PROOPIOMELANOCORTIN PHYSIOLOGICAL ROLES: PITUITARY VERSUS HYPOTHALAMIC FUNCTIONS

by

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LIST OF ABBREVIATIONS

A	Amygdala
A	Dominant Agouti Locus
Ac	Acetylated
ACTH	Adrenocorticotrophic Hormone
AGRP	Agouti Gene-Related Protein
AL	Anterior Lobe
α-MSH	alpha-Melanocyte-Stimulating Hormone
ANOVA	Analysis of Variance
ArcN	Arcuate Nucleus
A^W/A^W	White-bellied Agouti Mouse
A^Y/a	Lethal Yellow Mouse
B	Corticosterone
BAC	Bacterial Artificial Chromosome
bp	Base Pairs (nucleotides)
β-AR	beta-Adrenergic Receptor
β-End	beta-Endorphin
BMR	Basal Metabolic Rate
BrdU	5-Bromodeoxyuridine
cAMP	Cyclic 3', 5'-Adenosine Monophosphate
CART	Cocaine-Amphetamine-Response-Transcript
CeA	Central Nucleus of the Amygdala

CC	Central Canal
CLIP	Corticotropin-Like Intermediate Lobe Peptide
CNS	Central Nervous System
CRH	Corticotropin Releasing Hormone
C-terminal	Carboxy-terminal Domain
C57BL/6	Black Agouti-less (*a/a*) Inbred Mouse Line
DAB	Diaminobenzidine
DBH	Dopamine β-hydroxylase
DesAc	Des-acetyl (absence of acetylation)
DMH	Dorsomedial Nucleus of the Hypothalamus
DNA	Deoxyribonucleic Acid
E	Dominant Extension Locus
e	Recessive Extension Locus
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetate
EGFP	Enhanced Green Fluorescent Protein
Epi	Epinephrine
FDA	United States Food and Drug Administration
F1	First Generation Progeny
F2	Second Generation Progeny
Fisher's PLSD	Fisher's Protected Least Significant Difference (*Post Hoc* analysis)
GABA	gamma-Amino Butyric Acid
λ-MSH	gamma-Melanocyte-Stimulating Hormone

bGH	Bovine Growth Hormone
GnRH	Gonadotropin Releasing Hormone
GR	Glucocorticoid Receptor
HLH	Helix Loop Helix
HPA-axis	Hypothalamic-Pituitary-Adrenal Axis
HPG-axis	Hypothalamic-Pituitary-Gonadal Axis
icv	Intracerebroventricular
it	Intrathecal
ip	Intraperitoneal
IL	Intermediate Lobe
K_{ATP}	Adenosine Triphosphate-Sensitive Potassium Channel
KO	Knock Out
kb	Kilobases (nucleotides)
LacZ	β-galactosidase Gene
LH	Lateral Hypothalamus
LPH	Lipotropic Hormone
LPS	Lipopolysaccharide
MC#-R	Melanocortin # Receptor
$Mc1r^e/Mc1r^e$	Recessive Yellow Mouse
MOR	Mu-opioid Receptor
mRNA	Messenger Ribonucleic Acid
MTII	Melanotan II (cyclic heptapeptide)
N1	Congenic One Generation

N2	Congenic Two Generation
NE	Norepinephrine
Neo	Neomyosin Gene
NMU	Neuromedin U
NIH	National Institutes of Health
NPY	Neuropeptide Y
N-terminal	Amino-terminal Domain
NTS	Nucleus Tractus Solitarius
ob/ob	Leptin-deficient Mouse Model
OCT Compound	Optimal Cutting Temperature Compound
PC	Prohormone Convertase
PCR	Polymerase Chain Reaction
pHal*	10.2 kb *Pomc* Transgene with Oligo placed in 3rd exon
pHal Ex2*	10.2 kb *Pomc* Transgene with Oligo placed in 2rd exon
polyA	Polyadenylation Sequence
POMC	Proopiomelanocortin
$Pomc^{+/+}$	Proopiomelanocortin Wild-type Genotype
$Pomc^{+/-}$	Proopiomelanocortin Heterozygous Genotype
$Pomc^{-/-}$	Proopiomelanocortin Null Genotype
pref-1	Preadipocyte Factor-1
$pTyr^{C}/pTyr^{C\text{-}Ch}$	Heterozygous Hypomorphic "*Chinchilla*" Mutation at Tyrosinase Locus
PVH	Paraventricular Nucleus of the Hypothalamus
RER	Respiratory Exchange Ratio

RIA	Radioimmunoassay
rt-PCR	Reverse Transcriptase Polymerase Chain Reaction
SEM	Standard Error of the Mean
SNS	Sympathetic Nervous System
T-box	TATA box Transcription Factor
T_m	Melting Temperature
Tpit	T-box Transcription Factor (Corticotroph/Melanotroph Specific)
Tg/+	Transgenic Hemizygous Genotype
3V	3rd Ventricle
TH	Tyrosine Hydroxylase
UV	Ultra Violet (radiation)
VMN	Ventral Medial Nucleus of the Hypothalamus
VO_2	Volume of Oxygen
VTA	Ventral Tegmental Area
WT	Wild Type
ZF	Zona Fasciculata
ZG	Zona Glomerulosa

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"What is true will never change,

What man thinks is true will always change"

-Unknown

To my wife: Kari Lyn

To my daughter: Madeleine Grace

To my parents: John and Sonja Smart

ABSTRACT

The fact that the proopiomelanocortin (*POMC*) gene is a critical component of energy homeostasis and the stress response, two distinct yet not exclusively separate biological functions, distinguishes this gene as very intriguing and unique for study. The *POMC* gene encodes a preprohormone that is post-translationally processed into multiple bioactive peptides. The tissue specific regulation and tissue specific post-translational modifications provide a means for the broad spectrum of the gene's biological activities. Understanding the *POMC* gene's cell-specific regulation and the physiological functions of its encoded peptides has been an ongoing project of multiple labs spanning the last two decades. Initial studies predominately revolved around the role of the POMC peptide adrenocorticotropin-stimulating hormone's (ACTH) function as a key descending-component of the hypothalamic-pituitary adrenal axis. Recently, the majority of studies have transitioned to a focus on the hypothalamic POMC peptide, α-MSH, and its anorexigenic effects. This focus is with good reason. Within affluent societies, the rampant increase in obesity and overweight prevalence and the associated risk for several chronic diseases has accentuated the need for understanding the biological mechanisms of energy homeostasis. Using the murine rodent for its genetic advantages, this thesis attempts to further advance our understanding of the POMC system regarding its gene expression, neuron physiology, and biological functions.

Localization of the leptin receptor on POMC neurons together with the elevation in POMC mRNA following intracerebroventricular (icv) injections of leptin or neuropeptide Y (NPY) identified these hypothalamic neurons as a critical nodal point in the central regulation of energy homeostasis. However, due to the limited number of these neurons, POMC gene expression and regulation and the analyses of synaptic events in response to peripheral or central signals has been limited. We generated a transgenic C57BL/6 mouse line expressing enhanced green fluorescent protein (EGFP) under the transcriptional control of POMC genomic regulatory elements to facilitate the identification of these neurons in living brain slices and primary cultures. The generation of the POMC/EGFP transgenic mouse has led to the quantification of these hypothalamic neurons along with *in vivo* electrophysiological characterization of the leptin response. Additional electrophysiological studies using the POMC/EGFP mouse have provided a mechanism for the effects of the anorexic drug, D-fenfluramine by indirectly stimulating serotonin (5-hydroxytryptamine, 5-HT) receptors on POMC hypothalamic neurons. Most recently *in vivo* experiments with POMC/EGFP neurons showed that they were stimulated by the gut derived PYY_{3-36} hormone. Studies have also revealed the POMC neurons to be glucose-responsive neurons that express K_{ATP} inward-rectifying channels and the inhibitory neurotransmitter _–amino butyric acid (GABA). These studies described above have provided essential insight regarding hypothalamic POMC neurophysiology.

Whether the hypothalamic POMC peptides have functions that are exclusive to hypothalamic neurons or whether pituitary POMC cells provide a redundancy for these hypothalamic neurons was not known. Utilizing promoter-mapping data, we generated a transgene that would selectively rescue pituitary POMC while retaining the neuronal POMC deficiency when crossed onto a POMC null mutant that we obtained from the Hochgeschwender laboratory. Our studies revealed that replacing pituitary POMC (*Pomc*$^{-/-}$; Tg/+) was not sufficient to rescue the phenotypes caused by the ubiquitous absence of POMC peptides in the *Pomc*$^{-/-}$ mice. In fact some of the phenotypes present in the *Pomc*$^{-/-}$ mouse, such as obesity and diabetes, were accentuated in the mice lacking only CNS POMC. Obesity in the *Pomc*$^{-/-}$ mice resulted from a decrease in their basal metabolic rate (BMR). Replacing pituitary POMC and thus glucocorticoids resulted in a further depression in the mouse's BMR. Replacing pituitary POMC did not normalize the HPA axis but instead further suggested a regulation of the HPA axis, independent of glucocorticoids, by hypothalamic POMC neurons. The lack of CRH suppression in the presence of elevated circulating corticosterone in the *Pomc*$^{-/-}$; Tg/+ mice lead us to the previous conclusion. CRH is a known anorexigenic peptide and evidence supporting the opposing regulation of these neurons by POMC peptides, α-MSH and β-endorphin, makes this hypothalamic circuitry very intriguing for not only future stress axis studies but energy homeostasis studies as well.

CHAPTER I

AN INTRODUCTION TO THE PROOPIOMELANOCORTIN SYSTEM

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1. THE PROOPIOMELANOCORTIN GENE

1.1 *Discovery and history of the POMC Gene*

The discovery that one gene encoded more then one peptide was first realized in the cloning of the proopiomelanocortin (POMC) gene. Adrenocorticotropic hormone (ACTH), beta-endorphin (β-End) and the melanotropins (MSHs) are all derived from a single large precursor molecule, the POMC preprohormone, by individual processing through a series of co- and post-translational modifications (Mains, Eipper et al. 1977). The finding at the time that a single precursor encoded multiple yet distinct bioactive peptides was novel. Although the phenomenon of an existing inert precursor molecule was not realized until 1977 the concept was first perceived a decade earlier, as a result of analyses of structural forms and biological activities of beta-lipotropic hormone (β-LBH) [reviewed in (Chretien, Benjannet et al. 1979)]. The most surprising biological activity was the morphine-like response to C-terminal fragments of β-LPH, which later was attributed to the endogenous opioid, β-End. When the POMC preprohormone was first identified, the POMC peptides were known only to exist in the pituitary gland, but later the discovery of the POMC preprohormone led scientists to hypothesize that all neuropeptides were products of larger precursor molecules.

Later *in vivo* studies showed that POMC prohormone fragments lacked the classical endocrine effects while behavioral influences of these peptides were noticed. The various behavioral effects on motivation, attention, arousal, learning, memory, sleep, drug-seeking behavior, development of tolerance and drug dependency led to the

hypothesis that the central nervous system (CNS) must be a target of these hypophyseal hormones (De Wied and Jolles 1982). However, explaining how pituitary-expressed POMC peptides were able to reach targets within the CNS proved to be problematic. It was thought that small peptides might be able to reach their targets by crossing the blood-brain barrier via the bloodstream or by a retrograde transport from the pituitary gland. These questions have yet to be completely resolved but an alternative explanation surfaced from the finding that POMC peptides were also expressed in the CNS (Akil, Watson et al. 1978; Watson, Akil et al. 1978; Watson, Richard et al. 1978). A subset of neurons found in the arcuate nucleus (ArcN) was shown to express POMC mRNA and peptides with their projections extending throughout the limbic system (Watson, Akil et al. 1978; Gee, Chen et al. 1983). The finding of POMC peptides in the CNS, once thought only to be expressed in the pituitary gland, led investigators to try and understand the importance of pituitary derived POMC peptides versus hypothalamic derived POMC peptides. The task of unveiling and assigning individual roles to these peptides expressed in the CNS versus the pituitary, which began two decades earlier, is still being addressed today and is a major emphasis of this thesis.

1.2 *Murine Pomc Gene Expression and Ontogeny*

Pomc is expressed most abundantly in corticotroph and melanotroph cells of the pituitary but identical *Pomc* expression to that in the pituitary is also present in the brain (Orwoll, Kendall et al. 1979; Palkovits, Mezey et al. 1987). POMC preprohormone and its derived peptides are produced in neurons of the hypothalamic arcuate nucleus (ArcN) along with

the commissural nucleus of the solitary tract (NTS) in the brainstem (Berry and Haynes 1989). POMC neurons undergo final cell division at embryonic day twelve (e12) preceding other neurons in the ArcN (Shimada and Nakamura 1973; Zachary, Woll et al. 1987; Rius, Barg et al. 1991). Expression of the POMC peptides in the ArcN coincides with neurogenesis while other well-known hypothalamic peptides (vasopressin and oxytocin) are not expressed until after neurogenesis (Shimada and Nakamura 1973). In the spinal cord POMC mRNA and peptides, alpha-melanocyte stimulating hormone (α-MSH) and ß-endorphin (β-End), are transiently expressed (Rius, Barg et al. 1991). The transient or early ontogeny of the POMC mRNA and peptides is indicative of a developmental role in embryogenesis discussed later in this chapter.

Central POMC neurons project to nuclei within the forebrain, hypothalamus, thalamus, mesencephalan and medulla. Some of the POMC neurons send projections that terminate on the primary capillary bed of the hypothalamohypophyseal portal system much like corticotrophin-releasing hormone (CRH) neurons originating in the hypothalamic paraventricular nucleus (PVN). POMC peptides released onto the primary capillary bed known as the median eminence can then be transported via the hypothalamohypophyseal portal vasculature to the neurohypophysis also referred to as the posterior lobe of the pituitary gland.

The early expression of hypothalamic POMC peptides, first detected at embryonic day 10.5 (e10.5) shortly after neurogenesis, suggests a possible developmental role for this peptidergic system (Elkabes, Loh et al. 1989). Interestingly, the interconnection between

hypothalamic POMC and the pituitary gland could provide a neuro-anatomical mechanism for stimulating undifferentiated pituitary cells into POMC expressing corticotrophs and melanotrophs. These pituitary POMC expressing endocrine cells cannot be immunocytochemically detected until embryonic days e12.5 and e14.5, respectively two to four days after hypothalamic expression (Finley, Lindstrom et al. 1981; Palkovits, Mezey et al. 1987; Elkabes, Loh et al. 1989).

Although the highest levels of POMC expression is in the pituitary and hypothalamus, POMC expression can be detected at low levels in several tissues including the pancreas, testis, kidney, liver, lung, duodenum, colon, stomach, lymphocytes, spleen and skin (Smith and Funder 1988). My studies described within this thesis were confined and focused to that of pituitary and hypothalamic POMC peptides, and to understanding their separate and distinct physiological roles within these two tisssues. Further analyses are needed to evaluate the roles of POMC peptides in the other tissues of POMC mutant mice.

1.3 *Processing of the POMC Prohormone*

POMC mRNA is processed into two types of neuropeptides, the melanocortins (mainly ACTH and α-MSH) and an opioid (β-Endorphin). As mentioned previously, the principal sites of POMC expression are corticotrophs and melanotrophs of the pituitary gland and subsets of neurons in the arcuate nucleus (ArcN) of the hypothalamus and in the caudal nucleus of the solitary tract (NTS) within the dorsal vagal complex of the

medulla. This section will serve only to introduce major points relevant to the mouse models discussed in this thesis.

For the most part, all the POMC peptides are processed from the prohormone by enzymatic cleavage at basic amino acid residues. The majority of POMC prohormone processing occurs in secretory vesicles with the exception of the N-terminal signal peptide being cleaved by a peptidase in the endoplasmic reticulum (ER), the glycosylation in the ER and Golgi apparatus, and the phosphorylation and sulfation also in the Golgi apparatus (Eipper and Mains 1980; Hoshina, Hortin et al. 1982; Bennett, Brubaker et al. 1983; Bourbonnais and Crine 1985). A variety of studies, most of which where conducted in a corticotroph tumor cell line (AtT-20 cells), revealed how the POMC prohormone is processed into its bioactive peptides (Eipper and Mains 1977; Chretien, Benjannet et al. 1979; Eipper and Mains 1980; Policastro, Phillips et al. 1981; Estivariz, Carino et al. 1988; Estivariz, Morano et al. 1988; Bicknell, Lomthaisong et al. 2001). Endoproteolytic cleavage of POMC by a prohormone convertase (PC1/PC3) in corticotrophs yields ACTH, βLPH, and an NH2-terminal peptide (Pro-λMSH) (Figure 1). The processing of these peptides from the prohormone is done by PC1/PC3 recognizing a dibasic residue: Lys/Arg. The NH2-terminal peptide (Pro-λMSH) and/or further proteolytic derivatives have been postulated to mediate paracrine growth and secretory effects within the anterior lobe and to stimulate mitogenesis in the adrenal cortex (Seger and Bennett 1986; Denef and Van Bael 1998; Denef, Roudbaraki et al. 2001). A small proportion of β-LPH is cleaved to yield λ-LPH and $\beta\text{-End}_{1\text{-}31}$. However, in melanotrophs of the intermediate lobe virtually all β-LPH is cleaved by PC2 to λ-LPH and $\beta\text{-End}_{1\text{-}31}$.

Figure 1

Processing of the POMC prohormone in anterior pituitary, intermediate pituitary, and hypothalamic neurons.

Proopiomelanocortin Gene

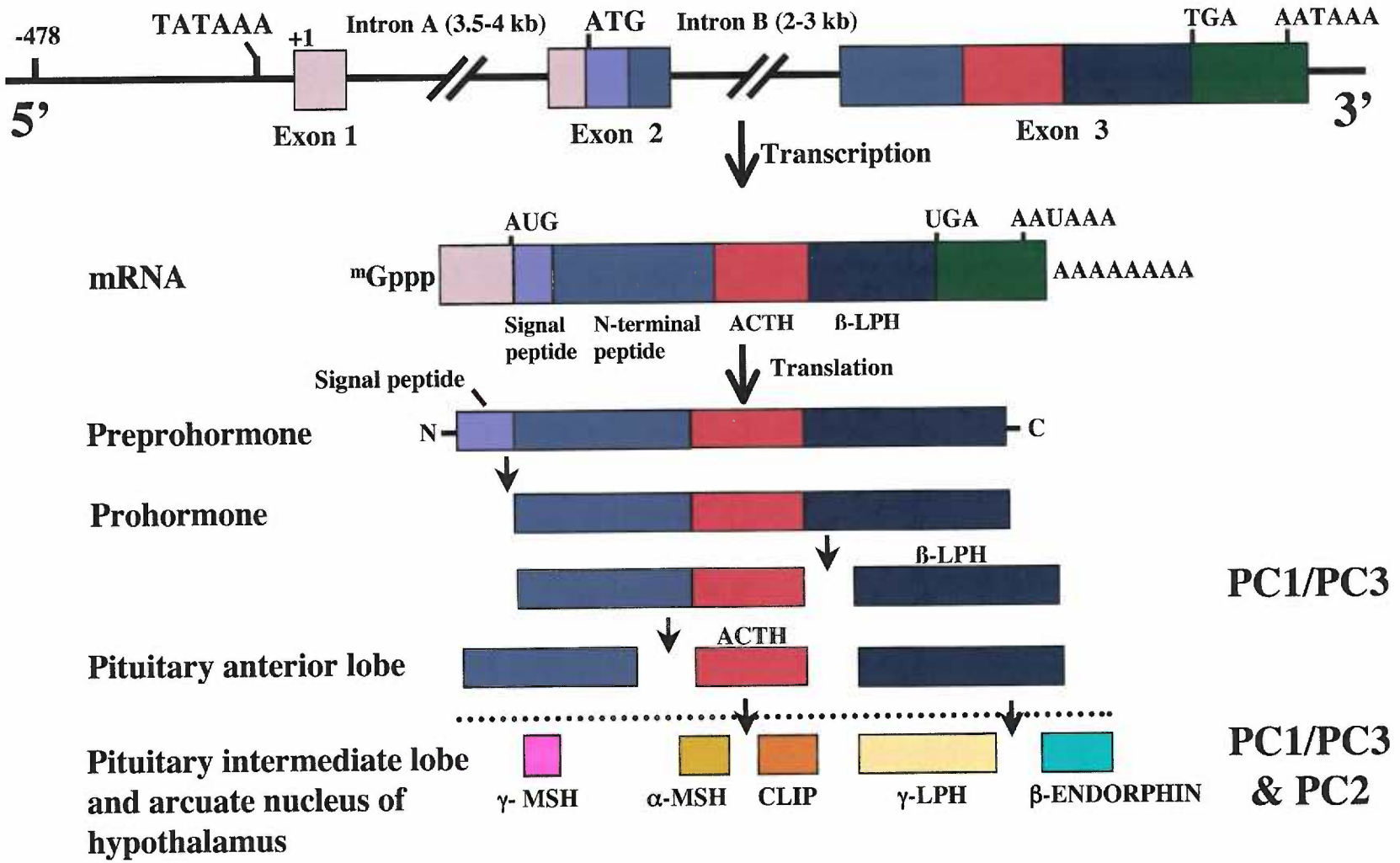


Figure 1

Unlike PC1/PC3, the PC2 endopeptidase activity is a result of PC2's recognition of all dibasic residue combinations: Arg/Arg, Lys/Lys, Arg/Lys, and Lys/Arg.

Within the pituitary gland PC1/PC3 is the primary processing enzyme in anterior lobe corticotrophs while PC2 is the processing enzyme in intermediate lobe melanotrophs. The ratio of PC1/PC2 present within POMC cells, which fluctuates more during ontogeny, determines the processing of α-MSH and β-End_{1-31} (Marcinkiewicz, Day et al. 1993). The latter peptide is acetylated on its NH2-tyrosine and truncated by the action of a carboxypeptidase to yield Ac-β-End_{1-27} and Ac-β-End_{1-26}. Most of the acetylation is thought to occur in the intermediate lobe of the pituitary gland (Glembotski 1982; Glembotski 1982). Depending on the peptide being acetylated, this posttranslational modification results in either a more or less active peptide (Guttmann and Boissonnas 1961; Smyth, Massey et al. 1979). The acetylated opioid peptides have a greatly reduced affinity for μ and δ opioid receptors and their biological function has not been satisfactorily determined. α-MSH is acetylated on its NH2-serine and amidated on its COOH-terminal to yield a fully bioactive peptide. β-MSH is an additional endoproteolytic product of human γ-LPH but is not generated from rodent γ-LPH because it lacks the necessary consensus amino acid sequence for cleavage by a PC.

Endoproteolytic processing of POMC in ArcN neurons closely mirrors the processing in intermediate lobe melanotrophs. α-MSH, ACTH, and β-End are all processed in POMC neurons. The neuronal α-MSH is fully amidated, but there is minimal NH2-acetylation of peptides or COOH-terminal shortening of β-End_{1-31} in the CNS (Smith and Funder

1988; Castro and Morrison 1997; Pritchard, Turnbull et al. 2002). Additionally, λ-MSH and/or λ-3-MSH are processed from the NH2-POMC domain in the ArcN.

A variety of peripheral tissues have been shown to express POMC mRNA, however it has remained difficult to detect any significant amount of peptides (Smith and Funder 1988). One tissue in which POMC peptides have been detected is in the skin (Slominski, Paus et al. 1992; Slominski, Ermak et al. 1995). The expression of POMC peptides in the skin suggests possible paracrine and/or autocrine roles of these peptides. The POMC peptide α-MSH and MC1R are known regulators of skin and hair pigmentation.

2. *POMC* GENE REGULATION

2.1 *POMC Promoter*

A standard method to characterize the *cis* DNA elements and transcription factors that control POMC gene transcription has been the expression of fusion genes, consisting of POMC promoter sequences and a reporter sequence, in transfected cell lines. The most commonly used cell line is AtT20, originally derived from a radiation-induced pituitary tumor (Furth, Gadsen et al. 1953). These cells have many characteristics of primary corticotrophs. Small cell lung cancers often ectopically express POMC and cell lines derived from these tumors have also been used for the analysis of POMC gene expression (Picon, Bertagna et al. 1999). It is clear, however, that neither of these cell lines are

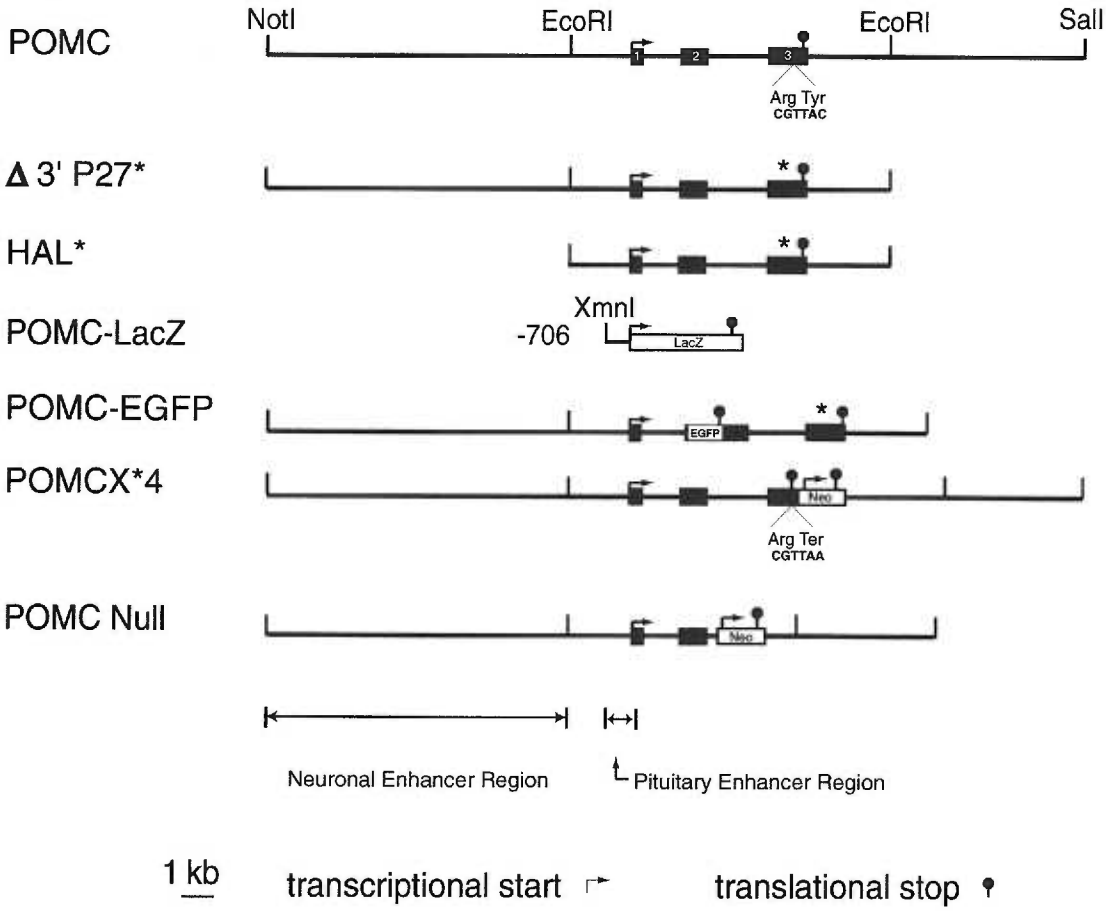
adequate models for elucidation of the mechanisms governing neural-specific expression of the *Pomc* gene. For this reason, the Low laboratory has focused on a transgenic mouse approach for the identification of cell-type specific regulatory elements in the POMC gene.

The Drouin laboratory was the first to report the generation of transgenic mice expressing a neomycin-resistance coding sequence (*neo*) from 770 bases of the rat POMC promoter (Tremblay, Tretjakoff et al. 1988). Although the expression pattern in pituitary and regulation by adrenalectomy and dexamethasone were consistent with accurate cellular expression of the transgene, the low levels of transcript from the *neo* reporter prevented the direct demonstration of corticotroph and melanotroph specific transcription. The Low laboratory initially approached this problem using an *E. coli* lacZ reporter gene encoding ß-galactosidase, which was useful for both the precise cellular identification of transgene expression and quantitation of transcriptional activity by an enzymatic assay utilizing a fluorigenic substrate. These studies demonstrated that 770 bases of the rat POMC promoter from nucleotide -706 in the 5' flanking sequences to +64 in the 5' untranslated region of exon 1 were sufficient to direct lacZ expression selectively to pituitary corticotrophs and melanotrophs (Figure 2, POMC-LacZ) (Hammer, Fairchild-Huntress et al. 1990). The ontogeny of transgene expression also closely approximated the time course of endogenous POMC gene expression in the two lobes of the pituitary gland. However, there was no detectable expression of the -706POMC-lacZ transgene in the brain suggesting that additional genomic sequences were necessary for neuronal transcription.

Figure 2

Restriction maps of the mouse POMC gene locus, transgene constructs utilized to map the location of tissue-specific regulatory elements, and targeted POMC alleles that either selectively prevent ß-endorphin production or result in the absence of all POMC peptides.

Figure 2


POMC
NotI
EcoRI
EcoRI
SalI
1
2
3
Arg Tyr
CGTTAC
Δ 3' P27*
HAL*
POMC-LacZ
XmnI
-706
LacZ
POMC-EGFP
EGFP
POMCX*4
Neo
Arg Ter
CGTTAA
POMC Null
Neo
Neuronal Enhancer Region
Pituitary Enhancer Region
1 kb
transcriptional start
translational stop

Additional studies from the Low laboratory analyzed the rat POMC promoter in more detail to define the minimal elements essential for pituitary expression (Liu, Hammer et al. 1992; Liu, Mortrud et al. 1995). Accurate cell specific colocalization was verified in all instances by double-label immunofluorescence histochemistry. Truncations of the rat POMC promoter to nucleotide -323 or -234 had minimal effects on both the ratio of expression-positive to total transgenic pedigrees and qualitative levels of reporter expression in the positively expressing lines. Further deletion of the 5' flanking sequences to nucleotide -160 abolished expression in the pituitary gland. Transfection studies in AtT20 cells had identified important regulatory elements in the core promoter region of the POMC gene. However, the Low lab demonstrated that the enhancer-less promoter and TATA box from the HSV1 thymidine kinase gene were equivalent to the native POMC promoter sequences between nucleotides -34 and +64, in the context of upstream flanking sequences of POMC, to support pituitary cell specific expression and normal up regulation by adrenalectomy. Based on these promoter truncation studies in transgenics and additional data from DNAse I protection assays and gel shift assays utilizing POMC oligonucleotide probes and fractionated nuclear proteins from AtT20 cells, the Low laboratory designed a series of more discrete mutations in the rat POMC promoter. This second generation of transgenic experiments identified two functionally important protein-binding sites at nucleotide positions -262/-253 and -202/-193 of the POMC gene (Liu, Mortrud et al. 1995). At least one of these sites had to be intact to support detectable pituitary expression of the transgenes. In addition, the ubiquitous transcription factor SP1 appeared to play a supportive role in POMC transgene expression through its interaction with DNA sites at positions -201/-192 and -146/-136.

None of the transgene constructs that were studied selectively directed expression to only pituitary melanotrophs or corticotrophs, suggesting that both cell types share one or more essential components for POMC gene transcription. In common with most published transgenic promoter analyses, the Low lab observed a large quantitative and qualitative range of expression levels among independent pedigrees for each construct. It has been suggested that these mosaic patterns of gene expression are influenced by repressive effects of multiple-copy transgene integration in some cases (Garrick, Fiering et al. 1998). In general, basal expression of the transgenes appeared to be greater in individual melanotrophs than corticotrophs, regardless of the type of reporter molecule.

The data from the Low laboratory transgenic promoter analyses revealed a number of inconsistencies with the mapping of functional POMC promoter elements by transfection studies in AtT20 cells. These differences may be due in part to the more stringent requirement in transgenic mice for the definition of cell-specific expression and the importance of chromatin remodeling during *in vivo* development. However, there is good agreement between both kinds of studies for the key importance of DNA elements between nucleotides -160 and –323 in the 5' flanking domain of the POMC gene. This region contains a site between -173/-160 that appears to mediate the transcriptional activating effects of both CRH and the cytokine leukemia inhibitory factor (LIF), although it is neither a binding site for CREB, AP-1 or STAT proteins (Bousquet, Ray et al. 1997). In contrast, the POMC TATA box, a putative AP-1 binding site in exon 1, and more distal sites in the POMC 5' flanking sequences that bind helix-loop-helix (HLH)

proteins and confer a synergistic activation of POMC expression in AtT20 cells do not appear to be essential for pituitary cell-specific expression in the *in vivo* paradigm (Therrien and Drouin 1993).

Recent data from the Drouin laboratory has identified yet another *trans*-acting factor, Tpit. Tpit is a novel transcription factor belonging to the T-box gene family and its discovery was a result of noticing the sequence similarities between a critical *cis*-acting sequence in the *Pomc* promoter along with the consensus binding-site for T-box (Lamolet, Pulichino et al. 2001). This transcription factor is exclusively expressed in developing corticotrophs and melanotrophs and is capable of converting cells in the pars tuberalis from thyrotropes to corticotropes. While differentiation of corticotrophs does occur in the absence of Tpit, this transcription factor is essential for POMC expression in mature cells. Deletion analyses of the Tpit gene in a designed mutant mouse has shown that the lack of Tpit results in a diminished number of pituitary POMC cells with a marked increase in gonadotrophs and thyrotrophs (Maira, Couture et al. 2003). Additional data from Drouin's lab suggests an antagonistic interaction between Tpit and the gonadotroph-restricted factor (SF1), and it may be this antagonism that regulates which cells differentiate into POMC expressing corticotrophs and melanotrophs (Pulichino, Vallette-Kasic et al. 2003). Mutations of the *Tpit* gene in both humans and mice result in ACTH deficiencies (Pulichino, Vallette-Kasic et al. 2003).

2.2 *Hormonal Regulation of POMC*

Leptin, an adipocyte secreted hormone, acts to stimulate POMC neurons while synergistically inhibiting NPY neurons localized to the ArcN of the hypothalamus (Elias, Aschkenasi et al. 1999; Cowley, Smart et al. 2001). Leptin and hypothalamic POMC neurons are discussed in more detail in the following chapter.

Glucocorticoids are potent regulators of energy homeostasis and POMC expression both in the hypothalamus and in the pituitary gland (Roberts, Budarf et al. 1979). During fasting and when energy levels are in deficit, circulating levels of glucocorticoids (corticosterone in mice) increase which stimulates food consumption and energy storage. Glucocorticoids modulate carbohydrate metabolism, converting stored proteins to carbohydrates. Within the CNS, these regulatory effects are carried out by attenuation of central anorexogenic and/or catabolic effectors like POMC, CART and CRH by glucocorticoids (Schwartz, Woods et al. 2000). In the pituitary, glucocorticoids inhibit anterior lobe POMC expression while in the intermediate lobe glucocorticoids seem to upregulate POMC expression [reviewed in (Autelitano, Lundblad et al. 1989)].

In addition to leptin and corticosterone, gonadal steroids are known to influence food intake and body weight. Estrogen behaves very similarly to that of leptin: decreasing food intake and body weight and increasing metabolism. While estrogens are catabolic, androgens tend to be anabolic: increasing food intake and body weight [reviewed in

(Mystkowski and Schwartz 2000)]. Estrogens have been shown to be inhibitory to POMC and CRH, yet testosterone is stimulatory to POMC (Wilcox and Roberts 1985; Blum, Roberts et al. 1989; Tong, Zhao et al. 1990). While there are definite interactions between gonadal steroids and energy homeostasis it is believed that these steroids play more of a modulatory role rather then an essential role in maintaining energy homeostasis. However, it may be these modulatory roles that are responsible for sexually dimorphic phenotypes seen in some of our mutant mouse models.

