Evidence for an alternative insulin transporter at the blood-brain barrier

William A. Banks1,2, Cassidy Noonan3,4, Elizabeth M. Rhea1,2\*

1 Department of Medicine, Division of Gerontology and Geriatric Medicine, University of Washington, Seattle, WA 98195

2 Geriatric Research Education and Clinical Center, Veterans Affairs Puget Sound Health Care System, Seattle, WA 98108

3 Research and Development, Veterans Affairs Puget Sound Health Care System, Seattle, WA 98108

4 University of Washington, Seattle, WA 98195

\*Corresponding author: Elizabeth M. Rhea; meredime@uw.edu; 1660 S. Columbian Way, Seattle, WA 98108

Keywords: insulin, transport, blood-brain barrier

Abstract:

Accumulating evidence suggests there is an alternative insulin transporter besides the insulin receptor at the blood-brain barrier (BBB), responsible for shuttling insulin from the circulation into the brain. In this review, we summarize key features of the BBB and what makes it unique compared to other capillary beds; summarize what we know about insulin BBB transport; provide an extensive review about diseases, physiological states, and serum factors tested in modifying insulin BBB transport; and lastly, highlight potential alternative transport systems that may be or have been tested in mediating insulin BBB transport. Identifying the transport system for insulin at the BBB will help control central nervous system (CNS) insulin levels in multiple diseases and conditions including Alzheimer’s disease (AD) and obesity where CNS availability is limited.

1. Introduction

The ability of insulin to act within the brain has been known since the early 20th century. However, as blood substrate entry into the brain is regulated by the blood-brain barrier (BBB) interface, evidence of insulin crossing the brain barriers was identified decades later. It is now well recognized that the majority of insulin acting within the brain crosses the BBB via a saturable, receptor-mediated transport system that is affected by various physiological states. Once present within the brain, insulin must navigate the brain parenchyma to reach various cell types and act as a ligand by binding its receptors, including the insulin receptor, insulin-like growth factor 1 receptor, and hybrids of the two, activating intracellular signaling cascades. Insulin signaling within the central nervous system (CNS) is important not only for regulation of metabolism but also cognition. CNS insulin signaling can become dysfunctional with age and in neurodegenerative diseases such as Alzheimer’s disease [1]. Insulin BBB transport could be a regulator of CNS insulin signaling since it is one of the mediators of CNS insulin levels. Without sufficient ligand, receptor signaling becomes impaired. Therefore, understanding more about the transport system at the BBB for insulin will aid in combating such deficiency in aging and Alzheimer’s disease.

1. Blood-brain barrier (BBB)
2. Components of the BBB

Conceptually, the BBB can be thought of as those structures which inhibit or otherwise regulate the exchange of substances between the CNS and blood. These barriers include the vascular BBB, the choroid plexus, the tanycytic barrier located between the circumventricular organs and adjacent brain tissue, the meningeal barrier, and the barriers of the cranial nerves such as the blood-retinal barrier [2]. Likely, all these barriers participate in insulin/CNS interactions, but it is the vascular BBB that has been most studied in this regard.

The physical wall that forms the BBB is comprised of brain endothelial cells (BECs) and occurs in the arteriolar, capillary, and venule portions of the cerebral vasculature [3]. These cells are in constant communication with other cells of CNS, forming the neurovascular unit (NVU). The NVU includes microglia, astrocytes, neurons, and mast cells, but it is the astrocytes and pericytes which have received the most attention in regard to their interactions with the BBB. Pericytes are anatomically connected by gap junctions with the BEC. Astrocytes form a sheath around the BBB capillaries and are separated from the abluminal surface of the BEC by the basement membranes. It is the pericytes and astrocytes which induce the BECs to express BBB characteristics, including the formation of tight junctions and the loss of fenestrae and micropinocytosis [4]. The cells of the NVU also modulate other BBB functions, such as cytokine secretions and transporter functions [4].

The BBB is also in communication with the circulating immune cells and, by way of secretions into blood, with the peripheral tissues. This communication can also affect various functions of the BBB. For example, lipopolysaccharide, a fragment of the cell wall of gram negative bacteria that is a potent stimulator of the innate immune system, increases insulin transport across the BBB by inducing nitric oxide release from immune cells [5, 6].

1. Roles of the BBB

The most widely appreciated role of the BBB and for which it was named is that of limiting the unregulated leakage of substances from blood into the CNS. Unlike other capillary beds, that of the CNS has greatly reduced transcytosis, few fenestrations, and adjacent BEC cell membranes are cemented together by tight junctions [7]. Thus, paracellular (between cells) and transcellular (across a cell) leakage is essentially absent in the healthy BBB so that no ultrafiltrate is produced by the capillary bed of the CNS. This lack of an ultrafiltrate protects brain tissue from blood-borne substances, both endogenous and xenobiotic, which would be toxic to those tissues. The physical barrier is reinforced for some substances by the presence of brain-to-blood efflux systems which prevent circulating substances from entering or remaining in brain tissue. For example, the anti-helminthic ivermectin is prevented from entering the CNS by the brain-to-blood transporter p-glycoprotein. In animals that do not express p-glycoprotein at their BBB, ivermectin is a potent neurotoxin [8]. The BBB can also be an enzymatic barrier, digesting substances such as the monoamines which could otherwise enter the brain from the circulation [9].

