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Leptin Reduces Pathology and Improves Memory in a Transgenic Mouse Model of Alzheimer's Disease

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Abstract

We have previously reported anti-amyloidogenic effects of leptin using *in vitro* and *in vivo* models and, more recently, demonstrated the ability of leptin to reduce tau phosphorylation in neuronal cells. The present study examined the efficacy of leptin in ameliorating the Alzheimer's disease (AD)-like pathology in 6-month old CRND8 transgenic mice (TgCRND8) following 8 weeks of treatment. Leptin-treated transgenic mice showed significantly reduced levels of amyloid-β $(Aβ)_{1-40}$ in both brain extracts (52% reduction, p=0.047) and serum (55% reduction, p=0.049), as detected by ELISA, and significant reduction in amyloid burden (47% reduction, p=0.041) in the hippocampus, as detected by immunocytochemistry. The decrease in the levels of Aβ in the brain correlated with a decrease in the levels of C99 C-terminal fragments of the amyloid-β protein precursor, consistent with a role for β-secretase in mediating the effect of leptin. In addition, leptin-treated TgCRND8 mice had significantly lower levels of phosphorylated tau, as detected by AT8 and anti-tau-Ser^{396} antibodies. Importantly, after 4 or 8 weeks of treatment, there was no significant increase in the levels of C-reactive protein, tumor necrosis factor-α, and cortisol in the plasma of leptin-treated TgCRND8 animals compared to saline-treated controls, indicating no inflammatory reaction. These biochemical and pathological changes were correlated with behavioral improvements, as early as after 4 weeks of treatment, as recorded by a novel object recognition test and particularly the contextual and cued fear conditioning test after 8 weeks of treatment. Leptin-treated TgCRND8 animals significantly outperformed saline-treated littermates in these behavioral tests. These findings solidly demonstrate the potential for leptin as a disease modifying therapeutic in transgenic animals of AD, driving optimism for its safety and efficacy in humans.

Keywords

Alzheimer's disease; amyloid-β; CRND8; leptin; tau

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INTRODUCTION

Leptin is an adipocyte-derived hormone which controls feeding behavior through specific receptors within the hypothalamus [1]. Additionally, leptin has important physiological roles in the control of fat storage or mobilization, the reproductive system, the immune system, bone homeostasis, antioxidant defense [2], insulin sensitivity [3–5], and neuronal activity and/or protection [6]. Leptin receptors have been identified in peripheral tissues and in neurons in many brain regions, including at a high density in the hippocampus [6–8], which is particularly vulnerable in Alzheimer's disease (AD) [9,10]. Direct injection of leptin into the hippocampus of rodents can improve memory processing and modulate long term potentiation and synaptic plasticity [11].

Recent studies have demonstrated the potential beneficial effects of leptin as an AD therapeutic [12]. Leptin treatment of neuronal cells reduces the amount of amyloid-β (Aβ) secreted into the medium in a time- and dose-dependent fashion [13,14]. This effect was coincident with a change in the lipid profile of membranes affecting lipid rafts, β-secretase (BACE) activity and proteolytic processing of the amyloid-β protein precursor (AβPP). This may be attributed to the lipolytic action of leptin which could also explain the ability of leptin to facilitate the lipoprotein receptor-like protein (LRP)-dependent uptake of apolipoprotein E (apoE)/Aβ complexes [13]. Leptin has also been shown to reduce tau phosphorylation at AD-relevant phospho-epitopes in neuronal cells with a potency two orders of magnitude greater than insulin [15], achieved through modulation of AMPK [14,15] and GSK-3β [16], and without any observed toxicity.

Herein we investigated whether prolonged leptin treatment of the CRND8 transgenic animal AD model can lower Aβ in brain extracts, as previously reported for the Tg2576 mouse [13]. The CRND8 mice overexpress the human AβPP gene containing the Swedish (K670N and M671L) and the Indiana (V717F) familial AD (FAD) mutations [17]. They exhibit early-onset, progressive cognitive defects and amyloid plaque deposition starting from 3 months of age [17,18], thus providing a robust model to study the potential therapeutic value of leptin.

In contrast to the Tg2576 studies, treatments here were initiated, and were continued, within the post-plaque period, allowing the evaluation of the effect of treatment on the brain amyloid burden. Also, the effect of leptin treatment on cognitive performance was evaluated, using two different experimental paradigms addressing hippocampal functionality. Lastly, we wanted to examine whether the ability of leptin to affect tau phosphorylation *in vitro* can be recapitulated *in vivo* as well.

MATERIALS AND METHODS

Reagents and antibodies

AβPP 643–695 mAb was purchased from Millipore (Billerica, MA). Rabbit anti-PPARγ and -SOCS3, Tau (pSer^{396}) mAb, and tau (tau46) mAb were purchased from Cell Signaling. PHF-tau mAb (clone AT8) was purchased from Pierce Biotechnology (Rockford, IL). PHF-1 mAb was a gift from Dr. Peter Davies, Albert Einstein College of Medicine (Bronx, NY). Rabbit anti-tau (pThr^{181}) was purchased from Sigma-Aldrich. Rabbit anti-α-tubulin, anti-leptin, and anti-leptin receptor (OB-R) were purchased from Affinity BioReagents (Golden, CO).

Animals and housing

CRND8 mice (n=22, 4 months old) carrying the AβPP695 gene with double mutations at KM670/671/NL (Swedish mutation), along with V717F (Indiana mutation) on a C3H/He-C57BL/6 background and wild-type mice (n=20) were used in this study. All animals were group housed upon arrival and provided *ad libitum* access to food and water and maintained

on a 12 hour light/dark cycle. All animals were treated following approved protocols by The Institutional Animal Care and Use Committee (IACUC) of Case Western Reserve University and experimental groups were determined in a random fashion. All animals were weighed 3 times during the study as a general measure of health status.

Leptin pump implantation

Pump implantations were carried out as described previously [13]. Briefly, mice were anesthetized with intraperitoneal injection of Avertin, and then surgically fitted with a subcutaneous Alzet miniosmotic pump (model 2004, Durect Corp., Cupertino, CA, USA). 13 of the CRND8 animals received a daily dose of 20 μg leptin in PBS (0.25 μl/h of 3.33 mg/ml recombinant murine leptin), and 9 were infused with PBS; all wild-type mice were infused with PBS. Refilled osmotic pumps replaced old ones at 4 weeks for a total of 8 weeks of treatment.

