Pharmacological and pathophysiological roles of carnitine/organic cation transporters (OCTNs: SLC22A4, SLC22A5 and Slc22a21)

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Pharmacological and pathophysiological roles of carnitine/organic cation transporters (OCTNs: SLC22A4, SLC22A5 and Slc22a21)

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ABSTRACT:
The carnitine/organic cation transporter (OCTN) family consists of three transporter isoforms, i.e., OCTN1 (SLC22A4) and OCTN2 (SLC22A5) in humans and animals and Octn3 (Slc22a21) in mice. These transporters are physiologically essential to maintain appropriate systemic and tissue concentrations of carnitine by regulating its membrane transport during intestinal absorption, tissue distribution, and renal reabsorption. Among them, OCTN2 is a sodium-dependent, high-affinity transporter of carnitine, and functional defect of OCTN2 due to genetic mutation causes primary systemic carnitine deficiency (SCD). Since carnitine is essential for beta-oxidation of long-chain fatty acids to produce ATP, OCTN2 gene mutation causes a range of symptoms, including cardiomyopathy, skeletal muscle weakness, fatty liver, and male infertility. These functional consequences of Octn2 gene mutation can be clearly seen in an animal model, *jvs* mouse, which exhibits the SCD phenotype. In addition, although the mechanism is not clear, single nucleotide polymorphisms of OCTN1 and OCTN2 genes are associated with increased incidences of rheumatoid arthritis, Crohn's disease and asthma. OCTN1 and OCTN2 accept cationic drugs as substrates and contribute to intestinal and pulmonary absorption, tissue distribution (including to tumor cells), and renal excretion of these drugs. Modulation of the transport activity of OCTN2 by externally administered drugs may cause drug-induced secondary carnitine deficiency. Octn3 transports carnitine specifically, particularly in male reproductive tissues. Thus, the OCTNs are physiologically, pathologically and pharmacologically important. Detailed characterization of these transporters will greatly improve our understanding of the pathology associated with common diseases caused by functional deficiency of OCTNs.

Key words: OCTN; SLC22A4; SLC22A5; transporter; carnitine; organic cation; carnitine deficiency; tetraethylammonium

Introduction

The carnitine/organic cation transporters OCTNs are classified as organic ion transporters in subfamily of 22A of the solute carrier (SLC) transporter superfamily, which includes organic cation transporters (OCTs), organic anion transporters (OATs) and carnitine/organic cation transporters (OCTNs). OCTs and OATs are categorized as transporters for cationic and anionic molecules (including xenobiotics), respectively; they can significantly affect drug disposition, and accordingly, they have to be considered as potential sites of drug-drug interaction during the clinical development of new drugs [1]. OCTNs are unique in recognizing zwitter-ionic carnitine (beta-hydroxy-gamma-trimethylaminobutyric acid) as an endogenous substrate, and they also recognize various xenobiotics, mainly cationic compounds. In mice, three isoforms, Octn1 (Slc22a4), Octn2 (Slc22a5) and Octn3 (Slc22a21), have been identified, while in humans there are two isoforms, OCTN1 (SLC22A4) and OCTN2 (SLC22A5) [2,3,4].

In humans, OCTN1 was discovered in 1997, and then OCTN2, the sodium ion-dependent carnitine transporter, was found in 1998 [2,3]. Systemic carnitine deficiency (SCD) arising from deficiency of OCTN2 was found as an inherited disease OMIM212149 in 1999 [5]. Immediately thereafter, cDNA cloning of human OCTNs and murine Octn1, 2 and 3 was achieved and the transporter molecules were functionally characterized [4] (Table 1). Here, the functional characteristics and roles of OCTNs in tissues will be comprehensively reviewed, focusing especially on OCTN2, which has been better characterized, partly because of its well-established physiological role as a sodium ion-dependent high-affinity carnitine transporter. The relationship of these transporters with various diseases is also considered, and their pharmacological and toxicological relevance is discussed

1: Characteristics of OCTN1

Initially, OCTN1 was found as a gene specifically expressed in fetal but not adult liver, and having only 30% similarity in primary amino acid sequence to other organic cation transporters (OCTs) with 11 to 12 putative membrane-spanning domains [2]. Accordingly, it was thought to be a novel transporter showing similar characteristics to OCTs. But, uniquely, it has a nucleotide-binding site motif, although ATP dependence of OCTNs has not been reported. Therefore, it appeared to be a *N*ovel organic cation transporter from a functional viewpoint, because of the *N*ucleotide-binding site motif [2]; this is the reason why this transporter is named OCT*N*. When OCTN1 is expressed in HEK293 cells, it exhibits transport activity for the organic cation tetraethylammonium (TEA). OCTN1-mediated TEA transport is pH dependent, with a lower activity at acidic pH, suggesting that OCTN1 may be an organic cation/proton exchange transporter. In addition, OCTN1 is localized at

the apical membrane of renal tubular epithelial cells [6]. Accordingly, considering that the tubular luminal pH is more acidic than the intracellular pH, it is conceivable that OCTN1 functions as an organic cation/proton exchanger in kidney, mediating the excretion of cationic compounds into urine. Such a transporter had long been suspected to be present on the basis of membrane physiological studies [2,6]. Although the physiological role of OCTN1 is not well established, it preferentially transports the anti-oxidant ergothioneine in a sodium ion-dependent manner. Accordingly, it has been suggested that the physiological role of OCTN1 is intracellular accumulation of ergothioneine for relief of oxidative stress [7,8]. In addition, OCTN1 was suggested to be involved in the development of inflammatory or autoimmune diseases such as rheumatoid arthritis and Crohn's disease [9,10,11]. Octn1 gene (*Slc22a4*) knockout mice are associated with the phenotype of intestinal tissue damage, suggesting that OCTN1 has an essential physiological role in the intestines, and a functional defect is associated with disease [12]. These ideas are consistent with the fact that Octn1-knockout mice exhibit a significant change in disposition of ergothioneine, based on metabolome analysis [12]. As described above, OCTN1 may also contribute to drug transport in the kidney, because it accepts drugs such as quinidine, pyrilamine, verapamil and oxaliplatin as substrates, as well as TEA [13,14] (Table 1). It is also reported that renal clearance of the anticonvulsant gabapentin is decreased in individuals with single nucleotide polymorphism (SNP) leading to non-synonymous mutation of leucine at codon 503 to phenylalanine [15]. This mutation causes a decrease in OCTN1-mediated transport of gabapentin, as verified *in vitro*, suggesting the *in vivo* pharmacokinetic relevance of OCTN1 as a contributor to renal secretion of this drug. Although there is some experimental evidence that OCTN1 has other pharmacological and pathophysiological roles, most of these findings require confirmation, and the extent of OCTN1's contribution also remains to be clarified.

2: Sodium-dependent, high-affinity carnitine transporter, OCTN2

OCTN2 is responsible for transport of carnitine, which is partially biosynthesized from lysine and methionine and is essential for beta-oxidation of long-chain fatty acids in mitochondria. Without carnitine, fatty acids are unable to cross the inner membrane of a mitochondria, where beta-oxidation reaction occurs, since fatty acids are translocated as acylcarnitine esters (formed by carnitine palmitoyl transferase 1, CPT1) into mitochondria *via* carnitine acylcarnitine translocase (CACT) [16]. Skeletal muscle and cardiac muscle utilize fatty acids as an energy source; therefore, a deficiency of carnitine in these tissues leads to cardiomyopathy and muscle weakness. In *juvenile visceral steatosis* (*jvs*) mice, which exhibit a SCD phenotype, alterations in intestinal absorption, tissue distribution and elimination of carnitine have been observed [17,18]. Pathologically, *jvs* mice display severe lipid accumulation in the liver, hypoglycemia, hyperammonemia, and growth retardation [17]. Table 2 shows PK parameters after administration of [^{3}H]carnitine in normal and *jvs*

mice, based on our previous studies. Comparison of the PK parameters reveals that total clearance is increased in *jvs* mice, while volume of distribution, tissue-to-plasma concentration ratio and bioavailability after oral administration are significantly decreased [18]. Such alterations in PK parameters of carnitine in *jvs* mice can be explained by decreased membrane permeability in intestinal and other tissues and reduced reabsorption in kidney, leading us to hypothesize that membrane transporter function for carnitine is impaired in these tissues. Further *in vitro* study has demonstrated decreased membrane permeability in primary-cultured fibroblast cells prepared from *jvs* mice [19]. Finally, it was confirmed that OCTN2/Octn2 is a molecular determinant of membrane permeability of carnitine, and the transporter was characterized as a high-affinity, sodium ion-dependent transporter of carnitine [3]. We confirmed that carnitine transport activity is impaired due to mutation of the Octn2 gene in *jvs* mice and mutation of the OCTN2 gene in SCD patients [5]. The Octn2 gene in *jvs* mice exhibits a single nonsynonymous mutation in codon 352 that results in a change of the amino acid residue from leucine to arginine, and this mutation resulted in loss of carnitine transport activity when expressed *in vitro* in cultured cells [5]. In addition, Japanese individuals and familial members who are affected by carnitine deficiency have been reported to have various mutations in the OCTN2 gene, all of which are associated with loss or decrease of carnitine transport activity [5]. OCTN2 is now generally accepted to be the causal gene of the genetic disease SCD (OMIM212149).

