Amylinergic control of food intake in lean and obese rodents

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Originally published at:
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Abstract

Obesity develops despite a complex and seemingly well orchestrated network that controls eating, energy expenditure and ultimately body weight; many of the involved signals are derived from the gastrointestinal tract. It is assumed that this network as an entity aims at maintaining body weight and body adiposity at a relatively constant level, but the control mechanisms seem to fail at least if an individual is chronically exposed to an oversupply of food. This article summarizes recent findings about the role of amylin in the control of eating in lean and obese rodents. The article gives some short background information about the well investigated adiposity and satiating signals leptin and cholecystokinin, respectively; this will provide the framework to discuss aspects of amylin physiology and pathophysiology in the control of eating in leanness and obesity. This discussion also involves the mechanisms mediating amylin’s eating inhibitory effect in the area postrema and the interactions between amylin and leptin. Further, we discuss the effect of high fat diets on amylin release and amylin action in lean and obese rats. The last part of this article raises the question whether amylin interacts with the reward system in the forebrain.
Amylinergic control of food intake in lean and obese rodents

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Keywords: amylin; satiation; adiposity; high-fat; hyperamylinemia; reward
ABSTRACT

Obesity develops despite a complex and seemingly well orchestrated network that controls eating, energy expenditure and ultimately body weight; many of the involved signals are derived from the gastrointestinal tract. It is assumed that this network as an entity aims at maintaining body weight and body adiposity at a relatively constant level, but the control mechanisms seem to fail at least if an individual is chronically exposed to an oversupply of food. This article summarizes recent findings about the role of amylin in the control of eating in lean and obese rodents. The article gives some short background information about the well investigated adiposity and satiating signals leptin and cholecystokinin, respectively; this will provide the framework to discuss aspects of amylin physiology and pathophysiology in the control of eating in leanness and obesity. This discussion also involves the mechanisms mediating amylin’s eating inhibitory effect in the area postrema and the interactions between amylin and leptin. Further, we discuss the effect of high fat diets on amylin release and amylin action in lean and obese rats. The last part of this article raises the question whether amylin interacts with the reward system in the forebrain.
Introduction

The maintenance of food intake and body weight is primarily controlled by a complex neural network comprising the hypothalamus and the hindbrain, which is also thought to be influenced by reward regions such as the nucleus accumbens and the ventral tegmental area. These brain nuclei not only communicate with each other, but they also receive and integrate neural and humoral information generated in the gut and other peripheral organs, which reflect the dynamic energy status and needs of the organism. Research from the past two decades suggests that while this system is well in balance to maintain energy homeostasis when an organism experiences daily moderate fluctuations in food intake, or even challenged by more severe circumstances such as occasional cycles of feast and famine, it is not designed to cope with prolonged periods of energy excess [1]. So, what exactly is happening in these networks, and in the periphery, when intake of highly palatable and energy–dense foods outweighs energy expenditure, resulting in obesity?

It is well-established that obesity leads to elevated circulating levels of adiposity signals, such as insulin and leptin, which in turn leads to insulin- and leptin-resistance. Increased levels of adiposity and of adiposity signals can also modulate the release and sensitivity to various gut hormones involved in the control of energy balance, including glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK), and amylin. More recent evidence suggests that obesity and exposure to foods high in fat may actually modify the wiring of this central network, potentially contributing to the insensitivity to many of these adiposity signals thought to prevent excess fat accumulation.

This review focuses on the specific role of amylin in this network. Various aspects of amylin release and sensitivity are discussed in the framework laid out by examples from the literature depicting how obesity or a high fat diet can modify central signaling of adipose and gut-derived hormones, in particular leptin...
Based on the background of leptin’s and CCK’s roles in this network, we summarize recent studies on amylin physiology and pathophysiology.

**Lessons learned from leptin**

Leptin, a hormone primarily produced and secreted by adipose tissue, produces its effects on food intake and body weight by acting on the long form leptin receptor (LRb), which is located throughout the central nervous system; notably, it is present in the hypothalamic arcuate nucleus (ARH), the lateral hypothalamic area (LHA), the ventromedial nucleus of the hypothalamus (VMH), the dorsomedial nucleus of the hypothalamus (DMH), the ventral tegmental area (VTA), and the nucleus of the solitary tract (NTS) [2-5]. A large number of publications indicate that leptin may influence eating and energy metabolism via these multiple brain sites (summarized in [6]) but the necessity or sufficiency of leptin receptors in these brain areas for leptin action is only incompletely understood. While the phenotype of many of these LRb-expressing neurons remains unclear, leptin receptors in the ARH are expressed by two distinct populations: pro-opiomelanocortin (POMC)-containing neurons and neuropeptide Y (NPY) and agouti-related peptide (AgRP)-containing neurons [7, 8]. Elevated levels of leptin stimulate POMC expression, activating the melanocortin network to inhibit food intake [9, 10]. In contrast, leptin inhibits the production of AgRP and NPY, which also reduces the drive the feed induced by these peptides [11].

Janus Kinase-2 (Jak2) is activated when leptin binds to LRb. Jak2 activation leads to autophosphorylation and to the phosphorylation of various tyrosine residues located on the intracellular domain of the LRb. The phosphorylation of these residues leads to the activation of several signaling pathways. One pathway involves the phosphorylation of signal transducer and activator of transcription-3 (STAT3), following which this transcription factor translocates into the nucleus to regulate gene expression. The presence of phosphorylated STAT3 (pSTAT3) is often used as a marker of LRb functionality, because at least in the ARH, STAT3 is required for leptin-induced POMC expression [12].

