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***The role of central PRCP in the metabolism
regulation***

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Introduction

The role of the central melanocortin system in the regulation of energy metabolism has received much attention during the past decade since gene mutations of key components in melanocortin signaling cause monogenic forms of obesity in animals and humans. In the arcuate nucleus of the hypothalamus the prohormone proopiomelanocortin (POMC) is posttranslationally cleaved to produce α -melanocyte stimulating hormone (α -MSH), a peptide with anorexigenic effects upon activation of the melanocortin receptors (MCRs). α -MSH undergoes extensive post-translational processing and its in vivo activity is short-lived due to rapid degradation. The enzymatic process that controls α -MSH inactivation is not completely understood. Recent evidence suggests that prolyl carboxypeptidase (PRCP) is an enzyme responsible for α -MSH degradation. PRCP is widely expressed in the body in organs such as the liver, lung, kidney and brain, with a variety of known substrates such as plasma prekallikrein, bradykinin, angiotensins II and III, suggesting its role in the processing of tissue-specific substrates. As with many key melanocortin peptides, gene mutation of PRCP causes a change in the metabolic phenotype of rodents. The purpose of our study is to evaluate the role of PRCP in energy metabolism.

1. Obesity epidemic

The obesity epidemic has received considerable attention worldwide. Numerous studies have documented that the instances of obesity are climbing around the world. Obesity rates have been rising in virtually all population groups, regardless of the demographic or socio-economic status [1-3]. The growth in the population fraction with unhealthy body weight

was particularly high in the U.S., but it has reached worrisome proportions in other developed and developing countries as well. The prevalence of obesity in U.S. adults more than doubled between the mid-1970s and late 1990s with the most dramatic growth occurring in the 1980s [2]. It increased more than three-fold among children and adolescents over that same period [4]. As a result obesity now is a major public health problem in the United States [5]. Approximately 30.4% of adults (≥ 20 years) are obese and 16% of children (6–19 years) are overweight [6] (Figure 1). Adult obesity is associated with excess mortality and a myriad of health problems and overweight children are at greater risk of becoming overweight adults [7]. Worldwide, the estimated number of overweight adults exceeds one billion, of which at least 300 million qualify as obese.

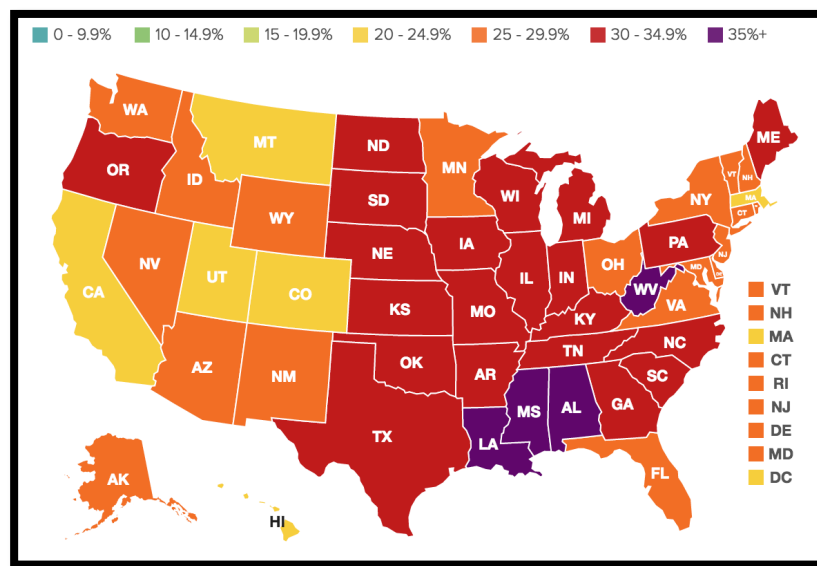


Figure 1: Percent of obese adults in the U.S.A (Body Mass index of 30+)

The terms overweight and obesity are defined as a process characterised by excessive accumulation of body fat with multiple organ specific consequences that could impair health. The most common method of assessing the degree of overweight/obesity in adults

is Body Mass Index (BMI). This is a simple index of weight-height that is calculated by dividing the weight of a person in kilograms by the square of their height in metres (kg/m^2). The international classification of overweight/obesity according to BMI is presented below in Figure 2

Classification	BMI (kg/m^2)	
Underweight	< 18.50	< 18.50
Severe thinness	< 16.00	< 16.00
Moderate thinness	16.00 - 16.99	16.00 - 16.99
Mild thinness	17.00 - 18.49	17.00 - 18.49
Normal range	18.50 - 24.99	18.50 - 22.99
		23.00 - 24.99
Overweight	≥ 25.00	≥ 25.00
Pre-obese	25.00 - 29.99	25.00 - 27.49
		27.50 - 29.99
Obese	≥ 30.00	≥ 30.00

Figure 2: International classification of overweight/obesity

National obesity prevalence is monitored using the National Health and Nutrition Examination Survey (NHANES), a database of measured heights and weights from a representative sample of the US population. Because NHANES is limited by changes in survey methodology over time and by the fact that some people, including, possibly, the super-obese, are less likely to come for the examinations, this data source is preferable to other databases that rely on self-reported data.

There is evidence going back a number of decades that show an increase in the prevalence of overweight and obesity instances in higher income countries, to the point that almost half of the adult population is now overweight ($\text{BMI} \geq 25\text{-}29.9 \text{ Kg/m}^2$) or obese ($\text{BMI} \geq 30 \text{ Kg/m}^2$)s. More recently, reports indicate that this scenario is also occurring in some middle income countries [8, 9] such as Central and South America (Mexico, Peru and Bolivia), the Gulf and Middle East (Egypt, Saudi Arabia), as well as Africa and Asia [9] to include a few (Figure 3). This is associated with an increase in the cost of treatment of obesity-related diseases.

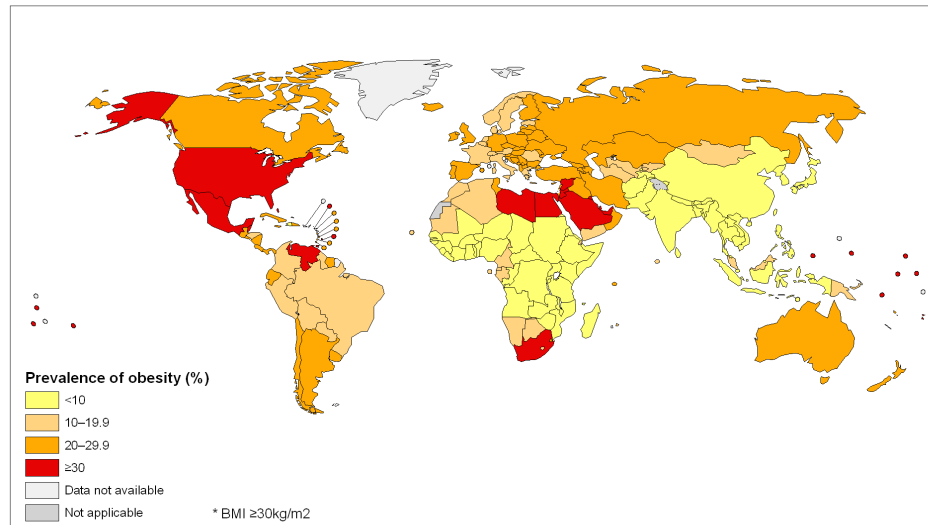


Figure 3: Prevalence of obesity, ages 20+, ages standardized both sexes, 2008

In epidemiology, two related but different measures describe the distribution of a disease in a particular population: prevalence and incidence. Prevalence is a measure of the proportion of the total population affected by the disease in question at a certain point in time [10]. The incidence of the disease is the number of new cases occurring in a population over a defined time interval (refers to new cases of disease occurring among previously unaffected individuals). Prevalence depends on both the incidence and the duration of the problem [10]. A problem with long duration is that it will have a high probability of being encountered at that designated time when prevalence is estimated [10]. The prevalence of obesity in a general population may be growing because of both the higher incidence and the longer survival of subjects with obesity.

The reported continuous occurrence of obesity in all age groups and in many countries of the world is most likely due to the lack of priority intervention strategies to prevent or reverse obesity at the population level, thus causing the upsurge of obesity cases year after year leading with time to increased prevalence.

Complex and interrelated factors are thought to be responsible for the current obesity epidemic, which is hastened by economic growth, modernization, urbanization and globalization of the food market. While the genetic background of populations is comparatively stable, serious societal changes and universal nutrition transitions have paved the way to more diverse diets with higher proportions of fats and sugars, accompanied with a decline in physical activity [11, 12].

Over time, stronger associations have been made between socioeconomic status (SES) and obesity, but inconsistent relationships have been reported between these two factors, depending on the degree of economic development of the country [13]. In higher-income countries, SES tends to be inversely related to obesity, particularly among females [14, 15]. Conversely, in lower-income countries, obesity was once viewed as a problem only among the prosperous population segments in men, women and children [15, 16]. However, a growing body of literature suggests that the SES-obesity gradient is shifting inversely as the Gross Development Product (GDP) Per Capita increases in a variety of developing countries with low and middle income, occurring in women first [17].

The trend in low and middle income countries is starting to resemble that in their high-income counterparts [17, 18]. This change is attributed to several transitions brought about with the increase in the GDP resulting from the country's economic advancement. These transitions are demographic (younger to older population distribution, rural to urban), epidemiological (infectious diseases to non-communicable diseases), technological (low to high automation and motorization), and nutritional (traditional foods to more processed energy-dense alternatives) [19].

1.1 Consequences of obesity

The spread in obesity is troubling because obesity is associated with increased morbidity and mortality. Its adverse health consequences may exceed those of smoking or alcohol abuse [20]. Obesity has become one of the major contributors to the global burden of disease and disability. It is one of the main risk factors for a number of life threatening non-communicable disease such as heart disease, type 2 diabetes, hypertension, stroke and certain types of cancers (endometrial, breast and colon) [21]. Cardiovascular diseases (heart disease and stroke) kill 17 million people globally each year and deaths due to diabetes is predicted to increase by more than 50% worldwide in the next ten years. Other medical conditions related to overweight and obesity include gastrointestinal diseases, sleep apnea and respiratory problems, osteoarthritis, low back pain, metabolic syndrome, reproductive hormone abnormalities, and various psychological consequences such as poor self-esteem, depression, body dissatisfaction, and psychological discomfort [22, 23].

1.2 Financial Effects of Obesity

Through the increased risk of adverse health conditions and work disability, obesity expands the financial burden of public transfer programs and private health plans. The costs are considerable. At the individual level, obesity is associated with health care expenditures that are on average about one-third above medical costs of otherwise similar individuals with normal weight [24]. At the aggregate level, obesity-related direct and indirect economic costs exceed \$100 billion per year [25], and this number is expected to grow. In relative terms, obesity accounts for about 6-10% of U.S. health care spending [26] and 2.0-3.5% in other Western countries [27]. In the U.S., the burden of obesity-

attributable costs falls disproportionately large on public health care, draining out resources of public programs like Medicare and Medicaid [27]. The dynamic estimates describe the cost implications of obesity in a similar fashion, suggesting that obesity accounts for 27% of the growth in real U.S. health care spending over 1987-2001 [28].

1.3 Effects of Obesity on Employment and Wages

A recent review in 2009 on the stigma of obesity reported that obese people face discrimination in employment settings, health care facilities and educational institutions [29]. Discrimination against obese people among Americans rose by 66% between 1995 and 2004 [30] on a par with racial discrimination [30]. This discrimination has psychological consequences that can lead to depression, body dissatisfaction and low self-esteem among obese people [29].

Increase in obesity levels not only has grave consequences for individuals with increased risk of many co-morbidities, but it also has cost implications.

1.4 Environmental factors

Environment strongly influences obesity. This includes lifestyle behaviors such as what and how much a person eats, as well as his or her level of physical activity. People tend to eat high-fat foods and put taste and convenience ahead of nutrition. Lack of exercise is an important factor. Sedentary Lifestyle Syndrome, or ‘SeDS’, is the term developed by more than 200 of the nation's leading physiologists to diagnose the growing epidemic of physical inactivity and its relationship to chronic, preventable diseases. ‘Sedentary’ is defined as engaging in less than 30 minutes of moderate physical activity, equivalent to brisk

walking, each day. More than 60% of US adults do not engage in the recommended amount of physical activity. Approximately 25% of US adults are not active at all. Inactivity increases with age and is more common among women than men and among those with lower income and less education. In the US, only 22% of adults are regularly physically active five times per week for at least 30 minutes, 15% get the recommended amount of vigorous activity at least three times per week for at least 20 minutes, and 25% do no physical activity at all in their leisure time. Among young people aged 12–21, almost 50% are not vigorously active on a regular basis. Female adolescents are much less physically active than male adolescents. Physical activity declines dramatically with age during adolescence.

2. Hypothalamic circuitry regulating food intake

The amount of stored fuel in the body is regulated in a very accurate manner. To accomplish this, energy intake must be matched to energy expenditure over time. Energy homeostasis is a tightly regulated process and an imbalance between its components, food intake and energy expenditure causes metabolic dysfunctions including obesity, a major risk factor for comorbidities including type 2 diabetes, hypertension and stroke, and cardiovascular diseases. Compelling evidence has been gathered during the last 100 years to suggest that a major player in the regulation of energy homeostasis as well as the underlying cause for fat accumulation in peripheral tissues, arises from the Central Nervous System (CNS) [31, 32]. From the dawn of modern neurobiology at the end of the 19th century, both intellectual as well as experimental attempts have been made to identify and isolate the brain's role in energy metabolism regulation. For example, based on the

emerging experimental observations on brain stem regulation of respiration at the time, Sherrington suggested that a similar blood link between the periphery and brain should exist for the regulation of food intake. Conclusions on signals from the periphery to the brain regarding feeding regulation were also drawn by others implicating gut-born humoral signals for appropriate brain responses to changing metabolic needs [33]. It is still remarkable to realize that these minds, and undoubtedly many more in or before the late 19th and early 20th centuries without the luxury of modern technology and widespread availability of knowledge, had the intellectual capability to predict basic principles that, 100 years later, proved to be correct. These past 100 years, however, have not elapsed without other advancements in the understanding of the brain-obesity relationship.

The involvement of various hypothalamic regions in the regulation of energy homeostasis stems from degeneration studies in the 1940s and 1950s: destruction of the hypothalamic ventromedial (VMH), paraventricular (PVH), and dorsomedial (DMH) nuclei induced hyperphagia [34]. In contrast, discrete lesions placed in the lateral hypothalamus (LH) [34] reduced food intake. While these approaches may be considered crude by today's standards of research, they were pivotal in pinpointing the hypothalamus as a major regulator of energy homeostasis. In fact, in retrospect to our current knowledge of specific neurotransmitters and neuromodulator-containing hypothalamic neuronal populations, these lesion studies were strikingly precise in distinguishing between sub-regions of the hypothalamus that house circuits that either promote or suppress feeding. The degeneration studies [35] confirmed the proposition that humoral signals arising from the periphery may exist to inform brain sites about overall energy needs.

A fundamental breakthrough came in 1994 and 1995 when the gene encoding the adipose signal leptin was discovered and its product tested on leptin deficient obese ob/ob mice [36]. Subsequently, receptors for leptin were cloned and localized to structures of the hypothalamus [37], some of which had been implicated in metabolism regulation by lesion studies. However, leptin binding and its receptors accumulated predominantly in a ventromedial hypothalamic structure, the arcuate nucleus [37], an area that was not specifically suggested by the lesion studies.

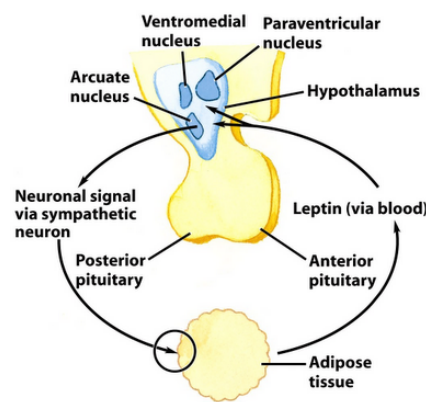


Figure 4: Regulation of leptin on hypothalamic nucleus

The appreciation of this small hypothalamic nucleus gained momentum regarding energy homeostasis when, due partially to serendipity, the melanocortin system was discovered as a player in obesity. The melanocortin system is comprised of neurons expressing pro-opiomelanocortin peptide (POMC), the Neuropeptide Y and Agouti-related peptide (NPY/AgRP) and neurons expressing melanocortin receptors (MCRs). In addition to data obtained from lesioning experiments, there has been considerable information obtained on the mechanisms of hypothalamic regulation of obesity by examining genetic models of obesity. The discovery that the cause of obesity in the ob/ob mouse was leptin deficiency, and that leptin receptor mutations were the cause of obesity in fa/fa rats and db/db mice

enable investigation of levels of hypothalamic peptides in hyperphagic, hypometabolic obese animals (Figure 5). The level of NPY mRNA and protein was increased in the hypothalamus of several obese animal models and the role of hypothalamic NPY in energy expenditure has been a subject of intense study for many years. Another spontaneously occurring obesity mutation, the agouti yellow mouse, produced evidence for another neuropeptide system being involved in regulating food intake and energy expenditure. The agouti protein binds to melanocortin receptors in the hypothalamus and blocks the anorectic effect of the normal agonist of this receptor α -MSH, a peptide derived from POMC.

In a quest to better understand the role of various melanocortin receptors in the determination of skin color [38], the striking revelation was made that when melanocortin receptor 4 (MC4R) was eliminated, animals became morbidly obese [39]. Since the melanocortin system had long been known to exist in neurons of the arcuate nucleus, it was reasonable to test whether leptin exerts its effect on metabolism by the mediation of the arcuate nucleus melanocortin system, and this was found to be the case [40]. Further analysis of components of the central melanocortin system delivered an attractive model, which today is considered to be the *primus movens* of metabolism regulation.

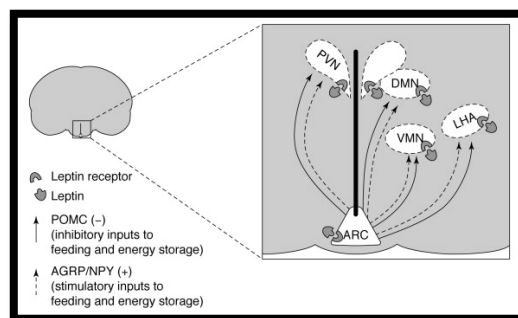


Figure 5: The central melanocortin system is defined as arcuate nucleus POMC neurons, arcuate nucleus AGRP/NPY neurons, and their downstream target sites expressing melanocortin 3 and/or melanocortin 4 receptors, such as the various hypothalamic nuclei indicated. AGRP, agouti- related protein; ARC, arcuate nucleus; DMN, dorsomedial nucleus; LHA, lateral hypothalamic area; NPY, neuropeptide Y; PVN, paraventricular nucleus; VMN, ventromedial hypothalamic nucleus.

2.1 Hypothalamic melanocortin system and control of energy metabolism

The critical role that the arcuate nucleus melanocortin system (consisting of POMC and NPY/AgRP cells [41]) plays in mediating leptin's effect on metabolism provided an exceptionally attractive and simple model of the central feeding center. In this model, activation of the POMC neurons by leptin [42] triggers release of α -MSH from POMC axon terminals, which in turn, activates MCRs leading to suppressed food intake and increased energy expenditure (Figure 6). Simultaneously, leptin suppresses the activity of arcuate nucleus NPY/AgRP neurons [42], which otherwise, through the release of AgRP, antagonize the effect of α -MSH on MCRs [43]. The NPY/AgRP system not only antagonizes anorexigenic melanocortin cells at their target sites where MCRs are located, but it also very robustly and directly inhibits POMC perikarya [44]. Both NPY, as well as the small inhibitory amino acid neurotransmitter, GABA, are involved in this inhibition, which occurs through basket-like synaptic innervations of POMC cells by NPY/AgRP terminals [44]. This unidirectional interaction between the NPY/AgRP and POMC perikarya is of critical significance, because it provides a tonic inhibition of the melanocortin cells whenever the NPY/AgRP neurons are active. Because there is no constitutive direct feedback mechanism from the POMC cells to disengage NPY/AgRP neurons, this simple anatomical constellation may provide the easiest explanation as to why the baseline blueprint of feeding circuits is more likely to promote feeding than satiety (Figure 6). While this bias towards positive energy balance is a necessity from an evolutionary perspective, it is also a likely contributor to the etiology of metabolic disorders, such as obesity.

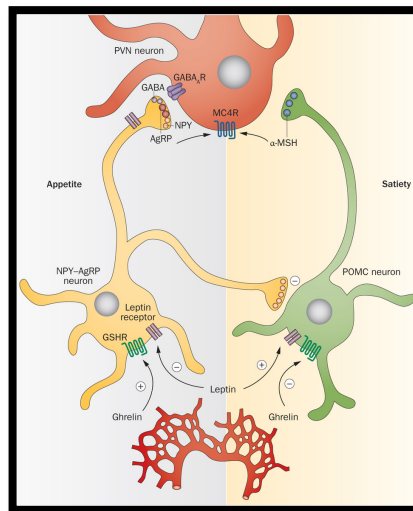


Figure 6: The primary neurons of the melanocortin system are the orexigenic NPY-AgRP neurons and the anorexigenic POMC neurons, which both send projections to the paraventricular nucleus. NPY-AgRP neurons also send inhibitory projections to neighbouring POMC cells. Activation of POMC cells by leptin triggers the release of α -MSH which binds to MC4R and promotes satiety. AgRP neurons are inhibited by leptin but stimulated by ghrelin, which promotes feeding and silences firing of POMC neurons. The effect of the NPY-AgRP cells is mediated by release of GABA, NPY and AgRP. Abbreviations: α -MSH, α -melanocyte stimulating hormone; AgRP, agouti-related protein; GABA, γ -aminobutyric acid; GABA_AR, GABA receptor; GHSR, growth hormone secretagogue receptor (also known as ghrelin receptor); MC4R, melanocortin receptor 4; NPY, neuropeptide Y; POMC, proopiomelanocortin; PVN, paraventricular nucleus.

The projections of the two components of the melanocortin system, POMC and NPY/AgRP neurons, grossly overlap both within and outside of the hypothalamus, although they are rarely very long. The common targets of the NPY/AgRP and POMC projections are the hypothalamic paraventricular nucleus (PVN), and the lateral hypothalamic and perifornical areas, all of which express melanocortin receptors MC3/4R [45]. The interplay between AgRP and α -MSH fibers on melanocortin receptor-expressing neurons in the PVN is suggested to be critical for metabolism regulation [45]. The signals that target the PVN can then be distributed in various directions as the PVN projects to brain stem nuclei, the spinal cord, cortex (via various routes), and thalamus, as well as to the portal vessels connecting the hypothalamus to the anterior pituitary.

Besides the PVN, other areas to which melanocortin neurons project include the medial preoptic area (MPA), the periaqueductal gray (PAG), the nucleus of the solitary tract (NTS) and the parabrachial nucleus (PBN).

An important target area of the mediobasal hypothalamic POMC neurons is the preganglionic sympathetic system in the thoracic segment of the spinal cord [46]. These POMC projections are unopposed by NPY/AgRP efferents [46] and are thought to play an important role in melanocortin regulation of the sympathetic nervous system [46]. Interestingly, POMC neurons that give rise to this spinal cord projection are not homogeneously distributed within the mediobasal hypothalamus. They are mainly located in the retrochiasmatic area of the hypothalamus and in an area lateral from the arcuate nucleus [46]. In fact, when analyzed closely, the appearance of POMC neurons is not homogenous throughout the mediobasal hypothalamus; within the arcuate nucleus, and specifically in its more medial aspects, POMC neurons are smaller compared to the laterally located, larger melanocortin cells. This enlarged soma size is consistent with neurons that have long projections, such as those POMC cells that project to the spinal cord.