The lack of an ultrafiltrate may protect the CNS from circulating toxins, but the ultrafiltrate is the major route by which most tissues receive their nourishment from the blood. Thus, the BBB has other mechanisms to provide the CNS with nutrients. The most prominent of these are the transport mechanisms. The BBB contains many transporters, and it is likely there are still more to be discovered. These transporters deliver to the brain the glucose, amino acids, free fatty acids, vitamins, and other nutrients needed by the brain. The transporters of the BBB also play a homeostatic role for the CNS by regulating electrolyte balance, bicarbonate levels, and as exemplified by p-glycoprotein eliminating from the CNS both endogenous and exogenous toxins. The BBB also participates in brain-body communication by regulating the transport of various informational molecules, including insulin.

1. Types of transport systems at the BBB

Transport systems can be categorized in various ways and the strict definitions from cellular biology [10, 11] are not always applied in the BBB literature. Pharmacokinetically, transporters demonstrate saturation and biochemically, are typically transmembrane glycoproteins. Many BBB transporters, such as the glucose transporter 1 (GLUT-1) which transports glucose across the BBB, use facilitated diffusion. These systems are energy independent, often are channels, and transport substances bidirectionally from the side of higher concentration to the side of lower concentration. Active transporters require energy or an electrochemical gradient to function, can be unidirectional, and can transport substances against a concentration gradient. Channels refer to a protein architecture which when active is open simultaneously to the extracellular and intracellular environments. Channels can undergo conformational changes and so become inactive (closed to both environments), whereas pores are always open and active. Carriers typically open and close so that they can be open to one environment and closed to the other. As a rule, channels can only engage in facilitated diffusion, whereas carriers can engage in facilitated diffusion or active transport. Channels, pores, and carriers typically transport substances into or out of the cytoplasm and transcellular transport would depend on a set of transporters located at both the luminal and abluminal cell membranes.

Receptor-mediated refers to a binding site for the ligand on the transporter and is a hallmark of carriers. Endocytosis refers to the internalization into the cell cytoplasm of the carrier protein with its ligand within a vesicle formed by the cell membrane. Those endosomes can be routed to various cellular structures, including back to the cell membrane. The term receptor-mediated endocytosis is often, but not always, used specifically to refer to endocytosis involving clathrin. Clathrin-independent endocytic mechanisms include potocytosis (internalization of caveolae), adsorptive transcytosis, pinocytosis and phagocytosis.

Transcytosis occurs when the endosome moves from one membrane of a polarized cell to the other (e.g., from apical to basal or luminal to abluminal). Transcytosis, therefore, requires that a cell have distinctive regions to its membrane as in the case of barrier cells; otherwise, exocytosis is membrane recycling. The BBB field tends to label any transport of a large molecule as receptor-mediated transcytosis, even when there is no evidence of involvement of vesicles or clathrin. The assumption is that vesicles are required to move larger substances, but enzymes and cytokines can be exported via carriers, as exemplified by interleukin 1 (IL-1) and p-glycoprotein [12]. Transport of insulin across the BBB is assumed to be clathrin-dependent but has not been directly tested in vivo. Insulin endocytosis is clathrin dependent for most cells [13], including endothelial cells [14], and insulin transcytosis across the blood-retinal barrier is clathrin-dependent [15].

1. Insulin BBB Transport

As established above, it is now well acknowledged that insulin can directly cross the BBB. This occurs in a saturable, somewhat specific, receptor-mediated process [16]. That is, at euglycemic doses of insulin, the rate of radioactive insulin BBB transport decreases [16]. In terms of specificity, of the serum ligands tested, like leptin [17], few have inhibited insulin BBB transport, suggesting the transport system for insulin may be specific for insulin. The saturability nature of the transport system suggests that it is receptor-mediated. The transport system is similar across species as human and rat insulin both cross the murine BBB [18] and unlabeled human insulin is able to inhibit the blood to brain passage of radioactive rat insulin [16]. There is great variability in the transport system throughout the brain as some regions have extremely fast transport [19]. Lastly, inactive insulin, either via freeze/thaw or heat-denatured methods, is unable to cross the BBB [20, 21]. Therefore, structural changes of insulin, such as deamidation, are likely necessary for recognition by the insulin transporter.

Over the years, insulin BBB transport has been investigated using various techniques. However, due to the small amounts of insulin transported across the BBB, there can be technical difficulties in some of the techniques. The ability to measure low amounts of immunoactive insulin present in brain compared to blood, and the need to inject high insulin concentrations of immunofluorescent tracer are some examples. Therefore, radioactivity is a great alternative to measure pharmacokinetics of insulin transport. By investigating insulin BBB transport, independent researchers have identified this transport system is impacted somewhat not surprisingly by metabolic changes, during development and pregnancy, and by exercise and perhaps surprisingly by Alzheimer’s disease and inflammation. There have also been factors and/or states that have had no effect on insulin transport, like loss of the BEC insulin receptor. We have summarized this literature in Table 1 and refer readers to the specific references mentioned for each factor/disease state/intervention investigated.

