Zebrafish Slc5a12 encodes an electroneutral sodium monocarboxylate transporter (SMCTn): A comparison to the electrogenic SMCT (SMCTe/Slc5a8)

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Running title: SMCT transporters in zebrafish

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We have identified and characterized two different sodium-coupled monocarboxylate cotransporters (SMCT) from zebrafish (Danio rerio): electrogenic (zSMCTe) and electroneutral (zSMCTn). zSMCTn is the 12th member of the zebrafish Slc5 gene family (zSld5a12). Both zSMCT sequences have ~50% homology to human SLC5A8 (hSMCT). Transport function and kinetics were measured in Xenopus oocytes injected with zSMCT cRNAs by measurement of intracellular Na+ activity ([Na+]i) and membrane potential. Both zSMCTs-oocytes increased [Na+]i with addition of monocarboxylates (MC), such as lactate, pyruvate, nicotinate and butyrate. Using two electrode voltage clamp experiments, we measured currents elicited from zSMCTe after MC addition. MC-elicited currents from zSMCTe were similar to hSMCT currents. In contrast, we found no significant MC-elicited current in either zSMCTn- or control-oocytes. Kinetic data show that zSMCTe has a higher affinity for lactate, nicotinate and pyruvate (KmL-lactate = 0.17 ± 0.02 mM, Kmnicotinate = 0.54 ± 0.12 mM @ -150mV) than zSMCTn (KmL-lactate =1.81 ± 0.19 mM, Kmnicotinate = 23.68 ± 4.88mM). In situ hybridization showed that both zSMCTs are expressed in 1, 3 and 5 day old zebrafish embryos. Both zSMCTs are abundantly expressed in the brain, eyes, intestine and kidney. Within the kidney, zSMCTn mRNA is expressed in pronephric tubules whereas zSMCTe mRNA is more distal in pronephric ducts. zSMCTn is expressed in exocrine pancreas but zSMCTe is not. Roles for Na+ coupled monocarboxylate cotransporters have not been described for the brain or eye. In summary, zSMCTe is the zebrafish SLC5A8 ortholog and zSMCTn is a novel, electroneutral SMCT (zSlc5a12). Slc5a12 in higher vertebrates is likely responsible for the electroneutral Na+/lactate cotransport reported in mammalian and amphibian kidneys.

INTRODUCTION

The liver and the kidneys are the major sites of lactate uptake and metabolism. Normally the blood lactate concentration is ~1.2 mM; however, lactate levels can increase to 10 mM during maximal exercise. Lactate is reabsorbed in kidney such that only trace amounts of lactate are normally excreted in the urine. Lactate in the
glomerular ultrafiltrate is almost completely reabsorbed by the proximal nephron (1).

The movement of lactate and other monocarboxylates across plasma membranes is thought to occur by proton-linked monocarboxylate transporters (MCT) (2,3). The MCT gene family (Slc16) now comprises 14 members, of which only the first four (MCT1–MCT4) have been demonstrated experimentally to mediate the proton-linked transport of metabolically important monocarboxylates, such as lactate, pyruvate and short chain fatty acids. MCTs are expressed in a number of tissues including kidney (2,3). However, a significant body of data indicates an important role for luminal Na⁺/monocarboxylate cotransport in the reabsorption of filtered lactate by the proximal tubule. Some studies have concluded that luminal Na⁺/lactate transport is electroneutral (4,5), whereas other reports have shown that the process is electrogenic (6-8). In the kidney, absorption of pyrazinoate, an antituberculosis drug, and nicotinate (aka, niacin), used in treatment of dyslipidemias (9-11), occurs via an electroneutral Na⁺/monocarboxylate transport system (12) suggesting multiple transporters. Thus, transport of these compounds and structurally similar compounds by SMCT transporters is likely of immediate clinical importance.

SLC5A8, a member of the Na⁺/glucose cotransporter family, was recently reported to function as a sodium monocarboxylate transporter (13,14). The Slc5 gene family encodes versatile cotransporters of organic solutes, ions, and potentially water (15). In humans, eleven SLC5 genes have been identified (15). SLC5 members function as Na⁺ and solute (e.g., glucose, myo-inositol, iodide and multivitamins) cotransporters (15). Although the functions of some of the family members are well understood, e.g., Slc5a1 (SGLT1; the Na⁺/glucose cotransporter) and Slc5a5 (NIS; the Na⁺/I⁻ cotransporter), others family members (SLC5A9-A12) have not been well characterized (15,16).

SLC5A8 was originally cloned by Rodriguez et al. (17) in an attempt to identify iodide transporters that might play a functional role in the physiology of thyroid gland. This gene codes for a protein with 46% identity in amino acid sequence with NIS (Slc5a5) (18). The SLC5A8 protein is expressed in the apical membrane of thyroid follicular cells and was hypothesized to mediate passive transport of iodide independent of Na⁺, consequently Slc5a8 was originally named the “apical iodide transporter” (AIT) (17).

Recently, the SLC5A8 gene was shown to play an important role in controlling the development of colon cancers (19) and classical papillary thyroid carcinomas (PTC-cf) (20). Silencing of SLC5A8 is a common and early event in both tumors. Promoter methylation that inactivates SLC5A8 is found in ~60% of colon cancer cell lines and primary colon cancers and >80% adenomas (19). In the case of PTC-cf, SLC5A8 silencing by methylation was found in 90% of thyroid (follicular – most common type) carcinomas and occurred in about 20% of other papillary thyroid carcinomas (20). SLC5A8 is thus also hypothesized to be a tumor suppressor gene. We reasoned that such important functions of SLC5A8 should be evolutionarily preserved and could be crucial in development and differentiation, particularly of epithelial tissues. In fact, Costa and coworkers have found that Slc5a8 (Vito) is first expressed at the blastopore lip (the beginning of neurulation and gastrulation) in Xenopus tadpoles (21).

Human SLC5A8 and mouse Slc5a8 protein functions have previously been studied in Xenopus laevis oocytes. As a transporter, SLC5A8 is an electrogenic Na⁺/monocarboxylate cotransporter (SMCT) moving substrates such as short-chain fatty acids (propionate, butyrate, valerate) as well as monocarboxylates such as lactate, pyruvate, nicotinate and pyrazinoate (13,14,22). Yet, these substrates are apparently moved with varied affinity and coupling to Na⁺ (13,14,22).

Slc5a8 was also cloned from mouse kidney (23). Not surprisingly, mouse Slc5a8 expressed in Xenopus oocytes is electrogenic, transporting the same substrates as human SMCT (13,14). Thus, Slc5a8 is the first transporter known to be
expressed in mammalian colon and kidney that has the ability to mediate the electrogenic absorption of lactate and other monocarboxylates with Na⁺ (13,23,24). The Kᵣ and V_max for MC substrates of SLC5A8 indicate that it is a high-affinity and low capacity transporter which could easily be saturated by MCs in the renal ultrafiltrate or in the colonic lumen. Thus, an electroneutral Na⁺/MC cotransporter, particularly with high transport capacity, would allow MC absorption without excess loss in the urine or stool. Yet the molecular identity of electroneutral Na⁺/lactate transport proteins in kidney remains unknown.

In the present study we identified, localized, and functionally characterized two zebrafish SMCTs. One is the electrogenic SMCT (Slc5a8) and the other is a novel, electroneutral form of Na⁺/monocarboxylate cotransporter (SMCTn) with high transport capacity. SMCTn is the zebrafish ortholog of Slc5a12 recently reported as a second electrogenic Na⁺/monocarboxylate cotransporter (32). Our data demonstrate that both SMCTn and the electrogenic SMCT (SMCTe / Slc5A8) are present in kidney and brain of the teleost Danio rerio (zebrafish) and have different kinetic properties. Both proteins increase intracellular Na⁺ in response to a variety of MC’s: short-chain fatty acids, lactate, pyruvate, nicotinate, acetoacetate and 3-β-hydroxybutyrate. Slc5a8 mRNA is also found in the gut consistent with its original description in human.

