Amino Acid Transporters in the Regulation of Insulin Secretion and Signalling

Short Title: Amino Acid Transporters and Insulin Signalling

Kiran Javed[1] and Stephen J. Fairweather[1]*

[1]Research School of Biology, Australian National University, Canberra, ACT, 2601, Australia.

*Corresponding author

Emails: stephen.fairweather@anu.edu.au

Abstract
Amino acids are increasingly recognised as modulators of nutrient disposal, including their role in regulating blood glucose through interactions with insulin signalling. More recently, cellular membrane transporters of amino acids have been shown to form a pivotal part of this regulation as they are primarily responsible for controlling cellular and circulating amino acid concentrations. The availability of amino acids regulated by transporters can amplify insulin secretion and modulate insulin signalling in various tissues. In addition, insulin itself can regulate the expression of numerous amino acid transporters. This review focuses on amino acid transporters linked to the regulation of insulin secretion and signalling with a focus on those of the small intestine, pancreatic β-islet cells and insulin responsive tissues, liver and skeletal muscle. We summarise the role of the amino acid transporter B^0AT1 (SLC6A19) and peptide transporter PEPT1 (SLC15A1) in the modulation of global insulin signaling via the liver secreted hormone Fibroblast Growth Factor 21 (FGF21). The role of vesicular vGLUT (SLC17) and mitochondrial SLC25 transporters in providing glutamate for the potentiation of insulin secretion is covered. We also survey the roles SNAT (SLC38) family and LAT1 (SLC7A5) the amino acid transporters play in the regulation of and by insulin in numerous affective tissues. We hypothesize the small intestine amino acid transporter B^0AT1 represents a crucial nexus between insulin, FGF21, and incretin hormone signalling pathways. The aim is to give an integrated overview of the important role amino acid transporters have been found to play in insulin-regulated nutrient signalling.

Introduction

Amino acids are vital nutrients for sustaining human life and are utilized by many essential biochemical pathways. These pathways depend on amino acid concentrations established using various inputs and outputs: dietary intake, protein synthesis, protein degradation, the synthesis of bioactive molecules, and the catabolism and anabolism of amino acids in different tissues (1). The pools of 20 canonical amino acids also serve as essential mediators of various intracellular and global signalling pathways. Nowhere has interest in amino acids been more apparent than in their emergence as modulators of insulin secretion and as targets of insulin signalling, the latter phenomenon being first recognized several decades ago in rodents and humans (2, 3). More recently, specific roles for amino acids have been elucidated in the regulation and reduction of glucose disposal (4-6), as co-activators of the major nutrient signalling pathway mTORC1 (7-11) and in the potentiation of insulin secretion (12-14). Furthermore, a rise in plasma amino acids, especially branched chain amino acids (BCAAs, i.e. leucine, isoleucine, valine) and aromatic amino acids, have also been tightly associated with insulin resistance (5, 6, 15, 16) and type 2 diabetes (17-20).

The movement of amino acids across the biological membranes of cells is controlled by integral membrane transporter proteins, also known as carriers. As a consequence amino acid transporters are responsible for the modulation of cellular and circulating amino acids concentrations and also, therefore, any amino acids regulating insulin secretion and signalling. Simply stated, anywhere amino acids play an important role in regulating insulin; amino acid transporters will also play a vital role. That said, specific roles for amino acid transporters in insulin regulation is a more recently recognised phenomenon, the elucidation of which was previously hampered by the incomplete catalogue of characterized human amino acid transporters (21-23) and one requiring the increased convergence of two research fields: nutrient signalling and transporter physiology. The amino acid transporters covered here are all solute carriers, utilising the electro-chemical energy of ion gradients to drive metabolite transport in either the same (symporter) or opposite (antiporter/exchanger) direction across a membrane. They can also be facilitated diffusers, using their own substrate electro-chemical gradient to drive transmembrane translocation. The human genome encodes 65 amino acid and peptide solute carriers (1), classified throughout this review by their Solute Carrier (SLC) designation in brackets following the introduction of their common protein name (22, 24). Advances in the characterization of amino acid transporters highlighting their role in the regulation of insulin signalling are summarised in Fig. 1 and Table 1 (25). We begin with the emerging role of small intestine neutral amino acid/peptide transporters B^0AT1 (SLC6A19) and PEPT1 (SLC15A1) in helping to uncover the role of insulin and the hormone Fibroblast Growth Factor 21 (FGF21) in the initiation of dietary amino acid sensing. We then detail the role of vesicular glutamate transporters vGLUTs (SLC17), mitochondrial exchangers (SLC25) and glutamate transporter EAAT2 (SLC1A2) in the amplification of insulin secretion from pancreatic β-cells. Lastly, we examine evidence from amino acid transporters of the SLC38 family and the neutral amino acid exchanger LAT1-4F2hc (SLC7A5-SLC3A2) in regulating liver, skeletal muscle and pancreas insulin responses. We focus on research where a direct link between the regulation of insulin signalling and an amino acid transporter has been made. The purpose is to provide an up to date summary of the role of amino acid transporters in insulin regulated metabolism and, where relevant, their potential as future treatment targets for metabolic disorders such as type II diabetes mellitus (TIIDM).

Epithelial amino acid transporters B^0AT1 (SLC6A19) and PEPT1 (SLC15A1)

Dietary protein restriction has gained increasing interest due to its significant beneficial impact on metabolic health, including improved glucose tolerance, energy expenditure, and reduced body weight (26-28). Conversely, long-term high protein diets have been associated with the onset of insulin resistance (29, 30). Dietary protein restriction curtails the development of insulin resistance and hypertriglyceridemia in mice by instigating the release of the hormone FGF21 in response to reduced hepatic amino acid supply (31, 32). FGF21 is an important metabolic regulator that improves insulin sensitivity, induces ketogenesis and inhibits gluconeogenesis (33-35). Recently, dietary intervention studies in mice highlighted the role of BCAAs in mediating the beneficial effects of protein restriction (36, 37). However, it was later shown that total amino acid restriction, not only BCAA restriction, from the diet was equally important for the beneficial effects of protein restriction. Maida *et al*. showed that BCAA repletion in protein restricted obese and wild type mice restored liver mTORC1 levels but that FGF21 levels remained elevated (37, 38). These results suggested the dietary loss of multiple amino acids, and not just BCAAs, are required to induce FGF21 and its beneficial metabolic effects. mTORC1 is a serine/threonine kinase that plays an important role in amino acid sensing and growth regulation [reviewed in detail (25, 39, 40)]. mTORC1 is considered a negative regulator of insulin signalling in overfed subjects where over-expression of mTORC1 eventually leads to insulin desensitization; mainly due to increase in BCAA supply (41). Dietary restriction by single amino acids, particularly methionine and leucine, also lead to various degrees of upregulated circulating FGF21 (31, 37, 38, 42-44).

