Brain, Behavior, and Immunity 60 (2017) 151–160

Contents lists available at ScienceDirect

Brain, Behavior, and Immunity

journal homepage: www.elsevier.com/locate/ybrbi

Full-length Article

Leptin resistance elicits depressive-like behaviors in rats

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ARTICLE INFO

Article history:
Received 7 July 2016
Received in revised form 22 September 2016
Accepted 10 October 2016
Available online 12 October 2016

Keywords:
Obesity
Adiposity
Pegylated leptin receptor antagonist
C-reactive protein
Triglycerides
Forced swim test
Inflammation
Activated T cells
Spleen

ABSTRACT

There is a growing appreciation that the complications of obesity extend to the central nervous system (CNS) and include increased risk for development of neuropsychiatric co-morbidities such as depressive illness. The neurological consequences of obesity may develop as a continuum and involve a progression of pathological features which is initiated by leptin resistance. Leptin resistance is a hallmark feature of obesity, but it is unknown whether leptin resistance or blockage of leptin action is casually linked to the neurological changes which underlie depressive-like phenotypes. Accordingly, the aim of the current study was to examine whether chronic administration of a pegylated leptin receptor antagonist (Peg-LRA) elicits depressive-like behaviors in adult male rats. Peg-LRA administration resulted in endocrine and metabolic features that are characteristic of an obesity phenotype. Peg-LRA rats also exhibited increased immobility in the forced swim test, depressive-like behaviors that were accompanied by indices of peripheral inflammation. These results demonstrate that leptin resistance elicits an obesity phenotype that is characterized by peripheral immune changes and depressive-like behaviors in rats, supporting the concept that co-morbid obesity and depressive illness develop as a continuum resulting from changes in the peripheral endocrine and metabolic milieu.

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1. Introduction

Obesity is defined as a body mass index (BMI) of greater than 30 and is associated with a host of co-morbidities, including cardiovascular disease, type 2 diabetes mellitus (T2DM) and the metabolic syndrome (MetS). In addition to peripheral complications, clinical and epidemiological studies indicate that there is an association between obesity and neuropsychiatric disorders such as depressive illness, illustrating that the complications of obesity extend to the CNS (Simon et al., 2006; Luppino et al., 2010; Fabricatore and Wadden, 2006; McElroy et al., 2004; Andersen et al., 2010; Stunkard et al., 2003). Moreover, there is growing appreciation that while a complete obesity phenotype may provide the greatest risk for the development of neuropsychiatric disorders (Onyike et al., 2003), the pathological features associated with obesity develop as a continuum. While a number of factors have been suggested as mechanistic links in this continuum between obesity and mood disorders (Raison et al., 2006; Reagan, 2012), leptin resistance may be an initiating factor in this co-morbidity.

Leptin is synthesized and secreted by adipocytes and is transported across the blood-brain barrier (BBB) via a saturable transport system (Banks, 2004). In the hypothalamus, the actions of leptin are well characterized and include the regulation of food intake, metabolism, body weight, and body composition (For review, see (Schwartz et al., 2000)). Beyond the hypothalamus, leptin is also known to regulate hippocampal synaptic plasticity (For reviews, see (Harvey, 2007; Fadel et al., 2013)). However, in obesity phenotypes leptin transport across the BBB is impaired (Banks, 2004; Burguera et al., 2000; Banks et al., 1999), leading to a leptin-deficient state in the CNS. These findings have led to the suggestion that reduced CNS leptin activity may be a mechanistic link between obesity and major depressive illness (Lu, 2007). In support of this hypothesis, *ob/ob* mice, which lack the gene coding for leptin, exhibit increased immobility time in the forced swim test (FST) compared to wild-type controls, a behavioral change that

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http://dx.doi.org/10.1016/j.bbi.2016.10.008
0889-1591/Published by Elsevier Inc.

is indicative of behavioral despair in rodents (Collin et al., 2000; Yamada et al., 2011). Additionally, *db/db* mice, which lack functional leptin receptors, exhibit increased immobility time in the FST and altered anxiety-like behaviors (Sharma et al., 2010). Our recent studies have demonstrated that an obesity/MetS phenotype also induces depressive-like behaviors (Grillo et al., 2011b) and that these behavioral deficits are reversed by food restriction paradigms that restore leptin sensitivity (Grillo et al., 2014). Collectively, these studies suggest that leptin resistance may be a key initiating factor in the development of neuropsychiatric disorders in obesity. However, a causal relationship between leptin resistance and the increased risk of co-morbid depressive illness in obesity remains to be established.

In addition to leptin resistance, our previous studies illustrated that increases in peripheral inflammation are associated with depressive-like behaviors (Grillo et al., 2014), supporting the concept that a pro-inflammatory state in obesity may elicit depressive symptoms (For reviews, see (Shelton and Miller, 2010; Soczynska et al., 2011)). As leptin may also play an important role in signaling cytokine production (Lord et al., 1998), leptin resistance could be an important initiating factor in peripheral inflammation which potentially leads to the development of depressive-like behaviors. To test this hypothesis, the goal of the current studies was to determine whether leptin resistance or central and peripheral blockage of leptin action induced by chronic administration of a pegylated leptin receptor antagonist (Peg-LRA) elicits depressive-like behaviors in adult male rats by altering peripheral immune responses.

2. Materials and methods

2.1. Animal protocol

Adult male Sprague Dawley rats (CD strain, Harlan) weighing 225–250 g were individually housed with *ad libitum* access to food and water, in accordance with all guidelines and regulations of the WJB Dorn VA Animal Care and Use Committee. Animals were maintained in a temperature-controlled room, with a light/dark cycle of 12/12 h (lights on at 7:00 a.m.). Rats were acclimated to the animal facility for one week during which time baseline body weights and food intake measurements were obtained daily at 10:00 a.m.; these measures were performed daily for the duration of the study. On Days 1 through 17 rats received intraperitoneal (i.p.) injections of the Peg-LRA at a dose of 7 mg/kg daily at 5:00 p.m.; control rats received daily i.p. vehicle (sterile 0.4% $NaHCO_3$, pH = 8.0) injections. Non-pegylated and mono-pegylated rat superactive leptin antagonist (D23L/L39A/D40A/F41A) mutant was prepared according to the protocol used for preparation of non-pegylated and mono-pegylated mouse and human superactive leptin antagonists (D23L/L39A/D40A/F41A) mutant (Shpilman et al., 2011; Jamroz-Wisniewska et al., 2014). In vitro and in vivo activity of this dose of the Peg-LRA was demonstrated in Jamroz-Wisniewska et al. (2014). Behavioral testing was initiated on Day 10 of treatment. Rats were sacrificed on Day 17 following leptin administration as described below.

2.2. Endocrine analyses

All plasma endocrine analyses were performed in duplicate as described in our previous studies (Grillo et al., 2015; Grillo et al., 2014) Tail bleeds were performed 10 days following initiation of Peg-LRA or vehicle treatment under non-fasting conditions to isolate plasma for endocrine analysis. Blood glucose levels were measured by glucose oxidase method (Pointe Scientific, Inc., Canton, MI). Plasma triglycerides were determined using an enzymatic kit (modified Trinder) according to the manufacturer's instructions (Pointe Scientific, Inc., Canton, MI, USA). Plasma insulin levels were measured by enzyme-linked immunosorbent assay (ELISA, Millipore, Billerica, MA). Using plasma isolated at the time of sacrifice, plasma C-reactive protein levels were determined by ELISA (BD Biosciences, San Diego, CA). ELISA plates were analyzed according to the manufacturer's instructions using a Tecan SPECTRAFluor plate reader (Tecan U.S., Inc., Durham, NC). Plasma cytokine levels were measured using a Bio-Plex rat cytokine panel according to the manufacturer's instructions (Bio-Rad, Hercules, CA). Plates were analyzed using a Bio-Plex system coupled to Bio-Plex Manager software.