ELISAs

Mouse leptin, insulin, C-reactive protein (CRP), and tumor necrosis factor-α (TNFα) levels in serum, collected at the end of the study, were determined using the Quantikine Mouse Leptin Immunoassay (R&D Systems; Minneapolis, MN), the Mouse Insulin ELISA Kit (Millipore), the Mouse C-Reactive Protein ELISA Quantitation Kit (Genway; San Diego, CA), and the Mouse TNFα ELISA kit (R&D Systems; Minneapolis, MN), respectively. Human $A\beta_{1\text{-}40}$ serum levels were determined using the $A\beta_{1\text{-}40}$ Colorimetric Immunoassay kit (Invitrogen; Carlsbad, CA). All assays were performed according to manufacturer's specific instructions, n=6. Levels of all serum markers were calculated from a standard curve developed with OD at 450 nm versus serial dilutions of known concentration.

Immunocytochemistry

At necropsy, the brain was removed and divided along the midline into two halves. One half was frozen on dry ice and the other half was immersion fixed in 10% neutral buffered formalin and processed in paraffin wax. Brains in paraffin blocks were sagitally sectioned serially (50 μm) across the hippocampus and were immunostained using 4G8 as the primary antibody (recognizes the 17–24 amino acid segment within Aβ) as previously described [19]. After washing, a goat anti-mouse secondary antibody was incubated for an additional 30 min at room temperature, and sections were visualized with avidin-biotin-HRP complex (Vectastain Elite ABC kit, Vector, Burlingame, CA) and diaminobenzidine tetrahydrachloride (DAB) in H_2O_2. Quantification of Aβ deposition was carried out using a Zeiss Axiocam (Munchen-Hallbergmoss, Germany) and compatible image analysis software, Axiovision (Carl Zeiss Vision GmbH, Munchen-Hallbergmoss, Germany). For each animal, quantification for Aβ deposition was as previously described [20]. Briefly, using a 5× objective, a single field encompassing the entire hippocampus or cortex was manually selected. Positive areas of immunostaining were detected by the computer and expressed as the percent area stained relative to the entire cortical or hippocampal area. Areas of vascular amyloid deposition were not included. The values obtained from all sections per animal were averaged. Sections were analyzed for the number of plaques, the size of plaques, and the amyloid burden, defined as the percentage of the area stained by the antibody, n=8–9.

Immunoblotting

Frozen brain samples were weighed, minced with a scalpel and then transferred to an equal volume of 10% PBS (pH 7.4). Tissues were homogenized using a dounce homogenizer and proteins extracted using the T-PER tissue extraction reagent (Pierce), supplemented with protease/phosphatase inhibitors (Pierce), at a ratio of 1 g tissue per 10 mL extraction reagent. Samples were briefly centrifuged at 10,000 rpm for 5 min and the supernatant was transferred

to a fresh tube. DNAse (Pierce) was added to each sample and incubated at 37°C for 30 min. Total protein was determined with the Coomassie (Bradford) Protein Assay Kit (Pierce) and samples (25 μg) were analyzed by immunoblot as described previously [15]. All primary antibodies, except tau-pSer^{396}, total tau (all 1:500), and PHF-tau AT8 (1:200), and secondary antibodies were used at final dilutions of 1:1,000 and 1:10,000, respectively. All experiments were performed in triplicate, n=3.

Behavioral assessment

Behavioral testing for established measures of cognitive performance [21] was performed after 4 and 8 weeks of treatment.

Trace fear conditioning—The contextual and cued fear conditioning tests measure the ability to remember an unpleasant (conditioned) stimulus and to connect it with a certain environment (context). Contextual fear conditioning is a form of learning that is generally thought to be hippocampus-dependent whereas cued fear conditioning is thought to be hippocampus-independent [22,23]. This protocol is carried out over 2 days.

Day 1 – Training: On the first day animals are allowed to habituate in the chamber (Med Associates, Burlington, VT) for 2 min and are then presented with a white noise (80dB) for 30 sec; this stimulus is designated as the conditioned stimulus (CS). After a 2.5 second interval, the animals are administered a 0.5mA shock; this is designated as the unconditioned stimulus (US). This procedure is repeated 4 times.

Day 2 – Contextual/Altered context/cued Testing: 24 h after training, animals are placed back in the original chamber and freezing bouts are scored during 5 min to determine the associations of the US with the context (contextual). Freezing measurements are automated using appropriate software (Med Associates, Burlington VT) designed to gather 30 observations in 5 min. After contextual freezing is measured, animals are returned to their home cage for 1 h. The chamber environment is modified (new walls, flooring and odor cues) and the animal is introduced in the "new" chamber for 6 min. Freezing rate is quantified as described in the contextual test for 3 min in the absence of the CS (altered context). For the remaining 3 min, the animal is presented with the CS in the altered context and scored for freezing behavior as described previously, to determine the cued fear conditioning score.

Novel object recognition—The novel object recognition test was carried out in a multiple open-field box (20″ × 20″ × 17″ × 4) (San Diego Instruments, San Diego, CA). Before training, mice were individually habituated by allowing them to explore the open-field box for 5 min on the day prior to testing. During the training session, two identical novel objects were placed into the open-field 16″ away from each other and the animals were allowed to explore for 10 min. Exploration of the object was considered to be when the head of the animal was facing ½ cm from the object or touching the object. If the animal used the object as a prop to explore the environment, this was not considered an exploration. The time each animal spent exploring each object was recorded. The animals were returned to their home cages immediately after training. One hour after the training the animals were re-introduced into the open-field that contained one novel object and one previously explored object. The objects were of similar exploratory level/physical complexity (i.e., if the old object had a hole, the new one did also) and similar size. During the retention test, animals were introduced into the open-field box and allowed to explore freely for 5 min. Time spent and frequency spent with both objects was recorded in addition to rearing and grooming frequency and duration. The open-field box and objects were thoroughly cleaned with 70% ethanol after each session to avoid possible instinctive odorant. A discrimination index (total time spent with new object/total time of object exploration) was used to measure recognition memory.

Statistical analysis

Statistical data analyses were performed with analysis of variance and Tukey-Kramer multiple comparisons test. Densitometric analyses were performed using the UN-SCAN-IT gel 6.1 software (Silk Scientific; Orem, UT). $p<0.05$ was considered statistically significant.