2.1.1 The NPY/AgRP neurons and the orexigenic pathway

The NPY/AgRP neurons within the arcuate nucleus (ARC) are probably the most established orexigenic population in the CNS. However, earlier studies reported that mice with NPY and/or AgRP deficiency have normal food intake and body weight [47]. Similarly, ablation of AgRP neurons during development resulted in normal body weight [48]. More recent studies used toxins to ablate the NPY/AgRP neurons in adults and confirmed the orexigenic role of these neurons [49]. Acute activation of NPY/AgRP neurons using optogenetic or pharmacogenetic stimulation methods also resulted a robust increase in food intake [50]. These series of experiments have established the NPY/AgRP neurons as the major orexigenic population in the CNS.

The NPY/AgRP neurons release the neuropeptide NPY (agonist at the Y receptors), AgRP (inverse agonist at the MC4Rs), and the inhibitory neurotransmitter GABA. The metabolic effects of NPY on Y receptors are somewhat complicated, and many are not consistent with the orexigenic role of NPY/AgRP neurons [51]. The ability of AgRP to antagonize the central melanocortin pathways received much attention, since this was considered to be the underlying mechanism of orexigenic effects. However, the orexigenic effects of NPY/AgRP neurons are not affected by the deletion of MC4Rs [52], and it is now regarded that the NPY/AgRP neurons increase food intake independent of the central melanocortin system. Currently, GABA release is believed to mediate most of the orexigenic effects of the NPY/AgRP neurons. For instance, deletion of vesicular GABA transporter (*vgat*) genes specifically in the AgRP neurons leads to lean phenotype [53], which confirms the importance of GABAergic neurotransmission in the orexigenic effects.

Multiple “anorexigenic” neuronal populations within the CNS receive the “inhibitory” GABAergic input from the NPY/AgRP neurons. The ARC POMC neurons receive GABAergic input from the NPY/AgRP neurons, which form a local circuit of appetite regulation within the ARC [42]. Indeed, when the *vgat* gene is deleted in the NPY/AgRP neurons, the frequency of inhibitory postsynaptic current (IPSC) is not increased by ghrelin, a hormone produced by the stomach, which excites the NPY/AgRP neurons [53].

2.1.2 The POMC neurons and the anorexigenic pathway

The POMC gene is expressed in the brain as well as in the periphery such as in the pituitary gland, immune system and skin. Within the brain, POMC is expressed in the hypothalamus and in the NTS of the brainstem [54]. The critical role of POMC in the regulation of metabolism has been evidenced by studies showing that in humans,

individuals with POMC gene mutations display early-onset obesity [55, 56]. A similar obese phenotype occurs also in POMC-deficient mice [57]. However, while the acute ablation of POMC neurons in adult mice results in an obese phenotype with hyperphagia [58], postnatal ablation of POMC neurons leads to an obese phenotype with reduced energy expenditure but no hyperphagia [59]. On the other hand, reactivation of central POMC at different stages of development in neural specific POMC deficient mice reduces food intake and weight gain, and attenuates comorbidities such as hyperglycemia and hyperinsulinemia [60].

The POMC gene encodes several peptides that are the products of a complex post-translational process. The bioactive peptides of the POMC gene include adrenocorticotrophin (ACTH), β -endorphin, α -, β - and γ -MSH. Among these peptides, α -MSH is the most well-known anorexigenic peptide which mediates the effects on food intake and energy expenditure through its binding and activation of MCRs [43]. The biosynthesis of POMC-derived peptides, as the majority of mammalian neuropeptide hormones, meets the criteria of the “prohormone theory”, which begins with the mRNA translation process into a large, inactive precursor polypeptide, followed by posttranslational proteolysis to release different products [61]. In the case of POMC, this is achieved through differential processing by the action of different prohormone convertases (PCs), which results in biological and functional diversity within the central nervous system. Once encoded from the corresponding mRNA, POMC follows the intracellular trafficking of a secreted protein through the Golgi complex, ultimately reaching secretory granules in which the end products of processing are stored before being secreted by exocytosis. During this trafficking, POMC is proteolytically processed into a number of physiologically important peptides, including endorphins, ACTH, and α -MSH.

2.1.3 The melanocortin receptor-expressing neurons

Receptors of melanocortins are G-protein-coupled receptors with characteristic seven transmembrane domains. So far, five melanocortin receptors have been identified in humans – MC1R–MC5R [62]. The encoded receptors bind four ligands: α -, β - and γ -MSH and the ACTH. The melanocortin receptors are Gs-coupled and signal via the adenylate cyclase-cAMP-protein kinase A second messenger pathway (Figure 7). However, depending on the cell type and the melanocortin receptor expression, signal transduction pathways other than cAMP may be activated which include the inositol triphosphate-diacyl glycerol- protein kinase C (IP3-DAG-PKC) pathway, extracellular Ca^{2+} influx, MAP kinase pathway, and the JAK/STAT pathway [63].

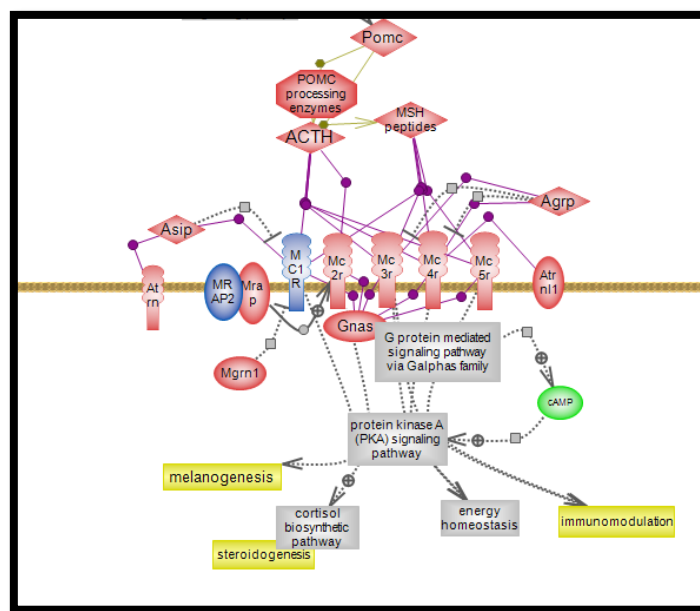


Figure 7: Melanocortin receptors and their endogenous ligands

The paraventricular nucleus of the hypothalamus (PVN) is a representative anorexigenic center in addition to POMC neurons, and the central melanocortin pathways.

Expression of the MCR genes is tissue-specific; While MC1R, MC2R and MC5R are mainly expressed in peripheral tissues, MC3R and MC4R are expressed in the Central Nervous System and have been shown to play a role in metabolism regulation.

2.1.3a MC4R

The melanocortin receptor type 4 was first cloned by Gantz et al in 1993 [62]. MC4R is a 332 amino acid protein encoded by a single gene, localized to chromosome 18q21.3. Using Northern blot analysis and in situ hybridization, MC4R was originally found to be expressed primarily in the brain. MC4R expression was notably absent in the adrenal cortex, melanocytes, and placenta [62]. MC4R is widely distributed in the central nervous system, especially in the cortex, hippocampus, amygdala, septum, corpus striatum, nucleus accumbens, hypothalamus, nucleus tractus solitarius, visual and motor nuclei of the brainstem, and the dorsal horn of the spinal cord. α -MSH, β -MSH, and ACTH are the known agonists and AgRP is the known reverse agonist of the MC4R.

Since MC4R is highly expressed in the hypothalamus and has a strong affinity for α -MSH it is believed to be a strong candidate for energy balance, appetite control, and body weight regulation. In rodents and in humans, mutations of the MC4R gene causes obesity [55]. Humans with MC4R mutations, showing severe, early onset childhood obesity, represent 4–6% of the obese population [64]. *Mc4r* knockout mice have been shown to also develop obesity syndrome characterized by hyperphagia, hyperglycemia and hyperinsulinemia [65]. The restoration of the MC4R gene selectively in the PVN of MC4R knockout mice by viral or genetic manipulation produced mice displaying lower body weight and reduced food intake compared to the MC4R knockout controls [66]. However, the restoration of the

receptor is not sufficient to bring energy expenditure to the level of wild type controls. Thus, this suggests that melanocortin regulation of energy expenditure occurs elsewhere in the hypothalamus or through receptors other than MC4R [66]. In addition, MC4R neurons also express Single-minded 1 (SIM1), a transcription factor that is critical for PVN development and one of only six genes implicated in human monogenic obesity. Haploinsufficiency of SIM1 is associated with hyperphagic obesity and increased linear growth in both humans and mice. Additionally, SIM1 heterozygous mice also show hyperphagia and obesity in response to a high-fat diet [67]. Thus, the phenotype of SIM1 haploinsufficiency is similar to that of agouti yellow (Ay), and MC4R knockout mice, both of which are defective in hypothalamic melanocortin signaling.

It has been shown that MC4R mRNA is expressed in autonomic control regions projecting to postganglionic neurons innervating peripheral metabolic tissues [68, 69]. Through sympathetic nerve innervation, CNS-MC4R has been shown to increase lipolysis in white adipose tissue (WAT) [70], insulin sensitivity in muscle, thermogenesis in brown adipose tissue (BAT), fatty acid oxidation in liver and decrease insulin release in pancreatic β -cells [71].

2.1.3b MC3R

The MC3R gene, similarly to the MC4R gene, plays a crucial role in energy homeostasis [72], but associations of its polymorphisms/mutations with human obesity are not as evident as in case of the MC4R gene [73]. MC3R is encoded by a single gene localized to the q13.2–q13.3 region of chromosome 20. Since this locus is associated with type 2 noninsulin-dependent diabetes mellitus (NIDDM), *Mc3r* could represent a candidate gene for NIDDM. MC3R is unique in that it binds to α -, β - and γ -MSH with similar affinities. Using Northern blot hybridization and polymerase chain reaction, Gantz et al first showed that MC3R is expressed in brain, placental, and gut tissues but not in melanoma cells or in adrenal glands [43]. MC3R expression in the brain is highest in the hypothalamus especially in the arcuate nucleus, ventromedial nucleus, preoptic nucleus, lateral hypothalamic area, and posterior hypothalamic area. Agouti-related protein (AgRP), which is normally expressed in the hypothalamus, is a potent reverse agonist of both MC3R and MC4R [43]. However, POMC and AgRP neurons in the arcuate nucleus of hypothalamus selectively express MC3R, and not MC4R, suggesting that MC3R might function as a presynaptic autoreceptor regulating the release of melanocortins. Physiological support for this hypothesis was provided by Cowley et al, [42] who demonstrated that the selective MC3R agonist, D-Trp- γ -MSH inhibited firing of GFP-labeled POMC neurons in the PVN. Also, Marks et al showed that peripheral administration of the MC3R agonist stimulates feeding via MC3R-mediated inhibition of the ARC POMC neurons [74].

An association between MC3R and human obesity has been identified by linkage studies. Several sequence variants have been found in the *Mc3r* coding region and in 5' flanking sequences. *Mc3r* variants are associated with subtle changes in weight, leptin levels, and

insulin-glucose ratios, but none of these explain human morbid obesity [75]. A novel heterozygous mutation I183N in *Mc3r* was identified in two obese patients of the same family [76]. Functional characterization of I183N showed that this mutation completely abolished the activity of the mutated receptor to stimulate intracellular cAMP production, suggesting that I183N might play an important role in obesity. Similarly, Tao et al showed that a novel mutation I335S in *Mc3r* results in complete loss of ligand binding and signaling suggesting that this mutation might contribute to obesity [77]. However, a recent study evaluating the functional consequences of all mutations found in *Mc3r* and *Mc4r* in severely obese North American adults concluded that *Mc4r*, but not *Mc3r* mutations are associated with severe obesity in this population [78]. Thus, the significance of *Mc3r* mutations in human obesity is still not conclusively established to date.

2.2 POMC processing

POMC, a prohormone with molecular weight of 31 kDa, is ubiquitously expressed in various tissues of mammals [79]. POMC expression in the central nervous system, however, is limited to the ARC, NTS, and corticotrophs and melanotrophs of the anterior pituitary. The 1200 base pair POMC transcript encodes for the 267 amino acid prohormone with an N-terminal signal peptide of 26 residues [80]. As this precursor peptide passes through the Golgi stacks, it is targeted, via a specific signal peptide, into regulated secretory granules. POMC undergoes extensive posttranslational modification within these secretory granules mediated by a family of serine proteases, the prohormone convertases (PCs).

POMC is cleaved by prohormone convertase 1 (PC1) to produce 22 kDa pro-ACTH and β -lipoprotein hormone (β -LPH). Pro-ACTH is further cleaved by PC1 to produce the N-terminus of POMC-joining peptide and 4.5 kDa ACTH. Prohormone convertase 2 (PC2) cleaves ACTH to ACTH 1–17 and corticotropin-like intermediate lobe peptide (CLIP) and β -LPH to γ -lipoprotein hormone (γ -LPH) and β -endorphin. γ -LPH and the N-terminus of POMC are further modified to produce β -MSH and γ -MSH, respectively (Figure 8).

α -MSH is a tridecapeptide derived from POMC. The synthesis of α -MSH from POMC involves several specific enzymes in addition to PC1 and PC2. First, carboxypeptidase E cleaves the C-terminal basic amino acid residues of ACTH 1–17. The peptide is then amidated by peptidyl α -amidating monooxygenase (PAM) to produce desacetyl α -MSH (Des- α -MSH). Finally, Des- α -MSH is acetylated by N-acetyltransferase (NAT) to produce acetylated α -MSH (Act- α -MSH). Act- α -MSH is more potent than Des- α -MSH in activating melanocortin receptor signaling and in reducing food intake, effects that are likely due to rapid degradation of Des- α -MSH. Guo et al have shown that total hypothalamic α -MSH levels are decreased in leptin-deficient *ob/ob* mice and increased in leptin-treated *ob/ob* and C57BL/6J mice. Leptin specifically enhances hypothalamic levels of Act- α -MSH without significantly affecting the amounts of Des- α -MSH, possibly by activating NAT in the POMC neurons [81].

While the maturation process of α -MSH has been largely studied, little is known on its fast degradation process. Only recently, it has been reported that PRCP, also known as lysosomal Pro- X carboxypeptidase, is a key enzyme responsible for the degradation and inactivation of α -MSH in the brain, [82] (Figure 8). Evidence was provided that hypothalamic PRCP is in an anatomical position to determine the efficacy of released α -MSH, thereby controlling the output and thus the efficacy of the melanocortin system. In

support of this idea, mice lacking PRCP display a lean phenotype when exposed to either a regular diet or high fat diet. Furthermore, in support of its function, PRCP has been previously associated with metabolic syndrome in human males [83].

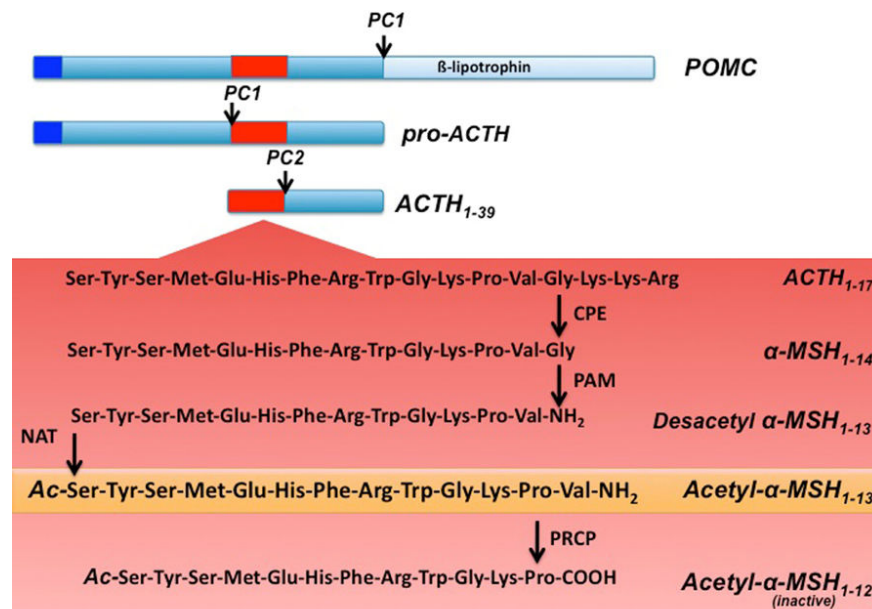


Figure 8: Schematic illustration of hypothalamic POMC processing. Prohormone convertase 1 (PC1) cleaves the 32 kDa preprotein into proACTH and β-lipotrophin . ProACTH is then further converted by PC1 to form ACTH1–39. Prohormone convertase 2 (PC2) will then cleave ACTH1–39, to form ACTH1–17. ACTH1–17 will be first processed by carboxypeptidase E (CPE) and then α-amidating mono-oxygenase (PAM) to form desacetyl α-MSH1–13. An n- actelyltransferase (NAT) will then convert desacetyl-α-MSH1–13 into the more active form acetyl-α-MSH1–13. Finally acetyl- α-MSH1–13 will be degraded by prolyl carboxypeptidase (PRCP) into the inactive product acetyl- α-MSH1–12.

2.2.1a Enzymatic properties of PRCP

PRCP is an enzyme in the family of the carboxypeptidase (CPs) that is a highly conserved enzyme in eukaryotes with high amino acid homology between mouse, rat, and human. PRCP is a serine protease with a unique protease structure that includes a novel helical structural domain, which caps the catalytic active site [84]. The enzyme cleaves the last amino acid at the C-terminal part of the target proteins if the substrate contains a proline as a penultimate amino acid [85]. Furthermore, substrate specificity studies have shown that

PRCP has preferences for the amino acid sequences Xxx-Pro- Phe-OH and Xxx-Pro-Val-OH, where Xxx can be any amino acid [85].

PRCP was first identified over 40 years ago during studies of bradykinin metabolism in kidney. It is expressed in many other tissues including lung, liver, heart, and adipose as well as brain, suggesting possible multiple tissue- and/or cellular specific roles, with a variety of substrates. Within tissues, PRCP has been found in lysosomes and extracellularly either as a membrane-bound or as a soluble form [85]. PRCP is a single chain protein of approximately 58 KDa. The cDNA for human PRCP was cloned from a human kidney library and based on that, the molecular weight of the mature protein should be around 51 kDa, indicating the presence of about 12% carbohydrate by weight. This is consistent with the existence of six potential N-linked glycosylation sites. The deduced protein sequence contains 496 amino acids and includes a 30 residue signal peptide and a 15 amino acid propeptide [86]. The C-terminal half of the sequence contains an interesting “serine repeat” in which Ser is repeated as the 26th residue 6 out of 9 times [86]. In the other three repeats, Ser is replaced by Thr, Gly or Glu. When the Ser residues are aligned to yield 26 residue repeats, the positions of some of the other residues also fall into a pattern. The significance of this repeat region is not clear, but it might be involved in the maintenance of a secondary or tertiary structural motif or in the formation of the homodimer. The gene encoding human PRCP is on chromosome 11, while in the mouse it is on chromosome 7. PRCP is inhibited by compounds that react with the active site serine residue. However, this action is not true for all serine protease inhibitors, such as aprotinin, or by chelating agents or inhibitors that react specifically with the active site cysteine in sulfhydryl proteases. Particularly, PRCP is not inhibited by non-specific SH-reactive

agents, but is relatively sensitive to the prolylendopeptidase inhibitor, Cbz-Pro-prolinal, because of its specificity for peptides with penultimate proline residue [86].

2.1.2b Function

The PRCP-catalyzed reaction was initially found to be part of the pathway for angiotensin II (Ang II) metabolism in renal tissues, whereas PRCP appeared to control the total amount of Ang II. Oda and others demonstrated that PRCP metabolizes Ang II to angiotensin 1–7 (Ang 1–7) [87]. The activation of Ang 1–7 receptor Mas (a G-protein-coupled protein) by Ang 1–7 results in the generation of nitric oxide (NO) and prostaglandins. Thus, Ang 1–7 counteracts Ang II function, providing evidence that PRCP regulates the negative effects of Ang II such as high blood pressure and heart failure. In addition, the activation of the Ang 1–7 receptor Mas may also lead to diminished cell proliferation through down-regulation of the phosphorylation and activation of Erk1 and Erk2 in the Erk1/Erk2 Map kinase signaling pathway [88]. In theory, the PRCP inhibitors target the production of pro-inflammatory prostaglandins and promote proliferation through the Ang 1–7 receptor Mas-dependent pathway. This represents a novel approach to suppress unwanted inflammation-causing prostaglandins.

Later, it was shown that PRCP is one of several enzymes that convert Ang II to a unique bioactive molecule. *In vitro* studies showed that angiotensin-converting enzyme 2 (ACE2) is an exopeptidase that converts Ang II to Ang 1–7 at a much faster rate than PRCP.¹² This data suggests that Ang II is a poor substrate for PRCP. Clinical studies have provided reliable evidence that ACE2 is an essential regulator of angiotensin I (Ang I), Ang II, and angiotensin-induced cardiac hypertrophy.¹³ Recent studies clearly show increased

myocardial levels of Ang II and a significant decrease in Ang 1–7 in ACE2-deficient hearts, suggesting that the role of PRCP in metabolizing Ang II may be insignificant. Taken together, these observations suggest that PRCP is a redundant catalyst contributing to alternate pathways for Ang II metabolism.

While the well-established cardiovascular and renal actions of Ang II are attributed to the angiotensin type 1 receptor (AT1R), much less is known about angiotensin III and its cardiovascular effects. For more than 30 years, it was known that PRCP metabolizes Ang III to Ang 2–7 [87]. Soon after, studies demonstrated that Ang III, is a pressor agent whose response, like that of Ang II, is mediated by AT1 receptors [89]. Apparently, Ang III has multiple effects on renal function in the diseased kidney and can enhance renal disease through the overproduction of aldosterone, leading to arterial hypertension and/or atrial fibrillation. Aldosterone maintains blood volume, pressure, and electrolyte balance. Its production is known to be regulated by renin, an enzyme produced in the kidneys. Renin increases in response to low blood pressure, decreased blood flow to the kidneys, or sodium deficiency. The elevation of renin results in an increase in synthesis and secretion of aldosterone. Studies indicate that Ang III also activates the secretion of aldosterone [89]. Recently, we have demonstrated that recombinant PRCP (rPCRCP) metabolizes Ang III to Ang 2–7, removing phenylalanine (Phe) [90]. It is tempting to speculate that PRCP might funnel the generation of angiotensin 3–4 (Ang 3–4) through Ang 2–7. If the over- secretion of aldosterone by Ang III is viewed as a trigger of arterial hypertension, then inactivation of Ang III by PRCP might lead to a decrease in blood pressure. Further studies are required to determine whether PRCP is critically important for regulating Ang III- induced hypertension and preserving renal structure and function. This is an important area of

research to pursue given the increasing prevalence of cardiovascular disease and stroke in the older population.