**Table 1.** Impact of disease, physiological states, and serum factors on insulin BBB transport

|  |  |  |  |  |  |
| --- | --- | --- | --- | --- | --- |
| **Study** | **Disease/Intervention** | **Model** | **Model**  | **Summary**  | **Reference** |
| **METABOLIC FACTORS** |
| 1 | Diabetes- induced | streptozotocin (ip) | Mouse | **↑** | [22] |
| 2 | Diabetes- induced | alloxin (iv) | Mouse | **↑** | [22] |
| 3 | Hyperglycemia (non-diabetic) | D-glucose (ip) | Mouse | **↔** | [22] |
| 4 | Obesity | high-fat diet | Dog | **↓** | [23] |
| 5 | Obesity  | retired breeders | Mouse | **↓** | [21] |
| 6 | Starvation in obesity | fasting (48 hr) | Mouse | **↑** | [21] |
| 7 | Triglycerides | cardiac perfusion | Mouse | **↑** | [21] |
| **DEVELOPMENTAL FACTORS** |
| 8 | Newborn/Infancy | newborn, 3 wks | Rabbit | **↑** | [24] |
| 9 | Pregnancy | late pregnancy, BCSFB | Mouse | **↓** | [25] |
| 10 | Pregnancy | late pregnancy | Rat | **↑** | [26] |
| 11 | Age | C57B/6J (12, 24 mo) | Mouse | **↓** | [27] |
| 12 | Aging | SAMP8 (12 mo) | Mouse | **↔** | [28] |
| 13 | Alzheimer’s | APP/PS1 (6 mo) | Mouse | **↑** | [29] |
| 14 | Alzheimer’s | APP/PSN1 (6 mo) | Mouse | **↑** | [30] |
| 15 | Alzheimer’s | moderate/severe AD, BCSFB | Human | **↓** | [31] |
| **PHYSIOLOGICAL STATES** |
| 16 | Iron Deficiency | nutritional iron-deficiency | Rat | **↑** | [32] |
| 17 | Exercise | voluntary running wheel (24 hrs) | Mouse | **↑** | [33] |
| **INSULIN RECEPTOR LOSS** |
| 18 | Insulin receptor inhibition | S961 | Mouse, BECs | **↓** | [20] |
| 19 | Insulin receptor loss/inhibition | EndoIRKO; S961 | Mouse | **↔** | [34] |
| 20 | Insulin receptor inhibition | S961 | BECs | **↔** | [35] |
| 21 | Insulin receptor inhibition | S961 | Mouse | **↔** | [30] |
| **GENETICS** |
| 22 | Young ApoE mice | apoE3/apoE4, male/female | Mouse | **↔** | [36] |
| 23 | Aged ApoE mice | apoE3/apoE4, male/female, HFD | Mouse | **↓** | [37] |
| **FACTORS/DRUGS/OTHER** |
| 24 | IGFs | IGF-1, IGF-II (perfusion) | Mouse | **↓** | [38] |
| 25 | Leptin | iv, co-injection | Mouse | **↔** |  [16] |
| 26 | Aluminum | ip | Rat | **↑** | [39] |
| 27 | Aluminum | ip | Mouse | **↑** | [16] |
| 28 | P-glycoprotein inhibitor | Verapamil iv, co-injection | Mouse | **↔** | [16] |
| 29 | Amino Acid | Tyrosine, iv, co-injection | Mouse | **↔** | [16] |
| 30 | Norepinephrine | iv, co-injeciton | Mouse | **↔** | [40] |
| 31 | Rapamycin | rapamycin (ip, 2 wks) | Mouse | **↔** | [41] |
| 32 | Rosiglitazone | iv, pre-treatment | Mouse | **↔** | [42] |
| 33 | CCK (Cholecystokinin) | ip, fasted (16 h) | Rat | **↑** | [43] |
| 34 | Acute estrogen | OVX female, male, ip (48 hr) | Rat | **↔** | [44] |
| 35 | Chronic estrogen | Male, ip (5 wks) | Rat | **↔** | [44] |
| 36 | Inflammation | LPS, ip (16, 24 h) | Mouse | **↑** | [45] |
| 37 | nNOS | 3x ip LPS, inhibitor (4 h post) | Mouse | **↓** | [46] |
| 38 | iNOS, eNOS | 3x ip LPS, inhibitor (4 h post) | Mouse | **↑** | [46] |
| 39 | Dexamethasone | oral (7 d) | Dog | **↓** | [47] |

apoE: apolipoprotein E, BCSFB: blood-cerebrospinal fluid barrier, CCK: Cholecystokinin, EndoIRKO: endothelial insulin receptor knock-out, eNOS: endothelial nitric oxide synthase, HFD: high-fat diet, IGF: insulin-growth factor, iNOS: inducible nitric oxide synthase, ip: intraperitoneal, iv: intravenous, LPS: lipopolysaccharide, nNOS: neuronal nitric oxide synthase, OVX: ovariectomized

The insulin receptor was long thought to serve as the protein responsible for shuttling insulin from the circulation, across the BEC, and release within the brain parenchyma. S961 is a potent, selective antagonist for the insulin receptor, and not IGF-1R or hybrid receptors [48] and has regularly been used to investigate the role of the insulin receptor in various processes, including transport. However, we recently showed that loss or inhibition of the insulin receptor in BECs in mice did not affect the transport rate of insulin across the BBB [34]. Since then, others have supported this finding, showing inhibition of the BEC insulin receptor did not impact insulin transport in an in vitro model [35] and in mice noted in a preprint [49]. In an exciting new paper describing a novel, in vivo insulin PET tracer, co-administration of S961 also had no effect on brain insulin uptake in mice [30]. These data suggest there is another protein(s) responsible for transporting insulin across the BEC. However, to date, identification of this protein is unknown.

