THE EFFECT OF FASTING ON ANTI-INFLAMMATORY MEDIATORS, IL-1RA AND IL-1R2, AND THEIR ROLE IN RESISTANCE TO SICKNESS BEHAVIORS IN MICE

BY

JENNIFER J. JOESTING

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Animal Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

Adviser:

Professor Gregory G. Freund

ABSTRACT

Dietary regimens involving fasting have long been linked to beneficial health outcomes including; reduction in heart disease, diabetes, improved mood and cognition, and resistance to sickness behaviors such as anorexia and fever. The mechanisms underlying fasting-induced health benefits and alterations in immunity and sickness behavior continues to remain debated despite over 20 years of interest. This research project confirms that acute dietary restriction (24 h water-only fast) is able to attenuate IL-1β-induced anorexia, in addition to other sickness behaviors at early stages of the acute phase response. We previously reported that fasting was able to reduce gene expression of inflammatory IL-1α in the brain, here we looked at liver and adipose in addition to brain and uncovered a major up-regulation of IL-1 endogenous inhibitors, IL-1RA and IL-1R2 in peripheral tissues. These findings imply that attenuation in sickness behaviors observed may be due to counter-regulation of IL-1 via up-regulation of IL-1R2 and/or IL-1RA shown in metabolically active organs, which is capable of blunting the centrally-mediated effects of induced peripheral challenge. Our results further demonstrate that the mechanisms involved in IL-1R2 and IL-1RA up-regulation are independent of IL-1, TLR-4, IL-4 and glucocordicoid signaling. Here we also demonstrate that free fatty acids (FFA) are increased in the plasma as a consequence of fasting. Using palmitic acid injection to mimic fastings FFA increase, we elucidated a novel mechanism by which IL-1R2 is up-regulated. This method showed increased IL-1R2 gene transcripts in the liver of mice. FFA signaling, which produces an immune response, is shown here to be TLR-4-independent, implicating free fatty acid receptor 1 (FFAR1) as the key signaling receptor initiating the anti-inflammatory result of fasting documented in this study.

ACKNOWLEDGMENTS

There are many people to thank for helping to make this project possible. I am very thankful to my adviser, Dr. Gregory G. Freund, for allowing me to do research in his lab and continue my education, and also for his guidance and support throughout this project. Additional thanks to my committee members, Dr. Jeffrey Woods and Dr. F. William Simmons, for agreeing to join the 'race to my Masters' without much notice. Thanks to all members of the Freund Lab, past and present, for their assistance, mentoring and counseling on occasion. Special thanks to my husband Clint and daughter Gracie, to my parents and family, and to my great friends who encouraged and supported me throughout this process. I appreciate all of your time, energy, resources and love. And finally, thanks to baby girl Joesting #2 (due 4/16/13) for hopefully waiting to come into this world until after my defense and thesis acceptance.

TABLE OF CONTENTS

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW……..1

CHAPTER 2: OBJECTIVES……..3

CHAPTER 3: METHODOLOGY……4

CHAPTER 4: RESULTS……..8

CHAPTER 5: TABLES, FIGURES AND FIGURE LEGENDS……..16

CHAPTER 6: DISCUSSION……32

CHAPTER 7: CONCLUSIONS……38

REFERENCES……39

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Within the last 20 years, the importance of body weight has become a physiological metric that has permeated the cultural psyche of nearly all industrialized nations. Weight loss has become a near societal obsession (Fraser, 1997). Many popular diet programs initially use significant calorie restriction (CR) to facilitate relatively rapid weight-loss (Volpe, 2006) including the recent trend of detox and/or cleansing diets which may incorporate water only fasting for up to 1 wk. In a broad context, however, fasting is a critical element of many religious and cultural practices (Brett & Nesbit, 2012; Salim, Al Suwaidi, Ghadban, Alkilani, & Salam, 2013) and is recommended by practitioners of alternative and complementary medicine as therapy for a variety of ailments (Fuhrman, Sarter, & Calabro, 2002; Goldhamer et al., 2002; Michalsen, 2010; Schmidt et al., 2010). Clinically, dietary regimens that incorporate intermittent fasting (IF) have gained significant popular attention (The Fast Diet: Lose Weight, Stay Healthy, and Live Longer with the Simple Secret of Intermittent Fasting, Dr. Michael Mosley) with human studies showing a potential to reduce heart disease and diabetes (Horne et al., 2008) and to improve mood (Michalsen et al., 2006; Michalsen, 2010). Since fasting regimens appear to benefit cognition function in aging mice (Martin, Mattson, & Maudsley, 2006) and in the 3xTgAD mouse model of Alzheimer's disease, (Halagappa et al., 2007) interest in CR and IF as a way to ward off human cognitive aging has also emerged (Mattson, 2010).

While overeating and obesity are associated with a variety of maladies including type 2 diabetes (T2D), heart disease, stroke (Kopelman, 2007), cancer (Louie, Roberts, & Nomura, 2013) Alzheimer's disease (Lee, 2011), anxiety/mood disorders (Kaczmarczyk et al., 2013; Simon et al., 2006), and declined cognition (Bressler et al., 2013), precisely how overnutrition contributes is not entirely clear. Emerging

evidence indicates that oxidative and metabolic stress in conjunction with dyslipedemia and excess glucose stimulates the unfolded protein response (Scheuner & Kaufman, 2008) and activates the NLRP3 inflammasome (Vandanmagsar et al., 2011). Thus, IL-1-mediated inflammation is a key consequence of overnutrition, whether indirectly provoked by cellular injury (Gregor & Hotamisligil, 2011) or directly triggered by excess nutrients acting as danger signals (Lumeng, 2013). Overnutrition-associated IL-1 is implicated in pancreatic beta cell loss in T2D, atherogenesis in heart disease and stroke and neurodegeneration in Alzheimer's disease (Cai & Liu, 2012) but, in general, IL-1 is a critical effecter of sickness symptoms that include loss of appetite, locomotion and social/environmental engagement (Kelley et al., 2003). Thus, ameliorations that inhibit IL-1 bioaction may positively impact health in those with either psychological (Maes, Song, & Yirmiya, 2012; Rossi et al., 2012) and/or overtly organic diseases (Dayer, 2003; Sloan-Lancaster et al., 2013).

We recently demonstrated that mice fasted for 24 hrs have reduced gene expression of brain IL-1α (Lavin et al., 2011), indicating that CR may be a physiologic way to negatively regulate IL-1 bioaction. Although starvation appears detrimental to immunity, as evidenced by lipopolysaccharide (LPS)-induced death in bees and mice (Faggioni, Moser, Feingold, & Grunfeld, 2000; Moret & Schmid-Hempel, 2000), fasting appears to mitigate LPS-induced sickness symptoms in rats (Inoue, Somay, Poole, & Luheshi, 2008). In addition, fasting offers protection from ischemic (Mitchell et al., 2010) and hypoxic injuries (Go, Prenen, & Korf, 1988) which we and others have shown are mediated in large part by IL-1 (Chiu et al., 2012; Johnson, O'Connor, Hartman, Tapping, & Freund, 2007). Therefore, we examined if the beneficial effects of fasting are due to IL-1 counter-regulation in both the systemic and neuroimmune systems.

CHAPTER 2

OBJECTIVES

1. Determine how fasting alters the immune system and if there are any cognitive or behavioral effects associated with fasting.

2. Determine what happens to a previously fasted immune system after being challenged with IL-1β, and elucidate whether these changes are associated with alterations in sickness behaviors with this immune stimuli.

3. Determine the mechanism by which fasting alters the immune system.

CHAPTER 3

METHODOLOGY

Materials- All reagents and chemicals were purchased from Sigma-Aldrich (St Louis, MO) except as noted. All primers were purchased from Applied Biosystems (Foster City, CA)

Animals- Procedures were conducted on protocols approved by the University of Illinois Institutional Animal Care and Use Committee. IL-1 receptor 1 (IL-1R1) knock out (KO), toll-like receptor (TLR-4) KO, IL-4 KO and C57BL/6J (wild type (WT)) mice were purchased from Jackson Laboratory (Bar Harbor, ME) and bred in-house. All KO mice were on a C57BL/6 backbone. Mice were group housed (×8 cage) in standard shoebox cages (length 46.9 cm; width 25.4 cm; height 12.5 cm) and allowed water and food (Harlan XXX (Indianapolis, IN) *ad libitum* except where otherwise noted. Housing temperature (72 °F) and humidity (45–55%) were controlled as was a 12/12 h reversed dark-light cycle (light = 2200-1000 h). Animals were sacrificed using CO_2 except for those in which corticosterone was measured which were sacrificed using ketamine/xylazine.

Fasting and mouse weight- As previously described (Lavin et al., 2011; York et al., 2012), 2 days prior to fasting, mice were singly housed. Fasting was initiated by placing mice in a new cage without food but with ad libitum water. Mice were fasted for 24 h starting at 0900 h. Mouse weight was recorded immediately before and immediately after fasting using an Ohaus Adventurer Pro digital scale (Parsippany, NJ).

Injectables- Millipore recombinant mouse IL-1β (Billercia, MA) in 100 µl of sterile Cellgro PBS (Manassas, VA) (vehicle) was administered intraperitoneally (IP) at a dose of 0.9 µg/mouse immediately

after fasting. Mifepristone in BioUltra 400 polyethylene glycol (PEG) (vehicle) (PEG) was administered subcutaneously (SC) at a dose of 25 mg/kg immediately prior to fasting. Palmitic acid in 50 µL of castor oil (vehicle) was administered IP at a dose of 30 µMoles.

Quantitative PCR (qPCR)- As previously described (Lavin et al., 2011), animals were perfused with 30 mLs of ice cold PBS and RNA isolated from homogenized tissues using Qiagen RNeasy Lipid Tissue Mini Kits (Valencia, CA). RNA was reverse transcribed using the Applied Biosystems High-Capacity cDNA Reverse Transcription Kit. The TaqMan Gene Expression primers used were: CD11b (Mm00434455_m1), TNF-α (Mm00443258_m1), IL-1α (Mm99999060_m1), IL-1β (Mm99999061_mH), IL-1R1 (Mm00434237_m1), IL-1RA (Mm01337566_m1) and IL-1R2 (Mm00439622_m1). qPCR was performed on an Applied Biosystems 7900 HT Fast Real-Time PCR System using Applied Biosystems TaqMan Universal PCR Master Mix. To compare gene expression, a parallel amplification of endogenous RPS3 (Mm00656272_m1) was performed. Reactions with no reverse transcription and no template were included as negative controls. Relative quantitative evaluation of target gene to RPS3 was performed by comparing ΔCts, where Ct is the threshold concentration.

Locomotion- Spontaneous locomotor activity was measured as previously described (Lavin et al., 2011, York et al., 2012). At the times indicated, mice were video recorded in their home cage for 5 min under red light using a using a Sony Night Shot capable video camera (Minato-ku, Tokyo). Distance moved was quantified using Noldus Information Technology EthoVision XT 7 automated tracking software (Leesburg, VA).

Social Behavior- Social exploration of a novel juvenile was measured as previously described (Sherry, Kramer, York, & Freund, 2009). In brief, a 4-wk old novel, conspecific male juvenile (challenge)

mouse enclosed in a 3 x 3 x 3 inch wire mesh cage was placed in the home cage of the adult (test) mouse for 5 minutes. Test mouse-initiated exploratory behavior of the challenge mouse enclosure was video recorded under red light using a using a Sony Night Shot capable video camera. Time spent exploring the challenge mouse (nose within a 2 cm region of interest (ROI) drawn around the caged juvenile) was quantified using Noldus Information Technology EthoVision XT 7 automated tracking software (Leesburg, VA).

Blood glucose- Mouse tail blood glucose was determined in duplicate using an Abbott Laboratories AlphaTRAK Blood Glucose Monitoring System (North Chicago, IL) by methods we have previously described (Lavin et al., 2011).

Serum and liver IL-1R2 and IL-1RA- Serum IL-1R2 was measured using a Millipore Milliplex MAP kit (Billerica, MA) following manufactures instructions. Bead-based fluorescent reporter signal was detected on a Luminex 100 System (Austin, TX). Serum IL-1RA was measured colorimetrically in by ELISA using the R&D Mouse IL-1RA/IL-1F3 conjugate kit (Minneapolis, MN). Liver IL-1R2 and IL-1RA were measured from freeze-fractured liver by methods we have previously described (Chiu et al., 2012). In brief, PBS perfused livers were liquid nitrogen frozen in freeze fracture buffer containing 10% glycerol, 50 mM NaCl, 1 mM EDTA, 50 mM HEPES (pH 7.4) plus a 1:200 dilution of Millipore protease inhibitor cocktail (Billerica, MA). The fractured liver was homogenized using the TissueLyser II (Qiagen, Valencia, CA) at a rotational frequency of 30/sec for 2 min. Lysates were clarified at 16,000 x g for 15 min at 4º C and the supernatant protein concentrations determined using the Bio-Rad DC Protein Assay (Hercules, CA). Liver IL-1R2 and IL-1RA was expressed as pg of IL-1R2 per ng of supernatant protein.

Plasma corticosterone and free fatty acids (FFAs)- Corticosterone was measured colorimetrically by ELISA using the Abcam corticosterone ELISA kit (Cambridge, MA) in plasma. FFAs were measured colorimetrically in plasma using the Randox Nefa Assay (Antrim, Ireland) and a Beckman-Coulter AU680 (Brea, CA) following manufactures instructions.

Statistics- Individual behavioral experiments were performed on separate cohorts of mice. Biochemical/qPCR experiments were performed on cohorts of mice not subjected to behavioral testing. All data are presented as mean ± SEM. Data were analyzed using Sigma Plot 11.2 (Systat Software,Chicago, IL). To test for statistical differences, one-way and two-way ANOVAs were used with or without repeated measurements where relevant. Tukey's test was used for post-hoc pair-wise multiple comparison procedures. Pearson product moment correlation was used to compute correlation coefficient. Where indicated, raw data was transformed to attain normality or equal variance via log(abs), ^2 or RANK simple transforms. All statistical analysis included testing for time point x treatment interactions. Statistical significance was denoted at $p<0.05$.

CHAPTER 4

RESULTS

1. Effect of fasting on gene expression

Table 1 shows that CD11b, TNF-α, IL-1α, IL-1β, IL-1R1, IL-1RA and IL-1R2 gene transcripts after fasting are differentially expressed in brain regions (hypothalamus, hippocampus, cortex), and peripheral tissues (liver, adipose). In all brain regions, fasting caused CD11b gene transcript to significantly downregulate ($p<0.001$ all brain regions). Whereas both adipose and liver upregulated CD11b ($p<0.001$ adipose, $p=0.032$ liver; transformed to RANK for normality). TNF-α was consistently downregulated in all tissues ($p=0.001$ hypoth, $p=0.002$ hippo, $p<0.001$ cortex, $p=.004$ adipose, $0=.045$ liver). IL-1α was significantly downregulated in all brain regions ($p<0.001$ all brain regions), but adipose and liver transcript was not significantly different due to fasting. IL-1β gene message was significantly downregulated in the hypothalamus ($p<0.006$ hypoth) and also in adipose tissue ($p=.008$ adipose), but was not significantly different in any other tissues. IL-1R1 was significantly upregulated in all tissues measured ($p=0.008$ hypoth,$p= 0.031$ hippo, $p<0.001$ cortex, $p<0.001$ adipose, $p=0.017$ liver). IL-1RA message was significantly upregulated in hippocampus ($p=0.046$ hippo) as well as in the liver ($p<0.001$ liver), but was downregulated in the adipose ($p=0.014$), and remained unchanged in hypothalamus and cortex. IL-1R2 was not significantly different in any brain regions, but was significantly upregulated in both adipose and liver ($p=0.008$ adipose, $p=0,016$ liver), both transformed to RANK for equal variances.

2. IL-1R2 and IL-1RA protein increase in liver after 24 h fast.

Table 2 shows that, in agreement with up-regulated gene transcript of IL-1R2, mice subjected to a 24 h fast had increased IL-1R2 protein in the liver, $p=0.004$; data transformed to log(abs) for normality. Also in agreement with up-regulated gene transcript of IL-1RA, mice subjected to a 24 h fast had increased IL-

1RA protein in the liver, p<0.001; data transformed to log(abs) for equal variance. No significant difference in serum IL-1R2 or IL-1RA protein due to fasting.

