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Effects of weaning and weaning weight on neuroendocrine regulators of feed intake in pigs

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ABSTRACT: A depression in feed intake and growth often occurs in the weaned pig. Spray-dried plasma is often added to nursery diets in an attempt to stimulate feed intake during this lag. The current study evaluated gene expression of appetite regulators in hypothalamus and adipose tissue 4 d after weaning. Barrows (2 wk of age) were cross-fostered to a sow (SOW, n = 8) or weaned and fed a nursery diet containing either 0 or 7% spray-dried plasma (NP, n = 8, and SDP, n = 8, respectively). Piglets were allocated such that 2 size groups existed within each experimental group: small (3.5 to 4.3 kg of BW piglets) and large (4.6 to 5.7 kg of BW piglets) subsets, based on weaning weight (WW), existed within each experimental group: small (3.5 to 4.3 kg piglets) and large (4.6 to 5.7 kg piglets). Animals were killed 4 d after weaning for tissue collection. There was a weaning group × WW interactive effect (P < 0.05) on hypothalamic neuropeptide Y messenger RNA expression, such that expression was least in the small SDP piglets. No WW or weaning group effects were seen on adipose leptin, hypothalamic leptin receptor, or hypothalamic proopiomelanocortin gene expression. An effect of WW was seen on hypothalamic neuropeptide Y, agouti-related protein, orexin, and type 2 orexin receptor gene expression, such that large pigs expressed greater amounts of these transcripts (P < 0.002). Strong positive correlations in gene expression were found among all of these genes, whose products are known to stimulate appetite. Partial correlation controlling for initial WW revealed that preweaning size explained most if not all of these associations. These data suggest that the postweaning expression of appetite-regulating genes is more dependent on preweaning conditions than on weaning diet.

Key words: appetite, growth, neuroendocrine, piglet, weaning

INTRODUCTION

The 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). The biological mechanisms underlying the postweaning growth lag are poorly understood. Suppressed intake may result from stressors encountered at weaning, such as maternal separation, relocation to new housing, introduction into new social groups, and changing to a dry diet (Hampson and Kidder, 1986; Ekkel et al., 1995). Few studies have evaluated metabolic and endocrine responses of the pig during weaning (Carroll et al., 1998; Matteri et al., 2000). Typical of the endocrine profile seen during inadequate feed intake (Buonomo and Baile, 1991), weaning the piglet increases concentrations of GH and decreases serum concentrations of IGF-I and IGF-II (Carroll et al., 1998; Matteri et al., 2000). Importantly, although weaning clearly decreases feed intake, associated changes in the expression of appetite-controlling genes such as leptin, neuropeptide Y (NPY), orexin, type 2 orexin receptor, proopiomelanocortin (POMC), and agouti-related protein remain to be determined. An inadequate understanding of the effects of weaning on these
gene products limits our capability to develop targeted strategies for maintaining early postweaning performance.

The objective of the current study was to evaluate the expression of appetite-regulating genes in response to weaning. To produce contrasting states of growth and feed intake, pigs were not weaned (cross-fostered to a lactating sow within the same farrowing room), weaned onto a standard starter diet, or weaned onto a diet containing spray-dried plasma. The use of spray-dried plasma has been reported to significantly improve feed intake, intestinal development, and growth in weaned pigs (Coffey and Cromwell, 1995; de Rodas et al., 1995; Touchette et al., 1997, 1998; Matteri et al., 2000).

MATERIALS AND METHODS

All animals were treated in accordance with the guidelines of the University of Missouri Animal Care and Use Committee.

Experimental Design

A total of 24 barrows (14 d of age), with an initial BW of 4.69 ± 0.13 kg, were allotted to 1 of 3 treatments groups (8 pigs per group) as follows:

- SOW: pigs were cross-fostered to a sow in 1 farrowing crate, such that the sow was not the natural mother to any of the pigs;
- NP: pigs were contained in 1 pen and given a standard phase I diet containing 0% spray-dried plasma; and
- SDP: pigs were contained in 1 pen and given a standard phase I diet containing 7% spray-dried plasma.

