Amino acid transporters as modulators of glucose homeostasis

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Abstract

Amino acids modulate glucose homeostasis. Cytosolic levels of amino acids are regulated by amino acid transporters, modulating insulin release, protein synthesis, cell proliferation, cell fate and metabolism. In β-cells, amino acid transporters modulate incretin-stimulated insulin release. In the liver, amino acid transporters provide glutamine and alanine for gluconeogenesis. Intestinal amino acid transporters facilitate the intake of amino acids induce causing protein restriction when inactive. Adipocyte development is regulated by amino acid transporters through activation of mTORC1 and amino acid-related metabolites. The accumulation and metabolism of branched-chain amino acid in muscle depends on transporters. The integration between amino acid metabolism and transport in tissues is critical for the maintenance and function of tissues and cells involved in glucose homeostasis.

Amino acids and their metabolites are powerful signalling molecules.

Amino acids regulate metabolism through mechanisms involving allosteric regulation, mitochondrial metabolism, mTORC1 and GCN2 [1-3]. The recognition of these pharmaceutical-like effects has resulted in the development of therapeutic amino acids mixtures for the treatment of a variety of multifactorial diseases [4,5]. Essential amino acids have to reach all cells through transport processes because critical enzymes for their biosynthesis are lacking in mammalian cells. Thus, transport processes are found at the start of metabolic pathways to ensure a tight communication between amino acid levels of blood plasma and the cytosol and to allow amino acids or their breakdown products to enter mitochondria. Amino acids play an important role as biomarkers of diabetes but also as active metabolites that modulate glucose and lipid homeostasis [6]. Elevated levels of branched-chain amino acids (BCAA) in particular are highly correlated with insulin resistance and are prognostic of future risk of T2D [6,7]. Genetic analysis supports a causal relationship between BCAA levels and T2D [8]. Reduction of branched-chain amino acids, for instance after bariatric surgery, is accompanied by enhanced glucose tolerance. Protein restriction is strongly associated with the improvement of metabolic health in mice and is driven by limiting essential amino acids [9-11]. In contrast to these observations, other studies have shown that supplementation of leucine improves muscle function and is accompanied by better insulin sensitivity [12-14]. Despite these strong influences of amino acids on metabolic health, the role of amino acid transport in these processes is less well investigated [2]. In addition to mediating plasma membrane amino acid permeability, transporters are also critical for subcellular amino acid distribution, such as the oxidative metabolism of amino acids inside mitochondria [15], or the communication between lysosomes and the cytosol [16]. This review will cover the role of amino acid transporters in tissues that are critical for nutrient metabolism and glucose homeostasis. A summary of the general principles of amino acid transport in mammalian cells is provided in Box 1 and Fig. 1. These principles suggest that a combination of loaders, harmonizers and controllers establishes a relation between plasma and cytosolic amino acid concentration. Most amino acids are found in blood plasma in the range of 0.05-0.2 mM [17]. The exceptions at the upper end are glutamine (0.33-0.63 mM) and alanine (0.23-0.42 mM). Both amino acids are non-essential and are produced as acceptors of amino-groups in many tissues and play an important role in interorgan transfer [18,19]. Aspartate (<0.02 mM) and methionine (0.01-0.04 mM) are the exceptions at the

lower end. Aspartate is mainly sequestered intracellularly due to lack of plasma membrane transport or due to highly accumulative transport. Methionine is of low abundance in food and used as a precursor for the synthesis of cysteine, choline and creatine [3]. Intracellular amino acid concentrations are typically 3-50-fold higher than those in blood plasma [20]. Most of these concentrations are determined by the equilibrium of the participating transport processes (Box 1) but the cytosolic concentrations of some amino acids with low membrane permeability are set by metabolism which can lead to higher cytosol/plasma ratios. The sum of tissue and plasma amino acids is determined by the balance between nutrient intake, synthesis (in the case of non-essential amino acids), and breakdown [1,20]. Amino acid transporters modulate glucose homeostasis through a variety of mechanisms, which will be discussed for different cell types in the following.

Box 1 Principles of amino acid transport in mammalian cells

Mechanistically, transporters are classified as ATP driven (primary active) or driven by substrate and ion gradients (secondary active) [21]. Secondary active transporters are further subdivided as symporters, antiporters and uniporters. Physiologically, however, transporters are better classified as loaders, harmonizers and controllers [22] (Fig. 1). Loaders actively accumulate a select group of amino acids inside the cytosol. Harmonizers ensure that these amino acids are exchanged against other amino acids that are not covered by loaders, often including essential amino acids (aka tertiary active transport). To avoid osmotic stress, controller transporters of low affinity release amino acids at a slow rate, becoming faster as the cytosolic concentration rises. Typically, intracellular amino acid levels are 3 to 50-fold higher than those in blood plasma. Such a set of transporters generates a stable equilibrium, which allows efflux of amino acids when proteolysis prevails and uptake of amino acids, after food intake. The diagram shows several transporters that are relevant to insulin sensitivity.

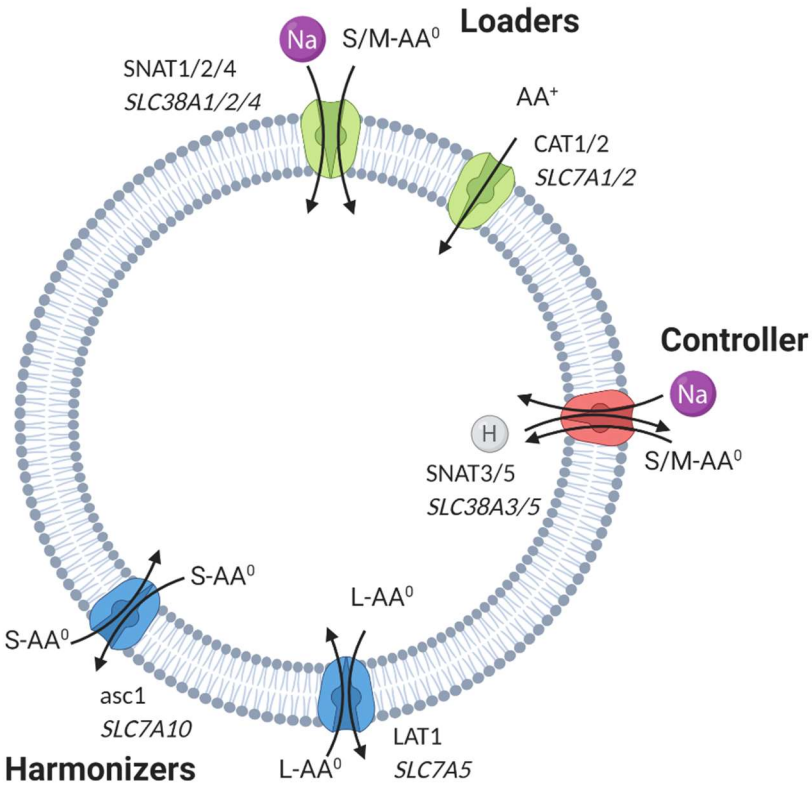

Fig. 1 Amino acid transporters in mammalian cells. Image created with Biorender.com.