Based on membrane physiological studies, it has been suggested that several types of carnitine transporters are expressed in various cells and tissues, including high- and low-affinity types and sodium ion-dependent and -independent types [20] (Table 1). Among them, OCTN2 is classified as a high-affinity, sodium ion-dependent carnitine transporter that is essential to maintain appropriate systemic and tissue carnitine concentrations, since loss of functional OCTN2 leads to SCD.

3: Role of OCTN2 in carnitine transport in somatic tissues

OCTN2 is expressed in various tissues. It is abundant in kidney, skeletal muscle, placenta, heart, prostate and thyroid, and to lesser extent, in small intestine, liver, lung, brain, trachea, pancreas and others [3], suggesting that the expression of OCTN2 is associated with beta-oxidation capacity of tissues. The tissue distribution of OCTN1 and OCTN2 mRNA (or protein) expression is summarized in Table 1. The putative roles of OCTN2 based on its intracellular protein localization in each tissue are summarized in Figure 1.

3-1: Kidney; In *jvs* mice, the plasma concentration of carnitine is significantly decreased. Carnitine is excreted into urine as a result of glomerular filtration, because it does not bind to serum protein; however, most of the filtered carnitine is reabsorbed in proximal tubules. In accordance with this, *jvs*

mice exhibit increased carnitine urinary excretion, suggesting that OCTN2 is involved in renal reabsorption of carnitine [18,19,21]. OCTN2 protein has been immunolocalized at the apical membrane of the proximal tubular epithelial cells [22]. In addition, transport studies using membrane vesicles prepared from OCTN2-transfected cultured cells exhibit sodium ion dependence with an overshoot uptake in the presence of an inward-directed sodium ion gradient, in a membrane potential-dependent manner [22]. Since carnitine is co-transported with a sodium ion in each transport cycle via OCTN2/Octn2, this transport is electrogenic. Similar characteristics of carnitine transport have been observed in brush-border membrane vesicles isolated from kidney cortex [23]. Sodium ion-dependent electrogenic transport is consistent with reabsorptive transport at the renal tubules. In kidney epithelial cells in primary culture, sodium dependence of carnitine uptake from the luminal side across the apical membrane has been reported in rats and humans [24,25]. Apical membrane localization of OCTN2 protein might be controlled by interaction with PDZ protein, PDZK1 [26], because OCTN2 and PDZK1 proteins are co-localized in brush-border membrane of kidney tubular cells and coexpression of OCTN2 with PDZK1 results in an apparent increase in transport activity of OCTN2, but with unchanged affinity and membrane surface expression. The precise mechanism of the increased activity of OCTN2 has not been established, but protein-protein interaction should be considered as an important determinant of efficient physiological reabsorption of carnitine mediated by OCTN2. In summary, available evidence indicates that OCTN2 serves as a transporter for reabsorption of carnitine at the apical membrane of renal tubular epithelial cells.

3-2: Intestine; The nature of the intestinal absorption mechanism of carnitine is controversial. Some studies show saturable and sodium ion-dependent apical uptake of carnitine [27,28], while others suggest no contribution of transporters [29]. However, a recent study in Caco-2 cells showed sodium ion-dependent carnitine uptake with similar characteristics to those of OCTN2 [30]. The mRNA expression of OCTN2 is significant in human intestinal tissues along duodenum through colon, and the expression level is similar to that of another important nutrient transporter, PEPT1 [31, 32]. Accordingly, OCTN2 is likely expressed in intestinal tissues and contributes to absorption of carnitine. In addition, *jvs* mice exhibit a lowered absorption of carnitine after oral administration, resulting in reduced carnitine AUC in plasma (Table 2) [17]. *In vitro* transport studies in isolated intestinal tissues and enterocytes from *jvs* mice showed reduced uptake of carnitine compared with normal mice [33], whereas uptake of carnitine by enterocytes from normal mice was sodium ion-dependent and saturable with a K_m of 12 μM, which is similar to the K_m of mouse Octn2-mediated carnitine transport, 22 μM [4] (Table 1). In addition, mouse Octn2 protein is localized at the apical membrane of the intestine in mice [34]. As in kidney, the apical membrane localization may be regulated by PDZK1, since Octn2 expression at the apical membrane is

decreased in Pdzk1-knockout mice [34]. Based on these results, OCTN2 and Octn2 are considered to contribute to intestinal absorption of dietary carnitine.

3-3: Skeletal muscle and heart; Carnitine is stored in muscle tissues, so that its intramuscular concentration is maintained at high levels [35]. In heart, OCTN2 protein is present in vascular endothelium [36]. As shown in Table 2, in *jvs* mice the carnitine distribution into muscular tissues, in particular heart and skeletal muscle, is significantly reduced [18]. Systemic carnitine deficiency causes cardiomyopathy and skeletal muscle weakness, and carnitine uptake in cultured muscle cells involves a saturable process with K_m lower than 10 μM, which can be ascribed to a high-affinity transporter [37-39]. These observations suggest that OCTN2 is important for carnitine uptake in these tissues and a functional defect has a critical effect on normal tissue function. The expression of OCTN2 protein in vascular endothelium in heart is likely to play a role in accumulation of plasma carnitine into heart tissues [36].

3-4: Liver; One of the symptoms of SCD is fatty liver, suggesting that carnitine is essential for normal liver function. In *jvs* mice, hepatic uptake of carnitine is lowered, showing a significant increase in K_m to 475 μM as estimated from the saturation kinetics [40]. Normal mouse liver exhibits biphasic uptake kinetics with K_m values of 4.6 μM and 404 μM for the high- and low-affinity components, respectively. Accordingly, *jvs* mouse lacks the high-affinity transporter for carnitine, suggesting that Octn2 is responsible for high-affinity carnitine uptake in liver. Biosynthesis of carnitine occurs in liver, kidney and brain, since expression of the enzyme gamma-butyrobetaine dioxygenase, which produces carnitine from gamma-butyrobetaine, is limited to these tissues. Since gamma-butyrobetaine is produced in many tissues and released into the blood circulation, these three tissues may utilize gamma-butyrobetaine as a precursor of carnitine biosynthesis [41]. Our laboratory has shown that OCTN2 transports gamma-butyrobetaine as a substrate in a sodium ion-dependent manner with K_m 12.9 μM [42]. Hence, OCTN2 may be involved in controlling the intracellular pool of this precursor. However, uptake of gamma-butyrobetaine is not exclusively due to OCTN2 in liver; the contribution of OCTN2 has been estimated to be approximately 30%. We have found that gamma-butyric acid transporter subtype GAT2 is involved in supply of gamma-butyrobetaine to the liver [42,43]. In *jvs* mice, administration of gamma-butyrobetaine, but not carnitine, increased carnitine concentration in brain, liver, and kidney [44]. However, in normal mice, such an increase of carnitine concentration in these tissues was not observed after administration of gamma-butyrobetaine. In addition, the hepatic concentration of free fatty acids was decreased after gamma-butyrobetaine treatment only in *jvs* mice, but not in normal mice. These results together suggest that GAT2 is an alternative transporter responsible for taking up gamma-butyrobetaine into the liver for biosynthesis of carnitine in hepatocytes to supply other tissues, when OCTN2 is not functional due to genetic mutation, as

observed in SCD. Usually, carnitine is administered to patients who show low systemic carnitine concentrations. However, for those who suffer from SCD, it might be better to supply gamma-butyrobetaine but not carnitine itself, since these patients have significantly reduced carnitine transport activity, but retain GAT2 activity.