Pigs were allocated by size, resulting in groups of small (3.95 ± 0.07 kg of BW, 3.5- to 4.3-kg range) and large (5.10 ± 0.09 kg of BW, 4.6- to 5.7-kg range) animals distributed across weaning groups (4 small and 4 large/group).

Pigs were checked at least once daily for health problems and feed availability. All pigs were allowed access to food and water at all times. Body weights were recorded daily for each pig. The phase I diets were formulated to contain equal amounts of digestible essential AA and also ME and are described in detail by Matteri et al. (2000).

On d 4 of treatment, all pigs were euthanized by electrical stunning followed by exsanguination. Animals were euthanized randomly with regard to treatment group from 0800 to 0930 h. Hypothalamic and s.c. adipose tissues were quickly removed and placed on dry ice until stored at −80°C for subsequent RNA isolation. The collected hypothalamic tissue was bounded by the cranial edge of the optic chiasm, the caudal edge of the mammillary bodies, and dorsally by the hypothalamic sulcus. The adipose tissue was collected from the back of the neck at the site of decapitation. A blood sample was also collected for serum hormone analyses, the results of which were previously published (Matteri et al., 2000) and reviewed briefly in the discussion section of this manuscript.

Riboprobe Generation

Polymerase chain reaction was used to amplify complementary DNA (cDNA) for NPY, orexin, type 2 orexin receptor, long-form leptin receptor, agouti-related protein, and POMC; adipose leptin expression was also measured (note that orexin as the term is used in this manuscript refers to the prepro-orexin transcript; the gene products orexin-A and orexin-B are both end products of this transcript).

Hybridization Assays

Total cellular RNA was isolated by using a commercial reagent (TRI Reagent, Molecular Research Company, Cincinnati, OH) and was transferred to a nylon membrane with a slot-blot apparatus (BioDot SF, BioRad Laboratories, Hercules, CA). Quality and concentration of RNA samples were determined by UV spectrophotometry at 260 and 280 nm, and all samples were brought to a final concentration of 2 μg/mL. Hybridization to specific biotinylated complementary RNA probes for use in chemiluminescence-based hybridization detection. Primer sequences used for PCR amplification of cDNA, references describing the initial probe generation for each target, and Northern blot data are shown in Table 1. The sequence of the porcine type 2 orexin receptor cDNA fragment, which had not been previously characterized, has been submitted to GenBank (accession number AF059740).

Statistical Analysis

Homogeneity of variance was confirmed throughout the data set by F-test. Body weight data were evaluated by ANOVA (weaning weight [WW] × weaning group with repeated measures over time). Expression data for feed intake regulators were analyzed by 2-way ANOVA (WW × weaning group). Weaning weight was removed from the model for any endpoints for which there was not a
significant effect, and the results from the simplified model were presented. Fisher’s LSD test was used to compare means. These analyses were performed with Statview (SAS, Cary, NC). To better understand the apparent interrelationships among the expression of appetite-regulating genes, partial correlations were performed, controlling for initial pig WW. This statistical analysis was performed with SAS.

It can be argued that pen and treatment are confounded, and therefore, the results are due to the effect of pen; however, we have not observed an effect of pen in previous studies in these facilities (Carroll et al., 2002; Touchette et al., 2002; Frank et al., 2003).

### RESULTS

Daily BW for each treatment are illustrated in Figure 1A. Cross-fostered pigs gained more BW over the 4-d experimental period than did those pigs fed dry feed, regardless of SDP content (P < 0.001, Figure 1B). At d 4, however, the SDP group gained the same amount as the SOW group; the NP group did not experience that gain (P < 0.001, Figure 1C). There was no effect of WW on these gain-related endpoints. Body weight data from this study (along with serum concentrations and gene expression of growth-related gene products) have been previously reported and discussed (Matteri et al., 2000).

