



8-2009

Effects of Weaning and Syndyphalin-33 on Appetite Regulators in Swine

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Recommended Citation

Cooper, Tabatha Anne, "Effects of Weaning and Syndyphalin-33 on Appetite Regulators in Swine." Master's Thesis, University of Tennessee, 2009.
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To the Graduate Council:

I am submitting herewith a thesis written by Tabatha Anne Cooper entitled "Effects of Weaning and Syndyphalin-33 on Appetite Regulators in Swine." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Animal Science.

Cheryl J. Kojima, Major Professor

We have read this thesis and recommend its acceptance:

Arnold M. Saxton, Brynn H. Voy

Accepted for the Council:

Dixie L. Thompson

Vice Provost and Dean of the Graduate School

(Original signatures are on file with official student records.)

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and recommend its acceptance:

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Brynn H. Voy

Accepted for the Council:

Carolyn R. Hodges, Vice Provost and
Dean of the Graduate School

Effects of Weaning and Syndyphalin-33 on Appetite Regulators in Swine

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Tabatha Anne Cooper

August 2009

Acknowledgements

I wish to express my appreciation to my major professor and mentor, Dr. Cheryl Kojima, for the opportunity to pursue a graduate degree in Animal Science at the University of Tennessee. Through her knowledgeable guidance and friendship I had a wonderful experience where I grew not only in academics but as a person. My future successes in life will always be due in part to the time that we spent together.

I would also like to thank my committee members, Drs. Arnold Saxton and Brynn Voy, for their continued support. I also wish to express my appreciation for Dr. Alan Mathew, Linda Miller, Eddie Jarboe, and Leczek Wojakiewicz for their friendship and help whenever needed.

Special thanks to all the graduate students. I would especially like to thank Brian Campbell, who was always willing to lend a hand with research, an ear to vent to or a shoulder to cry on; Sarah Jo Jenkins, for her help with research and for her camaraderie; and Karianne Chung, Cristina Campistol, and Rilla Reese for their continued friendship throughout this process.

Finally, I want to thank my husband and best friend, Ryan who has always supported and believed in me. Without him, and the rest of my family, this would not have been possible.

Thank you all.

Abstract

The synthetic met-enkephalin syndyphalin-33 (SD-33) increases feed intake in sheep and recently-weaned pigs. An experiment focused on changes in hypothalamic levels of mRNA transcripts from the following genes: μ -opioid receptor (*MOR*), neuropeptide Y (*NPY*), orexin (hypocretin; *HCRT*), melanocortin 4 receptor (*MC4R*), and agouti-related protein (AGRP). There was no effect of treatment on BW or FI at any point in the study. There is a strong positive correlation ($p < 0.001$) between *MOR* and *MC4R* that remains regardless of treatment or time. Weaning resulted in a numerical increase in hypothalamic *MOR* mRNA expression. There was a treatment effect of *MOR* expression levels 4 days postweaning. Hypothalamic *NPY* expression was unchanged through time or treatment. In VEH pigs, weaning resulted in increased hypothalamic expression of the *HCRT* gene at d 1 and d 7 postweaning ($P_{(\text{time})} < 0.01$). In SD pigs, an increase in *HCRT* expression (relative to d 0) was observed at d 4 postweaning. Expression of *HCRT* did not significantly differ between treatment groups at d 1 and d 4, but *HCRT* expression was greater in VEH pigs than in SD pigs at d 7 ($P_{(\text{time} \times \text{treatment})} < 0.05$). Expression of hypothalamic *MC4R* did not change through time, but a trend ($P_{(\text{treatment})} = 0.07$) was observed such that expression was less in SD pigs than in VEH pigs on d 1 and d 4 postweaning, possibly due to a numerical increase in expression in VEH pigs on d 1 relative to d 0.

Expression of *AGRP* was decreased 1 d postweaning in VEH pigs but not in SD pigs. On d 4, levels of *AGRP* expression were lower in SD pigs than in VEH pigs, and lower than d 0 levels as well. On d7, *AGRP* expression was less in VEH pigs than in SD pigs ($P_{(\text{time} \times \text{treatment})} < 0.01$). Syndyphalin-33 has the potential to alleviate the negative effects of stress during weaning, and has the potential to increase feed intake through the melanocortinergeric pathway. Further investigation is needed to more fully elucidate the complex mechanisms by which syndyphalin-33 may act to abrogate the post-weaning growth lag in pigs.

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Introduction

Syndyphalin-33 (**SD-33**; Tyr-D-Met(O)-Gly-N-methylphenethylamide) is a synthetic enkephalin with prolonged analgesic activity (Kiso et al., 1981). In pigs, rats, and sheep, administration of SD-33 via oral, subcutaneous, or intravenous routes resulted in transient increases in circulating concentrations of growth hormone (**GH**; Buonomo et al., 1991). Recently, SD-33 was found to increase feed intake in adult sheep 48 h after intravenous administration, but this effect was lost when the animals were challenged with lipopolysaccharide (**LPS**; Obese et al., 2007). In all three studies, the effects of SD-33 relating to analgesia, circulating GH concentrations, and appetite were all blocked by naloxone, suggesting that they were mediated at least in part through the μ -opiate receptor. The discrepancy in the timing of effects (immediate analgesia and stimulation of GH, but 48 h before significant increases in feed intake) is not yet understood.

The recently-weaned pig often exhibits decreased feed intake, increased susceptibility to disease, and poor growth (Matteri et al., 2000; Kojima et al., 2007; Kojima et al., 2008). This post-weaning growth lag can be manifested as mortality, particularly in conditions of suboptimal herd health or management. Many factors may determine the severity and duration of the post-weaning lag; evidence suggests that weaning weight may modulate the expression of key regulators of appetite during this period (Kojima et al., 2007).

Preliminary data in our lab suggests that a single intramuscular injection of syndyphalin-33 can increase feed intake in the recently-weaned pig (Kojima et al., submitted). SD-33 may offer some protection during the weaning process, compensating for an extreme decrease in appetite, and increasing overall health and well being of the animal during this critical period.

As an opioid molecule, SD-33 likely influences the regulation of expression of many appetite-regulating genes. An experiment was conducted to investigate the potential for SD-33 to alter hypothalamic expression of key appetite-regulating genes during the first 7 d postweaning. The experiment focused on changes in hypothalamic levels of mRNA transcripts from the following genes: μ -opiate receptor (*MOR*), neuropeptide Y (*NPY*), orexin (hypocretin; *HCRT*), melanocortin 4 receptor (*MC4R*), and agouti-related protein (*AGRP*).

Literature Review

Syndyphalin-33

Syndyphalin-33 (SD-33; Tyr-D-Met(O)–Gly-N-methylphenethylamide) was first synthesized in 1981 by scientists at the University of Tokushima, Japan (Kiso et al., 1981). A tripeptide, SD-33 represents the “minimal segment” of Met-enkephalin (an endogenous opioid peptide) that exhibits prolonged analgesic activity (Baulieu and Kelly, 1990). To evaluate the similarity of SD-33 to morphine, another opioid compound known for its analgesic properties, the authors conducted a tail-flick test in mice. A single subcutaneous injection of SD-33 was administered at 2, 4 and 8 mg/kg, doses comparable to 1, 2 and 4 mg/kg injections of morphine, respectively. The tail-flight response (in seconds) was recorded over a period of 180 minutes. SD-33 was similar to morphine in both the potency and duration of analgesia. The authors believed that although SD-33 no longer resembled an enkephalin, its ability to be stable in the mouse model made it a valuable opioid peptide.

Buonomo and colleagues evaluated the effect of syndyphalin-33 on the release of growth hormone in sheep, pigs and rats (Buonomo et al., 1991). Sheep were injected intravenously with SD-33 in a dose of .05, .10 and .20 $\mu\text{mol/kg}$. As compared to controls, GH (measured in ng/ML) was elevated in all groups five minutes after i.v. injection and remained elevated for 45 minutes. When sheep were given the i.v. injections two hours apart, SD-33 treated animals again had significantly higher GH levels than did control animals. Rats and 40-kg pigs were given a single

subcutaneous injection of 0.0, 0.50, 1.0 and 2.0 $\mu\text{mol/kg}$ or 0.0 or 0.50 $\mu\text{mol/kg}$ SD-33, respectively. Plasma GH concentrations were elevated in treated pigs relative to controls in a dose dependent manner, peaking at 30 minutes postinjection and remaining elevated at 60 minutes postinjection. Rats were given an oral gavage of SD-33 in a dose of 0.0, 1.0, 10.0 or 100.0 $\mu\text{mol/kg}$. An increase in GH levels was observed within 15 minutes, and for the rats given the dose of 100.0 $\mu\text{mol/kg}$, remained elevated for 2 hours post-administration. Pigs were given an oral gavage of SD-33 in a dose of 0.0, 0.125, 0.50 or 2.0 $\mu\text{mol/kg}$. The GH levels were elevated for all treatment groups relative to controls within 30 minutes of administration and remained elevated for two hours. To determine if SD-33 was actually working through the μ - opiate receptor, the authors challenged rats with both SD-33 and the opioid antagonist naloxone. Naloxone was able to completely block the elevation of GH concentrations by SD-33 at both the 1.0 and 10.0 mg/kg dose.

In 2007, Obese and others evaluated the effect of syndyphalin-33 on long-term feed intake in sheep (Obese et al., 2007). Three experiments were performed. Sheep were given an intravenous injection of SD-33 at a dose of 0.0, 0.05 or 0.1 $\mu\text{mol/kg}$. Feed intake was measured for 48 hours. Feed intake at 24 hours was greater in sheep receiving 0.1 $\mu\text{mol/kg}$ dose of SD-33 relative to controls. After 48 hours, both the 0.1 and 0.05 $\mu\text{mol/kg}$ groups had eaten significantly more feed than the control group. The next experiment tested the ability of naloxone to block the effects of SD-33. Naloxone was effective in blocking the stimulatory effect of SD-33 on feed

intake. Lastly, sheep were given either saline or SD-33 in conjunction with or without an immune challenge in the form of lipopolysaccharide (LPS). There was no difference in intake in LPS-challenged sheep with or without SD-33: although SD-33 increased the feed intake of adult sheep, it did not counteract the decrease in FI associated with an immune challenge.