3. RECEPTORS OF POMC PEPTIDES

3.1 *Melanocortin Receptors*

Five distinct members of a G-protein coupled melanocortin receptor gene family have been cloned (reviewed in (Cone, Lu et al. 1996)). MC1-R is expressed on melanocytes within the skin and its activation by Ac-αMSH-NH2 leads to increased membrane adenylyl cyclase activity by interaction with $G_{s\alpha}$ and subsequently greater production of the brown-black eumelanin pigment and less of the yellow-red phaeomelanin (Table 1). MC2-R is the classical ACTH receptor expressed on zona glomerulosa and fasiculata cells of the adrenal cortex and it has very low affinity for the various melanocortin peptides other than ACTH and ACTH1-24 (Mountjoy, Robbins et al. 1992). Two additional members of the gene family, MC3-R and MC4-R are expressed exclusively in the CNS in partially overlapping but distinct neuro-anatomical areas with particularly

Table 1

POMC Peptides and their Cognate G-Protein Coupled Receptors

Peptide	Cell of origin	Receptor	Target site	Function
ACTH	Corticotrophs	MC2-R	Adrenal cortex	Cort. secretion
Pro-γMSH	Corticotrophs	MC3-R, ?	Pituitary cells	Paracrine action
Ac-αMSH-NH_2	Melanotrophs	MC1-R	Melanocytes	Pigmentation
Ac–β–END1-27	Melanotrophs	μ,δ ↑ K_d	Peripheral sites	Modulation ?
DesAc-αMSH	Pituitary	MC5R	Exocrine glands	Secretion
γMSH, γ_3MSH	ARC neurons	MC3-R	Limbic system	Homeostasis
DesAc-αMSH-NH_2, ACTH	ARC neurons	MC4-R	Hypothalamus	Homeostasis
β–END1-31	ARC neurons	μ = δ > κ	Diffuse CNS	Analgesia, reward

Shown in the table are the POMC peptides relevant to this thesis matched up with their known receptors and the target site of these peptides. Also listed is the POMC cell type of the various peptides matched up with their known or believed physiological functions.

high abundance in the hypothalamus and limbic system (Roselli-Rehfuss, Mountjoy et al. 1993; Mountjoy, Mortrud et al. 1994). The MC3-R has the highest affinity for λ-MSH, although it is also activated by other melanocortins, while the MC4-R is activated most selectively by α-MSH and ACTH. Together these receptor subtypes mediate most of the described melanocortin effects on appetite, metabolism, thermoregulation, autonomic outflow, and behavior. The MC5-R is the most ubiquitously expressed of the melanocortin receptors and its best documented function is the stimulation of exocrine function including the sebaceous, preputial, Harderian, and lacrimal glands (Chen, Kelly et al. 1997).

3.2 *Mu–Opioid Receptor (MOR)*

Three opioid receptor genes have been cloned corresponding to the μ, δ, and κ receptors originally defined by their pharmacological profiles (Mansour, Watson et al. 1995; Hayward and Low 1999). The μ opioid receptor is postulated to mediate much of morphine-induced analgesia and physical dependence. Classical pharmacological experiments have shown that the various opioid receptor subtypes can mediate feeding but these experiments are limited due to the unspecificity of the endogenous ligands for the opioid receptors. β-End_{1-31} has the highest and nearly equivalent affinity to both μ and δ receptors (Pasternak 1993). However, each of these subtypes has significant affinity for other opioid peptides derived from the proenkephalin and prodynorphin precursors (Raynor, Kong et al. 1994). Two independent μ receptor deficient mouse lines were generated in separate labs (Matthes, Maldonado et al. 1996; Sora, Takahashi et

al. 1997). The μ receptor KO mice lacked morphine- induced analgesia seen in wild type siblings confirming that the μ receptor is the receptor mediating morphine-induced analgesia. No phenotypes exist in the μ receptor KO mice that suggest a disruption in energy homeostasis.

4. MUTATIONAL ANALYSIS OF POMC FUNCTION

4.1 *Hypomorphic and Constitutively Active MC1-R Gene Alleles*

Classical genetic studies indicated that two independent loci, extension (*E*) and agouti (*A*), primarily controlled the ratio of eumelanin and phaeomelanin in melanocytes, and hence the color of individual hairs. The extension locus is cell-autonomous and approximately a decade ago was demonstrated to encode the MC1-R or MSH receptor (Robbins, Nadeau et al. 1993). Numerous naturally occurring mutations at the extension locus have been characterized genetically because of the relative ease in tracing the mutant alleles during backcrosses and intercrosses by observation of coat color. Recessive mutations of the mouse extension locus (*e*) result in a yellow coat color due to low levels of tyrosinase activity in melanocytes and a high phaeomelanin/eumelanin pigment ratio. The *e* allele encodes a nonfunctional MC1-R resulting from a frame-shift mutation and premature translational stop codon between the fourth and fifth transmembrane domains. Dominant mutations of the mouse *e* locus result in black coat color and are epistatic to agouti. The sombre (E^{so}) allele encodes a MC1-R containing a

single activating point mutation (Robbins, Nadeau et al. 1993). This MC1-R is constitutively active in the absence of MSH and therefore not antagonized by agouti protein, accounting for the genetic epistasis (Ollmann, Lamoreux et al. 1998). These original data and the analyses of other mutant *E* alleles have been the basis of structure-function models to explain receptor activation by agonist ligand binding. Interestingly, identical mutations introduced into the other highly homologous MC-R subtypes have generally not resulted in receptor activation, thus emphasizing the important functional differences among these related G-protein coupled receptors and the danger of over extrapolating data concerning ligand interactions from one receptor subtype to another.

4.2 *Null Mutation of the MC4-R Gene*

Targeted deletion of the MC4-R has shown it to play an important role in energy homeostasis. MC4-R is the predominant melanocortin receptor in the brain and genetically engineered mice homozygous for a null MC4-R allele are obese, hyperphagic, and exhibit increased longitudinal growth (Huszar, Lynch et al. 1997), all features shared with the $A^{Y/a}$ yellow mouse. These data and the inability of the potent non-selective MSH analogue MTII to decrease food intake in MC4-R knockout mice (Marsh, Hollopeter et al. 1999) indicate that the MC4-R is essential for the anorectic effects of central melanocortins. Wild type mice given intraperitoneal administration of MTII exhibit increased metabolic rates while MC4-R knockout (KO) mice given MTII have unchanged metabolic rates suggesting that MC4-R may also regulate metabolism in addition to feeding (Chen, Metzger et al. 2000). Additionally, the Palmiter lab has shown

a metabolic defect in the MC4-R KO mice (Ste Marie, Miura et al. 2000). MC4-R KO mice that were pair-fed with wild type siblings were shown to have an increase in body weight, while non-pair-fed MC4-R KO mice show increases in body weight that precedes their hyperphagia which suggests that the hyperphagia seen in the MC4-R KO mice may be secondary to their metabolic defect. The anorexia and increase in metabolism seen in acute and chronic illnesses, medically referred to as cachexia, is absent in MC4-R deficient mice treated with lipopolysaccharide (LPS) or implanted with cachexigenic sarcomas (Marks, Ling et al. 2001). Additional studies with the MC4-R deficient mice revealed that increased thermogenesis in response to hyperphagia was lacking in these mice, identifying the MC4-R as a critical component of diet-induced thermogenesis (Butler, Marks et al. 2001).

4.3 *Null Mutation of the MC5-R Gene*

Transgenic mice deficient in the MC5-R do not exhibit any phenotypes that are directly associated with weight regulation or feeding but they do exhibit thermoregulatory defects and fail to repel water from their coat. These phenotypes were shown to be a result of reduced production and secretion of lipids from sebaceous glands in MC5-R deficient mice (Chen, Kelly et al. 1997). Additionally, the mice exhibited decreased production and attenuated stress-induced synthesis of porphyrins by the Harderian glands and decreased protein secretion from lacrimal glands. All of these exocrine glands, including the preputial gland, are normally sites of high MC5-R expression. The results suggest the existence of a hypothalamic-pituitary-exocrine axis that potentially regulates pheromone

production from exocrine glands in coordination with the behavioral arousal state of the mouse.

4.4 *Null Mutations of the MC3-R Gene*

Null mutations of the *Pomc* (Yaswen, Diehl et al. 1999) and *Mc4-r* (Huszar, Lynch et al. 1997) genes, and transgenic over expression or mis-expression of the MC4-R antagonists agouti and AGRP (Klebig, Wilkinson et al. 1995; Ollmann, Wilson et al. 1997) all produce phenotypes that can be attributed to diminished MC4-R signaling. Although these genetic models have provided great insight into the role of POMC and MC4-R in energy homeostasis, little specific information can be extracted regarding a contributory role of MC3-R signaling. The MC3-R, like the MC4-R, is expressed in the CNS but in a more restricted distribution including ArcN and VMN. Two groups have independently published reports of knockout mice lacking the MC3-R (Butler, Kesterson et al. 2000; Chen, Marsh et al. 2000). MC3-R deficient mice exhibit nearly normal growth curves and body weight, but carcass analyses reveal a significant increase in white adipose mass. Additionally, MC3-R deficient mice are more susceptible than controls to developing obesity on a high fat diet. In contrast to the obesity phenotype exhibited in MC4-R deficient mice, which can mostly be explained by alterations in feeding behavior, the MC3-R deficient mice apparently have a predominant metabolic defect involving energy partitioning. They have higher feed efficiency and decreased lean mass. Mice lacking both the MC3-R and MC4-R gain more weight than single MC4-R knockouts (Chen, Marsh et al. 2000). The MC3-R is localized presynaptically while the MC4-R is

postsynaptically localized suggesting that the two receptors cannot compensate for the lack of the other receptor. The fact that mice deficient in both genes have an additive weight phenotype reveals that these two receptors are involved in two independent pathways. Additional studies are needed to define the specific neural circuits responsible for the divergent actions of melanocortin peptides, MC3-R, and MC4-R on the regulation of food intake versus metabolic rate and energy expenditure.

4.5 *Null Mutations of the Opioid Receptor Genes*

Each of the three genes encoding the classical opioid receptor subtypes has been deleted by homologous recombination in mice (Hayward and Low 1999). Surprisingly, there have been no published reports regarding the effects of these mutations on food intake and energy homeostasis. Based on our findings in the ß-endorphin-deficient mice, we would predict that μ receptor knockout mice are the most likely to have a phenotype relevant to the physiologic regulation of body weight and fat mass.

4.6 *Null Mutation of the POMC Gene*

The original POMC null mice on the mouse 129 genetic back ground are described later in chapter 3 of this thesis.

4.7 *ß-Endorphin-Deficient Mice*

ß-Endorphin-deficient mice were generated by the introduction of a point mutation into the POMC gene by homologous recombination in embryonic stem cells (Rubinstein, Japon et al. 1993). The nucleotide insertion at the amino-terminal tyrosine of ß-End introduced a premature translational stop-codon into exon 3 (Figure 1, construct POMCX*4). Homozygous mice carrying this mutated allele express normal levels of POMC mRNA in pituitary and ArcN. The mRNA encodes a COOH-truncated POMC prohormone that is processed normally to ACTH and other melanocortin peptides (Rubinstein, Mogil et al. 1996). The mice have no detectable immunoreactive ß-End and have been used to infer the normal physiological function of this specific endogenous opioid peptide. Basal activity of the hypothalamic-pituitary-adrenal axis is normal and the β-End-deficient mice have normal corticosterone responses to a variety of stressors. β-End-deficient mice exhibit unaltered nociceptive thresholds and unaltered anti-nociceptive responses to ip morphine administration. However, βEnd-deficient mice fail to exhibit endogenous opioid-mediated stress-induced anti-nociception and have increased levels of non-opioid (naloxone insensitive) stress-induced anti-nociception (Rubinstein, Mogil et al. 1996). Complimentary studies in enkephalin-deficient mice have demonstrated that they retain endogenous opioid stress-induced anti-nociception (Konig, Zimmer et al. 1996). Taken together, these data provide convincing genetic evidence that ß-End derived from the POMC prohormone, and not the more abundant enkephalin peptides, is the natural opioid mediating stress-induced anti-nociception. The most likely relevant neuro-anatomical substrate is the projection of ArcN POMC neurons

to the central peri-aqueductal gray known to modulate descending anti-nociceptive pathways to the brainstem and spinal cord.

More recent studies have demonstrated that the ß-End-deficient mice have altered anti-nociceptive responses to morphine administered icv or it. The mutant mice are more sensitive to the anti-nociceptive action of morphine injected centrally into the lateral ventricle but less sensitive to morphine injected into the spinal compartment, compared to wild-type control mice (Mogil, Grisel et al. 2000). The μ-specific agonist DAMGO produced qualitatively identical results indicating the importance of the μ-opioid receptor in the phenotype. Total binding sites for the μ, δ, and κ receptors, assessed by quantitative autoradiography, were normal in the ß-End-deficient mice suggesting that subtle alterations in μ-receptor signaling may be the mechanism for the opposing alterations in central and spinal sensitivity to morphine. An electrophysiological study demonstrated a normal dose-response to DAMGO for the induction of an inwardly rectifying K+ current (GIRK) in mediobasal hypothalamic neurons of the mutant mice (Slugg, Hayward et al. 2000) consistent with the suggestion that μ-receptor number, affinity, and coupling to G proteins are not altered by the complete absence of ß-End.

Opioids, including β-End, have generally been shown to increase food intake in pharmacological studies (Morley 1987). Paradoxically, β-endorphin-deficient mice exhibit a sexually dimorphic obesity phenotype due to increased white fat mass in males (Appleyard, Hayward et al. 2003). The male mice are slightly hyperphagic and have a normal basal metabolic rate. This phenotype appears to be centrally mediated since

rescue of ß-endorphin expression to the pituitary alone does not reverse the phenotype while re-expression in both the pituitary and hypothalamus does. POMC derived melanocortins and ß-End are involved in a parallel, redundant anorexigenic pathway within the hypothalamus but β-End additionally plays a role in an opioid reward pathway. One hypothesis is the existence of a reward pathway that controls feeding behavior mediated by β-End. Studies utilizing operant responding for food reinforcers, support this latter hypothesis (Hayward, Pintar et al. 2002).

5. POMC PEPTIDE FUNCTIONS

5.1 *Hypothalamic Pituitary Adrenal Axis*

The classic stress axis involves corticotropin-releasing hormone (CRH) that is secreted from parvicellular neurons in the paraventricular nucleus (PVN). These parvicellular neurosecretory cells terminate at the median eminence where they release CRH into the primary plexus from which the peptide enters the hyophyseal portal veins. CRH then acts on the corticotrophs in the anterior pituitary to release ACTH (Vale, Spiess et al. 1981). Finally, ACTH secreted into the systemic circulation reaches the adrenal gland where it stimulates the production of corticosterone. POMC neurons are placed in a manner lending to an important role in hypothalamic-pituitary-adrenal (HPA) axis modulation. Although POMC involvement in this system is not fully understood, POMC projections of the arcuate nucleus to the paraventricular (PVN) (Lu, Barsh et al. 2003),

anteroventral periventricular (AVPv) nucleus as well as the previously described median eminence provide an intriguing mechanism for modulating stress responses at nodal central control points. Further anatomical evidence of POMC mediation of stress is that POMC neurons impinge on the nucleus of the tractus solitarius: a brain stem nucleus. Brain stem nuclei integrate autonomic system functions that are modulated during stress. Interestingly, integral players in the stress response (glucocorticoids and CRH) stimulate POMC mRNA in arcuate nucleus consistent with regulatory feedback loops through these hypothalamic neurons. Further, lesioning the arcuate nucleus in neonatal rats blocks the characteristic stress-induced prolactin secretion (Johnston, Spinedi et al. 1984).

Abundant data implicate opioids as mediators of stress and reproductive systems. Sex steroids administered exogenously and both female and male castration regulate POMC mRNA expression in hypothalamic neurons. These hypothalamic neurons project from the arcuate to the medial pre-optic area (MPOA) of the hypothalamus. The stress-induced inhibition of the hypothalamic pituitary gonadal (HPG) axis may result from direct or indirect inhibition of gonadotropin-releasing hormone (GnRH) neurons in the MPOA via projections from POMC neurons in the arcuate nucleus. These and other data support opiate involvement in modulating neuroendocrine systems in the hypothalamus. Although mice deficient in ß-End fail to support neuroendocrine modulation via ß-End, we have not been able to rule out any subtle compensatory mechanisms that may be masking ß-endorphin mediated actions.

5.2 *Energy Homeostasis*

Classical ablation or disconnection experiments utilizing electrolytic and chemically generated lesions or knife cuts of the mammalian brain revealed a number of discrete areas within the medial-basal hypothalamus to be nodal-points in the regulation of energy homeostasis [reviewed in (Elmquist, Elias et al. 1999)]. One principal area characterized in this fashion is the ArcN located at the base of the hypothalamus and adjacent to the third ventricle. The ArcN extends rostro-caudally from the posterior border of the optic chiasm to the anterior border of the mammillary nuclei. A subset of hypothalamic neurons distributed throughout the ArcN express the POMC gene. These POMC neurons densely innervate other brain areas implicated in energy homeostasis, motivated behavior, and autonomic control including the dorsomedial nucleus of the hypothalamus (DMH), paraventricular nucleus of the hypothalamus (PVN), lateral hypothalamic area (LH), ventral tegmental area (VTA), parabrachial nucleus, and NTS.

The lesion studies showed that disruption of the mammalian ArcN correlated with an obese phenotype and led to the concept that this area of the hypothalamus contained a satiety center. Later efforts focused on determining the specific neuronal phenotype in the ArcN that was involved in the regulation of energy homeostasis. An early indication of the importance of POMC peptides was the detection of MSH binding by autoradiography in the VMN, another hypothalamic nucleus involved in regulating appetite and feeding (Tatro 1990). In addition, central or peripheral injections of

melanocortin peptides were shown to affect food intake. Melanocortins appear to have dual roles in energy balance, depending on the site of action. Administration of ACTH and Ac-αMSH directly into adipose tissue increases lipolysis whereas intramuscular injection of DesAc-αMSH increases food intake (Boston 1999). Furthermore, peripheral DesAc-αMSH inhibits pituitary production of ACTH and Ac-αMSH, which may lead to increased lipogenesis (Mountjoy and Wong 1997). MSH analogues administered icv or injected stereotaxically directly into the PVN of rodents have potent anorexigenic effects and stimulate metabolic rate (Farooqi, Yeo et al. 2000; Vergoni and Bertolini 2000; Millington, Tung et al. 2001; Lim, Li et al. 2002). Exogenous opiates and endogenous opioid peptides have also been shown to affect food consumption and food preference, suggesting a role for POMC-derived β-End, in addition to the melanocortin peptides, as a modulator of energy homeostasis (Reid 1985; Levine, Grace et al. 1994; Hope, Chapman et al. 1997). The physiology of this complex neuroendocrine system is virtually impossible to replicate in *in vitro* models. Thus, transgenic mice and mice with naturally occurring mutants or targeted genetic alterations have been extremely informative tools for the analysis of POMC peptides and their receptors in the control of food intake, energy partitioning, and body weight.

One of the first mutant mouse models of obesity to be studied was the lethal yellow (A^{Y}/a) mouse (Duhl, Stevens et al. 1994). The mutation arose spontaneously in the C57BL/6 inbred strain and is associated with a striking yellow coat color and the development of obesity and hyperphagia. Genetically, the mutation was shown to be dominant to the extension (*E*) locus, later identified as the gene encoding the MC1-

receptor (Robbins, Nadeau et al. 1993). Cloning of the *agouti* gene revealed that it encodes a secreted protein characterized by multiple pairs of intra-chain disulfide bonds in its C-terminal domain. Agouti has high affinity binding for the MC1-R, antagonizes receptor activation by MSH (Lu, Willard et al. 1994), and is normally transiently expressed only in the hair follicles accounting for the yellow banding of individual black hairs in the fur of many mammals. The mutant A^{Y} allele results from a 120 kb genomic deletion and juxtaposition of the agouti coding sequences to the *Raly* gene locus and subsequent ubiquitous expression of agouti protein from the *Raly* promoter (Duhl, Stevens et al. 1994). These observations led to the agouti-obesity hypothesis, which states that agouti protein expressed within the brain antagonizes the action of central MSH to a neural MC receptor involved in regulation of energy balance. Some of the experimental models reviewed in this chapter provide essential data in support of this hypothesis, which has been further validated by the discovery and cloning of an agouti homologue, agouti gene-related protein (AGRP). AGRP is expressed in the nervous system and functions as an antagonist of central MC receptors (Ollmann, Wilson et al. 1997).

Genetic linkage studies and quantitative trait loci analyses have strongly implicated the *POMC* gene locus as an important determinant of weight homeostasis in humans of many different ethnic populations, although specific alleles associated with obesity have not yet been demonstrated (Comuzzie, Hixson et al. 1997; Hager, Dina et al. 1998; Hixson, Almasy et al. 1999; O'Rahilly, Farooqi et al. 2003). Recently, mutations within the coding region of the *POMC* gene that alter peptide activity have been identified (Krude,

Biebermann et al. 1998; Krude and Gruters 2000; Challis, Pritchard et al. 2002; Krude, Biebermann et al. 2003). However, a small number of children from cosanguineous parents have been found to have null mutations in the *POMC* gene resulting in the absence of detectable circulating ACTH (Krude, Biebermann et al. 1998; Krude and Gruters 2000). These children presented with a syndrome of red hair, adrenal insufficiency and severe, early-onset obesity. Alternatively, mutations in regulatory regions of the gene may decrease the level of *POMC* expression in the brain although such cases have yet to be found. In addition, both dominant and recessive mutations in the MC4-R gene have been found in the human population, and MC4-R mutations have been proposed to play a role in as many as 5% of pediatric obesity cases (Farooqi, Yeo et al. 2000; Yeo, Farooqi et al. 2000).

5.3 *Development*

Both *in vivo* and *in vitro*, ACTH and αMSH have neurotrophic properties that benefit neural growth during development and regeneration. Additionally, the robust early expression (e10.5) of POMC in cell bodies and fiber tracts, at the same time as hypothalamic neurogenesis, suggests a role for POMC peptides as trophic and/or differentiating factors during ontogeny of the hypothalamus (Shimada and Nakamura 1973). Immuno-neutralization of POMC peptides leads to impaired brain development during the late gestation period (Zagon and McLaughlin 1987). Exogenous administration of ACTH accelerates eye opening and motor behavior in up to 3-day-old rats, while administration of ACTH after day 3 tends to prolong eye opening (De Wied

and Jolles 1982). Furthermore, administration of ACTH induces nerve sprouting in early rat development (Frischer and Strand 1988). Coinciding with the developmental influence of melanocortins, opioids have also been shown to alter hypothalamic development. An opioid antagonist given to rat pups resulted in increased dendritic growth and dendritic spine formation in the brain (Hauser, McLaughlin et al. 1987). Interrupting opioid-opioid receptor interactions by the immuno-neutralization of β-End during embryogenesis and neonates of rat pups up to 10 days old dramatically altered neuro-ontogeny (Zagon and McLaughlin 1986; Zagon and McLaughlin 1987). Recently, the developmental pattern of central melanocortin receptors 3 and 4 (MC3-R & MC4-R) revealed MC4-R to be the prominent receptor expressed during the entire fetal period (Kistler-Heer, Lauber et al. 1998). Expression of MC4-R, shown by mRNA *in situ* hybridization, occurred at a time when neural circuits are known to be forming, but the mRNA message levels transiently peaked at these early embryonic time points. As mentioned earlier, transient expression of spinal cord POMC mRNA and peptides, α-MSH and ß-End, has also been reported (Elkabes, Loh et al. 1989). However, while the transient or early ontogeny of the melanocortin receptor's mRNA and POMC peptides are indicative of a developmental role in embryogenesis, it should be noted that studies of the POMC null mice have yet to reveal any gross anatomical defects other than atrophic adrenal glands.

Peripherally, the POMC derived peptide ACTH may also play a vital role in the maturation of the neuromuscular junction (Cossu, Cusella-De Angelis et al. 1989). ACTH appears to accelerate muscle strength, increase muscle contractility, and maintain

motor units during nerve reinervation following nerve lesion (Sanes, Marshall et al. 1978; Saint-Come, Acker et al. 1985; Berry and Haynes 1989). *In vitro* studies show ACTH as a stimulator of adrenal cortical blastomere formation (Strand, Rose et al. 1991). Maturation of peripheral organs such as the adrenal glands (Durand, Cathiard et al. 1984) is thought to be induced by POMC but pituitary POMC is not detected until e12.5 in the anterior lobe and e14.5 in the intermediate lobe. Adrenal blastomeres are present at e11, suggesting that central POMC is providing maturational signals (Estivariz, Carino et al. 1988; Estivariz, Morano et al. 1988; Cossu, Cusella-De Angelis et al. 1989; Davis, Burgess et al. 1989). While this thesis is not able to completely rule out a developmental role of CNS POMC in adrenal maturation, we do show that pituitary POMC in the absence of central POMC is sufficient for the development of ACTH-responsive adrenal glands.

5.4 *Pigmentation*

In mammals, skin, coat and hair color are determined by the relative ratio of phaeomelanin (yellow/red pigment) to eumelanin (brown/black pigment) produced by melanocytes. In fur-bearing mammals, both MC1-R and agouti, an endogenous MC1-R antagonist expressed in specialized cells in the dermis, affect this ratio. Activation of the MC1-R by peripheral α-MSH stimulates eumelanin synthesis. Conversely, antagonism of α-MSH action by agouti favors phaeomelanin synthesis (Ollmann, Lamoreux et al. 1998). Expression of *agouti* is temporally and spatially regulated (Vrieling, Duhl et al. 1994). Temporal expression of *agouti* accounts for the agouti-banding pattern; spatial

regulation accounts for differences in dorsal and ventral coat color seen in some mammals. Additional reports hypothesize that agouti protein promotes melanocortin-receptor internalization, or that it functions as an inverse agonist of melanocortin receptors (Siegrist, Willard et al. 1996; Siegrist, Drozdz et al. 1997).

Mutations of MC1-R also have profound effects on pigmentation. Both gain-of-function and loss-of-function mutations of MC1-R have been shown to alter pigmentation in a range of species (Robbins, Nadeau et al. 1993). MC1-R is also highly polymorphic in humans (Schaffer and Bolognia 2001). Certain allelic variants of the gene in humans are associated with red hair and pale skin (Valverde, Healy et al. 1995). Although human pigmentation is genetically complex, complete POMC loss-of–function mutations in humans exhibited a deficiency syndrome that included red hair (Krude, Biebermann et al. 1998; Krude, Biebermann et al. 2003).

In humans, α-MSH and ACTH produced locally in the skin have a major role in pigmentation [reviewed in (Thody 1999)]. The production of both peptides is upregulated in the keratinocyte by ultra-violet (UV) radiation, and then act as paracrine factors that stimulate the melanocyte to produce eumelanin. α-MSH is also produced by the melanocyte and may act as an autocrine factor that affects eumelanin synthesis and melanocyte morphology and as a paracrine factor that protects the melanocyte against immune system damage. MC1-R has also been reported to be upregulated by UV radiation. The contribution of centrally produced melanocortins on pigmentation has yet

to be described, although the centrally produced forms of these peptides circulate at an extremely low concentration.

CHAPTER II

EXPRESSION OF ENHANCED GREEN FLUORESCENT PROTEIN IN MURINE PROOPIMELANOCORTIN CELLS

Portions of this chapter have been published in or are in preparation for:

Cowley*, **Smart***, *et al.* (2001) "Leptin activates anorexigenic POMC neurons through a neural network in the arcuate nucleus." *Nature* **411**, 480-484. (*Co-First authors)

Overstreet,.....**Smart**, *et al.* (2004) "A transgenic marker for newly born granule cells in dentate gyrus." *Journal of Neuroscience* **23** (13): 3251-3259.

de Souza,.....**Smart**,..... "Phylogenetic conservation of the modular enhancer architecture that directs expression of the proopiomelanocortin gene to the hypothalamus and brain stem of transgenic mice." In preparation for submission to *Molecular & Cellular Biology*.

1. ABSTRACT

Proopiomelanocortin neurons in the arcuate nucleus of the hypothalamus and nucleus tractus solitarius have prominent roles in the modulation of energy homeostasis, autonomic function, and the stress response. However, the analysis of signal transduction events and gene expression in these important peptidergic neurons has been limited because there are only a few thousand POMC neurons in the entire brain. To facilitate the identification of these neurons in living brain slices or primary cultures, we generated a transgenic C57BL/6 mouse line expressing enhanced-green-fluorescent protein (EGFP, Clonetech) under the transcriptional control of POMC genomic regulatory elements. Bright green fluorescence was readily identified in the basal hypothalamus and in the NTS of the brain stem: the two CNS regions where POMC is expressed. Double-labeled immunofluorescence with a β-endorphin antisera revealed >99% colocalization of EGFP fluorescence and β-endorphin staining within the ArcN. Pituitary intermediate lobe (IL) melanotrophs and anterior lobe (AL) corticotrophs also exhibited intense green fluorescence. The neuron-specific expression pattern of this POMC-EGFP transgene further confirms the localization of critical genomic regulatory elements in the 13kb of 5' flanking sequence of the POMC gene (Young, Otero et al. 1998). Additional POMC-EGFP transgenic mice with truncated and/or spliced 5' flanking sequences have subsequently been generated and have further localized the regulatory elements essential for POMC neuronal expression.

2. INTRODUCTION

Abundant data from naturally occurring human mutations, engineered murine genomic mutations, and pharmacological studies have identified POMC neurons as a critical CNS component regulating feeding and metabolism. The ability to study these neurons *in vivo* and unveil how the POMC gene is neuronally regulated has been complicated due to the limited number of these cells. The discovery and cloning of the adipocyte hormone leptin has led to significant advances in our understanding of how levels of peripheral energy stores are regulated by the brain (Zhang, Proenca et al. 1994). Leptin administration to leptin-deficient humans, and the analogous *ob/ob* mice effectively reduces hyperphagia and obesity (Campfield, Smith et al. 1995; Farooqi, Jebb et al. 1999). However, most human obesity is associated with elevated leptin, suggesting that obese humans are less sensitive to leptin. In addition to its regulation of long term physiological processes like energy balance and reproduction, leptin also exerts rapid effects on neuronal activity (Haynes, Morgan et al. 1997; Haynes, Morgan et al. 1999; Kim, Small et al. 2000). To understand the relationships between the acute and chronic effects of leptin as well as the causes of leptin resistance it is necessary to precisely characterize the cellular and neuronal mechanisms of leptin action.

Leptin's effects on energy homeostasis are mediated in part by leptin receptors expressed on proopiomelanocortin (POMC) and neuropeptide Y (NPY) neurons in the arcuate nucleus of the hypothalamus (ARC) (Hakansson, Brown et al. 1998; Cone 1999; Kalra, Dube et al. 1999). NPY is a potent orexigen and decreases metabolic rate, while the

POMC peptide α-MSH inhibits feeding and increases metabolic rate (reviewed in (Cone 1999)). Leptin may act on different neuronal subtypes in different ways. Acute leptin treatment presumably activates POMC neurons, since increased immunoreactivity for the immediate early gene c-fos in POMC neurons results from icv administration of leptin. Conversely, leptin does not similarly activate NPY cells in the ARC (Elias, Aschkenasi et al. 1999). Furthermore this second population of ARC neurons appears to be directly inhibited by leptin (Glaum, Hara et al. 1996; Spanswick, Smith et al. 1997; Elias, Aschkenasi et al. 1999). Understanding how peripheral signals like leptin are able to relay information to the CNS is essential in understanding energy homeostasis.

The POMC gene is expressed in both endocrine cells of the pituitary gland and neurons of the basal hypothalamus. POMC gene promoter mapping studies have identified the DNA flanking regions essential for pituitary POMC expression (Liu, Hammer et al. 1992). In addition, the DNA flanking regions essential for neuronal POMC expression were subsequently cloned from a cosmid mouse genomic library (Young, Otero et al. 1998). The DNA flanking regions obtained from these clones were 17 kb in size. Although the DNA flanking *cis* elements for pituitary expression have been mapped to –700 bp, the *cis* elements for neuronal expression have not been mapped as precisely. The mapping of these essential POMC neuronal *cis* elements will eventually lead to a better understanding of how this gene is regulated and consequently the discovery of POMC gene transcriptional factors. Understanding how the POMC gene with its anorexigenic properties is regulated will help us unveil and understand putative key factors in the balance between energy storage and utilization.

3. METHODS

Transgene construction. The transgene contains the entire transcriptional unit of the mouse *Pomc* gene together with 13 kb of 5' flanking sequences and 2 kb of 3' flanking sequences. An enhanced green fluorescent protein (EGFP) (Clontech, CA) cassette was introduced into the 5' untranslated region of exon 2 within a mouse POMC genomic clone. The EGFP cassette contains its own Kozac's consensus translation initiation site along with SV40 polyadenylation site downstream of the EGFP gene directing proper processing of the 3' end of the EGFP mRNA.

Creation of transgenic mice. The transgene DNA was removed from its vector backbone by *Not*I/*Sal*I enzymatic digestion. Transgene DNA was purified by gel electrophoresis followed by electro-elution from the agarose gel, phenol/chloroform extraction and ethanol precipitation. DNA was then further purified and concentrated with an ELUTIP-d kit (Schleicher & Schuell). DNA was quanitated by U.V. spectroscopy and an injection concentration of 500 pmol of DNA in 5 mM Tris/ 0.1 mM EDTA/ pH 7.4 was used for injections.

Pronuclear microinjections of linearized DNA into one-cell stage embryos of the inbred C57BL/6J (Jackson Laboratories, ME) mouse strain were performed (Brinster and Palmiter 1984). One founder was generated and bred to wild type C57BL/6J to produce F1 hemizygotic mice. In addition, F2 progeny and subsequent generations of mice

homozygous for the transgene were also generated. The F2 mice were used to establish and maintain a breeding colony. All founder mice were initially screened by genomic DNA dot blots. After a mouse line was established, subsequent generations were screened using PCR. Standard PCR genotyping was conducted on DNA extracted and prepared from tail clippings.

PCR genotyping of pups was conducted on DNA extracted and prepared from tail clippings (described in (Barnes 1994)). Touchdown PCR was used to favor amplification of desired amplicons and to limit artifactual amplicons and primer-dimers (protocol desribed in (Don, Cox et al. 1991)). Multiple cycles were programmed so that the annealing segments in sequential cycles are run at incremental lower temperatures starting at the suspected melting temperature (T_m). To avoid low-T_m priming during the earlier cycles it was imperative to apply a hot start modification to the PCR protocol (D'Aquila, Bechtel et al. 1991). The programmed PCR cycle was 94°C for 10min; [94°C-30sec; 64°C minus 1°C per cycle-30 sec; 72°C-30 sec] X 15 cycles; [94°C-30sec; 50°C-30 sec; 72°C-30 sec] X 15 cycles; 72°C-3min; hold at 4°C. The following primers were used and were identified as EGFP-3 (5'-GAACTCCAGCAGGACCATGT-3') T_m=60°C and EGFP-5 (5'-AGAAGAACGGCATCAAGGTG-3') T_m=58°C which gave a PCR product of 199 bp. A standard PCR reaction mix was used as described in the manual "PCR Primer" (Dieffenbach and Dveksler 1995).

Southern blot. One centimeter of tail was removed for DNA extraction from mice that were anesthetized with 2,2,2-tribromoethanol (0.15 mls/30g of a 2% w/v solution). The

tails were placed into 0.5 ml of a [0.5mg/ml] proteinase K digestion buffer at 55°C overnight on a rotating 'nutator'. The next day the mouse hair was centrifuged out of the digestion buffer and 0.45 ml of isopropanol was added to the buffer to precipitate the DNA. Salts were washed out of the DNA with 70% EtOH, allowed to air-dry and then dissolved in a neutral pH Tris buffer. The DNA was then put through a phenol/chloroform extraction to remove any proteins or debris that would hinder a genomic DNA enzymatic digest and then salt precipitated with 10% sodium acetate and 2X volume ethanol. DNA was quantitated using U.V. spectroscopy so that equal amounts of DNA (20 μg) per mouse would be digest and loaded per well. Genomic DNA was digested overnight with BamHI because only one BamHI site exist in the pHal Ex2* transgene. With multiple insertion transgenes, they most commonly insert into the mouse's genomic DNA at one locus in a head to tail orientation. A genomic DNA digest using a restriction site that cuts once in the transgene will give a DNA band the size of the transgene. DNA was loaded and run overnight at 30 mV on a 0.6% w/v low melting point agarose gel made and run in 0.5X Tris-buffered saline. Gel was denatured, neutralized and transferred onto nylon membrane following a standard Southern blot protocol (Southern 1975). We used a random-primed POMC exon 3 [^{32}P]-DNA probe made from a linearized 700 bp DNA segment. Using one probe that recognized both the endogenous gene and the transgene allowed us to do a ratio analysis on the band intensities. Averaging this ratio from 40 Southern blot lanes (each lane representing one hemizygous POMC/EGFP mouse) gave us a transgene copy number of 18 per hemizygous mouse.