Levels of messenger RNA (mRNA) for hypothalamic orexin, type 2 orexin receptor, NPY, and agouti-related protein were greater in large as compared with small pigs (P < 0.001, Figure 2). A weaning group × WW interaction (P < 0.05) was detected for hypothalamic NPY mRNA expression, such that expression was least in the small SDP pigs. Expression of genes encoding adipose leptin, hypothalamic long-form leptin receptor, or hypothalamic POMC expression was not affected by weaning group or WW (P > 0.1).

Partial regression analysis was performed for gene expression data controlling for pig WW (Table 2). The strength of the relationship between the expression levels of any 2 genes was in most cases due in large part to the association of gene expression to initial pig WW. After controlling for pig WW, only the relationships between the expression of type 2 orexin receptor and POMC and between agouti-related protein and POMC remained very strong (P < 0.001). Relationships between expression of orexin and type 2 orexin receptor, orexin and agouti-related protein, type 2 orexin receptor and agouti-related protein, and type 2 orexin receptor and leptin receptor remained (P < 0.05) but were weakened considerably by controlling for WW.

### DISCUSSION

One of the greatest challenges in swine nutrition is to decrease the immediate postweaning growth lag by stimulating feed intake. A better understanding of the effect of weaning on the regulation of appetite and growth is necessary for the development of targeted approaches to enhance postweaning performance. We have previously observed changes in serum concentrations of GH (elevated), IGF-I (decreased), and IGF-II four days after weaning in young pigs (Carroll et al., 1998), likely influenced by the well-established effects of depressed

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Table 1. Details of complementary RNA probes used in hybridization assays

<table>
<thead>
<tr>
<th>Probe</th>
<th>Reference</th>
<th>Primers</th>
<th>Length (bp)</th>
<th>Northern analysis approximate size (kb)</th>
<th>Human messenger RNA size (kb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NPY</td>
<td>(Salfen et al., 2003)</td>
<td>GCCACCATGCTAGGTAACAA ATGTGGTGATGGGAAAT</td>
<td>311</td>
<td>0.8</td>
<td>0.551</td>
</tr>
<tr>
<td>Long-form leptin receptor</td>
<td>(Lin et al., 2000)</td>
<td>TCGGAGATATCAGTTGGA</td>
<td>313</td>
<td>5.0</td>
<td>0.515</td>
</tr>
<tr>
<td>POMC</td>
<td>(Daniel et al., 1999)</td>
<td>GTGGGAGATCCGAGATTGT CTCCTCTCTGCGGTCTCTC</td>
<td>343</td>
<td>1.5</td>
<td>1.245, 1.295</td>
</tr>
<tr>
<td>Agouti-related protein</td>
<td>(Salfen et al., 2003)</td>
<td>GGGGCACTGAAGAAGACAAGGTACCGGTCGCGGCAAGTA</td>
<td>233</td>
<td>1.5</td>
<td>0.467, 0.764</td>
</tr>
<tr>
<td>Orexin</td>
<td>(Dyer et al., 1999)</td>
<td>AGGCCCTGCAGACTTGCTGCGAGACTCGTCTTTATTGCC</td>
<td>370</td>
<td>0.9</td>
<td>0.577</td>
</tr>
<tr>
<td>Type 2 orexin receptor</td>
<td>(C. J. Dyer and R. L. Matteri, ARS, USDA, Columbia, MO, previously unpublished)</td>
<td>GGGGTGAGTGCTGACCGAAGCCA CAGTGCAGTGGGAACACATC</td>
<td>275</td>
<td>2.0</td>
<td>1.815</td>
</tr>
<tr>
<td>Leptin</td>
<td>(Ramsey et al., 1998)</td>
<td>ATGGCGCTGAGGCACCTGC</td>
<td>501</td>
<td>4.5</td>
<td>3.424</td>
</tr>
<tr>
<td>28S ribosomal RNA</td>
<td>(Salfen et al., 2003)</td>
<td>GACCGTGAAAGCGGGCCCTCGAGTGTATTACCTACT</td>
<td>230</td>
<td>not performed</td>
<td>0.380</td>
</tr>
</tbody>
</table>

1 The reference cited for each riboprobe describes its initial development. Primers are described 5′-3′; the forward primer is above the reverse primer. The human transcript data were taken from the National Center for Biotechnology Information reference sequence database.