Dietary protein is broken down into amino acids and peptides on the apical side of small intestine from where they are absorbed into the blood. The main BCAA transporter of the small intestine is the Broad neutral (^{0}) Amino acid Transporter (B^0AT1) (45, 46) (Fig. 1A). It mediates the uptake of all neutral amino acids[1] at the apical membrane of intestinal epithelial cells with transport driven by the symport of one sodium ion per amino acid (47-51) (Table 1). It transports all neutral amino acids with similar V_{max}[2] values but variable K_m values that range from 1mM to11mM for neutral amino acids (48, 49). BCAAs and methionine show the highest affinity whereas tryptophan is the least preferred substrate. The transporter is trafficked to the intestinal apical membrane by the ancillary proteins Angiotensin Converting Enzyme (ACE2) and, to a lesser extent, Aminopeptidase N (APN) (46, 52, 53). B^0AT1 is also expressed in kidney epithelial cells where it is activated by the ACE2 homolog collectrin (TMEM27) (54-56). B^0AT1 mRNA is also marginally expressed in pancreas, stomach, liver and colon (57-59).

It was not until research beginning in 2011 that B^0AT1 was recognised as the missing component linking dietary neutral amino acid to the beneficial health effects of protein

[1] Neutral amino acids are the 15 of 20 proteinogenic amino acids which have no net elemental charge at physiological pH (7.2-7.4) and include: Leu, Ile, Met, Phe, Val, Trp, Tyr, Ala, Gly, Ser, Thr, Gln, Asn, Cys, Pro.

[2] At various points we use the technical measures of transport kinetics V_{max} and K_m: the former refers to the maximal rate of transport activity (capacity) the latter is the Michaelis constant, the substrate concentration required to induce the half maximal rate measure. Given certain assumptions, the Michaelis constant is a measure of relative transport affinity, with the lower the number the higher the transport affinity.

restriction which are mediated by increased insulin sensitivity and circulating FGF21 levels (60, 61). A global B^0AT1 KO mouse showed reduced weight gain on a high fat diet and reduced expression of downstream mTORC1 targets in the intestine, liver, muscle and adipose tissue, indicative of lost BCAA activation (60, 61). B^0AT1 (-/-) mice exhibited enhanced insulin sensitivity as measured by lower postprandial glucose levels in the absence of insulin secretion. These mice also replicated the effects of protein restriction by inducing the upregulation of FGF21 in liver and serum (60), with FGF21 levels comparable or higher to those observed in protein-restricted mice and rats (62). Induction of FGF21 in mice liver also occurs during dietary methionine and leucine restriction but to lesser extent than the B^0AT1 (-/-) mouse [(63-66) reviewed in (67-69)]. Increased energy expenditure due to methionine restriction has been shown to occur only in male mice (66) but the sex-specific effects of FGF21-mediated protein and AA restriction in humans remain largely unstudied. Both methionine and leucine are major substrates of B^0AT1 and the requirement of multiple neutral amino acids for effective and cumulative FGF21 induction is consistent with the transporter's role as the primary uptake pathway for multiple neutral amino acids. The reduced plasma glucose recorded in these B^0AT1 KO mice seemed to be a result of a 50% reduction in intestinal uptake (61) but without any downregulation of known sugar transporters (70). However, the plasma glucose lowering effect of FGF21 has also been attributed to activation of brown adipose tissue (BAT), the browning of white adipose tissue (WAT), and increased hepatic energy consumption, particularly in obese and male rodents (35, 71-74). The cause of blunted insulin secretion in B^0AT1 (-/-) mice was unknown but possibly due to reduced postprandial glucose levels as a result of FGF21 upregulation or delayed absorption of glucose or a combination of both (see perspectives). As all studies involving B^0AT1-mediated neutral AA restriction have been conducted in mice models caution is warranted when translating these results to human, especially as many of the metabolic effects of FGF21 observed in rodents has not been replicated in primates [reviewed in (75, 76)]. The other significant phenotype of the B^0AT1 (-/-) mouse is the stimulated secretion of the incretin hormones glucagon like peptide (GLP-1) and gastric inhibitory polypeptide (GIP) (60, 77), the role of which in potentiating insulin release is discussed in the next section.

Our lab and others have proposed the effects of dietary protein restriction in the B^0AT1 KO mouse could be replicated by inhibiting B^0AT1, making it a potential target for the alleviation of metabolic disorders that would benefit from dietary restriction of neutral amino acids, such as TIIDM (78) and phenylketonuria (79). Several inhibitors of B^0AT1 have been identified but none yet with clinical significance (78-81). B^0AT1 (-/-) mice displayed hyper-excretion of neutral amino acids in urine and both major substrates of B^0AT1 but plasma levels remained normal, perhaps as a result of decreased oxidation of amino acids (1, 61). Mutations in human B^0AT1 result in Hartnup disorder, which is characterized by malabsorption of amino acids (57-59). Other than the hyper-excretion of neutral amino acids, Hartnup disorder patients are mostly asymptomatic (82), possibly due to modern dietary protein intake being many times the daily requirements of 80-100 g (83). Compensatory uptake is probably mediated by the peptide transporter PEPT1.

As the major small peptide transporter of the small intestine PEPT1 (SLC15A1) provides additional intestinal absorption capacity for neutral amino acids in the form of di- and tri-peptides (84) (Fig. 1A). PEPT1 is a high capacity, low affinity transporter (K_m of 0.2 -10mM) also expressed at limited levels in the colon, kidney, pancreas and bile duct epithelial cells (85-88) (Table 1). It uses the H^+ electrochemical gradient to cotransport peptides (84, 89). Recently, peptide uptake by PEPT1 was shown to stimulate GLP-1 secretion (90) and improved glucose homeostasis in healthy, obese and hyperglycemic mice (91). PEPT1 (-/-) mice also displayed reduced weight gain and fat deposits on a high fat diet (92). In contrast to the phenotype of B^0AT1 (-/-) mice, where increased GLP-1 levels were observed (60), the release of GLP-1 was blunted in PEPT1 (-/-) organoids (90). This discrepancy of GLP-1 levels between PEPT1 (-/-) and B^0AT1 (-/-) mice is most likely explained by the expression of PEPT1 in intestinal L cells and B^0AT1 in intestinal K cells (93). Intestinal L cells are located in distal part of the intestine and are usually referred to as GLP-1 producing cells (94). The absence of PEPT1 in the KO mouse probably ablates the ability of L cells to sense an increased gut protein load and secrete GLP-1 in response. On the other hand, the absence of B^0AT1 would lead to an increase in protein load on the luminal side of the intestine, leading to GLP-1 release from L cells.

