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Peptides and the blood–brain barrier

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Abstract

The demonstration that peptides and regulatory proteins can cross the blood–brain barrier (BBB) is one of the major contributions of Dr. Abba J. Kastin. He was the first to propose that peptides could cross the BBB, the first to show that an endogenous peptide did so, and the first to describe a saturable transport system at the BBB for peptides. His work shows that in crossing the BBB, peptides and regulatory proteins act as informational molecules, informing the brain of peripheral events. Brain-to-blood passage helps to control levels of peptides with the brain and can deliver information in the brain-to-blood direction. He showed that the transporters for peptides and proteins are not static, but respond to developmental and physiological changes and are affected by disease states. As such, the BBB is adaptive to the needs of the CNS, but when that adaption goes awry, the BBB can be a cause of disease. The mechanisms by which peptides and proteins cross the BBB offer opportunities for drug delivery of these substances or their analogs to the brain in the treatment of diseases of the central nervous system.

Keywords

Peptides; Blood–brain barrier; AJ Kastin; Delta sleep-inducing peptide; Regulatory proteins; Cytokines; Leptin

How do peripherally administered peptides affect behavior? Can peptides circulating in the blood stream impact brain function independently of the vagus and other components of the afferent nervous system? Can peptides cross the blood–brain barrier (BBB)?

These and related questions were being asked by Abba Kastin when I joined his lab 35 years ago. They were tough questions without obvious approaches to answer them. And they were controversial, especially the idea that peptides could cross the BBB.

Peptides and the blood–brain barrier: the first ten years

That the newly discovered class of substances termed peptides could have profound effects on behavior was beyond question. Abba's mentor, Andrew Schally, had won the Nobel Prize just the year before for showing that the hypothalamic factors that controlled pituitary functions were peptides [48]. Abba had been instrumental in showing that the release of

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thyroid stimulating hormone (TSH) from the pituitary was controlled by the hypothalamic secretion of thyrotropin releasing hormone [1]. TSH, in turn, controlled the thyroid's release of thyroxine, a hormone whose excess or absence had powerful effects on behavior. But Abba's work was showing that hypothalamic and pituitary hormones had effects on behavior not likely mediated through the hypothalamic-pituitary-end organ axes. This was shown elegantly in experiments conducted in hypophysectomized animals and also with hypothalamic peptide fragments that did not induce pituitary secretions, but were nonetheless behaviorally active [32].

But if peptides had extra-pituitary effects [37], how could they mediate those effects? Abba was the first to suggest that peptides could cross the BBB [35,36,47] and the first to attempt experiments to determine whether they could or could not cross the BBB.

Why it was assumed so widely among both BBB experts and non-experts that peptides could not cross the BBB and why the suggestion that they might cross was met with severe skepticism is still puzzling to me, even after all these years. Maybe it was because it was clearly established that large proteins as typified by albumin did not cross the BBB. It may have been reasoned that since proteins are composed of amino acids and do not cross the BBB, then peptides must not be able to cross the BBB either, since they are also composed of amino acids. That such reasoning was flawed should have been evident in that it was already proven false in another case: proteins played largely structural roles (note: this era predated the discovery of “regulatory proteins”), whereas peptides had regulatory effects. It may also have been that the term “barrier” was taken too literally for a tissue that is more properly viewed as a regulatory interface between the blood stream and CNS; Abba was to later highlight the dangers of reification in the discovery of the functions of peptides [33,38]. To this day, newly discovered classes of substances (e.g., cytokines and other regulatory proteins, antisense molecules) are assumed to not be able to cross “the barrier”. Whatever the reasoning, for at least two decades after Abba first proposed that peptides could cross the BBB, it remained an untested assumption by many that they could not cross, with many discussion sections in papers on the behavioral effects of peptides concluding that while the mechanism by which the peptide was affecting the CNS was unknown, at least it could be assumed that they could not cross the BBB to exert the effects.

