release in the PFC, stimulating microglia activity inducing local cytokine production and decreasing mBDNF levels. PFC activation may disinhibit amygdala and increase mBDNF levels and freezing behavior. Peripheral cytokines also stimulate the afferent vagus nerve that projects to dorsal complex and NTS through activating catecholamine neurons in the NTS and RVLM leads to increase CRF release in the PVN and activation of HPA axis

Abbreviations Ang II: angiotensin II; AT1R: angiotensin II type 1 receptor; CRF: corticotropin-releasing factor; CVO: circumventricular organs; HPA: hypothalamic-pituitary-adrenal; LC: locus coeruleus; mBDNF: mature brain-derived neurotrophic factor; MI: myocardial infarction; NTS: nucleus tractus solitarius; PFC: prefrontal cortex; PVN: paraventricular nucleus; RVLM: rostral ventrolateral medulla; SFO: subfornical organ.

HIGHLIGHTS

- OVX female rats post-MI develop depression-like behavior with increase in cytokines in CNS
- PTX prevents neuroinflammation and prevents the behavioral changes after MI
- PTX had no impact on the progression of cardiac dysfunction in OVX female rats post-MI

INTRODUCTION

Patients with cardiovascular disease such as heart failure (HF) have a high prevalence of depression (Rutledge et al., 2006), and those with depression have a worse outcome (Rutledge et al., 2006; Huffman et al., 2013; Moraska et al., 2013). Older women with HF are at a higher risk of developing depression and have a worse prognosis than men (Gottlieb et al., 2004; Wassertheil-Smoller et al., 2004; Rutledge et al., 2006; Wright et al., 2014). A myriad of psycho-social factors play an important role in this adverse interaction between cardiac and mental disease, particularly in women. In addition, biological factors have been identified to play a major role, in particular, enhanced systemic and brain inflammation which occurs in patients with HF and depression (Deswal et al., 2001; Liu et al., 2012), and in animal models of HF which develop depression-like behaviors (Wann et al., 2009; Bah et al., 2011b; Najjar et al., 2018).

On the other hand, studies in humans and animals suggest that estrogens exert a strong protective effect against depression. Postmenopausal women have a higher incidence of depression, which can be alleviated by hormone replacement therapy (Wise et al., 2008; Toffol et al., 2015). Similar changes occur after ovariectomy (OVX) in rodents (Borrow and Cameron, 2014; Park et al., 2014; Najjar et al., 2018). Recently we reported that adult male rats with HF post-MI develop depression-like behavior (Najjar et al., 2018), consistent with several studies (Grippo and Johnson, 2002; Wann et al., 2009; Bah et al., 2011b), associated with significant increases in cytokines in the plasma, paraventricular nuclei (PVN) in the hypothalamus and the medial prefrontal cortex (PFC). In contrast, female rats with the same degree of HF post-MI showed a similar increase in cytokines in the PVN, but none in the PFC and did not develop depression-like behavior. However, they did after OVX. Estrogen

replacement prevented both cytokines activation in the PFC and depression-like behavior (Najjar et al., 2018). Altogether, these findings suggest that ovariectomy and the resulting low estrogen levels enables an increase in cytokines levels in the PFC in female rats post-MI and thereby the development of depression-like behavior. In the present study, we tested this hypothesis by inhibiting neuroinflammation using pentoxifylline (PTX). PTX is a methylxanthine derivative, that acts as a competitive nonselective phosphodiesterase inhibitor which raises intracellular cAMP, and reduces the phagocytic activity of macrophages and lysosomal enzyme released by polymorphonuclear cells (D'Hellencourt et al., 1996). PTX blocks nuclear factor kappa-B-induced mRNA transcription of IL-1α, IL-1β, TNF-α, and IFN-ɣ (Windmeier and Gressner, 1997; Zhang et al., 2004). In small clinical trials in -mainly male- patients with HF, PTX treatment decreased circulating TNF- α and CRP, and improved functional status and ejection fraction (Sliwa et al., 1998, 2004; Skudicky et al., 2000, 2001). Several pre-clinical studies using only male rats, also reported that PTX can reduce inflammation and improve HF. For example, treatment with PTX subcutaneously for three weeks in chronically stressed male rats prevented anhedonia and decreased the kynurenine/serotonin ratio in the PFC, and plasma TNF-α (Mohamed et al., 2013). Five to six weeks of oral or IP PTX treatment decreased IL-1β, IL-6, and TNF-α levels in plasma and PVN of male rats post-MI, lowered plasma NE and improved cardiac dysfunction (Kang et al., 2006; Guggilam et al., 2007). Similarly, central infusion of PTX in male rats post-MI for four weeks decreased IL-1β, IL-6 and TNF-α levels in the PVN, and plasma Ang II and NE levels associated with less progression of cardiac dysfunction (Kang et al., 2008, 2010). PTX treatment also prevented depression-like behavior and the increase in plasma IL-1β in male rats two weeks after cardiac ischemia-perfusion (Bah et al., 2011b). Whether PTX would

have similar beneficial effects in female rats, has not yet been assessed. We hypothesized that PTX treatment of OVX female rats post-MI also would inhibit neuroinflammation and thereby prevent depression-like behavior and improve HF. To test this hypothesis, after OVX, female rats underwent MI or sham MI surgery and were treated with oral PTX or vehicle for ten weeks.

MATERIALS AND METHODS

Experimental animals

All experimental procedures conform to the *Guideline for the Care and the Use of Laboratory Animals* published by the US National Institutes of Health and were approved by the University of Ottawa Animal Care Committee. Nine to eleven weeks old female Wistar rats (Charles River, Montréal, QC, Canada) weighing 200-250g were housed in pairs at controlled temperature and humidity on a 12:12 light-dark reverse cycle, with the dark cycle starting at 3:00 AM. They were provided with standard chow and tap water *ad libitum*.

Experimental groups and design

After acclimatization for 5-7 days, female rats underwent OVX and then housed individually until the end of the study. Two weeks after OVX surgery, rats were randomized to either MI or sham MI surgery. One week before the MI surgery, treatment with PTX (Sigma, Oakville, ON, Canada) or vehicle was started at 30mg/kg daily by spatulation with 1.5 g peanut butter base, pasted on the side of the cage, and continued for ten weeks post-MI. Treatment of PTX with this dose in male rats with HF decreased IL-1β, TNF-α, and cyclooxygenase-2 (COX-2) levels in the PVN associated with a decrease in plasma IL-1β and TNF-α levels (Kang et al., 2006). The MI surgeries were performed blinded for the treatment allocation.

Rats were randomized into four groups: 1) OVX-sham MI +Veh (n=6), 2) OVX-sham MI+PTX (n=10), 3) OVX- MI+ Veh (n=20), 4) OVX- MI +PTX (n=20). In the OVX-MI+Veh group, 11 rats survived the MI surgery, and 4 were excluded due to MI size < 20 % of the LV. In the OVX-MI+PTX group, 13 rats survived the MI surgery, and 3 rats were excluded due to MI size <20 % of the LV. A sham OVX group was not included, because the inflammatory response to such surgery is short-

lived (eg(Hovens et al., 2014, 2016), and limited time to study the behaviour of more animals during the dark cycle.

Behavioral tests were carried out from 7 to 9 weeks after MI or sham MI surgery between 7:00 AM - 2:00 PM. In week ten, echocardiographic and hemodynamic parameters were measured.

Surgical procedures

One hour before surgeries, rats were premedicated with slow release buprenorphine (1mg/kg subcutaneous) (Chiron Compounding Pharmacy Inc., Guelph, ON, Canada). After induction of anesthesia with 2% isoflurane, rats were intubated, and after opening the pericardium via thoracotomy, the left anterior descending coronary artery (LAD) permanently ligated. Sham rats had similar surgery without LAD ligation.

Bilateral ovariectomies were performed under 2% isoflurane anesthesia, by a longitudinal incision on each flank to remove the ovaries.

Behavior tests

All behavioral tests were carried out during the dark cycle except the sucrose preference test which assesses the sucrose consumption over 24 hours. All behavior studies were recorded by a digital camera linked to an Ethovision 11.5xT video Track system (Noldus Information Technology, Leesburg, VA, USA). During testing for different types of behavior such as freezing, swimming and immobility, rats were exposed to dim lights to enable precise detection of specific behavior. Both hand score and software Ethovision V11.5 were used to score freezing behavior for the fear conditioning test and immobility for forced swim test. The correlation between the

two methods was >90%, and the presented results are the ones calculated by the software.

The sequence of the behavior tests was as follows: Sucrose preference test (SPT), Morris water maze test (MWM), forced swim test (FST), and fear conditioning test (FCT). Each test was separated by at least 24 hours from the following test. Details and references for each of the tests were recently reported (Najjar et al., 2018), and briefly summarized here:

Tests to assess depression-like behavior

SPT is used to evaluate anhedonia (reduced motivation or ability to experience pleasure). Rats were habituated to the presence of two bottles, one with 2% sucrose and the other one with tap water for three days. Thereafter 1% sucrose and tap water were used to assess sucrose preference for seven days. Bottles were daily switched to avoid location preference.

FST is used to assess despair behavior. Each rat was placed in a transparent cylinder filled with 10 liters of tap water at 25±2°C for 10 min. Immobility was identified as the lack of all movement except to keep floating.

Tests to assess learning and memory

MWM: The rat is randomly placed into one of four locations in the water maze and uses external visual cues to learn the location of the hidden platform. During training to learn the platform location, the rats were allowed to swim for one min to reach the hidden platform. If the rat failed to reach the platform, it was guided to it and allowed to stand for 15 sec and then removed from the maze. Each training session consisted of four trials on the first day, and three on the following three days, with two sessions/day separated by one hr. Spatial memory was assessed by a probe

trial on day three and day five, for which the hidden platform was removed. For each probe trial, the rat was placed in the water maze to swim for one min and the time was measured that the rat spent in the target quadrant where the platform was located.

FCT consists of three consecutive days of testing. Day one is to acquire fear behavior, starting with 30 sec tone as the conditioned stimulus (CS) combined with two sec unconditioned stimulus (US) shock 0.5mA at the end of 30 sec and perfromed in two cycles separated by one min apart. Day two is the contextual conditioning test to assess associative memory by evaluating freezing in the same environment as day one. On day three, the cued conditioning test assesses the associative memory to the CS. Three minutes of habituation to the new environment (pre-tone) were followed by the same CS tone for three min. Freezing behavior is presented for the first two min on day one, 6 min on day two, and 2x3 min on day three.

Echocardiography and hemodynamic measurements

A Visual Sonics Vevo 770 System (Visual Sonics, Toronto, ON, Canada) was used to measure cardiac diameters and ejection fraction (EF) under 2% isoflurane anesthesia. A Millar catheter was then placed in the left ventricle (LV) to measure LV peak-systolic and end-diastolic pressures, and maximum/minimum rate of pressure change in the LV (dP/dt_{max} and dP/dt_{min}) for 30- 60 s.

Tissue collection

Three hours after hemodynamic measurements were obtained, trunk blood was collected into pre-chilled polyethylene tubes containing EDTA, and centrifuged at 3000 rpm for 30 min at 4°C to obtain plasma for cytokine levels. Hearts were removed and kept in cold saline, followed by separation of the right ventricle (RV). The LV was longitudinally dissected from the septum, and 2-3 cuts performed on the

apex to flatten the LV. The area of LV infarction was measured by planimetry, and the infarct size expressed as a percent of the LV area. The brain was removed, and snap-frozen in methyl butane at -20° C. All tissues and plasma were kept at -80°C until further analysis.

Cytokine assays

Cytokines (IL-1α, IL-1β, IL-2, IL-6, IL-10, and TNF-α) were measured by using multi-plexed beads-based immunoassay set with cytokines reagent kit (171304070M; Bio-Rad, Mississauga, ON Canada). PFC and PVN samples were collected by brain punches, as previously described (Najjar et al., 2018). Plasma and brain samples were centrifuged at 13,000g for 15 min at 4°C, 1:4 diluted plasma and brain tissue samples with 0.5mg/mL protein were used to interact with the conjugated beads for 1.5 hr at room temperature (RT). This was followed by incubation with biotinylated antibodies for one hr at RT to interact with cytokine of interest. The mixture was incubated with streptavidin-phycoerythrin for 10 min at RT. Lastly, the Bioplex Protein Array System and related software Bio-Plex Manager version 5.6 were used to determine the levels of cytokines. Plasma levels are shown as pg/mL and brain levels as pg/mg.

Western Blot Analysis

20 µg of total protein from the PFC was separated by 12% SDS-PAGE gel for immunoblotting of mature BDNF (mBDNF), and then transferred on to PVDF membranes (Bio-Rad, Mississauga, ON, Canada). The blots were blocked for 1 hr at RT in 5% milk in Tris-buffered saline with 0.1% Tween-20 (TBST), cut below 37 kDa, and probed with rabbit monoclonal anti-BDNF (1:3000, Ab108319; Abcam Inc., Toronto, ON, Canada) at 4° C overnight. Then the blots were incubated with goat anti-rabbit IgG-HRP(1:5000, 111-035-144; Jackson ImmunoResearch, West Grove,

PA, USA) for 1 hr at RT. After washing the blot was developed with Luminata ECL reagent (Millipore Sigma, Etobicoke, ON, Canada) and visualized with ChemiDoc XRS+ imager(Bio-Rad). The top part of the blot was probed for GAPDH. The expression of the mBDNF protein was calculated as the ratio of its band density relative to the density of the protein loading control GAPDH in the same sample.

Statistical analysis

The data followed a normal distribution, as assessed by Wilks' Lambda test and presented as mean ± SE in Tables, and in most figures as mean ± 95% confidence interval (CI) with the individual data points (Krzywinski and Altman, 2014). The MI x PTX interaction was assessed by two-way ANOVA, followed by Bonferroni *post hoc test*. Two-way repeated measures ANOVA was used to compare freezing behavior in the context and cued tests of the FCT compared to their baseline, and spatial learning during training days in the MWM, followed by Bonferroni *post hoc test*. Correlation analyses for cytokines were performed by Pearson correlation analysis. $P<0.05$ was considered statistically significant.

RESULTS

Cardiac function

OVX caused the expected increase in body weight and a remarkable decrease in uterus weight. MI did not affect these weights. OVX-MI+Veh and OVX-MI+PTX rats developed similar MI size, and developed a marked increase in LVESV and LVEDV and decreases in cardiac index and EF. Hemodynamic measurements showed significant decreases in LVPSP, dP/dt_{max} and dP/dt_{min}, and an increase in LVEDP. PTX did not significantly affect any of these parameters of cardiac dysfunction (Table 3.1).

Table 3.1. Anatomical, echocardiographic and hemodynamic measurements in ovariectomized female rats with or without PTX at 10 weeks post-MI

Groups	**OVX-Sham MI +Veh n=6**	**OVX-Sham MI+PTX n=10**	**OVX-MI+Veh n=7**	**OVX-MI+PTX n=10**
Weight (BW)(g)	483 ± 16.7[a]	472 ± 9.8	430 ± 11.8	475 ±16.3
Uterus (mg/100g)	32 ± 2.4[b]	31 ± 1.5	37 ± 1.7	34± 2.2
MI size%	None	None	28 ±2.2	27 ± 1.5
LV/BW (mg/100g)	150 ± 1.7	165 ± 5.2	180 ± 4.8*	166 ± 6.7
RV/BW (mg/100g)	31 ± 0.9	36 ± 1.1	40 ± 5.7	37 ± 2.7
Echocardiographic parameters				
LVESV (µL/100gBW)	9 ± 1.2	8 ± 1.0	40 ± 4.8*	41 ± 6.5*
LVEDV (µL/100gBW)	56 ± 9.8	55 ± 3.9	75 ± 7.5*	69 ± 8.4
CI (ml/min/kg)	182 ±18.3	174 ± 11.1	128 ± 16.9*	127 ± 17.2*
EF (%)	82 ± 2.7	85 ± 0.9	45 ± 3.1*	46 ± 2.1*
Hemodynamic parameters				
LVPSP (mmHg)	132 ± 3.4	131 ± 3.4	114 ± 6.5*	113 ± 5.8*
LVEDP (mmHg)	4 ± 0.3	2.6 ± 0.2	11 ± 1.2*	12 ± 1.1*
LV dP/dt (+) (mmHg/s)	8708 ± 406	8120 ± 192	6545 ± 426*	6040 ± 332*
LV dP/dt (-) (mmHg/s)	7586 ± 352	6886 ± 209	5280 ± 465*	5102 ± 384*

[a] Body weight of sham OVX rats of the same age is in the 360 g range, and [b] uterus weight in the 160 mg/100g range (Najjar et al, 2018).

Values are mean ±SEM. Two-way ANOVA was done for MI and PTX interaction followed by Bonferroni *post hoc test*. LV/BW, MI effect, F=5.9, P=0.02, PTX effect, F=0.1, NS, MI x PTX interaction, F=2.6, NS. RV/BW, MI effect, F=4.1, P=0.05, PTX effect, F=0.2, NS, MI x PTX interaction, F=0.8, NS. LVESV, MI effect, F=47, P<0.001; PTX effect, F=0.01, NS, MI x PTX interaction, F=0.02, NS. LVEDV, MI effect, F=5.3, P<0.05, PTX effect. F=2.2, NS, MI x PTX interaction, F=0.7, NS. CI, MI effect, F=269, P<0.001, PTX effect, F=0.4, NS, MI x PTX interaction, F=0.3, NS. EF, MI effect, F=12, P<0.001. PTX effect, F=0.9, NS, MI x PTX interaction, F=0.8, NS. LVPSP, MI effect, F=11, P<0.01, PTX effect, F=001, NS, MI x PTX interaction, F=0.02, NS. LVEDP, MI effect, F=103, P<0.001, PTX effect, F=0.08, NS, MI x PTX interaction, F=1.5, NS, dP/dt(+), MI effect, F=40, P<0.001, PTX effect, F=1.8, NS, MI x PTX interaction, F=0.18, NS. dP/dt(-), MI effect, F=31, P<0.001, PTX effect, F=1.1, NS, MI x PTX interaction, F=0.9, NS. $^{*}P<0.05$ versus OVX- sham MI groups

ABBREVIATIONS: BW: body weight; LV: left ventricle; RV: right ventricle; LVESV: LV end systolic volume; LVEDV: LV end diastolic volume; CI: Cardiac Index; EF: ejection fraction; LVPSP: LV peak systolic pressure; LVEDP: LV end diastolic pressure; LV dP/dt(-): minimum rate of rise of LV pressure; LV dP/dt(+): maximum rate of rise of LV pressure.

Depression-like Behavior

Sucrose Preference test:

The OVX-MI+Veh group demonstrated a decrease in sucrose intake by 17% compared to OVX-sham MI+Veh group, which was prevented by PTX treatment (Fig 3.1A). All OVX groups showed a similar total liquid intake (Fig 3.1B).

Forced Swim test:

The OVX-MI+Veh displayed a significant increase in immobility compared to the OVX-sham MI+Veh group. PTX treatment decreased the despair behavior induced by OVX per se, as well as by OVX+MI (Fig 3.2.).

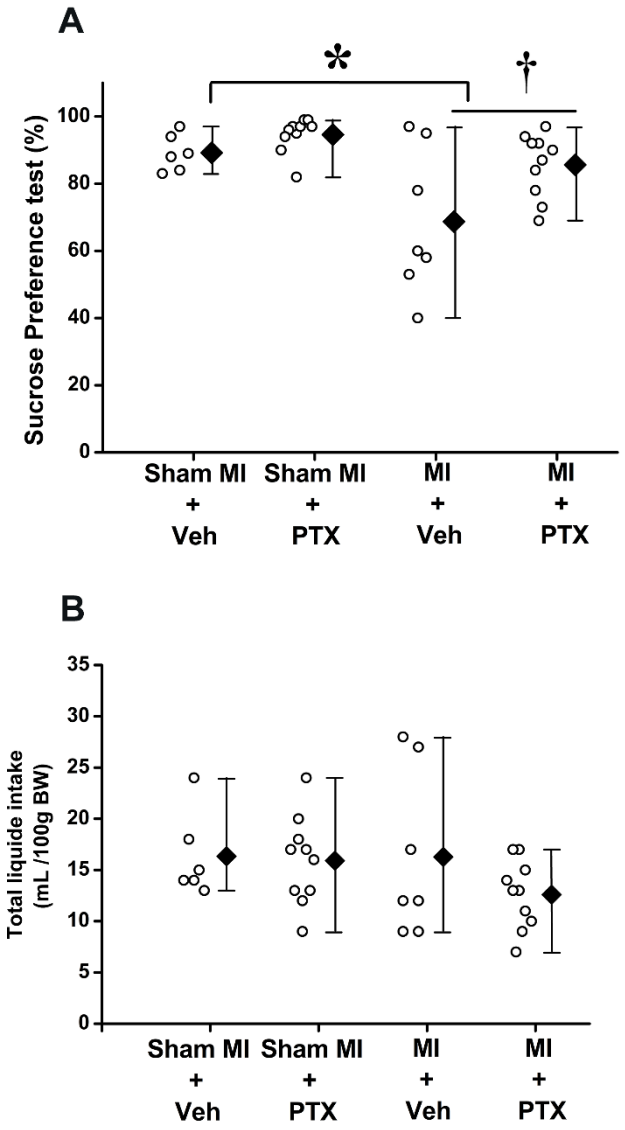


Figure 3.1. Percent sucrose intake and total liquid intake during the last three days of the Sucrose preference test. Values are mean ±95% CI (n=6-10/ group). Two-way ANOVA for PTX x MI interactions, followed by Bonferroni *post hoc test*. (**A**) Effects of PTX and MI on sucrose preference in OVX female rats. MI effect, F=12.2, P=0.002, PTX effect, F=7.0, P=0.013, MI x PTX interaction, F=1.8, NS. (**B**) Total liquid intake. There was no significant difference between groups.