β-islet cell glutamate transporters vGLUT (SLC17 family)

Both cytosolic glutamate and leucine can potentiate insulin secretion from pancreatic β-islet cells (95-101). The availability and effect of glutamate is determined by the complex intersection between glucose and amino acid metabolism, including the control of glutamate flux by various intracellular membrane transporters (102, 103). Although its role was previously questioned (104-107), intracellular glutamate is now thought to provide a crucial intersection between glucose- and incretin-stimulated insulin secretion (100). Critical players in this mechanism are the human vesicular glutamate (GLUT) transporters vGLUT1 (SLC17A7) and vGLUT2 (SLC17A6), which are responsible for loading glutamate into intracellular vesicles – a necessary event in the potentiation of insulin secretion (108, 109) (Fig. 1E). vGLUT1 and 2 are highly selective L-glutamate transporters (110, 111) with a K_m of 1-5 mM or slightly lower (112-121) and they require Cl^- for transport activity (110-114, 119-123) (Table 1). First identified as neuronal transporters, vGLUT2 has also been located outside the CNS in primary afferent neurons of the intestine and in pancreatic β-cell (109, 120, 124-126). In β-islet cells, it is vGLUT1 and 2-mediated loading of insulin granular vesicles that is essential for the potentiation of insulin secretion (100, 108, 109).

The primary stimulus for insulin release is the uptake of glucose into β-cells by the facilitated diffuser GLUT2. This stimulus sets off a regulatory cascade terminating in insulin release at the plasma membrane. Potentiation of this process by glutamate begins with the release of the incretin hormones GLP-1 and GIP from enteroendocrine L and K cells following a meal (127-130). Circulating GLP-1 and GIP bind the β-cells plasma membrane incretin receptor, leading to the cyclisation of AMP (cAMP) and instigating a PKA signalling cascade (131, 132) (Fig. 1E). PKA has numerous targets involved in recruitment and fusion of insulin granules to the membrane (133, 134). Gheni *et al*. identified cytosolic β-cells glutamate as an essential signal mediating incretin-induced potentiation of insulin secretion in rodents models

of diabetes, obesity and in insulin-producing cell lines (109, 135). Incretin signalling induced the loading of glutamate into secretory granules, which was subsequently necessary for enhanced insulin release. Furthermore, vGLUT1 was required for elevating glutamate concentrations in insulin-containing granules; an event that also preceded insulin release (108, 109, 135). This amplification of insulin secretion did not occur when vGLUT1 is absent from β-cells as demonstrated by using a SLC17A7 (-/-) mouse (109); vGLUT2, however, can compensate for the loss of vGLUT1 (108). Interestingly, the source of cytosolic glutamate required for potentiation was provided by the mitochondrial malate-aspartate shuttle, which indicated the important role of mitochondrial-cytosol glutamate flux in the process (see following section). Membrane permeable analogues of glutamate were shown to be sufficient to enhanced insulin secretion, demonstrating the presence of the amino acid as necessary and sufficient in the pathway (109). Glutamate-mediated potentiation of insulin secretion is impaired very early in the development of TIIDM in humans and rodent models (109, 136-140).

How glutamate uptake into secretory granules amplifies insulin secretion remains unresolved and is hampered by a lack of understanding of the transport mode vGLUT1 uses to load vesicles. Under most experimental conditions the membrane potential ($\Delta\psi$) is the major driving force (111-113, 121, 123, 141), suggesting the uniport of anionic glutamate. However, the proton gradient established by the V-ATPase can also drive vGLUT function, consistent with a glutamate:H^+ antiport mechanism (114, 121-123, 141). However, no testing of how the extent of glutamate loading or the physical properties of vesicles themselves effect insulin secretion has been conducted. The elevation of cytosolic glutamate required for secretory granule loading establishes the source of glutamate as an important question and highlights the roles of other β-cells amino acid transporters that will now be addressed.

Mitochondrial exchangers (SLC25 family): role in β-cells glutamate availability

Despite earlier evidence for direct glutamate uptake by plasma membrane transporters (142, 143), substantial research now suggests synthesis from glucose as the major source of cytosolic glutamate in β-cells (109, 144, 145). Intracellular β-cell glutamate can be synthesised from D-glucose via TCA cycle α-ketoglutarate by utilising cytosolic/mitochondria aspartate aminotransferase 1 (AST1) or mitochondrial glutamate dehydrogenase (GDH) (Fig. 1E). Glutamine has also been proposed as a significant alternative source of cytosolic glutamate (103, 108, 146). Both glutamate and glutamine provide reducing equivalents for NADH production in the TCA cycle and ultimately the synthesis of ATP to facilitate glucose-stimulated insulin secretion via closure of the K_{ATP} channels [reviewed in (147)]. As a result, mitochondrial inner membrane carriers play a pivotal role in the maintenance of cytosolic and mitochondrial glutamate concentrations in β-cells required for potentiation of insulin secretion.

Glutamate transport across the mitochondrial inner membrane occurs via the malate-aspartate shuttle and the mitochondrial Glutamate Carrier (SLC25A22, GC1) (100, 108, 109, 147-154) (Fig. 1E). The malate-aspartate shuttle involves two mitochondrial transporters: the malate-α-ketoglutarate antiporter (OGC, SLC25A11) and Aspartate Glutamate Carrier 1 (AGC1,

SLC25A12). Both exchangers are widely expressed, AGC1 more heavily in electrically excitable tissue but absent from liver, while GC1 is prominent in the liver and pancreas but only weakly expressed in the brain (155) (Table 1). The physiological role of GC1 is to import cytosolic glutamate into the mitochondria along with H^+ symport or OH^- antiport. Hence, GC1-mediated transport it is electroneutral and exhibits a relatively high K_m of ~4-5 mM (154). AGC1 exchanges an aspartate from the mitochondrial matrix with a glutamate and a proton from the cytosol (156). The exported aspartate is converted by an aminotransferase GOT1 into oxaloacetate. Oxaloacetate in turn is converted into malate which serves as an exchange substrate of OGC. Upon transport back into the mitochondrial matrix, malate provides the reducing equivalent to produce NADH for oxidative phosphorylation. Glutamate is co-transported with a proton by AGC1, meaning each transport cycle is electrogenic if aspartate-glutamate are exchanged but is electroneutral if identical substrates are exchanged. When mitochondria are respiring glutamate uptake is heavily favoured by AGC1 and GC1, due to strong negative potential and pH chemical gradient components of the proton motive force across the mitochondrial inner membrane (157).