The question of peptide penetration: major technical challenges

Another factor that slowed the progress of the BBB-peptide field was a lack of established methods for examining this question. Oldendorf had recently introduced his brain uptake index (BUI) method that revolutionized the study of the BBB [40]. It allowed the brain's uptake of amino acids, glucose, and many other substances to be quantified, compared, and categorized [40–42]. With this method, Oldendorf and colleagues demonstrated that amino acids were transported across the BBB but by transporters that were specific for categories (e.g., the large neutral amino acids) and that glucose was rapidly transported by a system that also carried other hexoses. Oldendorf and colleagues also showed that morphine, methodone, codeine, and heroine all crossed the BBB in proportion to their lipid solubilities [43]. However, the BUI was not very sensitive; it was only useful for substances that had very large rates of uptake across the BBB. It could, for example, easily detect the uptake of

methadone, codeine, and heroine, but not of morphine. When applied to peptides, the BUI could not reliably detect their uptake by brain [43]. Therefore, if peptides did cross the BBB, they did not do so in large amounts.

Another technical problem was that peptides have very short half-lives in the blood stream. This meant that if they entered the brain, they must do so rapidly. This presented both a conceptual dilemma (how could they affect behavior for hours if they were cleared from the blood after a few minutes?) and a technical one: any method for detection needed to be done over a short time course and to be able to distinguish intact peptide from degradation products.

A low entry rate (at best) and a short time available to enter greatly favored the idea that peptides did not cross the BBB, at least not in amounts sufficient to affect brain function. But other work countered the assumptions that these findings depended on [38]. First, it was clear that peptides were very potent and that not much peptide would need to cross the BBB to exert effects on brain. In this sense, peptides were similar to morphine in that morphine had very profound effects on the CNS, yet so little crossed that it could not be detected by the BUI method. Second, a peptide's effects could last for hours or even days after its administration, long after it was cleared from the blood stream. Peptides then challenged long-held assumptions about how substances injected into the blood stream could affect the brain [38].

The first challenge in determining whether peptides could cross the BBB was to find methods that could address the technical difficulties in studying peptides. There were only about a dozen studies on peptides and the BBB with about half of those concluding that they could cross and about half that they could not. However, all these studies were flawed in the sense that they had alternative explanations from their conclusions. For example, those studies that concluded that peptides did not cross did not use very sensitive methods. Those studies that did conclude that peptides could cross often used a sensitive approach, such as radioactively labeled peptides, but did not show that the radioactivity in the brain exceeded levels explicable by the vascular space of the brain or that the radioactive label was still attached to the peptide.

Technical challenges and delta sleep-inducing peptide

At this point in the evolution of the field, there were basically two dichotomous choices: the first, to choose to study uptake into brain tissue or uptake into cerebrospinal fluid (CSF); the second, to use radioactively labeled peptides or to follow immunoactivity with radioimmunoassays. Each of these choices had advantages and disadvantages. The main advantage of using radioactivity was its great sensitivity, but its main disadvantage was that the radioactive label could become detached from the peptide so that one was no longer assessing peptide penetration. We overcame this difficulty by using column chromatography to confirm that the radioactivity taken up into brain tissue or CSF was still attached to the peptide. The disadvantage of radioimmunoassay was that an immunoactive fragment might be crossing the BBB rather than the intact peptide. However, the advantage of our radioimmunoassay for delta sleep-inducing peptide (DSIP) was that it required eight of the nine amino acids for cross reactivity and we could easily determine with column

chromatography whether it was the nonapeptide or the octapeptide that was crossing the BBB. A major advantage of CSF was that any material recovered from the CSF had clearly crossed the BBB (vascular contamination from “bloody” taps is readily assessed by a variety of methods), but the main disadvantage at that time was that some questioned how relevant CSF levels were to levels at the brain receptor. The main advantage of using brain tissue was that it was not subjected to the “relevancy” question as was CSF. The main disadvantage of using brain tissue is that its vascular space will contain peptide that has not crossed the BBB but will contaminate the tissue upon homogenization. We accounted for the vascular contribution in two ways, either by injecting a vascular marker (e.g., radioactive albumin or inulin) that allowed us to compute the contribution of vascular contamination in the brain tissue or by washing out the vascular space of the brain prior to assessment.

With these options and incorporating these solutions, we performed all possible combinations of studies: radioimmunoassay using brain tissue, radioactivity using brain tissue, radioimmunoassay using CSF, radioactivity using CSF [4,12,31,34]. In all cases, we found intact peptide entering the CNS in excess of vascular markers. This clearly demonstrated that the small peptide DSIP could cross the BBB.