The reference cited for each riboprobe describes its initial development. Primers are described 5′-3′; the forward primer is above the reverse primer. The human transcript data were taken from the National Center for Biotechnology Information reference sequence database.

NPY = neuropeptide Y; POMC = proopiomelanocortin.
feed intake on somatotropic function (Vance et al., 1992; Straus, 1994). Having established this time frame, we evaluated early postweaning changes in gene expression related to appetite control. Matteri et al. (2000) described growth data (serum hormone concentrations and gene expression of known regulators of growth) observed in this study. Briefly, levels of specific mRNA for growth regulatory genes did not differ between nursed and dry-fed piglets 4 d after weaning. Serum IGF-I and IGF-II concentrations were decreased in both NP and SDP compared with SOW. Small pigs had lower levels of liver IGFBP acid labile subunit, muscle IGF-II, and muscle GH receptor mRNA, as well as serum concentrations of IGF-II. In contrast, adipose tissue IGF-I and IGF-II mRNA levels were greatest in the small piglets.

Little information exists concerning the regulatory influences of weaning, nutrition, and development on expression of porcine appetite-regulating genes such as NPY, leptin, orexin, and agouti-related protein. Neuropeptide Y is a hypothalamic peptide with potent appetite-stimulating activity (Billington et al., 1991). The expression of NPY is elevated in undernourished animals (Brady et al., 1990; McShane et al., 1993) and is regulated in part by leptin (Stephens et al., 1995), an appetite-suppressing hormone secreted by fat cells (Zhang et al., 1994). Leptin production corresponds with body fat content, thus creating a monitor of fatness by which to regulate appetite (Considine et al., 1996). Orexin has been shown to increase feed intake in rats (Sakurai et al., 1998; Muroya et al., 2004), pigs (Dyer et al., 1999), and sheep (Sartin et al., 2001). Like NPY, it is expressed at increased levels in undernourished rats (Sakurai et al., 1998). Recent evidence suggests that orexin directly regulates NPY-containing and other glucose-responsive neurons in various regions of the hypothalamus (Muroya et al., 2004).

The agouti-related protein is a melanocortin receptor antagonist that blocks the appetite-suppressing effects of α-melanocyte-stimulating hormone, a product of the POMC transcript (Kalra et al., 1999). Other products of the POMC transcript, the endorphins, stimulate feed intake (Morley et al., 1983; Levine and Billington, 1989). Expression of POMC in the paraventricular nucleus is increased by NPY (Kalra et al., 1995), and there is evidence that agouti-related protein is co-released with NPY in the paraventricular nucleus (Broberger et al., 1998; Hahn et al., 1998). A potential role for agouti-related protein could be to alleviate the appetite-suppressing effects of 1 POMC product (α-melanocyte-stimulating hormone) while allowing another product (the endorphins) to stimulate appetite. In the arcuate nucleus, POMC expression is decreased by NPY (Garcia de Yebenes et al., 1995). We did not see WW- or treatment-specific changes in POMC expression, perhaps because we used complete hypothalamic samples containing both the paraventricular and arcuate areas. Another consideration is that most research in this area (including those publications just discussed) has been performed using rodent models; species differences may certainly apply.