2.3. Open field test

On day 11 of the Peg-LRA treatment paradigm, Peg-LRA rats (N = 10) or Vehicle (N = 10) rats were placed in a 76 cm × 76 cm square, gray Plexiglas chamber with 45 cm walls and a floor grid for a five minute open field test. The test was conducted during the early light portion of the light/dark cycle and allows for the assessment of locomotor behaviors, indicated by distance traveled and velocity. Performance was recorded and analyzed using Ethovision 7, an automated system from Noldus Information Technology Inc. (Leesburg, VA).

2.4. Sucrose preference

On Day 12 of the Peg-LRA paradigm the sucrose preference test was performed as described previously (Grillo et al., 2011b; Grillo et al., 2014). Rats were exposed for 24 h to two identical bottles containing water and 1% sucrose solution. The next day the rats were water-deprived for 6 h (1:00 p.m.–7:00 p.m.) before testing their preference for sucrose (1%) or water in a three-hour two-bottle choice beginning at 7:00 p.m.

2.5. Forced swim test

The FST was modified from the test originally developed by Porsolt et al. (1978) as described in our previous studies (Grillo et al., 2011b). Testing for both days was done in the early light hours of the light/dark cycle. Animals were placed in a clear Plexiglas cylinder, 30 cm in diameter and 60 cm in height, filled with 35 cm of room temperature water (25 °C) in a separate room with no visual or audible stimuli. The first day consisted of a fifteen minute pretest (Day 14 of treatment), followed 24 h later by a five minute test (Day 15 of treatment). Animals were viewed and scored later by a scorer blind to the treatment group via video camera for three behaviors: immobility, climbing and swimming. Immobility behaviors were defined as little to no movement of the rat. Climbing was defined as vertical movement in contact with the side of the container. All other movements were defined as swimming. Behaviors were noted by the scorer every three seconds.

2.6. Immunoblot analysis of leptin signaling

A subset of Peg-LRA-treated rats (n = 5) and control rats (n = 5) were given an i.p. injection of leptin (5 mg/kg; National Hormone and Peptide Program, Torrance CA) sixty minutes prior to sacrifice. Adipose tissue, liver and spleens were collected to measure changes in fat mass and immune status as described below. The hypothalamus was also isolated for immunoblot analysis of leptin signaling. Briefly, 50 μg of total membrane fractions were separated by SDS/PAGE (10%), transferred to nitrocellulose (NC) membranes and blocked in TBS plus 10% nonfat dry milk. NC membranes were then incubated with primary antisera for the phosphorylated form of MAPK (Cell Signaling Technologies #9101) and the phosphorylated form of STAT3 (Cell Signaling

Technologies #9131) in TBS/5% nonfat dry milk. After overnight incubation at 4 °C, blots were washed with TBS plus 0.05% Tween 20 (TBST) and incubated with peroxidase-labeled, anti-rabbit secondary antibodies. NC membranes were then washed with TBST and developed using enhanced chemiluminescence reagents (GE Healthcare). Normalization for protein loading was performed using a mouse monoclonal primary antibody selective for actin (Sigma Chemical Company). Computer assisted microdensitometry of autoradiographic images was determined on the MCID image analysis system (Imaging Research, INC., St. Catherines, Canada), as previously described (Grillo et al., 2015).

2.7. Immunohistochemical analysis of leptin signaling

A subset of Peg-LRA-treated rats (n = 5) and control rats (n = 5) were anesthetized, placed in the stereotaxic apparatus and given intracerebroventricular injection of leptin (1 μg) at the following coordinates: AP: −0.8 mm; L: −1.4 mm; DV: −3.8. Sixty minutes later rats were transcardially perfused with phosphate buffer (PB) followed by 4% paraformaldehyde in PB. Rat brains were then processed for pSTAT3 immunohistochemistry as described in our previous studies (Grillo et al., 2011a) using established protocols (Levin et al., 2004; Peterson et al., 2000). Briefly, free-floating sections were washed in potassium phosphate-buffered saline (KPBS) at room temperature, followed by incubation in 1% NaOH, 1% H_2O_2 in KPBS. Sections were washed in KPBS and then incubated in 0.3% glycine/KPBS. Sections were then incubated in 0.03% SDS/KPBS solution for 10 min, washed in KPBS and then blocked in 4% normal horse serum (NHS)/0.4% Triton X/KPBS. Sections were then incubated in this buffer with pSTAT3 primary antisera (Cell Signaling Technology, #9131) overnight at 4 °C, followed by 1 h incubation at room temperature. Sections were washed in 1% NHS/0.02% Triton/KPBS and then incubated with biotinylated anti-rabbit IgGs for 1 h at room temperature. Sections were washed, incubated with ABC reagents for 1 h at room temperature, washed in KPBS and developed using diaminobenzidine as a substrate.

2.8. Adipose tissue histochemical analysis

A portion of epididymal adipose tissue was excised from each rat, fixed overnight in 10% formalin, dehydrated with alcohol, and embedded in wax. Paraffin sections were stained with hematoxylin and eosin (H&E). Tissue content of type I and III collagens was examined using the picro-sirius red stain kit (Abcam, Cambridge, MA, USA, #150681). Immunohistochemical staining for the macrophage marker F4/80 was performed in epididymal adipose tissue using rabbit-anti F4/80 (polyclonal) from Santa Cruz Biotech (M-300, Dallas, TX). Color detection was visualized with a Vectastain avidin-biotinylated enzyme complex detection kit (R&D Systems, Minneapolis, MN), and 3,3′-diaminobenzidine followed by counterstaining with hematoxylin. The surface area of 30 adipocytes per rat were determined (manual trace) from H&E stained slides using ImageJ software (National Institutes of Health, Bethesda, MD) and then averaged to represent mean adipocyte size for each rat.

2.9. Flow cytometric analyses

Spleens were harvested and immediately processed for flow cytometric analyses for macrophages, activated macrophages, T helper cells, activated T cells and cytotoxic T cells. Single cell suspensions were generated and red blood cells lysed by RBC cell lysis buffer (BD Biosciences) prior to cell staining. Aliquots of cells from individual rat spleens were stained with the following monoclonal anti-rat antibodies (BD Biosciences and AbD Serotec) in various 4-color combinations diluted in 1% fetal bovine serum in phosphate buffered saline (PBS; Sigma Aldrich): CD3 (clone 1F4), CD4 (clone OX-35), CD8a (clone OX-8), CD11b (clone WT.5), CD25 (clone OX-39), CD163 (clone ED2), and RT1B (clone OX-6). Cell fluorescence was measured using FACSAria (BD Biosciences) and data analyzed using FlowJo software (Treestar, Inc).

