This is to certify that the
thesis entitled

DIFFERENTIAL GENE EXPRESSION IN THE FRONTAL CORTEX AND HIPPOCAMPUS OF PIGLETS WEANED AT DIFFERENT AGES

presented by

Rosangela Poletto

has been accepted towards fulfillment
of the requirements for the

M.S. degree in Animal Science

Major Professor's Signature

May 4th, 2005

Date

MSU is an Affirmative Action/Equal Opportunity Institution

DIFFERENTIAL GENE EXPRESSION IN THE FRONTAL CORTEX AND HIPPOCAMPUS OF PIGLETS WEANED AT DIFFERENT AGES

By

Rosangela Poletto

A THESIS

Submitted to
Michigan State University
In partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Animal Science

2005

ABSTRACT

DIFFERENTIAL GENE EXPRESSION IN THE FRONTAL CORTEX AND HIPPOCAMPUS OF PIGLETS WEANED AT DIFFERENT AGES

By

Rosangela Poletto

Early-weaned piglets show abnormal behaviors, aggression, and cognitive deficits. Therefore, it was hypothesized that early-weaned piglets experience aberrant expression of stress response genes in the frontal cortex (FC) and hippocampus (HP), brain areas involved with cognitive function and social behavior. The objective was to examine the effects of early and conventional weaning on gene expression in the FC and HP of piglets either socially isolated or kept with their littermates. Early (EW; $n = 6$) and conventionally-weaned (CW; $n = 6$) piglets were weaned at 10 and 21 days after birth, respectively. Non-weaned piglets of both age groups, 12 (NW; $n = 6$) and 23 (NW; $n = 6$) days, remained with their dams. Half of CW, EW, and NW animals were socially isolated for 15 minutes at 12 (EW) and 23 (CW) days of age, immediately before euthanasia. Brain areas were collected and RNA extracted. A porcine brain library cDNA microarray and quantitative real-time RT-PCR were used to measure the expression of stress-responsive genes. Social isolation suppressed gene expression in the frontal cortex of piglets at 12 and 23 days of age, while early weaning suppressed hippocampal gene expression. These results may help to elucidate some of the biological basis for cognitive deficits and behavioral changes previously reported in early-weaned piglets.

With love and care, I dedicate this achievement to my family, who have always believed in my dreams and supported my work with love, understanding, and patience.

ACKNOWLEDGMENTS

I am thankful to several people who contributed to the completion of this degree. I would like to thank my major professor Dr. Adroaldo Jose Zanella in addition to my committee members Dr. Paul M. Coussens, Dr. Mark Breedlove, and Dr. George W. Smith.

Sincere thanks to the Animal Behavior and Welfare Group for their assistance, support, advisement and friendship. I also appreciate the assistance with the statistical analyses provided by Juan P. Steibel. I would like to thank the Michigan State University Swine Teaching and Research Center for providing animals for the research and the Center for Animal Functional Genomics for essential technical assistance during the development of this work.

I am also thankful for funds that contributed to the performance of the research and completion of the degree, the Michigan Agricultural Experiment Station and the United States Department of Agriculture / National Research Initiative Grant # 2001-0224 (A.Z.).

Enormous thanks to my family who always strongly supported me with words and love, even though far in body but close in mind. I appreciate the patience and support of my fiancée Ricardo. A special thanks to my second family, Robert, Cheryl, and Jordan Leece for their care.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

KEYS TO SYMBOLS OR ABBREVIATIONS

μg – Microgram

μM – Micromole

μl – Microliter

11β-HSD1 – 11Beta-Hydroxysteroid Dehydrogenase 1

11β-HSD2 – 11Beta-Hydroxysteroid Dehydrogenase 2

14-3-3 protein – Tyrosine 3-monoxygenase / Tryptophan 5-monooxygenase Activation Protein

3' – 3' Primer End

5' – 5' Primer End

5-HT – Serotonin

ARP2/3 – Actin Related Protein 2/3 Complex

BLAST – Basic Local Alignment Search Tool

cDNA – Complementary Deoxyribonucleic Acid

CPE – Carboxypeptidase E

CWC – Conventionally-Weaned Control

CWI – Conventionally-Weaned Socially Isolated

DBI – Diazepam Binding Inhibitor

$dT_{(18)}$ – Deoxythymine (18 nucleic acid bases)

dUTP – Deoxyuridine Triphosphate

EST – Expressed Sequence Tag

EWC – Early-Weaned Control

EWI – Early-Weaned Socially Isolated

GC – Glucocorticoid

GR – Glucocorticoid Receptor

HPA – Hypothalamic-Pituitary-Adrenal

kDa – Kilo Dalton

LOESS – Locally Weighted Regression and Smoothing Scatter plots

mg – Milligram

MR – Mineralocorticoid Receptor

mRNA – Messenger Ribonucleic Acid

ng – Nanogram

NWC – Non-Weaned Control

NWI – Non-Weaned Socially Isolated

OAZ2 – Ornithine Decarboxylase Antienzyme 2

ODC – Ornithine Decarboxylase

PA – Polyamines

PBL – Porcine Brain Library

PEA-15 – Phosphoprotein Enriched in Astrocytes 15kDa

Q-RT-PCR – Quantitative Real Time Reverse Transcriptase Polymerase Chain Reaction

RNA – Ribonucleic Acid

SEM – Standard Error of the Mean

CHAPTER ONE

Introduction

Domestic pigs (*Sus scrofa*) raised in a free range environment are naturally weaned at approximately 17 weeks of age (Jensen and Recen, 1989). Better understandings of the nutritional needs of young pigs, and constraints dictated by the intensification of swine production systems have caused dramatic changes to the weaning age. For disease management purposes, the swine industry in the United States (US) has adopted a segregated early weaning system, which consists of weaning the animals at ages younger than 21 days and moving the animals to facilities physically separated from the breeding and farrowing units. Recent data indicate that in the US, 15% of piglets are weaned at less than 16 days of age, and approximately 64% of the animals are weaned younger than 20 days of age (NAHMS, 2001).

From a production perspective, there are important advantages of practicing early weaning of pigs. Early disease control is one of the major reasons supporting early weaning, since the animals are moved to a totally "clean" environment post-weaning (segregated early weaning) at the peak of their passive immunity (Hunter, 1986; Orgeur et al., 2001). Improvements in nutritional managements have also favored increasing weight gain and growth rates of early-weaned piglets (Kim et al., 2001; Patience et al., 2000). However, several studies indicate that early weaning may be deleterious to pig behavior and welfare (Gardner et al., 2001; Hay et al., 2001; Hohenshell et al., 2000; Weary et al., 1999; Worobec et al., 1999; Yuan et al., 2004).

Early-weaned piglets perform abnormal behaviors, such as belly nosing (Gardner et al., 2001) and bar chewing (Worobec et al., 1999). These animals also vocalize more (Weary et al., 1999) and exhibit increased levels of aggression (Hohenshell et al, 2000; Yuan et al., 2004) when compared to piglets weaned on later ages. Moreover, early weaning is a stressful event which increases plasma and urinary cortisol levels (Hay et al., 2001; Hohenshell et al., 2000) in addition to causing a transitory reduction in weight gain and growth (Hay et al., 2001). Hunger and nutritional deficits (Carroll et al., 1998; Worobec et al., 1999) may contribute to an impairment of neuronal mechanisms as a consequence of, for instance, deficiency of important amino acids responsible for neurotransmitter biosynthesis (e.g. Fernstrom, 2000; Kaufman et al., 2000). The occurrence of abnormal behaviors (Gardner et al., 2001; Worobec et al., 1999), increased aggression (Hohenshell et al, 2000; Yuan et al., 2004) and cognitive deficits (Laughlin & Zanella, 2003; Souza and Zanella, 2003) suggest that stress of early weaning, which is an adverse experience for the animals, alters the development and function of both the neuroendocrine and autonomic systems (Kaufman et al., 2000; Plotsky et al., 2001). For instance, corticotrophin release hormone, which is produced in response to stress, in addition to trigger release of cortisol from adrenal glands trough activation of the hypothalamic-pituitary-adrenal (HPA) axis, also increases the release of norepinephrine in the brain (Kaufman et al., 2000).

A large body of scientific evidence indicates an age-dependent sensitivity of brain pathways to stress hormones (Kanitz et al., 1998; Kaufman et al., 2000; Plotsky et al., 2001). In pigs the first three weeks of age are critical "windows" of brain plasticity to alter behavior and brain development (Hemsworth et al., 1986; Weaver et al., 2000). For

instance, piglets neonatally handled showed permanent alteration of HPA axis function with increased plasma corticosteroid binding globulin binding capacity, decreased basal cortisol concentrations and increased adenocorticotropin hormone concentrations at the time of death (7 months of age). Additionally, handled animals showed greater locomotion during open field test and reduced body weight compared to non-handled piglets (Weaver et al., 2000)

Brain areas such as the hippocampus, which plays an important role in memory processes, and the frontal cortex, which facilitates behavioral organization and cognition, are targets of stress hormones, such as corticosteroids. Both hippocampus and frontal cortex have abundant levels of corticosteroid receptor binding sites (De Kloet, 2004; Diorio et al., 1993). Therefore, hippocampus and frontal cortex functions of piglets may be disrupted by stressful events (e.g. weaning, social isolation, and handling) primarily during the sensitive period of brain development (Hemsworth et al., 1986; Weaver et al., 2000). In a study performed with rats, Ordyan and colleagues (2001) examined corticosterone binding in the hippocampus and frontal cortex of animals that received glucocorticoid (GC) treatment at three weeks of age compared to one week old animals. Only animals treated at the third week showed prolonged corticosterone secretion at 30 days of age, and lower cerebral corticosteroid binding at 90 days of age, suggesting greater sensitivity to elevated levels of GC at early brain developmental phase. The presence of high levels of corticosteroids in these brain areas, induced by stress stimuli, has been credited with causing behavioral and cognitive deficits (Cook and Wellman, 2004; Mizoguchi et al., 2004). For instance, acute stress impairs learning acquisition of new information as well as memory of rats exposed to a hippocampal-dependent task

(Diamond et al., 1999). Moreover, acutely stressed rats show notable inhibition of long-term potentiation in the frontal cortex and hippocampus, as indicated by deficits in the acquisition and processing of information (Rocher et al., 2004). Changes in the frontal cortex caused by chronic stress affect spatial learning and memory in rats (Abidin et al., 2004; Radley et al., 2004). Additionally, the intensification of aggressive behavior is also mediated by stress hormones through altered neuronal morphology and function of frontal cortex (Blair, 2004) and hippocampus (Wood et al., 2003).

Stressful events trigger the hypothalamic production of corticotrophin-releasing hormone, which stimulates the pituitary gland to produce adrenocorticotrophin hormone (ACTH). This hormone stimulates the adrenal glands to synthesize corticosteroids (e.g. cortisol) and release them into the bloodstream. This physiological pathway is named the HPA axis (de Kloet, 2004). Cortisol crosses the blood brain barrier and affects the hypothalamus, hippocampus and frontal cortex, among other areas of the brain, by binding to glucocorticoid receptors (GR) and mineralocorticoid receptors (MR). Cortisol has higher affinity for MR, and when binding to MR, the stress response is buffered as corticosteroids are prevented from binding to GR (Perreau et al., 1999). One of the primary outcomes of central GC action is to mediate a negative feedback system which suppresses HPA axis activity when GC levels are elevated. The disruption of the HPA axis system is characterized by attenuated GC negative feedback, which is characterized by decreased expression of GR and MR (Mizoguchi et al., 2003). The enzymes 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2) are important pathways for GC metabolism (Holmes et al., 2003), and play a role in protecting the brain from deleterious effects of high concentrations of GC (Seckl, 1997). As dehydrogenases,

both enzymes inactivate GC by converting it on inactive 11-keto derivative cortisone. The 11β-HSD2 exclusively functions as a dehydrogenase (Seckl, 1997). However, 11β-HSD1 can also function as a reductase, regenerating active GC by transforming circulating cortisone.

The presence of GC in the brain is necessary for a wide range of normal physiological, behavioral and cognitive functions. Central levels of GC can be elevated in situations that may or may not be stressful, such as maternal care, changes in mood, fear and anxiety, attention, learning and memory (Erickson et al., 2003). In stressful circumstances, high levels of cortisol bind to GR activating a series of cellular events which regulate the expression of several neuronal genes (Herman, 1993). Taken together, available literature supports the hypothesis that early environmental manipulations, mainly during the first weeks of age, can result in permanent changes in the HPA axis function and behavior of piglets (Hemsworth et al., 1986; Weaver et al., 2000), given that early exposure to high levels of GC causes neuronal death (Lee et al., 2002) and alters organization of stress sensitive pathways (Mirescu et al., 2004).

The negative outcomes of stress on neuronal function in the frontal cortex and hippocampus can be intensified when individuals endure an additional stressful stimulus (Silva-Gomez et al., 2003). For instance, socially isolated early-weaned piglets show cognitive deficits compared to non-socially isolated animals (Laughlin & Zanella, 2002, Souza & Zanella, 2004). As a stressor, social isolation also elicits the negative feedback response controlled by the HPA axis, raising cortisol levels in pigs (Kanitz et al., 2004) and in rats (Weiss et al., 2004). Social isolation also impairs learning and memory in rhesus monkeys (Washburn & Rumbaugh, 1991), and rats (Rudy et al., 1999) and

induces fear in rats (Molina-Hernandez et al., 2001) and anxiety in pigs (Kanitz et al., 2004) and rats (Weiss et al., 2004).

Cerebral changes caused by stress, at the cellular level, can be studied in detail with the help of state of the art molecular biology techniques. Gene expression microarray can be used to characterize change in global gene expression in various tissues (Xiang & Chen, 2000), following exposure to stress. A porcine brain library cDNA microarray (Nobis et al., 2003) is currently available for screening changes in gene expression in 866 genes within the brain of pigs. Also, quantitative real time reverse transcriptase polymerase chain reaction (Q-RT-PCR) is extensively used to examine expression of specific genes. Q-RT-PCR has high sensitivity and accuracy in detecting low-abundance mRNA in various tissues. Microarray hybridization and Q-RT-PCR are viable approaches for examining underlying neuronal changes that may affect animal behavior and welfare in piglets.