Both GC1 and AGC1 play substantial roles in glutamate-induced insulin secretion (145, 153, 158-161). Although the increase in cytosolic glutamate from mitochondrial glucose oxidation has been demonstrated by several labs (99, 100, 109, 148, 158, 162), the idea remained controversial for some time [(see (104, 163)]. Several lines of evidence have now confirmed mitochondrial glutamate synthesis as vital for the potentiation of β-cell insulin secretion. These are, 1) the appearance of cytosolic [^{13}C]labelled glutamate isotopomers from [U-^{13}C] glucose in metabolic flux experiments (160, 164); 2) inhibition or genetic silencing of AGC1 and malate-aspartate shuttle enzymes (108, 148, 159, 165); 3) GC1 ablation (158); 4) demonstration of glutamate dehydrogenase (GDH) as a key enzyme in mitochondrial glutamate synthesis for the potentiation of insulin secretion (151, 166, 167). The physiological functioning of GC1 and AGC1 in respiring mitochondria leads to a net entry of glutamate into the mitochondrial matrix for GC1 and the recycling of glutamate as part of the malate-aspartate shuttle for AGC1. Glutamate production in the mitochondrial matrix by GDH could potentially provide an increase in cytosolic glutamate by increasing the overall rate of the shuttle and increasing the apparent steady-state concentration of all intermediates, including glutamate. Indeed, the evidence demonstrating a net accumulation of cytosolic glutamate from the malate-aspartate shuttle seems convincing (108, 109). However, an alternative mechanism for net glutamate production hypothesized mitochondria-exported isocitrate is oxidized in the cytosol to form α-ketoglutarate, which then undergoes transamination probably by utilising aspartate. This reactions series would provide an additional input into the malate-aspartate shuttle which would explain the simultaneous decrease of aspartate and increase of cytosolic glutamate following glucose stimulation of islet β-cells (168). The importance of GDH for glucose-stimulated glutamate synthesis as a singular source for enhanced insulin secretion is also contradicted by several studies (168-170). For example, Li *et al*. (168) found in human GDH transgenic mice that both glutamine and oxidative deamination of glutamate by leucine-stimulated GDH was associated with enhanced insulin secretion, a process inhibited by high glucose. These results suggested

alternative pathways for enhanced insulin secretion besides glucose-derived glutamate (see SLC38 and LAT1 sections).

It is important to emphasize these mitochondrial carriers are vital for potentiation of insulin secretion but only indirectly through their role in balancing cytosolic to mitochondrial glutamate concentrations. Insulin secretion can be enhanced simply by the elevation of cytosolic glutamate independently of the source of that glutamate (104, 151, 163). For example, supplementation of cytosolic glutamate using a membrane-permeable analogue enhances insulin secretion independently of glutamate supplied by the mitochondria (98, 99, 101, 151).

Other β-islet cell glutamate transporters: EAAT2 (SLC1A2)

mRNA transcripts and protein of the glutamate transporter EAAT2 (SLC1A2) have been detected in the plasma and secretory granular membranes of β-cells (124, 125, 171, 172). The transporter is part of the SLC1 family of excitatory amino acid and small neutral amino acid transporters (173). Primarily expressed in neuronal glial cells, it is responsible for the reuptake of glutamate from excitatory synapses and accounts >90% of total glutamate uptake in the brain (174) (Table 1). EAAT2 accumulates glutamate using the symport of 3 Na^+ molecules and one proton in exchange for one K^+ molecule. It can transport both C_α enantiomers of aspartate in addition to glutamate and also mediates an anion conductance, which is thermodynamically uncoupled from glutamate transport (175-179).

EAAT2 has been hypothesised to play two roles in the mechanism of incretin-dependent insulin secretion (180) (Fig. 1E). Feldmann *et al.* showed potentiation of insulin release resulted from inhibition of EAAT2, which implied the transporter was effluxing glutamate at the plasma membrane (148). This reversal of the transporter from its normal role accumulating glutamate is difficult to reconcile with the known thermodynamic drivers of EAAT2 [see (173)]. A second potential role of EAAT2 was proposed by Gammelsaeter *et al*. who showed that EAAT2 was co-expressed with vGLUT3 (SLC17A8) in secretory granules (172). An EAAT2 KO mouse displayed increased granular glutamate concentration but decreased rate of insulin exocytosis. The authors hypothesised a co-ordinated role of EAAT2 and vGLUT3 in recycling glutamate through secretory granules to mediate insulin secretion (181). Any role for EAAT2 in insulin secretion is, however, strongly disputed (145). A pancreatic-specific knockout of EAAT2 revealed no effect on β-cell viability or glucose-stimulated insulin secretion (145). Furthermore, no EAAT2 protein or mRNA was detected in β-cells.

SLC38 family of neutral amino acid symporters

SLC38 family transporters mediate sodium dependent influx and efflux of small neutral amino acids in all human tissues (182). Three SLC38 members, SNAT2 (SLC38A2), SNAT3 (SLC38A3) and SNAT5 (SLC38A5) play direct roles in the regulation and mediation of insulin signalling. SNAT2 is a ubiquitously expressed sodium-dependent symporter of, predominately, small neutral amino acids alanine, serine, glycine, and cysteine but can also transport glutamine, asparagine, methionine, proline, and histidine (183, 184) (Table 1). SNAT2 is regulated by various metabolic signals such as amino acid availability, amino acid

starvation, hypertonic stress, and insulin (185-190). The increase of amino acid uptake by insulin was first identified in rat skeletal muscle (185, 191) but was subsequently shown to stimulate plasma clearance of leucine in humans (3). Both increased glutamine uptake and SNAT2 plasma membrane expression occurred via rapid trafficking of transporter-containing endosomal pools to the plasma membrane following exposure to insulin in rat myocytes (185, 186, 192) (Fig. 1D). Essential amino acids also increased SNAT2 mRNA expression in humans in an mTORC1-dependent manner (193). Due to the co-stimulatory effects of insulin and leucine on mTOR signalling (9), these results suggest a common activation pathway for increased SNAT2 expression. Despite the evidence for insulin-induced increase of glutamine uptake (185, 186) earlier insulin-induced increases in plasma clearance for non-SNAT2 substrates [e.g. leucine (3)] have not been explained. The upregulation of other amino acid transporter mRNA such as LAT1 (SLC7A5) which could transport leucine has been noted in myocytes (193). For an extensive overview into the role of SNAT2 and amino acids in skeletal muscle metabolism the reader is directed to several excellent reviews (194, 195).

SNAT2 also plays a potential role in the development of ER stress response in pancreatic β-islets during the progression of TIIDM in mice (196) (Fig. 1E). Peripheral tissue insulin resistance increases β-cell synthesis of insulin and cell proliferation, thereby inducing the ER stress. The unfolded protein response (UPR) is also induced as a direct result of increased insulin synthesis (197). Prolonged UPR activation leads to apoptosis, which limits circulating insulin levels and leads to TIIDM (5). To overcome the translational repression associated with ER stress, an anabolic transcriptional pathway upregulates the expression and activity of SNAT2 and other transporters. The upregulation of SNAT2 was termed ‘self-defeating’ as the response leads to increased protein synthesis, thereby aggravating the ER stress, subsequent apoptosis of β-cells and exacerbating TIIDM progression (196).