We next asked the question of how DSIP could cross the BBB. We were fortunate in that we had several analogs of DSIP whose immunoactive or radioactive forms could be readily distinguished with the aid of column chromatography. We found that whatever peptide was injected into the blood was the peptide that was recovered from the CNS [12,31,34]. This, along with the results using radioactivity, ruled out the possibility that peptide appearing in the CNS originated there, having been stimulated by the increased levels in the blood. As neither the analogs nor the radioactive peptides were produced endogenously, their detection in the CSF after intravenous administration could have only occurred by passage across the BBB.

The work with DSIP and its analogs showed the surprising finding that not all the peptides crossed to the same degree. This meant that their entry could not be easily explained by residual leakiness of the BBB. We were able to show that protein binding affected the ability of the various DSIP-related peptides to enter the CSF with mostly the unbound fraction available for passage [12]. In later studies, we showed that uptake of DSIP peptides into the CSF correlated with their lipid solubility [14] and that uptake into brain was by a non-saturable mechanism [13]. We concluded that the DSIP peptides crossed the BBB by the non-saturable mechanism of transcellular diffusion.

Examination of other peptides

The next question posed was whether other peptides could cross the BBB. We examined 18 radioactively labeled peptides including the analog N-Tyr-DSIP, using radioactive erythrocytes and albumin as vascular markers [7]. We found that 10 peptides had an uptake that was statistically greater than either of the vascular makers. Therefore, BBB permeability to peptides seemed to occur for many peptides and was not limited to DSIP. However, the peptides, like the DSIP analogs, did not all cross to the same degree. We, therefore, determined the effects of molecular weight, lipid solubility, protein binding, and several aspects of charge on the degree to which the 18 peptides could cross the BBB. For the

majority of peptides, lipid solubility correlated with the degree of peptide penetration. Thus, transcellular diffusion seemed to be a mechanism by which many peptides could cross the BBB. This was later confirmed by work from other laboratories, most notably that of TP Davis, who showed that analogs of opiate peptides that were more lipid soluble crossed the BBB better and were more behaviorally active [49,50].

Peptide transport system-1: the first BBB transporter for peptides

There were 4 peptides of the 18 whose entry into brain was much less than predicted by the physicochemical characteristics [7]. These were all small (4–5 amino acid residue) peptides with an N-terminal tyrosine. Eventually, we established that these were all ligands for a brain-to-blood transporter [5], which we named peptide transport system-1 (PTS-1). Work with Abba eventually described six more PTSs (see Table 1), including PTS-3, the first blood-to-brain peptide transporter.

PTS-1 was used to describe a number of features that have since been shown to be generic to many BBB transporters, especially those that have peptides or regulatory proteins as their ligands [10]. First, they usually have as ligands a small number of compounds that have some a similarity or theme to their structure. As already noted, for PTS-1, this is a small (4–5 amino acid resides) peptide with an N-terminal tyrosine. Second, the transporters are not static but adapt to various conditions or are impacted by disease states. PTS-1, for example, had an activity rate that was altered by aging [6] and in mice physically dependent to alcohol [9]. Third, transporters are modulated or regulated by other substances. For example, leucine acts as an uncompetitive inhibitor of PTS-1 [8], whereas aluminum is a noncompetitive inhibitor [16]. PTS-1 was unidirectional, transporting its ligands in the brain-to-blood direction [23], but not in the blood-to-brain direction, whereas other peptide transporters, such as PTS-6, were bidirectional [20].

Thus, after about a decade of work, Abba's team had established that peptides could cross the BBB and could do so either by using transporters or transmembrane diffusion. Transporters could be directed in the brain-to-blood or blood-to-brain direction or be bidirectional. As shown in Abba's lab as well as in those of others, the BBB permeation of a peptide correlated with its having effects on brain after its peripheral administration.