We previously demonstrated that early-weaned piglets when socially isolated for 15 minutes showed reduced capacity to learn a spatial task (Laughlin & Zanella, 2002), and had an impaired ability to recognize familiar animals (Souza & Zanella, 2004). These changes indicate that some aspects of cognition may be affected by the inability of piglets to cope with stress when weaned at this stage of brain development. Connecting changes in gene expression in the brain to behavioral alterations in response to stressors will facilitate a better understanding of the mechanisms underlying neuronal and behavioral effects of stress in early-weaned piglets. Thus, our overall objective is to understand how stressful events, such as early weaning and social isolation, differently affect brains of early-weaned (10 days old) and conventionally-weaned piglets (21 days old). Our

specific objective is to study changes in expression of stress-responsive genes in the frontal cortex and hippocampus of early- and conventionally-weaned piglets that were either subjected to 15 minutes of social isolation or kept with littermates. Hence, we hypothesize that early-weaned piglets experience aberrant expression of stress-responsive genes in the frontal cortex and hippocampus.

CHAPTER TWO

Effects of early weaning and social isolation on the expression of glucocorticoid and mineralocorticoid receptor and 11β-hydroxysteroid dehydrogenase 1 and 2 mRNAs in the frontal cortex and hippocampus of piglets

Abstract

Pigs weaned younger than 21 days of age show behavioral abnormalities, aggressive behavior, impaired learning and memory, and social recognition deficits. Our aim in this experiment was to investigate whether age, weaning and (or) social isolation impacts the expression of genes regulating glucocorticoid response [glucocorticoid receptor (GR), mineralocorticoid receptor (MR), 11β-hydroxysteroid dehydrogenases 1 and 2 (11β-HSD1 and 11β-HSD2)] in the frontal cortex and hippocampus, brain areas involved in behavior organization and memory. Early- (EW; n = 6) and conventionally-weaned (CW; n = 6) piglets were weaned 10 and 21 days after birth, respectively. Non-weaned (NW; n = 6/group) piglets of both age groups remained with their dams. Two days after weaning, immediately before euthanasia and tissue collection, half of CW, EW, and NW animals were socially isolated for 15 minutes at 12 (EW) and 23 (CW) days of age. Frontal cortex and hippocampus were collected and RNA extracted. Differences in amounts of 11β-HSD1, 11β-HSD2, GR and MR mRNAs in both brain areas were determined by quantitative real time RT-PCR and data subjected to multivariate linear mixed models analysis and effects of age, weaning status and social isolation were tested. When compared with non-weaned pigs at 12 days of age, the

hippocampi of early-weaned piglets showed a decrease in expression of the four genes ($P < 0.01$), but no differences in expression were observed in CW compared to NW animals at age 23 ($P > 0.1$). Social isolation decreased the expression of 11β-HSD1, 11β-HSD2, GR and MR mRNAs ($P < 0.05$) in the frontal cortex of both 12 and 23 days old pigs. As an age effect, 12 day old piglets showed higher MR mRNA in the frontal cortex ($P < 0.01$) and lower 11β-HSD2 ($P < 0.05$) and GR ($P < 0.001$) mRNA in the hippocampus compared to 23 days old animals. The results indicate that early weaning affected the hippocampus of piglets at 12 days of age, and social isolation affected frontal cortex of piglets regardless of age. These results may be correlated with behavioral and cognitive changes reported in early-weaned piglets.

1. Introduction

Early maternal separation has been extensively studied and shown to cause long term psycho-physiological effects on brain and behavior of humans and animals (Kuhn and Schanberg, 1998; Sanchez et al., 2001). Maternal separation interferes with the proper development of both psychobiological and neuroendocrine regulatory mechanisms in the developing brain (Kanitz et al., 2004; Kaufman et al., 2000; Plotsky et al., 2001). Moreover, maternal separation affects the individual's behavior by eliminating the predictability and controllability provided by the mother-infant interaction (Plotsky et al., 2001). The early weaning of piglets can provide a useful model for studying early maternal separation effects on behavior and neuroendocrine mechanisms. Previous studies revealed that piglets weaned younger than 21 days of age show abnormal behaviors (Gardner et al., 2001; Orgeur et al., 2001) and intensified aggression

(Hohenshell et al., 2000; Yuan et al., 2004) later in life. Early-weaned piglets, when exposed to fifteen minutes of social isolation, show cognitive deficits compared to non-socially isolated animals (Laughlin & Zanella, 2002; Souza & Zanella, 2004). The behavioral outcomes and cognitive deficits reported may result from modifications in responsiveness of neuronal mechanisms during a sensitive period of brain development (Plotsky et al., 2001; Tuchscherer et al., 2004). An age-dependent sensitivity of the brain to stress hormones, such as corticosteroids, has been reported to occur in piglets during the first three weeks after birth (Hemsworth et al., 1986; Hemsworth and Barnett, 1992; Weaver et al., 2000).

Hippocampus and frontal cortex functions include cognition and behavioral organization (e.g. Cao et al., 2004; Kesner et al., 1996, 2004; Ongur & Price, 2000). Basal concentrations of endogenous corticosteroid hormones like glucocorticoids (GC) are essential for maintaining these functions (Erickson et al., 2003). However, hippocampus and frontal cortex can be affected by stressful events since both areas contain high density binding sites for corticosteroids. Thus, high concentrations of corticosteroids in the hippocampus during brain development cause behavioral and cognitive deficits that may not be reversible (McEwen, 1997), as well as cognitive deficits in the frontal cortex by altering neuronal morphology and function (Cook and Wellman, 2004; Mizoguchi et al., 2004).

Stressful challenges, such as early weaning and social isolation, are responsible for activation of the stress axis resulting in increased GC levels (Hay et al., 2001; Hohenshell et al., 2000, Kanitz et al., 2004). One of the primary outcomes of increased central GC action is to trigger a negative feedback system which suppresses further

activation of the HPA axis, preventing serious neuronal damage directly caused by GC (Lee et al., 2002). Therefore, at early brain developmental stages, the disruption of the GC negative feedback system, which is characterized by decreased GR expression in the hippocampus and frontal cortex, may be responsible for brain dysfunction (Mizoguchi et al., 2003, 2004). The GC action is mediated by cytoplasmic mineralocorticoid (MR) and glucocorticoid receptors (GR) which bind GCs with different affinities in the brain (Erickson et al., 2003). Mineralocorticoid receptors, when activated, buffer the activation of the stress response by preventing GC from binding to GR (Perreau, 1999), which once heavily bound mediates various neuronal changes (Herman, 1993, Meaney et al., 1996). Therefore, during early stages of development, the disruption of the HPA axis may compromise the developing brain and be accountable for some aspects of cognitive impairment and abnormal behaviors (Mizoguchi et al., 2003, 2004). Glucocorticoids indirectly regulate the activity of the enzymes 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and type 2 (11β-HSD2), which modulate GC action (Holmes et al., 2003). When acting as dehydrogenases, both enzymes protect the brain from the deleterious effects of excessive GC by rendering them inactive (Seckl, 1997).

Therefore, our research focus is to understand the molecular events associated with the cognitive deficits reported previously in early-weaned piglets subjected to social isolation, a short-term stressor (Laughlin & Zanella, 2002; Souza & Zanella, 2004). We hypothesize that spatial and social cognitive impairments experienced by early-weaned piglets are preceded by aberrant expression of stress sensitive genes in the frontal cortex and hippocampus. Our aim was to investigate the effects of weaning at two ages, 10 and 21 days, on 11β-HSD1, 11β-HSD2, GR and MR mRNA abundance in the frontal cortex

and hippocampus of piglets, when the animals were either socially isolated or kept with their littermates.

2. Materials and Methods

2.1 Animals

Twenty-four Large White female piglets from six litters were used in this experiment. Females were used as the experimental animals instead of males because male piglets are subjected to castration on the first days after birth, which is an adverse experience that may interfere with later stress response. The animals were housed at the Swine Teaching and Research Center, Michigan State University. Housing and feeding routines followed standard swine production practices. All animal housing and experimental procedures were approved by the Michigan State University All-University Committee on Animal Use and Care.

Four littermates from each litter were randomly assigned to one of four treatment groups belonging either to early or conventional weaning model. The early weaning model consisted of early-weaned control (EWC; $n = 3$), early-weaned socially isolated (EWI; $n = 3$), non-weaned control (NWC; $n = 3$) and non-weaned socially isolated (NWI; $n = 3$) treatments. The conventional weaning model consisted of conventionally-weaned control (CWC; $n = 3$), conventionally-weaned socially isolated (CWI; $n = 3$), non-weaned control (NWC; $n = 3$) and non-weaned socially isolated (NWI; $n = 3$) treatments. Early-weaned piglets (EW) were weaned 10 days after birth and the littermates were kept together until day 12 after birth. Conventionally-weaned piglets (CW) were weaned 21 days after birth and weaned littermates were kept together until day 23 after birth. Non-

weaned animals were left with their dams until days 12 or 23. On days 12 or 23 after birth, piglets assigned to EWI and CWI, respectively, and NWI groups, were socially isolated for 15 minutes and immediately euthanized. Social isolation was performed in a pen located in the same room as the dams and littermates but with no visual contact. Control animals (EWC, CWC and NWC) were then taken from their pens and immediately euthanized. Euthanasia was preceded by induction of general anesthesia with 5% isoflurane using a Moduflex Coaxial anesthesia machine (Dispomed, Quebec, Canada). Immediately after reaching a profound general anesthesia state, euthanasia was performed using an intracardiac injection of Sodium Pentobarbital (86mg/kg; Fatal Plus®, Vortech Pharmaceuticals, USA).

2.2 Tissue preparation and RNA extraction

Brains were removed from all animals within 10 minutes of euthanasia. Frontal cortices and hippocampi from all animals were dissected out of both brain hemispheres and placed in RNA*later*™ (Ambion, Austin, TX) to protect RNA integrity, and then snap frozen in liquid nitrogen. Samples were then stored at –80°C. The RNA*later*™ was removed from the samples according to manufacturer's guidelines. Total RNA from individual frontal cortex and hippocampus samples was isolated using Ribo Pure Kit (Ambion) following the manufacturer's instructions. Quantity and purity of isolated RNA samples were analyzed using an RNA 6000 Nano Chip on an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE).

2.3 Quantitative Real-Time RT-PCR

Quantitative real-time reverse transcriptase polymerase chain reaction (Q-RT-PCR) was performed to examine the relative abundance of 11β-HSD1, 11β-HSD2, GR and MR mRNA in the frontal cortex and hippocampus of piglets. Q-RT-PCR was performed as described previously (Gibson et al., 1996; Heid et al., 1996). A total of 2 μg of total RNA from each sample was reverse transcribed with oligo $(dT)_{18}$ and SuperScript II reverse transcriptase (Invitrogen Life Technologies Corp., Carlsbad, CA). A ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Rockland, DE) was used to determine quality and quantity of the resulting complementary DNA. Primer sequences for Q-RT-PCR were designed using Primer Express 2.0 Software (Applied Biosystems) and synthesized by Qiagen (Valencia, CA). Primer sequences for tested and control genes are shown in Table 1.

Table 1. Primer sequences used for Q-RT-PCR to analyze gene expression in the hippocampus and frontal cortex of piglets.

Gene name	GenBank Accession no.	Forward primer (5'→ 3' end)	Reverse primer (5' → 3' end)
11β-HSD1	AF414124	GCAGCTTTGCGCCAAGAG	GATTTCCCAGCAGGAGAGAAGTC
11β-HSD2	AF414125	GCGAAAGCTTCCCACTGAAC	AGGGTCTGTTTGGGCTCATG
GR	AF141371	GATCATGACCGCACTCAACATG	TTGCCTTTGCCCATTTCAC
MR	U88893	GCGCAATCTGGAAGGTTCTTC	CGGGAGCGTGTTTTTTAAGC
Beta actin	AF054837	CTCCTTCCTGGGCATGGA	CGCACTTCATGATCGAGTTGA
18S	AY265350	GGCTCATTAAATCAGTTATGGTTCCT	AGCTCTAGAATTACCACAGTTATCCAAG

A total of 30ng of template cDNA and 12.5µl of SYBR Green (Applied Biosystems, Foster City, CA) were employed in each real-time reaction. Primer mix (forward and reverse) was used in a concentration of 5µM. *Sus scrofa* 18S ribosomal RNA and beta actin were selected as control genes for frontal cortex and hippocampus samples, respectively and used for normalization purposes. All Q-RT-PCR reactions were performed in triplicate using template from individual animals in each reaction. A relative standard curve was used as the Q-RT-PCR quantification method (Livak, 1997; Johnson et al., 2000). The standard curve was constructed using the following amounts of cDNA (in duplicate): 95ng, 40ng, 10ng, 0.1ng and 0.01ng. A single control sample was chosen to be used as template for the standard curve, which was constant for each tissue and for each group of samples (12 or 23 days). Standard curves for the control genes and genes of interest were incorporated into every run. Q-RT-PCR was performed and analyzed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems).

2.4 Q-RT-PCR data analysis

The relative standard curve quantification software yielded 3 expression values for the tested and reference genes in each sample. These three technical replicates were averaged and the result for each tested gene was divided by the corresponding value of the control to obtain the normalized gene expression in each biological sample. For further analysis, the log-transformation of the normalized quantity for each sample was considered as the response variable. The expression of the four genes was analyzed simultaneously using a multivariate linear mixed model (Littell et al., 1996). Social isolation, age (12 and 23 days), weaning (weaned and non-weaned), gene (11β-HSD1,

11β-HSD2, GR and MR) and all possible interactions were set as fixed effects. Litter was considered as a random effect. All the (co)variances were estimated and the model assumptions (e.g. normality) were assessed. The main or simple effect contrasts were computed depending on the significance of the higher order interactions. The main effects are represented by the average difference in expression of two conditions. For instance, the difference of all socially isolated versus non-socially isolated individuals averaged across genes, weaning and age. The simple effects are represented by the differences between two groups within two levels of another factor. For instance, the simple effect of age within gene is the difference between the expression at two ages within each gene averaged across social isolation and weaning. Fold changes (ratios) were obtained by back transforming the linear estimates. The back transformation of the SEM was obtained by taking the anti-log of the mean plus or minus the standard error of the mean (SEM). For presentation of the results in which the gene expression was lower in the first treatment compared to the second, the ratio (< 1) was divided into -1 to give a negative relative expression value (-1/ratio). Fold changes showing with $P < 0.05$ were considered statistically significant. Fold change values are presented as the means with the corresponding SEM.

3. Results

The piglets studied in this experiment were weaned at two ages. Early-weaned animals were weaned at 10 days of age and samples collected at 12 days of age. Conventionally-weaned animals were weaned at 21 days after birth, and frontal cortex and hippocampus collected at 23 days of age. When examining mRNA levels for 11β-

HSD1, 11β-HSD2, GR and MR using real-time RT-PCR, a significant effect of weaning on gene expression was observed only in the hippocampus of early-weaned animals (interaction of age by weaning; $P = 0.004$). Early-weaned piglets showed suppressed expression of the four tested genes in the hippocampus by an average fold of -2.52 ($P = 0.004$), when compared with non-weaned piglets at 12 days of age (Table 2). Conversely, no significant changes in expression of the four genes were detected in the hippocampus of conventionally-weaned piglets when compared with non-weaned piglets at 23 days of age (Table 2). Interestingly, when examining the mRNA abundance for 11β-HSD1, 11β-HSD2, GR and MR in the frontal cortex, there were no evidences of significant differences in mRNA levels in either early-weaned compared with non-weaned piglets at age 12, or in conventionally-weaned compared with non-weaned piglets at age 23 (Table 2). The interaction of age by weaning was not significant for this brain area ($P > 0.1$).