SNAT3 is a Na^+-dependent symporter and H^+ antiporter, transporting glutamine, asparagine and histidine with high expression in liver, kidney, rat brain, adipose tissue, eye and muscle (182, 198) (Table 1). In the liver, SNAT3 is involved in the glutamine uptake from periportal hepatocytes and release from the perivenous hepatocytes into the circulation (199) (Fig. 1B). Glutamine plays an important role in energy metabolism in the liver as one of the major sources for gluconeogenesis via α-ketoglutarate and the TCA cycle (200). Ablation of SNAT3 in mice reduced the amount of intracellular glutamine and spared glutamine from gluconeogenesis due to the reduction in liver glutaminase 2 protein levels; the first step in the conversion pathway (201). Reduction in gluconeogenesis is the probable cause of reduced plasma glucose (< 2.8mM) and subsequent decreased insulin levels. Unexpectedly, plasma glutamine levels in these mice seem to be unaltered whereas intracellular leucine levels were reduced (201). One explanation for these results is that in WT animals, intracellular glutamine accumulation through SNAT3 is utilized as a efflux substrate by LAT1 in exchange for the uptake of leucine (202). Hence the loss of glutamine uptake in SNAT3 (-/-) mice resulted in a lost capacity for leucine accumulation. The decreased levels of intracellular leucine and plasma insulin also explain the reduced expression of downstream mTORC1 pathway targets in SNAT3 (-/-) mice, which could not be expected from glutamine alone as it is not a known activator of mTORC signalling (201). Consistent with a significant role for SNAT3 in insulin-mediated gluconeogenesis, direct insulin application onto WT mouse hepatocytes displayed decreased SNAT3 mRNA (203). This downregulation would reduce

the pool of glutamine available for hepatic glucose production. However, such a link may be tissue specific as insulin perfusion does not affect SNAT3 function in muscle (204). SNAT3 has also been reported as playing a role in β-cell glutamine acquisition (205). In this context, SNAT3 may provide an additional extracellular source of glutamate for β-islet cells as a consequence of the intra-cellular conversion of glutamine to glutamate (see β-cell sections) (102, 206, 207).

SNAT5 has similar properties to SNAT3, also co-transporting 1 Na^+ ion in exchange for a proton. It accepts glutamine, asparagine, histidine and alanine as major substrates and is expressed in intestine, kidney, retina, lung, pancreas and cervix (208). It also has been shown to be responsible for the uptake of glutamine in hepatocytes (208, 209). SNAT5 has raised recent interest due to a role in the regulation of amino acid homeostasis by α-cell-liver glucagon signalling. We include this topic here because glucagon and insulin actions are intricately linked in the regulation of global metabolism. Elevated serum glucagon is also symptomatic of TIIDM progression (210, 211). SNAT5 was shown to be upregulated at the plasma membrane of α-cells in an mTORC1-dependent manner (212, 213). This upregulation was caused predominately by elevated circulating levels of glutamine and alanine, which induces increased glucagon secretion and expansion (hyperplasia) of α-cell numbers (212, 214). The more general elevation of circulating amino acids was induced by inhibition of the G-protein Coupled Glucagon Receptor (GCGR) in the liver. The normal activation of the GCGR in hepatocytes controls liver gluconeogenesis utilising amino acid catabolism in the process. Therefore, inhibition of the GCGR leads to a downregulation of amino acid uptake and catabolism, and the concurrent elevation of plasma amino acid levels (212, 214, 215). The process is hypothesised to work as a feedback loop for glucagon, linking amino acid utilisation in the liver to the sensing of certain elevated amino acids by α-cells in order to kick start further glucagon release. This was confirmed by demonstrating that both GCGR (-/-) and SNAT5 (-/-) mice independently caused hyperplasia of pancreatic α-cells as the SNAT5-dependent uptake of amino acids activates mTORC1 leading to cell proliferation and compensatory additional glucagon release (212, 215). Unexplained by current understanding of this mechanism is how the SNAT5 substrates alanine and glutamine stimulate α-cells proliferation when they are not known activators of mTORC1. It is unknown if inhibiting SNAT5 may represent a potential treatment to alleviate hyperglycaemia as knocking out the transport in mice reduces α-cell mass and glucagon secretion (212, 216) and the interference with long-term glucagon signalling may entail adverse consequences [see (210)]. One publication has also implicated SNAT5 in β-cells as playing a substantial role in increasing intracellular glutamate and incretin-enhanced insulin release –potentially another alternative pathway for β-cells acquisition of glutamate (146) (see β-cell sections). Intracellular glutamine may also serve as an efflux substrate for the neutral amino acid exchanger LAT1 in exchange for leucine.

The neutral amino acid exchanger LAT1-4F2hc (SLC7A5-SLC3A1)

LAT1 (SLC7A5) is a major transporter of BCAAs in many non-epithelial cells and is involved in insulin signalling, including insulin secretion directly through its cytosolic accumulation of leucine (217) (Fig. 1E). LAT1 is a heteromeric amino acid transporter

requiring the ancillary subunit 4F2hc (SLC3A2) (218). It exchanges neutral amino acids more rapidly than accumulative Na^+-dependent amino acid transporters and is best viewed as a 'harmoniser' of cytosolic BCAA concentrations (1, 45, 219) (Table 1). The apparent affinity for large neutral amino acid such as leucine, isoleucine and methionine *in vitro* is 100-fold higher on the cytosolic side compared to the extracellular side (220). LAT1 expression is ubiquitous but specifically noted in the brain, spleen, placenta, testis, colon, pancreas, adipocytes, and skeletal muscle (221-223).

It has been recently proposed that elevated levels of circulating BCAAs in TIIDM patients could be due to downregulation of BCAAs catabolism in visceral adipose tissue and liver (224, 225). The increased levels of BCAAs act as anaplerotic substrates and cause an overload of mitochondrial substrates, which in turn could downregulate oxidation of fatty acids in muscle (226, 227). Since LAT1 is the major transporter controlling intracellular BCAA concentrations in adipocytes, it is likely to play an important role in the efflux of BCAAs resulting from any downregulated catabolism (Fig. 1C). One study also highlighted the role of LAT-1 in muscle tissue by showing that glucose can reduce the mRNA levels of LAT1 in myocytes after inactivating the regulator of cellular energetics, 5' adenosine monophosphate-activated protein kinase (AMPK) (228). Downregulation of LAT-1 could reduce the uptake of BCAAs in skeletal muscle and increase BCAA levels in plasma. This hypothesis requires testing *in vivo* to confirm its physiological significance.