The possible actions of another substrate of PRCP, plasma prekallikrein (PK, Fletcher factor), have recently begun to receive much attention. When the complex of high molecular weight kininogen (HK) and PK binds to endothelial membrane, PK is rapidly converted to kallikrein by PRCP. The formed kallikrein then cleaves HK to liberate bradykinin (BK), which leads to NO and prostaglandin-I₂ formation, as well as subsequent vasodilation, by activating constitutive bradykinin B₂ and inducible bradykinin B₁ receptors. The PRCP-dependent PK activation pathway might be considered an additional mechanism to preserve the availability of NO and prostacyclin as vasodilatory agents in vascular smooth muscle. We proposed that chronic PRCP inhibition might elevate blood pressure. In accordance, we have found that *PRCPgt/gt* mice have mild hypertension, suggesting a causative relationship between PRCP levels and signs of hypertension .

Local skeletal muscle ischemia and acidosis are shown to increase the generation of BK and prostaglandins, the two circulating products of the PRCP-induced cell activation [91]. The increased acidotic response during exercise and inflammatory mediators such as BK and prostacyclin have been shown to cause abnormal exercise-related symptoms and autonomic responses in congestive heart failure syndrome [91]. Nonetheless, the long-term elevated concentrations of NO and prostacyclin through PRCP-dependent pathways may be detrimental and eventually responsible for cardiovascular diseases such as congestive heart disease. Since BK and Prostaglandins exacerbate the genesis of the symptoms of exercise intolerance in heart failure, the inhibitors of PRCP might be effective in ameliorating the exercise-limiting symptoms.

Clinical studies demonstrate that PRCP is involved in the pathogenesis of inflammatory conditions such as rheumatoid arthritis and infection [92]. Melanocortin peptides have numerous effects on the host such as the modulation of fever, inflammation and appetite [92].

In addition to its cardiovascular and proinflammatory functions, PRCP, as reported by a genome-wide association study (GWAS), is a risk factor for the metabolic syndrome because it was significantly associated with coronary heart disease in men [83]. This report supports recent data produced in rodents showing that central PRCP is an important regulator of energy metabolism because it is responsible for the degradation and thus inactivation of hypothalamic α -MSH [82]. In peripheral tissues such as in the kidney, PRCP has been shown to have an acidic pH optimum (5.0). However, PRCP retains significant enzymatic activity also at neutral pH [82]. Subsequently, it was shown that, at neutral pH, α -MSH is a better PRCP substrate than angiotensin II. α -MSH is a 13 amino acid neuropeptide in which a proline in position 12 precedes a valine in position 13. By removing this valine residue, PRCP produces α -MSH1–12 from α -MSH1–13. The removal of the last amino acid is of physiological importance.

α -MSH1–13 is a potent anti-inflammatory agent. In addition to the specificity of cleavage, the cellular release of pro-inflammatory mediators seemed to be critical to PRCP actions. In theory, agents that increase production and effects of α -MSH1–13 could be used to counteract the effects of pro-inflammatory mediators such as bradykinin and cytokines.

Obesity is known to cause inflammation and insulin resistance in the vasculature and non-vascular tissues involved in glucose metabolism. Evidence suggests that hyperglycemia may contribute to defective NO-dependent vasodilation in diabetes [93]. The inducible NO synthase (iNOS) expression is elevated in adipose tissue of obese people compared to

those of lean people and is a mediator of inflammation and a key enzyme in insulin resistance [93]. The colocalization of α -MSH1–13 receptors (MC4R) with iNOS has been reported, suggesting a role for α -MSH1–13 in obese people [93]. The inactivation of α -MSH1–13 by PRCP provides a positive feedback loop for postprandial enhancement of food intake and inflammation by inhibiting α -MSH1–13 function. However is demonstrated by the inability of α -MSH1–12 to decrease food intake when administered centrally as well as by its inability to increase the action-potential frequency of hypothalamic MC4R-expressing neurons, compared to α -MSH1–13 peptide [82]. Further support for the role of PRCP in the degradation of α -MSH1–13 is provided by studies using the *PRCPgt/gt* null mice. These mice have been shown to be sensitive to peripheral administration of α -MSH and to have higher α -MSH1–13 levels in the hypothalamus compared to WT controls [82].

Although α -MSH is so far the only substrate involved in the regulation of food intake that has been identified as a PRCP substrate, we cannot exclude the possibility that more peptides with a proline as the penultimate amino acid and a function associated with body-weight regulation could be degraded by PRCP. However, data from human and rodent studies suggest that PRCP inhibition might provide a mechanism to increase central bioavailability of α -MSH, and thus reduce food intake.

2.1.2c A potential role for PRCP in leptin resistance

A postulated cause for the development of obesity is leptin resistance. As fat stores increase during the development of obesity, circulating leptin levels are continuously rising. However, these elevated leptin levels or administration of exogenous leptin to obese

subjects does not lead to decreased body weight. This discrepancy between leptin levels and the metabolic phenotype is attributed to leptin resistance. A primary site of leptin resistance is suggested to be the POMC neurons. Indeed, intracellular signaling cascades triggered by leptin receptor activation were found to be impaired in diet-induced obese animals [94]. Specifically, activation of the transcription factor STAT3 by leptin is diminished in these animals, an event that is attributed to the inhibitory action of SOCS3 on STAT3 phosphorylation [94]. Additionally, ex vivo studies revealed that leptin induced secretion of α -MSH1–13 is reduced from hypothalamic slices taken from diet-induced obese mice [95]. An alternative explanation for the development of leptin resistance was the decreased ability of leptin from the circulation to reach hypothalamic neurons. Despite of all this elegant and logical data, several parameters of POMC neurons in diet induced obesity show alterations completely consistent with elevated leptin levels. For example, the wiring of the melanocortin system and transcript levels of POMC and AgRP/NPY of obese animals in response to a hypercaloric environment reflects an adequate and possibly maximal responsiveness of these cells to leptin [96]. The synaptic organization triggered in these animals shows similar trends of changes to those that were described previously for highly leptin sensitive ob/ob mice in response to leptin administration. Although reactive gliosis was also detected in response to high fat diet specifically associated with arcuate nucleus capillaries [96], this gliosis does not appear to block the effect of leptin on the synaptic input organization of the POMC neurons and POMC, NPY and AgRP transcript levels, and hence, may not be the cause of leptin resistance as proposed earlier. On the other hand, it is conceivable that assays using hypothalamic slices to measure α -MSH release [95] may be skewed by gliosis, an event that could be a structural obstacle for the release of measurable α -MSH into the medium, thereby limiting the usefulness of these

assays for the study of α -MSH release kinetics. Thus, if neurobiological attributes of leptin action are present in the hypothalamus of diet-induced obese animals, it is a reasonable suggestion that the degradation of released α -MSH1–13 may interfere with the ability of MC4R activation in POMC target sites. As described above, PCRP location and function in the hypothalamus makes this carboxypeptidase, although previously unsuspected, a player in the emergence of systemic leptin resistance as it relates to the regulation of food intake, energy expenditure and glucose homeostasis.

2.1.2d Pharmacological aspects

Obesity and related metabolic dysfunctions, such as type 2 diabetes, have emerged as a leading cause of morbidity and mortality in developed societies. The development of obesity and type 2 diabetes may be triggered or abrogated by the mechanisms of the CNS. The hypothalamic melanocortin system and its major anorexigenic peptide α -MSH have an important role in such disorder, as revealed by the dramatic development of obesity after selective inactivation of the neuronal *Pomc* gene in mice, and the presence of POMC mutations in humans with severe early-onset obesity [97]. Thus, the α -MSH target MC4 receptor is an attractive candidate for a drug target to treat obesity, as it not only affects several aspects of feeding behavior, but the activation of the MC system also increases insulin sensitivity and energy expenditure [98].

Pharmaceutical development of MC4 receptor-specific drugs have been the object of many studies [99]. However, it has been challenging to design selective MC4 receptor agonists that would completely lack affinity for the MC3 receptor [99]. Nevertheless, mixed MC3/4 agonists might provide a therapeutic advantage since, as shown in MC3R knockout mice,

reduction in MC3R activity is associated with an increased adipogenesis. However, studies in rodents and humans have revealed some side effects of the use of MC receptor agonists. For example, administration of MC4 receptor agonists is associated with penile erections as well as flushing, which has resulted in a new application area for these drugs in erectile dysfunction. Also, preclinical studies have identified roles for the melanocortin system in blood pressure regulation (MC receptor agonists have a depressor effect most probably via the nucleus tractus solitarius in brainstem), in the inflammatory responses (where agonists limit thermogenic responses to pyrogens), and in pain processing (where antagonists suppress pain sensation) [100]. Thus, the development of MC4 receptor agonists in the treatment of obesity should be carefully monitored to avoid undesired side effects due to melanocortin function in other systems.

Because of these side effects, PRCP becomes a very promising drug target. It has been already reported that inhibition of PRCP activity by small molecule protease inhibitors administered peripherally or centrally decrease food intake in wild type, as well as obese animals [82]. The pharmacological manipulation of the melanocortin system accomplished by inhibiting α -MSH catabolism represents a highly innovative approach that may help to combat metabolic disorders such as obesity.

The local metabolism of neurotransmitters and neuro- modulators are generally considered an optimum drug target with a lower incidence of side/non-specific effects compared to direct agonists. This is the case, among others, of the actual pharmacotherapy for many pathologies of the CNS such as depression and Alzheimer's disease. The discovery of PRCP as a pharmacological target may allow, indeed, to selectively focus on the benefits derived from the enhancement of melano- cortin activation in discrete brain areas by its

endogenous ligand, avoiding the whole set of possible central and systemic effects due to the use of MC receptor agonists.

In conclusion, further studies on PRCP will shed light on a novel central regulatory element in the regulation of energy metabolism. Differential activity levels of PRCP in specific brain areas in different metabolic condition by affecting α -MSH levels will, in turn, affect the efficacy of melanocortin signaling. Thus, understanding PRCP role in metabolism regulation is important to reveal whether PRCP can be used as new drug targets for metabolic disorders, including obesity and type 2 diabetes.

3. Hormonal regulation of the hypothalamic melanocortin system

3.1 Leptin

POMC is a regulator of appetite, energy expenditure and glucose metabolism [101]. Therefore, its own regulation must be important for the control of energy homeostasis. The hormone leptin is a major regulator of POMC. Leptin is an anorexigenic hormone produced and released by the white adipose tissue in the amount proportional to the mass of fat in the body [102]. Leptin interacts with six types of receptors (*Ob-Ra*, *-Rb*, *-Rc*, *Rd*, *Re*, and *Rf*) encoded by a single leptin receptor gene (*Ob-R*) [103]. However, only the long form of leptin receptors (*Ob-Rb*), consisting of an extracellular and an intact cytoplasmic domain, mediates the anorexigenic effect of leptin [104]. Deficiency in leptin or leptin receptors induces a morbid obese phenotype characterized by hyperphagia, hyperglycemia, hyperlipidemia, and reduced energy expenditure in both rodents and humans [105]. Neuron-specific *Ob-R*-deficient (*db/db*) mice displayed an obese phenotype while hepatocyte-specific *db/db* mice are normal, suggesting that the direct effect of leptin in the

brain is essential for metabolism regulation. Mice lacking Ob-Rb in POMC neurons, AgRP neurons or both POMC/AgRP neurons displayed increased body weight and fat mass [106], although their phenotypes are milder compared to those of whole brain neuron-specific Ob-Rb deficient mice, suggesting the involvement of other leptin receptor-expressing neurons in mediating the effect of leptin on energy homeostasis.

Leptin regulates POMC and NPY/AgRP neurons at different levels. For example, leptin increases POMC mRNA levels while decreasing NPY/AgRP mRNAs. Besides the transcriptional regulation, leptin directly depolarizes (activates) POMC neurons while simultaneously hyperpolarizing (inactivates) NPY/AgRP neurons. In addition, systemic leptin administration to leptin deficient (*ob/ob*) mice rapidly induced synaptic input reorganization onto NPY/AgRP and POMC neurons, suggesting that the synaptic rearrangement is an important event for leptin-induced behavioral changes [107]. A recent study demonstrated that AgRP neurons are critical in mediating metabolic syndrome in *ob/ob* mice since ablation of AgRP neurons in leptin deficient (*ob/ob*) mice showed reduced food intake and improved glucose tolerance [108].

3.2 Insulin

In addition to leptin, other hormones such as insulin are also regulators of POMC neurons. Insulin, produced from pancreatic β -cells, plays a fundamental role in the regulation of glucose homeostasis by modulating glucose uptake in peripheral organs. However, since insulin receptors (IR) are widely expressed in the brain, accumulated evidence has indicated a role for insulin in the CNS. Indeed, brain specific-IR deficient mice develop obesity with increased body weight, fat mass, and food intake [109]. However, mice with

selective deletion of IR in the arcuate melanocortin system, either POMC or AgRP neurons, did not display alteration in energy homeostasis, while only a defective suppression on hepatic glucose production was found in mice with selective deletion of IR in AgRP neurons [110]. In addition, re-expression of IR in either POMC or AgRP neurons of L1 mice, a genetic mouse model with significant reduction of IR in the ARC, suggested that insulin signaling in AgRP neurons negatively regulates hepatic glucose production while IR activation in POMC neurons positively regulates hepatic glucose production and energy expenditure [111]. Interestingly, when both leptin and IR were ablated in POMC neurons, systemic insulin resistance despite increased pancreatic insulin secretion was observed in these mice. In addition, the obese phenotype observed in POMC-specific ObR knockout mice was ameliorated when both ObR and IR were selectively deleted from POMC neurons, suggesting that insulin and leptin signaling in POMC neurons may have opposing effects in the regulation of body weight but additive effects on glucose homeostasis. In support of this, leptin and insulin responsive neurons are expressed in distinct subpopulations of POMC neurons [112]. Thus, the divergent effects of leptin and insulin on energy homeostasis may be due to the activation of different POMC subpopulations.

3.3 Ghrelin

Ghrelin, the hunger hormone, is predominantly secreted by specialized endocrine cells of the stomach when the stomach is empty. Ghrelin is synthesized as a preprohormone and several processes are required to generate its active form. After removing the signal peptide from the preprohormone, ghrelin precursor is acylated at the third serine with n-

octanoic acid by an enzyme called ghrelin o-acyltransferase (GOAT) [113] and then it is cleaved by prohormone convertase 1/3 to produce the active 28-amino-acid acylated ghrelin. Ghrelin exerts its orexigenic effects through the growth hormone secretagogue receptor (GHSR). The physiological function of Ghrelin and GHSR on feeding has been demonstrated by ghrelin and GHSR deficient mice. Both ghrelin- and GHSR-deficient mice are resistant to high-fat diet-induced obesity but only when mice are exposed to the diet shortly after post-weaning and not in adulthood [114].

The strongest expression of GHSR has been observed in the hypothalamus (Figure 9). This observation, together with ghrelin-induced high c-fos expression in the hypothalamus, indicated this site of the CNS as the main site of ghrelin's action. In the ARC, GHSR is predominantly expressed in NPY/AgRP neurons. Accordingly, peripheral and central administration of ghrelin induced c-fos expression in NPY/AgRP neurons [115]. In addition, ghrelin increases NPY and AgRP mRNA expression levels and the electrical activity of NPY/AgRP neurons. Although ghrelin inhibits POMC neuronal activity, no GHSR expression has been reported in POMC neurons, therefore suggesting that this inhibitory effect may be mediated by the activation of NPY/AgRP neurons (Figure 9). In addition, ghrelin positively regulates prolyl carboxypeptidase, the enzyme responsible for α -MSH degradation [116], thus further increasing the orexigenic tone. Ghrelin-induced food intake is mediated by NPY/AgRP neurons. For example, administration of neutralizing antibodies or antagonists of both NPY and AgRP blunted the orexigenic effects of ghrelin. Consistently, ghrelin's effect on feeding is abolished in NPY/AgRP double-deficient mice [117]. NPY/AgRP neurons-mediated ghrelin's effect is evident in diet-induced obese mice (DIO). DIO mice show decreased expression of *Npy* and *AgRP* mRNA levels, decreased *Goat* mRNA levels in the stomach and

decreased hypothalamic *GHSR* expression while being ghrelin resistance. Interestingly, while ghrelin resistance has been observed in DIO mice, *ob/ob* mice retain their sensitivity to ghrelin, suggesting that elevated leptin levels may be involved in the development of ghrelin resistance. In support of this, a recent study showed that central leptin administration in *ob/ob* mice induced ghrelin resistance [118]. AgRP-selective re-expression of GHSR in whole body of GHSR-deficient mice partially restores the orexigenic response to ghrelin. In addition, AgRP-specific vesicular GABA transporter knockout mice showed impaired ghrelin-mediated feeding response and impaired inhibitory postsynaptic potentials in POMC neurons, indicating that GABA release from AgRP neurons is an important mediator of ghrelin's effect on food intake. Of note, uncoupling protein 2 (UCP2) is a mediator of ghrelin's action on feeding behavior in both the hypothalamus and the ventro tegmental area (VTA). In *Ucp2* deficient mice, intrahypothalamic administration of ghrelin showed a blunted effect on food intake and intra-VTA ghrelin injection also attenuated food intake [119].

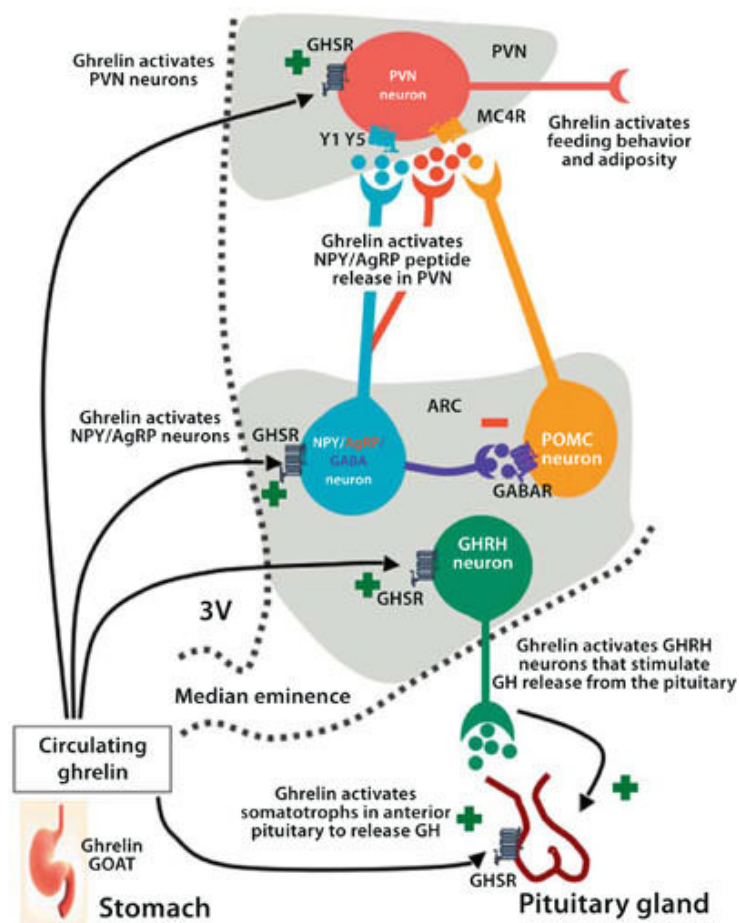


Figure 9: Melanocortin system in the arcuate nucleus of the hypothalamus. The primary neurons of the melanocortin system are the orexigenic NPY–AgRP neurons and the anorexigenic POMC neurons, which both send projections to the paraventricular nucleus. NPY–AgRP neurons also send inhibitory projections to neighbouring POMC cells. AgRP neurons are stimulated by ghrelin, which promotes feeding and silences firing of POMC neurons. The effect of the NPY–AgRP cells is mediated by release of GABA, NPY and AgRP.

3.4 Circulating nutrients

Nutritional signals have been known to regulate energy balance by hypothalamic neurons sensitive to the metabolic status. Circulating nutrients such as glucose, amino acids and fatty acids, affect POMC neurons. Several metabolic fuel sensors have been shown to be activated in POMC neurons according to the metabolic state of the organism. A considerable amount of work has been devoted to decipher the mechanism by which “nutrient sensing” occurs in the regulation of whole body energy metabolism. A large

amount of data has accumulated to suggest that specific mechanisms exist for the “sensing” of the levels of various circulating nutrients, such as carbohydrates (glucose), fatty- and amino acids, by the brain. These sensors include mammalian target of rapamycin (mTOR) kinase, Sirtuin1 (Sirt 1), AMP-activated protein kinase (AMPK) and glucokinase. In addition to PI3K cascade regulation, FoxO1 has recently been shown to be regulated by a NAD⁺ -dependent deacetylase, Sirt1 [61]. Sirt1 is expressed in POMC neurons and its activity levels are increased by fasting. Cakir and collaborators recently found that the fasting- induced decrease of FoxO1 acetylation and POMC expression are Sirt1-dependent [61]. Furthermore, they also showed that Sirt1 regulates S6 Kinase signaling, which has previously been shown to be under the control of mTOR kinase activity. mTOR kinase activity, which is inversely altered by changes in the AMP/ATP ratio, leads to the phosphorylation of its downstream targets including S6K1, S6 ribosomal protein (S6), and eukaryotic initiation factor 4E-binding protein (4E-BP1). The chronic activation of mTOR, specifically in POMC neurons, has been found to induce a defect in the development of POMC projections in a cell-autonomous manner, as well as an increase in the size of the soma. As mTOR signaling is critical for protein synthesis in general, it is reasonable to assume that alteration in mTOR activity will affect many neuronal parameters, such as membrane potential and synaptic events. Although Sirt1 and mTOR seem to regulate POMC neurons, it remains to be established whether they act independently or whether one is upstream to the other. Furthermore, recent data has reported that in peripheral tissues such as muscle, Sirt 1 is regulated by AMPK- the activity of which changes inversely to that of mTOR and according to changes in the AMP/ATP ratio. As for Sirt 1, fasting induces an increase of AMPK by increasing its phosphorylation on Thr172 within its catalytic α subunit. The activation of AMPK in the

hypothalamus has been shown to be involved in the fatty acid metabolic pathway [120]. Another sensing mechanism specific for glucose that was demonstrated first in pancreatic beta cells, and then in hypothalamic neurons, is mediated by glucokinase. However, the mechanism by which glucokinase regulates glucose-sensing appears to be independent of ATP, as shown by several studies [120]. Any of the aforementioned mechanisms, in turn, must trigger an orchestrated and predictable alteration of the activity of the POMC neuronal circuits that alter behavior, as well as autonomic and endocrine functions so that adaption to the changing metabolic milieu can occur. Thus, when one considers fuel sensing, it is from the perspective of the whole body responses to circulating carbohydrates, fatty acids or amino acids. The idea that there is a “sensor” of these fuels on the neurons of the hypothalamus independent of fuel metabolism has never been proven to exist. In contrast, the utilization of specific fuels by hypothalamic neurons in support of the whole body metabolism of that class of nutrient as a way of “fuel sensing” is more in line with available data. For example, glucose is considered the main driver of POMC neuronal firing [121]. POMC neurons increase their firing when glucose levels rise and this, in turn, affects hepatic glucose homeostasis and insulin action [121]. This is entirely consistent with the putative role of POMC cells as satiety signaling neurons, since at satiety, circulating glucose levels are elevated and POMC neurons exhibit increased firing. In turn, the increase in glucose oxidation induces a rise of free radicals which seems to play an important role as a driving force of POMC neuronal firing. Conversely, during negative energy balance when glucose levels are lower and circulating fatty acids increase, POMC neurons are silent, while NPY/AgRP neurons show increased firing. The intracellular mechanism of fatty acid oxidation occurs without an elevation of ROS levels [119].