Immunohistochemistry. Adult POMC/EGFP transgenic mice were anesthetized with 0.5 ml of 2% w/v 2,2,2-tribromoethanol (Avertin, Alrich, MO) and perfused transcardially with 4% paraformaldehyde borate buffer pH 9.5. Coronal sections were cut on a vibratome at a thickness of either 30 μm or 50 μm into 0.02M potassium phosphate buffered saline pH=7.4 (KPBS). Free-floating sections were blocked for 24 h at 4°C in KPBS containing 0.4% Triton X-100 and 2% normal horse serum (Vector Laboratories, Burlingame, CA) with gentle agitation. Sections were then incubated for 24 h at 4°C in KPBS containing 0.4% Triton X-100 and 2% normal horse serum and primary antibody of 1:2500 v/v rabbit anti-β-endorphin (a gift from Dr. Oline Ronnekleiv original source Dr. M.R. Brown) or 1:25000 v/v rabbit anti-NPY (Alanex Corp.) or 1:2000 v/v rabbit anti-ACTH (a gift from Dr. Richard Allen) or 1:1000 v/v mouse monoclonal anti-TH (Incstar) with gentle agitation. Sections were again rinsed in KPBS and incubated for 2-4 h at RT in KPBS containing 0.4% Triton X-100 and 2% normal horse serum and 10μg/μl biotinylated horse anti-mouse/rabbit serum (1:200; Vector Laboratories, CA) with gentle agitation. Sections were again rinsed with KPBS and incubated for 1 h at RT in KPBS containing 0.4% Triton X-100 and 1:500 v/v Cy-3 conjugated streptavidin (Jackson Immunoresearch Laboratories, Inc. West Grove, PA) with gentle agitation. Sections were then rinsed in KPBS for 3 h to reduce background. After rinsing, sections were mounted and then air-dried onto gelatin-coated slides in a rostral to caudal orientation. Slides were then placed in a 4 μg/μl bis-benzimide solution (Sigma) for 5 min at RT to stain cell nuclei. Slides were then rinsed and air-dried before cover slipping with 'vectashield' mounting media (Vector Laboratories, CA).

Cell counting and co-localization. After tissue sections were processed for anti-β-endorphin immunohistochemistry, sections were mounted on slides, air-dried and cover slipped with 'vectashield' mounting media to prevent photo bleaching. These sections (30μm) were mounted in a caudo-rostral orientation to assure accurate mapping of the POMC neurons. Sections were viewed under a 20X objective for EGFP fluorescence (green) and immunohistochemistry staining (red).

Care and use of animals. All animals for these studies were maintained under controlled temperature and photoperiod conditions (14 h of light, 10 h of dark; lights on, 0500 h) with food and water *ad libitum*. Maintenance of all animals and all surgical procedures used were approved by the Institutional Animal Care and use Committee and were in keeping with guidelines established by the National Institutes of Health (NIH).

4. RESULTS

4.1 *Generation and characterization of the initial POMC/EGFP (-13/+8 POMC/EGFP) transgenic mouse line*

In order to study *in vivo* physiological responses to peripheral signals like leptin, we constructed reporter genes to be introduced into the murine genome via standard transgenic technology. Utilizing a "Clontech Inc, CA." cassette containing the sequence for enhanced green fluorescent protein (EGFP), we constructed a variety of POMC/EGFP

transgenes. These transgenes were designed using POMC neuronal enhancer/promoter sequences to inertly mark POMC expressing cells while also mapping the gene's promoter regions essential for neuronal expression. The first POMC/EGFP transgene (-13/+8 POMC/EGFP) that was constructed contained genomic sequences located between -13kb and +2kb of the POMC gene (Figure 1). In order to eliminate epistatic modifications to phenotypes caused by genetic heterogeneity found among inbred mouse lines, we used C57BL/6 mouse oocytes for pronuclear injections. The C57BL/6 inbred mouse is commonly used as a background strain for many mutant mouse lines, and is commonly used in mouse behavioral studies. Many of the mutant mouse lines of the POMC system have been studied on this genetic background; furthermore, the C57BL/6 inbred mouse strain is susceptible to diet induced obesity lending this strain advantageous for studying a type of obesity that is prevalent among human populations.

Pronuclear injections resulted in the generation of one male founder. This founder mouse, as well as subsequent POMC/EGFP founder mice, was identified by a genomic DNA dot blot. A radionucleotide ^{32}P-labeled random-primed DNA probe was generated from EGFP specific sequences and used to hybridize with tail DNA on the dot blot. The male founder was then bred to C57BL/6 females to generate F1 progeny for transgene analysis. Genotyping of F1 progeny was done utilizing PCR techniques with EGFP sequence-specific primers (see methods). A quantitative-Southern-blot, described in greater detail in the methods, of mouse tail DNA using a ratio analysis of the endogenous POMC gene and the transgene revealed the transgene copy number to be 18 copies per hemizygous mouse (Figure 2).

Figure 1

The POMC/EGFP transgene was constructed by subcloning the coding sequence for enhanced green fluorescent protein and a polyA adenylation sequence from bGH into an untranslated region of exon 2 of a mouse POMC genomic clone. The POMC/EGFP transgene injected into mouse oocytes was ~21 kb in length. Both the transcriptional start and stop site are marked on the wild type allele (top) and the POMC/EGFP transgene construct (bottom) depicted in this schematic.

Figure 1

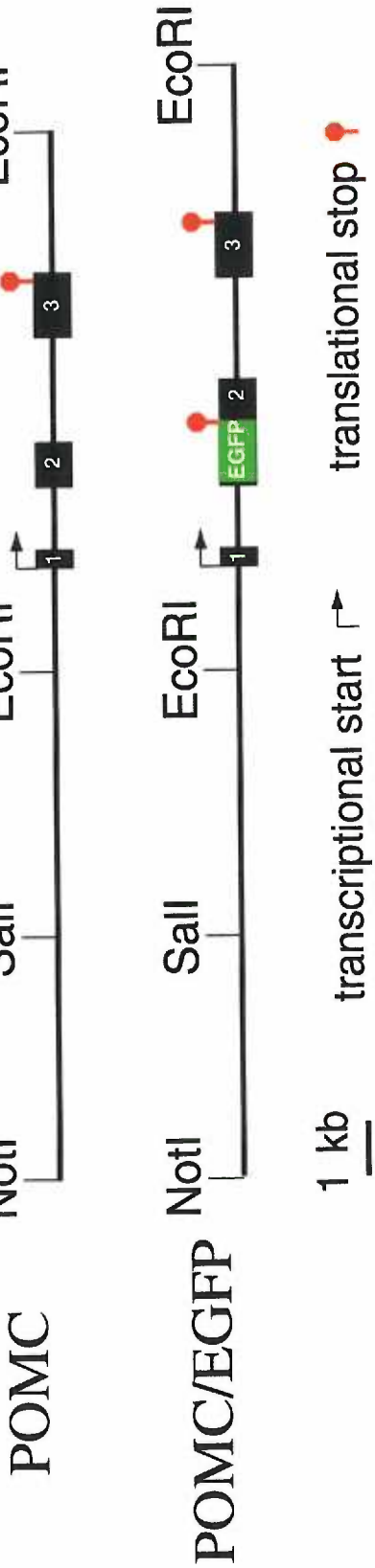

POMC
NotI
SalI
EcoRI
EcoRI
1
2
3
POMC/EGFP
NotI
SalI
EcoRI
EcoRI
1
EGFP
2
3
1 kb
transcriptional start
translational stop

Figure 2

Using a radionucleotide labeled DNA probe generated from exon 3 of the POMC gene, we could detect the endogenous alleles and the inserted POMC/EGFP transgene by a quantitative genomic-Southern-blot. A densitometric ratio was obtained from 40 lanes with each lane containing restriction enzyme (BamHI) digested genomic tail DNA. Each lane represents one hemizygous POMC/EGFP transgenic mouse and a ratio between the two bands revealed the transgene copy number to be 18 copies. 10.5 kb, wild type allele; 11.5 kb, transgenic allele.

Figure 2

EGFP Genomic Southern Blot

Using positive F1 progeny, initial immunohistochemistry studies revealed that the expression of EGFP in hypothalamic neurons mirrored the expression of the POMC peptide, β-endorphin (Figure 3). Under ultraviolet (450–480 nm) excitation, bright green fluorescence (509 nm) was seen in the two CNS regions where POMC is expressed: the ArcN and the NTS (Figure 4). Coronal sections of e14.5 mouse embryos revealed fluorescent cells in the basal hypothalamus along with the anterior and intermediate lobes of the pituitary gland (Figure 5). Expression of EGFP in these three cell types at an embryonic developmental stage when POMC has also been reported suggests the transgene is regulated similarly to that of the endogenous POMC gene (Elkabes, Loh et al. 1989).

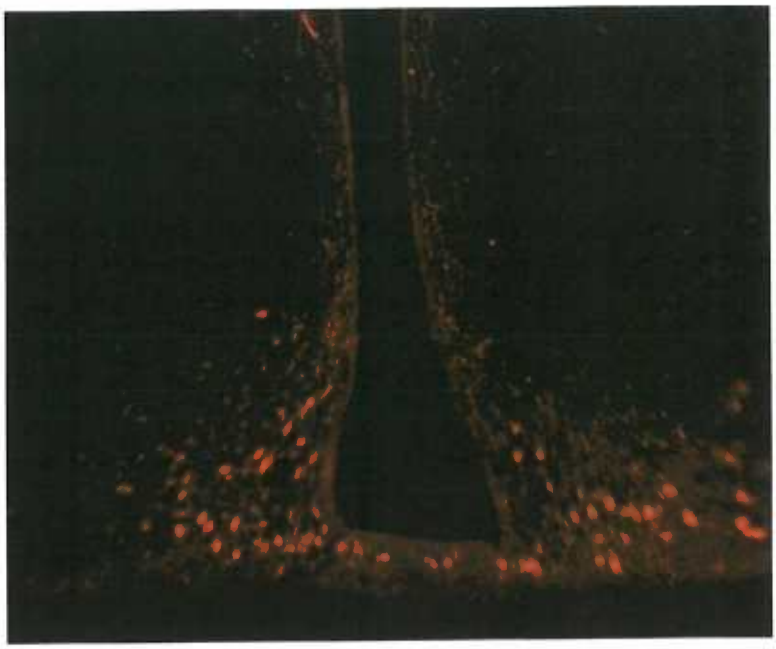
4.2 *EGFP expression confined to hypothalamic POMC neurons is used to count and map these neurons in the mouse*

EGFP excitation in POMC neurons was clearly distinguished from adjacent, non-fluorescent neurons visualized under infrared optics (not shown) for a short period of time to avoid damaging the cells/neurons and their circuitry. But before electrophysiological studies could be conducted on these fluorescent neurons, we needed to assure ourselves that the fluorescence was confined to only hypothalamic POMC neurons. Standard immunohistochemistry protocols, with traditional diaminobenzidine (DAB) peroxidase reactions, are very harsh on cellular proteins and greatly diminish the fluorescence of existing fluorophores. In order to circumvent this problem, we utilized a rabbit polyclonal β-endorphin antiserum, a secondary biotinylated goat anti-rabbit IgG,

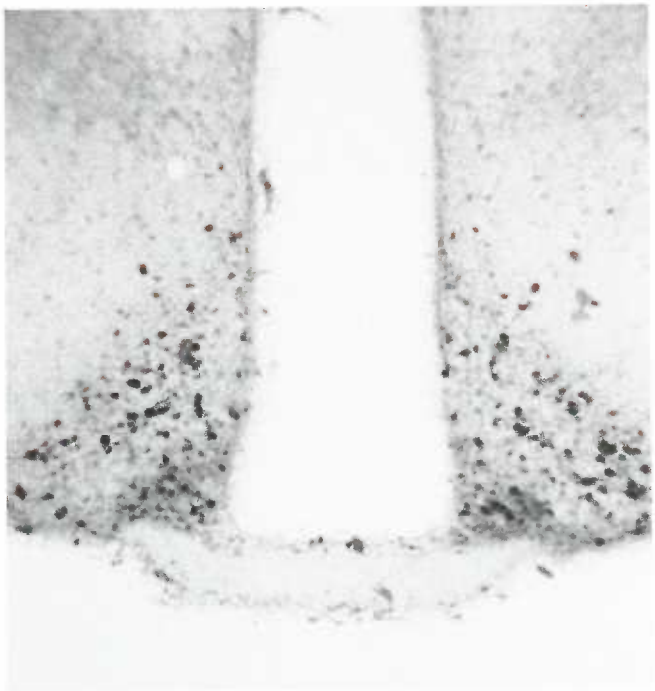
Figure 3

Immunohistochemistry for β-endorphin is shown in panel (**A**) and for EGFP in panel (**B**) on coronal sections of mouse hypothalami. Both immunohistochemical analyses were done with primary rabbit polyclonal antisera for either β-endorphin (panel (**A**)) or EGFP (panel (**B**)) followed with a biotinylated secondary antibody and peroxidase DAB redox reaction.

Figure 3



anti- β-endorphin



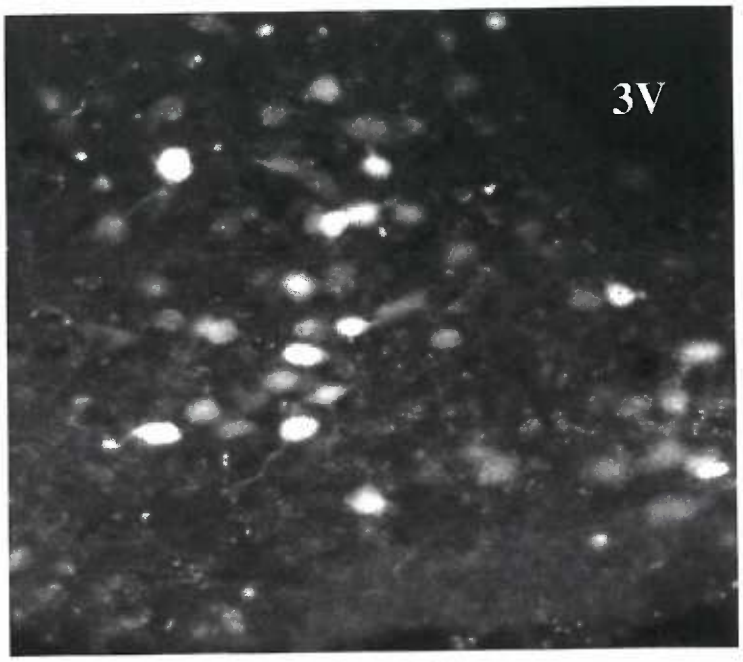
anti-EGFP

Figure 4

Analysis of adult coronal brain sections also revealed strong EGFP expression in both the ArcN and NTS. 50 μm coronal sections visualized by ultraviolet excitation (450-480 nm) revealed bright green fluorescent neurons in the basal hypothalamus and caudal brain stem: the two CNS regions where POMC is expressed. EGFP fluorescence can also be seen in processes extending two to three cell body lengths from fluorescent soma. **SolM**= Medial Solitary Tract; **CC**= Central Canal; **3V**= Third Ventricle.

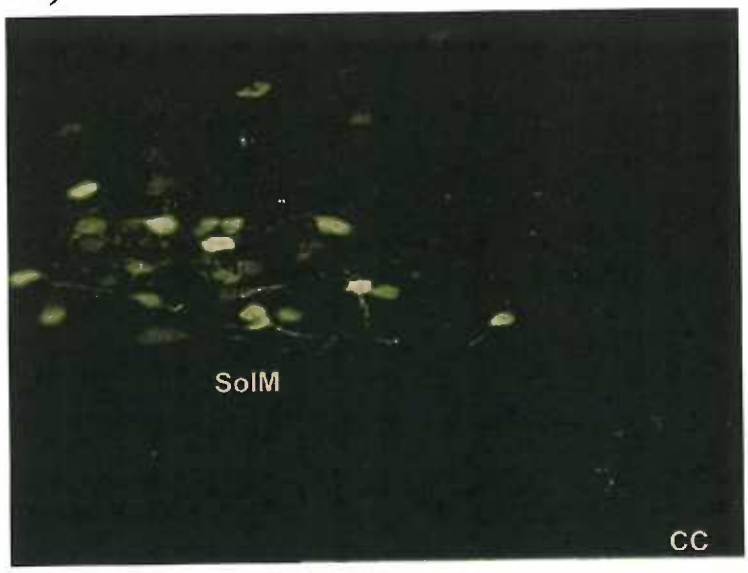
Figure 4

A)



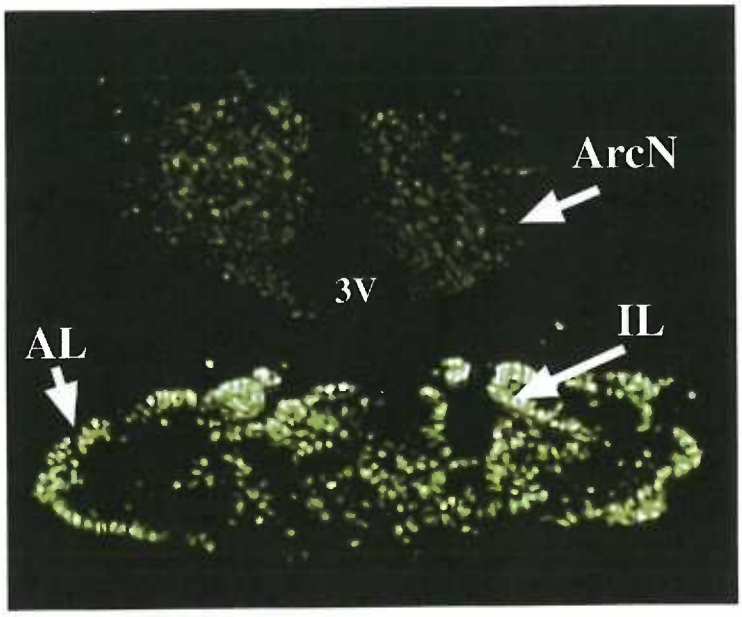
ArcN

B)



NTS

Figure 5



Basal Hypothalmus and Pituitary Gland
Embryonic Day (e14.5)

and then a tertiary strepavidin-Cy3 conjugate as the fluorophore: thus allowing the use of dual fluorophores, EGFP & Cy3, within the same cell. This immunofluorescence analysis revealed >99% colocalization of EGFP and POMC peptides within the ArcN with only six neurons lacking evidence of co-expression (n=1 mouse) (Figure 6). Additional immunofluorescent studies revealed close apposition of both tyrosine hydroxylase (TH) and NPY stained terminals on EGFP expressing POMC neurons, and no evidence of co-localization of the TH or NPY immunoreactivity with EGFP.

Total fluorescent cell counts were performed on coronal hypothalamic sections of three POMC/EGFP mice. The average number of POMC neurons from the three mouse hypothalami was 3148 ± 62 (mean ± s.e.m.; n=3). The POMC neurons from one mouse hypothalamus were plotted on line drawings obtained from "The Mouse Brain in Stereotaxic Coordinates" (Franklin and Paxinos 1997). The POMC neurons are distributed through the entire ArcN; interestingly, these POMC neurons in the mouse tend to be found both medially and ventrally within the ArcN, in contrast to the rat where they are mostly found in the lateral ArcN and the NPY neurons are found in the medial arcuate nucleus (Figure 7).

4.3 *Ectopic expression of EGFP in the CNS*

While EGFP expression in the hypothalamus was confined to POMC neurons, ectopic expression of EGFP was detected in a number of cell populations outside of the hypothalamus. Green fluorescent cells were first noticed in the hippocampus in a discrete

Figure 6

A) EGFP expression was visualized by ultraviolet excitation at 450-480 nm and **B)** in the same section immunofluorescence for β-endorphin antisera (530-560 nm) shows transgene expression exclusively in arcuate nucleus POMC neurons. **C)** Superimposed images revealed >99% co-localization of EGFP and β-endorphin within the ArcN. Less then 1% of EGFP fluorescent neurons did not contain β-endorphin staining, thus demonstrating a lack of ectopic expression within the hypothalamus. **D)** Immunohistochemistry for tyrosine hydroxylase (TH) shows that the hypothalamic dopaminergic neurons do not co-localize with the EGFP fluorescent POMC neurons.

Figure 6

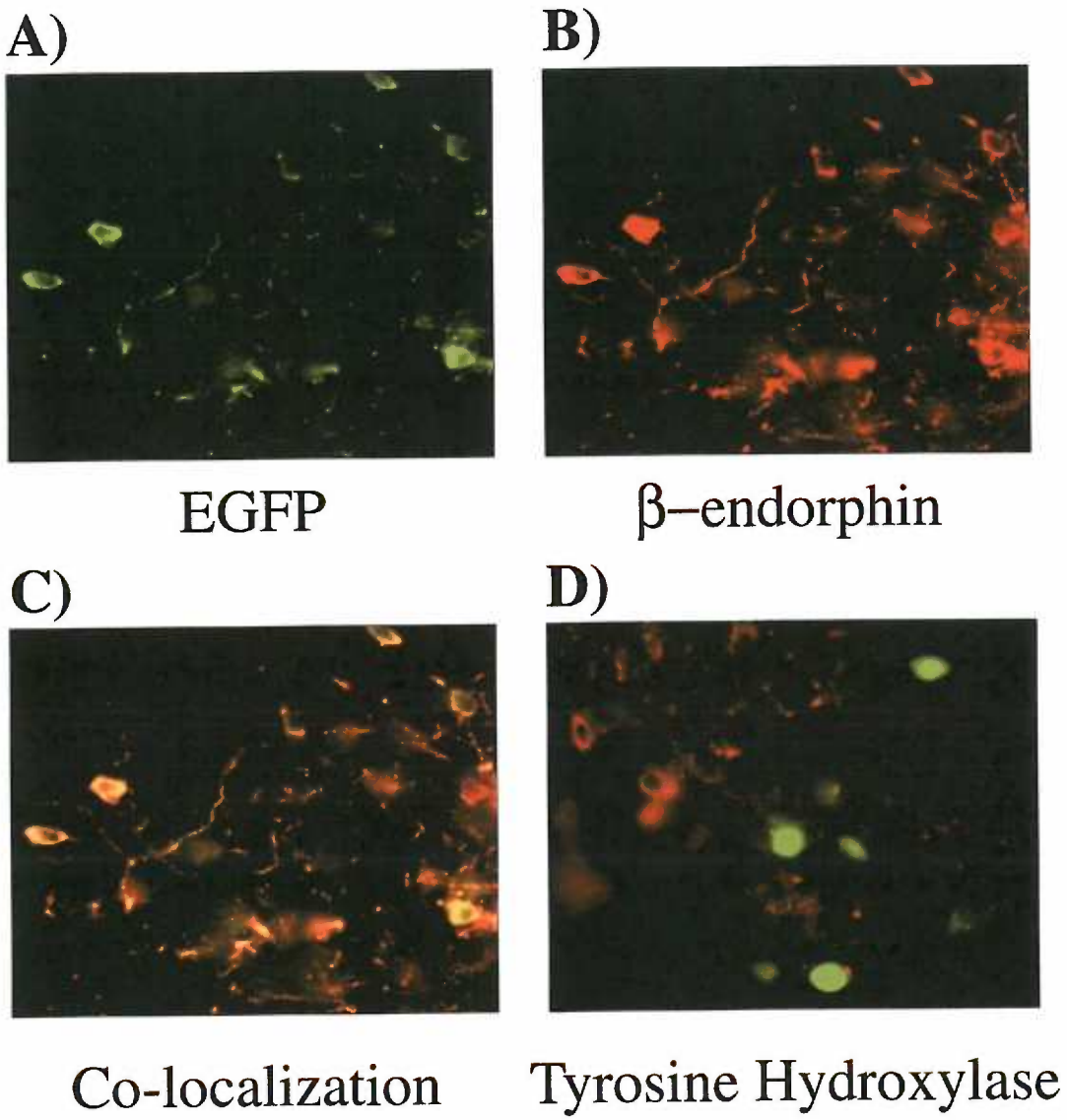
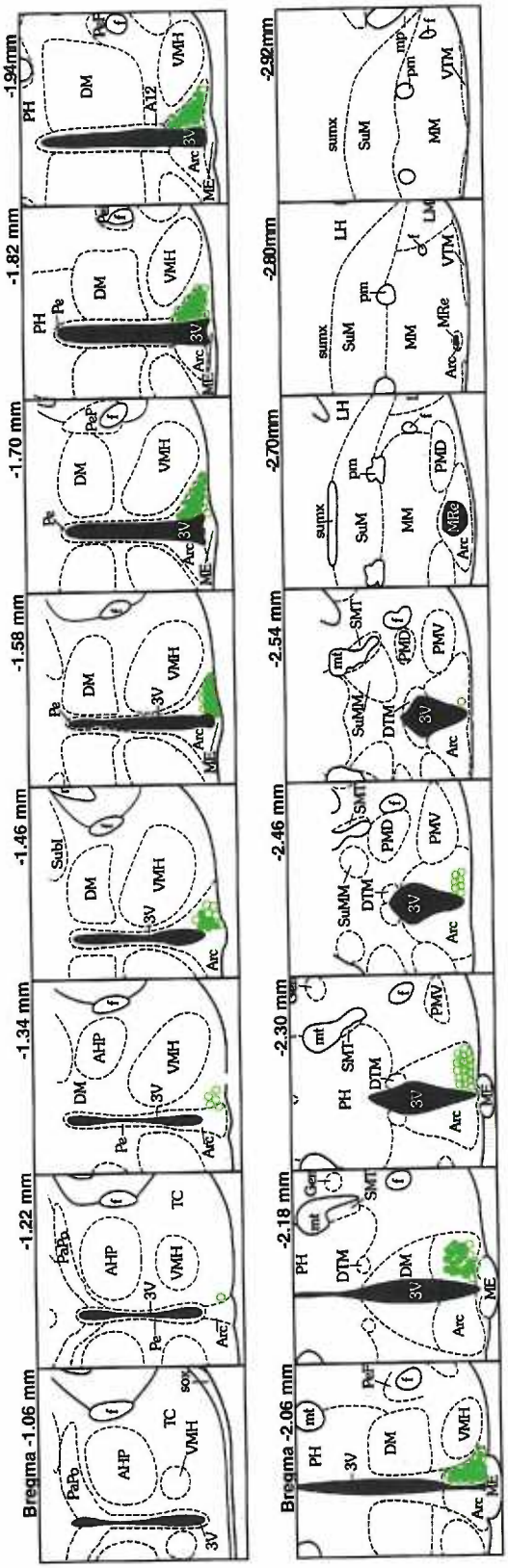

A)
B)
EGFP
β–endorphin
C)
D)
Co-localization
Tyrosine Hydroxylase

Figure 7

The bright fluorescence of neurons in the POMC/EGFP transgenic mice allowed for easy mapping and counting of hypothalamic neurons. Hypothalamic coronal sections (30μm) were mounted on gelatin-coated slides from three male hemizygous transgenic mice. Each section from all three mice was counted for fluorescent cell bodies as well as anatomical mapping of these neurons within the hypothalamus. POMC/EGFP neurons are distributed throughout the entire arcuate nucleus.

○ = 5 cells; ● = 10 cells

Figure 7

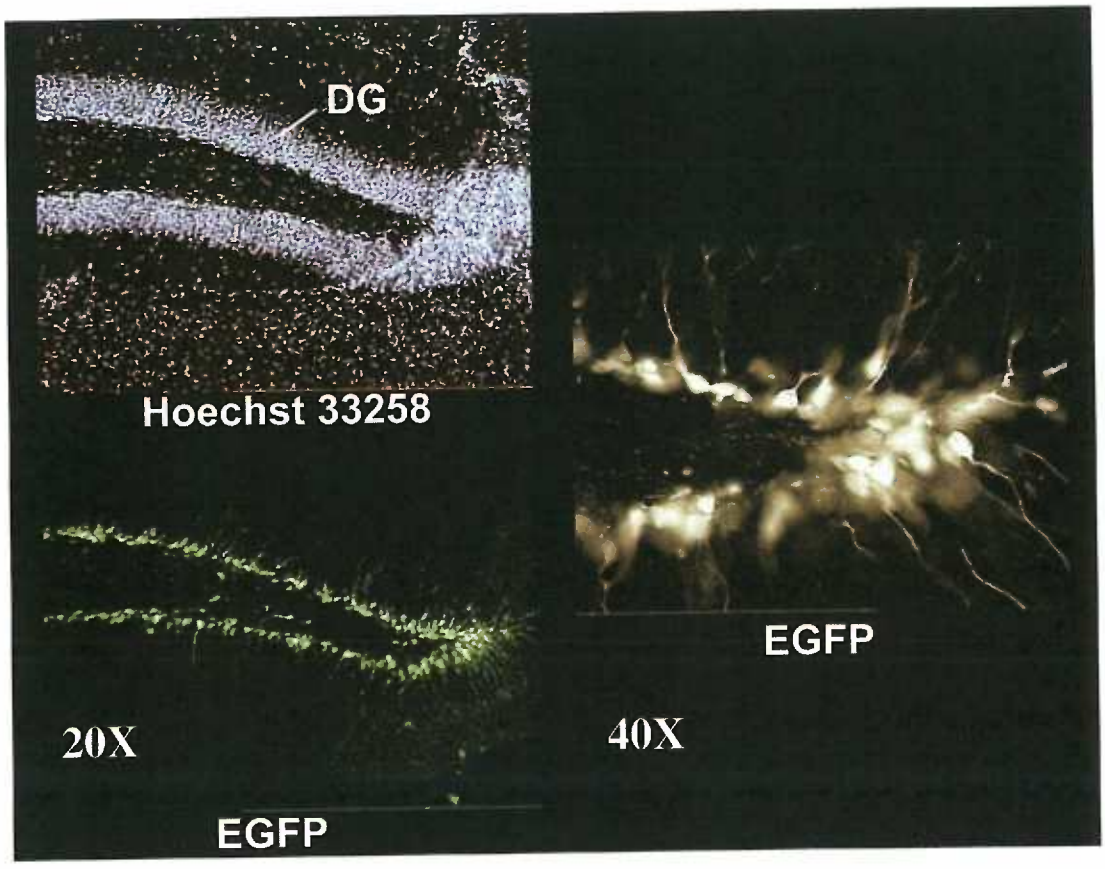

Bregma -1.06 mm
-1.22 mm
-1.34 mm
-1.46 mm
-1.58 mm
-1.70 mm
-1.82 mm
-1.94mm
Bregma -2.06 mm
-2.18 mm
-2.30 mm
-2.46 mm
-2.54 mm
-2.70mm
-2.80mm
-2.92mm
PaDo
AHP
TC
VMH
3V
Pe
Arc
Subl
DM
PH
mt
SMT
DTM
SuMM
PMD
PMV
ME
Gen
PaP
sox
sumx
SuM
LH
pm
MM
MRe
VTM
mp
A12

group of cells in the dentate gyrus sending projections that extend partially up into the molecular layer (Figure 8). The use of a polyclonal anti-EGFP antibody increased the sensitivity for EGFP detection in the axons projecting to the stratum lucidum of CA3 and short dentrites extending to the inner molecular layer (data not shown). EGFP was not observed in the more superficial granule layers of the dentate gyrus or in any other region of the hippocampus. Initially these cells were thought to be basket cells due to the number, location, and distribution of these fluorescent cells. However, subsequent data from a collaborating lab has identified these cells as a population of newly born granule cells by both morphological and electrophysiological analyses along with using bromodeoxyuridine (BrdU) labeling, a thymidine analog (data not shown). Cells that incorporate BrdU during S phase were detected using anti-BruD-specific antibodies following membrane permeablilization (Gratzner 1982). Co-localization of the BrdU and EGFP confirmed that the fluorescent cells were not post-mitotic. As these transgenic mice age, the number of EGFP fluorescent cells diminish and eventually in older mice (sometime after 12 months of age) fluorescent cells in the hippocampus are markedly diminished in number. At the same time in these older mice, hypothalamic POMC neurons still express the EGFP protein. The BrdU labeling also showed that these newly born granule cells migrate out of the dentate gyrus presumably as or after they differentiate. After the cells migrate out of the dentate gyrus they no longer express the EGFP protein suggesting that in the flourescent stem cells permissive factors/conditions exist that allow for transgene expression. Subsequent transgenes carrying various segments of 5' DNA flanking regions have identified a small region of DNA that is

Figure 8

Ectopic expression was detected in a discrete population of cells in the hippocampus. Fluorescent cell bodies can be seen in the inner granule cell layer of the dentate gyrus using a FITC filter to detect EGFP green fluorescence. Total cell nuclei are visualized by the DNA-binding fluorescent dye Hoechst 33258. A higher power detail of the tip of the dentate gyrus is also shown.

Figure 8



Hippocampus

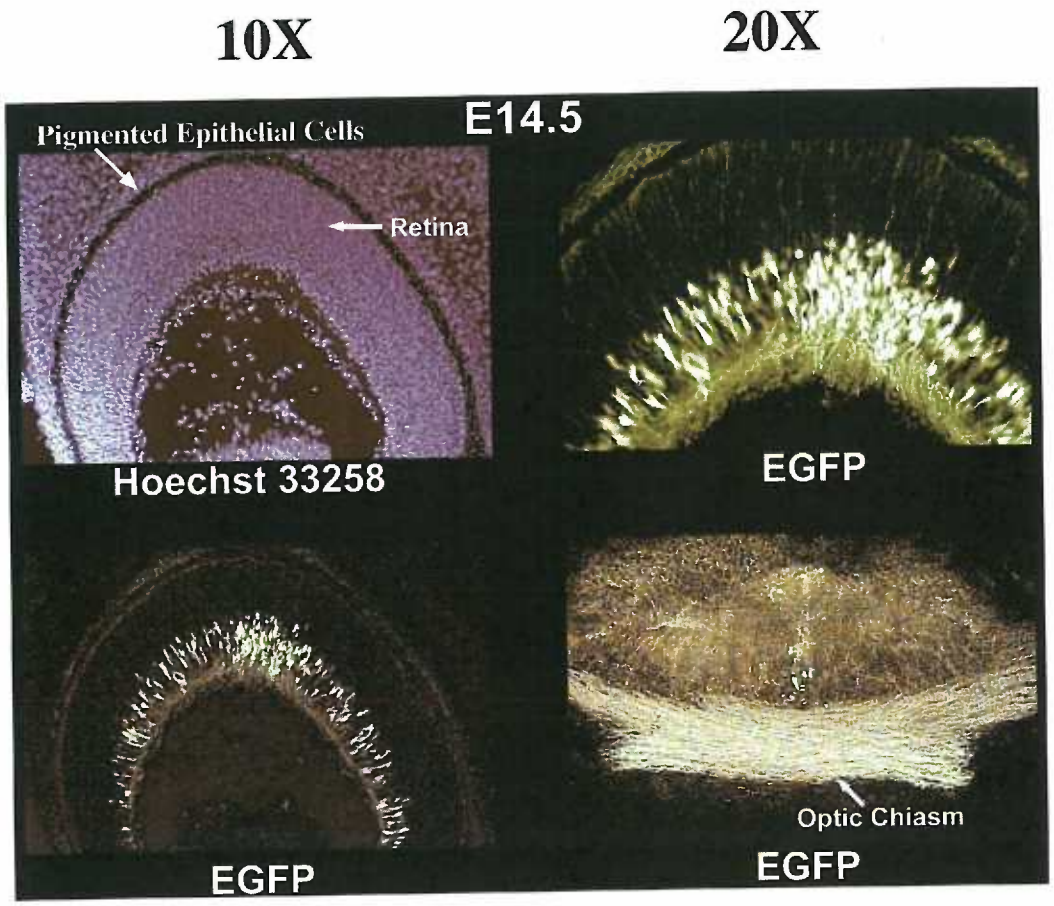
required for the reporter genes to express in the hippocampal granule cells (see section 4.4). No evidence of endogenous POMC mRNA or POMC peptides expressed in this brain region has been verified. In light of these findings, a closer developmental analysis of POMC expression in the CNS may be warranted. Currently, our hypothesis is that DNA silencing elements have been excluded in the construction of the transgenes due to their distance from the promoter sequences used in our reporter gene constructs, thereby allowing hippocampal expression. Recent data, in which transgenic mice have been generated using bacterial artificial chromosome (BAC) vectors containing reporter cassettes, has identified a BAC containing the *Pomc* gene that expresses in the hippocampus (Gong, Zheng et al. 2003). In contrast to the arcuate nucleus, we were unable to detect POMC immunoreactivity in the dentate gyrus using the polyclonal antibodies (as previously described) raised against different POMC-derived peptides, even in colchicines-treated mice (data not shown). *In situ* hybridization performed with a mouse exon 3 riboprobe (described in chaper V) failed to detect POMC mRNA in the dentate gyrus from early postnatal, 3 week-old, and adult transgenic and non-transgenic mice.

