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PREPRO-THYROTROPIN RELEASING HORMONE EXPRESSING NEURONS IN THE JUXTAPARAVENTRICULAR REGION OF THE LATERAL HYPOTHALAMUS ARE ACTIVATED BY LEPTIN AND ALTERED BY PRENATAL GLUCOCORTICOID EXPOSURE

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Abstract

The neuropeptide thyrotropin-releasing hormone (TRH) is recognized to play an important role in controlling energy balance through direct effects on the CNS, although mechanisms explaining the phenomenon are poorly understood. To begin to understand the effects of TRH on CNS control of energy balance, we first mapped neurons expressing the TRH precursor peptide, prepro-TRH (ppTRH) in the paraventricular nucleus of the rat hypothalamus and the surrounding regions. We identified a population of ppTRH-expressing neurons in the juxtaparaventricular region of the lateral hypothalamus (LHAjp) which were stimulated by the satiety signal leptin (2.5 μg/kg, IP). Using a model of fetal glucocorticoid (GC) exposure in which pregnant rats were treated with the synthetic GC dexamethasone (DEX) during gestational days 18–21, it was observed that such exposure resulted in reduced numbers of ppTRH-ir neurons in the LHAjp in adult male and female rats, and was accompanied by increased food intake. Our data provide further insight into the biological role of the LHAjp, as well as the potential involvement of TRH neurons within this region in metabolic disease associated with fetal glucocorticoid exposure.

Keywords

Dexamethasone; Hyperphagia; Thyrotropin Releasing Hormone; Juxtaparaventricular Region of the Lateral Hypothalamus; Energy Balance

1. Introduction

The neuropeptide thyrotropin-releasing hormone (TRH) plays a key role in the regulation of energy balance (Lechan and Fekete, 2006). In rats, TRH is synthesized as a 255 aa precursor peptide, prepro-TRH (ppTRH), from which 5 copies of TRH are spliced. The major population of neurons that express ppTRH are found in the paraventricular nucleus of the hypothalamus (PVN) (Segerson et al., 1987). However, additional populations of ppTRH-expressing neurons have also been identified in other regions of the brain, including the

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perifornical (Pf) region of the lateral hypothalamus (Merchenthaler, 1991; Wittmann et al., 2009a; Wittmann et al., 2009b). TRH plays a regulatory role in maintaining energy homeostasis by exerting a neuroendocrine influence on the synthesis and release of thyroid stimulating hormone by the pituitary gland. In addition to providing neuroendocrine control of the pituitary gland, non-hypophysiotropic populations of ppTRH neurons act within the brain to regulate functions such as locomotor activity, thermogenesis, and food intake (Choi et al., 2002; Schuhler et al., 2007; Sharp et al., 1984), but the precise loci for these effects remain poorly understood.

Consistent with a role in modulating feeding frequency, TRH-expressing neurons in the PVN have been shown to respond to the satiety signal leptin (Lechan and Fekete, 2006). Such observations raise the possibility that leptin may also influence populations of neurons outside the PVN. In the present study, ppTRH-expressing neurons in the PVN and surrounding areas were mapped by immunohistochemistry, revealing a population of such cells in the juxtaparaventricular region of the lateral hypothalamus (LHAjp). Although the existence of this ppTRH-ir neuronal population has been previously described, the biological roles played by these neurons are not understood (Merchenthaler, 1991; Wittmann et al., 2009a; Wittmann et al., 2009b). Given the role for TRH in regulating feeding, we administered the satiety signal leptin to adult male rats which resulted in activation of ppTRH-ir neurons in the LHAjp, suggesting a possible role for these neurons in feeding activity. This possibility is supported by retrograde tracing experiments in which projections to hypothalamic satiety centers including the arcuate nucleus of the hypothalamus (ArcN), and the ventromedial (VMH) and dorsomedial (DMH) hypothalamic regions, were found between the anterior PVN and the LHAjp, while no such projections were observed to arise from the remaining regions of the perifornical TRH neurons (Wittmann et al., 2009b).