STAT3
also regulates suppressor-of-cytokine-signaling-3 (SOCS-3), which acts as a negative feedback signal to inhibit LRb activity. Leptin binding to LRb can also lead to the activation of signal transducer and activator of transcription-5 (STAT5) and extracellular signal-regulated kinase (ERK) signaling pathways, and leptin is known to inhibit AMP-activated protein kinase in both the ARH and the paraventricular nucleus of the hypothalamus (PVH), as well as in the NTS of the hindbrain [13, 14]. Except for the pSTAT3 and SOCS-3 pathways, the functional relevance of the other intracellular signaling systems is largely unknown [6].

**Obesity and leptin resistance**

At the time of its discovery, leptin was thought to potentially be the answer to the obesity epidemic. However, it was soon recognized that while obese Lep\textsuperscript{ob/ob} mice which lack leptin remain very sensitive to leptin and lose weight when treated with exogenous leptin, most obese humans and rodents are not deficient in leptin, and actually demonstrate elevated levels of the circulating hormone. It is now known that obesity-induced hyperleptinemia contributes to leptin resistance (summarized in [6]), which in turn renders exogenous leptin quite ineffective in treating common obesity. Leptin-induced leptin resistance may be due to increased SOCS-3 induction, shifting the balance between pSTAT3 activation (and hence increased signaling) and SOCS-3 induction (which limits leptin action). However, the exact mechanism by which leptin resistance occurs is not understood, and a reversal of this resistance could potentially be very beneficial in the treatment of obesity.

Leptin resistance is typically characterized by a lack of behavioral or cellular responses to exogenously administered leptin at doses that produce effects in lean animals. Thus rodents that do not demonstrate a decrease in food intake or body weight, or an activation of leptin signaling pathways (e.g. phosphorylation of STAT3) when injected with leptin are classified as leptin resistant. Proposed causes of leptin resistance include defective leptin receptor signaling, defective
neuronal signaling downstream of the LRb, and decreased leptin transport across the blood brain barrier (summarized in [6]).

Most rodent models of leptin-resistance depend on an obese state, which is typically achieved by maintaining the animals on a high-fat diet (HFD). Obese rodents first develop resistance to peripherally injected leptin, and with prolonged access to a HFD, resistance to centrally injected leptin also emerges [15]. Most mice and rats will gain adiposity when placed on a HFD containing 60% of calories from fat; interestingly, when maintained on a more moderate HFD (~30% calories from fat), some strains, notably Sprague-Dawley (SD) rats, will demonstrate a bimodal pattern of body weight gain which results in clearly distinct diet-induced obesity (DIO)-prone and –resistant (DR) subpopulations [16]. Experiments utilizing outbred rodents maintained on HFD can assess the role of the diet on changes in central networks controlling homeostasis. Additionally, experiments involving, for example, SD rats selectively-bred to be DIO or DR can assess underlying traits that predispose these animals to obesity prior to any access to a diet high in fat [17]. Use of both the outbred and selectively bred DIO and DR rodents has shed considerable light on how HFD, or the genetic predisposition to gain fat when fed a HFD, alter leptin sensitivity. To name just one example, adult DIO rats show reduced expression of LRb mRNA, decreased leptin binding, and demonstrate decreased STAT3 phosphorylation when being administered leptin [18–20].

Decreased leptin-induced pSTAT3 activation is typically used as a correlate for cellular leptin resistance. In general, any decrease in intracellular LRb signaling is part of cellular leptin resistance which also occurs in DIO. As already mentioned, increased SOCS-3 contributes to an overall attenuation of leptin signaling. Another intracellular event associated with reduced leptin action is an increase of protein tyrosine phosphatase 1B (PTP1B); the mechanism how leptin increases PTP1B is unknown but PTP1B, similar to SOCS-3, decreases JAK2 phosphorylation and subsequently LRb signaling [21]. Interestingly, both SOCS-3
and PTB1B seem to be upregulated by high-fat feeding which may at least in part explain the link between increased dietary fat and leptin resistance [22, 23].

It is still a matter of debate whether leptin reaches its target neurons directly or whether it needs to be transported across the blood-brain barrier. Such transport into the brain by a saturable, regulated transporter may in fact play a role in the development of leptin resistance [24]. DIO rats have a reduced ratio of cerebrospinal fluid (CSF) leptin versus serum leptin; hence, the amount of leptin that reaches the LRb may not be sufficient for leptin action in obese [25]. Studies showing that central injections of leptin can at least partly improve leptin sensitivity in DIO rats are in line with the idea that defective leptin transport represents one component in leptin resistance [15, 26]. Diets high in fat, which obviously contribute to DIO, or elevated triglycerides may also decrease leptin transport directly [27].

**Leptin’s effects on hypothalamic neuronal connection development**

Recent evidence suggests that changes in circulating leptin levels alter signaling in the adult rodent brain, and that consumption of a HFD can actually modify how leptin-sensitive networks of the ARH are organized [28-30]. Interestingly, changes in circulating leptin also play a role in the development of critical connections within the central network controlling energy homeostasis in rodent pup brains. By using DiI to label the developing axonal connections between leptin-sensitive ARH and other hypothalamic nuclei, Bouret and colleagues have elegantly shown that leptin is required for the normal development of neuronal connections between the ARH and PVH, the DMH, and the LHA [31]. Leptin-deficient Lep$^{ob/ob}$ mouse pups show a significant decrease in fiber density of ARH projections to these hypothalamic targets compared to their wild type littermates at various points of postnatal development. This decrease can be rescued, however, when neonatal pups are treated with exogenous leptin [31]. It is interesting to consider that despite this decrease in the outgrowth of leptin-sensitive ARH neurons, Lep$^{ob/ob}$ mice remain very responsive to the weight-
reducing effects of leptin as adults. Maintenance of leptin sensitivity in adult Lep\textsuperscript{ob/ob} mice suggests at least two possible explanations. This phenomenon can be potentially explained by the fact that not all leptin-sensitive pathways are defective in Lep\textsuperscript{ob/ob} mice, particularly projections from the PVH and DMH [31]; these additional leptin-sensitive projections may compensate for the lack of ARH projections in congenital leptin deficiency. Alternatively, this may question the relevance (or necessity) of these ARH to PVH projections for leptin's behavioral effects in general.

