Role of leptin in modulation of corticotropin-releasing factor neurons in the brain of *Xenopus laevis*

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Acknowledgments

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Abstract

Leptin is a hormone produced by adipocytes that signals to the brain energy stores and acts on hypothalamic feeding control centers to suppress appetite. An important anorexigenic pathway that may mediate leptin actions on feeding involves the stress neuropeptide corticotropin-releasing factor (CRF). CRF is the central mediator of stress responses, controlling the hypothalamic-pituitary-adrenal (HPA) axis. CRF also functions as a neurotransmitter/neuromodulator, influencing stress-related behaviors and feeding. Previous studies in mammals have suggested that leptin's actions on feeding may be mediated by CRF neurons. However, how leptin influences secretion and whether that influence is indirect or direct is unknown. We hypothesize that leptin directly acts on CRF neurons in the anterior preoptic area (POA), a brain region in the frog homologous to the mammalian paraventricular nucleus (PVN). To investigate this hypothesis, leptin's influences on CRF expression were tested and immunohistochemistry was performed to localize leptin receptor and CRF neurons using changes in pSTAT3 immunoreactivity as a proxy for leptin receptor in juvenile *Xenopus laevis*. Colocalization data obtained using fluorescent and confocal microscopy demonstrated that leptin receptor is expressed on CRF neurons in the POA. The effects of acute leptin injection were examined by injecting frogs with either leptin or saline and performing immunohistochemical analyses for changes in CRF-ir in the POA. A decrease in CRF-ir in parvocellular CRF neurons was observed. We hypothesize that this is due to a leptin-induced secretion of CRF to further modulate feeding.

Introduction

Leptin

Leptin is an important hormone for long-term communication of energy status as well as short-term reduction of food intake. It was first discovered in mutant mice that were found to feed excessively and be extremely obese. These mice were deficient in proper leptin signaling caused by mutations in either the hormone itself *(ob/ob)* or its corresponding receptor *(db/db)* (Zhang et al., 1994, Tartaglia et al., 1995). Leptin is secreted by adipocytes and travels to its primary site of action, the hypothalamus, where it targets orexigenic and anorexigenic neurons that produce neuropeptide Y and alpha-melanocyte-stimulating hormone, respectively (Harris 2000). The downstream food intake and metabolic effects of leptin are mediated by leptin binding to the long form of the leptin receptor and activating the Janus kinase 2 (JAK)/signal trasnducer and activator of transcription 3 (STAT3) pathway (Baumann et al., 1996).

In *Xenopus laevis*, leptin is widely distributed throughout tissues including fat, the brain, and the pituitary gland (Crespi and Denver, 2006). Although frog leptin is only 35% similar to human leptin, the tertiary structure is conserved, and the frog hormone plays a similar appetite-suppressing role and may function as a circulating indicator of energy stores like in mammals. Our lab found that intracerebroventricular (i.c.v.) injections of leptin into juvenile frogs were potently anorexigenic and consequently decreased food intake (Crespi and Denver, 2006).

Corticotropin-releasing factor

Corticotropin-releasing factor (CRF) is the primary neuroregulator of the vertebrate stress response, controlling the activity of the hypothalamic-pituitary-adrenal (HPA) axis. In mammals it is produced in the paraventricular nucleus (PVN) of the hypothalamus and is released into the pituitary portal circulation where it travels to the pituitary gland to stimulate the release of

adrenocorticotropic hormone (ACTH). CRF is also expressed throughout the brain where it may act as a neurotransmitter/neuromodulator, influencing stress-related behaviors and appetite/feeding. In response to stress, activation of the CRF neurons in the PVN results in ACTH secretion and subsequent release of glucocorticoids from the adrenal cortex. In *X. laevis*, the homologous region to the PVN is the anterior preoptic area (POA), and it is also here that our lab has observed stress-induced activation of the HPA axis and CRF neurons (Yao et al., 2004). The hormone is highly conserved among vertebrates and only differs by 3 amino acids in the primary sequence between humans and frogs.

CRF and leptin in feeding

In addition to modulating the stress axis, CRF plays an important role in energy balance through its actions on food intake. As in mammals, i.c.v. injections of CRF have been shown to decrease food intake in tadpoles and frogs (Crespi and Denver, 2004). Furthermore, our lab found that co-injection with the antagonist αhelical CRF (αhelCRF) could attenuate the anorectic effect of CRF (Crespi and Denver, 2004). This action is adaptive in stressful situations where the animal's focus must be redirected from foraging for food to escape or survival. Because leptin also acts on the hypothalamus to promote anorexia, CRF may be a potential target for leptin.

