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AgRP, physiological role of an inverse agonist

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Abstract

The melanocortin (MC) system distinguishes itself by the presence of both endogenous agonists—melanocortins as well as endogenous inverse agonists, namely agouti and agouti-related protein (AgRP). The melanocortin system plays an important role in the regulation of pigmentation and body weight. Endogenous inverse agonists provide a means of fine-tuning physiological processes. Disturbances in the balance between melanocortin receptor constitutive activity, melanocortins, and AgRP may contribute to susceptibility for disorders in energy balance (obesity and anorexia).

Keywords: Melanocortin receptor; AgRP; Pigmentation; Obesity; Anorexia

1. Introduction

The melanocortin (MC) system regulates pigmentation and body weight. We summarise here how endogenous inverse agonists were identified for the melanocortin system and how these inverse agonists affect function. We provide a model that illustrates the importance of inverse agonists for fine-tuning physiological responsiveness such as the regulation of body weight.

2. Pigmentation: extension and agouti

Two major loci affecting coat colour are extension and agouti. Before the cloning of the genes that mediate the effects of these loci on pigmentation, it was suggested that

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extension encoded a receptor for a putative ligand that was encoded by the agouti locus because extension was dominant over the agouti locus. In 1992, the extension locus was demonstrated to encode the MC1 receptor (MSH receptor). A truncated MSH receptor leads to light coat colour, while activating mutations of the receptor lead to dark coat colour [1]. MSH via the MSH receptor increases cAMP levels, which subsequently stimulate tyrosinase. In melanocytes, this results in the synthesis of dark melanin (eumelanin) instead of yellow melanin (pheomelanin). Thus, the extension locus encodes the MSH receptor. The MSH receptor is expressed in skin melanocytes and mediates the stimulatory effects of MSH on pigmentation.

The gene contributing to the effects of the agouti locus on pigmentation was cloned and called the *agouti* gene [2]. The *agouti* gene encodes a paracrine signaling molecule that causes hair follicle melanocytes to synthesise pheomelanin, the yellow pigment, instead of the black/brown pigment eumelanin. Dominant alleles of agouti, such as A^y (which has ectopic overexpression of agouti protein due to a recombination event in the promoter region of agouti), display a yellow fur. Agouti was demonstrated to be an antagonist at the MSH receptor [3], which explains the effect of agouti on pigmentation.

Thus, for the MSH receptor, evidence was provided for the existence of both an endogenous agonist, MSH, as well as an antagonist, agouti.

One of the first lines of evidence that agouti might act as an inverse agonist came from genetic crossing experiments. Loss-of-function alleles of the extension locus (MSH receptor), such as in the e/e mouse, have a yellow fur. In order to investigate whether agouti has an effect on pigmentation independent from the MSH receptor, Ollmann et al. [4] crossed viable yellow mice (overexpressing agouti) with e/e mice. There was no effect of agouti in the absence of the MSH receptor on pigmentation. Furthermore, a phenotypic difference was found between the overexpression of agouti in lethal yellow (Ay/a) mice and the loss of MSH receptor function in recessive yellow (Mc1re/Mc1re) mice. The dominant agouti allele (viable yellow) had a cream colour, whereas the e/e mouse had a darker fur, irrespective of the presence or absence of the dominant agouti allele. The results indicate that a functional MSH receptor is required for the pigmentary effects of agouti, but also suggest that agouti protein can act as "an agonist" of the MSH receptor in a way that differs from α-MSH stimulation. To resolve at the genetic level whether agouti acts as an inverse agonist, the described experiment should be repeated in a mutant that has no endogenous agonist for the MSH receptor (thus a POMC $-/-$, provided that ACTH and MSH are the only endogenous agonists at the MSH receptor).

3. Agouti is not a pure competitive antagonist

In vitro experiments using B16 melanoma cells (which express MSH receptors) and a derivative thereof without functional MSH receptors (the G4F subline) demonstrated that an MSH receptor is essential for the effects of agouti on signal transduction in these cells [5]. Furthermore, it was demonstrated that agouti did not behave as a pure antagonist for MSH. Agouti affected cAMP levels, tyrosinase activity, as well as receptor down-regulation in the absence of MSH [6]. Recently, a more direct evidence demonstrating that agouti acts as an inverse agonist at the MSH receptor was obtained: basal adenylate

cyclase activity and melanin production were decreased by agouti in B16 melanoma cells [7]. Thus far, direct evidence that the wild-type MSH receptor displays constitutive receptor signalling is still lacking.