3-5: Blood-brain barrier; Supplementation of carnitine and/or acetylcarnitine may have benefit in the central nervous system (CNS) to improve diseases such as Alzheimer's disease, depression, and chronic fatigue syndrome, presumably by supplying acetyl moieties, because it enhances mitochondrial function and antioxidant effect in the CNS [45-47]. Accordingly, carnitine/acetylcarnitine needs to be translocated into brain across the blood-brain barrier. OCTN2 is indeed expressed in brain [3] and the presence of OCTN2 in brain capillary endothelial cells that constitute the blood-brain barrier has been demonstrated [48-51]. Studies using our *in vivo* and *in vitro* cultured cell models for blood-brain barrier transport have indicated that acetylcarnitine is significantly taken up by brain from the blood side, and the endothelial cellular uptake of acetylcarnitine has similar characteristics to those of OCTN2 [48]. An *in situ* brain microdialysis study suggested the movement of acetylcarnitine from blood to CNS [49]. OCTN2 and other amino acid transporters are likely involved in blood-brain barrier transport of carnitine [50], while OCTN2 might exists on the brain side but not the blood side, and so may contribute to transport of carnitine/acetylcarnitine from brain to blood [51]. Although the precise contribution of OCTN2 at the blood-brain barrier remains unclear, it should contribute to CNS function by regulating concentrations of carnitine and acetyl moiety in the brain.

3-6: Mammary glands and placenta; Carnitine and acylcarnitine are present at higher concentrations in the fetal circulation than in the maternal circulation, and the fetus has limited ability to synthesize carnitine. Therefore, it is possible that maternal carnitine is supplied to the fetus across the placental barrier [52]. In placenta-derived choriocarcinoma cells, sodium ion-dependent transport of carnitine with K_m 12 μM has been reported [53]. The transport system has affinity for both L- and D-carnitine, acetylcarnitine and other compounds, but has no affinity for choline. These characteristics are similar to those of OCTN2. Gene expression and protein localization of OCTN2 protein at the apical membrane of syncytiotrophoblast have been observed [54]. Furthermore, apical membrane vesicles, but not basolateral membrane vesicles, prepared from syncytiotrophoblast are able to mediate sodium-dependent uptake of carnitine with K_m 21 μM. In *jvs* mice, carnitine accumulation in fetal tissues is significantly lower than in normal mice; however, significant up-regulation of fatty acid oxidation-related enzymes is also observed in placental tissues of *jvs* mice [55]. This observation suggests that OCTN2 is essential to maintain energy supply in placenta and fetus, and the reduced carnitine level may be compensated by increased enzyme activity of fatty acid oxidation. Neonates also need carnitine to generate energy

via beta-oxidation of long-chain fatty acids, in particular to maintain brain function, since they are not able to synthesize enough carnitine. Accordingly, carnitine should be supplied to neonates in milk. Indeed, the concentration of carnitine in milk (0.32 mM) after 24 h nursing was approximately 10 times that in plasma in rats [56]. Interestingly, the carnitine concentration declines gradually during lactation, which may suggest that the neonate acquires increased activity to synthesize carnitine. There is a report that carnitine transport in mammary gland exhibits sodium ion dependence in rats [57]. Carnitine transport was inhibited by D-carnitine and acetylcarnitine but not by choline, showing similar characteristics to those of OCTN2, though the obtained K_m was relatively high (132 μM). In MCF12A cells derived from human mammary gland epithelial cells, high- and low-affinity transport processes for carnitine with K_m values of 5.1 μM and 15.9 mM, respectively, are present; it seems likely that the high-affinity component is mediated by OCTN2 [58]. OCTN2 protein is detected in the alveolar epithelia of mammary gland, apparently at a sufficient level to supply carnitine to milk. In mice, Octn1, 2, and 3 are all expressed in mammary gland [59]. Their intracellular localization in mammary gland is not known; however, Octn1 is expressed mainly in blood vessel endothelium and secretory alveolar apical membrane, Octn2 in secretory alveoli, and Octn3 in interstitial space and blood vessel endothelium. Their expression level in epithelial ducts is increased in pregnant and lactating mice, compared to virginal mice, and then the levels decrease after cessation of lactation, so that the expression profile of OCTN transporters is similar to the change of carnitine concentration in milk. Accordingly, carnitine transporters including OCTNs are thought to transfer carnitine to milk from maternal blood.

4: Roles of OCTNs in male reproductive tissues and germ cells

In male reproductive tissues, carnitine and acetylcarnitine concentrations are higher than in plasma and carnitine is likely an important nutrient for maturation and motility of spermatozoa [60]. OCTNs are present in testis and have been suggested to contribute to carnitine/acetylcarnitine handling [2,3]. The structure of male reproductive tissues and localization of expression of OCTNs/Octns are illustrated in Figure 2. Spermatozoa are produced in testis and move to epididymis. They mature to fertile sperms during the transition to epididymis. Spermatozoa are immotile in the proximal part of epididymis, where carnitine concentration in epididymal plasma is low. However, with increase of carnitine/acetylcarnitine concentration along the epididymis from caput to corpus and cauda, the number of motile spermatozoa increases [60].

Mouse Octn3 is selectively expressed in the testis [4], and human CT2 (SLC22A16), which is not classified into the OCTN family but has about 33% similarity to mouse Octn3, and shows similar functional characteristics of sodium ion independence and high affinity for carnitine, is also expressed in testis [61]. In Sertoli cells, which form a part of the blood-testis barrier, carnitine

transport is sodium ion-dependent and exhibits biphasic kinetics, suggesting contributions of OCTN2 and $ATB^{0,+}$, which was firstly found as amino acid transporter, to high- and low-affinity transport, respectively [62-64].

Uptake of carnitine by primary-cultured rat epididymal cells in suspension shows high- and low-affinity transport components, and the sodium ion dependence and inhibitor susceptibility of the high-affinity transport are similar to those of OCTN2 [65]. Octn2 protein is localized to the basolateral side of epithelial cells, with higher expression at the distal caput, corpus and proximal cauda in rats [66]. Although rat Octn1 expression in epididymal tissues is low, it is detectable in testis and epididymal tissues from caput to cauda [66]. Human CT2 is a sodium ion-independent transporter, like mouse Octn3, and is expressed at the luminal membrane of epididymal epithelial cells [61]. Accordingly, Octn3/CT2 should play a role in transport of carnitine in this tissue. However, in a carnitine transport experiment with suspended epididymal epithelial cells, sodium ion-dependent transport was observed, showing a discrepancy between the expression of transporters (Octn3/CT2) and the observed activity (sodium-dependent carnitine uptake) [65]. In experiments using suspended epididymal tissue, it is easier to detect basolateral transport than luminal transport [65]. Accordingly, it is possible that only sodium ion-dependent uptake of carnitine, which corresponds to basolateral uptake, might have been detected using this experimental method, and that sodium ion-independent transport of carnitine is actually present at the luminal side of epididymal tissues, but was not detected with this method. Carnitine is highly accumulated in epididymal plasma. This may be explained by the following mechanisms: OCTN2-mediated sodium ion-dependent active transport imports carnitine from the blood into epithelial cells, from which it is released to the lumen via sodium ion-independent CT2 transporter. However, in mice Octn2 is localized to the luminal membrane of epididymal epithelial cells, particularly the distal corpus and cauda [67, 68]. There may be several reasons for this, including the presence of splice variants and species difference. Although further studies are required, it is clear that OCTN2 is essential to maintain a high concentration of carnitine in the distal part of epididymal luminal plasma, based on the above findings and the observation of male infertility in *jvs* mice due to obstructive azoospermia [69].