Table 2. Average change in relative expression of stress-responsive genes in the frontal cortex and hippocampus of weaned compared with non-weaned piglets.

Tissue	Weaning status	Fold change	SEM	P-value
Frontal cortex	EW	1.35	[0.913, 1.914]	NS
	CW	-1.48	[-2.196, -0.808]	NS
Hippocampus	EW	-2.52	[-3.337, -1.244]	< 0.01
	CW	1.50	[1.1299, 1.904]	NS

Note. Positive and negative values indicate higher or lower gene expression, respectively, in weaned compared with non-weaned piglets at 12 and 23 days of age. SEM = standard error of the mean. NS = not significant.

At 12 or 23 days of age, a group of either weaned (EWI and CWI) or non-weaned (NWI) piglets were exposed to 15 minutes of social isolation. Social isolation significantly suppressed 11β-HSD1, 11β-HSD2, GR, and MR mRNA by a mean fold of -1.94 ($P < 0.05$) in the frontal cortex of both 12 and 23 days old piglets, regardless of whether the animals were weaned or non-weaned (Table 3). The interaction of gene with social isolation was not significant ($P = 0.32$). Intriguingly, for all four genes tested in the hippocampus, no effects of social isolation were observed, and no significant interactions of social isolation with either age or weaning were detected ($P > 0.1$) (Table 3).

Table 3. Main effect of social isolation on the relative mRNA abundance for tested genes in the brain areas examined.

Tissue	Average Fold change	SEM	P-value
Frontal cortex	-1.94	[-2.553, -1.092]	< 0.05
Hippocampus	1.15	[0.942, 1.438]	NS

Note. Negative values indicate lower gene expression in socially isolated compared with non-socially isolated animals regardless to the weaning status. SEM = standard error of the mean. NS = not significant.

Exclusively looking at age effects, 12 day old piglets showed significantly higher levels of MR mRNA ($P = 0.007$) in the frontal cortex when compared to 23 day old piglets (Figure 1). Additionally, simple effect comparisons of age within gene indicated that 12 day old piglets had lower expression of 11β-HSD2 ($P = 0.03$) and GR ($P =$

0.0009) in the hippocampus (Figure 2) when compared to 23 day old piglets (interaction of gene by age; $P = 0.006$).

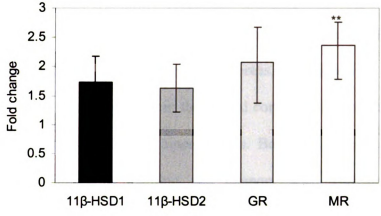

Figure 1. Relative levels of gene expression in the frontal cortex of 12 day old compared with 23 day old piglets. Positive values indicate higher mRNA levels in 12 day old compared with 23 day old piglets. The error bars represent the standard errors of the mean (SEM). ** $P < 0.01$.

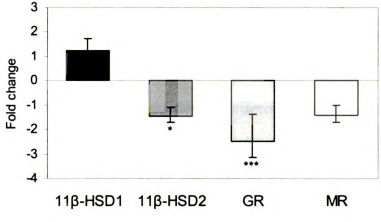

Figure 2. Relative levels of gene expression in the hippocampus of 12 day old compared with 23 day old piglets. Positive and negative values indicate higher or lower mRNA levels, respectively in 12 day old compared with 23 day old piglets. The error bars represent the standard errors of the mean (SEM). *$P < 0.05$, ***$P < 0.001$.

4. Discussion

Our data illustrate distinct regulation of mRNA expression for 11β-HSD1 and 2, GR and MR in the frontal cortex and hippocampus of piglets. Interestingly, early weaning affected the studied genes in the hippocampus of piglets, but did not affect frontal cortex expression of the same genes. Whereas, 15 minutes of social isolation affected expression of genes examined in the frontal cortex of piglets, regardless of age, but did not affect expression in the hippocampus. Both brain areas are involved in cognitive function, control of learning and memory and behavioral organization processes (McEwen, 1997; Radley et al., 2004).

As described in other species, maternal separation also causes activation of the HPA axis in piglets as demonstrated by studies which reported that weaning increased cortisol levels in piglets when performed at different ages (Hay et al., 2001; Hohenshell et al., 2000; Kanitz et al., 2004). Our results showed that piglets weaned at 10 days of age (early-weaned) had consistently suppressed mRNA abundance for 11β-HSD1 and 2, GR and MR in the hippocampus at 48 hours post-weaning (12 days of age) compared to non-weaned piglets. However, there may be a confounding, and (or) additive effect of age with weaning. When exclusively considering age effects, 12 day old piglets were found to have lower levels of 11β-HSD2 and GR than 23 day old animals. The decreased expression of GR mRNA in this brain area may suggest that younger animals may experience difficulties in reacting to raised GC levels, and consequently may be less competent in activating the negative feedback to regulate HPA axis function. The disruption of the GC negative feedback system may be associated with decreased GR expression in hippocampus and may be associated with brain dysfunction (Mizoguchi et

al., 2003, 2004). Also, the decreased levels of 11β-HSD2 mRNA may indicate greater vulnerability of younger piglets to high GC levels since this enzyme inactivates circulating GC (Holmes et al., 2003). Other studies showed that, during the early postnatal period, 11β-HSD2 expression correlates with GR expression in brain regions where GCs strongly affect neuronal division, growth and maturation (Robson et al., 1998).

The suppression of GR expression found in the hippocampus of early-weaned piglets partially support the work of Kanitz and colleagues (1998) who found a decrease in GR binding in the hippocampus of piglets four days after weaning (at 35 days of age), compared to 2 hours before weaning. Decreased GR expression in the hippocampus suggests potential attenuation of negative feedback, which is responsible for suppressing further activation of HPA axis and GC synthesis (Mizoguchi et al., 2003). Our results are also in agreement with findings from a previous study in which rats exposed to 24 hours of maternal deprivation developed reduced MR and GR mRNA in the hippocampus (Vazquez et al., 1996). Moreover, we observed a suppression of the mRNA for dehydrogenases 11β-HSD1 and 2 mRNA levels in the hippocampus of early-weaned piglets. It is important to mention that the regulation of mRNA for both enzymes in the brains of either weaned or socially isolated piglets has not being previously reported. Based on studies performed mostly with rodents, it is known that both enzymes play a role in GC metabolism, protecting the brain from the deleterious effects of excessive GC (Seckl, 1997). This indicates that the hippocampus of early-weaned piglets may be exposed to greater concentrations of active GC, which may be directly responsible for

negative effects on cognition, due mostly to hippocampal neuronal death (Antonawich et al., 1999; Lee et al., 2002).

Twelve day old piglets showed significantly higher MR mRNA levels in the frontal cortex when compared with 23 day old piglets. Unfortunately, limited information is known about the role of MR in the frontal cortex, but Diorio et al. (1993) reported GR and MR binding in the frontal cortex of rats and suggested that the frontal cortex mediates an inhibitory effect of GC on stress-induced HPA activity. Therefore, it is plausible to argue that the function of MR in the frontal cortex may be similar to that found in other brain areas in mediating GC negative feedback (de Kloet, et al., 1993; Diorio et al., 1993). In the hippocampus, the beneficial effects of GC in neuronal transmission and integrity are mediated by MR binding (de Kloet et al., 1993). No differences in expression of either GR or the dehydrogenases were detected in the frontal cortex of 12 compared to 23 day old animals.

In addition to early weaning, the impact of 15 minutes of social isolation, a short-term stressor, was tested in this experiment. We limited social isolation to only 15 minutes because our goal was to minimize nutritional deprivation and repeat the same protocol used in a previous study which reported cognitive deficits in early-weaned piglets (Laughlin & Zanella, 2002; Souza & Zanella, 2004). Fifteen minutes of social isolation significantly suppressed expression of 11β-HSD1 and 2, GR and MR mRNAs in the frontal cortex while no effect was observed in the hippocampus. In this case, we could speculate that social isolation, primarily a psychological stress, encompasses social separation from littermates and dam and exposure to a novel environment (Hennessy et al., 2004). Previous research using longer periods of social isolation showed different

patterns of gene expression in both frontal cortex and hippocampus of piglets. For instance, Kanitz et al. (2004) reported that 2 hours of daily social isolation from 3 to 11 days of age in piglets caused changes in behavioral, neuroendocrine and immune regulation and produced long-term effects on HPA axis activity. Also, according to Lapiz and colleagues (2003), social isolation, when applied as an early adverse event for long periods, causes long-term effects on the behavior and neurobiology involving hippocampus and frontal cortex functions. Short-term periods of social isolation may not be enough to cause changes in the hippocampal mRNA abundance for genes examined. Previous studies performed in piglets showed that repeated social isolation significantly altered GR binding in the hippocampus (Kanitz et al., 2004; Tuchscherer et al., 2004).

When comparing the differential responses to weaning versus social isolation observed, one should consider that the weaning period consisted of 48 hours of "stress", while 15 minutes of social isolation could be characterized as an acute stressor. Therefore, the intensity and duration of the stress stimuli may be accountable for the differences in regulation of gene expression in the frontal cortex versus the hippocampus. However, the reasons for observed differences between frontal cortex and hippocampus, in regards to regulation of expression of 11β-HSD1 and 2, GR and MR, in response to distinct stressors (SI and EW) are unclear. Both brain areas are targets of GC and are intrinsically involved in stress response regulation (Meaney et al., 1996). However, it is intriguing that the negative feedback effects of GC on HPA axis activity in the frontal cortex of rats vary according to the nature of the stress (Diorio et al., 1993).

In summary, our results indicate that early weaning affected hippocampal expression of 11β-HSD1 and 2, GR and MR in piglets at 12 days of age while no effects

were observed in conventionally-weaned piglets. Thus, piglets at 10 days of age may be more vulnerable to weaning stress than 21 day old animals. Furthermore, fifteen minutes of social isolation suppressed the expression of all four tested genes examined in the frontal cortex, but not hippocampus, regardless of whether the piglets were 12 or 23 day old. These changes may be correlated to some behavioral and cognitive deficits previously reported in early-weaned piglets. Thus, early maternal separation in piglets may have the same long-term negative effects as observed in other species (Sanchez et al., 2001), but further studies should address if changes in mRNA abundance would also be confirmed at the level of receptor binding and enzyme activity.

CHAPTER THREE

Investigation of changes in global gene expression in the frontal cortex of early-weaned and socially isolated piglets using microarray and quantitative real-time RT-PCR

Abstract

We hypothesize that early-weaned piglets experience aberrant expression of stress-responsive genes in the frontal cortex (FC), a key brain area involved in cognitive function and behavior organization. To test this hypothesis early-weaned piglets (EW; n = 6) were weaned 10 days after birth, while non-weaned (NW; n = 6) piglets were left with their dams. Half of EW and NW animals were socially isolated (SI) for 15 minutes at 12 days of age when euthanasia and frontal cortex collection were performed. The effects of SI and EW were examined by gene expression profiling using cDNA microarray hybridizations, generated from a porcine brain cDNA library. A total of 108 genes were differentially expressed ($P < 0.05$, fold change > 1.25) among the four comparisons. Forty-two genes had known functions, and 24 of these have important brain-related functions. Quantitative real-time polymerase chain reaction (Q-RT-PCR) was used confirm regulation of expression of a subset of 6 genes, with important brain function selected from the microarray outcomes. In non-weaned animals a significant suppression of mRNA abundance for carboxypeptidase E, 14-3-3 protein and phosphoprotein enriched in astrocytes 15kD was observed in response to SI. Also, in early-weaned animals, diazepam binding inhibitor and actin related protein 2/3 complex

mRNA levels were suppressed in response to SI. Results suggest that social isolation of non- and early-weaned piglets may impact the expression of genes involved in regulation of neuronal function, development and protection in the frontal cortex of young pigs.

1. Introduction

Weaning is a stressful event that may psychologically and physiologically challenge several animal species. In particular, weaning stress in piglets causes increases in plasma (Hohenshell et al., 2000) and urinary cortisol levels as well as temporary reductions in weight gain and growth (Hay et al., 2001). In addition, several studies have reported that early-weaned piglets perform abnormal behaviors (Gardner et al., 2001; Orgeur et al., 2001; Worobec et al., 1999), show more vocalization (Weary et al., 1999) and escape behaviors (Worobec et al., 1999) in addition to increased levels of aggression (Hohenshell et al., 2000; Orgeur et al., 2001; Yuan et al., 2004) relative to animals weaned later. Some of these behaviors suggest correlations between early weaning stress and increased stress hormone levels, which may negatively impact development of the central nervous system.

A large body of scientific evidence indicates an age-dependent sensitivity of brain pathways to stress hormones such as cortisol (Kanitz et al., 1998; Kaufman et al., 2000; Plotsky et al., 2001). In support of this hypothesis, early adverse experiences, such as maternal separation, may have long-term affects on brain function and behavior (Hay et al, 2001; Plotsky et al., 2001). Maternal separation interferes with proper development of both the autonomic and neuroendocrine systems (Kanitz et al., 2004; Kaufman et al., 2000; Plotsky et al., 2001) and affects behavior as a consequence of eliminating the

predictability and controllability provided by the mother-infant interaction (Plotsky et al., 2001).

Previous studies reported that early-weaned piglets when socially isolated for 15 minutes at 2 days post-weaning showed impaired spatial learning and memory (Laughlin & Zanella, 2002) and social recognition deficits (Souza & Zanella, 2004) compared to non-isolated animals. Moreover, impaired learning and memory are consequences of social isolation in rhesus monkeys (Washburn & Rumbaugh, 1991) and rats (Rudy et al., 1999). Social isolation pre- and post-weaning also activates the hypothalamic-pituitary-adrenal (HPA) axis, characterized by an increase in cortisol levels in pigs (Kanitz et al., 2004) and corticosterone in rats (Weiss et al., 2004), which may produce long-term neuroendocrine and behavioral effects (Hellemans et al., 2004; Tuchscherer et al., 2004). Social isolation of rats induces fear-like behavior (Molina-Hernandez et al., 2001), as well as anxiety when the animals were tested in the elevated plus maze (Weiss et al., 2004). Also, piglets socially isolated for 2 hours/day from day 3 to day 11 of age show behavioral, neuroendocrine and immunological changes, which may be indicative of depressed and anxious states (Kanitz et al., 2004). Altered behavioral responsiveness to opiates and psycho-stimulants has also been described as consequence of social isolation in rats (Kosten et al., 2000). These outcomes may result from modification of neuronal mechanisms in response to environmental challenges during a period of sensitive postnatal brain development (Tuchscherer et al., 2004).