Weaning

Currently, pigs are weaned (separated from the dam) at approximately 21 days of age, depending on the particular management practices of the facility (Holden and Ensminger, 2006). The stress of weaning causes reduced weight gain or actual weight loss directly after weaning. In addition to the stress of being separated from the dam, change to an unfamiliar food source and an unfamiliar way of obtaining that food also lead to weight loss (Libbrandt et al., 1975; Bark et al., 1986). The age, weight, and sex of the animal are all factors contributing to how severely the animal is affected by the stressors of weaning (Libbrandt et al., 1975; Cooper et al., 2009). Weaning also causes the animal to become immunosuppressed (Kojima et al., 2008). There is an increase in somatotropin (GH) concentrations and a decrease in IGF-I and IGF-II (Carroll et al., 1998; Matteri et al., 2000). After weaning, there is increased plasma and urinary levels of cortisol concentrations as well as decreased plasma corticosteroid-binding globulin (Le Dividich and Seve, 2000; Heo et al., 2003; Kojima et al., 2008). Factors such as weaning weight and sex also attribute to the immune status of the piglet (Matteri et al., 2000; Bruininx et al., 2001; Kojima et

al., 2007; Cooper et al., 2009). Mortality rate in the nursery phase (defined as the period from weaning to 60 lbs.) was 2.9% in 2006 (USDA, 2008). The causes of death are listed in table 1.

Appetite

The regulation of appetite is important in maintaining energy balance in the body. The role of the hypothalamus in appetite has been extensively reviewed by Arora and Anubhuti (2006). The hypothalamus can be broken down into several regions, or “nuclei”. The arcuate nucleus controls the body’s energy balance and is the feeding control center, integrating various hormonal signals (Funahashi et al., 2000). The arcuate nucleus produces neuronal peptides such as Neuropeptide Y (NPY), Agouti-related protein (AGRP) and α -melanocyte stimulating hormone (α -MSH; Arora and Anubhuti 2006). The paraventricular nucleus integrates signals from the thyroid and the hypothalamic-pituitary axis involved in nutrition. Some of the neuronal pathways that communicate between the two include NPY, orexin, and α -MSH (Neary et al., 2004). The ventromedial nucleus of the hypothalamus is a target for leptin and acts as a center for controlling satiety (Sato et al., 1997). The dorsomedial hypothalamic area processes information from other areas of the hypothalamus such as the medial and lateral hypothalamic nuclei, and also produces orexins (Elmqvist et al., 1998). In addition, the lateral hypothalamic area contains glucose-sensitive neurons that help regulate feeding and produces orexins (Bernardis et al., 1996). The brain stem includes the nucleus of tractus solitaries

(NTC). The NTC contains a high number of NPY-binding sites, and controls the frequency of meals as well as the size of meals, and gives fullness signals once the meal is complete (Harfstrand et al., 1986; Kawai et al., 1984; Williams et al., 2001). A schematic representation of each area of the hypothalamus and the hypothalamic and peripheral neuropeptides associated with each is shown in figure 1.

Opioids and Opioid Receptors

The body produces endogenous opioids that can be classified into three families. Each family of ligands is produced by a single gene. These genes are the proopiomelanocortin, proenkephalin and prodynorphin genes (Akil et al., 1984). There are three classical opioid receptor types: mu (μ), kappa (κ), and delta (δ). All three of the opioidreceptor subtypes are involved in analgesia (Zöllner and Stein, 2007). Synthetic opioids such as morphine can also bind to the opiate receptors and have similar effects to endogenous opioids (Kiso et al., 1981).

An extensive review by Bodnar (2004) describes how endogenous opioids affect feeding behavior and appetite. Agonists of all three opioid receptor types have been shown to increase feed intake (Lowy et al., 1983; Gosnell et al., 1986). The route by which these effects occur can be through changes in transcription and/or activity of AGRP, NPY, peptide YY and the melanocortin 3 and 4 receptors (Olszewski et al., 2001; Kotz et al., 1993; Hagan et al., 1993; Grossman et al., 2003). According to Glass (1999), opioids produced in different parts of the brain cause differing types of food responses. Opioids in the hindbrain are classified as sensory and metabolic in

nature, whereas opioids associated with the amygdala regulate the emotional response to food, and opioids in the hypothalamus are associated with maintaining homeostasis (Glass et al., 1999). The effect that opioids have on the appetite pathway can change depending on the energy needs of the animal (Margules et al., 1978).

Effects of stress and opiates on μ -opioid receptor expression

During stress, the body will release endogenous opioids, and decrease the number of available opioid receptors through negative feedback mechanisms (Seeger et al., 1984). In some cases however, stress can increase expression of the μ -opioid receptor, as was observed in young rats experiencing stress due to hot plate exposure (Torda et al., 1978). In the hypothalamus of these rats, the numbers of opiate receptor sites were significantly higher than controls. The authors speculated that stressing the young underdeveloped brain of these animals led to the development of an over-functioning defense system.

Effects of Stress and Opioids on Specific Appetite Regulators

Neuropeptide Y (NPY) is a 36 amino acid hypothalamic peptide that exhibits appetite-stimulating activity (Tatemoto et al., 1982; Billington et al., 1991). The mRNA expression of NPY increased in the arcuate nucleus of the hypothalamus in rats when deprived of food and during periods of food restriction. The change in NPY mRNA expression was most notable in the male rats as compared to females

in this particular study (Brady et al., 1990). Hypothalamic NPY gene expression was 88% higher in ewe lambs that were nutrient-deprived as compared to lambs that were adequately nourished (McShane et al., 1993). The weanling pig produces NPY in a body weight-dependent manner, such that pigs with greater weaning weights have increased NPY mRNA levels 4 days postweaning (Kojima et al., 2007). Stress will alter the expression of NPY: when rats are stressed, they exhibit increased expression of NPY in the arcuate nucleus (Makino et al., 2000). Appetite is stimulated by μ -opioid agonists through up-regulating activity of NPY (Kotz et al., 1993; Pomonis et al., 1997; Dodo et al., 2005). Rats that were injected simultaneously with NPY and a μ - and κ -opioid receptor antagonist showed decreased feed intake while the same injections with a δ -opioid receptor antagonist showed no effect on feed intake (Kotz et al., 1993). In male rats, the increase in feed intake caused by the injection of NPY directly into the paraventricular nucleus could be blocked with naloxone (Pomonis et al., 1997). In male broiler chicks, a similar study was performed to investigate the effects of μ -, κ - and δ -opioid receptor antagonists on NPY-induced feeding. In agreement with Kotz et al. (1993), the most dramatic effects were seen when the μ -opioid receptor antagonist was administered (Dodo et al., 2005). These studies indicate that the μ -opioid receptor in particular, and possibly others, is effecting NPY-induced feeding.

The hypocretin or prepro-orexin gene produces two products, orexin-A and orexin-B. While only 46% similar in amino acid sequence, both orexin-A and orexin-

B will stimulate feed intake when administered directly into the hypothalamus of rats (Sakurai et al., 1998). Also, the mRNA of these peptides is up-regulated when rats are fasted, indicating their role in the appetite pathway (Sakurai et al., 1998). Increased feed intake has also been observed in pigs when given an intramuscular injection of orexin-B (Dyer et al., 1999), and in sheep when given orexin-B intravenously (Sartin et al., 2001). The weanling pig produces orexin in a body weight dependent manner, such that pigs with greater weaning weights have greater mRNA levels of orexin 4 days postweaning (Kojima et al., 2007). Stress can affect orexin production: immobilization stress in young rats will increase mRNA expression levels of orexin (Ida et al., 2000). The administration of morphine in mice depressed the hypocretin/orexin arousal system (Li et al., 2008).

The melanocortin 4 receptor (MC4R), one of five melanocortin receptors, is a G protein-coupled receptor found throughout the central nervous system. Opioids will decrease expression of the MC4R gene (Chaki et al., 2005). The Proopiomelanocortin (POMC) gene products α -MSH is an MC4R agonist, while agouti-related protein (AGRP) is an MC4R antagonist (Cone, 2005). When α -MSH is bound to MC4R there is an appetite-suppressing effect on feed intake; this effect can be blocked by the antagonist AGRP (Kalra et al., 1999). Agouti-related protein is a neuropeptide produced by the same neurons of the hypothalamus that produce NPY and has potent appetite stimulating properties that are long lasting (Shutter et al., 1997; Ollmann et al., 1997). Outside of the melanocortin pathway, the variant

AGRP-(18-132) has long lasting orexinogenic effects that may involve the regulation of as yet unidentified gene products at transcriptional, translational and/or post-translational levels (Hagan et al., 2000). Pigs with heavier weaning weights have greater levels of hypothalamic AGRP mRNA 4 days postweaning when compared to pigs with lighter weaning weights (Kojima et al., 2007). Stress will effect the interactions between MC4R, AGRP, and α -MSH (collectively known as the melanocortin system). In rats, AGRP mRNA expression in the arcuate nucleus is decreased following a stressful event, contributing to increased sensitivity for α -MSH and subsequent decreased feed intake (Kas et al., 2005). Forced swimming and restraint in rats increased POMC gene expression and suppressed feeding, but pretreatment with an MC4R antagonist blocked the anorectic and anxiogenic effects of these stressors, suggesting that the melanocortinergic pathway is heavily involved in stress-related anorexia (Liu, 2007).

A model using opiate administration to alleviate stress has been investigated in infant Rhesus monkeys. Opiate administration was found to be an effective method of elevating separation-induced stress in these animals (Kalin et al., 1988). Administration of morphine and fentanyl (both μ -opioid receptor agonists) during a surgical procedure resulted in decreased postoperative cortisol concentrations relative to controls in young (20 kg) pigs (Malavasi et al., 2006), but it is unclear if this was due to a direct effect of the opioids or a response to differing levels of post-operative pain (an indirect effect due to the analgesic properties of the opioids).

Conclusions

The appetite pathway is disrupted by the stress of weaning in the young pig. Opiates may represent a novel mechanism to ease the stress of weaning, increase feed intake, and increase overall production gains. The following experiment explores the effects of the synthetic opioid syndyphalin-33 on weaning stress and appetite regulators in the pig.

Materials and Methods

All animal procedures were reviewed and approved by the University of Tennessee Animal Care and Use Committee.

