Symposium 1

Evolution of the melanocortin receptors and Agouti related peptides

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The largest group of receptors is the GPCR coupled receptor superfamily found in five main families in the human genome. The melanocortin receptors belong the *Rhodopsin* family of GPCRs forming a distinct cluster with long independent evolutionary history. We have previously cloned and expressed melanocortin receptors in range of species. We found for example that the agnathan, river lamprey has receptors, designated MCa and MCb, that show orthology to the MC1 and MC4 receptor subtypes, respectively. The lamprey MCa receptor has relatively high affinity for ACTH derived peptides similarly to the fish MC receptors, supporting a hypotheses that ACTH like peptides could have acted as the "original" ligand at the MC receptors. Several evolutionary important genomes have been sequenced providing additional sequence information on the MC receptors and the Agouti related peptides. We provide new hypotheses about the evolutionary events that led to multiple subtypes of the MC receptors as well as studies on the ancient origin of agouti related peptides.

Symposium 1

Fish melanocortin system

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Melanocortin signalling is mediated by binding to a family of G protein-coupled receptors that positively couple to adenylyl cyclase. Tetrapod species have five melanocortin receptors (MC1R-MC5R). The number of receptors diverges in fish. Zebrafish has six MCRs, with two copies of the MC5R, while pufferfish have 4 receptors with no MC3R and one copy of MC5R. Fish genomes also exhibit orthologue genes for agouti signalling protein (ASP) and agouti related protein (AGRP). AGRP expression is confined to a small area in the hypothalamus but ASP is expressed in the skin. Fish MC2R is specific for ACTH and requires the cooperation of accessory proteins (MRAP) to reach its functional expression. The four other MCRs distinctively bind MSHs. The interaction α-MSH/MC1R plays a key point in the control of the pigmentation and mutations of MC1R are responsible for reduced melanization. Both MC4R and MC5R are expressed in the hypothalamus and central MC4R expression is thought regulates energy balance through modulation of feeding behaviour. In addition, peripheral melanocortin system also regulates lipid metabolism by acting at hepatic MC2R and MC5R. Both sbMC1R and MC4R are constitutively expressed in vitro and both agouti signalling peptide (ASP) or agouti related protein (AGRP) work as inverse agonist but only after inhibition of phosphodiesterase system. Accordingly, overexpression of AGRP and ASP transgenes promote obesity and reduced melanization in zebrafish, respectively.

Symposium 1

Epigenetic changes in pro-opiomelanocortin in fetal hypothalamic feeding centres occur as a consequence of maternal undernutrition

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Maternal undernutrition influences the development of obesity and diabetes in adult offspring. However, the mechanisms responsible for consequent obesity in offspring are not known. We hypothesized that maternal undernutrition would cause epigenetic changes in POMC and glucocorticoid receptor (GR) genes in the hypothalamus.

In a sheep model of periconceptional undernutrition ewes were undernourished (UN) for 60 days pre- and 30 days post-mating or given normal feed (N). Brain tissues were dissected at post mortem (day 135). POMC and GR histone H3-K9 acetylation and promoter region methylation were examined as epigenetic markers of transcription along with mRNA expression.

In the stress axis (foetal hippocampus and anterior pituitary) there was no evidence of changes in promoter methylation or expression of the POMC and GR genes. However, in the hypothalamus there was increased H3K9 acetylation (1.6 fold increase, $p=0.0003$) and marked hypomethylation of the POMC gene in the UN group (62% decrease, $p=0.0003$). In comparison no change in POMC mRNA expression was detected. Interestingly there was increased H3K9 acetylation (1.6 fold increase, $p=0.00025$) and hypomethylation (53% decrease, $p=0.028$) of the GR promoter in the UN group. This was associated with a 4.7 fold increase in hypothalamic GR expression ($p=0.041$).

This study provides evidence that periconceptional undernutrition is associated with marked epigenetic changes in hypothalamic POMC and GR genes. While expression of POMC was unchanged, the epigenetic change in GR expression in the undernourished group may contribute to the fetal programming of predisposition to obesity and glucose intolerance, via altered GR regulation of neuropeptides.

Symposium 2

Adropin - a novel nutritionally-regulated liver secreted peptide identified from the analysis of melanocortin receptor mutant mice that controls muscle respiration and glucose metabolism

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The liver is a source of secreted proteins involved in energy homeostasis. Adropin is a small secreted peptide which in the periphery is most abundantly expressed in the liver. The adropin transcript was identified during our analysis of the fatty liver phenotype of melanocortin receptor knockout mice[1]. Adropin expression in liver is regulated by nutrition and is suppressed with fasting. Deregulation of hepatic adropin expression is observed in diet and genetic models of obesity. An association of the deregulation of adropin expression with the Metabolic Syndrome was suggested by experiments showing improvements in glucose homeostasis and fatty liver disease by adropin therapy. We have generated C57BL/6J adropin knockout mice (AdrKO). AdrKO are viable and exhibit no evidence of increased mortality. However, AdrKO are insulin resistant and glucose intolerant, and ehxibit hypertriglyceridemia. The insulin resistant phenotype of AdrKO is likely "partial", while insulin-stimulation of glucose disposal is impaired inhibition of endogenous glucose production is improved. We were anticipating that adropin would stimulate energy expenditure. Remarkably, adropin is a potent inhibitor of oxidative metabolism in skeletal muscle. Improvements in glucose homeostasis and obesity associated with adropin therapy may therefore be due to a restraint on mitochondrial oxidation, forcing the use of less efficient pathways for ATP production. [1] Kumar KG et al. Cell Metabolism 2008 Dec; 8(6):468-81

Symposium 2

MC3-R ablation creates Cushing's Syndrome in the mouse

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Deletion of the melanocortin-3 receptor (MC3-R) in mice causes an obesity syndrome associated with increased visceral adiposity, a reduction in lean mass, increased sensitivity to salt induced hypertension, and a decreased inflammatory response to adiposity. Here we report that deletion of the MC3-R also results in elevated basal corticosterone (3-5 fold), and blunts the upregulation of serum corticosterone normally induced by fasting. Additionally, we show that deletion of MC3-R results in decreased femur length, decreased cortical area, cancellus area, medialateral moment of inertia, and bone mineral density. Cushing's syndrome results from chronically elevated corticosteroids and is associated with increased visceral adiposity, muscle atrophy, salt and water retention and induced hypertension, immunosuppression, decreased linear growth, and decreased bone formation. Consequently, we propose that ablation of the MC3-R causes a murine model of Cushing's disease. Further analysis of HPA axis function in the MC3-R null mouse shows normal upregulation of the axis, as measured by assays for serum corticosterone, following other stressors such as restraint or LPS treatment. Thus, the MC3-R appears to play a role in tonic inhibition of the HPA axis and in specifically communicating stressors related to nutrient status.

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*BR and KLJE contributed equally to this study.

Symposium 2

Cardiovascular Effects of Melanocortins

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Melanocyte stimulating hormones (MSH's, melanocortins) have important roles in feeding and energy metabolism, and in inflammation. Recent observations have uncovered major functions for these peptides, particularly γ-MSH, in cardiovascular regulation and sodium metabolism. Both α- and γ-MSH acutely elevate blood pressure and heart rate through central stimulation of sympathetic nervous outflow. This action of α-MSH is mediated by the melanocortin 4 receptor (MC4R), whereas central sympathetic nervous stimulation by γ-MSH does not involve its receptor MC3R but rather is likely due to activation of a sodium channel in the CNS. In contrast, γ-MSH deficiency in rodents, or disruption of MC3R, leads to marked salt-sensitive hypertension, again through a central mechanism: a small dose of exogenous peptide delivered into the cerebroventricular system of mice with γ-MSH deficiency restores blood pressure to normal. This salt-sensitive hypertension is accompanied by the development of hyperglycemia and hyperinsulinemia, and provision of exogenous γ-MSH corrects not only the hypertension but also the abnormal glucose metabolism. The mechanism linking these two consequences of a high salt diet is likely due to activation of the sympathetic nervous system, as α-adrenergic receptor blockade also corrects both consequences of the high salt diet. The study of MSH peptides in blood pressure regulation offers a new opportunity to gain insight into the mechanisms underlying salt sensitivity and its link to insulin resistance, and to new therapies for these independent cardiovascular risk factors.

Symposium 2

Regulation of glucose metabolism via the hypothalamic melanocortin system

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Hypothalamic melanocortin receptors play a central role in regulating energy balance and peripheral insulin action. In particular, peripheral signals of energy status such as leptin and insulin act on the melanocortin circuitry of the arcuate nucleus by modulating the expression and release of two neuropeptides, AGRP and α-MSH, which respectively inhibit or activate melanocortin receptors. Chronic modulation of hypothalamic melanocortin receptors leads to significant changes in body composition, which are accompanied by dramatic changes in insulin action on both glucose uptake and production. On the other hand, short-term intracerobroventricular infusions of α-MSH does not improve insulin-mediated glucose uptake, but increases hepatic gluconeogenesis and enhances hepatic expression of gluconeogenic enzymes. Conversely acute ICV administration of a synthetic melanocortin antagonist (SHU9119) does not acutely alter glucose fluxes, nor enzymes expression, but blocks the effects of α-MSH on these parameters. Like α-MSH, short-term ICV leptin induces hepatic gluconeogenesis, but does not increase hepatic glucose production. However, in the presence of melanocortin blockade (with SHU9119), ICV leptin decreases hepatic glucose output. These results and the recent evidence obtained with selective knockouts in AGRP- and α-MSH-producing neurons support a complex and pivotal role of the melanocortin system in regulating peripheral insulin action independent of its effects on energy balance and body composition.

Symposium 2

Melanocortin-4 receptors on glutamatergic, but not on oxytocin or CRH neurons regulate feeding in mice

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Melanocortin-4 receptor (MC4R) deficiency is associated with morbid obesity, hyperphagia, hyperinsulinism and increased linear growth in both humans and rodents. We have shown previously that the anorectic action of Mc4R is mediated largely through Sim1-positive neurons in the hypothalamus and/or amygdala. To test the hypothesis that these effects are transmitted by oxytocin neurons, we have used Cre-loxP technology to re-express Mc4Rs selectively in oxytocin neurons in a Mc4R-deficient background (i.e. by crossing cre-expressing mice with mice homozygous for the loxTB-MC4R allele (Balthasar et al., Cell, 2004)). To our surprise, selective reexpression of Mc4Rs in oxytocin neurons fails to rescue the obesity associated with Mc4R deficiency. When Mc4Rs are selectively re-expressed in CRH neurons (a second candidate site for the anorectic effects of Mc4R), again we find no effect on bodyweight. However, when Mc4Rs are re-expressed in vGlut2-containing glutamatergic neurons, complete rescue of the Mc4R-deficiency phenotype is observed. These data demonstrate that direct effects of Mc4R activity in oxytocin and CRH neurons have little impact on the altered energy balance seen in Mc4R deficiency. Importantly, Mc4R activity in vGlut2-positive neurons appears to be responsible for the majority of the Mc4R-mediated effects of melanocortins in the central nervous system. Thus, the neurons mediating the effects of MC4Rs on food intake must be both Sim1- (Balthasar et al., Cell, 2005) and vGlut2-positive. These findings should facilitate the search for the key neuron(s) mediating the effects of MC4Rs on food intake.

Symposium 3

Effects of melanocortins on adrenal gland physiology

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The melanocortin-2-receptor (MC2R) is a critical component of the hypothalamic-pituitary-adrenal axis. The importance of MC2R in adrenal physiology is exemplified by the condition familial glucocorticoid deficiency (FGD), a potentially fatal disease characterised by isolated cortisol deficiency. *MC2R* mutations cause ~25% of FGD. More recently the discovery of a MC2R accessory protein MRAP, mutations of which account for ~15% of FGD, has provided insight into MC2R trafficking and signalling. MRAP is essential for the functional expression of MC2R. MRAP2, a novel homolog of MRAP, can also facilitate MC2R cell surface expression and function. Like MRAP, MRAP2 is a small transmembrane domain glycoprotein capable of homodimerising. In addition, MRAP/MRAP2 can heterodimerise. The presence of MRAP2 adrenal expression could therefore suggest a possible role for MRAP2 in adrenal physiology, which has yet to be elucidated. Importantly, new data shows that the MRAPs can interact with all the other melanocortin receptors (MC1,3,4,5R). In contrast to MC2R, this interaction results in reduced MCR surface expression and signalling. MRAP2 is predominantly expressed in brain. Hypothalamic expression has been demonstrated for both MRAP and MRAP2. The ability of MRAPs to modulate different members of the MCR family in a bidirectional manner is intriguing. Furthermore, central nervous system expression of MRAPs points to a role beyond MC2R mediated adrenal steriodogenesis.