The role of amino acid transporters in β-cells and α-cells

Amino acids can enhance glucose stimulated insulin release in primary islets and β-cell lines [23], in particular leucine, isoleucine, alanine and arginine. In addition, they modulate insulin release through mTORC1, AMPK and incretin signalling. These mechanisms combine to a strong enhancement of insulin release *in vivo* [24]. Glucose stimulated insulin secretion is correlated with cytosolic and mitochondrial ATP production. For glycolysis to run proportional to cytosolic glucose concentration an efficient transport of NADH into mitochondria is required, which in β-cells is mediated by the malate-aspartate shuttle (Fig. 2). A key component of the shuttle is the aspartate-glutamate carrier Aralar [25], which is also essential for the incretin-amplified insulin release [26]. The malate-aspartate shuttle indirectly mediates the import into mitochondria of NADH generated in the cytosol through reversible conversion of oxaloacetate to malate, which is accompanied by conversion of NADH to NAD and vice versa. The conversion of oxaloacetate to malate is linked to aspartate/glutamate exchange through transamination of oxaloacetate to aspartate and of glutamate to 2-

oxoglutarate. The 2-oxoglutarate is then swapped for malate via the oxoglutarate carrier across the mitochondrial membrane to complete the shuttle. The exported 2-oxoglutarate can be converted to glutamate via aminotransferases.

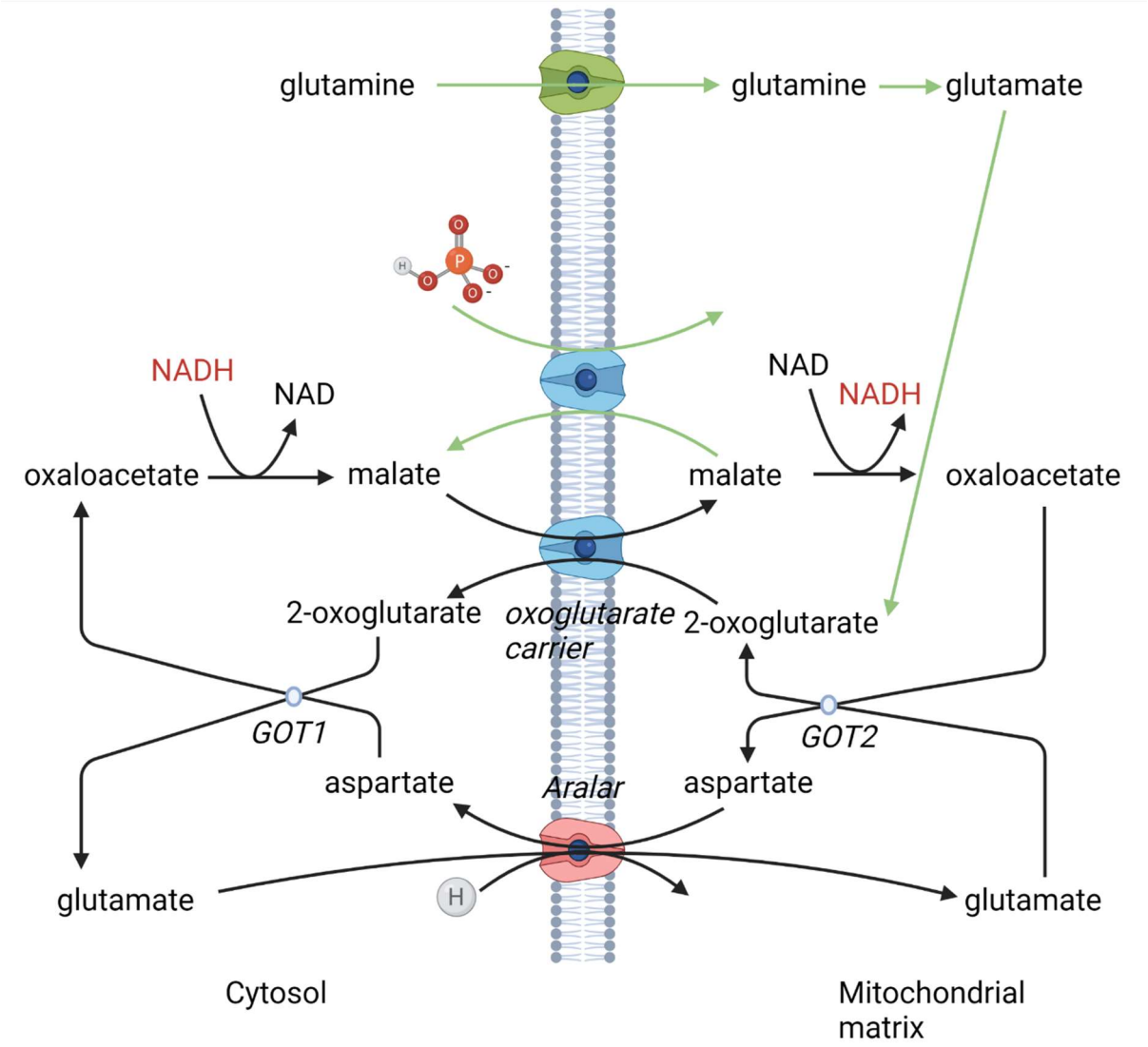

Fig. 2 Malate-aspartate shuttle and associated reactions. The reactions of the malate-aspartate shuttle are shown by black arrows. Transporters and enzymes are shown in italics. Associated reactions that can support glutamate production are shown by green arrows. Indirect translocation of NADH is shown in red. GOT1/2, glutamate-oxaloacetate transaminase 1/2. Image created with Biorender.com.

Cytosolic glutamate has been shown to enhance glucose-stimulated insulin release in β-cells [27,28], but these observations have been challenged by others [29,30]. Using two different

β-cell lines, Gheni et al. [31] modified the proposal by showing that cytosolic glutamate increases the incretin-amplified insulin release but not the basic glucose-induced insulin release. Two amino acid transporters were involved in this effect, namely the mitochondrial aspartate-glutamate exchanger Aralar (SLC25A12) and the vesicular glutamate transporter VGLUT1 (SLC17A7).

Subsequently, glutamate is imported into insulin granules via VGLUT1 (Fig. 3). Blocking VGLUT1 abolished incretin-amplified insulin secretion [31]. The mechanism depended on the incretin GLP-1 via protein kinase A signalling. In agreement with the *in vitro* results, Zucker fatty rats, which do not show incretin-stimulated insulin secretion, also did not show glucose-stimulated production of glutamate.