6. Aims

PRCP represents a novel central regulatory element in the modulation of energy metabolism. Although PRCP was discovered several decades ago, its role in the regulation of energy homeostasis is novel and its site of action in the regulation of metabolism has not been determined. PRCP is expressed in different neuronal populations of the CNS, including the NPY/AgRP- and the MC4R-expressing neurons, where their role in controlling energy metabolism is ill-defined. Therefore, these studies were carried out to elucidate the role of PRCP in specific neuronal populations in the regulation of metabolism.

AIM 1: TO ELUCIDATE THE ROLE OF PRCP IN NPY/AGRP EXPRESSING NEURONS OF THE ARCUATE NUCLEUS.

Previous studies have shown that PRCP is expressed in NPY/AgRP neurons and its activity is modulated by ghrelin [116], an important regulator of NPY/AgRP neuronal function. Thus, to understand the role of PRCP in this neuronal population, we generated and studied mice with selective ablation of PRCP in NPY/AgRP neurons using the LoxP-cre system.

AIM 2: TO ELUCIDATE THE ROLE OF PRCP IN MC4R-EXPRESSING NEURONS.

Previous studies have shown that PRCP is expressed in MC4R neurons. Thus, to understand the role of PRCP in this neuronal population, we generated and studied mice with selective ablation of PRCP in MC4R neurons using the LoxP-cre system.

AIM 1: TO ELUCIDATE THE ROLE OF PRCP IN NYP/AGRP-EXPRESSING NEURONS OF THE ARCUATE NUCLEUS.

7. Material and Methods

7.1 Animals

All animal studies were approved by Yale University Institutional Animal Care and Use Committee. All mice studied were on a C57Bl6 background, that being the transgenic mice back-crossed for ≥ 10 generations. Animals had a free access to either a standard chow diet (SD; Harlan Teklad no. 2018, 18% calories from fat) or a 45% high-fat diet (HFD; Harlan Teklad no. 93075) for ≤ 13 weeks from weaning. All animals were kept in temperature and humidity controlled rooms, in a 12/12h light/dark cycle, with lights on from 7:00AM-7:00PM. Food and water were provided ad libitum, unless otherwise stated. Fasted animals were food deprived overnight (about 16 h). For intraperitoneal (i.p) ghrelin injection, animals received a single dose of either ghrelin (10 nmol/mouse; PolyPeptide Laboratories, INC, Torrance, CA; cat.# SC1356) or the equivalent volume of vehicle (saline) just before the beginning of the dark phase. Food was removed and animals sacrificed after three hours from the injection. To determinate the feeding behaviour of wild-type and PRCP $-/-$ after ghrelin administration (intraperitoneal, 10 nmol) at the beginning of the dark phase food was measurement at 1,2 and 3 hours after injection.

7.2 Generation of mice with selective deletion of PRCP in AgRP neurons

We used the cre/lox technology to generate mice in which PRCP will be selectively ablated in AgRP neurons ($PRCP^{fl/fl}$ - $AgRP$ -cre). The construct was developed by the EUCOMM (European Mouse Model Cell Repository; see www.eucomm.org). Once received the PRCP floxed mice ($PRCP^{fl/fl}$) were generated by the Yale Genome Editing Center. By crossing the $PRCP^{fl/fl}$ mice with the AgRP-cre mice that express Cre recombinase under the control of AgRP promoter, we then produced mice with selective deletion of PRCP in AgRP neurons (Figure 10). As control mice, $PRCP^{+/+}$ -AgRP-cre positive littermates were used. All mice studied were on a mixed background (129SvEv, FVB and C57BL/6J).

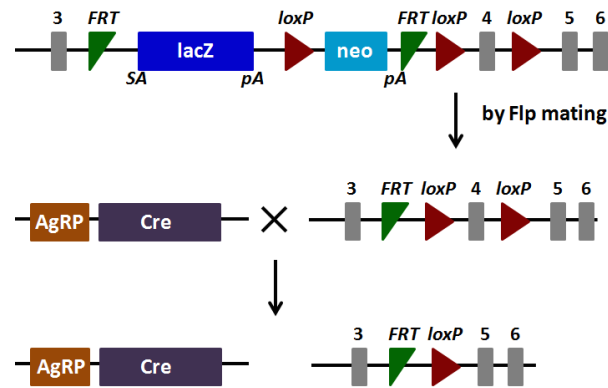


Figure 10: Generation of Mice Used in This Study. Schematic diagram showing AgRP-cre and $PRCP^{fl/fl}$ constructs

1.3 Genotyping

Genomic DNA was isolated from tails or yolk sacs by standard methods. PRCP mice were genotyped by polymerase chain reaction (PCR parameters: 42 cycles, 93°C for 30 s, 56°C for 1 min, and 72°C for 5 min). Amplification of a wild-type (WT) allele generated a 3.6-kb product, and a 4.1-kb product in the case of a mutant allele using the following primers: PRCP2 FRT5: 5' -ccttcagggcctcagtc -3' , PRCP2 FRT3: 5' -cagccatatttaaacattgagat -

3. Chrd alleles were genotyped as previously described [122]. AgRP-cre alleles were identified by PCR analysis for cre sequences using the following primers: M263, 5' - atggctaatecgccatcttccagca -3' ; and M262, 5' -gccctggaaggattttgaagca -3' (PCR parameters: 35 cycles, 94° C for 30 s, 55°C for 45 s, and 72°C for 55 s).

7.4 Metabolic chamber recordings

Adult male mice in SD or HFD (n=11) were acclimated in metabolic chambers (TSE Systems, Germany – Core Metabolic Phenotyping Center, Yale University) for 4 days before the start of the recordings. Mice were continuously recorded for 3 days with the following measurements being taken every 30 minutes: water intake, food intake, ambulatory activity (in X and Z axes), and gas exchange (O₂ and CO₂) (using the TSE LabMaster system, Germany). VO₂, VCO₂ and energy expenditure were calculated according to the manufacturer's guidelines (PhenoMaster Software, TSE Systems). The respiratory exchange rate (RER) was estimated by calculating the ratio of VCO₂/VO₂. Values were adjusted by body weight to the power of 0.75 (kg^{-0.75}) where mentioned. For the fasting response study, the same mice used above were acclimated to the cages for 4 days and then food was removed from the cages 2 hours before the dark cycle. The metabolic parameters (cited above) were recorded during ZT 12–18 and the data was analyzed as above. Body composition was measured in vivo by MRI (EchoMRI; Echo Medical Systems, Houston, TX).

7.5 Real-time PCR

Total RNA from the liver was extracted from AgRP PRCP^{fl/fl} and PRCP^{+/+}-AgRP-cre animals ($n = 10$ for each group in SD) using Trizol solution (Invitrogen). Phosphoenolpyruvate carboxykinase 1 (PCK1) and glucose-6-phosphatase (G-6-Pase) mRNA levels in the liver were measured by real-time PCR. A High Capacity cDNA Reverse transcription Kit (Applied Biosystems) was used for the reverse transcription. Real-time PCR (LightCycler 480; Roche) was performed with diluted cDNAs in a 20- μ l reaction volume in triplicates. Primers used for this study are as follows: cat. no. Mm 00494069_m1 for PCK1, cat. no. Mm 00839363_m1 for G-6-Pase, and cat. no. 4319413E-0810041 for 18S rRNA (Applied Biosystems). The calculations of average Cp values, SDs, and resulting expression ratios for each target gene were based on the Roche LightCycler 480 software.

7.6 Glucose and insulin tolerance tests

Glucose tolerance test was performed in 16- to 17-hours-fasted animals. After the level of blood glucose was determined, fasted animals were injected intraperitoneally with 20% glucose (10 ml/kg; DeltaSelect) in saline. Blood glucose levels were then monitored at 15, 30, 60, and 120 minutes from the injection.

Insulin tolerance test was performed with mice fed ad libitum. After determination of basal blood glucose levels, each animal received an intraperitoneal injection of insulin, 0.75 U/kg (Actrapid; Novo Nordisk). Blood glucose levels were then measured at 15, 30, 60, and 120 min after insulin injection.

7.7 cfos Immunostaining

After overnight fasting, mice were anesthetized and transcardially perfused with 0.9% saline with heparin followed by fixative (4% paraformaldehyde, 15% picric acid, 0.1% glutaraldehyde in PBS). Brains were collected and post fixed overnight before coronal sections were taken at every 50 μm

Sections were washed and incubated with the rabbit anti-cfos antibody (Santacruz, 1:2000), and the chicken anti-eGFP antibody (Life Technologies Corporation, 1:5000) in PB containing 4% normal goat serum, 0.1% glycine, and 0.2% Triton X-100 for 24 hours at room temperature. After several washes with phosphate buffer (PB), sections were incubated in the secondary antibodies (biotinylated goat anti-rabbit immunoglobulin G [IgG]; 1:250 in PB; Vector Laboratories and goat antichickens Alexa-fluor 488; 1:200 in PB; Life Technologies) for 2 hours at room temperature, then rinsed in PB five times, 10 minutes each time. Sections were then mounted with VectaShield antifade (Vector Laboratories). Fluorescent images of five to seven brain sections were captured with confocal microscope and analyzed by imaging Software (Image J).

7.8 Electron Microscopy analysis

Ad libitum 12 h fasted mice were anesthetized and transcardially perfused with 0.9% saline followed by fixative (4% paraformaldehyde, 15% picric acid, 0.1% glutaraldehyde in 0.1 M PB. Brain coronal sections were either immunostained double-stained with anti-cfos (1:10000) and anti- eGFP primary antibodies (1:5000). After several washes with PB, sections will be incubated in the secondary antibody (biotinylated goat anti-rabbit IgG; 1:250 in PB; Vector Laboratories) for 2 hr at room temperature, then rinsed in PB 3 times

10 min each time, and incubated for 2 hr at room temperature with avidin–biotin–peroxidase (ABC; 1:250 in PB; ABC Elite kit, Vector Laboratories). The immunoreaction was visualized with nickeldiaminobenzidine (NiDAB). Please note that NiDAB visualizes both cfos and eGFP (AgRP). However, because of their location (AgRP is localized in the cytoplasm while cfos is in the nucleus) we were able to distinguish activated and non-activated AgRP neurons. Sections were then osmicated (1% osmium tetroxide) for 30 min, dehydrated through increasing ethanol concentrations (using 1% uranyl acetate in the 70% ethanol for 30 min), and flat-embedded in araldite between liquid release-coated slides (Electron Microscopy Sciences, Fort Washington, PA). After capsule embedding, blocks of the ARC were trimmed. Ribbons of serial ultrathin sections will be collected on Formvar-coated single slot grids and examined using a Philips CM-10 electron microscope. Mitochondria morphology in activated AgRP neurons in fed and fasted mice was blindly analyzed from randomly selected sections, and Image J software was used to normalize cytoplasmic area so that mitochondrial density was expressed as number of mitochondria per square micrometers of cytosol [123].

7.9 Statistical analysis

We used software packages to analyze the data (Matlab R2009a and PASW Statistics 18.0) and plot the figures. First, we tested the homogeneity of variance across the different experimental conditions using Levene's or Barlett's test. When the p value was greater than 0.05 in these tests, homogeneity was assumed, and a parametric analysis of variance test was used. The student's t test was used to compare two groups. One-, two-, or three-way ANOVA were used as the other tests unless stated otherwise. Multiple comparisons were performed as described below. For repeated measures analysis we used a mixed

model ANOVA with time as a “within-subject repeated-measures” factor and treatments/genotype as a “between subject” factor. Significant effects were followed with Fisher’s PLSD post-hoc test with Bonferroni’s correction. When homogeneity was not assumed, the Kruskal–Wallis nonparametric ANOVA was used and the Mann–Whitney U test was used to determine post-hoc significance of differences between groups. Fisher’s exact test was used to find differences in the number of cells activated by ghrelin in the electrophysiology recordings. A value of $p < 0.05$ was considered statistically significant. All data is showed as mean \pm SEM unless stated otherwise.

**AIM 1: TO ELUCIDATE THE ROLE OF PRCP IN
NYP/AGRP-EXPRESSING NEURONS OF THE ARCUATE
NUCLEUS.**

8. Results

8.1 Selective deletion of PRCP in AgRP neurons alters energy balance in a gender-specific manner

To determine the effect of AgRP-selective PRCP deletion on whole-body physiology, we assessed their metabolic phenotype. Analysis of body weight from weaning to three months of age showed that when fed on a standard chow diet, *PRCP^{fl/fl}-AgRP-cre* male mice had significant lower body weight at all time points (4, 8 and 12 weeks of age) compared to their wild type controls (*PRCP^{+/+}-AgRP-cre* and *PRCP^{fl/fl}* male mice) (Figure 11, A). The difference in body weight was due to a significant reduction in fat mass in *PRCP^{fl/fl}-AgRP-cre* mice compared to controls (Figure 11, B) while no difference in lean mass was observed between the 2 experimental groups at all time points examined (Figure 11, C).

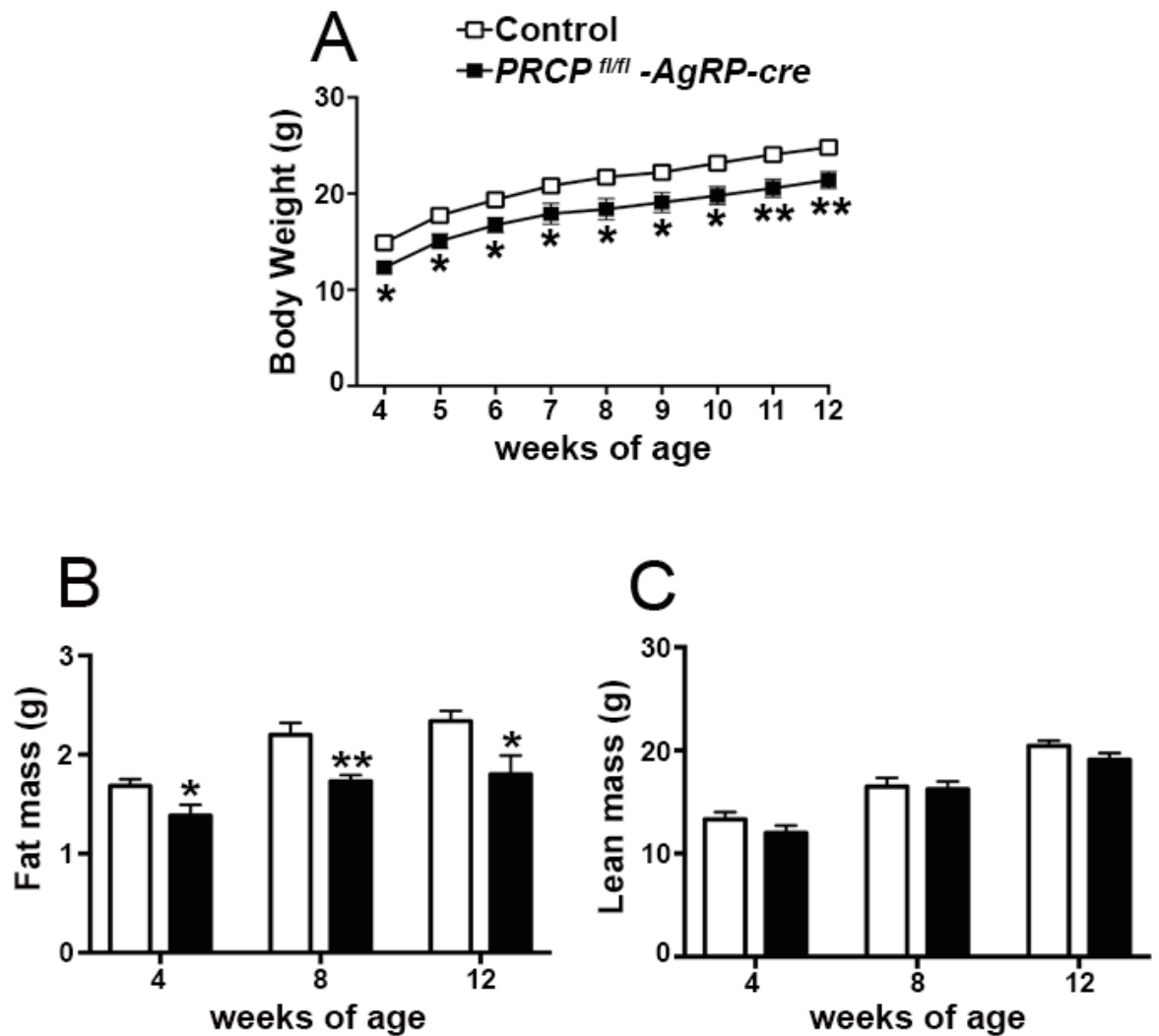


Figure 11: Deletion of PRCP in AgRP neurons in male is associated with a leaner phenotype. Analysis of (A) body weight ($n = 15$ per group), (B) fat mass and (C) lean mass at 4, 8 and 12 weeks of age ($n = 15$ per group), showed that male *PRCP^{fl/fl} -AgRP-cre* mice were leaner compared with littermate controls. Data in all graphs are shown as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$.

Interestingly, although a slight decrease in fat mass was observed in *PRCP^{fl/fl} -AgRP-cre* female mice compared to female controls at 4 and 8 weeks of age (Figure 12, B), no significant differences in body weight were found (Figure 12, A). No differences in lean mass at all time points were observed (Figure 12, C). This data suggests a gender difference in the role of PRCP in the modulation of metabolism.

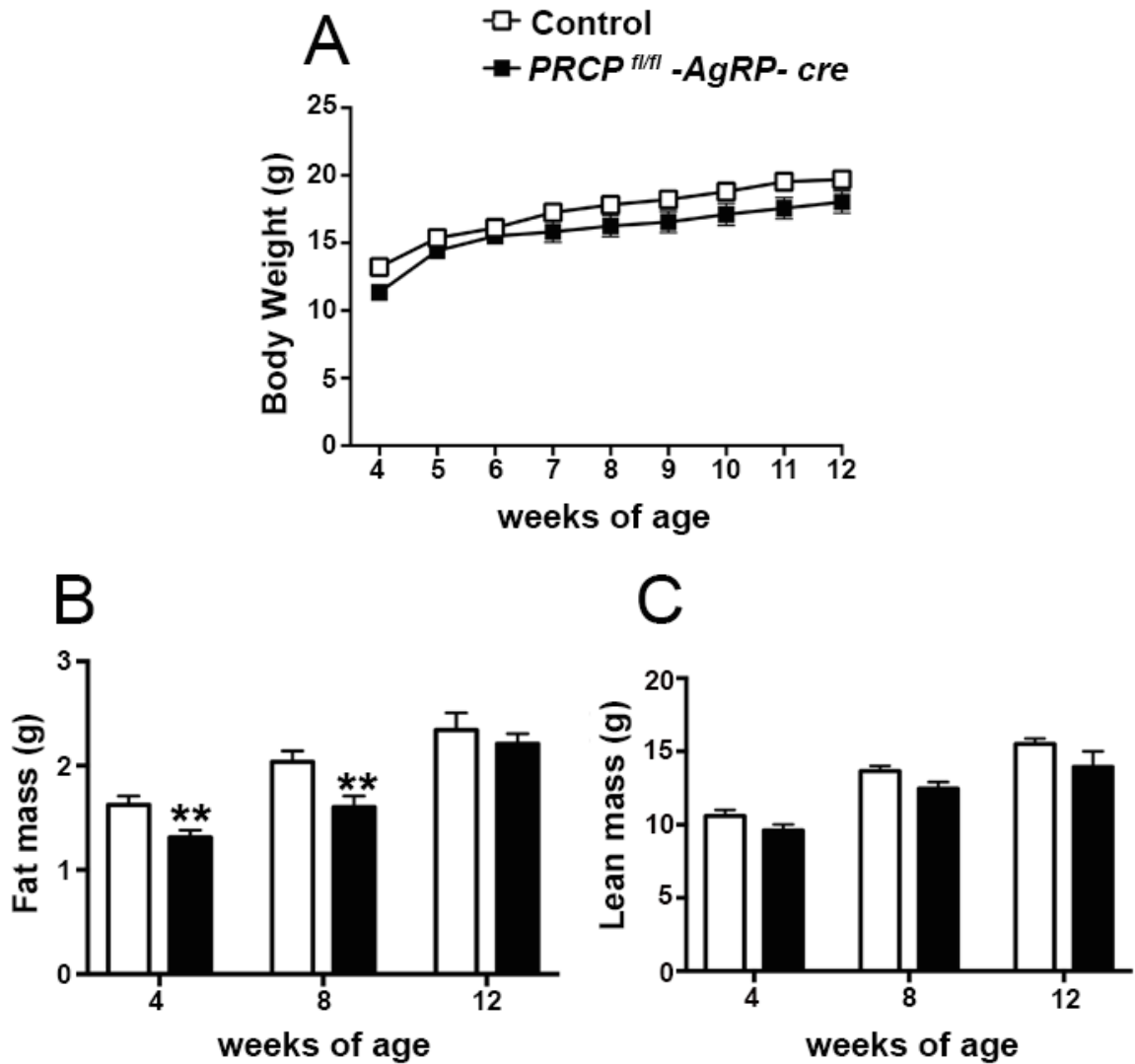


Figure 12: Loss of PRCP in AgRP neurons in female is associated with a decrease of fat mass. Analysis of (A) body weight ($n = 15$ per group), (B) fat mass and (C) lean mass ($n = 15$ per group) at 4, 8 and 12 weeks of age showed that female *PRCP^{fl/fl}-AgRP-cre* mice showed a decrease in fat mass compared to control mice. Data in all graphs are shown as mean \pm SEM. ** $P < 0.01$.

To understand whether the reduced body weight observed in *PRCP^{fl/fl}-AgRP-cre* male mice was due to a differential energy intake, we then measured feeding in a 24-hour period. *PRCP^{fl/fl}-AgRP-cre* male mice showed a significant difference in 24 hours-feeding compared to control mice (Figure 13, A). This difference in food intake was further tested

by analysis of 24 hours feeding after an overnight fasting (Figure 13, B). No differences in food intake were observed in female mice (data not shown).

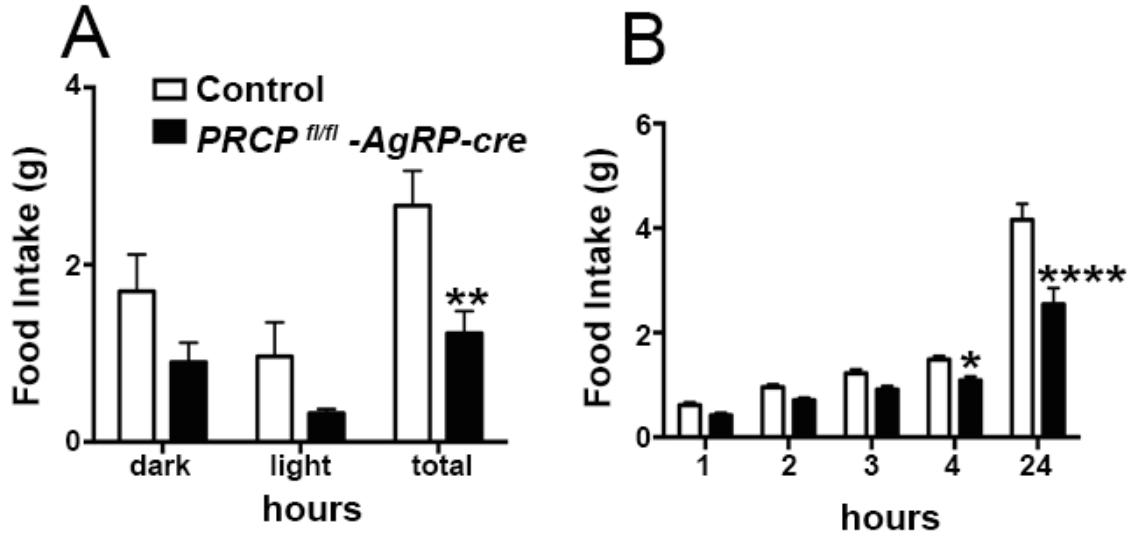


Figure 13: *PRCP^{fl/fl}-AgRP-cre* mice have reduced feeding. Analysis of 24hours food intake in (A) *PRCP^{fl/fl}-AgRP-cre* and control male mice ($n = 10$ per group). Analysis of 24 hours food intake after an overnight fasting (B) in *PRCP^{fl/fl}-AgRP-cre* and control male mice ($n = 10$ per group). Data in all graphs are shown as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; **** $P < 0.0001$ compared with controls.