Symposium 3

The role of the MC5 receptor in muscle

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The role of the central melanocortin system in the regulation of energy balance and body weight is well established. The effect of the melanocortin system on glucose homeostasis is less clear. In general it appears that chronic systemic activation of the central melanocortin system causes lipolysis and improved glucose tolerance. In contrast short term activation of the melanocortin system may cause increases in blood glucose levels. We and others have shown that α-MSH (derived from the POMC precursor) can be detected in the blood of humans. In this study we sought to determine if peripheral α-MSH levels were regulated, and what peripheral α-MSH might do to energy balance.

We have found that α-MSH secretion, from the pituitary, is elevated post prandially and by both glucose and insulin in vitro. Alpha-MSH infusion decreases glucose excursions during a glucose tolerance test, and increases thermogenesis in muscle. In vitro treatment of muscle biopsies with α-MSH increases the phosphorylation of AMPK.

This work suggests that α-MSH, secreted by the intermediate lobe of the pituitary, may increase glucose utilization in muscle post-prandially.

Symposium 3

Genetics and genomics of melanocortin pigment patterns: from Akitas to Zebras

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Stripes and spots in multicellular animals are a prominent feature in nature for which the melanocortin 1 receptor (Mc1r) provides a fulcrum upon which different types of pigment are balanced. Melanocortin pathways have been well-characterized from the genetics of mouse coat color, but patterned control of those pathways in animals such as cheetahs and zebras is still a mystery.

We developed a highly sensitive and robust methodology—EcoP15I-tagged Detection of Gene Expression, or EDGE—that is suitable for detecting and comparing gene expression among tissues from animals for whom fully assembled and annotated genomes do not yet exist. In a pilot study, we applied EDGE to mice carrying a loss-of-function mutation for the Mc1r, and discovered an unexpected connection between melanocortin signaling and innate immunity. We also carried out an EDGE analysis of patterned skin from dogs (yellow and black brindled stripes), cheetahs (yellow and black spots), and zebras (white and black stripes). In brindled dogs, the striping pattern is caused by a segmental duplication that leads to gene silencing, and our results suggest that epigenetic alterations in gene expression are confined to the duplicated segment. In zebras, the striping pattern is limited to hair rather than skin, and our results indicate that alterations in hair color are accompanied by alterations in hair structure.

Symposium 3

A melanocortin-5 receptor antagonist inhibits differentiation of human sebaceous glands and production of sebum-specific lipids

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The melanocortin-5 receptor (MC5R) is expressed only in differentiated sebocytes of the human sebaceous gland. MC5R knockout mice were shown to have abnormal water repulsion and thermoregulation due to reduced production of sebaceous lipids. Here we show the suppression of sebum production by the inhibition of the sebaceous MC5R with the MC5R antagonist JNJ-10229570. The compound blocked the binding of NDP-alpha-MSH to the receptor in MC5R-transfected cells in a dose-dependent manner and inhibited the production of sebum-specific lipids by cultured primary human sebocytes, as shown by high performance thin layer chromatography (HPTLC). Topical treatment of human skin transplanted onto severe combined immunodeficient (SCID) mice with JNJ-10229570 led to marked reduction in sebaceous gland and cell size, and differentiation level within the gland, and strongly reduced sebum secretion. We suggest that MC5R antagonists such as JNJ-10229570 could be used as topical sebum suppressive agents for the treatment sebaceous gland disorders such as acne.

Symposium 4

The impact of MC4R mutations

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MC4R mutations leading to a reduced function are considered to entail monogenic obesity, thus implying a large effect size on the quantitative phenotype BMI. Heterozygous individuals without obesity are assumed to reflect the reduced penetrance of these mutations. A comparison of mean BMI of family members with and without the respective mutation of the index patient revealed that the male and female mutation carriers were `only´ 4.5 and 9.5 kg/m² heavier than non- carriers. The gender difference is similar to findings in Mc4r knockout mice obtained in some but not all studies. It is difficult to assess if the effect sizes of single mutations differ; our family data imply that this is the case. Haplotype analyses of the genomic region comprising the MC4R, which were also based on SNPs downstream of the MC4R (identified by recent genome wide association studies), indicate that linkage disequilibrium extends into the 5´prime end of the gene. The implications of this finding need to be assessed.

Symposium 4

The genetic epidemiology of melanocortin 4 receptor (*MC4R*) variants

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MC4R mutations are the commonest cause of monogenic obesity. While rare *MC4R* mutations lead to monogenic forms, growing evidence shows that common *MC4R* variants contribute to obesity in the general population. Candidate gene studies have focussed on the V103I and I251L *MC4R* variants that both affect MC4R function *in vitro*. Individual association studies, typically small and underpowered, found no association between V103I (103I:~4%) or I251L (251L:~2%) and the risk of obesity in the general population. However, large-scale meta-analyses confirmed that both variants reduced the risk of obesity in carriers of the I103 (-20%,P<0.01) or L251 (-50%,$P<10^{-4}$) alleles.
Recently, genome-wide association (GWA) studies identified a common variant (MAF~27%) at 188kb downstream of *MC4R* showing robust association with BMI and obesity in adults and children. The minor allele increases BMI by 0.22kg.m^{-2} (~650g) ($P<10^{-14}$)and obesity risk by 12% in adults. Interestingly, this variant also showed association with increased height, consistent with the phenotype seen for rare *MC4R* mutations. Although MC4R is the nearest gene and phenotypic associations are consistent with *MC4R* mutations, it has not been established whether this variant indeed reflects MC4R function.
Thus, common *MC4R* variants contribute to variation in BMI and obesity risk in the general population. Of particular interest is the finding from GWA studies that suggests that the region downstream of MC4R contributes to its regulation.

Symposium 4

The genetics of ACTH insensitivity

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ACTH insensitivity or Familial Glucocorticoid Deficiency (FGD) is a potentially lethal autosomal recessive disorder in which affected subjects usually present in early childhood with the clinical consequences of glucocorticoid deficiency. Mutations in the *MC2R* occur in ~25% of patients, the majority of which are missense mutations and mainly lead to impaired folding and trafficking of the receptor which accumulates in the endoplasmic reticulum. Subsequently a second FGD locus was identified on chromosome 21 in a novel gene encoding a small single transmembrane domain protein now known as the melanocortin 2 receptor accessory protein (MRAP). Mutations - mainly nonsense or major splice site mutations - in MRAP lead to a relatively early onset form of FGD . MRAP functions as a specific MC2R trafficking factor and co-receptor for ACTH. *MRAP* mutations account for ~20% of patients. A further locus for FGD on chromosome 8 was recently shown to include the *STAR* gene, and specific mutations in *STAR* were associated with FGD. These mutations incompletely disable the protein, which normally functions to promote mitochondrial cholesterol uptake. *STAR* mutations only account for ~5% of FGD cases indicating that approximately half of all patients have disease resulting from unidentified genes. A number of candidate loci for these have been identified and a high throughput sequencing approach is underway to discover defective genes in these regions.

Symposium 4

Human MC1R genetics and model systems

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Polymorphism in the MC1R gene makes a large contribution to pigmentation differences between individuals within the European population. We have performed a systematic functional analysis of 9 common MC1R variant receptors and correlated these results with the strength of the genetic association of each variant allele with pigmentation phenotypes. In vitro analysis using transfected melanoma cells revealed that some MC1R variant receptors displayed reduced levels on the cell surface, which corresponded to impaired cAMP coupling. Other variants demonstrated normal cell surface expression but had reduced functional responses, indicating that altered G-protein coupling may be responsible for this loss of function. Wildtype and variant receptor co-expression studies revealed a dominant negative effect of some variant receptor alleles on the surface level of the wildtype receptor, which also resulted in reduced cAMP accumulation. Comparison of the in vitro receptor characteristics with skin and hair colour data of individuals both homozygous and heterozygous for MC1R variant alleles revealed parallels between variant MC1R cell surface expression, functional ability, dominant negative activity and their effects on human pigmentation. We have also established a large bank of clonal neonatal melanocyte cultures to examine the function of MC1R variant alleles in monoculture and in melanocyte-keratinocyte coculture models. In these studies we are examining the behaviour and functional abilities of melanocytes with homozygous MC1R variants in comparison to wildtype strains in a more physiologically relevant environment.

Symposium 4

Postnatal *Sim1* deficiency causes hyperphagic obesity and reduced *Mc4r* and *Oxytocin* expression

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Single-minded 1 (*SIM1*) mutations are one of the few known causes of monogenic obesity in both humans and mice. While the role of *Sim1* in the formation of the hypothalamus has been described, its post-developmental, physiologic functions have not been well established. Here we demonstrate that postnatal CNS deficiency of *Sim1* is sufficient to cause hyperphagic obesity. We conditionally deleted *Sim1* after birth using α-calcium/calmodulin-dependent protein kinase II-Cre (CamKII-Cre) lines to recombine a floxed *Sim1* allele. Conditional *Sim1* heterozygotes phenocopied germline *Sim1* heterozygotes, displaying hyperphagic obesity and increased length. We also generated viable conditional *Sim1* homozygotes, demonstrating that adult *Sim1* expression is not essential for mouse or neuron survival, and revealing a dosage-dependent effect of *Sim1* on obesity. Using stereologic cell counting, we showed that the phenotype of both germline heterozygotes and conditional *Sim1* homozygotes was not attributable to global hypocellularity of the paraventricular nucleus (PVN) of the hypothalamus. We used retrograde tract tracing to demonstrate that the PVN of germline heterozygous mice projects normally to the dorsal vagal complex (DVC) and the median eminence (ME). We showed that conditional *Sim1* homozygotes exhibit a decrease in hypothalamic oxytocin (*Oxt*) and PVN melanocortin 4 receptor (*Mc4r*) mRNA.

Symposium 5

Yet even more ligands, structures and modes of action at the melanocortin receptors

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Melanocortin receptor signaling is regulated by an ever increasing spectrum of endogenous ligands. In addition to the agonist α-MSH and the inverse agonists agouti signaling protein (ASIP) and agouti-related protein (AgRP), recent work shows that β-defensins act at MC1R and MC4R, probably as neutral antagonists. Here we focus on two aspects of ligand-MCR recognition. First, we address the molecular details of ASIP's selectivity for MC1R in the skin. AgRP and ASIP are homologous and bind with nanomolar affinity to MC3R and MC4R in the central nervous system, but only ASIP binds to MC1R. To explain this phenomenon, there must be unique molecular contacts mediating the ASIP-MC1R interaction. Using a series of loop-swapped AgRP/ASIP chimeric proteins, we identify a tight association conferred by the ASIP C-terminal loop, which is shown to be essential for receptor recognition and antagonism. The second study examines the molecular features in β-defensin 3 required for MCR recognition. Although elimination of key basic residues in ASIP and AgRP completely abrogates binding, we show through a series of mutagenesis studies that β-defensin binding is tolerant of all single, basic amino acid substitutions. By extending mutagenesis to examine highly charged patches on the β-defensin surface, we demonstrate that binding is largely controlled by strong electrostatics. These findings help explain the differential functions of α-MSH, ASIP, AgRP and the β-defensins, and uncover new principles for MCR recognition.

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Symposium 5

Design of novel melanocortin receptor ligands

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Though much is known about the multiple biological functions of the melanocortin receptors, there is an unmet need for potent, highly receptor selective and biologically stable agonist and antagonist ligands especially for the MC1R, MC3R, MC4R and MC5R receptors. These receptors all have as their native ligand α-melanocyte stimulating hormone, and appear to have essentially the same pharmacophore, namely the tetrapeptide sequence –His-Phe-Arg-Trp- with the His residue perhaps not required as a pharmacophore element for all of these four receptors. This conundrum provides a very difficult challenge. How to develop selective agonist and antagonist ligands when the receptors appear to utilize essentially the same pharmacophore elements. We have taken several approaches to solve this problem. Our primary hypothesis is that each receptor must utilize different conformational and topographical properties in the agonist and antagonist ligand-receptor complex. Thus we have examined a wide variety of conformation and topographic constraints, and in the process we have developed a number of selective agonist and antagonist ligands for these receptors. More recently we also have discovered some allosteric ligands for these receptors that may have very useful agonist and antagonist properties. A discussion of some of our successes will be presented, along with a discussion of future perspectives.

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Symposium 5

Ligand-receptor interactions that differenitate mMC3R agonist versus antagonist receptor pharmacology

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The melanocortin ligands all contain the His-Phe-Arg-Trp core sequence important for molecular recognition and receptor functional activity. We have previously reported tetrapeptides possessing mixed MC3R antagonist/partial agonist and MC4R agonist pharmacological profiles (*J.Med. Chem.* 51:5585 2008). Selected compounds, in conjunction with mMC3R mutagenesis, were used to probe putative ligand-receptor interactions important for the differentiation of MC3R agonist versus antagonist receptor pharmacology.