4. Cloning of AgRP

Ectopic overexpression of agouti, such as in viable yellow mice, does not only result in yellow pigmentation, but also in obesity [8]. In vitro experiments revealed that agouti is not only an "antagonist" at the MSH receptor, but also at the MC4 receptor [3]. Inhibition of MC4 receptor activity by agouti protein in the brains of viable yellow mice was suggested to underlie the obesity observed in these mice. Several groups then reasoned that a brain homologue of agouti should exist. Agouti is a conotoxin-like protein because it shares the spacing of 10 cysteine residues, which are important for the structure of this class of proteins. Based upon on the spacing of the cysteines in agouti, two groups, independently of one another, cloned an agouti homologue, called agouti-related protein (AgRP), from EST clones [9,10]. AgRP was indeed found to be expressed in brain exclusively in the arcuate nucleus of the hypothalamus. The agonists for MC receptors, the melanocortins, are also produced in this hypothalamic nucleus (products from the *POMC* gene). It was found that POMC and AgRP were expressed in distinct subsets of neurons in the arcuate nucleus. POMC and AgRP neuron fibers project side by side to other brain areas that express MC receptors [11]. It is likely that the activity of MC receptors is controlled by the activity of POMC and AgRP neurons. Although, in the literature, AgRP is mostly referred to as an antagonist for MSH, there is evidence that AgRP affects MC receptor activity independent of MSH [12].

5. AgRP is an inverse agonist

Because agouti does not act as a pure antagonist, and AgRP and POMC neurons project to the same brain areas, we investigated whether AgRP acts as an inverse agonist. B16G4F melanoma cells (which lack MSH receptor expression) were transfected with MC4 receptors and stable cell lines were identified with different MC4 receptor numbers. There was a clear relationship between the number of MC4 receptors expressed at the cell surface and spontaneous adenylate cyclase activity (Fig. 1A) [13]. AgRP was able to suppress this spontaneous signalling (Fig. 1B). We therefore concluded that AgRP acts as an inverse agonist at the MC4 receptor. This finding was confirmed by others [14].

6. MC4 receptor and obesity

We demonstrated that, at least in vitro, MC4 receptors display constitutive receptor activity. Thus, expression of an MC4 receptor on the cell surface already elicits a signal. Heterozygous mutations in the MC4 receptor have been associated with extreme obesity in humans [15,16]. It has been argued that these mutations result from haploinsufficiency