**EXPERIMENTAL PROCEDURES**

**cDNA Cloning of zebrafish SMCT**

**zSlc5a8 and zSlc5a12** — A BLAST search of the EST database revealed two Danio rerio cDNAs that were homologous to human SLC5A8, yet clearly distinct from one another. The cDNAs corresponding these EST (IMAGE clones zSMCTe (zSlc5a8, #7212813), zSMCTn (Slc5a12, #6793401) were obtained from the IMAGE consortium (Research Genetics, Genome Systems) and sequenced (GenBank: AY727859 for zSMCTe) and AY727860 for zSMCTn). The complete EST inserts were sequenced (W.M. Keck lab, New Haven, CT) and then subcloned in pGEMHE Xenopus oocyte expression vector between EcoRI (5’) and XbaI (3’).

**Oocyte isolation and injection**

Oocytes were removed from female Xenopus laevis (Xenopus Express, Beverly Hills, FL) as previously described (25). Excised lobes of oocytes were placed into a Ca²⁺-free buffered saline solution (200 mOsm) and defolliculated by collagenase digestion as previously described (26). Capped zSlc5a8 and zSlc5a12 cRNA was synthesized using a linearized cDNA template and the T7 mMessage mMachine (Ambion, Austin, TX). Oocytes were injected with 50 nl of 0.5ng/nl (25 ng/oocyte) of cRNA of one of the two different zSMCTs, hSMCT or water. The oocytes were maintained at 18°C in filtered ND96 medium (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM HEPES-Tris, pH 7.5) (13). Oocytes were studied 3-10 days after injection. All experiments were performed in accordance with the regulations of the Institutional Animal Care and Use Committee of Case Western Reserve University.

**Electrophysiology**

All monocarboxylate solutions (1mM: lactate, pyruvate, propionate, butyrate) were prepared in ND96 (96 mM NaCl, 2 mM KCl, 1.8 mM MgCl₂, 1 mM CaCl₂, 5 mM HEPES, pH 7.5) as previously described (13). Unless otherwise stated, MCs were all used at 1 mM. To examine the kinetics of lactate and nicotinate transport of zSMCTn, a low chloride ND96 was used in order to maintain the same tonicity for all solutions (~200 mOsmol). For lactate curves, we used (in mM) 66 NaCl base solution with 30 to 0 Na-gluconate, and 0 to 30 Na-lactate. For nicotinate curves, we used 56 NaCl base with 50 to 0 Na-gluconate and 0 to 50 Na-nicotinate. All solutions also contained 2 KCl, 1.8 CaCl₂, 1 MgCl₂, 5 HEPES and were pH 7.5.

**Ion selective microelectrode.** Ion selective microelectrodes were used to monitor intracellular Na⁺ concentration ([Na⁺]) of zSMCTs and water injected oocytes as previously described (25-27). Intracellular [Na⁺] was measured as the difference
between the Na\(^+\) electrode and a KCl voltage electrode impaled into the oocyte; and membrane potential (\(V_m\)) was the potential difference between the KCl microelectrode and an extracellular calomel (26,28). Na\(^+\) microelectrodes were calibrated using 10 mM NaCl and 100 mM NaCl solutions followed by point calibration in ND96 (96 mM Na\(^+\)) as previously performed (26,28). Intracellular Na\(^+\) microelectrodes had slopes of at least -50 mV/pNa.

**Two electrode voltage clamp.** Oocyte membrane currents were recorded using an OC-720C voltage clamp (Warner Instruments, Hamden, CT), filtered at 2-5 kHz, digitized at 10 kHz and recorded with the Pulse software, and data analyzed using the PulseFit program (HEKA, Germany) as previously described (25,29). For periods when I-V protocols were not being run, oocytes were clamped at a holding potential (\(V_h\)) of -50 mV; and current was constantly monitored and recorded at 1Hz. I-V protocols consisted of 400 ms steps from \(V_h\) to -150 mV and +50 mV in 20 mV steps as previously described (13). The I-V protocols were run in the absence and in the presence of a particular substrate. The substrate-specific current is determined by subtraction of the pre-substrate current from the substrate current. All of the experiments were performed at room temperature. Test substrates bathed oocytes for 1-3 min. For kinetic analysis, oocytes were exposed to only one monocarboxylate at one concentration and then discarded.

**Calculations.** Oocytes were perfused with ND96 for 5 min at which time “Initial [Na\(^+\)]” is measured. The solution was switched to different monocarboxylates for 8-10 min (i.e. [Na\(^+\)], and \(V_m\) or \(I\) plateau) and the “Final [Na\(^+\)]” is measured. Oocytes came from at least four separate donor animals.

**\(^{22}\)Na\(^+\) Uptakes.** Function of zSMCTe and zSMCTn were assessed by measuring \(^{22}\)Na\(^+\) uptake in groups of 15-20 oocytes 4 days after water or cRNA injection. \(^{22}\)Na\(^+\) uptake were performed as previously described (30). Briefly, \(^{22}\)Na\(^+\) uptake was measured with the following protocol: a 30-min incubation period ND96 with 1 mM ouabain, 100 \(\mu\)M amiloride, 100 \(\mu\)M bumetanide, followed by a 60-min uptake period in ND96. To analyze the pyruvate transport kinetics, we varied pyruvate concentration from 0 - 5mM in ND96 for zSMCTe and from 0 - 20mM for zSMCTn. These uptake solutions also contained 1 \(\mu\)Ci/ml of \(^{22}\)Na\(^+\) (Perking Elmer Life Sciences) and the same inhibitors used during incubation period. Uptakes were performed at 30°C. At the end of the uptake period, oocytes were washed five times in ice-cold ND96 solution without isotope to remove extracellular tracer. Next, individual oocytes were dissolved in 10% SDS and tracer activity was determined by beta scintillation counting. RNA injected oocytes were compared with water-controls subjected to identical conditions, using oocytes from the same donor. Kinetic analysis was performed by estimating the \(K_m\) values for pyruvate of each SMCT. The \(K_m\) values were calculated from log [ion] versus \(V/V_{\max}\) plots using GraphPad Prism (GraphPad Software, Inc., San Diego, CA).

**RNA in situ hybridization**

Single label in situ hybridization was carried out as describe (31). Reagents were obtained from Roche Molecular Biochemicals. Briefly, zebrafish embryo groups were incubated at 70°C with digoxigenin-UTP labeled antisense RNA probe for zSMCTe or zSMCTn in hybridization solution containing 50% formamide, detected with an anti digoxigenin antibody conjugated to alkaline phosphatase (AP), and visualized with a combination of 4-nitro blue tetrazolium (NBT) and 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (blue precipitate). We alkali-hydrolyzed cRNA probes with 0.2M Na\(_2\)CO\(_3\) (pH 10.2) for 14 minutes at 60°C to obtain 200-300 bp fragments (size verified by RNA gel and [cRNA] determined by UV spectrometry). AP buffer is 100 mM Tris-HCl (pH 9.5), 50 mM MgCl\(_2\), 100 mM NaCl, 0.1% Tween-20. The developing solution was 225 \(\mu\)g/ml NBT and 175 \(\mu\)g/ml BCIP in AP buffer. Endogenous AP activity was inhibited by adding 2 mM levamisole (Sigma) to all solutions after antibody incubation. Following in situ hybridization, embryos were postfixed in 4%
paraformaldehyde, dehydrated through a graded ethanol series, distained with undiluted methyl salicylate (Sigma, #M6752), digitally photographed (27) and stored in mineral oil.

**Fish embryos sections:** After in situ hybridization, 5 dpf embryos were postfixed in 4% paraformaldehyde for 1h at room temperature. Fixed embryos were incubated overnight in 30% sucrose in PBS and then embedded in OCT. 15-μm cryosections were cut with a Leica cryostat and mounted on gelatin-coated slides. Embryo sections were observed using a Zeiss Axiovert 25 microscope and acquired with an AxioCam digital camera and AxioVision software (Carl Zeiss, Germany) as previously (27).

**Statistical analysis.** The results are presented as means±SE. The significance of the differences between groups were tested by one-way ANOVA with multiple comparison using Bonferroni correction or by the Kruskal-Wallis one-way analysis of variance on ranks with the Dunn method for multiple comparison procedure, as needed.

**RESULTS**

**Molecular cloning of the zSMCTs.**

We found two cDNAs for the zebrafish (*Danio rerio*) homologs of the human electrogenic Sodium/Mono carboxylate cotransporter (hSMCTe / SLC5A8). One cDNA (zSMCTe) encodes a 610-amino acid protein (GenBank #AY727859), while the other cDNA (zSMCTn) encodes a 623 amino acid protein (GenBank #AY727860). zSMCTe and zSMCTn are 46% identical. Both zSMCT sequences are roughly equally divergent from mammalian Slc5a8. zSMCTe has 55% and 56% amino acid identity and zSMCTn has 51% and 53% amino acid identity to human and mouse Slc5a8, respectively. zSMCTn shows 64% identity to mouse Slc5a12 whereas zSMCTe is 48% identical to mammalian Slc5a12 (Figure 1).