2.10. Statistical analyses

For all endpoint measures, statistical analysis was performed using a two-tailed unpaired t-test with $\alpha = 0.05$ as the criterion for statistical significance.

3. Results

3.1. Peg-LRA treatment elicits metabolic and endocrine changes characteristic of an obesity phenotype

Peg-LRA administration significantly increased food intake (Fig. 1, Panel A) and elicited significant increases in body weight relative to vehicle-treated controls (Fig. 1, Panel B). These differences in food intake and bodyweight were not evident prior to the first day of Peg-LRA treatment, which is indicated by the arrow in Panels A and B. It is interesting to note that these differences in food intake and body weight were not evident on the first days of Peg-LRA treatment. These results contrast observations in mice in which the weight gain was observed after one day of treatment (Shpilman et al., 2011). This species difference likely reflects the fact in contrast to mice that begin to exhibit a plateau in body weight increases, rats continue to gain weight during the treatment paradigm in this study, as also shown by Jamroz-Wisniewska et al. (2014). An additional possible explanation is that development of leptin resistance in rats is slower compared to mice. A linear regression analysis revealed that 61.3% of the variance in bodyweight following Peg-LRA administration can be attributed to changes in food intake ($\beta = 0.783$, $t = 15.82$, $p < 0.001$). Peg-LRA-treated rats exhibited plasma glucose levels that were similar to those observed in vehicle-treated control rats (Fig. 1, Panel C). However, Peg-LRA rats exhibited significant increases in plasma insulin levels (Fig. 1, Panel D), suggesting that these animals were insulin resistant. Peg-LRA rats also exhibited significant increases in plasma triglycerides (Fig. 1, Panel E). Consistent with previous results in mice (Solomon et al., 2014; Elinav et al., 2009; Shpilman et al., 2011), these results demonstrate that Peg-LRA treatment elicits an obesity phenotype when administered to rats.

3.2. Peg-LRA administration increases adiposity but does not induce changes in adipose tissue inflammatory cell infiltration or fibrosis

Increases in body weight elicited by the Peg-LRA likely result from increases in fat mass. Specifically, Peg-LRA rats exhibited significant increases in mesenteric fat, epididymal fat and kidney fat compared to control rats (Table 1). Hematoxylin and eosin (H&E) staining of epididymal fat was performed to determine adipocyte size and immune cell infiltration. Compared to control rats (Fig. 2, Panel A), adipocyte size was increased in Peg-LRA-treated rats (Fig. 2, Panel B). Computer-assisted analysis determined that adipocyte size was significantly increased in Peg-LRA rats compared to vehicle-treated controls (Fig. 2, Panel C). Despite the increases in adipocyte size, there was no evidence of an increase in the infiltration of inflammatory cells by H&E staining. This observation was confirmed by F4/80 immunohistochemical analysis, which did not detect macrophage infiltration into epididymal adipocytes (data not shown). As adipocyte expansion has been linked to fibrosis, we also measured collagens type 1 and III. Simi-

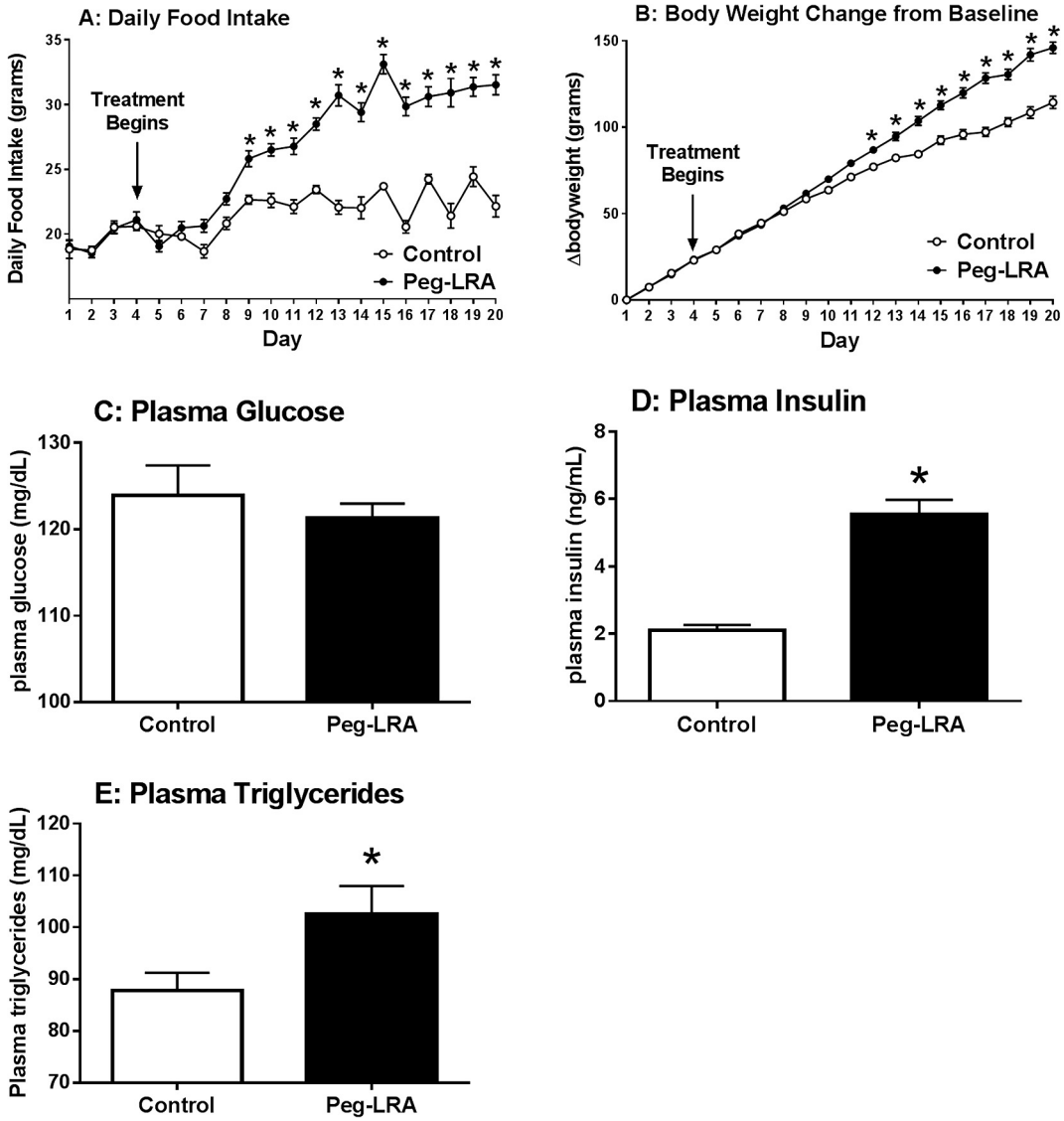

Fig. 1. Peg-LRA administration elicits endocrine and metabolic changes that are characteristic of an obesity phenotype. Panel A: Peg-LRA administration significantly increases food intake ($F(1, 18) = 61.95$, $p < 0.001$). Panel B: Peg-LRA administration significantly increases bodyweight ($F(1, 18) = 43.99$, $p < 0.001$). Panel C: There is no effect of Peg-LRA administration of plasma glucose levels. Panel D: Peg-LRA administration increases plasma insulin levels compared to vehicle-treated controls ($t(16) = 7.43$, $* = p < 0.0001$). Panel E: Plasma triglycerides are significantly increased in Peg-LRA rats compared to vehicle-treated controls ($t(18) = 2.31$, $p < 0.05$). [$* = p < 0.05$].