RESULTS

Effect of leptin administration on insulin and pro-inflammatory proteins

The majority of patients with AD have some form of insulin resistance, hyperinsulinemia, or type II diabetes [24]. Thus, the first set of studies examined the levels of leptin detectable in the serum of TgCRND8 mice, and explored whether an increase in leptin correlates with changes in insulin levels (Figure 1). Leptin-treated transgenic animals showed significantly ($p<0.05$) elevated levels of leptin (Figure 1A; left, light gray bar) compared to saline-treated animals (left, dark gray bar). The levels detectable in the saline-treated TgCRND8 were comparable to wt littermates (right bar). There was no significant difference in insulin levels observed in leptin- or saline-treated mice (Figure 1B).

Leptin has similar structural and functional characteristics to the cytokines [25], sharing post-receptor pathways and participating in the immune response to pathogens and infections. Thus, we next explored whether leptin administration promotes upregulation of inflammatory proteins. CRP is a protein whose levels rise dramatically during inflammatory processes occurring in the body, thus serving as a biomarker for inflammation [26]. There was no detectable difference in CRP levels observed in leptin- or saline-treated animals ($p>0.05$) (Figure 1C). Furthermore, we were unable to detect significant changes in the levels of plasma cortisol and TNFα in transgenic animals treated with leptin compared to those treated with saline or compared to saline-treated wt animals. In all animal groups the levels of these metabolites was close to the lower limit of the assays (see Methods).

Pathways regulated by leptin administration in brain

The post-receptor binding of leptin triggers the JAK/STAT pathway to induce gene transcriptional changes via activation of Janus tyrosine kinase 2 (JAK2), the signal transducer and activator 3 (STAT3), and the suppressor of cytokine signaling 3 (SOC3) [27] in central and peripheral tissues. We next investigated the levels of leptin in the brains of leptin- and saline-treated mice, and examined whether known downstream effectors of leptin, specifically SOCS3 and peroxisome proliferator-activated receptor-γ (PPARγ), are increased (Figure 2). As expected, leptin levels were significantly ($p<0.05$) higher in the brains of leptin-treated animals compared to saline-treated (Figure 2A, top row; Figure 2B, light gray bar). However, there was no significant change in expression of the long isoform of the leptin receptor (OB-R) in leptin-treated brains compared to control (Figure 2A, second row; Figure 2C). There was a non-significant increase ($p>0.05$) (Figure 2D) in expression of SOCS3 in leptin-treated TgCRND8 animals (Figure 2A, third row).

PPARγ is a transcription factor known to regulate BACE, a key enzyme in AβPP processing [28] and implicated in modulating leptin's action [29]. Leptin-treated transgenic animals displayed a significant ($p<0.05$) increase in PPARγ levels compared to control (Figure 2A, fourth row; Figure 2E, light gray bar). These results are in agreement with previous work [29].

Aβ levels and plaque deposition in leptin-treated TgCRND8 mice

The extracellular accumulation of Aβ in the form of plaques is a hallmark pathological feature of AD and the amount deposited depends on the rates of its production, secretion, aggregation, and clearance. We have previously demonstrated that treating neuronal cells with leptin reduces

the amount of Aβ secreted into the medium in a time- and dose-dependent fashion [13]. This was also reflected in the amount of extractable Aβ found in the brains of Tg2576 mice that underwent an 8 week treatment with leptin compared to saline-treated animals [13]. TgCRND8 mice overexpress the human AβPP gene containing the Swedish (K670N and M671L) and the Indiana (V717F) familial AD (FAD) mutations [17]. The aforementioned leptin treatments were initiated at 10 months of age, when typically the levels of total brain Aβ start rising and were completed by 12 months of age, just when the first Aβ deposits make appearance in the Tg2576 mice. In the current studies with the TgCRND8 mice, the entire treatment (4–6 months of age) was performed during the post-plaque period which starts around 3 months of age. A significant ($p<0.05$) reduction in $A\beta_{1\text{-}40}$ levels in both brain (Figure 3A, gray bar) and serum (Figure 3B, gray bar) of the leptin-treated TgCRND8 mice was found.

We then investigated whether leptin treatment altered the processing of brain AβPP into the C99 C-terminal fragment (CTF) of AβPP, derived by the action of BACE and which is a direct precursor of Aβ (Figure 3C), or the C83 CTF of AβPP, which is a non-amyloidogenic product derived by the action of α-secretase. A significant ($p<0.05$) reduction in the ratio of C99 fragment to the C83 species (Figure 3D, top panel) and total CTFs (bottom panel) was observed in leptin-treated animals versus saline-treated controls. This is consistent with leptin's involvement in modulating BACE activity, as reported [13].

Additionally, immunohistochemical examination of paraffin-embedded brain sections (Figure 4) revealed that 8 weeks of leptin treatment significantly ($p<0.05$) reduced the amyloid burden in the hippocampus (Figure 4A) of 6-month old TgCRND8 mice, compared to age- and gender-matched saline-treated transgenic mice. This is a region particularly enriched in functional (OB-Rb) leptin receptors. The significantly ($p=0.0406$; n=6) decreased (~50%) amyloid burden in the hippocampus (quantified as % area stained with 4G8 antibody) was parallel to a decrease (~25%) ($p=0.1583$; n=6) in the average size of plaques (Figures 4B and 4C), despite an insignificant change in the overall number of plaques in that region (data not shown). Examination of the cortex (Figure 4D) showed a similar pattern of staining.

Leptin-treated transgenic animals show reduced tau phosphorylation

Neurofibrillary tangles (NFT) are intraneuronal aggregates of highly phosphorylated tau protein that correlate closely with cognitive loss in AD [30]. The abnormal phosphorylation of tau protein leads to disrupted microtubule function, abnormal protein trafficking, the formation of NFTs, and eventual neuronal death [30]. TgCRND8 or Tg2576 mice do not develop NFTs; however, increased brain tau phosphorylation has been reported in Tg2576 mice [31]. We report here that tau is hyperphosphorylated in the TgCRND8 mice as well (Figure 5). We previously have shown that leptin, when compared to insulin, is two orders of magnitude more potent at reducing tau phosphorylation at AD-relevant phospho-epitopes in neuronal cells [15]. Therefore, we assessed phospho-tau levels in the brains of leptin- or saline-treated transgenic or wt mice (Figure 5). The saline-treated, transgenic mice expressed relatively high levels of phospho-tau at all epitopes examined (Figure 5A; Figures 5B–5E, left dark gray bars). Interestingly, leptin-treatment significantly ($p<0.05$) reduced phospho-tau (left light gray bars) at each AD-relevant epitope to levels observed in the brains of wt animals (right bars).