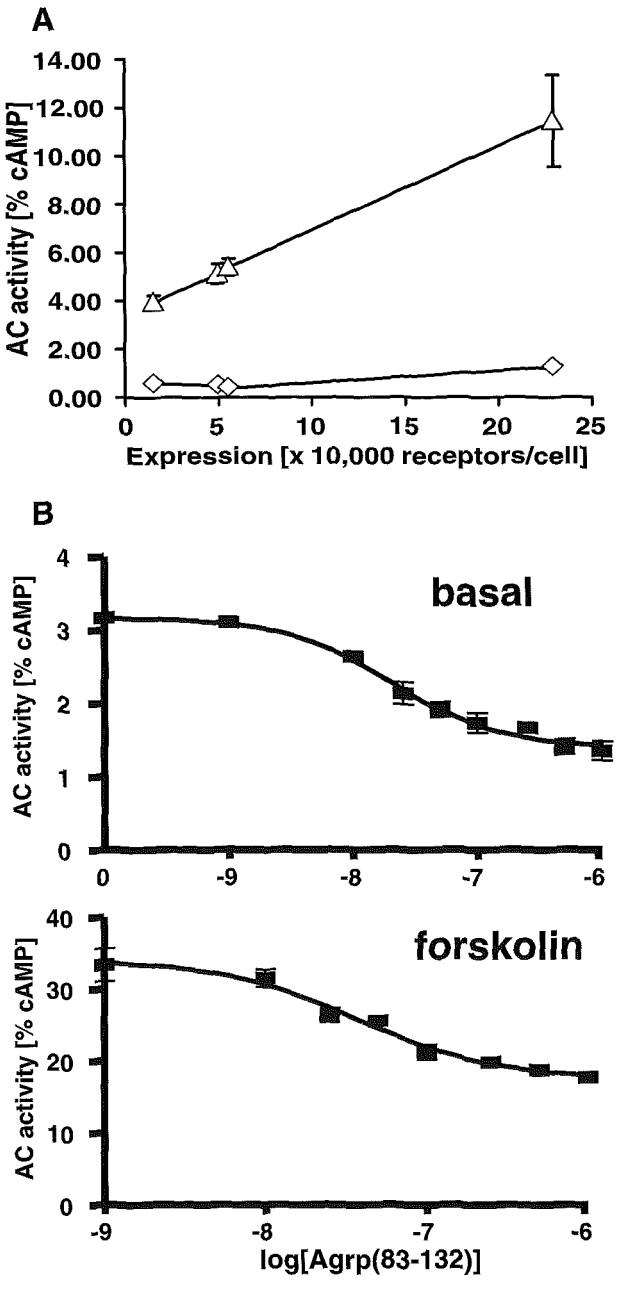


Fig. 1. (A) Human MC4 receptors display constitutive activity. B16G4F melanoma cells were transfected with the human MC4 receptor. Clones of cells expressing different levels of MC4 receptor were generated. Basal (◇) and forskolin-induced (△) adenylate cyclase activities were determined and plotted against the number of receptors (B_{max}). (B) AgRP acts as inverse agonist B16G4F cells expressing the human MC4 receptor were treated with AgRP (83-132). Basal and forskolin-induced adenylate cyclase activities were determined.

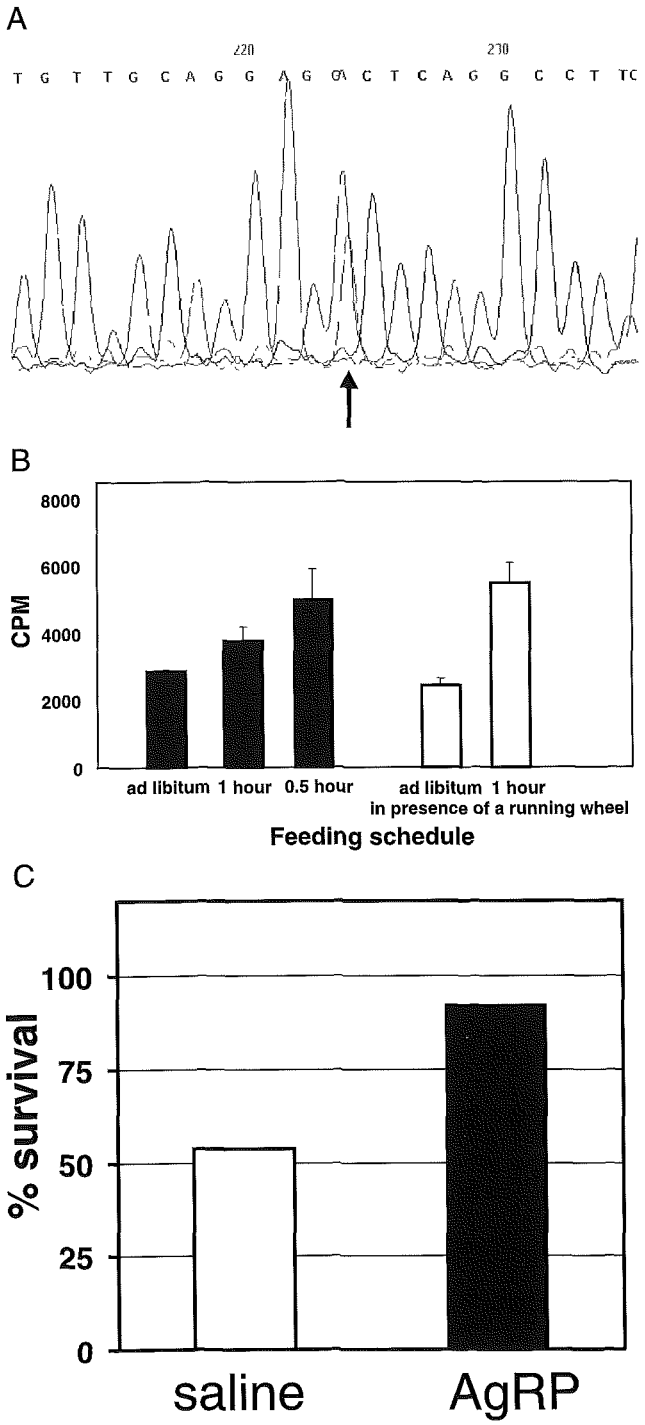
[17]. One may expect that a lower receptor number at the cell surface (due to haploinsufficiency) does not only result in a low efficacy of MSH, but also in a lesser degree of constitutive receptor signalling. Since decreased MC4 receptor activity results in increased food intake, this may explain the obesity observed in individuals carrying mutations in the MC4 receptor gene. Thus, modest changes in MC4 receptor activity, such as heterozygous MC4 receptor mutations in humans and rats, appear to be reflected in changes in body weight.