Expression of OCTNs in spermatozoa is interesting, since their expression shows site selectivity [70]. In mice, epididymal spermatozoa show partial sodium ion-dependent transport with K_m values of 23.6 μM and 6.57 μM for carnitine and acetylcarnitine, respectively. The uptake activity is higher in spermatozoa obtained from corpus than in those from caput or cauda, both in the presence and absence of sodium ions. Accordingly, a contribution of sodium ion-dependent or -independent transporters is possible. Immunocytochemical studies of the expression of mouse Octn1, 2 and 3 proteins in spermatozoa from caput, corpus and cauda showed selective expression of Octn2 and 3 in

the tail of spermatozoa, though expression of Octn1 protein is low (Figure 3). Octn3 expression is higher in the spermatozoa obtained from corpus and cauda than caput. Furthermore, although Octn2 and 3 are present in spermatozoa, their localizations are distinct, since they are expressed in the principal piece of the tail and middle piece of the tail, respectively (Figure 3). Several reports have suggested passive uptake of carnitine/and acetylcarnitine by these sperm cells. However, the selective expression of OCTN proteins and the uptake characteristics observed in spermatozoa indicate that OCTN transporters are responsible for the uptake of carnitine and acetylcarnitine to fertile spermatozoa. Expression profiles of Octns in male reproductive tissues and germ cells are illustrated in Figure 2.

5: OCTNs and autoimmune diseases.

Because of the physiologically essential role of carnitine in beta-oxidation of long-chain fatty acids to produce ATP, functional defect of OCTN2 leads to failure to maintain an appropriate systemic concentration of carnitine by reabsorption of filtered carnitine and thus to an inadequate supply of carnitine to various tissues as described above, directly resulting in carnitine deficiency-related diseases such as skeletal muscle weakness, cardiomyopathy, male infertility, and others. The symptoms observed in *jvs* mice clearly demonstrate the importance of OCTN2-mediated carnitine transport in the corresponding tissues [71]. In addition to these symptoms due to carnitine deficiency, current evidence suggests an association of OCTNs with various diseases, including rheumatoid arthritis, Crohn's disease and asthma, though the mechanisms involved remain enigmatic. It has been reported that SNP in intron 1 of the OCTN1 gene affects the expression of OCTN1 by lowering affinity for the transcription factor RUNX1 [9,10]. In addition, the *RUNX1* gene is independently associated with autoimmune diseases such as rheumatoid arthritis [9]. Similarly, SNPs in OCTN1 and OCTN2 genes are associated with susceptibility to Crohn's disease [10]. In Octn1-knockout mice, the absorption and disposition of its substrate ergothioneine are changed and these mice exhibit a higher susceptibility to intestinal inflammation [11]. Since OCTN1 protein is expressed in intestinal epithelial cells and its functional defect significantly affects the intestinal disposition of ergothioneine, OCTN1 might be related to many intestinal diseases, though the mechanisms remain to be clarified [72]. More recently, pharmacogenomic attempts to understand the relevance of OCTN1 and OCTN2 to susceptibility to Crohn's disease have uncovered a significant association in Caucasians, but not in an East Asian population [73]. Asthma is a disease that may involve heritability and alteration in several associated genes. *SLC22A5* may be one of them, based on SNP analysis in asthmatics and unaffected persons [74]. Expression of OCTN1 and OCTN2 in airway epithelial cells has been reported to be localized at the luminal membrane of epithelial cells and the

transporters are functional, since the substrates ergothioneine, carnitine and the anti-cholinergic drug ipratropium are taken up by cells with similar characteristics to those observed *in vitro* for OCTN1/2-expressing cells [75,76]. Although little is yet known about the mechanism, it is possible that OCTN1/2 expressed in respiratory tissues is involved in the normal physiological function of airways and its disruption may lead to asthma.

6: Drug transport and pharmacological and toxicological relevance of OCTNs

6-1: Substrate selectivity of OCTNs and their difference; OCTN1 and OCTN2 preferentially transport ergothioneine and carnitine/acetylcarnitine, respectively. They also accept various xenobiotics/drugs as substrates (Table 1). OCTN1 was first found as a transporter resembling the OCT family (SLC22A1, 2, and 3), and it mediated transport of TEA [2]. OCTN2 was the second member to be identified of the organic cation transporters that recognize TEA as a substrate, and the OCTNs were initially classified as polyspecific cation transporters, based on substrate specificity and inhibition profile [77]. However, we have shown that OCTN2 is a physiologically important carnitine transporter [3], while OCTN1 is an ergothioneine transporter [6,7]. Further, mouse Octn3 displays negligible transport of the organic cation TEA, and preferentially transports carnitine [4]. Similarly, CT2 is selective for carnitine and acylcarnitine and does not transport organic cations, thus showing similar specificity for carnitine-related compounds to mouse Octn3 [61]. Mouse Octn3 is a sodium ion-independent transporter for carnitine, and is distinct from OCTN2. Structural analysis of human OCTN2 and mouse Octn3 suggests that the sodium ion dependence of carnitine transport is associated with a few key amino acid residues, e.g. glutamine at codon 180 and glutamine at codon 207 of OCTN2, that are common to human, rat and mouse OCTN2s, but not to mouse Octn3, in which both of these residues are histidine [78]. This may be related to the fact that OCTN1 and OCTN2, but not Octn3, contribute to xenobiotic/drug transport. Experimentally, quinidine, pyrilamine, verapamil, oxaliplatin, gabapentin, and ipratropium are known to be substrates of OCTN1 [2,12,13,14, 76]. Substrate drugs of OCTN2 include oxaliplatin, verapamil, spironolactone, mildronate, pyrilamine, TEA, quinidine, cephaloridine, imatinib and valproate [13,36,76,79,80,81,82] (Table 1). There are also many inhibitors of OCTN1 and OCTN2 and those inhibitors may also be substrates of OCTNs, though there is no direct evidence for this. However, there are some conflicting results with regard to substrates [83]. Further studies, such as molecular modeling of binding of substrates and inhibitors, in addition to precise functional studies are needed [84].

6-2: Characteristics of substrate recognition of OCTN2; There are some interesting reports on the multiplicity of substrate recognition of OCTN2; for example, transport of carnitine is sodium ion-dependent, but transport of cationic xenobiotics is sodium ion-independent [79,85].

Physiologically, the electrochemical potential of sodium ion is inwardly directed and it is clear that carnitine is taken up by cells via OCTN2, but not transported out of cells via OCTN2. Interestingly, in *jvs* mice, which lack functional Octn2, the changes in tissue distribution and urinary excretion of the organic cation TEA from normal mice were distinct in part from those observed for carnitine (Table 2) [86]. Although the tissue distribution of TEA in most tissues was decreased in *jvs* mice similarly to that of carnitine, it was significantly increased in kidney (Kp values in kidney: for TEA, 9.89 in wild-type and 25.0 in *jvs* mice; for carnitine, 31.6 in wild-type and 10.8 in *jvs* mice). In contrast, urinary secretory clearance of TEA was decreased in *jvs* mice from 6.25 to 3.43 mL/min/kg, while total clearance of carnitine was increased in *jvs* mice from 2.45 to 6.7 mL/min/kg (Table 2). Accordingly, renal handling of TEA in *jvs* mice is quite different from that of carnitine. Considering the renal handling of TEA and carnitine, which are actively secreted and reabsorbed, respectively, such an observed change in TEA disposition implies that renal secretion of TEA is decreased in *jvs* mice, while reabsorption of carnitine is decreased in *jvs* mice. Thus, OCTN2 appears to contribute bidirectionally to secretion of cationic compounds, as well as contributing to reabsorption of carnitine. This apparent discrepancy may be due to the difference in sodium ion dependence of OCTN2-mediated transport between TEA and carnitine. Since TEA transport mediated by OCTN2 shows pH dependence but not sodium ion dependence and OCTN2 is expressed at the apical membrane of tubular epithelial cells [77], OCTN2-mediated TEA transport is partly driven by an inwardly directed proton gradient and TEA is exchanged with protons via OCTN2. This mechanism is distinct from sodium ion-dependent carnitine transport via OCTN2. The hypothetical mechanisms of OCTN2-mediated sodium ion-dependent carnitine and pH-dependent TEA transport are shown in Figure 4. Sodium ion requirement for carnitine transport may be mechanistically explained by the presence of the carboxyl moiety in carnitine, but not TEA, and the sodium ion-binding site on OCTN2 is close to the site that recognizes the carboxyl moiety of carnitine, while this site is not directly involved in binding of TEA on OCTN2 [85]. This model is shown in Figure 5. The idea that the binding sites of carnitine and cationic compounds are not identical, but are partly shared, can explain the difference in the apparent effects of mutations of OCTN2 protein on the transport activity towards these two types of compounds [85].