Animals and diets

Crossbred pigs (Landrace X Duroc X Hampshire) were farrowed in standard farrowing pens and processed according to usual University of Tennessee Experiment Station practice between 4 and 7 d of age. Standard processing procedures included needle teeth clipping, tail docking, iron supplementation, ear tagging, and castration of males. Piglets were kept in farrowing pens with their dams until weaning, with creep feed (Diet 554PE, Tennessee Farmers Cooperative, La Vergne, TN) available after 7 d of age.

Experimental Design

On day 0 (weaning day), 77 pigs (barrows and gilts, 19 ± 3 d of age, 7.16 ± 1.03 kg, 3 replications) were removed from the dam, weighed and allocated into four time groups: d 0 (weaning day), and d 1, 4 and 7 (postweaning). Tissue samples were collected on d 0 from unweaned animals ($n = 19$) and were considered a pre-weaning baseline. The remaining groups were further allocated into the following treatment groups: **SD**, receiving $0.5 \mu\text{mole/kg}$ SD-33 in a single intramuscular injection of 0.5 mL or less of saline; or **VEH**, receiving a single intramuscular injection of 0.5 mL saline. After administration of treatments, animals were weaned into individual pens (1.3 m^2) with nursery feed and water provided *ad libitum*. Body weight and

feed intake measurements were taken on d 1, 4 and 7 post-weaning, and average daily feed intake was calculated for each interval. Tissue samples were collected on d 1, 4 and 7 on animals within each treatment group (n = 8 or 9).

Tissue Collection and Processing

Animals were humanely euthanized and the hypothalamus was collected as previously described (Kojima et al., 2007) and stored in 5 mL RNAlater solution (Ambion, Forest City, CA) at -20°C until processed. For the isolation of mRNA from hypothalamus, tissue samples were homogenized in 10-20 volumes of TRI Reagent Solution (Ambion, Foster City, CA) and allowed to incubate for 5 minutes. After homogenization, bromochloropropane and isopropanol were used to extract total mRNA. Samples were then washed with ethanol and suspended in RNase-free water. To remove DNA contamination, samples were incubated with DNase using the Turbo DNA-Free kit (Ambion, Forest City, CA). Reverse transcription was performed with the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Forest City, CA) as per manufacturer recommendations. All cDNA samples were characterized (concentration and 260/280 nm absorbance ratio) and stored at -20°C until gene expression analysis was performed.

Gene Expression Analysis

Gene expression in the hypothalamus was analyzed using the quantitative real-time polymerase chain reaction technique with the following parameters: 2 min at 50°C, 8.5 min at 95°C, 45 cycles of 15 sec at 95°C followed by 1 min at 60°C,

indefinite hold at 4°C. For each gene of interest, primer efficiency was determined using a dilution series as described by Pfaffl (2001). In each reaction, a total volume of 20 µl was used including 10 µl Applied Biosystems Gene Expression Master Mix (Forest City, CA), 7 µl water, 2 µl cDNA template, and 1 µl Taqman Gene Expression assay primer/probe mix (Applied Biosystems, Forest City, CA). Table 1 shows the primer and probe sequences, efficiency and amount of cDNA used for each gene of interest. Threshold values were chosen so that data points were measured in the log section of the fluorescence curve. Each sample was run in duplicate, with the final C_T value being the average of both readings. The selected internal reference was 18S rRNA, and all target genes were normalized to it by calculating ΔC_T . The ΔC_T value was then transformed linearly as described by Livak and Schmittgen (2001) to obtain a relative expression value.

Statistical Analyses

Variables were analyzed in SAS using mixed model analysis of variance. Log transformations were performed on gene expression variables to maintain homogeneity of variance. Pig was the experimental unit. Body weight and daily feed intake were analyzed with repeated measures. Correlations and their associated values were analyzed for relationships among pre-weaning body weight and appetite genes both pre-weaning and 24 hours post weaning. A scatter plot and related correlation value were obtained for MC4R and MOR over all treatment groups. Gene expression data was analyzed with time and treatment as main effects

and the interaction between the two factors was also evaluated. Least squares means were compared using Fisher's protected least significant difference. A significance level of $P < 0.05$ was used for all testing; trends were reported if $P < 0.10$. Preliminary analysis detected no replicate effect, and so replicate was removed from the model. Pre-weaning body weight and sex were evaluated as covariates and were found to have no effect and were removed from the model. All figures depict raw means and standard errors.

Results

Feed Intake and Body Weight

As expected, average daily feed intake in VEH and SD pigs was minimal during the first 24 h post-weaning (Figure 2). Increases in average daily feed intake were evident for both treatment groups when measured from d 1 to d 4, and from d 4 to d 7, relative to the previous interval ($P < 0.05$). No significant difference in average daily feed intake due to treatment was observed at any time. Body weights of pigs in both treatment groups remained unchanged 1 d postweaning relative to d 0 weights (Figure 3). Similar to what was seen in average daily gain, increases in body weight were evident for both treatment groups when measured from d 1 to d 4, and from d 4 to d 7, relative to the previous interval ($P < 0.05$). No significant difference in body weight due to treatment was observed at any time.

Gene Expression

Weaning resulted in a numerical increase in hypothalamic *MOR* mRNA expression on d 1 and d 4 post-weaning in VEH pigs such that on d 4, *MOR* expression was significantly greater in VEH pigs than in SD pigs ($P < 0.05$; Figure 4). There was no difference between *MOR* expression levels in SD pigs at any time, although a numerical decrease in expression at d 4 contributed to the difference in expression observed between treatment groups at that time ($P_{(\text{time} \times \text{treatment})} = 0.01$). Expression of *MOR* at d 7 did not differ between treatment groups or from pre-wean levels.

Hypothalamic NPY expression was unchanged through time or treatment, but a trend ($P_{(\text{time} \times \text{treatment})} = 0.07$) was observed such that while NPY expression was less in SD than VEH pigs at d 1 and d 4 postweaning, expression was greater in SD pigs compared to VEH pigs at d 7 (Figure 5).

In VEH pigs, weaning resulted in increased hypothalamic expression of the *HCRT* gene at d 1 and d 7 postweaning relative to pre-wean levels ($P_{(\text{time})} < 0.01$; Figure 6). In SD pigs, an increase in *HCRT* expression (relative to d 0) was observed at d 4 postweaning. Expression of *HCRT* did not significantly differ between treatment groups at d 1 and d 4, but *HCRT* expression was greater in VEH pigs than in SD pigs at d 7 ($P_{(\text{time} \times \text{treatment})} < 0.05$).

Expression of hypothalamic *MC4R* did not change through time, but a trend ($P_{(\text{treatment})} = 0.07$) was observed such that expression was less in SD pigs than in VEH pigs on d 1 and d 4 postweaning, possibly due to a numerical increase in expression in VEH pigs on d 1 relative to d 0 (Figure 7). Levels of expression of *MC4R* were not different from d0 levels in either treatment group at d4 or d7 postweaning.

Expression of *AGRP* was decreased 1 d postweaning in VEH pigs but not in SD pigs, relative to d 0 (Figure 8). On d 4, levels of *AGRP* expression were lower in SD pigs than in VEH pigs, and lower than d 0 levels as well. On d7, *AGRP* expression was less in VEH pigs than in SD pigs at that time, and less than prewean levels as well ($P_{(\text{time} \times \text{treatment})} < 0.01$).

Correlations

Relationships between body weight and expression of appetite genes at d 0 (pre-weaning) were evaluated (Table 3). Body weight was positively associated with MC4R expression ($r = 0.544$; $p = 0.0146$), and tended to be positively associated with MOR expression ($r = 0.404$; $p = 0.0865$). A strong positive correlation ($r = 0.801$; $p < 0.0001$) existed between MC4R and MOR. Expression of NPY tended to be positively correlated with MC4R expression ($r = 0.418$; $p = 0.0753$), and was positively associated with MOR expression ($r = 0.694$; $p = 0.0006$). Expression of HCRT was found to be positively associated with AGRP expression ($r = 0.818$; $p < 0.0001$).

Correlations between transcript abundance of appetite genes was assessed at 1 d postweaning were also evaluated (Table 4). A positive relationship between expression of MC4R and MOR was observed at 1 d postweaning ($r = 0.515$; $p = 0.0333$), and a trend where MC4R and NPY expression were positively associated was also noted ($r = 0.421$; $p = 0.0930$). There was a strong positive correlation ($r = 0.764$; $p = 0.0002$) between expression of MC4R and HCRT. Additionally, positive relationships were also observed between MOR and NPY expression ($r = 0.612$; $p = 0.0077$) and between HCRT and AGRP expression ($r = 0.604$; $p = 0.0051$).

A strong positive correlation between expression of MOR and MC4R existed across time and treatment ($r = 0.665$; $p < 0.001$; Figure 9).

Discussion

Inhibition of piglet growth rate caused by weaning is well recognized and clearly associated with reduced feed intake (Bark et al., 1986; McCracken et al., 1995). In this experiment, weaning resulted in low feed intake and no weight gain over the first day postweaning, but pigs recovered so that both average daily feed intake and body weights were greater by d 4 and were still increasing by d 7. We did not observe an increase in average daily feed intake or body weight due to treatment with syndyphalin-33, although feed intake was numerically higher in SD animals after d 1. In a previous study in which pigs were given syndyphalin-33 then weaned into group pens, cumulative feed intake did not significantly increase (relative to intake of controls) until 9 d postweaning (Kojima et al., submitted). Obese et al. (2007) observed an increase in feed intake 48 h postinjection in adult wethers injected i.v. with SD-33 at a dosage of 0.05 mmole/kg. Differences in the timing of response may arise due to differences in age, species, route of administration, dosage, and level of concurrent stress. It should be noted that in both growing pigs (50 kg barrows; Buonomo et al., 1991) and recently-weaned pigs (Kojima et al., submitted), a single injection of SD-33 at 0.5 μ mole/kg resulted in similar increases in circulating growth hormone. The mechanism behind the prolonged, cumulative effect on feed intake is unclear. The actions of SD-33 on regulation of hypothalamic gene expression may occur much earlier than when the actual outcome (increased feed intake) is observed.