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Symposium 5

A High-Throughput Screen for Allosteric Modulators of the MC4-R

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Allosteric modulators of GPCRs may restore normal spatio-temporal activity of physiological systems without the toxicity resulting from potent orthosteric agonism. Since haploinsufficiency of the melanocortin-4 receptor causes severe obesity in children, we reasoned that positive allosteric modulators of this receptor might be used to increase receptor signaling, and thus provide a novel therapeutic approach to melanocortin obesity syndrome. With the Vanderbilt High Throughput Screening Core, we designed a high throughput screen for allosteric modulators utilizing the Promega pGLO system for real-time detection of cAMP. We optimized this assay based on analysis of luminescence from a clonal hMC4R cell line using an EC_{20} dose of a-MSH in a 384 well plate system and the Hammamatsu FDSS6000 plate reader. The assay was shown to be suitable for high throughput screening (Z'=0.5). We then performed a prescreen on the Spectrum collection of 2,000 biologically active compounds (Microsource), composed of current pharmaceuticals (50%), natural products (30%), and other bioactive compounds (20%). This prescreen identified 62 agonists and positive allosteric modulators of MC4-R signaling, and 60 inhibitors of MC4-R signaling. These included anticipated hits, such as adenylyl cyclase agonists and PDE inhibitors. This diversity of compounds demonstrated the value of the screen for identification of allosteric modulators of the MC4-R. We are currently investigating the specificity and mechanism of action of two selected novel structural families of positive modulators identified in this screen, and screening the larger 160,000 molecule Vanderbilt Drug Library. This work was supported by NIH RO1 DK070332 (RDC).

Symposium 5

Effects of deficiency of the MC4R-coupled G protein (Gsα) on energy homeostasis

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The central melanocortin system promotes negative energy balance (reduced food intake, increased energy expenditure), and melanocortin receptor MC4R mutations lead to obesity and glucose intolerance/insulin resistance in humans and mice (MC4RKO knockout). In Albright hereditary osteodystrophy, a monogenic obesity disorder linked to heterozygous mutations of Gsα, the G protein that mediates receptor-stimulated cAMP generation, obesity develops only when the mutation is on the maternal allele. Likewise, mice with maternal (but not paternal) germline Gsα mutation develop obesity and diabetes. These parent-of-origin effects are due to Gsα imprinting, with preferential expression from the maternal allele in some tissues. To better understand the mechanisms of the metabolic imprinting effect of Gsα, we have generated several mouse models with disruption of Gsα expression in either the whole central nervous system (CNS) or in specific neurons. Mice with disruption of the maternal Gsα allele throughout the CNS (mBrGsKO) develop severe obesity and diabetes, and a similar, although less severe, phenotype is also observed in maternal Gsα mutation restricted to the paraventricular nucleus of the hypothalamus (PVH). In contrast, mice with Gsα deficiency in the ventromedial hypothalamus are unaffected. These results are consistent with Gsα being imprinted in PVH. Unlike MC4RKO mice, mBrGsKO mice exhibit reduced energy expenditure without hyperphagia. Consistent with this, administration of MC4R agonist MTII to mBrGsKO mice show impaired melanocortin stimulation of energy expenditure with no impairment in the anorexic effect of the agonist. This suggests that melanocortins may regulate energy homeostasis in the CNS through both Gsα-dependent and independent pathways.

Symposium 6

AMP kinase action in melanocortin neurons

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The AMP-activated protein kinase (AMPK) pathway has been implicated in the regulation of whole body energy homeostasis. Furthermore, hypothalamic AMPK has been suggested to act as a key brain energy sensing mechanism responding to hormones and nutrients. However, the precise neuronal populations and cellular mechanisms involved are unclear. To directly address this question, we have generated a series of mouse models including POMCα2KO and AgRPα2KO mice which lack AMPKα2 in pro-opiomelanocortin- (POMC) and agouti-related protein- (AgRP) expressing neurons, key regulators of energy homeostasis. POMCα2KO mice developed obesity due to reduced energy expenditure and hyperphagia but remaine sensitive to leptin. In contrast, AgRPα2KO mice developed an age-dependent lean phenotype with increased sensitivity to a melanocortin agonist. Electrophysiological studies in AMPKα2-deficient POMC or AgRP neurons revealed normal leptin or insulin action but lack of response to alterations in extracellular glucose. These findings suggest that glucose-sensing signalling mechanisms in POMC and AgRP neurons are distinct from those pathways utilised by leptin or insulin and indicate that while AMPK plays a key role in hypothalamic function it does not act as a general sensor and integrator of hormonal signalling in the mediobasal hypothalamus. To further explore the role of the AMPK pathway in the hypothalamic regulation of energy homeostasis we are currently studying mice with targeted or global deletion of additional kinases in the pathway. These studies are also providing new insights into the roles of this signalling cascade in the regulation of food intake and glucose homeostasis.

Symposium 6

Signal integration in Arcuate Nucleus POMC/AgRP-Neurons

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POMC and AgRP-neurons in the arcuate nucleus of the hypothalamus integrate a wide variety of signals, including hormones and nutrient components from the periphery of the organism to regulate an integrated response in control of energy homeostasis. Here the melanocortin system not only controls feeding, but also energy expenditure and locomotor activity. The presentation will focus on the molecular basis of intracellular signal integration in POMC and AgRP neurons in the orchestration of energy homeostasis and on the mechanisms, how obesity associated signals interfere with these pathways.

Symposium 6

Deciphering the neuronal circuit underlying the anorexia produced by ablation of AgRP neurons

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Acute ablation of neurons expressing agouti related protein (AgRP neurons) in adult mice with diphtheria toxin results in severe anorexia. Anorexia can be overcome by 14-day minipump administration of bretazenil, a GABA-A receptor partial agonist, into the parabrachial nucleus (PBN). This treatment suppresses Fos activation in PBN and allows permanent adaption to the loss of AgRP neurons. Because Fos is also activated in the PBN by nausea, we asked whether ondansetron, an anti-nausea drug that antagonizes serotonin 5HT3c receptors, also overcomes the anorexia induced by AgRP neuron ablation. Chronic delivery of ondansetron by minipump into the 4th ventricle also allows mice to survive after ablation of AgRP neurons. However, delivery of ondansetron directly into the PBN is ineffective, whereas delivery into the nucleus tractus solitarius (NTS) is effective. Pre-treating mice with LiCl, which mimics nausea, prior to ablation of AgRP neurons also prevents severe anorexia. We suggest that LiCl treatment activates the same adaptive process that is engaged after ablation of AgRP neurons. Thus, suppression of PBN activity by bretazenil or ondansetron prior to AgRP neuron ablation or initiating adaptive process by pretreatment with LiCl can suppress anorexia elicited by AgRP neuron ablation.

Symposium 6

Cellular and molecular mechanisms in POMC neurons regulating energy balance and glucose homeostasis

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Metabolic cues such as leptin and insulin directly act on key collection of neurons both within and outside the hypothalamus to regulate food intake and body weight and glucose homeostasis. In addition, it is now established that classic neurotransmitters may also act on the same neuronal groups to regulate energy balance. However, the inherent complexity of these CNS circuits has made it extremely difficult to definitively identify the key neurons that are required to maintain glucose homeostasis and energy balance. Over the past several years the ability to manipulate gene expression in a neuron-specific fashion has become feasible. We will describe some our recent findings using mouse models that allow neuron-specific manipulation of genes regulating energy balance and glucose homeostasis. We will focus on the role of insulin and leptin to regulate hypothalamic POMC neurons. We will also describe results using a novel mouse model to investigate the role of serotonin action on hypothalamic POMC neurons to regulate food intake, body weight and glucose homeostasis.

Symposium 6

Arcuate nucleus controlled locomotor activity is depending on melanocortin signaling

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POMC- and AgRP-expressing neurons in the arcuate nucleus (ARC) of the hypothalamus play a critical role in the regulation of energy and glucose homeostasis. They are targets of leptin and insulin in the control of food intake and energy expenditure. We have previously demonstrated that overactivation of the transcription factor STAT3 specifically in AgRP neurons controls locomotor activity, thus assigning AgRP neurons in the ARC an unexpected role in this response. AgRP neurons are not only characterized by their ability to modulate melanocortin signaling via inverse agonism of the MC4 receptor but they also express other neurotransmitters such as GABA. To directly address the contribution of melanocortin dependent signaling in mediating the effect of activated STAT3 signaling in AgRP neurons in the control of locomotor activity, we have crossed mice expressing the constitutively active version of STAT3 in AgRP neurons with those overexpressing the mutant agouti (Ay mice). In contrast to STAT3-C^{AgRP} mice on a BL6 background, locomotor activity and body weight regulation are indistinguishable between Ay/Ay mice and STAT3-C^{AgRP} mice on an Ay background. Collectively, these data indicate that activation of locomotor activity via overactivation of STAT3 signaling in AgRP neurons depends on functional MC4 receptor signaling. Further work will have to elucidate the neuroanatomical target for MC4 receptor action with respect to the control of locomotor activity.

Symposium 7

The role of POMC and AgRP in the control of energy homeostasis; studies using rodent models

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Human genetic data indicate impaired synthesis or processing of POMC results in obesity. We have used a mouse model of POMC deficiency (Pomc null) to explore the role of POMC-derived peptides in energy homeostasis and adrenal biology. The phenotype of Pomc null mice recapitulates the clinical syndrome seen in humans congenitally lacking POMC, resulting in hyperphagia, obesity and hypoadrenalism. Loss of only one copy of the Pomc gene is sufficient to render mice susceptible to the effects of high fat feeding, emphasizing an important gene-environment interaction predisposing to obesity. Our studies indicate that POMC-derived peptides have influences on the response to a high fat diet, including a major influence on the dietary preference for fat. Pomc-null mice are unusual in that obesity and hyperphagia develop in the face of glucocorticoid (GC) deficiency. These mice appear hypersensitive to the adverse metabolic effects of GCs, developing hypertension, an exacerbation of both hyperphagia and obesity and a profound insulin resistance. Initial observations suggested that these adverse effects may be driven, at least in part, by an increased expression of the melanocortin antagonist agouti-related protein (AgRP). However, on-going studies in mice lacking both AgRP and POMC reveal that the metabolic phenotype seen with GC therapy is independent of AgRP action. Further, preliminary data from studies in Agrp/Pomc null mice indicate AgRP does not appear to have inverse agonist action in vivo.

Symposium 7

Plasticity of the melanocortin neurocircuitry

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The past twenty years have witnessed tremendous advances in the understanding of the central mechanisms regulating food intake and energy balance. Some of the most striking discoveries have included descriptions of the relationship between the peripheral hormones, leptin and ghrelin, and the hypothalamic melanocortin circuitry that respond to changes in peripheral metabolic signals, and that regulate metabolism through their multiple output pathways. In addition, the sophistication of research tools afforded by genetically engineered animals has provided a degree of certainty to the data that is unparalleled. Finally, much insight has been gained of the potential mechanisms that underlie the dynamic functioning of hypothalamic circuits. An attempt will be made to provide a synopsis of these advances, leading to the idea that synaptic plasticity as an important factor in the regulation of food intake and energy homeostasis.

Symposium 7

Melanocortins and serotonin interactions

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The central serotonin system is an established modulator of energy balance. Therefore, it is unsurprising that former (e.g. d-fenfluramine), current (e.g. sibutramine), and drug discovery (e.g. lorcaserin) obesity treatments target serotonin pathways. Pharmacological and genetic research implicates the Gq-coupled serotonin 2C receptor (5-HT2CR) and the Gi-coupled serotonin 1B receptor (5-HT1BR) specifically in these effects. Through a combination of functional neuroanatomy, genetic, feeding, and electrophysiology studies in rodents, we found that 5-HT2CR and 5-HT1BR agonists require melanocortin pathways to exert their effects on appetite. Specifically, we observed that 5-HT2CR agonists activate neurons expressing the endogenous anorectic melanocortin agonist pro-opiomelanocortin (POMC)/alpha-melanocyte stimulating hormone (alpha-MSH) and that serotonin and 5-HT1BR agonists inhibit the activity of neurons expressing the endogenous orexigenic melanocortin antagonist agouti-related peptide (AgRP) in the arcuate nucleus of the hypothalamus. In the brain, alpha-MSH and AgRP compete for action at the melanocortin 3 (MC3) and melanocortin 4 (MC4) receptors. We observed that activation of the MC4Rs, but not the MC3Rs, is required for d-fenfluramine, 5-HT2CR and 5-HT1BR agonists to influence feeding. A model is presented in which activation of the melanocortin system is downstream of serotonin and is necessary to produce the complete anorectic effect of serotonergic compounds.