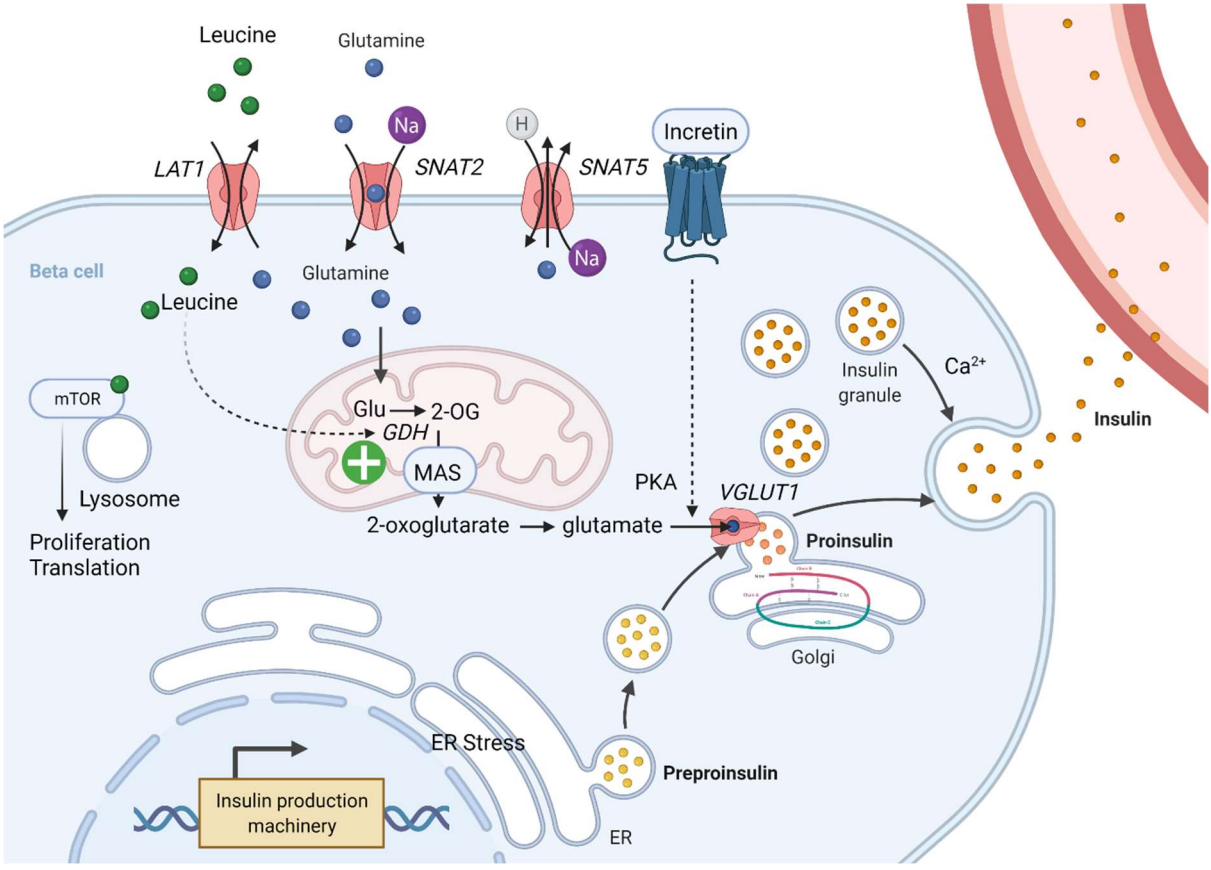

Fig. 3 Amino acid transporters in β-cells. Key elements of the incretin-stimulated insulin release are shown. Extracellular glutamine is used to generate 2-oxoglutarate inside mitochondria, which is exported to generate glutamate. Cytosolic glutamate is loaded into insulin granules to stimulate insulin release. GDH, glutamate dehydrogenase; MAS, malate-aspartate-shuttle; PKA, protein kinase A. Image created with Biorender.com.

The cyclic nature of the malate-aspartate shuttle in combination with the net production of glutamate from mitochondrial 2-oxoglutarate raises the question of the source of glutamate without returning it into mitochondria via the shuttle. Cellular glutamate levels in β-cells can be controlled by changing the levels of its precursor glutamine [29,32,33]. Knock-down of the glutamine transporter SNAT5 (SLC38A5) increased cytosolic glutamate levels, due to its role as a controller, and increased incretin responsiveness in β-cell lines [34].

Expression levels of SNAT5 are increased in ob/ob and KK-Ay mice, models of obesity and type 2 diabetes. The β-cells also express the functionally related transporter SNAT3 [35], which appears to be downregulated in db/db islets [36]. The malate-aspartate shuttle is a closed circuit and removal of any of its components impairs its function. Export of 2-oxoglutarate from mitochondria only requires a return of malate, for instance by the dicarboxylic acid carrier (Fig. 2). Thus, the malate-aspartate shuttle and glutamate production could run in parallel, by using an overlapping set of transporters and enzymes. Alternatively, glutamate can be elevated by the addition of alanine, which also has an insulinotropic effect in β-cells [37]. In addition, alanine depolarises β-cells due to Na^+-alanine cotransport most likely via SNAT2 (SLC38A2) [38].

Leucine is the only amino acid that can stimulate insulin release itself, while glutamine does not [39]. It is thought that leucine promotes insulin release through allosteric stimulation of glutamate dehydrogenase (GDH), which facilitates glutamate production from glutamine (Fig. 3) [23,39]. As a result, glutamine can enhance the effect of leucine. The large neutral amino acid transporter LAT1 (SLC7A5) is the major transporter for leucine in β-cells and is required for leucine-stimulated insulin secretion. Inhibition of LAT1 using 2-aminobicyclo[2.2.1]heptane-2-carboxylic acid (BCH) or silencing of LAT1 reduced leucine-stimulated insulin secretion [40]. Of note, BCH is a leucine analogue and can be used to stimulate glutamate-dehydrogenase and insulin secretion when applied alone [39]. The difference between the two actions is most likely related to the incubation time with short incubation times inhibiting transport and longer incubation times stimulating GDH.

The role of the LAT1 transporter in the release of insulin is supported by experiments in Drosophila [41]. Insulin-like-peptide producing cells (IPC) in brains from starved larvae showed a strong response to 20 mM leucine and isoleucine, while IPCs from fed larvae did not respond. The Drosophila mini-disc protein is one of two LAT1-like transporters and is

expressed in IPCs. Knockdown of this transporter in IPCs abolished the response to leucine. Application of 20 mM leucine resulted in a rapid release of insulin-like peptides from IPCs, which was also abolished by knockdown of the minidisc protein. In further alignment with mammalian cells, the stimulation of insulin-like peptide release was dependent on glutamate dehydrogenase.

In summary, the large neutral amino acid transporter LAT1 facilitates the import of leucine to activate glutamate dehydrogenase (GDH). This enhances insulin release by β-cells. Glutamine import is mediated by SNAT2 (SLC38A2), which in turn can be used as an exchange substrate to import leucine (Fig. 3). Glutamine is transported into mitochondria and can be used as an anaplerotic substrate of the TCA cycle [42] allowing the export of 2-oxoglutarate, which can be converted into glutamate in the cytosol. Loading of glutamate into insulin-containing granules is mediated by VGLUT1 and is required for the incretin action in β-cells. SNAT5 releases glutamine thereby reducing the generation of glutamate. The mechanism by which elevated glutamate levels contribute to enhanced loading or release of insulin remains to be elucidated.