In mammals, leptin has been shown to influence CRF levels. Intracerebroventricular administration of leptin in rats was found to increase CRF protein levels in the hypothalamus through radioimmunoassay (Uehara et al., 1998). A similar result was reported for CRF mRNA in response to i.c.v. leptin injection using in situ hybridization (Schwartz et al., 1996). Further evidence also suggests that leptin's anorectic actions are mediated by CRF. Studies delivering leptin and αhelCRF intracranially into rats have demonstrated an attenuated feeding-inhibition response (Okamoto et al., 2001, Uehara et al., 1998). Pretreatment of rats with CRF antibody

displayed the same results in reducing the leptin attenuation of appetite (Okamoto et al., 2001). Additional studies in mice have shown an increase in c-fos-like immunoreactivity (c-FLI) in the PVN following leptin injection but an attenuation of this response when co-injected with αhelCRF (Masaki et al., 2003). The authors speculated that because c-Fos is a marker of neuronal activation, the suppression of c-FLI with a CRF antagonist might indicate that CRF neurons mediate leptin pathways. Overall, these data in mammals suggest a role for leptin in modulating energy balance through the regulation of CRF.

Objective

In this study I examined CRF neurons as potential targets for leptin signaling, and I investigated the effects of leptin on CRF expression using the frog *X. laevis* as the model organism. To do this, the hypothesis that leptin receptor is expressed on CRF neurons was tested through a colocalization experiment. Fluorescent immunohistochemistry was performed on juvenile frog brain to detect pSTAT3 and CRF in the POA. pSTAT3 was used as a proxy for the receptor because our lab's leptin receptor antibody still requires validation. pSTAT3 is a transcription factor activated in the JAK/STAT pathway by ligand binding to leptin receptor and thus is a specific indicator of leptin receptor expression. Leptin's acute effects on CRF expression were also examined quantifying changes in CRF immunoreactivity (ir) following leptin injection. Based on the effects seen in mammals, I hypothesize that leptin injections will increase CRF levels to potentiate feeding suppression.

Materials and Methods

Animal and tissue preparation

Juvenile *X. laevis* were derived from in-house breeding (original population from Xenopus I, Dexter, MI, USA) and housed in large communal tanks with well water at 22-25°C. Animals were fed pellet food and kept on a 12:12 hr light/dark cycle.

Tissue preparation was performed according to Yao et al., 2004. For immunohistochemistry, animals were killed instantaneously by severing the spinal cord. Heads were collected and fixed overnight at 4°C in 4% paraformaldehyde. Following fixation, the brains were dissected and post-fixed for 1hr after which they were cryoprotected overnight at 4°C in 30% sucrose. The brains were then snap frozen in isopentane and stored at -80°C. Samples were sliced into 12µm thick tissue sections using a cryostat and tissue section slides were stored at -20°C until processed for immunohistochemistry.

Subcutaneous (s.c.) leptin injection and shaking stressor

Previous work from our lab (Yao et al., 2004) showed that in the unstressed state, CRF-ir in the POA was low, but could be increased dramatically by exposure of frogs to a physical stressor (shaking stressor). In order to detect CRF-ir so as to determine if CRF and leptin-induced pSTAT3 are colocalized in the POA, we subjected frogs to shaking stress for 4 hours. One hour prior to sacrifice, we administered intralymph sac subcutaneous (s.c.) injections of recombinant Xenopus leptin (rxLeptin) to activate STAT3 (previously shown in our lab – C. Hu, unpublished data). rxLeptin used for injections was previously produced in *E. coli* and purified following the methods of Crespi and Denver, 2006.

Juvenile frogs weighing approximately 9g were placed in pairs into small white polypropylene containers with 100ml of purified water. The containers were then placed onto an

orbital shaker and subjected to 4 hours of shaking at 120rpm. In the third hour, frogs were removed and injected s.c. with rxLeptin (1μg/g body weight (BW) in 0.6% saline or an equivalent volume of 0.6% saline. One hour post-injection (4 hours after the onset of the stressor), animals were sacrificed for immunohistochemistry.

To assess the acute effects of leptin on CRF-ir, juvenile frogs with body weights ranging from 8 to 16 g were injected s.c. with either 1μg/g BW rxLeptin or an equal volume of 0.6% saline. Animals were housed in two tanks to separate the leptin and saline injected groups. Care was taken to ensure adequate space and minimal outside disturbance. Animals were sacrificed 5 hours after injection for immunohistochemistry.

Immunohistochemistry

Immunohistochemistry was performed using two methods: immunofluorescence and horseradish peroxidase/diaminobenzidine (HRP/DAB). For determining if leptin receptor (pSTAT3-ir) and CRF were colocalized, tissue sections were stained for CRF and phospho-STAT3 (pSTAT3) through sequential immunofluorescence. Sections were first rehydrated in PBS and then subjected to a 2 minute antigen retrieval step in heated 0.01M citric acid. Blocking was performed for 2 hours in Pierce SuperBlock blocking buffer containing 5% normal goat serum (NGS). To stain for CRF neurons, sections were incubated overnight at 4°C in rabbit anti-xCRF antibody (1:20) followed by a 1 hour incubation in a goat anti-rabbit fluorescein (FITC) secondary (1:150). Leptin receptor was detected using a 3 hour mouse anti-pSTAT3 antibody (1:100, Cell Signaling) incubation followed by a 1 hour incubation in a goat anti-mouse Cy3 secondary (1:150).