Table 1
Fat pad weight in control and Peg-LRA rats.

	Control	Peg-LRA	p value
Mesenteric fat (mg)	3186 ± 72.0	4906 ± 231.2	0.001
Perirenal fat (mg)	975 ± 142.6	2813 ± 351.4	0.004
Epididymal fat (mg)	2221 ± 70.42	3661 ± 81.98	0.0001

larly, there was no increase in adipose tissue fibrosis in the Peg-LRA-treated rats compared to control rats as determined by picro-sirius red staining (data not shown).

3.3. Peg-LRA elicits leptin resistance to peripheral but not central administration

To confirm that Peg-LRA treatment blocked leptin transport across the BBB as described in previous studies (Elinav et al., 2009), we examined hypothalamic leptin signaling following an i.p. injection of leptin. As shown in Fig. 3, Panel A, immunoblot analysis determined that peripheral administration of leptin (5 mg/kg) elicited the expected increases in the phosphorylated form of p44/42 MAPK in control rats, while leptin-mediated phosphorylation of p44/42 was attenuated in Peg-LRA-treated rats. Autoradiographic analysis confirmed that leptin receptor signaling in response to peripheral leptin administration was significantly reduced in the hypothalamus of Peg-LRA rats when compared to control rats (Fig. 3, Panel B). Similarly, western blot analysis revealed that leptin-stimulated phosphorylation of STAT3 was reduced in the hypothalamus of Peg-LRA rats compared to vehicle-treated controls (Fig. 3, Panel C), which was confirmed by subsequent autoradiographic analysis (Fig. 3, Panel D).

If the Peg-LRA specifically blocks BBB transport of leptin, hypothalamic signaling to direct CNS administration of leptin should remain intact. To test this hypothesis, we also examined signaling in response to intracerebroventricular (icv) administration of leptin. Immunohistochemical analysis demonstrated that icv leptin treatment (1 μg) elicited the expected increases in phosphorylated STAT3 in both control rats (Fig. 4, Panel A) and in

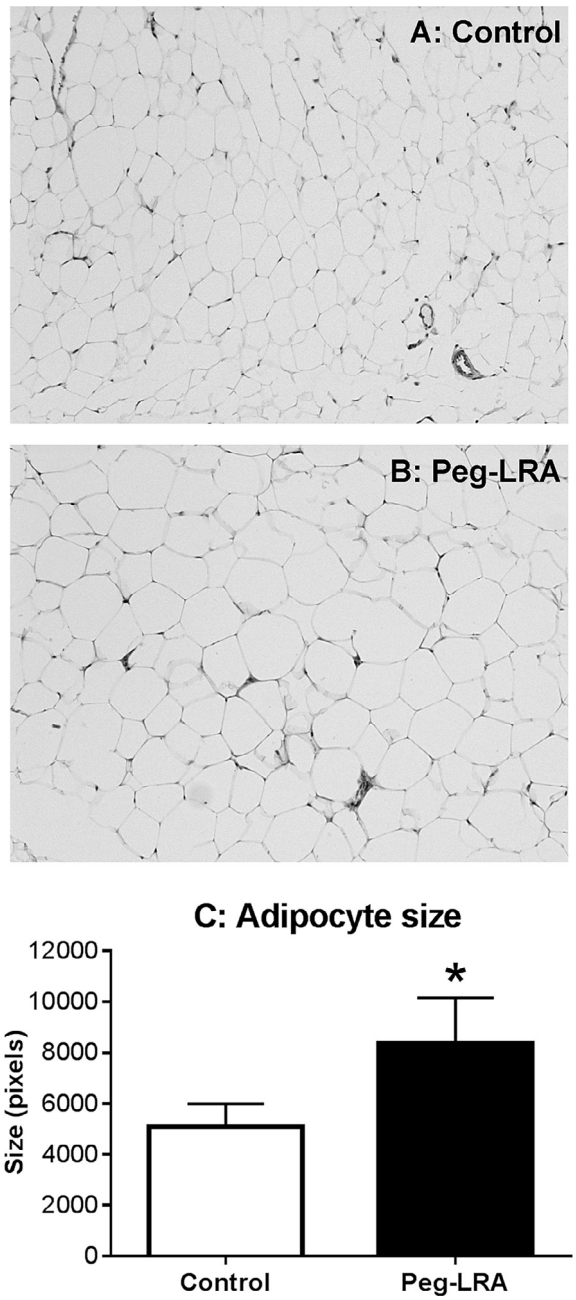

Fig. 2. Peg-LRA treatment increases adipocyte size but is not associated with macrophage infiltration. Panel A: Representative photomicrograph of H&E staining of epididymal fat in vehicle-treated control rats. Panel B: Representative photomicrograph of H&E staining in epididymal fat of adipocytes in Peg-LRA-treated rats. Panel C: Non-biased stereological analysis of adipocytes determined that Peg-LRA significantly increases adipocyte size relative to vehicle-treated controls ($t(7) = -3.645$, $p < 0.01$). [$* = p < 0.05$].