Expression of LAT1 in β-cells facilitates leucine uptake, which acts as an allosteric activator of Glutamate Dehydrogenase (GDH) (229, 230), enhancing insulin secretion via increased glutamate mitochondrial production and islet cell proliferation (151, 217, 231). Activation of mTORC1 signalling is also promoted by LAT1-mediated leucine uptake into β-cells (217). The originally identified activator of GDH was the leucine analogue, and LAT1 substrate/inhibitor, BCH (2-Aminobicyclo[2.2.1]heptane-2-carboxylic acid) (229, 232-235). The importance of leucine-mediated GDH activation to insulin secretion is confirmed by children with Hyperinsulinemia Hypoglycaemia Familial 6 (HHF6) syndrome (102, 167, 236, 237) (OMIM 606762). Patients exhibit abnormal insulin secretion caused by dominant activating mutations in GDH, which replicates leucine-induced GDH potentiation of insulin secretion and can be exacerbated by leucine-induced hypersecretion of insulin. A likely candidate of obligatory efflux via LAT1 is glutamine, which is present at high cytosolic concentrations (238) (Fig. 1E). LAT1 and other SLC7 heteromeric amino acid transporters may also be directly responsible for mTORC1 activation due to their ability to be re-directed from the plasma membrane to lysosomes by the proteins LAPTM4b and girdin (239, 240). The activation of GDH by leucine is well correlated with an increase in β-cell glutaminolysis, suggesting glutamine availability is being increased (229, 232, 241).

Perspectives and future directions

Several lines of research over the past 20 years have demonstrated amino acid transport plays a significant role in modulating the stimulation and sensitivity of insulin in numerous tissues. One unresolved question is why incretin hormone release in the B^0AT1 KO mouse does not lead to a stimulation of insulin release from the pancreas, as would be expected by our

understanding of the canonical mechanism of the incretin-insulin axis? We hypothesize this could be due to the actions of increased circulating FGF21 in B^0AT1 KO mice (Fig. 2). Although direct administration of FGF21 was shown to stimulate insulin secretion in diabetic mice (242), no mechanism directly linking protein-restricted FGF21 levels and β-cell signalling has been established to our knowledge. Any such mechanism could form part of a wider adaptive response to protein restriction orchestrated by FGF21-insulin signalling cross-talk (243, 244). The insulin sensitization effect of FGF21 results from enhanced glucose utilisation by white adipose (WAT) and cardiac tissue without any increase in plasma insulin concentration (43, 66). A very similar phenotype is observed in B^0AT1 KO mice (60, 61). Increased insulin sensitivity thus requires secretion of FGF21 due to upstream restriction of neutral AAs, or a caloric:protein imbalance sensed by the liver. This mechanism is supported by the unique endocrine role of FGF21 being induced by both protein restriction (28, 31, 37, 43, 44, 60, 245, 246) and carbohydrate overload (247, 248) but not caloric variation *per se*, suggesting the imbalance in caloric:protein dietary ratio as the underlying stimulus (27, 68, 249). It appears likely that most of the beneficial and anti-type II diabetic effects observed in B^0AT1 KO mice are mediated by FGF21 and these effects are also missing from FGF21-deficient mice (28).

Due to its established signalling roles in adipose tissue and the hypothalamus (67, 68), it is also possible FGF21 suppression of insulin secretion is mediated through indirect autonomic nervous system or endocrinal signals, with FGF21 binding to a tissue-specific Fibroblast Growth Factor Receptor (FGFR)-β-Klotho receptor sub-type (250). Alternatively, the lack of incretin effect could be caused by reduced leucine uptake in B^0AT1 KO mice, or reduced β-cell mTORC1 signalling, and subsequent lack of allosteric GDH activation. The increase insulin sensitivity observed in the absence of B^0AT1 may be partially caused by a reduction of serum glucagon levels and resulting reduction in liver gluconeogenesis.

The integrated regulation of insulin signalling and potential existence of an unexplored FGF21-amino acid-insulin pathway is interesting but hardly the only unresolved research problem raised by this review. The exploration of the role of various amino acid transporters in insulin-affected tissues such as skeletal muscle, adipose and liver, has barely begun and many novel areas of research connecting amino acid transporters to insulin controlled metabolism remain to be investigated. The necessity of mTORC1 signalling in conjunction with insulin response pathways in many cells makes amino acid transporters such as LAT1 and SNATs obvious focus for future research. The liver itself could represent the site for integration of amino acid endocrine signalling networks that signal energy balance and restriction of protein independent of dietary caloric content. As amino acid transporters represent the primary entry pathway into most tissues and are often metabolic bottlenecks, they represent many of the missing links in signalling networks that respond to protein restriction or modulate the downstream effect of insulin, FGF21 and other global endocrine hormones. Many of the important transporter-mediated effects outlined in this review have not yet been verified as biologically relevant in humans, for example, the research findings on B^0AT1, vGLUTs and SNAT5 in α-cells are so far confined to rodent models. As a result the important roles several amino acid transporters play in global insulin signalling and the

significant role of amino acids in global metabolism mean this field is likely to become increasingly significant for human medical research in coming years.

Table 1. Amino acid transporters involved in the regulation of insulin secretion and signaling.

Transporter	Gene	Accession number (UniProt)	Location	Substrates	Tissue Expression	Mechanism	KO Phenotype Relevant to Insulin Signalling	Ref
B^0AT1	SLC6A19	Q695T7	PM	All neutral	intestine, stomach, kidney, liver, prostate	S: $1Na^+$	Upregulated FGF21, GLP-1, GIP-1 Reduced body weight, postprandial glucose levels, insulin secretion, Altered gut microbiota	(60, 61)
PEPT1	SLC15A1	P46059	PM	di, tri peptides	small intestine, kidney, pancreas, bile duct, liver	S:H^+	Reduced body weight gain on HFD, blunted GLP-1 secretion, reduced fat stores	(92, 251)
vGLUT1	SLC17A7	Q9P2U7	V	E	brain (neurons only), endocrine	U or A: H^+ $(Cl^-)^*$	Reduced incretin-mediated insulin secretion in global KO of vGLUT1. β-cell specific KOs shown vGLUT1 and 2 are redundant and can both compensate for incretin – induced insulin secretion in a triple KO of all 3 vGLUTs	(108, 109)
vGLUT2	SLC17A6	Q9P2U8	V	E	brain (neurons only), endocrine	U or A: H^+ $(Cl^-)^*$		
$EAAT2^{\pm}$	SLC1A2	P43004	PM	D,E	Brain (astrocytes, Bergmann glia, neurons), liver, pancreas	S: $3Na^+/1H^+$ A:$1K^+$	Contradictory results. Glutamate content in secretory granules is higher than in WT mice. β-cell specific KO mice show no effect.	(145, 172)
GC1	SLC25A22	Q9H936	Mitochondria IM	E,D	Ubiquitous	S: $1H^{+\dagger}$	No KO phenotype	(158)
AGC1	SLC25A12	O75746	Mitochondria IM	E,D	Heart, skeletal muscle, brain, kidney, pancreas[L-]	A:H^+ $(Ca^{2+})^{\ddagger}$	Deficiency in humans and mice leads to severe neurological symptoms; no pancreas-specific KO reported	(153, 156, 159)
SNAT2	SLC38A2	Q96QD8	PM	A,S,G,C,Q,N, H,P	Ubiquitous	S:$1Na^+$	Sub-lethal due to cyanotic dyspnea	(252)