To quantitate CRF-ir following leptin injection we used a chromogenic substrate (HRP/DAB). Tissue sections were incubated for 15 minutes in 0.3% hydrogen peroxide to

quench endogenous peroxidase activity and blocked with 5% NGS in Pierce SuperBlock for 2 hours. Sections were then incubated overnight at 4°C in anti-xCRF (1:20). For the goat anti-rabbit secondary incubation and subsequent HRP substrate development, the Vectastain elite ABC (rabbit) and Vector VIP kits were used and steps were performed according to the manufacturer's protocol. pSTAT3-ir was assessed with the same protocol but slides were incubated in anti-pSTAT3 (1:200, Cell Signaling). Unless otherwise stated, all steps were performed at room temperature.

For colocalization experiments images were captured using a Leica TCS SP5 confocal microscope at 63x magnification. Optical sections (1μm thick) were captured to verify colocalization of leptin receptor on CRF neurons. Fluorescence and bright field microscopy was also employed using an Olympus IX81 inverted microscope.

Morphometric Analysis

Following HRP/DAB immunohistochemistry, images were randomized and analyzed using MetaMorph software (version 7.6) according to the methods of Meng et al., 2004, 2008. For each experimental sample, an anatomically matched section of the POA was analyzed by hand-drawing a region of interest of approximately equal area. CRF-ir was quantified by setting the threshold above which positive reactivity was counted automatically. Mean density was calculated as the area of positive staining divided by the area the selected region. For pSTAT3-ir, nuclei count was determined. Parameters for nuclei size and intensity above threshold were input to MetaMorph, and positive nuclei were highlighted for manual counting. Statistical analyses were performed with a Student's t-test using SYSTAT10 from SPSS Science.

Results

Activation of CRF-ir by shaking stress

To improve visualization of CRF neurons, juveniles were subjected to shaking stress. Previous work in our lab has shown that shaking stress activates the HPA axis and increases CRF-ir in the POA. This was verified, and a statistically significant (P=0.001), 3-fold difference was seen between unstressed animals and animals subjected to a 4 hour shaking stressor (Figure 1).

Leptin injection increases pSTAT3-ir in the frog POA

Because we used pSTAT3 as an indicator of leptin receptor signaling, it was necessary to ensure that shaking stress did not activate pSTAT3. Animals subjected to shaking stress without leptin injection did not display detectable pSTAT3-ir whereas pSTAT3-positive nuclei were labeled only in stressed animals that had received leptin injections (Figure 2). This was confirmed in both the parvocellular and magnocellular neurons of the POA. Presence of pSTAT3 only with leptin injection indicated that for these samples, pSTAT3-ir was specific to neurons expressing leptin receptor.

Colocalization of leptin receptor and CRF

Leptin receptor was observed on CRF neurons in the POA using epifluorescence microscopy, and confirmed by confocal microscopy (Figures 3,4). Juvenile frogs were injected with leptin and subjected to shaking stress to enhance visualization of both pSTAT3 and CRF. Overlap of pSTAT3 and CRF staining was detected in the POA, demonstrating that leptin receptor is expressed on CRF neurons. Colocalization was seen in both parvocellular and magnocellular POA neurons. Only a fraction of CRF-positive neurons were pSTAT3-positive

and the converse was also true. Quantitation indicated that an average of 41% of neurons expressing leptin receptor were CRF neurons (n=3). Confocal microscopy and captured z-stack images was used to confirm the colocalization of CRF and pSTAT3 (Figure 3). These data suggest that leptin receptor is expressed on a subset of CRF neurons as well as other neuron populations in the frog POA.

Effects of acute leptin injection on CRF-ir

Changes in CRF-ir following leptin injection of juveniles were assessed through immunohistochemistry. As a control, frogs were injected with saline to account for CRF-ir changes due to injection only. Animals were collected 5 hours post-injection. From prior experiments, pSTAT3 is known to be activated by one hour after leptin injection and CRF-ir is increased after 4 hours of shaking stressor. Images of magnocellular and parvocellular CRF neurons were captured and from these, immunoreactivity quantified. In parvocellular CRF neurons, CRF-ir was significantly decreased (P=0.011) by 20% with leptin administration (Figure 5). No effect of leptin injection on CRF-ir was seen in the magnocellular neurons.

Discussion

Leptin and CRF play important roles in regulating energy balance and food intake in vertebrates. The expression of leptin receptor on CRF neurons and demonstration that leptin can change neuronal CRF protein levels are significant in explaining how leptin's anorectic actions can occur. Studies in mammals have suggested that leptin's actions on feeding may be mediated by CRF neurons. Here we show for the first time in a non-mammalian vertebrate that leptin can act directly on CRF neurons through leptin receptor and influence CRF levels.

Colocalization of CRF neurons and pSTAT3 (leptin receptor) was observed in the POA indicating that leptin can act on these neurons. However, not all CRF neurons visualized were pSTAT3-positive. This may mean that only a spatially regulated subset of neurons is involved in the feeding circuitry and hence expresses leptin receptor. A follow-up in situ hybridization experiment detecting leptin receptor and CRF could be performed to enhance the current results. Another possible explanation is that there was a technical limitation in detecting pSTAT3. An antigen retrieval step is required to visualize pSTAT3 staining, but because CRF is leached from the cell with acid treatment, the incubation time for double labeling was shortened to reduce this occurrence and not all pSTAT3-ir may have been identified.