In addition to food intake, we also measured locomotor activity and energy expenditure in male and female *PRCP^{fl/fl}-AgRP-cre* mice and controls. Male *PRCP^{fl/fl}-AgRP-cre* mice showed a significant reduction in 24 hours locomotor activity, which was due to a significant reduction during the dark cycle (Figure 14, A). No difference in EE was observed in *PRCP^{fl/fl}-AgRP-cre* male mice compared to controls (Figure 14, B and C). In addition, no changes in vO_2 , vCO_2 and respiratory exchange rate between *PRCP^{fl/fl}-AgRP-cre* and control mice were found (Figure 14, D-F).

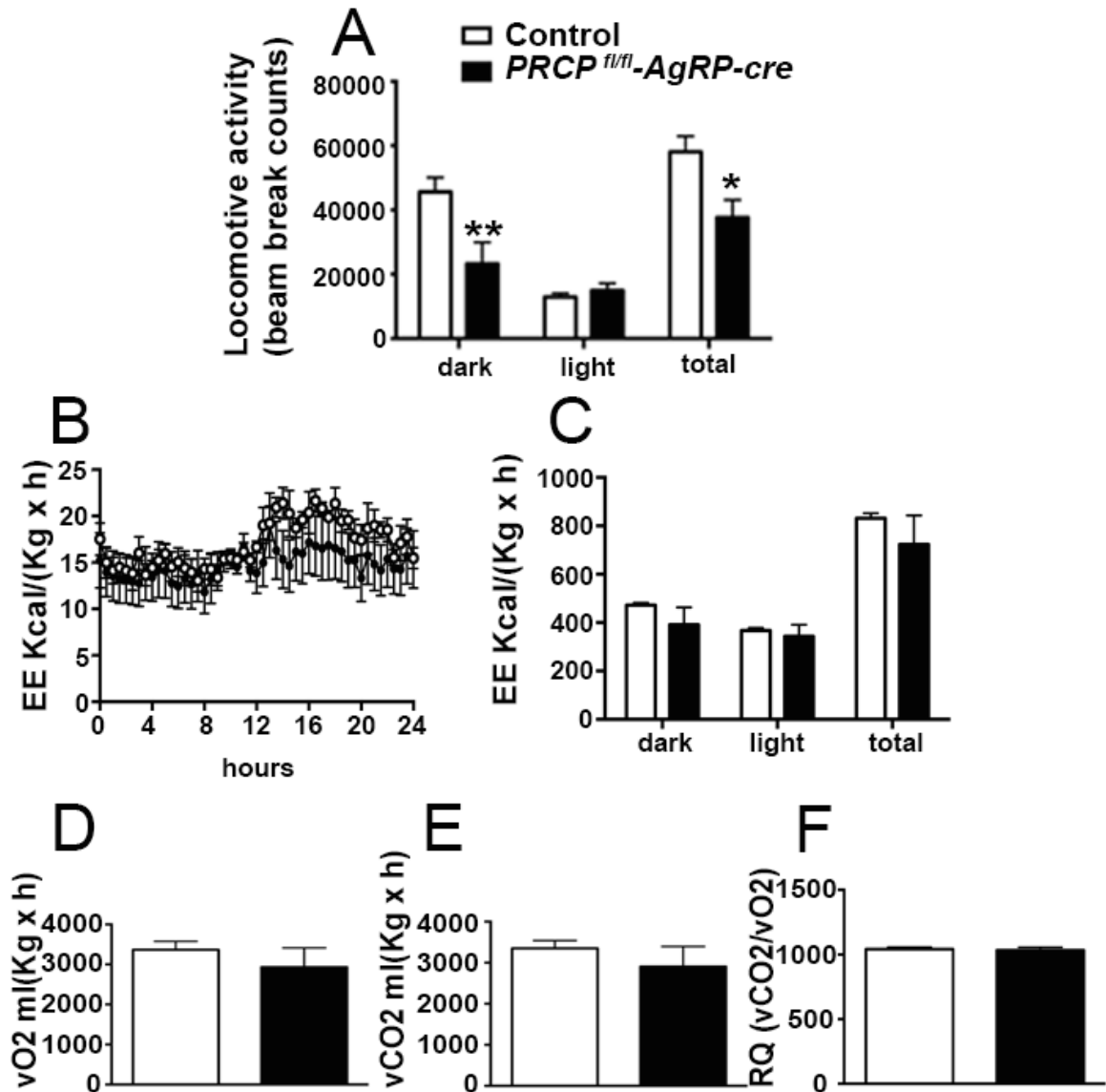


Figure 14 : *PRCP^{fl/fl}-AgRP-cre* male mice have decreased in locomotor activity and EE. (A) Decreased locomotor activity during dark and total cycles of male *PRCP^{fl/fl}-AgRP-cre* mice compared with male controls ($n = 5-6$). (B and C) The results of EE analysis after normalization by (A) lean mass between male *PRCP^{fl/fl}-AgRP-cre* and control mice ($n = 5-6$ per group). (D and E) The results of (D) VO_2 and (E) VCO_2 analysis in male *PRCP^{fl/fl}-AgRP-cre* and control mice using ANCOVA ($n = 5-6$ per group). (F) No difference in respiratory quotient (RQ) was found between the 2 groups. Data in all graphs are shown as mean \pm SEM. * $P < 0.05$ compared with controls.

Contrary to males, locomotor activity in females did not show differences between *PRCP^{fl/fl}-AgRP-cre* and control mice (Figure 15, A-C). No significant difference in EE, vO_2 , vCO_2 and respiratory exchange rate were found between *PRCP^{fl/fl}-AgRP-cre* and control female animals (Figure 15, D-F).

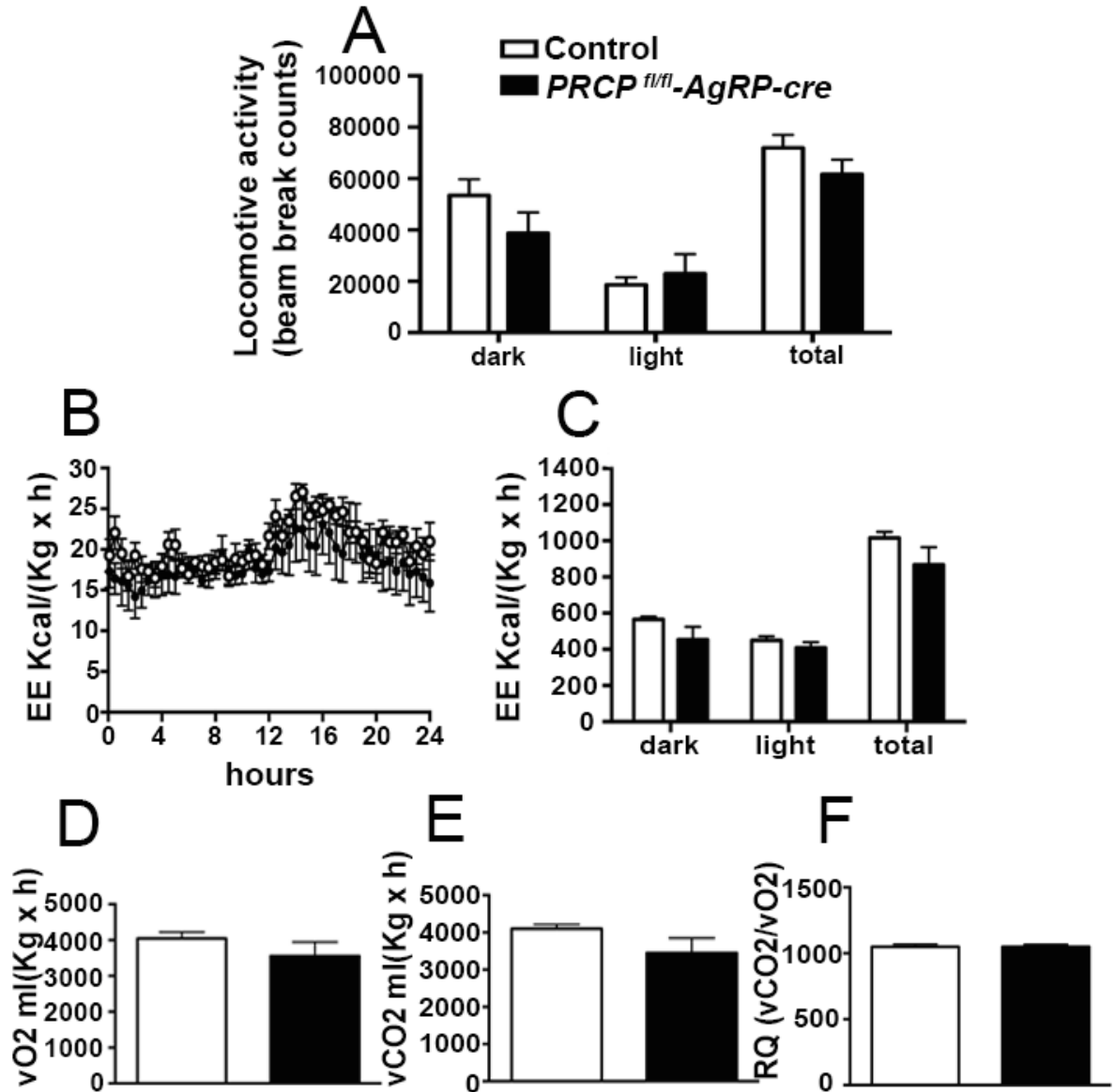


Figure 15: *PRCP^{fl/fl}-AgRP-cre* female mice showed no difference in locomotor activity and EE. (A) Decreased locomotor activity during dark and total cycles of female *PRCP^{fl/fl}-AgRP-cre* mice compared with male controls ($n = 5-6$). (B ,C) No difference in EE analysis after normalization by (A) lean mass in female *PRCP^{fl/fl}-AgRP-cre* compared to control mice ($n = 5-6$ per group). (D,E and F) No difference in (D) VO₂ and (E) VCO₂ and respiratory quotient (F) was found between the 2 groups.

8.2 Gender-specific effect of selective deletion of PRCP in AgRP neurons on insulin sensitivity

The ARC plays an important role in glucose metabolism. To test whether PRCP in the ARC has relevance to systemic glucose control, we analyzed glucose homeostasis in *PRCP^{fl/fl}-AgRP-cre* and control male mice. A significant improvement in glucose

tolerance was observed in *PRCP^{fl/fl}-AgRP-cre* mice compared to controls at 4, 8 and 12 weeks of age (Figure 16, A-C).

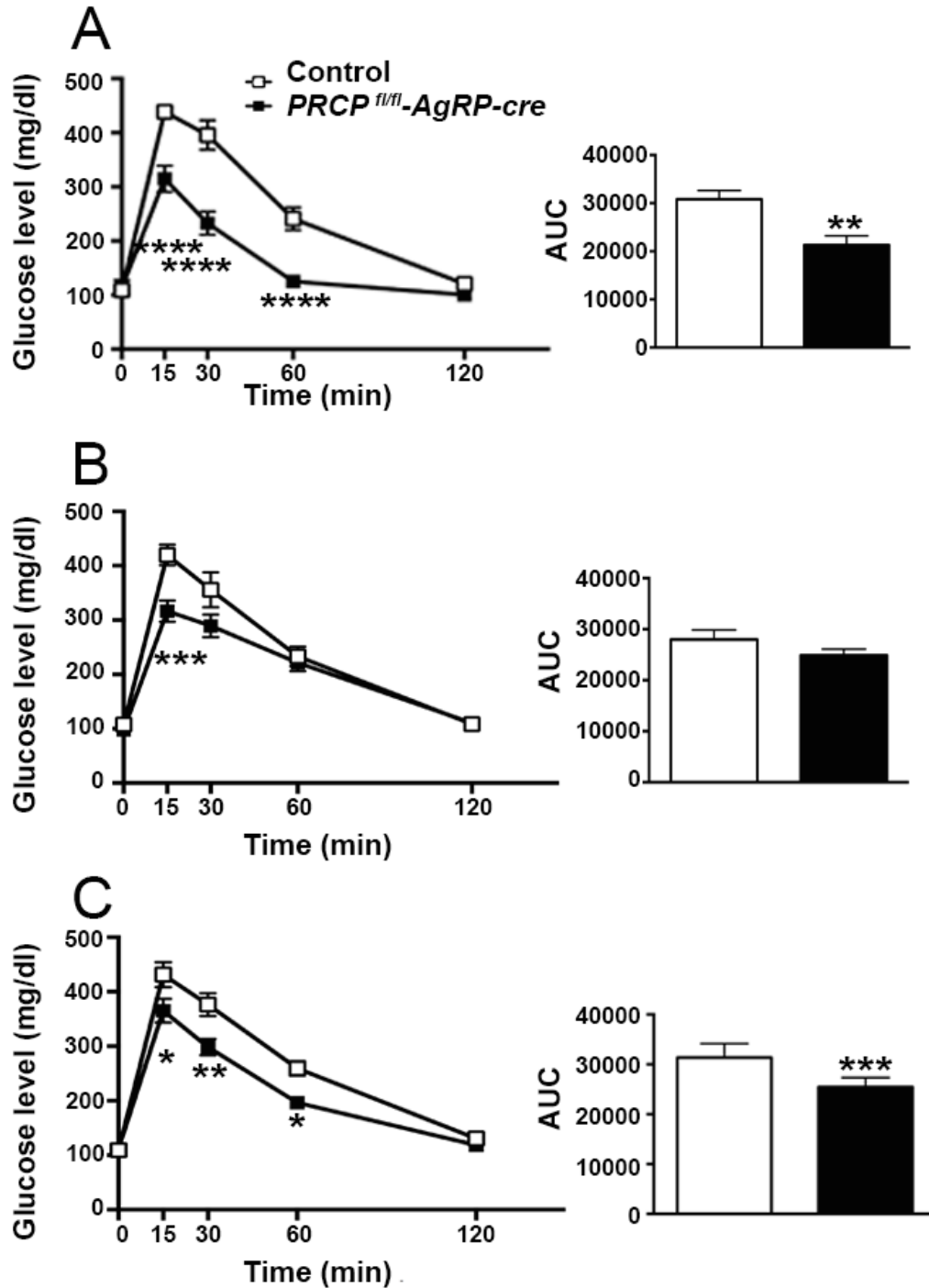


Figure 16: *PRCP^{fl/fl}-AgRP-cre* male mice have improved glucose metabolism. (A,B and C) Decreased glucose levels during a GTT in male *PRCP^{fl/fl}-AgRP-cre* animals ($n = 15$) compared with controls ($n = 15$) at 4, 8 and 12 weeks of age, respectively. Data in all graphs are shown as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ compared with controls.

Moreover, a significant difference in insulin sensitivity was observed between *PRCP^{fl/fl}-AgRP-cre* and control male mice at 4, 8 and 12 weeks of age (Figure 17, A-C).

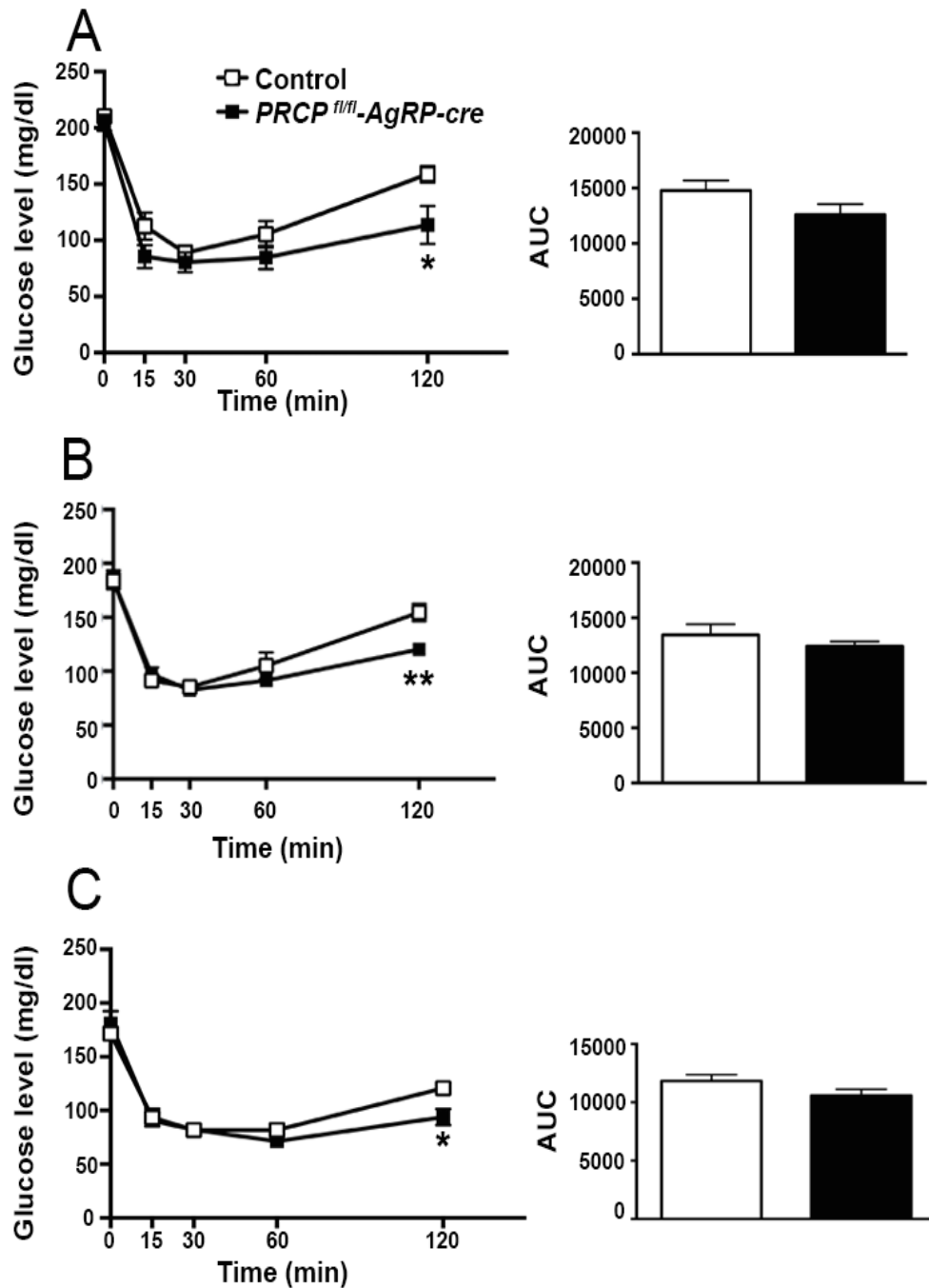


Figure 17: *PRCP^{fl/fl}-AgRP-cre* male mice have improved insulin tolerance. (A,B and C) Significant difference in insulin tolerance in male *PRCP^{fl/fl}-AgRP-cre* animals ($n = 15$) compared with controls ($n = 15$) at 4, 8, and 12 weeks of age, respectively. Data in all graphs are shown as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; compared with controls.

Because AgRP neurons have been implicated in the regulation of hepatic glucose production [124], we then analyzed the mRNA levels of hepatic gluconeogenesis enzymes, such as phosphoenolpyruvate carboxykinase (Pepck) (Figure 18, A) and glucose-6-phosphatase (G6pase). The mRNAs of both enzymes were significantly reduced in the liver of *PRCP^{fl/fl}-AgRP-cre* male mice compared with control male mice (Figure 18, B), indicating an increased hepatic insulin sensitivity in these mice.

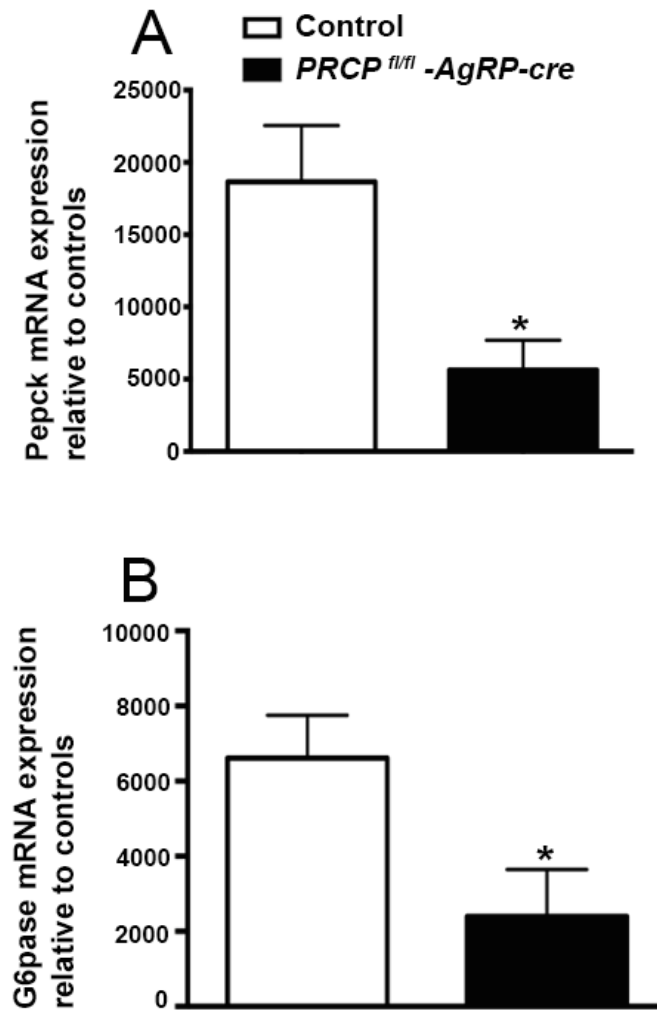


Figure 18: Effect of PRCP deletion on liver gluconeogenesis. (A and B) Graphs shown a significant decrease in the liver gluconeogenesis of *PRCP^{fl/fl}-AgRP-cre* male mice compared with matched controls, as shown by the liver mRNA levels of (A) *Pepck* ($n = 10$ per group) and (B) *G6pase* ($n = 10$ per group). Data in all graphs are shown as mean \pm SEM. * $P < 0.05$ compared with controls.

Contrary to males, *PRCP^{fl/fl}-AgRP-cre* female mice did not show changes in glucose metabolism (Figure 19, A-C). A slight decrease in insulin sensitivity was found in *PRCP^{fl/fl}-AgRP-cre* female mice at 8 weeks of age compared to control mice (Figure 19, D and E). However, this difference was not observed at 12 weeks of age (Figure 19, F).