More recent data showed that the disruption in ARH projections is not limited to leptin-deficient or leptin-receptor deficient rodents [32]. Selectively bred DIO SD pups also show significant reductions in ARH to PVH projections [33]. This effect is further carried into adulthood, as these rats show decreased AgRP-immunoreactive fibers in the PVH, suggesting a decrease in NPY/AgRP activity in this ARH target site [33]. Because adult DIO rats have been described as having reduced LRb expression and leptin binding [18, 19], it is possible that these traits may contribute to the blunted development of these projections, and eventually to the low leptin sensitivity seen in these animals. Of note, it was recently shown that selectively bred DIO pups are not necessarily fated to become obese. DIO pups reared in large litters (16 vs. 10 pups/dam) are not only protected from becoming obese adults, but also show increased AgRP-immunoreactive fibers in the PVH and elevated leptin-receptor binding in the ARH [34]. These studies indicate that the development of the neuronal network that is involved in the control of eating seems to depend on prenatal, most likely genetic, factors, but also on the postnatal environment, potentially via epigenetic effects.

**Effects of diet and obesity on CCK**

In addition to modifying adiposity signals, like leptin, obesity reportedly alters the efficacy of satiating factors as well. Cholecystokinin (CCK), which is mainly produced in the duodenum and jejunum, and which is rapidly released in
response to local nutrients, is considered the prototypical satiation signal [35, 36]. CCK acts on vagal afferents, which project to the NTS in the hindbrain, and administration of CCK to humans and animals inhibits food intake by reducing meal size and duration [35, 37]. The idea that a diet high in fat might modify the effects of satiating hormones, particularly CCK, was first addressed by Covasa and Ritter [38]. They demonstrated that peripheral administration of CCK reduced food intake significantly less in rats adapted to a diet containing at least 34% fat by weight, than in rats maintained on a low fat diet (5% by weight). They also showed using isocaloric diets that this effect was specific to the increased ingestion of fat, and not just caused by a higher energy intake [38]. Interestingly, the HFD was also shown to reduce CCK-induced inhibition of gastric emptying in rats. This effect was also observed if rats on a low fat diet were infused intraduodenally with oleate [39], once again indicating a critical role of fat ingestion in the blockade of CCK’s effects. HFD-induced reduction in CCK sensitivity is also accompanied by decreased neuronal c-Fos expression in the NTS following CCK administration, supporting the hypothesis that HFD reduces vagal responsiveness to CCK [40].

**Amylin signaling**

The previous paragraphs shortly summarized reports on how the effectiveness of leptin and CCK may be affected by adiposity or by specific feeding situations, such as exposure to high-fat diets. Similar phenomena may also be relevant for other signals involved in the control of energy balance. Understanding these phenomena is important to develop effective and safe treatments for obesity. In the following, we therefore discuss newly elaborated aspects of amylin physiology and pathophysiology in the control of eating in leanness and obesity.

Amylin is secreted from the pancreatic β-cells in response to nutrient consumption. Amylin plays a critical role in the control of nutrient flux and postprandial glucose regulation, and has inhibitory effects on gastric acid secretion, gastric emptying, pancreatic glucagon secretion, digestive enzyme
secretion, and eating [reviewed in 41, 42]. The most investigated function of amylin is its role as a physiological signal of satiation. Peripheral amylin administration quickly and dose-dependently inhibits eating in rats, causing a decrease in meal size, while having no effect on the subsequent intermeal interval [43, 44]. The satiating effect of peripheral amylin seems to be mediated by neurons in the area postrema (AP), a circumventricular organ located in the caudal hindbrain. Lesions of the AP, but not blockade of neural afferents projecting to the brain, abolish amylin’s inhibitory effect on eating in rats [45, 46]. The role of the AP in mediating amylin’s actions seems not restricted to the eating inhibitory effect because amylin’s effect to inhibit gastric emptying is also absent in AP-lesioned rats [47]. Injection of the amylin antagonist, AC187, into the AP stimulates eating by an increase in meal size [48]. Furthermore, electrophysiological and immunohistochemical studies confirmed the direct influence of amylin on AP neurons [49-52]. Functional amylin receptors comprise a calcitonin receptor (CT-R) core and receptor-activity modifying proteins (RAMPs) [53, 54]. RAMPs regulate transport of core receptors to the cell surface, and are involved in ligand specificity of the receptor. All components of the amylin receptor, including the CT-R, RAMP1, and RAMP3, are expressed in the AP [55, 56].