The double labeling results also indicated that not all neurons expressing leptin receptor were CRF neurons. We hypothesize that these may be thyrotropin-releasing hormone (TRH) neurons since in mammals TRH is implicated in feeding control (Harris 2000). TRH is an important regulator of thyroid-stimulating hormone (TSH) in the hypothalamic-pituitary-thyroid (HPT) axis, but like CRF, TRH is anorexigenic and plays a role in energy regulation (Valassi et al., 2008). TRH neurons are present in the PVN and receive input from neurons in the arcutate nucleus expressing peptides involved in feeding control, such as neuropeptide Y, a-melanocyte-

stimulating hormone (a-MSH) and agouti-related protein (Shibusawa et al., 2008). During periods of starvation, leptin levels fall coinciding with a suppression of the HPT axis, but it has been demonstrated in mice that this can be rescued with leptin injection to partially restore thyroid hormone and pro-TRH levels (Ahima et al., 1996). In vitro hypothalamic rat neuron experiments have shown that leptin can stimulate TRH release and leptin receptor is expressed on TRH neurons (Nillni et al., 2000). It is thus reasonable to predict that in *X. laevis* leptin may act directly on TRH neurons as well. The neurons expressing leptin receptor but not CRF in this report could be TRH neurons. Colocalization data could be obtained for leptin receptor and TRH neurons in *X. laevis* to test this hypothesis and acute leptin injections could be performed to observe how leptin injections can influence TRH expression.

Contrary to my prediction that leptin injection would increase CRF-ir, leptin injection decreased CRF-ir in parvocellular neurons of the POA. CRF contribution to the anorectic condition could occur either through gene transcription and biosynthesis or secretion from neurons. We would expect an increase in CRF-ir with an increase in biosynthesis. However, since we observed a decrease, it is more likely leptin increased CRF secretion in the short term. In addition, preliminary RT-qPCR data indicates that there is no change in CRF mRNA due to leptin injection in juvenile frogs. Whole brain samples were processed for this test, but because CRF mRNA changes will most likely be small relative to global changes, a future experiment could extract only the hypothalamic region. This would test the hypothesis that leptin does not increase CRF biosynthesis.

Because there is not yet a demonstrated way of detecting secretion in neurosecretory neurons in *X. laevis*, an alternative way of investigating leptin's role in CRF modulation would be through a behavioral assay. Because leptin is known to inhibit feeding, animals could be

injected with leptin and α-helical CRF, a CRF antagonist. Feeding behavior would be assessed through measurement of beef liver or pellet food consumed in a defined period of time. If leptin's anorectic effect were mediated by CRF, the decrease in feeding would be attenuated by injection of a CRF antagonist.

Release of CRF rapidly elevates plasma corticosterone (CORT) concentration, which in turn exerts negative feedback effect on CRF biosynthesis and secretion (Yao et al., 2004). We did not measure plasma CORT in this experiment, but future studies will investigate whether leptin injection alters circulating CORT. If CORT is increased following leptin injection (i.c.v.), this would support that leptin increases CRF secretion.

No change in mean density of CRF-ir was found in magnocellular neurons. This suggests that the effects of leptin could be localized to a specific part of the POA, but the presence of leptin receptor on magnocellular neurons seems to indicate that some response should be anticipated in this region (Figure 4A,B). The sample size may not have been large enough or more likely, due to the small number of magnocellular neurons detected, the technique used was not sensitive enough to detect a change in CRF-ir. A larger sample size could be gathered to try to verify the results presented here.

Leptin and CRF in Metamorphosis

Many amphibians undergo metamorphosis, a process that involves the transition from a larval to adult stage. This process involves extensive tissue remodeling, such as, in the case of *Xenopus laevis*, a switch from an herbivorous to carnivorous diet, tail resorption, formation of lungs, and development of stereoscopic vision. Because the decision to undergo metamorphosis is an energetic commitment, the timing is very important, and the animal must establish sufficient energy stores before initiating.

Metamorphosis is triggered by a rise in the thyroid hormone triiodothyronine (T_3), which mediates the changes that occur during this process. In mammals, TRH is responsible for stimulating the release of TSH, which in turn stimulates T_3 release from the thyroid gland. This, however, turns out not to be the case in nonmammalian vertebrates. In these animals, CRF is the primary regulator of the hypothalamo-pituitary-thyroid (HPT) axis, stimulating pituitary TSH secretion and thus the secretion of T_3 (Denver et al., 2002). The role of CRF as a TSH releasing factor is important in controlling the onset and the rate of metamorphosis because environmental and hormonal factors that influence the activity of CRF neurons can alter the timing of metamorphosis. We hypothesize that leptin influences this decision by signaling that there are sufficient body stores to undergo remodeling. From the findings in this paper, we believe that leptin could also play a role in metamorphosis and acting as an indicator of body condition/energy balance, could signal to the brain via CRF neurons to influence CRF release and metamorphic timing.