Figure 1. Body weight (A), total BW gain (B), and BW gain on last day (C) of pigs either cross-fostered (SOW) or weaned and fed a standard diet containing 0 or 7% spray-dried plasma protein (NP and SDP, respectively) for 4 d. Means and SE are shown. a,bMeans with different letters differ (P < 0.05). Body weight data from this study (along with serum concentrations and gene expression of growth-related gene products) have been previously reported and discussed (Matteri et al., 2000).
Weaning and weaning weight effects on appetite regulators

Figure 2. Gene expression of hypothalamic neuropeptide Y (NPY; A), orexin (B), agouti-related protein (C), and type 2 orexin receptor (D) in pigs either cross-fostered (SOW) or weaned and fed a standard diet containing 0 or 7% spraydried plasma protein (NP and SDP, respectively) for 4 d before euthanasia. Two different weaning weight (WW) classes of pigs were utilized: small (3.5 to 4.3 kg) and large (4.6 to 5.7 kg). Expression is measured as relative densitometric units normalized to 28S ribosomal RNA. Means and SE are shown, as well as probability values for effects from the main factors (treatment, WW) and their interaction. MRNA = messenger RNA.

The lack of associations between BW and adipose leptin or hypothalamic leptin receptor expression supports the concept that leptin expression may be regulated independently of adiposity or feed intake during early development (Hassink et al., 1996; Nakavisut et al., 1997; Ahima et al., 1998). The positive relationships between WW and hypothalamic NPY, orexin, type 2 orexin receptor, and agouti-related protein expression are suggestive of individual predispositions (either genetically or environmentally induced) to eat and grow. Although it is tempting to speculate that bigger piglets are larger because they eat more due to hypothalamic stimulation of appetite, this relationship must be better characterized by further investigation before that claim may be made.

Increases in both feed intake (Coffey and Cromwell, 1995; de Rodas et al., 1995; Touchette et al., 1998) and intestinal health (Touchette et al., 1997) have been reported in weanling pigs fed spray-dried plasma. Consistent with these previous studies, the present data revealed an increase in BW gain over the last day of the study (from 3 to 4 d after weaning). This increase in growth was not associated with significant changes in most of the appetite regulators examined in this study; changes in appetite regulator gene expression might not be detectable when evaluated at a single time point if the neural stimulation of appetite preceded the increase in intake in the SDP group. Although the SOW group was not weaned, those pigs did experience such stresses as social mixing and having to establish a new teat order. These events may have disrupted feed intake in this group as well, contributing to the failure to observe differences in gene expression due to treatment. The weaning group WW interactive effect on NPY expression observed in the current study, in which NPY mRNA levels were least in small SDP pigs, may be explained by compensatory feedback regulation of NPY following a satiating meal. The increased BW gain seen in these pigs on d 4 indicates that they had ingested a substantial amount of feed before euthanasia. A similar study in which pigs are euthanized at times before d 4 postweaning would allow for the observation of changes in neuro-
Table 2. Correlations of messenger RNA expression of appetite regulators on d 4 after cross-fostering or weaning and partial correlations controlling for initial BW