* P <0.05 vs OVX-Sham MI +Veh, † P <0.05 vs OVX-MI+Veh

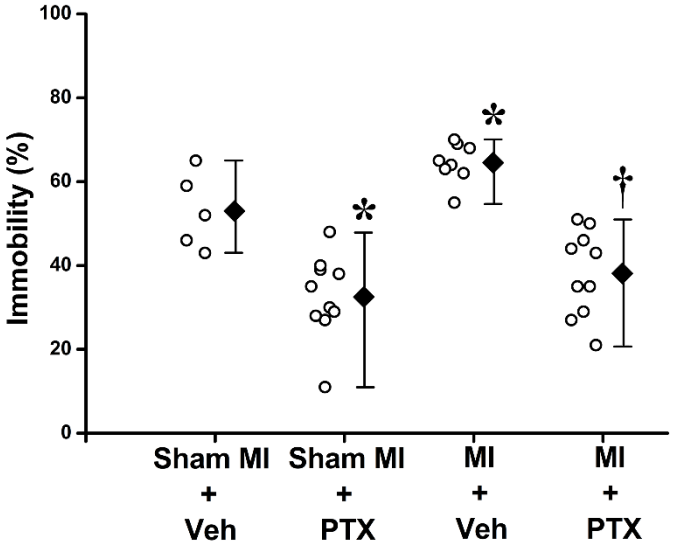


Figure 3.2. Percent immobility in the forced swim test. Values are mean ±95% CI (n=5-10/group). Two-way ANOVA for MI x PTX interaction, followed by Bonferroni *post hoc test*. MI effect: F=6.8, P =0.014, PTX effect: F=51.8, P <0.001, MI x PTX interaction, F=0.8, NS.

* P <0.05 vs OVX-Sham MI +Veh, † P <0.05 vs OVX-MI+Veh

Learning and Memory

Morris Water Maze:

All groups demonstrated a similar pattern of spatial learning, and decrease in the latency time to reach the hidden platform during the four days of training (Fig 3.3.A). MI groups showed a lower ($P<0.05$) spatial memory in probe 1 and 2 (Fig 3.3.B). All groups exhibited a comparable locomotor activity on the first day of training (Table 3.2).

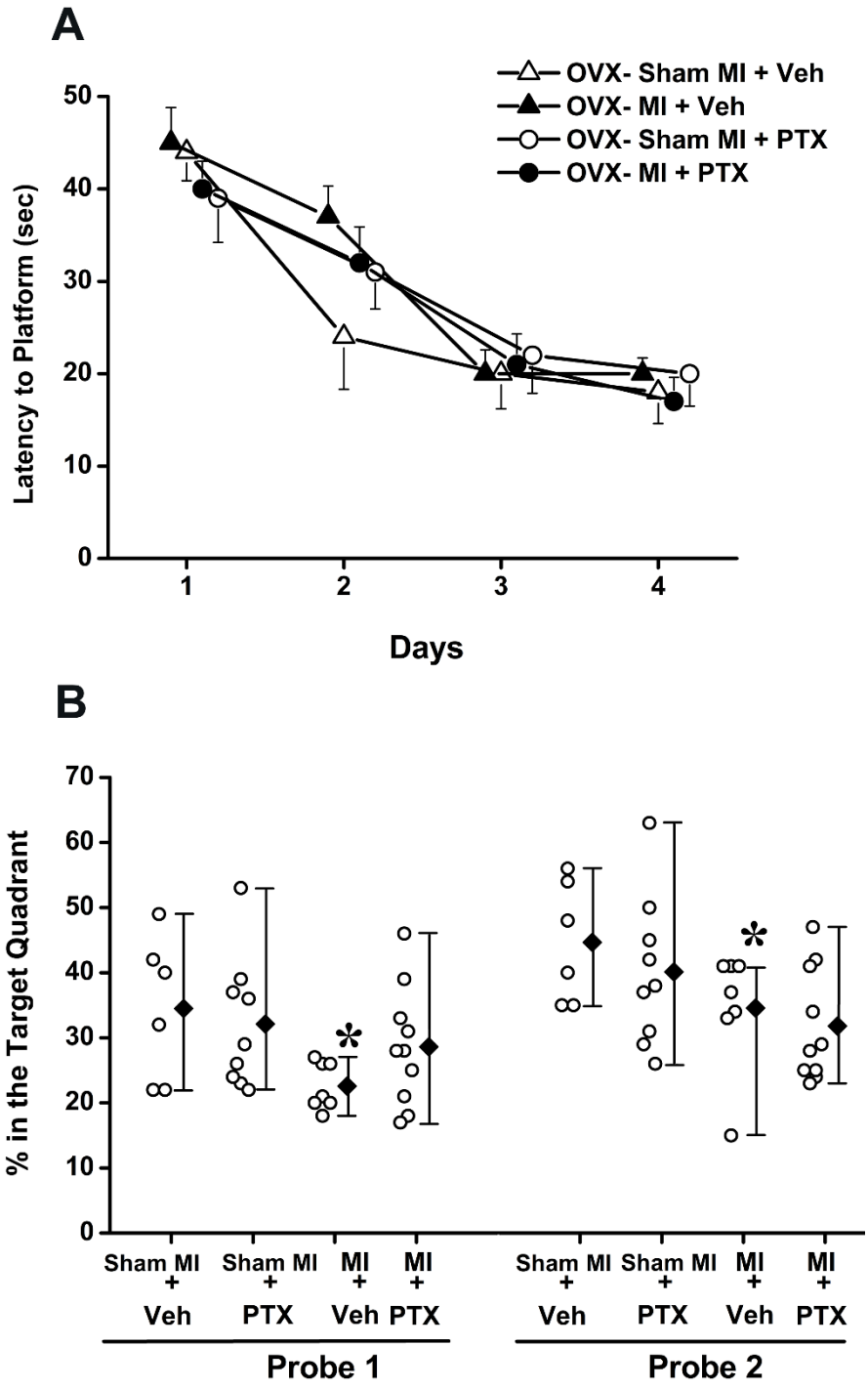

A
OVX- Sham MI + Veh
OVX- MI + Veh
OVX- Sham MI + PTX
OVX- MI + PTX
Latency to Platform (sec)
50
40
30
20
10
0
1
2
3
4
Days
B
% in the Target Quadrant
70
60
50
40
30
20
10
0
Sham MI + Veh
Sham MI + PTX
MI + Veh
MI + PTX
Sham MI + Veh
Sham MI + PTX
MI + Veh
MI + PTX
Probe 1
Probe 2

Figure 3.3. Learning and Spatial memory in the Morris water maze test. Values are mean ±SEM (panel A), and mean ±95% CI (panel B) (n=6-10/group). Repeated measures ANOVA was done to compare all groups during training days, and two-way ANOVA for MI x PTX interactions, followed by Bonferroni *post hoc test*. (**A**) Latency to the platform on training days. All groups showed a similar, significant decrease in latency of time compared to day 1 of training, F=38.4, P<0.001. (**B**) Probe 1 and 2, done on the third day and the fifth day respectively. Time spent in the target quadrant, where the platform was located in the training sessions, is expressed as a percent. Probe 1, MI effect, F=4.5, P=0.042, PTX effect, F=0.7, NS, MI x PTX interaction, F=1.0, NS. Probe 2, MI effect, F=4.9, P=0.034, PTX effect, F=0.5, NS, MI x PTX interaction, F=0.01, NS.

* P <0.05 vs OVX-Sham MI +Veh

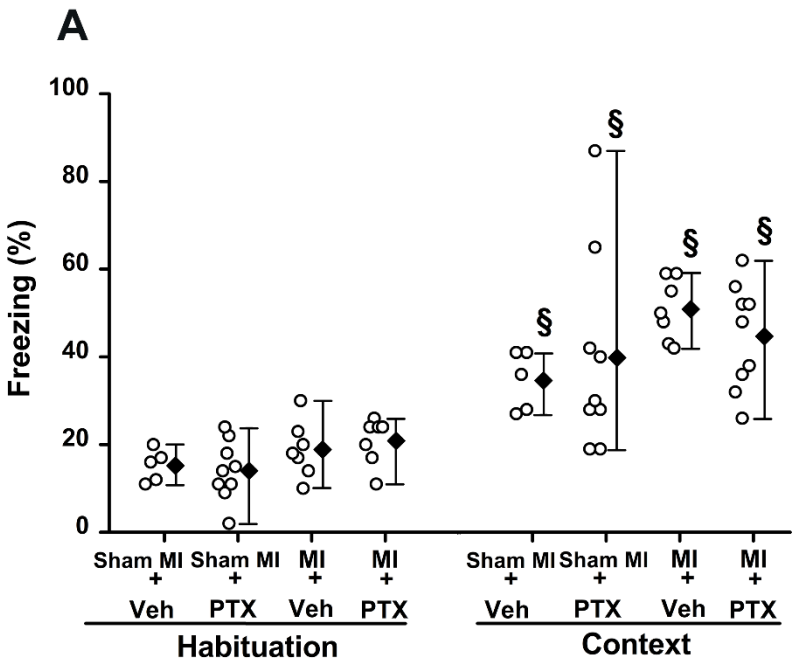
Table 3.2. Locomotor activity in the first training session of the Morris Water Maze, in ovariectomized female rats with or without PTX at 10 weeks post-MI.

Groups	Total path length (m)	Speed (cm/sec)
OVX-Sham MI +Veh **(n=6)**	13 ± 1.3	24 ± 2.3
OVX-Sham MI +PTX **(n=9)**	17 ± 0.9	29 ± 1.5
OVX-MI +Veh **(n=7)**	14 ± 1.1	30 ± 0.7
OVX-MI+PTX **(n=10)**	14 ± 0.9	26 ± 1.5

Values are mean ± SEM. Two-way ANOVA was done for MI x PTX interactions. No significant differences in the locomotor activity were observed.

Fear Conditioning test:

OVX-groups displayed a modest freezing behavior during the habituation period and pronounced freezing in the contextual conditioning test (day2). Neither MI nor PTX treatment affected freezing behavior in the context conditioning test (Fig 3.4.A). In the cued conditioning test, the OVX-sham MI+Veh group exhibited a high freezing behavior (~60%) compared to sham OVX-sham MI group (~20%, (Najjar et al., 2018), that was not attenuated by PTX. OVX-MI+Veh group displayed a further increase in freezing behavior compared to OVX-sham MI+Veh. PTX treatment prevented the increase of freezing behavior induced by MI (Fig 3.4.B).


A
Freezing (%)
100
80
60
40
20
0
§
Sham MI + Veh
Sham MI + PTX
MI + Veh
MI + PTX
Habituation
Context

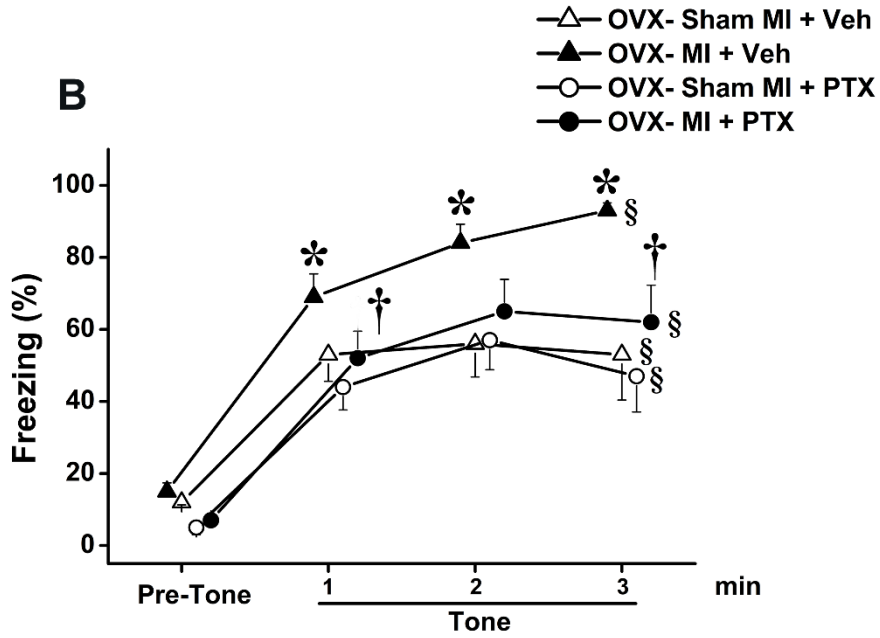

OVX- Sham MI + Veh
OVX- MI + Veh
OVX- Sham MI + PTX
OVX- MI + PTX
B
Freezing (%)
100
80
60
40
20
0
*
†
§
Pre-Tone
1
2
3
min
Tone

Figure 3.4. Freezing behaviour by OVX female rats at 10 weeks post-MI in the fear conditioning test. Values are mean ±95% CI (panel A) and SEM (panel B) (n=6-10/group). Freezing behavior is expressed as a percent of time freezing over the specified period. Repeated measures ANOVA was done to compare the baseline on day 1 of habituation or pre-tone on day 3 to the context or cued freezing respectively for all groups, and two-way ANOVA for MI x PTX interactions. (**A**) Habituation on day 1 during the first 2 min of acquisition. Context on day 2 to assess contextual fear, includes all 6 min of the test. There was no MI (F=4.1, P=0.08) or PTX effect (F=0.01, NS) or MI x PTX interaction (F=0.4, NS). (**B**) Cued fear conditioning test consists of 3 min habituation as the pre-tone period followed by the tone period for 3 minutes. MI effect, F=6.9, P=0.014, PTX effect, F=4.4, P=0.045, MI x PTX interaction, F=1.4, NS.

§ P<0.05 versus freezing behaviour during habituation (**A**) or pre-tone (**B**), * P <0.05 vs OVX-Sham MI +Veh, † P <0.05 vs OVX-MI+Veh

Cytokine levels

Plasma (Fig 3.5.):

The OVX-MI+Veh group exhibited an increase in the levels of IL-1α, IL-1β, IL-2, IL-6, and TNF-α, as well as IL-10 compared to the OVX-sham MI+Veh group. PTX treatment prevented the increases in plasma cytokines in OVX-MI rats.

PVN (Fig 3.6.):

In the PVN of OVX-MI+Veh rats, clear increases in IL-1β, IL-2, IL-6, TNF-α, and IL-10 were found compared to the OVX-sham MI+Veh group. PTX prevented these increases.

PFC (Fig3. 7):

In the PFC of the OVX-MI+Veh group, IL-1β, IL-2, IL-6, and TNF-α increased significantly. PTX treatment decreased IL-1β, IL-6, and TNF-α in the PFC of OVX-MI rats.

Correlation Analyses (Figs 3.8 and 3.9)

In the sham MI groups, no significant correlations were observed between cytokine levels in plasma, PVN and PFC. In contrast, in the MI groups TNF-α levels in plasma, PVN and PFC correlated significantly, as did IL-6 and IL-10 in plasma vs PVN and PVN vs PFC.

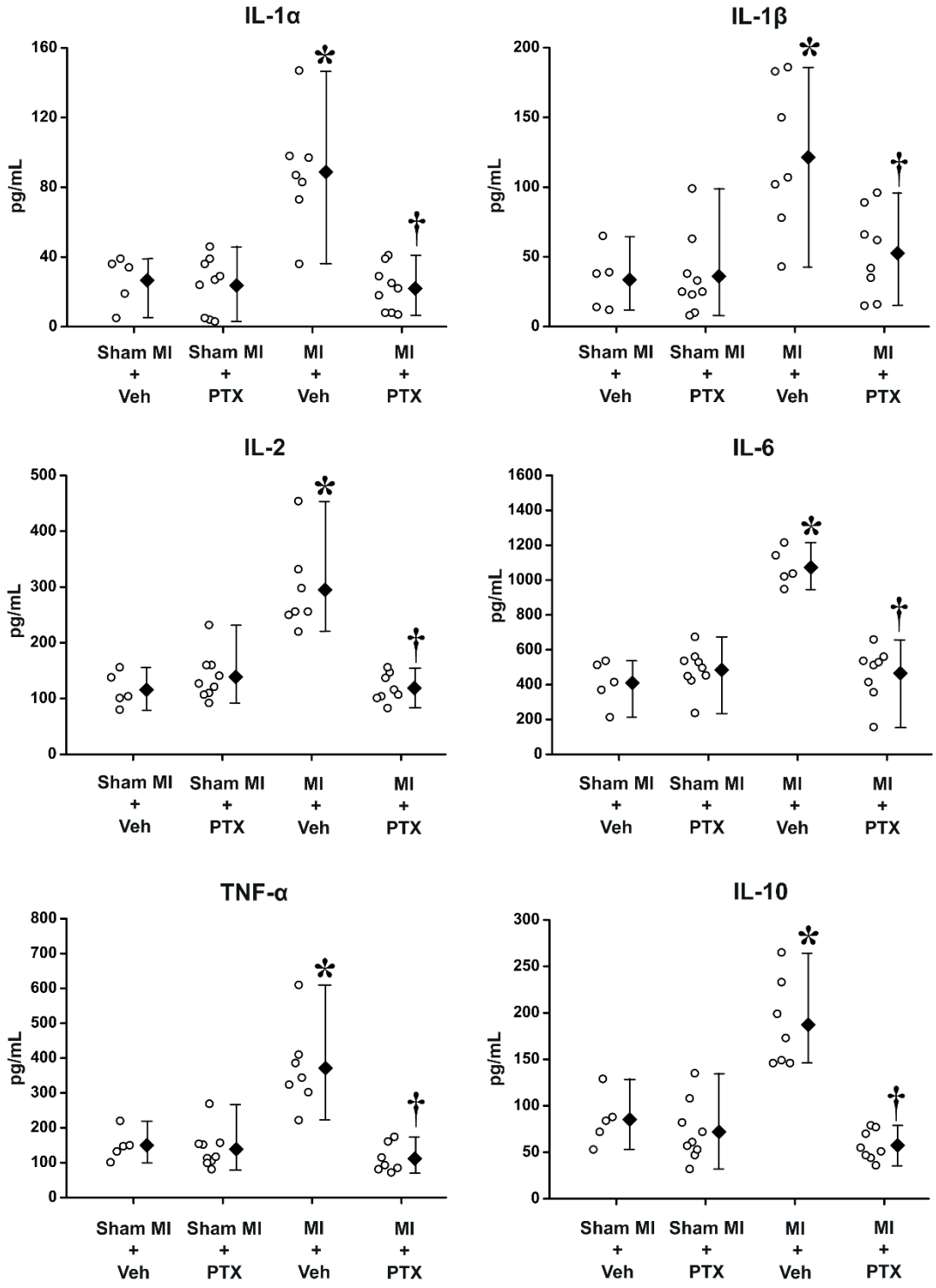

IL-1α
pg/mL
160
120
80
40
0
IL-1β
200
150
100
50
IL-2
500
400
300
200
IL-6
1600
1400
1200
1000
800
600
TNF-α
700
IL-10
250
Sham MI + Veh
Sham MI + PTX
MI + Veh
MI + PTX

Figure 3.5. Cytokine levels in the plasma of OVX female rats with or without PTX at 10 weeks post-MI. Values are mean ±95% CI (n=5-9/group). Two-way ANOVA for MI x PTX interaction, followed by Bonferroni *post hoc test*. IL-1α, MI effect, F=15.2, P<0.001, PTX effect, F=17.0, P<0.001, MI x PTX interaction, F=17.1, P<0.001. IL-1β, MI effect, F=14.0, P<0.001, PTX effect, F=2.1, NS, MI x PTX interaction, F=2.8, NS. IL-2, MI effect, F=20.1, P<0.001, PTX effect, F=20.9, P<0.001, MI x PTX interaction, F=30.9, P<0.001. IL-6, MI effect, F=25.3, P<0.001, PTX effect, F=21.9, P<0.001, MI x PTX interaction, F=26.5, P<0..001. TNF-α, MI effect, F=13.2, P<0.001, PTX effect, F=28.5, P<0.001, MI x PTX interaction, F=22.7, P<0.001. IL-10, MI effect, F=10.7, P=0.003, PTX effect, F=28.6, P<0.001, MI x PTX interaction, F=17.7, P<0.001

* *P* <0.05 vs OVX-Sham MI +Veh, † *P* <0.05 vs OVX-MI+Veh

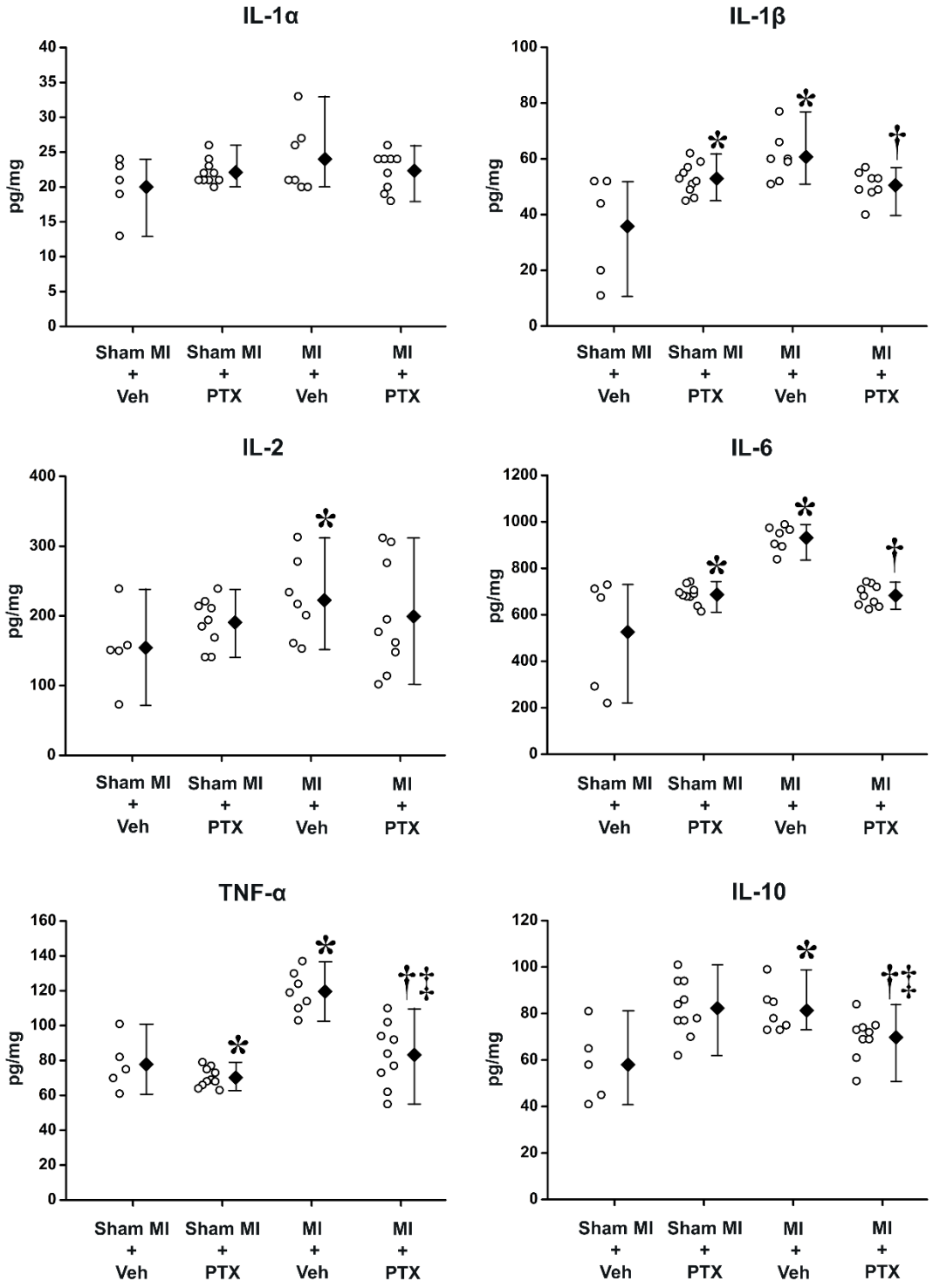

IL-1α
pg/mg
40
35
30
25
20
15
10
5
0
Sham MI + Veh
Sham MI + PTX
MI + Veh
MI + PTX
IL-1β
pg/mg
100
80
60
40
20
0
Sham MI + Veh
Sham MI + PTX
MI + Veh
MI + PTX
IL-2
pg/mg
400
300
200
100
0
Sham MI + Veh
Sham MI + PTX
MI + Veh
MI + PTX
IL-6
pg/mg
1200
1000
800
600
400
200
0
Sham MI + Veh
Sham MI + PTX
MI + Veh
MI + PTX
TNF-α
pg/mg
160
140
120
100
80
60
40
20
0
Sham MI + Veh
Sham MI + PTX
MI + Veh
MI + PTX
IL-10
pg/mg
120
100
80
60
40
20
0
Sham MI + Veh
Sham MI + PTX
MI + Veh
MI + PTX

Figure 3.6. Cytokine levels in the PVN of OVX female rats with or without PTX at 10 weeks post-MI. Values are mean ±95% CI (n=5-9/group). Two-way ANOVA for MI x PTX interaction, followed by Bonferroni *post hoc test*. IL-1α, MI effect, F=4.0, P=0.05, PTX effect, F=0.3, NS, MI x PTX interaction, F=3.4, P=0.07. IL-1β, MI effect, F=14.6, P<0.001, PTX effect, F=2.5, NS, MI x PTX interaction, F=20.6, P<0.001. IL-2, MI effect, F=2.3, NS, PTX effect, F=1.0, NS, MI x PTX interaction, F=3.6, P=0.07. IL-6, MI effect, F=36.5, P<0.001, PTX effect, F=0.2, NS, MI x PTX interaction, F=37.6, P<0.001. TNF-α, MI effect, F=39.6, P<0.001, PTX effect, F=15.7, P<0.001, MI x PTX interaction, F=12.8, P<0.001. IL-10, MI effect, F=4.0, P=0.05, PTX effect, F=5.1, P<0.05, MI x PTX interaction, F=25.7, P<0001.