3. Fasting effects social exploratory behavior in mice

Fig. 1A shows that a 24 h fast causes significant increased social interaction in mice (Fed 165.2± 13.9 vs. Fasted 210.5± 6.22). Main effect significant at p=0.015. Fig. 1B shows that fasting has no significant effect on locomotor behavior in mice (Fed 3155.0± 267.4 vs. Fasted 2916.3± 190.6). No significance at p=0.484. There was no significance seen with additional adverse behavior tests or cognitive deficits observed when tested with these other paradigms: Open Field, Novel Object Recognition, or Y-Maze (data not shown).

4. Fasting induces resistance to IL-1β-induced sickness behavior.

Fig. 2A shows that fasted mice are resistant to decreased social exploratory behavior of a juvenile 2 h after IL-1β injection (Fed Saline 123.048 ± 16.493s vs. Fasted Saline 172.414 ± 9.737s vs. Fed IL-1β 62.721± 13.082s vs. Fasted IL-1β 112.900 ± 22.296s); main effects of treatment (P<0.001) and state (P=0.004), no treatment-state interaction (P=0.980). Fig. 2B demonstrates that fasting a mouse prior to injecting with inflammatory IL-1β induces resistance to sickness as shown by resistance to decreased locomotor activity at 2 h, spontaneous locomotor activity was measured 2 h post injection (Fed Saline 1213.374 ± 36.764cm vs. Fasted Saline 1012.946 ± 115.244cm vs. Fed IL-1β 131.176± 74.121cm vs. Fasted IL-1β 731.617 ± 110.055cm); main effects of treatment (P<0.001) and state (P=0.046), treatment-state interaction (P<0.001). Fig. 2C Shows that mice subjected to a 24 h fast were resistant to IL-1β-induced sickness weight loss. Mice were weighed 2 h post injection and compared to their initial weight (0 h) prior to treatment (Fed Saline -0.400 ± 0.204g vs. Fasted Saline -0.300 ± 0.108g vs. Fed IL-1β -1.280± 0.180g vs. Fasted IL-1β -0.240 ± 0.067g); main effects of treatment (P=0.016) and state (P=0.002), treatment-state interaction (P<0.007). Fig. 2D Shows that fasting mice for 24 h prior to IL-1β

immune challenge induces resistance to IL-1β-induced decrease in blood glucose. Blood glucose was measured 2 h post injection and compared to their initial blood glucose (0 h) prior to treatment (Fed Saline -12.000 ± 18.898mg/dl vs. Fasted Saline -5.750 ± 18.368mg/dl vs. Fed IL-1β -111.200 ± 14.900mg/dl vs. Fasted IL-1β -11.400 ± 7.619mg/dl); main effects of treatment (P=0.004) and state (P=0.003), treatment-state interaction (P<0.007).

5. Effect of challenging mice with IL-1β on brain region cytokine transcripts

Fig. 3A demonstrates that challenging mice peripherally with IL-1β induces IL-1R2 gene transcript changes in specific brain regions regardless of a prior 24 h fast. 2 h post injection, brain regions were harvested (Hypothalamus-Hypoth, Hippocampus-Hippoc, Cortex-Cortex), gene transcripts were measured as relative fold ΔmRNA (Hypoth; Fed, Saline 1.000 +0.119 vs. Fasted, Saline 1.272 +0.036 vs. Fed, IL-1β 1.817 +0.189 vs. Fasted, IL-1β 1.877 +0.233); main effects of treatment (P<0.001) and state (P=0.182), no significant treatment-state interaction (P=0.300). (Hippoc; Fed, Saline 1.000 +0.172 vs. Fasted, Saline 1.150 +0.103 vs. Fed, IL-1β 1.811 +0.245 vs. Fasted, IL-1β 2.290 +0.312); main effects of treatment (P<0.001) and state (P=0.169), no significant treatment-state interaction (P=0.720). (Cortex; Fed, Saline 1.000 +0.030 vs. Fasted, Saline 1.274 +0.068 vs. Fed, IL-1β 1.579 +0.124 vs. Fasted, IL-1β 1.900 +0.104); main effects of treatment (P<0.001) and state (P=0.002), no significant treatment-state interaction (P=0.614). Fig. 3B demonstrates that challenging mice peripherally with IL-1β induces IL-1RA gene transcript changes in specific brain regions regardless of a prior 24 h fast. 2 h post injection, brain regions were harvested (Hypothalamus-Hypoth, Hippocampus-Hippoc, Cortex-Cortex), gene transcripts were measured as relative fold ΔmRNA (Hypoth; Fed, Saline 1.000 +0.442 vs. Fasted, Saline 0.461 +0.037 vs. Fed, IL-1β 52.130+2.897 vs. Fasted, IL-1β 34.184 +3.063); main effects of treatment (P<0.001) and state (P<0.001), no significant treatment-state interaction (P=0.747), data transformed to RANK for normality. (Hippoc; Fed, Saline 1.000, +0.412 vs. Fasted, Saline 0.870, +0.267 vs. Fed, IL-1β

49.634 +8.322 vs. Fasted, IL-1β 31.402 +5.176); main effects of treatment (P<0.001) and state (P=.245), no significant treatment-state interaction (P=0.525). (Cortex; Fed, Saline 1.000, +0.063 vs. Fasted, Saline 1.118 +0.175 vs. Fed, IL-1β 23.641 +3.136 vs. Fasted, IL-1β 16.506 +2.57); main effects of treatment (P<0.001) and state (P=.339), no significant treatment-state interaction (P=0.082). Fig. 3C demonstrates that challenging mice peripherally with IL-1β induces IL-1α gene transcript changes in specific brain regions regardless of a prior 24 h fast. 2 h post injection, brain regions were harvested (Hypothalamus-Hypoth, Hippocampus-Hippoc, Cortex-Cortex), gene transcripts were measured as relative fold ΔmRNA (Hypoth; Fed, Saline 1.000 +0.179 vs. Fasted, Saline 0.707 +0.036 vs. Fed, IL-1β 13.471 +0.613 vs. Fasted, IL-1β 10.354 +1.21); main effects of treatment (P<0.001) and state (P=0.013), no significant treatment-state interaction (P=0.700). (Hippoc; Fed, Saline 1.000, +0.065 vs. Fasted, Saline 0.739 +0.063 vs. Fed, IL-1β 7.638 +0.557 vs. Fasted, IL-1β 7.665 +1.57); main effects of treatment (P<0.001) and state (P=.210), no significant treatment-state interaction (P=0.200). (Cortex; Fed, Saline 1.000, +0.074 vs. Fasted, Saline 0.546 +0.074 vs. Fed, IL-1β 8.907 +0.955 vs. Fasted, IL-1β 7.347 +1.362); main effects of treatment (P<0.001) and state (P=0.01), no significant treatment-state interaction (P=0.168). Fig. 3D demonstrates that challenging mice peripherally with IL-1β induces IL-1β gene transcript changes in specific brain regions regardless of a prior 24 h fast. 2 h post injection, brain regions were harvested (Hypothalamus-Hypoth, Hippocampus-Hippoc, Cortex-Cortex), gene transcripts were measured as relative fold ΔmRNA (Hypoth; Fed, Saline 1.000 +0.412 vs. Fasted, Saline 0.425 +0.067 vs. Fed, IL-1β 72.810 +14.91 vs. Fasted, IL-1β 69.313 +9.665); main effects of treatment (P<0.001) and state (P=0.061), no significant treatment-state interaction (P=0.091). (Hippoc; Fed, Saline 1.000, +0.134 vs. Fasted, Saline 0.977, +0.044 vs. Fed, IL-1β 11.289 +1.355 vs. Fasted, IL-1β 12.606 +2.138); main effects of treatment (P<0.001) and state (P=.715), no significant treatment-state interaction (P=0.583). (Cortex; Fed, Saline 1.000, +0.053 vs. Fasted, Saline 0.849 +0.110 vs. Fed, IL-1β 11.119 +1.708 vs. Fasted, IL-1β

10.391 +1.480); main effects of treatment (P<0.001) and state (P=.347), no significant treatment-state interaction (P=0.693).

6. Substantial up-regulation in liver and adipose IL-1R2 gene expression and in liver IL-1RA gene expression and their correlations to percent weight loss.

Fig. 4A shows that IL-1R2 is substantially upregulated in the liver after a 24 h fast. There is a significant difference in average fold change in mRNA in liver fed vs. fasted (1.082± 0.095 vs. 27.975± 13.853) significant main effect, p<0.001 run on ΔCT values transformed to ^2 for normality. Fig. 4B shows that percent weight loss is negatively correlated to ΔCT value, data ran by Pearson Correlation test. The more weight the mouse lost during a 24 h fast, the lower the ΔCT value in PCR was for IL-1R2 gene expression in liver, and liver IL-1R2 correlation coefficient was -0.530, p=0.002.. Fig. 4C shows that IL-1R2 is substantially upregulated in adipose with a significant difference in average fold change in mRNA in adipose fed vs. fasted (1.118± 0.089 vs. 69.577± 21.829) significant main effect, p<0.001 run on ΔCT values transformed to RANK for normality. Fig. 4D shows that percent weight loss is negatively correlated to ΔCT value, data ran by Pearson Correlation test. The more weight the mouse lost during a 24 h fast, the lower the ΔCT value in PCR was for IL-1R2 gene expression in adipose. Adipose IL-1R2 correlation coefficient was -0.601, p<.001. Fig. 4E shows that IL-1RA is also substantically upregulated in the liver after a 24 h fast and there was a significant difference in average fold change in mRNA in liver fed vs. fasted (1.219± 0.140 vs. 33.792± 6.292) significant main effect, p<0.001. Fig. 4F shows that percent weight loss was not significantly correlated to ΔCT value for IL-1RA in liver, correlation coefficient -0.306, p=0.094. There was no significant correlation in any groups to age of mouse at fast (between 9-14wks) and ΔCT value for IL-1R2 or IL-1RA, data not shown. There was also no correlation between starting weight at time of fast and ΔCT value for IL-1R2 or IL-1RA, data not shown.

7. IL-1R2 and IL-1RA gene expression on CD11b+, CD11b- and hepatocytes isolated from liver.

Fig. 5A shows that IL-1R2 is primarily expressed only in those cells with CD11b when compared to CD11b- cells. Although there was no significance between fed or fasted in either isolated cell group, CD11b+ cells had significantly more expression of IL-1R2 than CD11b- cells (CD11b+ Fed 1.000, +0.206-0.171 vs. CD11b+ Fasted .902, +0.285-0.216 vs. CD11b- Fed 0.001, +0.000- 0.000 vs. CD11b- Fasted 0.001, +0.000-0.000); main effects of cell type ($P<0.001$) and state ($P=0.024$), no significant cell type-state interaction ($P=0.444$). Fig. 5B shows that IL-1RA is primarily expressed only in those cells with CD11b when compared to CD11b- cells. Although there was no significance between fed or fasted in either isolated cell group, CD11b+ cells had significantly more expression of IL-1RA than CD11b- cells (CD11b+ Fed 1.000, +0.221-0.181 vs. CD11b+ Fasted .620, +0.051-0.047 vs. CD11b- Fed 0.027, +0.002-0.002 vs. CD11b- Fasted 0.022, +0.004-0.003); main effects of cell type ($P<0.001$) and state ($P=0.058$), no significant cell type-state interaction ($P=0.454$). Fig. 5C shows that IL-1R2 expression is not different due to the fasting state in isolated hepatocytes (Fed 1.000, +0.337-0.252 vs Fasted 0.847 +0.223-0.176). Fig. 5D shows that IL-1RA expression is upregulated after a 24 h fast in hepatocytes (Fed 1.000, +0.199-0.166 vs. 7.146 +2.509-1.857) $p=0.001$. Fig5E shows that allowing a piece of liver to sit in buffer for 6 h after harvest, compared to extracting RNA within 1 h, significantly decreases IL-1R2 message (1.000, +0.219-0.180 vs. 0.013, +0.001-0.001), $p<0.001$, and also significantly decreases IL-1RA message (1.000, +0.175-0.149 vs. 0.437, +0.022-0.021), $p=0.009$.

8. Effect of fasting on gene expression of IL-1R2 and IL-1RA in liver and adipose of knockout mouse models.

Table 3 shows that IL-1R2 and IL-1RA gene transcripts after fasting in the liver and adipose are not impacted by knockout of IL-1R1, TLR-4 or IL-4 genes in mouse models. In IL-1R1 KO mice, fasting significantly up-regulated IL-1R2 mRNA by 8 fold in the liver ($p=0.021$), and by 18 fold in the adipose ($p=.007$). Fasting of IL-1R1 KO mice also significantly up-regulated IL-1RA mRNA in the liver by 16 fold

(p<0.001), and had no significant difference in adipose IL-1RA. In TLR-4 KO mice, fasting significantly up-regulated IL-1R2 mRNA by 7 fold in the liver (p<0.005), and by 9 fold in the adipose (p=.002), data transformed to RANK for equal variances. Fasting of TLR-4 KO mice also significantly up-regulated IL-1RA mRNA in the liver by 16 fold (p<0.001), and had no significant difference in adipose IL-1RA. In IL-4 KO mice, fasting significantly up-regulated IL-1R2 mRNA by 8 fold in the liver (p<0.050), and by 22 fold in the adipose (p=.022). Fasting of IL-4 KO mice also significantly up-regulated IL-1RA mRNA in the liver by 13 fold (p<0.001), and had no significant difference in adipose IL-1RA. None of these groups were statistically different from their WT controls (data not shown).

9. Effect of fasting on plasma corticosterone levels in mice.

Fig. 6A confirms that a 24 h fast causes significant increased plasma corticosterone levels in mice (Fed 368.120± 28.590 vs. Fasted 754.828± 84.421). Main effect significant at p=0.004. Fig. 6B shows that this increase in plasma corticosterone is significantly positively correlated (correlation coefficient 0.899) with percent body weight lost by the mouse in a 24 h fast, (p=0.005).

10. Effect of inhibiting glucocorticoid receptor activity on IL-1R2 and IL-1RA gene transcripts in liver.

Fig. 7A demonstrates that inhibiting glucocorticoid signaling via GCR antagonist (Mifepristone) does not effect IL-1R2 gene transcripts in the liver after a 24 h fasting period. Mouse liver was harvested after a 24 h fast and IL-1R2 gene transcripts were measured as relative fold ΔmRNA (Fed None 1.000, +0.103 -0.094; vs. Fasted None 4.247, +0.623 -0.543; vs. Fed Mifepristone 1.789, +0.299 -0.256; vs. Fasted Mifepristone 4.790, +0.609 -0.540; vs. Fed PEG 1.327, +0.126 -0.115; vs. Fasted PEG 3.471, +0.661 -0.555); main effects of treatment (P=0.058) and state (P<0.001), no significant treatment-state interaction (P=0.160). Fig. 7B also demonstrates that inhibiting glucocorticoid signaling via GCR antagonist (Mifepristone) does not effect IL-1RA gene transcripts in the liver after a 24 h fasting period. Mouse liver was harvested after a 24 h fast and IL-1RA gene transcripts were measured as relative fold

ΔmRNA (Fed None 1.000, +0.144 -0.126; vs. Fasted None 8.939, +1.691 -1.422; vs. Fed Mifepristone 1.236, +0.157 -0.140; vs. Fasted Mifepristone 9.521, +1.587 -1.260; vs. Fed PEG 1.086, +0.152 -0.133; vs. Fasted PEG 5.182, +1.395 -1.099); main effects of treatment (P=0.077) and state (P<0.001), no significant treatment-state interaction (P=0.161).

11. Effect of fasting and palmitic acid injection on NEFA levels in mice.

Fig. 8A shows fasting causes plasma NEFAs to double (Fed 0.927± 0.104 vs. Fasted 2.207± 0.161), and this was significant at p<0.001. Fig. 8B shows that palmitic acid injection mimics fasting plasma NEFAs increase 2 h after palmitic acid injection (Vehicle 0.881± 0.165 vs. Palmitic 1.63± 0.103); main effects of treatment (P=0.003).