Secondary structure of zSMCT proteins was predicted using TMHMM (www.cbs.dtu.dk/services/TMHMM). This analysis predicts that zSMCTe has 13 transmembrane spans, as indicated for hSMCT (19). The zSMCTe protein contains 3 potential N-linked glycosylation sites in extracellular loops (N112, N439, N485), 19 Ser, 8 Thr and 4 Tyr like potential phosphorylation sites but only 7 Ser (S477, S523, S541, S556, S568, S570, S588), 2 Thr (T75, T84) and one Tyr (Y80) are outside of the membrane, increasing the probability these are actual phosphorylation sites.

A similar sequence analysis of zSMCTn also predicted 13 transmembrane spans. There are two potential N-linked glycosylation sites in extracellular loops (N478, N614). Several potential phosphorylation sites are predicted but only some are not in the membrane making them accessible to kinases: seven Ser (S111, S216, S232, S266, S303, S314, S379, S547, S597), three Thr (T217, T495, T587) and two Tyr (Y12, Y82). BLAST analysis of the zSMCTn sequence against the human genome database (http://www.ncbi.nlm.nih.gov/genome/seq/HsBlast.html) resulted in a hypothetical protein MGC52019 (SLC5A12; NT_086780.1 and NT_009237.17) with ~65% identity to zSMCTn. Recently, Srinivas and coworkers identified and cloned mouse Slc5a12 indicating that mammalian homologs are not just putative (32). Thus, zSMCTn is the zebrafish ortholog of mouse Slc5a12 and zSMCTe is the ortholog of human SLC5A8 (Figure 1).

**Functional expression of zSMCT proteins in Xenopus oocytes.**

We analyzed the function of both zSMCT clones by simultaneous measurement of membrane potential (V_m) and intracellular Na⁺ concentration ([Na⁺]) with ion selective microelectrodes in *Xenopus* oocytes injected with zSMCTn or zSMCTe cRNA. Using two electrode voltage clamp experiments, we also examined currents elicited by monocarboxylates for both SMCT cotransporters.

All MCs tested induced a significant increase of intracellular Na⁺ in both zSMCT oocytes ($\Delta$[Na⁺]zSMCTe = 2.76 ± 0.29 mM, n= 8; $\Delta$[Na⁺]zSMCTn = 2.28 ± 0.33 mM, n=9). In contrast, control oocytes displayed only minor increments.
of intracellular Na\(^+\) (\(\Delta[Na^+]\)\(_{\text{control}}\) = 0.31 ± 0.10 mM; n=6). Comparison to the human transporter indicates that hSMCTe transports slightly more Na\(^+\) (\(\Delta[Na^+]\) = 3.47 ± 0.77 mM) than both zSMCT clones expressed in oocytes.

**Figure 2A-D** show individual experiments of simultaneous measurement of [Na\(^+\)]\(_i\) (top traces) and \(V_m\) (bottom traces) in oocytes. These experiments show that addition of 1 mM MC\(^b\) to a zSMCTe oocyte (**Figure 2C**) increases [Na\(^+\)]\(_i\) from 2.9 to 6.5 mM (\(\Delta[Na^+]\)\(_i\) = 3.6 mM) and in a zSMCTn oocyte (**Figure 2D**) increases [Na\(^+\)]\(_i\) from 3.8 to 5.8 mM (\(\Delta[Na^+]\)\(_i\) = 2.0 mM). When human SMCTe is expressed, [Na\(^+\)]\(_i\) increases a bit more with MC addition (**Figure 2B**), i.e., from 5.2 to 11.9 mM (\(\Delta[Na^+]\)\(_i\) = 6.6 mM), whereas [Na\(^+\)]\(_i\) of the water-injected oocyte (**Figure 2A**) was only slightly altered (3.5 to 4.0 mM, \(\Delta[Na^+]\)\(_i\) = 0.5 mM). Increases of [Na\(^+\)]\(_i\) for all SMCT clones (hSMCTe, zSMCTe and zSMCTn) occurred only in the presence of a transported monocarboxylate. When the MC tested was removed, the [Na\(^+\)]\(_i\) increase stopped. **Figure 2A** also shows that the [Na\(^+\)]\(_i\) increment elicited by butyrate is dependent on extracellular Na\(^+\), i.e., when extracellular Na\(^+\) was removed induce a rapid fall of [Na\(^+\)]\(_i\) in all SMCT oocytes but not in controls despite the continued butyrate presence (zSMCTe = -0.58 mM, zSMCTn = -1.25 mM, hSMCTe = -0.45 mM; water = -0.13 mM). These data indicate that Na\(^+\) and MCs move together and are capable of moving into as well as out of the oocytes.

While it is tempting to compare Na\(^+\) transport rates of zSMCTe vs. zSMCTn from **Figure 2**, it is however, difficult to compare these rates since the transporters have different affinities and capacities (see below). Additionally, zSMCTe and zSMCTn are subject to different electrochemical driving forces, i.e., zSMCTe is voltage dependent and voltage affected while zSMCTn is not. These Na\(^+\) transport data in **Figure 2C** vs. data in **Figure 2D** appear to indicate that zSMCTe does not readily reverse as zSMCTn with the removal of a given MC. A similar result is observed with human SLC5A8 (**Figure 2B**). One consideration is that Slc5a8 clones are voltage dependent, meaning that inward transport is higher at more negative voltages, while outward transport is reduced at more negative voltages. On the other hand, an electroneutral transporter like zSMCTn/zSLC5a12 does not have its transport driven by voltage but rather is “concentration gradient” driven (ln([substrates]\(_{\text{out}}\)/[substrates]\(_{\text{in}}\)). Thus, the transport driving force for SMCTe clones would predict lower Na\(^+\) and MC outward transport due to the electrical driving force (2 x \(V_m\)). This electrical driving force does not influence zSMCTn-mediated transport activity. If these SMCTe and SMCTn experiments were both performed under voltage clamp conditions, the data would be more comparable. Nevertheless, MC metabolism or altered intracellular MC affinity for transport-site might also have a role in these differences.

The [Na\(^+\)]\(_i\) increase for zSMCTe-oocytes (**Figure 2C**) is concurrent with large depolarization (\(\Delta V_m\): butyrate = 46mV, propionate = 51mV, L-lactate = 50mV, pyruvate = 42mV; average responses in **Figure 2G** bottom), similar to that observed with hSMCTe-oocytes (**Figure 2B**) (\(\Delta V_m\): butyrate = 45mV, propionate = 29mV, L-lactate = 30mV, pyruvate = 34mV; average responses in **Figure 2F** bottom). In contrast, zSMCTn oocytes (**Figure 2D**) do not show any change in \(V_m\) (\(\Delta V_m\): butyrate = 1.0 mV, propionate = 1.0 mV, L-lactate = 2.0 mV, pyruvate = 2.0 mV; average responses in **Figure 2H** bottom) similar to water-injected control oocytes (\(\Delta V_m\): butyrate = 0mV, propionate = 1mV, L-lactate = 1mV, pyruvate = 1mV) (**Figure 2A**; average response in **Figure 2E** bottom).