SNAT3	SLC38A3	Q99624	PM	Q,N,H	Eye, liver, kidney, brain, pancreas, adipose, skeletal muscle	S:Na^+ A:H^+	Stunted growth, hypoglycaemia, reduced hepatic amino acids, urea cycle dysregulation, death at 20 days	(201, 205)
SNAT5	SLC38A5	Q8WUX1	PM	Q,N,H,A	stomach, brain, liver, lung, small intestine, spleen, colon, kidney	S:Na^+ A:H^+	Minimal abnormal phenotype, pancreatic α-cells proliferation inhibited during glucagon stimulation	(146, 212, 214)
LAT1-4F2hc	SLC7A5-SLC3A2	Q01650	PM	H,M,L,I,V,F, Y,W	pancreatic β cells, brain, ovary, testis, placenta, spleen, colon, blood-brain barrier, foetal liver, activated lymphocytes, tumour cells	A	Lethal phenotype	(217, 253)

Abbreviations: PM = Plasma membrane, V = Vesicular (membranes), IM = Inner Membrane; S = symport, A = antiport, U = uniport

* Whether the transport mechanism involves proton antiport is suggested but unproven [see (254, 255)]. The stoichiometry and exact role of chloride as a substrate remains unclear.

± Role in β-islet cells is disputed.

† Also displays glutamate: glutamate exchange which limits the maximal rate of proton-equivalent transport.

⊢ Pancreas expression is low despite its functional importance; AGC2 (SLC25A13) has higher pancreatic expression, see (155).

‡ Exchanges cytosolic glutamate and a proton for mitochondrial aspartate under respiring conditions; depends of Ca^{2+} for substrate release.

Figure 1. **Amino acid transporters in the regulation of insulin signalling**.
A: Dietary intake gives rise to neutral amino acids (AA) and peptides that are first taken up by B^0AT1 and PEPT1, respectively from the lumen of the intestine. Intestinal L cells secrete GLP-1 & GIP due to increase in neutral AA or nutrients in general. Neutral AAs are then released into the circulation through LAT-1. **B&C**: BCAAs and other AAs can activate mTOR/S6K pathway that results in insulin desensitization under nutrient stress. Glutamine can serve as substrates of GNG and contribute to EGP in the liver. On the other hand, low levels of AA can decrease mTOR/S6 signalling and upregulate FGF21 that can subsequently increase Glc uptake and browning of WAT in adipose tissue after binding to FGFR1-4/β-Klotho. **D**: In muscle, Insulin upregulates SNAT2 expression through endosomal pools. LAT1 and SNAT3 may also regulate intracellular AA levels that could regulate the uptake of glucose for storage. **E**: In pancreas, SNAT3/5 mediates the uptake of glutamine which could provide a potential source of glutamate and finally loaded into vesicles by vGLUT1/2. The efflux of glutamate by EAAT2 is controversial (see text). SNAT2 is upregulated during ER stress in response to hyper-production of insulin. Leucine entering pancreas through LAT1 allosterically regulates the production of glutamate in mitochondria. Glutamate is pumped out in the cytosol through AGC1 and can stimulate the secretion of insulin by glucose and hormonal mediated pathways. Black lines indicate the route and pathways. Broken lines indicate an as yet unknown mechanism; upward arrow indicates upregulation or increased levels. Abbrev. αKG, alpha ketoglutarate; AA, amino acid; BCAA, branched-chain amino acid; cAMP, cyclic adenosine monophosphate; Cl, chloride ion; EGP, endogenous glucose production; FGF21, fibroblast growth factor 21; FGFR, fibroblast growth factor receptor; Glc, glucose; GDH, glutamate dehydrogenase; GIP, gastric inhibitory peptide; GLP-1, glucagon like peptide 1; Gln, glutamine; Glu, glutamate; GNG, gluconeogenesis; g6p, glucose- 6-phosphate; H, hydrogen ion; mTOR, mammalian target of rapamycin; Na, sodium ion; pKA, protein kinase A; TCA, tricarboxylic acid cycle; WAT, White adipose tissue

Figure 2. Global integration of dietary amino acid transport and FGF21-insulin networks. Reduced uptake of neutral amino acids in the lumen of the intestine by B^0AT1 causes protein restriction and secretion of FGF21 in the liver. FGF21 is the major mediator behind the global effects of protein restriction resulting in increased glucose uptake in heart and white adipose tissue (WAT) and browning of WAT. We hypothesize (?) that FGF21 acts directly at the pancreatic β-cells or indirectly through neuronal-endocrinal mechanisms to suppress insulin secretion at the same time as increasing insulin sensitivity through its known actions in cardiac and adipose tissue.

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Abbreviations
AMPK, 5’ adenosine monophosphate-activated protein kinase; AA, Amino acids; ACE2, Angiotensin converting enzyme 2; BAT, brown adipose tissue; BCAA, Branched chain amino acid; BCH, 2-Aminobicyclo[2.2.1]heptane-2-carboxylic acid; CNS, Central Nervous System; ER, Endoplasmic Reticulum; FGF21, Fibroblast Growth Factor 21; FGFR, Fibroblast Growth Factor Receptor (1-4); GCGR, G-protein Coupled Glucagon Receptor; GDH, Glutamate Dehydrogenase; GIP, Gastric Inhibitory Peptide; GLP1, Glucagon like peptide 1; HHF6, Hyperinsulinemia Hypoglycaemia Familial 6 syndrome; IGF1, Insulin-like growth factor 1; KO, Knock out; mTORC, mammalian target of rapamycin; PKA, Protein kinase A; TIIDM, Type II Diabetes Mellitus; WAT, white adipose tissue.