Symposium 7

Brain region specific roles of MC receptors

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Reduction of melanocortin signaling in the brain results in obesity. However, where in the brain reduced melanocortin signaling mediates this effect is poorly understood. Therefore, we determined the effects of long term inhibition of melanocortin receptor activity in specific brain regions, namely the paraventricular nucleus (PVN), the ventromedial hypothalamus (VMH), the lateral hypothalamus (LH) and accumbens shell (Acc). Melanocortin signaling was inhibited by recombinant adeno-associated viral (rAAV) vector mediated overexpression of the inverse agonist Agouti-related peptide (AgRP). Overexpression of AgRP in the PVN, VMH or LH increased food intake, bodyweight, percentage white adipose tissue and leptin concentration. In these areas food intake was increased due to an increase in meal size. Overexpression of AgRP in the Acc did not have any effect on the measured parameters. Although the orexigenic peptides AgRP and NPY are co-released from neurons of the arcuate nucleus, the effects of AgRP clearly differ from those of NPY, which suggests complementary roles for these neuropeptides in energy balance.

Symposium 7

Involvement of melanocortins in food-motivated behavior

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Rats offered a free-choice high-fat high-sugar (cHFHS) diet developed leptin resistance accompanied by decreased proopiomelanocortin mRNA levels in the arcuate nucleus (ARC). Both these effects promote hyperphagia, which may result from either increased hunger signaling or increased motivation to eat. Indeed, we found that rats on a cHFHS diet were more motivated to work for a sugar reward than rats on a chow diet. We therefore determined the role of melanocortins in food-motivated behavior using a progressive ratio schedule of reinforcement. Our data revealed that, in satiated rats, α-melanocyte stimulating hormone (α-MSH) dose dependently decreased, whereas agouti-related protein (AgRP) dose dependently increased the motivation to work for a sugar reward. Interestingly, when sugar pellets were freely available, we did not observe an effect of AgRP on sugar intake, suggesting that rats want more sugar even though they do not choose to eat more of it under free feeding conditions. Since dopamine is an important mediator of food-motivated behavior, we determined whether the AgRP-induced increase in food-motivated behavior could be blocked by a dopamine antagonist. Indeed, the effects of AgRP on food-motivated behavior could be blocked by flupentixol, whereas it did not affect the AgRP-induced increase in total food intake. Thus, in addition to its effects on food intake, the melanocortin system also plays a role in the incentive motivational properties of sucrose.

Symposium 8

Design and efficacy profile of highly potent MC4R agonists with high brain receptor occupancy

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Despite significant genetic and pharmacological validation as an excellent obesity target and over ten years of intensive research in academia and industry, clinical proof-of-concept for the MC4R agonist mechanism has not been attained thus far. In fact, one of our first generation candidates, MK-0493, showed did not demonstrate statistically significant effects relative to placebo after 12 weeks in a fixed-dose study and also after 18 weeks of stepped-titration dosing (Krishna, R.; et al. *Clin Pharmacol Ther.* **2009**, 86(6), 659-66). Other than nausea/emesis, there was no target engagement marker for MK-0493. Subsequent research in our laboratories led to the discovery of MK-1661 which demonstrates a robust efficacy profile in all preclinical species tested. In DIO rats, MK-1661 attained significantly higher *ex vivo* brain receptor occupancy than MK-0493. Furthermore, in PET distribution studies in rhesus monkeys, MK-1661 had brain levels ~3.4X greater than MK-0493. This presentation will discuss the discovery and efficacy profile of MK-1661.

Symposium 8

Human MC4R deficiency

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The identification and characterisation of patients with mutations in the melanocortin 4 receptor (MC4R) gene has provided insights into the role of melanocortin pathways in the regulation of eating behaviour, intermediary metabolism, growth and cardiovascular physiology. We report data on the phenotypic study of over 200 patients with MC4R mutations from our cohort of severely obese patients. We show that in humans, MC4R plays a key role in modulating sympathetic nervous system mediated changes in blood pressure. The long term effects of MC4R disruption on cardiovascular function have also been studied.

Symposium 8

In vitro and in vivo profiling of a novel potent melanocortin 4 receptor peptide agonist (Palatin Peptide A)

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Melanocortin 4 receptor (MC4R) agonists are of significant clinical interest for treatment of erectile dysfunction and female sexual dysfunction. Agonism at the melanocortin 1 receptor (MC1R) could result in skin darkening and is hence undesirable. Furthermore, pressor effects have been observed pre-clinically and clinically with MC4R agonists. A potent MC4R peptide agonist (with selectivity against MC1R) was discovered and profiled in the rat for erectogenic potential, pharmacokinetics, body weight, and cardiovascular safety. Erectogenic results demonstrated a minimally effective dose of 0.3 mg/Kg dosed either subcutaneously or intravenously. No significant decreases in body weight were observed at this dose. Furthermore, results in telemetered rats at this dose shown no significant increase in diastolic or systolic blood pressure suggesting an acceptable therapeutic window.

Symposium 8

Utilize conjugated hMC1R selective ligands for the earlier diagnosis and treatment of melanoma

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Magnetic nanoparticles possess unique magnetic properties and the ability to function at the cellular and molecular level of biological interactions making them an attractive platform as contrast agents for magnetic resonance imaging (MRI) and as carriers for drug delivery. To better address specific clinical needs, Magnetic nanoparticles with higher magnetic moments, non-fouling surfaces, and increased functionalities are now being developed for applications in the detection, diagnosis, and early treatment of malignant tumors, cardiovascular disease, and neurological disease. Through the incorporation of highly specific selective hMC1R ligands and other functional moieties, such as organic or inorganic fluorophores and permeation enhancers, the applicability and efficacy of these magnetic nanoparticles could be greatly increased.Here we report the conjugation of hMC1R selective peptides specifically targeting on melanoma cell utilizing both of organic and inorganic fluorophore. The receptor-ligand interactions provide an effective strategy to improve the residence time in melanoma cells. Specific selective hMC1R ligands have been investigated to increase the site specific accumulation of integrated nano-particles. Finally ,these hybrid magnetic nanoparticles will serve as photothermal agents that could kill tumors by cooking them to death when energized by light.
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Symposium 9

Regulation of Melanocortin Receptor Signaling by Accessory Proteins

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The five members of the melanocortin (MC) receptor family mediate responses to diverse physiological stimuli. Expression of MC2, or ACTH, receptors on the plasma membrane requires either MRAP or MRAP2, homologous accessory proteins found in the adrenal gland. MRAPs are small, single transmembrane proteins that form highly unusual structures, anti-parallel homodimers. MRAP dimers are found in a complex with MC2 receptor. In the absence of an accessory protein, the MC2 receptor fails to reach the plasma membrane, but in the presence of either MRAP or MRAP2 the receptor is localized on the cell surface. When MC2 receptor is expressed with MRAP, ACTH stimulates a robust increase in cyclic AMP at subnanomolar concentrations. When the receptor is expressed with MRAP2, supraphysiological concentrations of ACTH are necessary to elicit a small cyclic AMP increase. MRAPs act by regulating the affinity of the MC2 receptor for agonist. When both MRAP and MRAP2 are expressed with MC2 receptor in the same cell, MRAP2 acts as an endogenous dominant negative inhibitor to decreases the potency of ACTH. The balance of stimulatory and inhibitory accessory proteins can control the sensitivity of the MC2 receptor to its natural agonist, ACTH. Interestingly, endogenous inverse agonists modulate the affinity of several other MC receptors for agonist. These features set the MC receptors apart from other members of the large G protein coupled receptor family.

Symposium 9

New aspects of MC4R biology

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A large number of G protein-coupled receptors (GPCRs) rely on their N-terminal domains for ligand recognition and activation. In contrast, most of our knowledge regarding GPCR activation was derived from studies investigating diffusible physiological or pharmacological ligands interacting directly with their core transmembrane regions. This raises the question of the functional and evolutionary link between N-terminal mediated and direct GPCR activation. We addressed this issue by dissecting the molecular interactions underlying both modes of activation in the Melanocortin-4 Receptor (MC4R). We find that constitutive activation of this receptor by its N-terminal domain specifically requires amino-acids outlining a conserved Class A GPCR activation domain while α-MSH, the high-affinity physiological agonist of this receptor, requires a separate sets of residues for activation. The additional observation that AGRP, the physiological inverse-agonist of MC4R, can independently modulate the activation by the N-terminal domain suggests that a number of constitutively active orphan GPCR could have physiological inverse agonists as sole regulators.

Symposium 9

Structure, function and regulation of the melanocortin receptors

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Melanocortin receptors (MCRs) belong to the seven-transmembrane (TM) domain proteins that are coupled to G-proteins and signaled through intracellular cyclic adenosine monophosphate. Many structural features conserved in other GPCRs are found in the MCRs. There are five MCR subtypes and each of the MCR subtypes has a different pattern of tissue expression and has its own signature profile regarding the relative potency of different melanocortin peptides. α-, β-, and γ-MSH and ACTH are known endogenous agonist ligands for the MCRs. Agouti and AGRP are the only known naturally occurring antagonists of MCRs. We have determined the molecular basis of all five human melanocortin receptors for different ligand binding affinity and potency using chimeric and mutated receptors. Our studies indicate that hMC1R, hMC3R, hMC4R and hMC5R utilize orthosteric sites for non selective agonists, a-MSH and NDP- a-MSH, high affinity binding and utilize allosteric sites for selective agonist or antagonist binding. Our experiments also indicate that agonists can induce different conformation changes of MCRs, which then lead to the activation of different signaling pathways, even when the expression level of receptor and the strength of stimulus-response coupling are the same. Furthermore, our results indicate that molecular determinants of hMC2R for ACTH binding and signaling are different from that of other MCRs. This finding may provide new information for the design of drugs targeting MCRs.

Symposium 9

The multifunctions of the melanocortin 1 receptor: regulation of pigmentation and activation of DNArepair, antioxidant, and survival pathways

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Activation of the melanocortin 1 receptor (MC1R) by α-melanocortin (α-MSH) is known to stimulate the synthesis of eumelanin, the brown-black pigment in mammalian melanocytes. We showed that activation of the human MC1R by its agonists α-MSH or adrenocorticotropic hormone (ACTH) increased melanogenesis and proliferation of human melanocytes (HM) *in vitro,* and that stimulation of the cAMP pathway, the main signaling pathway for the MC1R, rescued HM from ultraviolet radiation (UVR)-induced growth arrest, and was required for the pigmentary response. More recently, we found that activation of the MC1R enhanced nucleotide excision repair in HM and reduced the generation of reactive oxygen species, oxidative DNAdamage and apoptosis in response to UVR. The effects of α-MSH required the expression of functional MC1R and were independent of pigmentation, as they were evident in tyrosinase-negative albino HM. The effects of α-MSH on DNAdamage and repair occurred earlier than the increase in melanin, suggesting that the former effects are critical for maintaining survival and genomic stability of HM, and the latter is important for protection against subsequent UVR exposure. Given the importance of the MC1R in the UVR response of HM, we synthesizedtetra- and tripeptide analogs of α-MSH that mimicked α-MSH in all its effects on HM. These analogs with improved stability and MC1R selectivity can be an effective strategy for prevention of sun-induced skin cancer, particularly melanoma.

Symposium 9

The melanocortin 4 receptor localizes and may function at the primary cilium

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Primary cilia are cellular organelles that are present on most vertebrate cells and are host to a number of G protein-coupled receptors (GPCRs). Disruption of primary cilia causes obesity, among other phenotypes, in humans and rodents. The underlying cause of obesity resulting from ciliary dysfunction is undetermined.

The most common cause of monogenic obesity itself is mutation of the melanocortin-4 receptor (MC4R), a GPCR that is crucial in maintaining energy balance. We therefore examined the subcellular relationship between this GPCR and the primary cilium. We found that cells in neuronal populations that express MC4R are ciliated, and that the MC4R localizes at the primary cilium in vitro. Expression of the MC4R in primary neurons also results in MC4R localization at the primary cilium.

More than 80 mutations of the MC4R are currently known. Functional receptor deficiencies were previously described to explain the association with obesity of most patients carrying these mutations. However, we found a significant reduction in receptor localization to primary cilium as a single defect in some of the inexplicable mutations of the MC4R. Reduced ciliary expression provides a potential new explanation for the obesity association in these and other naturally-occurring MC4R mutations.