1. Alternatives for the insulin transporter

The involvement of another protein besides the insulin receptor for transporting insulin across the BBB makes evolutionary sense. As the receptor is involved in many intracellular signaling cascades and acts as a tyrosine kinase, it further supports the primary role to be a signaling protein rather than a transporter. The impact of BEC insulin receptor signaling should not be overlooked when investigating insulin interactions at the BBB. Recent proteomic studies of immortalized BECs support how critical the insulin receptor is in BECs, necessary for a multitude of functions, including regulation of a variety of BBB transporters, the transferrin receptor, and the tight junction protein claudin-5 [50]. A separate transporter would allow not only transport across the BEC while still allowing for these critical intracellular signaling events via the receptor. Endothelial cell intracellular insulin signaling is a critical metabolic event. There is evidence in other receptor/transport systems supporting different proteins to accomplish these two independent events, as described next. We also discuss other alternatives for the insulin transporter that have been hypothesized.

1. Evidence for alternative transporters to canonical receptors at the BBB

Insulin binding to the luminal surface of BECs has two fates. It may activate the intracellular machinery that affects cellular functions (here termed the signaling receptor) or it may be transported across the BBB (here termed the transporter binding site). Binding to either the signaling receptor or the transporter binding site results in insulin endocytosis and exocytosis. In the case of signaling receptor binding, the exocytosis is at the luminal membrane of the BEC and in the case of transporter binding site, the exocytosis is to the abluminal membrane of the BEC. The question arises as to whether the protein forming the signaling receptor is the same protein as the transporter binding site. As we have previously reviewed, it seems that the usual situation is that the signaling receptor protein and the BBB transporter binding site are usually different proteins, as exemplified by prolactin [51], epidermal growth factor, Tyr-MIF-1, the enkephalins, pituitary adenylate cyclase activating polypeptide, and thyroid hormones [52]. Our data argues that a similar dichotomy exists for insulin. We found that the insulin antagonist S961 binds avidly to the BEC, but is not transported across the BBB [34]. This means that the transporter binding site differs sufficiently from the signaling receptor as to not recognize S961 as a ligand. We also found that S961 blocked the ability of radioactive insulin to bind to BECs, but not its ability to cross the BBB. Finally, mice with loss of the signaling receptor in BECs demonstrated poor binding to BECs, but unimpaired transport activity. These studies are consistent with the insulin signaling receptor and the transporter binding site being different proteins.

1. Insulin-like growth factor-1 receptor (IGF-1R)

Could IGF-1R be insulin’s transporter binding site? Insulin and IGF-1 each bind to the other’s receptors, although much less avidly. IGF-1R is expressed at the BBB and choroid plexus [53]. IGF-1 crosses the BBB and inhibits the transport of radioactive insulin transport across the BBB, just as insulin inhibits the transport of radioactive IGF-1 [38, 54]. Both insulin and IGF-1 transport are reduced in obese animals and affected by triglycerides [55, 56]. However, cross inhibition studies suggest that there is a separate insulin-favoring transporter and an IGF-1-favoring transporter [38, 57]. Triglycerides increase insulin transport across the BBB but inhibit transport of IGF-1 [55, 56]. Furthermore, evidence suggests that IGF-1R does not transport IGF-1 across the BBB [58], but that low-density lipoprotein receptor-related protein (LRP)-1 is involved at the vascular BBB and LRP-2 at the choroid plexus [56, 59]. Therefore, IGF-1R is not a candidate for being the BBB insulin transport protein.

1. Low-density lipoprotein receptor-related proteins (LRP)

The LRP family of proteins are structurally similar but participate in a wide range of physiological processes including lipid metabolism, neurodevelopment, and transport of nutrients [60]. Megalin, also known as LRP2, is the largest sized protein in the family and can bind a wide variety of ligands. While it can play a role in reabsorption of various molecules in the proximal renal tubule, including insulin [61], it can also act as a cell signaling transducer within the CNS [60, 62]. LRP8, also referred to as apolipoprotein E receptor 2 (apoER2), has been recognized as a signal transducer critical in brain development [63]. Both of these have been suggested to play a role in insulin transport in peripheral systems.

LRP2/Megalin can regulate insulin transport in kidney proximal tubule cells [61] and can take up other hormones as well, including leptin [64] and IGF-1 [65]. Receptor-associated protein (RAP) is a 39 kDa protein that is a natural inhibitor of ligand binding to LRP2. We used this non-specific inhibitor of LRP2, RAP, and reported insulin BBB transport was unchanged [34]. However, Orlando et al has also reported the RAP does not affect insulin binding to proximal tubule cells, compared to excess, unlabeled insulin [61]. Therefore, a more specific inhibitor of LRP2 would aid in fully identifying a role for LRP2 in insulin BBB transport. Further evidence suggests leptin is also not transported across the BBB via LRP2 [66], despite its role in transport at the choroid plexus [67]. IGF-1 is also transported across the choroid plexus by LRP2 [65].

LRP8/ApoER2 is not only a receptor for apoE but also acts as the primary receptor for the critical brain development protein Reelin [63]. ApoER2 is involved in long-term potentiation, learning, and memory. In the last few years, due to the AD risk gene allele, ApoE4, the role for ApoER2 in AD has begun to be explored. Post-translational proteolytic cleavage of ApoER2 [68] and pre-translational splicing [69] is dysfunctional in AD. Additionally, the risk allele ApoE4 impairs the trafficking of the insulin receptor, resulting in decreased insulin signaling [70]. How ApoER2 may fit into this pathway remains to be determined.

