Regular Article

Monocarboxylate Transporters 1 and 2 Are Responsible for L-Lactate Uptake in Differentiated Human Neuroblastoma SH-SY5Y Cells

Tomoya Sakuma,[a,#] Yuto Mukai,[a,#] Atsushi Yamaguchi,[a,b] Yudai Suganuma,[a] Keisuke Okamoto,[a] Ayako Furugen,[a] Katsuya Narumi,[a] Shuhei Ishikawa,[c] Yoshitaka Saito,[b] and Masaki Kobayashi*[,a]

[a] *Laboratory of Clinical Pharmaceutics & Therapeutics, Division of Pharmasciences, Faculty of Pharmaceutical Sciences, Hokkaido University, Kita 12, Nishi 6, Kita-ku, Sapporo 060–0812, Japan:* [b] *Department of Pharmacy, Hokkaido University Hospital, Kita 14, Nishi 5, Kita-ku, Sapporo 060–8648, Japan: and* [c] *Department of Psychiatry, Hokkaido University Hospital, Kita 14, Nishi 5, Kita-ku, Sapporo, 060–8648, Japan.*
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L-Lactate transport *via* monocarboxylate transporters (MCTs) in the central nervous system, represented by the astrocyte-neuron lactate shuttle (ANLS), is crucial for the maintenance of brain functions, including memory formation. Previously, we have reported that MCT1 contributes to L-lactate transport in normal human astrocytes. Therefore, in this study, we aimed to identify transporters that contribute to L-lactate transport in human neurons. SH-SY5Y cells, which are used as a model for human neurons, were differentiated using all-*trans*-retinoic acid. L-Lactate uptake was measured using radiolabeled L-lactate, and the expression of MCT proteins was confirmed Western blotting. L-Lactate transport was pH-dependent and saturated at high concentrations. Kinetic analysis suggested that L-lactate uptake was biphasic. Furthermore, MCT1, 2 selective inhibitors inhibited L-lactate transport. In addition, the expression of MCT1 and 2 proteins, but not MCT4, was confirmed. In this study, we demonstrated that MCT1 and 2 are major contributors to L-lactate transport in differentiated human neuroblastoma SH-SY5Y cells from the viewpoint of kinetic analysis. These results lead to a better understanding of ANLS in humans, and further exploration of the factors that can promote MCT1 and 2 functions is required.

Key words astrocytes-neuron lactate shuttle, monocarboxylate transporter, SH-SY5Y cell, lactate

INTRODUCTION

L-Lactate is an essential regulator of brain function. L-Lactate is the product of anaerobic metabolism and effluxes from the cell, whereas under aerobic conditions, it is also used for synthesizing acetyl-CoA for use in the tricarboxylic acid cycle. In the central nervous system, the astrocyte-neuron lactate shuttle (ANLS), in which L-lactate is released from astrocytes and taken up by neurons for energy metabolism, is important for brain energy metabolism.[1)] Additionally, L-lactate plays an important role in neuronal excitability, plasticity, and memory consolidation.[2)] Therefore, understanding the kinetics of L-lactate in the brain is important.

Monocarboxylate transporter (MCT)1, MCT2, and MCT4 are the major pH-dependent L-lactate transporters that are expressed in various organs, including the small intestine, skeletal muscle, lung, liver, and brain.[3,4)] In the brain, it has been reported that the knockdown of MCTs in rats impaired long-term memory formation.[5)] Interestingly, topical administration of L-lactate to the hippocampus restored long-term memory formation in MCT1 and MCT4 knockdown mice, but not in MCT2 knockdown mice, indicating that each isoform may have a different role. Thus, MCTs may affect brain function, and it is important to confirm their functional isoforms.