The average \(V_m\) change (\(\Delta V_m\)) (**Figure 2**, bottom columns) in presence of various MCs for nine zSMCTn-oocytes (**Figure 2H**) was not different between the average \(\Delta V_m\) for six control oocytes (**Figure 2E**). Yet both datasets are significantly different than the average \(\Delta V_m\) for eight zSMCTe-oocytes (**Figure 2G**) and for nine hSMCT-oocytes (**Figure 2F**). **Figure 2** (middle columns) also shows the average \(\Delta[Na^+]\)\(_i\) in response to 1 mM of different MCs for water-controls (**Figure 2E**), hSMCTe oocytes (**Figure 2F**), zSMCTe oocytes (**Figure 2G**), and zSMCTn oocytes (**Figure 2H**). These
data show that the $\Delta [\text{Na}^+]_i$ of zSMCTe-oocytes in presence of pyruvate is significantly different with respect to other MCs ($p = 0.05$; $\Delta [\text{Na}^+]_i$: butyrate = 0.58 ± 0.12 mM, propionate = 0.47 ± 0.04 mM, L-lactate = 0.64 ± 0.08 mM, pyruvate = 0.96 ± 0.11 mM). However, $\Delta [\text{Na}^+]_i$ data for zSMCTn-oocytes is significantly different regarding $\Delta [\text{Na}^+]_i$ between pyruvate and L-lactate ($p = 0.05$; $\Delta [\text{Na}^+]_i$: butyrate = 0.54 ± 0.11 mM, propionate = 0.6 ± 0.12 mM, L-lactate = 0.82 ± 0.15 mM, pyruvate = 1.03 ± 0.24 mM). Interestingly, hSMCTe oocytes do not display any significant differences of $[\text{Na}^+]_i$ movement for the MCs tested ($\Delta [\text{Na}^+]_i$: butyrate= 0.87 ± 0.23 mM, propionate = 0.83 ± 0.12 mM, L-lactate = 0.69 ± 0.17 mM, pyruvate = 0.85 ± 0.22 mM). This analysis suggests that the affinity of zSMCTe for pyruvate and zSMCTn for pyruvate/L-lactate is different than for other MCs.

These results indicate like human SMCTe, zSMCTe electrogenically transports Na+, and that Na+ transport is elicited by monocarboxylates. Moreover, these results illustrate that zSMCTn is a new electroneutral Na+/monocarboxylate cotransporter which is the molecular ortholog of mouse Slc5a12 (32).

**Current-voltage (I-V) relationship zSMCT current.**

The above data indicate that zSMCTe is electrogenic and the zSMCTn is electroneutral, with both transporting Na+ and MCs. We confirmed this difference by measuring currents elicited by Na+ and butyrate (1 mM) addition to oocytes expressing zSMCTe (**Figure 3A**) or zSMCTn (**Figure 3B**). **Figure 3A** shows that the zSMCTe oocyte displays a -250 nA current with butyrate addition (close diamond). Na+ removal from the bath solution completely reduced this current (open diamond), indicating that butyrate transport is Na+ dependent. The zSMCTn oocyte (**Figure 3B**) did not show any current change with butyrate addition with or without Na+, indicating that the Na+ monocarboxylate of zSMCTn (**Figure 2D, H**) is electroneutral. Water-injected controls showed no monocarboxylate elicited currents (not shown).

**Figure 3C** shows the currents (I) elicited from the addition of different monocarboxylates to zSMCTe expressing oocytes. These currents elicited by addition of 1 mM of each substrate @ -150 mV were (in µA) pyruvate = -1.10 ± 0.12, L-lactate = -1.05 ± 0.12, propionate = -1.09 ± 0.15, butyrate = -1.08 ± 0.16 (n=6 oocytes in duplicate). Again, the butyrate current was eliminated in the absence of extracellular Na+ (average I (µA) @ -150mV: 0Na+ butyrate= -0.14 ± 0.04). The zSMCTe currents induced by different monocarboxylate are similar to hSMCTe currents (I @ -150mV hSMCTe = -1.2µA) (13,33).

A recent study by Srinivas and coworkers (32) reported the cloning of mouse Slc5a12 as a low-affinity, electrogenic Na+ /monocarboxylate cotransporter (SMCT2). Our zSMCTn clone is most closely related to this mouse Slc5a12. Therefore, we tested if zSMCTn might be electrogenic if presented with 40 mM nicotinate (**Figure 3E**) or 30 mM L-lactate (**Figure 3G**) and compared the zSMCTn responses to those of water-injected control oocytes (**Figure 3D, F**, respectively). We verified that there was no osmolality differences in our test solutions by maintaining constant [Cl-] and replacing gluconate with lactate or nicotinate. These voltage clamp data indicate that between -150 and +50 mV, there is no difference between water-injected and zSMCTn-injected oocytes (not shown), yet **Figure 2D, H** clearly demonstrate robust changes of $[\text{Na}^+]_i$.

**L-lactate transport and nicotinate transport.**

Srinivas et al. (32) reported that they had insufficient protein expression to allow kinetic analysis of mouse Slc5a12. Since we have robust Na+ transport mediated by zSMCTn (Slc5a12 ortholog), we compared the affinities of zSMCTn and zSMCTe for L-lactate and nicotinate by measuring either $[\text{Na}^+]_i$ responses (**Figure 4**) or current-responses (**Figure 5**) to varying concentrations. For zSMCTn, Na+ intracellular
changes during a 5 minute exposure ($\Delta$Na$^+$/5min) to varying concentrations of L-lactate (0-30 mM) or nicotinate (0-50mM) were measured using Na$^+$ selective microelectrodes in a non-voltage clamped configuration (Figure 4A, B). To account for potential biologic variation, we used at least seven zSMCTn-injected oocytes for each MC concentration from at least three different frogs.

Figure 4 shows steady-state kinetics calculated from the Michaelis-Menten equation for lactate (Figure 4A), nicotinate (Figure 4B) and pyruvate (Figure 4C, D). The data show that zSMCTn has $\sim$15 times greater affinity for lactate ($K_m$ L-lactate = 1.81 ± 0.19 mM) and pyruvate ($K_m$ pyruvate = 2.03 ± 0.36 mM) than nicotinate ($K_m$ Nicotinate = 23.68 ± 4.88 mM). The Na$^+$ transport capacity of zSMCTn oocytes was lower for lactate ($V_{max}$ L-lactate = 3.1 ± 0.09 mM Na$^+$/5min) than nicotinate ($V_{max}$ Nicotinate = 4.8 ± 0.05 mM Na$^+$/5min). Figure 4C shows that the maximal 22Na$^+$ transport of zSMCTe and zSMCTn for pyruvate is $\sim$18 nmol/oocyte/h ($V_{max}$), the apparent affinity differs significantly ($K_m$ = 0.34 vs. 2.0 mM respectively). At 2 mM pyruvate, zSMCTe transports more Na$^+$ than zSMCTn ($V_{max}$ L-lactate = -697 ± 21nA @ -150mV) than nicotinate ($V_{max}$ Nicotinate = -513 ± 14nA @ -150mV). Thus, the zSMCTe affinity for lactate and nicotinate are close to those reported for mammalian SMCT (Slc5a8), i.e., L-lactate = 235 ± 24 μM and nicotinate = 296 ± 88 μM (13,14,22,23). Taken together these data demonstrate that affinity of zSMCTn for L-lactate and nicotinate are $\sim$10 times and $\sim$30 times lower than the affinity of zSMCTe for the same compounds, respectively (compare Figure 4 to Figure 5).

Nicotinate alters L-lactate transport by zSMCTn

Under normal physiological circumstances multiple monocarboxylates are present. We were particularly interested in the interrelationship of nicotinate ($K_m$ $\sim$24mM) and L-lactate ($K_m$ $\sim$2mM) mediated Na$^+$ transport via zSMCTn. To do this, we measured [Na$^+$], in unclamped zSMCTn oocytes and varied the order of L-lactate and nicotinate addition. Figure 6A is a representative zSMCTn experiment in which we first exposed a zSMCTn-oocyte to 10 mM nicotinate (below $K_m$) for 5 minutes, then to 10 mM L-lactate (above $K_m$). Figure 6C is a similar zSMCTn experiment in which the oocyte was exposed first to 10 mM L-lactate then 10 mM nicotinate. In both experiments, L-lactate elicits a large increase of
[Na$^+\text{]}_i$ (Figure 6A: $\Delta[\text{Na}]_i = 1.36 \text{ mM}$ and Figure 6C: $\Delta[\text{Na}]_i = 2.72 \text{ mM}$). Likewise, 10 mM nicotinate elicited obvious [Na$^+\text{]}_i$ increases (Figure 6A: $\Delta[\text{Na}]_i = 0.96 \text{ mM}$ & Figure 6C; $\Delta[\text{Na}]_i = 0.91 \text{ mM}$). The proportion of $\Delta[\text{Na}]_i$ increase with L-lactate was decreased by ~30% after the exposure to nicotinate. The average rates for the “nicotinate-lactate” protocol were 0.15±0.05 mM Na$^+\text{}/\text{min}$ for nicotinate and 0.33±0.04 mM Na$^+\text{}/\text{min}$ for lactate. For the “lactate-nicotinate” protocol the rate of nicotinate-elicited [Na$^+\text{]}_i$ transport was constant (0.16±0.02 mM Na$^+\text{}/\text{min}$) while that rate of lactate-elicited [Na$^+\text{]}_i$ transport was dramatically increased (0.57±0.04 mM Na$^+\text{}/\text{min}$). That is, pre-exposure to L-lactate did not diminish the rate or extent of Na$^+$ transport elicited by nicotinate.