While the exact signaling pathway of amylin is unclear, work from our laboratory provides evidence for the role of specific intracellular pathways in mediating amylin action [49, 57, 58]. Amylin, like other satiating hormones, induces a strong c-Fos response in distinct brain nuclei, including the AP, NTS and lateral parabrachial (PBl); however, it is important to mention that the functional relevance of c-Fos activation in all these cases is unknown [51, 59]. More recent data indicate that exogenous amylin administration induces both the accumulation of cyclic guanosine monophosphate (cGMP) and the phosphorylation of ERK (pERK) in the AP [49, 57, 58]; both may be involved in mediating effects of amylin on food intake, but the necessity of these events for amylin’s satiating properties is still being delineated.
The noradrenergic neurons also play a critical role in encoding amylin information from the AP to other regions of the hindbrain and the forebrain, as neurotoxic lesions of these neurons result in a reduction in the hypophagic effect of exogenous amylin [60]. Noradrenergic neurons in the AP send significant projections to the PBl, which potentially acts as a key relay station between the hindbrain and the hypothalamus [57, 61]. The PBl, which demonstrates strong amylin-induced c-Fos [51] and is required for the full induction of amylin’s anorectic effect [62], sends projections to the LHA, ARH, VMH, DMH, and the PVH [61]. Two other forebrain nuclei receiving projections from the PBl and activated by amylin administration include regions of the bed nuclei of the stria terminalis (BST) and the central nucleus of the amygdala (CEA) [51, 52, 61]. Induction of c-Fos in the CEA requires an intact PBl [63], however the involvement of the CEA or the BST in the behavioral effects of amylin remains undefined.

In addition to amylin’s role as a satiation factor, amylin meets the main criteria to be considered an adiposity signal. Similar to leptin and insulin, the basal circulating level of amylin correlates positively with adipose mass [64]; the basal plasma levels of amylin are higher in obese rodents, but amylin levels throughout the development of obesity have typically not been reported [64, 65]. Basal and glucose-stimulated plasma amylin levels are also elevated in obese humans [66, 67]. In principle, these findings support an association between body adiposity and plasma amylin. Future studies will have to test whether changes in body adiposity result directly in changing amylin levels, and whether adiposity and amylin levels follow the same temporal pattern (see [68] for further discussion).

Chronic amylin infusion reduces food intake and body weight, primarily caused by a decrease in spontaneous meal size [44], and chronic central amylin infusion lowers body weight in both under- and overweight rats [69], i.e. irrespective of prior manipulation of body weight. The decrease in body mass was specifically
due to a reduction in body adiposity. Overall, these experiments suggest that the central amylin level appears to be an important determinant for the attained level of body weight or body adiposity. Additionally, and in support of this hypothesis, chronic peripheral or central infusion of amylin antagonists increases body weight and body fat mass [70]. Finally, the amylin knockout mouse is heavier than wildtype controls [71], but it is not yet known whether amylin replacement reverses this phenotype, as would typically be expected.

Together, these data present amylin as a putative adiposity signal, and suggest that amylin secretion may also be altered by increased body adiposity. However, it remains unclear how adiposity modifies amylin sensitivity and function; further, it is unclear whether amylin’s role as satiating and as adiposity signals are processed differently by the brain [72].

**Amylin and leptin interactions**

There is a growing body of evidence supporting the existence of amylin and leptin interactions in the regulation of energy homeostasis. In response to acute administration, central leptin increased the acute eating-inhibitory effect of peripheral amylin in rats [73]. Similar observations were reported in more chronic experimental designs. While non-genetically obese rodents and humans are generally not sensitive to the weight-reducing effects of leptin, when leptin is co-administered with amylin, or its analog pramlintide, greater weight loss can be achieved than if either peptide were administered alone [74]. Amylin appeared to overcome the leptin insensitivity of obese rats and restore leptin sensitivity to normal. The body weight loss in rats that were pair-fed to the amylin-treated rats and that only received leptin was not more pronounced than in rats that were infused with amylin alone. In other words, additional exogenous amylin was necessary to increase the potency of leptin and to reduce the body weight gain in obese rats.

Further analysis demonstrated that the interaction between amylin and leptin to reduce body weight is synergistic, and not just additive [75]. Pair-fed control rats,
matched to rats treated with amylin and leptin for 28 days, demonstrated that the weight loss induced by the combination treatment was primarily a function of decreased food intake, though the combination treatment did yield a significantly greater percentage of fat mass loss than pair-feeding [75]. The study also revealed that leptin and amylin co-administration resulted in preferential utilization of fat as an energy substrate, and that decreased hepatic lipogenesis and increased lipid utilization may also underlie the weight loss effects [75].

While the mechanisms underlying amylin/leptin synergy are not entirely understood, it has been proposed that the two hormones share common neuronal pathways to generate such an effect on body weight [76]. Initial studies showed that while DIO rats did not mount a hypothalamic pSTAT3 response to peripherally administered leptin, pretreatment with amylin for seven days restored STAT3 phosphorylation in parts of the hypothalamus (e.g., the VMH), but not in the hindbrain [74]. It appears unlikely that the AP, amylin’s primary direct target region, is directly involved in the amylin/leptin interaction. After single injections, leptin did not enhance the amylin-induced activation of AP neurons as gauged by c-Fos expression [76]. Further, rats that were pretreated with amylin for one week showed increased pSTAT3 formation in the AP after acute leptin [74], but the effect was relatively weak and a very large dose of leptin was utilized in that study.

Based on our and other studies [74, 76], we therefore believe that the amylin/leptin interaction resides in the hypothalamus, presumably after polysynaptic input from the AP. Amylin increased the effect of leptin to induce pSTAT3 formation in the ARH in lean rats [76], and at least high doses of leptin resulted in increased pSTAT3 signaling in the VMH in obese, amylin-pretreated rats [74]. In other words, amylin restored the leptin-induced immunoreactivity of pSTAT3 in the VMH of obese rats to a level that is seen in leptin-treated lean rats. These results are consistent with data showing that acute amylin treatment upregulated leptin receptor expression about 3-fold in the rat hypothalamus [72]. Further, leptin binding as determined by receptor autoradiography in the rat brain
was increased in the ARH by combination treatment with amylin and leptin, and it was increased by amylin alone in the VMH and the DMH [76]. Finally, these results are also consistent with reduced leptin receptor expression in the mediobasal hypothalamus in amylin-deficient mice [76]. It is plausible that the effects of amylin on pSTAT3 expression and leptin receptor expression are causally linked and are part of a common neuromechanism, but this has not been investigated yet.