12. Liver IL-1R2 gene transcription levels after palmitic acid injection

Fig. 9A shows that IL-1R2 is upregulated in the liver of mice 2 h after they are injected with palmitic acid. Palmitic injected mice had significantly more expression of IL-1R2 in liver than vehicle controls (Vehicle 1.000, +0.213-0.175 vs. Palmitic 5.06, +3.07-1.91); significant main effect (P=0.01). Fig. 9B shows that IL-1R2 is significantly increased in the liver 2 h after palmitic acid regardless of TLR-4. (WT, Vehicle 1.000, +0.113-0.101 vs. WT. Palmitic 3.267, +0.592-0.501 vs. TLR-4, Vehicle 0.97, +0.085- 0.078 vs. TLR-4, Palmitic 2.91, +0.558-0.468); main effects of treatment (P<0.001) no significant effect of genotype (P=0.645), no significant treatment-genotype interaction (P=0.75).

CHAPTER 5

TABLES, FIGURES AND FIGURE LEGENDS

Table 1
Effect of fasting on gene expression

Tissue	Hypothalamus		Hippocampus		Cortex		Adipose		Liver	
	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted	Fed	Fasted
CD11b	1.000 (0.05,0.05)	0.577* (0.03,0.03)	1.000 (0.01,0.01)	0.599* (0.03,0.03)	1.000 (0.04,0.04)	0.540* (0.04,0.03)	1.000 (0.06,0.06)	3.732* (0.58,0.50)	1.000 (0.10,0.09)	6.016* (2.69,1.64)
TNF-α	1.000 (0.18,0.15)	0.479* (0.03,0.02)	1.000 (0.09,0.08)	0.614* (0.06,0.05)	1.000 (0.07,0.07)	0.607* (0.02,0.02)	1.000 (0.28,0.22)	0.170* (0.03,0.02)	1.000 (0.10,0.09)	0.660* (0.08,0.07)
IL-1α	1.000 (0.06,0.06)	0.567* (0.03,0.03)	1.000 (0.05,0.05)	0.565* (0.02,0.02)	1.000 (0.10,0.09)	0.496* (0.02,0.02)	1.000 (0.58,0.36)	0.295 (0.04,0.04)	1.000 (0.14,0.12)	0.857 (0.03,0.03)
IL-1β	1.000 (0.17,0.14)	0.487* (0.05,0.05)	1.000 (0.08,0.07)	0.791 (0.06,0.05)	1.000 (0.12,0.11)	0.9018 (0.10,0.09)	1.000 (0.36,0.26)	0.167* (0.03,0.03)	1.000 (0.12,0.11)	.979 (0.33,0.25)
IL-1R1	1.000 (0.10,0.09)	1.484* (0.12,0.11)	1.000 (0.20,0.16)	1.600* (0.18,0.10)	1.000 (0.03,0.03)	1.319* (0.05,0.05)	1.000 (0.11,0.10)	2.334* (0.35,0.30)	1.000 (0.12,0.11)	3.130* (0.95,0.69)
IL-1RA	1.000 (0.32,0.24)	0.920 (0.24,0.19)	1.000 (0.11,0.09)	1.600* (0.33,0.27)	1.000 (0.14,0.12)	1.066 (0.11,0.10)	1.000 (0.24,0.19)	0.254* (0.07,0.06)	1.000 (1.55,0.64)	68.482* (38.6,24.7)
IL-1R2	1.000 (0.12,0.11)	1.104 (0.04,0.04)	1.000 (0.09,0.09)	0.880 (0.09,0.08)	1.000 (0.04,0.04)	1.061 (0.06,0.05)	1.000 (0.25,0.02)	83.424* (79.0,40.5)	1.000 (0.14,0.12)	9.467* (19.3,6.35)

Results are expressed as relative fold change in mRNA (ΔmRNA), means (SEM upper, SEM lower); n=4-9
*** p< 0.05, significant (One-way ANOVA)**

Table 2
Effect of fasting on protein in liver (pg IL-1R2 or IL-1RA/ng total protein) and protein in serum (pg/mL)

	IL-1R2		IL-1RA	
	Fed	Fasted	Fed	Fasted
Liver	8,475.2 ±1,837.4	27,140.4* ±8,993.1	29,054.4 ±3,580.0	71,722.6* ±11,176.4
Serum	11,112.2 ±392.7	10,646.6 ±350.4	661.9 ±112.7	693.6 ±95.7

Results are expressed as means (±SEM); Liver IL-1R2, n=7-8. Liver IL-1RA, n=13-16. Serum IL-1R2, n=8. Serum IL-1RA, n=8
*** p< 0.05, significant (One-way ANOVA)**

Table 2 *IL-1R2 and IL-1RA protein increase in liver after 24 h fast*. Mice were subjected to either a 24 h fast (Fasted), or continued to feed *ad lib* (Fed). IL-1R2 results are expressed as pg IL-1R2/ng total liver protein, means (± SEM); n=7-8. *p <0.05. IL-1RA results are expressed as pg IL-1RA/ng total liver protein, means (± SEM; n=13-16. *p<0.001. Serum IL-1R2 and IL-1RA levels are not significant, results are expressed as pg/mL.

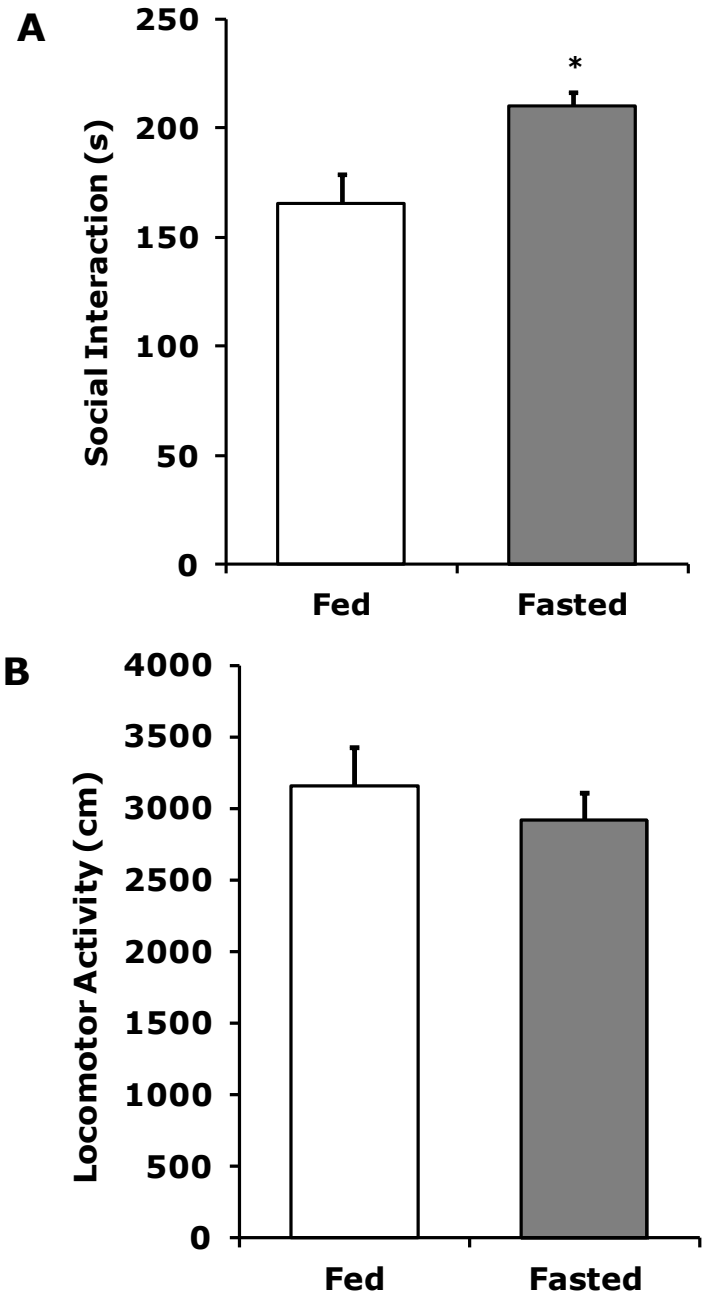

Figure 1. *Fasting effects social exploratory behavior, but not locomotor activity in mice.* Mice were subjected to either a 24 h fast (Fasted), or continued to feed *ad lib* (Fed). (A) Mice were introduced to a novel juvenile mouse and allowed to explore for 5 minutes. Results are expressed as seconds (s) spent being socially interactive, means (±SEM); n=8. Significant main effect (*p <0.05). (B) Locomotor activity was tracked for a 5 minute interval. Results are expressed as centimeters (cm) moved, means (±SEM); n=6.

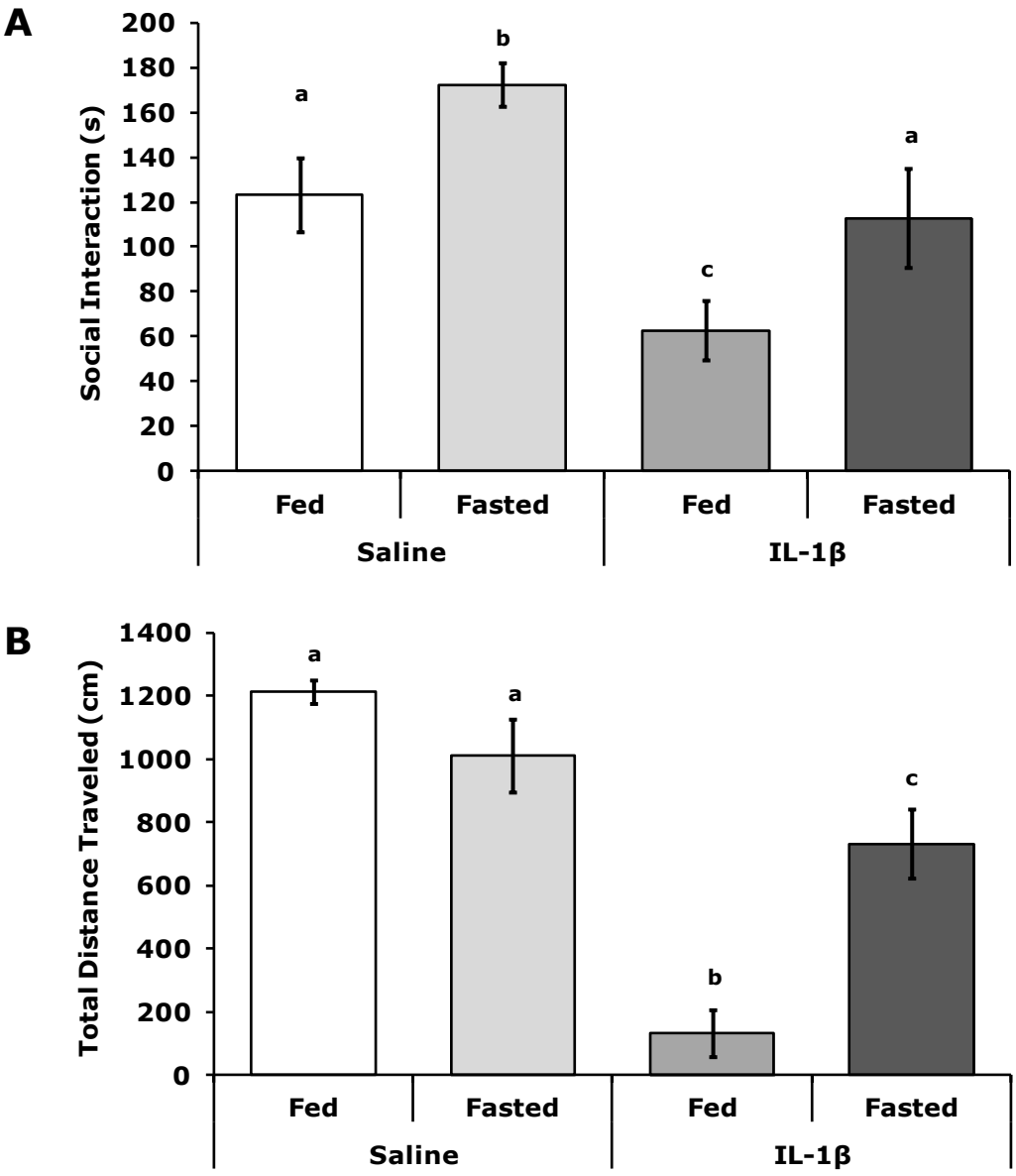

Figure 2 *Fasting induces resistance to IL-1β-induced sickness behavior.* Mice were subjected to either a 24 h fast (Fasted), or continued to feed *ad lib* (Fed) prior to IL-1β or vehicle (Saline) at 0 h. (A) A juvenile was introduced 2 h post injection, and social interaction was measured as seconds (s) spent investigating juvenile for a duration of 5 minutes, means (±SEM); n=8. Bars without a common superscript are different (*p <0.05). (B) 2 h post injection, spontaneous locomotor activity was measured for 5 minutes. Results are expressed as total distance traveled in centimeters (cm), means (± SEM); n=4. Bars without a common superscript are different (*p <0.05).

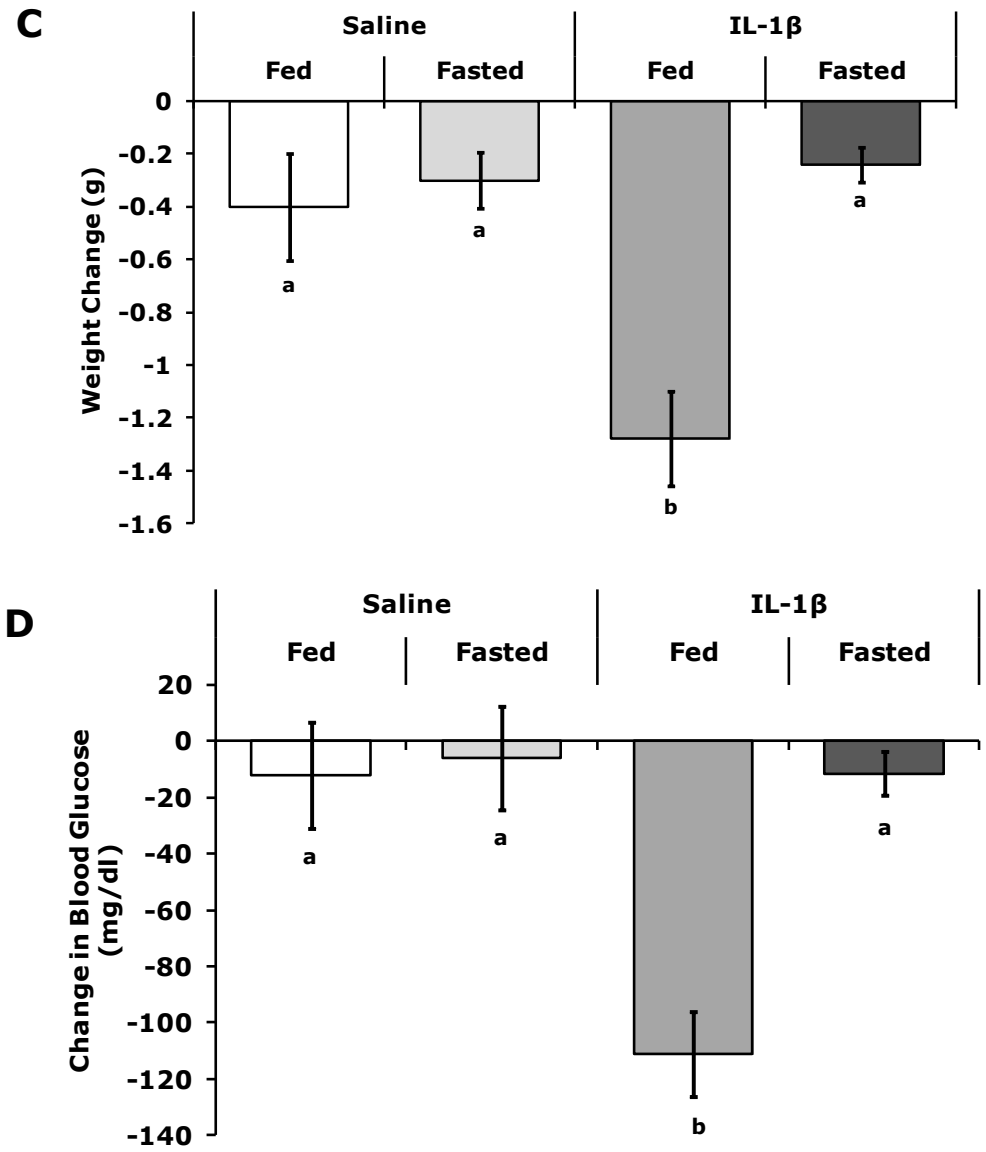

Figure 2 (cont.) *Fasting induces resistance to IL-1β-induced sickness behavior.* Mice were subjected to either a 24 h fast (Fasted), or continued to feed *ad lib* (Fed) prior to IL-1β or vehicle (Saline) at 0 h. (C) Mice were weighed 2 h post injection and compared to their weight at 0 h. Results are expressed as weight change from 0 h in grams (g), means (± SEM); n=4-5. Bars without a common superscript are different (*p<0.05). (D) Blood Glucose was measured 2 h post injection and compared to 0 h blood glucose measurement for each individual mouse. Results are expressed as change in blood glucose (mg/dl), means (± SEM); n=4-5. Bars without a common superscript are different (p<0.05).