Maintenance of β-cell mass and proliferation are also dependent on amino acids [43]. Both SNAT2 and LAT1 have been shown to support mTORC1 signalling and proliferation in β-cells [40,44] (Fig. 3). The role of SNAT2 has been discussed controversially [44,45]. Medras et al. showed that high doses of glutamine could partially restore β-cell function and insulin production in STZ treated rats [45]. This was accompanied by a 30-fold increase of mRNA of the glutamine transporter SNAT2. Krokowski et al. [44], by contrast, showed that chronic ER stress in Min6 cells induces several amino acid transporter genes, including SNAT2 and LAT1, involving the integrated stress response mediators PERK and ATF4. This could further exacerbate ER stress by enhancing translation due to increased amino acid supply. In Akita mice, which have elevated ER stress in β-cells due to a mutation in the proinsulin gene that causes misfolding, the expression of several amino acid transporters was increased. Suppression of protein synthesis reduced ER stress and apoptosis in Min6 cells. The different results can be reconciled by acknowledging that amino acids are crucial for the proliferation of β-cells for instance after a brief treatment with STZ. However, up-regulation of amino acid transporters may be counterproductive during chronic ER stress in highly active islets in insulin-resistant individuals.

Arginine stimulates insulin secretion directly through depolarisation caused by its transport [46] and indirectly through the generation of nitric oxide [47]. Analysis of proteins that bind to galectin-lattices on the surface of Min6 cells revealed a tight association with arginine transporter CAT3 (SLC7A3) stabilising its surface expression [48]. Disruption of this interaction reduced NO production and reduced insulin secretion.

The role of glutamate as a secretagogoue of insulin has been discussed controversially in the past [23,29,37], but a more specialised role as a mediator of the incretin-stimulated insulin release appears to be supported by the data. The role of leucine as an allosteric activator of glutamate dehydrogenase, by contrast, is well established.

Glucagon is produced and released by α-cells in pancreatic islets. Liver is the main target of glucagon and the main site of amino acid oxidation. Mouse models with interrupted glucagon signalling have a 3-6 fold increase of most serum amino acids implying reduced metabolism [49]. Vice versa, humans with glucagon-producing tumours have low serum amino acids and elevated ureagenesis [50]. Elevated amino acid levels stimulate the proliferation of mouse α-cells but not of β- or δ-cells. The amino acid transporter SNAT5 (SLC38A5) was identified as one of the mediators of this response and found to be upregulated in α-cells [51,52]. The effect appears to be mouse-specific but could be replaced by expression of the redundant transporter SNAT4 (SLC38A4) in human islets [53]. Similar to β-cells, glutamine, alanine and arginine are the most potent secretagogues in α-cells [49].

Amino acid transport in liver

The liver is the main organ to maintain plasma glucose concentrations during fasting via glycogenolysis and gluconeogenesis. The main sources for gluconeogenesis are lactate, glucogenic amino acids and glycerol. Arteriovenous differences show that glutamine and alanine are the main glucogenic amino acids released by muscle during fasting. These are extracted by the liver and converted into glucose. The liver has high expression levels of SNAT2(SLC38A2), SNAT3(SLC38A3) and SNAT4 (SLC38A4) that co-localise with gluconeogenic enzymes [54,55] (Fig. 4). SNAT2 transports glutamine and alanine, SNAT3 glutamine and SNAT4 mainly alanine [56]. Homozygous SNAT3 mutant mice die around 20 days after birth [57] and showed reduced levels of alanine and elevated levels of histidine. This suggests that SNAT3 is an important transporter for the metabolism of histidine. Importantly, plasma glucose levels were significantly lower in SNAT3 deficient animals, suggesting that SNAT3

plays an important role in providing glutamine for gluconeogenesis. Lower levels of alanine further suggest that it was used as an alternative gluconeogenic precursor, while glutamine permeability was reduced. Insulin levels were lower, most likely in response to low plasma glucose levels. The neutral amino acid transporter SNAT2 could mediate the uptake of alanine to compensate for the lack of SNAT3 [58]. The transporter has a carbohydrate responsive element in its promoter (ChREBP) and its expression is downregulated by a carbohydrate-rich diet. Consistently, the transporter is upregulated by glucagon involving a cAMP response element (CRE) in the proximal promoter of the SLC38A2 gene [59].

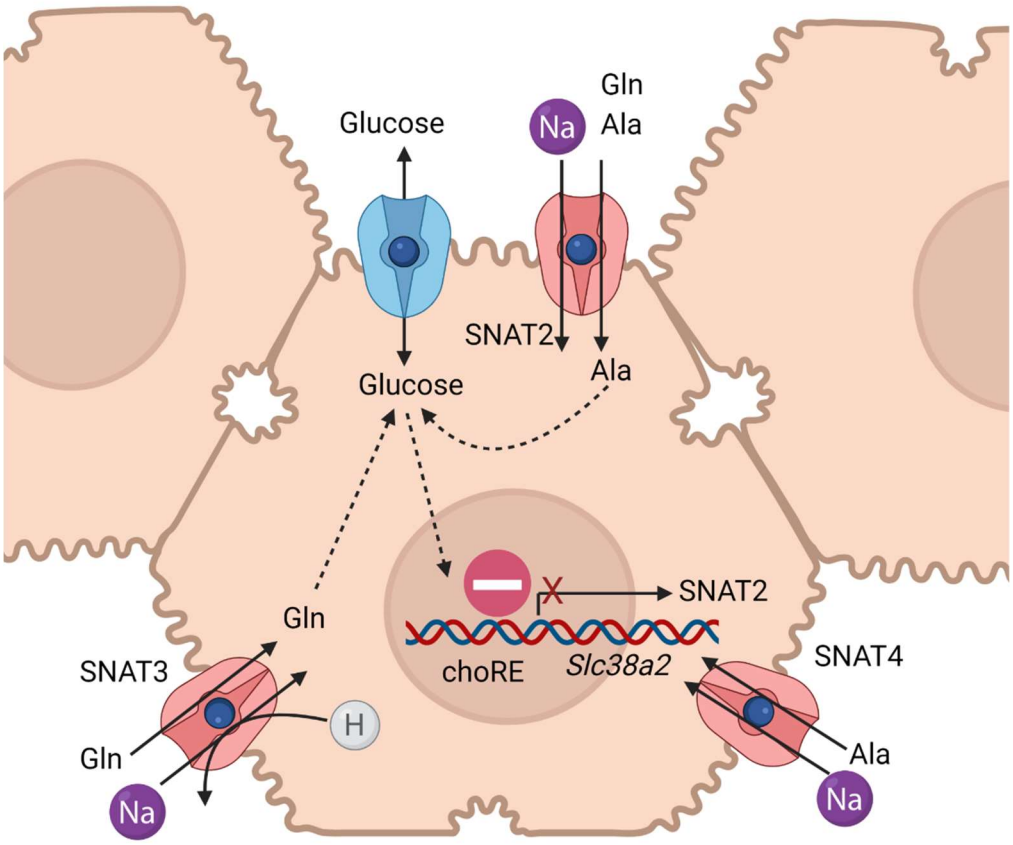

Fig. 4 Regulation of glucose production in liver by amino acid transport. SNAT2 and SNAT3 are co-localised with gluconeogenic enzymes. SNAT4 is located primarily in perivenous hepatocytes. SNAT3 imports glutamine and histidine into hepatocytes, while SNAT4 mainly transports alanine. SNAT2 transports both amino acids. SNAT2 transcription is regulated by a carbohydrate response element in its promoter. Image created with Biorender.com.