Several studies have been conducted on pH-dependent L-lactate transport in the central nervous system of rodents.[6–8)] It has been reported that MCT1 and MCT2 are expressed in the GT1-7 mouse hypothalamic GnRH neuronal cell line, and MCT1, MCT2 and MCT4 are expressed in hypothalamic primary cell culture of rats.[8)] Some reports have analyzed for the pH-dependent and concentration-dependent L-lactate uptake in neuronal cell lines of rodents,[6–8)] but it has not been clear which transporters are involved in the uptake of L-lactate. Kinetic analysis and expression characteristics have also been performed on cells other than neurons, and it is widely known that MCT1 contributes to the release of L-lactate from vascular endothelial cells and MCT1 or MCT4 from astrocytes.[9)] From the results of these studies in rodents, MCT isoforms with confirmed protein expression did not necessarily transport L-lactate in each tissue. In the human brain, MCT1 is expressed in vascular endothelial cells and astrocytes[10)]; MCT2 is found in neurons in the white matter and gray matter and in some astrocytes[11)]; and MCT4 is found in the basolateral cytoplasmic membrane of a small number of epithelial cells, astrocytes, and the capillary wall.[12)] We previously reported that MCT1 contributes to L-lactate transport in human astrocyte-derived cells (NHA) using kinetic analysis and inhibitory experiments.[13)] In the central nervous system, MCT2 is thought to play an important role in L-lactate transport because MCT2 is expressed in human neurons. However, there are no verification of L-lactate transport in human neurons, and the carriers of L-lactate remain unclear. Consequently, we aimed to identify transporters that contribute to L-lactate transport in human neurons.

Differentiated SH-SY5Y human neuroblastoma cells are often used as human neuronal models.[14)] In this study, we performed a kinetic analysis to elucidate L-lactate transport carriers in human neuroblastoma SH-SY5Y cells.

[#] These authors contributed equally to this work.

* To whom correspondence should be addressed. e-mail: masaki@pharm.hokudai.ac.jp

MATERIALS AND METHODS

Chemicals [^{14}C]-labeled L-lactic acid sodium salt (NEC599050UC) was purchased from PerkinElmer, Inc. (U.S.A.). All the compounds used were of the highest purity available.

Cell Culture and Differentiation The SH-SY5Y cells were purchased from KAC Co., Ltd. (Japan). SH-SY5Y cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham F-12 (Nacalai Tesque, Japan) supplemented with 15% fetal bovine serum, 1% penicillin–streptomycin antibiotic (Sigma-Aldrich, U.S.A.), and 1% MEM nonessential amino acids (Gibco, U.S.A.). The medium was changed every 2 d and passaged when subconfluent. No more than 25 passages were used in any experiment. As a differentiated condition, cells were seeded at the appropriate density, cultured for 2 d, and the medium was replaced with Neurobasal™-A Medium containing 10 μM all-*trans*-retinoic acid (Wako, Japan), B-27™ Supplement (Gibco, Thermo Fisher, U.S.A.), GlutaMAX Supplement (Gibco, Thermo Fisher) and 1% penicillin–streptomycin antibiotic. Thereafter, the medium was replaced every 2 d and used for various experiments 7 d after seeding. We examined whether this manipulation resulted in changes in cell morphology and mRNA levels of differentiation markers.

RT-PCR and Quantitative Real-Time PCR (qPCR) Total RNA was extracted using ISOGEN II (Nippon Gene, Tokyo, Japan). cDNA was synthesized from the total RNA using ReverTra Ace (TOYOBO, Japan). qPCR was performed using KAPA SYBR Fast qPCR kit (Kapa Biosystems, U.S.A.) and LightCycler® 480 System II (Loche, U.S.A.). To determine the housekeeping genes for the normalization of gene expression levels, we investigated the mRNA level changes of several commonly used genes (Supplementary Fig. 1). Thus, we decided to use 18S ribosomal RNA (rRNA), which shows no changes during differentiation, as a housekeeping gene. The primer sequences used for qPCR amplification are listed in Supplementary Table 1. The qPCR thermocycling protocol was as follows: 40 cycles of denaturation at 95 °C for 5 s; annealing at 57 °C for 20 s; and extension at 72 °C for 1 s. The software calculated the relative amount of the target and housekeeping genes based on threshold cycles.