<table>
<thead>
<tr>
<th>Variables</th>
<th>r</th>
<th>P-value</th>
<th>Partial r (controlling for d 0 BW)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>d 0 wt NPY</td>
<td>0.652</td>
<td>&lt;0.0001</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>d 0 wt ORX</td>
<td>0.607</td>
<td>&lt;0.001</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>d 0 wt OR2</td>
<td>0.77</td>
<td>&lt;0.0001</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>d 0 wt AGRP</td>
<td>0.634</td>
<td>&lt;0.001</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>d 0 wt POMC</td>
<td>0.301</td>
<td>&lt;0.1</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>d 0 wt OB</td>
<td>-0.064</td>
<td>NS2</td>
<td>0.42</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>d 0 wt OBR</td>
<td>0.368</td>
<td>&lt;0.05</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>NPY ORX</td>
<td>0.736</td>
<td>&lt;0.0001</td>
<td>0.42</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>NPY OR2</td>
<td>0.697</td>
<td>&lt;0.0001</td>
<td>0.159</td>
<td>NS</td>
</tr>
<tr>
<td>NPY AGRP</td>
<td>0.773</td>
<td>&lt;0.0001</td>
<td>0.303</td>
<td>NS</td>
</tr>
<tr>
<td>NPY POMC</td>
<td>0.518</td>
<td>&lt;0.01</td>
<td>0.177</td>
<td>NS</td>
</tr>
<tr>
<td>NPY OB</td>
<td>-0.018</td>
<td>NS</td>
<td>0.006</td>
<td>NS</td>
</tr>
<tr>
<td>NPY OBR</td>
<td>0.488</td>
<td>&lt;0.001</td>
<td>0.282</td>
<td>NS</td>
</tr>
<tr>
<td>ORX OR2</td>
<td>0.708</td>
<td>&lt;0.0001</td>
<td>0.466</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ORX AGRP</td>
<td>0.736</td>
<td>&lt;0.0001</td>
<td>0.497</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>ORX POMC</td>
<td>0.503</td>
<td>&lt;0.01</td>
<td>0.346</td>
<td>NS</td>
</tr>
<tr>
<td>ORX OB</td>
<td>0.156</td>
<td>NS</td>
<td>0.085</td>
<td>NS</td>
</tr>
<tr>
<td>ORX OBR</td>
<td>0.526</td>
<td>&lt;0.01</td>
<td>0.232</td>
<td>NS</td>
</tr>
<tr>
<td>OR2 AGRP</td>
<td>0.76</td>
<td>&lt;0.0001</td>
<td>0.523</td>
<td>&lt;0.05</td>
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<tr>
<td>OR2 POMC</td>
<td>0.62</td>
<td>&lt;0.0001</td>
<td>0.702</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>OR2 OB</td>
<td>0.173</td>
<td>NS</td>
<td>0.209</td>
<td>NS</td>
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<tr>
<td>OR2 OBR</td>
<td>0.573</td>
<td>&lt;0.001</td>
<td>0.461</td>
<td>&lt;0.05</td>
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<tr>
<td>AGRP POMC</td>
<td>0.75</td>
<td>&lt;0.0001</td>
<td>0.691</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>AGRP OB</td>
<td>-0.049</td>
<td>NS</td>
<td>-0.073</td>
<td>NS</td>
</tr>
<tr>
<td>AGRP OBR</td>
<td>0.366</td>
<td>&lt;0.05</td>
<td>-0.043</td>
<td>NS</td>
</tr>
<tr>
<td>POMC OB</td>
<td>0.018</td>
<td>NS</td>
<td>0.046</td>
<td>NS</td>
</tr>
<tr>
<td>POMC OBR</td>
<td>0.388</td>
<td>&lt;0.05</td>
<td>0.196</td>
<td>NS</td>
</tr>
<tr>
<td>OB OBR</td>
<td>0.312</td>
<td>&lt;0.1</td>
<td>0.233</td>
<td>NS</td>
</tr>
</tbody>
</table>

1NPY = neuropeptide Y; ORX = orexin; OR2 = type 2 orexin receptor; AGRP = agouti-related protein; POMC = proopiomelanocortin; OB = leptin; OBR = long-form leptin receptor.
2NS = not significant.

endocrine expression as these animals undergo the weaning process.

The positive relationships observed between pre-WW and postweaning hypothalamic agouti-related protein, orexin, type 2 orexin receptor, and NPY gene expression may reflect physiological roles of these factors in the regulation of growth in the young pig (acknowledging that changes in gene expression at the level of the transcript do not necessarily imply changes at the level of protein function). We have determined that levels of specific mRNA-encoding proteins that regulate appetite do not differ between nursed and dry-fed pigs 4 d after weaning. The increase in growth produced by dietary SDP over the final day of this study was not accompanied by significant changes in appetite-related gene expression. These data raise important questions relating to the temporal relationships between postweaning changes in gene expression and function. Continued studies of the effects of weaning on the physiology of the pig will provide a better understanding of the regulation of appetite, growth, and adaptation to stressors encountered within the production environment.

LITERATURE CITED


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