This could increase its expression in SNAT3 ko mice. The upregulation of SNAT2 by glucagon could contribute to the enhanced oxidation of amino acids in the presence of this hormone by increasing cytosolic amino acid concentrations (Fig. 1). Surprisingly, SNAT2 liver mRNA levels dramatically increase in STZ treated rats [55]. The mechanism of this upregulation remains to be established, but it contradicts the down-regulation expected by elevated levels of glucose. SNAT4 is a liver-specific transporter, expressed at high levels in perivenous hepatocytes. As a result, its expression only partially overlaps with gluconeogenic enzymes, which are enriched in periportal hepatocytes. Inhibitors of SNAT2 could potentially be used to reduce elevated fasting levels of glucose in diabetes but this concept remains to be tested.

Amino acid transport in adipose tissue

The transporter asc-1 (SLC7A10) has been identified as a member of a regulatory network in subcutaneous adipose tissue that is involved in metabolic syndrome, but its expression is inversely correlated with body mass index, insulin, insulin resistance, glucose and triglycerides [60]. Subsequently, the transporter was identified as a surface marker for white adipocytes [61]. The mechanism by which asc-1 modulates adipocyte differentiation (Box 2) and function is, however, controversial.

Box 2 Adipocyte differentiation

Mature adipocytes are classified into white, beige and brown. Brown and beige adipocytes have the metabolic capacity to convert fat into heat through the expression of uncoupling protein 1 (UCP1). Many of these cells develop during embryogenesis, but some can also develop from stem cells and pre-adipocytes that can be isolated from dispersed adipose tissue because of their higher density. Hypertrophic growth (enlargement of individual adipocytes through lipid expansion) of visceral and subcutaneous adipocytes is linked to the development of insulin resistance, while hyperplastic growth (proliferation of adipocyte precursors) is considered healthy [62]. Peroxisome proliferator-activated receptor gamma (PPARγ) is the key transcription factor that is required and sufficient for adipocyte differentiation and can be activated by the drug Rosiglitazone [62].

Jersin et al. confirmed that asc-1 was inversely correlated with high obesity and waist-to-hip ratio [63]. The loss of fat increased asc-1 expression in subcutaneous WAT. Blocking asc-1

increased the lipid content in cultured pre-adipocytes. Transcriptome analysis revealed the upregulation of genes involved in lipid biosynthesis and down-regulation of mitochondrial functions. Inhibition of asc-1 reduced cellular glutathione levels and increased the generation of reactive oxygen species (ROS). Moreover, inhibition of asc-1 abrogated the insulin-stimulated glucose uptake. Knock-out of slc7a10 isoform b in *Zebrafish* resulted in higher weight gain upon overfeeding and larger adipocytes. The phenotype in mice is more complex due to severe neurological complications, including reduced body size, hepatosteatosis, hypoglycaemia, and reduced body temperature [64].

Arianti et. al. [65], propose that asc-1 is a marker of brown adipocytes, while it is missing in preadipocytes. The transporter was found to be expressed at higher levels in adipocytes from deep neck tissue, in particular when they were differentiated into brown adipocytes. The transporter accumulated glycine, serine and threonine at the expense of alanine. The addition of the asc-1 inhibitor BMS-466442 blocked the UCP-1 mediated proton leak and creatine-dependent futile cycling. This suggests that amino acids play an important role in regulating thermogenesis. Consistent with this notion, the expression of genes associated with adipocyte browning, such as PGC1α and UCP1, was reduced after incubation with BMS-466442. Thus, low levels of the transporter would be associated with the limited functionality of brown adipocytes.

Suwandhi et al.[64] suggest that asc-1 expression suppresses the expression of genes associated with browning in preadipocytes. Single-cell transcriptomics revealed a population of pre-adipocytes with high or low expression of the small amino acid transporter asc-1 (SLC7A10). In the population of immature adipocytes, asc-1 expression was found to indicate a state favouring the development into white adipocytes [64]. A combination of insulin plus rosiglitazone can induce UCP-1 expression in asc-1-negative cells, but not in asc-1-positive cells. Consistently, silencing of asc-1 in preadipocytes, increased the expression and protein levels of UCP-1, particularly when rosiglitazone was added to induce differentiation. Thus, asc-1 actively suppresses brown/beige adipocyte gene expression and loss of asc-1 in white preadipocytes promotes differentiation of beige adipocytes [64]. When asc-1 was inhibited in proliferating preadipocytes with compound BMS-446442 [66], the cells differentiated into beige adipocytes. In addition to small non-essential amino acids, such as alanine, serine and cysteine, asc-1 also transports D-serine a metabolite crucial for neural activity [67]. Due to its

harmonizer activity, asc-1 can mediate efflux or uptake of D-serine. Knockdown of serine racemase, which converts L-serine to D-serine, reduced expression UCP-1 in proliferating preadipocytes. Together this suggests that asc-1 may efflux D-serine produced by serine racemase. Cells with high levels of intracellular D-serine (asc-1-negative) differentiate into beige adipocytes, while low D-serine levels promote the formation of white adipocytes.

While all studies agree that asc-1 plays an important role in adipocyte differentiation, the detailed results are not easily reconciled. D-serine is a tempting candidate to explain the involvement of asc-1 because other amino acids such as serine, threonine and glycine can be transported by a variety of other transporters. The putative role of D-serine is reminiscent of the role of L-proline in stem cell differentiation [68,69]. During development, cells maintain a low-proline status as embryonic stem cells, switching to a high proline status which increases proliferation and results in a mesenchymal-like state of high motility and pluripotency. Alternatively, asc-1 could provide glycine and serine as precursors to generate pyruvate as a transamination receptor for BCAA metabolism, similar to the mechanism suggested in muscle cells [70]. A third possibility might be the transport of alanine. Adipocytes recycle a significant fraction of fatty acids after lipolysis, which requires 3-phosphoglycerol. This metabolite is generated in a process called glyceroneogenesis [71], for which alanine is a precursor. Alanine is released from muscle during fasting, and spares the use of glucose for this process.

Insulin sensitivity of adipose tissue is also affected by lysosomal amino acid transport and its role in mTORC1 activation (Fig. 5). Hyperactivation of mTORC1 suppresses insulin signalling through phosphorylation of insulin receptor substrate (IRS-1) [72,73]. mTORC1 is tightly controlled by lysosomal amino acid transport processes that not only involve bona fide lysosomal amino acid transporters but also plasma membrane transporters that are redirected to the lysosomal membrane. Amino acid transporters LAT1 (SLC7A5) and ASCT2 (SLC1A5) can be recruited to the lysosome by a DRAM-1 dependent mechanism that also involves SCAMP3 [74]. The two transporters were proposed earlier to activate mTORC1 by a tertiary transport mechanism that would elevate cytosolic leucine concentrations [75]. It appears more likely, however, that lysosomally redirected LAT1 and ASCT2 activate mTORC1 because the process is DRAM-1 dependent, while cytosolic amino acid levels remained the same in the absence and presence of DRAM-1 [74]. This is consistent with results showing activation of mTORC1 at times when cytosolic amino acid levels were barely changed [76].