Ectopic expression of EGFP was also visualized in the retina of a mouse embryo at age e14.5 (Figure 9). Bright fluorescence could be detected extending from the retina through the optic nerve to the optic chiasm. Retinal ganglion cells are the only cells whose processes make up the optic nerve and so it is these cells that are most likely expressing EGFP. Attempts to visualize EGFP fluorescence in adult mouse retinas has

Figure 9

Ectopic expression could also be seen in retinal ganglion cells in e14.5 mouse embryos. Bright green fluorescence was detected in the optic chiasm that contains only retinal ganglion processes.

Figure 9


10X
20X
E14.5
Pigmented Epithelial Cells
Retina
Hoechst 33258
EGFP
EGFP
Optic Chiasm
EGFP

been unsuccessful. Further studies would be needed to determine if this expression is transient and/or if the expression in these cells is similar to those in the hippocampus.

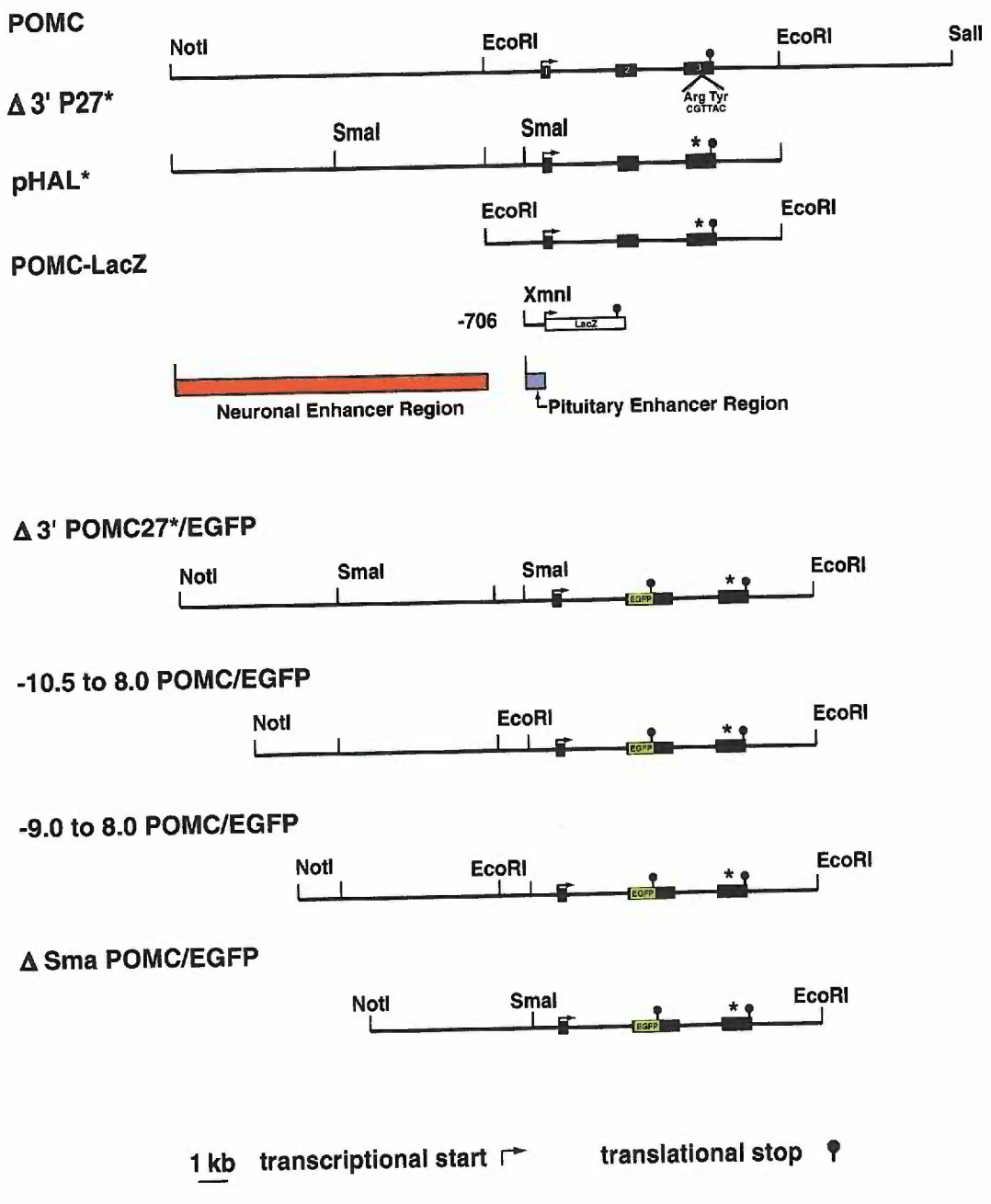
4.4 *Mutational analysis of the Pomc 5' flanking region*

Following the generation of the first POMC/EGFP transgenic mouse line (Δ3'POMC27*/EGFP) described earlier in this chapter, three additional transgenic lines have subsequently been generated (Figure 10). Transgenic mice produced with –10.5 to 8.0 POMC/EGFP showed identical neuronal expression to the Δ3' POMC27*/EGFP transgenic mice, while –9.0 to 8.0 POMC/EGFP did not express in the CNS but pituitary expression was present in these mice. These data have mapped essential neuronal *cis* regulatory elements to a 1.5 kb region between –10.5 to –9.0 of the 5' flanking sequence of the *Pomc* gene. All the above described transgenes, including the pHal* transgene that has shown to express POMC peptides only in the pituitary (Rubinstein, Mortrud et al. 1993), show either EGFP or mRNA expression (pHal*) in the hippocampus. Conversely, the ΔSma POMC/EGFP transgenic mice, containing the same sequences as the Δ3'POMC27*/EGFP mice minus 6 kb of sequence between the SmaI sites, does not express in the hippocampus but does express in the hypothalamus, brain stem and pituitary. These data map a hippocampal regulatory element to –2 to –0.8 kb of sequence between the first EcoRI site and the second SmaI site just a couple kb 5' to the POMC promoter region (Figure 10).

Figure 10

Wild type allele is shown at the top followed by transgene constructs with variable lengths of DNA flanking regions of the *Pomc* gene. Δ3' P27* was first shown to express in the CNS followed by Δ3' POMC27*/EGFP which is identical except for the EGFP cassette in exon 2. pHal* and –9.0 to 8.0 constructs only express in the pituitary while –10.5 to 8.0 POMC/EGFP construct expresses in the pituitary and the CNS. Identifying a region –10.5 to –9.0 as containing neuronal *cis* elements. POMC-LacZ reporter gene confirmed pituitary expressing *cis* elements to be contained in –706 bp of 5' flanking sequence. ΔSma POMC/EGFP was the only transgene that expressed in the both CNS and pituitary POMC cells but did not express EGFP in the hippocampal dentate, thus localizing sequence directing dentate expression to a region that is –2 to –0.8 from the POMC promoter region.

Figure 10


POMC
NotI
EcoRI
EcoRI
SalI
Arg Tyr
CGTTAC
Δ3' P27*
SmaI
SmaI
pHAL*
EcoRI
EcoRI
POMC-LacZ
XmnI
-706
LacZ
Neuronal Enhancer Region
Pituitary Enhancer Region
Δ3' POMC27*/EGFP
NotI
SmaI
SmaI
EcoRI
EGFP
-10.5 to 8.0 POMC/EGFP
NotI
EcoRI
EcoRI
-9.0 to 8.0 POMC/EGFP
NotI
EcoRI
EcoRI
Δ Sma POMC/EGFP
NotI
SmaI
EcoRI
1 kb
transcriptional start
translational stop

5. DISCUSSION

A caveat of pronuclear DNA injections into mouse oocytes, with endogenous or novel genes, is that the location and copy number of the transgene being inserted is unpredictable and can sometimes result in adverse side effects such as disrupting an existing gene (Brinster and Palmiter 1984; Palmiter and Brinster 1985). However, no obvious behavioral phenotypes have been noticed in the POMC/EGFP mouse. The Rubinstein lab used quantitative rt-PCR to measure *Pomc* and *EGFP* mRNA after leptin administration. Unpublished results have shown that both *Pomc* and *EGFP* mRNA levels increase in parallel after leptin injections. These data suggest the POMC/EGFP transgene is regulated in a similar fashion as the *Pomc* endogenous gene. Such analyses will be useful for studying the regulation of the POMC gene in the presence or absence of promoter and/or putative neuronal transcriptional/regulatory elements. Consequently, fluorescent protein tagged cells can be mechanically sorted to create an enriched POMC cell population that can be easily identified for such *in vitro* studies and further *in vitro* manipulations.

The ease of identifying fluorescent cells in fixed tissue provided an unambiguous method for promoter analyses in transgenic founder lines. Using transgenes containing specific deletions of 5' DNA flanking sequences, we have been able to narrow the region containing the essential *cis* regulating elements for neuronal POMC expression to 1.5 kb of 5' flanking sequence. In addition, ectopic hippocampal expression from a variety reporter transgenes has identified cryptic sequences localized between –2 and –0.8 kb of

the 5' flanking region upstream of the POMC promoter. Our failure to detect POMC immunoreactivity or POMC mRNA in the mouse hippocampus was consistent with all known POMC gene brain expression studies and therefore indicates that the hippocampal EGFP expression was ectopic. Furthermore, the fact that independent pedigrees carrying EGFP, as well as other reporter genes (Rubinstein, Mortrud et al. 1993), exhibited hippocampal expression revealed that expression was not due to chromosomal influences. Interestingly, this necessary region for dentate expression of reporter genes was dispensable for appropriate expression of transgenes in POMC cells of the hypothalamus, the brain stem, and the pituitary.

In vivo identification of this sparsely populated group of POMC neurons led to physiological experiments that previously were technically very difficult. With the help of our collaborators in the Cone lab, electrophysiological observations allowed us to propose a model of the dual mechanisms of leptin action in the ArcN and the interactions between NPY/GABA and POMC neurons (Cowley, Smart et al. 2001). In this model leptin directly depolarizes the POMC neurons, and simultaneously hyperpolarizes the NPY/GABA neurons, probably at their presynaptic terminals. This hyperpolarization diminishes GABA release and thereby disinhibits the POMC neurons. Together the direct and indirect effects of leptin result in an activation of POMC neurons and an increased frequency of action potentials. Additionally, both POMC and NPY neurons express autoreceptors for some of their respective neuropeptide products and activation of these autoreceptors may provide an ultra short feedback loops that further modulate the effects of leptin on POMC neurons. Thus it appears that there are two classes of

neurons accounting for leptin sensitivity in the brain, those that are activated (depolarized) to release anorexigenic peptides, and those that are inhibited (hyperpolarized) by signals that indicate energy repletion. Identifying POMC neurons with EGFP provides an invaluable new tool for determining complex actions on these important neurons. Furthermore, an understanding of the acute effects of leptin provides new targets to test for defects in central leptin action, an important component of leptin resistance.

CHAPTER III

LACK OF PROOPIOMELANOCORTIN PEPTIDES RESULTS IN OBESITY AND DEFECTIVE ADRENAL FUNCTION BUT NORMAL MELANOCYTE PIGMENTATION IN THE MURINE C57BL/6 GENETIC BACKGROUND

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1. ABSTRACT

Mice deficient in proopiomelanocortin peptides (*Pomc*$^{-/-}$) generated on a 129 (A^W/A^W) genetic background were backcrossed onto the C57BL/6 (*a/a*) genetic background. These mice exhibit most of the phenotypic characteristics previously reported on the 129 genetic background (Yaswen, Diehl et al. 1999). Adult mice became obese, their adrenals were atrophied, and they had undetectable plasma corticosterone in basal and stressed states. The partial perinatal lethality previously reported was also present on the C57BL/6 background. In addition, we found that both male and female homozygote (-/-) adults are fertile, but when homozygous males were intercrossed with homozygous females, all the pups died in the perinatal period. Attempts to rescue the perinatal lethality of pups from homozygous breeder pairs by supplementing the mother's drinking water with glucocorticoids were unsuccessful. Furthermore, failure to stimulate adrenal development and corticosterone production/release with daily exogenous adrenocorticotropin-stimulating hormone (ACTH) injections indicates an adrenal dependence on POMC peptides for normal development and function during adrenal maturation. While the original *Pomc*$^{-/-}$ mice, bred on a mixed white-bellied agouti (A^W/A^W) 129 genetic background, had patchy alternations in their coat color, they clearly were not a uniform yellow like the *lethal yellow* (A^y/a) mice. Our *Pomc*$^{-/-}$ mice bred onto the C57BL/6 (*a/a*) genetic background had a black coat color indistinguishable from that of the wild-type C57BL/6 mice, further suggesting that the POMC peptide melanocyte-stimulating hormone (α-MSH) is not essential for the production of eumelanin (black/brown) pigmentation.

2. INTRODUCTION

Over the last ten years, the role of POMC peptides as central regulators of energy homeostasis has become very apparent (Elmquist, Elias et al. 1999). Early evidence centered on the detection of MSH binding to hypothalamic nuclei involved in regulating energy homeostasis (Tatro 1990). Since then, a variety of mutant mouse models have shown central αMSH to be an anorexigenic peptide, whereas the POMC opioid, β-endorphin may play a more complex role in a reward pathway that reinforces feeding behavior [Huszar, 1997 #56;Butler, 2000 #62;Chen, 2000 #1;Appleyard, 2003 #48;Hayward, 2002 #49]. In addition to the energy homeostatic role of central POMC peptides, a large body of evidence exists implicating central POMC involvement in stress response, reproduction, and ontogeny of the hypothalamus, pituitary and adrenal glands (Allen, Pintar et al. 1984; Zagon and McLaughlin 1987; Estivariz, Carino et al. 1988; Estivariz, Morano et al. 1988; Berry and Haynes 1989; Marsh, Hollopeter et al. 1999; Bicknell, Lomthaisong et al. 2001). However, further experiments are needed to conclusively establish these hypothesized roles of central POMC peptides from other peptidergic systems. Additionally, *in vivo* experiments using a POMC transgene are described in later chapters that help delineate the roles of central POMC peptides from those of peripheral POMC peptides released from the pituitary gland corticotrophs and melanotrophs. This chapter identifies specific physiological alterations in the *Pomc*$^{-/-}$ mutation on the C57BL/6 murine genetic background.

3. ORIGINAL POMC NULL MUTATION

A mouse strain, deficient in all POMC peptides, was generated by the Hochgeschwender and Brenan laboratories using homologous recombination of a replacement-type *Pomc* allele containing a deletion of exon 3 and insertion of *neo* (see Figure 2; chapter 1). This mutant strain was generated on a mixed white-bellied agouti 129 genetic-background (A^W/A^W $pTyr^C/pTyr^{C\text{-}Ch}$) and develops hyperphagia and obesity similar to that seen in the *lethal yellow* (A^y/a) mice and MC4-R deficient mice (Yaswen, Diehl et al. 1999). However, in contrast to these and other previously described obesity models, $Pomc^{-/-}$ mice have no detectable levels of circulating corticosterone or epinephrine. Analysis of adult suprarenal fat pads revealed what appeared to be an adrenal remnant but no clearly identifiable adrenal glands. A possible result of the adrenal absence was the partial perinatal lethality seen in offspring of heterozygous breeder pairs. The loss of circulating α-MSH, the MC1-R agonist, was predicted to eliminate the production of eumelanin (black/brown) pigmentation in melanocytes of the $Pomc^{-/-}$ mice; however, they had only modest increases in phaeomelanin (yellow) pigmentation, most noticeable on their bellies.

4. INCREASED ADIPOSITY AND LINEAR GROWTH

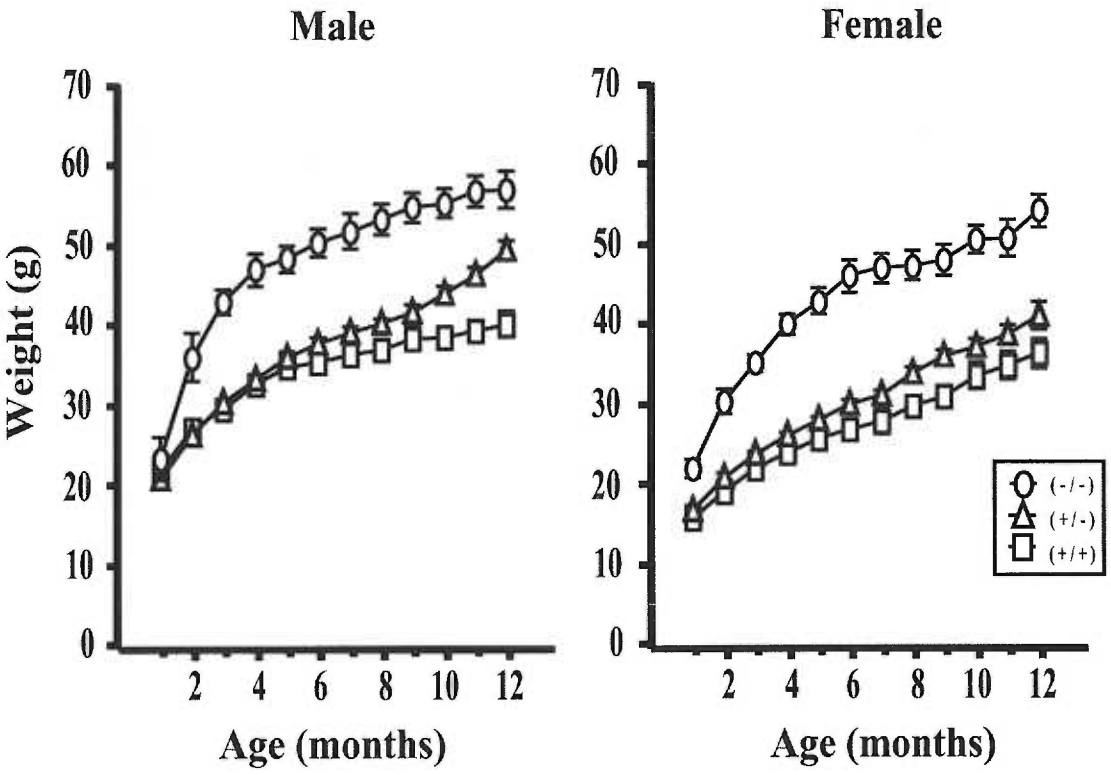
To better understand the contribution of POMC peptides to hair follicle pigmentation, we backcrossed the *Pomc*$^{-/-}$ mutant allele from the agouti (A^W/A^W) 129 genetic background on to the C57BL/6 (*a/a*) genetic background for two successive generations. Additionally, the C57BL/6 genetic background allows us to study proposed POMC physiological functions on a more commonly used inbred mouse line and one that is predisposed to diet-induced obesity, thus eliminating confounds due to genetic heterogeneity when comparing data to the melanocortin receptor knockouts and other spontaneous mouse obesity models.

Analysis of growth curves of *Pomc*$^{-/-}$ mice (Figure 1) reveals an increase in body mass very similar to that seen in the *lethal yellow* (A^y/a) and MC4-R deficient mice. Further analysis showed that the increased size of the *Pomc*$^{-/-}$ mice was due to both an increase linear growth and increased mass of white fat depots, again similar to those of the *lethal yellow* and MC4-R deficient mice (Boston, Blaydon et al. 1997; Huszar, Lynch et al. 1997). There were no obvious differences in the growth curves of *Pomc*$^{-/-}$ mice on the 129 versus C57BL/6 genetic background. However, all murine mutant models besides the *Pomc*$^{-/-}$ mice have circulating glucocorticoids. In addition, when the *lethal yellow* or the leptin-deficient *ob/ob* mice are adrenalectomized, these mice loose a significant amount of their white-fat mass, suggesting that glucocorticoids play a significant role in the development of obesity in these mutant mouse models (Boston, Blaydon et al. 1997). *Pomc*$^{-/-}$ mice develop an obesity phenotype in the absence of circulating glucocorticoids.

Figure 1

Male and female mice were group housed, fed a standard diet of rodent chow (5% fat, 19% protein, and 5% fiber weight; 3.4kcal/g), and weighed monthly. *Pomc*$^{-/-}$ mice became significantly heavier than wild-type littermates at 6-8 weeks old for males and 4-6 weeks old for females. Heterozygous males developed later onset obesity at about 8-10 months, whereas heterozygous females had a smaller increase in body mass at 8-10 months. (O, *Pomc*$^{-/-}$; Δ, *Pomc*$^{+/-}$; □, *Pomc*$^{+/+}$; N= 25 to 105.)

Figure 1


Male
Female
Weight (g)
70
60
50
40
30
20
10
0
2
4
6
8
10
12
Age (months)
Age (months)
(-/-)
(+/-)
(+/+)

suggesting a role for POMC peptides regulating energy homeostasis independent of corticosterone.

5. ADRENAL ATROPHY AND HORMONE DEFICIENCIES

The adrenal gland, composed of an outer cortical shell and an inner medullary core, secretes the steroid hormones corticosterone, aldosterone, and androgens together with the catecholamines: norapinephrin and epinephrine. Although the *Pomc*$^{-/-}$ mutant mice on the 129 genetic background fail to develop any distinguishable adrenal glands, the C57BL/6 mutant mice have easily identifiable, but severely hypoplastic adrenals. *Pomc*$^{-/-}$ mice adrenal glands were approximately 15% (in females) to 30% (in males) the weight of their respective wild-type littermates (Figure 2). The greater reduction in the size of female adrenal glands compared with male adrenal glands eliminates the sexual dimorphism found in wild-type mice. Morphological analysis of paraffin embedded adrenal sections revealed an attenuated, dystrophic cortical layer possibly due to the absence of ACTH stimulated glucocorticoid production (Figure 3). Adrenal cells that synthesize and release glucocorticoids are localized in the zona fasciculata of the cortex. The zona fasciculata in the *Pomc*$^{-/-}$ adrenals had less eosin staining and the cellular columns were more densely packed and less defined, again presumably a result of no glucocorticoid synthesis and cytoplasmic storage. The *Pomc*$^{-/-}$ mouse was originally reported to lack detectable circulating epinephrine; yet, immunohistochemistry revealed an attenuated medulla that was immuno-positive for tyrosine hydroxylase (TH).

Figure 2

Adrenals were removed, trimmed of fat, and weighed as a pair from each mouse. Male *Pomc*$^{-/-}$ mice adrenals were 70% smaller than those from their wild type and heterozygous littermates. Female *Pomc*$^{-/-}$ mice adrenals were 85% smaller than those from their wild type and heterozygous littermates. In addition, female *Pomc*$^{+/-}$ heterozygous mice adrenals were slightly, but significantly, smaller than those from wild type littermates. (***P <0.0001 and ** P <0.01 compared to wild-type; N= 5 to 15.)

Figure 2

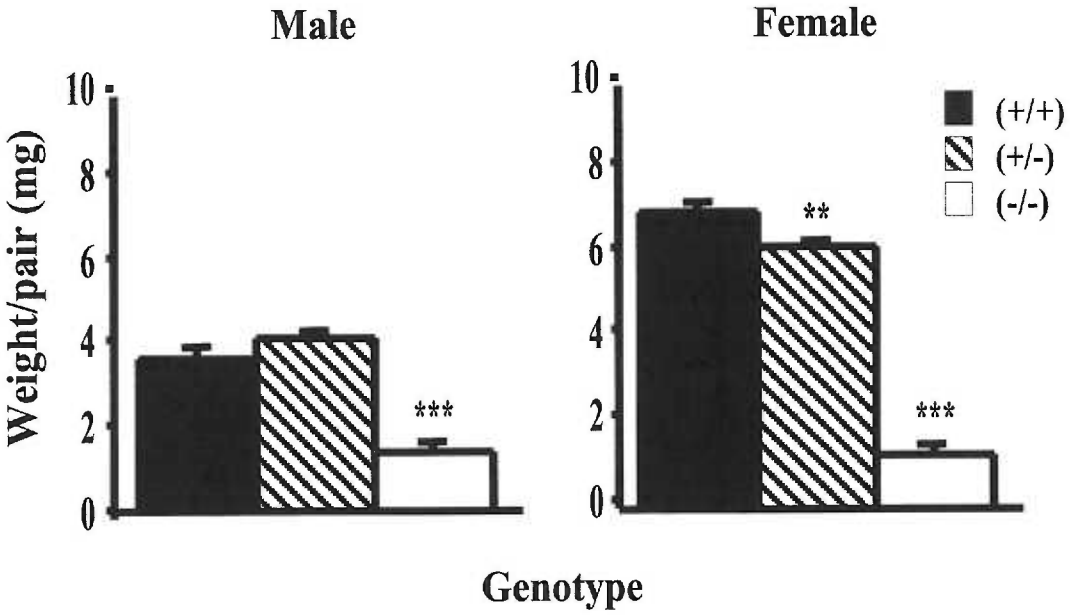
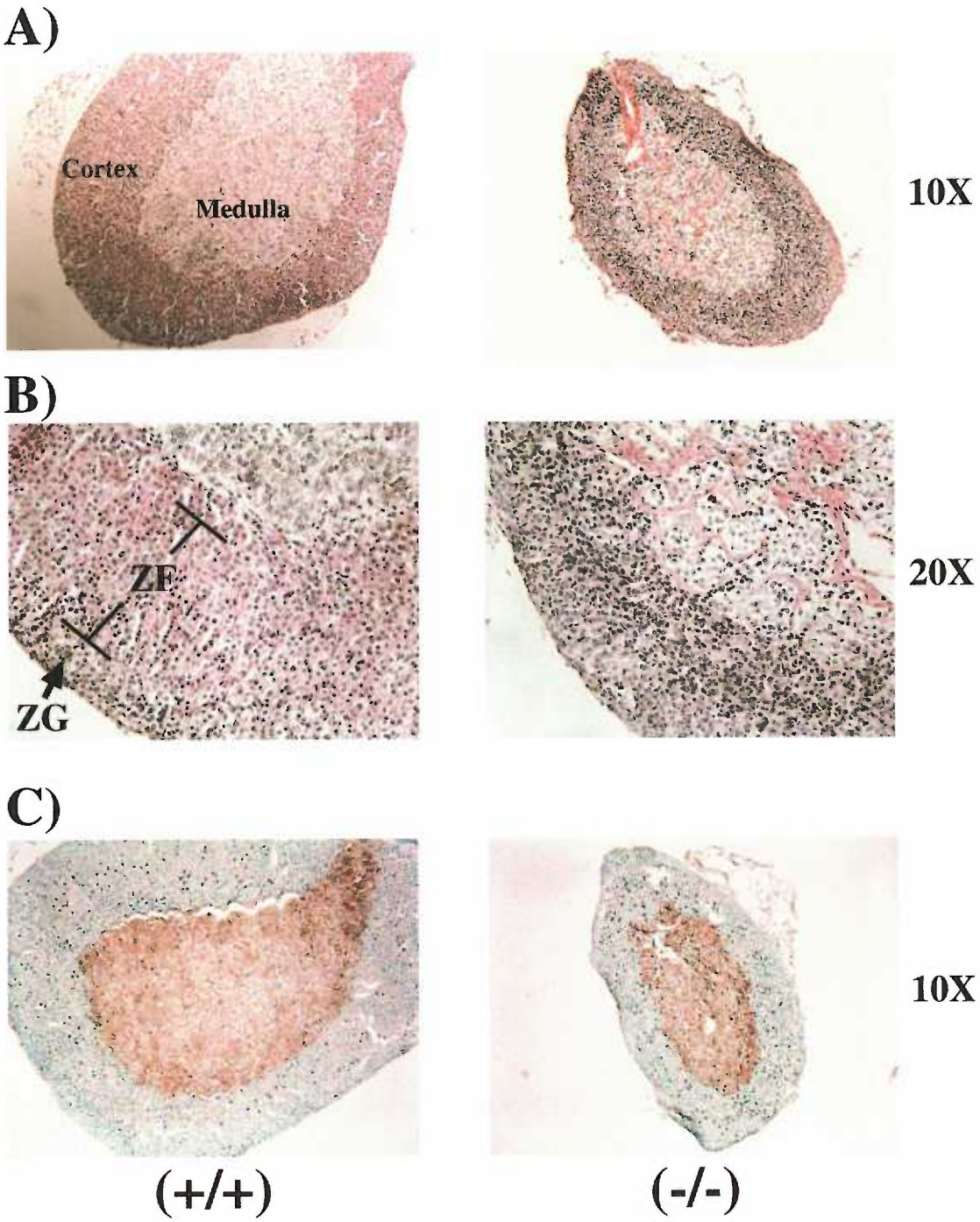


Figure 3

Paraffin embedded male adrenal glands from *Pomc*$^{+/+}$ and *Pomc*$^{-/-}$ were sectioned and stained with hematoxylin and eosin for morphological analyses along with immunohistochemistry for tyrosine hydroxylase (brown reaction) in the medulla. **(A)** At a magnification of 10X the smaller sized null adrenals still contain a distinguishable adrenal cortex and medulla. **(B)** Higher magnification of the cortex shows an attenuated and dystrophic cortical layer. Eosin, a dye staining cytoplasmic material, seems to be less abundant in the null cortex. In addition, the defined cell columns seen in the zona fasciculata (ZF) of wild type adrenals are less defined and the cells are more densely packed. **(C)** Immunohistochemistry for tyrosine hydroxylase (brown reaction product) can be seen in the medulla of both wild type and null adrenals. The cortex was counter-stained with methelyne blue.

Figure 3



The presence of tyrosine hydroxylase suggests a disruption in catecholamine production downstream of TH. Despite the presence of these hypoplastic adrenals on the C57BL/6 genetic-background, plasma collected from unstressed, and restraint-stressed, or LPS-challenged *Pomc*$^{-/-}$ mice still have no detectable levels of corticosterone (data not shown). Furthermore, treatment of *Pomc*$^{-/-}$ mice with exogenous ACTH (1-24) twice daily for 2 weeks failed to elicit any corticosterone secretory response, whereas wild-type responded promptly to the same dose of exogenous ACTH 3 hours after dexamethasone suppression of their HPA axis (Figure 4). The adrenal deficiencies in these mice strengthen the hypothesis that normal POMC expression is needed for complete adrenal maturation; however, the role of NH_2-terminal peptides in addition to ACTH remains an open question (Bicknell, Lomthaisong et al. 2001).

We hypothesize that the lack of adrenal hormones, and not POMC peptides *per se*, is responsible for the non-Mendelian production of viable *Pomc*$^{-/-}$ offspring (Figure 5). Both heterozygous and homozygous male and female mice were shown to be fertile. Further evaluation of heterozygous breeder pairs revealed that transmission of the *Pomc*$^{-/-}$ allele was normal but that 50-70% of the homozygous *Pomc*$^{-/-}$ mice die in the perinatal period, typically within the first hour after birth. Homozygous breeder pairs produced full-term litters with no pup surviving the perinatal period. Mice lacking corticotrophin-releasing hormone (CRH) are also unable to make corticosterone and exhibit a similar perinatal lethality (Muglia, Jacobson et al. 1995). The CRH$^{-/-}$ lethality can be rescued by administration of glucocorticoids to the mother's drinking water. However, an identical attempt to rescue the lethality in the homozygous *Pomc*$^{-/-}$ breeder pairs proved

Figure 4

EDTA-plasma corticosterone levels were measured using a ^{125}I-labeled corticosterone radioimmunoassay kit from ICN. Dexamethasone (10 μg/mouse) was given i.p. immediately following blood draw at baseline time point A. At all other time points, mice received either saline or 1 μg ACTH (1-24) i.p., 30 min prior to blood collection from a tail vein. All null male mouse EDTA-plasma samples were 2X aliquot volume in order to lower the RIA detection level from 25 ng/ml to 12 ng/ml for these samples, yet at all time points the null plasma samples were still below the assay sensitivity (12 ng/ml). (**A**) Dexamethasone injection (time 0). (**B**) 2.5 h after dexamethasone (saline or ACTH injection). (**C**) 3.0 h after dexamethasone and 30 min after saline or ACTH. (**D**) 7 days after twice daily ACTH injections (1 μg/injection). (**E**) 14 days after twice daily ACTH injections (1 μg/injection). N = 2 to 6; ***P <0.001 compared to baseline values at point A, Fisher's PLSD.

Figure 4

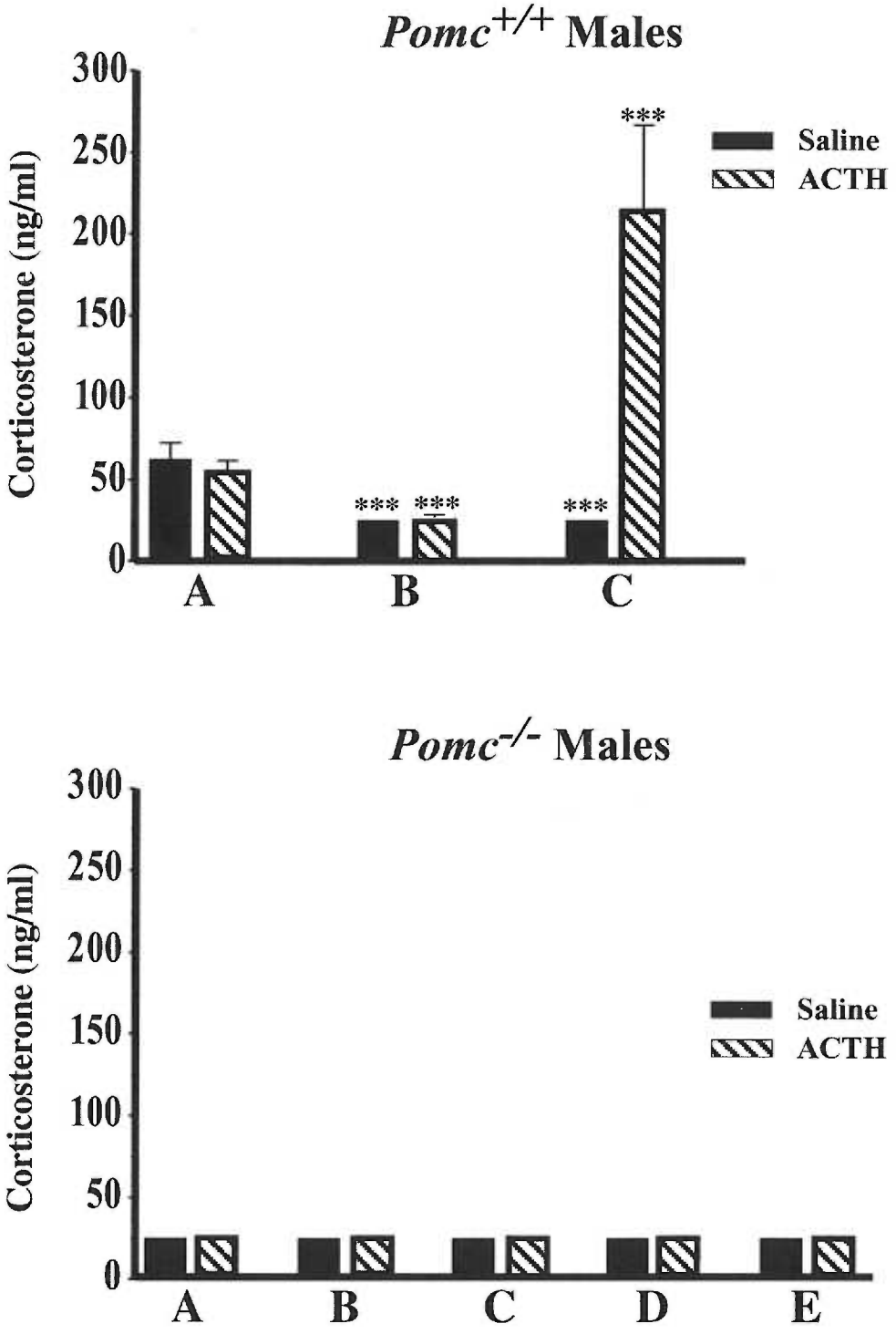

Pomc+/+ Males
Corticosterone (ng/ml)
300
250
200
150
100
50
0
***
***
***
***
Saline
ACTH
A
B
C
Pomc-/- Males
Corticosterone (ng/ml)
300
250
200
150
100
50
0
Saline
ACTH
A
B
C
D
E

Figure 5

Offspring numbers and percentages of total offspring for each genotype are shown for two types of breeder pairs: heterozygous *Pomc*$^{+/-}$ male X heterozygous *Pomc*$^{+/-}$ female, and homozygous *Pomc*$^{-/-}$ male X heterozygous *Pomc*$^{+/-}$ female. The latter breeding pair was used to help generate sufficient numbers of *Pomc*$^{-/-}$ mice for our experiments.