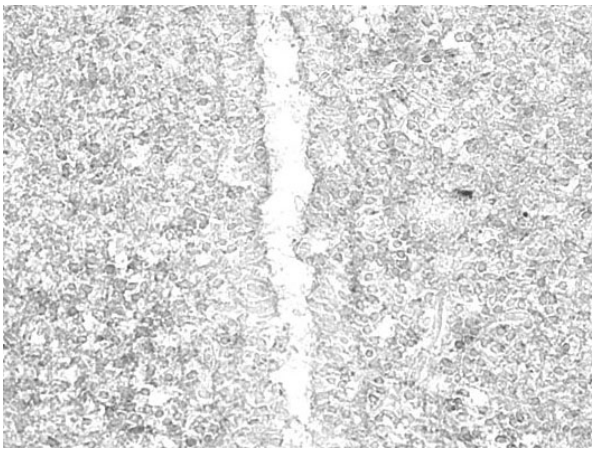
Conclusions

The results presented here indicate that leptin receptor is expressed on CRF neurons and is involved in CRF modulation. We hypothesize that CRF mediates actions of leptin on appetite in the frog. We also hypothesize that leptin influences the timing of metamorphosis through its actions on CRF neurons. Activation of leptin receptor could stimulate the secretion of CRF to mediate leptin's anorectic effect. In metamorphosis, sufficient energy stores would cause leptin activation of CRF neurons, and CRF release would act to stimulate TSH secretion, causing a rise in T_3, triggering metamorphosis. Future directions could investigate the mechanism by which CRF mediates leptin signaling (secretion or synthesis) and which neurons participate in the feeding circuitry since differential colocalization was observed. We could examine the

developmental implications of the interaction between leptin and CRF. Knocking down the leptin receptor signaling by using a non-signaling leptin mutein or overexpressing a truncated leptin receptor without the intracellular signaling domain could be used to see the effects on metamorphosis. We would expect that, barring outside stress, the tadpoles would become much larger than usual before metamorphosing.

Figures

A



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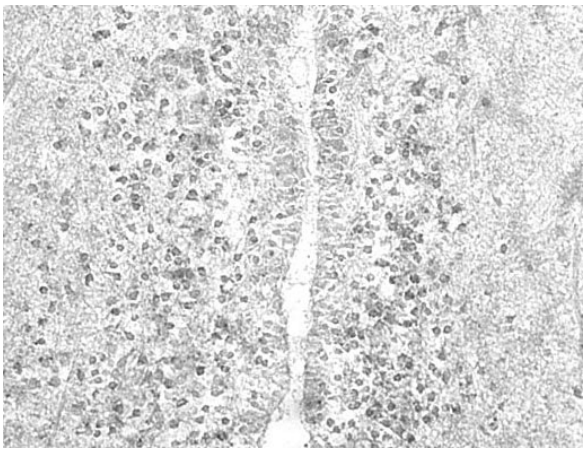

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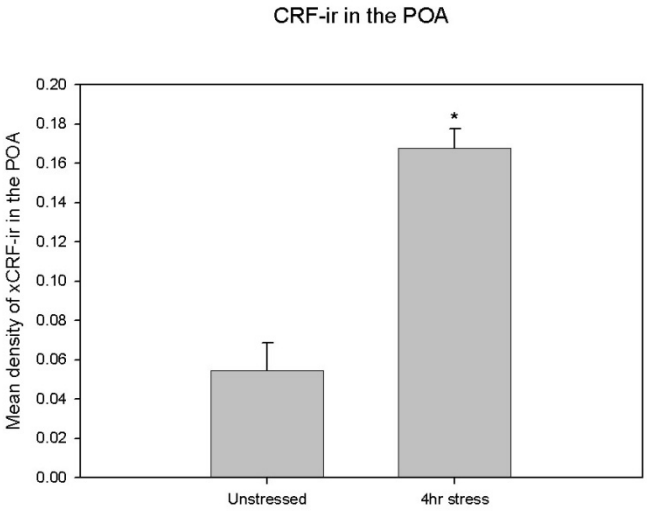

Figure 1: Activation of CRF by shaking stress
Immunohistochemical stain for CRF in POA of juvenile *X. laevis* (A) Unstressed animal (n=4) (B) Animal subjected to a 4 hour shaking stressor (n=4) (C) MetaMorph analysis of mean density demonstrating increase in CRF-ir following a 4 hr shaking stress. Data presented are the mean ± SEM. Significant difference from control is denoted (*P<0.001). Images captured at 10x magnification.