Adverse fetal environments, such as exposure to synthetic glucocorticoids (GC), are recognized to result in metabolic abnormalities in the offspring (Cleasby et al., 2003; Drake et al., 2010). These findings have resulted in concern over the clinical practice of using the synthetic GC dexamethasone (DEX) to promote lung development in preterm infants (Watterberg, 2010). Because neurons within the LHAjp may be involved in energy balance, the possibility exists that abnormal development of this region plays a role in the permanent metabolic derangements arising from fetal DEX exposure. In adult rats which were exposed during gestational days (GD) 18–21 to DEX, we show that increased food consumption was accompanied by decreased numbers of ppTRH-immunoreactive (ir) neurons and fibers in the LHAjp. Given the projections extending from the TRH neurons in the LHAjp to satiety centers such as the ArcN, VMH, and DMH, the possibility exits that abnormal development of the TRH neurons in the LHAjp may be one mechanism explaining the pathology which arises in adulthood following fetal GC exposure.

2. Results

2.1. ppTRH-ir Neurons are found in the LHAjp

Populations of ppTRH-ir neurons in the PVN and surrounding area were mapped using IHC. Distinct populations of ppTRH neurons were found in the PVN, the perifornical (Pf) region, located immediately surrounding the fornix (f) (Figure 1A), including the LHAjp (Figure 1B), which extends laterally from the PVN to the fornix, as described previously (Hahn and Swanson, 2010).

2.2. ppTRH-ir neurons in the LHAjp are Leptin Responsive

Two hours following the administration of leptin (Figure 2A–B), or saline (Figure 2C–D) to male rats, co-localization of ppTRH and cFos within the LHAjp was measured by dual IHC

(white arrowheads). Estimates of co-labeled neurons indicate a significant ($p < 0.05$) increase in the percentage of cFos labeling in ppTRH-ir neurons (Figure 2E). Estimates of total ppTRH-ir neurons within the LHAjp showed that there was no change in the number of ppTRH-expressing neurons after leptin administration (Figure 2F).

2.3. Fetal DEX Exposure Reduces the Number of ppTRH-ir Neurons in the LHAjp

ppTRH-ir neurons were measured bilaterally through the LHAjp in brains harvested from adult rats which had been exposed during fetal development to either DEX or vehicle. Total numbers of immunoreactive neurons were compared between male vehicle-exposed (Figure 3A), male DEX-exposed (Figure 3B), female vehicle-exposed (Figure 3C) and female DEX-exposed (Figure 3D) offspring. Two-way ANOVA revealed a main effect of fetal DEX exposure ($F(1, 16)=17.97$; $p=0.0002$) on ppTRH-expressing neurons within the LHAjp, however, no sex effect was observed, nor was a significant interaction between fetal DEX exposure and sex observed (Figure 3E). *Post hoc* comparison of total neuron counts revealed significantly fewer ppTRH-positive neurons in the LHAjp in both adult male and female offspring following gestational exposure to DEX when compared with their vehicle-exposed counterparts (Figure 3E).

PreproTRH fiber density was estimated within the LHAjp in male and female offspring (Figure 3A–D), and main effects of fetal DEX exposure ($F(1, 18)=21.58$; $p=0.0002$) and sex ($F(1, 18)=5.412$; $p=0.0319$), on ppTRH fiber counts were observed (Figure 3F). No significant interaction between these variables was present, however, *post hoc* comparison of ppTRH-expressing fibers in the LHAjp revealed significantly fewer immunopositive fibers in both male and female offspring which had been exposed to DEX during development (Figure 3F).

2.4. Fetal DEX Exposure Increases Food Consumption

The fetal DEX exposure paradigm employed in this study resulted in decreased birth weight in both male and female offspring (Figure 4A). Birth weights were compared using two-way ANOVA, which revealed a main effect of fetal DEX exposure ($F(1, 90)=113.1$; $p<0.0001$) and sex ($F(1,90)=29.36$; $p<0.0001$), with no interaction effect. *Post hoc* comparison of birth weights revealed that fetal DEX exposure resulted in reduced birth weight in both male and female offspring when compared to the vehicle-exposed group (Figure 4A).

Body weight was monitored throughout the study, and comparison of weight at PD60 (Figure 4B) by two-way ANOVA revealed main effects of both fetal DEX exposure ($F(1, 20)=16.43$; $p=0.0006$) and sex ($F(1, 20)=454.4$; $p<0.0001$), with no significant interaction effect. *Post hoc* comparison of PD60 body weight revealed a significant decrease in body weight in DEX-exposed male, but not female adult offspring. Weight gain during the final week of the study is reported as Final Growth Velocity (Figure 4C), and was compared by two-way ANOVA which revealed a main effect of sex ($F(1, 20)=18.00$; $p=0.0004$), but not fetal DEX exposure.