The finding that amylin and leptin may at least in part interact via the VMH is also interesting in the context of our previous studies that showed that amylin’s eating-inhibitory effect in rats is reduced by administration of the histamine H1 receptor antagonists pyrilamine or chlorpheniramine into the VMH [77]; further, the acute effects of both amylin and leptin on eating were blunted in H1 receptor-deficient mice [78, 79]. Hence, the potential role of histamine and of H1 receptors in the functional interaction of amylin and leptin in the VMH clearly deserves to be studied.

Interestingly, the synergistic effect of amylin and leptin appears to be dependent on the extent of obesity. The amylin and leptin synergy is not as evident nor as powerful in reducing body weight in severely obese rats (~800g) as compared to more mildly obese rats (~450g) [80], but amylin alone was still effective. In other words, amylin is not able to overcome extreme leptin resistance that may be associated with massive obesity. These findings have important clinical implications, suggesting that pharmacological treatments may differ for severely obese patients. Hence, it may be postulated that the synergy between amylin and leptin is most prominent within a designated range of body adiposity, but that amylin alone is still effective when higher pharmacological doses are administered chronically.

**Amylin sensitivity in the obese state**
Provided our growing understanding of how adiposity signals and satiating hormones, like leptin and CCK, are affected by obesity and the chronic intake of diets high in fat, and given the therapeutic potential of amylin-based therapies, investigations into how amylin sensitivity may be altered by obesity or by the intake of HFD are imperative. We recently tested the hypothesis that obesity reduces sensitivity to amylin. Furthermore, we evaluated if factors causing obesity (e.g., consumption of a high-fat or palatable diet) and factors associated with obesity (e.g., hyperamylinemia) lead to a change in amylin sensitivity, independently of obesity. Our results suggest that amylin sensitivity is actually quite dynamic, depending on factors such as diet macronutrient composition, diet palatability and metabolic state [81, 82]. In all cases, at least high doses of amylin still caused a significant effect on eating.

We first addressed if the acute anorexic response to amylin is altered in rats either maintained on a HFD to induce obesity or chronically food-restricted. Over the course of the fourteen-week study, sensitivity to acute amylin injection was determined by injecting vehicle or amylin (5, 20, or 50 μg/kg, s.c.) and monitoring food intake. We observed that when maintained on a 60% HFD (by calories), rats retained amylin sensitivity comparable to rats fed a standard chow diet for up to eleven weeks, at which point the significant amylin-induced reduction in food intake was lost. These data demonstrate that intake of HFD diet alone does not seem to alter amylin sensitivity; rather, the metabolic changes that occur following prolonged HFD intake appear to modify amylin’s efficacy. While it is not clear what accounts for the shift in amylin sensitivity following prolonged HFD, we did observe significant increases in visceral and subcutaneous adiposity in these rats, as well as increases in circulating leptin and amylin. Elevated amylin does not appear to be solely responsible for the shift (see below), however, since plasma metabolites (e.g., free fatty acids or triglycerides) were not measured during the HFD feeding period, it remains unclear whether a change in metabolites or combined changes in metabolite and hormone levels may contribute to reduced amylin sensitivity.
The third cohort of rats, which was food restricted to 80% of the standard chow group and provided their daily allotment of chow immediately following amylin administration, showed a strong resistance to amylin’s effects. However, if the food-restricted rats were pre-fed prior to administration of a high dose of amylin, sensitivity was restored and food intake was significantly reduced. This suggests that strong restriction-induced hunger signals can override amylin-induced satiation in an acute, short-term experiment. This observation is consistent with an earlier study showing that hypoglycemia can reduce the amylin-induced slowing of gastric emptying, suggesting that certain physiological states can override some effects of amylin [83].

These findings showing that amylin sensitivity seems shifted based on metabolic status are also interesting in the context of the reported amylin and leptin interactions; DIO rats maintained on a HFD for up to fourteen weeks, and food restricted rats, which have either very high or very low circulating leptin levels, respectively, were the least responsive to acute amylin administration. Thus, though amylin sensitivity is evident in even very obese rodents chronically administered with relatively high doses of amylin, it is possible that optimal amylin responsiveness occurs within a specific range of body adiposity and/or circulating plasma leptin levels.

We have also collected behavioral data suggesting that food palatability can alter amylin sensitivity, at least temporarily. When naïve rats are fed palatable chocolate liquid Ensure, they demonstrate resistance to central or peripheral amylin (5 pmol, i3v or 5 μg/kg, i.p.) within the first three days of Ensure access. However, if the rats are maintained on the Ensure for three weeks, or switched back to a chow diet, then amylin sensitivity was restored [81]. It is important to mention, however, that the intake of novel Ensure can be inhibited with higher doses of centrally administered amylin (10 or 100 pmol, i3v), suggesting that
Ensure does not cause amylin immunity, but rather causes a right-shift in the dose-response curve.