Consistent with a cross-talk between insulin signalling and amino acid signalling, AKT phosphorylation was enhanced and IRS-1 phosphorylation decreased in DRAM-1 knock-out mice. The mice showed superior glucose tolerance after being maintained on a high-fat diet for 22 weeks, had reduced body weight and fat. Mechanistically, mTORC1 appears to inhibit the differentiation from pre-adipocytes to adipocytes [77].

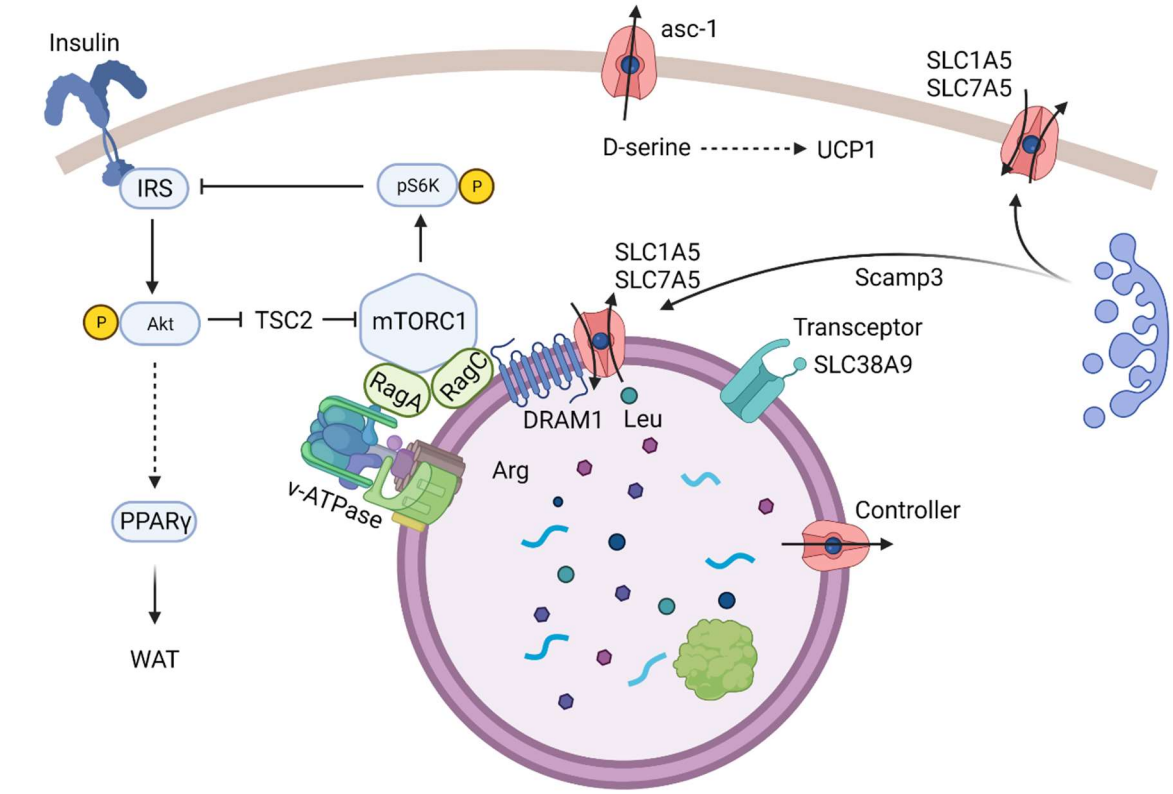

Fig. 5 Amino acid transporters in adipose tissue. Plasma membrane transporters ASCT2 (SLC1A5) and LAT1 (SLC7A5) can be redirected to lysosomes by a DRAM1- and Scamp3-dependent mechanism. In lysosomes they play a prominent role in activating mTORC1. Overactivation of mTORC1 impairs insulin signalling through phosphorylation of insulin receptor substrate and reduces development of white adipose tissue. High levels of D-serine favour expression of UCP1 and development into brown adipocytes. Release of D-serine via asc-1 promotes development into white adipose tissue (WAT). Image created with Biorender.com.

Amino acid transport in the intestine

Low protein diets and protein restriction increase metabolic health in rodents [10,78,79] and also improve type 2 diabetes [80,81]. Mechanistically, it is thought that amino acid restriction increases the expression of FGF21 in liver [82], which in turn increases energy expenditure relative to food intake by a variety of mechanisms. An important factor is the upregulation of uncoupling protein (UCP-1) in adipose tissue resulting in browning and increased energy expenditure [83]. The involvement of FGF21 has been confirmed in humans [11,80,84]. Essential amino acids are the key drivers of this response. A diet low in branched-chain amino acids increases hepatic insulin sensitivity, activates ketogenesis and increases energy expenditure by activating FGF-21 [11]. Whether this mechanism involves the activation of GCN2 is controversial [11,85,86], but lack of branched-chain amino acids results in a broad reprogramming of metabolism. This suggests that reducing the dietary intake of branched-chain amino acids could be used to treat obesity, reduce hepatic steatosis and improve insulin sensitivity. Other studies suggest a limiting role for the essential amino acid threonine [9] or point to the involvement of methionine [87,88].

In the intestine, the amino acids associated with the beneficial effects of protein restriction are absorbed across the apical membrane by the neutral amino acid transporter B^0AT1 (SLC6A19) [89]. Branched-chain amino acids, methionine, tryptophan and threonine are substrates of this transporter. Transport measurements in inverted sections of the mouse intestine suggest the absence of alternative pathways [90]. However, nutritional protein is also absorbed as di- and tripeptides by the intestinal peptide transporter PepT1 [91]. The fraction of total protein absorbed by each pathway is difficult to evaluate as brush-border peptidases form metabolic complexes with amino acid transporters [92]. A global knock-out of B^0AT1, however, mimics all features of protein restriction [93]. In this mouse model FGF-21 was highly upregulated and UCP-1 expression was increased in inguinal white adipose tissue. Accordingly, oxygen consumption was higher in the ko mice compared to weight-matched wild-type controls. Two metabolic phenotypes were observed in these mice. First, an overload of amino acids in the lumen of the intestine caused by reduced absorption [94]. This increased the release of incretins such as GLP-1 and GIP. Secondly, a protein restriction phenotype that activated GCN2 in the liver, a known signal that leads to FGF21 expression [95], and decreased mTORC1 activation across all tissues. Appearance of threonine in the

portal vein after food intake was reduced in B^0AT1 ko mice and could be responsible for this phenotype [9]. The starvation response in the liver was accompanied by increased ketogenesis, reduced triglyceride levels and reduced levels of gluconeogenesis. A shift to increased glucose consumption was observed in heart muscle. This is at variance with studies in rats, where restriction of branched-chain fatty acids caused a shift towards fatty acid utilisation [96]. In a subsequent study, it was shown that lack of B^0AT1 reduced the amount of steatosis in the liver on a high-fat/fructose diet [97]. Despite the increase of incretins, insulin secretion during an oral glucose tolerance test in B^0AT1 ko mice was nearly absent. Moreover, glucose tolerance and insulin sensitivity were improved in B^0AT1 ko mice [93]. This observation agrees with studies of dietary reduction of branched-chain amino acids in humans which also reduced insulin secretion [98]. As a result, blockade of B^0AT1 in humans may improve diabetic states [99]. Inhibitor development for this transporter has been reported by several groups [100-102].