Following weaning of the offspring at PD21, food intake was monitored daily for the duration of the study (Figure 4D), and was normalized to body weight and compared by two-way ANOVA. This analysis revealed a main effect of fetal DEX on food consumption ($F(1, 108)=13.43$; $p=0.0004$). However, *post hoc* analysis showed that this effect reached significance only in the male offspring.

3. Discussion

Hypophysiotropic populations of ppTRH-ir neurons in the PVN are recognized to influence energy balance through modulation of the HPT axis. However, non-hypophysiotropic

populations of such neurons are also involved in regulating behavior, including food consumption, through effects within the brain (Choi et al., 2002; Suzuki et al., 1982). To provide a further understanding of how TRH acts locally within the brain, we mapped ppTRH-expressing neurons in the PVN and surrounding regions of the hypothalamus, which revealed a population of such neurons in the LHAjp which were activated by the satiety signal leptin.

The LHAjp lies lateral to the PVN, and is immediately adjacent to the fornix. Although the biological role for this region of the hypothalamus remains unclear, existing studies have reported connections between this population of neurons and the ArcN, VMH, and DMH (Wittmann et al., 2009b). Given the established role for the these nuclei in regulating food intake, projections to these regions from the TRH-ir neurons in the LHAjp suggest that this popoulation of neurons may be involved in feeding behavior. This hypothesis is supported by the observation that ppTRH-ir neurons in the LHAjp are activated in response to the satiety signal leptin. These data suggest two possibilities: that the ppTRH neurons of the LHAjp are responsive to leptin via transynaptic regulation from the ArcN, or that these neurons are directly stimulated by leptin. Recent studies have suggested that leptin receptor is found in the LHAjp, although the current lack of antibodies with sufficient selectivity for leptin receptor makes it difficult to determine if leptin receptors are expressed by ppTRH neurons themselves (Scott et al., 2009). The studies presented here regarding ppTRH/cFos coexpression also indicate that cFos was not induced in all ppTRH-ir neurons. Possible explanations for this observation are that cFos induction was below our detection limits, or alternatively, that multiple populations of ppTRH-ir neurons exist in the LHAjp, and not all of these are responsive to stimulation by leptin. This latter possibility is intriguing given that among the TRH-expressing neurons within the Pf neuronal population which were mapped using the retrograde tracer, only those within the LHAjp were observed to project to the ArcN, VMh, and DMH (Wittmann et al., 2009b). Taken together, the report by Wittmann et al., (2009) indicates projections from TRH neurons within the LHAjp to hypothalamic regions which are heavily involved in food intake (Wittmann et al., 2009b).

In addition to identifying a population of leptin sensitive neurons in the LHAjp, we also conducted studies in adult rats that were exposed during late gestation to DEX or a safflower oil vehicle. This paradigm has been used to model elevated fetal GC exposure following extreme maternal stress or clinical administering synthetic GC (Watterberg, 2010). Recent studies have suggested that such a treatment can result in offspring with increased risk for metabolic abnormalities (Carbone et al., 2012a; Cleasby et al., 2003; Drake et al., 2010). Specific abnormalities relevant to the observations presented here include reports that similar DEX-exposure paradigms result in a leaner body composition (Cleasby et al., 2003), suggesting increased use of energy stores. The possibility that fetal DEX exposure results in greater energy expenditure is also supported by observations from our laboratory indicating that such exposure results in decreased core body temperature in adult female rats, which suggests less efficient thermogenesis in these animals (Carbone et al., 2012b). In our studies, exposure to DEX over the last 4 days of gestation resulted in reduced birth weight of the offspring. However, by early adulthood (PD60) the weight difference between DEX-exposed and control female offspring was no longer apparent, although reduced body weight in the male offspring following DEX exposure was still present. Since the rate of weight gain in all female, but not male, Sprague Dawley rats slowed considerably by PD60, this may explain the complete catch-up growth observed in the DEX exposed female offspring. In contrast to body weight, 2-way ANOVA of food consumption following weaning of the offspring indicated that fetal DEX exposure increased food intake, although post hoc analysis showed that this effect only reached significance in the males. These data are consistent with reports in both rat and mouse models which demonstrate that prenatal stress results in elevated food consumption in the offspring (Luque and Kineman, 2007; Pankevich

et al., 2009); however, this is the first report to our knowledge implicating fetal exposure to a synthetic GC in alterations in this behavior.