7. Association of an AgRP variant and anorexia nervosa

Would increased MC4 receptor activity be associated with decreased food intake and body weight loss such as those observed in anorexia nervosa?

The melanocortin system acts downstream of leptin [18]. Leptin is an adipose tissue-derived hormone. Plasma leptin levels are proportional to stored fats in adipose tissues. Leptin stimulates leptin receptors expressed in POMC neurons in the arcuate nucleus. Increased activity of POMC neurons results in the release of MSH in projection areas expressing MC4 receptors. Loss-of-function mutations in the genes encoding leptin, leptin receptor, POMC, or MC4 receptor all result in obesity in both rodents and humans. Leptin receptors are also expressed in AgRP neurons. However, leptin decreases the activity of AgRP neurons and AgRP suppresses MC4 receptor activity. During starvation, fats from adipose tissues are burned and plasma leptin levels drop. Subsequently, the activity of AgRP neurons increases and, due to release of AgRP, MC4 receptor activity decreases. This then contributes to an increased drive to eat.

During periods of starvation, one may expect that due to low leptin levels, AgRP neurons are active, resulting in increased food intake. Anorexia nervosa patients have very low leptin levels [19]. We reasoned that the loss of function of AgRP would result in inadequate suppression of MC4 receptor activity. We therefore screened the AgRP coding region for mutations. We found an AgRP variant that results in a nonconservative amino acid change (Ala67Thr AgRP). This AgRP variant was shown to occur more frequently in anorexia nervosa patients than in controls (Fig. 2A) [20]. Thus, we provided suggestive evidence for a decreased activity of an inverse agonist associated with anorexia nervosa. We found that rats exposed to an animal model for anorexia nervosa (the activity-based anorexia model) increased the density of MC receptors in an area that had been associated with food intake—the ventromedial hypothalamus (Fig. 2B) [21]. This was a surprising result since it indicated that during starvation, spontaneous receptor activity per cell would increase and these rats would become even more sensitive to the anorectic effects of MSH. Therefore, it would be essential to suppress this MC receptor activity with AgRP. We found that AgRP mRNA levels in the arcuate nucleus of rats exposed to the model were strongly increased. We then tested the efficacy of AgRP for its potential to suppress the anorectic behavior in rats exposed to the model. We found that the treatment of these rats with AgRP increased survival rate, suggesting that the endogenous increased AgRP levels were inadequate to suppress MC receptor activity (Fig. 2C) [21]. This further supports the idea that patients suffering from cachexia-induced anorexia and anorexia nervosa should be considered for treatment with MC4 receptor inverse agonists.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8. Putative role of AgRP as inverse agonist in vivo

Definite proof for in vivo relevance of inverse agonism of AgRP is still lacking. This would require the demonstration that MC4 receptors in vivo display constitutive receptor activity. The presence of endogenous agonist can only be excluded when experiments are performed in tissues derived from animals lacking endogenous agonist. Alternatively, pure selective and competitive MC4 receptor antagonists should be developed in order to investigate whether the effect of AgRP differs from that of a competitive antagonist. Another issue is that we cannot exclude that AgRP affects other signal transduction pathways than the cAMP pathway that we have studied. Thus, it remains difficult to prove the relevance of inverse agonism of AgRP in vivo.