Mice lacking the intestinal peptide transporter PepT1 have no particular phenotype on a normal diet, but stay much leaner on a high-fat diet. The lower fat mass could not be explained by higher energy consumption, but rather by energy loss in faecal matter [103]. Content analysis revealed high concentrations of glycerol and fatty acids, but low levels of carbohydrates and amino acids on a high-fat diet. On a high carbohydrate diet, levels of carbohydrates and amino acids were elevated. Plasma glucose levels were significantly lower in PepT1 ko mice on both diets and fatty acid levels in the liver were reduced. PepT1 is also involved in appetite control. Food intake on a high protein diet was more blunted in PepT1 ko mice than in wild-type mice. Surprisingly leptin levels were found to be lower in PepT1 ko mice. Plasma BCAA were significantly higher on a high protein diet, but the effect was much reduced in PepT1 ko mice [104]. The phenotype of PepT1 ko mice is surprisingly different to that of B^0AT1 ko mice, particularly the effect on plasma glucose. This could cause indirect metabolic effects that help to explain the differences.

Amino acid transport in muscle

Protein synthesis in muscle is regulated by a complex interaction between insulin, amino acids, mTORC1 and exercise [105,106]. Insulin is required for mTORC1 activation through inhibition of TSC2 but it also directly stimulates protein synthesis through releasing inhibition of eIF2B. When mTORC1 is activated through amino acids and insulin, it stimulates protein

synthesis via phosphorylation of 4E-BP1. In a negative feedback loop, chronic stimulation of mTORC1 suppresses insulin signalling [72,73] as outlined for adipose tissue (Fig. 5).

A multitude of studies demonstrated the role of leucine or isoleucine in the stimulation of glucose uptake into muscle cells and the improvement of glucose tolerance [12,107-111]. This appears surprising given the beneficial effects observed for low protein diets and restriction of essential amino acids. Dietary protein, in particular branched-chain amino acids, stimulates protein synthesis, regulate body weight and glucose homeostasis [73,105]. Essential amino acids have been used to support exercise and build-up of muscle mass for many years and have not increased the incidence of T2D in exercising individuals who are mostly young [112]. It appears likely that the increase of branched-chain amino acids before the onset of diabetes is caused by a reduction of its metabolism [6,113,114]. This is supported by elevated levels of circulating branched-chain ketoacids (BCKA) in insulin-resistant individuals [115,116], which are generated before the rate-limiting oxidation of BCKA. The beneficial effects of protein restriction are focused on liver and adipose tissue, while the beneficial effects of leucine supplementation are focused on muscle tissue. However, one mechanism suggests that high BCAA oxidation depletes intracellular glycine pools, impairing acyl-glycine export from muscle [117], thereby increasing muscular fat content. Depending on the design of the experiment, leucine supplementation and branched-chain amino acid restriction can show beneficial effects on glucose tolerance. Other studies have reported that metabolites of branched-chain amino acid oxidation impair glucose uptake into muscle [118]. In the case of α-ketoiscaproate, this can be explained by its reversible conversion to leucine, which activates mTORC1. Alternatively, increased oxidation of BCKA in mitochondria may prevent the oxidation of fatty acids resulting in insulin resistance. Muscle has a very low capacity to oxidise BCKA directly but can use circulating acyl-carnitines derived from other tissues [119]. This can be compensated by exercise, which increases oxidative capacity [120]. The same could be true for the valine metabolite β-hydroxyisobutyric acid (HIB), which has been shown to increase the accumulation of fatty acids in muscle cells [121]. The final product of valine metabolism β-aminoisobutyrate (BAIBA) by contrast has opposite effects, inducing browning of adipose tissue and increasing hepatic β-oxidation [122].

Amino acid transporters mediate the rapid uptake of BCAA into muscle cells. This is mediated by an indirect transport mechanism in which polar amino acids are loaded into muscle cells

by Na^+-amino acid cotransporters (SNAT1/2) followed by an exchange process in which glutamine and histidine are exchanged for BCAA via the large neutral amino acid transporter LAT1 [123]. Human cultured myotubes express 25 different amino acid transporters, the combination of which equilibrate amino acid concentrations between blood plasma and the myotube cytosol [124]. Due to the driving force of the loader transporters, amino acid concentrations in the cytosol are significantly higher than in the media. Alanine and glutamine can be released via exchangers and controllers (Fig. 6), such as ASCT1 (SLC1A4) and SNAT3 (SLC38A3), respectively.