1. Amino Acid Transporter Involvement

Amino acids are transported across the BBB involving both facilitative systems and active transporters. Some of these transporters are selective for a single substrate or group of substrates while others are non-selective [71]. Recently, it was identified in a high-throughput screen that the amino acid transporter, *SLC7A1*, also known as CAT-1, could regulate leptin transport across an iPSC- derived BEC model [72]. It is therefore possible that the same, or another amino acid transporter, could regulate insulin transport across the BBB. Transport of amino acids could modify transporter expression, activity, and cellular distribution. Additionally, it is possible that the amino acid itself could aid as a co-factor for the insulin transporter. While insulin is known to impact amino acid transport, either directly or indirectly [73], the converse is less understood. The amino acid-derived hormone norepinephrine did not affect insulin BBB transport contrary to a 2-3 fold increase of leptin BBB transport [40]. In an in vitro co-culture model of astrocytes and brain endothelial cells, L-glutamate enhances insulin transcytosis [20]. L-arginine, in the presence of LPS, also enhances insulin BBB transport [46]. L-arginine is a nitric oxide precursor and nitric oxide has been shown to regulate insulin BBB transport, as discussed next.

1. Involvement of Nitric Oxide Synthase (NOS)

Nitric oxide is a common secondary messenger that helps orchestrate multiple signaling pathways. Synthesis of nitric oxide from L-arginine is primarily converted by NOS, present in multiple different cell types. One of the more common roles is to act as a vasodilator, relaxing the smooth muscle cells around the blood vessels. In the brain, there are three main NOS enzymes: endothelial NOS (eNOS), neuronal NOS (nNOS), and inducible NOS (iNOS). NOS and nitric oxide have an important role at the BBB, regulating its structure and function. Under inflammatory-stimulated conditions in vivo, NOS inhibitors enhance insulin BBB transport, specifically nitric oxide coming from nNOS [46]. In an in vitro co-culture model, astrocytic inhibition of NOS decreases insulin transcytosis [20]. This suggests the source of the nitric oxide stimulation can regulate insulin transport. The transport system is also suspected to involve calcium signaling as pre-treating astrocytes with a calcium donor enhanced insulin transport across a BEC model [20]. As these second messengers are complex, further investigation on the interaction and the NVU cell types involved in regulating insulin BBB transport are warranted.

1. Conclusions

We have presented evidence there is an alternative insulin transport system at the BBB than the insulin receptor. Identification of this transport system will be critical in treating diseases with deficient CNS insulin signaling, such as Alzheimer’s disease or dysregulated metabolism, as insulin availability could be a contributing factor. While there are ways to deliver exogenous insulin to the CNS, such as via intranasal insulin [74], that have proven to be beneficial, preventing and/or restoring the endogenous insulin BBB transport system would likely be more effective and potentially even prevent a deficiency in the first place. Insulin clearly has multiple impacts not only within the CNS but also in regulating BBB function, that any slight modification of this signaling has downstream detrimental effects. Whether this transport system is unique to the BBB or is similar to other peripheral endothelial beds remains to be determined. Leveraging multiple genetic data sets could hopefully shed light on potential targets for the transport system, but proteomic data will also be necessary. In a recent proteomics study, protein levels of the insulin receptor were detected at similar levels between rat microvessels isolated from various regions including white matter, cortical grey matter, and spinal cord [75]. While protein expression level does not necessarily translate to activity of a transporter, equivalent expression of the insulin receptor across brain regions does not support the high variability of insulin transport rate across regions. Additionally, if the transport system involves co-factors, the identification of the transporter could prove to be even more difficult. It is likely the abundance of the transporter(s) within BECs is low, given the limited entry of insulin into the CNS, which will further add to the difficulty. Despite these difficulties, recent technological advances in microvessel isolation, omics-based discovery approaches, and cell culture screening tools will help elucidate the transport system for insulin.

REFERENCES

[1] Rhea EM, Banks WA, Raber J (2022) Insulin Resistance in Peripheral Tissues and the Brain: A Tale of Two Sites. *Biomedicines* **10**.

[2] Neuwelt E, Abbott NJ, Abrey L, Banks WA, Blakley B, Davis T, Engelhardt B, Grammas P, Nedergaard M, Nutt J, Pardgridge W, Rosenberg GA, Smith Q, Drewes LR (2008) Strategies to advance translational research into brain barriers. *Lancet Neurology* **7**, 84-96.

[3] Sweeney MD, Zhao Z, Montagne A, Nelson AR, Zlokovic BV (2019) Blood-Brain Barrier: From Physiology to Disease and Back. *Physiological reviews* **99**, 21-78.

[4] Vangilder RL, Rosen CL, Barr TL, Huber JD (2011) Targeting the neurovascular unit for treatment of neurological disorders. *Pharmacol Ther* **130**, 239-247.

[5] Banks WA, Dohgu S, Nakaoke R, Lynch JL, Fleegal-DeMotta MA, Erickson MA, Vo TQ (2008) Nitric oxide isoenzymes regulate LPS-enhanced insulin transport across the blood-brain barrier. *Endocrinology* **149**, 1514-1523.

[6] Xaio H, Banks WA, Niehoff ML, Morley JE (2001) Effect of LPS on the permeability of the blood-brain barrier to insulin. *Brain Research* **896**, 36-42.

[7] Reese TS, Karnovsky MJ (1967) Fine structural localization of a blood-brain barrier to endogenous peroxidase. *J Cell Biol.* **34**, 207-217.

[8] Mealey KL, Bentjen SA, Gay JM, Cantor GH (2001) Ivermectin sensitivity is associated with a deletion mutation of the mdr1 gene. *Pharmacogenetics* **11**, 727-733.