The frontal cortex in humans, monkeys and rats is responsible for regulation of executive function, cognition and learning and memory, among other functions (Ongur & Price, 2000). A study performed by Rocher and colleagues (2004) demonstrated that

acutely stressed rats show notable inhibition of long-term potentiation in the frontal cortex, as indicated by deficits in the acquisition and processing of information. Moreover, changes in the frontal cortex caused by chronic stress affect spatial learning and memory in rats (Abidin et al., 2004). Kesner et al. (1996) described significantly impaired spatial learning during task performance in rats with lesions in the frontal cortex areas. These changes were also responsible for modifying social behaviors (Seguin, 2004), including aggression (Blair, 2004). Moreover, Lyons & Schatzberg (2003) showed that maternal availability alters the development of frontal cortex regions involved in reward-related memory in squirrel monkeys.

Currently, information on the impact of stress on development of the brain in young pigs is limited (Hay et al., 2001; Kanitz et al., 1998, 2004; Tuchscherer et al., 2004). Associating changes in brain gene expression to behavioral changes in response to stressors would allow for a better understanding of potential mechanisms disrupted by stress. In a previous experiment, we found that the expression of stress-response genes in the frontal cortex was affected by fifteen minutes of social isolation but not by weaning age (Chapter 2). Therefore, to further investigate potential mechanisms correlated with early weaning and social isolation stress in the frontal cortex, a novel and unique porcine brain cDNA microarray (Nobis et al., 2003) was utilized to determine effects of weaning and social isolation on gene expression profiles in the frontal cortex of piglets. In addition, quantitative real-time reverse transcriptase polymerase chain reaction (Q-RT-PCR) was employed to more precisely measure expression of a set of genes selected according to their function, from the microarray outcome. We hypothesize that early-

weaned piglets experience aberrant expression of stress-sensitive genes in the frontal cortex.

2. Material and Methods

2.1 Animals and housing

Twelve Large White female piglets, from three litters were used in this experiment. Only females were used as the experimental animals instead of males because male piglets are subjected to castration on the first days after birth, which is an adverse experience that may interfere with later stress responses. The animals were housed at the Swine Teaching and Research Center at Michigan State University. Housing and feeding routines followed standard swine production practices. Animal housing and experimental procedures were approved by the Michigan State University All-University Committee on Animal Use and Care and were in compliance with United States Department of Agriculture standards. Four female littermates from each of the three litters were randomly assigned to one of four treatment groups: non-weaned control (NWC; n = 3), non-weaned and socially isolated (NWI; n = 3), early-weaned control (EWC; n = 3), and early-weaned and socially isolated (EWI; n = 3). Early-weaned piglets were weaned 10 days after birth and the animals from each litter were kept together until 12 days of age. Non-weaned animals were left with their dams until day 12 after birth. On day 12, piglets assigned to EWI and NWI groups were visually and individually socially isolated from other pigs for 15 minutes, then immediately euthanized. Control animals, EWC and NWC, were taken from the pens with littermates or dams respectively and immediately euthanized. General anesthesia was induced with 5% isoflurane using a

Moduflex Coaxial anesthesia machine (Dispomed, Quebec, Canada). Immediately after reaching deep general anesthesia state, euthanasia was performed using an intracardiac injection of sodium pentobarbital (86mg/kg; Fatal Plus®, Vortech Pharmaceuticals, USA).

2.2 Tissue preparation and RNA extraction

Brains were removed from all animals within 10 minutes of euthanasia. Frontal cortices were dissected out and placed in RNA*later*™ (Ambion, Austin, TX), to protect RNA integrity, and snap frozen in liquid nitrogen. Samples were moved from liquid nitrogen and stored at –80°C. Prior to isolating mRNA, the RNA*later*™ was removed from the samples according to the manufacturer's guidelines. Total RNA from frontal cortex samples was isolated using Ribo Pure Kit (Ambion) following the manufacturer's instructions. Quantity and purity of RNA samples were analyzed using an RNA 6000 Nano Chip Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Wilmington, DE).

2.3 Microarray Loop Design

The samples were arranged in three loops (Kerr & Churchill, 2001) representing three biological replicates per treatment. Each loop accommodated samples from littermates (Figure 1). The decision was made *a priori* to compare: *a*) EWC vs. EWI, *b)* EWI vs. NWI, *c)* NWI vs. NWC, and *d)* NWC vs. EWC. Comparison *a* directly addressed the effect of social isolation in early weaned animals. The effect of social isolation in non-weaned animals was directly tested using comparison *c*. Finally,

comparisons *b* and *d* were designed to directly examine the effect of early weaning in socially isolated and control animals respectively.

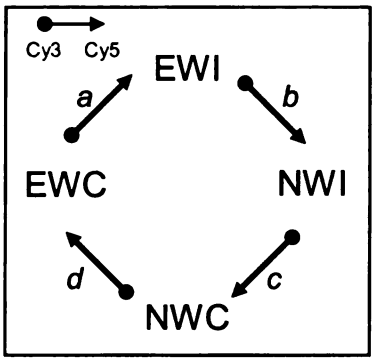

Figure 1. Loop design applied to the cDNA microarray experiment (representing samples from one litter). Three litters were used for this experiment. The arrows indicate the labeling order (dyes Cy3/Cy5) on each array. EWC = early-weaned control, EWI = early-weaned and socially isolated, NWI = non-weaned and socially isolated, NWC = non-weaned control.

2.4 cDNA Microarrays

A porcine brain library (PBL) cDNA microarray containing 866 expressed sequence tags (EST) was used for gene expression profiling. The PBL microarray is described in Nobis et al. (2003). During the data analysis for this experiment we found 858 unique porcine brain ESTs spotted in triplicate and another 8 ESTs spotted six times, resulting in a total of 866 instead of 877 unique features as previously reported (Nobis et al., 2003).

For this study, 12 cDNA microarray hybridizations were performed according to Figure 1. Microarray hybridizations were performed essentially as described (Nobis et al.,

2003). Briefly, 8 μg of total RNA from each frontal cortex sample was used as a template. The cDNA was produced using a reverse transcription reaction incorporating a modified dUTP into the cDNA (BD Atlas PowerScript Fluorescent labeling kit; BD Biosciences, Alameda, CA). Oligo$(dT)_{18}$ was used as a primer, and 0.6 ng of synthetic lambda Q-gene RNA was added to each reaction as a positive control for cDNA synthesis and hybridization. First-strand cDNA was labeled with Cy3 and Cy5 dyes (Amersham Biosciences, Piscataway, NJ) according to instructions for the BD Atlas PowerScript Fluorescent labeling kit (BD Biosciences). Dye-labeled products were purified using QIAquick spin columns (Qiagen, Valencia, CA), recombined and concentrated to 10 μl using Microcon 30 spin concentrators (Millipore, Bedford, MA). SlideHyb solution (Ambion) was added to the probe to reach a final volume of 110 μl. Microarray hybridizations were performed using GeneTAC HybStation (Genomic Solutions, Ann Arbor, MI). Final microarrays were scanned using a GeneTAC LS IV microarray scanner (Genomic Solutions). Images were processed and final intensity reports created using GeneTAC Integrator 4.0 software (Genomic Solutions).

Log intensities were background subtracted and LOESS (Yang et al., 2002) normalized for each array. A moderated t-test (Smyth, 2004) was used to assess significant differences in gene expression between treatment comparisons. Genes differentially expressed in at least one comparison at $P < 0.05$ and exhibiting fold change greater than or equal to 1.25 (Nobis et al., 2003) were submitted to basic local alignment search tool (BLAST; Altschul et al., 1990) analysis on our database (http://nbfgc.msu.edu) under PBL, to disclose identities. For the BLAST analysis, the expectation values (E value) were set at a minimum of 10^{-14} for gene homology. The biological functions of

these genes were determined based on PubMed literature (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi) and AmiGO search (http://www.godatabase.org/cgi-bin/amigo/go.cgi). Genes differentially expressed were clustered in ontogeny groups according to functions of proteins encoded (e.g. structure, development and protection).

2.5 Quantitative Real-Time RT-PCR

Six genes encoding proteins with known relevant brain-related functions, which were differentially expressed in the cDNA microarray hybridization, were selected for further investigation using Q-RT-PCR procedures. The selection criteria consisted of differential expression at $P < 0.05$ and a 1.25 minimum fold change based on microarray outcome and functions related to neuronal function, structure and protection, mechanisms potentially affected by stress. The selected genes were differentially expressed in at least one of the four comparisons. Four of the six genes chosen were differentially expressed in two of the four comparisons.

Q-RT-PCR was performed as described previously (Gibson et al., 1996; Heid et al., 1996). Briefly, a total of 2 μg of total RNA from each sample was reverse transcribed using oligo $(dT)_{18}$ and SuperScript II reverse transcriptase (Invitrogen Life Technologies Corp., Carlsbad, CA). Reverse transcribed cDNA was quantified using ND-1000 spectrophotometer (NanoDrop Technologies Inc., Rocklnd, DE). A total of 30 ng of cDNA was employed in each real-time reaction. Forward and reverse primer sequences were designed with Primer Express 2.0 Software (Applied Biosystems, Foster City, CA) and synthesized by Qiagen. The oligonucleotide sequences of the primers are summarized in Table 1. To confirm that primer dimer products were not influencing final

Ct values, control reactions without template but with each set of primers were performed, with the anticipated result that no product was amplified. Q-RT-PCR was performed and analyzed on an ABI Prism 7000 Sequence Detection System (Applied Biosystems). *Sus scrofa* 18S ribosomal RNA was selected as the control gene to be used for normalization purposes based on preliminary test reactions, as expression of this control gene did not change relative to treatments. All reactions were performed using template from individual animals in triplicate. Relative quantification methods were determined based on the primer amplification efficiency tests (Livak, 1997; Livak & Schmittgen, 2001). Control primer 18S ribosomal RNA amplification efficiency was equal to 1. Relative gene expression quantification using $2^{-(\Delta\Delta Ct)}$ method (Livak & Schmittgen, 2001) was applied to measure mRNA abundance for actin related protein 2/3 complex (ARP2/3), diazepam binding inhibitor (DBI), phosphoprotein enriched in astrocytes 15kDa (PEA-15) and tyrosine 3-monoxygenase/tryptophan 5-monooxygenase activation protein (14-3-3 protein) (GenBank accession nos. NM_005719, AJ301366, NM_003768, and NM_145690 respectively; Table 1), which showed amplification efficiencies equal to 1. A relative standard curve method (Livak, 1997) was applied to examine expression of carboxypeptidase E (CPE) and ornithine decarboxylase antienzyme 2 (OAZ2) mRNAs (GenBank accession nos. NM_001873 and NM_002537 respectively; Table 1), which showed amplification efficiencies different from 1. The four treatment comparisons used in the cDNA microarray experiment were also considered for Q-RT-PCR data analysis (Figure 1). Hence, for this study, changes in gene expression result from the comparison of the first treatment group relative to the second for each contrast. For presentation of results in which gene expression was lower in the

first treatment compared to the second, the ratio (< 1) was divided into -1 to give a negative relative expression value (-1/ratio). Values are presented as the mean ± standard error of the mean (SEM). Statistical significance of relative changes in gene expression was assessed using paired t-tests. Genes with $P < 0.05$ were considered differentially expressed.

Table 1. Primers used for Q-RT-PCR analysis of gene expression in the frontal cortex of piglets.

GenBank accession no.	Forward primer (5' – 3' end)	Reverse primer (5' – 3' end)
NM_005719	ATGGACCCCGACACCAAAC	GTCCTTTGAACTGACTCCTGATAGG
AJ301366	GGAAGTTAAGAACCTTAAGACCAAACC	TCGCTTGTTTGTAGTGGCTGTAG
NM_003768	TGTTGCACTGATCCCCAGTTC	CCAAGGTGCCAGGATATTGAG
NM_145690	ACATCGGATACCCAAGGAGATG	TTGGAAGGCCGGTTAATTTTC
NM_001873	GCTGAGCTACGGTGGGAACTC	AAATCATCCAATAAACCCTCCTAAAG
NM_002537	CCTTCAGCTTCTTGGGCTTT	TCTGGCCGAGAGGGAACAC

3. Results

3.1 cDNA Microarray

The BLAST analysis of the results from the cDNA microarray hybridizations revealed 103 unique ESTs differentially expressed ($P < 0.05$ and at least 1.25 fold change) among the four comparisons. Only 67 of the unique sequences display significant similarity with known genes in the GenBank database (Table 2). Forty-two of these genes had known function from which five were identified as ribosomal proteins. Twenty-five EST sequences matched only genomic or EST sequences in the database were classified as genes with unknown function. Six of 42 genes were identified as

playing known roles in neuronal function, structure and protection, functions relevant to our model, and had their relative expression further examined by Q-RT-PCR.

Table 2. Genes with identified by BLAST analysis of differentially expressed ESTs on the PBL cDNA microarray.