It has been reported previously that the chocolate flavor of Ensure plays a critical role in why rats overeat when it is offered [84]. We also found that the chocolate flavor of the Ensure seems to be a key element in our effect, because three-day feeding with neutral-flavored Ensure, which is nutritionally identical to chocolate-flavored Ensure, did not result in amylin resistance. These results suggest that the novelty of the palatable Ensure creates a strong signal to ingest that reduces the satiating effects of acute amylin; this alludes to the possibility that amylin’s effect on food intake can be influenced by the rewarding properties of food.

Finally, because rats maintained on 60% HFD demonstrated elevated circulating amylin levels in the study previously described, we addressed if hyperamylinemia is a contributor to decreased amylin sensitivity in obese rodents. It was recently shown that hyperleptinemia is required for the induction of leptin resistance in rats [85], and increased circulating levels of CCK have also been implicated in reduced sensitivity to CCK administration [86]. Increased circulating levels of amylin are present in obese rats and humans [64, 66, 67, 87], and previous studies suggest that high circulating levels of amylin also reduce amylin’s ability to slow gastric emptying [47]. Thus, we hypothesized that in rats, in which circulating baseline amylin was increased through chronic amylin administration, the sensitivity to acute exogenous amylin administration would be decreased. Furthermore, we hypothesized that the reduction in amylin sensitivity is proportional to the amylin concentration of chronically infused rats.

Hence, we chronically infused lean rats for one week with amylin to increase their baseline amylin concentration to levels typically observed in obesity (30-40 pmol/l versus 10-15 pmol/l in lean rats). We then challenged these rats with acute peripheral amylin and assessed subsequent food intake. Interestingly, and in contrast to our hypothesis, we observed that exogenous amylin administration
decreased food intake dose-dependently under all conditions; in other words, amylin acutely decreased eating to a similar extent in all rats, regardless of circulating amylin levels [82]. While chronic infusion of physiological doses of amylin did cause subtle reductions in food intake and body weight, the data suggest that the rapid rise in circulating amylin following an acute injection, which more closely mimics meal-induced amylin release, may be more critical for amylin’s effect on food intake, regardless of prevailing basal amylin levels. Thus, at least under our experimental conditions, we saw no decrease in amylin sensitivity by chronically elevated amylin concentrations. Downregulation of amylin receptors or a decrease in post-receptor signaling following hyperamylinemia therefore appears to be unlikely.

To summarize, we observed reduced amylin sensitivity under some but not all experimental conditions that, in the case of leptin, would typically be associated with leptin resistance. While there is still much to be investigated, we believe that the mechanisms underlying the known instances of amylin and leptin insensitivity are likely to be quite different. In the case of leptin resistance, central leptin receptor function is compromised, particularly in the ARH, the primary site of leptin action. In contrast, at least when gauged by c-Fos expression, amylin induced AP activation, where amylin targets CT-Rs, is similar between rats fed standard chow or those fed HFD demonstrating either a DIO or DR profile. Thus reduced amylin sensitivity may not be caused by direct changes in amylin receptor function. Future studies will have to investigate whether intracellular signaling systems like cGMP and pERK which seem to be involved in amylin action [49, 58], are affected by body adiposity or diet composition. Similar studies should also include other brain areas, which are activated subsequent to primary AP action; to mention just one example, it could be tested whether amylin-induced noradrenaline release, which seems to be involved in mediating amylin action [60], is reduced in obese rats or in rats exposed to specific diets.

Additionally, while decreased leptin transport across the blood brain barrier
appears to play a role in leptin resistance [25], amylin sensitivity is not likely regulated by BBB transport. Amylin crosses the BBB [88, 89], but the exact nature of this non-saturable transport or whether it is reduced in obesity is still unknown. However, because much more evidence supports the notion that amylin exerts its effects on food intake and metabolism via the AP, which lacks a functional BBB, a change in amylin transport in obesity would likely be less influential on amylin action, as compared with changes in leptin and insulin transport across the BBB, which can profoundly alter their action [71, 90].

Recent evidence suggests that hyperleptinemia is required for the development of leptin resistance [85], while the experiments discussed above show that lean rats, whose plasma amylin has been elevated and clamped to match that of obese rats, respond to acute amylin injections in a manner very similar to lean rats infused with saline [82]. Despite some potential differences in contributors to leptin and amylin insensitivity, it also should be noted that a decrease in amylin sensitivity may be related to changes in leptin sensitivity; namely, as suggested previously, that amylin controls energy homeostasis by engaging central networks downstream of the AP which are also employed by leptin. Thus, when leptin resistance leaves these networks latent, or if leptin levels are too low to activate these networks, amylin may perhaps not work as efficiently.

**Amylin secretion in the obese state**

In addition to assessing amylin sensitivity, we have also recently looked at how obesity or maintenance on a HFD might alter amylin release in response to nutrient consumption. While it is known that basal levels of amylin are elevated in obese humans and in various rodent models of obesity, yet to be described is whether the meal-contingent release of amylin is affected by diet composition and, more importantly, whether the release pattern differs between obese and non-obese animals. Furthermore, the amylin-to-insulin concentration ratio, which is approximately 0.01 in lean humans and rodents [91, 92], is also elevated in the
obese state, but it has not been tested if this ratio is also affected by the diet composition of a meal and if the ratio specifically of the meal-contingent release is different in obese rats. To address these issue, we measured levels of amylin and insulin in hepatic portal vein blood samples collected from outbred SD rats maintained for eight weeks on either standard chow or HFD and classified as either DIO or DR. We observed that both diet and obesity affect amylin release. DR rats on the HFD that actually weighed less than the DIO and chow-fed rats, showed significantly elevated basal amylin levels, compared to chow-fed rats. Both HFD-fed groups, DR and DIO, demonstrated an earlier meal-induced rise in amylin concentration compared to the chow-fed group. While insulin concentrations in response to a fixed-calorie meal were generally comparable between the groups, the slightly lower levels observed in the DR group did have an overall effect on the amylin:insulin ratio of the three groups. DR rats had significantly higher basal amylin:insulin concentration ratios than both DIO and chow-fed rats, and this ratio remained higher than the chow-fed rats during the meal. The differential meal-induced secretion patterns observed in DIO, DR, and chow-fed controls suggest that the ratio at which amylin and insulin are secreted during a meal is influenced by both body composition and diet composition (Boyle et al, unpublished observations).