* *P* <0.05 vs OVX-Sham MI +Veh, † *P* <0.05 vs OVX-MI+Veh, ‡ *P*<0.05 vs OVX-Sham MI+PTX.

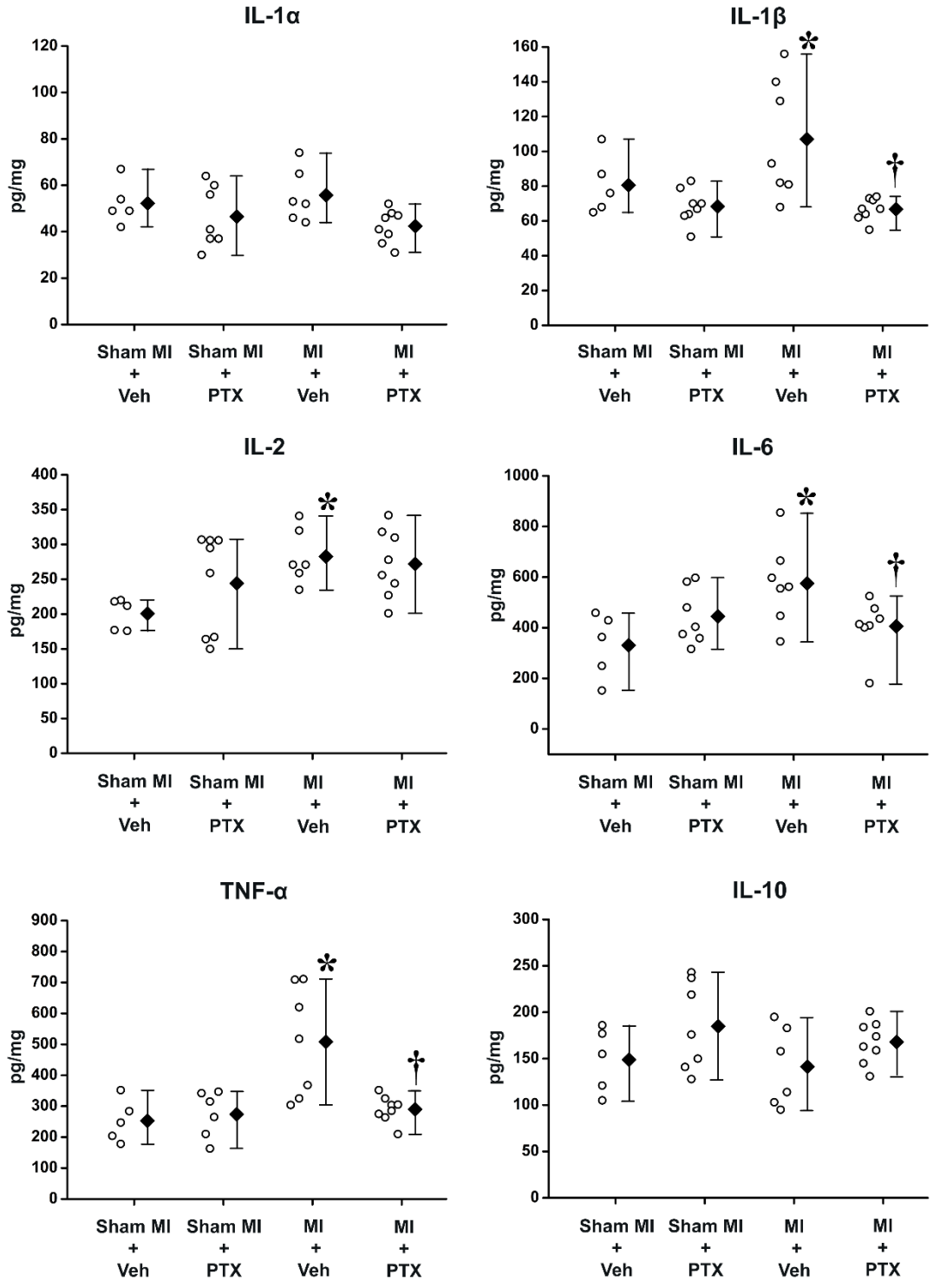

IL-1α
IL-1β
IL-2
IL-6
TNF-α
IL-10
pg/mg
Sham MI + Veh
Sham MI + PTX
MI + Veh
MI + PTX
*
†

Figure 3.7. Cytokine levels in the PFC of OVX female rats with or without PTX at 10 weeks post MI. Values are mean ±95% CI (n=5-9/group). Two-way ANOVA for MI x PTX interaction, followed by Bonferroni *post hoc test*. IL-1α, MI effect, F=0.5, NS, PTX effect, F=3.8, P=0.006, MI x PTX interaction, F=1.4, NS. IL-1β, MI effect, F=2.7, NS, PTX effect, F=12.2, P<0.001, MI x PTX interaction, F=20.6, P<0.001. IL-2, MI effect, F=7.6, P<0.01, PTX effect, F=0.6, NS, MI x PTX interaction, F=1.8, NS. IL-6, MI effect, F=2.1, NS, PTX effect, F=0.8, NS, MI x PTX interaction, F=8.5, P<0.01. TNF-α, MI effect, F=7.6, P=0.01, PTX effect, F=3.5, P=0.07, MI x PTX interaction, F=5.6, P=0.03, IL-10, MI effect, F=0.1, NS, PTX effect, F=1.8, NS, MI x PTX interaction, F=0.6, NS

* *P* <0.05 vs OVX-Sham MI +Veh, † *P* <0.05 vs OVX-MI+Veh.

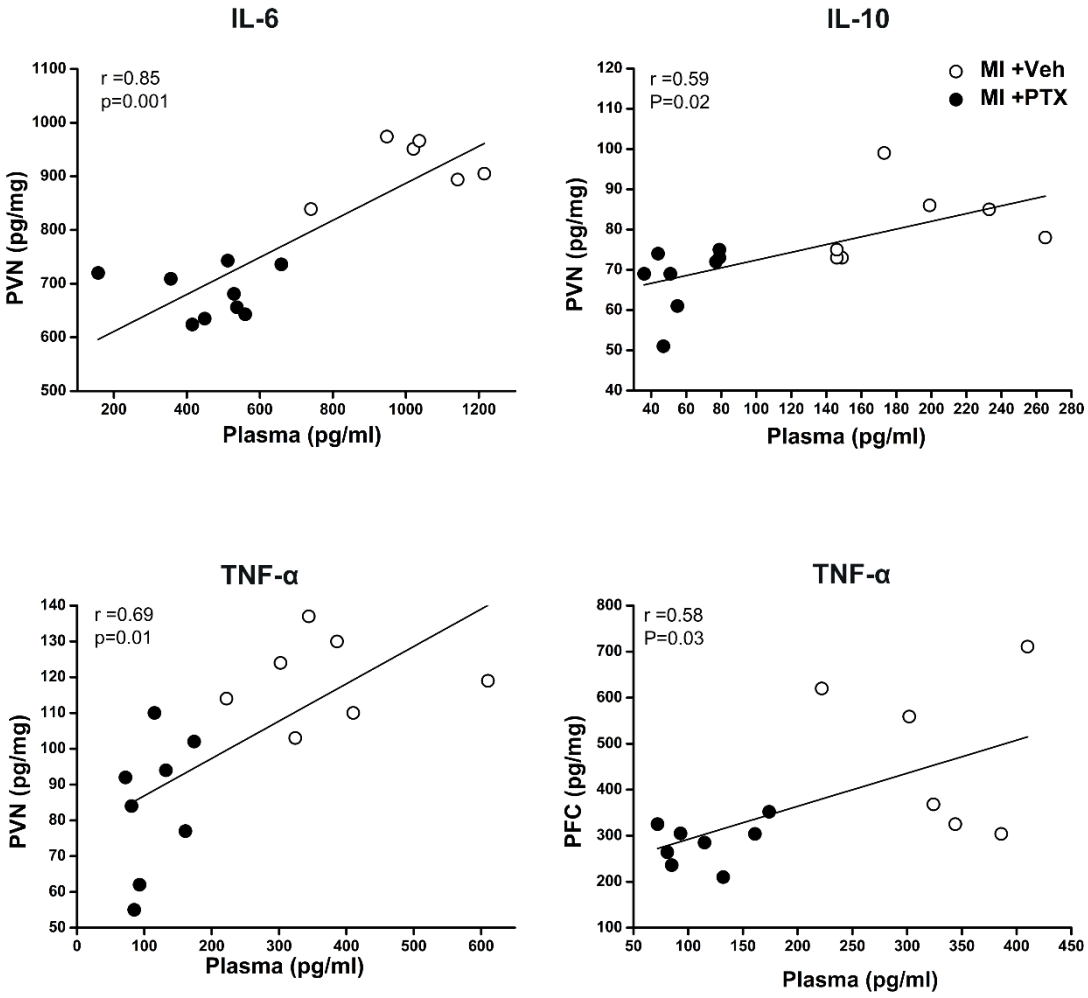


Figure 3.8. Significant correlations between cytokine levels in Plasma and PVN or plasma and PFC. Other cytokines did not have significant correlations.

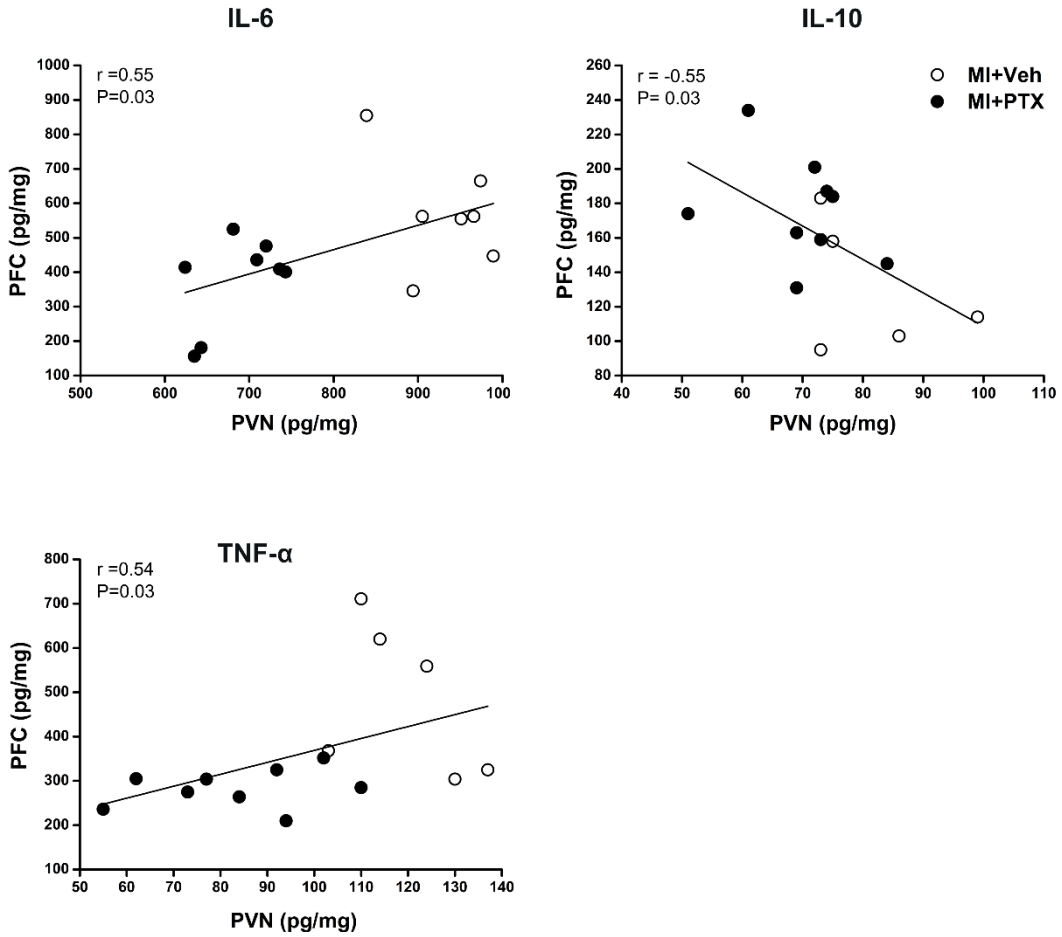


Figure 3.9. Significant correlations between cytokine levels in the PFC and PVN. Other cytokines did not have significant correlations.

mBDNF protein levels

The OVX-MI +Veh group showed a significant decrease in the mBDNF expression compared to OVX-Sham MI+Veh. PTX prevented this decrease (Fig 3.10).

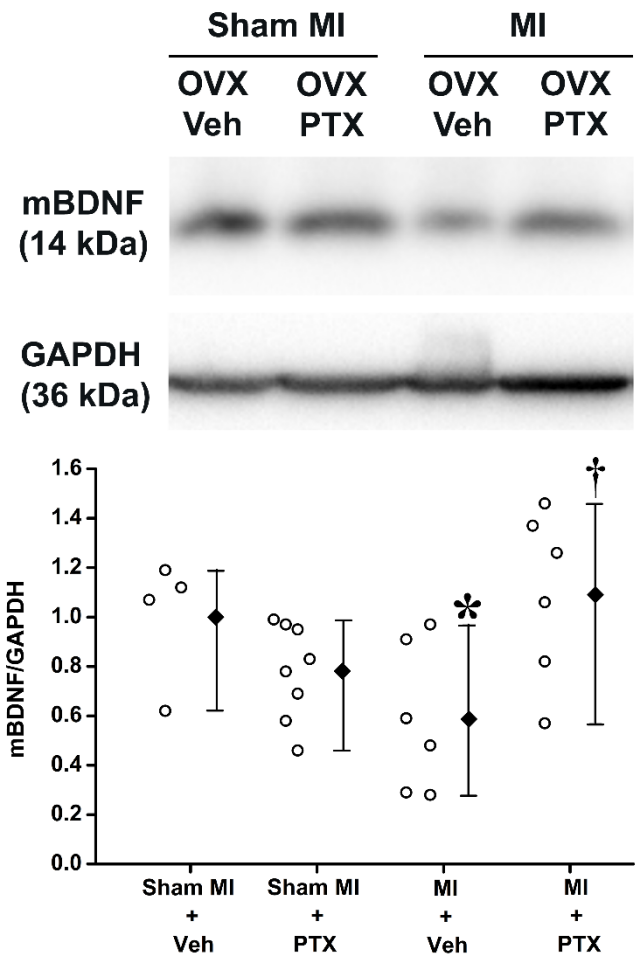


Figure 3.10. mBDNF expression in the PFC of OVX female rats with or without PTX at 10-weeks post MI. Values are mean ±95% CI (n=5-8/group). Two-way ANOVA for MI x PTX interaction, followed by Bonferroni *post hoc test*. MI effect, F=0.63, P=.44, PTX effect, F=.65, P=0.43, MI x PTX interaction; *F*=6.7, *P*=0.017

* *P* <0.05 vs OVX-Sham MI +Veh, † *P* <0.05 vs OVX-MI+Veh

DISCUSSION

The present study demonstrates that OVX female rats post-MI develop depression-like behaviors, and deficits in cued associated learning, and some deficit in spatial memory. These behavioral changes were associated with significant increases in cytokine levels in the plasma, PVN and PFC. Oral PTX treatment prevents most of these behavioral changes and decreases most cytokines in the PFC. In contrast, cardiac dysfunction post-MI was not attenuated by PTX, despite the prevention by PTX of increases in cytokine levels in the plasma and PVN. These findings suggest that in OVX female rats neuroinflammation plays a major role in depression-like behavior induced by HF post-MI, but not in the progression of cardiac dysfunction post-MI.

Inflammation and Cardiac dysfunction

A variety of pre-clinical studies have demonstrated that inflammation contributes to cardiac dysfunction post-MI. In this study, cytokine levels increased in the plasma and the PVN of OVX female rats with HF post-MI, similar to what occurs in intact male and female rats with HF post-MI (Najjar et al., 2018; Yu et al., 2018a). After MI, the damaged extracellular matrix in the injured cardiac tissue activates matrix metalloproteinase (MMP) releasing TNF-α (Dobaczewski et al., 2010). TNF-α stimulates IL-1α, IL-1β, and IL-6 gene expression in cardiac fibroblasts (Turner et al., 2007). The release of pro-inflammatory cytokines (PICs) from the cardiac tissue (Mann, 2002) also activates and increases immune cell populations in the spleen which further increases the circulatory and cardiac PICs and contributes to continuing myocyte apoptosis and progressive cardiac remodeling and dysfunction (Ismahil et

al., 2014; Epstein et al., 2017). In addition, plasma PICs can via several mechanisms (Wei et al., 2014b; Yu et al., 2018b) activate central pathways, resulting in an increase of sympathetic outflow and further cardiac dysfunction post-MI (Zhang et al., 2001; Yu et al., 2018b).

Consistent with an important role of cytokines in the progression of HF post-MI, treatment of male rodents with an IL-1β and TNF-α antagonist or IL-10 agonist limits the reduction of EF and the increase of LVEDP post-MI (Berry et al., 2004; Stumpf et al., 2008; Toldo et al., 2014). Further supporting the role of systemic and central inflammation, systemic or central treatment with PTX inhibits the increase of IL-1β and TNF-α in the plasma and PVN of male rats post-MI, decreases plasma NE and attenuates the increase in LVEDP and the decrease in EF (Kang et al., 2006, 2010).

The present study is the first study evaluating in female rodents with HF the possible role of inflammation and effects of PTX for cardiac dysfunction post-MI. Oral PTX treatment prevented the increase of PICs levels in the plasma of OVX female rats with HF post-MI and lowered some to levels below those of sham OVX-sham MI rats. Similarly, PTX prevented the increase of most PICs in the PVN. However, PTX did not affect the progression of cardiac dysfunction, as assessed by echocardiography and hemodynamic measurements. These findings may indicate that peripheral and central inflammation do not contribute to progressive cardiac dysfunction in female rats post-MI. The mechanisms that mediate this sex difference are still unknown, but there are several factors that may contribute to such difference between male and female rats.

First, the extent of inflammation may differ. PICs in the plasma similarly increased in male and female rats with HF post-MI (Najjar et al., 2018; Yu et al., 2018a). Only IL-6 was ~45% higher in male than female rats post MI (Najjar et al., 2018). The extent of increases in PICs levels in the PVN of intact females and males ten weeks post-MI was also comparable. However, actual levels in the females were ~20% lower than in males (Najjar et al., 2018). Furthermore, the mRNA expression of IL-1β and TNF-α in the PVN of intact female rats was lower than of male rats at four weeks post-MI (Yu et al., 2018a). Collectively, the extent of inflammation post-MI in the PVN might be somewhat less in intact female rats compared to males. OVX and intact female rats also appear to show a similar pattern of change post-MI except for no increase in plasma and PVN IL-1α in OVX rats.

Secondly, sex differences may also occur in the heart. In the acute phase of MI, intact female rats showed fewer macrophages in the border zone of infarction associated with lower gene expression of IL-1β, IL-6, and MMP-9 compared to male rats (Fang et al., 2007). In contrast, the few studies that examined inflammation in the heart in the chronic phase post-MI, do not suggest sex differences. For example, at 14 days post-MI, increases in macrophages and MMP in the LV were similar in male and female mice (Cavasin et al., 2004). Intact female and male mice also show similar cardiac dysfunction up to ten weeks post-MI (Cavasin et al., 2004; Shioura et al., 2008).

Thirdly, the effect of PTX on peripheral or central inflammation may be different in male and female rats post-MI. Oral or IP PTX treatment in male rats post-MI prevented ~60% of the increase of IL-1β and TNF-α in the plasma, and ~50% of the increase in IL-1β and TNF-α in the PVN (Kang et al., 2006, 2008; Guggilam et al., 2007). In contrast to males, in OVX female rats post-MI, PTX decreased some

plasma PICs levels below those in sham OVX-sham MI rats and prevented increases in PICs levels in the PVN. Only IL-2 in the PVN was not affected by PTX treatment. IL-2 is a growth factor for T-cells and induces microglia NO production (Hanisch, 2002), which facilitates the activation of microglia (Kettenmann et al., 2011).