[9] Hardebo JE, Owman C (1990) Enzymatic barrier mechanisms for neurotransmitter monoamines and their precursors at the blood-brain barrier In *Pathophysiology of the Blood-Brain Barrier*, Johansson BB, Owman C, Widner H, eds. Elsevier, Amsterdam, pp. 41-55.

[10] Stein WD (1986) *Transport and diffusion across cell membranes*, Academic Press, Inc, London.

[11] Yeagle P (1987) Transport In *The Membranes of Cells* Academic Press, Inc., Orlando, pp. 191-215.

[12] Drach J, Gsur A, Hamilton G, Zhao S, Angerler J, Fiegl M, Zojer N, Raderer M, Haberl I, Andreeff M, Huber H (1996) Involvement of P-glycoprotein in the transmembrane transport of interleukin-2 (IL-2), IL-4, and interferon-gamma in normal human T lymphocytes. *Blood* **88**, 1747-1754.

[13] Fan JY, Carpentier JL, Gorden P, Van Obberghen E, Blackett NM, Grunfeld C, Orci L (1982) Receptor-mediated endocytosis of insulin: role of microvilli, coated pits, and coated vesicles. *Proc Natl Acad Sci U S A* **79**, 7788-7791.

[14] Azizi PM, Zyla RE, Guan S, Wang C, Liu J, Bolz SS, Heit B, Klip A, Lee WL (2015) Clathrin-dependent entry and vesicle-mediated exocytosis define insulin transcytosis across microvascular endothelial cells. *Mol Biol Cell* **26**, 740-750.

[15] Stitt AW, Anderson HR, Gardiner TA, Bailie JR, Archer DB (1994) Receptor-mediated endocytosis and intracellular trafficking of insulin and low-density lipoprotein by retinal vascular endothelial cells. *Invest Ophthalmol Vis Sci* **35**, 3384-3392.

[16] Banks WA, Jaspan JB, Huang W, Kastin AJ (1997) Transport of insulin across the blood-brain barrier: saturability at euglycemic doses of insulin. *Peptides* **18**, 1423-1429.

[17] Banks WA, Kastin AJ, Huang W, Jaspan JB, Maness LM (1996) Leptin enters the brain by a saturable system independent of insulin. *Peptides* **17**, 305-311.

[18] Banks WA, Jaspan JB, Kastin AJ (1997) Selective, physiological transport of insulin across the blood-brain barrier: novel demonstration by species-specific radioimmunoassays. *Peptides* **18**, 1257-1262.

[19] Banks WA, Kastin AJ, Pan W (1999) Uptake and degradation of blood-borne insulin by the olfactory bulb. *Peptides* **20**, 373-378.

[20] Gray SM, Aylor KW, Barrett EJ (2017) Unravelling the regulation of insulin transport across the brain endothelial cell. *Diabetologia* **60**, 1512-1521.

[21] Urayama A, Banks WA (2008) Starvation and triglycerides reverse the obesity-induced impairment of insulin transport at the blood-brain barrier. *Endocrinology* **149**, 3592-3597.

[22] Banks WA, Jaspan JB, Kastin AJ (1997) Effect of diabetes mellitus on the permeability of the blood-brain barrier to insulin. *Peptides* **18**, 1577-1584.

[23] Kaiyala KJ, Prigeon RL, Kahn SE, Woods SC, Schwartz MW (2000) Obesity induced by a high-fat diet is associated with reduced brain insulin transport in dogs. *Diabetes* **49**, 1525-1533.

[24] Frank HJ, Jankovic-Vokes T, Pardridge WM, Morris WL (1985) Enhanced insulin binding to blood-brain barrier in vivo and to brain microvessels in vitro in newborn rabbits. *Diabetes* **34**, 728-733.

[25] Azar AS, Brooks VL (2011) Impaired baroreflex gain during pregnancy in conscious rats: role of brain insulin. *Hypertension* **57**, 283-288.

[26] Shi Z, Hansen KM, Bullock KM, Morofuji Y, Banks WA, Brooks VL (2019) Resistance to the sympathoexcitatory effects of insulin and leptin in late pregnant rats. *J Physiol* **597**, 4087-4100.

[27] Zhou AL, Sharda N, Sarma VV, Ahlschwede KM, Curran GL, Tang X, Poduslo JF, Kalari KR, Lowe VJ, Kandimalla KK (2022) Age-Dependent Changes in the Plasma and Brain Pharmacokinetics of Amyloid-beta Peptides and Insulin. *J Alzheimers Dis* **85**, 1031-1044.

[28] Banks WA, Farr SA, Morley JE (2000) Permeability of the blood-brain barrier to albumin and insulin in the young and aged SAMP8 mouse. *J Gerontol A Biol Sci Med Sci* **55**, B601-606.

[29] Poduslo JF, Curran GL, Wengenack TM, Malester B, Duff K (2001) Permeability of proteins at the blood-brain barrier in the normal adult mouse and double transgenic mouse model of Alzheimer's disease. *Neurobiol Dis* **8**, 555-567.

[30] Taubel JC, Nelson NR, Bansal A, Curran GL, Wang L, Wang Z, Berg HM, Vernon CJ, Min HK, Larson NB, DeGrado TR, Kandimalla KK, Lowe VJ, Pandey MK (2022) Design, Synthesis, and Preliminary Evaluation of [(68)Ga]Ga-NOTA-Insulin as a PET Probe in an Alzheimer's Disease Mouse Model. *Bioconjug Chem* **33**, 892-906.