The stress of weaning numerically increased expression of the μ -opiate receptor in VEH pigs on d 1 and d4, but this was not evident in SD pigs; indeed, by d 4 the difference in expression levels between VEH and SD pigs was significant. While there is some evidence that stress (and subsequent release of endogenous opioids) would predict a down-regulation of the opiate receptors (Seeger et al., 1984), this response may be dependent on age. In young rats experiencing stress due to hot plate exposure, opiate receptor levels in the hypothalamus were significantly higher in the stressed animals as compared to controls. It was speculated that by stressing the young underdeveloped brain of these animals, the development of an over functioning defense system arose (Torda et al., 1978). Obese et al. (2007) observed that the increase in feed intake in adult wethers by SD-33 was blocked by naloxone, suggesting that the effect was mediated (at least in part) through μ -opiate receptors. In the present study, a down-regulation of hypothalamic *MOR* expression was observed in SD pigs relative to VEH pigs at d 4, supporting the notion that SD-33 binds the μ -opiate receptor as μ -opioids will readily down-regulate their receptors by many different mechanisms, including modulation of gene expression (Gach et al., 2008). It is impossible to say when the down-regulation first occurred, although it is tempting to speculate that it had actually began prior to sample collection at d 1, as evidenced by the numerical difference between treatment groups at that time. By d 7, *MOR* expression in VEH pigs had decreased to its lowest observed level while expression in SD pigs was no different from pre-wean

levels, suggesting that homeostatic mechanisms may have been activated to alleviate the effects of both weaning and SD-33 on *MOR* expression.

In the present study, a trend was observed where weaning tended to increase expression of *NPY* in VEH pigs more than in SD pigs at d 1 and d 4 postweaning, but *NPY* expression had decreased to pre-weaning levels by seven days postweaning in those animals. The expression patterns of *NPY* during the weaning process in the pig are largely unknown. Previously, we found that hypothalamic *NPY* mRNA expression at 4 d postweaning was positively associated with weaning weight (Kojima et al., 2007). When rats are stressed they also exhibit increased expression of *NPY* in the arcuate nucleus (Makino et al., 2000). Expression of *NPY* in SD animals did not appear to fluctuate over the course of the study. Opioid agonists have been shown to stimulate feed intake by up-regulating activity of *NPY* (Kotz et al., 1993; Pomonis et al., 1997; Dodo et al., 2005). It is possible that we missed modulation of expression that occurred before or after the times we chose for tissue collection. It is also unclear if the apparent increase in *NPY* expression in VEH pigs is a consequence of stress or a response to hunger, as transcription activity of the *NPY* gene will increase during fasting or feed-restriction (Shimokawa et al., 2003; minor et al., 2009).

Orexin (hypocretin) will increase feed intake in pigs (Dyer et al., 1999) as well as many other animals (as reviewed by Benoit et al., 2008). Similar to *NPY*, post-weaning levels of *HCRT* expression are dependant to some extent on weaning weight, such that heavier pigs have greater hypothalamic expression of *HCRT*

(Kojima et al., 2007). A stressful event such as immobilization stress in young rats will increase transcript abundance of *HCRT* (Ida et al., 2000). Here, the stress of weaning increased *HCRT* expression at d 1 and d 7 postweaning in VEH pigs, but not in SD pigs. Treatment with SD-33 may have resulted in the down-regulation of *HCRT* expression; Li and van den Pol (2008) showed that exogenous opiates such as morphine will depress the hypocretin/orexin arousal system.

Stress, in particular electrical foot shock in rats, will increase expression of the *MC4R* gene (Yamano et al., 2004). Opiates, on the other hand, will decrease expression of *MC4R* (Chaki et al., 2005). This coincides with what was observed when piglets were weaned and treated with SD-33. We observed an increase in *MC4R* expression due to weaning 1 d after the piglet was removed from the sow, but this increase was negated when the piglet received treatment with SD-33 at weaning. By 4 d postweaning, *MC4R* expression had essentially returned to pre-weaning levels in both treatment groups.

Agouti-related protein is an *MC4R* antagonist (Cone, 2005) and will increase feed intake (as reviewed by Pritchard and White, 2005). The stress of weaning caused an increase in expression of *AGRP* on day one followed by a sharp decrease in expression on day four followed by an increase by seven days post weaning. A stressful event will decrease the expression of *AGRP* (Kas et al., 2005). Opiates have been shown to increase activity of *AGRP* (Hagan et al., 2001). In the present study, we also observed an increase in the expression of *AGRP* due to the effects of an opiate.

The (83-132) splice variant of AGRP has long lasting orexinogenic effects (Hagan et al., 2000). We observed changes in AGRP and MC4R expression levels due to SD-33 at 1 d postinjection, yet previous studies in sheep (Obese et al., 2007) and pigs (Kojima et al., submitted) consistently describe a delayed response in feed intake. Hagan et al. (2000) suggests that the prolonged effects of AGRP - (83-132) may stem from its role in regulating transcription and synthesis of other proteins further downstream in the appetite pathway. Interestingly, AGRP has also been shown to increase activity at opioid receptors. When AGRP– (82-132) was given simultaneously with naloxone, the short-term orexinogenic affects of AGRP– (82-132) were decreased, but the long-term effects were not altered (Hagan et al., 2001). Although modulation of opioid action by AGRP – (82-132) appears to be of a short duration, it may contribute to the overall effects of SD-33. It is important to note that the primers we used for quantitating expression of AGRP were not designed to be specific for a particular splice variant, and so any effect of SD-33 on AGRP – (82-132) abundance is unknown.

Obese et al. (2007) observed that the increase in feed intake by SD-33 was blocked by naloxone, suggesting that the effect was mediated through μ -opioid receptors. Appetite is stimulated by μ -opiate agonists in many ways: by upregulating activity of the appetite stimulators *AGRP* and *NPY* (Hagan et al., 2001; Brugman et al., 2002) and by decreasing synthesis of the appetite suppressor α -melanocyte stimulating hormone (α -MSH), a product of the proopiomelanocortin

(*POMC*) gene (Wardlaw et al., 1996). Opiates also act to decrease expression of the *MC4R* gene, the product of which is the receptor which binds both α -MSH and AGRP (Chaki et al., 2005). Stress also regulates the expression of many of these regulators of appetite. Kas et al. (2005) observed that in rats, *AGRP* mRNA expression in the arcuate nucleus is decreased following a stressful event, contributing to increased sensitivity for α -MSH and subsequent decreased feed intake. Liu et al. (2007) observed that forced swimming and restraint increased *POMC* gene expression and suppressed feeding, but pretreatment with an *MC4R* antagonist blocked the anorectic and anxiogenic effects of these stressors, suggesting that the melanocortinerbic pathway is heavily involved in stress-related anorexia.

Several positive relationships were found to exist between body weight and the expression of appetite-regulating genes prior to weaning. Body weight was positively correlated with *MC4R* and *MOR* on d 0. But these become uncoupled at 24 h postweaning.

The positive relationships between *MC4R* and *NPY*, *MOR* and *NPY*, and *HCRT* and *AGRP* remain through the first 24 hours of weaning. The positive relationship between *HCRT* and *MC4R* becomes apparent 24 hours after weaning. This may be a result of the stress of weaning, as stress has been shown to increase transcriptional activity of both of these genes (Ida et al., 2000; Yamano et al., 2004). We observed a strong correlation between *MC4R* and *MOR* expression across all time and treatment, supporting the concept that the melanocortinerbic pathway is influenced

by opioid tone at all times. This is in agreement with Chaki et al. (2005) who observed that opioids down-regulated MC4R expression. As an opioid agonist, SD-33 would be expected to down-regulate transcription of its receptor as well as MC4R, resulting in a positive association between the expression of those two genes.

Opioid administration was found to be an effective method of elevating separation-induced stress in infant rhesus monkeys separated from their mothers (Kalin et al., 1988), and in speeding post-operative recovery (including recovery of appetite) in young (20 kg) pigs (Malavasi et al., 2006). Similarly, the synthetic enkephalin SD-33 also has the potential to alleviate the negative effects of stress during weaning in pigs, and has the potential to increase feed intake through effects on the melanocortinerbic pathway. Further investigation is needed to more fully elucidate the complex mechanisms by which syndyphalin-33 may act to abrogate the post-weaning growth lag in pigs.

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Appendices

Appendix A

Tables

Table 1. Percentage of Nursery Pig Deaths by Producer-identified Causes in 2006¹

Cause	%	Std. Error
Scours	12.5	1.1
Starvation	9.8	0.9
Respiratory Problems	44.2	2.3
CNS/Meningitis	18.7	1.9
Other identified problems	4.1	0.8
Unknown problem	10.7	1.1
Total	100	

¹Adapted from (USDA, 2008)

Table 2. Details of primers and probes used for real-time PCR

Gene ¹	Forward Primer Sequence	Reverse Primer Sequence	Probe Sequence	Efficiency (%)	Concentration (ng/μl)
18-S ²	GGCCTGCTTTGAACACTCTAATTTT	CCCCTCGATGCTCTTAGCT	CCCCGCGGGACT	102	25.00
18-S ³	GGCCTGCTTTGAACACTCTAATTTT	CCCCTCGATGCTCTTAGCT	CCCCGCGGGACT	102	1.00
18-S ⁴	GGCCTGCTTTGAACACTCTAATTTT	CCCCTCGATGCTCTTAGCT	CCCCGCGGGACT	94	10.00
MC4R	GTAATTCCATCATCGATCCCCTGAT	GGGATAGCAACAGATGATCTCTTTGA	CCGGAGCCAAGAACT	104	25.00
MOR	TGGTCTGCCTGTGATGTTTCATG	GGTGAGAGAATGTTAGTGCACAATC	AACCATTCCGGTACTTTGT	97	25.00
NPY	TCGGCGTTGAGACATTACATCA	GTCTCGGGACTAGATCGTTTTCC	CACCAGGCAGAGATAC	105	1.00
HCRT	GGCCGGCATCCTCACT	GCAGCCGGCCTTGCA	CCGCCGCTTGCCCA	92	1.00
AGRP	GCAGGCCGAGGCCAA	CGTGCCTTGCGTCCTC	CAGAGGTGCTAGATCCT	85	10.00

¹MC4R = Melanocortin 4 Receptor; MOR= μ -Opiate Receptor; NPY = Neuropeptide Y; HCRT = Hypocretin; AGRP = Agouti Related Protein.

²Used to normalize MC4R and MOR.

³Used to normalize NPY and HCRT.