Collectively, OVX female rats exhibit clear peripheral and central inflammation post-MI, but their inhibition by PTX does not attenuate cardiac dysfunction. The specific mechanisms involved in this sex difference between male and female rats are not obvious and require further studies.

Inflammation and depression-like behavior

In contrast to intact female rats, OVX female rats with HF post-MI demonstrate increases of cytokines in the PFC, decreases in mBDNF levels in the PFC, and clear evidence of anhedonia in the SPT and despair behavior in FST. Furthermore, associative memory for nociceptive stimuli is markedly enhanced in the cued conditioning test. PTX treatment prevents MI induced increases of cytokines in the PFC, and decrease of mBNDF in the PFC, as well prevents most aspects of depression-like behavior. Recall of nociceptive stimuli, reflected as freezing behavior was only partially improved. Male rats showed depression-like behavior two weeks post MI, assessed by SPT and FST, which was prevented by PTX treatment associated with a decrease of neural apoptosis in the PFC (Bah et al., 2011b). Estrogen replacement decreases PIC's levels and increases the anti-inflammatory cytokine IL-10 in the PFC and prevents the development of depression-like behavior in OVX female rats at ten weeks post-MI (Najjar et al., 2018). In contrast to PTX treatment, estrogen replacement completely prevents the enhanced freezing in the cued conditioning test (Najjar et al., 2018). Taken together, these studies suggest that the combined impact of OVX and HF post-MI on depression-like behavior symptoms is

largely mediated by neuroinflammation that can be prevented by PTX treatment. Enhanced freezing in the fear conditioning test reflects an enhanced adaptation response to stressful events through the amygdala and hippocampus (Rosen and Schulkin, 1998), and appears only partially (cued-conditioning) or not at all (context-conditioning) dependent on OVX and MI induced neuroinflammation.

Limitations of Present Study

Treatment with PTX was started one week before the MI to ensure adequate anti-inflammatory effects in the early, possibly critical, post MI period. Further studies need to establish whether treatment started in the early or chronic post MI phase also can minimize depression-like behavior. The present findings support the concept that anti-inflammatory actions mediate the behavioral effects of PTX, but do not exclude that "off-target" effects play a role (as well).

Cytokine levels increased in the plasma, PVN and PFC of OVX-MI rats with significant correlations for the PICs, TNF-α and IL-6 (Figs 3.8 and 3.9). These correlations are consistent with the concept that systemic inflammation drives neuroinflammation not only in the PVN (Wei et al., 2014b; Yu et al., 2018b), but also in the PFC. The actual mechanisms and pathways involved in activation of inflammation in the PFC have not yet been identified and require further study.

In **conclusion**, OVX female rats post-MI develop a marked depression-like behavior associated with an increase of neuroinflammation in the PFC and PVN. These findings extend our previous study that showed that intact females and OVX females treated with estrogens post-MI exhibit protection against depression-like behavior induced by MI. Oral PTX prevents the increase of cytokines in the plasma, PVN, and PFC of OVX female rats post MI, and prevents most aspects of depression-

like behavior, but in contrast to male rats does not inhibit the progression of cardiac dysfunction. These findings provide a better understanding of mechanisms contributing to sex-specific responses in HF and may contribute to new therapeutic strategies.

DISCLOSURES

No conflicts of interest, financial or otherwise are declared by the authors.

AUTHORS CONTRIBUTIONS

F.H.H.L designed the study, interpreted the data, and revised drafts of the manuscript. F.N and M.A performed the experiments, collected, analyzed and interpreted the data. F.N drafted the manuscript. D.L interpreted the data and critically revised the draft for intellectual content.

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Chapter 4:

General Discussion

4.1. Summary of findings:

Male rats developed depression-like behavior but not female rats post-MI. The degree of cardiac dysfunction and the increase in plasma and PVN cytokines levels were similar in male and female rats post-MI. However, cytokine levels increased only in the PFC of male rats post-MI associated with a decrease in mBDNF levels, and increase in mBDNF levels in the amygdala of male rats post-MI. These changes were not detected in female rats post-MI.

Ovariectomy induces modest depression-like behavior. MI combined with ovariectomy causes marked depression-like behavior symptoms as assessed by SPT and FST, and E_2 replacement prevents depression-like behavior induced either by MI or ovariectomy. In FCT, OVX female rats show an increase in freezing behavior in context and cued conditioning test, but MI augments OVX effect on freezing behavior only in the cued conditioning test. E_2 replacement prevents the augmentation of MI on freezing behavior in the cued conditioning test. PTX prevents all aspects of depression-like behavior in OVX female rats post-MI.

Plasma and PFC cytokines were not altered by OVX or E_2 replacement in sham MI female rats. MI increases plasma, PVN and also PFC cytokine levels in OVX female rats post-MI. E_2 replacement decreased PIC levels and increased anti-inflammatory (IL-10) cytokines in the PFC in OVX female rats post-MI, and did not affect plasma cytokines. E_2 replacement also prevents the decrease of mBDNF levels in the PFC of OVX-female rats post-MI. Amygdala mBDNF levels increased markedly in OVX female rats post-MI, and E_2 replacement did not alter these changes in the amygdala. Oral PTX markedly decreased plasma and PFC cytokines levels, and prevented the decrease in mBDNF levels in the PFC of OVX-female rats post-MI. Initiating E_2 before induction of MI did not attenuate HF progression post-MI or the

increase in plasma cytokine levels in OVX female rats post-MI. Oral PTX also did not affect cardiac dysfunction but partially prevented the increase in the PVN cytokine levels in OVX female rats post-MI.

4.2. Depression-like behavior post-MI affected by sex

Sham-MI female rats had less immobility phase in the FST compared to sham-MI male rats. Several studies showed that female rodents are more active in elevated plus maze test compared to their male counterpart, and spent 10-20% less time in the immobile phase than males in FST (Barros and Ferigolo, 1998; Brotto et al., 2000). Sex did not affect sucrose consumption in SPT, similar findings were reported by Sclafani et al., (1987). Females showed slower learning in MWM compared to males that may be due to females having a different navigation approach to reach the platform, as male and females rats have similar travel distance in training sessions, and there was no significant difference in probe trials (Astur et al., 2004). Gouweleeuw et al., (2016) assessed sex differences in behavioral changes at 3 weeks post-MI. Female post-MI rats spent more time in open arms of elevated plus maze test compared to males. This is the first study that highlights that sex affects depression-like behavior post-MI. Only male rats developed depression-like behavior post-MI with a reduction in sucrose consumption by ~ 30% and increased in immobility phase by ~ 30%, despite male and female rats post-MI developed similar cardiac dysfunction.

4.2.1. Sex affects neuroinflammation induced by MI

The number and morphology of microglia display sex differences, with microglia higher in the resting phase in the hippocampus, amygdala and parietal cortex of young adult (60 days) female rats compared to males but not in the PVN (Schwarz et al., 2012). Microglia activation by LPS, leading to increase IDO activity

in the CNS and decrease in tryptophan levels, and caused depression-like behavior was displayed only in male rats but not females. In contrast, females showed an increase in 5-HIAA levels in the PFC, hippocampus and amygdala 4 hr after LPS injection but not males (O'Connor et al., 2009; Pitychoutis et al., 2009). Our findings showed that cytokines increased similarly in the plasma and PVN of male and female at 10 weeks post-MI. This similarity might be related to the fact that PVN in male and female rats have comparable microglia number and only ER-β localized in the PVN of females which have low efficacy in inhibiting inflammation (Smith et al., 2011). Ovarian hormones may, therefore, have no effect on neuroinflammation in the PVN. The PFC of male rats post-MI revealed 2 folds increase in cytokines levels compared to the sham-MI male rats, while sham-MI female rats showed higher levels for IL-1α, IL-6 and the anti-inflammatory cytokine IL-10 compared to male sham-MI, but females post-MI showed no further increase in cytokines levels. Amygdala was protected from neuroinflammation induced by MI in males and females, which may be due to the higher levels of estrogen aromatase in the amygdala in both sexes compared to the PFC and hippocampus, which preserves E_2 levels in the amygdala (Barker et al., 2009).These findings suggest that ovarian hormones modulate cytokine production, and inhibit cytokine production in the PFC of females rats post-MI.

4.2.2. Monoamine and mBDNF levels affected by sex

5-HT turnover is higher in the PFC, amygdala and dorsal hippocampus of normal female rats compared to males (Beck and Luine, 2002; Bisagno et al., 2003; Dedovic et al., 2009). Female rats also display higher dopamine turnover in the PFC and hippocampus compared to males (Dalla et al., 2008a). After stress event (shock), 5-HT and dopamine extracellular levels were increased in the BLA of female rats compared to males, while in context freezing test male rats showed an enhanced

freezing behavior associated with a decrease in 5-HT levels in the BLA (Mitsushima et al., 2006). Females were protected against depression-like behavior induced by stress such as CMS. CMS male rats exhibited lower dopamine turnover in the PFC compared to CMS female rats. There was no sex difference for dopamine levels in the amygdala of CMS males and females. Lastly 5-HT turnover was higher in the hypothalamus of CMS males compared to CMS females (Beck and Luine, 2002; Luine, 2002; Drossopoulou et al., 2004; Dalla et al., 2008b). Whether sex affects neuroadaptation in HF post-MI has not yet been assessed.

Cytokine levels increased in the PFC of male rats post-MI. Stressed male rats showed higher mRNA cytokines expression (IL-6 and TNF-α) in the PFC compared to their female counterpart after 6 weeks of stress. The increase of cytokine expression was associated with activation of IDO enzyme and a decrease in BH4 cofactor, 5-HT and dopamine production (Haroon et al., 2012; Hudson et al., 2014). ICV infusion of IL-1β inhibits neurogenesis in the hippocampus of male rats (Koo and Duman, 2008). Our findings show that BDNF levels in the PFC of sham-MI male and female rats were comparable, whereas normal male rats had higher BDNF levels in the hippocampus compared to normal females (Bimonte-Nelson et al., 2008). In male rats, MI decreased BDNF levels in the frontal cortex and increased in the medial amygdala at 7 day post-MI, associated with an increase in the TUNEL positive cells (apoptosis) in the frontal cortex and hippocampus (Kaloustian et al., 2008). Bax/Bcl2 also increased significantly in the PFC of male rats at 3 weeks of myocardial ischemia-reperfusion injury (Wann et al., 2007). Our findings show that female rats post-MI maintained BDNF levels in the PFC, unlike males that tend to be decreased. Stressed male rats showed an increase in neuronal atrophy in the hippocampus but not females (Galea et al., 1997). On the other hand, male rats showed a significant

increase in amygdala mBDNF levels compared to females at 10 weeks post-MI, with preserved form neuroinflammation in both sexes. The increase of mBDNF levels in the amygdala contributed to the maladaptation for stress event (Yu and Chen, 2011) and enhances freezing behavior found in CMS male rats (Rosen and Schulkin, 1998; McEwen et al., 2016). BDNF enhances 5-HT synthesis and promotes the survival and sprouting of 5-HT fibers, ovarian hormones also increase mRNA BDNF levels, tryptophan synthesis and upregulate 5-HT2A receptors that may contribute to the protective effect of ovarian hormone against depression-like behavior (Cavus and Duman, 2003; Franklin and Perrot-Sinal, 2006). Only male rats post-MI increased cytokine levels in their PFC, which contributed to lower mBDNF levels and showed depression-like behavior symptoms. However, female sex showed protection against MI reducing mBDNF levels in the PFC and may modulate neuroadaptation that leads to depression-like behavior in HF post-MI.

4.3. Estrogens, neuroinflammation and depression-like behavior

Ovarian hormone deficiency induced depression-like behavior that was prevented by E_2 replacement. Similar findings were previously reported (discussed in section 1.4.). Whereas E_2 replacement had no protective effect for HF after MI, but it rescued OVX female rats from depression-like behavior induced by MI. This is the first study showing the extent of inflammation in the CNS induced by MI in OVX female rats post-MI. OVX sham MI female rats displayed mild symptoms of depression-like behavior, but OVX per se did not increase plasma or PFC cytokine levels. However, cytokines levels increased in the PFC of OVX female rats post-MI and E_2 replacement prevents the increasing of PICs while increases anti-inflammatory cytokine (IL-10) in the PFC, which might stimulate inflammation resolution. To address the anti-inflammatory role of E_2 in depression, we used oral PTX (anti-

inflammatory agent) treatment in OVX female rats post-MI, that prevented depression-like behavior, similar as in E_2 replacement with exception that PTX only partially prevents freezing behavior in cued conditioning test, and not the OVX effect on freezing behavior in cued conditioning test. Oral PTX treatment markedly prevents the increase of cytokine levels in PFC of OVX female rats post-MI, which suggest that PTX could prevent depression-like behavior. Therefore, E_2 replacement has an anti-inflammatory effect in developing depression-like behavior. OVX per se did not affect mBDNF levels in the PFC and amygdala of female rats. However, MI reduces mBDNF levels in the PFC and increases in the amygdala that prevented by E_2 replacement only in the PFC. Similar findings were found in oral PTX treatment. OVX decreases synaptogenesis and 5-HT levels, and increases IL-6 and IFN-ɣ levels in the hippocampus of female rats, which is prevented by estrogen replacement (Woolley et al., 1997; Harte-Hargrove et al., 2013; Xu et al., 2015) through several mechanisms such as: decreases the expression of NF-*k*B, decreases the production of cytokines, and decreases the phagocytic activity of microglia (discussed in section 1.4.4.).

Collectively, this is the first study showing that chronic E_2 replacement had an antidepressant effect for depression-like behavior after MI through neuroinflammation pathway, that can also be inhibited by anti-inflammatory agents such as PTX, and minocycline.

4.4. Estrogens, inflammation and HF post MI

Our findings show that OVX or estrogen replacement before MI did not affect cardiac dysfunction in OVX female rats at 10 weeks of MI. Initiating estrogen replacement 2 weeks after MI also had no benefit on cardiac dysfunction at 8 weeks

of MI (Almeida et al., 2018). MI and ovarian hormone deficiency also increased plasma cytokine levels, which were not prevented by E_2 replacement.

In pre-clinical studies, estrogens showed a variety of effects on cardiovascular regulation that depends on several factors such as, the abundance of ERs type, and the stimulating factor (hypo-/hypertension, MI). E_2 microinjection in the PVN of male rats attenuated glutamate-induced pressor effect via activating GABA receptors in the PVN (Gingerich and Krukoff, 2006). Chronic S.C. E_2 treatment for 10 days decreased c-fos in the SFO but not PVN and RVLM after bolus isoproterenol administration in OVX female rats (Krause et al., 2006, 2007). E_2 microinjection in the RVLM and NTS enhanced baroreflex sensitivity with an increase in vagal parasympathetic nerve activity and decrease in renal sympathetic nerve activity after bolus phenylephrine administration in OVX female rats (Saleh et al., 2000). OVX per se increases AT1R in the PVN and SFO up to 5 weeks as well as heart, kidneys and lung that was prevented by E_2 replacement (Dean et al., 2005). However, OVX decreases total glutamate receptors that expressed ER-β labeled neurons in the PVN of female rats, and ICV Ang II infusion for 2 weeks increased glutamate receptors in ER-β labeled neurons in the PVN of OVX female rats but not in intact female rats (Marques-Lopes et al., 2017). Specific knockdown of ER-β by AAV-siRNA-ERβ in the PVN and RVLM of intact females augments aldosterone/NaCl induced hypertension associated with an increase in reactive oxygen species detected by dihydroethidium in the PVN (Xue et al., 2013). Previous studies showed the effects of E_2 in preventing CVD developing, however E_2 replacement did not attenuate cardiac dysfunction after MI (Bridgman et al., 2005; Shioura et al., 2008; Almeida et al., 2018). Therefore, E_2 replacement may not decrease sympathetic activity after MI, which may be due to the ER-β expression in the PVN of females that is lower than males specifically for

neurons projecting to RVLM (Stern and Zhang, 2003). Estrogens also impair glucocorticoids negative feedback on the HPA axis (Weiser and Handa, 2009).

4.7. Conclusion

Female rats post-MI are protected against depression-like behavior, but ovarian hormone deficiency female rats post-MI develop marked depression-like behavior symptoms and decrease in neurogenesis that can be prevented by E_2 replacement. Neuroinflammation plays a major role in development of depression-like behavior in HF post-MI. Oral PTX treatment reduces cytokines levels in the PFC that contributed to lower mBDNF in the PFC and depression-like behavior. However, E_2 showed different modulation in decreasing cytokines that stimulate inflammation resolution, which needs further studies for better understanding. On the other hand, neither PTX treatment or E_2 replacement attenuate the progression of cardiac dysfunction in post-MI in OVX female rats, suggesting that other mechanisms might contribute to cardiac dysfunction induced by MI in females than inflammation per se. This also needs further study. Understanding the link between depression and HF and underlying mechanisms involved provides alternatives, possibly more effective treatments.

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6. Appendix

6.1. Detailed statistics of chapter 2: sex differences in depression-like behavior and neuroinflammation in rats post-MI: role of estrogens

6.1.1. (*Table 2.1*) Anatomical, echocardiographic and hemodynamic measurement in male and female rats at 10 weeks after MI

Variables	Male sham group	Male MI group	Female sham group	Female MI group
Number of rats/group	8	8	8	10
Body weight, g	605 ± 19.9	593 ± 13.2	368 ± 10.6‡	368 ± 11.2‡
MI size, %	-------	26 ± 1.5	-------	29 ± 1.5
LV/body wt, mg/100g	170 ± 5.0	184 ± 4.5	200 ± 5.2	235 ± 11.2†
Right ventricle /body wt mg/100g	42 ± 2.6	61 ± 12.9*	42 ± 1.7	57 ± 9.4†

Values are means ± SE. *$P < 0.05$ vs. the male sham group; †$P < 0.05$ vs. the female sham group; ‡$P < 0.05$ vs. male groups.

1. Body weight (g) of male and female rats 10 weeks post-MI

Two-way ANOVA evaluates sex x MI interaction

Variable	df	F	Sig.
MI effect	1	0.2	0.7
Sex effect	1	266.7	0.0001
MI x Sex	1	0.2	0.6
Pairwise comparison			
Variables		Sig	
Male groups vs. Female groups		0.0001	
Sham MI groups vs. MI groups		0.7	
Bonferroni post hoc analysis			
Variables		Sig	
Male group	Sham MI vs. MI	0.5	
Female group	Sham MI vs. MI	0.9	

2. MI size (%) of male and female rats 10 weeks post-MI

Student's t-test evaluate MI size: $F = 0.31$, $P = 0.7$

3. LV/Body wt (mg/100g) of male and female rats 10 weeks post-MI

Two-way ANOVA evaluates sex x MI interaction:

Variable	df	F	Sig.
MI effect	1	5.8	0.02
Sex effect	1	16.1	0.0001
MI x Sex	1	1.2	0.3

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.0001
Sham MI groups vs. MI groups	0.02

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.4
Female group	Sham MI vs. MI	0.016

4. RV/Body wt (mg/100g) of male and female rats 10 weeks post-MI

Two-way ANOVA evaluates sex x MI interaction:

Variable	df	F	Sig.
MI effect	1	10.3	0.003
Sex effect	1	0.08	0.8
MI x Sex	1	0.1	0.7

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.8
Sham MI groups vs. MI groups	0.003

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.02
Female group	Sham MI vs. MI	0.05

Echocardiographic measurements

Variables	Male sham group	Male MI group	Female sham group	Female MI group
Number of rats/ group	8	8	8	10
LV end-systolic volume, µL/100g body wt	9 ± 1.7	46 ± 4.3*	5 ± 1.2	50 ± 3.8†
LV end-diastolic volume, µL/100g body wt	63 ± 7.5	99 ± 5.4*	59 ± 4.5	102 ± 6.5†
Ejection fraction, %	86 ± 1.6	49 ± 1.9*	91 ± 1.6	52 ± 1.9†

Values are means ± SE. *$P < 0.05$ vs. the male sham group; †$P < 0.05$ vs. the female sham group; ‡$P < 0.05$ vs. male groups.

5. LV end systolic volume /body wt (µL/100g) of male and female rats 10 weeks post-MI

Two-way ANOVA evaluates sex x MI interaction:

Variable	df	F	Sig.
MI effect	1	135.9	0.0001
Sex effect	1	0.003	0.9
MI x Sex	1	1.5	0.2

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.9
Sham MI groups vs. MI groups	0.0001

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.0001
Female group	Sham MI vs. MI	0.0001

6. LV end diastolic volume /body wt (µL/100g) of male and female rats 10 weeks post-MI

Two-way ANOVA evaluates sex x MI interaction

Variable	df	F	Sig.
MI effect	1	38.1	0.0001
Sex effect	1	0.003	0.9
MI x Sex	1	0.3	0.6

Pairwise comparison		
Variables		Sig
Male groups vs. Female groups		0.9
Sham MI groups vs. MI groups		0.0001
Bonferroni post hoc analysis		
Variables		Sig
Male group	Sham MI vs. MI	0.001
Female group	Sham MI vs. MI	0.0001

7. Ejection fraction (%) of male and female rats 10 weeks post-MI

Two-way ANOVA evaluates sex x MI interaction:

Variable	**df**	**F**	**Sig.**
MI effect	1	442.7	0.0001
Sex effect	1	4.4	0.04
MI x Sex	1	0.1	0.7
Pairwise comparison			
Variables		Sig	
Male groups vs. Female groups		0.04	
Sham MI groups vs. MI groups		0.0001	
Bonferroni post hoc analysis			
Variables		Sig	
Male group	Sham MI vs. MI	0.0001	
Female group	Sham MI vs. MI	0.0001	

Hemodynamic measurements

Variables	**Male sham group**	**Male MI group**	**Female sham group**	**Female MI group**
Number of rats/ group	8	8	8	10
LV peak systolic pressure, mmHg	118 ± 1.9	107 ± 3.9*	125 ± 2.0	112 ± 3.5†
LV end-diastolic pressure, mmHg	3.6 ± 0.3	14.9 ± 1.6*	3.5 ± 0.3	17.1 ± 2.1†
LV dP/dt(+), mmHg/s	7499 ± 176	5864 ± 245*	8108 ± 105	6027 ± 260†
LV dP/dt(-), mmHg/s	6466 ± 130	4474 ± 245*	7092 ± 160	4899 ± 315†

Values are means ± SE. *$P < 0.05$ vs. the male sham group; †$P < 0.05$ vs. the female sham group; ‡$P < 0.05$ vs. male groups.