GenBank Accession no.	Gene Name	Gene Function
AJ301366	Sus scrofa partial mRNA for diazepam binding inhibitor (DBI)	Neurosteroidogenesis
NM_001873	Homo sapiens carboxypeptidase E (CPE)	Biosynthesis of neuropeptides
NM_145690/ BC000179	Homo sapiens tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein	Biosynthesis of neurotransmitters
NM_002537	Homo sapiens ornithine decarboxylase antizyme 2 (OAZ2)	Biosynthesis of polyamines
AF162445	Canis familiaris skeletal muscle chloride channel ClC-1 (CLCN1)	Cell function and metabolism
NM_005938	Homo sapiens myeloid/lymphoid or mixed-lineage leukemia 7 (MLLT7)	Cell protection
AB079894	Sus scrofa gene for T-cell receptor beta-chain	Cell protection
AJ251914	Sus scrofa MHC class I SLA genes, haplotype H01	Cell protection
AJ251829	Sus scrofa MHC class I SLA genomic region, haplotype H01	Cell protection
NM_003768	Homo sapiens phosphoprotein enriched in astrocytes 15 (PEA15)	Cell protection/apoptosis
NM_006406	Homo sapiens peroxiredoxin 4 (PRDX4)	Cell protection/catalysis
BC032148	Homo sapiens, troponin I	Cell structure
NM_000089	Homo sapiens collagen, type I, alpha 2 (COL1A2)	Cell structure/component
J04204	Bos taurus 32 kd accessory protein	Energy metabolism
X59048	Bovine mRNA heart mitochondrial oxidoreductase	Energy metabolism
Bc016812	Homo sapiens ATP synthase, H+ transporting, mitochondrial F1 complex	Energy metabolism
NM_005690	Homo sapiens dynamin 1-like (DNM1L)	Energy metabolism
NM_006876	Homo sapiens UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 6 (B3GNT6)	Energy metabolism
AJ504726	Sus scrofa mut gene for methylmalonyl-CoA mutase	Energy metabolism
X86791	Sus scrofa beta-globin gene	Energy metabolism
AF304202	Sus scrofa breed Landrace mitochondrion	Energy metabolism
L11869	Porcine growth hormone-releasing hormone receptor	Growth
NM_015286	Homo sapiens desmuslin (DMN)	Membrane protein
BC030589	Homo sapiens aldehyde dehydrogenase 1 family, member A2,	Metabolism of neurotransmitters
K00800	Bos taurus fibronectin mRNA	Neuronal development
NM_005719	Homo sapiens actin related protein 2/3 complex (ARP3/3)	Neuronal development
XM_114617	Homo sapiens beta 5-tubulin (OK/SW-cl.56)	Neuronal development
NM_004522	Homo sapiens kinesin family member 5C (KIF5C)	Neuronal development
BC015149	Homo sapiens, similar to microtubule-associated protein 4	Neuronal development
Z99716	Human DNA similar to neuronal-specific septin 3	Neuronal development
NM_145330	Homo sapiens mitochondrial ribosomal protein L33 (MRPL33)	Ribosomal protein
NM_000982	Homo sapiens ribosomal protein L21	Ribosomal protein
NM_001030	Homo sapiens ribosomal protein S27	Ribosomal protein
X03342	Human mRNA for ribosomal protein L32	Ribosomal protein

Table 2 (cont'd).

AK002787	Mus musculus adult male kidney cDNA ribosomal protein L13	Ribosomal protein
NM_016545	Homo sapiens immediate early response 5 (IER5), mRNA	Transcription
BC036013	Homo sapiens mitogen-activated protein kinase (MAPK)	Transcription
NM_032188	Homo sapiens MYST histone acetyltransferase 1 (MYST1)	Transcription
AF087481	Homo sapiens retinoblastoma binding protein 2 homolog 1 (RBBP2H1)	Transcription
NM_004264	Homo sapiens SRB7 suppressor of RNA polymerase B homolog (yeast) (SURB7)	Transcription
BC014269	Homo sapiens, similar to eukaryotic translation termination factor	Translation
AC090976	Bos taurus clone RP42-400M23, complete sequence	Unknown
Y17923	Bos taurus mRNA for lyncein	Unknown
AC023050	Homo sapiens 12 BAC RP11-428G5	Unknown
AC019171	Homo sapiens BAC clone RP11-173C1 from 2	Unknown
BC033863	Homo sapiens BAC clone RP11-455J15 from 7	Unknown
AC013449	Homo sapiens BAC clone RP11-558C24 from 2	Unknown
AF038540	Homo sapiens brain NSP-like 1 (NSPL1) (neuroendocrine-specific protein)	Unknown
AK057557	Homo sapiens cDNA FLJ32995 fis, clone THYMU1000136	Unknown
AC022392	Homo sapiens chromosome 10 clone RP11-150D20	Unknown
AC008378	Homo sapiens chromosome 5 clone CTC-209H22	Unknown
BC027921	Homo sapiens CXYorf1-related protein	Unknown
AL021408	Homo sapiens DNA sequence from PAC 523C21 on chromosome 6q23.1-23.3.	Unknown
AP001201	Homo sapiens genomic DNA, chromosome 11	Unknown
XM_114482	Homo sapiens heat shock 70kD protein 4 (HSPA4)	Unknown
BC024023	Homo sapiens LOC317671, mRNA	Unknown
AB020681	Homo sapiens mRNA for KIAA0874 protein	Unknown
AL359597	Homo sapiens mRNA; cDNA DKFZp547B146	Unknown
BC024316	Homo sapiens mRNA; cDNA DKFZp564H1916 (from clone KFZp564H1916)	Unknown
BC011583	Homo sapiens, clone IMAGE:3834272	Unknown
BC034275	Homo sapiens, clone IMAGE:4179671	Unknown
BC019624	Homo sapiens, Similar to hypothetical protein	Unknown
AL732326	Human DNA sequence from clone RP11-723P2 on chromosome X, complete sequence	Unknown
AL139255	Human DNA sequence from clone RP4-753C8 on chromosome 1p31.3-32.3.	Unknown
M29512	Pig unidentified hepatic protein mRNA	Unknown
AC097230	Sus scrofa clone RP44-254G1	Unknown

3.2 Q-RT-PCR

From the cDNA microarray experiment, six differentially expressed genes presenting relevant brain-related function were selected to carry out further studies using Q-RT-PCR. This method was chosen to allow more precise quantification of changes in expression of the selected genes. The genes selected for Q-RT-PCR examination were DBI, OAZ2, tyrosine 3-monoxygenase/tryptophan 5-monooxygenase activation protein (14-3-3 protein), CPE, PEA-15 and ARP2/3 (Table 2).

3.2.1 Effect of social isolation

The contrast between NWI and NWC allowed us to examine the effect of 15 minutes of social isolation on 12 day old non-weaned piglets. Interestingly, social isolation significantly suppressed mRNA levels of 14-3-3 protein and CPE in the frontal cortex of NWI versus NWC animals ($P < 0.05$). In addition, a significant decrease in PEA-15 mRNA abundance was detected in NWI animals ($P < 0.01$) compared to controls, as shown in Figure 2.

3.2.2 Effect of early weaning

Examining the contrast between EWC and NWC allowed investigating the effects of weaning (48 hours post-weaning). Our findings indicated that there is no evidence of effects caused by weaning in the expression of the tested genes in the frontal cortex of early-weaned compared to non-weaned control piglets.

3.2.3 Effect of social isolation in the presence of early weaning

The comparison EWI versus EWC highlighted the effect of social isolation in early-weaned piglets. The DBI mRNA expression was remarkably suppressed in EWI animals compared to early-weaned control animals ($P < 0.05$), and the levels of ARP2/3 mRNA were also significantly suppressed in EWI when compared to the non-socially isolated control group ($P < 0.05$; Figure 2). Based on the Q-RT-PCR results, there was no major effect of weaning or social isolation on gene expression in the frontal cortex when early-weaned piglets were compared to non-weaned animals exposed to fifteen minutes of social isolation (EWI versus NWI).

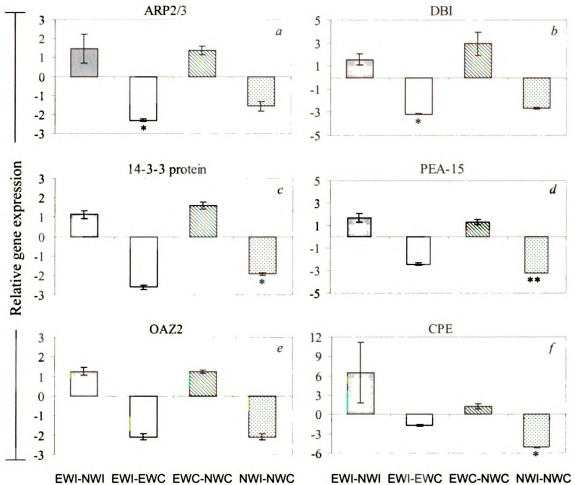
ARP2/3
DBI
14-3-3 protein
PEA-15
OAZ2
CPE
a
b
c
d
e
f
Relative gene expression
EWI-NWI
EWI-EWC
EWC-NWC
NWI-NWC

Figure 2.

Figure 2. Relative expression of stress-responsive genes in the frontal cortex of piglets subjected to early weaning and/or social isolation. (*a*) actin related protein 2/3 complex (ARP2/3), (*b*) diazepam binding inhibitor (DBI), (*c*) 14-3-3 protein, (*d*) phosphoprotein enriched in astrocytes 15kDa (PEA-15), (*e*) ornithine decarboxylase antienzyme 2 (OAZ2), (*f*) carboxypeptidase E (CPE). The ratio represents the first treatment group compared to the second in each contrast. Negative values indicate smaller expression of the first group relative to the second. Positive values indicate greater expression of the first group compared with the second. Each bar represents the mean (± SEM) change in gene expression for each treatment comparison. * $P < 0.05$; ** $P < 0.01$. EWC = early-weaned control, EWI = early-weaned and socially isolated, NWI = non-weaned and socially isolated, NWC = non-weaned control. Note different scales on the y axes of the plots.

4. Discussion

Our results show that expression of five of the six genes examined by Q-RT-PCR, associated with neuronal function, structure and protection, was significantly suppressed by 15 minutes of social isolation in either non-weaned or early-weaned piglets. Interestingly, this study partially agrees with a prior study in which the effects of weaning at two ages, and social isolation were investigated. We consistently found that the expression of the stress-responsive genes 11β-HSD1, 11β-HSD2, GR and MR mRNAs was reduced following 15 minutes of social isolation but not affected by weaning age (Chapter 2). In this experiment, however, some of the changes found as a result of social isolation may be confounded with the effects of 48 hours post-weaning. Potentially distinct effects on gene expression may be observed following longer periods of social isolation. It is also possible that the transitory nutritional deficits that early-weaned animals experience, at least for 2 days post weaning (Carroll et al., 1998), may have influenced some of our findings. Previous studies reported that early weaning stress experienced by piglets is associated with the occurrence of abnormal behaviors, intensified aggression and related neuroendocrine changes (Hay et al., 2001; Kanitz et al., 2004; Yuan et al., 2004). Additionally, social isolation pre- and post-weaning has also been reported to activate the stress axis, causing long-term neuroendocrine and behavioral effects (Hellemans et al., 2004; Tuchscherer et al., 2004).

Twelve day old non-weaned piglets, when exposed to fifteen minutes of social isolation, showed suppressed mRNA abundance of 14-3-3 protein in the frontal cortex, a brain area in which this protein is highly expressed (Takahashi, 2003). In the brain, 14-3-3 protein activates tyrosine and tryptophan hydroxylases, stabilizing the phosphorylation

state of these enzymes (Ichimura et al, 1988) and indirectly regulates the biosynthesis of neurotransmitters, such as serotonin (5-HT) and catecholamines (Ichimura et al., 1987). Albeit we did not measure 5-HT or catecholamine levels in the experimental animals, our results suggest that socially isolated animals may experience deficits in neurotransmitter biosynthesis. Changes in 5-HT and catecholamine levels in the prefrontal cortex can impair attentional control and reward-based learning (Rogers et al., 1999). For instance, acute reduction in 5-HT in the frontal cortex induces impaired decision-making and spatial working memory in primates (Clarke et al., 2004). Interestingly, previous studies showed that early-weaned piglets exposed to 15 minutes of social isolation 2 days after weaning showed cognitive deficits (Laughlin & Zanella, 2002; Souza & Zanella, 2004).

Effects of early weaning and social isolation on CPE mRNA also were examined in the frontal cortex. Carboxypeptidase E is a key enzyme in the biosynthesis of neuropeptides (Fricker et al., 1986) and directs neuropeptide precursors, such as pro-opiomelanocortin, proenkephalin and prodynorphin to the proper secretory pathways in neuroendocrine cells (Loh et al., 2002, 2004). In this study, a significant decrease in CPE mRNA levels was observed as an effect of social isolation in non-weaned animals compared to controls (NWI vs. NWC). This may suggest that social isolation interfered with neuroendocrine peptide metabolism in the frontal cortex mainly in non-weaned piglets. Abnormal behaviors in pigs are correlated with opioid receptor density in the brain (Zanella et al., 1996). Also, differences in stress response among pigs are influenced by neuroendogenous opioid systems (Loijens et al., 2002). Interestingly, CPE knockout mice show diminished reactivity to stimuli, impaired coordination and visual placing, in addition to other dysfunctions (Cawley et al., 2004). Further studies measuring

levels of neuropeptides in the frontal cortex of socially isolated piglets would help to confirm the biological significance of our findings.

Non-weaned piglets when subjected to social isolation also showed decreased PEA-15 mRNA in the frontal cortex, compared to non-socially isolated piglets. This gene is abundantly expressed in astrocytes and plays a role in controlling cell proliferation and apoptosis (Danziger et al., 1995; Renault et al., 2003). It either inhibits or enhances apoptosis depending on the pathway activated (Estelles et al., 1999). Interestingly, Kitsberg et al. (1999) demonstrated that astrocytes lacking PEA-15 rapidly showed signs of apoptosis when exposed to tumor necrosis factor (TNF), but astrocyte protection and survival was reinstated once PEA-15 levels recovered. Non-weaned piglets when socially isolated may be experiencing reduced neuronal protection in the frontal cortex, however further studies are needed to elucidate the role of PEA-15 in this brain area.

We also observed more than 3 fold decrease in DBI mRNA in the frontal cortex of early-weaned piglets exposed to social isolation versus early-weaned control. Diazepam binding inhibitor plays a major role in regulation of neurosteroidogenesis in the brain by binding to benzodiazepine receptors associated with GABA receptors (Costa & Guidotti, 1991; Ferrarese et al., 1993). The decrease in DBI gene expression in early-weaned piglets, when socially isolated suggests that this acute stressor may be negatively affecting neurosteroidogenesis in the frontal cortex. Lack of steroids in the brain impairs myelination (Chan et al., 1998) and affects modulation of neurotransmitter systems impairing cognitive processes, in particular learning and memory (Schumacher et al., 2003; Vallee et al., 2001). A previous study demonstrated that piglets weaned at 12 days of age, when socially isolated, showed lower levels of steroids such as cortisol and

corticosterone, in the hippocampus compared to non-weaned animals (Zanella et al., 2004). No study has been performed previously in piglets examining the expression of DBI in the brain.

Social isolation of early-weaned piglets also reduced mRNA abundance for the protein complex ARP2/3, which is involved with neuronal development (Meyer & Feldman, 2002). Goldberg and colleagues (2000) demonstrated that neuronal growth factor stimulated actin polymerization by recruiting the ARP2/3 complex. Thus, social isolation may adversely affect the neuronal development process in the frontal cortex of early-weaned piglets since reduction of the ARP2/3 expression in frontal cortex may be associated with abnormal development of the brain (Weitzdoerfer et al., 2002).

In summary, our findings suggest that social isolation is associated with reduction in mRNAs encoding for factors involved in biosynthesis of neurotransmitters and neuropeptides, and neurosteroidogenesis pathway; therefore potentially affecting proper neuronal function in the frontal cortex of young piglets. Results reveal potential pathways associated with impaired learning and memory and social recognition deficits reported in early-weaned piglets when socially isolated.