The blood–brain barrier and regulatory proteins

The 1990s saw an acceleration in the discovery of regulatory proteins. Many of the same issues that had been raised about peptides were now raised about regulatory proteins, including how they could affect brain function after peripheral administration. In particular, cytokines emerged as being involved in neuroinflammation and as connectors of the peripheral immune system and the central nervous system. But these substances were much closer to the size of albumin, the intravascular marker whose lack of permeation was a hallmark of the vascular BBB, than to that of peptides. With the exception of transferrin, molecules in this size range were assumed not to be able to cross the BBB. However, when Abba's team used the sensitive techniques developed to measure peptide permeation, interleukin-1 alpha (IL-1α) was found to cross the BBB 40 times faster than albumin [15]. This was an astonishing rate for a substance of this size and the amount of IL-1α crossing

the BBB was clearly enough to affect brain function; this was formally confirmed about a decade later [3]. IL-1α, IL-1β, and IL-1 receptor agonist were subsequently shown to share a saturable transport system across the BBB [21,29]. Other separate transporters were each found for IL-6 and TNF-alpha [17,28]. Abba and Weihong Pan and other labs have since expanded the number of cytokines shown to cross the BBB [46]. Like other BBB transporters, the transporters for cytokines adapt to physiological conditions and disease states. For example, the rate at which TNF crosses the BBB is greatly increased during the clinical phase of experimental autoimmune encephalomyelitis [44], an animal model of multiple sclerosis. Although the idea that cytokines could cross the BBB was controversial for at least a decade after Abba's lab had suggested it, it is now accepted as one of the main ways in which blood-borne cytokines can affect brain function, along with acting on afferent nerves, at circumventricular organs, and modulating the functions of the BBB [2].

Other fields were impacted by Abba's work on regulatory peptides, including those of nutrition and obesity. In the mid 1990s, Abba's lab showed that leptin, a 16 kDa protein secreted by adipose tissue, crossed the BBB by a using a transporter unique to it [19]. This provided the mechanism by which leptin secreted from fat and circulating in the blood could reach its receptors in the brain to affect feeding and the rate of caloric expenditure. Subsequently, Abba and Weihong Pan have shown that leptin interacts not only at receptors on neurons, but other cell types in the CNS, most notably brain endothelial cells and astrocytes, and that actions by these cells are counter regulatory to those induced at the arcuate nucleus [30,39,45].

Future directions

This brief overview shows that Abba's work established that peptides and regulatory proteins cross the BBB. By doing so, the BBB acts as a conduit transferring informational molecules between the CNS and peripheral circulation. This forms a means of brain-peripheral tissue communication and is important in many fields in addition to neuroimmunology, endocrinology, and nutrition. The BBB transporters for these substances are not static, but are influenced by changes in physiology and are altered in disease states. In some cases, the changes in response to disease are adaptive and in other cases are maladaptive and may induce or promote the disease. These findings are the basis for future work that will further elucidate how BBB/peptide/regulatory interactions are important in normal physiology and disease.

An area that has yet to be fully considered is the use of this information in developing drugs for the CNS. The discovery of transporters for peptides and regulatory proteins in particular holds great potential for the development of analogs that can be used to treat diseases of the CNS. As just one example, Abba noted early on that the tripeptide prolyl-leucyl-glycinamide (MIF-1) rapidly reversed the symptoms of depression in humans [26,27] and later confirmed that MIF-1 was transported very rapidly from the blood into the brain [11]. As the possibility of using peptides and regulatory proteins as CNS therapeutic agents is once again revisited, the seminal studies of Abba Kastin regarding the permeability of the blood–brain barrier to peptides and regulatory proteins are of fundamental importance.

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Abbreviations

BBB	blood–brain barrier
BUI	brain uptake index
CNS	central nervous system
CSF	cerebrospinal fluid
DSIP	delta sleep-inducing peptide
IL	interleukin
MIF-1	prolyl-leucyl-glycinamide
PTS	peptide transport system
TSH	thyrotropin stimulating hormone

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Table 1

PTS #	Direction	Main ligands	Minor ligands	Modulators	References
PTS-1	Brain-to-blood	Tyr-MIF-1 Met-Enk	Leu-Enk, dynorphin 1-8, beta-casomorphin, oxytocin	Aluminum, leucine (D > L)	[5]
PTS-2	Brain-to-blood	Arginine vasopressin	Vasotocins	Acute hydration	[18]
PTS-3	Blood-to-brain	Peptide-T analog		Aluminum	[24]
PTS-4	Bidirectional	LHRH		Luteinizing hormone	[25]
PTS-5	Brain-to-blood	Somatostatin cyclic analogs			[22]
PTS-6	Bidirectional	PACAP 38, PACAP27			[20]

LHRH = Luteinizing hormone releasing hormone; PACAP = pituitary adenylate cyclase activating polypeptide.