8. LV peak systolic pressure (mmHg) of male and female rats 10 weeks post-MI

Two-way ANOVA evaluates sex x MI interaction:

Variable	df	F	Sig.
MI effect	1	13.9	0.001
Sex effect	1	3.4	0.07
MI x Sex	1	0.2	0.7
Pairwise comparison			
Variables		Sig	
Male groups vs. Female groups		0.07	
Sham MI groups vs. MI groups		0.001	
Bonferroni post hoc analysis			
Variables		Sig	
Male group	Sham MI vs. MI	0.03	
Female group	Sham MI vs. MI	0.006	

9. LV end diastolic pressure (mmHg) of male and female rats 10 weeks post-MI

Two-way ANOVA evaluates sex x MI interaction:

Variable	df	F	Sig.
MI effect	1	73.6	0.0001
Sex effect	1	0.7	0.4
MI x Sex	1	0.5	0.5
Pairwise comparison			
Variables		Sig	
Male groups vs. Female groups		0.4	
Sham MI groups vs. MI groups		0.0001	
Bonferroni post hoc analysis			
Variables		Sig	
Male group	Sham MI vs. MI	0.0001	
Female group	Sham MI vs. MI	0.0001	

10. LV dP/dt (+) (mmHg/s) of male and female rats 10 weeks post-MI

Two-way ANOVA evaluates sex x MI interaction:

Variable	df	F	Sig.
MI effect	1	73.9	0.0001
Sex effect	1	3.2	0.08
MI x Sex	1	1.1	0.3

Pairwise comparison		
Variables		Sig
Male groups vs. Female groups		0.08
Sham MI groups vs. MI groups		0.0001
Bonferroni post hoc analysis		
Variables		Sig
Male group	Sham MI vs. MI	0.0001
Female group	Sham MI vs. MI	0.0001

11. LV dP/dt (-) (mmHg/s) of male and female rats 10 weeks post-MI

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	76.5	0.0001
Sex effect	1	4.8	0.036
MI x Sex	1	0.2	0.7
Pairwise comparison			
Variables		Sig	
Male groups vs. Female groups		0.04	
Sham MI groups vs. MI groups		0.0001	
Bonferroni post hoc analysis			
Variables		Sig	
Male group	Sham MI vs. MI	0.0001	
Female group	Sham MI vs. MI	0.0001	

6.1.2. (*Table 2.2*) Anatomical, echocardiographic and hemodynamic measurements in ovariectomized female rats without/with estrogen replacement at 10 weeks post-MI

Variables	**sham OVX-sham MI group**	**OVX-sham MI group**	**OVX-E2 sham MI group**	**OVX-MI group**	**OVX-E2-MI group**
Number of rats/group	8	12	10	11	9
Body weight, g	362 ± 7.2	466 ± 14.4*†	381 ± 12.7	437 ± 10.0*†	365 ± 10.1
Uterus/body weight, mg/100g	164 ± 13.1	38 ± 3.4*†	162 ± 14.1	37 ± 1.1*†	184 ± 14.6
MI size, %	-------	-------	-------	27 ± 1.4	27 ± 1.7
LV/body wt, mg/100g	204 ± 3.9	163 ± 3.7*	204 ± 9.1	183 ± 5.8‡	230 ± 7.2‡
Right ventricle /body wt mg/100g	46 ± 1.7	37 ± 1.4 †	42 ± 2.3	40 ± 3.5†	50 ± 2.4

Values are means ± SE. *$P < 0.05$ vs. the sham OVX-sham MI group; †$P < 0.05$ vs. OVX-17β-estradiol (E_2) groups; ‡$P < 0.05$ vs. the related sham MI group.

1. Body weight of OVX female rats with/without E_2 replacement 10 weeks post MI:

One-way ANOVA: F (4) = 15.2 P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	0.0001
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.004
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	3.3	0.08
OVX effect	1	40.8	0.0001
MI x OVX	1	0.3	0.6

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.001
Sham MI groups vs. MI groups	0.08

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.09
OVX + E_2 groups	Sham MI vs. MI	0.4

2. Uterus /body weight (mg/100g) of OVX female rats with/without E_2 replacement 10 weeks post MI:

One-way ANOVA: F (4) = 51.4 P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	0.0001
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.0001
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	df	F	Sig.
MI effect	1	3.1	0.3
OVX effect	1	222.5	0.0001
MI x OVX	1	3.2	0.3

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.0001
Sham MI groups vs. MI groups	0.07

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.9
OVX + E2 groups	Sham MI vs. MI	0.2

3. MI size (%) of OVX female rats with/without E_2 replacement 10 weeks post MI:

Student's t-test evaluate MI size: $F = 1.7$, $P = 0.2$

4. LV/body wt (mg/100g) of OVX female rats with/without E_2 replacement 10 weeks post MI:

One-way ANOVA: $F(4) = 16.4$, $P<0.0001$

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	0.0001
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.5
Sham OVX-Sham MI vs. OVX+E_2-MI	0.08

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	12.8	0.001
OVX effect	1	45.7	0.0001
MI x OVX	1	0.2	0.6

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.001
Sham MI groups vs. MI groups	0.001

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.029
OVX + E2 groups	Sham MI vs. MI	0.008

5. Right ventricle/body wt (mg/100g) of OVX female rats with/without E_2 replacement 10 weeks post MI:

One-way ANOVA: F (4) = 8.7, P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	0.023
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.2
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	df	F	Sig.
MI effect	1	1.6	0.2
OVX effect	1	15.3	0.001
MI x OVX	1	0.002	0.9

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.001
Sham MI groups vs. MI groups	0.2

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.3
OVX + E2 groups	Sham MI vs. MI	0.4

Echocardiographic measurements

Variables	sham OVX-sham MI group	OVX-sham MI group	OVX-E2 sham MI group	OVX-MI group	OVX-E2-MI group
Number of rats / group	8	12	10	11	9
LV end-systolic volume, µL/100g body wt	7 ± 0.7	8 ± 0.7	6 ± 0.4	44 ± 4.1*‡	47 ± 3.4*‡
LV end-diastolic volume, µL/100g body wt	68 ± 5.4	59 ± 1.9	59 ± 2.8	81 ± 6.0‡	103 ± 7.0*‡
Ejection fraction, %	89 ± 0.9	87 ± 0.9	90 ± 0.5	46 ± 2.5*‡	54 ± 2.4*‡

Values are means ± SE. *$P < 0.05$ vs. the sham OVX-sham MI group; †$P < 0.05$ vs. OVX-17β-estradiol (E_2) groups; ‡$P < 0.05$ vs. the related sham MI group.

6. LV end systolic volume /body wt (µL/100g) of OVX female rats with/without E_2 replacement 10 weeks post MI:

One-way ANOVA: F (4) = 76.8, P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.0001
Sham OVX-Sham MI vs. OVX+E_2-MI	0.0001

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	df	F	Sig.
MI effect	1	198.7	0.001
OVX effect	1	0.01	0.9
MI x OVX	1	0.6	0.4

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.9
Sham MI groups vs. MI groups	0.001

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.001
OVX + E2 groups	Sham MI vs. MI	0.001

7. LV end diastolic volume /body wt (μL/100g) of OVX female rats with/without E_2 replacement 10 weeks post MI:

One-way ANOVA: F (4) = 17.4, P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.0001
Sham OVX-Sham MI vs. OVX+E_2-MI	0.03

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	45.6	0.001
OVX effect	1	4.7	0.037
MI x OVX	1	4.6	0.038

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.037
Sham MI groups vs. MI groups	0.001

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.002
OVX + E2 groups	Sham MI vs. MI	0.001

8. Ejection fraction (%) of OVX female rats with/without E_2 replacement 10 weeks post MI:

One-way ANOVA: F (4) = 119.3, P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.0001
Sham OVX-Sham MI vs. OVX+E_2-MI	0.0001

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	458.6	0.0001
OVX effect	1	11	0.002
MI x OVX	1	2.2	0.1

Pairwise comparison	
Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.002
Sham MI groups vs. MI groups	0.0001

Bonferroni post hoc analysis		
Variables		Sig
OVX groups	Sham MI vs. MI	0.0001
OVX + E2 groups	Sham MI vs. MI	0.0001

Hemodynamic measurements

Variables	sham OVX-sham MI group	OVX-sham MI group	OVX-E2 sham MI group	OVX-MI group	OVX-E2-MI group
Number of rats/group	8	12	10	11	9
LV peak systolic pressure, mmHg	128 ± 1.5	127 ± 1.5	130 ± 3.2	121 ± 3.9‡	115 ± 3.9*
LV end-diastolic pressure, mmHg	2.7 ± 0.4	4.1 ± 0.5	3.5 ± 0.3	10.1 ± 0.7*‡	13.3 ± 0.8 *‡
LV dP/dt(+), mmHg/s	8646 ± 261	8773 ± 181	8716 ± 181	6445 ± 257 *‡	6064 ± 282*‡
LV dP/dt(-), mmHg/s	7322 ± 280	7366 ± 141	7572 ± 145	5425 ± 220*‡	5390 ± 271*‡

Values are means ± SE. *$P < 0.05$ vs. the sham OVX-sham MI group; †$P < 0.05$ vs. OVX-17β-estradiol (E_2) groups; ‡$P < 0.05$ vs. the related sham MI group.

9. LV peak systolic pressure (mmHg) of OVX female rats with/without E_2 replacement 10 weeks post MI:

One-way ANOVA: F (4) = 4.3, P<0.005

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	0.05

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	df	F	Sig.
MI effect	1	9.8	0.003
OVX effect	1	0.05	0.8
MI x OVX	1	1.8	0.2

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.8
Sham MI groups vs. MI groups	0.003

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.2
OVX + E2 groups	Sham MI vs. MI	0.004

10. LV end diastolic pressure (mmHg) of OVX female rats with/without E_2 replacement 10 weeks post MI:

One-way ANOVA: F (4) = 61.7, P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	0.8
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.0001
Sham OVX-Sham MI vs. OVX+E_2-MI	0.0001

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	df	F	Sig.
MI effect	1	183.9	0.0001
OVX effect	1	2.8	0.1
MI x OVX	1	11.7	0.001

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.1
Sham MI groups vs. MI groups	0.0001

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.0001
OVX + E2 groups	Sham MI vs. MI	0.0001

11. LV dP/dt(+) (mmHg/s) of OVX female rats with/without E_2 replacement 10 weeks post MI:

One-way ANOVA: F (4) = 37.9, P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.0001
Sham OVX-Sham MI vs. OVX+E_2-MI	0.0001

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	131.1	0.0001
OVX effect	1	1.0	0.3
MI x OVX	1	0.6	0.5

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.3
Sham MI groups vs. MI groups	0.0001

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.0001
OVX + E2 groups	Sham MI vs. MI	0.0001

12. LV dP/dt(-) mmHg/s of OVX female rats with/without E_2 replacement 10 weeks post MI:

One-way ANOVA: F (4) = 24.9, P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.0001
Sham OVX-Sham MI vs. OVX+E_2-MI	0.0001

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	104.4	0.0001
OVX effect	1	0.2	0.7
MI x OVX	1	0.4	0.6

Pairwise comparison		
Variables		Sig
OVX-groups vs. OVX+E_2 groups		0.7
Sham MI groups vs. MI groups		0.0001
Bonferroni post hoc analysis		
Variables		Sig
OVX groups	Sham MI vs. MI	0.0001
OVX + E2 groups	Sham MI vs. MI	0.0001

6.1.3. (*Fig.2.1*) Sucrose preference test in male vs. female rats and OVX female rats with/without E2 replacement post MI

Variables	Male sham MI	Male MI	Female sham MI	Female MI
Number of rats/group	8	8	8	10
Sucrose preference (%)	97 ± 1.1	70 ± 2.2 *	97 ± 0.5	94 ± 2.3
Total liquid intake (ml/100g BW)	16 ± 2.5	19 ± 1.6	16 ± 1.9	20 ± 1.4

Value are means ± SE. *P< 0.0001 vs. male sham MI

1. Sucrose preference (%)

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	67.7	0.0001
Sex effect	1	42.4	.0001
MI x Sex	1	42.4	0.0001
Pairwise comparison			
Variables		Sig	
Male groups vs. Female groups		0.001	
Sham MI groups vs. MI groups		0.0001	
Bonferroni post hoc analysis			
Variables		Sig	
Male group	Sham MI vs. MI	0.0001	
Female group	Sham MI vs. MI	0.3	

2. Total liquid intake (ml/100g BW)

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	2.7	0.1
Sex effect	1	0.02	0.9
MI x Sex	1	0.09	0.8

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.9
Sham MI groups vs. MI groups	0.1

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.2
Female group	Sham MI vs. MI	0.09

Variables	sham OVX-sham MI group	OVX-sham MI group	OVX-E2 sham MI group	OVX-MI group	OVX-E2-MI group
Number of rats/group	8	10	10	9	10
Sucrose preference (%)	96 ± 1.2	88 ± 2.5 [a]	97 ± 0.8	63 ± 8.8 [a b c]	98 ± 0.3
Total liquid intake (ml/100g BW)	22 ± 2.4	13 ± 1.3 [a]	17 ± 1.2	12 ± 2.7 [a]	17 ± 1.2

Value are means ± SE. [a] $P < 0.05$ vs. the sham OVX-sham MI group; [b] $P < 0.05$ vs. OVX-sham MI; [c] $P < 0.05$ vs. 17β-estradiol (E_2) MI group

3. Sucrose preference (%)

One-way ANOVA: $F(4) = 10.3$, P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	0.004
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.005
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	df	F	Sig.
MI effect	1	4.5	0.04
OVX effect	1	29.7	0.001
MI x OVX	1	5.7	0.02

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.001
Sham MI groups vs. MI groups	0.04

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.003
OVX + E_2 groups	Sham MI vs. MI	0.8

4. Total liquid intake (mL/100g BW)

One-way ANOVA: F (4) = 6.3, P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	0.002
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	0.4
Sham OVX-Sham MI vs. OVX-MI	0.001
Sham OVX-Sham MI vs. OVX+E_2-MI	0.3

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	df	F	Sig.
MI effect	1	0.3	0.6
OVX effect	1	6.8	0.013
MI x OVX	1	0.2	0.7

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.013
Sham MI groups vs. MI groups	0.6

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.5
OVX + E2 groups	Sham MI vs. MI	0.9

6.1.4. (*Fig. 2.2.*) Forced swim test in male vs female rats and OVX female rats with/without E2 replacement post-MI

1.Immobility (%) in male vs. female rats post-MI

Variables	Male sham MI	Male MI	Female sham MI	Female MI
Number of rats/group	8	8	8	10
Immobility (%)	42 ± 5.8	59 ± 3.4*	29 ± 4.6 ‡	29 ± 3.3 ‡

Value are means ± SE. * $P < 0.0001$ vs. male sham MI, ‡ $P < 0.0001$ vs. male groups

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	4.7	0.04
Sex effect	1	26.7	0.0001
MI x Sex	1	3.9	0.05

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.001
Sham MI groups vs. MI groups	0.04

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.008
Female group	Sham MI vs. MI	0.9

2. Immobility (%) in OVX female rats with/without E_2 treatment post-MI

Variables	sham OVX-sham MI group	OVX-sham MI group	OVX-E2 sham MI group	OVX-MI group	OVX-E2-MI group
Number of rats/group	8	11	10	10	10
Immobility (%)	39 ± 3.2	51 ± 4.3 [d]	24 ± 3.9	68 ± 5.0 [a b d]	32 ± 3.7

Value are means ± SE. [a] $P < 0.05$ vs. the sham OVX-sham MI group; [b] $P < 0.05$ vs. OVX-sham MI; [d] $P < 0.05$ vs. 17β-estradiol (E_2) groups

One-way ANOVA: F (4) = 21.5, P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	0.9
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	0.5
Sham OVX-Sham MI vs. OVX-MI	0.007
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	df	F	Sig.
MI effect	1	7.3	0.01
OVX effect	1	50.5	0.0001
MI x OVX	1	4.4	0.04

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.0001
Sham MI groups vs. MI groups	0.04

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.002
OVX + E2 groups	Sham MI vs. MI	0.7

6.1.5. (*Fig.2.3.*) Morris water maze in male vs female rats and OVX female rats with/without E2 replacement post-MI

Variables	Male sham MI	Male MI	Female sham MI	Female MI
Number of rats/group	8	8	8	10
Latency to the platform (sec)				
Day 1	37 ± 1.6	30 ± 3.4	42 ± 3.7‡	40 ± 3.5‡
Day 2	18 ± 2.5	22 ± 3.3	37 ± 3.5‡	28 ± 3.7‡
Day 3	14 ± 1.7	18 ± 3.4	28 ± 3.5 ‡	25 ± 4.6‡
Day 4	7 ± 1.1	12 ± 1.7	16 ± 2.6‡	19 ± 3.1‡
% time spent in the target quadrant				
Probe 1	27 ± 2.9	25 ± 2.2	26 ± 2	25 ± 6.8
Probe 2	42 ± 3.4	40 ± 3.6	36 ± 3	34 ± 3.1

Value are means ± SE. ‡ $P < 0.0001$ vs. male groups

1. Training days in male vs. female rats post-MI

Reapeated measures evalute taining days and MI x sex interaction

Variable	Df	F	Sig.
Training effect	3	69.2	0.0001
Training x MI	3	2.5	0.06
Training x sex	3	2.5	0.06
Training x MI x sex	3	1.9	0.14

Pairwise comparison

Variables	Sig
Day 1 vs day 2	0.002
Day 2 vs day 3	0.04
Day 3 vs day 4	0.001

Variable	Df	F	Sig.
MI effect	1	12	0.002
Sex effect	1	0.05	0.8
MI x Sex	1	0.9	0.4

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.8
Sham MI groups vs. MI groups	0.002

Bonferroni post hoc analysis			
Variables		Sig	
Male group	Sham MI vs. MI	Day 1	0.3
		Day 2	0.4
		Day 3	0.5
		Day 4	0.2
Female group	Sham MI vs. MI	Day 1	0.4
		Day 2	0.07
		Day 3	0.6
		Day 4	0.4

2. Probe 1 in male vs. female rats post-MI

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	0.007	0.9
Sex effect	1	0.5	0.5
MI x Sex	1	0.03	0.7

Pairwise comparison	
Variables	Sig
Male groups vs. Female groups	0.9
Sham MI groups vs. MI groups	0.5

Bonferroni post hoc analysis		
Variables		Sig
Male group	Sham MI vs. MI	0.6
Female group	Sham MI vs. MI	0.7

3. Probe 2 in male vs. female rats post-MI

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	0.9	0.3
Sex effect	1	4.8	0.03
MI x Sex	1	0.05	0.8

Pairwise comparison	
Variables	Sig
Male groups vs. Female groups	0.03
Sham MI groups vs. MI groups	0.3

Bonferroni post hoc analysis		
Variables		Sig
Male group	Sham MI vs. MI	0.6
Female group	Sham MI vs. MI	.04

Morris water maze test in OVX female rats with/without E_2 replacement post-MI

Variables	sham OVX-sham MI group	OVX-sham MI group	OVX-E2 sham MI group	OVX-MI group	OVX-E2-MI group
Number of rats/group	8	10	9	7	8
Latency to the platform (sec)					
Day 1	37 ± 3.3	36 ± 3.4	37 ± 3.6	43 ± 3.1	41 ± 3.1
Day 2	25 ± 2.1	30 ± 2	24 ± 2.7	33 ± 2.3	18 ± 2.2
Day 3	15 ± 2.7	17 ± 2.6	19 ± 2.9	18 ± 0.7	16 ± 2.8
Day 4	12 ± 2.1	16 ± 1.7	15 ± 3.1	20 ± 1.7	12 ± 1.8
% time spent in the target quadrant					
Probe 1	27 ±2.3	32 ±3	33 ±3.6	22 ±1.4 [a b c]	36 ±2.5
Probe 2	32 ±2.4	33 ±2.3	39 ±3.2	32 ±3.1	37 ± 2

Value are means ± SE. [a] $P < 0.05$ vs. the sham OVX-sham MI group; [b] $P < 0.05$ vs. OVX-sham MI; [c] $P < 0.05$ vs. 17β-estradiol (E_2)-MI group

4. Training days in OVX female rats with/without E2 replacement post-MI

Reapeated measures evalute taining days vs all groups including Sham OVX-sham MI

Variable	Df	F	Sig.
Training	3	65.5	0.0001
Training x groups	12	0.8	0.7
Pairwise comparison			
Variables		Sig	
Day 1 vs day 2		0.0001	
Day 2 vs day 3		0.0001	
Day 3 vs day 4		0.8	

Reapeated measures evalute taining days vs all groups excluding Sham OVX-sham MI

Variable	Df	F	Sig.
Training	3	44.7	0.0001
Training x MI	3	0.7	0.7
Training x OVX	3	1.7	0.4
Training x OVX x MI	3	1.6	0.2

Variable	Df	F	Sig.
MI effect	1	0.4	0.5
OVX effect	1	1.0	0.3
MI x OVX	1	3.4	0.07

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.3
Sham MI groups vs. MI groups	0.5

Bonferroni post hoc analysis

Variables		Sig	
OVX groups	Sham MI vs. MI	Day 1	0.9
		Day 2	0.5
		Day 3	0.4
		Day 4	0.4
OVX +E_2 groups	Sham MI vs. MI	Day 1	0.6
		Day 2	0.01
		Day 3	0.1
		Day 4	0.2

5. Probe 1 in OVX female rats with/without E2 replacement post-MI

One-way ANOVA: F (4) = 3.5, P=0.02

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.7
Sham OVX-Sham MI vs. OVX+E_2-MI	0.8

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	df	F	Sig.
MI effect	1	1.8	0.2
OVX effect	1	4.4	0.02
MI x OVX	1	5.7	0.02

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.02
Sham MI groups vs. MI groups	0.3

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.014
OVX + E2 groups	Sham MI vs. MI	0.5

6. Probe 2 in OVX female rats with/without E2 replacement post-MI

One-way ANOVA: F (4) = 1.4, P=0.2

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	0.5	0.5
OVX effect	1	2.7	0.08
MI x OVX	1	0.06	0.8

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.07
Sham MI groups vs. MI groups	0.5

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.8
OVX + E2 groups	Sham MI vs. MI	0.5

6.1.6. (*Table 2.3.*) Locomotor activity in the first training session of the Morris water maze