Figure 5

Breeder Pair:	(+/-) X (+/-)		
Offspring:	(+/+)	(+/-)	(-/-)
Number:	83	158	31
Percentage:	30.5%	58.1%	11.4%

Breeder Pair:	(-/-) X (+/-)		
Offspring:	(+/+)	(+/-)	(-/-)
Number:	0	93	27
Percentage:	0%	77.5%	22.5%

unsuccessful suggesting the *Pomc*$^{-/-}$ perinatal lethality may be a result of the combined lack of glucocorticoids and epinephrine in the *Pomc*$^{-/-}$ pups. *Pomc*$^{-/-}$ pups expressing a transgene that rescues pituitary POMC peptides and therefore adrenal corticosterone and presumably epinephrine production, but that are still void of central POMC peptides do not exhibit the perinatal lethality of their *Pomc*$^{-/-}$ siblings. These mice are described in further detail in chapters 4 and 5 of this thesis.

6. PIGMENTATION IS UNALTERED IN THE C57BL/6 GENETIC BACKGROUND

Information gained from the *recessive yellow* (*Mc1r*^{e} */Mc1r*^{e}) and the autosomal-dominant *lethal yellow* (*A*^{y}*/a*) mice would predict an inability to make eumelanin pigmentation in the *Pomc*$^{-/-}$ mice (Ollmann, Lamoreux et al. 1998). This prediction for the *Pomc*$^{-/-}$ mice is based on the absence of stimulation of the MC1-R by its natural POMC peptide agonist, α-MSH. In the *lethal yellow* mice, the constitutively activated expression of agouti peptide antagonizes the MC1-R, preventing stimulation by αMSH and inhibiting eumelanin synthesis. The original *Pomc*$^{-/-}$ mice on an agouti (*A*^{W}*/A*^{W}) 129 genetic background resulted in only subtle alteration of yellow hair pigmentation on the belly, much different from the coat color seen in the *lethal yellow* (*A*^{y}*/a*) mice. The presence of agouti in the 129 genetic background produces a banded hair shaft with both eumelanin and phaeomelanin pigmentation, complicating the analysis of coat color on this genetic background. To more closely evaluate the potential alterations in coat color

in absence of peripheral αMSH, the *Pomc*$^{-/-}$ allele was evaluated on an agouti-less genetic background, C57/BL/6 (*a/a*), to eliminate all phaeomelanin caused by antagonism of αMSH binding. Our studies show no distinguishable difference in *Pomc*$^{-/-}$ black coat color from wild-type (*Pomc*$^{+/+}$) or heterozygous (*Pomc*$^{+/-}$) littermates on the C57BL/6 background, including the presence of a few yellow hairs behind the ears, in the perianal area and around the female nipples seen on wild type C57BL/6 mice. Possible explanations for the black coat color in the absence of αMSH include endogenous basal activity of MC1-R, compensatory pathways that stimulate cAMP production and tyrosinase activity, thereby bypassing the MC1-R altogether, and alternative endogenous agonist ligands for the receptor.

7. POMC NEURONS, CORTICOTROPHS, AND MELANOTROPHS ARE STILL PRESENT IN *Pomc*$^{-/-}$ MICE

The inability to immunohistochemically detect POMC cells in *Pomc*$^{-/-}$ mice makes it difficult to know if the cells, void of the POMC peptides, are still present. To identify and study POMC neurons *in* vivo, we previously generated a transgenic C57BL/6 mouse line expressing enhanced green fluorescent protein (EGFP, Clontech) under the transcriptional control of POMC genomic regulatory elements (Cowley, Smart et al. 2001). The transgene was designed to only express EGFP under the *Pomc* promoter, but not to express any of the POMC peptides. We backcrossed this POMC-EGFP transgene onto the *Pomc*$^{-/-}$ mice to determine if the POMC neurons and POMC pituitary cells

developed in their normal numbers and locations. Our analysis revealed that POMC neurons along with pituitary corticotrophs and melanotrophs remain intact in the *Pomc*$^{-/-}$ mice (Figure 6). POMC neurons have been found to co-express another peptide called cocaine-amphetamine-response-transcript (CART), however, no other peptide has beenshown to co-expressed in corticotrophs and melanotrophs with POMC peptides (Elias, Lee et al. 1998). Whether CART neuron physiology has been altered and/or whether pituitary corticotrophs and melanotrophs void of POMC peptides have any physiological function in the *Pomc*$^{-/-}$ mouse has yet to be determined.

8. DISCUSSION

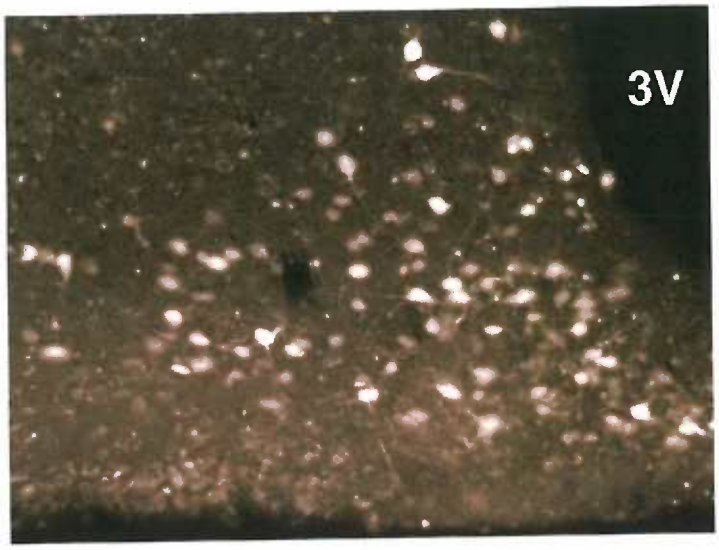
The heterogeneity found among the many inbred mouse lines can result in an epistatic modification of phenotypes when the same genetic mutations are analyzed (Butler and Cone 2002; Kozak and Rossmeisl 2002). Therefore, the back-crossing and analysis of the *Pomc*$^{-/-}$ allele are essential for understanding the function of the POMC peptides on the C57BL/6 genetic background, an inbred strain that has been used for functional analysis of melanocortin-receptor-deficient mice and other spontaneous obesity models. In addition, C57BL/6 mice are sensitive to diet-induced obesity and have been the subject of extensive behavioral and energy homeostatic studies. Although much of the data originally obtained from the mutant POMC allele on the 129 genetic background has been recapitulated in the C57BL/6 genetic background, we have found certain discrepancies in the phenotypes. Most notably, we find that the coat color of *Pomc*$^{-/-}$ on

Figure 6

(**A**) Coronal vibratome sections (50-μm thick) from a compound *Pomc*$^{-/-}$, *Pomc*-EGFP$^{Tg/+}$ mouse were viewed under UV light at 10X magnification on a Zeiss microscope with a FITC filter cube. Neurons expressing EGFP are brightly fluorescent and distributed in the arcuate nucleus of the basal hypothalamus in the same location as POMC neurons. (**B**) A pituitary section (12μm) exhibiting robust fluorescence in the intermediate lobe (IL) and the anterior lobe (AL) under UV light at 3X magnification. Fluorescent cells seen in both intermediate anterior lobes closely resemble those seen in melanotroph and corticotroph distributions.

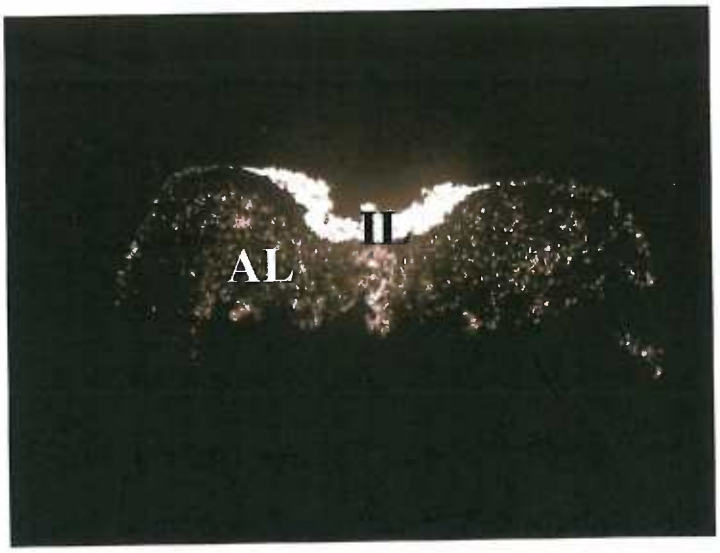
Figure 6

A)



Arcuate Nucleus

B)



Pituitary

the C57BL/6 background is indistinguishable from that of wild type littermates. In addition, we find that the adrenals are present in adult *Pomc*$^{-/-}$ mice on the C57BL/6 background, although they are very much reduced in size and still incapable of secreting corticosterone or responding to exogenous ACTH stimulation.

CHAPTER IV

PITUITARY REPLACEMENT OF PROOPIOMELANOCORTIN ON THE NULL BACKGROUND AUGMENTS OBESITY THROUGH METABOLIC SUPPRESSION AND HYPERPHAGIA

1. ABSTRACT

Null mutations of the proopiomelanocortin gene (*Pomc*$^{-/-}$) lead to obesity, but a definitive contribution of hypothalamic versus pituitary POMC peptide-deficiency in its pathogenesis has not been established. Obesity syndromes due to mutations of the melanocortin receptor-4 (MC4-R), which is only expressed in the central nervous system, support a principal role of hypothalamic POMC and modulation of neuronal circuits. However, it is possible that a loss of pituitary POMC contributes independently to altered energy balance either directly through peripheral actions of melanocortins or indirectly by the absence of ACTH, and secondarily of corticosterone, itself a potent regulator of metabolism and energy partitioning. Here we demonstrate that obese *Pomc*$^{-/-}$ mice backcrossed for two generations on the C57BL/6 genetic background were actually hypophagic when food intake was corrected for metabolic mass and they had decreased basal VO_2 consumption measured by indirect calorimetry. To decipher the roles of hypothalamic versus pituitary POMC, we generated *Pomc*$^{-/-}$ mice expressing a transgene (*Tg*) with a tissue-specific promoter that rescued production of all POMC peptides specifically in pituitary cells, and consequently adrenal glucocorticoid secretion, but which were still devoid of central POMC peptides (*Pomc*$^{-/-}$; *Tg*/+). The replacement of pituitary POMC markedly augmented body weight and white adipose tissue mass with a disproportionate increase of subcutaneous fat in both sexes and exacerbated glucose intolerance, particularly in females, compared to *Pomc*$^{-/-}$ mice. There were further significant reductions in basal metabolic rate and increases in absolute caloric

intake in both sexes, however food consumption corrected for metabolic mass was normalized to wild-type levels only in the male *Pomc*$^{-/-}$; *Tg/+* mice. These data confirm that hypothalamic POMC plays a critical role in energy homeostasis and pituitary POMC replacement cannot compensate for the absence of central neural POMC peptides. In fact, the exacerbation of obesity and diabetes secondary to insulin-resistance by a selective rescue of pituitary POMC expression indicates a counter-regulatory involvement relative to central POMC in the complex regulation of energy homeostasis and partitioning, most likely mediated by ACTH-dependent corticosterone secretion.

2. INTRODUCTION

Due to the growing prevalence of obesity within affluent societies during the last quarter century, understanding the underlying mechanisms of energy balance has become a health-care priority (Isomaa 2003). Recently, a gamut of studies have established that energy homeostasis is maintained by peripheral signals informing the central nervous system (CNS) of energy storage levels and metabolic status. Peripheral information processed centrally is then used to elicit the appropriate feeding and/or metabolic response. Hormones like leptin, insulin, and corticosterone relay this peripheral information to hypothalamic nuclei that in turn regulate energy consumption, utilization, and storage.

One of these nuclei, the arcuate nucleus of the hypothalamus, contains neurons expressing the gene encoding the prohormone proopiomelanocortin (POMC). POMC is post-translationally processed to the melanocortin peptides—adrenocorticotropin stimulating hormone (ACTH), α-melanocyte stimulating hormone (α-MSH), and λ-melanocyte stimulating hormone (λ-MSH)—and additionally the endogenous opioid, β-endorphin. Recently, our laboratory demonstrated that these POMC neurons in mouse hypothalamic slices could be activated by the adipocyte-generated hormone, leptin (Cowley, Smart et al. 2001). In addition, genetic disruption of the mouse or human *POMC* genes gives rise to early-onset obesity (Krude, Biebermann et al. 1998; Yaswen, Diehl et al. 1999). Furthermore, mutations of melanocortin receptor-4 (MC4-R), the most abundant melanocortin receptor expressed in the central nervous system (CNS), also

lead to both mouse and human obesity (Vaisse, Clement et al. 1998; Yeo, Farooqi et al. 1998). Such findings identify POMC neurons as a critical anorexigenic component in the hypothalamic peptidergic-pathways that regulate energy homeostasis.

While the majority of studies have focused on POMC's central roles in energy homeostasis, a complete understanding of pituitary POMC's role in energy balance has not conclusively been established. The POMC null mouse (*Pomc*$^{-/-}$) does not reveal whether pituitary POMC plays a role in energy homeostasis or whether this homeostatic role is exclusively a function of hypothalamic POMC neurons. By transgenic rescue of only pituitary POMC in the *Pomc*$^{-/-}$ background, we are able to address this question. The fact that the *Pomc*$^{-/-}$ mice develop obesity in the absence of circulating glucocorticoids, which are present and sometimes elevated in known obesity models, emphasizes the role of hypothalamic POMC in weight regulation. However, as we report in this paper, the replacement of pituitary POMC, and thus glucocorticoids, has profound effects on energy balance beyond what is seen in the *Pomc*$^{-/-}$ mice.

3. METHODS

Transgene construction. A 10.2 Kb mouse genomic clone containing all three POMC exons was used to construct the POMC rescue transgene *pHalEx2**. This genomic clone contains 2 Kb of 5' and 1.5 Kb of 3' flanking regions, the essential DNA elements needed for pituitary-specific expression, but does not contain the regulatory elements for

directing hypothalamic expression of the POMC gene (Rubinstein, Mortrud et al. 1993). A pair of complementary 23 bp oligonucleotides (5'-CCCGGGCTCGAGTTTAAAGCGCG-3' and 5'CGCGCTTTAAACTCGAGCCCGGG-3') was synthesized, annealed to form a blunt-ended double stranded DNA fragment, and inserted at the *Stu I* site in the 5' untranslated region of exon 2 by a ligation reaction. The inserted oligo was designed to detect transgene expression independently of the endogenous POMC alleles but not interfere with translation of POMC from mRNA. Additionally, the following restriction-enzyme sequences were introduced into exon 2 by the transgene oligo: *SrfI*, *SmaI*, *XhoI*, *DraI*, and *BssHII*. These restriction-sites were used for distinguishing PCR products obtained from wild type and transgenic alleles. In addition these sites will facilitate the introduction of additional sequences by directed ligations into new transgenes based on the *Pomc* genomic regulatory elements.

Care and use of animals. A colony of *Pomc* mutant mice was established as described previously (Smart and Low, 2003). Pituitary *Pomc-Tg* mice were generated by nuclear microinjection of the linearized, oligonucleotide marked *pHalEx2* Tg* into B6D2 F_2 hybrid embryos using standard techniques (Brinster and Palmiter 1984). The *pHalEx2* Tg* allele was backcrossed to the C57BL/6 genetic background for two generations and subsequently crossed onto the *Pomc*$^{-/-}$ genetic background by an additional two generations of heterozygous matings. Therefore the hybrid genetic background of mutant mice used in these experiments was approximately 80% C57BL/6, 10% DBA/2, and 10% 129. All animals for these studies were maintained under controlled temperature and photoperiod conditions (14 h of light, 10 h of dark; lights on, 0500 h) with food and water

provided *ad libitum* except for overnight fasting studies. All surgical procedures and other experimental studies were approved by the Institutional Animal Care and Use Committee and followed the guidelines established by the Public Health Service.

Genotyping. PCR genotyping was conducted on DNA extracted and prepared from tail clippings (described in (Barnes 1994)). Touchdown PCR was used to favor amplification of desired amplicons and to limit artifactual amplicons and primer-dimers (protocol described in (Don, Cox et al. 1991)). Multiple cycles were programmed so that the annealing segments in sequential cycles are run at incremental lower temperatures starting at the suspected melting temperature (T_m). To avoid low-T_m priming during the earlier cycles it was imperative to apply a hot start modification to the PCR protocol (D'Aquila, Bechtel et al. 1991). The programmed PCR cycle was 94°C for 10min; [94°C-30sec; 64°C minus 1°C per cycle-30 sec; 72°C-30 sec] X 15 cycles; [94°C-30sec; 50°C-30 sec; 72°C-30 sec] X 15 cycles; 72°C-3min; hold at 4°C. Three separate reactions were required to identify all three alleles: wild type, null, and transgenic. Wild type allele primers identified as POMWF (5'- GCTTGCATCCGGGCTTGCAAACT-3') T_m=68°C and POMWR (5'-AGCAACGTTGGGGTACACCTTC-3') T_m=72°C gave a 317 bp product. Null allele primers identified as POMMF (5'-TTGTTAACTTGTTTATTGCAGCTT-3') T_m=57°C and POMMR (5'-ATGAGAAATTGGGCCATGGGACTGAC-3') T_m=73°C gave a 210 bp product. Transgene allele primers identified as POMC Ex2 Sense (5'-TAGTGTTGGCTCAATGTCCT-3') T_m=53°C and POMC Ex2 Antisense (5'-GCTCTCCAGGCACCAGCTCC-3') T_m=63°C gave a 230 bp product from the

endogenous gene and 226 bp product from the transgene containing the inserted 253 bp oligonucleotide. A standard PCR reaction mix was used as described in the manual "PCR Primer" (Dieffenbach and Dveksler 1995).

In situ hybridization. mRNA *in-situ* hybridization was performed utilizing the antisense strand of the inserted 23 bp oligonucleotide as the radiolabeled probe, to confirm that the transgene was expressed in a normal pattern in the pituitary but not the hypothalamus of F_1 transgenic mice (data not shown). After crossing the transgene onto the *Pomc* null background, additional *in situ* hybridization experiments were performed with a single stranded RNA probe for *Pomc* mRNA, further confirming appropriate expression of the *pHal Ex2** transgene in the pituitary and not the hypothalamus (see chapter V).

Histology and immunohistochemistry. Mice were anesthetized and perfused with 4% paraformaldehyde to provide tissue fixation for histological analyses. 50 μm sections of brain and pituitary were collected with a vibratome. Immunohistochemistry was conducted as previously described using rabbit polyclonal antisera for β-endorphin or ACTH (Cowley, Smart et al. 2001) followed by biotinylated goat anti-rabbit serum, ABC Elite avidin-biotin-peroxidase complex (Vector Labs, Burlingame, CA), and diaminobenzidine (DAB) for the reaction product.

Growth charts, length, and tissue weights. Male and female mice of mixed genotypes were group housed, fed a standard diet of rodent chow (5% fat, 19% protein, and 5% fiber by weight; 3.4 kcal/g), and weighed weekly for 6 months. At 9 months final

weights were obtained and nose to anus lengths measured, then the mice were sacrificed and tissues dissected and weighed individually (liver, spleen, thymus, and heart) or in pairs (fat depots, adrenals, kidneys, and gonads).

Fasting glucose, insulin and leptin. Following a 24-hour fast, glucose was measured from tail blood using an Accuscan glucometer. Insulin and leptin levels were measured using EDTA-plasma with mouse-specific leptin and insulin RIAs (Linco, Inc.) according to the manufacturer's instructions. Even with a two-fold dilution of plasma samples, most values for *Pomc*$^{-/-}$; *Tg/+* males and females and occasional values for some of the other genotypes were above the highest concentration of the standard curves in both RIAs. Therefore to better estimate the real differences in these values from those in other genotype groups that were clustered just at the highest standard concentrations (2 ng/ml for insulin and 40 ng/ml for leptin), we manually re-plotted the standard curves onto semi-log graph paper and extrapolated from these curves a higher cutoff point that clearly delineated the populations of extreme outliers. All data points in these outlying clusters were then reassigned values of either 4 ng/ml for insulin or 80 ng/ml for leptin prior to statistical analysis. All other data points at or above the highest standard concentration but below the extreme outliers were assigned values of 2 ng/ml for insulin and 40 ng/ml for leptin.

Glucose tolerance test. Mice were fasted overnight 16-18 hours then given an i.p. bolus of glucose (2g/kg as dextrose 300 mg/ml). Blood glucose was measured from tail blood

using an Accuscan glucometer at 0, 10, 20, 30, 45, 60, 90, 120, 180 minutes post-injection.

Food consumption, basal metabolic rate, and respiratory exchange ratio. Mice 6-7 months old were individually housed, fed standard chow as previously stated, and the chow was weighed daily for 14 days allowing 9 days of habituation to the stress of individual housing and restabilization of feeding behavior. Food consumption was then averaged across days 10-14 and normalized to metabolic mass using the scaling formula [food intake (g)/body weight (g) $^{\wedge 0.75}$ x 100] (Pace, Rahlmann et al. 1980).

Basal metabolic rates were assessed using an indirect open-circuit calorimeter system (Oxymax, Columbus Instruments) consisting of 8 chambers with dimensions of 10.5 cm (W) x 20.5 cm (L) x 12.5 cm (H) that limit spontaneous locomotor activity. Mice were placed into individual chambers at 8:00 AM for six hours without access to food or water on two sequential days to allow for chamber habituation. Following habituation, a total of 12 measurements were recorded from each mouse with approximately 30 minutes between each measurement for a total experimental sampling period of 6 hours on the third day. The inlet fresh air-flow rate was 0.5 l/min with a sample flow rate of 0.4 l/min. Each chamber was sampled for 50 s with a resting time of 150 s. Individual basal oxygen consumption (VO_2) levels were established by averaging the three lowest of twelve VO_2 measurements. CO_2 production from each mouse was also recorded simultaneously and used to calculate the respiratory exchange ratio (RER) [VCO_2/VO_2] at each 30 min interval.

Statistics. Data were analyzed by 2-factor ANOVA using Stat View Power PC version for Macintosh (version 5.0.1; SAS Institute Inc., Cary, North Carolina, USA) except where otherwise stated. P values less than 0.05 were considered significant. *Post hoc* comparisons between groups were performed by Fisher's (PLSD).

4. RESULTS

4.1 *Transgenic rescue of pituitary POMC in Pomc null mice*

The original *Pomc*$^{-/-}$ knockout mice were generated on a hybrid white-belled agouti 129 genetic background (*A*^{w}/*A*^{w}; *pTyr*^{C}/*pTyr*$^{C\text{-}Ch}$) using homologous recombination to replace exon 3 with a *neomycin* cassette thus deleting the coding region of all known functional POMC peptides (Yaswen, Diehl et al. 1999). We backcrossed the *Pomc*$^{-}$ allele for two generations onto the non-agouti, wild-type tyrosinase C57BL/6 (*a/a*) background to evaluate possible epistatic contributions to phenotype (Smart and Low 2003). By backcrossing onto the C57BL/6 genetic background, an inbred mouse line more commonly used in energy homeostasis studies, we reduced the genetic heterogeneity when comparing our data with those of other spontaneous or genetically induced mouse obesity models. To replace pituitary POMC expression in the *Pomc*$^{-/-}$ background, we generated a strain of transgenic mice (*pHalEx2**) utilizing previous promoter mapping studies that identified critical DNA flanking regions essential for expression of the *Pomc*

gene in the pituitary but not in hypothalamic neurons (Rubinstein, Mortrud et al. 1993). A 23 bp oligonucleotide was inserted into the untranslated region of exon 2 thus allowing identification of the transgenic allele from that of the other alleles. The wild type (*Pomc*$^{+/+}$), null (*Pomc*$^{-/-}$), and *pHalEx2** transgenic (*Tg*) alleles are depicted schematically in Figure 1.

Hemizygous *pHalEx2** (*Tg/+*) transgenic mice and *Pomc*$^{+/-}$ mice were intercrossed to generate *Pomc*$^{+/-}$; *Tg/+* female breeders. These *Pomc*$^{+/-}$; *Tg/+* females were then bred with either *Pomc*$^{+/+}$ or *Pomc*$^{-/-}$ males to generate *Pomc*$^{+/+}$, *Pomc*$^{+/-}$, and *Pomc*$^{-/-}$ mice with and without the transgene (*Tg/+*) resulting in a total of six potential genotypes for both sexes. Combinations of PCR reactions using primers specific for each allele identified these six genotypes. Initially, mRNA *in-situ* hybridization with a transgene-specific oligonucleotide probe was used to demonstrate selective expression of the transgene in pituitary corticotrophs and melanotrophs but not in hypothalamic neurons (data not shown). Immunohistochemistry on coronal brain and pituitary sections then confirmed the restored production of POMC peptides β-endorphin (Figure 2) and ACTH (not shown) in *Pomc*$^{-/-}$; *Tg/+* pituitaries but not hypothalami.

4.2 *Effects on adrenal size, body weight, body length, and white adipose tissue by pituitary Pomc rescue in Pomc null mice*

The transgenic rescue of pituitary but not hypothalamic POMC restored adrenal function, demonstrated by a reversal of adrenal atrophy (Table 1) and production and secretion of

Figure 1

Schematic of wild type *Pomc*$^{+/+}$, null *Pomc*$^{-/-}$, and transgene *pHalEx2** (*Tg/+*) alleles are shown with a neomycin cassette replacing exon 3 in the *Pomc*$^{-/-}$ allele and a 23 bp oligo inserted into exon 2 indicated by (*****) in the *pHal Ex2** (*Tg/+*) allele. The arrow (shown above exon 1) represents the transcriptional start site. PCR primers used described in the **Methods** are indicated by the red arrows. Wild type allele primers set A,B = (317 bp product); Null allele primer set C,D = (210 bp product); and *Pomc Tg* allele primer set E,F = (230 bp wild type or 253 bp transgene products)

Figure 1

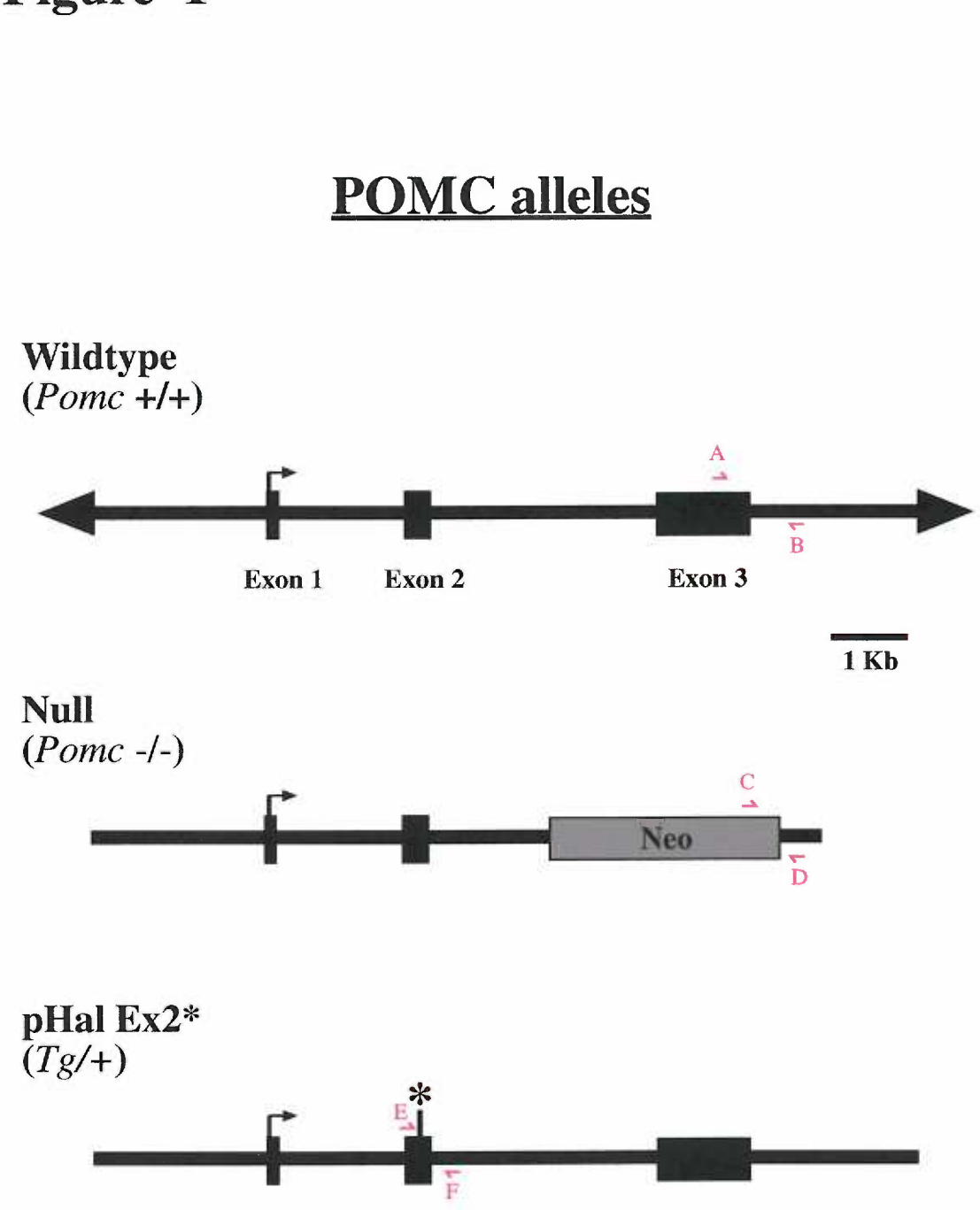
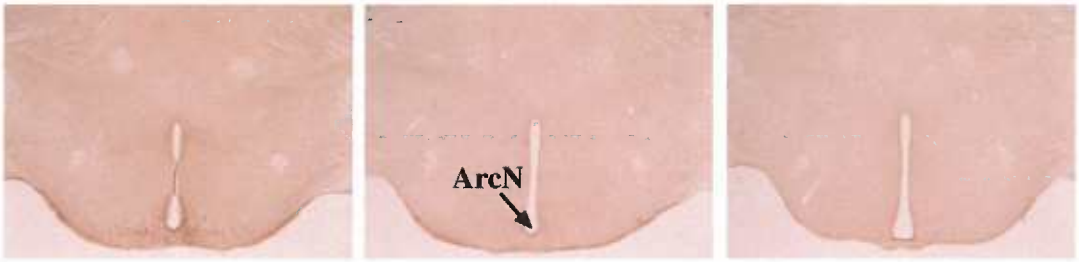

POMC alleles
Wildtype
(Pomc +/+)
A
B
Exon 1
Exon 2
Exon 3
1 Kb
Null
(Pomc -/-)
C
Neo
D
pHal Ex2*
(Tg/+)
*
E
F

Figure 2

Immunohistochemistry for β–endorphin in sections of hypothalami and pituitaries. The POMC peptide β–endorphin was detected in (**A**) *Pomc*$^{+/+}$ but not (**B**) *Pomc*$^{-/-}$ or (**C**) *Pomc*$^{-/-}$; *Tg/+* hypothalami. β-endorphin expression was detected in (**D**) *Pomc*$^{+/+}$ and (**F**) *Pomc*$^{-/-}$; *Tg/+* but not (**E**) *Pomc*$^{-/-}$ pituitaries confirming rescued peptide expression from the *pHal Ex2** transgene on the *Pomc*$^{-/-}$ background in pituitary but not hypothalamus.

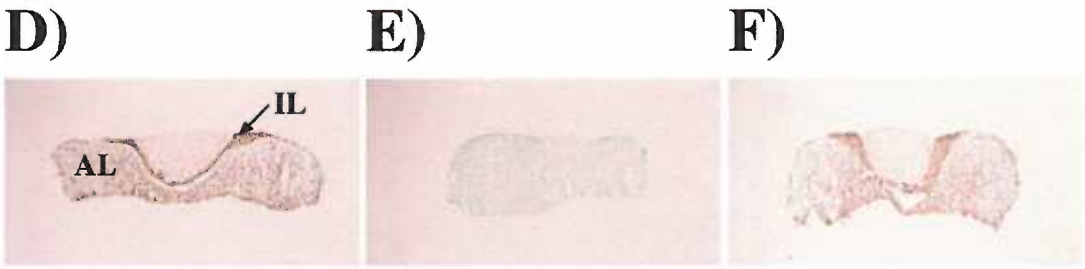
Figure 2

Hypothalamus



Pomc +/+ *Pomc* -/- *Pomc* -/-; Tg/+

Pituitary



Pomc +/+ *Pomc* -/- *Pomc* -/-; Tg/+

Table 1

Wet tissue weights of mice sacrificed at 9-11 months old

Male

Genotype (n)	Liver (g)	Spleen (mg)	Thymus (mg)	Kidney (mg)	Heart (mg)	Testes (mg)
Pomc$^{+/+}$; +/+ (5)	1.5 ± .1	89 ± 8	22 ± 3	466 ± 21	177 ± 17	232 ± 8
Pomc$^{+/+}$; *Tg*/+ (3)	1.8 ± .2	88 ± 7	27 ± 3	476 ± 25	174 ± 6	234 ± 4
Pomc$^{+/-}$; +/+ (11)	2.5 ± .2	95 ± 6	22 ± 1	573 ± 43	200 ± 8	228 ± 13
Pomc$^{+/-}$; *Tg*/+ (10)	2.9 ± .4 [a]	98 ± 8	23 ± 1	590 ± 66	212 ± 18	225 ± 11
Pomc$^{-/-}$; +/+ (6)	4.1 ± .5 [b]	256 ±16 [c]	28 ± 2 [a]	570 ± 46	224 ± 10 [a]	232 ± 13
Pomc$^{-/-}$; *Tg*/+ (9)	6.9 ± .5 [c]	164 ± 7 [c]	27 ± 3	679 ± 43 [a]	238 ± 11 [b]	214 ± 10

Main effect for genotype on weights of liver ($F_{5,38}$=22.2, $p < 0.0001$); spleen ($F_{5,44}$=22.6, $p < 0.0001$); thymus ($F_{5,29}$=2.1, $p < 0.09$); kidney ($F_{5,31}$=1.7, $p < 0.15$); and heart ($F_{5,31}$=3.1, $p < 0.02$). [a]p< 0.05; [b]p< 0.01; [d]p< 0.0001 compared to (*Pomc*$^{+/+}$; +/+) Fisher's PLSD

Female

Genotype (n)	Liver (g)	Spleen (mg)	Thymus (mg)	Kidney (mg)	Heart (mg)	Ovaries (mg)
Pomc$^{+/+}$; +/+ (7)	1.2 ± .1	96 ± 8	22 ± 3	347 ± 11	130 ± 17	8.8 ± 1.9
Pomc$^{+/+}$; *Tg*/+ (5)	1.3 ± .1	102 ± 7	20 ± 2	357 ± 4	139 ± 5	7.8 ± 1.2
Pomc$^{+/-}$; +/+ (6)	1.6 ± .1	108 ± 10	16 ± 1	371 ± 13	139 ± 4	8.0 ± .6
Pomc$^{+/-}$; *Tg*/+ (4)	1.6 ± .1	125 ± 16	21 ± 4	376 ± 20	145 ± 6	7.6 ± .7
Pomc$^{-/-}$; +/+ (5)	2.3 ± .3 [a]	227 ± 21 [d]	31 ± 3 [a]	404 ± 30 [b]	181 ±12 [d]	10.2 ± .4
Pomc$^{-/-}$; *Tg*/+ (7)	4.8 ± .6 [d]	161 ± 12 [c]	18 ± 3	467 ± 24 [d]	211 ± 9 [d]	10.2 ± .7

Main effect for genotype on weights of liver ($F_{5,28}$=22.9, $p < 0.0001$); spleen ($F_{5,28}$=14.8, $p < 0.0001$); thymus ($F_{5,28}$=3.2, $p < 0.02$); kidney ($F_{5,28}$=6.2, $p < 0.0006$); and heart ($F_{5,28}$=22.2, $p < 0.0001$). [a]p< 0.05; [b]p< 0.01; [c]p< 0.001; [d]p< 0.0001 compared to (*Pomc*$^{+/+}$; +/+) Fisher's PLSD

Values are the mean ± SEM. Adrenal, kidney, testes and ovaries values are the mean of tissue pairs. Significant differences in means from that of *Pomc*$^{+/+}$; +/+ mice are indicated in the tables with corresponding p-values found beneath each table.

glucocorticoids. Unexpectedly, the adrenal glands in *Pomc*$^{-/-}$; *Tg/+* male and female mice were significantly larger than their same sex wild type siblings, although basal serum corticosterone levels were only significantly higher in *Pomc*$^{-/-}$; *Tg/+* males compared to *Pomc*$^{+/+}$ males (data presented in Chapter V). Replacing pituitary POMC in the *Pomc*$^{-/-}$ mice had profound effects to further increase their body weights, but *pHalEx2* Tg* expression did not affect the weights of either *Pomc*$^{+/+}$ or *Pomc*$^{+/-}$ mice (Figure 3A). By age six months, both sexes of *Pomc*$^{-/-}$; *Tg/+* mice gained between 30% to 40% more weight than their *Pomc*$^{-/-}$ siblings. Significant separation of body weight was observed between 4 to 6 weeks of age in both the *Pomc*$^{-/-}$ and *Pomc*$^{-/-}$; *Tg/+* mice compared to *Pomc*$^{+/+}$ mice. Late onset obesity at 3 to 4 months of age was observed in the *Pomc*$^{+/-}$ males while only a trend was evident in *Pomc*$^{+/-}$ females as previously reported (Smart and Low 2003). The increase in body weight can partially be attributed to an increase in linear growth in both sexes of *Pomc*$^{+/-}$ and *Pomc*$^{-/-}$ mice; however, the pituitary *pHalEx2** transgene had no effect on linear growth on any of the genotypes except *Pomc*$^{+/+}$ females (Figure 3B). The fact that linear growth was not altered by the *pHalEx2** transgene indicates that it is the loss of hypothalamic POMC peptides, and not pituitary POMC peptides that directly or indirectly affects linear growth.