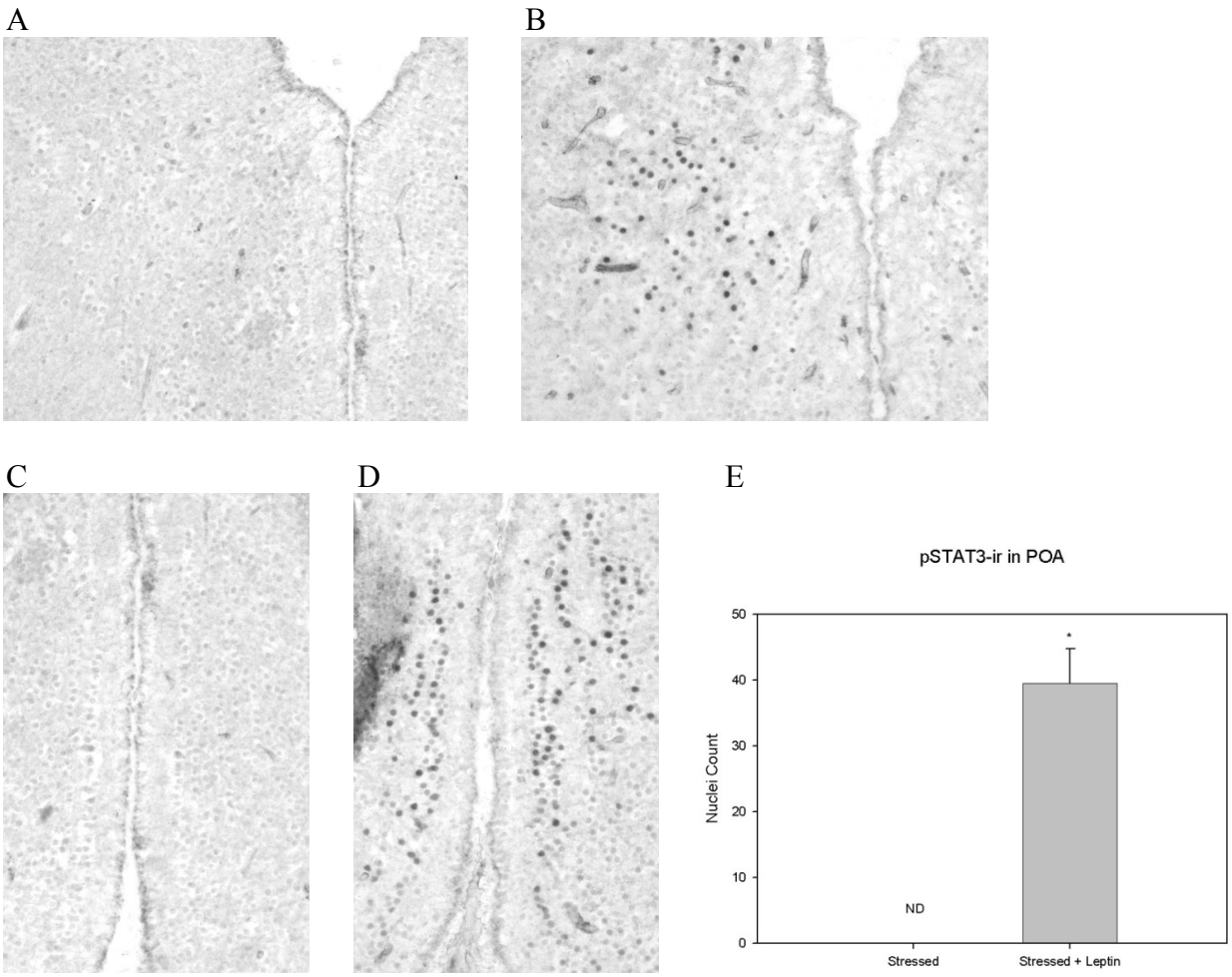

Figure 2: Activation of pSTAT3 by leptin injection but not stress
Representative images of immunohistochemical stain for pSTAT3 in POA of juvenile *X. laevis* subjected to either 4 hour shaking stressor or shaking stressor and leptin injection (A) Magnocellular neurons in stressed animal (n=3) (B) Magnocellular neurons in stressed and leptin injected animal (n=3) (C) Parvocellular neurons in stressed animal (n=4) (D) Parvocellular neurons in stressed and leptin injected animal (n=4) (E) MetaMorph quantification of pSTAT3-positive nuclei demonstrating increase in pSTAT3 following leptin injection but not shaking stress. ND=not detectable. Data presented are the mean ± SEM. Significant difference from control is denoted (*P<0.001). Images captured at 10x magnification.