Behavioral improvements of TgCRND8 mice treated with leptin

Novel object recognition—Animals of the three groups: a) TgCRND8 treated with leptin, b) TgCRND8 treated with saline, and c) wild-type treated with saline were tested in the Novel object recognition test after 4 and 8 weeks treatment duration. Leptin-treated TgCRND8 and wild-type mice spent statistically ($p<0.05$) more time with the novel object compared to the transgenic treated with saline (Fig. 6A). This indicated that there was an improvement in

working memory performance of the TgCRND8 mice after 4–8 weeks of leptin treatment, compared to saline treated TgCRND8. Leptin-treated transgenic animals were indistinguishable from the wild-type mice in this test, while saline-treated TgCRND8 mice performed very poorly (Fig. 6A) compared to both other groups. Thus, 1–2 month chronic leptin treatment abrogates impaired performance in this cognitive task in the 6-month old TgCRND8 mice.

Fear conditioning (FC)—In this test, an aversive training chamber was used for training and measurement of contextual and cued fear associated memory after repeated pairings of CS (auditory cue) and US (mild footshock). 24 h after training, animals were placed in the original chamber to test contextual fear conditioning or in a novel environment in the presence of the CS to test for cued fear conditioning. The x–axis represents % freezing time (Fig. 6B). TgCRND8 mice performed better in the contexual fear conditioning test after 8 weeks of leptin treatment, compared to saline-treated animals. This finding approached statistical significance ($p<0.06$). Additionally, leptin treatment resulted in a statistically significant ($p<0.05$) improvement in performance in the cued fear conditioning. There was no statistical significance when tested in the altered context and low freezing response (data not shown) indicating that the animals did not recognize the altered context.

DISCUSSION

In the present report, we describe the effects of chronic leptin-treatment in transgenic CRND8 mice on AD-like pathobiology and cognitive decline. Leptin-treated animals were found to have reduced levels of $A\beta_{1\text{-}40}$ in both brain and serum. In the brain, a reduction in the processing of AβPP through the amyloidogenic pathway, presumably due to modulation of BACE, was observed. Additionally, leptin-treated animals showed a significant decrease in amyloid burden in the hippocampus, which was associated with a decrease in the average size but not number of 4G8-stained amyloid plaques (Figure 4). Cortical areas were less affected by the treatment, despite the significant drop in the amount of total solubilized $A\beta_{1\text{-}40}$ in brain extracts (52% reduction in detergent-extractable $A\beta_{1\text{-}40}$ after 8 weeks of leptin treatment). One reason attributed to this difference could be the higher density of functional receptors usually found in the hippocampus compared to other regions. This on the other hand could be particularly advantageous for using leptin as an AD therapy, as it could positively impact the functionality of a brain structure particularly vulnerable to degradation in AD. It is speculated that a longer duration or higher dose of leptin may be required to permit global pathological changes as they relate to deposition of Aβ in plaques. Solubilized brain Aβ levels were significantly decreased by leptin treatment and perhaps this pool is more amenable to manipulation. In fact, this pool is likely to contain the pre-fibrillary oligomeric forms of Aβ [32], implied to be involved in neurotoxicity [33] and which levels could correlate to neuronal loss better than amyloid plaque number and density.

Leptin treatment has the potential to exacerbate a variety of inflammatory conditions, mainly due to the structural and functional characteristics it shares with many cytokines [25], and its participation in the immune response to pathogens and infections. We therefore examined whether chronic leptin administration produced changes in the inflammatory milieu by monitoring inflammatory biomarkers. Leptin did not significantly alter the levels of CRP (Figure 1C), TNFα or cortisol (data not shown) compared to saline treatment. These findings are assuring considering the implication of the immune system in the pathobiology of neurodegeneration in AD.

Interestingly, we did not discern a significant difference between serum leptin levels among wt and saline-treated CRND8 mice (Figure 1A). Leptin levels are known to decrease prior to the onset of dementia in AD patients [34], and we have previously shown that leptin levels are

decreased in AD Tg animals compared to age-matched wt littermates [13]. However, our previous studies utilized 8- and 12-month old Tg animals, while the present study utilized 4-month old Tg animals treated for up to 8 weeks. As leptin levels are seen to progressively decrease with age in AD, this age difference may account for the observed findings.

Leptin forms a feedback loop with SOCS3, a negative cytokine regulator which inhibits Jak2/STAT3 signaling following prolonged receptor (OB-R) activation [27]. Overactivation of SOCS3 signaling could lead to prolonged repression of inflammatory pathways and thus increase the risk for immunosuppression. Leptin-treated TgCRND8 and wt animals did not show elevated SOCS3 expression (Figure 2D), suggesting that chronic administration of leptin is unlikely to lead to immune defects.

Another downstream target of leptin signaling is PPARγ, and PPARγ levels have been shown to increase *in vivo* with leptin administration in peripheral tissues [29] as well in neuronal cultures [14]. Because we observed an approximate 2.5–3 fold increase in circulating leptin levels in the treated Tg animals compared to either saline-treated Tg or wt (Figure 1A), we believe that this transient increase in leptin triggers an increase in PPARγ expression while basal levels do not have a similar effect (Figure 2E). In addition, PPARγ expression seems to be independent of STAT3 signaling, since leptin-treated Tg and wt animals did not show elevated SOCS3 expression (Figure 2D).

Since PPARγ has been shown to regulate BACE [28], and, thereby, AβPP processing, this could be another mechanism by which leptin regulates Aβ homeostasis. In agreement with previous results [13], leptin-treated TgCRND8 displayed reduced levels of the amyloidogenic C99 AβPP fragment and $A\beta_{1\text{-}40}$ levels in both brain and serum (Figure 3). Furthermore, as leptin appeared to modulate Aβ, at least partially, through its ability to modulate BACE activity in lipid rafts [13], the current findings strongly implicate, although not directly prove, an interesting interaction between leptin and PPARγ in modulating Aβ.

Leptin treatment of TgCRND8 significantly reduced the levels of phospho-tau at all examined epitopes (Figure 5). Induction of hyperphosphorylation but not tau oligomerization is common in transgenic mice expressing AβPP. Oligomerization of tau and tangle formation has only been observed in the triple transgenic (3xTg) AD model [35] that expresses human tau isoforms. Thus, it would be of interest to study the effect of leptin treatment on tangle formation in the triple transgenic animals.