Most of the differences in body weight among the genotypes were accounted for by white adipose tissue. Analyses of white fat depots showed that the increased body weight correlated with increased mass of white fat depots (Figure 4). Interestingly, gonadal fat mass was largest in *Pomc*$^{-/-}$ mice as compared to their *Pomc*$^{-/-}$; *Tg/+* siblings, even though the latter mice had body weights that were 30% to 40% greater. In fact, the gonadal fat

Figure 3

The effects of rescued pituitary POMC expression on body weight and body length in the *Pomc*$^{-/-}$ background. (**A**) Body weight curves for male and female *Pomc*$^{+/+}$, *Pomc*$^{+/-}$, and *Pomc*$^{-/-}$ mice with and without the *pHalEx2** transgene (n = 9-44). (**B**) Body lengths of male and female mice at age nine months (n = 3-12). Data are reported as the mean ± SEM. * $p<0.01$; ** $p<0.001$; *** $p<0.0001$ compared to (*Pomc*$^{+/+}$; +/+), Fisher's PLSD.

Figure 3

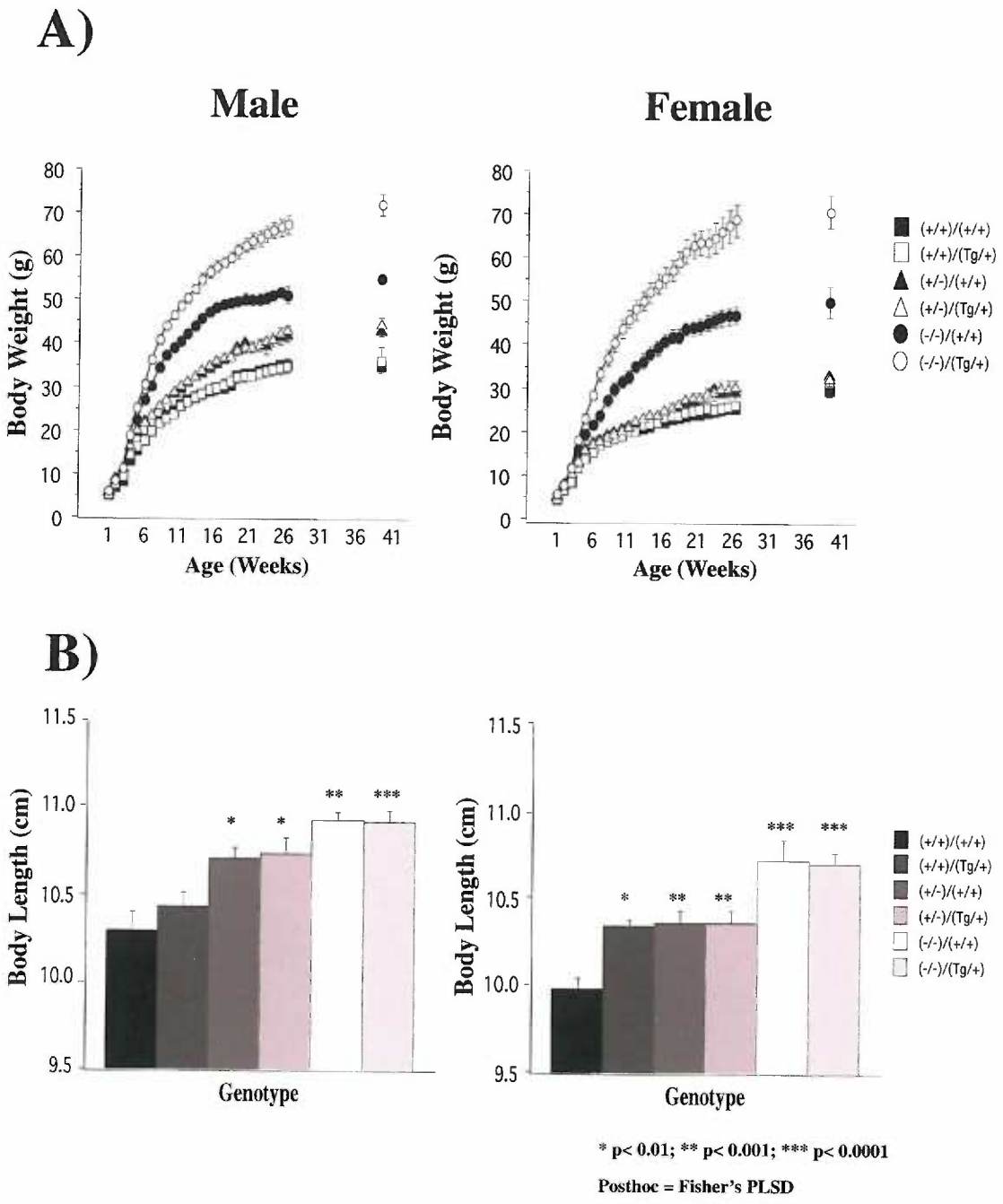
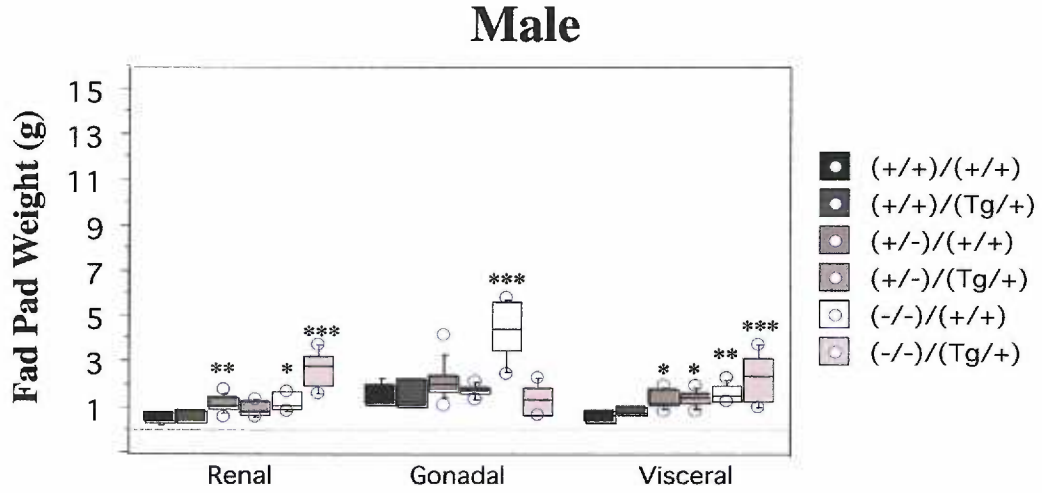
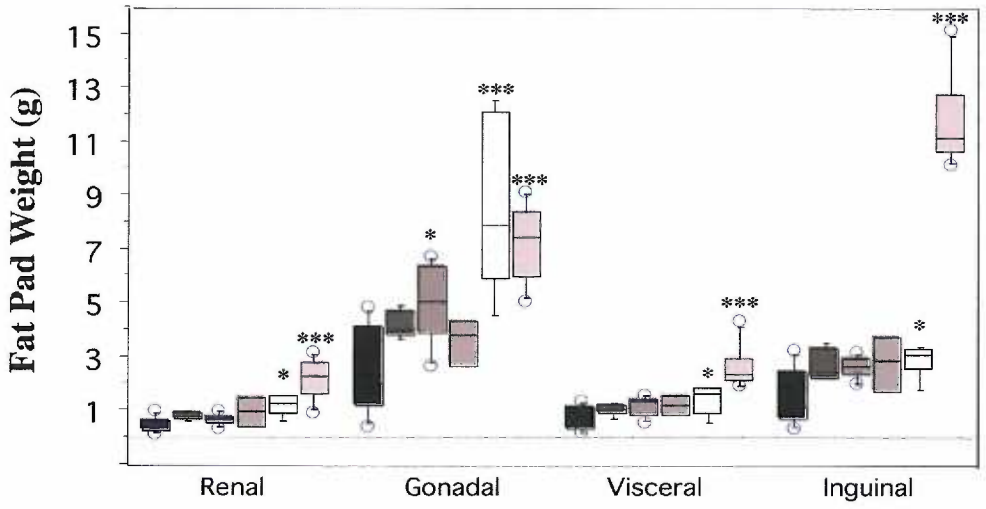

A)
Male
Female
Body Weight (g)
Age (Weeks)
(+/+)/(+/+)
(+/+)/(Tg/+)
(+/-)/(+/+)
(+/-)/(Tg/+)
(-/-)/(+/+)
(-/-)/(Tg/+)
B)
Body Length (cm)
Genotype
* p< 0.01; ** p< 0.001; *** p< 0.0001
Posthoc = Fisher's PLSD

Figure 4

White adipose tissue depots from male and female *Pomc*$^{+/+}$, *Pomc*$^{+/-}$, and *Pomc*$^{-/-}$ mice with and without *pHalEx2** transgene expression are shown as a box plot to compare variable distributions. The box and whisker plots shows the 10th, 25th, 50th (median), 75th, and 90th percentiles for renal, gonadal, visceral, and inguinal white adipose tissue depots from mice 9 to 11 months old (n = 3-11). Values above the 90th and below the 10th are plotted as points (O). Male inguinal adipose tissue was not weighed. *p<0.05; ** p<0.01; *** p<0.0001 compared to (*Pomc*$^{+/+}$; +/+), Fisher's PLSD.

Figure 4





*** p< 0.05; ** p< 0.01; *** p< 0.0001**

Posthoc = Fisher's PLSD

Conversely, the mass of renal, visceral, and inguinal white fat depots in the *Pomc*$^{-/-}$; *Tg/+* mice was greater than that measured in the *Pomc*$^{-/-}$ mice with the most striking difference in subcutaneous fat. The inguinal fat pads, representative of subcutaneous white fat deposits, were disproportionately larger in the female *Pomc*$^{-/-}$; *Tg/+* mice compared to the other genotypes. Although we did not weigh intrascapular white fat, we observed that an excessive amount of white fat had also accumulated dorsally in the *Pomc*$^{-/-}$; *Tg/+* mice. These differences in subcutaneous white fat depots accounted for a significant portion of the body mass differences between *Pomc*$^{-/-}$; *Tg/+* mice and their *Pomc*$^{-/-}$ siblings. Liver mass was also substantially increased with the visual appearance of fatty infiltration (Table 1).

4.3 *Pomc*$^{-/-}$ *and Pomc*$^{-/-}$*; Tg/+ mice exhibited type II diabetes that was more pronounced in Pomc*$^{-/-}$*; Tg/+ females*

The markedly exacerbated obesity seen in the *Pomc*$^{-/-}$; *Tg/+* mice led us to evaluate the possibility of associated endocrine abnormalities. Whole blood glucose measurements obtained at random times from *ad libitum* feeding mice at 3 to 4 months of age showed significantly higher levels of glucose in *Pomc*$^{-/-}$ and *Pomc*$^{-/-}$; *Tg/+* males and *Pomc*$^{-/-}$; *Tg/+* females (data not shown). At 6 months of age, mice were fasted overnight and whole blood glucose, plasma insulin and plasma leptin levels were measured (Table 2). Male *Pomc*$^{+/-}$; *Tg/+* and *Pomc*$^{-/-}$; *Tg/+* mice were hyperglycemic yet the females had normal fasting glucose levels. Furthermore, male *Pomc*$^{-/-}$; *Tg/+* mice and female *Pomc*$^{-/-}$ and *Pomc*$^{-/-}$; *Tg/+* mice were hyperinsulinemic. Predictably, hyperleptinemia was

TABLE 2

Fasting glucose, insulin, and leptin levels in 6-8 month old mice

Male

Genotype (n)	Glucose (mg/dl)	Insulin (ng/ml)	Leptin (ng/ml)
Pomc$^{+/+}$;(+/+) (8)	89 ± 7	0.73 ± .20	10.8 ± 2.3
Pomc$^{+/+}$;(Tg/+) (6)	94 ± 8	0.99 ± .30	26.3 ± 12.1
Pomc$^{+/-}$;(+/+) (13)	108 ± 7	0.86 ± .14	23.6 ± 3.4
Pomc$^{+/-}$;(Tg/+) (9)	107 ± 8	1.53 ± .20 [a]	33.5 ± 3.7 [b]
Pomc$^{-/-}$;(+/+) (9)	124 ± 8 [b]	1.15 ± .17	26.7 ± 4.0
Pomc$^{-/-}$;(Tg/+) (6-7)	125 ± 13 [b]	2.91 ± .54 [c]	70.5 ± 9.5 [c]

Main effects for genotype on glucose ($F_{5,45} = 2.79$, $p < 0.05$), insulin ($F_{5,46} = 8.97$, $p < 0.0001$), and leptin ($F_{5,46} = 11.3$, $p < 0.0001$). [a]$p< 0.05$; [b]$p< 0.01$; [c]$p< 0.0001$ compared to (*Pomc*$^{+/+}$; +/+) Fisher's PLSD.

Female

Genotype (n)	Glucose (mg/dl)	Insulin (ng/ml)	Leptin (ng/ml)
Pomc$^{+/+}$;(+/+) (12)	83 ± 5	0.46 ± .08	17.1 ± 1.7
Pomc$^{+/+}$;(Tg/+) (8)	78 ± 6	0.60 ± .20	21.8 ± 4.2
Pomc$^{+/-}$;(+/+) (22-23)	85 ± 6	0.36 ± .03	20.7 ± 2.2
Pomc$^{+/-}$;(Tg/+) (11)	88 ± 7	0.32 ± .06	22.5 ± 3.4
Pomc$^{-/-}$;(+/+) (4)	95 ± 10	0.93 ± .38 [a]	33.9 ± 2.1 [a]
Pomc$^{-/-}$;(Tg/+) (12)	89 ± 6	2.52 ± .33 [c]	68.6 ± 6.1 [c]

Main effects for genotype on insulin ($F_{5,64} = 27.6$, $p < 0.0001$) and leptin ($F_{5,64} = 29.9$, $p < 0.0001$). [a]$p< 0.05$; [b]$p< 0.01$; [c]$p< 0.0001$ compared to (*Pomc*$^{+/+}$; +/+) Fisher's PLSD.

Values are the mean ± SEM. Significant differences in means from that of *Pomc*$^{+/+}$; +/+ mice are indicated in the tables with corresponding p-values found beneath each table. Some individual values above the highest concentrations of the RIA standard curves (2 ng/ml for insulin and 40 ng/ml for leptin) were extrapolated before statistical analyses as described in the **Methods**.

observed in genotypes of both sexes that had a significant increase in body weight and white adipose tissue. Intraperitoneal glucose tolerance tests (2g/kg) were performed on mice at age 6-8 months. Male *Pomc*$^{+/-}$ and *Pomc*$^{-/-}$ mice with and without *pHalEx2** transgene expression were equally impaired in their hyperglycemic responses to the glucose challenge (Figure 5). Female *Pomc*$^{+/-}$, *Pomc*$^{+/-}$; *Tg/+*, and *Pomc*$^{-/-}$ mice all exhibited a mild glucose intolerance, but this shift compared to *Pomc*$^{+/+}$ mice was overshadowed by the markedly abnormal glucose tolerance of *Pomc*$^{-/-}$; *Tg/+* female mice. Unlike male *Pomc*$^{-/-}$; *Tg/+* mice, female *Pomc*$^{-/-}$; *Tg/+* mice were not hyperglycemic following an overnight fast yet they exhibited profound impairment after a glucose challenge.

4.4 *Obesity in Pomc*$^{-/-}$ *and Pomc*$^{-/-}$*; Tg/+ mice was due to distinct combinations of altered basal metabolic rate and food intake*

Mice 6 to 8 months of age were individually housed and food consumption was recorded daily over a 14-day period. All mice exhibited an initial stress-induced hypophagia, apparent on the first four to eight days of group separation (data not shown). Between days 5 to 8, daily food consumption increased and a steady-state plateau was established. Food consumed on days 10-14 was then measured and averaged for each mouse to calculate daily food intake by all genotypes (Figure 6). Absolute daily caloric intake was identical among male and female mice for all genotypes except for the *Pomc*$^{-/-}$; *Tg/+* mice, which consumed significantly more food than wild type siblings. The five-day averages were also corrected for metabolic mass according to the formula [food intake

Figure 5

Intraperitoneal glucose tolerance tests performed on 6 to 8 month old male and female mice after a 16-18 hour fast (n = 4-24). For clarity, results from the *Pomc*$^{+/+}$; *Tg/+* and *Pomc*$^{+/-}$; *Tg/+* groups are not shown but they did not differ from their corresponding groups without the *pHal Ex2** transgene (*Tg/+*) in either sex. Data are reported as the mean ± SEM.

Figure 5

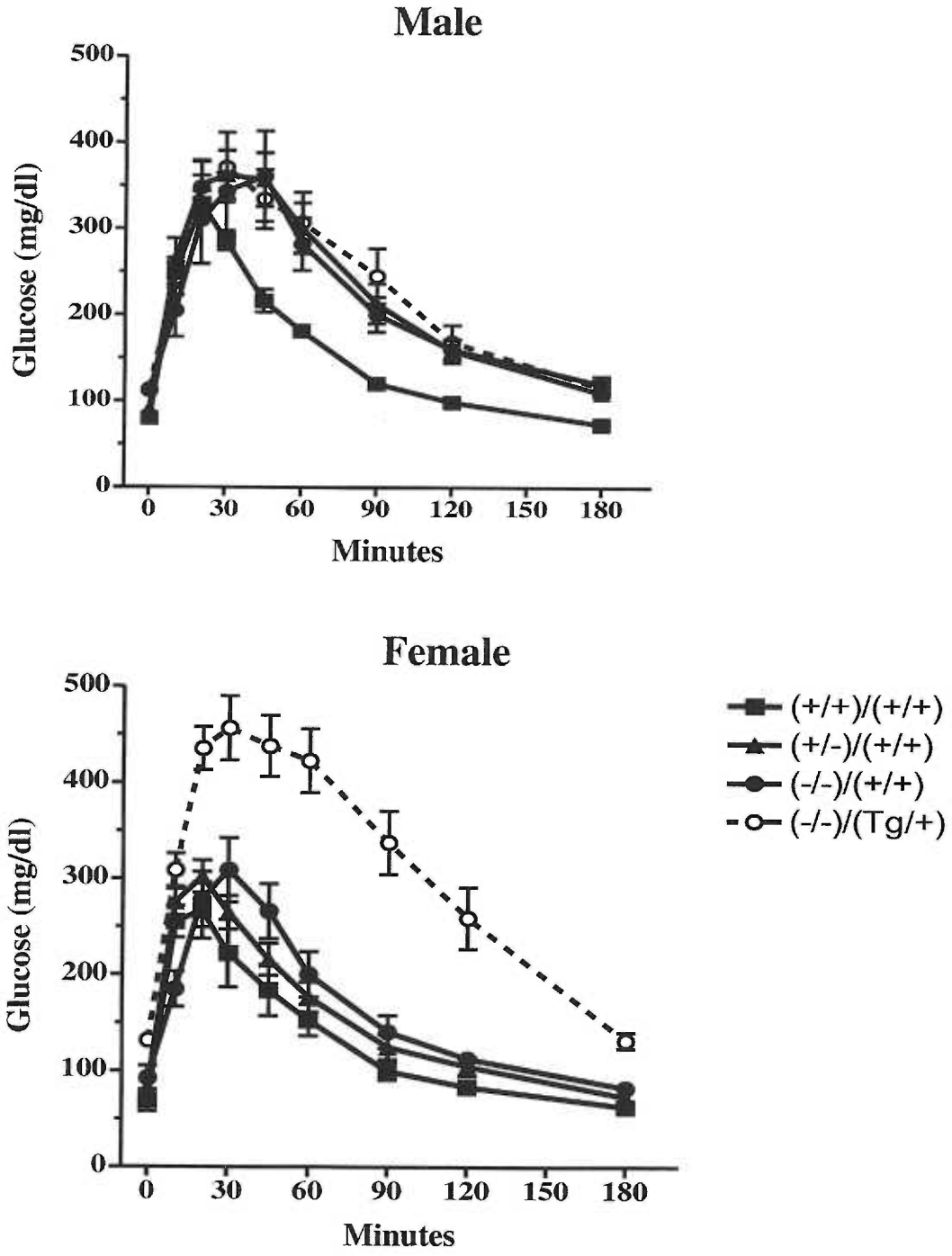
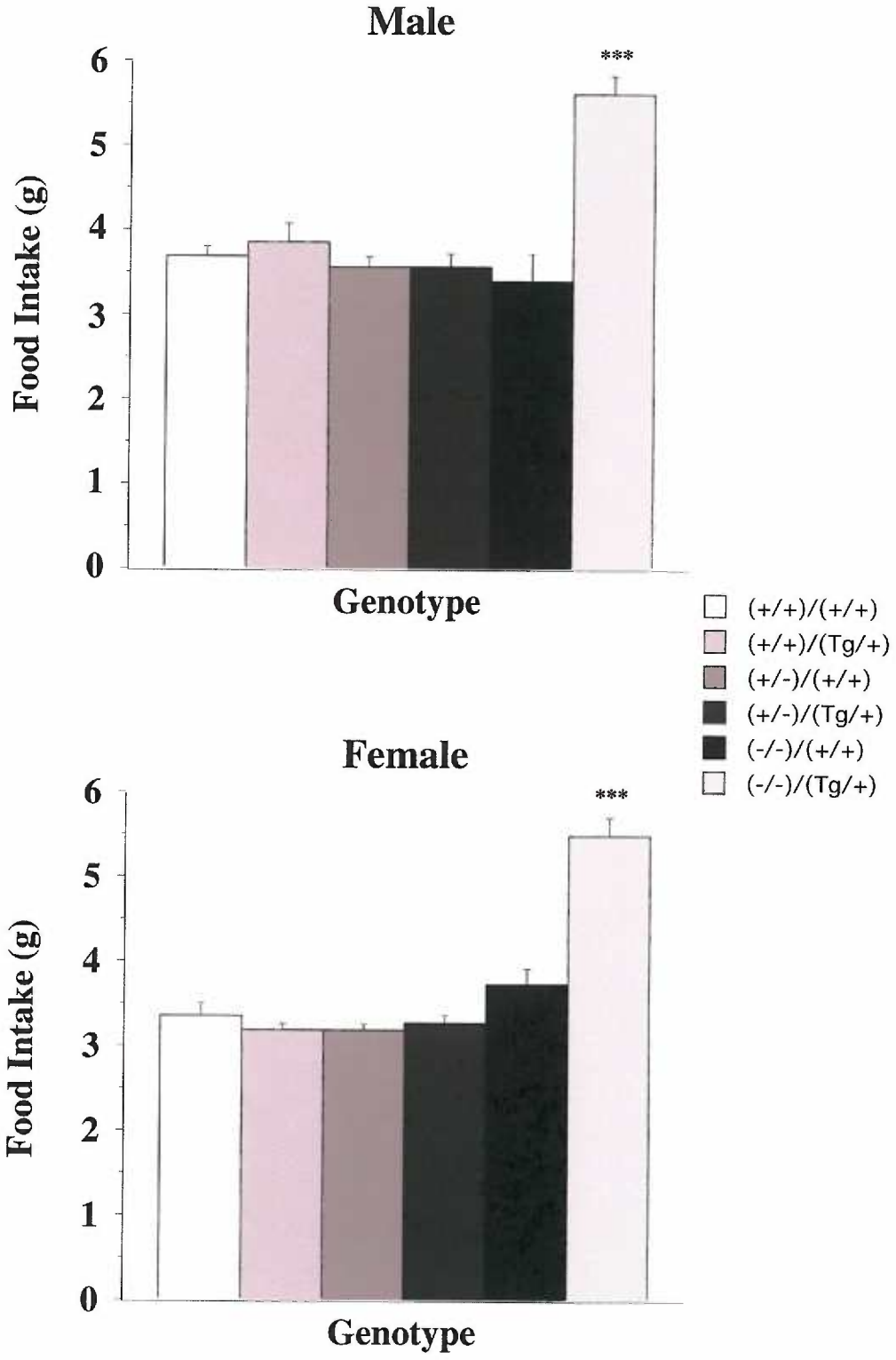

Male
Glucose (mg/dl)
500
400
300
200
100
0
0
30
60
90
120
150
180
Minutes
Female
Glucose (mg/dl)
500
400
300
200
100
0
0
30
60
90
120
150
180
Minutes
(+/+)/(+/+)
(+/-)/(+/+)
(-/-)/(+/+)
(-/-)/(Tg/+)

Figure 6

Average daily absolute food intake over five days for each mouse after nine days of acclimation to individual housing (n=6 to 24). Food intake measurements are shown as the absolute values. There were significant main effects of genotype for male ($F_{5,57} = 19.9$, $p < 0.0001$) and female ($F_{5,62} = 40.8$, $p < 0.0001$) mice. *** = $p<0.001$ compared to (*Pomc*$^{+/+}$; +/+), Fisher's PLSD.

Figure 6


Male
Food Intake (g)
6
5
4
3
2
1
0
***
Genotype
(+/+)/(+/+)
(+/+)/(Tg/+)
(+/-)/(+/+)
(+/-)/(Tg/+)
(-/-)/(+/+)
(-/-)/(Tg/+)
Female
Food Intake (g)
6
5
4
3
2
1
0
***
Genotype

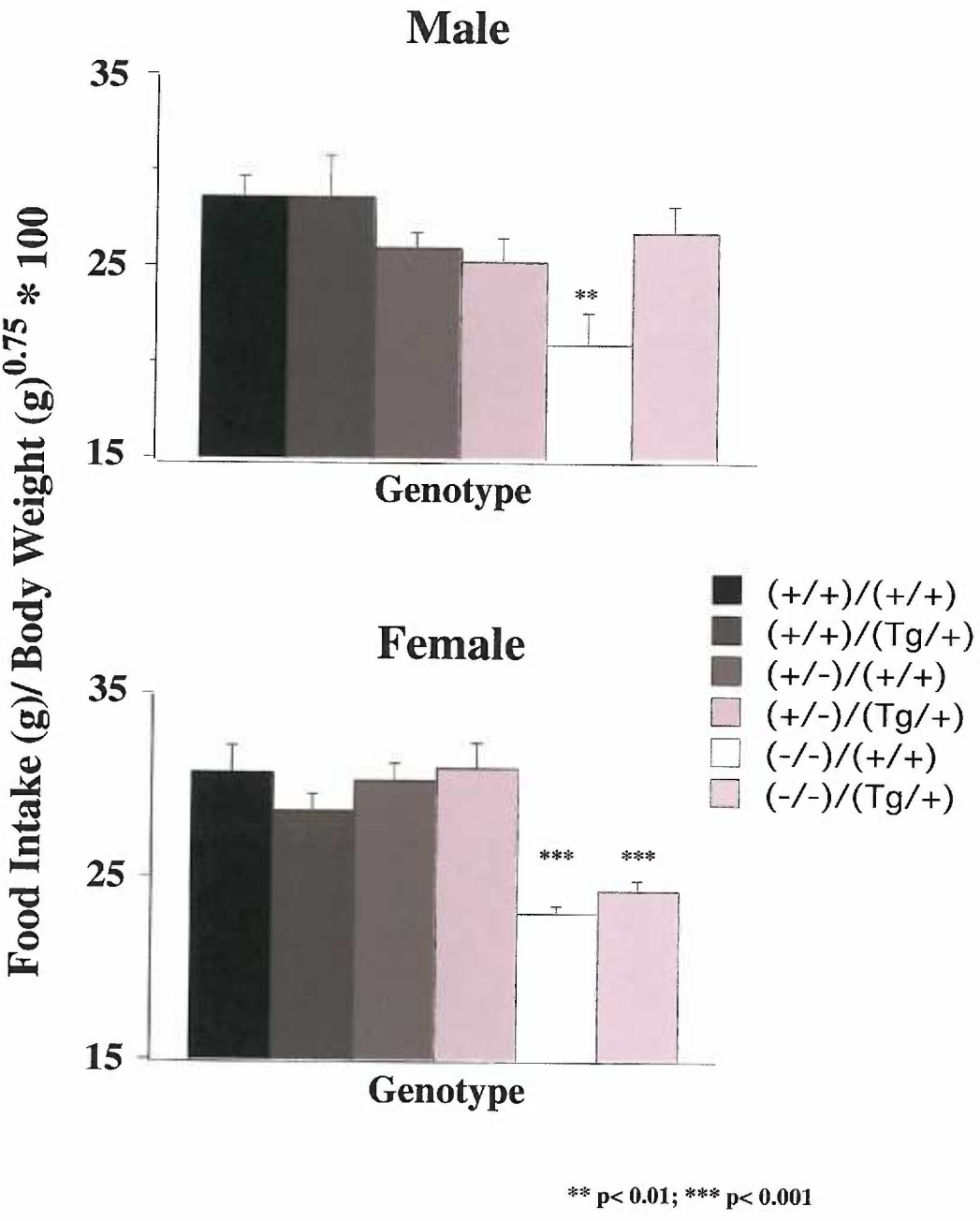
(g)/body weight (g) $^{\wedge 0.75}$ *100] (Pace, Rahlmann et al. 1980). In contrast to the values for absolute food intake, both male and female *Pomc*$^{-/-}$ mice and female *Pomc*$^{-/-}$; *Tg/+* mice had significantly lower food intake normalized to estimated metabolic mass than their wild-type siblings (Figure 7). Only the male *Pomc*$^{-/-}$; *Tg/+* mice had corrected food intake values that were indistinguishable from wild type animals, although the mutant males were clearly hyperphagic based on their absolute daily consumption of food.

To explain the maintenance of obesity in the mutant mice in the context of their measured caloric intake, we next assessed their basal metabolic rates by indirect calorimetry. Oxygen consumption (VO_2) was measured over a 6-hour period starting at 9:00 AM during the animals' most inactive phase of the diurnal light-dark cycle. 12 measurements were obtained from each mouse at 30 min intervals and the lowest three measurements over the 6-hour period were averaged to estimate basal levels of oxygen consumption. Basal VO_2 levels were indirectly correlated to body weight differences among genotypes for both male and female mice (Figure 8). An identical ranking of VO_2 levels by genotype was obtained when all 12 measurements instead of the lowest three were used in the calculations (data not shown). Furthermore, both male and female *Pomc*$^{-/-}$; *Tg/+* mice exhibited an elevated respiratory exchange ratio (RER), particularly in the earliest of the 12 measurements, compared with the other three genotypes (Figure 9). A higher ratio indicates less fatty acid oxidation and is indicative of a shift in the type of energy stores utilized and possibly a shift in energy partitioning (Elia and Livesey 1992). *Pomc*$^{+/+}$; *Tg/+* and *Pomc*$^{+/-}$; *Tg/+* mice were not analyzed in the Oxymax chambers.

Figure 7

Average daily food intake as shown in figure 6 that has been corrected for metabolic mass with the following formula [5 day average of food intake (g) / body weight (g)$^{\wedge 0.75}$ *100]. There were significant main effects of genotype for male ($F_{5,57} = 2.7$, $p < 0.0285$) and female ($F_{5,62} = 7.6$, $p < 0.0001$) mice. ** = $p<0.01$; *** = $p<0.001$ compared to (*Pomc*$^{+/+}$; +/+), Fisher's PLSD.

Figure 7



** p< 0.01; *** p< 0.001

Figure 8

Basal metabolic rate measured by volume of O_2 (VO_2) consumption corrected for body weight (ml/kg/hr) in Oxymax chambers. Data shown are the means ± SEM of the three lowest measurements from each mouse (n = 5-10) over approximately a 6-hour period. There were significant main effects of genotype for male ($F_{3,21}$ = 8.8, $p < 0.0006$) and female ($F_{3,23} = 24.7$, $p < 0.0001$) mice. * = $p<0.05$; *** = $p<0.0001$ compared to (*Pomc*$^{+/+}$; +/+), Fisher's PLSD.