[31] Craft S, Peskind E, Schwartz MW, Schellenberg GD, Raskind M, Porte D, Jr. (1998) Cerebrospinal fluid and plasma insulin levels in Alzheimer's disease: relationship to severity of dementia and apolipoprotein E genotype. *Neurology* **50**, 164-168.

[32] Ben-Shachar D, Yehuda S, Finberg JP, Spanier I, Youdim MB (1988) Selective alteration in blood-brain barrier and insulin transport in iron-deficient rats. *J Neurochem* **50**, 1434-1437.

[33] Brown C, Pemberton S, Babin A, Abdulhameed N, Noonan C, Brown MB, Banks WA, Rhea EM (2022) Insulin blood-brain barrier transport and interactions are greater following exercise in mice. *J Appl Physiol (1985)*.

[34] Rhea EM, Rask-Madsen C, Banks WA (2018) Insulin transport across the blood-brain barrier can occur independently of the insulin receptor. *J Physiol* **596**, 4753-4765.

[35] Hersom M, Helms HC, Schmalz C, Pedersen TA, Buckley ST, Brodin B (2018) The insulin receptor is expressed and functional in cultured blood-brain barrier endothelial cells but does not mediate insulin entry from blood to brain. *Am J Physiol Endocrinol Metab* **315**, E531-E542.

[36] Rhea EM, Torres ERS, Raber J, Banks WA (2020) Insulin BBB pharmacokinetics in young apoE male and female transgenic mice. *PLoS One* **15**, e0228455.

[37] Rhea EM, Hansen K, Pemberton S, Torres ERS, Holden S, Raber J, Banks WA (2021) Effects of apolipoprotein E isoform, sex, and diet on insulin BBB pharmacokinetics in mice. *Sci Rep* **11**, 18636.

[38] Reinhardt RR, Bondy CA (1994) Insulin-like growth factors cross the blood-brain barrier. *Endocrinology* **135**, 1753-1761.

[39] Banks WA, Kastin AJ (1985) Peptides and the blood-brain barrier: lipophilicity as a predictor of permeability. *Brain Res Bull* **15**, 287-292.

[40] Banks WA (2001) Enhanced leptin transport across the blood-brain barrier by alpha 1-adrenergic agents. *Brain Res* **899**, 209-217.

[41] Nguyen S, Banks WA, Rhea EM (2021) Effects of Rapamycin on Insulin Brain Endothelial Cell Binding and Blood-Brain Barrier Transport. *Med Sci (Basel)* **9**.

[42] Galindo DC, Banks WA, Rhea EM (2020) The impact of acute rosiglitazone on insulin pharmacokinetics at the blood-brain barrier. *Endocrinol Diabetes Metab* **3**, e00149.

[43] May AA, Liu M, Woods SC, Begg DP (2016) CCK increases the transport of insulin into the brain. *Physiol Behav* **165**, 392-397.

[44] May AA, Bedel ND, Shen L, Woods SC, Liu M (2016) Estrogen and insulin transport through the blood-brain barrier. *Physiol Behav* **163**, 312-321.

[45] Xaio H, Banks WA, Niehoff ML, Morley JE (2001) Effect of LPS on the permeability of the blood-brain barrier to insulin. *Brain Res* **896**, 36-42.

[46] Banks WA, Dohgu S, Lynch JL, Fleegal-DeMotta MA, Erickson MA, Nakaoke R, Vo TQ (2008) Nitric oxide isoenzymes regulate lipopolysaccharide-enhanced insulin transport across the blood-brain barrier. *Endocrinology* **149**, 1514-1523.

[47] Baura GD, Foster DM, Kaiyala K, Porte D, Jr., Kahn SE, Schwartz MW (1996) Insulin transport from plasma into the central nervous system is inhibited by dexamethasone in dogs. *Diabetes* **45**, 86-90.

[48] Schaffer L, Brand CL, Hansen BF, Ribel U, Shaw AC, Slaaby R, Sturis J (2008) A novel high-affinity peptide antagonist to the insulin receptor. *Biochem Biophys Res Commun* **376**, 380-383.

[49] Leclerc M, Bourassa P, Tremblay C, Caron V, Sugère C, Emond V, Bennett DA, Calon F (2021) Cerebrovascular insulin receptors are defective in Alzheimerˈs disease. *bioRxiv*, 2021.2012.2001.470582.

[50] Nagano H, Ito S, Masuda T, Ohtsuki S (2022) Effect of Insulin Receptor-Knockdown on the Expression Levels of Blood-Brain Barrier Functional Proteins in Human Brain Microvascular Endothelial Cells. *Pharm Res* **39**, 1561-1574.

[51] Brown RS, Wyatt AK, Herbison RE, Knowles PJ, Ladyman SR, Binart N, Banks WA, Grattan DR (2016) Prolactin transport into mouse brain is independent of prolactin receptor. *FASEB J* **30**, 1002-1010.

[52] Banks WA (2004) The source of cerebral insulin. *European Journal of Pharmacology* **490**, 5-12.

[53] Rosenfeld RG, Pham H, Keller BT, Borchardt RT, Pardridge WM (1987) Demonstration and structural comparison of receptors for insulin-like growth factor-I and -II (IGF-I and -II) in brain and blood-brain barrier. *Biochem Biophys Res Commun* **149**, 159-166.

[54] Pan W, Kastin AJ (2000) Interactions of IGF-1 with the blood-brain barrier in vivo and in situ. *Neuroendocrinology* **72**, 171-178.