Of particular interest was the finding that leptin-treated TgCRND8 mice showed improved cognitive performance in novel object recognition and fear conditioning tests compared to saline-treated littermates (Figure 6). This improvement may indirectly result from the reduced amyloid load found within the hippocampus of leptin-treated transgenic mice (Figure 4A), where a decreased burden would be less toxic to the neurons and thereby improve behavioral performance. Additionally, direct injection of leptin into the hippocampus of rodents has been shown to improve memory processing and modulate long term potentiation and synaptic plasticity [11]. Hence, leptin may improve cognitive performance in the transgenic mice by altering processing of AβPP and hence decreasing amyloid burden within the hippocampus and/or by directly promoting memory formation and synaptic plasticity.

Our data fully support the ability of leptin to ameliorate AD-like pathological pathways and, for the first time, demonstrate its efficacy for reverting or preventing the cognitive deterioration of the TgCRND8 mouse. Further, leptin treatment was not associated with inflammation, adding increased safety to the profile of our novel therapeutic for AD.

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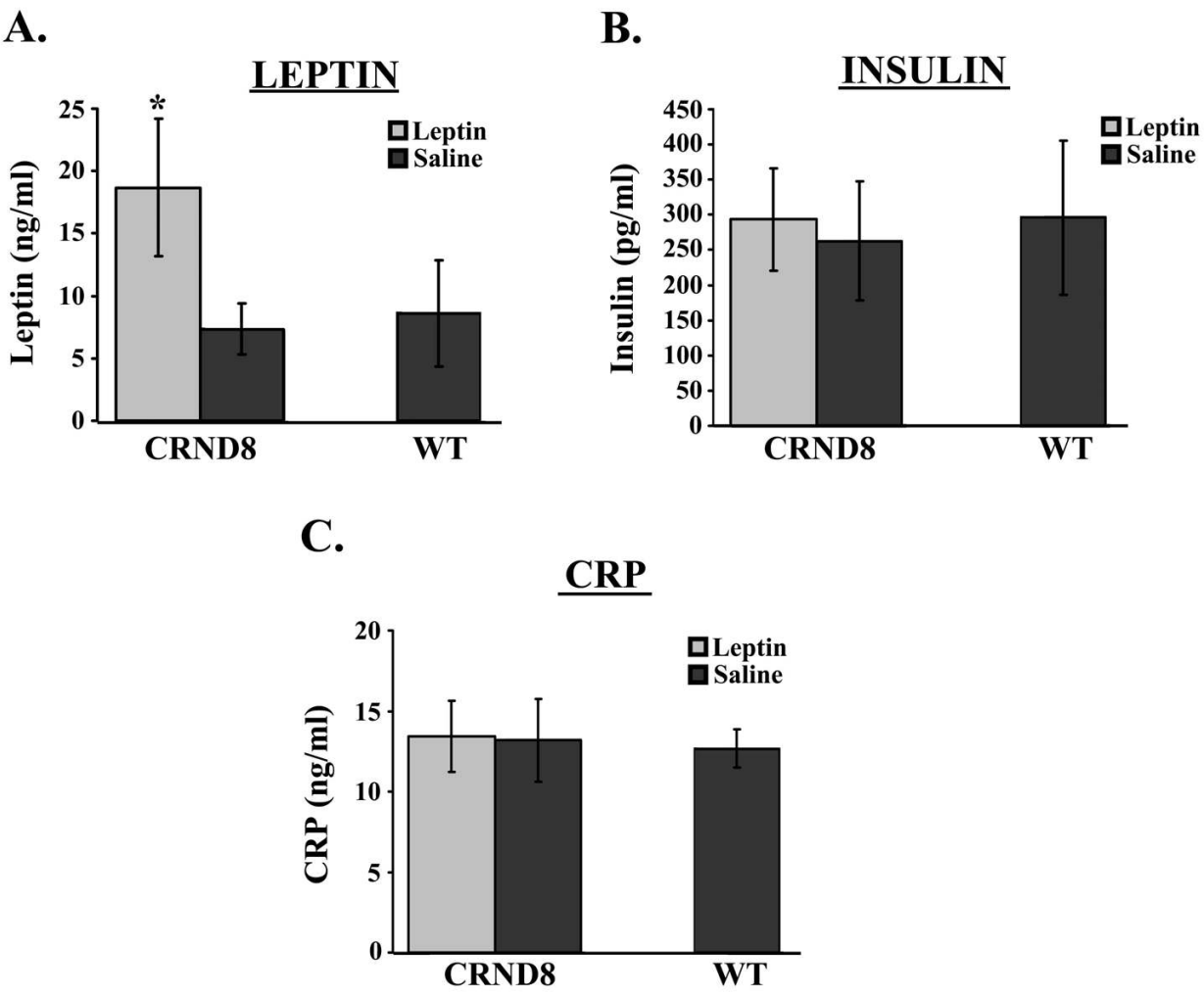

Figure 1.

Serum concentrations of leptin, insulin, and CRP in CRND8 and wt mice. Circulating levels of (**A**) leptin, (**B**) insulin, and (**C**) CRP were assessed in serum from leptin- or saline-treated CRND8 or wt mice by ELISA. Results (n=6) are presented as the mean concentration (ng/ml or pg/ml) ± SD. * vs. saline-treated CRND8.