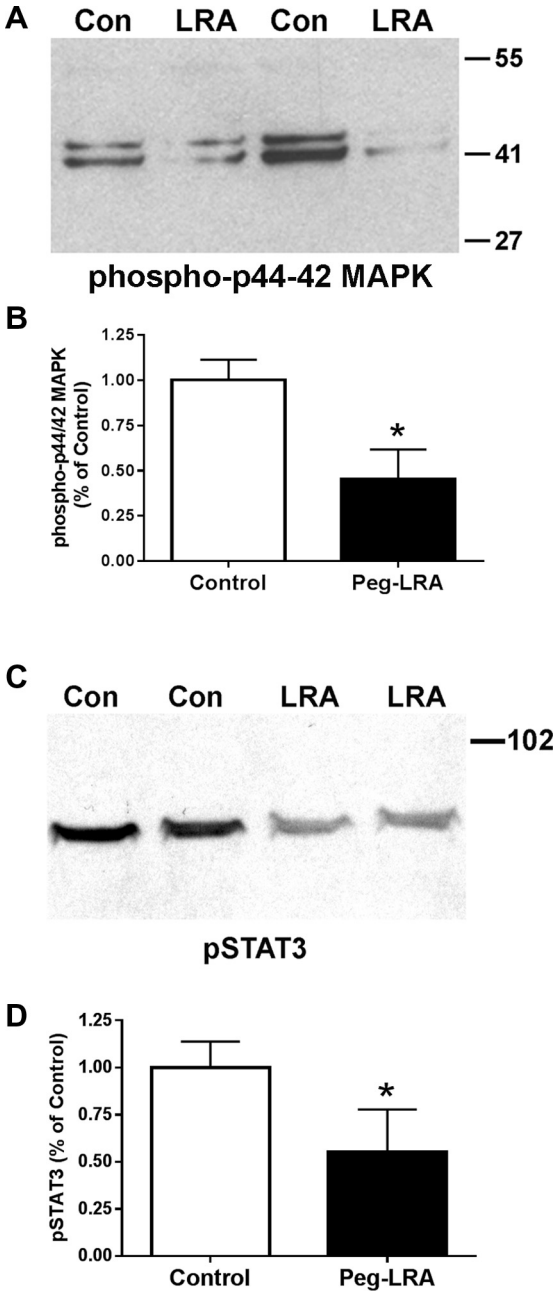

Fig. 3. Peg-LRA treatment inhibits hypothalamic leptin signaling following peripheral administration of leptin. Panel A: Representative immunoblot of phosphorylated p44/42 MAPK in the hypothalamus of control rats (Con) and Peg-LRA (LRA) rats 60 min following an i.p. injection of leptin (5 mg/kg). Panel B: Autoradiographic analysis of phosphorylated p44/42 MAPK in the hypothalamus of control rats (Con) and Peg-LRA (LRA) rats confirms that phosphorylated p44/42 MAPK is decreased in Peg-LRA rats relative to controls ($t(10) = 4.183$, $p < 0.01$). Panel C: Representative immunoblot of phosphorylated STAT3 in the hypothalamus of control rats and Peg-LRA rats 60 min following an i.p. injection of leptin (5 mg/kg). Panel D: Autoradiographic analysis confirms that leptin-stimulated pSTAT3 is decreased in Peg-LRA rats relative to controls ($t(10) = 2.62$, $p < 0.03$). [$* = p < 0.05$].

Peg-LRA rats (Fig. 4, Panel B) in the arcuate nucleus and the ventromedial nucleus of the hypothalamus. Higher power magnification identified robust pSTAT3 expression in the arcuate nucleus of vehicle-treated control rats (Fig. 4, Panel C) and Peg-LRA-treated rats (Fig. 4, Panel D), as well as robust labeling in the ventromedial nucleus of the hypothalamus of control rats (Fig. 4, Panel E) and Peg-LRA rats (Fig. 4, Panel F). Collectively, these results illustrate that the Peg-LRA blocks BBB transport of leptin to elicit a CNS leptin deficient state while not directly impacting CNS leptin receptor activity.

3.4. Peg-LRA treatment elicits behavioral despair in the forced swim test

We evaluated depressive-like behaviors in Peg-LRA rats compared to vehicle-treated control rats in the FST and the sucrose preference test. In the FST, an increase in immobility behaviors (and corresponding decrease in active behaviors such as swimming and climbing) is considered a measure of behavioral despair. In the

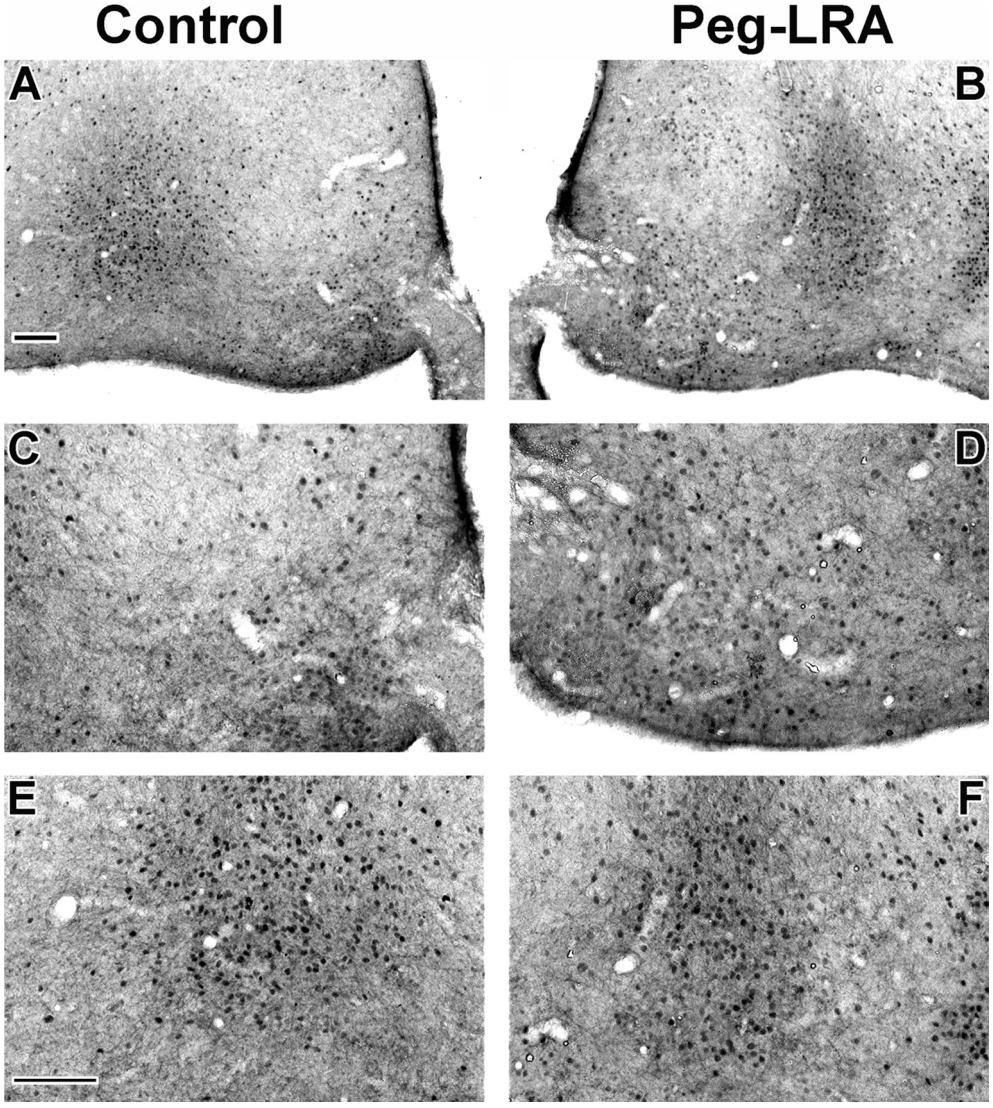

Fig. 4. Peg-LRA treatment does not affect hypothalamic leptin signaling following central administration of leptin. Rats received intracerebroventricular administration of leptin (1 μg) and 60 min later were perfused for pSTAT3 immunohistochemical analysis. Central leptin administration elicited robust increases in pSTAT3 in the hypothalamus of Control rats (Panel A) and Peg-LRA rats (Panel B). Higher power magnification illustrates that leptin-induced pSTAT3 levels are similar in the arcuate nucleus of Control rats (Panel C) and Peg-LRA rats (Panel D), as well as in the ventromedial nucleus of the hypothalamus of Control rats (Panel E) and Peg-LRA rats (Panel F). [scale bar shown in Panel A is for Panels A and B = 100 μm; scale bar shown in Panel E is for Panels C through F = 100 μm].

fifteen minute pretest, the levels of immobility and active behaviors did not differ between the two groups (Fig. 5, Panel A). However, Peg-LRA rats exhibited significant increases in immobility and reductions in active behaviors compared to vehicle-treated control rats during the 5 min test performed 24 h later (Fig. 5, Panel B). Peg-LRA rats also exhibited a decrease in the latency to float compared to control rats, although this decrease did not achieve statistical significance (Fig. 5, Panel C). It is important to note that the increases in immobility times observed in Peg-LRA rats are only apparent during the 5 min test session performed 24 h after the pre-test, which indicates that Peg-LRA-treated rats do not exhibit immobility (i.e. float) in the test phase simply because of their increased adiposity. Rather, the increases in immobility time specific to the test phase of the forced swim test are more likely indicative of behavioral despair. It could also be suggested that the increases in immobility were related to locomotor deficits in obese Peg-LRA rats. To test this possibility, locomotor activity of Peg-LRA rats and vehicle-treated control rats was assessed in the open field test. Peg-LRA rats did not exhibit any differences in total distance traveled (Fig. 5, Panel D) or in velocity in the testing arena (Fig. 5, Panel E), indicating that locomotor activity was unaffected by Peg-LRA treatment.

