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Blood-Brain Barrier and Feeding:

Regulatory Roles of Saturable Transport Systems for Ingestive Peptides

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Abstract

The two main ways for peptides in the peripheral body to enter the brain are by either saturable transport or passive diffusion across the blood-brain barrier (BBB). Saturable transport systems have the advantage of being responsive to physiological and pathological stimuli. Since saturable systems can regulate peptide entry into the brain, they have the potential to play controlling roles in feeding behavior. For therapeutic applications, however, saturable systems have the disadvantage of functioning as a threshold to limit access of large amounts of peptides into the brain. This pharmacological problem presumably would not be encountered for peptides crossing the BBB by passive diffusion, a process dependent on physicochemical properties. Thus, the gatekeeper function of the BBB can be expanded to a primary governing role, especially for entry of ingestive peptides subject to their respective saturable transport systems.

Keywords

blood-brain barrier; saturable transport; passive diffusion; urocortin; leptin; insulin; GALP; mahogany peptide

INTRODUCTION

The misguided dogma that peptides do not cross the blood-brain barrier (BBB) has finally been laid to rest. PubMed lists more than 4000 published papers dealing with the subject, and only a few of the ones from the early 1980's claim that peptides cannot penetrate the BBB.

This review, for the first time, organizes the relation of the ingestive peptides with the BBB into six categories: (1) peptides not entering the brain any faster than the vascular marker albumin; (2) peptides entering by passive diffusion without evidence of the self-inhibition characteristic of saturable transport; (3) peptides entering faster than albumin but only by perfusion in blood-free buffer; (4) peptides with saturable transport only evident when perfused in buffer; (5) peptides with saturable entry from blood; and (6) peptides with negligible entry in the basal state, but saturable transport from blood once activated. Those peptides with saturable blood-to-brain transport across the BBB are the most likely to play a pivotal role in feeding. This is summarized in Table 1.

NEGLECTIBLE ENTRY

There are several reasons why some peptides have negligible entry into the brain when measured by the highly sensitive method of multiple-time regression analysis (1). For this

method, the radioactively labeled peptide is injected as a bolus intravenously (iv), usually together with a differently labeled vascular marker such as albumin. Blood and brain from individual mice are obtained at several times in the period during which the peptide remains intact. Based on the exponential decay pattern of serum radioactivity, exposure time is calculated as the integral of serum radioactivity over time divided by the serum radioactivity at each experimental time. This represents the theoretical value correlated with each experimental time if the blood concentration of the peptide was constant. Thus the slope of the linear regression line reflects how fast the peptide permeates the BBB and the initial volume of distribution, the y-intercept, indicates the cerebral vascular volume of the peptide. As a further refinement, the radioactivity remaining in the cerebral vasculature is determined by the capillary depletion method with and without cardiac perfusion. To achieve effective separation of brain parenchyma from the microvasculature, brain cortical homogenate is thoroughly mixed with dextran to yield a final concentration of dextran of about 18.4 % and centrifuged with a swing bucket rotor [1]. This reflects the amount of the injected peptide that reaches the brain parenchyma.

Degradation and protein binding are the most obvious interfering factors to explain negligible entry by this method. For example, orexin B, unlike orexin A, is rapidly degraded in blood so that it cannot be detected in intact form in brain after injection in the peripheral circulation (2). A similar situation occurs for obestatin (3). Another potential confounding influence, not prevalent with ingestive peptides, is efflux. A peptide entering the brain could be rapidly transported out, so that the amount remaining in the brain would be negligible. This can be measured by a comparison of intracerebroventricular (icv) injection of a peptide with that of albumin, reflecting bulk flow resorption of cerebrospinal fluid (CSF) [841]. Although it might seem that use of an unlabeled peptide would be preferable in BBB studies, endogenous concentrations of the peptide would contribute a confounding effect, making interpretation difficult.

By contrast with orexin B and obestatin, adiponectin is very stable in blood. Although there is some controversy about its ability to enter the brain (4), neither our group nor that of Banks found it to significantly penetrate the BBB after iv injection (3;5).

It is difficult to conceive of much of a role of the BBB for the CNS effects of peptides in this category that don't cross the BBB.