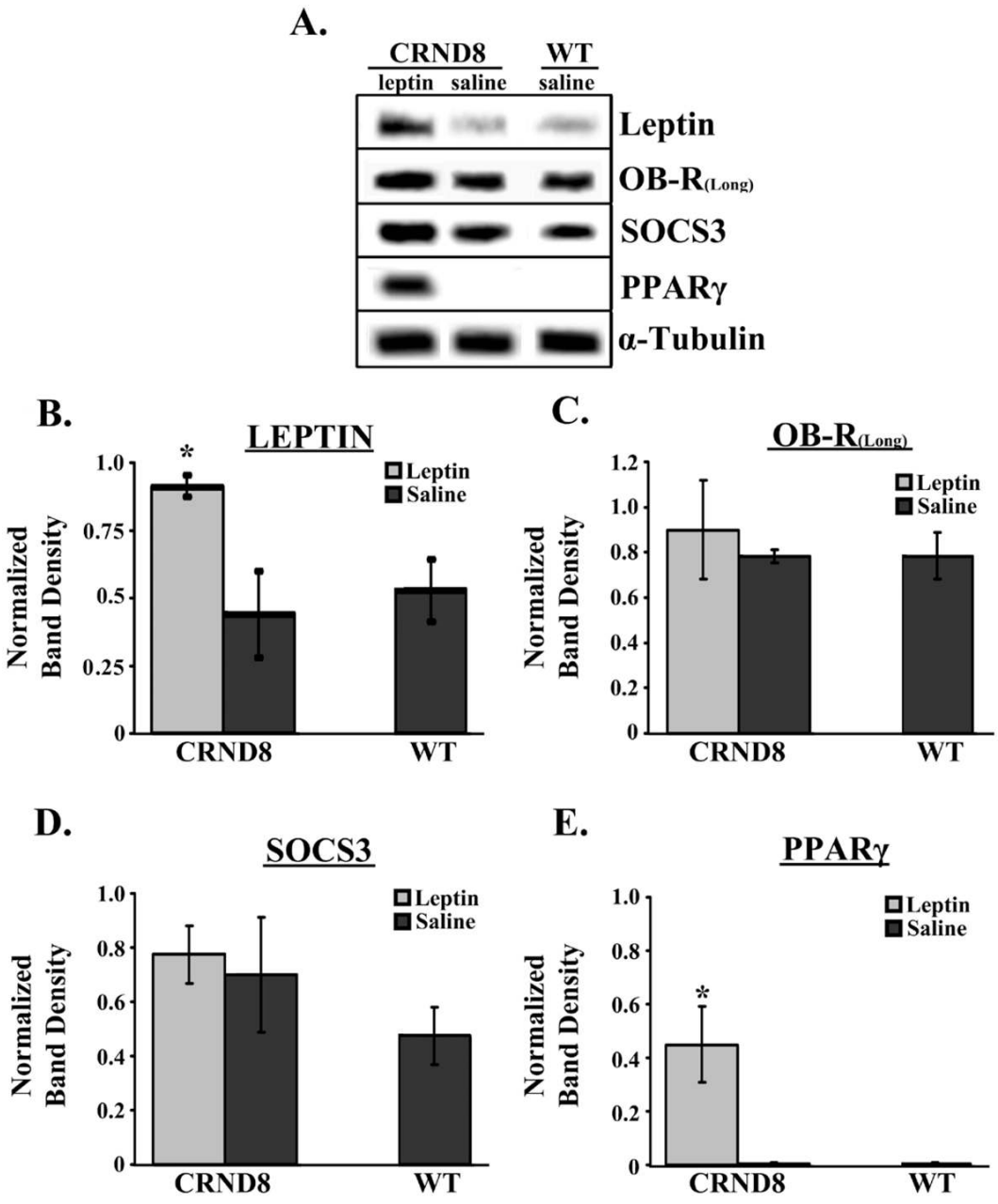

Figure 2.

Expression of leptin, leptin receptor (OB-R), and downstream signaling targets in CRND8 and wt mouse brains. (**A)** Brains from leptin- or saline-treated CRND8 or wt mice were harvested, and expression of (**B**) leptin, (**C**) leptin receptor, and (**D**, **E**) downstream signaling targets (SOCS3, PPARγ) were determined by immunoblot. Membranes were stripped and reprobed with α-tubulin antibody for normalization. Representative blots are shown, n=3. Normalized bands were analyzed by densitometry and are presented as the mean density ± SD. * vs. like saline-treated

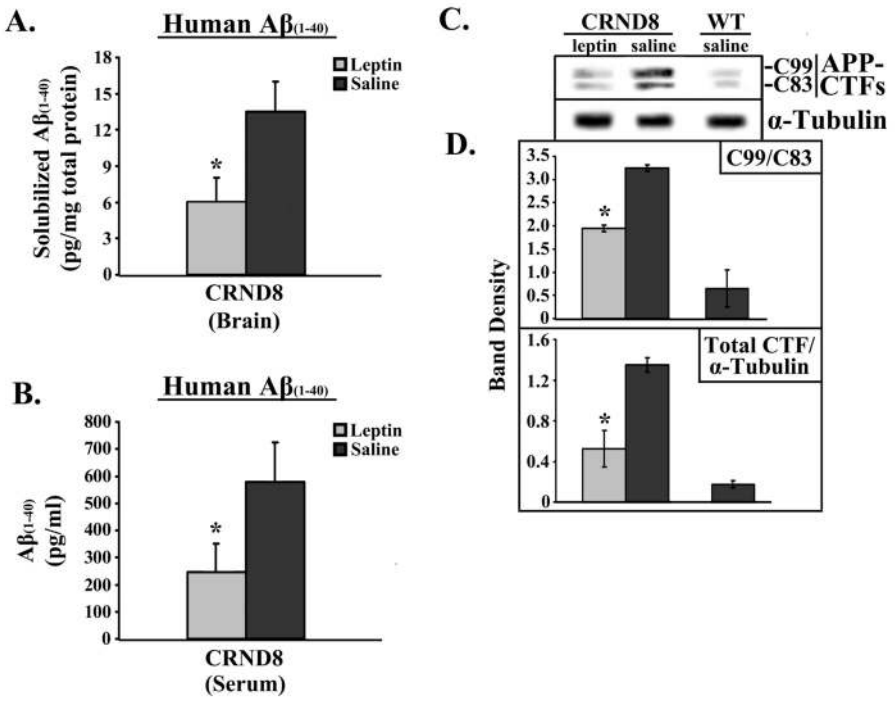

Figure 3.