CHAPTER FOUR

Discussion and Future Research Directions

Discussion

The impact of early weaning on occurrence of intensified aggression, abnormal behaviors, and neuroendocrine modifications in piglets has been previously documented (Gardner et al., 2001; Hay et al, 2001; Kanitz et al., 1998, 2004; Worobec et al., 1999; Yuan et al., 2004). The postnatal cerebral development of piglets is characterized by a period sensitive to stress, during which the brain may be more vulnerable to manipulations carried out at early ages, mainly during the first 3 weeks after birth (Hemsworth et al., 1986; Weaver et al., 2000). As described in Chapters Two and Three, early weaning suppressed expression of 11β-HSD1, 11β-HSD2, MR and GR in the hippocampus of piglets, while social isolation suppressed the expression of genes encoding 11β-HSD1, 11β-HSD2, MR and GR in the frontal cortex of piglets, regardless of age or weaning status. Fifteen minutes of social isolation also suppressed expression of 14-3-3 protein, PEA-15, CPE, DBI and ARP2/3 in the frontal cortex of either early- or non-weaned piglets. One effect of social isolation and early weaning is enhanced expression of glucocorticoids, particularly cortisol.

Glucocorticoids, such as cortisol, are potentially one class of indirect regulators of the changes in gene expression observed in the frontal cortex and hippocampus of piglets. The glucocorticoid receptors GR and MR are hormone-activated transcription factors that have the potential to influence gene expression in a wide variety of central nervous system neurons (e.g. Drouin et al., 1989; Herman, 1993; de Kloet et al., 1993; Shaaf et

al., 2000) interfering, for instance, with neuronal growth and viability (Diaz et al., 1998; Herman, 1993). Glucocorticoids are also important regulators of stress-induced immediate early gene expression in the brain (Senba & Ueyama, 1997). In this study, in addition to suppressed GR and MR gene expression, several other genes studied had lower mRNA levels in the frontal cortex and hippocampus in response to social isolation and early weaning (Figure 1).

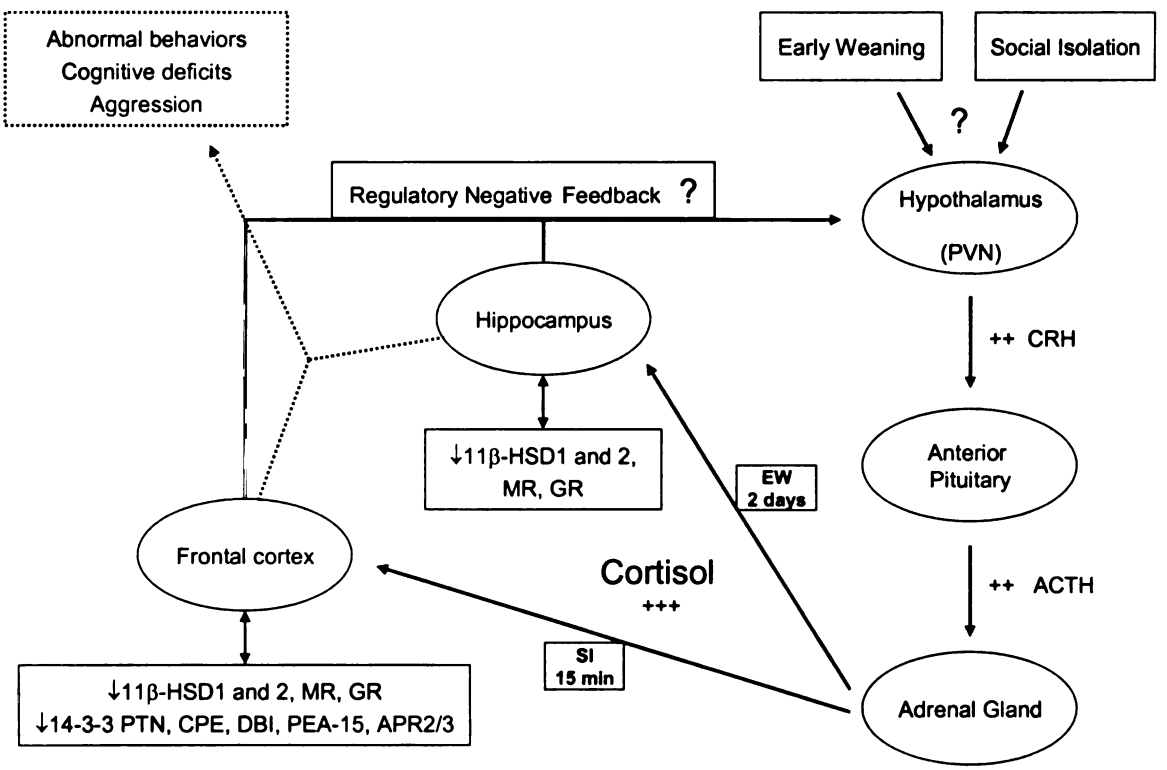

Figure 1. Diagram illustrating the potential effects of early weaning (EW) and social isolation (SI) on the hypothalamic-pituitary-adrenal axis and consequent changes in gene expression in the hippocampus and frontal cortex of piglets. Paraventricular nucleus (PVN); Corticotrophin release hormone (CRH); Adrenocorticotropin hormone (ACTH).

Despite progress in understanding the effects of increased cortisol on brain function and gene expression, biologists have yet to uncover the basic molecular processes leading to cognitive deficits. In this study, we employed techniques such as cDNA microarray hybridization and Q-RT-PCR to begin investigating changes in gene expression as a result of early weaning and social isolation. The porcine brain library cDNA microarray (Nobis et al., 2003) used in our work is a novel and powerful approach for screening gene expression profiles. Also, Q-RT-PCR is a sensitive and accurate technique that measures mRNA abundance in a small amount of sample (Bustin, 2000). One caveat to both of these techniques is that they measure only mRNA levels and therefore, interpretation of results presented on chapters 2 and 3 should be strictly limited to a discussion on mRNA levels for the genes encoding various proteins of interest and not directly used to infer changes in the proteins themselves.

A larger porcine cDNA brain library than the one used for this study (Nobis et al., 2003) would likely help to identify additional brain pathways affected by stress and may have implicated more pathways and mechanisms associated with behavioral and neuroendocrine changes resulting from weaning and social isolation stress. Also, a larger sample size would provide greater statistical power, and more effectively enable detection of differentially expressed genes. This would add significantly to the limited information available on potential genes involved with brain development and plasticity that are affected by early adverse experiences in piglets (Kanitz et al., 1998, 2004; Tuchscherer et al., 2004). To estimate sample sizes for future gene expression studies, power of tests were computed using the median standard deviation of gene expression for frontal cortex and hippocampus of piglets. For instance, as shown on Figure 2,

approximately 15 animals are needed to have an 80% probability of detecting a 2-fold change in gene expression in the frontal cortex of piglets. Also, as shown on Figure 3, approximately 8 animals are needed to have an 80% probability of detecting a 2-fold change in gene expression in the hippocampus of piglets.

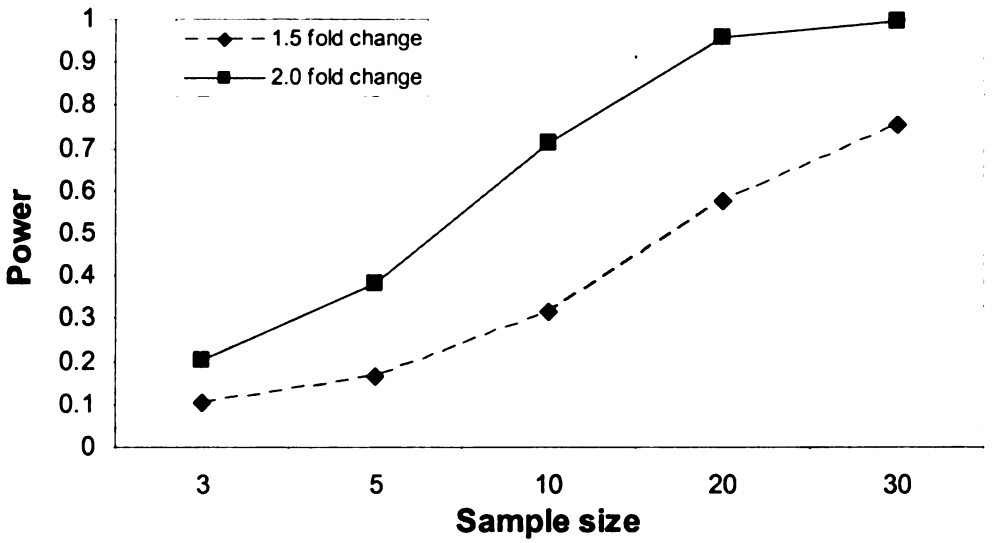

Figure 2. Power to detect 1.5 and 2-fold change in expression of genes in the frontal cortex of piglets as a function of sample size considering a median standard deviation equals to 0.84.

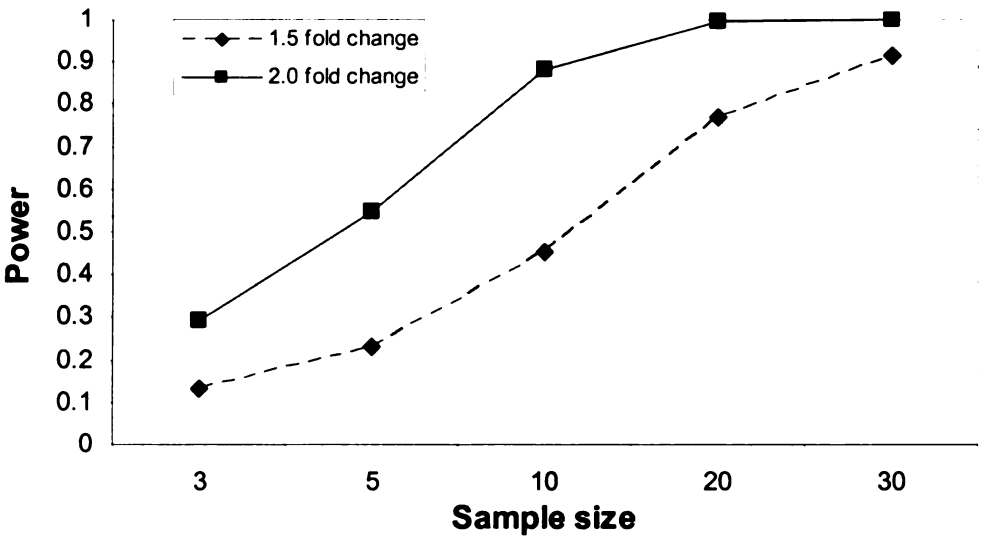

Figure 3. Power to detect 1.5 and 2-fold change in expression of genes in the hippocampus of piglets as a function of sample size considering a median standard deviation equals to 0.67.

In addition to neuroendocrine changes resulting from early adverse experiences (e.g. Kanitz et al., 1998, 2004; Tuchscherer et al., 2004), previous studies reported that when socially isolated for 15 minutes, early-weaned piglets showed impaired spatial learning and memory (Laughlin & Zanella, 2002) and social recognition deficits (Souza & Zanella, 2004) tested two days post-weaning. It was suggested that the stress of early weaning altered cellular and biochemical parameters in the frontal cortex and hippocampus of piglets during a sensitive period of brain development. Both, frontal cortex and hippocampus, play important roles in cognitive function, such as visual and spatial learning and memory (e.g. Abidin et al., 2004; Cao et al., 2004), and social behavior, including aggression (Kolb et al., 2004; Wood et al., 2003). Therefore, the

objective of this study was to directly investigate changes in expression of stress-response genes in the frontal cortex and hippocampus of early- and conventionally-weaned piglets subjected to additional stress through social isolation or when kept with littermates.

The age at which piglets were weaned affected expression of 11β-HSD1, 11β-HSD2, MR and GR in the hippocampus, but did not influence frontal cortex gene expression. When comparing weaned versus non-weaned piglets at ages 10 and 21, it was demonstrated that early weaning suppressed expression of stress-response genes (i.e. 11β-HSD1, 11β-HSD2, MR and GR) in the hippocampus. Interestingly, no effect on expression of the tested genes was observed when piglets were weaned at 21 days of age. These results suggest that the hippocampus of piglets may be more vulnerable to weaning stress at 10 days than at 21 days. If, in fact, mRNA levels are related or result in reduced protein levels, than lower expression of GR in the hippocampi of early-weaned compared to conventionally-weaned piglets, suggests that early-weaned animals would be exposed to higher levels of active unbounded glucocorticoids, such as cortisol. This effect could be aggravated since early-weaned piglets also showed reduced mRNA levels of the dehydrogenases 11β-HSD1 and 11β-HSD2 in the hippocampus. However, transitory nutritional deficits potentially experienced by the piglets during the two days post-weaning (Carroll et al., 1998) could also have contributed to the cognitive deficits, since for instance amino acids such as tyrosine and tryptophan available on the diet, are essential for biosynthesis of neurotransmitters (Fernstrom, 2000).

Our results suggest that the negative effects of early weaning can be exacerbated when piglets are challenged by an additional short-term stressor, such as social isolation. In this study, the fifteen minutes of social isolation was used as a stressor to replicate the

protocol that, in previous studies, induced cognitive deficits (Laughlin & Zanella, 2002; Souza & Zanella, 2004), and to minimize nutritional deprivation, but to cause a biologically relevant stressful situation for the piglets. Previous studies showed that social isolation increases circulating glucocorticoid levels in response to activation of the hypothalamic-pituitary-adrenal axis (e.g. Laughlin & Zanella, 2002; Kanitz et al., 2004). In our study only the immediate effects of short-term social isolation on frontal cortex and hippocampal gene expression were analyzed, thus reducing contributions from potential nutritional deficits.

Based on our results, overall, social isolation reduced the mRNA abundance for several genes in the frontal cortex of piglets, independently of the weaning status. In the first experiment (Chapter 2), social isolation suppressed mRNA levels for genes encoding 11β-hydroxysteroid dehydrogenase 1 and 2 (11β-HSD1 and 11β-HSD2, respectively), enzymes mainly responsible for inactivation of cortisol, and the mRNA abundance for genes encoding mineralocorticoid and glucocorticoid receptors (MR and GR respectively), which are responsible for mediating the stress axis response by binding glucocorticoids, in the frontal cortex of both 12 and 23 days old piglets. In the second experiment (Chapter 3), when using the PBL cDNA microarray and Q-RT-PCR to analyze expression profiles of genes in the frontal cortex of 12 days old piglets, whether weaned or not, it was found that 15 minutes of social isolation significantly suppressed the expression of genes encoding 14-3-3 protein, PEA-15, CPE, DBI and ARP2/3. If mRNA levels translate to changes in protein abundance, then neuronal development, function and protection may be impaired in socially-isolated piglets. Thus, the psychological stress, which encompasses social separation and exposure to a novel

environment (Hennessy et al., 2004) encountered by the piglets may have influenced their responses to social isolation.