ENTRY BY PASSIVE DIFFUSION

Most circulating peptides enter the brain by non-saturable passive diffusion. Differences in rates are usually ascribed to physicochemical properties such as lipophilicity, hydrogen bonding, conformation, and enzymatic activity (6). Differences in enzymatic activity at the BBB can occur in different parts of the brain, possibly explaining differences in regional permeation (7). Entry for the peptides in this category of non-saturable penetration is significantly faster than that of a vascular marker like albumin, but does not occur by leakage between the microvascular endothelial cells composing the BBB [1]. The term non-saturable means that permeation across the BBB by the radioactively labeled peptide is not affected by addition of an excess of the unlabeled peptide. As indicated, before a peptide can be considered to have crossed the BBB, it must be established that the radioactivity counted in the brain remains part of the peptide injected and that it reaches the brain parenchyma intact rather than being degraded or trapped in the cerebral vasculature (1).

Even when protein binding or aggregation decreases blood-to-brain entry, a peptide can still cross the BBB significantly faster than the vascular marker. This occurs with agouti-related protein (AgRP) (83-132) (8;9) and galanin-like peptide (GALP) (10).

Ingestive peptides entering the brain from blood by passive diffusion after iv injection include neuropeptide Y (11), orexin A (2), cocaine- and amphetamine-regulated transcript (CART) (12), α -melanocyte-stimulating hormone (MSH) (13-15), amylin (16), insulin-like growth factor (IGF)-1 (17-19), pituitary adenylate cyclase activating polypeptide (PACAP)-27 (20), cycloHis-Pro (21), glucagon-like peptide-1 (GLP-1, although the related exendin-4 may be slightly saturable at high doses) (22;23), peptide YY3-36 (24), GALP (10), nesfatin-1 (25;26), fibroblast growth factor (FGF)-21 (27), and the polypeptide growth hormone (GH) (28;29).

Even though less than 1% of an injected peptide enters the brain, it is sufficient to exert biological effects. This is illustrated by the ability of cycloHis-Pro to reverse ethanol induced narcosis after iv, intraperitoneal (ip), oral (po), or icv administration (21;30). Nevertheless, it would seem unlikely that the BBB plays an active role for peptides lacking a saturable transport system from blood to brain under physiological conditions. However, the situation with GALP, as discussed later, illustrates the dictum: beware of simple solutions.

ENTRY ONLY IN THE ABSENCE OF BLOOD

Under these circumstances peptides such as melanin-concentrating hormone (MCH), which essentially don't enter the brain from blood, are able to cross the BBB faster than albumin when infused in-situ in buffer (31). The negligible blood-to-brain transport of MCH is not explained by degradation, as occurs with orexin B and obestatin. Even though MCH aggregates as a trimer, after administration by in-situ perfusion in blood-free buffer it shows significant entry into brain, implying the existence of a substance interfering with its entry when injected into blood. For MCH, this probably is caused by protein binding, as confirmed by capillary zone electrophoresis [31].

In general, most peptides not showing blood-to-brain permeation of the BBB are routinely tested by in-situ perfusion so as to exclude the presence of interfering substances in blood as an explanation for the negligible entry. Although in-situ perfusion can give much information about BBB function, it is not physiological since blood is no longer present. The method entails clamping of the abdominal aorta and severing of the jugular veins of anesthetized mice before perfusion of the oxygenated blood-free buffer containing the radioactively labeled peptide and vascular marker at a rate of 2 ml/min for 5 min. There is a prewash to clear the cerebral vasculature of blood and a postwash to remove radioisotopes that had not entered the brain. The results are usually expressed as a brain/perfusate ratio of radioactivity per gram of brain.

SATURABLE ENTRY ONLY IN ABSENCE OF BLOOD

Some peptides entering the brain faster than albumin but without self-inhibition, may become saturable when interfering substances in blood are removed. This can be determined by the in-situ perfusion method just discussed (32). When perfused in blood-free media, but not evident after iv administration, adrenomedullin shows self-inhibition by excess unlabeled adrenomedullin (33). Saturable influx of GALP, also, occurs by in-situ perfusion but not by iv injection (10).

This situation of non-saturable entry from blood but saturable entry in buffer might occur with some of the other peptides mentioned in the preceding paragraphs of this review, but not all have been tested by the in-situ perfusion method. Of the peptides entering by passive diffusion from blood without saturability, NPY (11), orexin A (2), IGF-1 (18), CART (12), GLP-1 (22), exendin-4 (23), AgRP83-132 (8), nesfatin-1 (26), and GH (28) all were tested by in-situ perfusion. None of them showed self-inhibition, although entry was again faster than that of the vascular marker.