Levels of solubulized $A\beta_{1\text{-}40}$ and AβPP C-terminal fragments (CTFs) in CRND8 and wt mice. (**A**) Levels of detergent-soluble $A\beta_{1\text{-}40}$ present in the brains or (**B**) serum of leptin- or saline-treated CRND8 or wt mice were determined by ELISA. Levels of detergent-soluble brain $A\beta_{1\text{-}40}$ were normalized to the total amount of soluble brain protein. Results are presented as the mean concentration (brain – pg/mg total protein, n=5; serum – pg/ml, n=10) ± SD. (**C**) Brains from leptin- or saline-treated CRND8 or wt mice were harvested, and levels of AβPP CTFs (C99, C83) were determined by immunoblot. Membranes were stripped and re-probed with α-tubulin antibody for normalization. Representative blots are shown, n=3. (**D**) Normalized bands (ratios of C99/C83 and total CTFs/α-tubulin) were analyzed by densitometry and are presented as the mean density ± SD. * vs. saline-treated TgCRND8

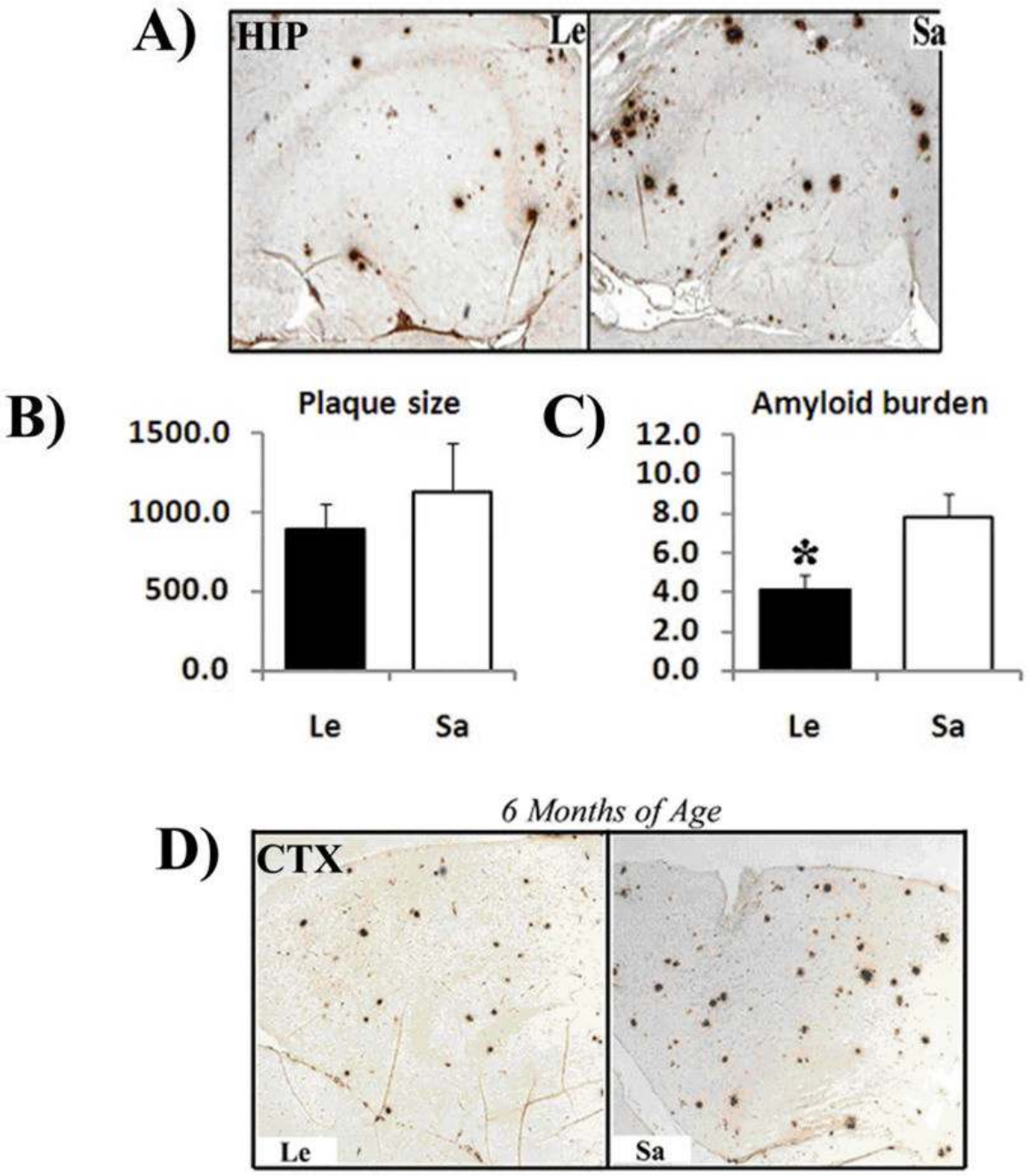

Figure 4.

Amyloid plaque deposition in leptin-treated TgCRND8 mice. (**A**) Brain slices were stained with the 4G8 antibody in the hippocampal region; Le - transgenic animals treated with leptin; Sa - transgenic animals treated with saline. (**B**) Bars represent average size of plaque (μm^2) ± S.E.M. or (**C**) % area stained in the region ± S.E.M.; n=8–9 per bar. (**D**) Cortical region stained with 4G8 antibody.

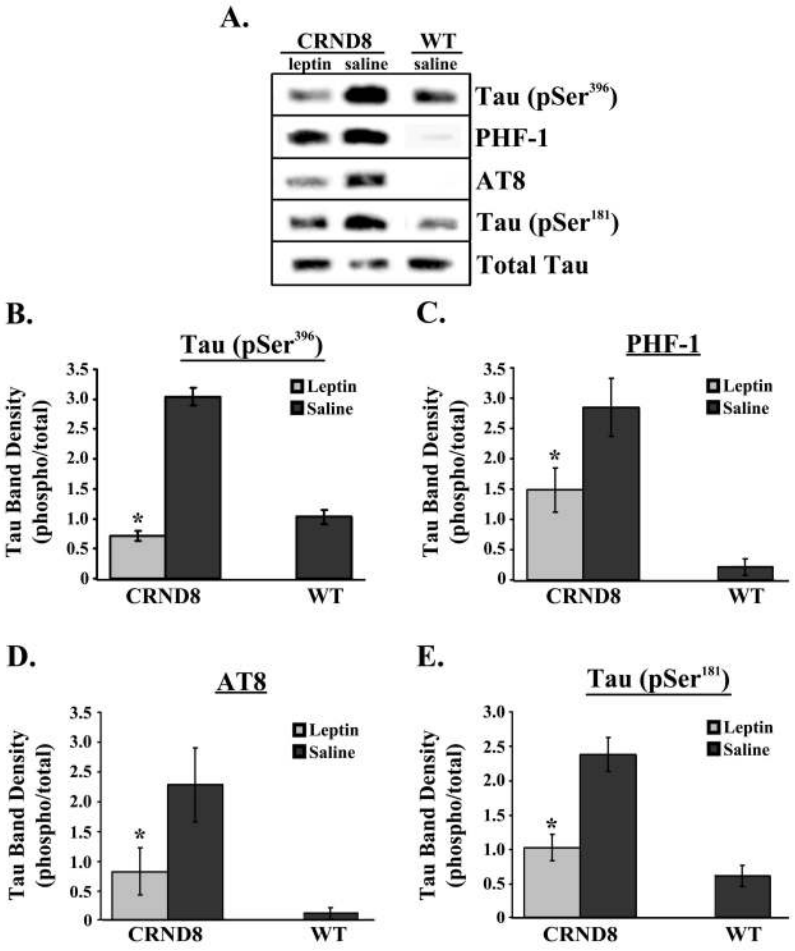

Figure 5.