Besides a reduction in ppTRH-ir neurons, our data also indicate that the number of ppTRH-ir nerve fibers are also reduced in the LHAjp in adult (PD60) rats which were exposed to DEX during GD18-21, although a sex difference was also reported. This observation likely reflects a less profound effect of fetal DEX exposure on the female ppTRH-ir fiber density. These data suggest the possibility that efferent and/or afferent projections between the LHAjp and other populations of ppTRH-ir neuronal populations are influenced by fetal DEX exposure. Based on the findings reported by Hanh and Swanson (2010), the efferent projections from the ppTRH-ir neurons in the LHAjp could potentially impact a variety of different regions, given the number of scattered connections revealed by the described anterograde tracing experiments (Hahn and Swanson, 2010). Regarding a possible influence of the LHAjp on appetite regulation, efferent projections from the LHAjp to the ArcN, VMH, and DMH and PVN were reported, further suggesting that the LHAjp may influence the function of these nuclei (Wittmann et al., 2009b).

The central administration of TRH reduces food intake (Choi et al., 2002; Suzuki et al., 1982). Although the mechanisms explaining this observation remain unclear, the presence of synaptic connections between the ArcN and the TRH-ir neurons in the LHAjp suggests that neurons within the LHAjp may influence appetite through this circuitry (Wittmann et al., 2009b). A potential mechanism behind CNS-mediated effects of TRH may therefore include activity at the ArcN, based on observations reported by Heuer et al. (2000) which documented the presence of *Trhr2* message in the ArcN using in situ hybridization (Heuer et al., 2000). The report by Heuer et al. (2000) thus suggests the possibility that TRH influences feeding behavior by modulating ArcN neuronal populations involved in appetite and satiety; however, further neuronal tract tracing and IHC studies are needed to confirm this.

In summary, the results presented here describe the existence of a population of ppTRH-expressing neurons in the LHAjp which respond to stimulation by the satiety signal leptin, indicating that the LHAjp may play a role in feeding behavior. This possibility is supported by our observations that the number of ppTRH-expressing neurons in the LHAjp is decreased in rats which were exposed during fetal development to DEX, and is accompanied by elevated food consumption. The data presented here thus allow further insight into the biological role of the LHAjp, including a potential role in the development of metabolic disease following fetal exposure to synthetic GC, as well as a potential mechanism by which TRH regulates energy balance through direct effects on the CNS.

4. Experimental Procedures

4.1. Animals

Timed pregnant rats were purchased from Charles River Laboratories (Wilmington, MA) to arrive at the Arizona State University Department of Animal Care and Technology (Tempe, AZ) at gestational day (GD) 7. Animals were acclimated to handling beginning on GD14, and received either 0.4 mg/kg DEX (Sigma-Aldrich Chemical Company, St. Louis, MO) or vehicle (2% ethanol in safflower oil) via subcutaneous injection on GD18-21. This dosing regimen was designed to fall within the range of clinical human exposure (0.1–0.5 mg/kg), but also remain consistent with existing reports using Sprague-Dawley rats in which dosing between 0.2 and 0.8 mg/kg resulted in abnormal neurodevelopment (Duksal et al., 2009; Kreider et al., 2006). Parturition occurred on GD 23, which was designated postnatal day (PD) 0. Litters were culled to 10 pups at birth (5 males, 5 females), and offspring were weighed weekly until the termination of the study. Pups were weaned at PD21, and food

consumption was monitored daily after weaning. Rats were allowed to mature until PD60 at which point all male offspring were killed. Two weeks prior to the termination of the study, female estrous cycle was recorded daily using vaginal swabs, confirming a 4 day cycle in all females, and female rats were killed only when in diestrus (PD 60–63) to limit any confounding effect of estrogen on ppTRH expression. Rats used for IHC experiments were anesthetized using isoflurane, and were intracardially perfused with heparin-supplemented phosphate-buffered saline (PBS), followed by 4% neutral-buffered paraformaldehyde. Brains were harvested and post-fixed overnight in 4% neutral-buffered paraformaldehyde, followed by infiltration with 30% sucrose and storage at 4°C until cryosectioning for immunohistochemistry. Prior to ppTRH and cFos co-localization experiments, adult male rats were administered 2.5 μg/kg recombinant rat leptin (Sigma-Aldrich Chemical Company, St. Louis, MO) or sterile saline via IP injection. Two hours post-injection these rats were perfused with 4% neutral-buffered paraformaldehyde, and brains were postfixed and stored in cryoprotectant until processing for IHC. For reverse transcriptase PCR experiments (RT-PCR), adult male rats were anesthetized with isoflurane and rapidly decapitated. Brains were quickly removed from the skull and snap frozen by immersion in methylbutane (−20°C), and were then stored at −80°C until processing for RNA. All procedures were approved by the Arizona State University Institutional Animal Care and Use Committee, under subcontract from the University of Arizona College of Medicine-Phoenix and were in keeping with National Institutes of Health Guidelines.