⁴Used to normalize AGRP.

Table 3. Correlations of messenger RNA expression of appetite regulators before weaning.

Variables ¹		r	p-value
x	y		
d 0 wt	MC4R	0.544	0.0146
d 0 wt	MOR	0.404	0.0865
d 0 wt	NPY	0.155	NS ²
d 0 wt	HCRT	0.028	NS
d 0 wt	AGRP	-0.030	NS
MC4R	MOR	0.801	<0.0001
MC4R	NPY	0.418	0.0753
MC4R	HCRT	-0.133	NS
MC4R	AGRP	0.007	NS
MOR	NPY	0.694	0.0006
MOR	HCRT	-0.199	NS
MOR	AGRP	-0.153	NS
NPY	HCRT	-0.0231	NS
NPY	AGRP	-0.097	NS
HCRT	AGRP	0.818	<0.0001

¹ d 0 wt= pre weaning body weight; MC4R= Melanocortin 4 Receptor; MOR= μ -Opiate Receptor; NPY= Neuropeptide Y; HCRT= Hypocretin/Orexin; AGRP= Agouti Related Protein.

² NS= not significant.

Table 4. Correlations of messenger RNA expression of appetite regulators 24 hours after weaning.

Variables ¹		r	p-value
x	y		
MC4R	MOR	0.515	0.0333
MC4R	NPY	0.421	0.0930
MC4R	HCRT	0.764	0.0002
MC4R	AGRP	0.283	NS ²
MOR	NPY	0.612	0.0077
MOR	HCRT	0.283	NS
MOR	AGRP	0.034	NS
NPY	HCRT	-0.006	NS
NPY	AGRP	-0.380	NS
HCRT	AGRP	0.604	0.0051

¹ MC4R= Melanocortin 4 Receptor; MOR= μ -Opiate Receptor; NPY= Neuropeptide Y; HCRT= Hypocretin/Orexin; AGRP= Agouti Related Protein.

² NS= not significant.

Appendix B

Figures

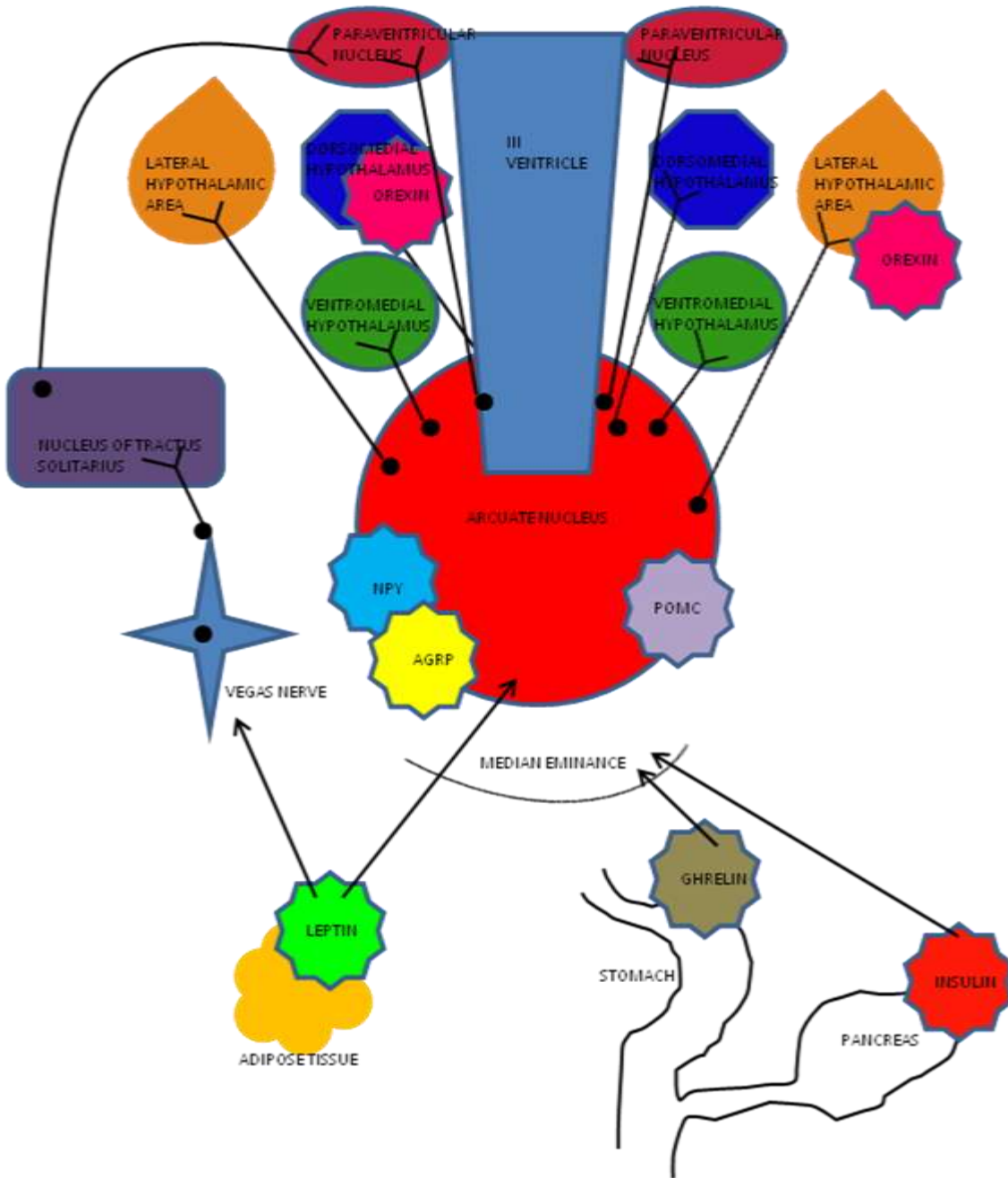


Figure 1. Hypothalamic regulation of appetite. Pathway depicting the central hypothalamic and peripheral neuropeptides and their sites of origin. Adapted from Arora et al., 2006.

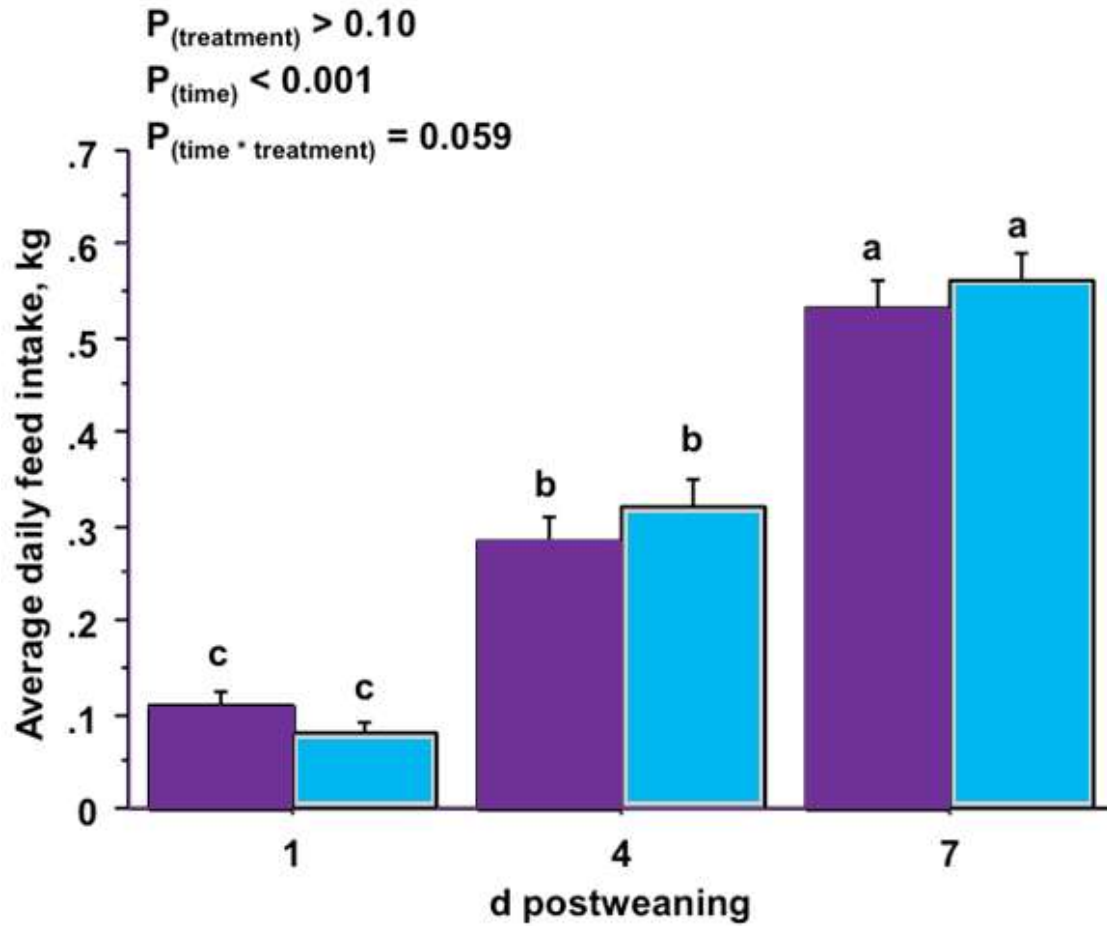


Figure 2. Average Daily Feed Intake. Average daily feed intake of pigs injected with saline (VEH; purple bars) or 0.05 $\mu\text{mol/kg}$ syndyphalin-33 (SD-33; blue bars) prior to weaning. Raw means with SE are shown. Within a graph, means with different letters differ ($P < 0.05$).