**Figure 6B and D** show average [Na$^+\text{]}_i$ responses of 5 experiments similar to Figure 6A (nicotinate 0.72 ± 0.18 mM ; L-lactate = 1.58 ± 0.19 mM) and 6 experiments similar to Figure 6C (L-lactate = 2.84 ± 0.21mM ; nicotinate 0.79 ± 0.08 mM). These data show that Na$^+$ transport amount by L-lactate was reduced in zSMCTn oocytes by ~40% after the exposure of nicotinate. These data also suggest that reduction of lactate movement via zSMCTn may be due to both MCs competing for the putative MC binding-site likely due to the obvious difference in $K_m$’s for the two substrates.

**Ketone body transport**

Ketone bodies are produced by the liver and used peripherally as an energy source during fasting and other lipolytic stress (1,2). The two main ketone bodies are acetoacetate (AcAc) and β-hydroxybutyrate (βOH-buty), while acetone is the third, less abundant, ketone body. We therefore evaluated the ability of the zSMCT clones to transport Na$^+$ in response to the presence of AcAc and βOH-buty (Figure 7). These ketoacids play important roles in basic metabolism (see Discussion and Figure 10A).

Here we show that both zSMCT can transport two of the main ketone bodies (AcAc and βOH-buty). Figure 7 shows individual oocyte experiments simultaneously measuring [Na$^+\text{]}_i$ (top traces) and $V_m$ (bottom traces) after the addition of 1.0 mM AcAc, βOH-buty and butyrate (buty): zSMCTn oocyte (Figure 7B) and zSMCTe oocyte (Figure 7C). Both clones in oocytes increase [Na$^+\text{]}_i$, with AcAc (zSMCTn: 2.88 to 3.71 mM, $\Delta[\text{Na}]_i = 0.83 \text{ mM}$; zSMCTe: 3.31 to 3.98 mM, $\Delta[\text{Na}]_i = 0.67 \text{ mM}$). Yet, a water-injected oocyte (Figure 7A) does not show any [Na$^+\text{]}_i$ increment (control from 3.23 to 3.16 mM, $\Delta[\text{Na}]_i = -0.07 \text{ mM}$). Only the zSMCTe oocyte showed a [Na$^+\text{]}_i$ increase with the addition of 1.0 mM βOH-buty (4.36 to 5.24 mM, $\Delta[\text{Na}]_i = 0.88 \text{ mM}$ ). Neither the zSMCTn nor the water-injected oocyte showed a [Na$^+\text{]}_i$ change in the presence of βOH-buty (zSMCTn: 3.46 to 3.23 mM, $\Delta[\text{Na}]_i = -0.23 \text{ mM}$; water-control, 3.16 to 3.16 mM, $\Delta[\text{Na}]_i = 0.00 \text{ mM}$).

However, the addition of 5 mM βOH-buty to a zSMCTn oocyte (Figure 7H) elicited an obvious [Na$^+\text{]}_i$ increase (2.22 to 2.76 mM; $\Delta[\text{Na}]_i = 0.54 \text{ mM}$). The same maneuver with the water-injected oocyte (Figure 7G) did not show a [Na$^+\text{]}_i$ increase (3.78 to 3.85 mM, $\Delta[\text{Na}]_i = 0.07 \text{ mM}$). Neither oocyte showed a significant $V_m$ change in presence of 5mM of βOH-buty (zSMCTn $\Delta V_m = 1 \text{ mV}$; water-control $\Delta V_m = 1 \text{ mV}$). These data suggest that zSMCTn has a lower affinity for βOH-buty that zSMCTe. Thus, zSMCTn is also an electroneutral Na$^+$/βOH-buty cotransporter.

The [Na$^+\text{]}_i$ increase in presences of ketone bodies and βOH-buty for zSMCTe-oocytes (Figure 7C) are coincident with large depolarizations (Δ$V_m$; AcAc = +23 mV, βOH-buty = +34 mV, butyrate = +59 mV). In contrast, zSMCTn oocytes (Figure 7B) did not show any $V_m$ change (Δ$V_m$; AcAc = -1 mV, βOH-buty = 0 mV, butyrate = 0 mV), similar to water-injected oocytes (Δ$V_m$; AcAc = +1 mV, βOH-buty = 0 mV, butyrate = 0 mV) (Figure 7A).

The average [Na$^+\text{]}_i$, change ($\Delta[\text{Na}]_i$) (Figure 7D-F, middle columns) from 6 zSMCTn-oocytes (Figure 7E) in presence of 1mM of either ketone body or butyrate show that AcAc ($\Delta[\text{Na}]_i = 1.04±0.06 \text{ mM}$) and butyrate = ($\Delta[\text{Na}]_i = 1.11±0.06 \text{ mM}$) are transported with a similar
apparent affinity and capacity, but βOH-butyrate (Δ[Na⁺] = -0.1±0.06 mM) is not obviously transported at this concentration. In contrast, the average Δ[Na⁺] of 6 zSMCTe-oocytes in presence of 1mM substrate shows a more obvious difference in the capacity and affinity for both ketone bodies and butyrate (Figure 7F) (Δ[Na⁺]: AcAc = 0.25±0.09 mM, βOH-buty = 0.50±0.10 mM, buty = 0.80±0.22 mM). If butyrate-elicited transport is 100%, then Na⁺ transport elicited by AcAc is 31% and βOH-buty is 63%. These data are significantly different from the lack of substrate-elicited Na⁺ transport in control oocytes (Figure 7D; Δ[Na⁺]: AcAc = -0.28±0.13, βOH-buty = -0.05±0.05, buty = -0.07±0.03).

Likewise, the average substrate-elicited voltage changes, i.e., ΔVₘ (Figure 7D-F, bottom columns) from 1mM MCs is negligible for zSMCTn-oocytes (ΔVₘ = mV ± SE.; AcAc = 1.0±0.06, βOH-buty = -0.1±0.06, buty = 1.0±0.06; n=6) (Figure 7E) and not different from control oocytes (AcAc = 0.0±0.23, βOH-buty = 0.0±0.34, buty = 0.0±0.24; n=5) (Figure 7D). And as observed with other substrates, Figure 7F illustrates that ΔVₘ’s elicited from zSMCTe-oocytes are significantly different from both control and zSMCTn oocytes (AcAc = +25±3.4, βOH-buty = +40±6.2, buty = +58±3.2; n=6). These data indicate that zSMCTn is an electroneutral Na⁺/ketoacid cotransporter and that zSMCTe is an electrogenic Na⁺/ketoacid cotransporter.

**In situ hybridization**

The distribution of zSMCTe and zSMCTn expression was determined by *in situ* hybridization in zebrafish embryo whole mounts with a digoxigenin-labeled, antisense cRNA probe for zSMCTe and zSMCTn (Figure 8 and Figure 9). Control zebrafish embryos show no signal in the organs with reactivity for either zSMCTe or zSMCTn transcripts (Figure 8A,D,G). Signals for zSMCTn and zSMCTe, respectfully, were detected in brain and eyes 24 h post fertilization (hpf) (Figure 8B, C), 3 days post fertilization (dpf) (Figure 8E, F) and 5 dpf (Figure 8H, I). Both zSMCTe (Figure 8I) and zSMCTn (Figure 8H) transcripts are also present in the swim bladder of 5 day old embryos. zSMCTe (Figure 8C, F, I) and zSMCTn (Figure 8E, H) transcripts were also present in the pronephros (embryonic kidney). While zSMCTn mRNA was in the pronephric tubule (early region of pronephros) (Figure 8H), zSMCTe mRNA was in pronephric ducts (late region of pronephros) (Figure 8I). Interestingly, zSMCTe transcripts appeared in the pronephros at 24 hpf (Figure 8C), while zSMCTn transcripts were first evident at 3 dpf (Figure 8E).

We speculate that this message and presumably protein distribution, is reflective of the absorptive capacity and function as well as the changing role of the pronephros between 24 and 96 hpf.