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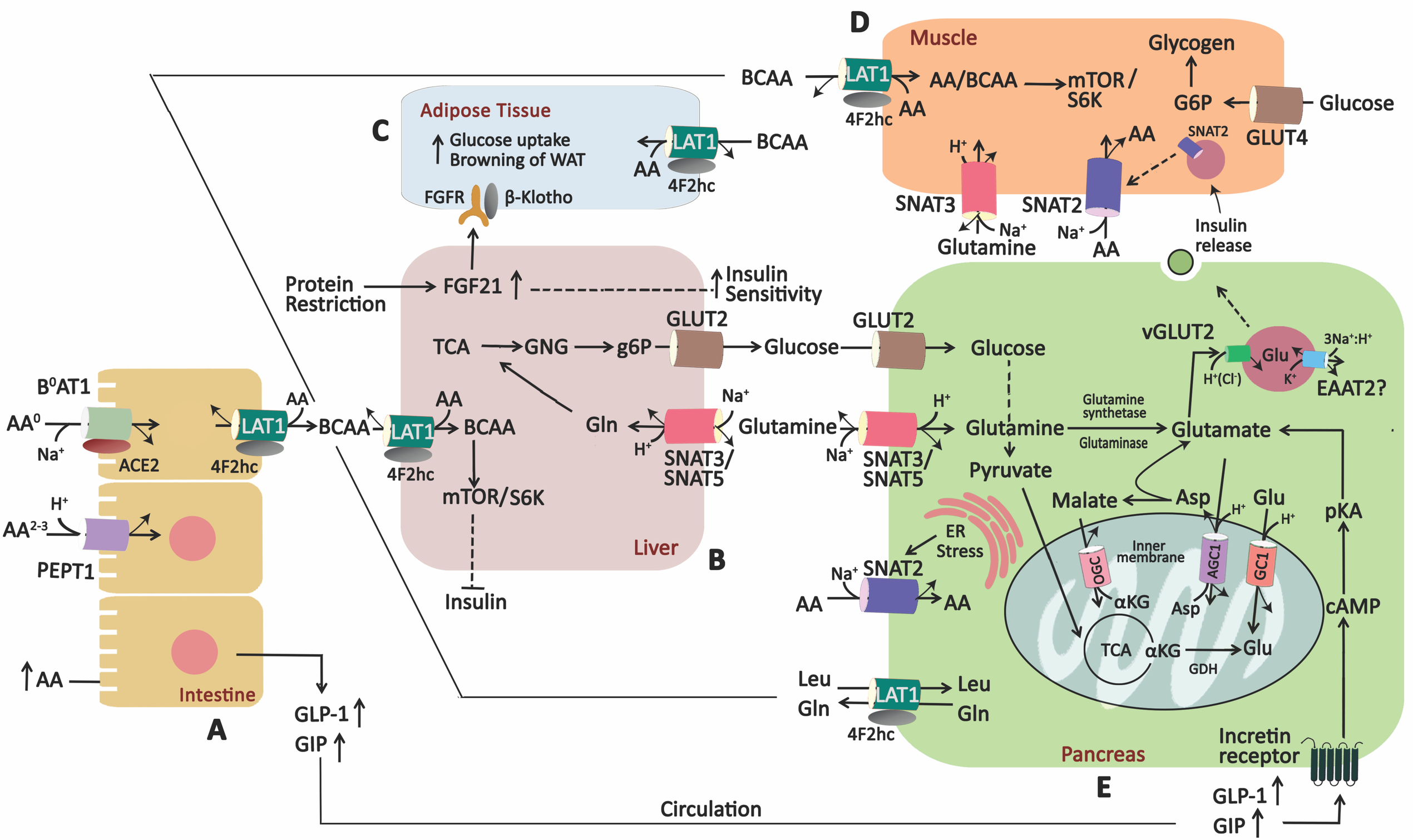
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Protein Restriction
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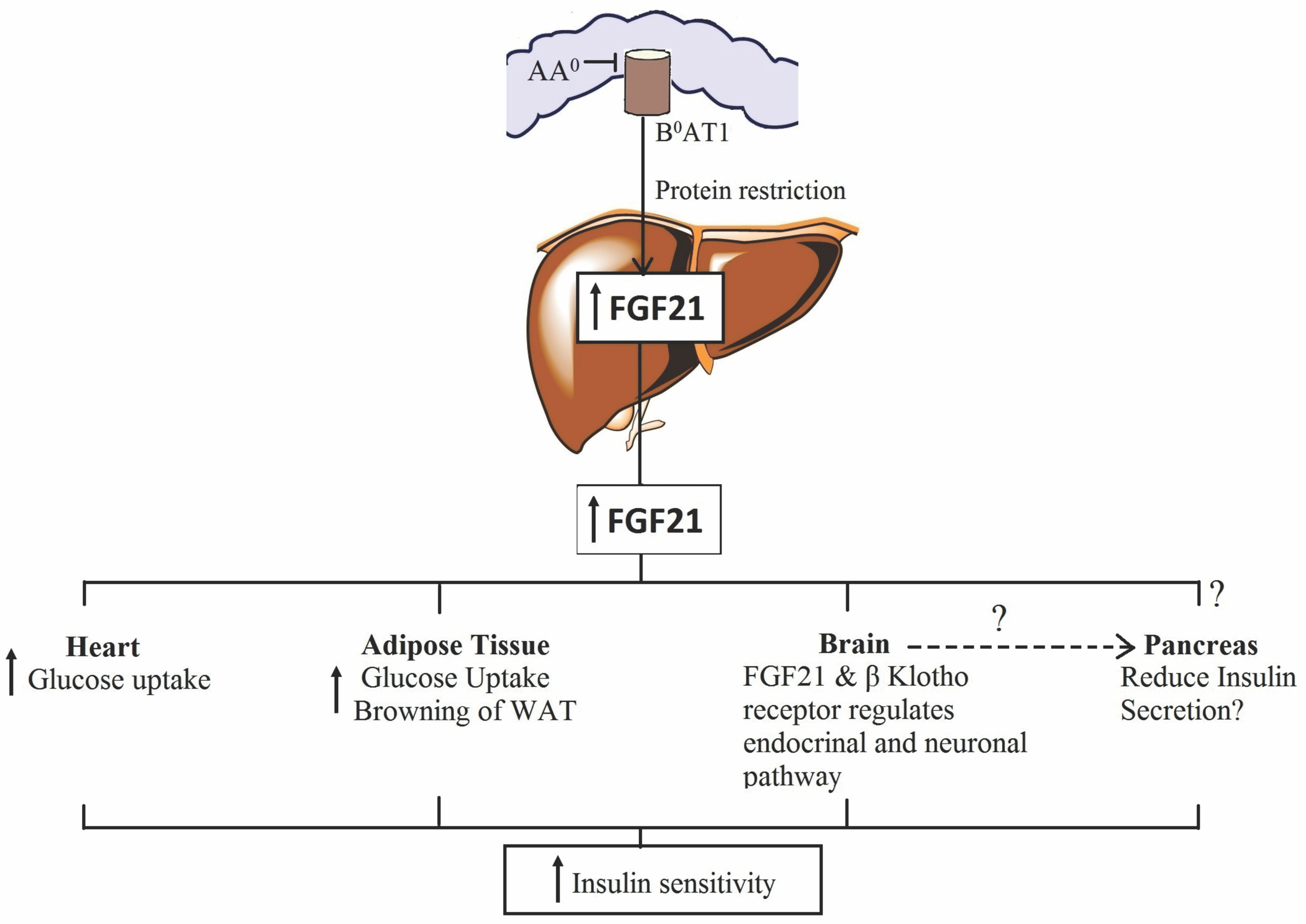
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