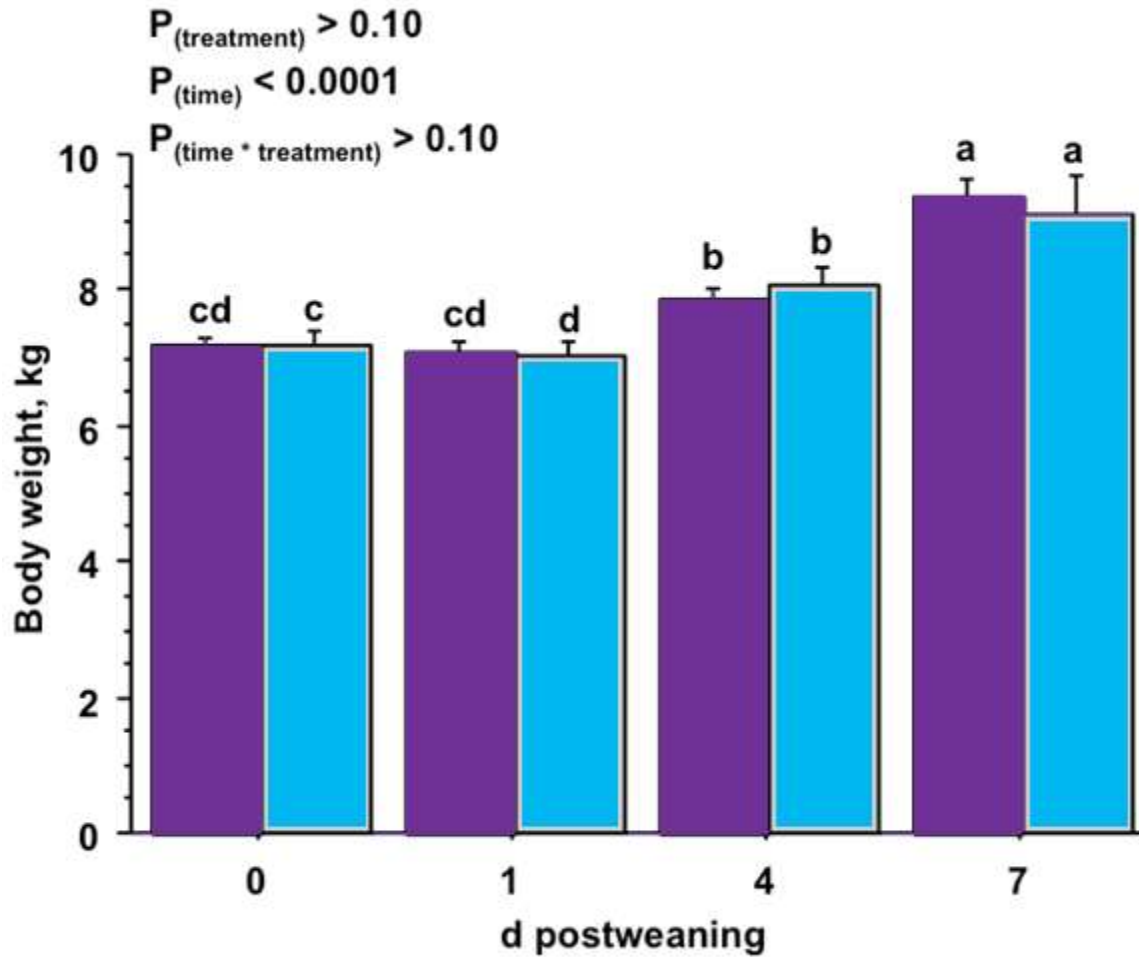


Figure 3. Average Daily Body Weight. Average daily body weight of pigs injected with saline (VEH; purple bars) or 0.05 $\mu\text{mol/kg}$ syndyphalin-33 (SD-33; blue bars) prior to weaning. Raw means with SE are shown. Within a graph, means with different letters differ ($P < 0.05$).

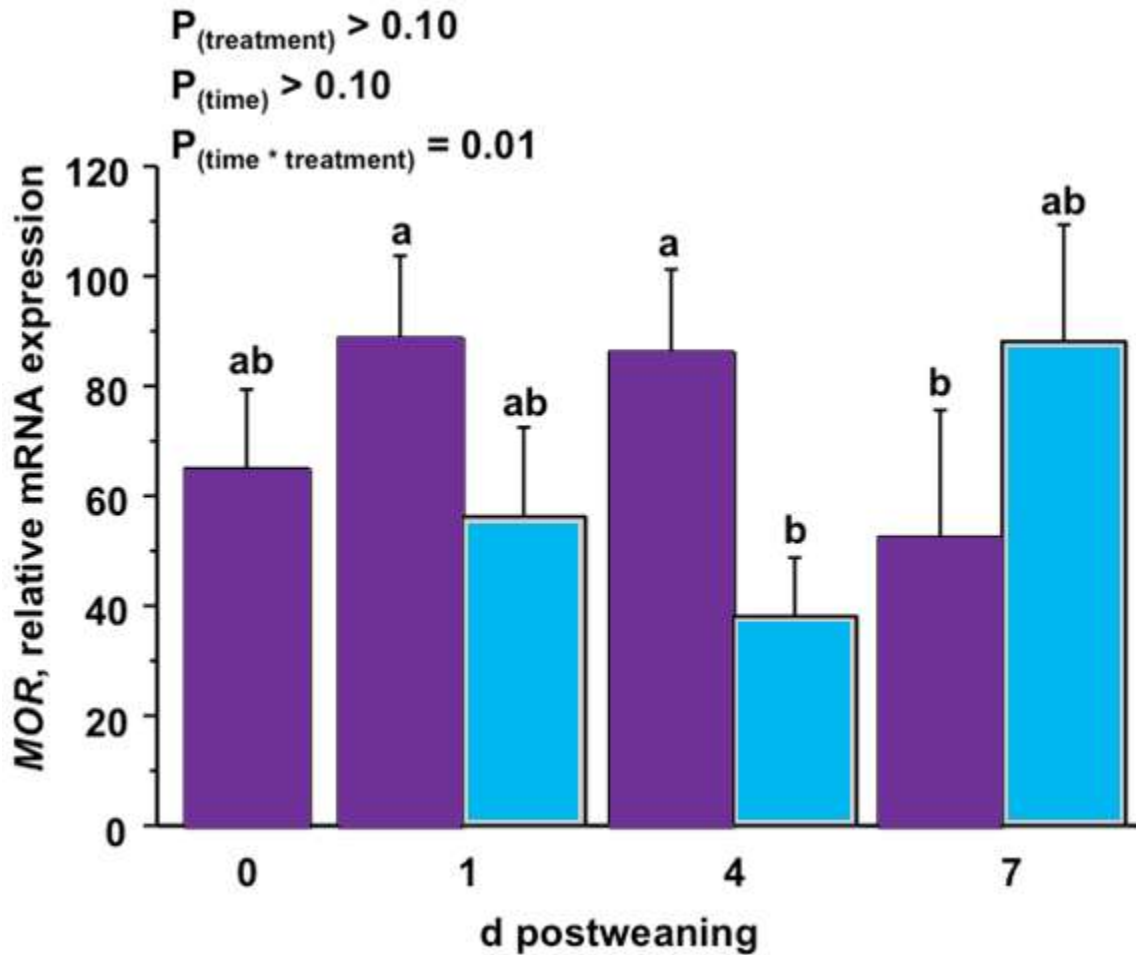


Figure 4. Relative hypothalamic transcript abundance of μ -Opiate Receptor. Relative hypothalamic transcript abundance of the MOR gene in pigs injected with saline (VEH; purple bars) or 0.05 $\mu\text{mol/kg}$ syndyphalin-33 (SD-33; blue bars). Raw means with SE are shown. Within a graph, means with different letters differ ($P < 0.05$).

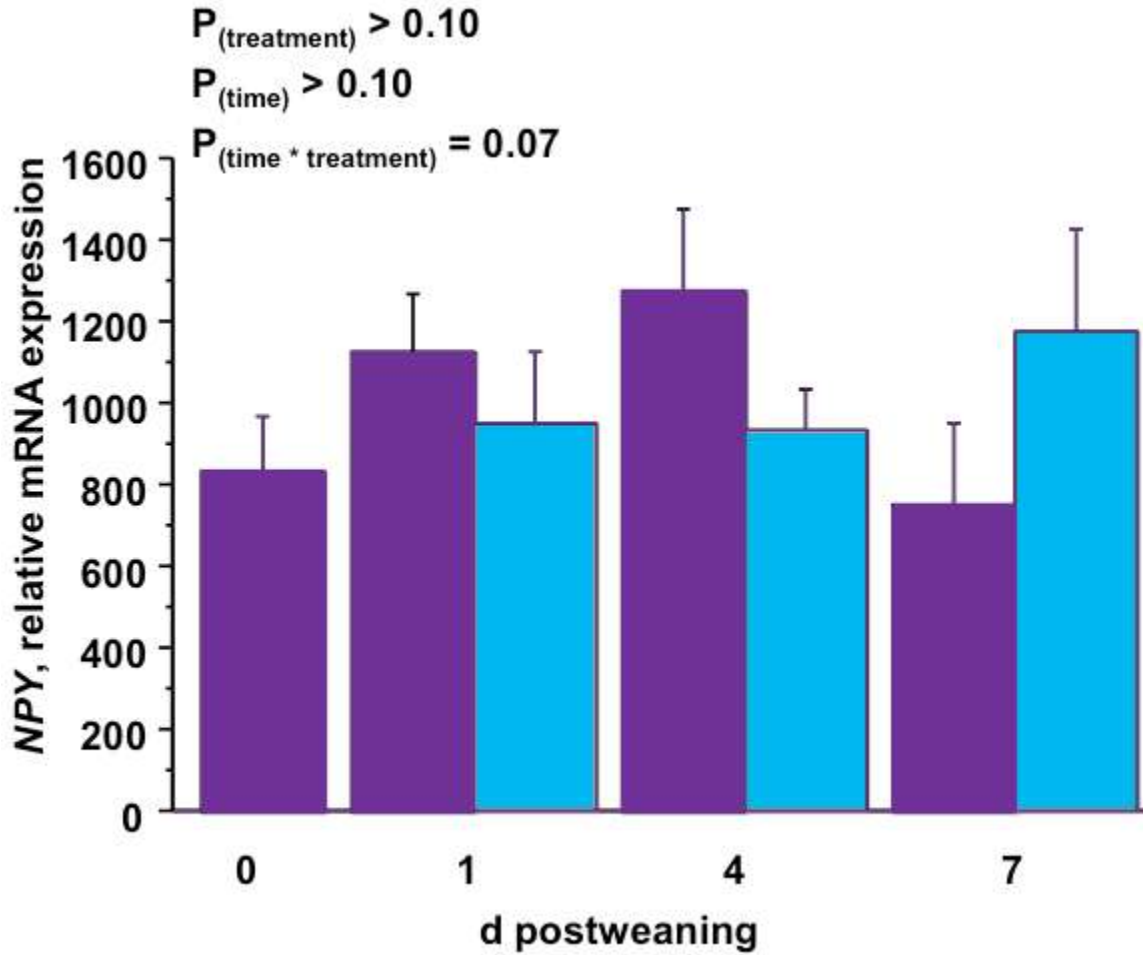


Figure 5. Relative hypothalamic transcript abundance of Neuropeptide Y. Relative hypothalamic transcript abundance of the NPY gene in pigs injected with saline (VEH; purple bars) or 0.05 $\mu\text{mol/kg}$ syndyphalin-33 (SD-33; blue bars). Raw means with SE are shown. Within a graph, means with different letters differ ($P < 0.05$).