Figure 8

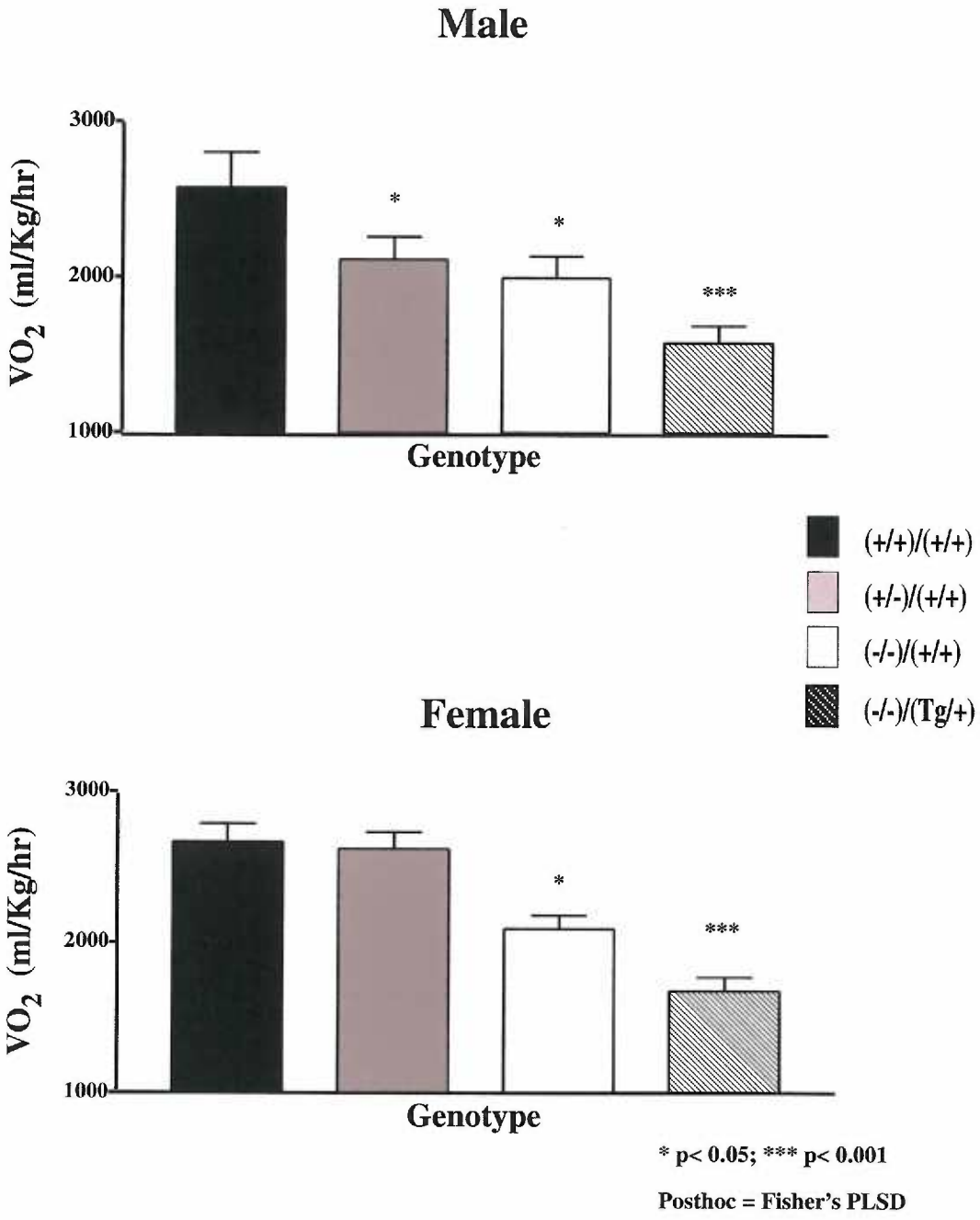
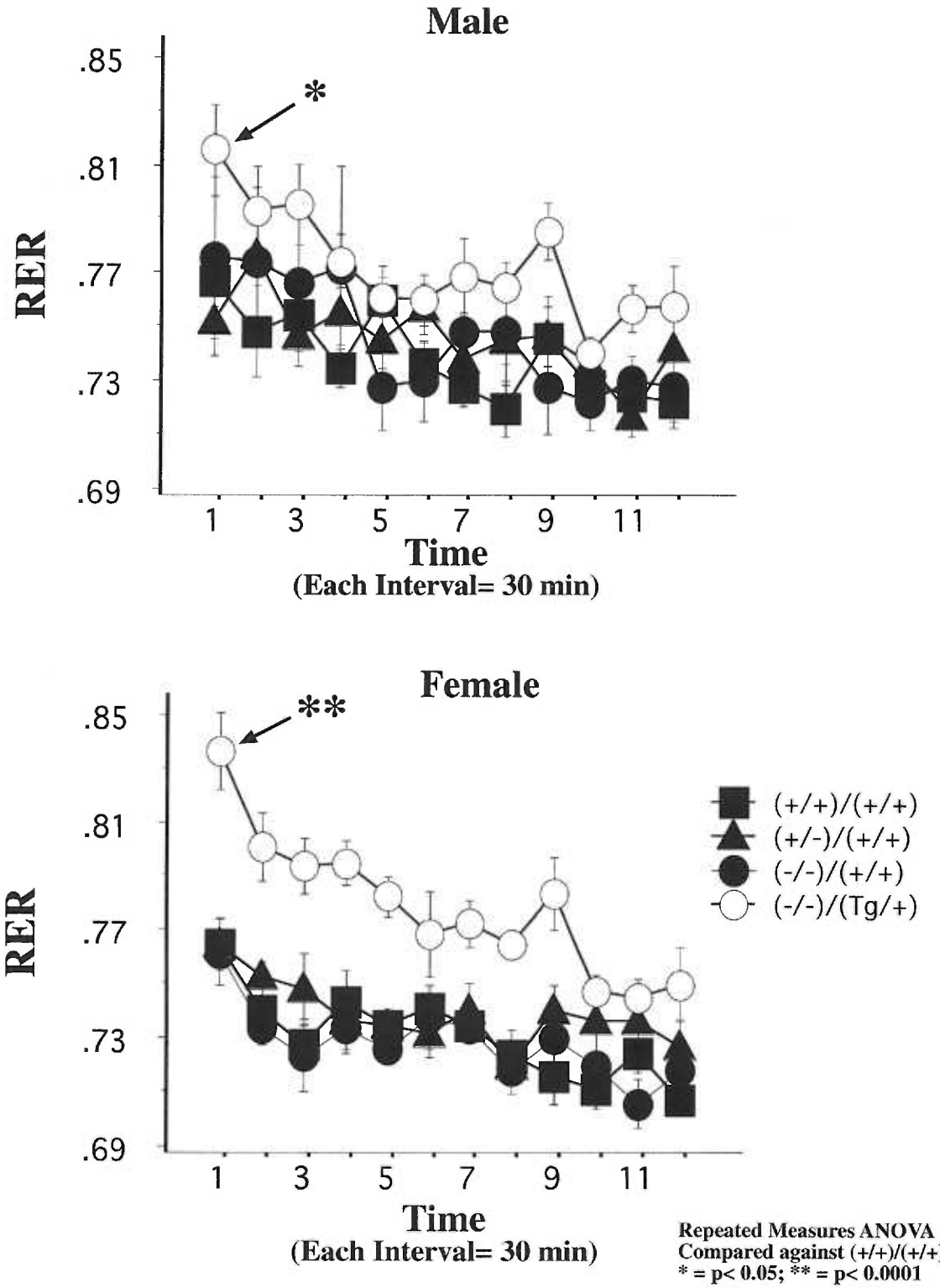

Male
3000
2000
1000
VO_2 (ml/Kg/hr)
*
*
***
Genotype
(+/+)/(+/+)
(+/-)/(+/+)
(-/-)/(+/+)
(-/-)/(Tg/+)
Female
3000
2000
1000
VO_2 (ml/Kg/hr)
*
***
Genotype
* p< 0.05; *** p< 0.001
Posthoc = Fisher's PLSD

Figure 9

Respiratory exchange ratio (RER) was higher in both male and female *Pomc*$^{-/-}$; *Tg*/+ mice compared to *Pomc*$^{+/+}$; +/+ siblings. For clarity, results from the *Pomc*$^{+/-}$; +/+ and *Pomc*$^{-/-}$; +/+ groups are not shown but they did not differ from their *Pomc*$^{+/+}$; +/+ siblings. Each time interval on the horizontal axis is equivalent to 30 minutes. There were significant main effects of genotype by a repeated measures ANOVA for male ($F_{3,21} = 2.9$, $p < 0.0614$) and female ($F_{3,23} = 19.1$, $p < 0.0001$) mice. * = $p<0.05$; ** = $p<0.0001$; Fisher's PLSD.

Figure 9



Repeated Measures ANOVA
Compared against (+/+)/(+/+)
* = p< 0.05; ** = p< 0.0001

normalization of food intake to metabolic mass using the conventional scaling exponent of mass to the three fourths power originally proposed by Kleiber (Kleiber's law), the *Pomc*$^{-/-}$ mice actually appeared to be hypophagic and hence more efficient in caloric utilization than their wild-type siblings. Estimates of basal metabolic rate by the measurement of VO_2 consumption were consistent with this contention and showed a 20% decrease compared to wild type siblings. Importantly, preliminary results of food intake and VO_2 consumption obtained from another cohort of mice at age 6 wk, when body weights are much more similar among genotypes but the rate of weight gain is at its maximal slope, showed the same pattern of comparable absolute daily food intake but decreased metabolic rate of *Pomc*$^{-/-}$ mice compared to *Pomc*$^{+/+}$ mice (data not shown). It is possible that the differences between our results and those of Yaswen, et al. (1999) regarding food intake are a reflection of the different genetic background of the mutant mice in each study. Whereas the other laboratory maintained the *Pomc*$^{-/-}$ allele on an F_2 129 hybrid background, the mice used in our experiments had a predominant C57BL/6 background.

Adipocytes are no longer considered passive storage vesicles for excess energy, but they are very dynamic endocrine cells that respond to a variety of stimuli (Shuldiner, Yang et al. 2001). In the *Pomc*$^{-/-}$; Tg/+ mice, the adipocyte's integration of both central and peripheral stimuli results in a markedly large increase in white fat depots. Adipocytes do however express the melanocortin receptor MC2 and *in vitro* studies have shown this receptor responds to ACTH by inducing lipolysis (Oelofsen and Ramachandran 1983; Boston and Cone 1996; Boston 1999). However, if indeed melanocortins are able to

induce lipolysis *in vivo*, it appears that this ability is masked by other stimuli. One such stimulus we believe could be that of glucocorticoids, which are elevated in *Pomc*$^{-/-}$; *Tg/+* mice (see following chapter) and are known transcription factors that can down regulate preadipocyte factor-1 (pref-1), an inhibitor to the differentiation of preadipocytes to mature adipocytes (Wolf 1999). In addition, glucocorticoids are known catabolic agents. Their inhibition of cellular uptake of amino acids and glucose and thus inhibiting protein synthesis results in a net decrease in metabolism (Brown 1994). Our data suggests the exaggerated obesity is caused by a reduction in basal metabolic rate (BMR) in the *Pomc*$^{-/-}$; *Tg/+* mice, and this BMR reduction is most likely due to the replacement of glucocorticoids. Although an increase in absolute food intake was observed, this phenotype may be secondary to the metabolic defect and similar to what was observed in the MC4-R KO mice (Ste Marie, Miura et al. 2000). Feeding studies preceding obesity along with pair-fed studies with wild type, *Pomc*$^{-/-}$, and *Pomc*$^{-/-}$; *Tg/+* siblings will need to be conducted to determine if hyperphagia is secondary to a the metabolic defect. While we believe our data indicates that the increase in white fat mass is due to a decrease in basal metabolism, we cannot exclude that a shift in energy partitioning may also be contributing to the obesity phenotype. Such pair-feeding studies will also help evaluate any differences in energy partitioning that may underlie the observed obesity phenotypes.

In humans, accumulation of intra-abdominal fat correlates with insulin resistance, whereas subcutaneous fat deposition correlates with increased circulating leptin levels (Havel 2001; Oberbauer, Runstadler et al. 2001; Cnop, Landchild et al. 2002). The

excessive amount of subcutaneous fat found in the *Pomc*$^{-/-}$; *Tg*/+ mice, presumably due to the replacement of circulating corticosterone in the absence of hypothalamic POMC, explains the very high fasting leptin levels seen in these *Pomc*$^{-/-}$; *Tg*/+ mice. The *Pomc*$^{-/-}$ mice did not have nearly the amount of subcutaneous fat as the *Pomc*$^{-/-}$; *Tg*/+ mice and 2 to 3 fold lower plasma leptin levels; yet conversely, they had very large gonadal fat pads, a subset of the mouse's intra-abdominal white fat, which in humans and mice tends to be directly proportional with insulin resistance (Masuzaki, Paterson et al. 2001; Cnop, Landchild et al. 2002). However, glucose-tolerance tests of *Pomc*$^{-/-}$ mice showed only a mild shift in their ability to clear glucose unlike the *Pomc*$^{-/-}$; *Tg*/+ mice.

Paradoxically, it may be the lack of circulating corticosterone in the *Pomc*$^{-/-}$ mice causing the disproportional accumulation of gonadal fat, and giving *Pomc*$^{-/-}$ mice exogenous glucocorticoids for an extended period of time and then subsequently measuring gonadal fat could help explain this phenotype. We hypothesize that glucocorticoids play an inhibitory role in the accumulation of intra-abdominal fat and are responsible for directing energy storage elsewhere like subcutaneous fat and/or the liver, where gluconeogenesis is stimulated by corticosterone. It should be noted that the *Pomc*$^{-/-}$; *Tg*/+ mice had very large fatty livers. The lack of glucocorticoids in the *Pomc*$^{-/-}$ mice could then lead to intra-abdominal fat accumulation. Intra-abdominal fat, as compared to subcutaneous fat, is known to have an increased lipolytic response via the stimulation of existing adrenergic receptors (Kahn and Flier 2000). An alternative hypothesis is that the *Pomc*$^{-/-}$ mice, which lack the catecholamine epinephrine (Yaswen, Diehl et al. 1999), would thus lack adrenergic induced lypolysis in gonadal fat leading to an increase storage

in this depot. Mice lacking dopamine β-hydroxlase (DBH), the rate-limiting enzyme in the production of norepinephrine (NE) and epinephrine (Epi), have an increased metabolic rate, are slightly hyperphagic, with normal amounts of white fat (Thomas and Palmiter 1997). The increase in metabolism seen in the DBH deficient mice may partially explain why the *Pomc*$^{-/-}$ mice show a higher BMR when compared with *Pomc*$^{-/-}$; *Tg/+* siblings.

A major up-regulator of leptin production is insulin. It is not surprising that mice having the highest fasting insulin levels and largest white fat depots also have the highest leptin levels. Both insulin and leptin inhibit feeding and increase metabolism. We previously have shown leptin's stimulatory effects on POMC neurons (Cowley, Smart et al. 2001). Mice lacking POMC would presumably be insensitive to leptin's anorexigenic effects that are mediated by these POMC neurons and could explain the decrease in metabolism seen in the *Pomc*$^{-/-}$ and *Pomc*$^{-/-}$; *Tg/+* mice. In addition, glucocorticoids have been shown to attenuate leptin effects on feeding and metabolism (Makimura, Mizuno et al. 2003). The reduced metabolism in *Pomc*$^{-/-}$; *Tg/+* mice from that of *Pomc*$^{-/-}$ mice may be due to glucocorticoid inhibiting the leptin effects on metabolism and feeding in the *Pomc*$^{-/-}$; *Tg/+* mice. If glucocorticoids were indeed suppressing leptin's anabolic effects it would be through a POMC independent pathway due to the lack of central POMC.

5.2 *Glucose tolerance and insulin resistance in Pomc$^{-/-}$ and Pomc$^{-/-}$; Tg/+ mice*

Diabetes, although a very complex endocrine disorder, can be induced by an increase in circulating glucocorticoid levels. The *Pomc*$^{-/-}$ mice, void of any circulating glucocorticoids, develop a very mild type II diabetic phenotype revealed by a mild glucose intolerance, *ad libitum* hyperglycemia, intra-abdominal obesity, and (in females only) hyperinsulinemia. These data are supported by research from the Hochgeschwender laboratory of the *Pomc*$^{-/-}$ mice on the 129 genetic background (Hochgeschwender, Costa et al. 2003). Evidence from the Palmiter laboratory suggests a tonic inhibitory role of NE and Epi on insulin secretion that is counteracted when glucose levels rise (Ste Marie and Palmiter 2003). It has been proposed that catecholamines inhibit both pancreatic β-cells directly and indirectly via inhibiting the stimulatory parasympathetic β-cell input. The loss of β-cell inhibition could explain the hyperinsulinemic phenotype in the *Pomc*$^{-/-}$ females that lack epinephrine. Furthermore, insulin release can be mediated by central POMC. Such inhibition was demonstrated by stereotaxic administration of the melanocortin receptor agonist MTII into the PVH resulting in a suppression of insulin release (Fan, Dinulescu et al. 2000). This insulin inhibition can be blocked with a non-specific α-adrenergic receptor anatagonist suggesting that this central POMC regulation of insulin release is through sympathetic input to the pancreas. The fact that the *Pomc*$^{-/-}$ mice endocrine abnormalities depicting type II diabetes develop in the absence of glucocorticoids suggests that glucocorticoids are not essential for the occurrence of type II diabetes. However, we know from

adrenalectomy studies that glucocorticoids play a role in the development of insulin resistance and thus glucocorticoids most likely expedite the development of this disease (Makimura, Mizuno et al. 2003). Furthermore, glucocorticoids are known mediators of metabolism and energy storage, and we hypothesize that the presence of glucocorticoids in the *Pomc*$^{-/-}$; Tg/+ mice accentuates both the obesity and progression of type II diabetes by decreasing the BMR. Interestingly, hyperglycemia is present in fasted *Pomc*$^{+/-}$; Tg/+ and *Pomc*$^{-/-}$; Tg/+ males but not females while glucose intolerance is much more pronounced in female *Pomc*$^{-/-}$; Tg/+ mice. The sexual dimorphisms in these mutant mice are most likely a result of sex steroids that have known opposing interactions on energy homeostasis (Machinal, Dieudonne et al. 1999; Anderson, McTernan et al. 2001; Dos Santos, Dieudonne et al. 2002).

5.3 *Conclusions*

In conclusion, we have demonstrated that central POMC is essential for normal energy balance and that the obesity phenotype in the *Pomc* null mice could not be rescued by genetically restoring pituitary POMC peptides. In fact, replacement of pituitary POMC augmented the obesity phenotype by a further suppression of basal metabolic rate. Restoration of pituitary ACTH and circulating glucocorticoids in the *Pomc* null background also resulted in more profound peripheral insulin resistance and glucose intolerance, the prime characteristics of type II diabetes mellitus. Both male and female *Pomc*$^{-/-}$; *Tg/+* mice had a three to four fold increase in circulating leptin levels

corresponding to their very pronounced obesity, further identifying hypothalamic POMC neurons as a critical substrate for the development and expression of leptin resistance.

CHAPTER V

TRANSGENIC REPLACEMENT OF PITUITARY POMC ON THE POMC NULL BACKGROUND UNCOVERS A MODULATORY ROLE FOR CENTRAL POMC NEURONS IN REGULATION OF THE HYPOTHALAMIC-PITUITARY-ADRENAL AXIS

1. ABSTRACT

Mice homozygous for a proopiomelanocortin null allele (*Pomc*$^{-/-}$) are devoid of all POMC peptides throughout development. Consequently, they have atrophic adrenal glands and undetectable corticosterone levels under basal or stressed conditions. More than half of the *Pomc*$^{-/-}$ offspring carried by heterozygous dams are stillborn or die within the first hours after birth. A selective transgenic rescue of pituitary POMC expression in the null background (*Pomc*$^{-/-}$; *Tg/+*) fully restored adrenal development and circulating corticosterone and prevented the characteristic partial perinatal lethality. However, adult male *Pomc*$^{-/-}$; *Tg/+* mice exhibited adrenal hypertrophy and elevated basal levels of both adrenocorticotrophic hormone (ACTH) and corticosterone (B) compared to their wild-type siblings. These mice responded to restraint stress with attenuated ACTH, but accentuated corticosterone release compared to control littermates. In contrast, female *Pomc*$^{-/-}$; *Tg/+* mice had normal basal and restraint-stress induced corticosterone levels compared to control littermates. Corticotropin-releasing hormone (CRH) synthesis and release from hypophyseotropic neurons in the paraventricular nucleus (PVN) of the hypothalamus are modulated by glucocorticoid feedback mechanisms. Therefore predictably, in the absence of circulating corticosterone, male *Pomc*$^{-/-}$ mice had very high CRH mRNA and immunoreactive peptide content in PVN neurons. CRH mRNA levels in the PVH of *Pomc*$^{-/-}$; *Tg/+* mice were significantly reduced compared to *Pomc*$^{-/-}$ mice, but remained inappropriately higher than control *Pomc*$^{+/+}$ mice in the context of their

elevated circulating corticosterone. Together these data indicate that the increased HPA axis activity of *Pomc*$^{-/-}$; *Tg/+* mice is driven primarily from the central nervous system and therefore suggest an important modulatory role for central POMC peptides in the set-point for hypothalamic CRH expression.

2. INTRODUCTION

The hypothalamic pituitary adrenal (HPA) axis is the biological core component utilized in response to external or internal stressors (Vale, Spiess et al. 1981). The PVN acts as a central nodal point for integrating stress levels and then releasing corticotropin-releasing hormone (CRH) from a subset of PVN parvicellular neurons onto the anterior pituitary gland. CRH stimulates anterior lobe POMC expressing corticotrophs that then produce and secrete adrenocorticotrophic hormone (ACTH). ACTH released into the peripheral circulation stimulates production and release of corticosterone from the outer cortical layer of the adrenal gland [reviewed in (Brown 1994)].

A disruption in the HPA axis has profound effects on the ability of an organism to respond to stressful stimuli. One example of HPA axis disruption is the mutant strain of mice deficient in CRH (*Crh* $^{-/-}$). Because of their glucocorticoid deficiency, homozygous pups exhibit defective surfactant production and lung maturation that leads to perinatal death (Muglia, Jacobson et al. 1995; Muglia, Bae et al. 1999). However, no developmental defects were noted in the hypothalamus, pituitary, or adrenal glands and replacement of glucocorticoids reverses the lung dysplasia and lethality in the *Crh*$^{-/-}$ pups. POMC was still expressed and ACTH content was at normal levels in the pituitary glands of these mutant mice, but the male adrenal glands and to a lesser extent the female adrenal glands exhibited a markedly atrophic zona fasciculata (ZF) (Muglia, Jacobson et al. 1995). The atrophic cortex in the *Crh*$^{-/-}$ mice is much like that seen in the *Pomc*$^{-/-}$ adrenals although the latter also have smaller medullary cores (Smart and Low 2003).

The *Crh*$^{-/-}$ mice have an impaired hormonal stress response however their behavioral stress response is normal (Venihaki and Majzoub 2002). One might predict similar phenotypes in the *Pomc*$^{-/-}$ mouse which lack circulating glucocorticoids.

In addition to the production of corticosterone, the adrenal gland also secretes steroid hormones aldosterone and androgens from the cortex and epinephrine from the medullary core. In humans, cortisol, the corticosterone equivalent, is required for life. However, *Pomc*$^{-/-}$ mice lacking corticosterone are viable yet only about half of the *Pomc*$^{-/-}$ offspring survive suggesting a perinatal role for adrenal steroids that could not be rescued with glucocorticoid replacement. We tested whether the transgenic replacement (*Tg/+*) of pituitary POMC in the *Pomc*$^{-/-}$ background was sufficient to rescue the HPA deficiencies or whether hypothalamic POMC was required for normal HPA function.

3. METHODS

Transgene construction and description. The construction and descritption of *pHalEx2* Tg* transgene (a pituitary only exprsessing POMC transgene) is detailed in the methods of chapter 4.

Care and use of animals. A colony of *Pomc* mutant mice was established as described previously (Smart and Low, 2003). Pituitary *Pomc-Tg* mice were generated by nuclear microinjection of the linearized, oligonucleotide marked *pHalEx2* Tg* into B6D2 F_2

hybrid embryos using standard techniques as described in Chapter IV. The *pHalEx2* Tg* allele was backcrossed to the C57BL/6 genetic background for two generations and subsequently crossed onto the *Pomc*$^{-/-}$ genetic background by an additional two generations of heterozygous matings. Therefore the hybrid genetic background of mutant mice used in these experiments was approximately 80% C57BL/6, 10% DBA/2, and 10% 129. Genotyping of offspring was done by PCR and described in the **Methods** of chapter IV. All animals for these studies were maintained under controlled temperature and photoperiod conditions (14 h of light, 10 h of dark; lights on, 0500 h) with food and water provided *ad libitum* except for overnight fasting studies. All surgical procedures and other experimental studies were approved by the Institutional Animal Care and Use Committee and followed the guidelines established by the Public Health Service.

Collection of plasma, brains and pituitaries for mRNA in situ hybridization and dissection of adrenal glands. Animals were individually housed for two days (allowing for cage habituation) before they were sacrificed between 0700 and 1000 during the light cycle. Mice were quickly removed from their cages and killed by decapitation in an adjacent room within 30 sec to obtain blood for measurement of basal, non-stressed hormone levels. Trunk blood was collected into tubes containing 10mM EDTA and the plasma immediately separated by centrifugation (12,000 rpm x 5 min), split into two tubes and frozen on dry ice for later analyses. Brains and pituitaries were immediately removed following decapitation. Brains were frozen in –78.5°C isopentane and stored at –80°C for later sectioning. Pituitaries were submersed in a cryomold containing OCT and frozen on dry ice. For restraint stress studies, mice were individually constrained

inside a 50 ml conical polypropylene tube for 20 minutes, and then killed by decapitation for trunk blood and tissue collection as described above. Adrenal glands of mice sacrificed at 6 to 8 months of age were completely dissected away from renal white fat and weighed wet as a pair.

Histology and immunohistochemistry. Histological analyses were performed on tissue fixed in 4% paraformaldehyde, and 50-μm sections of brain or agarose embedded pituitaries were collected with a vibratome. Free floating sections were incubated with 2% normal goat serum followed by incubation with a polyclonal rabbit anti-CRH primary antibody (1:4000, Peninsula, San Carlos CA) in KPBS buffer with 0.3% TritonX-100 at 4°C for 48 hr. The sections were then rinsed three times in KPBS and secondary biotinylated goat-antirabbit IgG was applied for 2 hours followed by an avidin-biotin horseradish peroxidase complex (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, California, USA) for 60 minutes. The reaction was revealed by the chromagen diaminobenzidine (DAB) containing 0.1% hydrogen peroxidase yielding a brown precipitate.

In situ hybridization. We used mRNA *in-situ* hybridization in F1 progeny, utilizing the inserted 23 bp oligo from the *pHal Ex2** transgene as a probe, to confirm that the transgene was expressed in a normal pattern in the pituitary but not the hypothalamus (data not shown). After crossing the transgene onto the POMC null background additional *in situ* hybridization studies were performed with a single stranded RNA probe. After linearization with NcoI, sense and antisense ^{35}S-labeled cRNA probes were

generated using T3 and T7 polymerase from a mouse *Pomc* partial exon3 clone (m*Pomc* Ex3 ribo). The probe is 700bp in length and is contained in the coding region of the *Pomc* gene that was subcloned into pGEM-7. The antisense probe recognizes both wild type and pHal Ex2* transgenic POMC mRNA and the POMC sense probe was used as a control. The POMC probes were separated from unincorporated nucleotides over NICK™ columns [Sephadex™ G-50 DNA Grade (Amersham Parmacia Biotech, Piscataway, NJ)] and stored in 1ml aliquots (5-10 million cpm/ml) of hybridization buffer (66% Formamide; 260mM NaCl; 1.318X Denhardt's solution; 13.16 mM Tris pH 8.0; 13.15% Dextran Sulfate). Slides with 16 μm coronal sections from cryostat sectioning were hybridized at 55°C-60°C for 24h. After hybridization, slides were washed four times with 4XSCC, RNase digested (30 min), rinsed again in a series of SSC & dithiothriol (DTT), dehydrated in a series of EtOH and vacuum dried and then placed on 8"X10" card for film exposure. CRH sense and antisense probes were made identical to that of the POMC probes using a 578 bp PstI/PstI DNA fragment that is contained in exon 2 of the *Crh* gene (gift of Dr. Audrey Seasholtz). CRH mRNA *in situ* hybridization was performed identically and in parallel to the POMC mRNA *in situ* hybridizations. Slides were always exposed with [^{14}C]-microscale slides to assure that the exposure time was in the linear range of the film or cassette (*in situ* procedure described in (Lechan, Wu et al. 1986; Fremeau, Autelitano et al. 1989)). Slides containing coronal brain sections were exposed overnight to a phosphor-imaging cassette. Slides containing pituitary sections were exposed to one-sided emulsion film (Biomax MR, Kodak) overnight. Film was then scanned at a resolution and density of 1200 pixels. Scanned images were analyzed using IP-Lab Gel and NIH image by quantifying a selected area that remained

constant from section to section and easily contained nuclei of interest. Background hybridization was quantified from a section not containing any visible hybridization using the same area size and was then used for subtracting background hybridization from analyzed sections. mRNA in the ArcN was analyzed on the three most robust sections from 4 mice per genotype. Total pituitary POMC mRNA was analyzed from the three most robust pituitary sections from 3 to 4 mice per genotype. CRH mRNA in the PVN was analyzed on the three most robust sections from 4 mice per genotype.

Hormone assays. Hormone levels were measured in EDTA-plasma using a double antibody ACTH IRMA kit (Nichol's Diagnostic Institute) and a single antibody corticosterone RIA kit (ICN). Assays were carried out according to the manufacturers' instructions with the exception that plasma samples from *Pomc*$^{-/-}$ mice were doubled in volume to increase the sensitivity of the kits by two-fold in an attempt to measure hormonal concentrations expected to be below the standard curve. After this adjustment, samples from *Pomc*$^{-/-}$ mice were still below the minimum detection level of the kits.

Statistics. Data were analyzed by 2-way ANOVA with genotype and sex as independent variables using Stat View Power PC version for Macintosh (version 5.0.1; SAS Institute Inc., Cary, North Carolina, USA) except where otherwise stated. *Post hoc* comparisons between groups were performed by Fisher's (PLSD), and P values < 0.05 were considered significant.

4. RESULTS

4.1 *Perinatal lethality was absent and adrenal gland size was restored in Pomc$^{-/-}$; Tg/+ pups*

Matings of compound heterozygous/hemizygous (*Pomc*$^{+/-}$; *Tg/+*) breeder pairs produced the expected Mendelian frequency of offspring with the exception of *Pomc*$^{-/-}$ pups. Significantly decreased numbers of the latter genotype compared to the 25% expected frequency has previously been reported (Yaswen, Diehl et al. 1999; Smart and Low 2003). There was a striking absence of perinatal lethality in the *Pomc*$^{-/-}$; *Tg/+* pups compared to *Pomc*$^{-/-}$ pups (Figure 1). Adrenal glands were removed from 6-8 month old male and female mice and wet weights of adrenal pairs were recorded (Figure 2). A marked sexual dimorphism was apparent in the *Pomc*$^{+/+}$, *Pomc*$^{+/+}$;*Tg/+*, *Pomc*$^{+/-}$, and *Pomc*$^{+/-}$;*Tg/+* groups of mice with the female glands approximately twice as large as their male counterparts. *Pomc*$^{-/-}$ mice of both sexes had atrophic adrenal glands of similar low weight. Pituitary POMC replacement in the *Pomc*$^{-/-}$; *Tg/+* mice resulted in enlarged adrenal glands in both sexes. This hypertrophy as a percentage of wild type controls was more dramatic in males than females, reflecting the fact that wild type female adrenal glands are normally larger then their male counterparts.

Figure 1

Offspring numbers along with the predicted and actual percentages of pups for each genotype from the first 73 pups born to the following breeder pairs: homozygous *Pomc*$^{-/-}$; +/+ males X heterozygous *Pomc*$^{+/-}$; *Tg*/+ females.

Figure 1

Breeder Pair:	(-/-) X (+/-)/(Tg/+)			
Offspring:	(+/-)/(+/+)	(+/-)/(Tg/+)	(-/-)/(+/+)	(-/-)/(Tg/+)
Number	23	26	2	22
Predicted (%)	25	25	25	25
Actual (%)	31	36	3	30

Figure 2

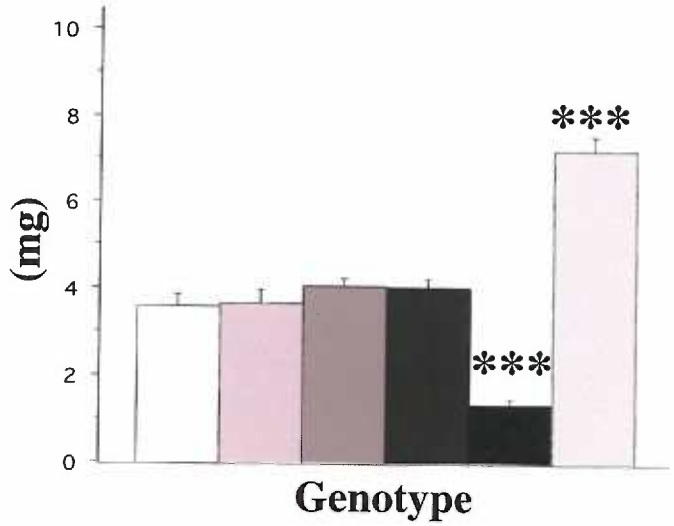
Adrenal gland weights of male and female *Pomc*$^{+/+}$, *Pomc*$^{+/-}$, *Pomc*$^{-/-}$ mice with and without the pHal Ex2* transgene. Adrenal glands were weighed as a pair at nine months of age (n=5-16). Data are reported as the mean ± SEM. There were significant main effects of genotype for male ($F_{5,68} = 42.5$, $p < 0.0001$) and female ($F_{5,59} = 32.0$, $p < 0.0001$) mice.

** $p<0.01$; *** $p<0.001$ compared to (*Pomc*$^{+/+}$; +/+), Fisher's PLSD.

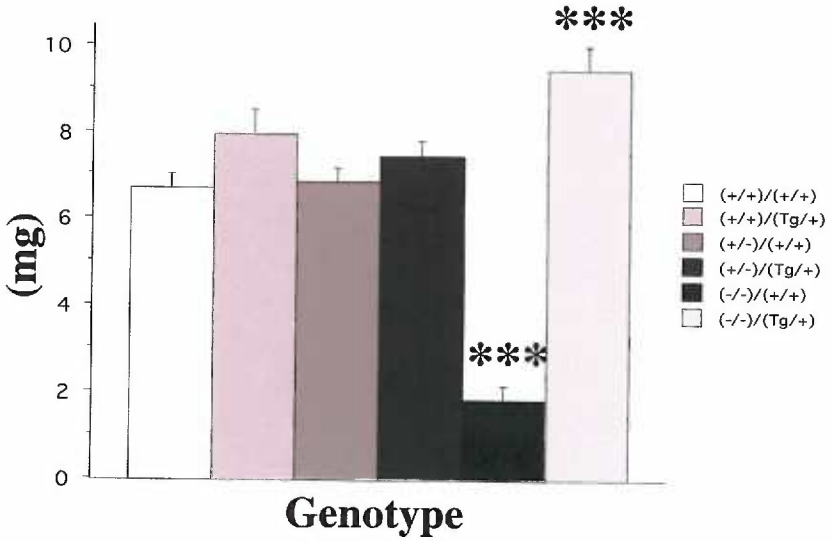
Figure 2

Adrenal gland size

Male



Female



4.2 *mRNA levels for Pomc mRNA in hypothalami and pituitary glands*

We performed mRNA *in situ* hybridizations on cryostat sectioned pituitaries and hypothalami to further confirm that the *pHalEx2** transgene selectively rescued POMC expression in the pituitary gland. It was possible to directly compare the levels of mRNA transcribed from the *pHalEx2** transgene and the endogenous *Pomc* alleles because the mouse *Pomc* exon 3 riboprobe hybridizes equally with the two mRNA species, which differ only in exon 2 sequences. The *pHal Ex2** transgene successfully rescued POMC mRNA expression in both the anterior and intermediate lobes of the pituitary gland but not the arcuate nucleus of the hypothalamus (Figure 3). Qualitatively, the distribution of *Pomc* mRNA throughout the anterior lobes of $Pomc^{-/-}$; *Tg/+* mice was comparable to wild type mice and to the expression patterns of previously reported transgene constructions containing analogous *Pomc* promoter regions (Hammer, Fairchild-Huntress et al. 1990; Rubinstein, Mortrud et al. 1993; Young, Otero et al. 1998; Low, Rubinstein et al. 2001). Quantitation of total *Pomc* mRNA hybridization signal in both pituitary lobes revealed no gross differences among any of the genotypes with the exception of a complete absence of specific signal in the $Pomc^{-/-}$ mice (Table 1). Keeping in mind that small differences may not be detected in the mRNA *in situ* hybridization assay, these data strongly suggest that expression from the *pHal Ex2** transgene and endogenous *Pomc* gene must be balanced by the same regulatory mechanisms to account for the absence of an additive or gain-of-function effect on *Pomc* mRNA levels in the compound $Pomc^{+/+}$; *Tg/+* mice and, conversely, normal *Pomc* mRNA levels in the compound $Pomc^{-/-}$; *Tg/+* mice.