Under normal circumstances peptides crossing the BBB must deal with conditions in blood. It might not be expected therefore that peptides in this group, in which saturability is only evident after in-situ perfusion, would be actively involved in the physiological regulation of feeding. GALP, however, shows otherwise. Food deprivation for 24 or 48 h decreases the blood-to-brain permeation of GALP across the BBB, accompanied by decreased plasma concentrations of GALP at 48 h (10). It is conceivable that high basal levels of GALP in blood might have saturated a latent transport system which would only become evident after food deprivation decreased circulating GALP; since addition of unlabeled GALP did not affect the influx of GALP in fasted mice, this does not seem to provide a likely explanation for the altered BBB transport of iv injected GALP.

A saturable system across the BBB for GALP was observed by in-situ perfusion, and this occurred despite evidence by SDS-gel electrophoreses and size-exclusion chromatography for aggregation of GALP as a trimer, (10). Perhaps the protein binding in blood, shown by capillary zone electrophoresis, is the primary regulator of GALP permeation across the BBB, but then why did pretreatment with glucose increase the blood-to-brain transport of GALP without affecting its concentrations in blood? Although a full explanation is elusive, these findings proved predictive of the unexpected anorectic effect of GALP (34;35).

SATURABLE ENTRY FROM BLOOD

After injection iv as a bolus, several peptides involved in feeding behavior cross the BBB by saturable transport systems to enter the brain in intact form. These include insulin (36-38), pancreatic polypeptide (PP)(39), PACAP-38 (40), corticotropin-releasing hormone (CRH) (41), mahogany1377-1428 (42), human (but not mouse) ghrelin (3;43), and the polypeptide leptin (44;45). These have the potential to be causally involved in feeding.

The saturable system for the blood-to-brain transport of leptin, shown in-vivo (44) and in-vitro (45), can be modulated by several manipulations. For example, pretreatment with glucose increases and food deprivation decreases the transport of leptin into the brain (46;47). Leptin influx shows a diurnal variation partially saturated at physiological concentrations of circulating leptin (48). Banks et al. have shown that the impaired transport of leptin in obesity, with its elevated concentrations in blood, occurs even by in-situ perfusion (49;50).

Banks and colleagues also reported that triglycerides, abundant in milk, inhibit leptin transport in-vivo, in-vitro, and by in-situ perfusion {15111494: **LB: add to leptin ref mg and give me revised alphabetical list}. It is known that circulating glucose can alter the BBB transport of insulin [38], urocortin [67], and GALP [10], besides that of leptin [47], whereas the amino acid leucine, like leptin, can increase the hypothalamic mammalian target of rapamycin (mTOR) signaling to decrease food intake and body weight {16690869}. Thus, altered concentrations of circulating triglycerides, glucose, amino acids and probably other metabolic fuels in obese subjects could further contribute to the leptin resistance of obesity as well as the regulation of food intake in general

The short form of the leptin gene (ObRa) has been considered the main transporter of leptin across the BBB, as shown in studies of rodents lacking this gene {1782, 1781}. Nevertheless, there is persistent, but reduced, blood-to-brain influx of leptin in Koletsky rats that lack ObRa [51], and in-situ brain perfusion studies have elegantly shown that their transport system for leptin remains intact, although there is partial saturation of the transporter resulting from obesity and its associated high blood concentrations of leptin [52]. In ob/ob mice lacking leptin and db/db mice lacking the signaling peptide ObRb, two forms of genetically induced obesity, normal saturable transport of leptin across the BBB persists (51). More recently, the endocytosis functions of ObRc and ObRd have been shown in cellular studies after overexpression of these receptor isoforms (52). After receptor-mediated endocytosis, leptin

can surprisingly remain intact intracellularly for at least 1 h. This illustrates sequestration of internalized leptin by intracellular organelles, perhaps serving a function to prolong the biological activity of leptin. Regardless, the relationship between intracellular signaling by leptin receptors and its impact on leptin trafficking has not been fully understood.

Differing from the membrane-bound forms of ObRs, ObRe and other soluble receptors generated by membrane shedding may exert antagonistic functions. This at least occurs in the BBB transport of leptin, where the soluble receptors can reduce the endocytosis of leptin by cultured cells, and decrease the BBB permeation of leptin in mice {2405}. In blood, the soluble receptors may serve as binding proteins to prolong the half-life of leptin {L157; L159}.