Though the specific drivers of change in amylin:insulin ratio need further investigation, our results are interesting in light of a recent report demonstrating that elevated fatty acids, which can result following long-term consumption of a diet high in fat, induce enhanced mRNA expression and release of amylin, but not insulin [93]. Furthermore, while the clinical relevance of the amylin:insulin secretory ratio remains largely unexplored, reports have shown that an elevated ratio is associated with chronic hyperglycemia in rats and with increased glucose intolerance in obese humans [94, 95].

**Amylin as a trophic factor**
We have also recently collected evidence that, like leptin, amylin during perinatal development may influence the formation of neuronal connections involved in food intake [96], specifically the development of projections from the AP to the NTS. This phenomenon may be comparable to the effect of leptin in the hypothalamus [31, 33, 97]. Bouret and colleagues [31, 33, 97] showed that genetically leptin-deficient Lep\textsuperscript{ob/ob} mice and leptin-resistant DIO rats have deficient projections from the hypothalamic ARH to the PVH. An early postnatal surge of leptin secretion, that is lacking in Lep\textsuperscript{ob/ob} mice, may be required for the normal development of the brain [31, 33].

Using a similar methodology, we tested whether amylin may also be an important trophic factor for the normal development of the mouse brain, namely hindbrain projections from the AP to the NTS. In fact, genetically amylin-deficient mice had a markedly reduced density of AP-NTS projections compared with controls when tested on postnatal day 10 [96]. Further experiments are necessary; at present, it is not clear whether amylin replacement therapy, similar to leptin [31], restores the normal pattern of AP to NTS projections. Further, despite the neuroanatomical defect in the brain of early postnatal amylin-deficient mice, it is currently unknown whether these projections may develop later in life and whether they play a specific functional role in amylin’s inhibitory effect on eating. Because amylin-deficient mice eat less after exogenous amylin administration, and because exogenous amylin triggers c-Fos expression to a similar extent as in control mice, it is possible that some structural or functional changes compensate for the defective AP to NTS connections that we observed in early postnatal amylin-deficient mice. Also, it has yet to be explored if the propensity of rats to develop DIO, or pre- and postnatal nutrition will modify amylin’s effect on these developing circuits, as seems to be the case for leptin [33, 34].

How the reward system comes into play

A final point to consider when addressing how obesity and HFD may influence how the brain perceives and responds to gut and adiposity hormones controlling
food intake, is how the central reward system comes into play. The mesoaccumbens dopamine (DA) system, which comprises the dopaminergic pathway projecting from the ventral tegmental area (VTA) to the nucleus accumbens (ACB), has been widely investigated in regard to its role in various components of reward, addiction, and food intake [98]. Numerous reports also suggest that this pathway is altered by obesity and the ingestion of highly palatable, high-energy diets.

Extensive pharmacological studies have shown that the ACB contributes to multiple, dissociable components of feeding behavior that are dependent on the engagement of specific neurochemical networks within the nucleus ([99-104]. For instance, it has been suggested that DA transmission within the ACB is involved in stimulating food-seeking behaviors prior to interaction with the goal object (“wanting”), while an opioid network enhances the organism’s interaction with the food once it has been obtained (“liking”) [105]. Increased DA efflux in the ACB accompanies consumption of novel palatable food [106], and when rats are allowed intermittent access to sucrose pellets [107]; however, when given repeated or chronic access to palatable food, the effect disappears. Thus, while consumption of palatable food initially causes significant increases in ACB DA release, this response dissipates over time as the novelty of the food wanes [107]. Such effects are very interesting in respect to our experiments where rats were exposed to liquid Ensure (discussed below).

The state of obesity also appears to effect DA levels in the ACB. Decreased basal and chow-induced extracellular DA levels in the ACB have been observed in DIO rats, as compared to lean rats [108]. Further, brain slices obtained from DIO rats demonstrated decreased electrically-stimulated DA release, suggesting a presynaptic deficit resulting from either decreased DA synthesis in VTA neurons [109], or decreased DA release and increased clearance in the ACB [108].

An increasing amount of research supports the idea that the effects of obesity or palatable and high energy diets on the mesoaccumbens DA system is partially
caused by modulation of neurons of the VTA and ACB by the circulating hormones controlling energy homeostasis, e.g. by leptin. Small doses of leptin administered directly to the VTA resulted in a suppression in food intake that corresponds to the anorectic effect of systemic or intracerebroventricular leptin [110]. A portion of dopaminergic neurons in the VTA have been shown to not only express LRb receptors [111], but also increased pSTAT3 following leptin administration [110, 112]. A small percentage of pSTAT3-positive neurons in the VTA were also shown to project to the shell of the ACB, suggesting that leptin may directly modulate DA release from the VTA to the ACB [112]. Furthermore, reduced electrically-stimulated DA release was observed in ACB brain slices of leptin-deficient Lep^{ob/ob} mice [112]. While decreased DA levels in leptin-deficient mice might appear to first contrast to the similar data described above in DIO rats, which are hyperleptinemic, it is actually not surprising that both models of rodent obesity, which are related to impaired function of the leptin system, show disruptions in mesoaccumbens DA release [113, 114]. While the mechanisms by which leptin can influence the mesoaccumbens DA system are not fully delineated, the effects of this hormone on a pathway intimately involved in food reward and palatability are evident.