6-3. OCTN-mediated absorption of inhaled drugs; Although it has not yet been proven, OCTNs might be involved in transport of inhaled drugs, influencing the efficacy and toxicity of these drugs. OCTNs are expressed in lung [2,3] and the anti-cholinergic ipratropium bromide (ipratropium) has been shown to be a substrate of OCTN2 using Caki-1 cells, which are derived from human kidney proximal cells expressing OCTN2 at the apical membrane [87]. The uptake of ipratropium by an artificial expression system of OCTN1 and OCTN2 was significantly higher than of non-transfected

cells and K_m values for its transport were 444 μM and 53 μM, respectively [76], although ipratropium is also transported by OCT1 and OCT2 [88]. Ipratropium is a quaternary cationic drug and is effective after inhalation for chronic obstructive pulmonary disease and asthma, so it must cross airway epithelial cells to exert its pharmacological activity. Since ipratropium is hydrophilic and is a substrate of OCTN2, its transport by OCTNs in human bronchial epithelial cell line BEAS-2B has been studied [76]. Ipratropium uptake by BEAS-2B cells via OCTNs was characterized by silencing OCTN2 mRNA expression; this resulted in a significant decrease of uptake, suggesting that OCTN2 expressed in the airway epithelial cells is involved in the absorption of ipratropium, and presumably also tiotropium bromide, after inhalation. A carnitine ester prodrug of prednisolone has been synthesized to target bronchial epithelial cells after delivery by inhalation, in the expectation of OCTN-mediated bronchial epithelial absorption [89]. Such prodrugs are likely transported by OCTN2 in BEAS-2B cells. OCTN2, and to a lesser extent OCTN1, may contribute to airway absorption of such inhaled drugs, for selective delivery to their pharmacological target(s). To date, pulmonary drug delivery has not been well characterized, but OCTNs and other transporters, including OCTs, may contribute to drug absorption from lung [90]. It is reported that the air-interfaced cultured cell line Calu-3 expresses OCTNs as well as OCTs [91], and is a suitable model to evaluate transport of inhaled drugs [92].

6-4. OCTN and platinum anticancer drug toxicity; Cisplatin and some other platinum anticancer drugs cause toxicity in kidney, and organic cation transporters are likely involved in this drug-dependent toxicity by causing them to accumulate in kidney tubular epithelial cells [93,94]. For example, the dose-limiting toxicity of oxaliplatin is neuropathy due to drug accumulation in dorsal root ganglion [95], and OCTNs might be involved in this accumulation [14]. Oxaliplatin is a substrate of OCTN1 and OCTN2, and HEK293 cells transfected with OCTN1 and OCTN2 exhibited higher sensitivity to oxaliplatin-induced toxicity. Further, OCTN1 and OCTN2 are both expressed in cultured dorsal root ganglion cells from rats. Hence, intracellular accumulation of oxaliplatin in dorsal root ganglion cells could be due to uptake by the OCTNs. In this regard, reducing the accumulation of oxaliplatin by administration of OCTN inhibitors may be useful to prevent oxaliplatin-induced neurotoxicity [14].

6-5. Drug-induced secondary carnitine deficiency; Drug-induced carnitine deficiency is sometimes caused by clinically administered drugs such as cefditren pivoxyl and valproic acid [96,97]. It has been considered that OCTN2-mediated reabsorption of carnitine might be inhibited by these drugs, resulting in a decrease of plasma concentration of carnitine. Although valproic acid, emetin and pivalic acid are known to cause secondary carnitine deficiency or to have an inhibitory effect on OCTN2-mediated carnitine transport, their inhibitory potencies may not be sufficient to account for the clinical observations [79]. An alternative possibility is that carnitine esters formed

with pivalic acid and valproic acid may not be reabsorbed well, resulting in decreased reabsorption of carnitine [98,99,100]. In addition, carnitine esters have the potential to inhibit OCTN2-mediated reabsorption of carnitine to some extent, so these drugs may cause drug-induced secondary carnitine deficiency.

7: Conclusion

The key physiological role of OCTNs appears to be transport of carnitine, which is required for energy supply through beta-oxidation of long-chain fatty acids. Any disturbance of carnitine homeostasis is likely to affect heart and skeletal muscle function, as well as male fertility. Furthermore, genetic variations of OCTN genes are associated with severe diseases, including autoimmune diseases such as rheumatoid arthritis, Crohn's disease, and asthma. Accordingly, studies of carnitine-related systems may provide clues to the mechanisms of these diseases. In addition, clinically used drugs that disturb carnitine homeostasis might lead to adverse effects, such as heart failure and myopathy. Therefore, a detailed understanding of OCTNs is expected to be clinically important in various respects. In addition, OCTNs contribute to the absorption and disposition of various therapeutic drugs, and may influence their pharmacokinetics and pharmacological activity. Accordingly, a better understanding of the interaction of drugs with OCTNs should be clinically beneficial, and in addition, there is the possibility of targeting OCTN transporters to modulate drug activity or side effects.

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Table 1

Characteristics of OCTNs and carnitine transporters

Name	Gene	Tissue expression	Substrate	K_m (L-carnitine)
OCTN1 (human)	SLC22A4	bone marrow, gut, heart, kidney, lung, placenta, prostate, skeletal muscle, trachea, *etc.*	*D*- and *L*-carnitine, acetylcarnitine, ergothioneine gabapentin, oxaliplatin, pyrilamine quinidine, verapamil, TEA. *etc.*	Low affinity K_m (TEA) human 436 μM mouse 452 μM
OCTN2 (human)	SLC22A5	brain, heart, intestine, kidney, liver, lung, pancreas, placenta, thyroid, trachea, *etc.*	*D*- and *L*-carnitine, acetylcarnitine, γ-butyrobetaine, cephaloridine, imatinib, mildronate, oxaliplatin, pyrilamine, quinidine spironolactone, TEA, valproate, verapamil, *etc.*	4.3 μM
octn2 (mouse)	slc22a5			22.1 μM TEA: 215 μM
CT2 (human)	SLC22A16	testis	carnitine	20.3 μM
octn3 (mouse)	Slc22a21			2.9 μM
$ATB^{0,+}$ (human)	SLC6A14	lung, hippocampus, salivary gland, mammary gland, stomach, prostate, pituitary, uterus, heart, colon, cecum, small intestine	Carnitine Gly Ala Leu Arg Pro *etc*.	
$atb^{0,+}$ (mouse)	slc6a14			mouse 830 μM

Table 2

Comparison of pharmacokinetic parameters of carnitine and TEA in normal and *jvs* mice

Parameters	Carnitine		TEA	
	wild	*jvs*	*wild*	*jvs*
$AUC_{1\text{-}4hr}$ (ng min/mL) iv	29.2	39.8	-	-
po	19.7	13.6	-	-
Bioavailability (%)	67.5	34.1*		
Vd_{ss} (L/kg)	8.16	1.10*	1.3	1.63
CL_{total} (mL/min/kg)	2.45	6.7**	-	-
$CL_{secretion}$ (mL/min/kg)	-	-	6.25	3.43*
Tissue to plasma ratio (Kp)				
Brain	1.14	0.8	0.081	0.039*
Lung	20.7	9.5*	5.29	3.7*
Heart	24.4	1.22*	0.961	1.35
Liver	29.5	8.34*	16.7	4.55*
Kidney	31.6	10.8*	9.89	25.0**
Gut	15.8	5.37*	6.32	5.7
Spleen	31.6	5.07*	5.90	2.47*
Muscle	2.16	0.54*	0.843	0.783

- indicates a significant decrease ($P<0.05$) and ** indicates a significant increase ($P<0.05$).

From References 17 and 86.

Figure legends

Figure 1: Schematic illustration of the involvement of OCTNs in carnitine disposition and role of carnitine in β-oxidation of long chain fatty acids in various tissues.