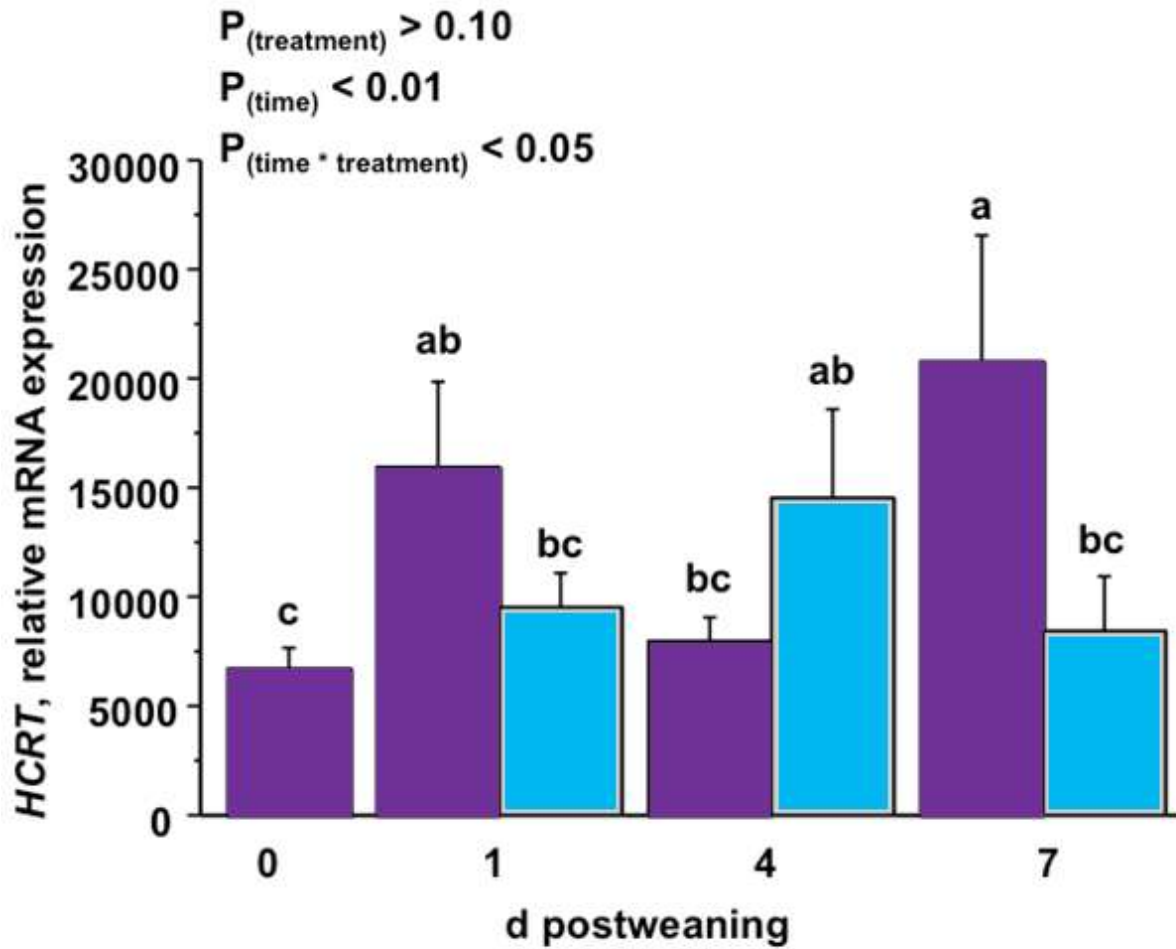


Figure 6. Relative hypothalamic transcript abundance of Orexin. Relative hypothalamic transcript abundance of the HCRT gene in pigs injected with saline (VEH; purple bars) or 0.05 $\mu\text{mol/kg}$ syndyphalin-33 (SD-33; blue bars). Raw means with SE are shown. Within a graph, means with different letters differ ($P < 0.05$).

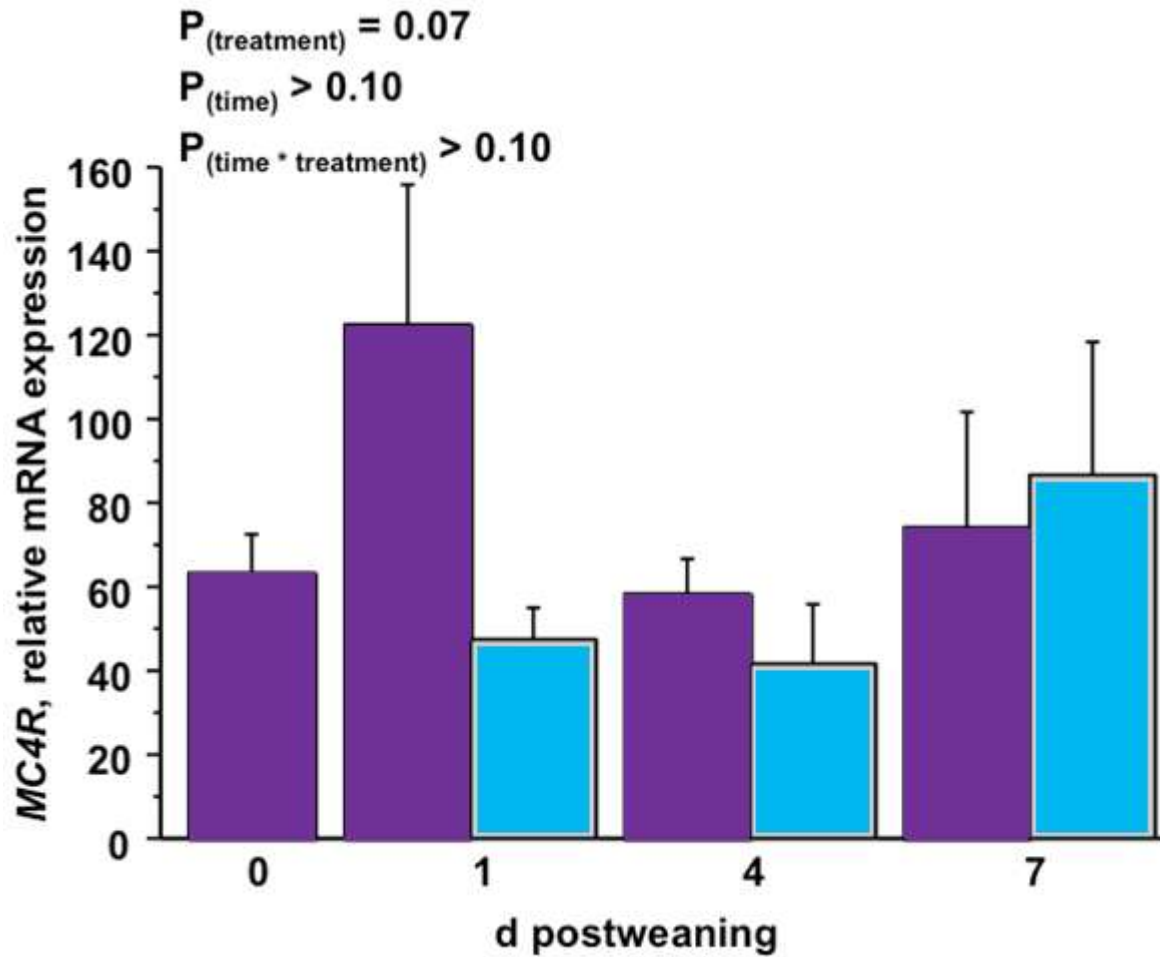


Figure 7. Relative hypothalamic transcript abundance of Melanocortin 4 Receptor. Relative hypothalamic transcript abundance of the MC4R gene in pigs injected with saline (VEH; purple bars) or 0.05 $\mu\text{mol/kg}$ syndyphalin-33 (SD-33; blue bars). Raw means with SE are shown. Within a graph, means with different letters differ ($P < 0.05$).