Overall, results from this study in association with previous research, indicate that the hippocampus of piglets weaned at 10 days of age may experience decreased mRNA expression of important genes encoding proteins that are mediators of the stress response, such as 11β-HSD1 and 2, GR and MR. When examining gene expression in the frontal cortex, the mRNA expression of several genes encoding proteins involved in neuronal development, function, and protection, in addition to 11β-HSD1 and 2, GR and MR, were found to be suppressed as a result of 15 minutes of social isolation. Therefore, our results demonstrated that social isolation affected the expression of genes examined in frontal cortex but not hippocampus, indicating that the effects of distinct stressors are additive in order to cause cognitive deficits in young piglets.

Future Research Directions

Further studies should focus on the investigation of long-term effects of early weaning on swine behavioral and neuroendocrine development. Studies exploring the relationship of early weaning stress and cognitive deficits, and their association with aggression, should be encouraged. One hypothesis that I would like to explore is that early weaning stress increases aggressive behavior as a consequence of neuronal exposure to high glucocorticoid levels during a sensitive period of brain development. Therefore, to investigate at brain level the long-term effects of weaning, brain areas such as hippocampus and frontal cortex should be further studied. As discussed earlier, both

brain areas play important roles in cognitive functions and social behavior including aggression (Kolb et al., 2004; Wood et al., 2003).

To investigate potential correlations between glucocorticoid exposure in early-weaned piglets, and mechanisms that contribute to greater vulnerability of early-weaned animals to stressors such as social isolation, I would first measure the levels of proteins encoded by the most important genes reported in this study, such as 11β-HSD1 and 2, GR and MR, 14-3-3 protein, DBI and possibly CPE in the frontal cortex and hippocampus of early-weaned and socially isolated piglets.

Second, a controlled experiment to test the effects of peripheral glucocorticoid levels on gene expression in the hippocampus and frontal cortex of early-weaned piglets (weaned at 10 days of age) should be carried out. For this study, the following treatment groups would be used: early-weaned plus social isolation, early-weaned treated with exogenous glucocorticoid plus social isolation, non-weaned plus socially isolation and non-weaning treated with exogenous glucocorticoid plus social isolation. The administration of cortisol, the exogenous glucocorticoid, to treated non-weaned and weaned animals should be performed n at day 10 after birth, the same day that weaning is performed. This set up would to replicate for non-weaned the state in which weaned animals are exposure to high cortisol level. One of the main objectives of this experiment would be to correlate the peripheral levels of cortisol with the levels of MR and GR binding, and also the enzymes 11β-HSD1 and 2 in the hippocampus and frontal cortex of early and non-weaned piglets.

To complement this experiment, behavioral observations should be taken, and spatial and cognitive tests, in addition to fear/anxiety tests performed as well. The

prediction would be that the cortisol treated early-weaned and non-weaned animals, when socially isolated, would show cognitive impairments as previously observed (Laughlin & Zanella, 2002; Souza & Zanella, 2004), and fear/anxious state (Kanitz et al., 2004; Molina-Hernandez et al., 2001; Weiss et al., 2004). Also, specific behavioral tests consisting of measuring specific brain area functions, such as frontal cortex and hippocampus would be useful to determine which brain area may be mediating the previously observed cognitive deficits (Laughlin & Zanella, 2002; Souza & Zanella, 2004).

A complementary tool for this last procedure would be the application of imaging techniques, such as photo-optic imaging (PET) scan or functional magnetic resonance imaging (fMRI) (Potchen, 2000), at different time points post-weaning, to provide information on the activated sites in the frontal cortex and hippocampus. However, currently, to apply these techniques for piglets, the animals would have to be anesthetized, and this procedure could influence the outcomes. Thus, to employ imaging tools, it may be valuable to train the animals to stand still while being tested.

Future studies should also control for the transitory nutritional deficits likely experienced by weaned piglets for at least two days post-weaning (Carroll et al., 1998). Amino acids, such as tyrosine and tryptophan, are essential for the biosynthesis of neurotransmitters, such as serotonin and catecholamines. Therefore, depletion of these neurotransmitters may lead to impaired neurosysnaptic transmission, resulting in cognitive deficits (e.g. Clarke et al., 2005) and aggression (e.g. Chiavegatto and Nelson, 2003). Thus, the use of high performance liquid chromatography could be applied for monitoring brain catecholamines and serotonin and metabolites concentrations.

Further behavioral and neuroendocrine studies would certainly facilitate a better understanding of the stress physiology and its responsiveness to weaning, providing insights of the long-term consequences of the stress caused by early weaning. In summary, future studies should investigate the correlation of behavioral alterations and cerebral mechanisms, correlated to early weaning stress response, to improve swine welfare.

COMPILED REFERENCES

Abidin I., Yargicoglu P., Agar A., Gumuslu S., Aydin S., Ozturk O., Sahin E. 2004. The effect of chronic restraint stress on spatial learning and memory: relation to oxidant stress. Inter. J. Neurosci., 114, 683-99.

Altschul S. F., Gish W., Miller W., Myers E. W., Lipman D. J. 1990. Basic local alignment search tool. Journal of Molecular Biology, 215, 403-10.

Antonawich F. J., Miller G., Rigsby D. C., Davis J. N. 1999. Regulation of ischemic cell death by glucocorticoids and adrenocorticotropic hormone. Neuroscience., 88, 319-25.

Blair, R. J. 2004. The roles of orbital frontal cortex in the modulation of antisocial behavior. Brain Cog., 55, 198-208.

Bustin S. A. 2000. Absolute quantification of mRNA using real-time reverse transcription polymerase chain reaction assays. J. Mol. Endoc., 25, 169-93.

Cao L., Jiao X., Zuzga D. S., Liu Y., Fong D. M., Young D., During M. J. 2004. VEGF links hippocampal activity with neurogenesis, learning and memory. Nat Genet., 36, 827-35.

Carroll J. A., Veum T. L., Matteri R. L. 1998. Endocrine responses to weaning and changes in post-weaning diet in the young pig. Dom. Anim. Endoc., 15, 183-94.

Cawley N. X., Zhou J., Hill J. M., Abebe D., Romboz S., Yanik T., Rodriguiz R. M., Wetsel W. C., Loh Y. 2004. The Carboxypeptidase E knockout mouse exhibits endocrinological and behavioral deficits. Endocrinology, 145, 5807-19.

Chan J. R., Phillips L. J., Glaser M. 1998. Glucocorticoids and progestins signal the initiation and enhance the rate of myelin formation. Proceedings of the National Academic of Science of the United States of America, 95, 10459-64.

Clarke H. F., Dalley J. W., Crofts H. S., Robbins T. W., Roberts A. C. 2004. Cognitive inflexibility after prefrontal serotonin depletion. Science, 304, 878-80.

Clarke H. F., Walker S. C., Crofts H. S., Dalley J.W., Robbins T.W., Roberts A.C. 2005. Prefrontal serotonin depletion affects reversal learning but not attentional set shifting. J. Neurosci., 25, 532-8.

Chiavegatto S., Nelson R.J. 2003. Interaction of nitric oxide and serotonin in aggressive behavior. Horm Behav., 44, 233-41.

Cook S. C, Wellman C. L. 2004. Chronic stress alters dendritic morphology in rat medial prefrontal cortex. J. Neurobiol., 60, 236-48.

Costa E., Guidotti A. 1991. Diazepam binding inhibitor (DBI): a peptide with multiple biological actions. Life Science, 49, 325-44.

Danziger N., Yokoyama M., Jay T., Cordier J., Glowinski J., Chneiweiss H. 1995. Cellular expression, developmental regulation, and phylogenic conservation of PEA-15, the astrocytic major phosphoprotein and protein kinase C substrate. Journal of Neurochemistry, 64, 1016-25.

De Kloet E. R., Sutanto W., van den Berg D. T., Carey M. P., van Haarst A. D., Hornsby C. D., Meijer O. C., Rots N. Y., Oitzl M. S. 1993. Brain mineralocorticoid receptor diversity: functional implications. J. Steroid Biochem. Mol. Biol., 47, 183–190.

De Kloet E. R. 2004. Hormones and the stressed brain. Ann N Y Acad Sci. 1018, 1-15.

Diamond D. M., Park C. R., Heman K. L., Rose G. M. 1999. Exposing rats to a predator impairs spatial working memory in the radial arm water maze. Hippocampus, 9, 542-52.

Diaz R., Brown R. W., Seckl J. R. 1998. Distinct ontogeny of glucocorticoid and mineralocorticoid receptor and 11beta-hydroxysteroid dehydrogenase type I and II mRNAs in the fetal rat brain suggest a complex control of glucocorticoid actions. J Neurosci., 18, 2570-80.

Diorio D., Viau V., Meaney M. J. 1993. The role of the medial prefrontal cortex (cingulate gyrus) in the regulation of hypothalamic-pituitary-adrenal responses to stress. J Neurosci., 13, 3839-47.

Drouin J., Nemer M., Charron J., Gagner J. P., Jeannotte L., Sun Y. L., Therrien M., Tremblay Y. 1989. Tissue-specific activity of the pro-opiomelanocortin (POMC) gene and repression by glucocorticoids. Genome, 31, 510-9.

Erickson K., Drevets W., Schulkin J. 2003. Glucocorticoid regulation of diverse cognitive functions in normal and pathological emotional states. Neurosci. Biobehav. Rev., 27, 233-46.

Estelles A., Charlton C. A., Blau H. M. 1999. The phosphoprotein protein PEA-15 inhibits Fas- but increases TNF-R1-mediated caspase-8 activity and apoptosis. Developmental Biology, 216, 16-28.

Fernstrom J. D. 2000. Can nutrient supplements modify brain function? American Journal of Clinical Nutrition, 71, 1669-75.

Ferrarese C., Appollonio I., Bianchi G., Frigo M., Marzorati C., Pecora N., Perego M., Pierpaoli C., Frattola L. 1993. Benzodiazepine receptors and diazepam binding

inhibitor: a possible link between stress, anxiety and the immune system. Psychoneuroendocrinology, 18, 3-22.

Fricker L. D., Evans C. J., Esch F. S., Herbert E. 1986. Cloning and sequence analysis of cDNA for bovine carboxypeptidase E. Nature, 323, 461-4.

Gardner J. M., de Lange C. F., Widowski T. M. 2001. Belly-nosing in early-weaned piglets is not influenced by diet quality or the presence of milk in the diet. J. Anim. Sci., 79, 73-80.

Gibson U. E., Heid C. A., Williams P. M. 1996. A novel method for real time quantitative RT-PCR. Genome Research, 6, 995-1001.

Gilad G. M., Gilad V. H. 2003. Overview of the brain polyamine-stress-response: regulation, development, and modulation by lithium and role in cell survival. Cellular and Molecular Neurobiology, 23, 637-49.

Goldberg D. J., Foley M. S., Tang D., Grabham P. W. 2000. Recruitment of the Arp2/3 complex and mena for the stimulation of actin polymerization in growth cones by nerve growth factor. Journal of Neuroscience Research, 60, 458-67.

Hay M., Orgeur P., Levy F., Le Dividich J., Concordet D., Nowak R., Schaal, B., Mormede, P. 2001. Neuroendocrine consequences of very early weaning in swine. Physiol. Behav., 72, 263-9.

Hayashi Y., Tanaka J., Morizumi Y., Kitamura Y., Hattori Y. 2004. Polyamine levels in brain and plasma after acute restraint or water-immersion restraint stress in mice. Neuroscience Letter, 355, 57-60.

Heid C. A., Stevens J., Livak K. J., Williams, P. M. 1996. Real time quantitative PCR. Genome Research, 6, 986-94.

Hellemans K. G., Benge L. C., Olmstead M. C. 2004. Adolescent enrichment partially reverses the social isolation syndrome. Brain Res., 150, 103-15.

Hemsworth P. H., Barnett J. L., Hansen C., Gonyou H. W. 1986. The influence of early contact with humans on subsequent behavioral response of pigs to humans. Appl.Anim. Behav. Sci., 15, 55-63.

Hemsworth P.H., Barnett J.L. 1992. The effects of early contact with humans on the subsequent level of fear of humans in pigs. Applied Animal Behaviour Science, 35, 83-90.

Hennessy M. B., Deak T., Schiml-Webb P. A., Wilson S. E., Greenlee T. M., McCall E. 2004. Responses of guinea pig pups during isolation in a novel environment may represent stress-induced sickness behaviors. Physiol Behav., 81, 5-13.

Herman J. P. 1993. Regulation of adrenocorticosteroid receptor mRNA expression in the central nervous system. Cell. Mol. Neurobiol., 13, 349-72.

Hohenshell L. M., Cunnick J. E., Ford S. P., Kattesh H. G., Zimmerman D. R., Wilson M. E., Matteri R. L., Carroll J. A., Lay D. C. Jr. 2000. Few differences found between early- and late-weaned pigs raised in the same environment. J. Anim. Sci., 78, 38-49.

Holmes M. C., Yau J. L., Kotelevtsev Y., Mullins J. J., Seckl J. R. 2003. 11 Beta-hydroxysteroid dehydrogenases in the brain: two enzymes two roles. Ann N Y Acad Sci., 1007, 357-66.

Hunter P. 1986. The immune system of the neonatal and weaner piglet: a review. J S Afr Vet Assoc., 57, 243-5.

Ichimura T., Isobe T., Okuyama T., Yamauchi T., Fujisawa H. 1987. Brain 14-3-3 protein is an activation protein that activates tryptophan 5-monooxygenase and tyrosine 3-monooxygenase in the presence of Ca2+,calmodulin-dependent protein kinase II. Federation of European Biochemical Societies Letter, 219, 79-82.

Ichimura T., Isobe T., Okuyama T., Takahashi N., Araki K., Kuwano R., Takahashi Y. 1988. Molecular cloning of cDNA coding for brain-specific 14-3-3 protein, a protein kinase-dependent activation of tyrosine and tryptophan hydroxylases. Proceedings of the National Academic of Science of the United States of America, 85, 7084-8.

Jensen P., Recen B. 1989. When to wean – observations from free-ranging domestic pigs. Appl. Anim. Behav Sci., 23, 49-60.