What about amylin? Although there is relatively little known about interactions between amylin and the reward pathways, enough data exist to advocate future studies in this direction. Most interesting are studies demonstrating that some of the highest densities of amylin binding sites are located in the ACB [115-117]. However, since there is no evidence of amylin synthesis or release in the ACB, the functionality of these receptors remains unknown. Of note, amylin is able to cross the blood brain barrier by specific non-saturable transport [88]. Hence, it is in principle possible that amylin may reach neurons at these brain sites and exert effects which may be independent of amylin’s action at its main primary target site in the AP.

Despite not knowing the physiological relevance of amylin binding in the ACB, it has been shown that amylin injected directly into the ACB produces a dose-
dependent reduction in food intake, which may be related to a more general diminished state of arousal or exploratory behavior [118]. Furthermore, while amylin is known to not cause taste aversion or sickness to produce its eating inhibitory effects [43, 119], it has been shown to decrease lever-pressing for milk in mice, suggesting that amylin decreases the motivation to obtain milk [120].

Finally, our recent data on the effect of amylin in rats that were exposed to a highly palatable liquid Ensure diet (see “Amylin sensitivity in the obese state”; [81]) may provide a direct link to reward related questions. In these experiments, amylin’s effect to reduce eating was markedly reduced in rats exposed to Ensure for few days; however, the effect was quickly reversible upon removal of Ensure, and the effect seemed to fade on more chronic exposure to Ensure. These findings provide interesting parallels to the studies showing increased DA efflux in the ACB on novel, but not on chronic exposure to palatable food [106, 107]. In other words, highly rewarding stimuli may produce a strong signal to the brain that cannot easily be overcome by amylin’s effect to acutely reduce eating, but that amylin is effective once this strong signal wanes. It would therefore be interesting to test whether acute blockade of the DA system in the ACB restores amylin’s effect to reduce eating even in rats with access to a novel, highly palatable stimulus.

Summary

Despite a complex and elaborate network that controls eating and energy metabolism, some of the components of this system appear to fail in obesity, i.e. when seemingly being needed most. Here, we discuss similarities and differences in the effects of amylin, leptin and CCK in obesity. We have to realize that at present, only very little is known about amylin pathophysiology in many of these respects. While leptin resistance, which is present in most cases of common obesity, has been traced back to defective transport systems for leptin across the blood brain barrier and to defects in LRb signaling, the exact reasons for a reduced sensitivity to the eating inhibitory effect of amylin are less clear.
Similar to leptin or CCK, chronic exposure to HFD seems to lower amylin sensitivity; however, whether reduced amylin receptor number or activity, or defects in intracellular signaling systems account for this effect, is unknown. Further, it is also not yet clear whether reduced sensitivity is specifically due to some dietary components rather than high energy content, as seems to be the case for CCK. Finally, amylin’s effect seems to be less prominent when rats are exposed to novel and highly palatable flavors, but the interactions between amylin and the (food) reward system require further studies.

In clear contrast to leptin, we showed that chronically elevated amylin levels do not seem to downregulate the sensitivity of the amylin signaling system because amylin effectively reduced eating despite prevailing hyperamylinemia, which was induced independently of obesity or exposure to specific HFDs. We also addressed the aspect whether obesity or the exposure to HFD influence the secretion of amylin; however, at least the meal-induced increase in plasma amylin was comparable between DIO and DR rats, or in rats on a HFD versus chow diet. Hence, our experiments did not provide evidence for reduced amylin secretion under such conditions.

Finally, we present recent findings on the trophic effects of leptin and amylin in their putative primary target areas. Similar to leptin, which acts as an important trophic factor for intra-hypothalamic neuronal projections, amylin may also be necessary for the proper development of brainstem pathways. However, the physiological relevance of this effect is unclear at present; in fact, this also seems to be the case for leptin because adult amylin-deficient mice react normally to exogenous amylin, just as leptin-deficient mice do to exogenous leptin. It has not yet been studied whether similar to leptin, amylin-sensitive pathways may also be affected in animal models of common obesity, such as the DIO rat.

This brings us to an important question. Does the amylinergic system “explain” the obesity epidemic? In other words, is overeating and subsequent obesity caused by a lack of amylin secretion or action? We believe that the vast majority of data would not support such a statement. Defects in meal-induced amylin
secretion are unlikely to occur, and reduced amylin sensitivity which has only been observed under specific experimental conditions, apparently can usually be overcome by higher amylin doses. Hence, the amylinergic system may be less sensitive under some conditions, but it is not insensitive. This is important for any potential use of amylin-based drugs for anti-obesity therapy.

Future studies will address in more detail the effect of specific dietary components and of obesity on the amylin secretory pathway, on amylin receptor components (CT-R; RAMP) and on amylin receptor signaling, i.e. the intracellular signaling systems mediating amylin's effects. Finally, because amylin-deficient mice show a normal response to exogenous amylin, the biological relevance of amylin's trophic effect on hindbrain neuronal projections also needs to be evaluated further.
ACKNOWLEDGEMENTS

The financial support of the Swiss National Science Foundation, Novartis Foundation for Medical and Biological Research, Ciba-Geigy Foundation, Vontobel Foundation, EMDO Foundation, Olga Mayenfisch Foundation and of the Zurich Center of Integrative Human Physiology are gratefully acknowledged.
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