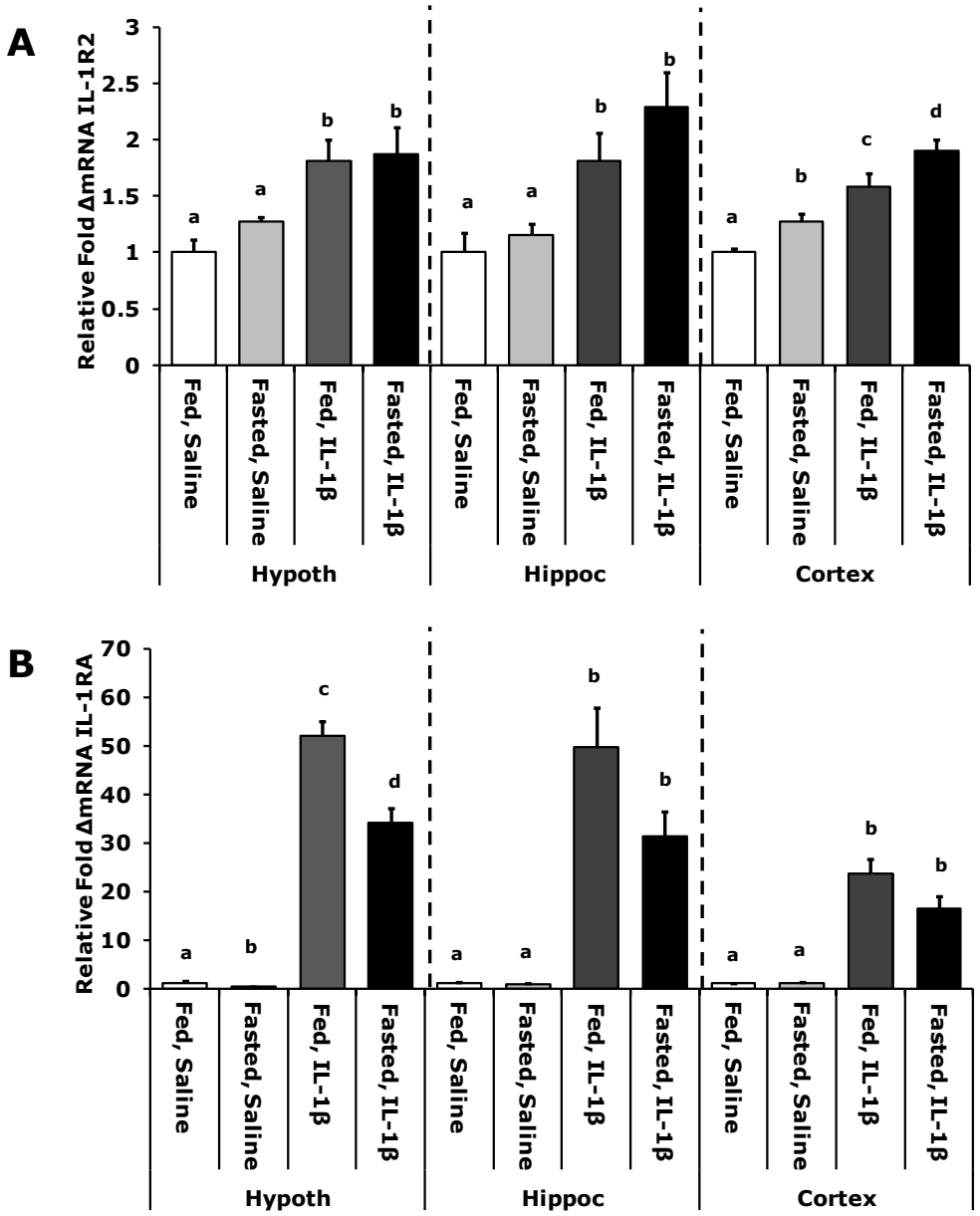

Figure 3. *Effect of challenging mice with IL-1β on brain region cytokine transcripts.* Mice were subjected to either a 24 h fast (Fasted), or continued to feed *ad lib* (Fed) prior to IL-1β or vehicle (Saline) at 0 h. 2 h post injection, brain regions were harvested (Hypothalamus-Hypoth, Hippocampus-Hippoc, Cortex-Cortex). (A) IL-1R2 gene transcripts were measured and compared to set control group within each separate region (Fed, Saline). Results are expressed as relative fold change in mRNA, means (+ SEM Upper); n=4. Bars without a common superscript are different (*p <0.05). (B) IL-1RA gene transcripts were measured and compared to set control group within each separate brain region (Fed, Saline). Results are expressed as relative fold change in mRNA, means (+ SEM Upper); n=4. Bars without a common superscript are different (*p <0.05).

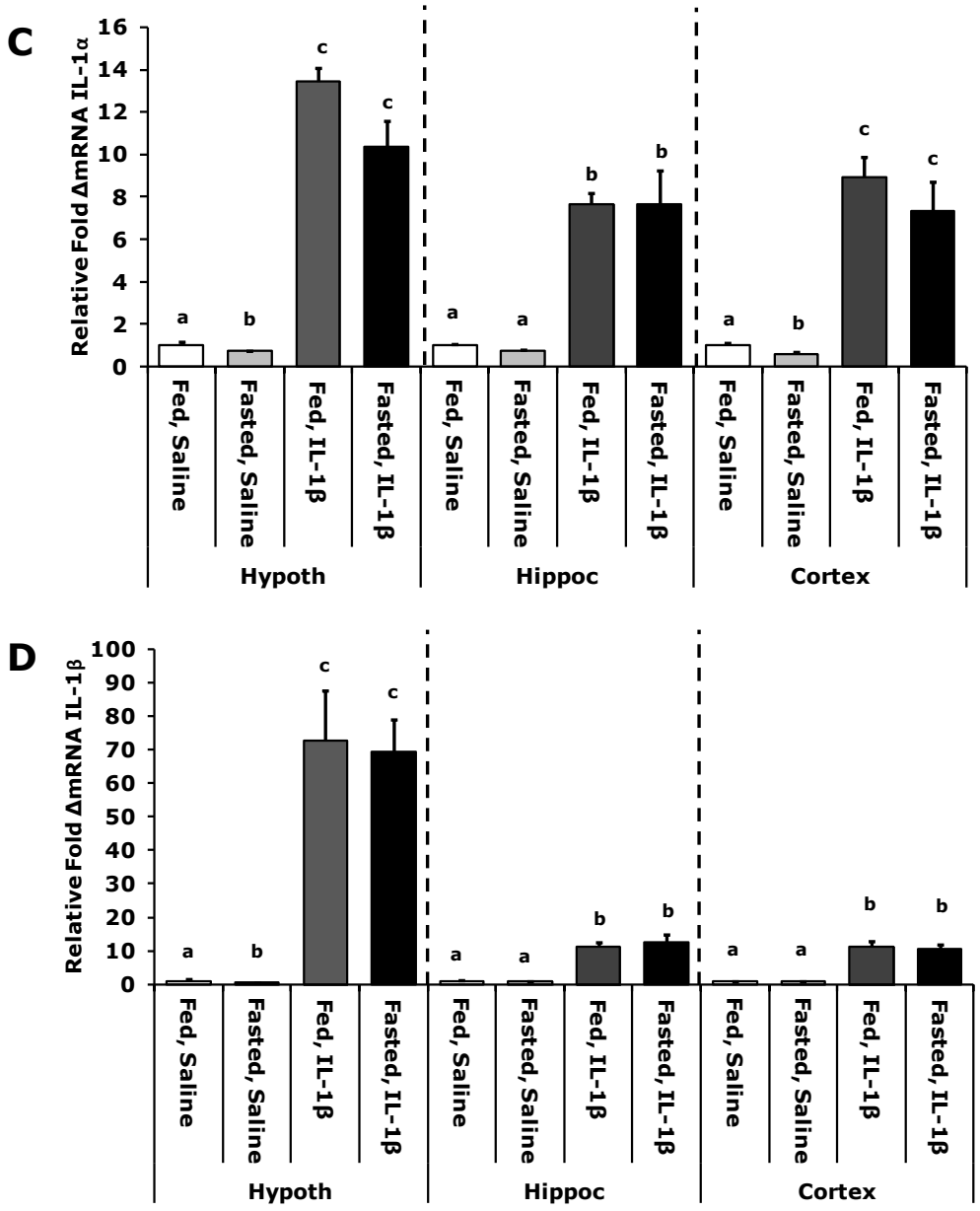

Figure 3 (cont.). *Effect of challenging mice with IL-1β on brain region cytokine transcripts.* Mice were subjected to either a 24 h fast (Fasted), or continued to feed *ad lib* (Fed) prior to IL-1β or vehicle (Saline) at 0 h. 2 h post injection, brain regions were harvested (Hypothalamus-Hypoth, Hippocampus-Hippoc, Cortex-Cortex).(C) IL-1α gene transcripts were measured and compared to set control group within each separate brain region (Fed, Saline). Results are expressed as relative fold change in mRNA, means (+ SEM Upper); n=4. Bars without a common superscript are different (*p <0.05). (D) IL-1β gene transcripts were measured and compared to set control group within each separate brain region (Fed, Saline). Results are expressed as relative fold change in mRNA, means (+ SEM Upper); n=4. Bars without a common superscript are different (*p <0.05).

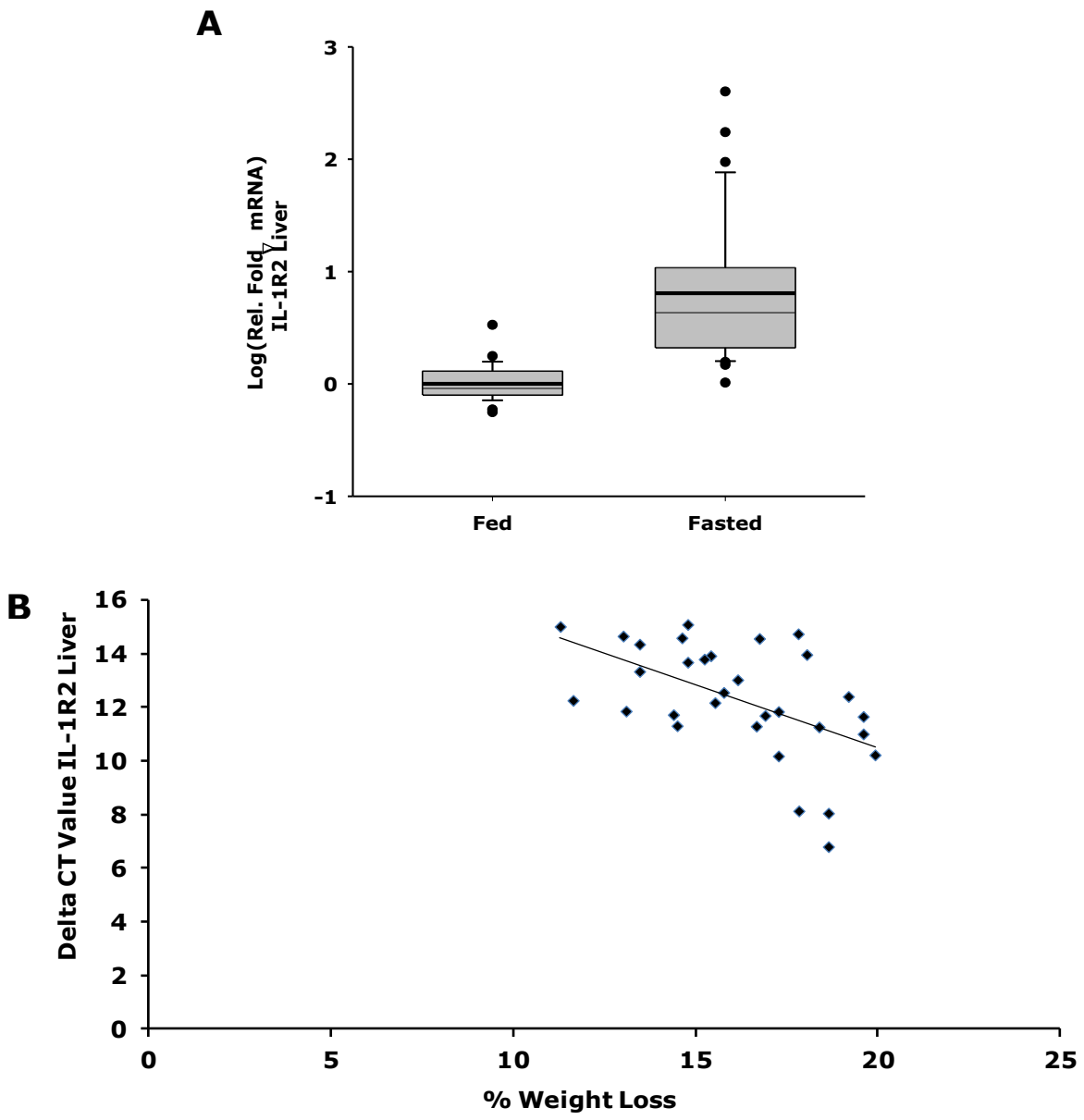

Figure 4. *Substantial up-regulation in liver and adipose IL-1R2 gene expression, and in liver IL-1RA gene expression and their correlations to percent weight loss.* These graphs represent a collective description of data from numerous cohorts of mice that were either fast (Fasted) for 24 h, or continued to feed *ad lib* (Fed). Box plots are representative of the summated relative fold changes in mRNA transformed to log; the box outlines the lower 1st quartile and upper 3rd quartile, the vertical bars represent the largest/smallest non-outlier observation, the thick horizontal line represents the mean of the relative fold change values and the thin horizontal line is the median, and black circles represent outliers (A) Liver IL-1R2 gene transcription results are summarized and expressed as log (average relative fold change (ΔmRNA)) for fed (n=29) and fasted groups (n=31) *p<0.001. (B) Percent weight lost per mouse was correlated to their dCT value for IL-1R2 in the adipose, significant correlation was observed p<0.001, n=30.