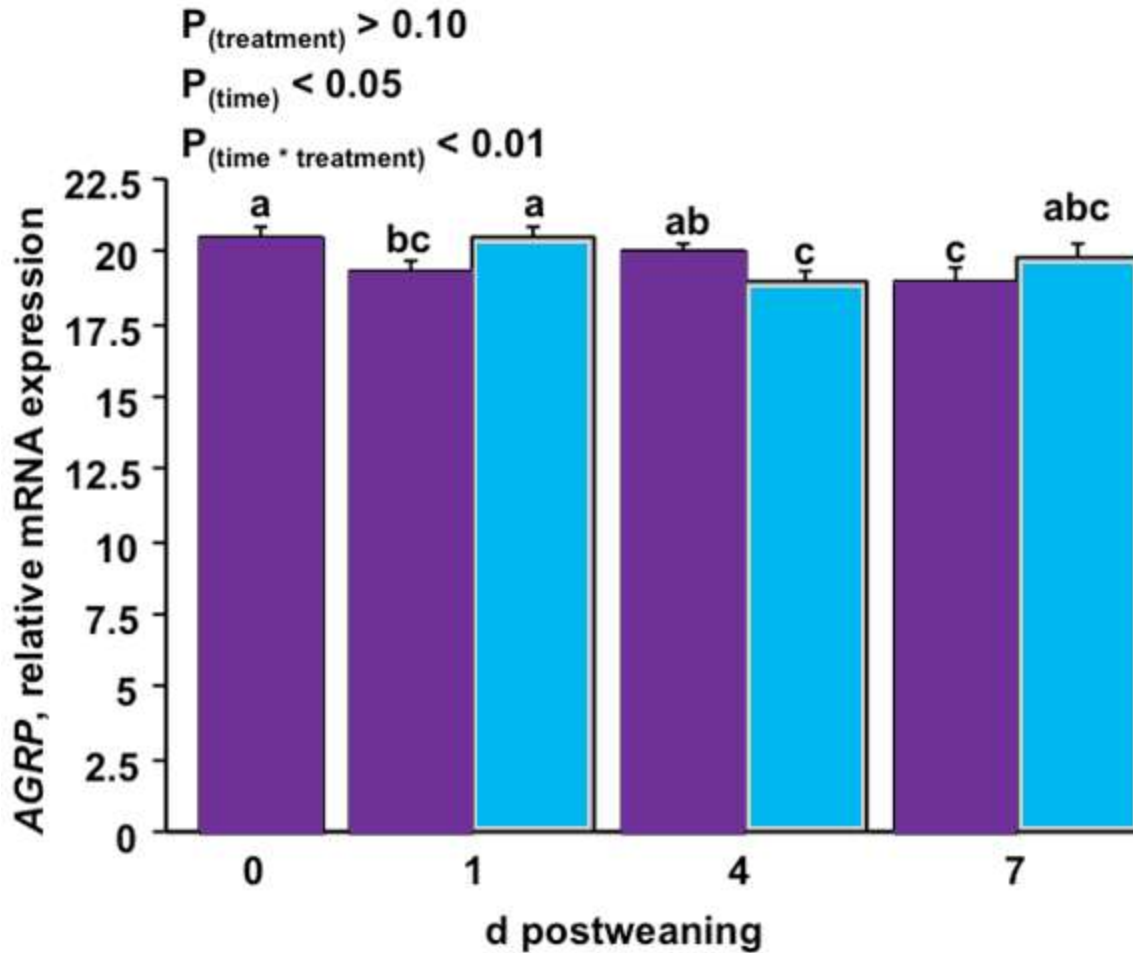


Figure 8. Relative hypothalamic transcript abundance of Agouti-Related Protein. Relative hypothalamic transcript abundance of the AGRP gene in pigs injected with saline (VEH; purple bars) or 0.05 $\mu\text{mol/kg}$ syndyphalin-33 (SD-33; blue bars). Raw means with SE are shown. Within a graph, means with different letters differ ($P < 0.05$).

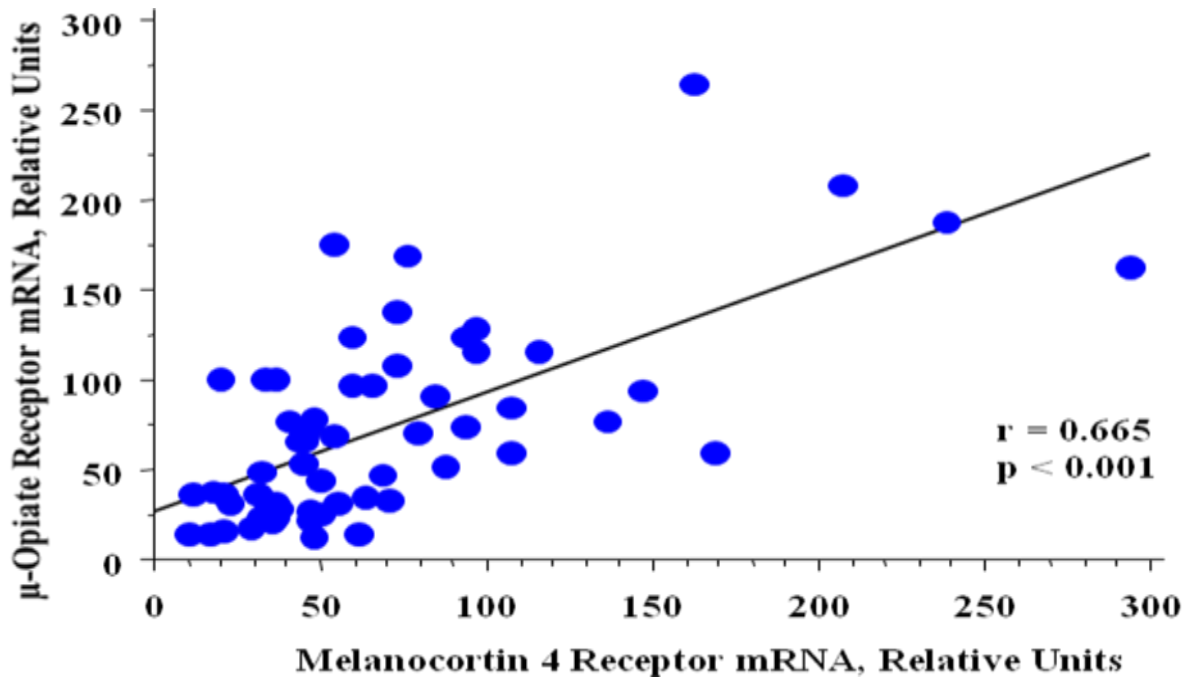


Figure 9. Linear regression of μ -Opiate Receptor and Melanocortin 4 Receptor relative hypothalamic transcript abundance. Data from all treatments across all time points are included in this analysis.

Appendix C

Procedures

Procedure 1. Isolation of total mRNA

1. Homogenize tissue sample in 10-20 volumes of TRI¹
2. Incubate at room temperature for 5 min.
3. Centrifuge at 12,000 x g for 10 min. @ 4°C
4. Transfer supernatant to a fresh tube²
5. Add 100 µl BCP³ per 1 ml TRI
6. Mix well and incubate at room temperature for 15 min.
7. Centrifuge at 12,000 x g for 10-15 min. @ 4°C
8. Transfer aqueous phase to a fresh tube
9. Add 500 µl isopropanol per 1 ml TRI
10. Mix well / vortex and incubate at room temperature for 10 min.
11. Centrifuge at 12,000 x g for 8 min. @ 4-25°C
12. Discard supernatant
13. Add 1 ml 75% ethanol per 1 ml TRI
14. Centrifuge at 7,500 x g for 5 min.
15. Remove ethanol and briefly dry
16. Dissolve total mRNA in buffer⁴

¹ TRI reagent solution (Ambion, Forest City, CA)

² Repeat this step if sample contains high lipid content

³ BCP= bromochloropropane

⁴ 50 µl H₂O

Procedure 2. Quality/Quantity analysis of total mRNA

1. Add 1 μl mRNA and 99 μl H_2O + several 100 μl H_2O (blanks) to a 96 well optically clear plate
2. Read samples at 260 nm¹ and 280 nm using mass spectrometry
3. Subtract the blank value obtained from the pure H_2O reading for both the 260 and 280 values
4. Obtain the quality by evaluating the 260/280 ratio² by dividing as such
5. Obtain the quantity by multiplying (260 nm value * 40 * 100)³

¹ nm= nanometers

² ideal 260/280 ratio is 2.0

³ ng/ μl

Procedure 3. Removal of DNA contamination¹

1. Bring all samples to 500 ng/μl concentration
2. Use 50 μl sample
3. Add 5 μl 10X TURBO DNase Buffer and 1 μl DNase
4. Mix by inversion
5. Incubate at 37°C for 30 min.
6. Add 5 μl re-suspended DNase Inactivation Reagent
7. Mix well
8. Incubate at room temperature for 2 min.
9. Centrifuge at 12,000 x g for 90 sec.
10. Transfer supernatant to RNase-free tube

¹Turbo DNA-Free kit (Ambion, Forest City, CA)

Procedure 4. cDNA synthesis by reverse transcription¹

1. Mix to obtain a total volume of 20.0 μ l
 - 10 μ l 2X Buffer
 - 1 μ l 20X Enzyme Mix
 - 2 μ l mRNA sample
 - 7 μ l nuclease-free H₂O
2. Incubate reaction at 37°C for 60 min.
3. Stop reaction by heating to 95°C for 5 min.
4. Hold at 4°C
5. Store cDNA² at -20°C

¹ High Capacity RNA-to-cDNA Kit (Applied Biosystems, Forest City, CA)

² cDNA= complementary DNA

Procedure 5. Determining real-time PCR primer efficiency

1. Create a serial dilution using pooled cDNA for each primer¹
2. Using standard cycling conditions², obtain the C_T value for each dilution point
3. Graph the log of the dilution on the X-axis and the C_T value on the Y-axis
4. Connect the points with a trend line and obtain the slope of the line
5. Use the formula: $10^{[(1/|\text{slope}|)-1]}$ to obtain the efficiency³

¹Use a wide dilution range ex: 1:10 – 1:100,000

² Explained in greater detail in procedure 6

³ Pfaffl, 2001

Procedure 6. Real-time PCR analysis

1. Mix to obtain a total volume of 20.0 μl
 - 10 μl Gene Expression Master Mix¹
 - 7 μl H₂O
 - 2 μl cDNA²
 - 1 μl Primer/Probe Mix³
2. Run⁴ using the following parameters
 - 2 min. at 50°C
 - 8.5 min. at 95°C
 - 45 Cycles of 15 sec 95°C/ 1 min. 60°C
 - Hold at 4°C
3. Obtain target and reference gene C_T values
4. Subtract the reference gene C_T from the target gene C_T = Δ C_T
5. Transform linearly to obtain relative expression value by using the formula:

$$(2^{-\Delta\text{CT}})^5$$

¹ Gene Expression Master Mix (Applied Biosystems, Forest City, CA)

² Use dilutions from the linear part of the efficiency graph from procedure 5

³ Taqman Gene Expression assay primer/probe mix (Applied Biosystems, Forest City, CA)

⁴ BIO-RAD iCycler

⁵ Livak et al., 2001

VITA

Tabatha Anne Cooper was born in Tampa, FL in November of 1984 to Thomas and Tracy Sawyer. She was raised in Florida and in Tennessee. Tabatha graduated from Farragut High School in 2003. She obtained her Bachelor of Science degree from The University of Tennessee, Knoxville with a major in Biology in 2007. Tabatha began her graduate career at the University of Tennessee in August of 2007, with a Major in Animal Science and a Minor in Statistics. She is a member of Gamma Sigma Delta. She served as the Animal Science Graduate Student Association President for the 2008-2009 academic year. And, she was the Animal Science Department representative on the Graduate Student Senate.