9. A model for a general role of endogenous inverse agonists

We propose here a role for constitutive receptor activity and inverse agonists based upon in vitro experiments. When we coexpress the vasopressin-2 (V2) receptor with the MC4 receptor, we observed a synergistic effect of MSH on the vasopressin dose–response curve (Fig. 3). Furthermore, AgRP behaved as an antagonist when we coexpressed the V2 receptor with MC4 receptor. We also observed that the expression of MC4 receptor without addition of MC receptor ligands potentiated the effect of VP on the V2 receptor. We conclude that the constitutive activity of MC4 receptors lowers the threshold for V2 receptors to increase cAMP levels. Although this in vitro experiment may seem artificial, it is easy to understand the impact of this finding for the in vivo situation. Neurons integrate information from inputs of other neurons that project on them. These neurons contain different signalling molecules. Anorectic neuropeptides (such as MSH and CRF) generally stimulate cAMP levels, whereas orexigenic neuropeptides (such as NPY and MCH) are negatively coupled to cAMP via their respective receptors. Thus, when these receptors are coexpressed in a cell, the level of cAMP will be determined by the balance of activity of these receptors. However, since we found that MC4 receptor strongly affects the sensitivity of other signalling molecules, one can imagine that MC4 determines the set point for sensitivity for other (an)orexigenic neuropeptides. Subtle changes in the activity of this receptor, for instance due to mutations that affect receptor activity modestly, may thus have a strong impact on body weight in the long term. Indeed, MC4 receptor knockouts are more sensitive to the anorexigenic effect of corticotropin hormone than

Fig. 2. (A) Discovery of an (Ala67Thr)AgRP polymorphism. Sequence analysis of the AgRP coding region in anorexia nervosa patients revealed a (heterozygous) polymorphism that results in a nonconservative amino acid. (B) Negative energy balance increased MC receptor binding in the ventromedial hypothalamus. Brain slices from rats exposed to different starvation schedules (rats with ad libitum access to food, fed 1 or 1/2 h/day; or rats with running wheels, fed ad libitum or for 1 h/day) were incubated with [^{125}-I]NDP-MSH. Specific binding (cpm/area) was taken as a measure for receptor density in the ventromedial hypothalamus, an area associated with regulation of food intake. (C) Treatment of rats exposed to a starvation model with AgRP (83-132) increased survival rate. Rats, supplied with running wheels, were fed 1 h/day for 1 week. Groups of rats were treated either intracerebroventricularly with saline, or AgRP(83-132) via osmotic minipumps.