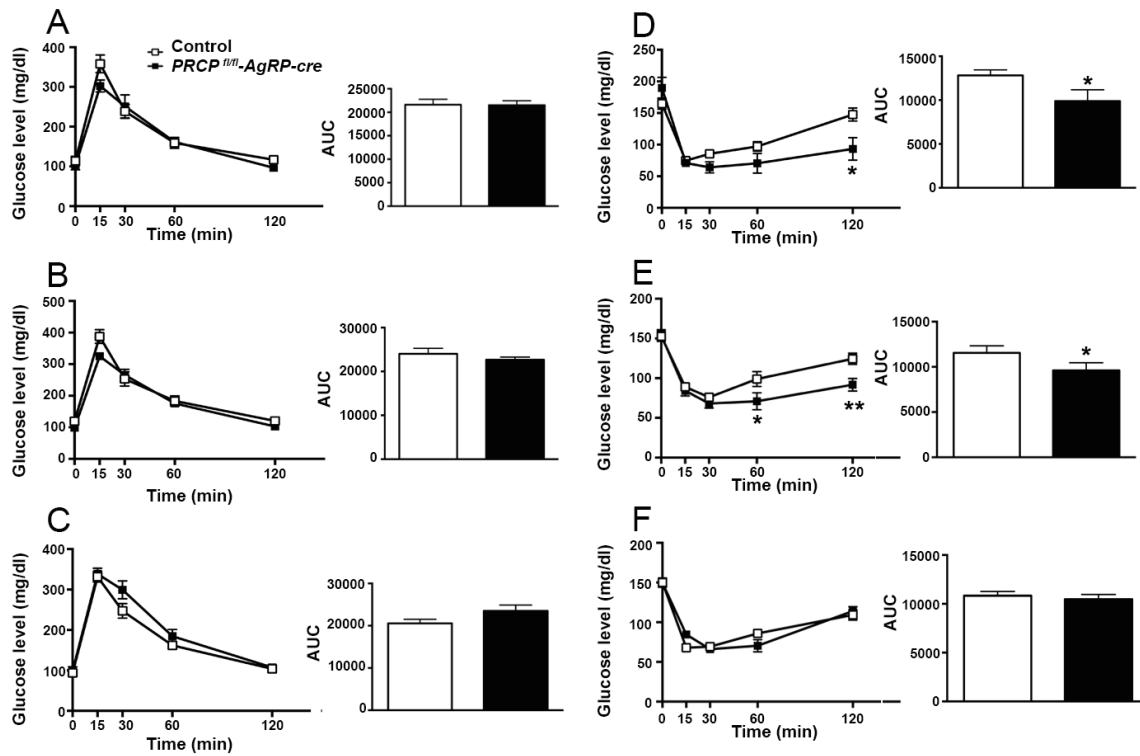


Figure 19: Effect of PRCP deletion in female on glucose homeostasis. Glucose (left) and insulin tolerance tests (right) in control and *PRCP^{fl/fl}-AgRP-cre* mice exposed to SD. (A,B and C) No difference was found in glucose tolerance between the 2 groups at 1,2 and 3 months old. (D,E and F): increased insulin sensitivity was found in *PRCP^{fl/fl}-AgRP-cre* mice when exposed to SD compared to control mice. Data in all graphs are shown as mean \pm SEM. *P < 0.05; **P < 0.01; compared with controls.

8.3 Selective deletion of PRCP in AgRP neurons induces decreased AgRP neuronal activation in male mice

To study the role of PRCP on AgRP cellular function, we then analyzed immunostaining for cfos, a marker of neuronal activation, in AgRP neurons. Analysis of double labeling in male mice revealed a significant decreased in the percentage of AgRP neurons immunopositive for cfos in *PRCP^{fl/fl}-AgRP-cre* mice compared to control mice (Figure 20,

A and B). No significant difference in the percent of *cfos* in AgRP neurons was observed between in *PRCP^{fl/fl}-AgRP-cre* and control female mice (Figure 20, C and D). Thus, this data suggests that selective deletion of PRCP in AgRP neurons of male mice significantly affects the activity levels of these neurons.

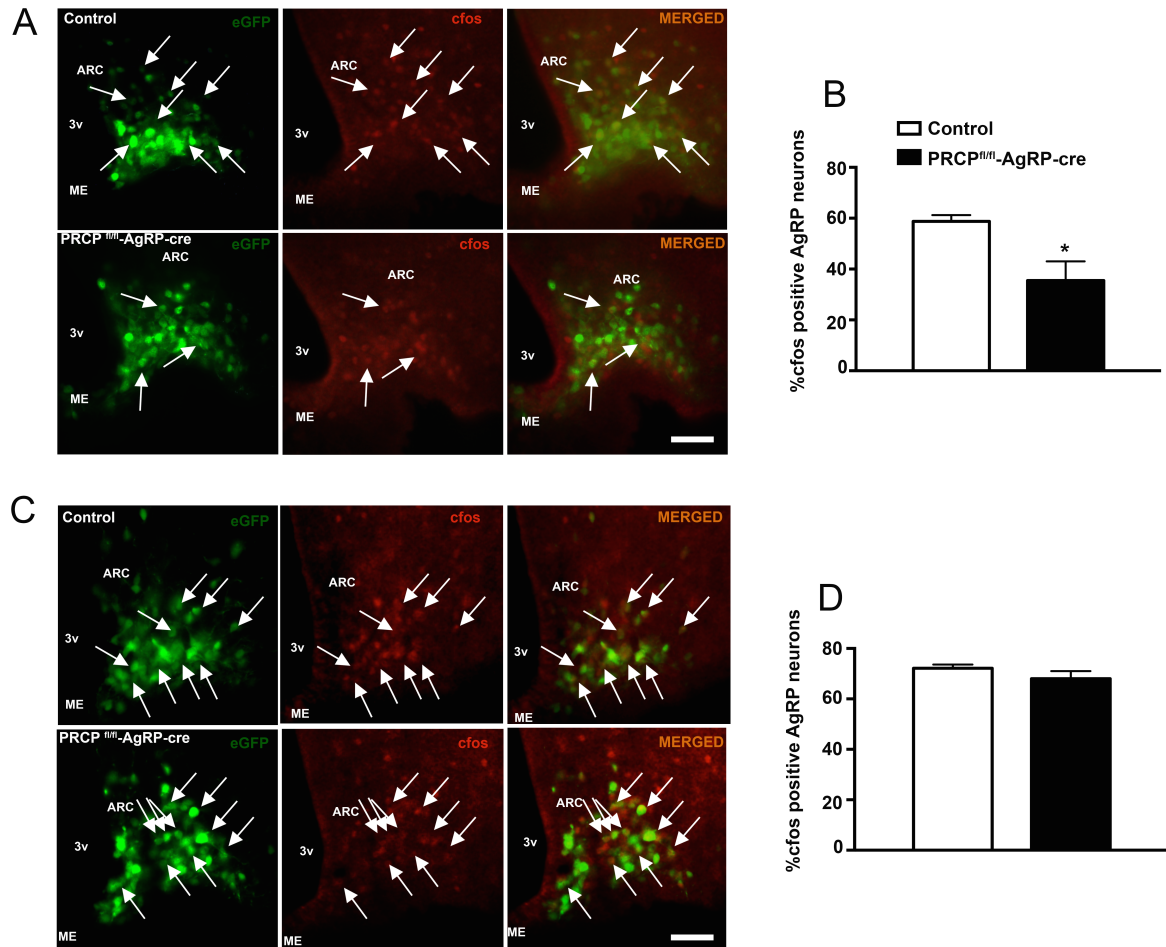


Figure 20 : PRCP mediated regulation of AgRP neurons. (A and B) Deletion of PRCP in male decrease *cfos* expression in AgRP neurons compared to controls mice (n=5 per group). (C and D) No difference in *cfos* expression between female *PRCP^{fl/fl}-AgRP-cre* and control mice was observed (n=5 per group). Scale bar in A and C (lower right panels) represents 75 μ m. All values denote mean \pm s.e.m. *P < 0.05.

8.4 PRCP in AgRP neurons mediates ghrelin-induced food intake

To determine whether the PRCP-dependent cellular responses are relevant for behaviour, we analyzed feeding behaviour in response to ghrelin administration (intraperitoneal, 10 nmol) in *PRCP^{fl/fl}-AgRP-cre* and control male mice at the beginning of the dark phase (Figure 21, A). At all time points examined (up to 24 hours) after injection, ghrelin-induced hyperphagia was strongly attenuated in *PRCP^{fl/fl}-AgRP-cre* compared to control mice (Figure 21, A). No difference in ghrelin-induced food intake was found in female mice (Figure 21, B).

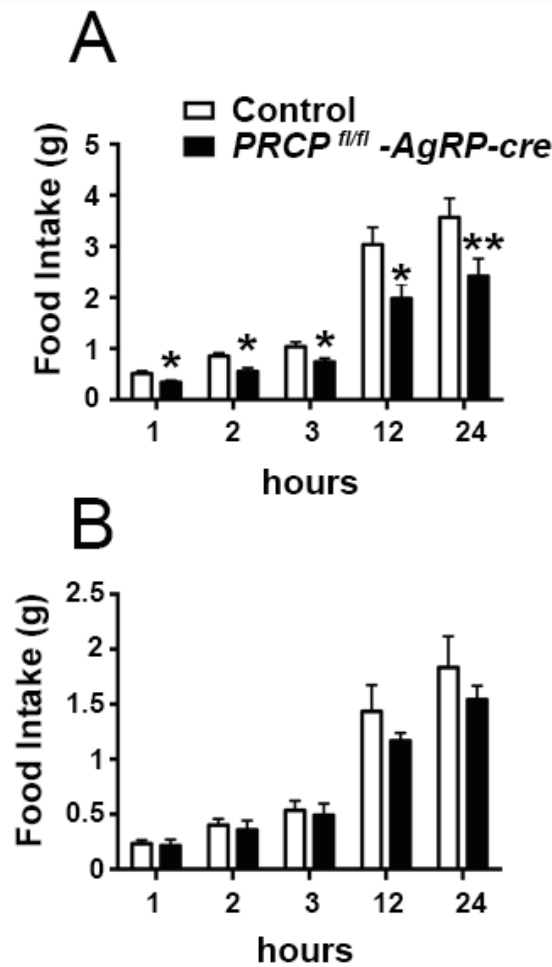


Figure 21: Effect of ghrelin-induced food intake in *PRCP^{fl/fl}-AgRP-cre* mice.(A) Peripheral injection of ghrelin in male *PRCP^{fl/fl}-AgRP-cre* mice decreased food intake compared to controls mice (mean \pm s.e.m., n = 10 per group). (B) No significant difference between female mice was found (mean \pm s.e.m., n = 10 per group). Data in all graphs are shown as mean \pm SEM. *P < 0.05; **P < 0.01; compared with controls.

8.5 Ablation of PRCP in AGRP neurons prevents hyperphagia and alters energy balance during high fat diet (HFD) feeding in both male and female mice

To assess whether a different diet composition would affect the metabolic outcomes, we then challenged the mice by exposing them to a high fat diet consisting of 45% fat. High fat diet was started in all mice at 6 weeks of age. As on standard chow diet, on HFD *PRCP^{fl/fl}-AgRP-cre* male mice showed a significant lower body weight compared to the male control at all time points examined (Figure 22, A). Contrary to the standard chow diet however, HFD feeding induced a significant difference in body weight gain in also *PRCP^{fl/fl}-AgRP-cre* female mice compared to female controls. This difference appeared as early as 6 weeks of HFD exposure (Figure 22, D). Consistent with the body weight measurements, significant changes in body composition were also observed (Figure 22, B and C; E and F). Specifically, a decrease in fat mass was observed in both male (Figure 22, B) and female (Figure 22, E) *PRCP^{fl/fl}-AgRP-cre* mice compared with control littermates. No differences in lean mass were observed between groups at each time point examined (Figure 22, male C and female F).

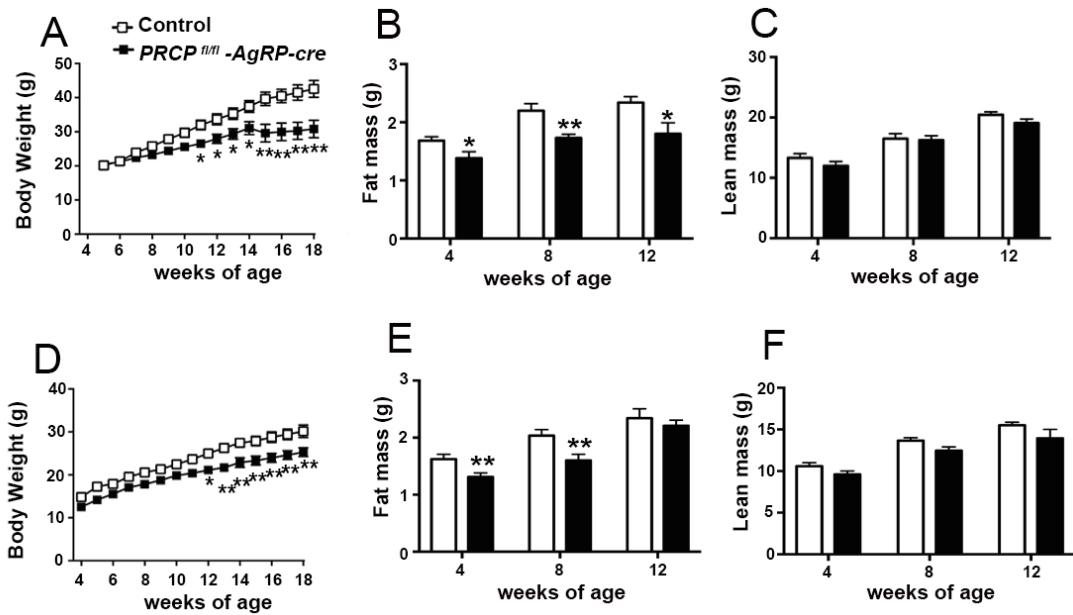


Figure 22: *PRCP^{fl/fl}-AgRP-cre* mice on HFD showed a leaner phenotype. Analysis of body weight in male (A) and female (D; $n = 10$ per group) in *PRCP^{fl/fl}-AgRP-cre* mice and their controls. Analysis of fat mass (B) and lean mass (C) in *PRCP^{fl/fl}-AgRP-cre* male mice and their controls ($n = 10$ per group). Analysis of fat mass (E) and lean mass (F) in *PRCP^{fl/fl}-AgRP-cre* female mice and their controls ($n = 10$ per group). Data in all graphs are shown as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

Analysis of 24 hour food intake showed that a significant decrease in food intake occurred in *PRCP^{fl/fl}-AgRP-cre* male mice compared to controls specifically during the dark phase (Figure 23, A). However, no significant differences in food intake were observed in female mice (Figure 23, B).

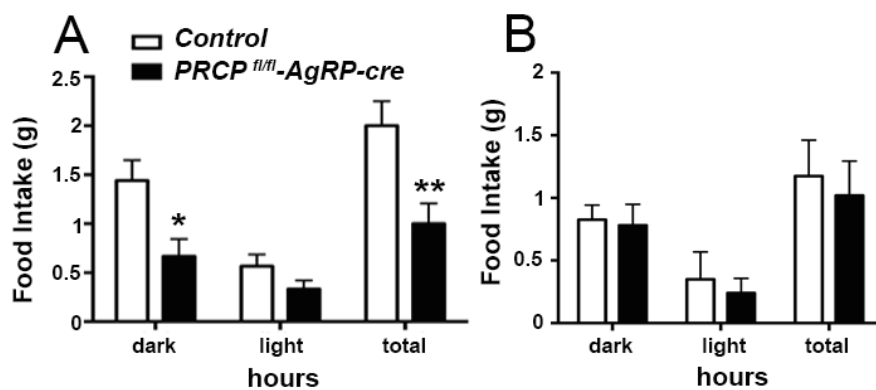


Figure 23: *PRCP^{fl/fl}-AgRP-cre* male mice on HFD have reduced feeding during 24 hours. Analysis of daily food intake in (A) male (B) female mice on HFD for 12 weeks ($n = 10$ per group) shows that food intake was significantly decreased during the dark cycle and total cycle in male mice, but not in female mice. Data in all graphs are shown as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$.

To assess whether fasting would alter food intake, we then analyzed 24 hour food intake after overnight food deprivation (16 hours) and found a slight, but not significant decrease in food consumption in *PRCP^{fl/fl}-AgRP-cre* female mice compared to controls (Figure 24, A-C).

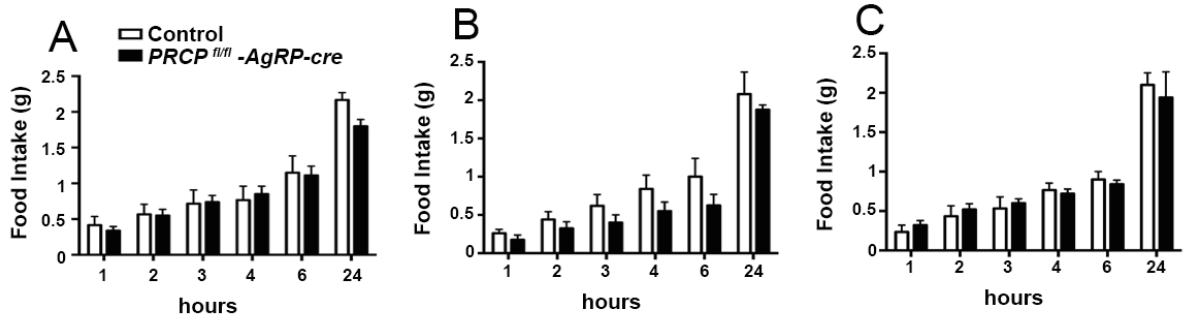


Figure 23: *PRCP^{fl/fl}-AgRP-cre* female mice on HFD do not show reduced feeding after fasting . (A,B and C) Analysis of food intake after fasting in *PRCP^{fl/fl}-AgRP-cre* and control female mice at 4, 8 and 12 weeks of HFD ($n = 10$ per group) does not show significant difference between the 2 groups.

On the other hand, food intake in *PRCP^{fl/fl}-AgRP-cre* male mice after an overnight fasting was significant lower than that of controls at all time points during HFD feeding (Figure 25, A-C).

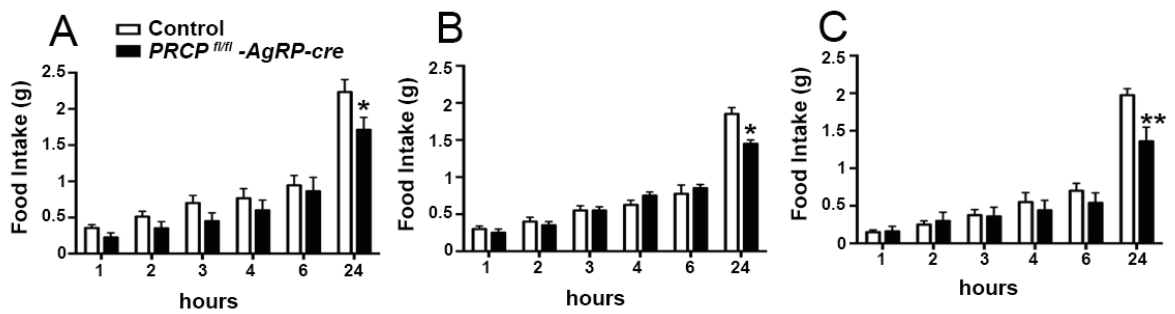


Figure 24: *PRCP^{fl/fl}-AgRP-cre* male mice on HFD have reduced feeding. (A,B and C) Analysis of daily food intake in male mice at 4, 8 and 12 weeks of HFD ($n = 10$ per group) shows that *PRCP^{fl/fl}-AgRP-cre* mice ate less than control mice 24 hr after fasting. Data in all graphs are shown as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$.

We then measured locomotor activity and EE in *PRCP^{fl/fl}-AgRP-cre* male mice and controls on HFD. The total locomotor activity of *PRCP^{fl/fl}-AgRP-cre* male mice on HFD

was significantly lower than that of controls (Figure 26, A) . No differences in EE (Figure 26, B and C), VO₂ (Figure 26, D), VCO₂ (Figure 26, E) and in the respiratory exchange rate (Figure 26, F) were observed in *PRCP^{fl/fl}-AgRP-cre* male mice compared with control mice.

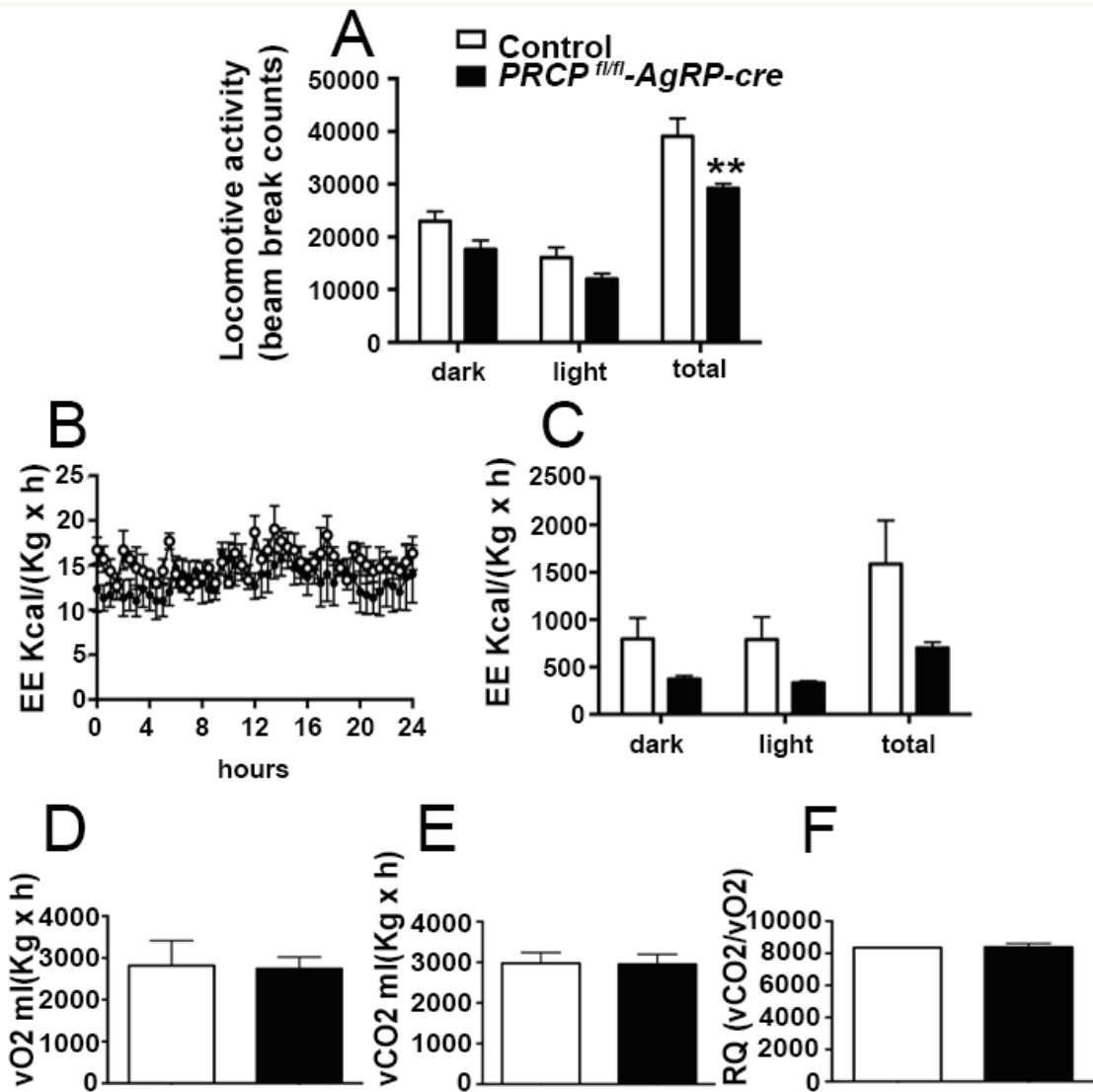


Figure 25: Energy metabolism in male *PRCP^{fl/fl}-AgRP-cre* and control mice on HFD. (A) Locomotor activity during metabolic cage studies was significant decreased during the total cycle in *PRCP^{fl/fl}-AgRP-cre* mice compared to control mice. EE (B and C) was not significantly lower in *PRCP^{fl/fl}-AgRP-cre* mice compared with control mice on HFD for 12 weeks. (n = 5 per group). No difference in VO₂ (D), VCO₂ (E) and respiratory quotient (RQ; F) was found between the 2 groups. Data in all graphs are shown as mean ± SEM. **P < 0.01.