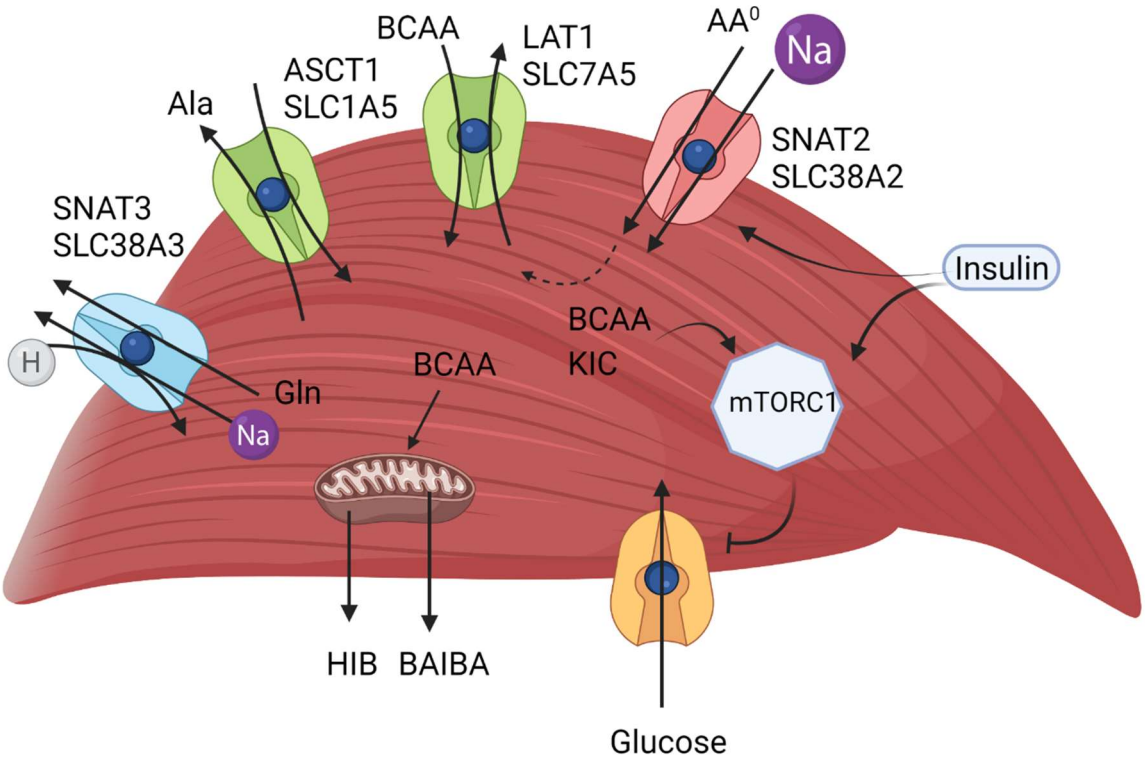

Fig. 6 Amino acid transport in muscle. Polar neutral amino acids are taken up into muscle via SNAT2. These can be used as exchange substrates to accumulate BCAA inside muscle to activate mTORC1. Muscle amino acid transport and protein synthesis is stimulated by insulin. BCAA and their metabolites β-aminoisobutyric acid (BAIBA) and hydroxisobutyric acid (HIB) modulate mitochondrial metabolism inside muscle and act as endocrine signals on other tissues. Image created with Biorender.com.

This set of transporters ensures that BCAA can be rapidly deaminated after intake and that alanine and glutamine can be released during fasting. Insulin is required for optimal stimulation of protein synthesis in muscle [105]. It is required for activation of mTORC1 via inhibition of the Tuberoslcerosis complex 2 (TSC2) [125]. As outlined above, BCAA bypass liver metabolism for optimal activation of mTORC1 in muscle. To coordinate protein synthesis with amino acid uptake several transporters are regulated by insulin. LAT1 mRNA abundance is up-regulated by insulin via a mTORC1-dependent mechanism [126], followed by LAT1 protein [127]. SNAT2 is regulated at multiple levels [128,129] but under the influence of insulin it is translocated from intracellular stores to the plasma membrane [129,130]. SNAT2 is a direct activator of mTORC1 coordinating the uptake of amino acids into muscle with signalling [76,131]. Insulin stimulation of alanine transport, presumably via SNAT2 is enhanced by exercise [132]. Through tertiary transport this mechanism could contribute to the suppression of protein degradation in muscle by insulin. Consistently, ceramide a mediator of insulin resistance, reduces SNAT2 activity and protein synthesis in muscle [133].

Conclusion: Amino acid transporters generate a stable equilibrium between plasma and cytosolic amino acid concentrations allowing the free exchange of amino acids without compromising protein translation and amino acid metabolism. As a result, changes in plasma amino acid concentrations due to nutritional intake are converted into elevated cytosolic amino acid concentrations. This can modulate insulin release and increase amino acid metabolism. The underlying mechanisms require further research (see outstanding questions). Plasma membrane amino acid transporters can be redirected to lysosomes, where they increase mTORC1 signalling which can result in insulin resistance. Blocking amino acid transport can be used to induce restriction of essential amino acids which in turn causes increased expression of FGF21. Blocking amino acid transport could be used to reduce gluconeogenesis, which may have a beneficial effect on the development of type 2 diabetes and obesity.

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Glossary

Aralar: Aka aspartate/glutamate exchanger, a mitochondrial antiporter exchanging aspartate and glutamate.

Asc-1: Alanine-serine-cystein transporter-1, a harmonizer exchanging small neutral amino acids, including D-amino acids.

ATF4: Activating tyranscription factor 4, a transcription factor that is activated under conditions of amino acid depletion and ER stress.

B^0AT1: Broad neutral amino acid transporter 1, a loader transporting all neutral amino acids into intestinal and renal epithelial cells.

BCAA: Branched-chain amino acids are the essential amino acids leucine, isoleucine and valine

BCKA: Branched-chain ketoacids, the transamination products of branched-chain amino acids.

DRAM-1: DNA damage regulated autophagy modulator 1, a lysosomal protein involved in recruitment of transporters to the lysosome.

eIF2B: eukaryotic initiation factor 2B. A factor required for the initiation of protein translation

ER stress: Secreted proteins, such as insulin, are folded after extrusion from the ribosome into the endoplasmic reticulum. Increased insulin production as a result of peripheral insulin resistance is thought to increase ER stress and cause damage to β-cells.

FGF21: An endocrine hormone of the fibroblast growth factor family that induces browning of adipose tissue and ketogenesis in liver.

GCN2: General control non-derepressible 2, a sensor of amino acid imbalance that regulates protein translation.

GLP-1: Glucagon-like peptide 1, a hormone released by endocrine cells of the intestine upon food intake to enhance glucose-stimulated insulin secretion (GSIS).

Incretin: Metabolic hormones, such as GLP-1, released by the intestine upon food intake.

LAT1: Leucine preferring amino acid transporter 1. A harmonizer that exchanges large neutral amino acids. Often used to import branched-chain amino acids and aromatic amino acids in exchange for glutamine.

mTORC1: mechanistic target of rapamycine. A complex contaning the mTOR kinase, which responds to amino acid availability and regulates protein synthesis and autophagy.

PepT1: A transporter for di- and tri-peptides in intestinal epithelial cells.

PERK: PRKR-like endoplasmic reticulum kinase, a protein kinase that is activate by ER stress and phosphorylating GCN2.

PGC1α: PPARγ coactivator 1α, a transcription factor important for the generation and maintance of mitochondria.

PPARγ: Peroxisome proliferator-activated receptor gamma is the key transcription factor required for adipocyte differentiation.

SLC: Solute carrier, in mammalian genomes, transporter genes are grouped into solute carrier families based on sequence similarity. Common names are often used for the encoded proteins indicating substrate and ion specificity.

SNAT1: Sodium-neutral amino acid transporter 1, a loader that imports small and polar neutral amino acids into the cytosol, including alanine and glutamine.

SNAT2: Sodium-neutral amino acid transporter 2, a loader that imports small and polar neutral amino acids into the cytosol, including alanine and glutamine. Highly regulated by amino acid availability, osmolarity and insulin.

SNAT3: Sodium-neutral amino acid transporter 3. A controller transporter that facilitates the transport of glutamine and histidine.

SNAT4: Sodium-neutral amino acid transporter 4, a loader that imports small neutral amino acids into the cytosol, including alanine and glycine.

SNAT5: Sodium-neutral amino acid transporter 5. A controller transporter that facilitates the transport of glutamine and other small amino acids.

STZ: Streptozotocin, a compound toxic to β-cells that is experimentally used to mimick type I diabetes. When used in low doses damage is partial and growth of β-cells can be restored.

TCA cycle: Tricarboxylic acid cycle, the main metabolic pathway to oxidise nutrients to generate CO_2.

876 UCP-1: Uncoupling protein 1, a proton translocator in the inner mitochondrial membrane that
877 converts the proton-motive force into heat instead of ATP production.

878 VGLUT1: vesicular glutamate transporter 1. A transporter located in vesicular membranes to
879 import glutamate.