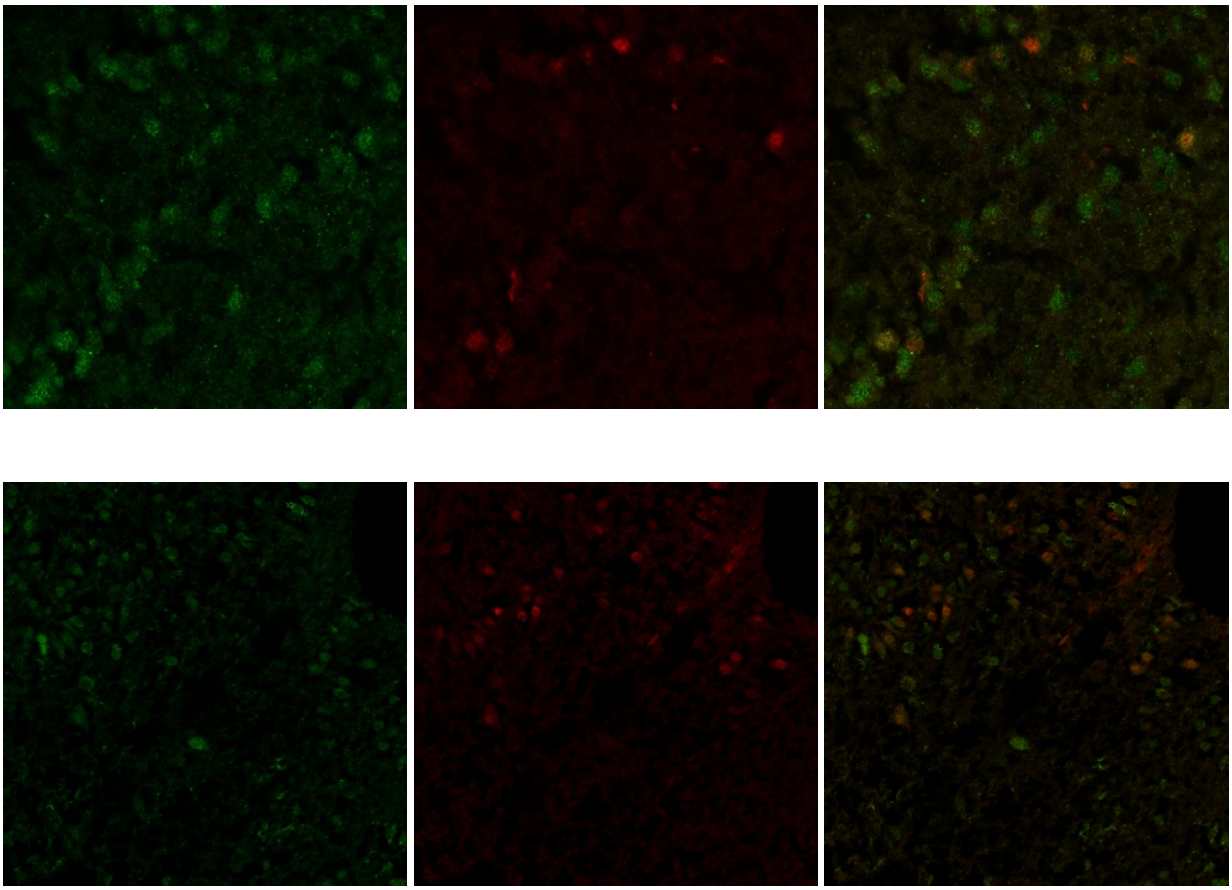

Figure 3: Localization of leptin receptor on CRF neurons
Confocal images depicting colocalization of pSTAT3 (Cy3) as a proxy for leptin receptor and CRF (FITC) in POA. Images captured at 63x magnification.