4.2. Immunohistochemistry (IHC)

Paraformaldehyde-perfused brains were sectioned at 35 μm using a Leica CM3050S Cryostat (Buffalo Grove, IL). Dual labeling experiments entailed cFos immunodetection using a rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and were visualized using the ABC method with 3-3 diaminobenzidine (DAB) in the presence of nickel ammonium sulfate (Ni-DAB) as the chromagen. Subsequently, following a series of six washes in PBS over the course of 48 hours, sections were blocked with PBS which contained 5% normal goat serum and 1% triton X-100, and a second round of IHC was used to detect ppTRH. For all experiments in which IHC was used to visualize ppTRH-ir neurons or fibers, a rabbit polyclonal antibody was used which is directed to preproTRH178-199 and has been previously validated for IHC and demonstrated to specifically detect ppTRH (Suzuki et al., 2001). Neurons and fibers which express ppTRH were visualized by the ABC method using DAB without the addition of nickel ammonium sulafate, resulting in a lighter brown reaction product. Figures indicating distinct ppTRH-ir neuronal populations were created using a montage of multiple images.

The use of Ni-DAB and DAB resulted in black nuclear staining (cFos) and brown cytosoplasmic staining (ppTRH), which allowed for estimates of dual labeling. Neuron and fiber imaging was performed using a Zeiss Axioskop light microscope equipped with Neurolucida v.7 Software (MicroBrightField, Williston, VT) and soma were counted bilaterally throughout the LHAjp in every fourth section, resulting in neuron counts through four sections per brain. Data are reported as an estimate of cFos and/or ppTRH neurons per LHAjp, however, these data were not corrected for double-counting errors as described by Abercrombie (1946) (Abercrombie, 1946), nor were they generated using a stereological technique, such as that described by Coggeshal and Lekan (1996) (Coggeshall and Lekan, 1996). Because the labeled neurons we were counting did not appear to change in size, and section thickness did not vary between groups, systematic error should be identical for all groups. Our results are thus meant to provide relative data on the expression of cFos and ppTRH within the LHAjp, and not provide an estimate of absolute numbers of ppTRH neurons.

PreproTRH-ir fiber density was estimated as previously described by Suzuki et al., (2001) by drawing a straight line from medial to lateral near the middle the PVN, and counting the immunoreactive fibers which intersected this line over an 250 μm segment (Suzuki et al., 2001). Fibers were estimated through the entire PVN using every fourth section, and are meant to provide a relative estimate of ppTRH-ir fiber density within the PVN.

4.4. Statistical Analysis

Statistical analyses were performed using GraphPad Prism v5.0b (GraphPad Software, Inc., La Jolla, CA). Two-way ANOVA was used to test for main effects and interactions between sex and fetal DEX exposure on food consumption ppTRH immunoreactivity in the LHAjp. For all experiments, *post-hoc* comparison of means was performed using the method of Bonferroni. Results of the two-way ANOVA are presented in respective results sections. Differences in growth velocity or cFos expression were compared by t-test. Differences were deemed significant if $p<0.05$.

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Abreviations

ArcN	arcuate nucleus
DEX	dexamethasone
LHAjp	juxtaparaventricular region of the lateral hypothalamus
PVN	paraventricular nucleus
ppTRH	prepro thyrotropin releasing hormone

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Highlights

- ppTRH-expressing neurons are present in the LHAjp.
- The ppTRH-ir neurons in the LHAjp are stimulated by leptin.
- ppTRH-ir nerves and fibers in the LHAjp are reduced by fetal DEX exposure.
- Fetal DEX exposure results in increased food consumption.