Symposium 10

Genomic landscape regulating pituitary POMC expression

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Pituitary expression of the POMC gene is largely driven by its proximal promoter that is highly conserved between species. This proximal promoter is active in both corticotrope and melanotrope cells, with a preference for the later in transgenic assays. We have identified a novel enhancer within the POMC locus that preferentially directs expression to corticotropes during development. This enhancer is different from the neural enhancer and does not appear to confer hormonal regulation.
Investigation of the POMC promoter led to the discovery of transcription factors that are critical for pituitary POMC expression and/or for differentiation of POMC lineages, incl. Pitx1 (pituitary homeobox 1) and Tpit (T-box of the pituitary). The importance of Tpit was highlighted in mouse studies that also identified it as a switch between POMC (corticotrope / melanotrope) and gonadotrope cell fates, and by the identification of human TPIT mutations that cause isolated ACTH deficiency. However, current data do not account for cell type specificity of corticotropes compared to melanotropes. We have identified a transcription factor that is responsible for cell fate switching between these two cell types. This factor was discovered through temporal- and lineage-specific expression profiling. Thus, corticotropes and melanotropes share a basic genetic program that is modulated by lineage-restricted regulatory factors acting through diverse regulatory domains of the POMC locus.

Symposium 10

Molecular and functional evolution of neuron-specific Pomc expression

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POMC expression in the mammalian brain is controlled by a distal upstream module containing two neuronal enhancers, nPE1 and nPE2. To study the functional role of these two enhancers we generated several transgenic mouse pedigrees expressing either a red or a green fluorescent protein under the transcriptional control of nPE1 or nPE2, respectively. Brain slices obtained from various double transgenic mice showed that nPE1 and nPE2 may drive reporter gene expression to identical POMC neurons. Despite their overlapping function, nPE1 and nPE2 seem to have originated from independent evolutionary processes; whereas nPE2 is under purifying selection in all Mammals, nPE1 is just present in Eutheria. To determine the evolutionary origin of these two enhancers we followed an *in silico* paleogenomic strategy based on paralog sequence searches in most available genomes. We found that nPE2 originated from the exaptation of a CORE-SINE retroposon in the lineage leading to mammals whereas nPE1 is derived from a MaLR, a member of the LTRs retrotransposon family widespread in placental mammals. In summary, two independent retroposition insertions upstream of POMC evolved into enhancer sequences that POMC neuron specific expression in the same subset of hypothalamic neurons, probably binding the same combination of transcription factors.

Symposium 10

Functional synergism between neuronal enhancers determines POMC tone in the CNS

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Previously, we identified two distinct neuronal-specific transcriptional enhancers located 10-12kb 5' of the basal *Pomc* promoter. To investigate their physiological roles, we produced mutant mice lacking one or both nPE sequences from the *Pomc* locus. Insertion of a loxP-flanked *neoR* selection cassette at either nPE site resulted in nearly complete silencing of *Pomc* expression in the hypothalamus and development of profound obesity. However, subsequent germ line Cre-mediated deletion of *neoR* revealed three distinct phenotypes corresponding to the absence of nPE1, nPE2, or both enhancers. Although either nPE1 or nPE2 alone is sufficient to drive expression of transgenes specifically to POMC neurons, deletion of nPE2 from its endogenous locus had no effect on *Pomc* mRNA levels, body weight, or adiposity compared to wildtype mice, even in response to a chronic high fat diet. In contrast, deletion of nPE1 caused mild obesity on a regular chow diet. Deletion of both enhancer sequences caused ~75% reduction in hypothalamic *Pomc* mRNA and more severe obesity, but still attenuated relative to the adiposity of mice with the *neoR* neuronal silencing alleles. Each of the six mutant strains retained full *Pomc* expression in the pituitary. Our data show that nPE1 and nPE2 play discrete functional roles in regulating neuronal *Pomc* expression and that they act in combination to control gene expression. Obesity magnitude in the mice was inversely proportional to *Pomc* mRNA levels. The unexpected hypomorphic, rather than null, phenotype in the double nPE-deficient mice suggests the possibility of a third, undiscovered neuronal *Pomc* enhancer.

Symposium 10

Hypothalamic proopiomelanocortin processing and regulation of energy balance and neuroendocrine function

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Hypothalamic proopiomelanocortin (POMC) neurons play a key role in regulating energy balance and neuroendocrine function. Much attention has been focused on regulation of POMC gene expression with less emphasis on regulated peptide processing. This is particularly important given the complexity of posttranslational POMC processing and the generation of several peptide products from the POMC precursor, including α-MSH and ß-EP, with opposing biological actions. α-MSH can attenuate the effects of ß-EP on gonadotropin and prolactin release in rodents and primates and with respect to feeding, α-MSH is inhibitory while ß-EP has stimulatory effects. ß-EP 1-31 can be cleaved by carboxypepitdase E (Cpe) to ß-EP 1-27 and 1-26 which have markedly reduced opioid activity. We have recently shown that Cpe may link FoxO1 (a transcription factor that mediates effects of insulin) in POMC neurons with regulation of food intake. Mice with FoxO1 ablation in POMC neurons have a lean phenotype with decreased food intake and increased Cpe expression in the arcuate. Analysis of POMC peptides in these mice revealed an anorexigenic profile with a selective increase of α-MSH and HPLC analysis demonstrated relatively more ß-EP 1-27 and ß-EP1-26 compared to ß-EP 1-31; this is opposite to the pattern seen in Cpe-deficient mice. Regulated Cpe-dependent cleavage of α-MSH and ß-EP may thus serve to maintain energy balance by both increasing α-MSH levels and by enhancing α-MSH activity.

Poster Session 1

P1.

Nutritional state influences effects of central alpha-MSH infusion in middle-aged rats

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Melanocortins (MC) are important in energy balance regulation. MCs decrease food intake (FI), increase metabolic rate (MR) and reduce body weight (BW). We have shown that the catabolic effects of a central leptin infusion vary in different nutritional states. As leptin stimulates the MC system via alpha-melanocyte stimulating hormone (αMSH), the question arises, whether the activity of the MC system changes similarly in different nutritional states.

Parameters of energy balance [FI, BW, core temperature (Tc), heart rate (HR)] of three groups of 6-month old male Wistar rats [calorie-restricted (CR6), normally fed (NF6) and a high-fat diet-induced obese (HF6) group] were recorded in a biotelemetric system during a 7-day (1 μg/μl/h) intracerebroventricular infusion of αMSH.

The MC-induced transient drop in FI was most pronounced in HF6, less in NF6 rats and weakest in the CR6 group. A consequent BW loss occurred in NF6, CR6, but not in HF6 rats. A simultaneous increase in the mean daytime Tc and HR was observed in all groups (indicating a rise in daytime MR). These changes were most pronounced in the CR6, but also remarkable in the NF6 and HF6 groups.

In contrast to the leptin effects, some of the αMSH-induced catabolic effects were maintained in HF6. In CR6, where leptin failed to decrease BW, αMSH caused a significant drop. Our data suggest that despite obesity-induced leptin resistance, MC-responsiveness is partly maintained. (OTKA 49321, PTE AOK-KA-34039-25/2009)

P2.

Mechanisms of stress-induced anorexia in mice with genetically decreased MC-4R activity

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Aim. Mutations decreasing central MC-4R activity increase basal feeding, and induce anorexia after stress. Stress-induced anorexia may be caused by: (1) activation of 2 type hypothalamic corticotrophin-releasing-factor receptors (CRFR2) mediating anorexigenic CRF effect; (2) inhibition of hypothalamic orexigenic neuropeptides AgRP and NPY activity; (3) changes in plasma corticosterone, insulin and glucose levels. The aim of the work was to investigate mechanisms underlying increased anorexigenic response to stress in mice with decreased MC-4R activity.
Methods. The agouti yellow C57Bl/6J Ay/a mice were used as a model of decreased MC-4R activity. Male Ay/a-mice and their a/a sibs (control) were exposed to 1-h restraint. All parameters were measured before and 0, 1, 3 h after stress.
Results. ??/?-mice had higher than a/a-mice basal food intake and plasma insulin levels. Stress reduced food intake and insulin levels in ??/?-mice only. Stress anorexia in ??/?-mice was not associated with elevated levels of corticosterone and glucose or reduced transcriptional activity of AgRP and NPY genes in hypothalamus. Stress decreased hypothalamic CRFR2 mRNA levels in a/a-mice only. So after stress CRFR2 mRNA levels were higher in ??/?- than in a/a-mice.
Conclusion. Stress-induced anorexia in mice with genetically decreased MC-4R activity might be associated with increased anoretic CRF signal due to elevated CRFR2 levels in hypothalamus.
The study was supported by the RFBR 08-04-00603, 09-04-00447

P3.

Protective effects of melanocortins on short-term changes in a rat model of traumatic brain injury

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A treatment for traumatic brain injury (TBI) remains elusive despite compelling evidence from animal models for a variety of therapeutic targets. Numerous animal models have been developed to address the wide spectrum of mechanisms involved in the progression of secondary injury after TBI.To further compromise the patient's prognosis is the lack of clinically effective treatments to assist recovery from injury. Those individuals that survive TBI continue to live for many years with disabilities conferring large emotional and financial burdens.We used a rat model of diffuse traumatic brain injury (TBI) the impact-acceleration model. In this study, we investigated the molecular and histological changes induced by TBI and the possible protective effects of melanocortins.

Brain tissue nitric oxide (NO) synthesis, by Griess reaction; phosphorylation level of two protein kinases ERK 1/2 and JNK, TNF-alpha expression by western blot; and brain histological damage were evaluated 24 and 48 hrs after insult.

Posttraumatic administration of melanocortin (3 and 6 hrs after injury) reduced TBI-induced upregulation of ERK and JNK phosphorylation, and TNF-alpha expression. These molecular changes were associated with a reduction in brain NO synthesis at both time points. These results were in agreement with a reduced brain tissue damage as highlighted by histopathological findings.

The findings of our study clarly indicate that anti-inflammatory effect of melanocortins could be useful for the treatment of diffuse TBI.

P4.

A pineal-specific agouti protein, AgRP2, regulates background color adaptation in teleosts

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Background color adaptation is widely used by teleosts as one of variety of camouflage mechanisms for avoidance of predation. Background color adaptation is known to involve light sensing by the retina and the pineal, and subsequent regulation of melanophore dispersion or contraction in pigment cells, most potently by melanocyte stimulating hormone (MSH) or melanin concentrating hormone (MCH), respectively. Here, we demonstrate that a teleost-specific agouti protein, AgRP2, is specifically expressed in the pineal. Pharmacological characterization of synthetic $AgRP_{(87-132)}$ and $AgRP2_{(93-136)}$ proteins using cloned zebrafish melanocortin receptors shows that while AgRP is an antagonist of the MC3-R and MC4-R receptors, AgRP2 is most potently a MC1-R antagonist, suggesting a role in pigmentary regulation. Injection of morpholino oligonucleotides against AgRP2 resulted in melanosome dispersion in zebrafish larvae, while those against AgRP did not. Furthermore, morpholinos against AgRP2 blocked the upregulation of hypothalamic MCH following exposure to a white background. By polymerase chain reaction, we then identified high levels of MC1-R mRNA in zebrafish hypothalamus. These data suggest that light detected by the pineal regulates AgRP2 release, which in turn regulates synthesis of MCH, possibly via direct pineal projection to hypothalamic MCH neurons. This identifies a novel physiological function for the agouti family of proteins and defines a neuroendocrine axis by which background light regulates pigmentation.

This work was supported by NIH grant DK075721 (R.D.C.), a Freedom to Discover Grant from the Bristol-Myers Squibb Foundation, (R.D.C.), and by NIH grants DK64265 (G.M.), DC04186 (M.W.), and HD22486 (M.W.).

P5.

Role of brain insulin signaling on tissue-specific glucose disposal

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Circulating insulin inhibits hepatic glucose production and stimulates peripheral glucose uptake. Hypothalamic insulin signaling is required for the inhibitory effects of circulating insulin on hepatic glucose production. In this study, we examined the central effects of circulating insulin on tissue-specific glucose uptake.
Tolbutamide, an inhibitor of ATP-sensitive potassium channels, was infused in the lateral ventricle (i.c.v.) in hyperinsulinemic euglycemic clamp conditions in chow-fed and in diet-induced obese C57Bl6/J mice. Whole body glucose uptake was measured by D-[^{14}C]glucose kinetics and tissue-specific glucose uptake by 2-deoxy-D-[^{3}H]glucose uptake.
I.c.v. administration of tolbutamide impaired the ability of circulating insulin to inhibit endogenous glucose production by ~20% ($P<0.01$). Surprisingly, i.c.v. tolbutamide infusion also diminished insulin-stimulated glucose uptake by muscle (-59%; $P<0.05$), but not by heart or adipose tissue. In contrast, in diet-induced obese mice, high fat feeding abolished this inhibitory effect of i.c.v. tolbutamide on insulin-stimulated glucose production and muscle glucose uptake.
In conclusion, circulating insulin stimulates glucose uptake in muscle in part through effects via ATP-sensitive potassium channels in the central nervous system, similarly to the effects on hepatic glucose production. In diet-induced obese mice, these effects of circulating insulin via the central nervous system are absent. These observations stress the role of the central effects of circulating insulin in normal physiological conditions and in diet-induced insulin resistance.