8.6 Insulin sensitivity in $PRCP^{fl/fl}$ -AgRP-cre mice

Analysis of glucose metabolism showed a greater glucose tolerance at 4, 8 and 12 weeks on HFD in both male and female $PRCP^{fl/fl}$ -AgRP-cre mice compared to littermate controls (Figure 27, male A-C; female D-F).

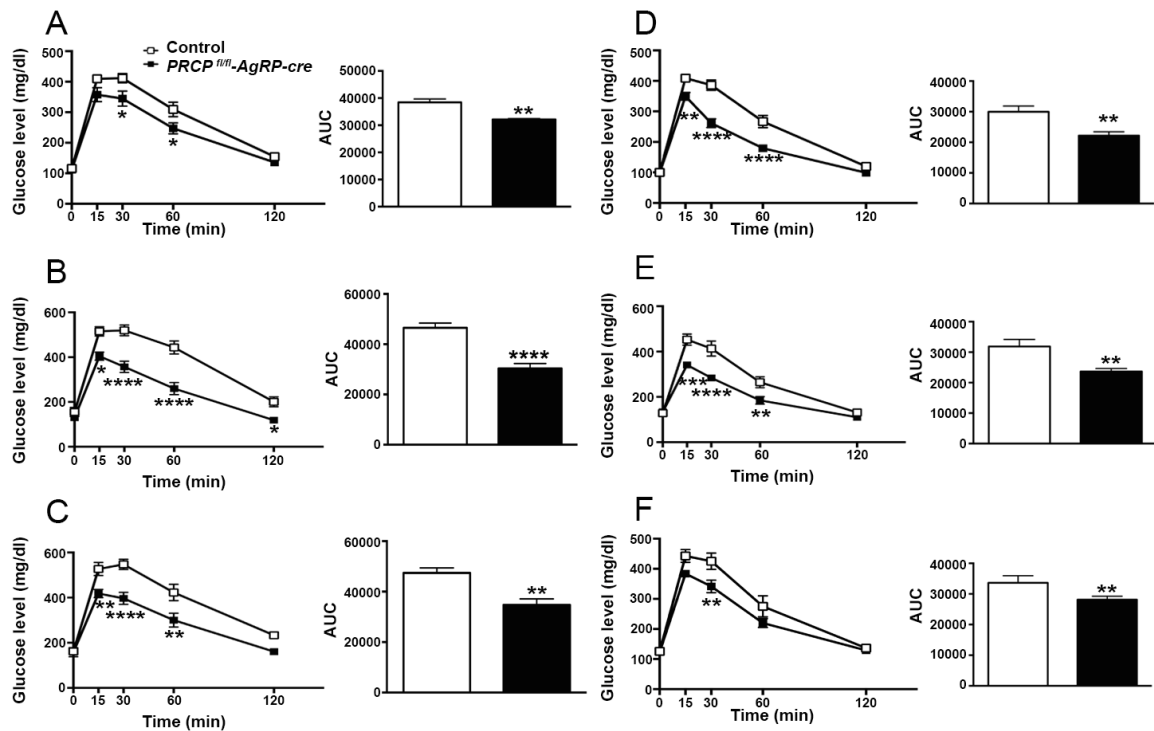


Figure 26: Effect of PRCP deficiency on glucose metabolism on HFD. (A,B and C) Graphs showing decreased circulating glucose levels in male $PRCP^{fl/fl}$ -AgRP-cre animals ($n = 15$) compared to controls ($n = 15$) at 4,8, and 12 weeks of HFD. (D,E and F) Similarly, significant differences were also found in female $PRCP^{fl/fl}$ -AgRP-cre mice compared to controls mice ($n=15$ per group). Data in all graphs are shown as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ compared to controls.

A significant increase in insulin sensitivity in $PRCP^{fl/fl}$ -AgRP-cre male mice was also observed (Figure 28, A-C). However, no differences in insulin sensitivity were observed in $PRCP^{fl/fl}$ -AgRP-cre female mice compared to controls at any of the time points examined (Figure 28, D-F).

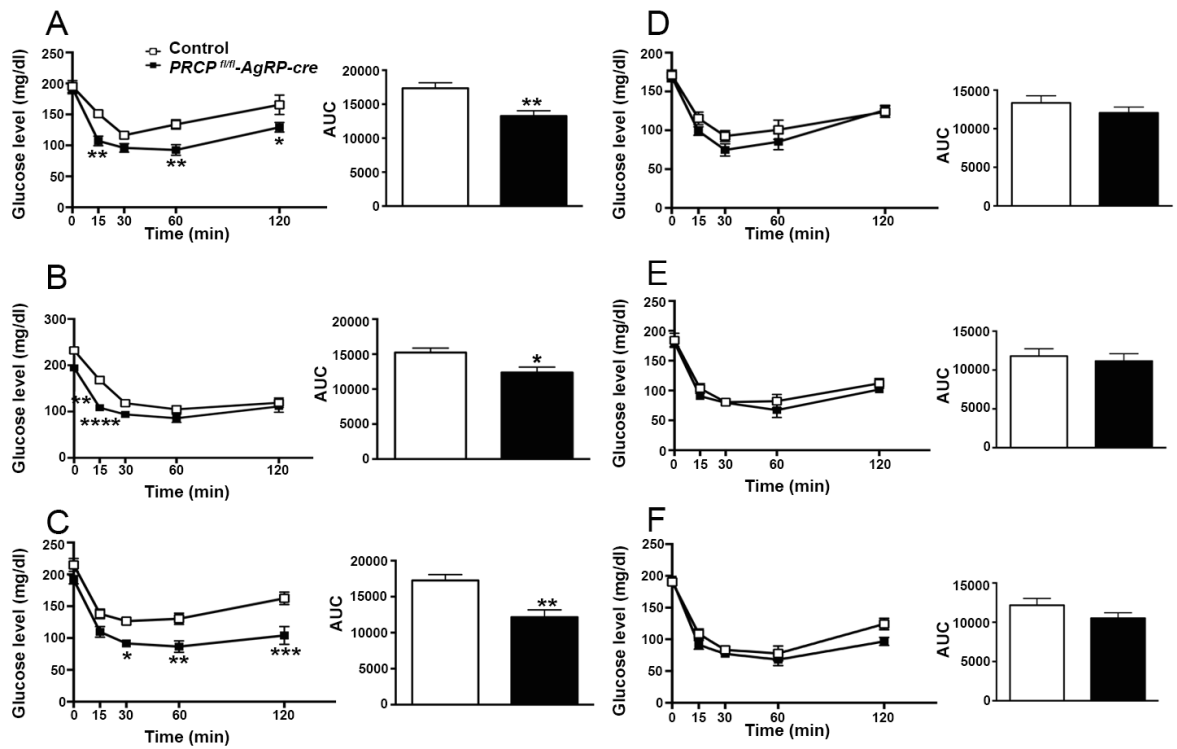


Figure 27: Insulin tolerance tests in $PRCP^{fl/fl}$ -AgRP-cre mice and control exposed to HFD. (A,B and C) Graphs showing significant differences in insulin sensitivity in $PRCP^{fl/fl}$ -AgRP-cre male mice compared to controls at 4, 8 and 12 weeks of HFD. (D,E and F) No differences in insulin sensitivity were found between female mice. Data in all graphs are shown as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ compared to controls.

In agreement with these results, a significant lower AgRP activation was observed in $PRCP^{fl/fl}$ -AgRP-cre male mice compared to controls as the percent of cfos-immunopositive AgRP neurons was significantly lower than that of wild type controls (Figure 29 , male A; female B).

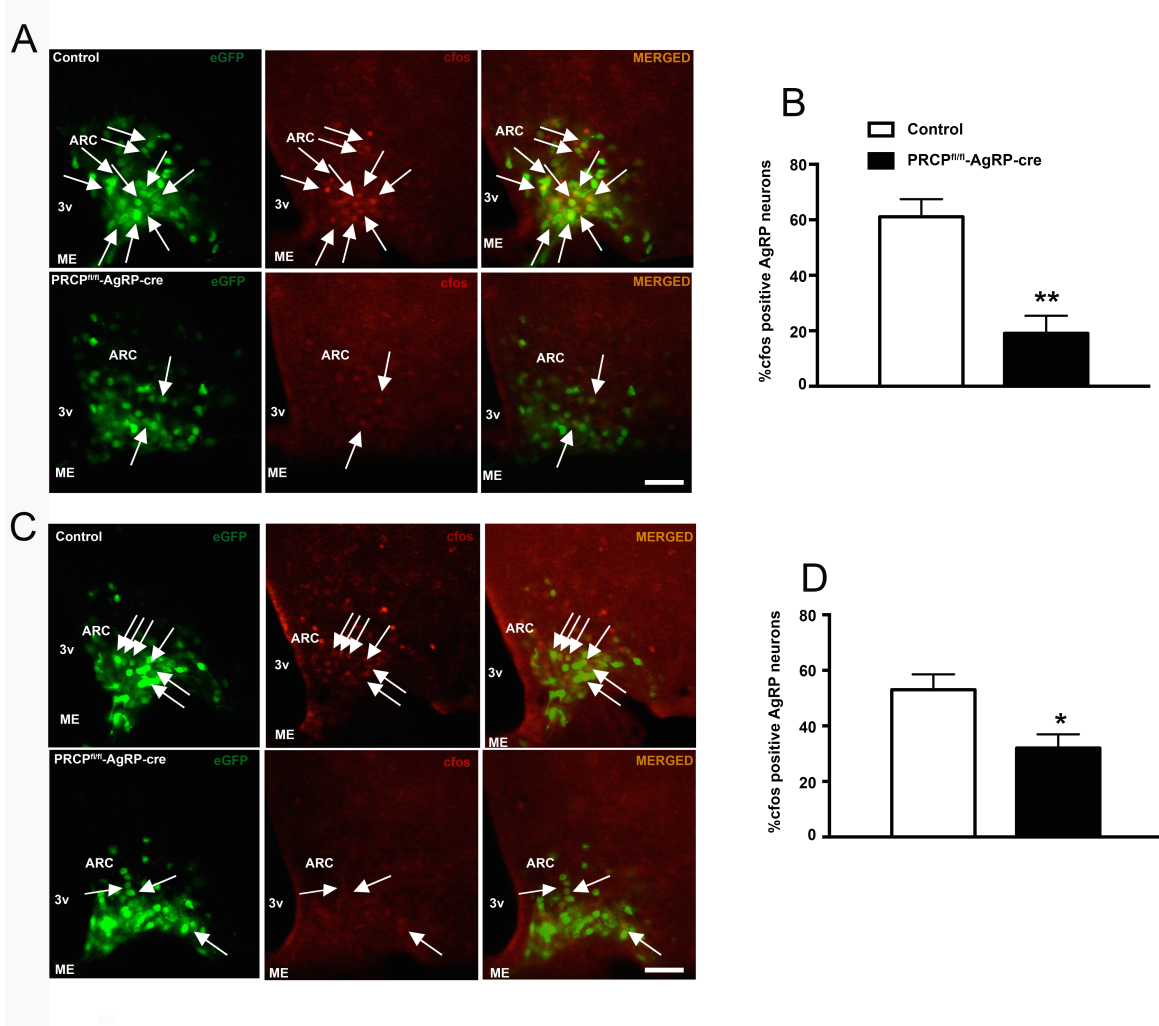


Figure 28 : PRCP mediated regulation of AgRP neurons on HFD. (A and B) Deletion of PRCP in male and (C and D) female decreases cfos expression in AgRP neurons compared to controls mice (n=5 per group). Scale bar in A and C (lower right panels) represents 75 μ m. All values denote mean \pm s.e.m. *P < 0.05; **P < 0.01.

8.7 Mitochondrial size in activated AgRP neurons of fasted animals

Mitochondria have been shown to play a critical role in regulating AgRP neuronal activation. Next, we analyzed mitochondrial size, form factor, density, coverage and aspect ratio in AgRP neurons of fasted *PRCP^{fl/fl}-AgRP-cre* and control male mice on HFD (Figure 30, A). In support of the decreased AgRP neuronal activation in fasted *PRCP^{fl/fl}-AgRP-cre* male mice, we found decreased mitochondrial density (Figure 30, B) and coverage (Figure

30, C) in AgRP neurons of *PRCP^{fl/fl}-AgRP-cre* mice compared to control mice. The observed decreases were not statistically significant possibly due to the group size. More materials are in the process to be analyzed. No differences in size (Figure 30, D), form factor, (Figure 30, E) and aspect ratio (the ratio between the major and minor axis of the ellipse equivalent to the mitochondrion) (Figure 30, F) were observed between the 2 experimental groups.

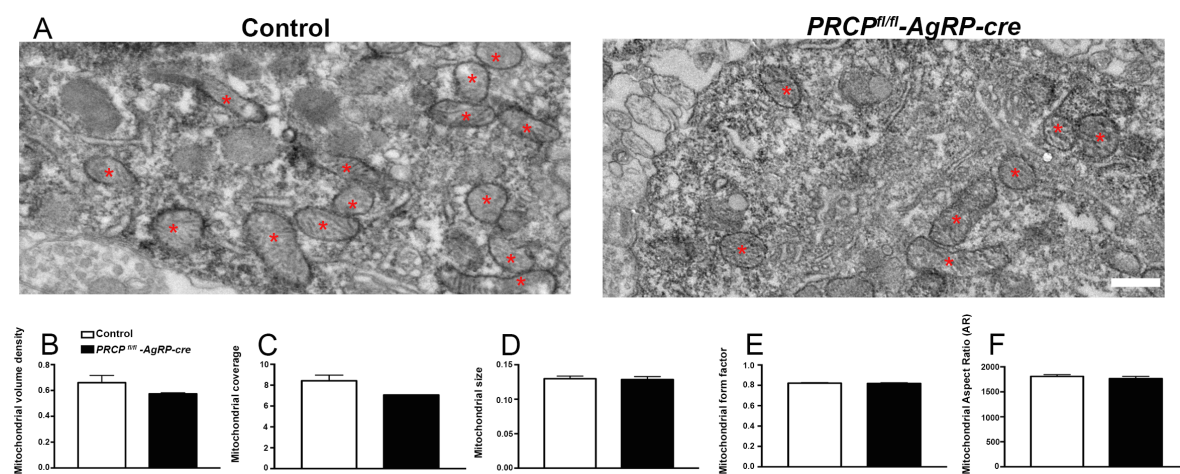


Figure 30 Analysis of mitochondrial size, form factor, density, coverage and aspect ratio in AgRP neurons of mice in HFD. (A) Electron micrographs showing representative sections of AgRP neurons in the arcuate nuclei of control (left panel) and *PRCP^{fl/fl}-AgRP-cre* mice (right panel). Red asterisks show mitochondria. Scale bar in A (right panel) represents 500 nm. (B) A decrease in mitochondrial density (C) and coverage in *PRCP^{fl/fl}-AgRP-cre* mice compared to control mice was observed. In both groups we found similar mitochondrial size (D), form factor, (E) and aspect ratio (F).

8.8 Increased interactions between ER and mitochondria in AgRP neurons of fasted animals

The existence of physical interaction between ER and mitochondria have been suggested to affect ER and mitochondria functions. ER stress, mitochondrial dysfunction and alterations in their interaction have been implicated in the pathogenesis of obesity and leptin resistance [125, 126]. While increased interactions between ER and mitochondria

have been shown to be associated with greater neuronal activation [125], decreased interactions have been reported in neurons with reduced neuronal activation [126]. Interestingly, analysis of mitochondria-ER interaction in our study showed that the number of mitochondria-ER contacts in AgRP neurons of *PRCP^{fl/fl}-AgRP-cre* mice is lower than that in AgRP neurons of control mice (Figure 31, A and B), which is an indication that PRCP may play a role in ER and mitochondrial functions.

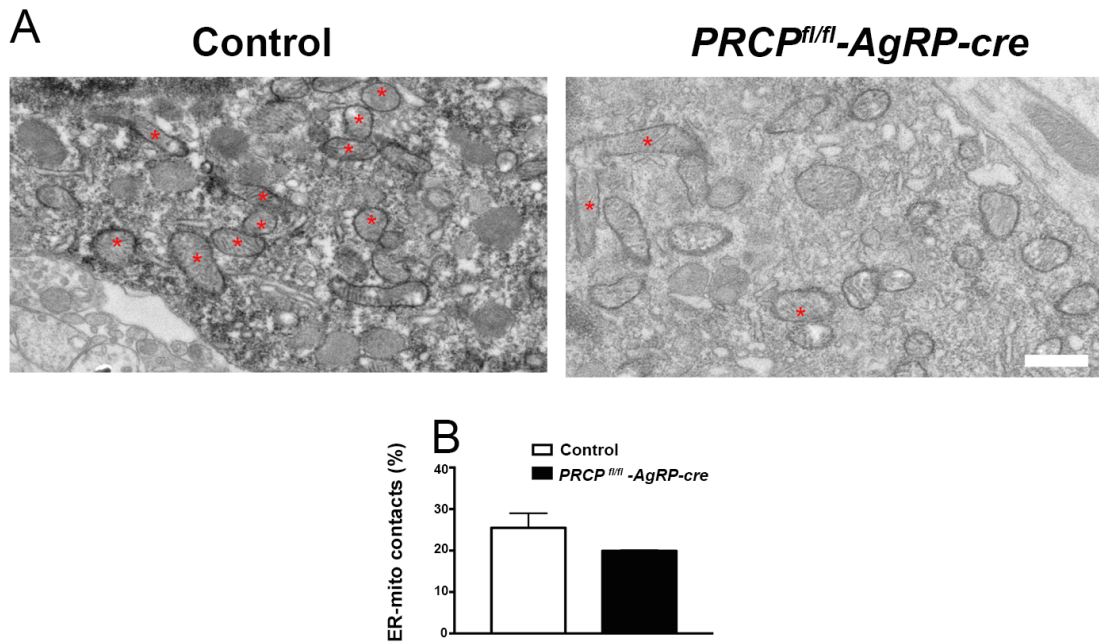


Figure 31: Analysis of mitochondria-ER interaction. (A) Electron micrographs showing representative sections of AgRP in the arcuate nuclei of a control (left panel) and *PRCP^{fl/fl}-AgRP-cre* mice (right panel). Red asterisks show mitochondria in close association with ER. Scale bar in A (right panel) represents 500 nm. (B) Quantification of mitochondria-ER interaction revealed lower numbers of mitochondria-ER contacts in AgRP neurons of *PRCP^{fl/fl}-AgRP-cre* animals compared to control animals ($n = 5$ per group).

AIM 2: TO ELUCIDATE THE ROLE OF PRCP IN MC4R- EXPRESSING NEURONS OF THE CNS.

9. Material and Methods

9.1 Generation of MC4R-PRCP KO mice

We used the cre/lox technology to generate mice in which PRCP will be selectively ablated in MC4R receptors ($PRCP^{fl/fl}$ - $MC4R$ -cre). We generated mice by crossing PRCP floxed mice ($PRCP^{fl/fl}$) described above with MC4R-cre that express Cre recombinase in exclusively in MC4R receptors (Figure 32). As control mice, $PRCP^{+/+}$ -MC4R-cre positive littermates were used. All mice studied were on a mixed background (129SvEv, FVB and C57BL/6J).

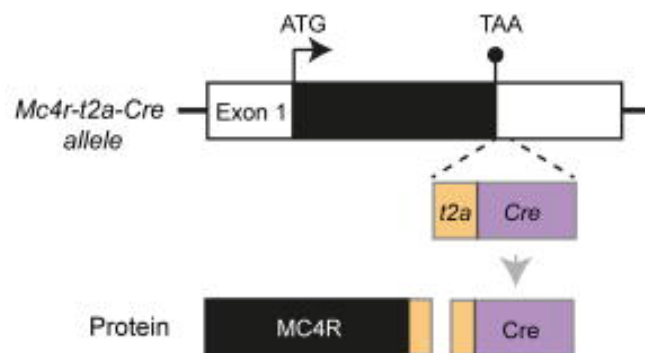


Figure 32: Generation of Mice Used in This Study. Schematic diagram showing MC4R-cre constructs.

9.2 Genotyping

Genomic DNA was isolated from tails or yolk sacs by standard methods. PRCP mice were genotyped by polymerase chain reaction (PCR parameters: 42 cycles, 93°C for 30 s, 56°C for 1 min, and 72°C for 5 min). Amplification of a wild-type (WT) allele generated a 3.6-

kb product, and a 4.1-kb product in the case of a mutant allele using the following primers:

PRCP2 FRT5: 5' -ccttcagggccctcagtc -3' , PRCP2 FRT3: 5' -cagccatatttaaattgagat -

3. MC4R-cre alleles were identified by PCR analysis for cre sequences using the following

primers: MC4R-2A Cre Stop R1, 5' -catgtcaattcataacgcccac -3' ; CRE 350, 5' -

ctgtcacttggtcgtggcagc -3' ; MC4R-2A Cre Stop F1 5' - gatcatgtgtaagccgac -3' .

9.3 Metabolic phenotype analyses

All the metabolic analyses were performed as described above.

9.4 Blood Pressure Measurement

The blood pressure of the animals were recorded in conscious mice via a noninvasive tail-cuff apparatus (ADInstruments, Sydney, Australia). After a 5-day training period, diastolic blood pressure (DBP) and systolic blood pressure (SBP) were monitored in the morning. Blood pressure was calculated as the average value of 3–4 successive measurements.

AIM 2: TO ELUCIDATE THE ROLE OF PRCP IN MC4R- EXPRESSING NEURONS OF THE CNS.

10. Results

10.1 Selective deletion of PRCP in MC4R-expressing neurons alters energy balance

Previous studies have shown that PRCP is expressed in MC4R neurons. Thus, to understand the role of PRCP in this neuronal population, we generated and compared *PRCP^{fl/fl}-MC4R-cre* mice to their wild type controls.

First, we analyzed body weight and body composition. On a standard chow diet, *PRCP^{fl/fl}-MC4R-cre* male mice showed significantly lower body weight than control mice starting at 10 weeks of age (Figure 33, A). Body composition analysis (Figure 33, B and C) showed that the fat mass was reduced already at 8 weeks of age (Figure 33, B) although at this time point no significant difference in body weight was found. No changes in lean mass were observed at any of the time points examined (Figure 33, C). No differences in body weight or composition were observed in *PRCP^{fl/fl}-MC4R-cre* and control female mice (Figure 33, D-F). Thus, similar to the *PRCP^{fl/fl}-AgRP-cre* mice, PRCP regulation of metabolism in MC4R neurons occurs in a sex-specific manner.