Johnson M. R., Wang K., Smith J. B., Heslin M. J., Diasio R. B. 2000. Quantitation of dihydropyrimidine dehydrogenase expression by real-time reverse transcription polymerase chain reaction. Anal Biochem., 278, 175-84.

Kanitz E., Manteuffel G., Otten W. 1998. Effects of weaning and restraint stress on glucocorticoid receptor binding capacity in limbic areas of domestic pigs. Brain Res., 804, 311-5.

Kanitz E., Tuchscherer M., Puppe B., Tuchscherer A., Stabenow B. 2004. Consequences of repeated early isolation in domestic piglets (Sus scrofa) on their behavioural, neuroendocrine, and immunological responses. Brain Behav. Immun., 18, 35-45.

Kaufman J., Plotsky P. M., Nemeroff C. B., Charney D. S. 2000. Effects of early adverse experiences on brain structure and function: clinical implications. Biol. Psych., 48, 778-90.

Kerr M. K., Churchill G. A. 2001. Experimental design for gene expression microarrays. Biostatistics, 2, 183-201.

Kesner R. P., Hunt M. E., Williams J. M., Long J. M. 1996. Prefrontal cortex and working memory for spatial response, spatial location, and visual object information in the rat. Cerebral Cortex, 6, 311-8.

Kim J. H., Heo K. N., Odle J., Han K., Harrell R. J. 2001. Liquid diets accelerate the growth of early-weaned pigs and the effects are maintained to market weight. J. Anim. Sci., 79, 427-34.

Kitsberg D., Formstecher E., Fauquet M., Kubes M., Cordier J., Canton B., Pan G., Rolli M., Glowinski J., Chneiweiss H. 1999. Knock-out of the neural death effector domain protein PEA-15 demonstrates that its expression protects astrocytes from TNFalpha-induced apoptosis. Journal of Neuroscience, 19, 8244-51.

Kolb B., Pellis S., Robinson T. E. 2004. Plasticity and functions of the orbital frontal cortex. Brain Cogn., 55, 104-15.

Kosten T. A., Miserendino M. J., Kehoe P. 2000. Enhanced acquisition of cocaine self-administration in adult rats with neonatal isolation stress experience. Brain Research, 875, 44-50.

Kuhn C. M., Schanberg S. M. 1998. Responses to maternal separation: mechanisms and mediators. International Journal of Developmental Neuroscience, 16, 261-70.

Lapiz M. D., Fulford A., Muchimapura S., Mason R., Parker T., Marsden C. A. 2003. Influence of postweaning social isolation in the rat on brain development, conditioned behavior, and neurotransmission. Neurosci Behav Physiol., 33, 13-29.

Laughlin K., Zanella A. J. 2002. Weaning age impairs spatial memory of pigs at increased but not basal levels of stress. J. Anim. Sci., 80, 83.

Lee A. L., Ogle W. O., Sapolsky R. M. 2000. Stress and depression: possible links to neuron death in the hippocampus. Bipolar Disorders, 4, 117-128.

Littell R.C., Milliken G.A., Stroup W.W., Wolfinger R.D. 1996. SAS System for Mixed Models, SAS Institute Inc., Cary, 647 pp.

Livak K. J. ABI Prism 7700 Sequence Detection System. User Bulletin no. 2. 1997. PE Applied Biosystems, AB website, bulletin reference: 4303859B 777802-002. http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf.

Livak K. J., Schmittgen T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods, 25, 402-8.

Loh Y. P., Maldonado A., Zhang C., Tam W. H., Cawley N. 2002. Mechanism of sorting proopiomelanocortin and proenkephalin to the regulated secretory pathway of neuroendocrine cells. Annals of the New York Academy of Sciences, 971, 416-25.

Loh Y. P., Kim T., Rodriguez Y. M., Cawley N. X. 2004. Secretory granule biogenesis and neuropeptide sorting to the regulated secretory pathway in neuroendocrine cells. Journal of Molecular Neuroscience, 22, 63-71.

Loijens L. W., Schouten W. G., Wiepkema P. R., Wiegant V. M. 2002. Brain opioid receptor density reflects behavioral and heart rate responses in pigs. Physiol. Behav., 76, 579-87.

Lyons D. M., Schatzberg A. F. 2003. Early maternal availability and prefrontal correlates of reward-related memory. Neurobiology of Learning and Memory, 80, 97-104.

McEwen B. S. 1997. Hormones as regulators of brain development: life-long effects related to health and disease. Acta Paediatr Suppl., 422, 41-4.

Meaney M. J., Diorio J., Francis D., Widdowson J., LaPlante P., Caldji C., Sharma S., Seckl J. R., Plotsky P. M. 1996. Early environmental regulation of forebrain glucocorticoid receptor gene expression: implications for adrenocortical responses to stress. Dev Neurosci., 18, 49-72.

Meyer G., Feldman E. L. 2002. Signaling mechanisms that regulate actin-based motility processes in the nervous system. Journal of Neurochemistry, 83, 490-503.

Mirescu C., Peters J. D., Gould E. 2004. Early life experience alters response of adult neurogenesis to stress. Nat. Neurosci., 7, 841-6.

Mizoguchi K., Ishige A., Aburada M., Tabira T. 2003. Chronic stress attenuates glucocorticoid negative feedback: involvement of the prefrontal cortex and hippocampus. Neuroscience, 119, 887-97.

Mizoguchi K., Ishige A., Takeda S., Aburada M., Tabira T. 2004. Endogenous glucocorticoids are essential for maintaining prefrontal cortical cognitive function. J. Neurosci., 16, 5492-9.

Molina-Hernandez M., Tellez-Alcantara P., Perez-Garcia J. 2001. Isolation rearing induced fear-like behavior without affecting learning abilities of Wistar rats. Prog. Neuro-Psychoph. Biol. Psych., 25, 1111-23

National Animal Health Monitoring System (NAHMS). 2001. Reference of Swine Health and Management in the United States, 2000. 16 pp.

Nobis W., Ren X., Suchyta S. P., Suchyta T. R., Zanella A. J., Coussens, P. M. 2003. Development of a porcine brain cDNA library, EST database, and microarray resource. Physiol. Gen. 16, 153-59.

Ongur D., Price J. L. 2000. The organization of networks within the orbital and medial prefrontal cortex of rats, monkeys and humans. Cerebral Cortex, 10, 206-19.

Ordyan N. E., Pivina S. G., Rakitskaya V. V., Shalyapina V. G. 2001. The neonatal glucocorticoid treatment-produced long-term changes of the pituitary-adrenal function and brain corticosteroid receptors in rats. Steroids, 66, 883-8.

Orgeur P., Hay M., Mormede P., Salmon H., Le Dividich J., Nowak R., Schaal B., Levy F. 2001. Behavioural, growth and immune consequences of early weaning in one-week-old large-white piglets. Reprod. Nutr. Dev., 41, 321-32.

Patience J. F., Gonyou H. W., Whittington D. L., Beltranena E., Rhodes C. S., Van Kessel A. G. 2000. Evaluation of site and age of weaning on pig growth performance. J. Anim. Sci., 78, 1726-31.

Perreau V., Sarrieau A., Mormede P. 1999. Characterization of mineralocorticoid and glucocorticoid receptors in pigs: comparison of Meishan and Large White breeds. Life Sci., 64, 1501-15.

Plotsky P. M., Sanchez M. M., Levine S. 2001. Intrinsic and extrinsic factors modulating physiological cope systems during development. In: D. M. Broom (Ed.), Dahlem Workshop on Coping with challenge: welfare in animals including humans (pp. 169-96). Berlin: Dahlem University Press.

Potchen E. J. 2000. Prospects for progress in diagnostic imaging. J Intern Med., 247, 411-24.

Radley J. J., Sisti H. M., Hao J., Rocher A. B., McCall T., Hof P. R., McEwen B. S., Morrison J. H. 2004. Chronic behavioral stress induces apical dendritic reorganization in pyramidal neurons of the medial prefrontal cortex. Neurosc., 125, 1-6.

Renault F., Formstecher E., Callebaut I., Junier M. P., Chneiweiss H. 2003. The multifunctional protein PEA-15 is involved in the control of apoptosis and cell cycle in astrocytes. Biochemistry and Pharmacology, 66, 1581-8.

Robson A. C., Leckie C. M., Seckl J.R., Holmes M.C. 1998. 11 Beta-hydroxysteroid dehydrogenase type 2 in the postnatal and adult rat brain, Brain Res. Mol. Brain. Res., 61, 1-10.

Rocher C., Spedding M., Munoz C., Jay T. M. 2004. Acute stress-induced changes in hippocampal/prefrontal circuits in rats: effects of antidepressants. Cereb. Cortex, 14, 224-9.

Rogers R. D., Blackshaw A. J., Middleton H. C., Matthews K., Hawtin K., Crowley C., Hopwood A., Wallace C., Deakin J. F., Sahakian B. J., Robbins T. W. 1999. Tryptophan depletion impairs stimulus-reward learning while methylphenidate disrupts attentional control in healthy young adults: implications for the monoaminergic basis of impulsive behaviour. Psychopharmacology, 146, 482-91.

Rudy J. W., Kuwagama K., Pugh C. R. 1999. Isolation reduces contextual but not auditory-cue fear conditioning: a role for endogenous opioids. Behav. Neurosc., 113, 316-23.

Sanchez M. M., Ladd C. O., Plotsky P. M. 2001. Early adverse experience as a developmental risk factor for later psychopathology: evidence from rodent and primate models. Dev Psychopathol., 13, 419-49.

Shaaf M. J., De Kloet E. R., Vreugdenhil E. 2000. Corticosteroid effects on BDNF expression in the hippocampus. Implications for memory formation. Stress, 3, 201-8.

Schumacher M., Weill-Engerer S., Liere P., Robert F., Franklin R. J., Garcia-Segura L. M., Lambert J. J., Mayo W., Melcangi R. C., Parducz A., Suter U., Carelli C., Baulieu E. E., Akwa Y. 2003. Steroid hormones and neurosteroids in normal and pathological aging of the nervous system. Progress in Neurobiology, 71, 3-29.

Seckl J. R. 1997. 11beta-Hydroxysteroid dehydrogenase in the brain: a novel regulator of glucocorticoid action? Front Neuroend., 18, 49-99.

Seguin J. R. 2004. Neurocognitive elements of antisocial behavior: Relevance of an orbitofrontal cortex account. Brain Cognition, 55, 185-97.

Senba E., Ueyama T. 1997. Stress-induced expression of immediate early genes in the brain and peripheral organs of the rat. Neurosci. Res., 29, 183-207.

Silva-Gomez A. B., Rojas D., Juarez I., Flores G. 2003. Decreased dendritic spine density on prefrontal cortical and hippocampal pyramidal neurons in postweaning social isolation rats. Brain Res., 983, 128-36.

Smyth G. K. 2004. Linear models and empirical Bayes methods for assessing differential expression in microarray experiments. Statistical Applications in Genetics and Molecular Biology, 3, 3.

Souza A. S., Zanella A. J. 2004. Can acute stress impair the ability of weaned piglets to recognize a familiar conspecific? In: L. Hanninem & A. Valros (Eds.), Proceedings of the 38th International Congress of the ISAE (p. 119). Helsinki: International Society for Applied Ethology.

Takahashi, Y. 2003. The 14-3-3 proteins: gene, gene expression, and function. Neurochemistry Research, 28, 1265-73.

Tuchscherer M., Kanitz E., Puppe B., Tuchscherer A., Stabenow, B. 2004. Effects of postnatal social isolation on hormonal and immune responses of pigs to an acute endotoxin challenge. Physiol. Behav., 82, 503-11.

Vallee M., Mayo W., Koob G. F., Le Moal M. 2001. Neurosteroids in learning and memory processes. International Review of Neurobiology, 46, 273-320.

Vazquez D. M., Van Oers H., Levine S., Akil H. 1996. Regulation of glucocorticoid and mineralocorticoid receptor mRNAs in the hippocampus of the maternally deprived infant rat, Brain Res., 731, 79-90.

Washburn D. A., Rumbaugh D. M. 1991. Impaired performance from brief social isolation of rhesus monkeys (Macaca mulatta): a multiple video-task assessment. J. Comp. Psychol., 105, 145-51.

Weary D. M., Appleby M. C., Fraser D. 1999. Responses of piglets to early separation from the sow. Appl. Anim. Behav Sci., 63, 289-300.

Weaver S. A., Aherne F. X., Meaney M. J., Schaefer A. L., Dixon W. T. 2000. Neonatal handling permanently alters hypothalamic-pituitary- adrenal axis function, behavior, and body weight in boars. J. Endocrinol., 164, 349-59.

Weiss I. C., Pryce C. R., Jongen-Relo A. L, Nanz-Bahr N. I., Feldon J. 2004. Effect of social isolation on stress-related behavioural and neuroendocrine state in the rat. Behav. Brain Res., 152, 279-95.

Weitzdoerfer R., Fountoulakis M., Lubec G. 2002. Reduction of actin-related protein complex 2/3 in fetal Down syndrome brain. Biochemical and Biophysical Research Communications, 293, 836-41.

Wood E. G, Trevor Young L., Reagan L. P., McEwen B. S. 2003. Acute and chronic restraint stress alter the incidence of social conflict in male rats. Horm. Behav., 43, 205–213.

Worobec E. K., Duncan I. J. H., Widoswki T. M. 1999. The effects of weaning at 7, 14 and 28 days on piglet behavior. Appl. Anim. Behav Sci., 62, 173-182.

Xiang C. C, Chen Y. 2000. cDNA microarray technology and its applications. Biotechnol Adv., 18, 35-46.

Yang Y. H., Dudoit S., Luu P., Lin D. M., Peng V., Ngai J., Speed T. P. 2002. Normalization for cDNA microarray data: a robust composite method addressing single and multiple slide systematic variation. Nucleic Acids Research, 30, e15.

Yuan Y., Jansen J., Charles D., Zanella A. J. 2004. The influence of weaning age on post-mixing agonistic interactions in growing pigs. Appl. Anim. Behav Sci., 88, 39-46.

Zanella A. J., Broom D. M., Hunter J. C., Mendl M. T. 1996. Brain opioid receptors in relation to stereotypies, inactivity, and housing in sows. Physiology & Behavior, 59, 769-75.

Zanella A. J., Hodges H., Palme R. 2003. Glucocorticoids and their metabolites in the hippocampus of weaned and unweaned piglets. In: V. Ferrante (Ed.). Proceedings of

the 37th International Congress of ISAE, Abano Terme, Italy (p. 116). Brescia: Fondazione Iniziative Zooprofillatiche e Zootecniche.

Zhou J., Atkins J. F., Gesteland R. F. 1999. Structure of human ornithine decarboxylase antizyme 2 gene. Gene, 232, 165-71.