P6.

No amelioration of the metabolic phenotype of POMC deficiency with additional AgRP deficiency

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The central melanocortin system is a key neuronal pathway involved in the regulation of energy homeostasis. We have shown that the loss of *Pomc* results in hyperphagia, increased fat and lean mass and hypoadrenalism. *Pomc* null mice are also hypersensitive to the adverse metabolic effects of corticosterone supplementation.
However it remains uncertain if the phenotype results from the loss of POMC peptides, the unopposed action of AgRP (a putative inverse agonist at central melanocortin receptors) or a combination of both.
We have generated a mouse model lacking both POMC and AgRP (*Pomc*$^{-/-}$/*Agrp*$^{-/-}$ "DKO"). DKO mice are identical to Pomc null mice with both having increased food intake, body weight and fat and lean mass. Similarly, corticosterone supplementation causes an identical increase in food intake and body weight with both DKO and Pomc null mice increasing bodyweight by 13.29 and 13.09% respectively compared to control.
Ongoing studies of icv administration to DKO mice suggest that AgRP leads to a significant increase in body weight but not food intake when compared to controls.
These data indicate that the hypersensitivity to the adverse metabolic effects of glucocorticoids seen in Pomc null animals is not due to a mechanism involving AgRP and that AgRP may not have an inverse agonist action within the central melanocortin system *in vivo*.

P7.

Peripheral α-MSH regulates fuel homeostasis in skeletal muscle

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There is little data on the source and physiologic relevance of systemic melanocortin peptides.

We confirmed the pituitary is the critical source of circulating α-MSH and that α-MSH levels vary according to meals. By studying healthy human subjects with very low or non-functioning pituitaries (hypopituitarism and after craniopharyngioma surgery), we displayed that α-MSH from the pituitary mainly contributes to circulating α-MSH.

We found, by in vitro (pituitary explant incubation with glucose and/or insulin) and in vivo experiments (α-MSH levels during a glucose tolerance test-GTT-) done in humans, monkeys and mice, that α-MSH secretion is increased by glucose and insulin. We showed that α-MSH infusion during GTT increases glucose disposal in lean but not obese mice. By incubating biopsies of soleus muscle with α-MSH and insulin we confirmed part of this effect was directly intramyocellular in origin. Thus, α-MSH stimulates glucose uptake in muscles of lean but not obese mice.

Using a model of diet-induced thermogenic response, we found that α-MSH infusion creates a direct thermogenic response in sheep muscle.

This suggests that pituitary secretion of α-MSH after a meal may mediate post-prandial thermogenesis and be part of a parallel system to supplement insulin mediated glucose uptake into skeletal muscle. Failure of α-MSH to act in muscles of obese insulin resistant mice suggests that failure of α-MSH mediated glucose uptake may lead to diabetes in some model systems.

P8.

α-MSH excites brainstem raphe pallidus nucleus (RPa) neurons by suppressing a K^+ conductance and activation of RPa melanocrotin receptor 4 (MC4-R) stimulates thermogenesis

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The central melanocortin system plays a critical role in regulation of thermogenesis. The RPa contains important sympathetic premotor neurons controlling thermogenesis. The MC4R-bearing neurons in the RPa connect with interscapular brown adipose tissue (IBAT). However, the functional implications of RPa MC4R signaling remain elusive. Here we report that α-MSH fibers are in close apposition to the RPa MC4R neurons and the neurons projecting to IBAT. Bath applied α-MSH increases the firing rate of a subset of RPa cells by a postsynaptic mechanism using loose path extracellular recordings in young rat brain slice preparations. α-MSH depolarizes the RPa cells postsynaptically under whole cell current-clamp recordings. Voltage-clamp recordings reveal that α-MSH reduces membrane conductance, and the conductance reduced by α-MSH is linear over the range of -30 to - 120 mV and reversed near the potassium equilibrium potential, suggesting that α-MSH excites RPa neurons by suppressing a K^+ conductance. Furthermore, we show that α-MSH excites the targeted RPa spinally projecting neurons by activating MC4R. Microinjecting MC3/4R agonist MTII into the RPa area dose-dependently stimulates oxygen consumption and IBAT temperature. These results provide neuroanatomical, cellular electrophysiological and functional evidence to support an idea that the RPa bears a novel action site in the melanocortin circuits regulating thermogenesis. Supported by NIH DK62179 and 3R01DK062179-06S1 (WF).

P9.

Gender differences in the melanocortin regulation of energy balance: role of estrogen receptor alpha on AgRP neurons

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The melanocortin system is a target for the treatment of obesity but gender disparities in maintaining energy balance remain to be established. Agouti-related peptide is an endogenous melanocortin 3 and 4 receptor antagonist that promotes hyperphagia. Males display a prolonged period of hyperphagia following ICV AgRP while females respond with an attenuated response but gain the same percent body weight as males over a period of 7 days. The differential effect of AgRP in males and females could be due to differences in MCR mRNA within the CNS. Using whole hypothalamus RNA, it was determined that no differences lie between males and females in receptor expression using real time PCR; however, ovariectomy resulted in a 25% increase in MC4R mRNA ($p<0.05$). Within the hindbrain, male and female MC4R mRNA did not vary while MC3R mRNA was 3-fold higher in OVX females ($p<0.05$). OVX rats given AgRP also showed a more "male-like" pattern of feeding and energy expenditure. It is known that hypothalamic expression of estrogen receptor alpha (ERα), AgRP, and NPY increase once females have been ovariectomized. Here, we demonstrated using double-label immunofluorescence that AgRP neurons express ERα in the arcuate nucleus (ARC) of the hypothalamus. We compared intact vs. OVX rats and the amount of co-localization between AgRP and ERα within the ARC. These findings reveal mechanisms into the gender differences that exist in the melanocortin regulation of energy balance.

P10.

Central NPY administration divergently regulates food intake and hepatic very low density lipoprotein (VLDL) production in mice

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Introduction In rats, central administration of NPY has been found to increase both food intake and hepatic VLDL-triglyceride (TG) production (Stafford et al. Diabetes 2008; 57: 1482). The aim of this study was to investigate whether central NPY administration also increases food intake and VLDL-TG production in mice. **Methods** Male C57Bl/6J mice received an intracerebroventricular (i.c.v.) cannula in the left lateral ventricle of the brain. One week later, after a 4 h fast, the mice received an i.c.v. injection of NPY (200 µg/kg BW) under temporary isoflurane anesthesia and food intake was measured during two subsequent hours after injection. Alternatively, after a 4 h fast, mice were anesthetized and received an i.c.v. injection of NPY (200 µg/kg BW) following an intravenous (i.v.) injection of trans^{35}S label (150 µCi/mouse) and tyloxapol (500 mg/kg BW), enabling the study of hepatic VLDL-^{35}S-apoB and VLDL-TG production, respectively. **Results** NPY was found to be effective in increasing food intake by +175% over 2 hours (0.91±0.15 g vs 0.33±0.14 g; n=9). The largest effect was observed during the first hour after injection, indicating a rapid effect of NPY on food intake. In contrast, the same dose of NPY did not acutely affect the VLDL-TG production (7.7±0.6 vs 7.3±1.1 mM/h; n=8-10) or VLDL-^{35}S-apoB production. **Conclusion** Altogether, these data show that in mice, central NPY is likely to divergently regulate food intake and hepatic VLDL production.

P11.

Melanocortin MC_4 receptor stimulation protects against stroke by counteracting the main ischemia-related mechanisms of damage

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Background and Aim. We repeatedly reported that melanocortin peptides have protective effects in severe hypoxic conditions. Here we investigated whether melanocortins produce neuroprotection in experimental models of ischemic stroke. *Methods and Results.* Global and focal cerebral ischemia (in gerbils and rats, respectively) caused impairment in spatial learning and memory, associated with activation of inflammatory and apoptotic pathways in the hippocampus and striatum, as well as in the liver. Treatment with nanomolar doses of the melanocortin analog [Nle^4, D-Phe^7] α-melanocyte-stimulating hormone (NDP-α-MSH) reduced central and peripheral injuries by modulating the excitotoxic, inflammatory and apoptotic responses, even if delayed up to 9-12 h after ischemia, with consequent dose-dependent improvement in subsequent functional recovery. The selective melanocortin MC_3 receptor agonist γ_2-MSH had no protective effects. Pharmacological blockade of MC_4 receptors and of peripheral nicotinic acetylcholine receptors, as well as bilateral cervical vagotomy, blunted the protective effects of NDP-α-MSH. *Conclusions.* Our data give evidence that melanocortins acting at MC_4 receptors protect, in a relevant time window, against the main ischemia-related mechanisms of cerebral and systemic injuries following ischemic stroke, with involvement of a vagus nerve-mediated protective pathway. These findings could have clinical relevance in the stroke setting.

P12.

Identification of melanocortin 2 receptor domains responsible for specific membrane transport and ligand selectivity

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Membrane expression of ACTH receptor MC2R is specifically limited to adrenal cells. In addition unlike the other members of the evolutionary related MCRs that recognize different melanocortin peptides, the MC2R solely binds ACTH. We used cassette substitution approach for systematic construction of chimeric MC2R/MC4R receptors to identify the domains determining the selectivity of MC2R in membrane trafficking and ACTH binding. We constructed 15 chimeric receptors replacing number of selected MC2R domains with corresponding regions of MC4R. We developed a new analysis method to quantify the localization of the recombinant receptors fused with enhanced green fluorescent protein in a cell membrane from the results of confocal fluorescent microscopy. NDP-MSH and ACTH binding and cAMP response were measured for all receptors. We have found that substitution of MC4R N-terminal part with homologues part from ACTHR significantly decreased the membrane trafficking of receptor. We have also identified another signal localized in TM3 and TM4 regions that is responsible for trapping MC2R in the endoplasmatic reticulum of the cell. We have also found that the TM4 and TM5 in the MC2R are involved in MC2R binding selectivity. Bypassing of these arrest signals may involve MRAP protein. We speculate that involvement of second pharmacophore K K R R of ACTH molecule in MC2R binding introduces conformational changes to receptor allowing the "main" pharmacophore H F R W to bind the receptor.

P13.

Fitting of fluorescence anisotropy and fluorescence intensity data by global numerical analysis for characterisation of ligand binding to MC4 receptor

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Present methodology combines fluorescence intensity and fluorescence anisotropy data for estimation kinetic parameters of ligands binding to receptors. The complexities of 7TM receptors activation and signalling do not allow to achieve analytical solution for these processes and therefore the numerical mechanistic models based approach has been applied. Such factors like receptor or ligand degradation, tracer photobleaching, basal drift of signal in process of measurements etc., could be integrated into the modelling algorithms for the fitting binding data of receptor ligand interactions. Using the multivariate analysis of two-way fluorescence data allow to avoid overparameterisation problem. Moreover, utilising the stochastic optimisation algorithms allow to obtain the true kinetic parameters for the mechanistic models from the data. This approach can be used also for screening assays based on fluorescence intensity/anisotropy read-out for estimation of kinetic binding properties of competitive ligands. As an example, this methodology was validated on the studies of interactions between human melanocortin 4 receptor subtype and Cy3B-NDP-MSH ligand on a new assay format where receptor were exposed on baculovirus particle surface.

P14.

Source of 5-HT influencing arcuate nucleus of the hypothalamus melanocortin neurons

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Serotonin (5-HT) has been shown to inhibit food intake by reciprocal actions on melanocortin neurons in the arcuate nucleus of the hypothalamus (ARC), stimulating pro-opiomelanocortin (POMC) neurons and inhibiting agouti-related peptide (AgRP) neurons. Ascertaining the location of 5-HT neurons projecting to the ARC would provide insight into signals that are relayed to melanocortin neurons *via* 5-HT neurons. To achieve this, the monosynaptic retrograde tracer Fluorogold (FG) was injected into the ARC unilaterally. Following 10 days of survival time, brains were assessed for FG and 5-HT immunoreactivity (IR). FG-labelled 5-HT neurons were observed in the dorsal (DR) and median (MnR) raphe nuclei, suggesting that these midbrain raphe nuclei are the principal source of 5-HT released in the ARC. Moreover, 5-HT synthesis (as measured by *Tph2* expression) was selectively reduced by fasting in the midbrain, but not the brainstem, suggesting that midbrain 5-HT neurons are sensitive to nutritional state. The hormone leptin also plays an important role in appetite and its receptor is expressed in the DR. We next investigated whether 5-HT neurons are responsive to leptin. However, we found no evidence that leptin directly influences brain 5-HT activity. We conclude that the principal sources of brain 5-HT projecting to melanocortin neurons in the ARC are 5-HT neurons in the MnR and DR and that key appetitive modulators of the activity of these 5-HT neurons remain to be clarified.