Figure 3

POMC mRNA *in-situ* hybridization in 30 μm cryostat coronal sections of $Pomc^{+/+}$; $Pomc^{+/-}$; and $Pomc^{-/-}$ male mice showing normal POMC mRNA expression in $Pomc^{+/+}$ pituitary and hypothalamus (**A,D**); no expression in $Pomc^{-/-}$ mice in either pituitary or hypothalami (**B,E**); and rescued pituitary POMC in $Pomc^{-/-}$;Tg/+ mice but still no expression in the hypothalamus (**C,F**).

Figure 3

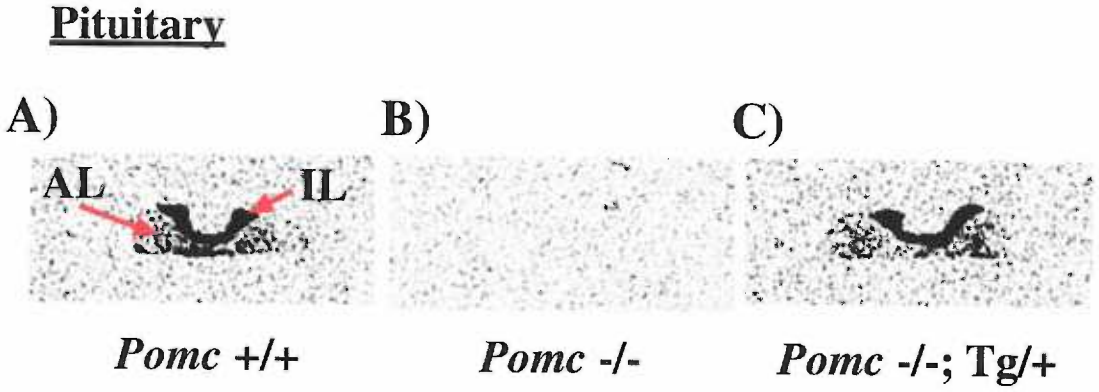

Pituitary
A)
B)
C)
AL
IL
Pomc +/+
Pomc -/-
Pomc -/-; Tg/+
Hypothalamus
D)
E)
F)
ArcN
Pomc +/+
Pomc -/-
Pomc -/-; Tg/+

Table 1

Basal POMC and CRH mRNA Levels

Genotype	ArcN (POMC mRNA)	Pituitary (POMC mRNA)	PVN (CRH mRNA)
Pomc$^{+/+}$; +/+	1050 ± 27	81359 ± 10167	646 ± 18
Pomc$^{+/+}$; *Tg*/+	1346 ± 477	51500 ± 3993	657 ± 77
Pomc$^{+/-}$; +/+	894 ± 162	59887 ± 8575	972 ± 146
Pomc$^{+/-}$; *Tg*/+	951 ± 92	87886 ± 8043	906 ± 77
Pomc$^{-/-}$; +/+	22.7 ± 2.0 [a]	109 ± 14.7 [a]	4238 ± 416 [a]
Pomc$^{-/-}$; *Tg*/+	20.6 ± 3.8 [a]	62108 ± 5277	1263 ± 350

[a] $p < 0.0001$ compared to (*Pomc*$^{+/+}$; +/+), Fisher's PLSD.

Quantification of mRNA *in situ* hybridization for male mice hypothalamic and pituitary POMC along with CRH mRNA levels in the PVN. Data are reported as the mean ± SEM.

4.3 *Basal HPA activity: ACTH and corticosterone hormone levels*

Male and female mice' basal non-stressed hormone levels were measured from EDTA-plasma of all six genotypes. Both male and female *Pomc*$^{-/-}$ mice had undetectable circulating levels of ACTH (as previously reported) while *Pomc*$^{-/-}$; *Tg/+* mice had elevated basal ACTH levels compared to control *Pomc*$^{+/+}$ mice (Figure 4A,B). The significantly elevated basal ACTH levels in the male *Pomc*$^{-/-}$; *Tg/+* mice were paralleled by a similar four-fold elevation in basal plasma B levels (Figure 4C). However, in female *Pomc*$^{-/-}$; *Tg/+* mice there was only a trend towards higher basal ACTH levels and basal plasma corticosterone levels were the same as wild type controls (Figure 4D).

4.4 *Restraint stress: ACTH and corticosterone hormone levels*

Mice of all genotypes except *Pomc*$^{-/-}$ responded briskly with elevated ACTH and corticosterone levels in response to an acute 20 min restraint stress compared to their corresponding basal, non-stressed levels. However, the magnitude of the ACTH stress response was blunted in both male and female *Pomc*$^{-/-}$; Tg/+ mice compared to wild type mice and the other responding groups (Figure 5A,B). Corresponding measurements of corticosterone revealed significantly greater elevations in male *Pomc*$^{-/-}$; Tg/+ mice compared to the stress response of other genotypes, while the female *Pomc*$^{-/-}$; Tg/+ mice had comparable elevations with the other genotypes of females (Figure 5C,D).

Figure 4

Basal HPA activity in male and female mice of all six genotypes. Mice were killed between 08:00 and 09:00 AM during there most inactive time after being individually housed for 3 days prior to being sacrificed. (**A,D**) The *pHal Ex2** transgene restored circulating ACTH and B in the *Pomc*$^{-/-}$ mice. (**A,C**) However, basal ACTH and B levels were significantly higher in male *Pomc*$^{-/-}$; Tg/+ mice then their wild type siblings (**B**) with only a higher trend in just ACTH levels in female *Pomc*$^{-/-}$; Tg/+ mice (n=3-8). Data are reported as the mean ± SEM. Main effects for genotype on basal plasma ACTH and corticosterone in males ($F_{5,25}$ = 5.6, $p < 0.0014$), ($F_{5,25}$ = 8.6, $p < 0.0001$) and females ($F_{5,27}$ = 2.8, $p < 0.0386$), ($F_{5,27}$ = 2.1, $p < 0.1022$), respectively. * $p< 0.01$; ** $p< 0.001$; *** $p< 0.0001$ compared to (*Pomc*$^{+/+}$; +/+), Fisher's PLSD.

Figure 4

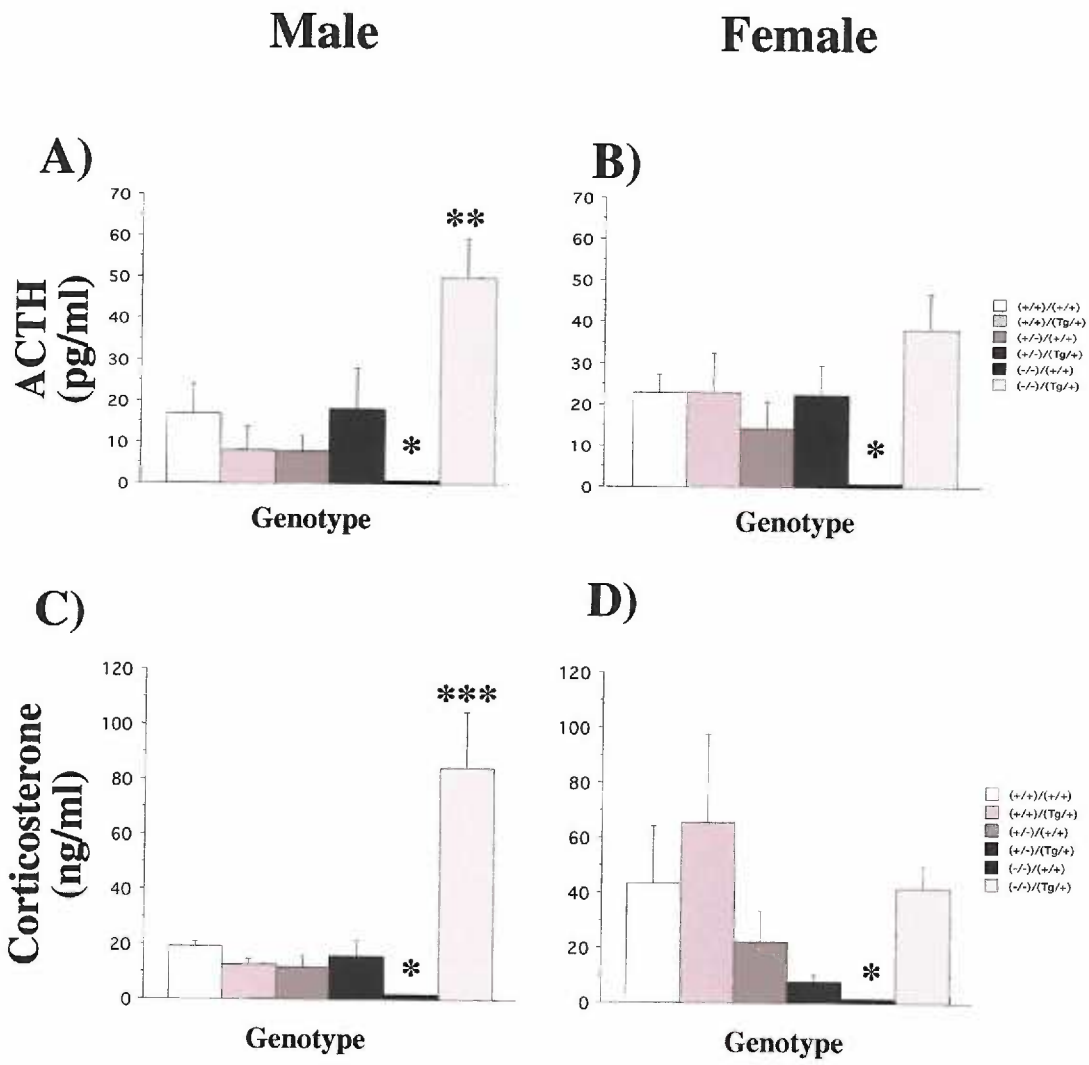
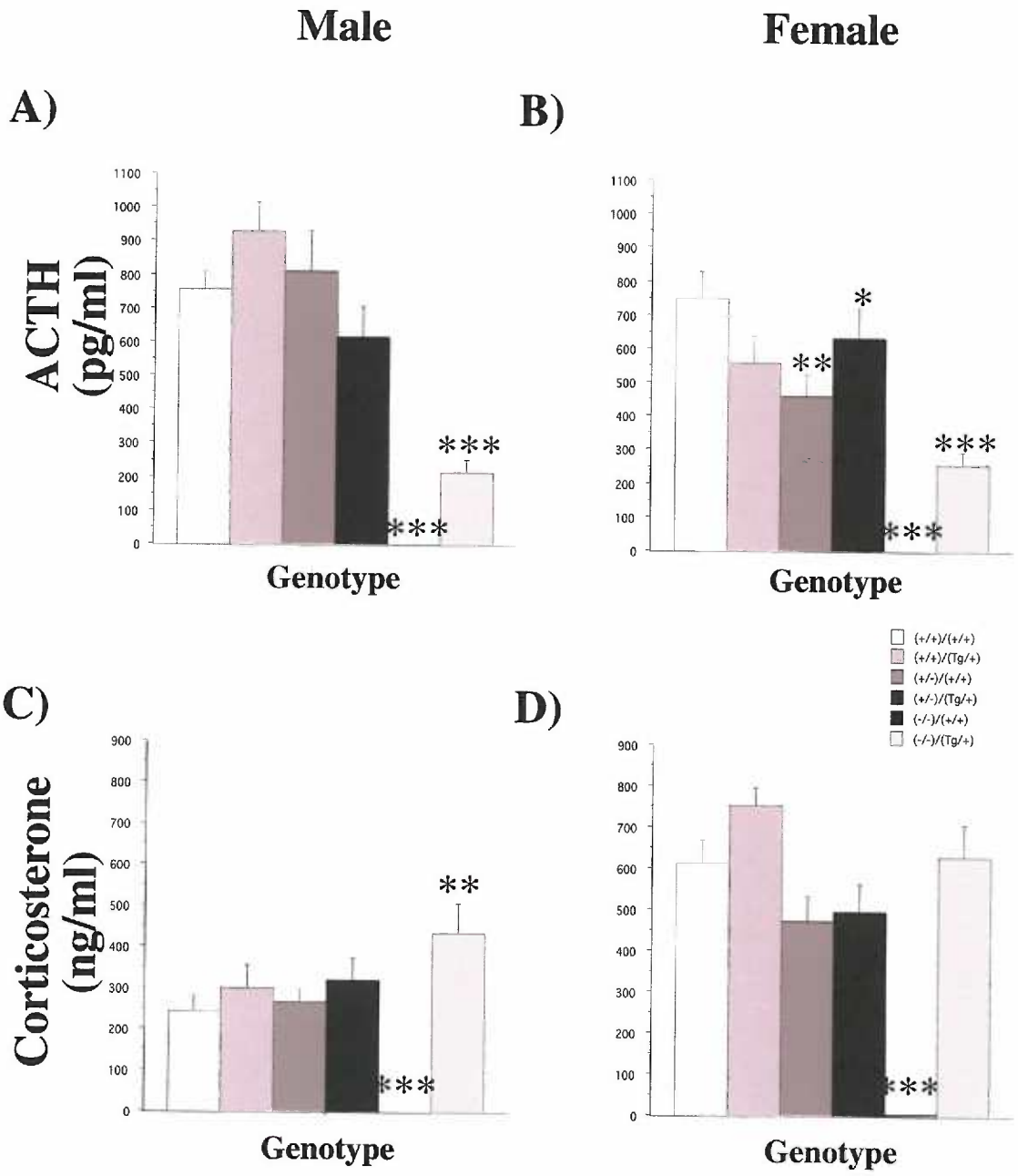

Male
Female
A)
B)
C)
D)
ACTH (pg/ml)
Corticosterone (ng/ml)
Genotype
(+/+)/(+/+)
(+/+)/(Tg/+)
(+/-)/(+/+)
(+/-)/(Tg/+)
(-/-)/(+/+)
(-/-)/(Tg/+)

Figure 5

HPA activity in male and female mice following 20 minutes of restraint stress. Mice were killed after a 20-minute restraint stress conducted during the light cycle. (**A**,**C**) ACTH levels were significantly lower while B levels were significantly higher in male *Pomc*$^{-/-}$; Tg/+ mice as compared with their wild type siblings. (**B,D**) Female *Pomc*$^{-/-}$; Tg/+ mice showed a blunted ACTH response to the 20 minute stress while there were no differences in circulating B levels in the female *Pomc*$^{-/-}$; Tg/+ mice (n=2 to 10). Data are reported as the mean ± SEM. Main effects for genotype on stressed ACTH and corticosterone levels in males ($F_{5,31} = 9.0$, $p < 0.0001$), ($F_{5,31} = 4.0$, $p < 0.0063$), and females ($F_{5,38} = 16.1$, $p < 0.0001$), ($F_{5,38} = 14.6$, $p < 0.0001$), respectively. * $p< 0.01$; ** $p< 0.001$; *** $p< 0.0001$ compared to (*Pomc*$^{+/+}$; +/+), Fisher's PLSD.

Figure 5


Male
Female
A)
B)
ACTH (pg/ml)
1100
1000
900
800
700
600
500
400
300
200
100
0
***
***
*
**
***
Genotype
C)
D)
Corticosterone (ng/ml)
900
800
700
600
500
400
300
200
100
0
**
***
***
Genotype
(+/+)/(+/+)
(+/+)/(Tg/+)
(+/-)/(+/+)
(+/-)/(Tg/+)
(-/-)/(+/+)
(-/-)/(Tg/+)

4.5 *CRH mRNA levels in the PVN were elevated in Pomc $^{-/-}$ mice and not fully suppressed in Pomc $^{-/-}$; Tg/+ mice*

Negative feedback from the periphery onto the CNS is essential for regulation of the HPA axis [reviewed in (De Kloet, Vreugdenhil et al. 1998)]. Predictably therefore, *Pomc*$^{-/-}$ mice lacking glucocorticoid feedback had markedly elevated steady-state CRH mRNA levels in the hypothalamic PVN (Figure 6). However, male *Pomc*$^{-/-}$; *Tg/+* mice with elevated plasma corticosterone had CRH mRNA levels that were two fold higher than wild type levels (Table 1) contrary to expectations that CRH expression would be suppressed by the glucocorticoids (Jingami, Matsukura et al. 1985). Although not statistically significant with the sample size used, heterozygous *Pomc*$^{+/-}$ mice had hypothalamic CRH mRNA levels that were intermediate to that of wild type and *Pomc*$^{-/-}$; *Tg/+* mice suggesting a mild gene dosage effect (Table 1).

Due to the stimulatory effects of glucocorticoids on CRH in the central nucleus of the amygdala (CeA) as opposed to the inhibitory effects of glucocortiocids on CRH in the PVN, we did observe mRNA levels in brain sections corresponding to the increase or decrease in plasma glucocorticoid levels that reflected this phenomenom. Thus, CRH expression in the CeA displayed a pattern opposite to that of CRH expression in the PVN of corresponding brain sections. CRH mRNA levels were normal in the CeA of *Pomc*$^{+/+}$ mice although relatively higher then that seen in the PVN of *Pomc*$^{+/+}$ mice (Figure 6A), CRH mRNA levels were suppressed in the CeA of *Pomc*$^{-/-}$ mice lacking glucocorticoids

(Figure 6B), and although not quantified CRH mRNA in the CeA appears restored to normal levels in the CeA of *Pomc*$^{-/-}$; *Tg/+* mice (Figure 6C).

Immunocytochemical analysis of CRH in the PVN paralleled the results of CRH mRNA *in situ* hybridization. As expected without colchicine pretreatment to inhibit axonal transport of peptides, there was almost no immunoreactive CRH detected in cell bodies of wild type *Pomc*$^{+/+}$ mice, but positive neuronal processes were present (Figure 6D). In contrast, both CRH-immunoreactive soma and processes were abundant and strongly labeled in the PVN of *Pomc*$^{-/-}$ mice (Figure 6E). Although not quantitative, there appeared to be more cell-body staining of CRH neurons in the *Pomc*$^{-/-}$; *Tg/+* mice compared to control, although clearly less than in the *Pomc*$^{-/-}$ mice (Figure 6F).

We next tested whether a selective absence of ß-endorphin, accompanied by normal levels of the melanocortin peptides encoded by POMC, would recapitulate the observed alteration of CRH expression in the PVN of *Pomc*$^{-/-}$; *Tg/+* mice lacking all central POMC peptides. Quantitative in situ hybridization for CRH was performed in C57BL/6 congenic male wild type and ß-endorphin-deficient mice (Rubinstein, Mogil et al. 1996). These mutant mice have previously been demonstrated to have normal adrenal gland size and normal ACTH and corticosterone levels. Steady-state levels of CRH mRNA were also identical between the wild type [429 ± 36 units (mean ± SEM, n = 6)] and ß-endorphin-deficient [444 ± 19 units (n = 5)] animals.

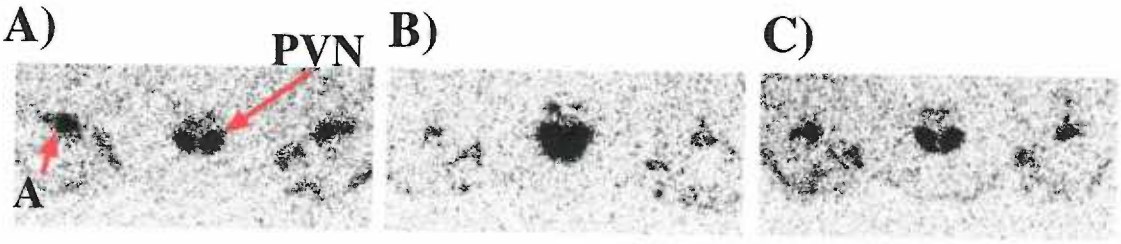
Figure 6

CRH mRNA *in-situ* hybridization and immunocytochemistry for CRH peptide in 30 μm cryostat coronal sections of $Pomc^{+/+}$; $Pomc^{+/-}$; and $Pomc^{-/-}$ male mice showing up regulated CRH peptide in $Pomc^{-/-}$ mice. **(A,C)** Hypothalamic sections showing CRH mRNA expression in the PVN and amygdale. **(D,F)** ICC of hypothalami shows very robust CRH staining without any colchicines treatments. **PVN**=paraventricular Nucleus; **A**=amygdale; **3V**=third ventricle

Figure 6

CRH mRNA In Situ Hybridization

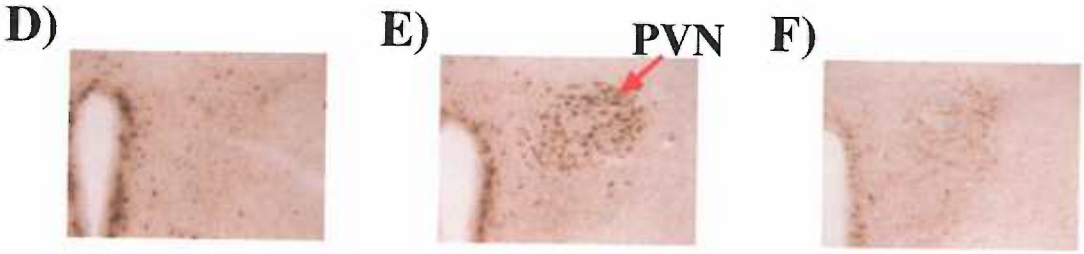
Hypothalamus



Pomc +/+ *Pomc* -/- *Pomc* -/-; Tg/+

CRH ICC

Hypothalamus



Pomc +/+ *Pomc* -/- *Pomc* -/-; Tg/+

5. DISCUSSION

It has long been hypothesized that POMC peptides play a critical role in the development and maturation of the adrenal gland, but whether these peptides were hypothalamic or pituitary derived was unknown (Estivariz, Iturriza et al. 1982; Estivariz, Carino et al. 1988; Estivariz, Morano et al. 1988; Bicknell, Lomthaisong et al. 2001). Mice deficient in all POMC peptides have atrophic, ACTH non-responsive adrenals (Yaswen, Diehl et al. 1999; Smart and Low 2003). The fact that replacing pituitary POMC onto the null background rescued adrenal size and the ability to secrete corticosterone established that pituitary POMC was sufficient for adrenal development and maturation. The *Pomc*$^{-/-}$; *Tg/+* mice adrenal glands were significantly larger then wild type siblings, yet this finding is not surprising, given that these mice had elevated basal corticosterone levels. It has long been known that humans with hypercortisolemia and rodents with chronic ACTH stimulation develop hyperplasic adrenal glands (Kovacs, Horvath et al. 1977; Carey, Varma et al. 1984; Gertz, Contreras et al. 1987). Additional studies will be needed to determine if the *Pomc*$^{-/-}$; *Tg/+* mice adrenal glands increase in size is due to hyperplasia or hypertrophy.

Unlike the *CRH*$^{-/-}$ mice, replacement of glucocorticoids via the mother's drinking water was unable to rescue the perinatal lethality in *Pomc*$^{-/-}$ pups born to heterozygous mothers (Muglia, Jacobson et al. 1995; Smart and Low 2003). *Pomc*$^{-/-}$ pups that carry the *pHal Ex2** transgene, born also to heterozygous mothers, do not exhibit this perinatal lethality, as demonstrated by normal Mendelian offspring numbers of *Pomc*$^{-/-}$; *Tg/+* pups. These

data, in combination with data from various catecholamine-deficient mice, have led us to hypothesize that the perinatal lethality in *Pomc*$^{-/-}$ pups is due to the lack of non-glucocorticoid adrenal hormones (Kobayashi, Morita et al. 1995; Thomas, Matsumoto et al. 1995; Zhou, Quaife et al. 1995). The most likely adrenal candidate is epinephrine, which is undetectable in the *Pomc*$^{-/-}$ mice (Yaswen, Diehl et al. 1999). When catecholamine-deficient mice were given isoproterenol, a β-adrenergic receptor (β-AR) agonist, via their mother's drinking water, the number of catecholamine-deficient pups surviving birth regained Mendelian proportions (Portbury, Chandra et al. 2003). During birth, pups moving through the birth canal experience various degrees of hypoxia that can lead to bradycardia. The current belief is that catecholamines, which increase during birth, protect the fetal heart during episodes of hypoxia that commonly occurs during labor (Slotkin and Seidler 1988; Portbury, Chandra et al. 2003). We hypothesize that the perinatal lethality that was seen in the *Pomc*$^{-/-}$ pups is due to the lack of epinephrine in these mice (Figure 1).

Although replacing pituitary POMC rescued some of the phenotypes in the *Pomc*$^{-/-}$ mice, analyses of the stress hormones under basal and acute stress conditions revealed an altered HPA axis when compared to wild type mice. Basal levels of ACTH and corticostrone (B) were elevated in male *Pomc*$^{-/-}$; *Tg/+* mice despite normal pituitary POMC mRNA. However, studies with *Crh*$^{-/-}$ mice have shown that changes in pituitary POMC mRNA levels are not necessarily mirrored by plasma ACTH concentrations but that plasma ACTH levels depend more on CRH stimulation of ACTH release from corticotrophs then from an increase of POMC mRNA levels (Jacobson, Akana et al.

1989; Muglia, Jacobson et al. 2000). Thus, the elevated basal CRH mRNA levels in the *Pomc*$^{-/-}$; *Tg/+* mice may stimulate a greater release of ACTH from corticotrophs rather then an increase in POMC mRNA. An increase in CRH stimulated ACTH release would explain the observed increase in basal plasma ACTH despite no gross differences in pituitary POMC mRNA.

The fact that we saw high levels of CRH mRNA in the face of elevated B suggests that corticosterone's ability to inhibit CRH mRNA levels in the brain has been disrupted and/or circumvented in the *Pomc*$^{-/-}$; *Tg/+* mice. Evidence from the Dallman laboratory indicates that corticosterone does not directly inhibit CRH levels in the PVN, and in fact when B was given icv it stimulated CRH mRNA levels in the PVN (Dallman, Akana et al. 2003). As to how B inhibits CRH in the PVN remains unknown, but our data identifies central POMC as a component of B's inhibitory effects on hypothalamic CRH levels. A direct projection of POMC neurons onto hypothalamic CRH neurons has recently been established and the effects of melanocortin peptide agonists on hypothalamic CRH expression as been stimulatory (Lu, Barsh et al. 2003). Pharmacological studies of μ-receptor agonists given centrally have had mixed results (Buckingham 1986; Buckingham and Cooper 1986; Buckingham and Cooper 1986; Tsagarakis, Rees et al. 1990; Hellbach, Gartner et al. 1998). MC4-receptors have been localized on CRH neurons along with autoradiographic evidence for the presence of μ-receptors in the hypothalamic PVN, and thus CRH regulation by POMC neurons is most likely a complex integration of expression, trafficking, and release of both melanocortins and β-endorphin that may have opposing effects (Lu, Barsh et al. 2003).

An alternative hypothesis is that the loss of central POMC has removed an indirect input onto CRH neurons rather then a direct input. Immunohistochemical analysis revealed α-MSH immunoreactivity in the dorsal raphe, a predominantly serotonergic nucleus (O'Donohue, Miller et al. 1979; Khachaturian, Lewis et al. 1984; Leger, Bonnet et al. 1994). In addition, CRH neurons also send projections to Raphe nuclei in the midbrain stimulating these serotonergic neurons. Furthermore, serotonergic neurons that innervate neuroendocrine control regions in both the hypothalamic paraventricular nucleus (Petrov, Krukoff et al. 1994) and arcuate nucleus inhibit feeding (Heisler, Cowley et al. 2003). This CRH serotonergic circuitry may explain the absence of hyperphagia in the *Pomc*$^{-/-}$ mice (described in chapter IV) due to their excessive CRH levels in the PVN. Serotonergic neurons in the dorsal Raphe nucleus also send projections back onto the hypothalamic PVN, and activation of the serotonergic receptor 5-HT_{2A} lead to CRH release and increased plasma ACTH levels (Van de Kar, Javed et al. 2001). Inhibition of these serotonergic neurons by POMC peptides would remove the serotonergic stimulation of CRH in the PVN, and providing an indirect regulation of CRH by POMC via the dorsal Raphe nucleus.

Although our data reveals a role of central POMC in the regulation of hypothalamic CRH, we cannot exclude other potential peripheral signals that may be altering hypothalamic CRH. For instance, the gut peptides like neuromedin U (NMU) and ghrelin injected into the PVN alter feeding, and *in vitro* experiments with hypothalamic explants revealed CRH stimulated secretion by NMU and ghrelin (Wren, Small et al.

2002; Wren, Small et al. 2002). Additional studies would be intriguing and are needed in understanding of such peripheral signals that may also affect the hypothalamic circuitry in our mouse models.

In conclusion, the present study emphasizes the importance of both hypothalamic and pituitary POMC in a normal functioning HPA axis. Our observations provide direct evidence that the lack central POMC disrupts the normal corticosterone-dependent inhibition of hypothalamic CRH. Furthermore, pituitary POMC plays an essential role in the survivability of pups during the perinatal period.

SUMMARY AND CONCLUSIONS

My hypotheses were that hypothalamic POMC peptides play essential roles in regulating energy homeostasis while pituitary POMC peptides stimulate glucocorticoid and melanin production, and furthermore that these distinct physiological functions cannot be compensated by POMC peptides from the other cell types. Because the POMC prohormone is selectively processed into multiple bioactive peptides in hypothalamic neurons and pituitary endocrine cells in a dissimilar manner, deciphering the physiological functions of these peptides in separate cell types was challenging. The goal of my studies was to selectively remove POMC peptides from one group of cells while leaving normal expression of POMC peptides in the other cells. Using murine models that were either ubiquitously deficient in POMC peptides or were only deficient in hypothalamic POMC, I was able to assess and compare loss of function phenotypes in the two models. My analyses have allowed me to conclude that hypothalamic POMC is a critical component in the regulation of energy homeostasis within the CNS that cannot be compensated by pituitary POMC peptides. In fact, replacement of pituitary POMC augmented the obesity phenotype by a further suppression of basal metabolic rate. Furthermore, mice expressing pituitary POMC and lacking hypothalamic POMC had more pronounced characteristics of type II diabetes mellitus then their ubiquitous POMC deficient siblings. Collectively, these data emphasize the interactions and communication that can occur between the CNS and periphery, and it's these interactions that need to be assessed when evaluating mouse models that have tissue specific deletions of specific genes.

The weight phenotypes in our mouse models revealed that normal energy homeostasis occurs only when central and peripheral POMC systems are intact. The same is true regarding the need for both hypothalamic and pituitary POMC for a normal stress response. My data reinforce previous studies showing that the loss of pituitary POMC results in an abnormal stress response, however this thesis provides novel evidence that not only pituitary POMC but also hypothalamic POMC is needed for a normal stress response. Our analyses have provided new insight into the hypothalamic regulation of CRH. In mice lacking central POMC, we have shown CRH is not down regulated in the presence of elevated circulating corticosterone as would be predicted. The disruption of normal hypothalamic circuitry has led to an abnormal hypothalamic-pituitary-adrenal axis even after pituitary POMC has been transgenically replaced. These data identified central POMC as a regulating component of the classical stress-axis. This thesis emphasizes the importance of both hypothalamic and pituitary POMC in a normal functioning HPA axis, however further genetic, pharmacological, and anatomical experiments are required to unveil the mechanism and circuitry underlying hypothalamic POMC regulation of CRH.

We don't know why hypothalamic POMC regulates CRH expression. One hypothesis is that during a mammal's fed state hypothalamic POMC expression increases not only to decrease food intake but also to directly or indirectly down regulate hypothalamic CRH. CRH down regulation in the PVN by POMC during the fed state may communicate that the mouse is not stressed due to lack of food as opposed to a mouse that would be

extremely stressed in a fasted condition. However, future experiments including i.c.v. injections of POMC agonists in the null rescued animals are necessary to test the above hypothesis.

The generation of mice lacking only central POMC has not only further defined the biological roles of POMC, but has also definitively identified which POMC cells carry out these biological functions. We accomplished this by comparing wild type mice, ubiquitous deleted POMC mice, and CNS only deleted POMC mice under identical experimental paradigms. The data obtained have led us to conclude that the anorexogenic effects of POMC are a function of hypothalamic POMC neurons. Our data have thus challenged previous claims that α-MSH may be acting as a peripheral anorexigenic agent (Yaswen, Diehl et al. 1999). Although our data do not conclusively exclude peripheral α-MSH as a lipolytic peptide, the fact that mice lacking only central POMC peptides have a greater white fat mass than mice lacking both central and peripheral POMC peptides argues against α-MSH having a peripheral lipolytic role. An alternative explanation is that the presence of glucocorticoids in the pituitary rescued POMC null (*Pomc*$^{-/-}$; *Tg/+*) mice masks any putative lipolytic effects of pituitary α-MSH. Although we uncovered no evidence indicating that α-MSH induced lypolysis, peripheral α-MSH may have altered the partitioning of energy stores due to the differences in white fat accumulation in mice with or without peripheral α-MSH. To test such a hypothesis one would need to selectively remove α-MSH without removing the other POMC peptides and circulating glucocorticoids.

While our data suggest that α-MSH is not a lipolytic agent, we knew from previous studies that α-MSH is a potent agonist for the MC1-R. *In vitro* stimulation of this receptor by α-MSH leads to an increase in adenylyl cyclase and the downstream signal transduction pathway initiating the production of eumelanin pigment. Because the *in vitro* antagonism of MC1-R blocks adenylyl cyclase production, and the *in vivo* antagonism of MC1-R on melanocytes results in the conversion of eumelanin to phaeomelanin production, we were surprised to find that mice lacking the endogenous agonist α-MSH had a uniform black coat color on the agouti-less C57Bl/6 genetic background. Our data have led us to conclude that in the C57BL/6 genetic background the endogenous MC1-R has sufficient basal activity to stimulate eumelanin production in the absence of α-MSH. Alternatively, one cannot rule out the existence of an additional unidentified endogenous agonist. A detailed histological and biochemical analyses of both skin and hair from our mouse models is pending. These data will help to identify any subtle difference in the skin and/or hair that was not apparent by visual observation.

The homeostatic roles of the POMC peptides described within this thesis emphasize the importance of appropriate *POMC* gene regulation within an organism. Using cosmid clones from genomic mouse DNA containing the *Pomc* gene, a variety of DNA reporter constructs were designed to express EGFP under the transcriptional control of the POMC promoter. These reporter constructs containing deleted distal sequences have identified DNA flanking regions containing *cis* elements that regulate neuronal versus pituitary expression of the *Pomc* gene. These constructs have helped us successfully map the essential neuronal *cis* elements for POMC expression to a region –10.5 to –9.0 kb from

the POMC promoter. Sequencing of this region will be essential for identifying known or novel transcriptional elements regulating POMC gene expression. Furthermore, these DNA sequences will be used to determine what *trans* elements bind to these regions, and thus further our understanding of POMC gene transcription. These putative *cis* and *trans* elements would be key molecular targets for understanding physiological events such as leptin desensitization, age-related obesity and age-related decreases in basal metabolic rates. Removal of these elements from a mouse could possibly recapitulate some or all of the phenotypes seen in our POMC mutants, and thus highlight pharmacological targets for therapeutic treatment of endocrine disorders like obesity and diabetes.

A large portion of this thesis centers on the role of POMC in relation to energy homeostasis. We validate our studies along with realizing the necessity to further our understanding beyond the data presented within this thesis by emphasizing the potential clinical impact of such research. The FDA sponsored task force, established to identify ways to assist and educate the general population in making wise dietary choices that benefit long-term health, pointed out that dietary factors and sedentary lifestyles contribute substantially to the burden of preventable illnesses and premature deaths in the United States. This task force revealed that dietary factors are associated with four of the ten leading causes of death: coronary heart disease, some types of cancer, stroke, and type II diabetes (NCHS 1997). The Centers for Disease Control and Prevention have projected using current trends that the number of obese United States citizens will jump from 20 percent of adults today to 80 percent by the year 2040, and the population of normal weight individuals will drop from 42 percent today to 5 percent in the year 2040

(Ford, Mokdad et al. 2003; Mokdad, Ford et al. 2003). Understanding how the body maintains a balance of energy intake, expenditure, and storage is crucial for developing preventative and/or therapeutic measures to alleviate elevate these public health problems. My data generated from mouse mutants not only show the integral role of central POMC in maintaining energy balance but also reveal the independent, although not exclusive, potent effects of glucocorticoids on energy balance and partitioning in the absence of central POMC. Ultimately, the complexity of the POMC system has allowed and will continue to allow scientists to propose multiple options for therapeutic treatments and prevention for obesity and its associated diseases.

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