SLC5A8 was originally cloned from human colon, and thus its expression was expected in the teleost gut. Figure 8I illustrates that zSMCTe mRNA is present in the gut at 5 dpf.

We further analyzed the zSMCTn and zSMCTe mRNA localization in different tissues by sectioning 5 dpf embryos after whole mount in situ hybridization (Figure 9). Structures were identified based on the zebrafish anatomy atlas at ZFIN (http://zfin.org/zf_info/anatomy.html).

Both SMCT mRNAs were detected in brain (Br), trabecular bar (TB), eyes (E) (Figure 9A,E), otic capsule (OC) (Figure 9B, F), stomach (S), gall bladder (GB), pronephric duct (PD), pronephric tube (PT) (early pronephric) (Figure 9C,G): swimming bladder (SB), and gut (G) (Figure 9D,H).

There are differences in zSMCTe and zSMCTn mRNA localization and relative amounts. zSMCTn mRNA is present in exocrine pancreas (P) (Figure 9C). And zSMCTn mRNA appears more abundant in the otic capsule (Figure 9B) than zSMCTe mRNA (Figure 9F).

**DISCUSSION**

We have identified and characterized two Na⁺/monocarboxylate cotransporters from *Danio rerio* (zSMCT): one electrogenic (zSMCTe) and another that is electroneutral (zSMCTn).
Molecular and functional characteristics indicate that zSMCTe is the ortholog of hSMCT/SLC5A8. The second transporter is a novel electroneutral SMCT, evidently the 12th member of the Slc5 gene/protein family (zSMCTn/Slc5a12). Like the hSMCT/SLC5A8 cotransporter (13,33), both zSMCTe and zSMCTn will transport a wide variety of monocarboxylates (short-chain fatty acids SCFA; pyruvate; lactate and nicotinate) in a Na⁺ dependent manner.

Reports from the 1980's indicated electroneutral Na⁺/MC transport in the luminal membrane of salamander proximal tubules (5) and membrane vesicles prepared from the cortex of rabbit kidney (4,12,34), with other data indicating the existence of electrogenic Na⁺/MC transport (6-8). That is, there has been experimental support for the existence of two kinds of Na⁺ coupled monocarboxylate transport in kidney. Our studies in zebrafish are the first to experimentally determine the molecular entities for both of these processes, electroneutral transport (zSMCTn/zSlc5a12) and electrogenic transport (zSMCTe/zSlc5a8), and demonstrate their coexistence in the kidney (Figure 8, Figure 9, Figure 10).

While in situ staining at both 3 and 5 dpf show pronephros staining, mRNAs for both zSMCT’s are expressed by 24 hpf in the eye and brain. It is well known that the MCT transporters (Slc16) are expressed in the retinal pigmented epithelia (RPE) and Müller cells (35,36), but the role of Na⁺ coupled monocarboxylate cotransporters has not been incorporated into models of the RPE or visual system. Likewise, monocarboxylate cotransport has been shown in a variety of CNS tissues and cells, yet the possibility of Na⁺ coupling to this monocarboxylate transport has not been evaluated. Interestingly, mRNA reactivity seems labile in the brain and eyes. Messages in the brain and eye seem readily degraded in embryos that are not freshly isolated and fixed (within 9 months, not shown).

Li and collaborators first identified human SLC5A8 as a tumor suppressor in colon cancer (19). In fact, transfection of colon carcinoma cell lines with SLC5A8, dramatically reduced the growth rate of these cancer cells (19). This tumor suppressor role appears to be recapitulated in several other epithelial and nervous tissues (20,37,38). In support of a differentiation promoting role, a search for genes involved in early development in Xenopus, by Costa and coworkers identified a transporter sequence Vito (21), aka, Xenopus Slc5a8, whose mRNA appears early in development (first evident at stage 11 in the superficial ectoderm of the blastopore lip). Vito (Slc5a8) is also abundant at the gastrula and neurulation stages. By stage 35 (organ development), Vito is abundant in the liver diverticulum, the pronephros and the tail tip (21). Based on their results, these investigators postulated that Vito (two isoforms) is associated with early ectodermal cell determination (21). These data seem consistent with the view that Slc5a8 promotes differentiation. We also predict that demethylation of the Slc5a8 promoter region activates Slc5a8 transcription. Our experiments here have only examined SMCT-mediated transport and SMCT-mRNA localization and thus do not discriminate between these possibilities.

It is well established that apoptosis provides a mechanism to remove damaged or oncogenic cells. It is plausible that restriction of metabolic fuels (lactate, pyruvate, etc.) is another form of cellular defense against growth deregulation (initial oncogenic step). Slc5a8 (zSMCTe) and Slc5a12 (zSMCTn) are Na⁺ coupled monocarboxylate cotransporters carrying lactate, pyruvate and other monocarboxylate metabolic fuels across epithelial membranes. Butyrate is a well known inhibitor of histone deacetylases which directly regulate the extent of winding or unwinding of specific genes on chromosomes (39). Thus, butyrate transport per se may also play a key role in the differentiation or tumor suppressor effects associated with SLC5A8. These cause and effect relationships of monocarboxylate transport, differentiation and tumor suppression await detailed studies to specifically address these critical issues.
Several studies have also reported the kinetics of renal Na\(^+\)/MC cotransport (7,8,12,34,40). These studies suggested the presence of two kinetically different Na\(^+\)/MC cotransport systems with one having higher affinity for MCs that the other. In the present report we show that zSMCTn has a lower affinity for MCs than zSMCTe or hSMCTe (Figure 4, Figure 5). Since zSMCTn is proximal to zSMCTe in the pronephros (see diagram in Figure 10B), its lower MC affinity is anticipated. The pronephros localization of zSMCTn and zSMCTe are likely reflective of their physiologic role in the uptake of MCs. The concentration of MCs from the glomerular filtrate that arrives in the early proximal tubule can be high (L-lactate, 1-10mM in mammals). Thus, efficient absorption of MCs occurs by having a low affinity but high capacity transport of MCs (SMCTn). The MC concentration in the tubule fluid decreases as absorption occurs. Consequently, efficient MC absorption in the later proximal tubule is accomplished by high-affinity MC transport (SMCTe) (Figure 10B).

Studies with salamander proximal tubule showed that luminal, electroneutral lactate transport is Na\(^+\) coupled and induces an intracellular alkalinization (41), whereas basolateral lactate transport is H\(^+\) coupled (42). In our experiments we observed a clear Na\(^+\) dependent, alkalinization mediated by SCFA (butyrate and propionate) in oocytes injected with zSMCTn. This alkalinization was not observed with the addition of 1 mM pyruvate, lactate or nicotinate, perhaps due to some endogenous oocyte mechanism for H\(^+\)/pyruvate or H\(^+\)/lactate cotransport which dissipated the alkalinization produced by zSMCTn. In SMCTe- and SMCTn-injected oocytes, alkalinization was consistently elicited by SCFA in our experiments.

There are 11 SLC5 genes identified in humans (15,16). Our genomic predictions indicated that a human hypothetical protein MGC52019 (SLC5A12) existed. This sequence is ~65% identical to zSMCTn. Recently Srinivas and coworkers reported a mouse sequence (GenBank #: AY964639; Slc5a12/SMCT2 (32)) which is the ortholog of this putative protein. The human chromosomal localization of SLC5A8 (12q22-24) implies that it is a positional gene candidate for association with non-insulin dependent type 2 diabetes (NIDDM2, MIM #: 601407) (43,44). It is attractive to speculate that defects in SMCT (SLC5A8) function could cause a saturation of the renal Na\(^+\) coupled monocarboxylate transport system and might be manifest by monocarboxylates, especially lactate, in the urine. In fact, Thirumurugan and coworkers have found that L-lactate excretion is increased in Fanconi syndrome (45), which encompasses generalized defects in proximal tubule function.