P15.

Murine and human tolerogenic dendritic cells induced by alpha-MSH generate functional regulatory T cells

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Alpha-MSH is able to induce immunosuppression and tolerance. To elucidate the mechanisms underlying α-MSH-induced immunomodulation we investigated whether α-MSH affects dendritic cell (DC)-T cell communication since this interaction plays an important role in the induction and regulation of immune responses. DC stimulated with α-MSH showed a reduced expression of co-stimulatory molecules but expressed increased levels of CD205 and IL-10, markers that are associated with DC-mediated induction of regulatory T cells (Treg). Interestingly, CD4+ T cells from co-cultures with α-MSH stimulated DC expressed markers characteristic for Treg, suppressed the proliferation of effector T cells in vitro, and inhibited contact allergy responses in DNFB sensitized mice upon adoptive transfer clearly demonstrating that α-MSH stimulated DC induced a regulatory phenotype in CD4+ T cells. Moreover, α-MSH stimulated DC ameliorated ongoing psoriasis-like skin inflammation in mice topically treated with imiquimod via the in vivo induction of functional Treg. Importantly, α-MSH also increased the numbers and suppressive activity of Treg from psoriasis patients via inducing tolerogenic DC. Together, these data demonstrate that α-MSH generated tolerogenic DC, which induced/expanded functional Treg in vitro as well as in vivo. Furthermore, in psoriasis patients α-MSH up-regulated the numbers as well as suppressor function of Treg and down-regulated the activity of Th-17 cells.

P16.

Why does mutation A^y not affect food intake in suckling mice?

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Mouse mutation A^y evokes hyperphagia due to disturbance of melanocortin regulation. We found that mutation did not affect food intake during suckling.

Aim: to study the influence of mutation A^y on hypothalamic expression of orexigenic neuropeptides and blood signals regulating this expression in pregnant and suckling mice.

Methods: Food intake (FI), levels of leptin, insulin, corticosterone, glucose in blood and mRNA of AgRP and NPY in hypothalamus were measured before mating, on days 7, 13, 18 of pregnancy, days 10, 21 postpartum in *a/a* (control) and A^y/a C57Bl/6J mice.

Results: Virgin A^y/a compared to *a/a* females had enhanced FI, the same blood parameters, the same NPY and decreased AgRP expression. In *a/a* mice, FI and hormone levels increased, glucose decreased, expression of NPY and AgRP increased during pregnancy, and FI continued to grow but levels of hormones and expression of NPY and AgRP decreased after parturition becoming equal with those in virgin females. Pregnant A^y/a as compared to *a/a* mice had FI higher in the first and lower in the second half of pregnancy, higher leptin levels and lower NPY and AgRP expression. Suckling A^y/a mice had equal FI, blood parameters and NPY and AgRP expression as *a/a* mice.

Conclusion: increase of NPY and AgRP expression in hypothalamus is essential to induce hyperephagia in pregnant but not in suckling mice. A^y mutation does not affect FI after parturition as MCR-independent signalling predominates in FI regulation during suckling.

P17.

Identification of neuronal populations involved in food anticipatory activity

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Rats develop anticipatory behaviour in expectance of food, which is suggested to reflect the drive to eat. This food anticipatory activity (FAA) occurs both with a restricted feeding paradigm as well as with a feeding schedule with restricted access to palatable food, but *ad libitum* access to normal chow. Previous Fos-studies have implicated that the hypothalamus, a homeostatic regulator of energy, is involved in anticipation to food in a restricted feeding paradigm, whereas the non-homeostatic regulators, e.g. corticolimbic structures, are activated during anticipation to a palatable meal. Which neuronal populations drive food anticipation is however not known yet. The aim of this study is, therefore,to identify the neural and molecular substrates of anticipation to food and their involvement in potentiation of over-consumption.
Rats subjected to either a restricted feeding schedule or a palatable feeding schedule showed clear anticipation to the meal. Plasma levels of leptin, ghrelin and insulin at the time of anticipation will be determined. Furthermore, colocalization of Fos and several neuropeptides, including NPY, orexin, and α-MSH, will be performed to investigate the role of these neuropeptides in anticipation to food or a palatable treat.

Poster Session 2

P18.

AP214, a new αMSH analogue, possesses anti-inflammatory activities

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Modulation of endogenous anti-inflammatory pathways constitutes a new strategy for the management of inflammatory processes. Targeting of MC1R or MC3R, using natural or synthetic agonists, has shown anti-inflammatory activity in several disease models. Research in this area is focused on the development of more specific ligands as well as to improve their half-life and stability. Here we studied the anti-inflammatory properties of the new peptide AP214, a MSH analogue with increased stability and binding affinity to MCR. Acute peritonitis was induced with 1 mg i.p. of zymosan A to male C57Bl6 mice (WT), MC1R-/- or MC3R-/- mice, 30 minutes after AP214 treatment (400 and 800mg/kg i.p.). Neutrophil recruitment was assesed by flow cytometry at 4 h. *In vitro*, peritoneal macrophages were stimulated with 25 μg/ml zymosan, and cytokine release measured by ELISA at 6 h. AP214 peptide displayed inhibitory activity at the 400μg/kg dose in WT (35%) and MC1R-/- mice (33.8%); however, this effect was lost in MC3R-/- mice. *In vitro*, AP214 (1-1000 nM) reduced IL-1b, TNF-a and IL-6 levels, in WT and in MC1R-/- mice, but an uneven effect was observed in MC3R-/- macrophages, so that AP214 no longer affected IL-1b secretion, whereas it fully retained its inhibitory properties on TNF-a and IL-6. Gene expression data revealed up-regulation of MC1R and MC5R in MC3-/- cells, suggesting compensatory mechanisms, and the involvement of other receptors (possibly MC5R) in the actions of AP214.

P19.

The effects of dimerization of the Melanocortin receptors on signal transduction

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Melanocortin receptors (MC3R and MC4R) are well known for their role in hypothalamic weight regulation. When stimulated with a-msh or blocked by the inverse agonist AgRP they are able to decrease or increase food intake, respectively. In addition to these receptors many other G-protein coupled receptors (GPCRs) are expressed in the brain. Over the past decade it has become clear that many of these GPCRs do not only function as a monomer but also can operate in a multimer complex. These GPCR homo- or heterodimers could prove promising drug targets that offer increased specific ligand binding and fewer side effects. In this study we investigate the possible interaction and dimerization of the melanocortin receptors with other GPCRs the striatum.

Using double fluorescent in situ hybridization (FISH) we evaluate the expression of the melanocortin receptors and aim to show coexpression with several other GPCRs in brain. We will apply BRET technology to investigate dimerization behavior for the melanocortin receptors with alternative GPCRs. Furthermore, reporter assays using CRELacZ showed that the MC4R is capable of increasing the activity of the vasopressin 2 receptor when coexpressed, whereas this was not found for the dopamine 1 receptor.

Identification of GPCR dimers in brain would add to the understanding of the complexity of signaling and may give new directions in finding new drug targets regarding brain-related disorders leading to more potent and specific drugs.

P20.

Central actions of alpha-MSH on parameters of energy balance in rats: age-related patterns

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Melanocortins (MC) are major catabolic mediators of energy balance: they suppress food intake (FI), enhance metabolic rate (MR) and decrease body weight (BW). During aging two major trends emerge: obesity in the middle-aged, later anorexia of aging. MC agonist alpha-melanocyte stimulating hormone (αMSH) may participate in these alterations.
In different age-groups of male Wistar rats, we studied the effects of intracerebroventricular administrations of αMSH (injection: 5 μg; 7-day infusion: 1 μg/μl/h) on parameters of energy balance: FI, BW, core temperature (Tc), heart rate (HR, indicator of MR) in a biotelemetric system.
Decline of FI and BW induced by injection or infusion was weakest in juvenile and middle-aged and most pronounced in young adult and old rats. Infusion-induced elevation of daytime Tc was modest in the young and old groups, both day- and nighttime values increased significantly in middle-aged animals. Tachycardia developed both in the middle-aged and in the old groups. It lasted for 4 days in the oldest rats, while middle-aged animals maintained it throughout the infusion.
Middle-aged rats are insensitive to the anorexic but not the metabolic effects of αMSH. On the other hand, old rats are particularly sensitive to MCs. These observations contribute to the explanation of the age-related trends in BW regulation. Effects of αMSH on different parameters of energy balance do not change parallel to one another during aging. (OTKA 49321, PTE AOK-KA-34039-25/2009)

P21.

Improved vascular function and altered cardiac phenotype in mice over-expressing melanocyte-stimulating hormones

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The central melanocortin system plays a crucial role in the regulation of energy homeostasis and cardiovascular functions. Here we identify that long-term activation of the melanocortin system affects autonomic nervous system function, vascular reactivity and cardiac growth. We applied echocardiography, blood pressure telemetry and isometric tension recordings to examine changes in cardiovascular functions of transgenic α- and γ-MSH over-expressing (MSH-OE) mice, and assessed the observed phenotype against age-matched wild type (WT) mice. MSH-OE mice did not differ in terms of arterial blood pressure, but displayed reduced heart rate, elevated cardiac vagal activity and improved baroreceptor-mediated responses. Echocardiographic examination indicated normal or even enhanced myocardial performance in MSH-OE mice. Interestingly, MSH-OE mice showed reduced cardiac hypertrophy associated with aging. Furthermore, we observed a decreased vasoconstrictory profile in large conduit arteries of MSH-OE mice, an effect that was dependent on increased NO bioavailability. The concept of improved vascular function was further strengthened by a Doppler echocardiographic finding of augmented vasodilatory responses in coronary arteries of MSH-OE mice. In conclusion, long-term activation of melanocortin signalling pathways could provide cardioprotective regulation by increasing cardiac vagal activity, improving vascular function, and ultimately restraining age-associated cardiac hypertrophy.

P22.

Spatial and temporal signalling of melanocortin 5 receptor: its putative role on lipolysis regulation

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Melanocortin 5 Receptor (MC5R) is expressed in a wide variety of peripheral tissues and seems to be implicated in the control oflipid metabolism and energy homeostasis. The exact mechanism underlying its action is still poorly understood and constitutes the basis of our work.
We recently showed that MC5R activates ERK1/2 by a PI3K-regulated but Akt, PKA and PKC-independent mechanism. The present work demonstrates that in MC5R-GFP stably transfected HeLa cells, ERK1/2 activation led to downstream phosphorylation of p90RSK and MSK-1. Activated ERK1/2 and p90RSK were mainly found at cytoplasm. Indeed, only 10% of both phosphorylated kinases translocated to nuclei, where ERK1/2-dependent expression of c-Fos was observed. Alpha-MSH treatment also increased CREB phosphorylation through the cAMP/PKA pathway.
Regarding the pitfalls of overexpressing systems, we further evaluated the alpha-MSH action in 3T3-L1 adipocytes, in which MC5R is endogenously expressed. Adipocytes response to alpha-MSH resulted in a loss of cytoplasmatic lipid content and releaseof glycerol by an ERK1/2 dependent mechanism. Similarly to MC5R-GFP transfected cells, sustained ERK1/2 phosphorylation occurs via PI3K, independently of Akt, leading to p90RSK and MSK-1 activation. Moreover, phosphorylated forms of ERK1/2 and p90RSK were mostly retained at the cytoplasm. Further approaches are being developed to establish the functional link between these signalling pathways and lipolysis.

P23.

Pleiotropy in the melanocortin system, coloration and behavioural syndromes

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In vertebrates, melanin-based coloration is often associated with variation in physiological and behavioural traits. We propose that this association stems from pleiotropic effects of the genes regulating the synthesis of brown to black eumelanin. The most important regulators are the melanocortin 1 receptor and its ligands, the melanocortin agonists and the agouti-signalling protein antagonist. On the basis of the physiological and behavioural functions of the melanocortins, we predict five categories of traits correlated with melanin-based coloration. A review of the literature indeed reveals that, as predicted, darker wild vertebrates are more aggressive, sexually active and resistant to stress than lighter individuals. Pleiotropic effects of the melanocortins might thus account for the widespread covariance between melanin-based coloration and other phenotypic traits in vertebrates.