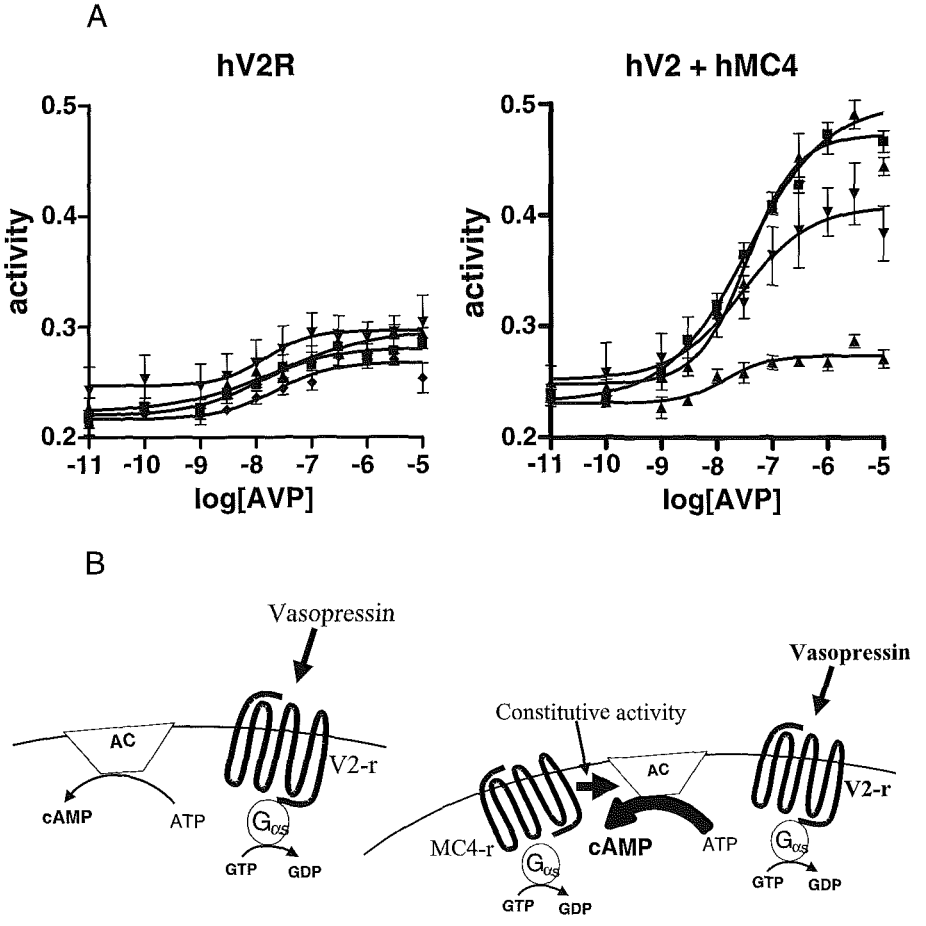

Fig. 3. (A) Constitutive activity of the MC4 receptor determines vasopressin responsivity. HEK 293 cells were transfected with either the vasopressin V2 receptor and a cAMP reporter gene (left panel), or cotransfected with the vasopressin V2 receptor, the human MC4 receptor, and a cAMP reporter gene (right panel). Cells were treated with vasopressin (AVP; ■: AVP alone): with or without AgRP (83-132) 1×10^{-9} M (▲), AgRP (83-132) 1×10^{-8} M (▼), AgRP (83-132) 2×10^{-7} M (♦). (B) Model depicting the increased cAMP response to vasopressin (represented by a thick arrow) when the human MC4 receptor (MC4-r) is present in cells expressing the V2 receptor (V2-r).

wild-type controls [22]. This supports that the MC4 receptor determines the sensitivity for other neuropeptides.

10. Concluding remarks

As far as we know, the melanocortin system is the only system in the brain where both endogenous agonists (melanocortins) and endogenous inverse agonists exist. From a

biological view, what explanations may be given for the presence of an endogenous inverse agonist? One is that the activity level of MC receptors is more important than that of other receptor systems. Therefore, the level of MC receptor activity is regulated by two separate populations of neurons expressing ligands with opposite activities.

We end by another intriguing explanation for the existence of endogenous inverse agonists. The fact that we know of AgRP is because of the cloning of agouti. Agouti was identified based upon the effect of the agouti locus on pigmentation. Of course, we cannot exclude that AgRP would have been found because of its effect on body weight. It is important to notice that we know about the inverse agonists (agouti and AgRP) because they affect phenotypes that are easily observed from the outside. Imagine that there would exist endogenous inverse agonists for the histamine H2 receptor, for the dopamine D2 receptor, or for the FSH receptor. How would we find these endogenous inverse agonists? Therefore, we propose to search for endogenous inverse agonists for those receptors that display a high degree of constitutive receptor activity. As far as we know, such a search has not been performed yet.

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Discussion 16

R. Leurs

I'm intrigued by the cross-talk of these two receptors. I have some data from our own laboratory showing cross-talk of different receptors at the level of constitutive activity. But you have tools that allow you to test this hypothesis in vivo as well. Can you test the effect of this inverse agonism? Perhaps you've done this, by modulation of food intake by CRH and similar systems.

R. Adan

We can do a lot of elegant experiments in vivo, but AgRP is also effective at the melanocortin-3 receptor, besides the melanocortin-4 receptor. The MC3 receptor shows a lesser degree of constitutive receptor activity. We need receptor subtype-selective neutral antagonists and inverse agonists to address the role of inverse agonism in vivo. I think another way to address it would be by putting in an electrode and performing electrophysiology. Because then if you would see a different response to a neutral antagonist as compared to an inverse agonist, you would have some evidence for constitutive signaling. In particular, we would be interested to expose rats to the activity based on anorexia model. We know that the brain melanocortin receptor density increases upon exposure to

this model, and then we should see a difference between AgRP and a neutral antagonist like SHU9119. But still you have the problem of the presence of endogenous agonists.