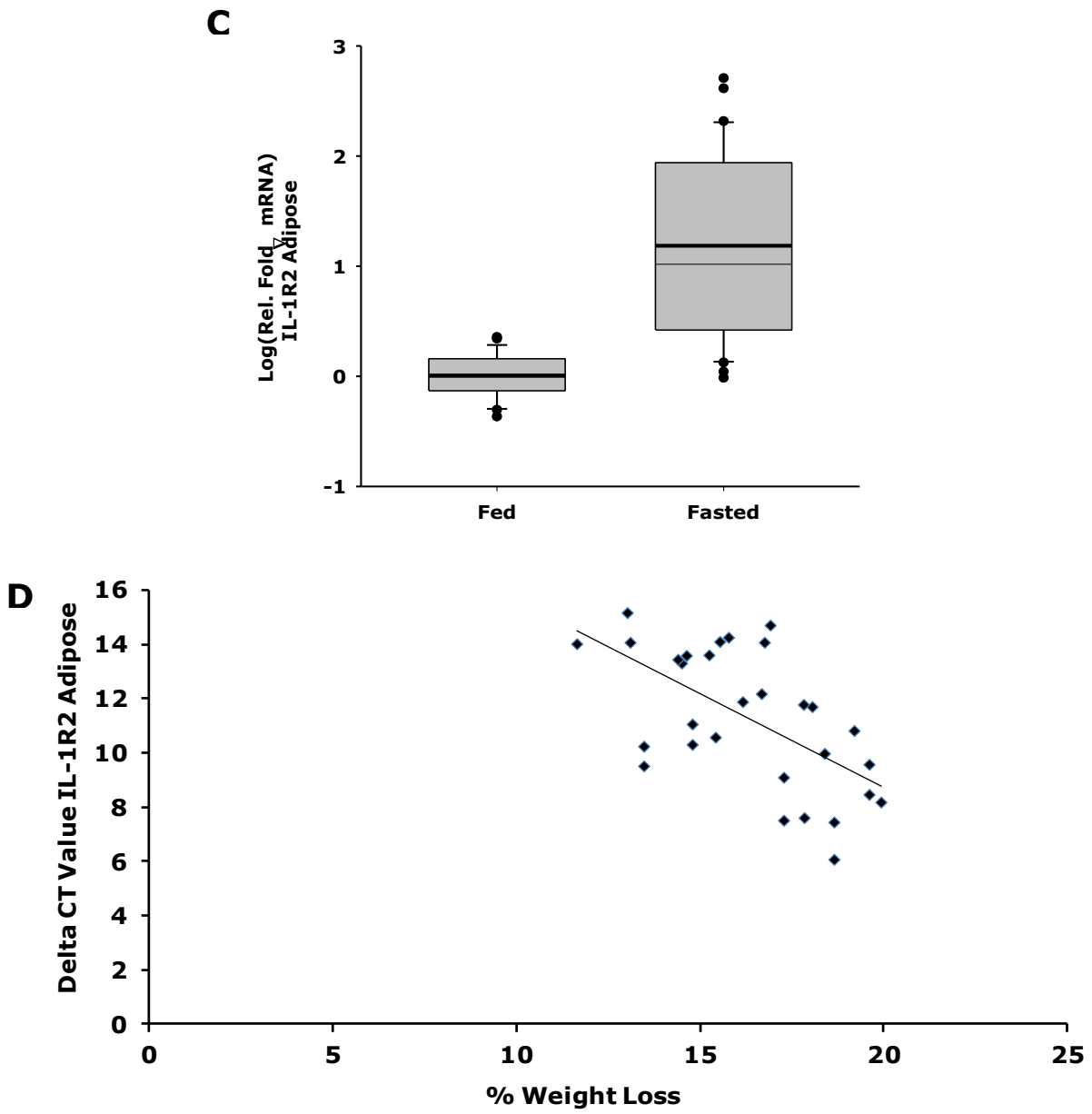

Figure 4 (cont.). *Substantial up-regulation in liver and adipose IL-1R2 gene expression, and in liver IL-1RA gene expression and their correlations to percent weight loss.* These graphs represent a collective description of data from numerous cohorts of mice that were either fast (Fasted) for 24 h, or continued to feed *ad lib* (Fed). Box plots are representative of the summated relative fold changes in mRNA transformed to log; the box outlines the lower 1st quartile and upper 3rd quartile, the vertical bars represent the largest/smallest non-outlier observation, the thick horizontal line represents the mean of the relative fold change values and the thin horizontal line is the median, and black circles represent outliers. (C) Adipose IL-1R2 gene transcription results are summarized and expressed as log (average relative fold change (ΔmRNA)) for fed (n=29) and fasted groups (n=30) *p<0.001.(D) Percent weight lost per mouse was correlated to their dCT value for IL-1R2 in the adipose, significant correlation was observed p<0.001, n=30.

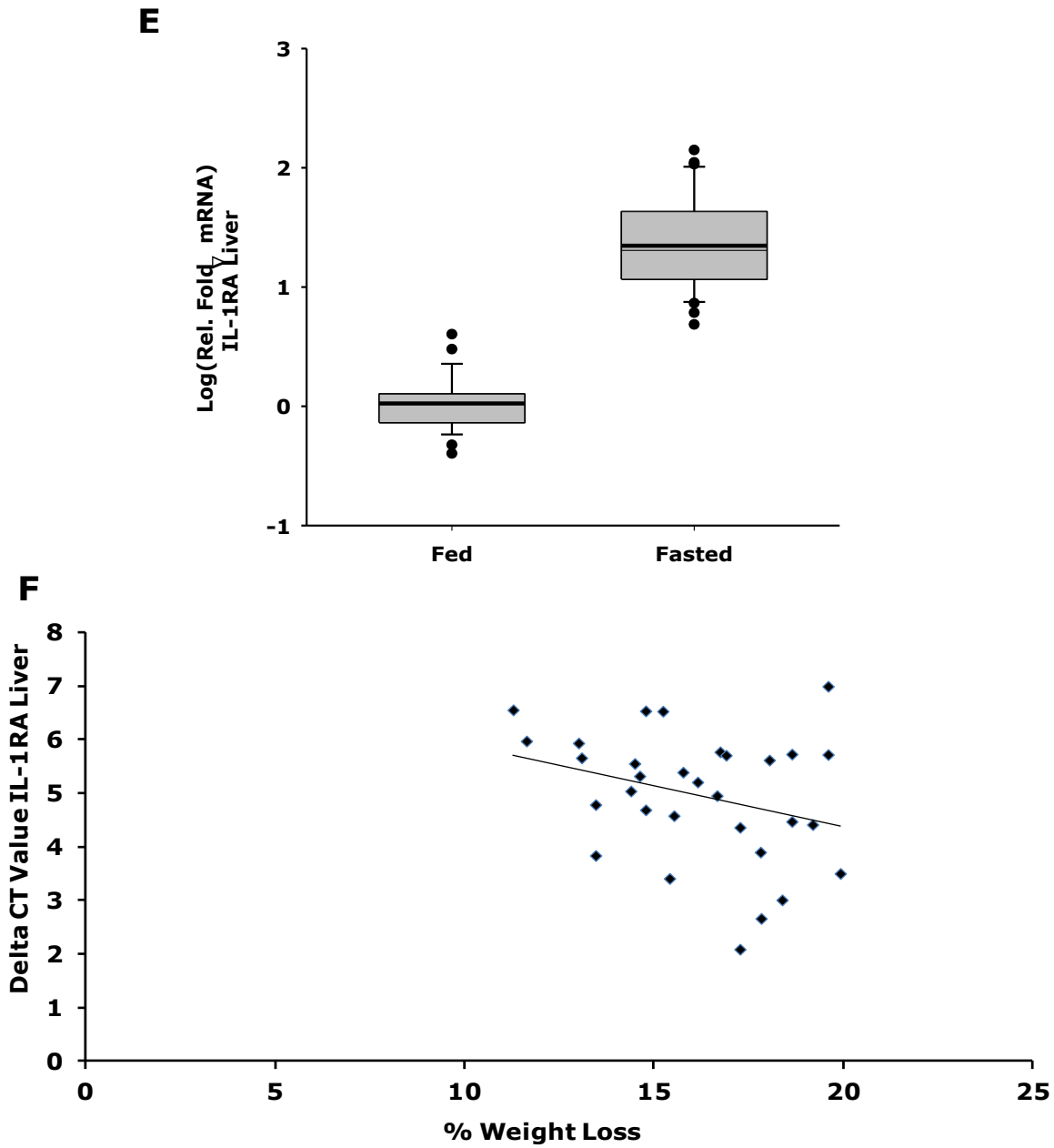

Figure 4 (cont.). *Substantial up-regulation in liver and adipose IL-1R2 gene expression, and in liver IL-1RA gene expression and their correlations to percent weight loss.* These graphs represent a collective description of data from numerous cohorts of mice that were either fast (Fasted) for 24 h, or continued to feed *ad lib* (Fed). Box plots are representative of the summated relative fold changes in mRNA transformed to log; the box outlines the lower 1st quartile and upper 3rd quartile, the vertical bars represent the largest/smallest non-outlier observation, the thick horizontal line represents the mean of the relative fold change values and the thin horizontal line is the median, and black circles represent outliers (E) Liver IL-1RA gene transcription results are summarized and expressed as log (average relative fold change (ΔmRNA)) for fed (n=29) and fasted groups (n=31) *p<0.001.(F) Percent weight lost per mouse was correlated to their dCT value for IL-1RA in the liver, no significant correlation, n=31.

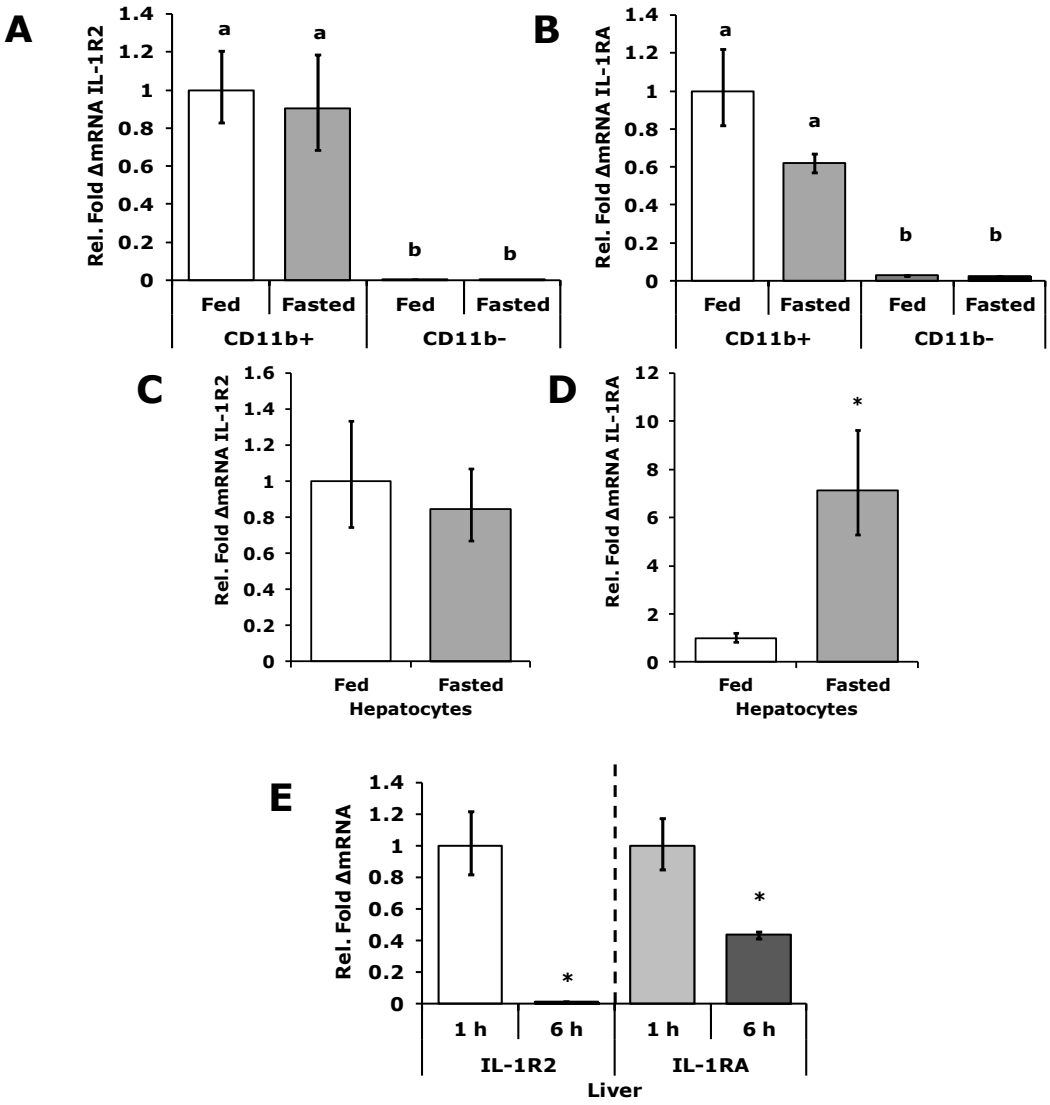

Figure 5. *IL-1R2 and IL-1RA gene expression on isolated liver cells.* Mice were subjected to either a 24 h fast (Fasted), or continued to feed *ad lib* (Fed), and cells were isolated. (A) RNA was extracted from CD11b+ and CD11b- cells isolated from liver, IL-1R2 results are expressed as relative fold change (ΔmRNA) compared to control CD11b+ Fed group, means (+ SEM Upper, - SEM Lower); n=8. Bars without a common superscript are different (*p <0.05). (B) IL-1RA results are expressed as relative fold change (ΔmRNA) compared to control CD11b+ Fed group, means (+ SEM Upper, - SEM Lower); n=8. Bars (C) RNA was extracted from hepatocytes isolated from liver and IL-1R2 gene expression was measured. Results are expressed as relative fold change (ΔmRNA), means (+ SEM Upper, - SEM Lower); n=4. (D) IL-1R2 gene expression was measured. Results are expressed as relative fold change (ΔmRNA), means (+ SEM Upper, - SEM Lower); n=4. *p <0.05. (E) Liver was harvested from mice and RNA was either extracted immediately, within 1 hour (1 h), or extracted 6 hours after harvest (6 h). IL-1R2 and IL-1RA gene expression was measured. Results are expressed as relative fold change (ΔmRNA), means (+ SEM Upper, - SEM Lower); n=4. *p <0.05.

Table 3
Effect of fasting on gene expression of IL-1R2 and IL-1RA in liver and adipose of knockout mouse models.

Genotype		IL-1R2		IL-1RA	
		Fed	Fasted	Fed	Fasted
IL-1R1 KO	Liver	1.000 (+0.33,-0.24)	8.306* (+7.09,-3.82)	1.000 (+0.08,-0.07)	16.271* (+2.06,-1.82)
	Adipose	1.000 (+0.24,-0.19)	18.783* (+37.2,-9.31)	1.000 (+0.14,-0.12)	0.589 (+0.87,-0.19)
TLR-4 KO	Liver	1.000 (+0.30,-0.23)	7.605* (+7.71,-3.83)	1.000 (+0.58,-0.37)	16.420* (+10.4,-6.37)
	Adipose	1.000 (+0.28,-0.24)	9.863* (+18.9,-6.48)	1.000 (+0.43,-0.31)	0.759 (+0.23,-0.17)
IL-4 KO	Liver	1.000 (+0.19,-0.16)	8.129* (+10.9,-4.66)	1.000 (+0.30,-0.23)	13.678* (+5.04,-3.68)
	Adipose	1.000 (+0.19,-0.16)	22.768* (+35.5,-13.8)	1.000 (+0.10,-0.09)	0.545 (+0.24,-0.17)

Results are expressed as relative fold change in mRNA expression (ΔmRNA), means (+SEM upper, -SEM lower); IL-1R1 KO n=4. TLR-4 KO n=7. IL-4 KO n=4.
*** p< 0.05, significant (One-way ANOVA)**

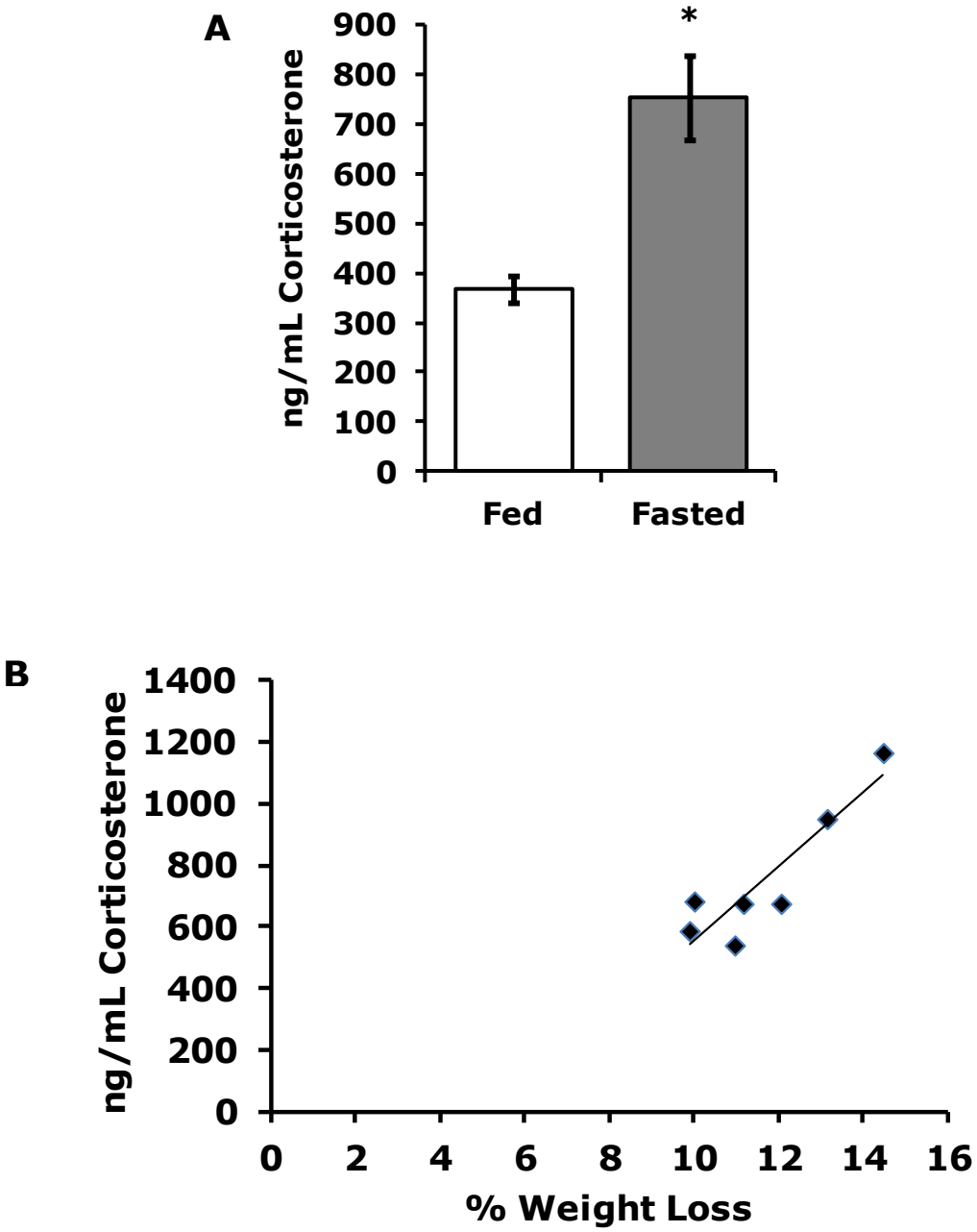

Figure 6. *Effect of fasting on plasma corticosterone levels in mice.* Mice were subjected to either a 24 h fast (Fasted), or continued to feed *ad lib* (Fed) before blood was harvested. (A) Corticosterone was measured and compared to control group (Fed). Results are expressed as ng/mL corticosterone, means (±SEM); n=5-7. Significant main effect (*p <0.05). (B) Corticosterone levels in ng/mL were positively correlated to percent weight lost by mouse; n=5-7, p=0.005.