P24.

Agouti protein regulates adrenal steroidogenesis via a mechanism independent of melanocortin receptor competitive antagonism in A^y mice

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Adrenocorticotropin (ACTH) a melanocortin type 2 receptor (MC2-R) agonist, is the major stimulator of adrenal corticosterone production. Lethal yellow (A^y) mutation causes the wild-type Agouti protein overexpression throughout the body, including adrenals. Agouti protein is the endogenous MC-Rs antagonist. It suggests their inhibitory role in adrenal steroidogenic regulation in A^y mice. However, we have demonstrated that Agouti protein increased ACTH-induced steroidogenesis in A^y mice. The aim of the study was to investigate possible mechanisms of Agouti protein stimulation effect on adrenal steroidogenesis in A^y mice. Chronic ACTH signaling reduction by Agouti protein might lead to compensatory increased adrenal MC2-R levels, which caused gain of adrenal reactivity to ACTH. Our results contradict the assumption: Agouti protein overexpression did not effect on MC2-R mRNA expression and ACTH-induced cAMP level in adrenal cells. We have shown that Agouti protein stimulated intracellular steroidogenic enzyme activity: corticosterone production induced by steroidogenic enzyme stimulators (dibutyryl-cAMP, progesterone) was increased in A^y adrenal cells. These data demonstrate that Agouti protein can modulate adrenal steroidogenesis in A^y mice via a mechanism independent of MC2-R competitive antagonism, consistent with existence of distinct receptors or additional physiological mechanisms for Agouti protein action. The study was supported by the RFBR 08-04-00603.

P25.

Melanocortins improve outcome in an experimental model of multiple organ dysfunction syndrome

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Background and Aim. Melanocortins have a life-saving effect in circulatory shock by counteracting the systemic inflammatory response, and through the activation of the vagus nerve-mediated cholinergic anti-inflammatory pathway.To gain insight into the potential therapeutic value of melanocortins, here we studied the melanocortin analog [Nle^4,D-Phe^7]α-MSH (NDP-α-MSH) in an experimental model of multiple organ dysfunction syndrome (MODS) that resembles human MODS.*Methods.* MODS was produced in mice by a single intraperitoneal (i.p.) injection of lipopolysaccharide followed, 6 days later (= day 0), by zymosan. After MODS (or sham MODS) induction, animals (pretreated or unpretreated with chlorisondamine) received i.p. NDP-α-MSH for 16 days. *Results.* NDP-α-MSH significantly reduced, at day 7, mRNA expression of tumor necrosis factor-α, and increased mRNA expression of interleukin-10, in the liver and lung of MODS mice; furthermore, at day 16 NDP-α-MSH significantly improved the histological picture of both organs, and survival rate. The peripheral nicotinic acetylcholine receptor antagonist chlorisondamine abrogated all beneficial effects of NDP-α-MSH. *Conclusions.*Our data indicate that melanocortins protect against MODS by counteracting the systemic inflammatory response, likely through brain activation of the cholinergic anti-inflammatory pathway. These findings could provide the potential for development of a new class of drugs for a novel approach to MODS.

P26.

Acetylation-induced changes in α-MSH activity may be related to concomitant expression of different melanocortin receptor subtypes

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Acetylation is known to modify α-MSH activity; the mechanism by which acetylation alters the interaction between ligands and receptors has not yet been elucidated. Studies using barfin flounder have provided useful information regarding the functions of α-MSHs with respect to the presence or absence of acetyl groups. Both α-MSH and desacetyl-α-MSH dispersed the pigments in xanthophores *in vitro*; the dispersion activity of the former was greater than that of the latter, indicating that acetylation increased this activity. In contrast, in melanophores, while desacetyl-α-MSH exhibited pigment-dispersing activity, α-MSH exhibited only negligible activity. When the expression of MC genes in isolated skin cells was determined using PCR, only *Mc1r* transcript was detected in xanthophores, whereas both *Mc1r* and *Mc5r* transcripts were detected in melanophores. Results similar to those in melanophores were also observed on cortisol release from the interrenal cells—desacetyl-α-MSH exhibited dose-dependent activity, whereas α-MSH exhibited negligible activity. In the interrenal cells, *Mc2r* and *Mc5r* transcripts were detected by *in situ* hybridization. It has been indicated that many GPCRs form heterodimers that may affect the affinity of the ligand for the corresponding GPCR. Taken together, the expression of two different *Mcr* subtypes in melanophores and interrenal cells indicates that a heterodimer consisting of MC1R and MC5R or MC2R and MC5R may lower the activity of α-MSH.

P27.

ENU-induced nonsense mutation results in loss of MC4R function in the rat

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The laboratory rat (Rattus norvegicus) is one of the preferred model organisms for studying human physiology and disease. However, availability of genetic modified rat models is limited. We make use of N-ethyl-N-nitrosourea (ENU) mutagenesis to introduce random point mutation in the germ line of male rats. Subsequently, the DNA of the offspring of these rats is screened for interesting mutations, like premature translational stop codons or missense mutations of functionally important residues in preselected genes-of-interest. Using this approach we have generated multiple rat knockout models for genes involved in a variety of biological processes, including the melanin-concentrating hormone precursor (PMCH).

Recently, we have re-sequenced a large collection of G protein-coupled receptors (GPCRs) in a mutagenized F1 population and identified 9 nonsense mutations, including in the gene encoding the melanocortin 4 receptor (MC4R). This mutation results in a truncation at C-terminus of the receptor at the fourth intracellular loop. We show in vitro that mutant MC4R is still produced, but fails to be transported to the plasma membrane and to transduce signal after agonist treatment. Homozygous mutant rats exhibited increased body weight, food intake, adiposity, and insulin and leptin levels. In conclusion, using ENU target-selected mutagenesis we successfully generated a bona fide MC4R knockout rat, which is highly complementary to existing mouse knockout models.

P28.

Neural correlates of food anticipatory activity in the ventromedial hypothalamus

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Food anticipatory activity (FAA) is characterized as increased locomotor behaviour in response to impending food availability. This behaviour is observed in rats that are maintained on a timed restricted feeding schedule (RFS) or when limited availability of food is cued. Various brain areas have been implicated in FAA, but there is no single area that is both sufficient and necessary for the expression of FAA (Ribeiro et al., 2009).

For a long time now, the ventromedial hypothalamus (VMH)- Arcuate region has been considered as a satiety centre. It contains glucose-sensing neurons, and subpopulations of VMH and Arcuate neurons respond to various humoral factors, including leptin and ghrelin. Lesion studies and activation of neuronal activity markers indicate that the VMH region is presumably part of the FAA circuitry. However, how FAA is regulated by neuronal activity in the VMH and how leptin an ghrelin modulate this activity is still unknown.

To assess how the VMH is involved in feeding-related behaviour we recorded neuronal firing in the VMH in rats that were kept on a random feeding schedule. Neuronal activity was measured during cue-induced anticipation and feeding behaviour. Systemic injections of leptin and ghrelin were administered to examine whether feeding-related activity in neurons is related to responsivity to either hormone.

P29.

Brain melanocortins and melanin-concentrating hormone: an axis linking nutrient and fluid balance

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This paper reviews seemingly obligatory relations between nutrient and fluid balance. A neuronal pathway involving the melanocortins αMSH and AGRP in the arcuate nucleus (Arc) of the hypothalamus projecting to the lateral hypothalamus (LH) may bridge this gap. In the fasted hypoleptinemic condition, increased expression of MCH (due to low melanocortin tone) and neuronal release of MCH underlies a drive towards a positive energy balance associated with optimal fluid homeostasis. On the other side of the spectrum, obesity may result from leptin resistance, leading to lowering of MC drive too. Since obesity is frequently associated with disrupted water balance, elevation of MCH expression in this event may be prevented via the hyper-osmotic stress signalled in the OVLT-SFO-MePN complex. As such, obesity development independent of MCH could underlie sustained disbalance between nutrient and fluid homeostasis. The latter is a risk factor towards perpetuation of cardio-metabolic diseases.

P30.

AAV-mediated knockdown of melanocortin receptor genes involved in obesity

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Obesity is an increasingly important health problem and there is an urgent need for the development of an effective treatment. The melanocortin 3 (MC3R) and 4 (MC4R) receptors play an important role in the development of obesity and could therefore be possible drug targets for the treatment of obesity. To unravel the precise role of the melanocortin receptors in obesity we will apply RNA interference using viral vector based technology in order to locally knock down MC receptors. Using the Gateway cloning technology we will clone a cDNA renilla fusion for both melanocortin receptors. Four short hairpin RNA's (shRNAs) will be designed and cloned into adeno-associated viral vectors. After testing the shRNAs, using a renilla-luciferase assay, the two best performing shRNAs will be used to generate virus. The high titer virus will be injected bilaterally into different brain areas expressing melanocortin receptors. To confirm injection locations and verify knockdown we will perform *in situ* hybridizations. This study may

P31.

N-terminal acylation with long chain fatty acids of melanocortin activating peptides changes receptor binding and PK properties

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The MC4 receptor is recognized as one of the most promising targets to therapeutically approach obesity. By activating the MC4 receptor it has been shown in rodents, that appetite is suppressed and the metabolic rate is increased, leading to a significant weight loss. *In vivo* studies in rodents and data from MC4 deficient humans underscore the importance of the MC4 receptor in body weight regulation; both mice and humans deficient in MC4 receptors are obese (Huszar et al. 1997 Cell 88, 131-141, Yeo et al. 1998 Nature Genetics 20, 111-112 and Vaisse et al. 1998 Nature Genetics 20, 113-114).

Here we report the *in vitro* and *in vivo* profile of peptides with and without an *N*-terminal acylation with a long chain fatty acid. The acylated peptides had improved *in vitro* potency towards the MC1 and MC4 receptor as well as prolonged halflife and prolonged acute effect *in vivo*. Daily subcutaneous administration of 3 mg/kg to diet induced obese rats of the acylated peptides resulted in a ~8% weight loss after 15 days.

P32.

Activity of the melanocortin system modulates effect of estradiol on insulin sensitivity

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Insulin resistance is the main link between obesity and type 2 diabetes (D2). Activation of the hypothalamic melanocortin (MC) system increases insulin sensitivity. Estradiol (E2) can also increase insulin sensitivity. The purpose of our work was to examine whether the activity of the MC system modulates E2 effect in regulation of insulin sensitivity. The Agouti yellow (Ay/a) C57Bl/6J mice were used as a model of decreased MC system activity. Body weight, food intake, plasma glucose, insulin levels, glucose tolerance were measured in 13 wk female Ay/a mice and their a/a sibs (control). There were three experimental groups: sham, ovariectomy (OVX) and OVX+E2. In sham operated Ay/a mice body weight, food intake, levels of glucose and insulin were increased and glucose tolerance was decreased as compared with a/a mice. Thus D2 metabolic syndrome developed in Ay/a mice. OVX increased and E2 treatment did not normalize food intake and body weight in mice of both genotype. OVX increased glucose and insulin levels, reduced glucose tolerance inducing D2 development in a/a mice, and E2 normalized these parameters acting as anti diabetic factor. OVX and E2 treatment did not effect on glucose and insulin levels and glucose tolerance in Ay/a mice in contrast to a/a mice. We concluded that suppression of MC signaling induced D2 development in Ay/a mice partly through preventing anti diabetic E2 effect.

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P33.

Effects of third ventricular administration of MC3R and MC4R antagonists in the rat

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The melanocortin-3 and 4 receptors are vital contributors for energy balance in the CNS. Their effects have been illustrated in mutagenic knockout models in mice. MC4R-/- mice show increased food intake, reduced energy expenditure, and increased body mass. MC3R-/- mice show decreased or normal food intake while fat mass is increased. This suggests that the MC3R influences substrate utilization. The combined MC3/4R -/- mouse is more obese than either single knockout. Previous studies have shown that administration of Agouti related peptide, an endogenous MC3/4R antagonist, increases food intake and decreases energy expenditure. These obesogenic effects of AgRP cannot be explained by a single receptor. We examined the effects of centrally-administered selective MC3R and MC4R antagonists. Body weight, food and water consumption were measured. For the first twenty-four hours following the injection indirect calorimetry data was collected. The MC3R antagonist (1μg) caused a significant increase in food intake for four days ($p<0.05$). Rats also showed an elevation in their respiratory quotient ($p<0.05$). This leads to the conclusion that the rats were storing fat while burning carbohydrates. Early tests with the MC4R (1μg) antagonist have shown insignificant differences in food intake and similar results as MC3R antagonist in respiratory quotient ($p<0.05$). We will continue to examine these effects as well as monitor blood pressure following injections of each antagonist.