We also examined sucrose intake in Peg-LRA rats and in vehicle-treated control rats. Twenty-four hours following a habituation period, Peg-LRA rats and control rats were provided access to water and a 1% sucrose solution and fluid intake was monitored for the first 3 h of the dark cycle. Total fluid intake (13.29 ± 1.1 ml Control; 13.63 ± 0.7 ml Peg-LRA; $p = 0.79$) and sucrose preference (Fig. 5, Panel F) did not differ in Peg-LRA rats when compared with vehicle-treated control rats.

3.5. Peg-LRA administration elicits increases in peripheral inflammation

Since obesity-induced increases in pro-inflammatory cytokines are proposed to contribute to the neurological complications of metabolic disorders, we examined plasma cytokine levels in Peg-LRA rats and in vehicle-treated control rats. As shown in Fig. 6,

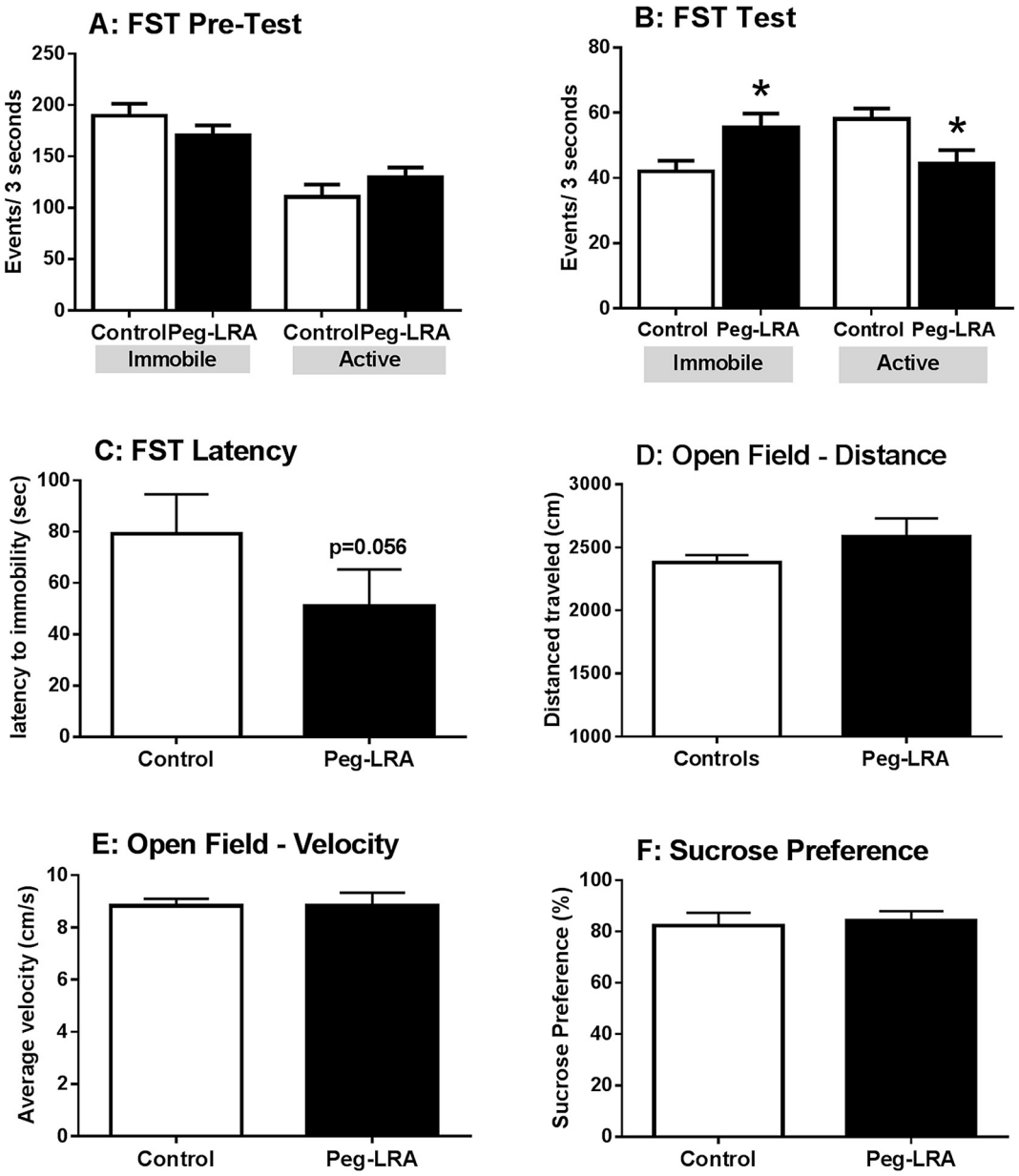

Fig. 5. Peg-LRA administration elicits behavioral despair in the forced swim test. Panel A: Peg-LRA rats do not show significant differences in active or immobile behaviors in the 15-min forced swim test pre-test. Panel B: Peg-LRA administration significantly increases immobility and decreases active behaviors ($t(18) = 2.556$, $p < 0.05$) relative to vehicle-treated controls in the 5 min forced swim test. Panel C: Peg-LRA administration does not significantly affect latency to float relative to vehicle-treated controls ($t(17) = 2.03$, $p = 0.0586$). Panel D: in the open field test, Peg-LRA administration does not affect total distance traveled relative to control rats. Panel E: Velocity in the open field test is not significantly different between Peg-LRA rats and control rats. Panel F: Peg-LRA administration does not affect sucrose preference relative to controls. [$* = p < 0.05$].

Peg-LRA rats exhibited significant increases in plasma levels of pro-inflammatory cytokines such as IL-1, IL-6 and TNF-α, as well as significant increases in anti-inflammatory cytokines such as IL-10. We also examined peripheral immune status in terms of cellular phenotype and activation status in the spleen. The percent of the lymphocyte population identified as macrophages did not differ between control and Peg-LRA rats (Fig. 7, Panel A); however, drug treatment significantly increased the percent of activated macrophages in the spleen (Fig. 7, Panel B). The percent of lymphocytes identified as T helper cells was significantly decreased with Peg-LRA treatment (Fig. 7, Panel C), as was the percent of activated T cells (Fig. 7, Panel D). In addition, there was a significant increase in the density of cytotoxic T cells (Fig. 7, Panel E). Peg-LRA rats also exhibited increases in plasma levels of C-reactive protein (Fig. 7, Panel F). Collectively, these data indicate that Peg-LRA treatment induced an obesity phenotype that was accompanied by an increase in activated pro-inflammatory peripheral immune status.