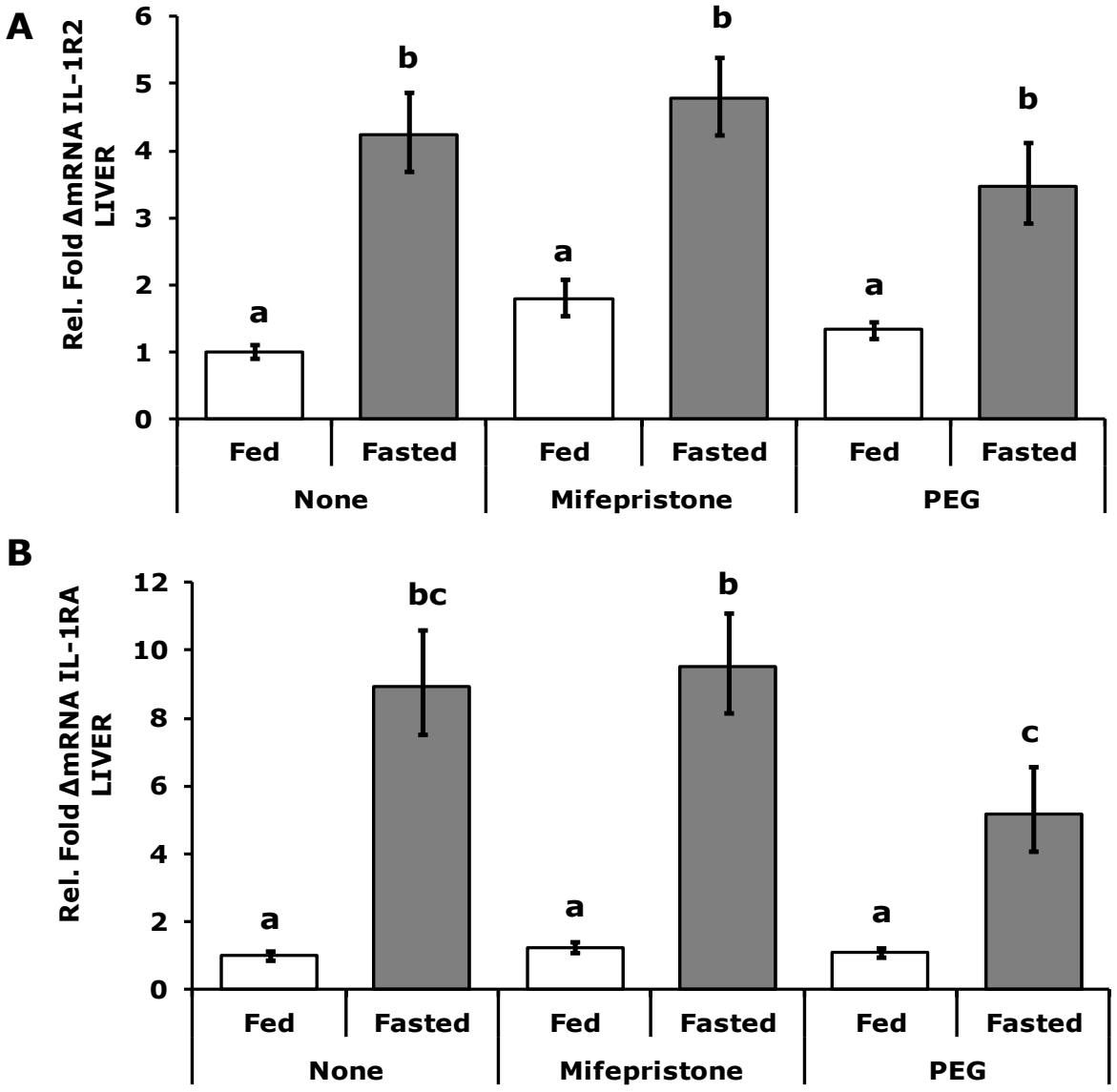

Figure 7. *Effect of inhibiting glucocorticoid receptor activity on IL-1R2 and IL-1RA gene transcripts in liver.* After injecting GCR antagonist (Mifepristone), vehicle (PEG) or sham scruffing with no injection (None), mice were subjected to either a 24 h fast (Fasted), or continued to feed *ad lib* (Fed). (A) Liver IL-1R2 gene transcripts were measured and compared to set control group (Fed None). Results are expressed as relative fold change in mRNA, means (+ SEM Upper, - SEM Lower); n=4-6. Bars without a common superscript are different (*p <0.05). (B) Liver IL-1RA gene transcripts were measured and compared to set control group (Fed None). Results are expressed as relative fold change in mRNA, means (+ SEM Upper, - SEM Lower); n=4-6. Bars without a common superscript are different (*p <0.05).

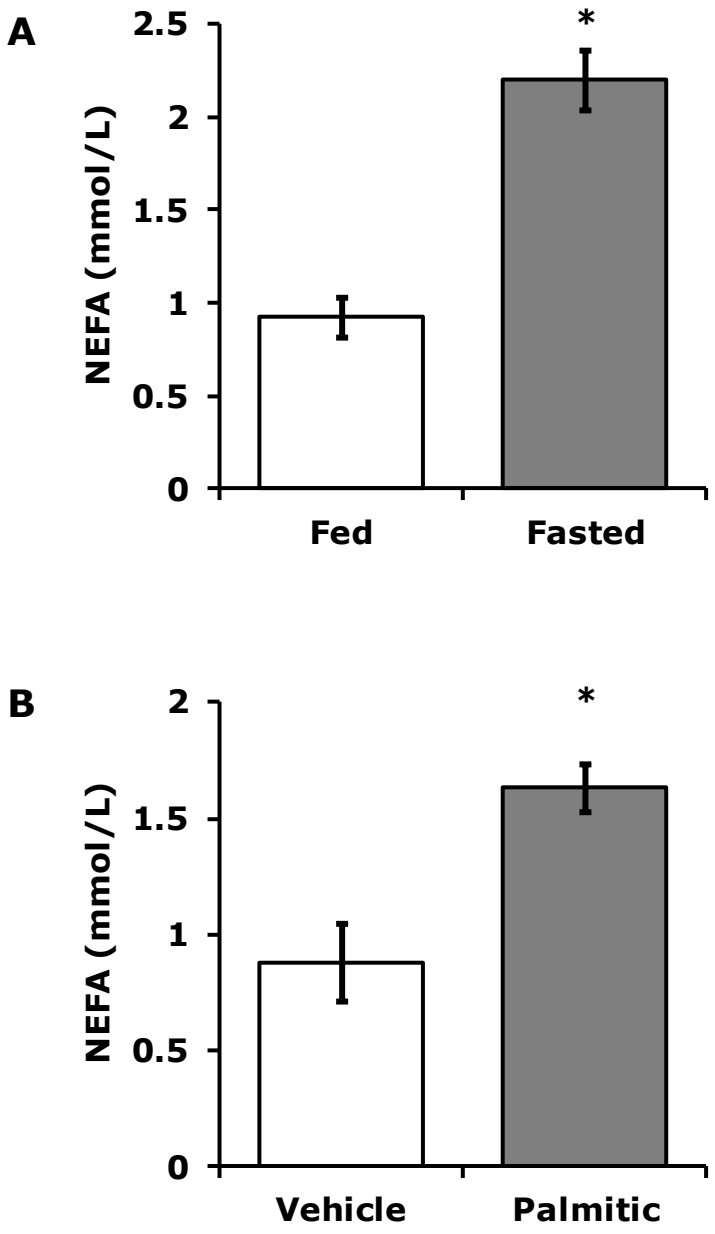

Figure 8. *Effect of fasting and palmitic acid injection on NEFA levels in mice*. (A) Mice were subjected to either a 24 h fast (Fasted), or continued to feed *ad lib* (Fed) before blood was harvested. Plasma NEFAs were measured and results are expressed as mmol/L, fed vs. fasted, means (±SEM); n=3-4. Significant main effect (*p<0.001). (B) Mice were injected with palmitic acid or vehicle control and 2 h after injection blood was harvested. Plasma NEFAs were measured and results are expressed as mmol/L, Vehicle vs. Palmitic, means (±SEM); n=6. Significant main effect (*p <0.05).

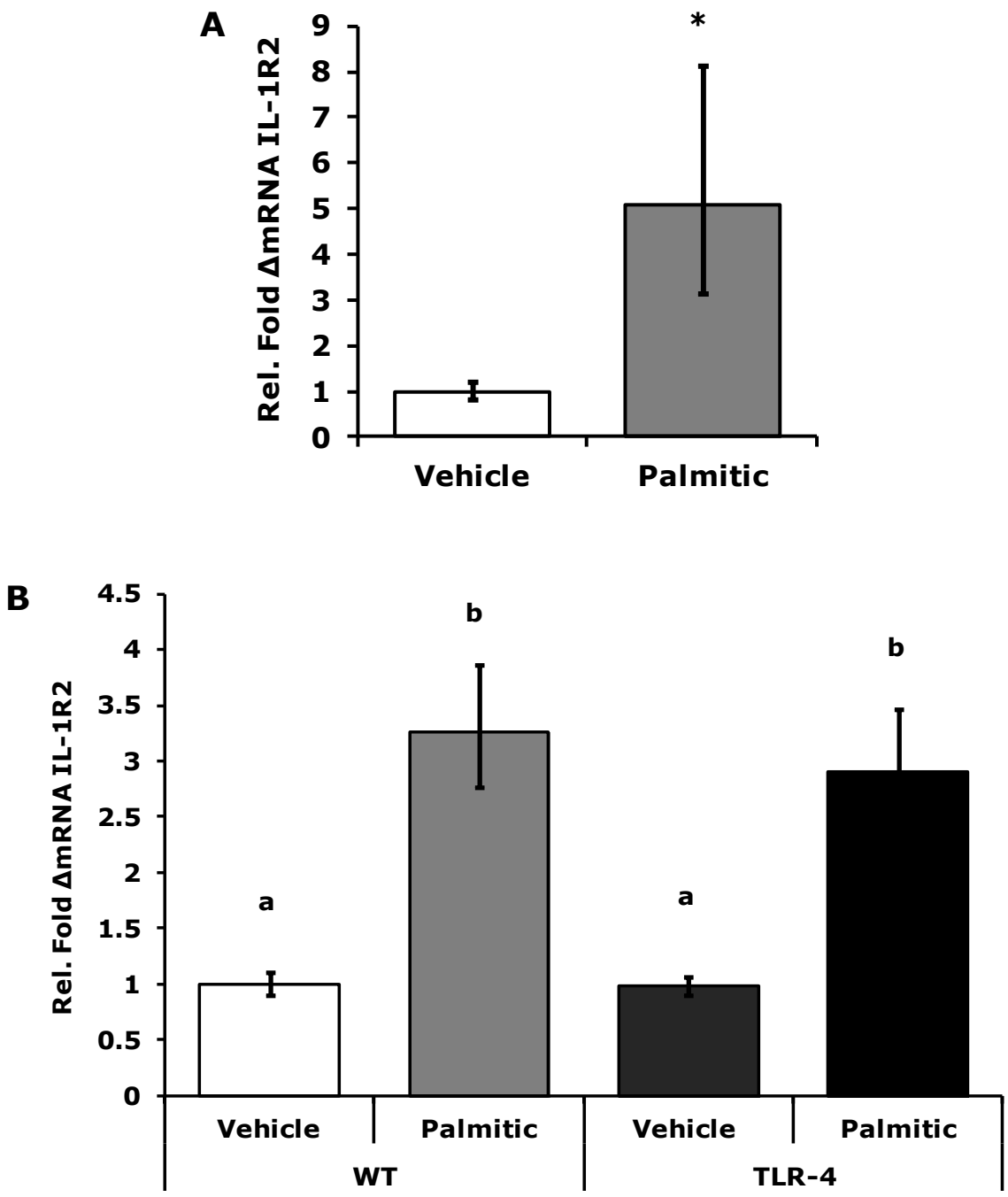

Figure 9. *Liver IL-1R2 gene transcription levels after palmitic acid injection*. Mice were injected with palmitic acid or vehicle control and 2 h after injection liver was harvested and RNA was extracted. (A) Results are expressed as relative fold change in mRNA, means (+ SEM Upper, -SEM Lower); n=6. Significant main effect (*p <0.05). (B) TLR-4 KO liver IL-1R2 gene transcripts were compared to control group (WT, Vehicle). Results are expressed as relative fold change in mRNA, means (+ SEM Upper, -SEM Lower); n=8. Bars without a common superscript are different (*p <0.001).

CHAPTER 6

DISCUSSION

This project was able to successfully identify immune regulators impacted by a 24 h fast. As evidenced in Table 1, fasting differentially regulated transcripts of cytokines in brain regions compared to liver and adipose. This decoupling of immune phenotype of the brain indicates that tissues involved in energy supply, during nutrient deprivation, may have the potential to prevent, treat or exacerbate disease. CD11b is a complement receptor and has mainly been shown to have a central role in host defense in which it enhances migration of leukocytes (ex. macrophages and microglia) from peripheral blood to sites of inflammation (Kataru et al., 2009). Here we show that CD11b gene transcription is significantly down-regulated in all brain regions, whereas CD11b is up-regulated in adipose and liver in response to fasting. These perturbations may indicate a brain preparatory shift to anti-inflammation. Different from CD11b, TNF-α gene transcription is consistently down-regulated throughout all tissues investigated. TNF-α is produced by macrophages and is known best for its role in promoting inflammatory responses, inducing fever and anorexia (MacEwan, 2002). It is logical for fasting to down-regulate genes responsible for inducing anorexia in times of nutrient deprivation.