	Number of rats/ group	Total path length, m	Speed, cm/s
Male vs Female rats			
Male sham group	7	14 ± 2.0	26 ± 1.5
Male MI group	8	15 ± 0.8	26 ± 1.3
Female sham group	8	11 ± 1.8	25 ± 1.8
Female MI group	9	15 ± 0.6	28 ± 0.8
Ovariectomized Female rats			
Sham OVX-Sham MI group	9	15 ± 1.0	28 ± 1.4
OVX-Sham MI group	12	15 ± 1.5	28 ± 1.7
OVX-E_2-Sham MI group	10	15 ± 0.5	31 ± 0.8
OVX-MI group	9	14 ± 0.9	28 ± 0.8
OVX-E2-MI group	9	16 ± 0.7	27± 1.1

Values are means ±SE. Two-way ANOVA was performed for sex x myocardial infarction (MI) and ovariectomy (OVX) x MI interactions. E2, 17β-estradiol. No significant differences in locomotor activity were observed

1. Total path length (m) male vs female rats post -MI

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	0.007	0.9
Sex effect	1	0.5	0.5
MI x Sex	1	0.03	0.7

2. Speed (cm/s) male vs female rats post -MI

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	0.08	0.8
Sex effect	1	0.7	0.1
MI x Sex	1	0.9	0.3

3. Total path length (m) in OVX female rats with/without E2 replacement post-MI

One-way ANOVA: F (4) = 2.4, P=0.065

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	0.5
Sham OVX-Sham MI vs. OVX-MI	0.04
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	df	F	Sig.
MI effect	1	1.3	0.3
OVX effect	1	2.9	0.09
MI x OVX	1	0.8	0.4

4. Speed (cm/s) in OVX female rats with/without E2 replacement post-MI

One-way ANOVA: F (4) = 2.6, P=0.052

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	0.4
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.04
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	df	F	Sig.
MI effect	1	1.3	0.3
OVX effect	1	3.1	0.08
MI x OVX	1	0.8	0.4

6.1.7. (*Fig.2.4.*) Fear conditioning test in male vs female rats and OVX female rats with/without E_2 replacement post-MI

Fear conditioning test in male vs female rats post -MI

Variables	Male sham MI	Male MI	Female sham MI	Female MI
Number of rats/group	7	8	8	10
Freezing (%) in habituation	18 ±2.9	11 ± 2	2 ± 0.5	2 ± 0.5
Freezing (%) in context test	19 ± 3.3	21 ± 3.3	7 ± 1.1§	9 ± 1.9§
Freezing (%) in Cued test				
Pre-Tone	5 ± 1.6	7 ± 1.1	3 ± 0.7	2 ± 0.7
Tone (1 min)	25 ± 9†	52 ± 7*†	32 ± 10.3†	26 ± 9†
Tone (2 min)	22 ± 6.4†	49 ± 10*†	36 ± 10.3†	21 ± 9†
Tone (3 min)	15 ± 6.5†	48 ± 10.5*†	20 ± 7.2†	20 ±9.6†

Value are means ± SE. § $P < 0.0001$ vs. First day habituation, *$P< 0.001$ vs. male sham group, † $P<0.001$ vs. pre-tone

1. Context fear conditioning test (% freezing behaviour) in male vs. female rats post-MI

Reapeated measures evalute taining days and MI x sex interaction

Variable	Df	F	Sig.
Context effect	1	15.9	0.0001
Context x MI	1	3.1	0.09
Context x sex	1	0.09	0.8
Context x MI x sex	1	2.02	0.2

Pairwise comparison

Variables	Sig
Habituation vs day 2	0.0001

Variable	Df	F	Sig.
MI effect	1	0.3	0.6
Sex effect	1	58.7	0.0001
MI x Sex	1	1.4	0.3

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.0001
Sham MI groups vs. MI groups	0.6

Bonferroni post hoc analysis			
Variables		Sig	
Male group	Sham MI vs. MI	Habituation	0.009
		Day 2	0.7
Female group	Sham MI vs. MI	Habituation	0.8
		Day 2	0.7

2. Cued fear conditioning test (% freezing) in male vs. female rats post-MI

Reapeated measures evalute taining days and MI x sex interaction

Variable	**Df**	**F**	**Sig.**
Cued effect	4	10.4	0.0001
Cued x MI	3	2.02	0.12
Cued x sex	3	1.7	0.2
Cued x MI x sex	3	1.3	0.3

Pairwise comparison

Variables	Sig
Pre-tone vs 1 min	0.0001
Pre-tone vs 2 min	0.0001
Pre-tone vs 3 min	0.0001

Variable	**Df**	**F**	**Sig.**
MI effect	1	1.5	0.2
Sex effect	1	1.6	0.2
MI x Sex	1	4.2	0.049

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.2
Sham MI groups vs. MI groups	0.2

Bonferroni post hoc analysis

Variables		Sig	
Male group	Sham MI vs. MI	Pre-tone	0.2
		1 min	0.04
		2 min	0.06
		3 min	0.02
Female group	Sham MI vs. MI	Pre-tone	0.3
		1 min	0.6
		2 min	0.3
		3 min	0.9

Fear conditioning test OVX female rats with/without E_2 post-MI

Variables	sham OVX-sham MI group	OVX-sham MI group	OVX-E2 sham MI group	OVX-MI group	OVX-E2-MI group
Number of rats/group	9	12	10	10	9
Freezing (%) in habituation	1 ± 0.5	3 ± 0.6	1 ± 0.5	15 ± 2.2 [a]	7 ± 1.4 [a]
Freezing (%) in context test	7 ± 1.4	51 ± 5.6 [a d] §	25 ± 3.4 [a]§	48 ± 3.6 [a d] §	28 ±5 [a]§
Freezing (%) in Cued test					
Pre-Tone	3 ± 0.6	5 ± 1.1	4 ± 0.8	11 ±1.3	2 ± 0.6
Tone (1 min)	27 ± 7.9†	46 ± 8.3†	32 ± 5.5†	78 ± 3.3 [a b c]†	38 ± 7†
Tone (2 min)	26 ± 6.7†	56 ± 10.8†	26 ± 6.7†	84 ± 2 [a b c]†	41 ± 7.5†
Tone (3 min)	19 ± 7.1†	43 ± 9.2†	22 ± 9.2†	90 ± 3.7 [a b c]†	39 ±8.9†

Value are means ± SE. § $P < 0.0001$ vs. First day habituation, [a] $P< 0.05$ vs. Sham OVX-Sham MI, [b] $P<0.05$ vs. OVX-Sham MI group, [c] $P<0.05$ the OVX +E_2-MI group, [d] P<0.05 vs OVX+E_2 groups, † $P<0.001$ vs. pre-tone

3. Context fear conditioning test (% freezing) in OVX female rats with/without E_2 replacement post-MI

Reapeated measures evalute taining days vs all groups including Sham OVX-sham MI

Variable	Df	F	Sig.
Context effect	1	194	0.0001
Context x groups	4	13.9	0.001
Pairwise comparison			
Variables		Sig	
Habituation vs day 2		0.0001	

Reapeated measures evalute taining days vs all groups excluding Sham OVX-sham MI

Variable	Df	F	Sig.
Context effect	1	190.6	0.0001
Context x MI	1	3.8	0.059
Context x OVX	1	15.9	0.0001
Context x OVX x MI	1	1.8	0.2

Variable	Df	F	Sig.
MI effect	1	2.9	0.09
OVX effect	1	25.7	0.0001
MI x OVX	1	0.002	0.9

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.0001
Sham MI groups vs. MI groups	0.09

Bonferroni post hoc analysis

Variables		Sig	
OVX groups	Sham MI vs. MI	Habituation	0.001
		Day 2	0.6
OVX +E_2 groups	Sham MI vs. MI	Habituation	0.008
		Day 2	0.6

4. Cued conditioning test (% freezing) in OVX female rats with/without E2 replacement post-MI

Reapeated measures evalute taining days vs all groups including Sham OVX-sham MI

Variable	Df	F	Sig.
Cued effect	3	97.1	0.0001
Cued x groups	12	5.4	0.0001

Pairwise comparison

Variables	Sig
Pre-tone vs I min	0.0001
Pre-tone vs 2 min	0.0001
Pre-tone vs 3 min	0.0001

Reapeated measures evalute taining days vs all groups excluding Sham OVX-sham MI

Variable	Df	F	Sig.
Cued effect	3	98.8	0.0001
Cued x MI	3	7.4	0.01
Cued x OVX	3	20.1	0.001
Cued x OVX x MI	3	0.2	0.7

Variable	Df	F	Sig.
MI effect	1	7.5	0.01
OVX effect	1	25.3	0.0001
MI x OVX	1	1.3	0.7

Pairwise comparison			
Variables		Sig	
OVX-groups vs. OVX+E_2 groups		0.001	
Sham MI groups vs. MI groups		0.01	
Bonferroni post hoc analysis			
Variables		Sig	
OVX groups	Sham MI vs. MI	Pre-tone	0.006
		1 min	0.01
		2 min	0.05
		3 min	0.01
OVX +E_2 groups	Sham MI vs. MI	Pre-tone	0.4
		1 min	0.5
		2 min	0.2
		3 min	0.2

6.1.8. (*Table 2.4*) Plasma cytokine levels increase in both male and female rats 10 wk post-MI

Cytokine, pg/ml	Male sham group n=4	Male MI group n=4	Female sham group n=5	Female MI group n=6
IL-1α	43 ± 5.4	96 ± 23.1*	41 ± 7.4	75 ± 5.4†
IL-1β	220 ± 32.9	223 ± 50.3	188 ± 44.3	145 ± 22.8
IL-2	88 ± 11.1	123 ± 8.8	95 ± 20.9	142 ± 23.8
IL-4	7 ± 0.6	12 ± 1.5*	7 ± 1.1	11 ± 0.6†
IL-5	79 ± 21.7	180 ± 26.0 *	83 ± 19.9	155 ± 7.0†
IL-6	145 ± 17.8	492 ± 184.1*	126 ± 46.3	279 ± 18.5†
IL-10	228 ± 35.8	442 ± 74.2*	255 ± 46.7	370 ± 27.5
IL-12	18 ± 3.4	47 ± 5.8*	21 ± 3.8	42 ± 2.2†
IL-13	28 ± 5.7	49 ± 8.9	35 ± 12.5	41 ± 1.4
TNF-α	127 ± 7.5	290 ± 16.7*	119 ± 15.9	262 ± 6.6†
IFN-ɣ	75 ± 14.8	169 ± 26.2*	62 ± 9.2	141 ± 4.9†

Values are means ± SE, * P <0.05 vs. male sham MI group , † P <0.05 vs. female sham MI group.

1. IL-1 α

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	14.9	0.002
Sex effect	1	1.1	0.3
MI x Sex	1	0.7	0.4

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.3
Sham MI groups vs. MI groups	0.002

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.007
Female group	Sham MI vs. MI	0.03

2. IL-1 β

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	0.3	0.6
Sex effect	1	2.1	0.2
MI x Sex	1	0.4	0.6

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.2
Sham MI groups vs. MI groups	0.6

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.9
Female group	Sham MI vs. MI	0.4

3. IL-2

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	0.5	0.5
Sex effect	1	0.2	0.7
MI x Sex	1	0.6	0.8

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.7
Sham MI groups vs. MI groups	0.06

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.8
Female group	Sham MI vs. MI	0.5

4. IL-4

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	19.1	0.001
Sex effect	1	0.05	0.8
MI x Sex	1	0.8	0.4

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.8
Sham MI groups vs. MI groups	0.001

Bonferroni post hoc analysis		
Variables		Sig
Male group	Sham MI vs. MI	0.03
Female group	Sham MI vs. MI	0.02

5. IL-5

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	22.5	0.0001
Sex effect	1	0.4	0.6
MI x Sex	1	0.6	0.5

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.6
Sham MI groups vs. MI groups	0.0001

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.002
Female group	Sham MI vs. MI	0.008

6. IL-6

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	24.7	0.0001
Sex effect	1	1.5	0.2
MI x Sex	1	0.1	0.9

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.2
Sham MI groups vs. MI groups	0.0001

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.006
Female group	Sham MI vs. MI	0.002

7. IL-10

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	12.7	0.003
Sex effect	1	0.2	0.6
MI x Sex	1	1.2	0.3

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.6
Sham MI groups vs. MI groups	0.003

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.008
Female group	Sham MI vs. MI	0.07

8. IL-12

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	42.5	0.0001
Sex effect	1	0.04	0.8
MI x Sex	1	1.2	0.3

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.8
Sham MI groups vs. MI groups	0.0001

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.0001
Female group	Sham MI vs. MI	0.001

9. IL-13

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	2.8	0.1
Sex effect	1	0.001	0.9
MI x Sex	1	0.8	0.4

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.9
Sham MI groups vs. MI groups	0.1

Bonferroni post hoc analysis		
Variables		Sig
Male group	Sham MI vs. MI	0.1
Female group	Sham MI vs. MI	0.6

10. TNF-α

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	158.7	0.0001
Sex effect	1	2.1	0.2
MI x Sex	1	2.1	0.2

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.2
Sham MI groups vs. MI groups	0.0001

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.0001
Female group	Sham MI vs. MI	0.0001

11. IFN-γ

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	39.4	0.0001
Sex effect	1	2.2	0.2
MI x Sex	1	0.3	0.6

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.2
Sham MI groups vs. MI groups	0.0001

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.0001
Female group	Sham MI vs. MI	0.001

6.1.9. (*Fig.2.5*) Cytokine levels in the PFC and PVN in male and female with HF at 10 wk post-MI

PFC in male vs female

Cytokine, pg/ml	Male sham group n=4	Male MI group n=4	Female sham group n=5	Female MI group n=6
IL-1α	28 ±11.9	42 ± 1.6	48 ± 3.4‡	47 ± 1.4
IL-1β	36 ± 6.7	53 ± 1.6*	45 ± 7.9	49 ± 0.8
IL-2	132 ± 4.2	262 ± 30.8*	137 ± 23.9	153 ± 23.8
IL-6	212 ± 50.7	340 ± 12.2*	315 ± 63.4‡	377 ± 8.6
IL-10	231 ± 31.3	375 ± 11.3*	356 ± 52.9‡	377 ± 8.6
TNF-α	114 ± 19	175 ± 5.9*	156 ± 13.2	156 ± 2.1

Values are means ± SE, * P <0.05 vs. male sham MI group , ** P <0.05 vs. female sham MI group, ‡ P<0.0001 vs. the male sham MI group.

1. IL-1 α

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	0.1	0.9
Sex effect	1	5.5	0.3
MI x Sex	1	0.2	0.6

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.3
Sham MI groups vs. MI groups	0.9

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.7
Female group	Sham MI vs. MI	0.8

2. IL-1 β

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	4.5	0.05
Sex effect	1	0.2	0.6
MI x Sex	1	12.9	0.002

Pairwise comparison		
Variables		Sig
Male groups vs. Female groups		0.6
Sham MI groups vs. MI groups		0.05
Bonferroni post hoc analysis		
Variables		Sig
Male group	Sham MI vs. MI	0.001
Female group	Sham MI vs. MI	0.3

3. IL-2

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	3.9	0.06
Sex effect	1	5.5	0.3
MI x Sex	1	3.8	0.06
Pairwise comparison			
Variables		Sig	
Male groups vs. Female groups		0.06	
Sham MI groups vs. MI groups		0.06	
Bonferroni post hoc analysis			
Variables		Sig	
Male group	Sham MI vs. MI	0.01	
Female group	Sham MI vs. MI	0.9	

4. IL-6

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	6.5	0.02
Sex effect	1	11.7	0.003
MI x Sex	1	0.5	0.5
Pairwise comparison			
Variables		Sig	
Male groups vs. Female groups		0.003	
Sham MI groups vs. MI groups		0.02	
Bonferroni post hoc analysis			
Variables		Sig	
Male group	Sham MI vs. MI	0.04	
Female group	Sham MI vs. MI	0.2	

5. IL-10

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	19.7	0.001
Sex effect	1	36	0.001
MI x Sex	1	25.3	0.001

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.001
Sham MI groups vs. MI groups	0.001

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.001
Female group	Sham MI vs. MI	0.8

6. TNF-α

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	4.0	0.06
Sex effect	1	0.3	0.6
MI x Sex	1	17.7	0.001

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.6
Sham MI groups vs. MI groups	0.06

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.001
Female group	Sham MI vs. MI	0.9

PVN in male vs female

Cytokine, pg/ml	Male sham group n=4	Male MI group n=4	Female sham group n=5	Female MI group n=6
IL-1α	23 ± 6	43 ± 3.5*	13 ± 2.7‡	34 ± 5.6**
IL-1β	27 ± 9.5	44 ± 3.2*	15 ± 1.8‡	34 ± 5.5**
IL-2	59 ± 17.2	101 ± 15.1*	38 ± 4.4	90 ± 1.1**
IL-6	114 ± 37.6	231 ± 8.2*	96 ± 27.9	189 ± 30**
IL-10	188 ± 32.2	326 ± 35.1*	181 ± 35.9	286 ± 32.9**
TNF-α	83 ± 16.6	131 ± 13.8*	73 ± 7.8	120 ± 11.1**

Values are means ± SE, * P <0.05 vs. male sham MI group , ** P <0.05 vs. female sham MI group, ‡ P<0.0001 vs. the male sham MI group.

1. IL-1 α

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	17.7	0.001
Sex effect	1	4.1	0.06
MI x Sex	1	0.03	0.8

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.06
Sham MI groups vs. MI groups	0.001

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.02
Female group	Sham MI vs. MI	0.004

2. IL-1 β

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	10.7	0.005
Sex effect	1	3.9	0.06
MI x Sex	1	0.07	0.8

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.02
Sham MI groups vs. MI groups	0.004

Bonferroni post hoc analysis		
Variables		Sig
Male group	Sham MI vs. MI	0.06
Female group	Sham MI vs. MI	0.01

3. IL-2

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	13.9	0.002
Sex effect	1	1.6	0.2
MI x Sex	1	0.2	0.7

Pairwise comparison	
Variables	Sig
Male groups vs. Female groups	0.2
Sham MI groups vs. MI groups	0.002

Bonferroni post hoc analysis		
Variables		Sig
Male group	Sham MI vs. MI	0.04
Female group	Sham MI vs. MI	0.006

4. IL-6

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	13.1	0.002
Sex effect	1	1.1	0.3
MI x Sex	1	0.2	0.7

Pairwise comparison	
Variables	Sig
Male groups vs. Female groups	0.3
Sham MI groups vs. MI groups	0.002

Bonferroni post hoc analysis		
Variables		Sig
Male group	Sham MI vs. MI	0.02
Female group	Sham MI vs. MI	0.03

5. IL-10

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	11.7	0.003
Sex effect	1	0.4	0.5
MI x Sex	1	0.2	0.6

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.5
Sham MI groups vs. MI groups	0.003

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.02
Female group	Sham MI vs. MI	0.04

6. TNF-α

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	14.3	0.002
Sex effect	1	0.7	0.4
MI x Sex	1	0.001	0.9

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.4
Sham MI groups vs. MI groups	0.002

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.02
Female group	Sham MI vs. MI	0.01

6.1.10. (*Table 2.5*) Cytokine levels in the amygdala of male and female rats are unchanged at 10 weeks post-MI

Cytokine, pg/mg	Male sham n=4	Male MI n=5	Female sham n=5	Female MI n=6
IL-1α	24 ± 6.0	29 ± 5.9	31 ± 2.3	29 ± 3.3
IL-1β	28 ± 7.5	36 ± 7.1	29 ± 6.0	33 ± 4.4
IL-2	49 ± 8.4	41 ± 3.8	50 ± 6.3	41 ± 2.0
IL-6	151 ± 45.0	205 ± 43.5	226 ± 16.8	201 ± 34.2
IL-10	217 ± 51.5	259 ± 44.0	267 ± 15.0	250 ± 28.5
TNF-α	124 ± 33.2	150 ± 27.6	226 ± 16.8	201 ± 34.0

Values are means ± SE; *n*, number of rats/group.

1. IL-1 α

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	0.02	0.8
Sex effect	1	0.4	0.5
MI x Sex	1	0.6	0.5

2. IL-1 β

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	0.5	0.5
Sex effect	1	0.06	0.8
MI x Sex	1	0.3	0.6

3. IL-2

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	2.5	0.1
Sex effect	1	0.003	0.9
MI x Sex	1	0.06	0.8

4. IL-6

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	0.0001	0.9
Sex effect	1	0.3	0.7
MI x Sex	1	2.1	0.2

5. IL-10

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	0.03	0.9
Sex effect	1	0.1	0.7
MI x Sex	1	0.8	0.4

6. TNF-α

Two-way ANOVA evaluates sex x MI interaction:

Variable	Df	F	Sig.
MI effect	1	0.2	0.7
Sex effect	1	5.3	0.03
MI x Sex	1	1.8	0.2

6.1.11. (*Table 2.6*) Plasma cytokine levels in ovariectomized female rats with or without E_2 replacement at 10 weeks post-MI

Cytokine, pg/ml	**Sham OVX-sham MI n=6**	**OVX-sham MI n=7**	**OVX-E2-sham MI n=7**	**OVX-MI n=10**	**OVX-E2-MI n=9**
IL-1α	74 ± 9.8	70 ± 9.1	52 ± 11.3	82 ± 9.8	104 ± 19.9
IL-1β	144 ± 46.1	213 ± 27.5	399 ± 86.5	135 ± 20.3	207 ± 60.1
IL-2	123 ± 16.4	131 ± 65.4	82 ± 9.0	193 ± 27.3*	138 ± 9.8*
IL-6	479 ± 85.1	439 ± 109.3	357 ± 82.0	928 ± 173.6*	630± 54.2*
IL-10	107 ± 26.2	82 ± 19.6	33 ± 5.9	131 ± 24.2*	203 ± 34.9*†
TNF-α	213 ± 49.1	149 ± 39.7	76 ± 6.9	288 ± 53.8*	396 ± 64.4*

Values are means ±SE; *n*, number of rats/group. **P* <0.05 vs. the related sham MI group; †*P* <0.05 vs. the OVX-MI group.