4. Discussion

The results of the current study demonstrate that blocking BBB transport of leptin via a leptin receptor antagonist elicits an obesity phenotype, as well as depressive-like behaviors and markers of peripheral inflammation in rats. Specifically, Peg-LRA administration increased food-intake, which resulted in an increase in body-weight. Peg-LRA treatment also increased adipose mass and adipocyte size, elicited insulin resistance and also increased several indices of peripheral inflammation. Peg-LRA treatment elicited behavioral despair in the FST, although sucrose preference was

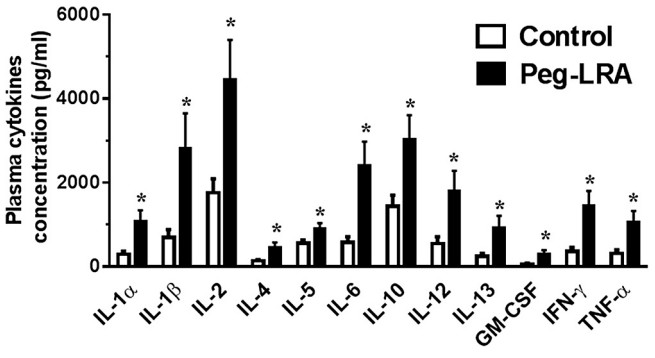

Fig. 6. Peg-LRA treatment increases plasma cytokine levels. Analysis of plasma cytokines using a rat Bio-Plex cytokine panel revealed that Peg-LRA administration elicited significant increases in plasma IL-1α ($t(10) = 2.90$, $p = 0.02$), IL-1β ($t(10) = 2.50$, $p = 0.03$), IL-2 ($t(10) = 2.74$, $p = 0.02$), IL-4 ($t(10) = 2.84$, $p = 0.02$), IL-5 ($t(10) = 2.38$, $p = 0.04$), IL-6 ($t(10) = 3.16$, $p = 0.01$), IL-10 ($t(10) = 2.54$, $p = 0.03$), IL-12 ($t(10) = 2.49$, $p = 0.03$), IL-13 ($t(10) = 2.39$, $p = 0.04$), GM-CSF ($t(10) = 2.44$, $p = 0.04$), IFN-γ ($t(10) = 2.91$, $p = 0.02$), and TNF-α ($t(10) = 2.77$, $p = 0.02$). [$* = p < 0.05$].

unaffected by the Peg-LRA. Interestingly, some measures that are normally characteristic of an obesity phenotype, namely macrophage infiltration into adipocytes, were not observed in Peg-LRA-treated rats. Such results support the concept that obesity develops as a continuum of endocrine, metabolic and immune changes and that the current study identifies a point in this continuum that is associated with some but not all features of an obesity phenotype. Such results suggest that distinct endocrine or immune features of the obesity profile might underlie specific depressive-like behavioral consequences.

As noted above, *ob/ob* mice and *db/db* mice exhibit behavioral deficits in the FST and anhedonia in the sucrose preference test, suggesting that leptin resistance may be a mechanistic link between obesity and depressive-like behaviors. The current study using a pharmacological approach without developmental confounds expands these findings by demonstrating that leptin resistance elicits certain aspects of a depressive-like phenotype. In parallel, studies have suggested that restoration of leptin signaling can reverse these behavioral deficits. For example, leptin administration dose-dependently reverses chronic stress-induced behavioral despair in the FST and decreases in sucrose preference (Lu et al., 2006). In addition, we have reported that restoration of leptin sensitivity reverses neuroplasticity deficits in leptin-resistant rats (Grillo et al., 2011b; Grillo et al., 2014). Conversely, a more recent study indicated that acute leptin administration did not reverse anhedonia or behavioral despair in mice with diet-induced obesity (Yamada et al., 2011). This may be partially due to impaired signaling at leptin receptors in obesity models (Levin et al., 2004). Another consideration is the relative contributions of peripheral leptin resistance, central leptin resistance and peripheral endocrine/metabolic changes in the development of depressive-like behaviors in obesity. While the results of the current study demonstrate that specific induction of leptin resistance initiates the development of an obesity phenotype that elicits depressive-like behaviors, these results cannot disentangle the potential role of leptin resistance vis-à-vis insulin resistance and other endocrine changes in co-morbid depressive illness in obesity. Indeed, as explained below, increases in plasma triglycerides, as well as increases in peripheral and CNS inflammation, are likely to be contributors to this comorbidity. Even more specifically as it relates to leptin resistance, it is likely that both peripheral and central leptin resistance contributes to the development of behavioral despair in Peg-LRA rats, although the obesity phenotype itself is very likely initially driven by the increases in food intake that results from hypothalamic leptin resistance. One way to differentiate between peripheral and CNS leptin resistance would be to more selectively induce leptin resistance in leptin receptor expressing limbic regions that have previously been implicated in the pathophysiology of depressive illness, such as the hippocampus. In this regard, we have previously reported that hippocampal-specific insulin resistance elicits deficits in hippocampal synaptic plasticity and learning independent of changes in body weight and peripheral glucose homeostasis (Grillo et al., 2015). Using a similar approach to induce region-specific leptin resistance would begin to differentiate between the contributions of peripheral and CNS endocrine and immune changes in the development of neuropsychiatric disorders in obesity.

As noted above, increases in plasma triglycerides are a feature of obesity that have previously been reported to impair hippocampal neuroplasticity. For example, elevation of plasma triglycerides impairs hippocampal-dependent learning and direct application of triglycerides to hippocampal slice preparations reduces stimulus-evoked long term potentiation (Farr et al., 2008). Mechanistically, triglycerides are proposed to reduce BBB transport of leptin (Banks et al., 2004), which provides an interesting point of convergence with the current studies using an antagonist that blocks BBB transport of leptin. While decreases in hypothalamic leptin signaling precede decreases in BBB leptin transport in diet-induced obesity rats (Levin et al., 2004), the current results demonstrate that blocking BBB leptin transport, while leaving leptin receptors responsive to direct CNS administration of leptin, is sufficient to induce behavioral despair in rodents. In the broader context these results suggest that increases in plasma triglyceride levels elicited by leptin resistance are an important contributor to the development of a CNS-leptin deficient state and thereby contribute to neuroplasticity impairments that include deficits in learning and memory and development of depressive-like behaviors.

Obesity is also recognized as a state of chronic mild inflammation and neuroinflammation is proposed to play a role in the pathogenesis of co-morbid neuropsychiatric disorders in obesity (For reviews, see (Shelton and Miller, 2010; Soczynska et al., 2011)). Conceptually, it has been proposed that increases in inflammation are observed under conditions of increased adiposity, which is linked to macrophage infiltration into an expanding population of adipocytes. In the current study we observed that Peg-LRA rats exhibit increases in both anti-inflammatory and pro-inflammatory cytokines. It is possible that increased plasma levels of cytokines such as IL-10 may be a compensatory response to increases in pro-inflammatory cytokines such as IL-6 and TNF-α. As such, an interesting future direction would be the examination of the time course of changes of pro-inflammatory versus anti-inflammatory cytokines in response to Peg-LRA treatment. Irrespective of such analyses, it is interesting to note that while Peg-LRA-treated rats do not exhibit macrophage infiltration, these rats do exhibit increased plasma levels of pro-inflammatory cytokines and CRP, as well as activated macrophages in spleen. In this way, our study has identified depressive-like behaviors at a point in the obesity spectrum that is associated with inflammation prior to macrophage infiltration into adipocytes.