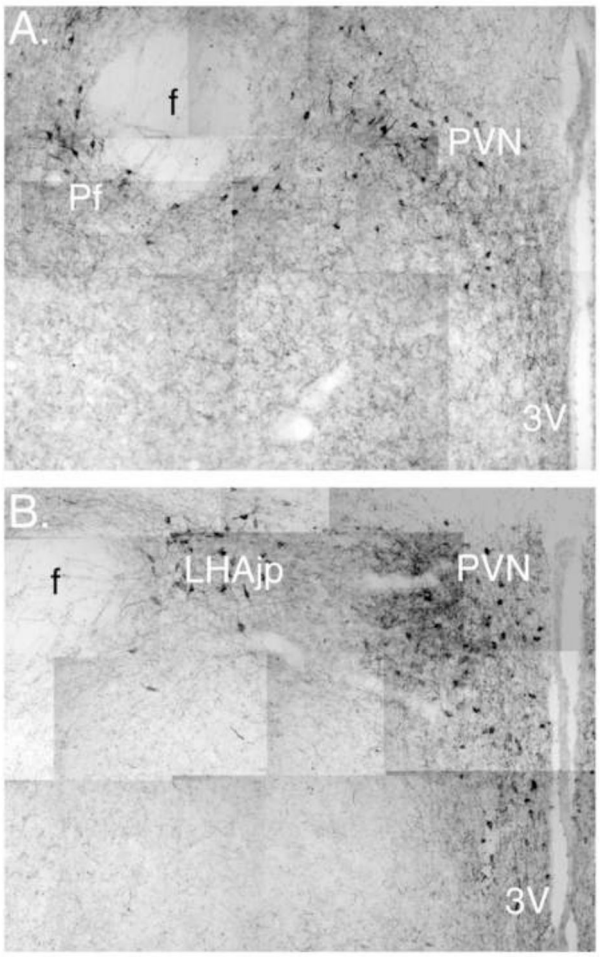

Fig. 1.

Photographic montage showing distribution of ppTRH-ir neurons in the PVN, perifornical area (A; Pf), and LHAjp (B). Panel A is reconstructed from photos taken at bregma −1.30 mm and photos in Panel B were taken at bregma −1.80 mm. Each photomicrograph was taken using a 20x objective and the region was reconstructed using Photoshop software. f = fornix, 3v = 3rd ventricle. Scale bar = 100 uM.

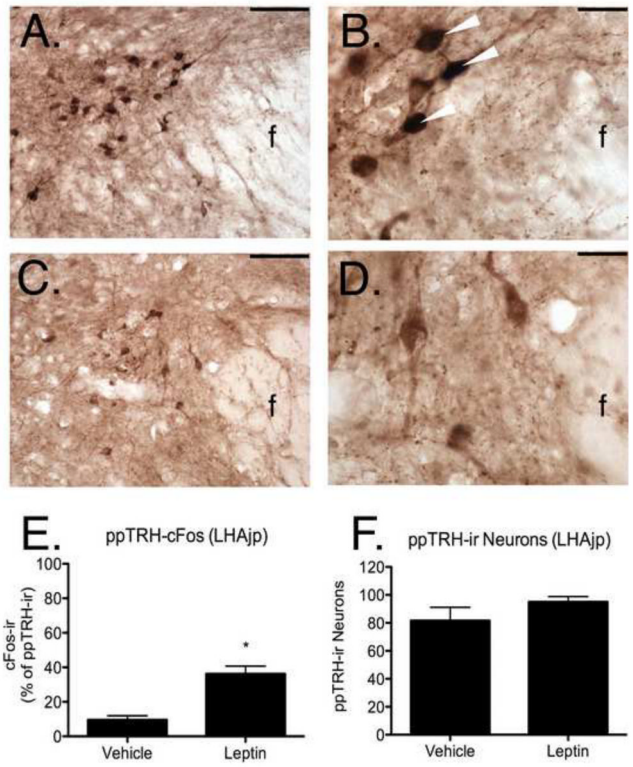

Fig. 2.

Coexpression of cFos and ppTRH (white arrowheads) was measured in the LHAjp following the administration of 2.5 ug/kg leptin (A–B) or sterile saline (C–D). Neuronal counts revealed an increase in the percentage of cFos/ppTRH colocalization following leptin administration (E). Graph showing the number of ppTRH-ir neurons in the LHAjp after leptin or vehicle administration. Panels A and C were taken using a 20× objective (100 μm scale bar), while panels D and E were taken using a 63× objective (25 μm scale bar). Data are presented as a mean ± SEM of 3 individuals (*=$p<0.05$; f = fornix).