1. IL-1 α

One-way ANOVA: $F(4) = 2.1$, $P=0.09$

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	df	F	Sig.
MI effect	1	12.1	0.003
OVX effect	1	2.9	0.1
MI x OVX	1	0.5	0.5

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.1
Sham MI groups vs. MI groups	0.003

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.06
OVX + E2 groups	Sham MI vs. MI	0.01

2. IL-1 β

One-way ANOVA: F (4) = 3.6, P=0.015

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	0.3
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	0.1
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	df	F	Sig.
MI effect	1	15.4	0.001
OVX effect	1	0.2	0.7
MI x OVX	1	1.8	0.2

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.7
Sham MI groups vs. MI groups	0.001

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.001
OVX + E2 groups	Sham MI vs. MI	0.09

3. IL-2

One-way ANOVA: F (4) = 2.5, P=0.067

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	0.2
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	4.5	0.04
OVX effect	1	3.8	0.06
MI x OVX	1	0.015	0.9

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.06
Sham MI groups vs. MI groups	0.04

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.12
OVX + E2 groups	Sham MI vs. MI	0.17

4. IL-6

One-way ANOVA: F (4) = 6.01, P=0.001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	0.7
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	0.2

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	5.0	0.04
OVX effect	1	0.4	0.5
MI x OVX	1	1.3	0.3

Pairwise comparison		
Variables		Sig
OVX-groups vs. OVX+E_2 groups		0.5
Sham MI groups vs. MI groups		0.04
Bonferroni post hoc analysis		
Variables		Sig
OVX groups	Sham MI vs. MI	0.03
OVX + E2 groups	Sham MI vs. MI	0.4

5. IL-10

One-way ANOVA: F (4) = 2.3, P=0.002

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	0.5
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	0.4

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	29.4	0.0001
OVX effect	1	0.6	0.5
MI x OVX	1	2.4	0.1
Pairwise comparison			
Variables		Sig	
OVX-groups vs. OVX+E_2 groups		0.8	
Sham MI groups vs. MI groups		0.0001	
Bonferroni post hoc analysis			
Variables		Sig	
OVX groups	Sham MI vs. MI	0.001	
OVX + E2 groups	Sham MI vs. MI	0.0001	

6. TNF-α

One-way ANOVA: F (4) = 8.7, P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	0.4
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	0.3
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	0.2

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	29.7	0.0001
OVX effect	1	0.016	0.9
MI x OVX	1	0.3	0.6

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.9
Sham MI groups vs. MI groups	0.0001

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.002
OVX + E2 groups	Sham MI vs. MI	0.001

6.1.12. (*Fig 2.6.*) Cytokine levels in the PFC of OVX female rats with or without E2 at 10 wk post-MI

Cytokine, pg/ml	**Sham OVX-sham MI n=6**	**OVX-sham MI n=7**	**OVX-E2-sham MI n=7**	**OVX-MI n=10**	**OVX-E2-MI n=9**
IL-1α	65 ± 16.8	71 ± 6.7	77 ± 6.4	86 ± 16	49 ± 3.1
IL-1β	92 ± 14.7	108 ± 6.7	106 ± 5.8	120 ± 11.2	90 ± 4.2
IL-2	147 ± 21.6	158 ± 25.6	164 ± 27	233 ± 27.1[c]	213 ± 18.5 [c]
IL-6	342 ± 24.5	325 ± 45.3	375 ± 30.6	555 ± 40.2 [c d]	326 ± 12.2
IL-10	142 ± 32.2	148 ± 9.8	163 ± 10.7	153 ± 19.2	280 ± 12.2 [c d]
TNF-α	327 ± 85.5	423 ± 49	370 ± 31	448 ± 63.9	114 ± 5.5 [c d]

Values are means ±SE; n, number of rats/group. [c] P <0.05 vs. the related sham MI group; [d]P <0.05 vs. the OVX-E_2 group.

1. IL-1 α

One-way ANOVA: F (4) = 1.4, P=0.3

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

2. IL-1 β

One-way ANOVA: F (4) = 1.4, P=0.3

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.3
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

3. IL-2

One-way ANOVA: F (4) = 3.6, P=0.02

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	0.02
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	0.1

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	5.4	0.03
OVX effect	1	0.09	0.8
MI x OVX	1	1.1	0.3

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.8
Sham MI groups vs. MI groups	0.03

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.03
OVX + E2 groups	Sham MI vs. MI	0.04

4. IL-6

One-way ANOVA: F (4) = 6.9, P=0.001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	0.005
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	0.003
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	0.1

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	4.4	0.05
OVX effect	1	0.6	0.4
MI x OVX	1	5.9	0.02

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.4
Sham MI groups vs. MI groups	0.05

Bonferroni post hoc analysis

Variables		Sig
Sham MI	OVX vs. OVX+E_2	0.3
MI	OVX vs. OVX+E_2	0.02

5. IL-10

One-way ANOVA: F (4) = 9.1, P<0.0001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	0.0001

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	12.3	0.002
OVX effect	1	16.7	0.0001
MI x OVX	1	12.5	0.002

Pairwise comparison		
Variables		Sig
OVX-groups vs. OVX+E_2 groups		0.0001
Sham MI groups vs. MI groups		0.002
Bonferroni post hoc analysis		
Variables		Sig
Sham MI	OVX vs. OVX+E_2	0.7
MI	OVX vs OVX+E_2	0.0001

6. TNF-α

One-way ANOVA: F (4) = 5.9, P<0.001

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	0.2

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	7.5	0.01
OVX effect	1	12.7	0.001
MI x OVX	1	6.2	0.02
Pairwise comparison			
Variables		Sig	
OVX-groups vs. OVX+E_2 groups		0.001	
Sham MI groups vs. MI groups		0.01	
Bonferroni post hoc analysis			
Variables		Sig	
Sham MI	OVX vs. OVX+E_2	0.5	
MI	OVX vs OVX+E_2	0.0001	

6.1.13. (*Fig 2.7.*) mBDNF protein expression in the PFC and amygdala of male and female rats with heart failure at 10 wks post-MI

mBDNF/β-Actin	**Male sham n=4**	**Male MI n=5**	**Female sham n=5**	**Female MI n=6**
PFC	1 ± 0.1	0.6 ± 0.05	1.0 ± 0.16	1.2 ± 0.13
Amygdala	1 ± 0.08	1.6 ± 0.17†	0.9 ± 0.2	0.7 ± 0.12

Values are means ± SE,† P <0.05 vs. female MI group.

1. PFC

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	0.4	0.5
Sex effect	1	5.1	0.03
MI x Sex	1	2.8	0.07

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.03
Sham MI groups vs. MI groups	0.5

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.08
Female group	Sham MI vs. MI	0.1

2. Amygdala

Two-way ANOVA evaluates sex x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	0.9	0.3
Sex effect	1	7.8	0.01
MI x Sex	1	5.4	0.03

Pairwise comparison

Variables	Sig
Male groups vs. Female groups	0.3
Sham MI groups vs. MI groups	0.01

Bonferroni post hoc analysis

Variables		Sig
Male group	Sham MI vs. MI	0.04
Female group	Sham MI vs. MI	0.3

6.1.13. (*Fig 2.8.)* mBDNF protein expression in the PFC and amygdala of OVX female rats with heart failure at 10 wk post-MI

mBDNF/β-Actin	**Sham OVX-sham MI n=7**	**OVX-sham MI n=9**	**OVX-E2-sham MI n=7**	**OVX-MI n=13**	**OVX-E2-MI n=9**
PFC	1 ± 0.1	0.9 ± 0.04	1.1 ± 0.07	0.8 ± 0.07 [b c]	0.9 ± 0.1
Amygdala	1 ± 0.1	0.8 ± 0.09	1.3 ± 0.1 [a b]	1.3 ± 0.1 [c]	1.4 ± 0.1 [a]

Values are means ± SE, [a] $P< 0.05$ vs. Sham OVX-Sham MI, [b] $P<0.05$ vs. OVX-Sham MI group, [c] $P<0.05$ the related sham MI group

1.PFC

One-way ANOVA: F (4) = 3.1, P=0.025

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	0.2
Sham OVX-Sham MI vs. OVX+E_2-MI	1.0

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	4.3	0.05
OVX effect	1	6.5	0.02
MI x OVX	1	0.6	0.5

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.016
Sham MI groups vs. MI groups	0.046

Bonferroni post hoc analysis

Variables		Sig
OVX groups	Sham MI vs. MI	0.04
OVX + E2 groups	Sham MI vs. MI	0.4

2. Amygdala

One-way ANOVA: F (4) = 3.6, P=0.014

Bonferroni post hoc analysis

Variables	Sig
Sham OVX-Sham MI vs. OVX-Sham MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-Sham MI	1.0
Sham OVX-Sham MI vs. OVX-MI	1.0
Sham OVX-Sham MI vs. OVX+E_2-MI	0.3

Followed by two-way ANOVA: (exclude Sham OVX-sham MI)

Variable	**df**	**F**	**Sig.**
MI effect	1	4.7	0.04
OVX effect	1	5.5	0.02
MI x OVX	1	1.6	0.2

Pairwise comparison

Variables	Sig
OVX-groups vs. OVX+E_2 groups	0.024
Sham MI groups vs. MI groups	0.04

Bonferroni post hoc analysis

Variables		Sig
OVX	Sham MI vs MI	0.013
OVX+E_2	Sham MI vs MI	0.6

6.2. Detailed statistics of chapter 3: role of myocardial infarction-induced neuroinflammation for depression-like behavior and heart failure in ovariectomized female rats

6.2.1. (*Table 3.1.)* Anatomical echocardiographic and hemodynamic measurements in ovariectomized female rats with or without PTX at 10 weeks post-MI

Groups	**OVX-Sham MI +Veh n=6**	**OVX-Sham MI+PTX n=10**	**OVX-MI+Veh n=7**	**OVX-MI+PTX n=10**
Weight (BW)(g)	483 ± 16.7[a]	472 ± 9.8	430 ± 11.8	475 ±16.3
Uterus (mg/100g)	32 ± 2.4[b]	31 ± 1.5	37 ± 1.7	34± 2.2
MI size%	None	None	28 ±2.2	27 ± 1.5
LV/BW (mg/100g)	150 ± 1.7	165 ± 5.2	180 ± 4.8*	166 ± 6.7
RV/BW (mg/100g)	31 ± 0.9	36 ± 1.1	40 ± 5.7	37 ± 2.7

Values are mean ± SEM. *P<0.05 vs. OVX-Sham MI groups

1. Body weight (g) of OVX female rats with or without PTX 10 weeks post-MI

Two-way ANOVA evaluates PTX x MI interaction

Variable	**df**	**F**	**Sig.**
MI effect	1	2.9	0.09
PTX effect	1	0.6	0.5
MI x PTX	1	1.8	0.2

2. Uterus (mg/100g) of OVX female rats with or without PTX 10 weeks post-MI

Two-way ANOVA evaluates PTX x MI interaction

Variable	**df**	**F**	**Sig.**
MI effect	1	1.9	0.09
PTX effect	1	0.3	0.6
MI x PTX	1	0.05	0.8

3. LV/BW (mg/BW) of OVX female rats with or without PTX 10 weeks post-MI

Two-way ANOVA evaluates PTX x MI interaction

Variable	df	F	Sig.
MI effect	1	5.9	0.02
PTX effect	1	0.1	0.7
MI x PTX	1	2.6	0.1
Pairwise comparison			
Variables		Sig	
Vehicle vs PTX		0.7	
Sham MI groups vs. MI groups		0.02	
Bonferroni post hoc analysis			
Variables		Sig	
Vehicle groups	Sham MI vs. MI	0.01	
PTX groups	Sham MI vs. MI	0.5	

4. RV/BW (mg/BW) of OVX female rats with or without PTX 10 weeks post-MI

Two-way ANOVA evaluates PTX x MI interaction

Variable	df	F	Sig.
MI effect	1	4.1	0.053
PTX effect	1	0.2	0.7
MI x PTX	1	0.8	0.4
Pairwise comparison			
Variables		Sig	
Vehicle vs PTX		0.7	
Sham MI groups vs. MI groups		0.053	
Bonferroni post hoc analysis			
Variables		Sig	
Vehicle groups	Sham MI vs. MI	0.04	
PTX groups	Sham MI vs. MI	0.4	

Echocardiographic measurements

Groups	**OVX-Sham MI +Veh n=6**	**OVX-Sham MI+PTX n=10**	**OVX-MI+Veh n=7**	**OVX-MI+PTX n=10**
LVESV (µL/100gBW)	9 ± 1.2	8 ± 1.0	40 ± 4.8*	41 ± 6.5*
LVEDV (µL/100gBW)	56 ± 9.8	55 ± 3.9	75 ± 7.5*	69 ± 8.4
CI (ml/min/kg)	182 ±18.3	174 ± 11.1	128 ± 16.9*	127 ± 17.2*
EF (%)	82 ± 2.7	85 ± 0.9	45 ± 3.1*	46 ± 2.1*

Values are mean ± SEM. *P<0.05 vs. OVX-Sham MI groups

5. LVESV (µL/100gBW) of OVX female rats with or without PTX 10 weeks post-MI

Two-way ANOVA evaluates PTX x MI interaction

Variable	**df**	**F**	**Sig.**
MI effect	1	47	0.0001
PTX effect	1	0.003	0.9
MI x PTX	1	0.02	0.9
Pairwise comparison			
Variables		Sig	
Vehicle vs PTX		0.9	
Sham MI groups vs. MI groups		0.0001	
Bonferroni post hoc analysis			
Variables		Sig	
Vehicle groups	Sham MI vs. MI	0.0001	
PTX groups	Sham MI vs. MI	0.0001	

6. LVEDV (µL/100gBW) of OVX female rats with or without PTX 10 weeks post-MI

Two-way ANOVA evaluates PTX x MI interaction

Variable	**df**	**F**	**Sig.**
MI effect	1	5.3	0.03
PTX effect	1	2.2	0.2
MI x PTX	1	0.7	0.4
Pairwise comparison			
Variables		Sig	
Vehicle vs PTX		0.2	
Sham MI groups vs. MI groups		0.03	

Bonferroni post hoc analysis		
Variables		Sig
Vehicle groups	Sham MI vs. MI	0.045
PTX groups	Sham MI vs. MI	0.3

7. CI (ml/min/kg) of OVX female rats with or without PTX 10 weeks post-MI

Two-way ANOVA evaluates PTX x MI interaction

Variable	**df**	**F**	**Sig.**
MI effect	1	269	0.0001
PTX effect	1	0.4	0.5
MI x PTX	1	0.3	0.6
Pairwise comparison			
Variables		Sig	
Vehicle vs PTX		0.5	
Sham MI groups vs. MI groups		0.0001	
Bonferroni post hoc analysis			
Variables		Sig	
Vehicle groups	Sham MI vs. MI	0.001	
PTX groups	Sham MI vs. MI	0.001	

8. EF (%) of OVX female rats with or without PTX 10 weeks post-MI

Two-way ANOVA evaluates PTX x MI interaction

Variable	**df**	**F**	**Sig.**
MI effect	1	12	0.001
PTX effect	1	0.9	0.3
MI x PTX	1	0.8	0.4
Pairwise comparison			
Variables		Sig	
Vehicle vs PTX		0.3	
Sham MI groups vs. MI groups		0.001	
Bonferroni post hoc analysis			
Variables		Sig	
Vehicle groups	Sham MI vs. MI	0.001	
PTX groups	Sham MI vs. MI	0.04	

Hemodynamic measurements

Groups	OVX-Sham MI +Veh n=6	OVX-Sham MI+PTX n=10	OVX-MI+Veh n=7	OVX-MI+PTX n=10
LVPSP (mmHg)	132 ± 3.4	131 ± 3.4	114 ± 6.5*	113 ± 5.8*
LVEDP (mmHg)	4 ± 0.3	2.6 ± 0.2	11 ± 1.2*	12 ± 1.1*
LV dP/dt (+) (mmHg/s)	8708 ± 406	8120 ± 192	6545 ± 426*	6040 ± 332*
LV dP/dt (-) (mmHg/s)	7586 ± 352	6886 ± 209	5280 ± 465*	5102 ± 384*

Values are mean ± SEM. *P<0.05 vs. OVX-Sham MI groups

9. LVPSP (mmHg) of OVX female rats with or without PTX 10 weeks post-MI

Two-way ANOVA evaluates PTX x MI interaction

Variable	df	F	Sig.
MI effect	1	11	0.002
PTX effect	1	0.002	0.9
MI x PTX	1	0.02	0.9
Pairwise comparison			
Variables		Sig	
Vehicle vs PTX		0.9	
Sham MI groups vs. MI groups		0.002	
Bonferroni post hoc analysis			
Variables		Sig	
Vehicle groups	Sham MI vs. MI	0.03	
PTX groups	Sham MI vs. MI	0.02	

10. LVEDP (mmHg) of OVX female rats with or without PTX 10 weeks post-MI

Two-way ANOVA evaluates PTX x MI interaction

Variable	df	F	Sig.
MI effect	1	103	0.001
PTX effect	1	0.08	0.8
MI x PTX	1	1.5	0.2
Pairwise comparison			
Variables		Sig	
Vehicle vs PTX		0.8	
Sham MI groups vs. MI groups		0.001	

Bonferroni post hoc analysis		
Variables		Sig
Vehicle groups	Sham MI vs. MI	0.001
PTX groups	Sham MI vs. MI	0.001

11. LVdP/dt (+) (mmHg/s) of OVX female rats with or without PTX 10 weeks post-MI

Two-way ANOVA evaluates PTX x MI interaction

Variable	df	F	Sig.
MI effect	1	40	0.001
PTX effect	1	1.8	0.2
MI x PTX	1	0.18	0.7

Pairwise comparison	
Variables	Sig
Vehicle vs PTX	0.7
Sham MI groups vs. MI groups	0.001

Bonferroni post hoc analysis		
Variables		Sig
Vehicle groups	Sham MI vs. MI	0.001
PTX groups	Sham MI vs. MI	0.001

12. dP/dt(-) (mmHg/s) of OVX female rats with or without PTX 10 weeks post-MI

Two-way ANOVA evaluates PTX x MI interaction

Variable	df	F	Sig.
MI effect	1	31	0.001
PTX effect	1	1.1	0.3
MI x PTX	1	0.9	0.4

Pairwise comparison	
Variables	Sig
Vehicle vs PTX	0.3
Sham MI groups vs. MI groups	0.001

Bonferroni post hoc analysis		
Variables		Sig
Vehicle groups	Sham MI vs. MI	0.001
PTX groups	Sham MI vs. MI	0.001

6.2.2. (*Fig3.1.*) Percent sucrose intake and total liquid intake in ovariectomized female rats with or without PTX at 10 weeks post-MI

Groups	**OVX-Sham MI +Veh n=6**	**OVX-Sham MI+PTX n=10**	**OVX-MI+Veh n=7**	**OVX-MI+PTX n=10**
Sucrose intake (%)	89 ± 2.3	95 ± 1.6	69 ± 8.2*	86 ± 2.8†
Total liquid intake (mL/100g BW)	16 ± 1.7	16 ± 1.4	16 ± 3.1	14 ±1.03

Values are mean ± SEM. *P<0.05 vs. OVX-Sham MI groups, † P<0.05 vs. OVX-MI+Veh

1.Sucrose intake (%)

Two-way ANOVA evaluates PTX x MI interaction

Variable	**df**	**F**	**Sig.**
MI effect	1	12	0.002
PTX effect	1	7	0.01
MI x PTX	1	1.8	0.2

Pairwise comparison

Variables	Sig
Vehicle vs PTX	0.01
Sham MI groups vs. MI groups	0.002

Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.004
PTX groups	Sham MI vs. MI	0.09

2.Total liquid intake (mL/100g BW)

Two-way ANOVA evaluates PTX x MI interaction

Variable	**df**	**F**	**Sig.**
MI effect	1	0.8	0.4
PTX effect	1	1.3	0.3
MI x PTX	1	0.8	0.4

6.2.3. (*Fig3.*2.) Percent immobility in the forced swim test in ovariectomized female rats with or without PTX at 10 weeks post-MI

Groups	**OVX-Sham MI +Veh n=6**	**OVX-Sham MI+PTX n=10**	**OVX-MI+Veh n=7**	**OVX-MI+PTX n=10**
Immobility (%)	55 ± 4.1	33 ± 3.2	63 ± 2.3*	38 ± 3.3†

Values are mean ± SEM. *P<0.05 vs. OVX-Sham MI groups, † P<0.05 vs. OVX-MI+Veh

1.Immobility (%)

Two-way ANOVA evaluates PTX x MI interaction

Variable	**df**	**F**	**Sig.**
MI effect	1	7	0.01
PTX effect	1	51.8	0.001
MI x PTX	1	0.8	0.4

Pairwise comparison

Variables	Sig
Vehicle vs PTX	0.001
Sham MI groups vs. MI groups	0.01

Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.03
PTX groups	Sham MI vs. MI	0.2

6.2.4. (*Fig3.*3.) Morris water maze test assessed learning and spatial memory in OVX female rats with or without PTX at 10 weeks post-MI

Groups	**OVX-Sham MI +Veh n=6**	**OVX-Sham MI+PTX n=9**	**OVX-MI+Veh n=7**	**OVX-MI+PTX n=10**
Latency to the platform (sec)				
Day 1	44 ± 3.1	39 ± 4.8	45 ± 3.8	40 ± 3.0
Day 2	24 ± 5.7	31 ± 4.0	37 ± 3.3	32 ± 3.9
Day 3	20 ± 3.8	22 ± 4.1	20 ± 2.6	21 ± 3.3
Day 4	18 ± 3.4	20 ± 3.5	20 ± 1.7	17 ± 2.6
% time spent in the target quadrant				
Probe 1	33 ± 4.5	32 ± 3.3	23 ± 1.4*	29 ± 2.9
Probe 2	42 ± 4.5	40 ± 3.6	35 ± 3.5*	32 ±2.7

Values are mean ± SEM. *P<0.05 vs. OVX-Sham MI groups

1. Training days

Reapeated measures evalute taining days and MI x PTX interaction

Variable	**Df**	**F**	**Sig.**
Training effect	3	99.4	0.0001
Training x MI	3	0.5	0.5
Training x PTX	3	1.0	0.3
Training x MI x PTX	3	0.003	0.9

Pairwise comparison

Variables	Sig
Day 1 vs day 2	0.002
Day 2 vs day 3	0.001
Day 3 vs day 4	0.001

Variable	**Df**	**F**	**Sig.**
MI effect	1	0.6	0.4
PTX effect	1	0.08	0.8
MI x PTX	1	1.3	0.3

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.8
Sham MI groups vs. MI groups	0.4

Bonferroni post hoc analysis			
Variables		Sig	
Vehicle group	Sham MI vs. MI	Day 1	0.8
		Day 2	0.054
		Day 3	1.0
		Day 4	0.6
PTX group	Sham MI vs. MI	Day 1	0.9
		Day 2	0.8
		Day 3	0.8
		Day 4	0.4

2. Probe 1

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	4.5	0.04
PTX effect	1	0.7	0.4
MI x PTX	1	1.0	0.3

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.4
Sham MI groups vs. MI groups	0.04

Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.04
PTX groups	Sham MI vs. MI	0.4

3. Probe 2

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	4.9	0.034
PTX effect	1	0.5	0.5
MI x PTX	1	0.01	0.9

Pairwise comparison

Variables	Sig
Vehicle groups vs PTX groups	0.5
Sham MI groups vs. MI groups	0.03

Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.04
PTX groups	Sham MI vs. MI	0.2

6.2.5. (*Table 3.2.*) Locomotor activity in the first training session of the Morris water maze test in OVX female rats with or without PTX at 10 weeks post-MI

Groups	Total path length (m)	Speed (cm/sec)
OVX-Sham MI +Veh **(n=6)**	13 ± 1.3	24 ± 2.3
OVX-Sham MI +PTX **(n=9)**	17 ± 0.9	29 ± 1.5
OVX-MI +Veh **(n=7)**	14 ± 1.1	30 ± 0.7
OVX-MI+PTX **(n=10)**	14 ± 0.9	26 ± 1.5

Values are mean ± SEM.