AD-related tau phosphorylation in TgCRND8 and wt mouse brain. (**A**) Brains from leptin- or saline-treated CRND8 or wt mice were harvested, and tau phosphorylation at (**B**) Ser^{396}, (**C**) PHF-1 ($Ser^{396/404}$), (**D**) AT8 ($Ser^{202/204}$), and (**E**) Ser^{181} were determined by immunoblot. Membranes were stripped and re-probed with total tau antibody for normalization. Representative blots are shown, n=3. Normalized bands were analyzed by densitometry and are presented as the mean density ± SD. * vs. saline-treated CRND8

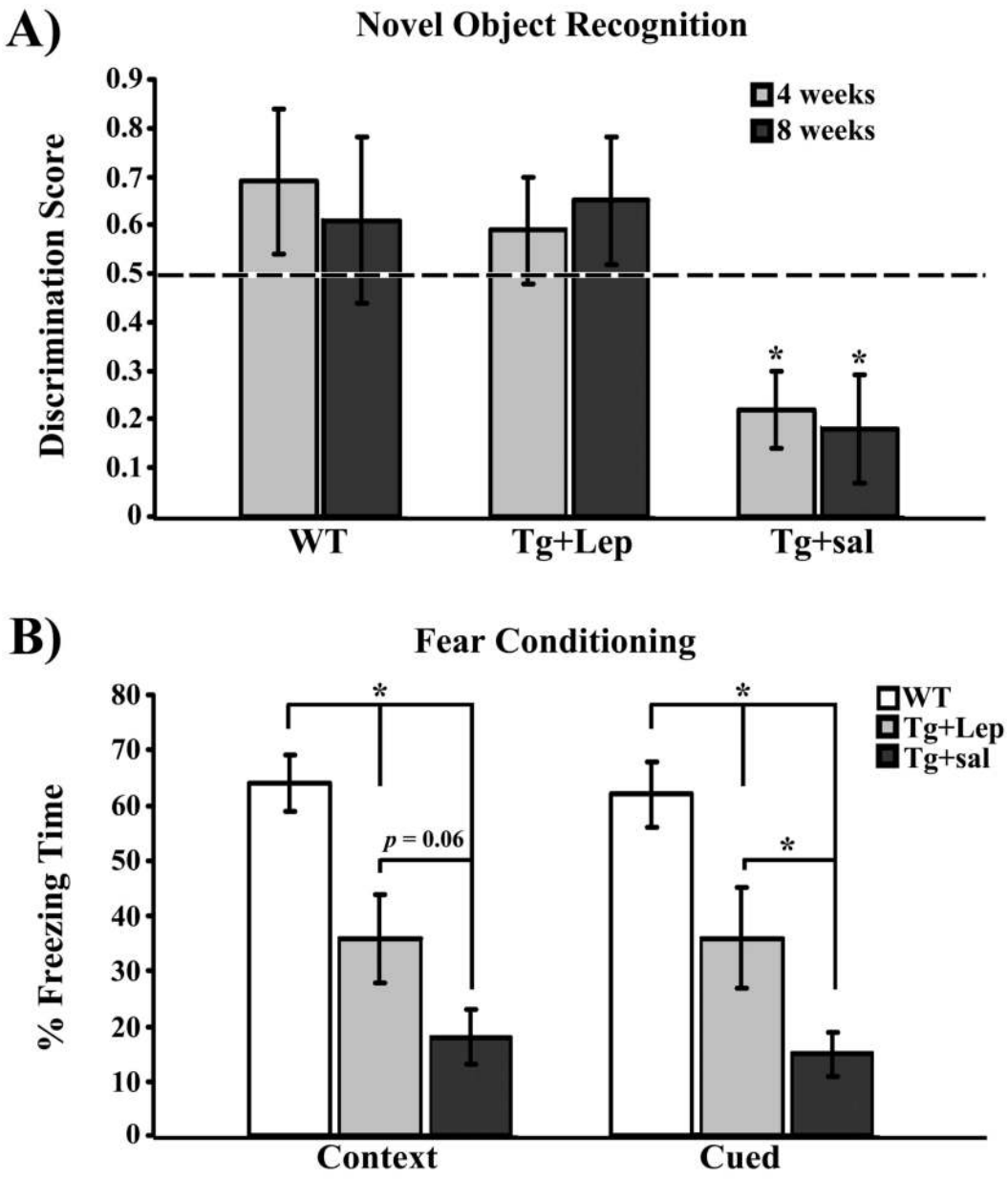

Figure 6.

Cognitive assessment of CRND8 and wt mice. (**A**) In the novel object recognition test, working memory was scored in wt and 4- or 8-week, leptin- or saline-treated transgenic mice as time spent exploring familiar versus novel objects. The dotted line indicates animals remembering (more than ½ of the total time spent between the two objects is with the new object); anything below the line shows memory impairment. $^{*}p$=0.01 for Tg-Lep vs Tg-saline;$^{*}p$=0.003 for Tg-sal vs WT. (**B**) In the fear conditioning test, aversive learning tasks were used to measure fear response to an unpleasant stimulus in wt and leptin- or saline-treated transgenic mice. Freezing was quantified in the original chamber before and after several training sessions (mild footshocks) for contextual fear conditioning or in a novel environment containing an auditory cue that was previously paired with a footshock. **Context**: $^{*}p$=0.009 for WT vs Tg +Lep;$^{*}p$=0.0001 for WT vs Tg+Sal; **Cued**:$^{*}p$=0.012 for WT vs Tg+Lep;$^{*}p$=0.0001 for WT vs Tg+Sal;$^{*}p$=0.04 for Tg+Lep vs Tg+sal.