Carnitine is supplied mainly from food and partially via biosynthesis. In kidney, carnitine is filtered and efficiently reabsorbed by OCTN2. Carnitine in plasma is taken up by OCTN2 and other transporters into tissues, where β-oxidation of fatty acids occurs. Then acylcarnitine is formed in the cells via CPT1 and crosses the mitochondrial inner membrane via carnitine acylcarnitine translocase CACT for β-oxidation. Carnitine is essential in this reaction. ATP production is lowered by carnitine deficiency, resulting in a range of symptoms, including cardiomyopathy, skeletal muscle weakness, fatty liver and male infertility.

Figure 2: Differential expression of Octns in male reproductive tissues of mouse.

Spermatozoa in seminiferous tubule, initially formed from germ cells in testis, move out of the testis via efferent ductules to epididymis. The epididymis is divided into three parts, the caput (upper part), corpus (middle part) and cauda (lower part) and the spermatozoon gains motility during passage from caput to cauda. The carnitine concentration in the luminal fluid in epididymis gradually increases from the upper to lower part. Octns are expressed as indicated and are considered to control carnitine concentration in seminiferous fluid in the epididymal lumen and in epithelial and Sertoli cells.

Figure 3: Differential expression of Octns in mouse spermatozoa.

Mouse spermatozoa obtained from caput, corpus and cauda were stained with antibodies to Octn1, Octn2, and Octn3. Each Octn protein was visualized using Alexa Fluoro594 goat anti-rabbit Ig-G conjugate after reaction with the corresponding anti-Octn antibodies (Red). Nuclei were stained with DAPI (blue). The staining of octn1 is low, but staining of Octn2 and Octn3 is high in corpus and cauda, compared with caput. Cellular localizations of Octn2 and Octn3 are in the tail and a distinct part of spermatozoa. Octn2 and Octn3 are stained in the principal piece and middle piece

of the tail, respectively. This difference in expression profiles may be related to the maturation of spermatozoa. For details, see reference 70.

Figure 4: Model of OCTN2-mediated transport of carnitine and organic cation TEA in renal tubular epithelial cells.

Carnitine in tubular lumen is taken up by cells in a sodium ion-dependent manner, while TEA transport is sodium ion-independent. The carnitine binding site is only functional when sodium ion is present on the same side. Accordingly, carnitine transport via OCTN2 is in the direction of reabsorption, whereas organic cations accumulated in cells via basolateral OCT2 are removed partly by OCTN2 in a partially pH-dependent manner. Accordingly, OCTN2 might act as a reabsorptive transporter for carnitine and a secretory transporter for organic cations via different mechanisms. The hypothetical model is described in more detail in reference 86.

Figure 5: Model of substrate binding sites of OCTN2. OCTN2 accepts zwitter-ionic carnitine, quaternary cation TEA, and acidic valproic acid. Furthermore, transport of carnitine is sodium ion-dependent, while that of TEA is sodium ion-independent. Valproic acid displays sodium ion-dependent affinity for OCTN2 [reference 85]. Accordingly, it is presumed that there are at three determinant sites on OCTN2 that independently accept positively and negatively charged substrates and sodium ion. The sodium ion-binding site is very close to the site that recognizes the negative charge of a substrate, since TEA (cationic charge only) does not show sodium ion-dependent transport. Because of the presence of substrate-dependent binding sites on OCTN2 protein, mutations of certain amino acid residues of OCTN2 have differential effects on transport of TEA and carnitine [reference 85].

Figure 1

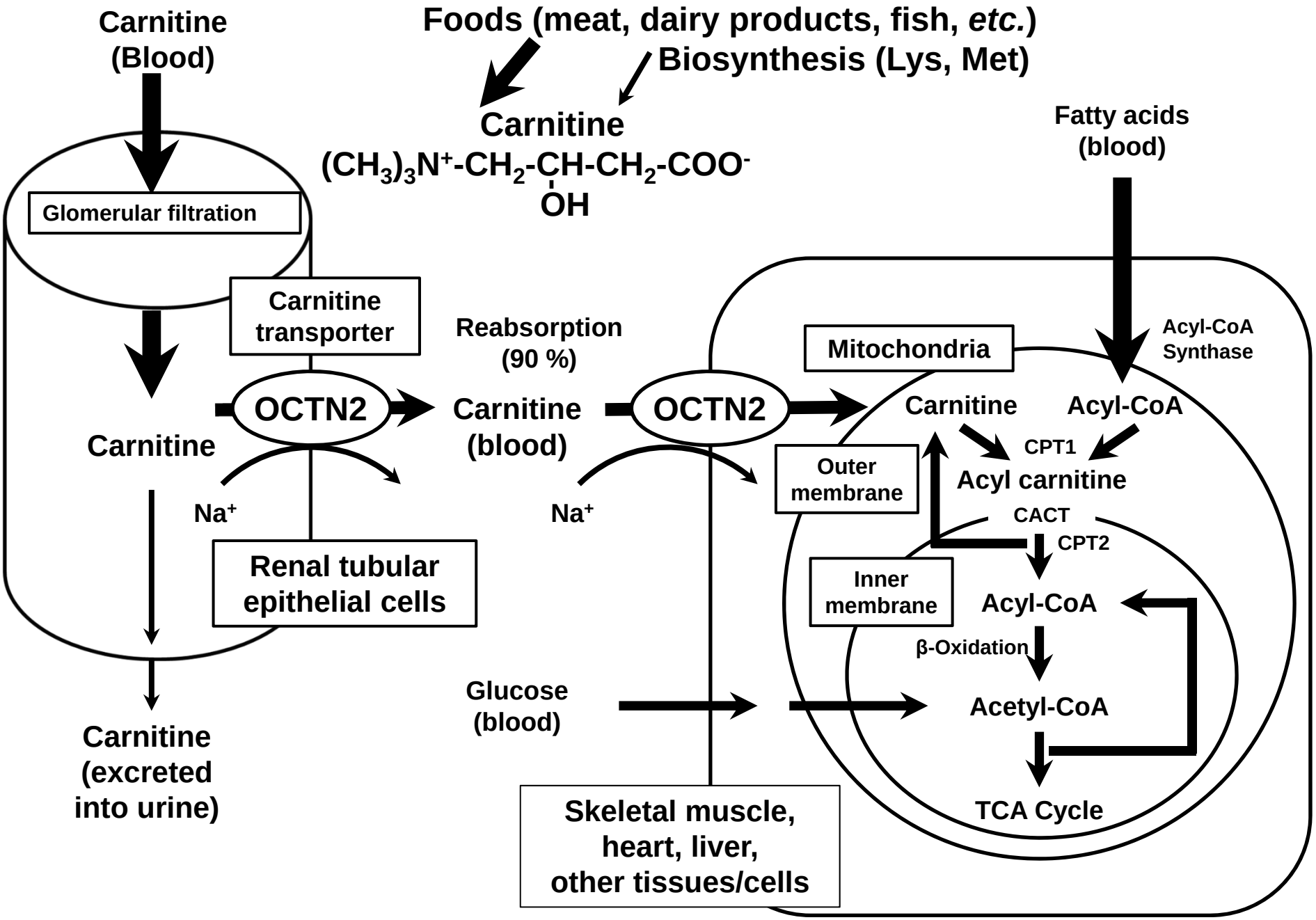

Carnitine
(Blood)
Foods (meat, dairy products, fish, *etc.*)
Biosynthesis (Lys, Met)
Carnitine
$(CH_3)_3N^+$-CH_2-CH-CH_2-COO^-
OH
Fatty acids
(blood)
Glomerular filtration
Carnitine
transporter
Reabsorption
(90 %)
Mitochondria
Acyl-CoA
Synthase
OCTN2
Carnitine
(blood)
OCTN2
Carnitine
Acyl-CoA
CPT1
Acyl carnitine
Outer
membrane
CACT
CPT2
Carnitine
Na+
Na+
Renal tubular
epithelial cells
Inner
membrane
Acyl-CoA
β-Oxidation
Glucose
(blood)
Acetyl-CoA
TCA Cycle
Carnitine
(excreted
into urine)
Skeletal muscle,
heart, liver,
other tissues/cells