When placed in the broader context, the current results suggest that there is a continuum of pathophysiological features in obesity that ultimately result in CNS complications including the development of depressive illness. For example, increases in body weight, body adiposity, leptin resistance and decreases in insulin sensitivity may be necessary components in this continuum that result in the development of behavioral despair, but these changes are not sufficient to elicit anhedonia. There are several additional potential explanations for the lack of an anhedonic response in Peg-LRA rats. To begin, Peg-LRA rats exhibit profound hyperphagia and this drive to consume calories may suppress the development of reduced

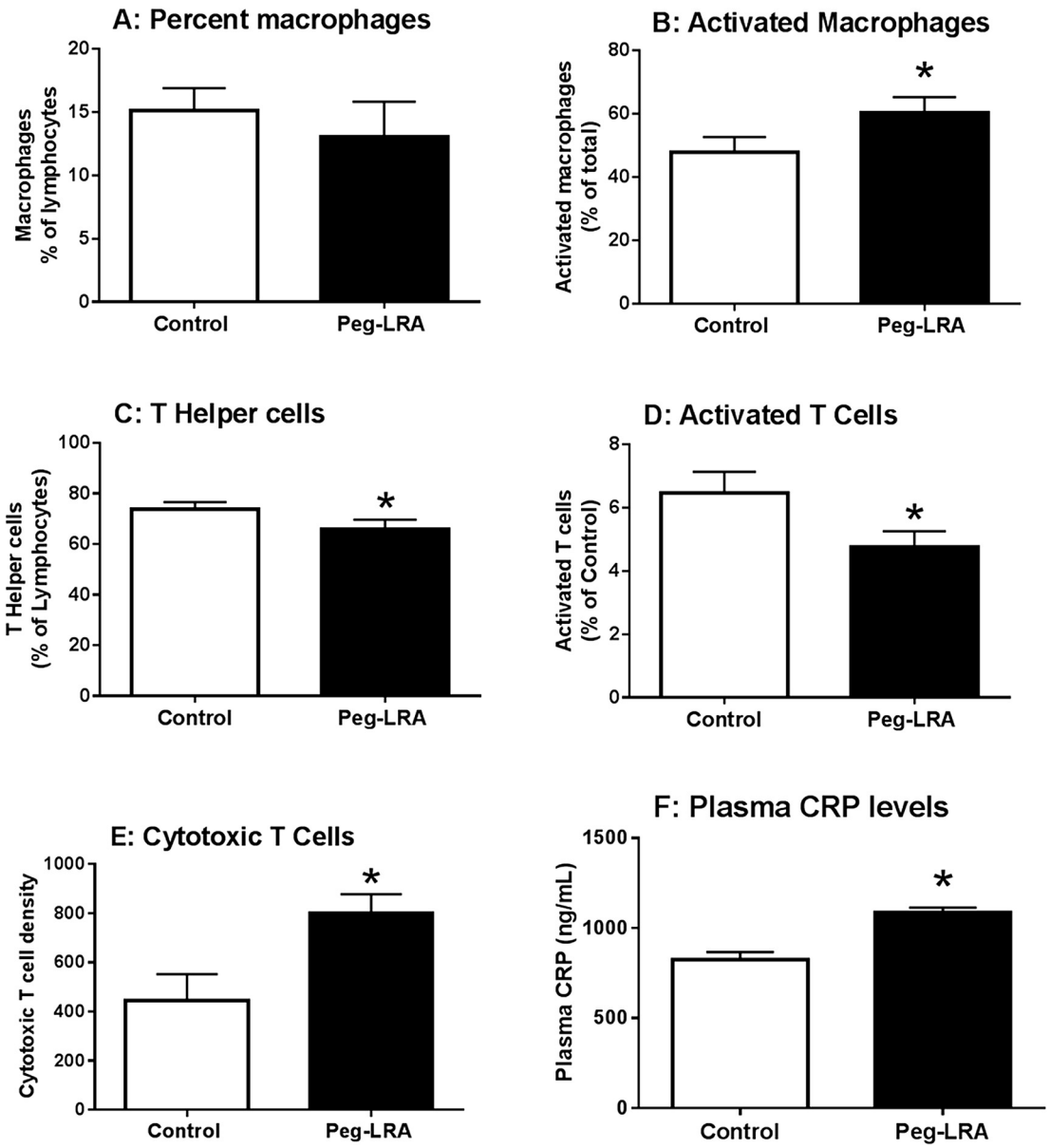

Fig. 7. Peg-LRA treatment increases indices of peripheral inflammation. Panel A: flow cytometry analysis determined that the percentage of lymphocytes isolated from spleen that exhibit a macrophage phenotype are unchanged in Peg-LRA rats compared to control rats ($t(10) = 0.6747$, $p = 0.51$). Panel B: the percentage of activated macrophages isolated from the spleen of Peg-LRA rats is significantly increased compared to control rats ($t(10) = 3.92$, $p = 0.0045$). Panel C: the percentage of spleen lymphocytes identified as T helper cells is significantly reduced in Peg-LRA rats ($t(10) = 3.79$, $p = 0.006$). Panel D: compared to controls, the percentage of activated T cells is significantly reduced in the spleen of Peg-LRA rats ($t(10) = 4.49$, $p = 0.0024$). Panel E: the density of cytotoxic T cells is significantly increased in the spleen of Peg-LRA rats ($t(10) = 5.94$, $p = 0.0005$). Panel F: ELISA determined that plasma C-reactive protein (CRP) levels are significantly increased in Peg-LRA rats compared to vehicle-treated control rats ($t(10) = 5.2$, $p = 0.0001$). [$* = p < 0.05$].

sucrose preference in Peg-LRA rats. Alternatively, it is possible that components of an obesity phenotype beyond leptin resistance are mechanistically responsible for the development of anhedonia. This concept of an obesity continuum is further supported by clinical observations indicating that there is a correlation between increased BMI and increased risk for developing co-morbid depressive illness (Onyike et al., 2003), as well as observations that obese patients are less responsive to antidepressant treatment compared to overweight patients and patients with BMIs in the normal range (Kloiber et al., 2007). While the literature is somewhat equivocal, these results support other studies indicating that obese individuals are more likely to exhibit treatment resistance, especially to serotonin selective reuptake inhibitors (Uher et al., 2009; Khan et al., 2007; Lin et al., 2014; Rizvi et al., 2014). These observations are interesting in view of the hypothesis that inflammation increases the activity of indoleamine 2,3-dioxygenase (IDO) in the CNS and thereby shifts metabolic pathways to reduce 5HT levels (Raison et al., 2006; Capuron and Miller, 2011). From a clinical perspective, longitudinal studies indicate that patients that undergo bariatric surgery experience a significant elevation in mood, which is often maintained for several years post-surgery (Burgmer et al., 2014; Hayden et al., 2011; White et al., 2015; Thonney et al., 2010; Rutledge et al., 2012). In view of these observations, even modest reductions in BMI that ameliorate the endocrine and metabolic components of the obesity milieu that include restoration of leptin sensitivity may be an effective strategy to successfully manage depressive illness symptomatology in obese individuals.

Acknowledgments

Supported by the Department of Veterans Affairs (IO1 BX001804 to LPR and 1101 BX001374 to MAW), and the University of South Carolina School of Medicine Research Development Fund.

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