In addition to the inhibition of leptin transport by metabolic disturbance (e.g., hyperlipidemia with elevated triglyceride) {15111494}, obesity itself (increased body weight and adiposity) elevates blood leptin concentrations and partially saturates the BBB transport. This has been shown by a careful examination of partial saturation of leptin transport at physiological concentrations by in-situ brain perfusion {763}. It also should be recognized that obesity and leptin concentrations may not be the ultimate decisive factors; rats bred to be obesity-prone can show defects in central leptin signaling at a young age (preceding the onset of obesity) even before the BBB transport of leptin is affected {2251}.

Neuroendocrine changes may also affect leptin transport. Although adrenalectomy does not affect leptin transport [46], ovariectomy reduces leptin entry from blood to the brain (53). At 5 weeks or more after ovariectomy of mice, a model of menopause, the amount of leptin crossing the BBB to reach the brain is greatly reduced, perhaps contributing to the increased body weight seen in this condition.

Like leptin, most cytokines are polypeptides that inhibit feeding. As seen with peptides (<100 amino acids), some of these polypeptides (100-200 amino acids) do not cross the BBB, some cross by passive diffusion (some being saturable by in-situ perfusion), and some enter the brain by saturable transport. This is reviewed elsewhere (54-57). We have used two of these anorectic cytokines that cross by saturable transport to explore the intriguing and largely unexplored question of how a peptide or polypeptide can cross the cells of the BBB in intact form without being degraded (58-61).

It must be emphasized that the arcuate nucleus of the hypothalamus, the main site of leptin's ingestive actions, lies within the BBB. The convincing anatomical evidence for this is summarized elsewhere {k818; bbb142}.

NEGLIGIBLE BASAL ENTRY, SATURABLE AFTER ACTIVATION

So far, urocortin is the only peptide found to require activation for substantial blood-to-brain permeation of the BBB. Its relation with the brain has been recently reviewed (62). A very potent anorectic peptide, urocortin appears to have a latent saturable transport system that can be activated by leptin (63), tumor necrosis factor (TNF) α (64), or pretreatment with glucose (65). Just as it would be reasonable for a fuel such as glucose to increase the BBB transport of a satiety peptide such as urocortin, it is not surprising that urocortin transport is inhibited by peripheral pretreatment with insulin, which reduces blood glucose (65).

The specific receptor for urocortin, CRH2, mediates the activating effect of leptin in-vivo (64) and in-vitro (66). However, both CRH1 as well as CRH2 are involved in the trafficking and signaling of urocortin (67). The activating effect of TNF α on urocortin transport is mediated by both the p55 and p75 TNF α receptors (64).

Urocortin, therefore, provides the first example of how transport of one small protein across the BBB can be regulated by another small protein involved in feeding behavior. While normal blood concentrations of leptin do not seem to facilitate urocortin influx across the BBB, higher levels such as would be seen in obesity, stimulate urocortin transport. Since leptin transport is already partially saturated in obesity (49;50), so that the effectiveness of leptin in decreasing feeding is limited, activation of the transport of another anorectic peptide would be a more efficient mechanism to decrease feeding. This probably represents a physiological adaptation for greater suppression of feeding behavior in obesity and highlights the potential regulatory function of the BBB.

CONCLUSION

The BBB may play a primary role in the regulation of feeding. Although this is most evident for peptides with a saturable blood-to-brain transport system across the BBB, it may also occur in other situations, as discussed in this review.

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Table 1**Peptides and the BBB**

<u>Negligible brain entry</u>	<u>Orexin B</u> <u>Obestatin</u> <u>Adiponectin</u> <u>MCH</u>
<u>Entry by passive diffusion</u>	<u>AgRP</u> <u>NPY</u> <u>Orexin A</u> <u>CART</u> <u>α-MSH</u> <u>Amylin</u> <u>IGF-1</u> <u>PACAP-27</u> <u>cHis-Pro</u> <u>GLP-1</u> <u>PYY3-36</u> <u>Nesfatin</u> <u>FGF-21</u> <u>GH</u>
<u>Entry only in absence of blood</u>	<u>MCH</u>
<u>Saturable entry only in absence of blood</u>	<u>Adrenomedullin</u> <u>GALP</u>
<u>Saturable entry from blood</u>	<u>Insulin</u> <u>PP</u> <u>CRH</u> <u>Mahogany</u> <u>hGhrelin</u> <u>Leptin</u>
<u>Negligible basal entry, saturable after activation</u>	<u>Urocortin</u>