Figure 2

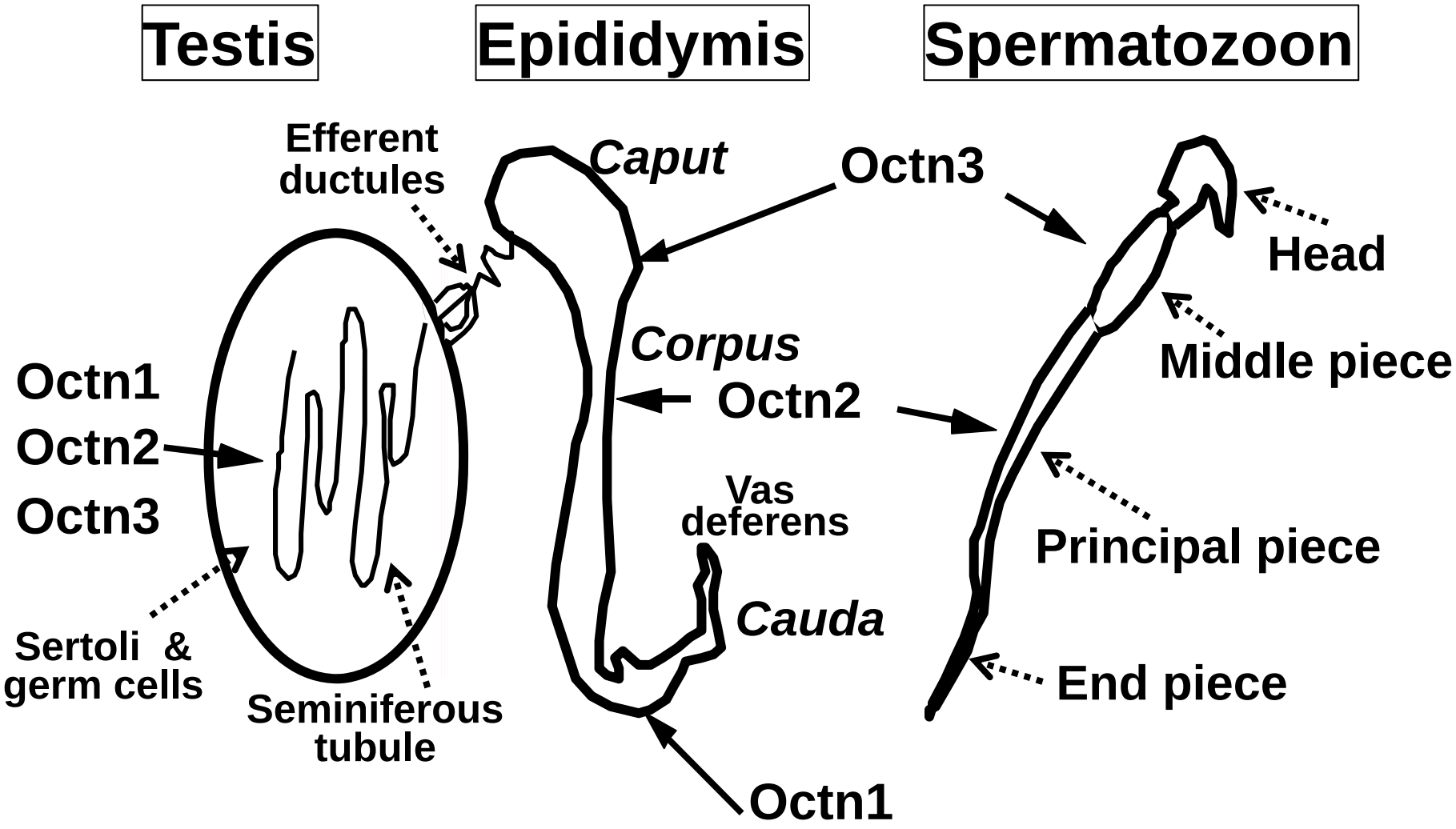

Testis
Epididymis
Spermatozoon
Efferent ductules
Caput
Octn3
Head
Middle piece
Corpus
Octn2
Octn1
Octn2
Octn3
Vas deferens
Principal piece
Cauda
Sertoli & germ cells
Seminiferous tubule
End piece
Octn1