[55] Urayama A, Banks WA (2008) Starvation and triglycerides reverse the obesity-induced impairment of insulin transport at the blood-brain barrier. *Endocrinology* **149**, 3592-3597.

[56] Dietrich MO, Muller A, Bolos M, Carro E, Perry ML, Portela LV, Souza DO, Torres-Aleman I (2007) Western style diet impairs entrance of blood-borne insulin-like growth factor-1 into the brain. *Neuromolecular Med* **9**, 324-330.

[57] Yu Y, Kastin AJ, Pan W (2006) Reciprocal interactions of insulin and insulin-like growth factor I in receptor-mediated transport across the blood-brain barrier. *Endocrinology* **147**, 2611-2615.

[58] Pulford BE, Ishii DN (2001) Uptake of circulating insulin-like growth factors (IGFs) into cerebrospinal fluid appears to be independent of the IGF receptors as well as IGF-binding proteins. *Endocrinology* **142**, 213-220.

[59] Nishijima T, Piriz J, Duflot S, Fernandez AM, Gaitan G, Gomez-Pinedo U, Verdugo JM, Leroy F, Soya H, Nuñez A, Torres-Aleman I (2010) Neuronal activity drives localized blood-brain-barrier transport of serum insulin-like growth factor-I into the CNS. *Neuron* **67**, 834-846.

[60] May P, Woldt E, Matz RL, Boucher P (2007) The LDL receptor-related protein (LRP) family: an old family of proteins with new physiological functions. *Ann Med* **39**, 219-228.

[61] Orlando RA, Rader K, Authier F, Yamazaki H, Posner BI, Bergeron JJ, Farquhar MG (1998) Megalin is an endocytic receptor for insulin. *J Am Soc Nephrol* **9**, 1759-1766.

[62] Willnow TE, Hilpert J, Armstrong SA, Rohlmann A, Hammer RE, Burns DK, Herz J (1996) Defective forebrain development in mice lacking gp330/megalin. *Proc Natl Acad Sci U S A* **93**, 8460-8464.

[63] D'Arcangelo G (2005) Apoer2: a reelin receptor to remember. *Neuron* **47**, 471-473.

[64] Hama H, Saito A, Takeda T, Tanuma A, Xie Y, Sato K, Kazama JJ, Gejyo F (2004) Evidence indicating that renal tubular metabolism of leptin is mediated by megalin but not by the leptin receptors. *Endocrinology* **145**, 3935-3940.

[65] Carro E, Spuch C, Trejo JL, Antequera D, Torres-Aleman I (2005) Choroid plexus megalin is involved in neuroprotection by serum insulin-like growth factor I. *J Neurosci* **25**, 10884-10893.

[66] Sandin ES, Folberth J, Muller-Fielitz H, Pietrzik CU, Herold E, Willnow TE, Pfluger PT, Nogueiras R, Prevot V, Krey T, Schwaninger M (2021) Is LRP2 Involved in Leptin Transport over the Blood-Brain Barrier and Development of Obesity? *Int J Mol Sci* **22**.

[67] Dietrich MO, Spuch C, Antequera D, Rodal I, de Yebenes JG, Molina JA, Bermejo F, Carro E (2008) Megalin mediates the transport of leptin across the blood-CSF barrier. *Neurobiol Aging* **29**, 902-912.

[68] Mata-Balaguer T, Cuchillo-Ibanez I, Calero M, Ferrer I, Saez-Valero J (2018) Decreased generation of C-terminal fragments of ApoER2 and increased reelin expression in Alzheimer's disease. *FASEB J* **32**, 3536-3546.

[69] Hinrich AJ, Jodelka FM, Chang JL, Brutman D, Bruno AM, Briggs CA, James BD, Stutzmann GE, Bennett DA, Miller SA, Rigo F, Marr RA, Hastings ML (2016) Therapeutic correction of ApoER2 splicing in Alzheimer's disease mice using antisense oligonucleotides. *EMBO Mol Med* **8**, 328-345.

[70] Zhao N, Liu CC, Van Ingelgom AJ, Martens YA, Linares C, Knight JA, Painter MM, Sullivan PM, Bu GJ (2017) Apolipoprotein E4 Impairs Neuronal Insulin Signaling by Trapping Insulin Receptor in the Endosomes. *Neuron* **96**, 115-+.

[71] Zaragoza R (2020) Transport of Amino Acids Across the Blood-Brain Barrier. *Front Physiol* **11**, 973.

[72] Shi Y, Kim H, Hamann CA, Rhea EM, Brunger JM, Lippmann ES (2022) Nuclear receptor ligand screening in an iPSC-derived <em>in vitro</em> blood-brain barrier model identifies new contributors to leptin transport. *bioRxiv*, 2022.2005.2003.490335.

[73] Banks WA, Owen JB, Erickson MA (2012) Insulin in the brain: there and back again. *Pharmacol Ther* **136**, 82-93.

[74] Rhea EM, Salameh TS, Banks WA (2019) Routes for the delivery of insulin to the central nervous system: A comparative review. *Exp Neurol* **313**, 10-15.

[75] Uchida Y, Yagi Y, Takao M, Tano M, Umetsu M, Hirano S, Usui T, Tachikawa M, Terasaki T (2020) Comparison of Absolute Protein Abundances of Transporters and Receptors among Blood-Brain Barriers at Different Cerebral Regions and the Blood-Spinal Cord Barrier in Humans and Rats. *Mol Pharm* **17**, 2006-2020.