Transport by SMCTs may be the major mechanism by which lipid-modifying drugs gain entry into cells, i.e., clinically useful. Since the 1950’s nicotinic acid (Niacin, Niasapan) has been used as a lipid-modifying drug (gram doses), in the treatment of dyslipidemias (46,47) or dyslipidemia with diabetes (11). Several hypotheses have been proposed regarding the basic mechanism for this niacin effect on blood lipids. These hypotheses include inter alia a vasodilation effect, a vitamin effect, decreased hepatic cholesterol synthesis, increased cholesterol oxidation, etc. Regardless of the underlying mechanisms, it is clear that the hypolipidemic effect of nicotinate requires the recently identified nicotinate receptor, (GPR109A / PUMA-G) (47-49). It is also well established that ligand-receptor interaction time is often determined by ligand degradation (acetylcholine receptors and acetylcholinesterase) or ligand uptake mechanisms. One particularly germane example is vesicular glutamate release at the CNS presynaptic terminals, stimulating post-synaptic glutamate receptors and rapid uptake of glutamate by adjacent glutamate transporters (SLC1 family) (50-54). In humans genomic DNA, GPR109A is at 12q24.31 (49) and SLC5A8 is at 12q22-24 (19). It is attractive to speculate that these genes are located in the same region of the genome so that their activities may be coordinated.

Another explanation for nicotinate’s role in dyslipidaemia therapy involves inhibition of...
lipolysis in adipose tissue, resulting in a decreased mobilization of free fatty acids (9-11). Nicotinate is efficiently transported with Na\(^+\) by both Slc5a8 (zSMCTe) (13,22) and Slc5a12 (zSMCTn) but with varied affinities and capacities (Figure 3, Figure 4, Figure 5). Moreover, as illustrated in Figure 6, the amount and rate of nicotinate transport by zSMCTn is significantly reduced compared to lactate transport. This transport reduction likely points to competition for the monocarboxylate binding-site of zSMCTn such that sterically “bulky” MCs compete with more simple MCs including short-chain fatty acids. These data support the idea that one possible therapeutic action of nicotinic acid in hyperlipidemia is to reduce the absorption of free fatty acid in tissues expressing SMCTs (Slc5a8, Slc5a12) or MCTs (Slc16a1-a4).

Lactate transport is also reported in brain, but this transport is attributed to H\(^+\)/MC cotransport (MCT, Slc16a1-4) (55,56). Monocarboxylates such as lactate, pyruvate and ketone-bodies appear to play an important role in brain energy metabolism (Figure 10A) as well as neuronal and glial intracellular pH buffering (57-59). Lactate has long been considered a waste product disposed of via the circulation; however, when lactate is accumulated it can be toxic for the brain. Monocarboxylates, together with other non-glucose substrates, have long been known to be substantial energy substrates for the developing brain (60). Na\(^+\)/MC cotransport in brain has not been previously reported. Nevertheless, we show clear evidence of both SMCT transcripts in the developing zebrafish brain (Figure 8, Figure 9). The roles of the SMCT transporters in the brain and eye remain to be explored, yet CNS function of these transporters may provide some clues to early CNS development or cellular “programming.”

Ketone bodies or ketoacids, such as acetoacetate (AcAc) and β-hydroxy-butyrate (βOH-buty) (Figure 10A), are always present in the blood and their levels increase during fasting and prolonged exercise. The metabolic pathway of hepatic ketone body formation (ketogenesis) and extrahepatic ketone body utilization (ketolysis) is especially important for the brain, which uses ketone bodies as a primary energy source when glucose is not available (3). Additionally, ketone bodies are used as substrates for lipid synthesis such as cholesterol for myelin for neonates and during suckling (1).

Physiologic levels of ketone bodies circulating in plasma range from <0.1 mM (postprandial) to 6 mM (prolonged fasting) and can reach 25 mM in diabetic ketoacidosis. Diabetes is the most common pathological cause of elevated blood ketoacids. In diabetic ketoacidosis (DKA), high levels of ketoacids are produced in response to low insulin levels and high levels of counter regulatory hormones. In acute DKA, the ketoacid ratio (βOH-buty :AcAc) rises from normal (1:1) to as high as 10:1. In response to insulin therapy, βOH-buty levels commonly decrease long before AcAc levels (1-3). The H\(^+\)/MC cotransporters (MCT) are involved in the transport of ketone bodies (4). However, the chromosomal localization of the human homolog of zSMCT (SLC5a8 and SLC5a12) are positional candidates for proteins involved in diseases like non-insulin dependent type 2 diabetes (NIDDM2 on 12q22-24, MIM # 601407) (43,44) and familial combined hyperlipidemia (FCHL) (11p14) (61); (62), respectively.

Our current data suggest that zSMCTe (Slc5a8) and zSMCTn (Slc5a12) have significant differences in affinities for the ketoacids AcAc and βOH-buty (Figure 7). Furthermore, these mRNAs and presumably proteins are present in several organs, including the kidney. The chromosome localization of the human SLC5A8 and SLC5A12 orthologs are positional candidates for non-insulin dependent type II diabetes and hyperlipidemia. We speculate that both SMCTe and SMCTn have important roles in the exacerbating diabetic ketoacidosis since reabsorption of these ketoacids by SMCT proteins after glomerular filtration would prevent normal elimination or metabolic use leading to increasing plasma levels of these ketoacids and acidosis.
Finally, it should be noted that even though zSMCTe is only ~50% identical to hSMCT (SLC5A8), the affinity of zSMCTe for lactate and nicotinate were quite similar to those reported for mammalian SMCT (13,22-24).

In summary, we have identified, cloned, functionally characterized and localized two distinct Na+/monocarboxylate cotransporters in the zebrafish, Danio rerio. Our data demonstrate that zSMCTe is the molecular and functional ortholog of human high-affinity SMCT (SLC5A8). In the first day of zebrafish development, zSMCTe is present, implicating that the presence of SMCT is important for normal development. The second transporter zSMCTn, is a novel, electroneutral and low-affinity Na+/monocarboxylate cotransporter, with a definite mammalian homolog (Slc5a12). Additionally, more than being merely renal or gut transporters, our zebrafish data demonstrate significant expression in the developing brain, eye and ear. The roles of the SMCT proteins in these tissues will likely lead to novel biochemical, physiologic and pathophysiologic insights.

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FOOTNOTES

a Abbreviations used: MCT, monocarboxylate transporter; SMCT, sodium monocarboxylate transport; MCs, monocarboxylates; NMDG, N-methyl-D-glucamine; [Na+], intracellular Na+ concentration.
b Several MCs were added and washed out in each experiment. The order of additions was randomized. Each MC solution bathed the oocyte for 5 minutes. These results did not differ significantly from experiments with a single MC addition. 22Na+ uptake experiments revealed that 1 mM MC elicited a linear accumulation between 5 and 90 min (not shown).
c The [Na+]i response of zSMCTn-injected oocytes to MCs was linear over at least the first 5 minutes.
d The average unclamped, voltages for zSMCTn oocytes (Figure 4) for these experiments was -45 ± 6 mV. Thus, a comparison of the zSMCTn-Kms to the zSMCTe-Kms at -50 mV (Figure 5) was used to calculate the 10- or 30-fold difference.
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FIGURE LEGENDS

Figure 1: Homology of known sodium monocarboxylate cotransporters (SMCTs).
Slc5a8 and Slc5a12 are most related to NIS (Slc5a5, not shown) in the Slc5 gene family. However, these two monocarboxylate transporters (SMCTe and SMCTn) form an independent branch of the Slc5 family. The two main branches of the monocarboxylate arm of the Slc5 gene family are outlined. The length of the horizontal lines approximates evolutionary distance. Phylogenetic tree was generated using the DNASTAR program (Madison, WI, USA). Figure illustrates that both sequences of zSMCT are approximately 50% identical to human and mouse SMCTe (Slc5a8) but zSMCTn is ~65% identical to human and mouse Slc5a12.

Figure 2: zSMCT expression – intracellular Na⁺ transport.
Simultaneous measurement of intracellular Na⁺ activity ([Na⁺]i) (top panels) and membrane potential (V_{m}) (bottom panels) for a water-injected control oocyte (A), hSMCTe-injected oocyte (B), zSMCTe-injected oocyte (C), and zSMCTn-injected oocyte (D). Oocytes were continually superfused as indicated in Methods. Monocarboxylates [1 mM of pyruvate (Pyr), lactate (Lac), propionate (Pro) or butyrate (Buty)] were added as indicated by horizontal lines. Na⁺ removal (0Na⁺) was a choline replacement. Average Na⁺ transport response (Δ[Na⁺] = end[Na⁺] - initial[Na⁺]) in presence of pyruvate (left hatched bar), lactate (horizontal hatched bar), propionate (right hatched bar), butyrate (open and gray bars) and 0Na+/butyrate (black bar) are indicated in the column graphs: water-injected oocytes (E), hSMCTe-injected oocytes, n=8 (F), zSMCTe-injected oocytes, n=9 (G) and zSMCTn-injected oocytes, n=9 (H). Data were collected from at least 4 frogs. *Statistical significance (P ≤ 0.05) for Na⁺ increment compared to butyrate response for that clone. ** Statistically significance difference (P ≤ 0.05) of Na⁺-free + butyrate between both groups (hSMCTe and zSMCT). Data are reported for 6-7 oocytes from at least 2 frogs. Individual oocyte experiments are noted with numeric labels (below time bars) indicating the oocyte number used.

