Chapter 7

Interfering with neuropeptidergic systems; a comparison of strategies

INTERFERING WITH NEUROPEPTIDERGIC SYSTEMS; A COMPARISON OF STRATEGIES

The central regulation of energy balance is a complex process, composed of multiple interacting pathways. To increase the knowledge of systems and brain areas involved in the regulation of energy balance, various strategies have been used. Already in the fifties lesion studies revealed that the hypothalamus plays an important role in the control of body weight (2-4). However, not until 1994, when the leptin gene was cloned (376), knowledge about the neural systems involved in the central regulation of energy balance expanded. Since then, a lot of genes have been found to play a role in the regulation of energy homeostasis and an array of genetic obesity models has become available. In addition, to gain more insight in how and where neuropeptidergic systems control energy balance, administration of agonists or antagonists either ICV or local has been used. In this thesis, it was aimed to further clarify the contribution of MC and NPY signaling in distinct brain areas in the development of obesity.

Despite the enormous amount of information that has been obtained following the various strategies mentioned here, there are some drawbacks one has to keep in mind (Table 1), especially when investigating the local role of neuropeptides. Below, the most important advantages and disadvantages of the different strategies used in the exploration of energy balance are discussed.

Table 1: Characteristic of the various strategies used to unravel the regulation of energy balance.

	germline genetic	conditional genetic	pharm infusion	viral vector
certainty normal development	-	+/-	+	+
local interference	+/-	+/-	+/-	+/-
long-term interference	+	+	-	+
stable gene expression	+	+	-	+

GENETIC INTERFERENCE

Germline transgenic and knock out studies are widely used to gain information about the contribution of a gene to energy homeostasis. Indeed, the technique is ideal to investigate the overall function of a gene. In addition, long-term absence of a gene can be studied. Examples of genes that are deleted to study their function in the control of body weight are leptin, NPY and AgRP (152;235;376).

However, conventional knock out strategies result in the deletion of a gene in all cells. Besides the fact that this prevents the exploration of site-specific effects of a gene, it

also does not represent a normal physiological situation. Furthermore, alterations in gene expression due to genetic interference can have secondary effects. Leptin for example is required for a normal development of arcuate projection pathways (254;255). Mice lacking a functional gene encoding leptin or the leptin receptor have therefore an altered neural system, which can exaggerate the effects of reduced leptin signaling. Moreover, the brain shows a massive plasticity during development, which is shown by the fact that AgRP/NPY neurons can be ablated during development, while they are required for normal feeding in adult mice (155;156). Due to this plasticity, it is possible for other systems involved in energy balance to compensate for the loss of a gene in development. This can explain why $NPY^{-/-}$ mice and $AgRP^{-/-}$ mice are relatively normal, while alterations in NPY or MC signaling in adult wild-type mice does have obvious effects.

More or less the same problems are met in transgenic studies. Ectopic overexpression of agouti results in a specific obesity phenotype (139). Nevertheless, it is possible that a part of these symptoms is due to an altered development. Furthermore, since A^y mice overexpress agouti both centrally and peripherally, it cannot be determined where and how agouti acts in the development of obesity.

Besides, one has to keep in mind that the effects of genetic alterations depend on the background of the mouse strain used (241;242;377;378). The 129 strain for example is much less sensitive to obesity than the C57Bl/6J (241;242). As a result, varying or even opposite findings can be obtained when the function of a gene is analyzed on different backgrounds. Indeed, depending on the background, deletion of the Y2 receptor has been reported to either increase or decrease food intake and body weight gain (185;249).

An improvement of genetic interference came with the introduction of conditional knockouts, which are used to delete a gene in a particular organ, cell type, or stage of development. The most widely used method is the Cre-loxP recombinase system which is for instance used to generate mice with a hypothalamic-specific deletion of the Y2 receptor (249). It is also used to create $MC4^{-/-}$ mice in which MC4 expression can be rescued specifically in the PVN by crossing the mice with *Sim1*-Cre transgenic mice (379). Another example of a conditional knock out strategy is the use of the Tet-On system, which allows inducible regulation of a gene (380), and is used to study the blockage of NPY expression in the adult mouse (232). Nevertheless, also in these models developmental changes can not be excluded.

PHARMACOLOGICAL INTERFERENCE

In contrast to the genetic modification of a gene, pharmacological administration of neuropeptides does not interfere with development. Moreover, ligands can be injected locally in the brain, allowing the investigation of the role of specific areas in a neuropeptidergic system. However, although ICV infusions for one week are feasible, this is

not true for local infusions. Thus, also with a pharmacological approach it is not possible to explore the long-term contribution of neuropeptide signaling in a specific target site in energy balance, i.e. in the development of obesity.

AAV-MEDIATED INTERFERENCE

In the present experiments, recombinant adeno-associated (rAAV)-induced overexpression of neuropeptides was used to further understand their local role in the central regulation of energy balance. rAAV-mediated gene expression overcomes the major shortcomings of both genetical and pharmacological strategies. As shown in table 1, rAAV-mediated gene expression can result in a stable long-term expression of the transgene, in a local area, without altering developmental processes.

Also rAAV studies have some disadvantages. Firstly, it is questioned whether the observed effects cannot be attributed to neuronal transport of either the virus itself or the transcribed product in the infected neuron. However, no agouti staining could be observed in target areas of the PVN, as the brain stem, after injection of rAAV-agouti, which could indicate anterograde transport of the transcribed transgene (chapter 2). In addition, no viral gene expression was observed in cell bodies of the Arc, which could indicate retrograde transport of the virus. Therefore, we can assume that neuronal transport to areas outside the injection area did not contribute to our results. To be absolutely sure that the transgene is only expressed in the target area, one could choose to make use of an area-specific promoter, as for example the *Sim1* promoter for PVN specific expression.

Secondly, it is argued that injection of rAAV infects multiple subsets of neurons, including neurons that normally do not express the transgene. To bypass this issue, gene-specific promoters could be used.

COMBINED INTERFERENCE

Table 1 shows that the strategies have overlapping advantages and disadvantages. Obviously, it depends on the research question which of the approaches will result in the most clear-cut answers. Probably the best models to further investigate long-term, site specific effects of neuropeptides are a combination of genetic models and interference with rAAV. An example of such a combination is used by Coppari *et al*. They have reexpressed the leptin receptor of FLPe-reactivatable leptin receptor null mice by injecting rAAV-FLPe specifically in the Arc (381). This resulted in an improvement of hyperinsulinemia and a modest decrease in body weight (381). Nevertheless, these animals do lack the leptin receptor during development, which is known to alter projection pathways from the Arc (254;255). To investigate the role of a gene in a specific area, it would therefore be better to generate an inducible knockout, which has a normal development, and with the use of rAAV, locally delete the gene.

Another way to explore the role of decreased neuropeptide signaling in the brain is to use rAAV-mediated RNA interference (382). An advantage of this method is that the gene of interest will not be silenced completely, which might mimic better the genetic variation in the human population. Especially combined with gene-specific or site-specific promoters this would be an excellent manner to further unravel the mechanisms regulating energy balance.