T. Schwartz

I totally agree with your conclusion that if we have a receptor with a decent degree of constitutive activity, it's very likely that there is some sort of endogenous regulation of that. We should look much more for those naturally occurring inverse agonists, and obviously most of our ways of looking are totally unable to see them. But one thing that will be interesting in your system is that you actually have these coreceptors, which means you can actually address the issue of specifically blocking the inverse agonist as opposed to the agonist.

R. Adan

The mutation we found in the AgRP is located in a domain that is binding to syndecan-3. Syndecan-3 acts as a kind of coreceptor because it binds AgRP with low affinity but it ensures that AgRP is close to the receptor. Currently, we obtained an expression vector for syndecan-3 in order to investigate whether the polymorphic variant we found has less affinity for syndecan-3.

T. Schwartz

You were coexpressing the receptor with a suppressant receptor to show that if you took the level up, then the other one would respond more, and so on. Could you say more specifically what you mean? And related to that, in order to see the MC constitutive activity, you always have to play the little trick with forskolin. Basically, if you just look at it, you cannot really see it unless you give forskolin. So, are these things related?

R. Adan

If we overexpress the receptor, we clearly can demonstrate the constitutive receptor activity, also without forskolin. But the V2 receptor, as far as I know in physiology, is never coexpressed with MC4. Now we are looking for other receptors normally coexpressed with MC4 to know whether we can see the same kind of things. Those experiments are on the way.

T. Schwartz

In your paper, the first one, you really had to give forskolin. Could you say something about what the mechanism is for that?

R. Adan

My interpretation would be that there are at least three binding sites at adenylate cyclase: a forskolin binding site, a binding site for α_s, and a binding site α_i. You enhance the sensitivity for G_s in one way or another by adding forskolin. That would be my interpretation, but I could well be wrong.

A. IJzerman

I should like to discuss the curve that showed the inhibitory effect of AgRP on forskolin-stimulated cAMP levels. One might argue that such effects are simply mediated by a G_i-coupled receptor.

R. Adan

We did also an experiment adding pertussis toxin and we observed that the efficacy of AgRP increased. So we had a higher potency of AgRP suppressing the forskolin effect.

A. IJzerman

And if you repeated the experiment in the presence of the competitive antagonist, SHU9119, would you see a rightward shift of the AgRP curve?

R. Adan

Yes, we've done that.

G. Milligan

With the mutations that you're observing in the MC4 receptor, even though they're quite rare, the ones that are associated with obesity. Is this quite similar to the story with the vasopressin receptor, where you can use pharmacological chaperonins to rescue the receptor? And is the SHU a small molecule that is membrane-permeant?

R. Adan

I think the take-home message for the pharmaceutical industry would be that if you're going to screen for agonists for the MC4 receptor in order to treat obesity, it's a very good idea to investigate whether this ligand stabilises the receptor at the cell surface because these mutations, as I showed, sometimes lead to only a 20% loss of receptor expression. And then these patients are heterozygous for these mutations. So a very subtle decrease in MC4 already is associated with obesity. So maybe it's even more important to make ligands that stabilise the receptor at the cell surface than agonism.

G. Milligan

In relation to the issue that came up about trying to screen for endogenous inverse agonists, I think one of the reasons that the industry is really failing to do this effectively is their love for the FLIPR. And the calcium mobilisation assay is really not well-suited for this type of screening. So it may just be a practical thing, where if we convince the guys who work for companies not to screen only in one way, then we may get the answers we are looking for.

P. Strange

In terms of understanding how inverse agonists work in this system, what do we know about the structure of AgRP and how it might interact with the receptor?

R. Adan

It has a conotoxin-like structure, thus it has 10 cysteines, and they form bridges in a very rigid structure. But we performed the side-directed mutagenesis with the MC4 receptor, in which by a single-point mutation at the top of transmembrane domain 6, we could change the pharmacological profile of the MC3 receptor into that of the MC4 receptor and vice versa. We had a few selective agonists that were selective for MC4, and we could flip over the selectivity by changing just one amino acid. And this was true for agonists but also for agouti. Thus, we could flip it also over for agouti. So the single amino acid is probably involved in agouti binding and in binding MSH in the same way, so there is at least overlap in the binding pocket.

T. Schwartz

It might be actually interesting that if mutants are causing obesity, 2% or 5% of children could be obese—that's a very significant amount.

R. Adan

Yes, it's the most frequent monogenic form of obesity we know.