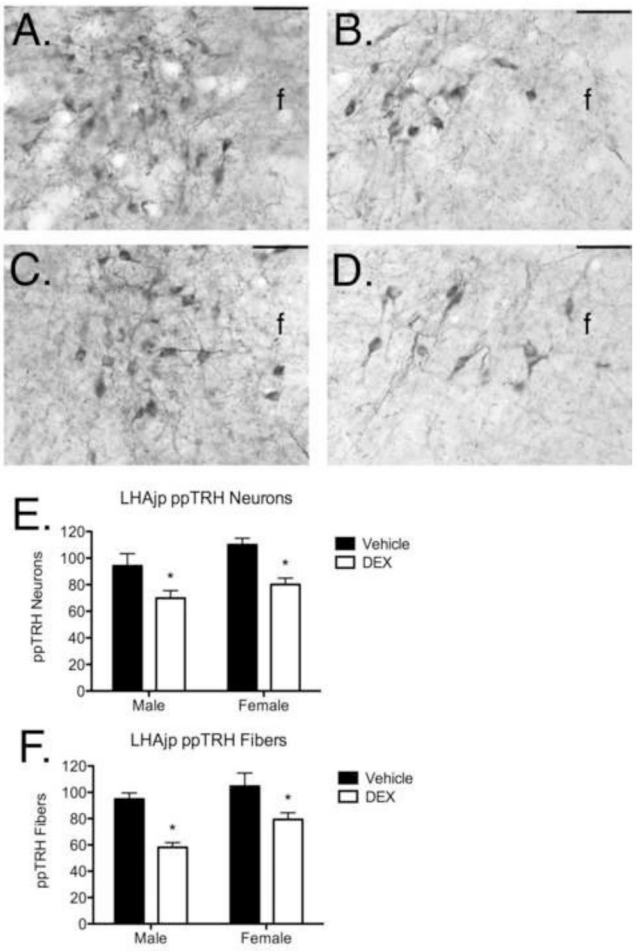

Fig. 3.

ppTRH-ir neurons and fibers within the LHAjp in adult male and female offspring which were exposed during gestation to DEX (male, A–B; female, C–D; 40× objective, 50 μm scale bar; f = fornix). Bilateral neuron counts were taken through the LHAjp in offspring which were exposed during late gestation to DEX (E), and ppTRH-ir fibers were counted through the LHAjp (F). Neuron and fiber counts are reported as a mean ± SEM of 6 brains (*$p<0.05$).

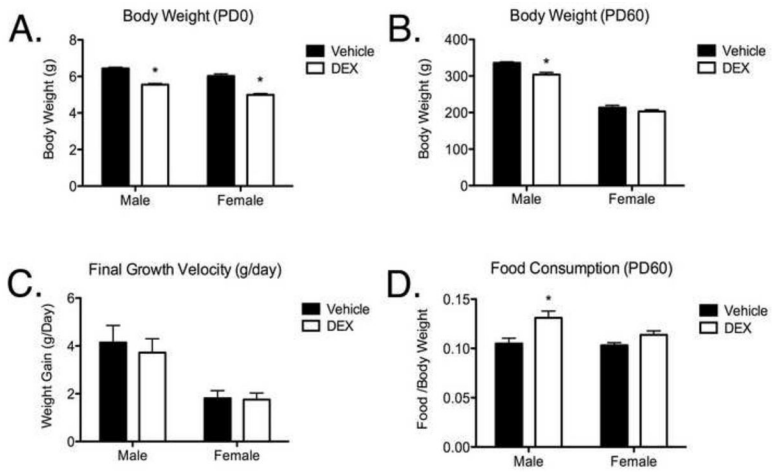

Fig. 4.

Birth weight in male and female offspring following exposure to DEX during GD18-21 (panel A). Body weight in male and female offspring at postnatal day 60 (B). Weight gain (g/day) was measured during PD 50–60, to estimate final growth velocities of male and female offspring (C). Effects of fetal DEX exposure on food consumption in both male and female adults (D). Food consumption is reported as the weight (g) food consumed per body weight (g) ± SEM of 6 animals. Weights are presented as mean (g) ± SEM of no fewer than six animals. Growth rate is reported as the mean weight gain (g/day) ± SEM of 6 animals. In all panels, significance between vehicle and DEX-exposed offspring is indicated by asterisk ($p < 0.05$).