1.Total path length (m)

Two-way ANOVA evaluates PTX x MI interaction

Variable	df	F	Sig.
MI effect	1	1.9	0.2
PTX effect	1	0.02	0.9
MI x PTX	1	1.3	0.2

2.Speed (cm/s)

Two-way ANOVA evaluates PTX x MI interaction

Variable	df	F	Sig.
MI effect	1	0.05	0.8
PTX effect	1	1.5	0.3
MI x PTX	1	0.6	0.2

6.2.6. (*Fig.3.4.*) Freezing behavior by OVX female rats at 10 weeks post-MI in the fear conditioning test

Groups	**OVX-Sham MI +Veh n=6**	**OVX-Sham MI+PTX n=9**	**OVX-MI+Veh n=7**	**OVX-MI+PTX n=10**
Freezing (%) in habituation	15 ± 1.7	14 ± 2.3	19 ± 2.4	21 ± 1.8
Freezing (%) in context test	34 ± 3.0 §	40 ± 7.6 §	51 ± 2.7 §	45 ± 3.0 §
Freezing (%) in Cued test				
Pre-Tone	12 ± 0.7	5 ± 2.0	15 ± 2.4	7 ± 1.8
Tone (1 min)	53 ± 7.4 §	44 ± 6.4 §	69 ± 6.4 §*	52 ± 7.5 §†
Tone (2 min)	56 ± 9.2 §	57 ± 8.2 §	84 ± 5.2 §*	65 ± 8.9 §
Tone (3 min)	53 ± 12.6 §	47 ± 9.9 §	93 ± 2.1 §*	62 ± 10.3 §†

Values are mean ± SEM. *P<0.05 vs. OVX-Sham MI + Veh , † P<0.05 vs. OVX-MI+Veh §P<0.05 vs. freezing behavior during habituation or pre-tone

1. Context test

Reapeated measures evalute taining days and MI x PTX interaction

Variable	**Df**	**F**	**Sig.**
Context effect	1	92.5	0.0001
Context x MI	1	0.8	0.4
Context x PTX	1	0.2	0.8
Context x MI x PTX	1	2.4	0.1

Pairwise comparison

Variables	Sig
Habituation vs day 2	0.0001

Variable	**Df**	**F**	**Sig.**
MI effect	1	4.2	0.052
PTX effect	1	0.01	0.9
MI x PTX	1	0.4	0.5

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.9
Sham MI groups vs. MI groups	0.052

Bonferroni post hoc analysis

Variables		Sig	
Vehicle group	Sham MI vs. MI	Habituation	0.3
		Day 2	0.08
PTX group	Sham MI vs. MI	Habituation	0.03
		Day 2	0.7

2. Cued test

Reapeated measures evalute taining days and MI x PTX interaction

Variable	Df	F	Sig.
Cued effect	4	94	0.0001
Cued x MI	3	3.8	0.012
Cued x PTX	3	1.2	0.3
Cued x MI x PTX	3	1.1	0.4

Pairwise comparison

Variables	Sig
Pre-tone vs min 1	0.001
Pre-tone vs min 2	0.001
Pre-tone vs min 3	0.001

Variable	Df	F	Sig.
MI effect	1	6.9	0.01
PTX effect	1	4.4	0.04
MI x PTX	1	1.4	0.2

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.04
Sham MI groups vs. MI groups	0.01

Bonferroni post hoc analysis

Variables		Sig	
Vehicle group	Sham MI vs. MI	Pre-tone	0.8
		Min 1	0.07
		Min 2	0.04
		Min 3	0.01
PTX group	Sham MI vs. MI	Pre-tone	0.4
		Min 1	0.3
		Min 2	0.5
		Min 3	0.2

6.2.7. (*Fig.3.5.*) Cytokine levels in the plasma of OVX female rats with or without PTX at 10 weeks post-MI

Groups	**OVX-Sham MI +Veh n=5**	**OVX-Sham MI+PTX n=9**	**OVX-MI+Veh n=7**	**OVX-MI+PTX n=9**
IL-1α (pg/mL)	75 ± 52.0	24 ± 5.4	89 ± 12.5*	22 ± 4.3†
IL-1β (pg/mL)	82 ± 50.5	36 ± 9.6	121 ± 20.4*	60 ± 12.0†
IL-2 (pg/mL)	116 ± 13.7	139 ± 14.0	295 ± 29.8*	122 ± 8.4†
IL-6 (pg/mL)	410 ±58.0	484 ± 39.7	1017 ± 62.5*	464 ± 48.3†
TNF-α (pg/mL)	150 ± 19.5	139 ± 18.6	371 ± 46.0*	114 ± 12.8†
IL-10 (pg/mL)	85 ± 12.5	72 ± 10.7	187 ± 17.8*	60 ± 5.5†

Values are mean ± SEM. *P<0.05 vs. OVX-Sham MI + Veh , † P<0.05 vs. OVX-MI+Veh

1. IL-1α

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	0.9	0.8
PTX effect	1	10.1	0.004
MI x PTX	1	0.12	0.7

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.004
Sham MI groups vs. MI groups	0.8

Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.001
PTX groups	Sham MI vs. MI	0.8

2. IL-1β

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	14	0.001
PTX effect	1	2.05	0.2
MI x PTX	1	2.8	0.1

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.001
Sham MI groups vs. MI groups	0.2

Bonferroni post hoc analysis		
Variables		Sig
Vehicle groups	Sham MI vs. MI	0.002
PTX groups	Sham MI vs. MI	0.1

3. IL-2

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	20.1	0.001
PTX effect	1	20.9	0.0001
MI x PTX	1	30.9	0.001

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.0001
Sham MI groups vs. MI groups	0.001

Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.0001
PTX groups	Sham MI vs. MI	0.4

4. IL-6

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	25.3	0.001
PTX effect	1	21.9	0.001
MI x PTX	1	26.5	0.001

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.001
Sham MI groups vs. MI groups	0.001

Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.001
PTX groups	Sham MI vs. MI	0.04

5. TNF-α

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	13.2	0.001
PTX effect	1	28.5	0.001
MI x PTX	1	22.7	0.001
Pairwise comparison			
Variables		Sig	
Vehicle groups vs. PTX groups		0.001	
Sham MI groups vs. MI groups		0.001	
Bonferroni post hoc analysis			
Variables		Sig	
Vehicle groups	Sham MI vs. MI	0.001	
PTX groups	Sham MI vs. MI	0.8	

6. IL-10

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	10.7	0.003
PTX effect	1	28.6	0.001
MI x PTX	1	17.7	0.001
Pairwise comparison			
Variables		Sig	
Vehicle groups vs. PTX groups		0.001	
Sham MI groups vs. MI groups		0.003	
Bonferroni post hoc analysis			
Variables		Sig	
Vehicle groups	Sham MI vs. MI	0.001	
PTX groups	Sham MI vs. MI	0.4	

6.2.8. (*Fig.3.6.*) Cytokine levels in the PVN of OVX female rats with or without PTX at 10 weeks post-MI

Groups	**OVX-Sham MI +Veh n=5**	**OVX-Sham MI+PTX n=10**	**OVX-MI+Veh n=7**	**OVX-MI+PTX n=9**
IL-1α (pg/mg)	19 ± 1.9	22 ± 0.6	24 ± 1.9	22 ± 0.9
IL-1β (pg/mg)	36 ± 8.5	53 ± 1.7	61 ± 3.3*	50 ± 1.7†
IL-2 (pg/mg)	154 ± 26.3	209 ± 21.3	222 ± 22.1*	199 ± 26.7
IL-6 (pg/mg)	525 ± 110.9	686 ± 12.4	931 ± 20.3*	683 ± 15.2†
TNF-α (pg/ mg)	78 ± 6.7	70 ± 1.	120 ± 4.4 *	83 ± 6.1†‡
IL-10 (pg/mg)	58 ± 7.2	82 ± 3.8	81 ± 3.6*	70 ± 3.1†‡

Values are mean ± SEM. *P<0.05 vs. OVX-Sham MI + Veh , † P<0.05 vs. OVX-MI+Veh, ‡ P<0.05 vs. OVX-Sham MI +PTX

1. IL-1α

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	4.0	0.054
PTX effect	1	0.3	0.6
MI x PTX	1	3.4	0.07

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.6
Sham MI groups vs. MI groups	0.054

Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.02
PTX groups	Sham MI vs. MI	0.8

2. IL-1β

Two-way ANOVA evaluates PTX x MI interaction:

Variable	Df	F	Sig.
MI effect	1	14.6	0.001
PTX effect	1	2.5	0.12
MI x PTX	1	20.6	0.001

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.12
Sham MI groups vs. MI groups	0.001

Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.001
PTX groups	Sham MI vs. MI	0.6

3. IL-2

Two-way ANOVA evaluates PTX x MI interaction:

Variable	Df	F	Sig.
MI effect	1	4.5	0.04
PTX effect	1	0.6	0.5
MI x PTX	1	3.1	0.09

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.5
Sham MI groups vs. MI groups	0.04

Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.02
PTX groups	Sham MI vs. MI	0.8

4. IL-6

Two-way ANOVA evaluates PTX x MI interaction:

Variable	Df	F	Sig.
MI effect	1	36.5	0.001
PTX effect	1	0.23	0.6
MI x PTX	1	37.6	0.001

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.6
Sham MI groups vs. MI groups	0.001

Bonferroni post hoc analysis		
Variables		Sig
Vehicle groups	Sham MI vs. MI	0.001
PTX groups	Sham MI vs. MI	0.9

5. TNF-α

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	39.6	0.001
PTX effect	1	15.7	0.001
MI x PTX	1	12.8	0.001
Pairwise comparison			
Variables		Sig	
Vehicle groups vs. PTX groups		0.001	
Sham MI groups vs. MI groups		0.001	
Bonferroni post hoc analysis			
Variables		Sig	
Vehicle groups	Sham MI vs. MI	0.001	
PTX groups	Sham MI vs. MI	0.03	

6. IL-10

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	4.0	0.054
PTX effect	1	5.1	0.032
MI x PTX	1	25.7	0.001
Pairwise comparison			
Variables		Sig	
Vehicle groups vs. PTX groups		0.032	
Sham MI groups vs. MI groups		0.054	
Bonferroni post hoc analysis			
Variables		Sig	
Vehicle groups	Sham MI vs. MI	0.001	
PTX groups	Sham MI vs. MI	0.02	

6.2.9. (*Fig.3.7.*) Cytokine levels in the PFC of OVX female rats with or without PTX at 10 weeks post-MI

Groups	**OVX-Sham MI +Veh n=5**	**OVX-Sham MI+PTX n=10**	**OVX-MI+Veh n=7**	**OVX-MI+PTX n=9**
IL-1α (pg/mg)	53 ± 4.1	46 ± 4.3	68 ± 12.1	42 ± 2.4
IL-1β (pg/mg)	81 ± 6.8	68 ± 3.2	107 ± 11.9*	67 ± 2.1†
IL-2 (pg/mg)	200 ± 8.8	244 ± 23.6	261 ± 23.5*	272 ± 1601
IL-6 (pg/mg)	330 ± 51.3	444 ± 36.3	575 ± 56.5*	362 ± 52.7†
TNF-α (pg/ mg)	253 ± 27.4	274 ± 24.2	431 ± 64.5*	290 ± 14.2†
IL-10 (pg/mg)	149 ± 14.0	184 ± 15.6	157 ± 20.5	168 ± 7.6

Values are mean ± SEM. *P<0.05 vs. OVX-Sham MI + Veh , † P<0.05 vs. OVX-MI+Veh

1. IL-1α

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	0.5	0.5
PTX effect	1	3.8	0.06
MI x PTX	1	1.4	0.3

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.06
Sham MI groups vs. MI groups	0.5

Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.24
PTX groups	Sham MI vs. MI	0.7

2. IL-1β

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	2.7	0.1
PTX effect	1	12.2	0.002
MI x PTX	1	20.6	0.001

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.002
Sham MI groups vs. MI groups	0.1

Bonferroni post hoc analysis		
Variables		Sig
Vehicle groups	Sham MI vs. MI	0.03
PTX groups	Sham MI vs. MI	0.9

3. IL-2

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	7.6	0.013
PTX effect	1	0.8	0.4
MI x PTX	1	8.5	0.008
Pairwise comparison			
Variables		Sig	
Vehicle groups vs. PTX groups		0.4	
Sham MI groups vs. MI groups		0.013	
Bonferroni post hoc analysis			
Variables		Sig	
Vehicle groups	Sham MI vs. MI	0.016	
PTX groups	Sham MI vs. MI	0.3	

4. IL-6

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	2.1	0.2
PTX effect	1	0.8	0.4
MI x PTX	1	8.5	0.008
Pairwise comparison			
Variables		Sig	
Vehicle groups vs. PTX groups		0.4	
Sham MI groups vs. MI groups		0.2	
Bonferroni post hoc analysis			
Variables		Sig	
Vehicle groups	Sham MI vs. MI	0.008	
PTX groups	Sham MI vs. MI	0.3	

5. TNF-α

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	7.6	0.012
PTX effect	1	3.5	0.07
MI x PTX	1	5.6	0.028

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.07
Sham MI groups vs. MI groups	0.012

Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.003
PTX groups	Sham MI vs. MI	0.7

6. IL-10

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	0.05	0.8
PTX effect	1	1.8	0.2
MI x PTX	1	0.6	0.5

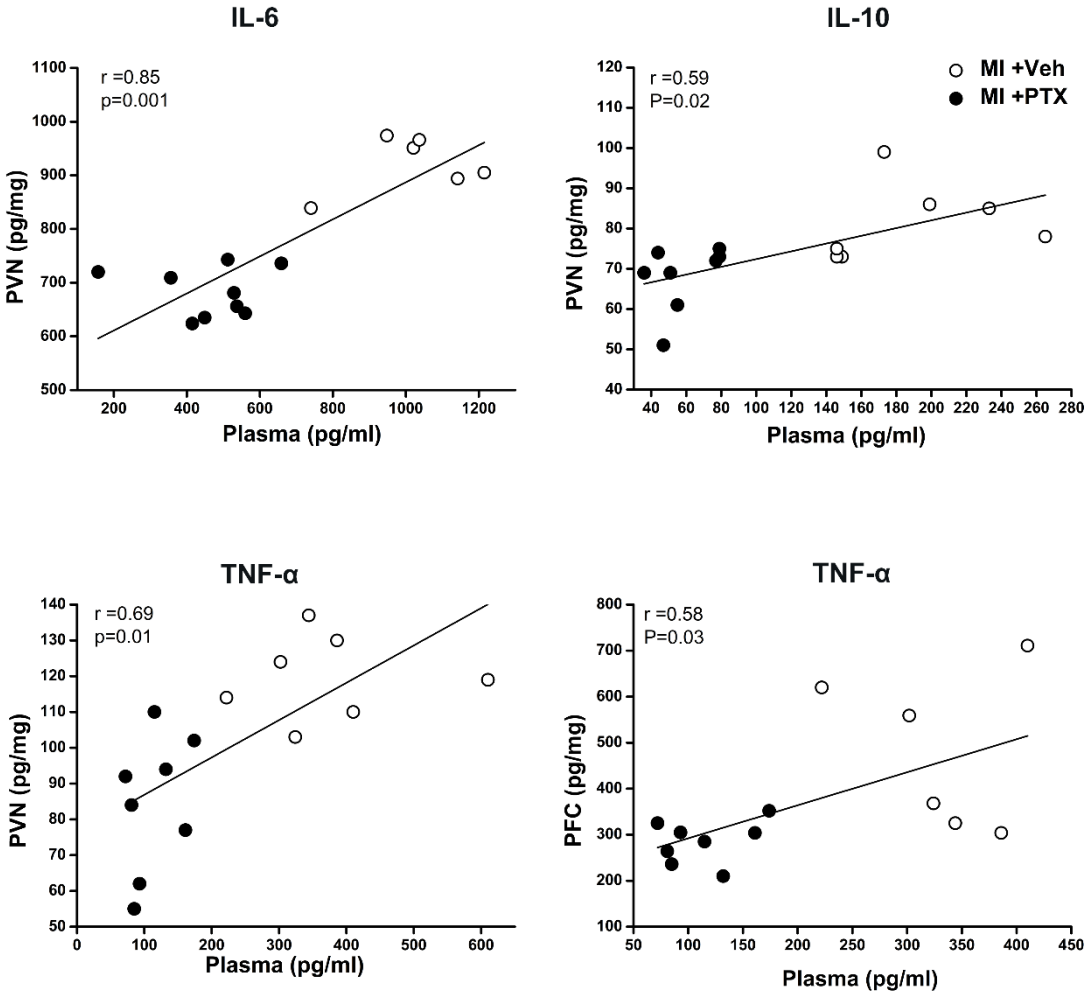
Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.2
Sham MI groups vs. MI groups	0.8

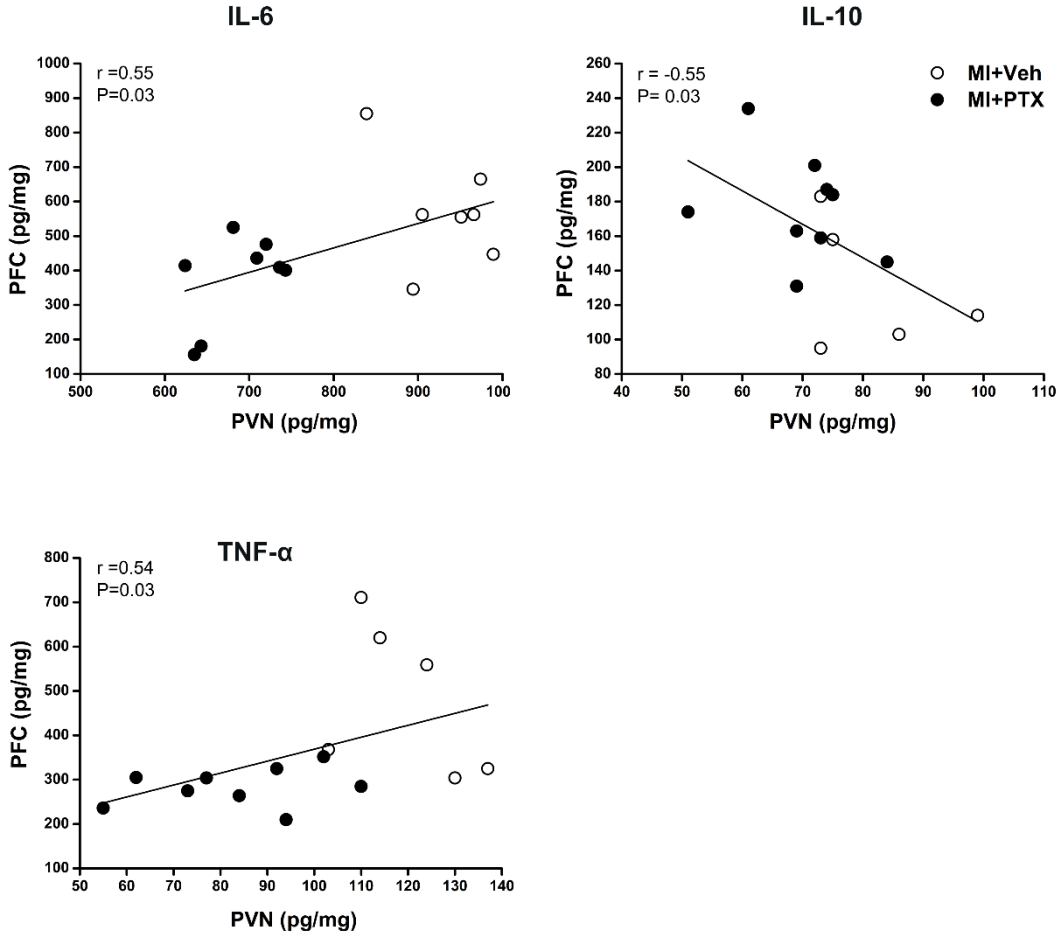
Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.7
PTX groups	Sham MI vs. MI	0.4

6.2.10. *(Figure 3.8.)* Significant correlations between cytokine levels in Plasma and PVN or plasma and PFC. Other cytokines did not have significant correlations.



6.2.11. *(Figure 3.9.)* Significant correlations between cytokine levels in the PFC and PVN. Other cytokines did not have significant correlations.



6.2.12. *(Figure 3.10.)* mBDNF expression in the PFC of OVX female rats with or without PTX at 10 weeks post-MI

Groups	**OVX-Sham MI +Veh n=4**	**OVX-Sham MI+PTX n=8**	**OVX-MI+Veh n=6**	**OVX-MI+PTX n=7**
mBDNF/β-Actin	1 ± 0.13	0.78 ± 0.06	0.59 ± 0.12*	0.93 ± 0.13†

Values are mean ± SEM. *P<0.05 vs. OVX-Sham MI + Veh , † P<0.05 vs. OVX-MI+Veh

1. mBDNF

Two-way ANOVA evaluates PTX x MI interaction:

Variable	**Df**	**F**	**Sig.**
MI effect	1	0.6	0.4
PTX effect	1	0.6	0.4
MI x PTX	1	6.7	0.01

Pairwise comparison

Variables	Sig
Vehicle groups vs. PTX groups	0.4
Sham MI groups vs. MI groups	0.4

Bonferroni post hoc analysis

Variables		Sig
Vehicle groups	Sham MI vs. MI	0.04
PTX groups	Sham MI vs. MI	0.6