Figure 3

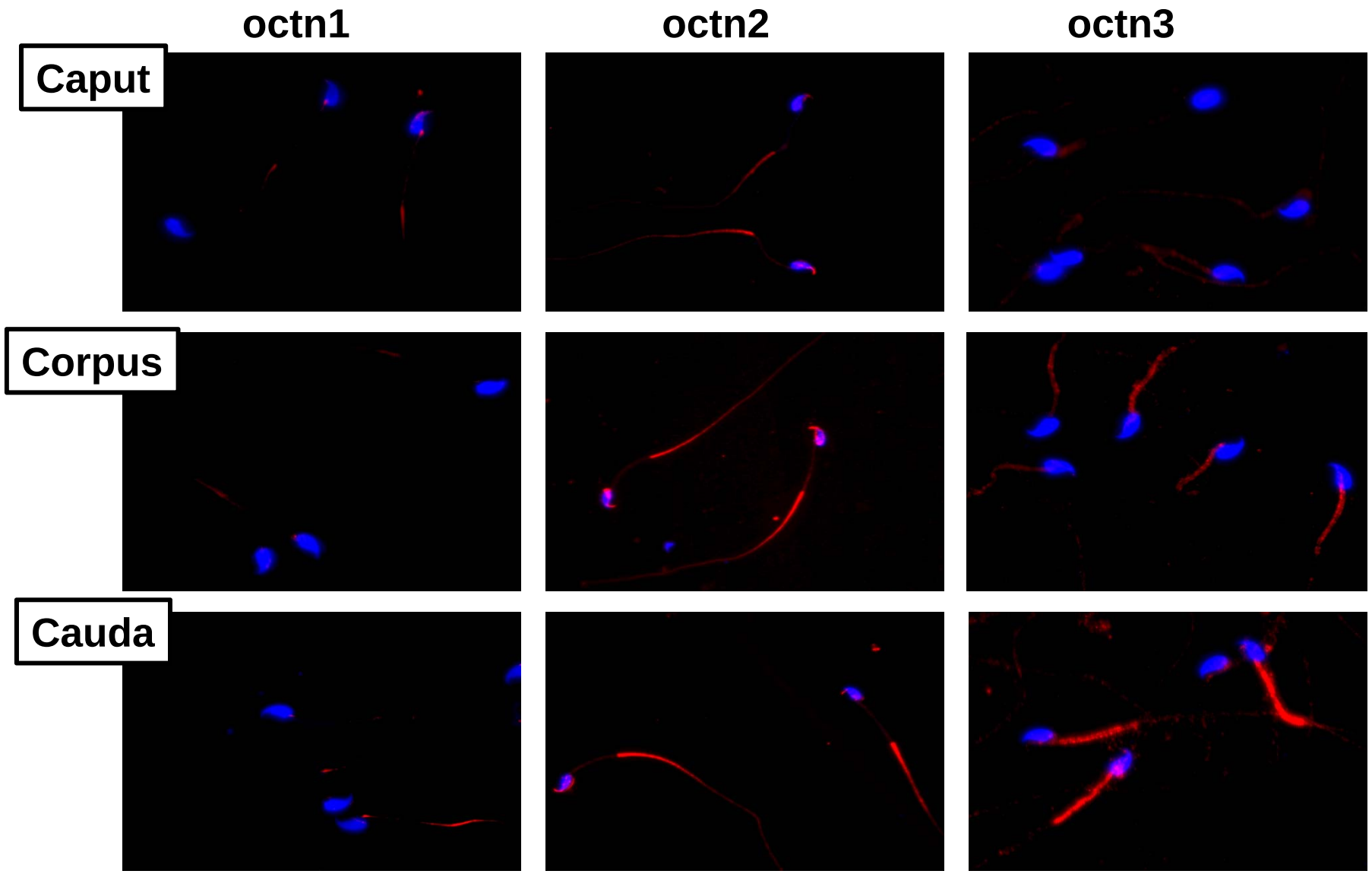


Figure 4

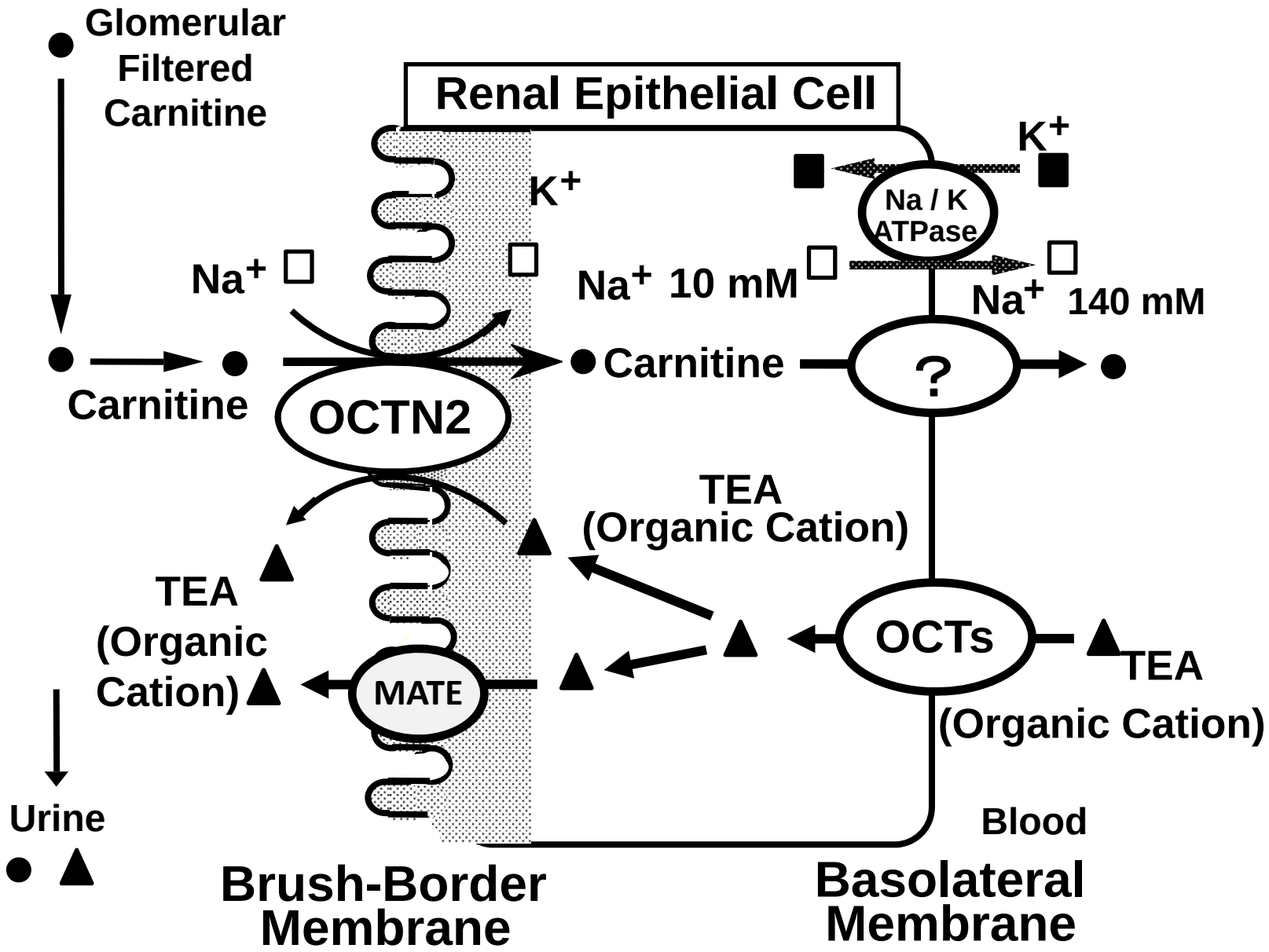
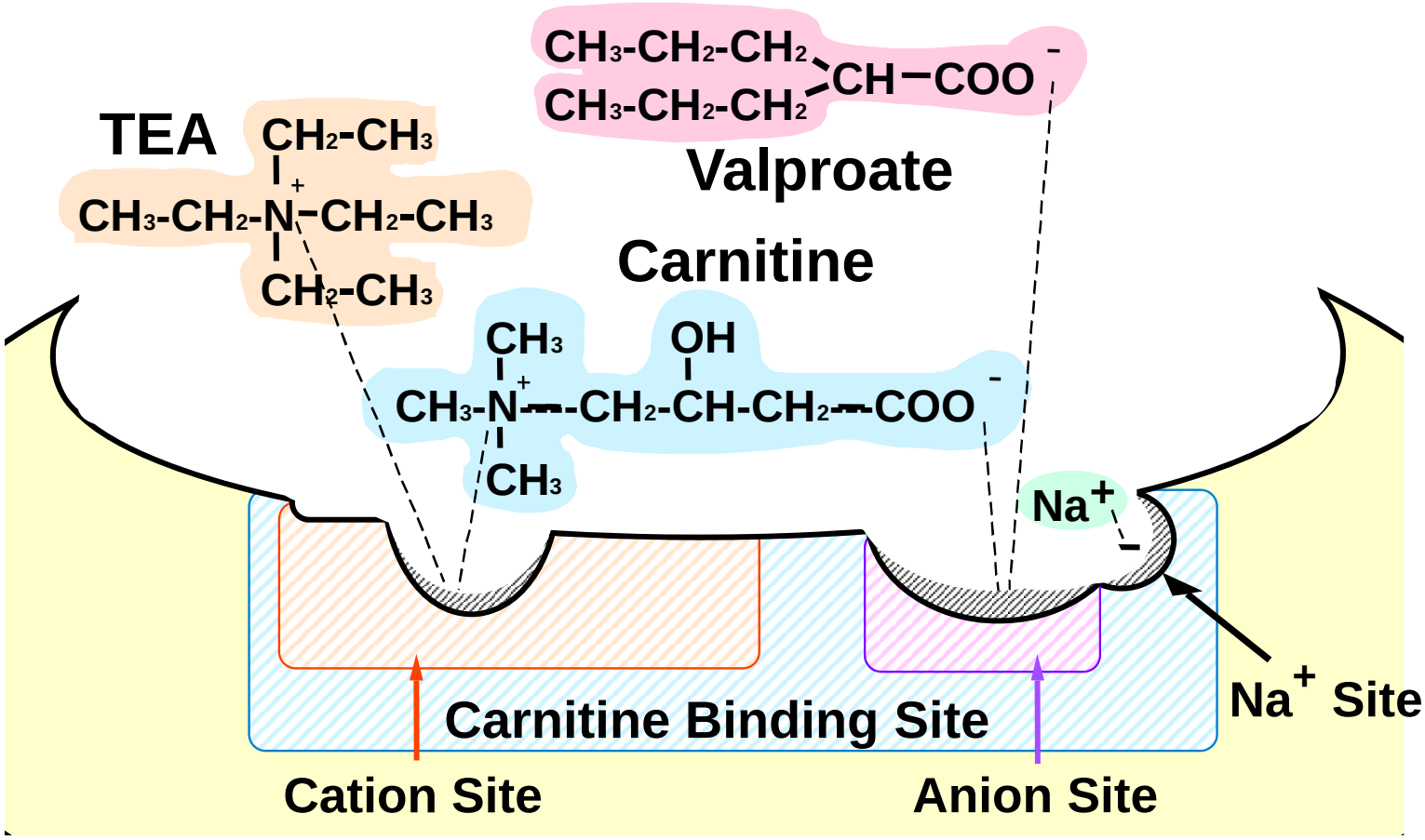

Glomerular
Filtered
Carnitine
Renal Epithelial Cell
K+
Na / K
ATPase
K+
Na+
Na+ 10 mM
Na+ 140 mM
Carnitine
OCTN2
Carnitine
?
TEA
(Organic Cation)
TEA
(Organic
Cation)
MATE
OCTs
TEA
(Organic Cation)
Urine
Blood
Brush-Border
Membrane
Basolateral
Membrane

Figure 5


TEA
CH3-CH2-N+-CH2-CH3
CH2-CH3
CH2-CH3
CH3-CH2-CH2
CH3-CH2-CH2
CH-COO-
Valproate
Carnitine
CH3
OH
CH3-N+-CH2-CH-CH2-COO-
CH3
Na+
Carnitine Binding Site
Cation Site
Anion Site
Na+ Site