Many changes were seen in transcription of the IL-1 family due to fasting. IL-1α was down-regulated in all brain regions without significantly affecting the peripheral tissues, and IL-1β was significantly decreased only in the hypothalamus and adipose. These markers indicate a general dampening of inflammation in these tissues. The IL-1 family is an important part of the innate immune system and its balance is extremely essential to mediate inflammation and tissue damage. Because uncontrolled IL-1 is implicated in the pathogenesis of a variety of inflammatory conditions and diseases

(Dinarello, Simon, & van der Meer, 2012), mechanisms tightly controlling its potentially potent activity at multiple levels is extremely important. For this reason, one way IL-1 is counterbalanced is by two distinct endogeneous inhibitors, IL-1RA and IL-1R2. IL-1RA transcription was shown to be up-regulated in the hippocampus and liver, but down-regulated in the adipose. IL-1R2 was not up-regulated in any of the brain tissues, but was instead substantially significantly up-regulated in both adipose and liver (Table 1). This IL-1R2 up-regulation was consistent throughout numerous experiments, but produced extremely variable fold changes which we found to be related to its correlation to percent body weight loss during the 24 h fast (Fig. 4). IL-1RA is a competitive inhibitor that can antagonize the pro-inflammatory effects via binding with near equal avidity as IL-1 to the signaling IL-1 receptor (IL-1R1) without causing activation of the cells (Symons, Young, & Duff, 1995). Although less is understood about IL-1R2, it has primarily been demonstrated to function as an important negative regulator, acting like a decoy receptor binding IL-1 both on the plasma membrane and as a soluble receptor in fluid phase, preventing the interaction of IL-1 with IL-1R1 (Arend, 2002; Bossu et al., 1995; Rauschmayr, Groves, & Kupper, 1997; Re et al., 1996). As Table 2 shows, these mRNA transcription up-regulations were confirmed to translate into an increase in liver protein of both IL-1RA and IL-1R2. Interestingly, these increases in the IL-1 inhibitor proteins were not seen in serum of fasted mice. These results suggest these normally secreted proteins are remaining intracellular or remaining in the interstitial fluid within the liver. More recently, studies have elucidated a new level of control for IL-1 in which IL-1R2 has been shown to interact with precursor form IL-1α intracellularly (Chang, Su, & Lee, 2009; Kawaguchi et al., 2006), and this interaction was just confirmed in Cell where it was found that IL-1R2 is bound to pro-IL-1α intracellularly during times of stress-induction, preventing IL-1 from further processing into its mature form. Binding of proIL-1α and IL-1R2 was able to control and prevent necrosis-induced sterile inflammation (Zheng, Humphry, Maguire, Bennett, & Clarke, 2013).

Sickness Behaviors are defined as adaptive, physiological and behavioral changes that occur in response to infection and inflammation (Hart, 1988). Furthermore, a dramatic reduction in food intake is commonly associated with illness during localized and systemic diseases (Hart, 1988; Konsman & Dantzer, 2001), but this disease-associated anorexia is dependent on the feeding status, as it has been shown that the anorexic effect is attenuated when restricting food intake prior to IL-1β or turpentine-induced sickness (Kent, Rodriguez, Kelley, & Dantzer, 1994; Larson, Romanoff, Dunn, & Glowa, 2002; Lennie, 1998; Mrosovsky, Molony, Conn, & Kluger, 1989). Acute starvation has also been shown to attenuate the fever response to LPS in rats (Inoue et al., 2008; Shido, Nagasaka, & Watanabe, 1989). Here we showed (Fig.2) that fasting prior to peripheral IL-1B challenge was able to attenuate weight loss and IL-1β- induced hypoglycemia at 2 h post injection. Fasted animals were also resistant to reduction in locomotor activity and social behavior. Proinflammatory cytokines, such as those included in the IL-1 family, can act at the periphery to induce its own synthesis and the synthesis of other cytokines allowing it to potentiate or oppose its effects, and these signals can induce the expression of transcripts of inflammatory cytokines in the brain in response to peripheral stimuli (Sims and smith, 2010). Here, we uncover a link between dietary restrictions effect being that of up-regulation of IL-1 family antagonistic mediators within the liver, which then blunts the centrally-mediated effects of induced peripheral challenge. The importance of the liver in transmitting cytokine signals from the periphery to the brain after i.p.- injected inflammatory stimuli has been shown previously by those investigating the effects of subdiaphragmatic vagotomy. The vagus nerve, by which the liver is innervated, has an important role in transmitting peripheral cytokine signals to the brain as evidenced by rodents injected i.p. with IL-1β, in which vagotomy was able to substantially attenuate any increase in IL-1β mRNA in the brain, and block behavioral effects of IL-1β injected i.p., whereas IL-1β mRNA increase in liver was not affected and the behavioral effects were not different if injected i.v. or i.c.v. (Bluthe, Michaud, Kelley, & Dantzer, 1996a; Bluthe, Michaud, Kelley, & Dantzer, 1996b; Hansen, Taishi, Chen, & Krueger, 1998). Although we did not

see an attenuation of IL-1α or IL-1β gene transcription 2 h after IL-1β peripheral challenge in any brain regions (Fig. 3), we did observe a significant increase in IL-1R2 message in fasted mice challenged with IL-1β in the cortex, and we also saw a significant decrease in fasted mice IL-1RA in the hypothalamus 2 h after IL-1B challenge. Because of the nature of IL-1RA inhibiting IL-1, sources have suggested that increased IL-1RA can actually be a marker of severity of inflammation rather than expression of anti-inflammation (Pihlajamaki et al., 2012). This implies for our results that the decrease in IL-1RA in the hypothalamus is indicative of a lesser inflammatory process occurring in the fasted mice compared to fed. This data suggests that the liver, which we have now found to be basally skewed anti-inflammatory via acute food deprivation, plays a major role in communicating inflammatory signals to the brain after an i.p. immune challenge.

When trying to elucidate a mechanism as to how fasting up-regulates these anti-inflammatory cytokines, we tried to look at which cells within the liver were expressing the messages. Fig. 5 shows that we were unable to find significant up-regulation of IL-1R2 or IL-1RA in either CD11b+ or CD11b- cell fractions, however, when we look at cellular fractions of hepatocytes, we were able to uncover IL-1RA up-regulation which is consistent with current findings (Gabay, Gigley, Sipe, Arend, & Fantuzzi, 2001). It was then discovered that because processing necessary to isolate the cells of the liver took up to 6 hours for PCR(without fixation), IL-1R2 was rapidly degraded due to IL-1R2 transcripts short half-life of only 110 min. (Zeisel et al., 2011), and this degradation is the probable cause of our inability to see IL-1R2 up-regulation in any cellular fraction. This was confirmed by setting out a piece of liver tissue for 6 h (the time it takes to isolate cells) and comparing it to a piece of liver that was immediately extracted, in which our results show after 6 h the IL-1R2 message is practically undetectable.

IL-1R2 expression has been shown to be induced by numerous agents in vitro including dexamethasone, prostaglandins, glucocorticoids, IL-4, IL-13, IL-27 and aspirin (Peters, Joesting, & Freund, 2012). Using knockout mouse models (Table 3), it was determined that IL-1R2 and IL-1RA gene transcription in the liver and adipose due to fasting is independent of IL-1 (as demonstrated by IL-1R1 KO), TLR-4, or IL-4 signaling. Food deprivation is also known to cause many hormonal effects that are implicated in influencing the immune response. These include; inhibited insulin secretion, decreased leptin, increased glucagon, epinephrine and glucocordicoids, and the main metabolic purpose of these changes are to reduce glucose use while stimulating lipolysis and ketogenesis (Finn & Dice, 2006; Schwartz & Seeley, 1997). We confirmed the glucocordicoid, corticosterone, to be elevated in our model in the serum of fasted mice, demonstrated in Fig. 6, and, as with IL-1R2 up-regulation, this increase in corticosterone was correlated to percent body weight loss during the 24 h fast. Although corticosterone seemed like the logical candidate signaling to the cells to increase IL-1R2, Fig. 7 proves otherwise, showing through inhibition of glucocorticoid receptors via mifepristone injection that IL-1R2 and IL-1RA up-regulation in the liver is independent of corticosterone signaling. This mimics results showing protection against renal ischemic reperfusion injury induced by preoperative fasting up to 3 days by mice is mediated by corticosterone-independent mechanisms as glucocorticoid receptor blockade does not interfere with the protective effects of fasting (Van Ginhoven, Van Den Berg, Dik, Ijzermans, & De Bruin, 2010).

An additional known effect of fasting is increased plasma Free Fatty Acids, where rats fasted for 24 h had a 75% increase in serum NEFA concentration and 120% increase after 48 h (Pires, Curi, & Otton, 2007). We confirmed that our fasting models have increased plasma NEFA levels after a 24 h fast (Fig. 8). We were able to mimic plasma fasting NEFA levels by injecting palmitic acid into the periphery. Using this technique, palmitic acid is able to stimulate the increase in IL-1R2 message in liver 2 h after

being injected (Fig. 9), and this effect of FFAs on IL-1R2 up-regulation is independent of TLR-4 signaling (as shown by TLR-4 KO models). Ruling out TLR-4 signaling which is the generally accepted signaling receptor responsible for detecting saturated fatty acids and provoking an inflammatory response (Milanski et al., 2009). Instead, the probable candidate recognizing FFAs, causing induction of these anti-inflammatory markers is free fatty acid receptor 1 (FFA1), which recognizes medium and long chain fatty acids. It has been shown that NEFAs can trigger apoptosis in different cells lines (Cury-Boaventura, Pompeia, & Curi, 2005; Finstad et al., 1998). Significant fasting-induced apoptosis has also been shown to occur in rat liver after 2 days of fasting. This model resulted in an approximate 19% loss in body weight (Tessitore, Tomasi, & Greco, 1999), and as stated previously, IL-1R2 has just been implicated as the protein responsible for preventing inflammation during apoptotic stress (Zheng et al., 2013). FFA1 is a GPR that: 1) recognizes fatty acids relevant to dietary ingestion; 2) is expressed on cells and tissues key to neuroimmunity namely macrophages and brain; 3) and likely signals through kinases critical to macrophage function and activation. These features of FFA1 implicate it as a regulator of immunobehaviors and warrants significant further study.

CHAPTER 7

CONCLUSIONS

In conclusion, this project shows fasting increases IL-1R2 in the liver and adipose, and IL-1RA in the liver, and these increases represent a novel way in which fasting is able to attenuate sickness behaviors resulting from a peripheral IL-1β challenge. We have been able to rule out numerous mechanisms that are known to increase transcription of IL-1R2 including IL-1, TLR-4, IL-4 and glucocordicoid signaling. Instead, this research project introduces a new mechanism by which IL-1R2 is up-regulated via FFAs. Using these results and new literature elucidating the role of IL-1R2, our current hypothesis is that IL-1R2 is up-regulated in peripheral tissues resulting from stress-induced (24 h fast) increase in epinephrine and FFA. Furthermore, these FFA are signaling "danger" to the cells via FFAR1, which is the cause of intracellular up-regulation of IL-1R2/IL-1RA in the form of a preparatory response for possible apoptosis and prevention of IL-1α-induced necrosis upon apoptosis. We anticipate that once a challenge has been sensed (i.e. peripheral IL-1β injection), the increased reservoir of anti-inflammatory contents within these cells is released, providing an initial blunting of IL-1 signaling and centrally-mediated sickness responses. More investigation is necessary to confirm this hypothesis. We have proposed experiments including looking at protein in the blood after fasted mice have been challenged with IL-1β, using cell culture and flow cytometry and/or immunostaining analysis to determine which form of IL-1R2 is up-regulated in response to FFA (intracellular, soluble, membrane bound), blocking epinephrine and/or FFA during a fast, and we also propose to determine just how much circulating IL-1R2 at any given time is bound to IL-1. The implications of this project are important because this may elucidate the mechanism behind the plethora of health benefits known to be induced by fasting, and also because of the numerous ailments known to be driven by inflammatory IL-1, in which new ameliorations that inhibit IL-1 bioaction may positively impact.

References

Arend, W. P. (2002). The mode of action of cytokine inhibitors. *The Journal of Rheumatology. Supplement, 65*, 16-21.

Bluthe, R. M., Michaud, B., Kelley, K. W., & Dantzer, R. (1996a). Vagotomy attenuates behavioural effects of interleukin-1 injected peripherally but not centrally. *Neuroreport, 7*(9), 1485-1488.

Bluthe, R. M., Michaud, B., Kelley, K. W., & Dantzer, R. (1996b). Vagotomy blocks behavioural effects of interleukin-1 injected via the intraperitoneal route but not via other systemic routes. *Neuroreport, 7*(15-17), 2823-2827.

Bossu, P., Visconti, U., Ruggiero, P., Macchia, G., Muda, M., Bertini, R., . . . Maurizi, G. (1995). Transfected type II interleukin-1 receptor impairs responsiveness of human keratinocytes to interleukin-1. *The American Journal of Pathology, 147*(6), 1852-1861.

Bressler, J., Fornage, M., Demerath, E. W., Knopman, D. S., Monda, K. L., North, K. E., . . . Boerwinkle, E. (2013). Fat mass and obesity gene and cognitive decline: The atherosclerosis risk in communities study. *Neurology, 80*(1), 92-99. doi: 10.1212/WNL.0b013e3182768910; 10.1212/WNL.0b013e3182768910

Brett, A. S., & Nesbit, R. M. (2012). A forty-day water-only fast by a pentecostal woman: Clinical and religious observations. *The American Journal of the Medical Sciences,* doi: 10.1097/MAJ.0b013e3182760349

Cai, D., & Liu, T. (2012). Inflammatory cause of metabolic syndrome via brain stress and NF-kappaB. *Aging, 4*(2), 98-115.

Chang, S. Y., Su, P. F., & Lee, T. C. (2009). Ectopic expression of interleukin-1 receptor type II enhances cell migration through activation of the pre-interleukin 1alpha pathway. *Cytokine, 45*(1), 32-38. doi: 10.1016/j.cyto.2008.10.013; 10.1016/j.cyto.2008.10.013

Chiu, G. S., Chatterjee, D., Darmody, P. T., Walsh, J. P., Meling, D. D., Johnson, R. W., & Freund, G. G. (2012). Hypoxia/reoxygenation impairs memory formation via adenosine-dependent activation of caspase 1. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 32*(40), 13945-13955. doi: 10.1523/JNEUROSCI.0704-12.2012; 10.1523/JNEUROSCI.0704-12.2012

Cury-Boaventura, M. F., Pompeia, C., & Curi, R. (2005). Comparative toxicity of oleic acid and linoleic acid on raji cells. *Nutrition (Burbank, Los Angeles County, Calif.), 21*(3), 395-405. doi: 10.1016/j.nut.2004.07.007

Dayer, J. -M. (2003). The pivotal role of interleukin-1 in the clinical manifestations of rheumatoid arthritis. *Rheumatology, 42*(suppl 2), ii3-ii10. doi: 10.1093/rheumatology/keg326

Dinarello, C. A., Simon, A., & van der Meer, J. W. (2012). Treating inflammation by blocking interleukin-1 in a broad spectrum of diseases. *Nature Reviews.Drug Discovery, 11*(8), 633-652. doi: 10.1038/nrd3800; 10.1038/nrd3800

Faggioni, R., Moser, A., Feingold, K. R., & Grunfeld, C. (2000). Reduced leptin levels in starvation increase susceptibility to endotoxic shock. *The American Journal of Pathology, 156*(5), 1781-1787. doi: 10.1016/S0002-9440(10)65049-3

Finn, P. F., & Dice, J. F. (2006). Proteolytic and lipolytic responses to starvation. *Nutrition (Burbank, Los Angeles County, Calif.), 22*(7-8), 830-844. doi: 10.1016/j.nut.2006.04.008

Finstad, H. S., Drevon, C. A., Kulseth, M. A., Synstad, A. V., Knudsen, E., & Kolset, S. O. (1998). Cell proliferation, apoptosis and accumulation of lipid droplets in U937-1 cells incubated with eicosapentaenoic acid. *The Biochemical Journal, 336 (Pt 2)*(Pt 2), 451-459.

Fraser, L. (1997). *Losing it: America's obsession with weight and the industry that feeds on it*. New York: Dutton.

Fuhrman, J., Sarter, B., & Calabro, D. J. (2002). Brief case reports of medically supervised, water-only fasting associated with remission of autoimmune disease. *Alternative Therapies in Health and Medicine, 8*(4), 112, 110-1.

Gabay, C., Gigley, J., Sipe, J., Arend, W. P., & Fantuzzi, G. (2001). Production of IL-1 receptor antagonist by hepatocytes is regulated as an acute-phase protein in vivo. *European Journal of Immunology, 31*(2), 490-499. doi: 2-H

Go, K. G., Prenen, G. H., & Korf, J. (1988). Protective effect of fasting upon cerebral hypoxic-ischemic injury. *Metabolic Brain Disease, 3*(4), 257-263.