Figure 3: zSMCTe but not zSMCTn elicited currents in presence of monocarboxylates.
Typical voltage clamp experiments are shown zSMCTe-oocytes injected (A) exposure to (1mM) butyrate (Buty) induced currents but not zSMCTn (B). I_{Buty} was measure before (gray diamond), during (0Na⁺/Butyrate, open diamond) and after (gray diamond) removal of bath Na⁺. Panel C show zSMTCe I-V relationships (holding potential = -50 mV) for 1 mM different MCs: butyrate (open shapes), propionate (close circle), lactate (close triangle), and pyruvate (reverse close triangle). Voltage step protocols were executed 30-60 seconds before a solution change (see Methods). Step protocols were run at peak currents. I_{MCs} were calculated as I (MCs) − I (ND96) and plotted vs. pulse voltage (C). Individual voltage clamp experiments with water-injected oocytes (D and F) and zSMCTn-injected oocytes (E and G) are shown to illustrate that exposure of these oocytes to high concentrations of nicotinate (Nico 40mM) and L-lactate (Lac 30mM) not elicit a current response.

Figure 4: zSMCTn – Steady-state kinetics of lactate, nicotinate and pyruvate transport.
Intracellular Na⁺ concentration (Δ[Na⁺]) induced by addition of various concentrations of L-lactate and Nicotinate during 5 minutes were done with simultaneous measurement of membrane potential (V_{m}) and intracellular Na⁺ concentration ([Na⁺]) using Na⁺ selective microelectrodes (A and B). The average Δ[Na⁺]/5min of different concentrations for lactate (A) and nicotinate (B) of at least 7 oocytes from 3 donor animals were fitted to the Michaelis-Menten equation from which the K_{m} values are shown. Panel
C shows the steady-state kinetics for pyruvate from $^{22}\text{Na}^+$ uptake experiments (1 h, see Methods). Panel D compares the raw $^{22}\text{Na}^+$ uptakes with 2 mM pyruvate for water-controls, zSMCTe and zSMCTn injected oocytes. Uptake results are from 15-20 individual oocytes in each group. Values shown represent mean $\pm$ SEM.

Figure 5: zSMCTe – Steady-state kinetics of lactate and nicotinate transport.
The currents induced by addition of various concentrations (closed shapes) of L-Lactate (A) and Nicotinate (E) were measured in the presence of 96 mM Na while the cell was clamped at a series of different membrane potentials. The Michaelis-Menten equation was fitted to the current measurements at different membrane potentials (closed shapes) for L-Lactate (B) and Nicotinate (F), from which the derived $I_{\text{max}}$ and $K_m$ values are shown in panel C and D for L-Lactate; G and H for Nicotinate, respectively. Values shown represent mean $\pm$ SEM; n = 7 oocytes, from 3 donor animals. Substrate concentrations or holding potential (mV) are indicated to the right of the appropriate panel.

Figure 6: Effect of nicotinate on L-lactate transport in zSMCTn-injected oocytes.
Examples of individual experiments of simultaneous measurement of $[\text{Na}^+]_i$ (A and C) and $V_m$ (not shown) for zSMCTn-injected oocytes are shown. Oocytes were continually superfused with [10mM] L-Lactate or Nicotinate (A, indicated by bars). In panel C the order of perfusion was switched. Panels B and D show the average Na$^+$ transport response ($\Delta[\text{Na}^+]_i$) in the presence of lactate (open bar) and nicotinate (black bar). *Statistical significance ($P \leq 0.05$) for $[\text{Na}^+]_i$ increase induced by the presence of Lactate without previous exposure to Nicotinate (D) vs. with previous exposure to Nicotinate (B). Data represent mean $\pm$ SEM for 6-7 oocytes from at least 2 frogs.

Figure 7: Ketone transport by zSMCT’s – intracellular Na$^+$
Simultaneous measurement of intracellular Na$^+$ concentration ($[\text{Na}^+]_i$) (top panels) and membrane potential ($V_m$) (bottom panels) for a water-injected control oocyte (A,G), zSMCTn-injected oocyte (B,H), and zSMCTe-injected oocyte (C). Oocytes were continually superfused as indicated in Methods. 1 mM (A,B,C) or 5mM (G,H) of acetoacetate (AcAc), $\beta$-hydroxybutyrate ($\beta$OH-buty) or butyrate (Buty) were added as indicated by horizontal lines. Average Na$^+$ transport response ($\Delta[\text{Na}^+]_i = \text{end}[\text{Na}^+]_i - \text{initial}[\text{Na}^+]_i$) in presence of AcAc (black bar), $\beta$OH-buty (gray bars) and Buty (open bars) are indicated in the column graphs: water-injected oocytes n=5 (D), zSMCTn-injected oocytes, n=6 (E) and zSMCTe-injected oocytes, n=6 (F). Data were collected from at least 2 frogs. *Statistical significance ($P \leq 0.05$) for Na$^+$ increment compared to Buty response for that clone. Individual oocyte experiments are noted with numeric labels (below time bars) indicating the oocyte number used.

Figure 8: Localization of zSMCTs mRNA in zebrafish embryos.
Whole mount in situ hybridization of embryos at 1 dpf (Panels A, B and C), 3 dpf (Panels D, E and F) and 5 dpf (Panels G, H, I). zSMCTn (Panels B, E, H), and zSMCTe (Panels C, F, I) mRNAs are present in the pronephros (pronephric tubule, PT and pronephric duct, PD), eye (E), brain (Br), swim bladder (SB), and gut (G) (blue staining and arrows). Note absence of staining in control embryos treated identically, but not exposed to probe (Panels A, D and G).

Figure 9: Localization of zSMCTn (A,B,C,D) and zSMCTe (E,F,G, H) in 5 pfd zebrafish embryos sections
15 $\mu$m cryosections of 5 dpf zebrafish embryo whole mounts hybridized in situ with zSMCTn and zSMCTe antisense RNA probes. SMCT transcripts (blue staining and arrow) were detected in brain (Br),
trabecular bar (TB), eyes (E), otic capsule (OC), stomach (S), gall bladder (GB), pronephric duct (PD), pronephric tubule (PT), swim bladder (SB), gut (G), and exocrine pancreas (P).

**Figure 10: Physiological role of SMCTs in the kidney.**
Panel A shows a diagram illustrating where the principle substrates of the SMCT transporters enter intermediate metabolism. Panel B illustrates that the proximal tubule reabsorbs MCs via Na\(^+\)/MCs in two ways: 1Na\(^+\):1MC\(^-\) electroneutral transport by SMCTn (blue) in the earlier proximal tubule where high affinity for MCs is not necessary because uptake occurs, and nNa\(^+\):1MC\(^-\) in the later proximal tube by SMCTe (green) where high affinity and coupling to multiple Na\(^+\) insures absorption.
Figure 1- Plata et al.
Figure 2 – Plata et al.
Figure 3 – Plata et al.
Figure 4- Plata et al.

A) zSMCTn

\[ V_{max} = 3.1 \pm 0.1 \]
\[ K_m = 0.340 \pm 0.06 \]

B) zSMCTn

\[ V_{max} = 4.8 \pm 0.1 \]
\[ K_m = 23.7 \pm 4.9 \]

C) zSMCTe

\[ V_{max} = 17.55 \pm 78 \]
\[ K_m = 0.340 \pm 0.06 \]

D) zSMCTn

\[ V_{max} = 19.3 \pm 0.9 \]
\[ K_m = 2.0 \pm 0.4 \]
Figure 5 – Plata et al.
Figure 6 - Plata et al.
Figure 7 - Plata et al.
Figure 8 – Plata et al.
Figure 9
Plata et al.
Figure 10 - Plata et al.
Zebrfish Slc5a12 encodes an electroneutral sodium monocarboxylate transporter (SMCTn): A comparison to the electrogenic SMCT (SMCTe/Slc5a8)
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