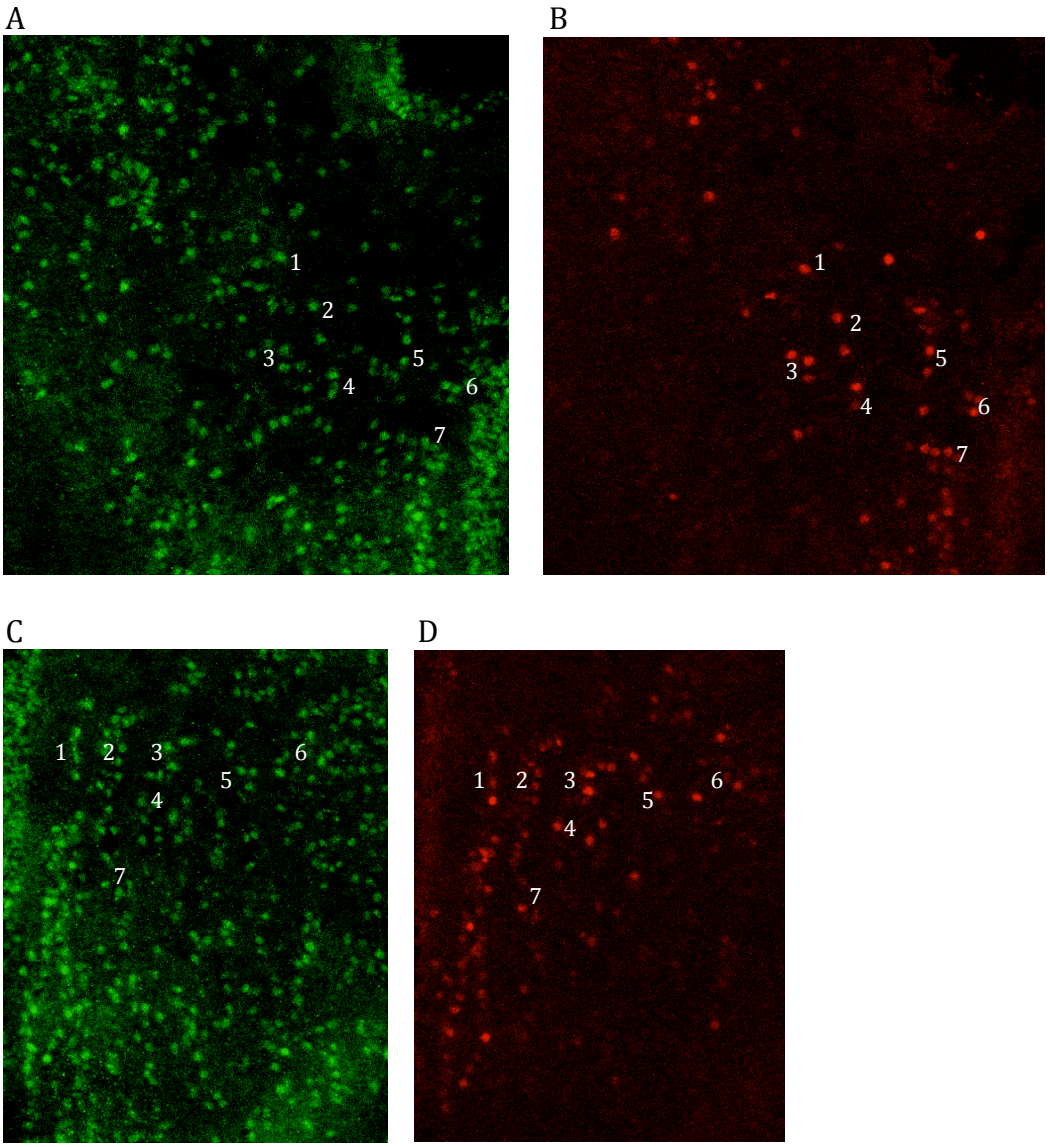

Figure 4: Localization of CRF neurons and pSTAT3
Fluorescent staining of leptin receptor and CRF in POA. Numbers indicate same neuron on corresponding images. Magnocellular neurons: (A) CRF, FITC (B) pSTAT3, Cy3. Parvocellular neurons: (C) CRF, FITC (D) pSTAT3, Cy3. Images captured at 10x magnification.

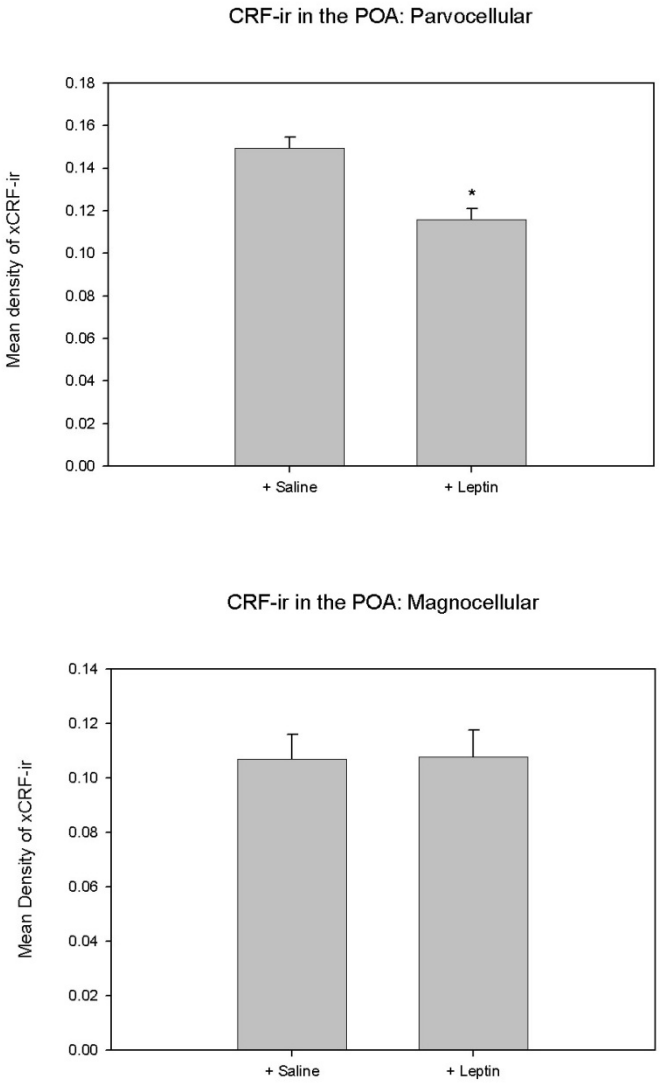

Figure 5: Effect of leptin injection on CRF-ir
Mean density of CRF-ir in POA of juvenile *X. laevis* injected with either 0.6% saline or 1µg/g BW leptin. CRF-ir was significantly decreased in the region containing parvocellular but not magnocellular neurons of the POA (n=3, 4 per treatment respectively). Data presented are the mean ± SEM. Significant difference from control is denoted (*P<0.05).

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