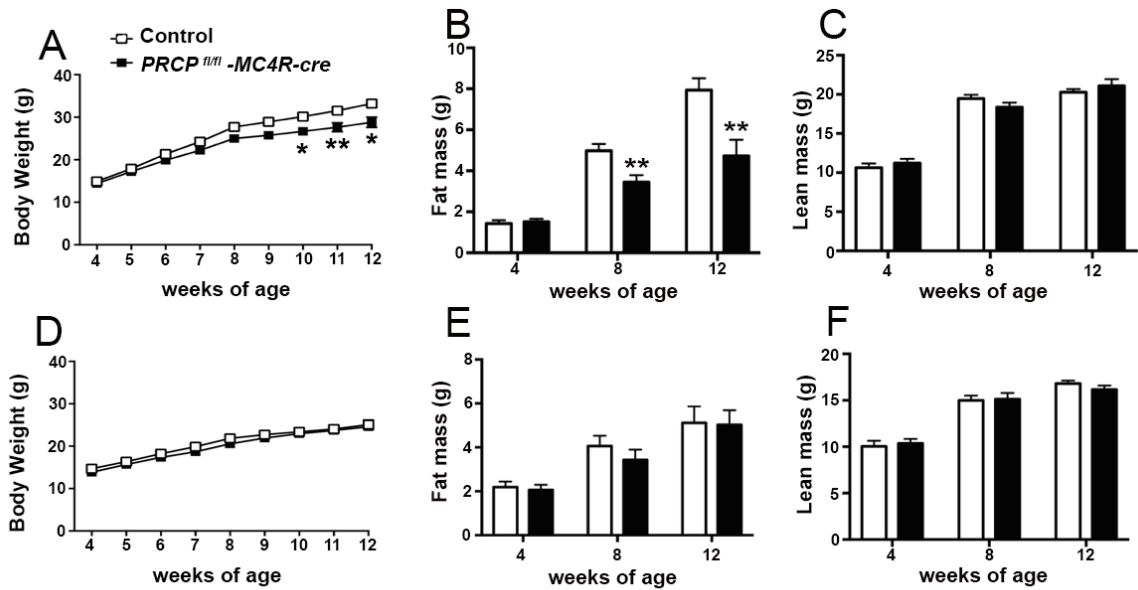


Figure 33: *PRCP^{fl/fl}-MC4R-cre* mice and energy metabolism. Graph showing body weight (A) fat mass (B) and lean mass (C) of *PRCP^{fl/fl}-MC4R-cre* male mice and wildtype controls at 4, 8, and 12 weeks of age. Graph showing body weight (D) fat mass (E) and lean mass (F) of *PRCP^{fl/fl}-MC4R-cre* female mice and wildtype controls at 4, 8, and 12 weeks of age. Data represent the mean ± SEM. *P < 0.05; **P < 0.01 compared to control group.

When food intake was analyzed after an overnight fasting, *PRCP^{fl/fl}-MC4R-cre* male mice showed a reduced feeding at 24 hours time point (Figure 34, A), while no differences were observed between females (Figure 34, B).

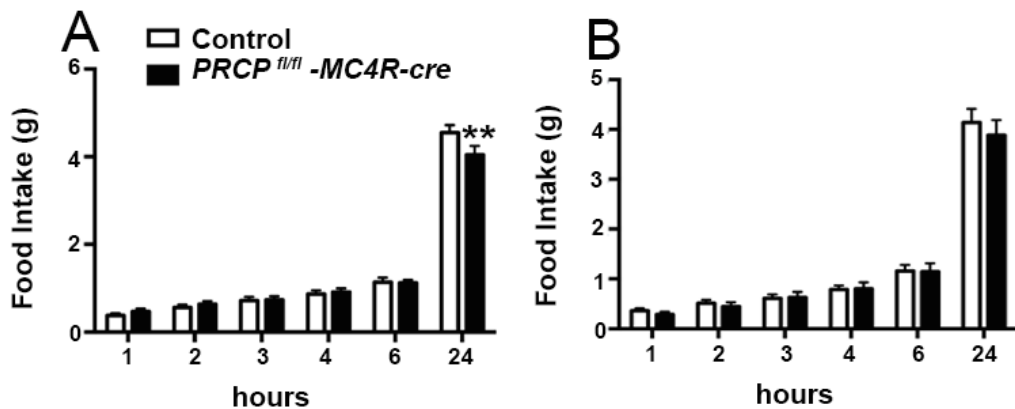


Figure 34: *PRCP^{fl/fl}-MC4R-cre* male mice have reduced fasting-induced food consumption. Analysis of fasting-induced food intake in male (A; n = 8 per group) and female (B; n = 9 per group) *PRCP^{fl/fl}-MC4R-cre* and control mice. Data in all graphs are shown as mean ± SEM. **P < 0.01 compared with controls.

10.2 Glucose homeostasis

To assess glucose metabolism, we next performed glucose and insulin tolerance tests. *PRCP^{fl/fl}-MC4R-cre* male mice showed a significant improvement in glucose metabolism at all ages examined (Figure 35, A-C). Interestingly, even when body weight and composition were similar to those of controls at 4 weeks of HFD, *PRCP^{fl/fl}-MC4R-cre* male mice showed an increased glucose tolerance (Figure 35, A), which is an indication that PRCP in MC4R neurons plays a role in the control of glucose metabolism. On the other hand, glucose metabolism was not affected in female mice (Figure 35, D-F).

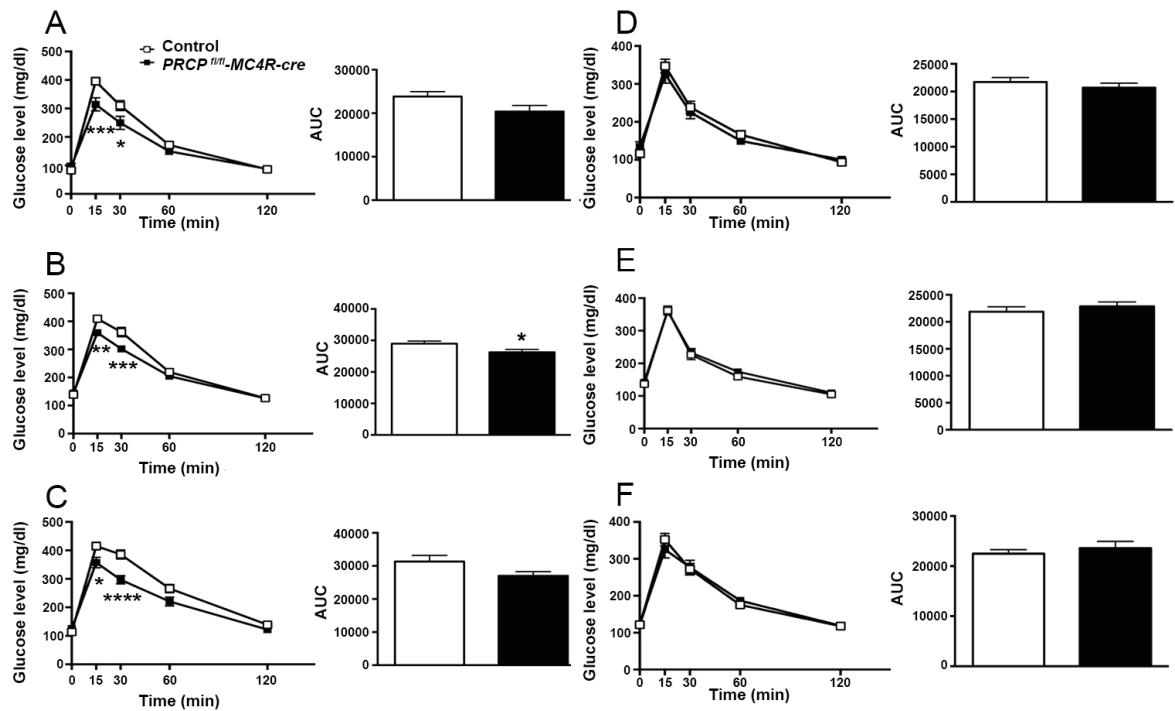


Figure 35: *PRCP^{fl/fl}-MC4R-cre* male mice show improved glucose metabolism. (A,B and C) Increased glucose tolerance in male *PRCP^{fl/fl}-MC4R-cre* animals ($n = 10$) compared to controls ($n = 10$) at 4 (A), 8 (B) and 12 (C) weeks of age. (D,E and F) Graphs showing no significant changes in glucose tolerance in female *PRCP^{fl/fl}-MC4R-cre* animals ($n = 10$) compared to controls ($n = 10$) at 4 (D), 8 (E) and 12(F) weeks of age. Data in all graphs are shown as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$; **** $P < 0.0001$ compared with controls.

Similar to glucose tolerance tests, insulin tolerance tests showed an increased insulin sensitivity in *PRCP^{fl/fl}-MC4R-cre* male mice (Figure 36, A-C), while no differences between the female groups (Figure 36, D-F).

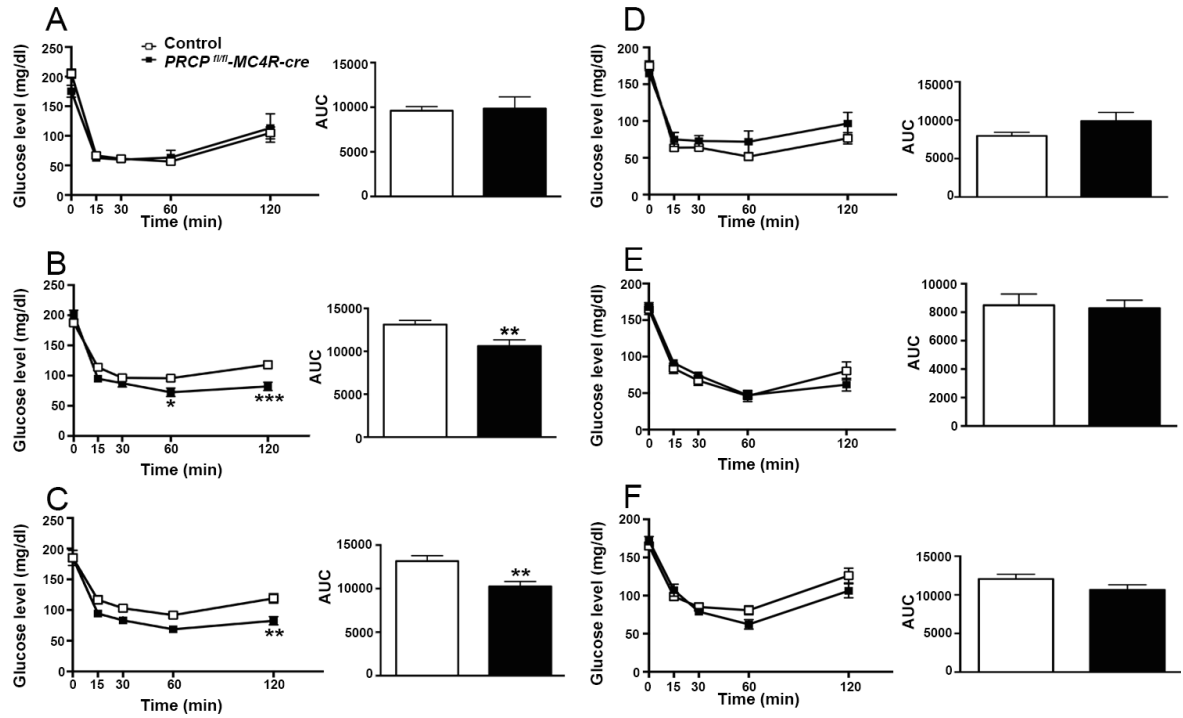


Figure 36: *PRCP^{fl/fl}-MC4R-cre* male mice show improved insulin sensitivity. (A,B and C) Increased insulinsensitivity in male *PRCP^{fl/fl}-MC4R-cre* animals ($n = 10$) compared to controls ($n = 10$) at 4 (A), 8 (B) and 12 (C) weeks of age. (D,E and F) Graphs showing no significant changes insulinsensitivity in female *PRCP^{fl/fl}-MC4R-cre* animals ($n = 10$) compared to controls ($n = 10$) at 4 (D), 8 (E) and 12(F) weeks of age. Data in all graphs are shown as mean \pm SEM. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ compared with controls.

10.3 Role of PRCP in the regulation of blood pressure

PRCP whole body knockout mice have been shown to develop hypertension [127]. On the other hand, MC4R whole body knockout mice while becoming obese, do not become hypertensive [128]. Thus, to assess whether PRCP in MC4R neurons may be involved in the control of blood pressure, we next monitored blood pressure in *PRCP^{fl/fl}-MC4R-cre* mice and their wildtype controls. As showed in Figure 37, compared to control group, *PRCP^{fl/fl}-MC4R-cre* male mice presented increased mean blood pressure (Figure 37, A)

which was due to the increase in both diastolic (Figure 37, B) and systolic blood pressure (Figure 37, C). No differences in blood pressure were found between female groups (Figure 37, D-F).

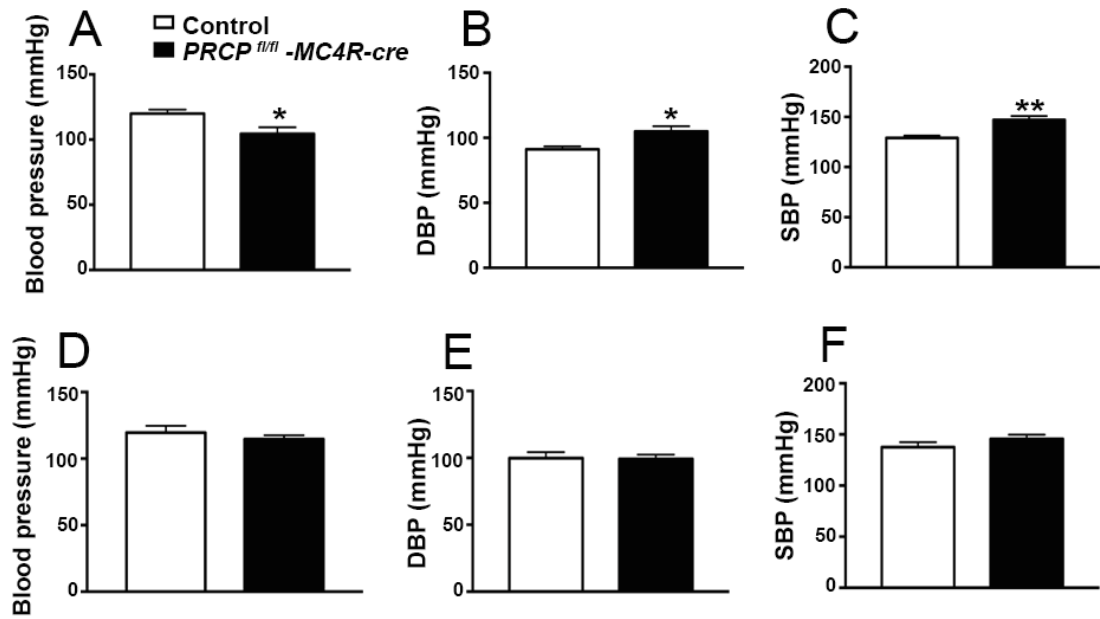


Figure 37: Graphs showing the results of the blood pressure measurements in male and female in *PRCP^{fl/fl}-MC4R-cre* mice and controls. (A) Mean, (B) diastolic and (C) systolic blood pressure were increased in *PRCP^{fl/fl}-MC4R-cre* mice compared to their controls (n=10 per group). (D-F) No difference was found in female mice. Data represent the mean ± SEM. *=P<0.05; **P < 0.01 compared to control group.

11. Discussion

Several studies have focused on the enzymes involved with the production of α -MSH [129, 130] but little is known about how α -MSH is degraded. Wallingford and colleagues have identified an enzyme, prolylcarboxypeptidase (PRCP), that inactivates α -MSH by removing the C-terminal valine residue [82]. The resulting truncated peptide, α -MSH 1–12, was shown to be ineffective in reducing food intake when injected icv compared to α -MSH 1–13. In agreement with a role for PRCP in α -MSH degradation, *Prcp* null mice had a lean phenotype and elevated levels of α -MSH in the hypothalamus. Small molecule inhibitors of PRCP activity have also shown to decrease food intake, possibly by crossing the brain blood barrier and acting on central PRCP. In the Central Nervous System (CNS) PRCP is expressed in several regions including specific nuclei of the hypothalamus and the brain stem. Within the hypothalamus, PRCP has been shown to be localized in the arcuate NPY/AgRP neurons while in the brain stem it was shown to be localized in MC4R-expressing neurons.

NPY/AgRP neuronal projections overlap with those of α -MSH-producing neurons, therefore, it is conceivable that PRCP may cleave α -MSH extracellularly at the synaptic level, thus increasing the antagonistic tone of the orexigenic neuropeptide agouti-related protein (AgRP), an endogenous inverse agonist of MC4R. However, in other areas where PRCP colocalizes with MC4R such as the brain stem, PRCP may cleave α -MSH after ligand-mediated receptor endocytosis.

Our findings in this thesis revealed a critical role of central PRCP in the regulation of energy metabolism. Using a transgenic mouse model with selective deletion of PRCP in specific neuronal population, such as the NPY/AgRP, we showed that *PRCP^{fl/fl}-AgRP-cre*

male mice develop a significant lower body weight compared to control mice when fed on a standard chow diet. When body composition was analyzed, a significant decrease of fat mass was observed in *PRCP^{fl/fl}-AgRP-cre* male mice compared to controls. To assess whether energy intake and expenditure were affected in this animal model, with the use of metabolic cages, we found that *PRCP^{fl/fl}-AgRP-cre* mice had reduced food intake and increased energy expenditure due to a significant increase in locomotor activity. As expected by an improved metabolic phenotype, *PRCP^{fl/fl}-AgRP-cre* mice also showed improved glucose metabolism having increased glucose tolerance and insulin sensitivity compared to control mice. In agreement and in association with an improved glucose metabolism, a significant decrease in hepatic gluconeogenic enzymes, such as *Pepck* and *G6Pase*, was also observed in our transgenic male mice compared to controls.

To assess the effect of PRCP deletion at the cellular levels, we then examined the activation levels of NPY/AgRP neurons following an overnight fasting. *PRCP^{fl/fl}-AgRP-cre* male mice had a significant reduction of cfos staining, a marker of neuronal activation, in the arcuate AgRP neurons compared to control mice. These data on the reduction of neuronal activation were further confirmed by the attenuation of peripheral administration of ghrelin-induced hyperphagia in *PRCP^{fl/fl}-AgRP-cre* mice compared to their controls. Interestingly, while all of these phenotypic differences were observed in males, no significant differences were found in female mice, indicating sex differences in PRCP effects on metabolism regulation. This data is in agreement with the genome-wide association study (GWAS), showing that PRCP is a significant risk factor for metabolic syndrome only in men [83].

To determine whether diet composition had an effect on the metabolic phenotype of our transgenic mouse model, we then exposed male and female *PRCP^{fl/fl}-AgRP-cre* mice

and control mice to a high fat diet (HFD). Similar to mice exposed to a standard chow diet (low in fat), HFD-fed male *PRCP^{fl/fl}-AgRP-cre* mice showed a leaner phenotype than the male controls due to decreased food intake and increased energy expenditure. Interestingly, when challenged with the HFD, female *PRCP^{fl/fl}-AgRP-cre* mice also showed significant differences compared to female controls. As for the standard chow diet-fed mice, glucose metabolism and insulin sensitivity were significantly improved in both male and female mice due to their improved metabolic phenotype compared to controls.

Mitochondria have been shown to play a critical role in the regulation of NPY/AgRP neurons [131-133]. Specifically, increased mitochondrial density is required to enable AgRP neurons to respond to peripheral signals such as ghrelin, and to induce feeding behavior. In agreement with this published data and with our results on the decreased AgRP neuronal activation following either an overnight fasting or peripheral ghrelin administration, we found that mitochondrial density and coverage were significantly decreased in male *PRCP^{fl/fl}-AgRP-cre* mice compared to their wild type controls.

ER stress, mitochondrial dysfunction and alterations in their interaction have been implicated in the pathogenesis of obesity and leptin resistance [125, 126]. While increased interactions between ER and mitochondria have been shown to be associated with greater neuronal activation [125], decreased interactions have been reported in neurons with reduced neuronal activation [126]. Our data on ER-mitochondria interactions in *PRCP^{fl/fl}-AgRP-cre* mice fed on HFD are in agreement with these published studies, as we have shown that NPY/AgRP neurons of *PRCP^{fl/fl}-AgRP-cre* mice are less activated and have present lower ER-mitochondria interactions compared to control mice. Thus, our observation that PRCP deletion in AgRP neurons affects mitochondria-ER interactions in

response to HFD highlights a role for PRCP in the etiology of ER stress. Future studies are warranted to understand the mechanism(s) by which PRCP affects these intracellular organelles.

Previous studies have shown that PRCP is not only expressed in the hypothalamic region [134], but in many other regions of the CNS. Several extra-hypothalamic areas showed considerable PRCP expression, including the hippocampus, cortex, amygdala and several nuclei of the brain stem. Many of these hypothalamic and extra hypothalamic areas where PRCP is expressed also express melanocortin receptors 3 and 4 (MC3R and MC4R, respectively), thus suggesting that PRCP may cleave α -MSH intracellularly after ligand-mediated receptor endocytosis. Strong expression of PRCP has been observed in the dorsomotor nucleus of the vagus (DMV) and in the nucleus of the solitary tract [135]. Both of these brain stem areas express high levels of MC4R, thus suggesting that PRCP may also regulate melanocortin signaling in the brain stem. Considering that the DMV and the NTS are important parasympathetic structures, PRCP in these nuclei may therefore play a role in the modulation of the autonomic nervous system (ANS). In support of this, whole body PRCP knockdown mice (*PRCP^{gt/gt}* mice) are hypertensive [127], while MC4R knockout mice as well as humans, although obese, do not develop hypertension. Intriguingly, a recent study [136] showed that increased electrical activity of DMV neurons is associated with lower melanocortin tone. Thus, decreased PRCP activity in this brain stem site, by increasing melanocortin signaling, may reduce the activity of these neurons, and therefore, decrease the parasympathetic tone. This, in turn, could result in a relative increase of the sympathetic tone. In support of this, *PRCP^{gt/gt}* mice not only are hypertensive, but they also

have increased thermogenesis as shown by the increased UCP1 mRNA levels in the brown adipose tissue (BAT) [134].

Thus, to evaluate the effect of PRCP in the control of the ANS we generated mice with selective deletion of PRCP in MC4R receptors. In support of a role for PRCP in the regulation of the ANS, we found that *PRCP^{fl/fl}-MC4R-cre* mice are hypethensive. Besides this cardiovascular phenotype, *PRCP^{fl/fl}-MC4R-cre* mice also display a lower body weight and fat mass compared to control mice starting at 8 weeks of age. Moreover, an improved glucose tolerance was also observed in *PRCP^{fl/fl}-MC4R-cre* mice compared to control mice as early as at 4 weeks of age when their body weight and fat mass were not significantly different than those of control mice, suggesting that the effect on glucose metabolism is independent of the body weight and composition. Many more studies on this animal model are needed to dissect out the role of PRCP in the MC4R neurons. These studies will be conducted during my postdoctoral training in the laboratory of Dr. Diano at Yale University.

In conclusion, in this project we have shown that PRCP plays a pivotal role in energy homeostasis in both NPY/AgRP neurons and in MC4R-expressing neurons of the CNS. We believe that PRCP activity levels in specific brain areas in different metabolic condition by affecting α -MSH levels will, in turn, affect the efficacy of melanocortin signaling. Thus, understanding PRCP role in metabolism regulation is important to reveal whether PRCP can be used as new drug target for metabolic disorders, including obesity and type 2 diabetes.

12. References

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