Goldhamer, A. C., Lisle, D. J., Sultana, P., Anderson, S. V., Parpia, B., Hughes, B., & Campbell, T. C. (2002). Medically supervised water-only fasting in the treatment of borderline hypertension. *Journal of Alternative and Complementary Medicine (New York, N.Y.), 8*(5), 643-650. doi: 10.1089/107555302320825165

Gregor, M. F., & Hotamisligil, G. S. (2011). Inflammatory mechanisms in obesity. *Annual Review of Immunology, 29*, 415-445. doi: 10.1146/annurev-immunol-031210-101322; 10.1146/annurev-immunol-031210-101322

Halagappa, V. K. M., Guo, Z., Pearson, M., Matsuoka, Y., Cutler, R. G., LaFerla, F. M., & Mattson, M. P. (2007). Intermittent fasting and caloric restriction ameliorate age-related behavioral deficits in the triple-transgenic mouse model of alzheimer's disease. *Neurobiology of Disease, 26*(1), 212-220. doi: 10.1016/j.nbd.2006.12.019

Hansen, M. K., Taishi, P., Chen, Z., & Krueger, J. M. (1998). Vagotomy blocks the induction of interleukin-1beta (IL-1beta) mRNA in the brain of rats in response to systemic IL-1beta. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 18*(6), 2247-2253.

Hart, B. L. (1988). Biological basis of the behavior of sick animals. *Neuroscience and Biobehavioral Reviews, 12*(2), 123-137.

Horne, B. D., May, H. T., Anderson, J. L., Kfoury, A. G., Bailey, B. M., McClure, B. S., . . . Intermountain Heart Collaborative Study. (2008). Usefulness of routine periodic fasting to lower risk of coronary artery disease in patients undergoing coronary angiography. *The American Journal of Cardiology, 102*(7), 814-819. doi: 10.1016/j.amjcard.2008.05.021; 10.1016/j.amjcard.2008.05.021

Inoue, W., Somay, G., Poole, S., & Luheshi, G. N. (2008). Immune-to-brain signaling and central prostaglandin E2 synthesis in fasted rats with altered lipopolysaccharide-induced fever. *American Journal of Physiology.Regulatory, Integrative and Comparative Physiology, 295*(1), R133-43. doi: 10.1152/ajpregu.90335.2008; 10.1152/ajpregu.90335.2008

Johnson, D. R., O'Connor, J. C., Hartman, M. E., Tapping, R. I., & Freund, G. G. (2007). Acute hypoxia activates the neuroimmune system, which diabetes exacerbates. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 27*(5), 1161-1166. doi: 10.1523/JNEUROSCI.4560-06.2007

Kaczmarczyk, M. M., Machaj, A. S., Chiu, G. S., Lawson, M. A., Gainey, S. J., York, J. M., . . . Freund, G. G. (2013). Methylphenidate prevents high-fat diet (HFD)-induced learning/memory impairment in juvenile mice. *Psychoneuroendocrinology,* doi: 10.1016/j.psyneuen.2013.01.004; 10.1016/j.psyneuen.2013.01.004

Kataru, R. P., Jung, K., Jang, C., Yang, H., Schwendener, R. A., Baik, J. E., . . . Koh, G. Y. (2009). Critical role of CD11b+ macrophages and VEGF in inflammatory lymphangiogenesis, antigen clearance, and inflammation resolution. *Blood, 113*(22), 5650-5659. doi: 10.1182/blood-2008-09-176776; 10.1182/blood-2008-09-176776

Kawaguchi, Y., Nishimagi, E., Tochimoto, A., Kawamoto, M., Katsumata, Y., Soejima, M., . . . Hara, M. (2006). Intracellular IL-1alpha-binding proteins contribute to biological functions of endogenous IL-1alpha in systemic sclerosis fibroblasts. *Proceedings of the National Academy of Sciences of the United States of America, 103*(39), 14501-14506. doi: 10.1073/pnas.0603545103

Kelley, K. W., Bluthé, R., Dantzer, R., Zhou, J., Shen, W., Johnson, R. W., & Broussard, S. R. (2003). Cytokine-induced sickness behavior. *Brain, Behavior, and Immunity, 17*(1, Supplement), 112-118. doi: 10.1016/S0889-1591(02)00077-6

Kent, S., Rodriguez, F., Kelley, K. W., & Dantzer, R. (1994). Reduction in food and water intake induced by microinjection of interleukin-1 beta in the ventromedial hypothalamus of the rat. *Physiology & Behavior, 56*(5), 1031-1036.

Konsman, J. P., & Dantzer, R. (2001). How the immune and nervous systems interact during disease-associated anorexia. *Nutrition (Burbank, Los Angeles County, Calif.), 17*(7-8), 664-668.

Kopelman, P. (2007). Health risks associated with overweight and obesity. *Obesity Reviews, 8*, 13-17. doi: 10.1111/j.1467-789X.2007.00311.x

Larson, S. J., Romanoff, R. L., Dunn, A. J., & Glowa, J. R. (2002). Effects of interleukin-1beta on food-maintained behavior in the mouse. *Brain, Behavior, and Immunity, 16*(4), 398-410.

Lavin, D. N., Joesting, J. J., Chiu, G. S., Moon, M. L., Meng, J., Dilger, R. N., & Freund, G. G. (2011). Fasting induces an anti-inflammatory effect on the neuroimmune system which a high-fat diet prevents. *Obesity (Silver Spring, Md.), 19*(8), 1586-1594. doi: 10.1038/oby.2011.73; 10.1038/oby.2011.73

Lee, E. B. (2011). Obesity, leptin, and alzheimer's disease. *Annals of the New York Academy of Sciences, 1243*(1), 15-29. doi: 10.1111/j.1749-6632.2011.06274.x

Lennie, T. A. (1998). Relationship of body energy status to inflammation-induced anorexia and weight loss. *Physiology & Behavior, 64*(4), 475-481.

Louie, S. M., Roberts, L. S., & Nomura, D. K. (2013). Mechanisms linking obesity and cancer. *Biochimica Et Biophysica Acta,* doi: 10.1016/j.bbalip.2013.02.008; 10.1016/j.bbalip.2013.02.008

Lumeng, C. N. (2013). Innate immune activation in obesity. *Molecular Aspects of Medicine, 34*(1), 12-29. doi: 10.1016/j.mam.2012.10.002

MacEwan, D. J. (2002). TNF receptor subtype signalling: Differences and cellular consequences. *Cellular Signalling, 14*(6), 477-492.

Maes, M., Song, C., & Yirmiya, R. (2012). Targeting IL-1 in depression. *Expert Opinion on Therapeutic Targets, 16*(11), 1097-1112. doi: 10.1517/14728222.2012.718331; 10.1517/14728222.2012.718331

Martin, B., Mattson, M. P., & Maudsley, S. (2006). Caloric restriction and intermittent fasting: Two potential diets for successful brain aging. *Ageing Research Reviews, 5*(3), 332-353. doi: 10.1016/j.arr.2006.04.002

Mattson, M. P. (2010). The impact of dietary energy intake on cognitive aging. *Frontiers in Aging Neuroscience, 2*, 5. doi: 10.3389/neuro.24.005.2010; 10.3389/neuro.24.005.2010

Michalsen, A. (2010). Prolonged fasting as a method of mood enhancement in chronic pain syndromes: A review of clinical evidence and mechanisms. *Current Pain and Headache Reports, 14*(2), 80-87. doi: 10.1007/s11916-010-0104-z; 10.1007/s11916-010-0104-z

Michalsen, A., Kuhlmann, M. K., Ludtke, R., Backer, M., Langhorst, J., & Dobos, G. J. (2006). Prolonged fasting in patients with chronic pain syndromes leads to late mood-enhancement not related to weight loss and fasting-induced leptin depletion. *Nutritional Neuroscience, 9*(5-6), 195-200.

Milanski, M., Degasperi, G., Coope, A., Morari, J., Denis, R., Cintra, D. E., . . . Velloso, L. A. (2009). Saturated fatty acids produce an inflammatory response predominantly through the activation of TLR4 signaling in hypothalamus: Implications for the pathogenesis of obesity. *The Journal of Neuroscience : The Official Journal of the Society for Neuroscience, 29*(2), 359-370. doi: 10.1523/JNEUROSCI.2760-08.2009; 10.1523/JNEUROSCI.2760-08.2009

Mitchell, J. R., Verweij, M., Brand, K., van de Ven, M., Goemaere, N., van den Engel, S., . . . de Bruin, R. W. (2010). Short-term dietary restriction and fasting precondition against ischemia reperfusion injury in mice. *Aging Cell, 9*(1), 40-53. doi: 10.1111/j.1474-9726.2009.00532.x

Moret, Y., & Schmid-Hempel, P. (2000). Survival for immunity: The price of immune system activation for bumblebee workers. *Science (New York, N.Y.), 290*(5494), 1166-1168.

Mrosovsky, N., Molony, L. A., Conn, C. A., & Kluger, M. J. (1989). Anorexic effects of interleukin 1 in the rat. *The American Journal of Physiology, 257*(6 Pt 2), R1315-21.

Peters, V. A., Joesting, J. J., & Freund, G. G. (2012). IL-1 receptor 2 (IL-1R2) and its role in immune regulation. *Brain, Behavior, and Immunity,* doi: 10.1016/j.bbi.2012.11.006; 10.1016/j.bbi.2012.11.006

Pihlajamaki, J., Kuulasmaa, T., Kaminska, D., Simonen, M., Karja, V., Gronlund, S., . . . Laakso, M. (2012). Serum interleukin 1 receptor antagonist as an independent marker of non-alcoholic steatohepatitis in humans. *Journal of Hepatology, 56*(3), 663-670. doi: 10.1016/j.jhep.2011.10.005; 10.1016/j.jhep.2011.10.005

Pires, J., Curi, R., & Otton, R. (2007). Induction of apoptosis in rat lymphocytes by starvation. *Clinical Science (London, England : 1979), 112*(1), 59-67. doi: 10.1042/CS20060212

Rauschmayr, T., Groves, R. W., & Kupper, T. S. (1997). Keratinocyte expression of the type 2 interleukin 1 receptor mediates local and specific inhibition of interleukin 1-mediated inflammation. *Proceedings of the National Academy of Sciences of the United States of America, 94*(11), 5814-5819.

Re, F., Sironi, M., Muzio, M., Matteucci, C., Introna, M., Orlando, S., . . . Mantovani, A. (1996). Inhibition of interleukin-1 responsiveness by type II receptor gene transfer: A surface "receptor" with anti-interleukin-1 function. *The Journal of Experimental Medicine, 183*(4), 1841-1850.

Rossi, S., Sacchetti, L., Napolitano, F., De Chiara, V., Motta, C., Studer, V., . . . Centonze, D. (2012). Interleukin-1beta causes anxiety by interacting with the endocannabinoid system. *The Journal of*

Neuroscience : The Official Journal of the Society for Neuroscience, 32(40), 13896-13905. doi: 10.1523/JNEUROSCI.1515-12.2012; 10.1523/JNEUROSCI.1515-12.2012

Salim, I., Al Suwaidi, J., Ghadban, W., Alkilani, H., & Salam, A. M. (2013). Impact of religious ramadan fasting on cardiovascular disease: A systematic review of the literature. *Current Medical Research and Opinion, 29*(4), 343-354. doi: 10.1185/03007995.2013.774270; 10.1185/03007995.2013.774270

Scheuner, D., & Kaufman, R. J. (2008). The unfolded protein response: A pathway that links insulin demand with beta-cell failure and diabetes. *Endocrine Reviews, 29*(3), 317-333. doi: 10.1210/er.2007-0039; 10.1210/er.2007-0039

Schmidt, S., Stange, R., Lischka, E., Kiehntopf, M., Deufel, T., Loth, D., & Uhlemann, C. (2010). Uncontrolled clinical study of the efficacy of ambulant fasting in patients with osteoarthritis. [Unkontrollierte klinische Studie zur Wirksamkeit ambulanten Heilfastens bei Patienten mit Arthrose] *Forschende Komplementarmedizin (2006), 17*(2), 87-94. doi: 10.1159/000285479; 10.1159/000285479

Schwartz, M. W., & Seeley, R. J. (1997). Neuroendocrine responses to starvation and weight loss. *N Engl J Med, 336*(25), 1802-1811. doi: 10.1056/NEJM199706193362507

Sherry, C. L., Kramer, J. M., York, J. M., & Freund, G. G. (2009). Behavioral recovery from acute hypoxia is reliant on leptin. *Brain, Behavior, and Immunity, 23*(2), 169-175. doi: 10.1016/j.bbi.2008.09.011; 10.1016/j.bbi.2008.09.011

Shido, O., Nagasaka, T., & Watanabe, T. (1989). Blunted febrile response to intravenous endotoxin in starved rats. *Journal of Applied Physiology (Bethesda, Md.: 1985), 67*(3), 963-969.

Simon, G. E., Von Korff, M., Saunders, K., Miglioretti, D. L., Crane, P. K., van Belle, G., & Kessler, R. C. (2006). Association between obesity and psychiatric disorders in the US adult population. *Archives of General Psychiatry, 63*(7), 824-830. doi: 10.1001/archpsyc.63.7.824

Sloan-Lancaster, J., Abu-Raddad, E., Polzer, J., Miller, J. W., Scherer, J. C., De Gaetano, A., . . . Landschulz, W. H. (2013). Double-blind, randomized study evaluating the glycemic and anti-inflammatory effects of subcutaneous LY2189102, a neutralizing IL-1beta antibody, in patients with type 2 diabetes. *Diabetes Care,* doi: 10.2337/dc12-1835

Symons, J. A., Young, P. R., & Duff, G. W. (1995). Soluble type II interleukin 1 (IL-1) receptor binds and blocks processing of IL-1 beta precursor and loses affinity for IL-1 receptor antagonist. *Proceedings of the National Academy of Sciences of the United States of America, 92*(5), 1714-1718.

Tessitore, L., Tomasi, C., & Greco, M. (1999). Fasting-induced apoptosis in rat liver is blocked by cycloheximide. *European Journal of Cell Biology, 78*(8), 573-579. doi: 10.1016/S0171-9335(99)80023-5

Van Ginhoven, T. M., Van Den Berg, J. W., Dik, W. A., Ijzermans, J. N., & De Bruin, R. W. (2010). Preoperative fasting induces protection against renal ischemia/reperfusion injury by a corticosterone-independent mechanism. *Transplant International : Official Journal of the European Society for Organ Transplantation, 23*(11), 1171-1178. doi: 10.1111/j.1432-2277.2010.01116.x; 10.1111/j.1432-2277.2010.01116.x

Vandanmagsar, B., Youm, Y. H., Ravussin, A., Galgani, J. E., Stadler, K., Mynatt, R. L., . . . Dixit, V. D. (2011). The NLRP3 inflammasome instigates obesity-induced inflammation and insulin resistance. *Nature Medicine, 17*(2), 179-188. doi: 10.1038/nm.2279; 10.1038/nm.2279

Volpe, S. L. (2006). Popular weight reduction diets. *The Journal of Cardiovascular Nursing, 21*(1), 34-39.

York, J. M., Blevins, N. A., Meling, D. D., Peterlin, M. B., Gridley, D. S., Cengel, K. A., & Freund, G. G. (2012). The biobehavioral and neuroimmune impact of low-dose ionizing radiation. *Brain, Behavior, and Immunity, 26*(2), 218-227. doi: 10.1016/j.bbi.2011.09.006; 10.1016/j.bbi.2011.09.006

Zeisel, A., Kostler, W. J., Molotski, N., Tsai, J. M., Krauthgamer, R., Jacob-Hirsch, J., . . . Domany, E. (2011). Coupled pre-mRNA and mRNA dynamics unveil operational strategies underlying transcriptional responses to stimuli. *Molecular Systems Biology, 7*, 529. doi: 10.1038/msb.2011.62; 10.1038/msb.2011.62

Zheng, Y., Humphry, M., Maguire, J. J., Bennett, M. R., & Clarke, M. C. (2013). Intracellular interleukin-1 receptor 2 binding prevents cleavage and activity of interleukin-1alpha, controlling necrosis-induced sterile inflammation. *Immunity, 38*(2), 285-295. doi: 10.1016/j.immuni.2013.01.008; 10.1016/j.immuni.2013.01.008