[^{14}C]-L-Lactate Uptake The SH-SY5Y cells were differentiated and cultured until they reached confluence. The measurement of L-lactate uptake was performed as previously described with minor modification.[13] Briefly, SH-SY5Y cells were plated and cultured as described in Cell culture and differentiation section. After washing, the cells were pre-incubated with the 0.5 mL of Hank's balanced salt saline (HBSS) buffer (25 mM D-glucose, 137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1.3 mM $CaCl_2$, 0.81 mM $MgSO_4$, 4.2 mM $NaHCO_3$, 10 mM Good's buffer), adjusted to pH 5.5 at 37 °C for 10 min. *N*-(2-Hydroxyethyl)-piperazine-*N*′-2-ethanesulfonic acid (HEPES) was used for pH 8.0–7.0 HBSS buffer, MES was used for pH 7.0–5.5 HBSS buffer and HOMOPIPES was used for pH 5.0 HBSS buffer. After pre-incubation, HBSS buffer containing [^{14}C]-labeled L-lactate of 0.64 μM was incubated at 37 °C. After removal of the added HBSS buffer, cells were washed twice with 1000 μL of HBSS buffer adjusted to pH 7.4 to stop reactions. In addition, AR-C155858 and AZD3965 were pre-incubated for 10 min, and AZD0095 and atorvastatin for 5 min because it takes time to show inhibitory effects or to match the incubation time used in a previous study.[15,16] After cells were lysed by adding 500 μL of 0.2 N NaOH -1% sodium dodecyl sulfate (SDS) solution, 3 mL of Ultima Gold™ scintillation cocktail (PerkinElmer, Inc.) was added, and a liquid scintillation counter was used to quantify [^{14}C]-L-lactic acid sodium salt using a liquid scintillation counter. All uptake values were corrected for protein content. Protein concentration was determined using a Pierce® BCA Protein Assay Kit (Thermo Scientific), in accordance with the manufacturer's instructions.

Western Blotting SH-SY5Y cell proteins were extracted using radio immunoprecipitation assay (RIPA) buffer (Cell Signaling Technology, U.S.A.), according to the manufacturer's instructions. Equal amounts of protein (10 μg) were separated using 10% SDS-polyacrylamide gel electrophoresis (PAGE) and transferred to polyvinylidene fluoride (PVDF) membranes. PVDF membranes were blocked with 1% fat-free dry milk for 1 h, followed by incubation with primary antibodies against MCT1 (sc-365501, Santa Cruz Biotechnology, U.S.A.), (1/200), MCT2 (ab198272, Abcam, U.K.), (1/1000), MCT4 (22787-1-AP, Proteintech Group, U.S.A.), (1/1000), and β-actin (ab179467, Abcam, U.K.), (1/5000) in both 0.5% fat-free dry milk in phosphate-buffered saline containing Tween 20 (PBS-T) at 4 °C overnight. Membranes were washed with PBS-T and incubated with secondary goat anti-mouse immunoglobulin G $(IgG)_l$-HRP antibody (1070-05, Southern Biotech, U.S.A.) (1/4000) or mouse anti-rabbit IgG-HRP (sc-2357, Santa Cruz Biotechnology, U.S.A.) (1/4000) in both 0.5% fat-free dry milk in PBS-T for 1 h at room temperature. Membranes were washed again with PBS-T, developed with ECL Prime Western blotting Detection Reagent (Cytiva, U.S.A.), and visualized using an Image Quant LAS 4000 mini.

Small Interfering RNA (siRNA) Transfection The siRNA transfection was optimized using Lipofectamine RNAiMAX (Thermo Fisher Scientific). According to the previous study,[17] the cells were treated by forward transfection to a final siRNA concentration of 7.5 nM on days 1 and 6 after seeding. The medium was replaced 24 h after each transfection. Stealth RNA interference (RNAi) Negative Control Medium GC Duplex was used as the negative control siRNA and HSS185929 was used as the MCT1 siRNA.

Data Analysis All data are expressed as mean ± standard error. Significant differences between the two groups of data were evaluated using the Student's *t*-test. Dunnett's method was used for multiple comparisons with the controls. Statistical significance was set at p-value <0.05 or 0.01. Curve fitting was performed to fit the following equation by nonlinear regression analysis using SigmaPlot 14.5 (Systat Software, U.S.A.):

$$V = \frac{V_{\max 1} * K_{m1}}{K_{m1} + [S]} + \frac{V_{\max 2} * K_{m2}}{K_{m2} + [S]}$$

where V is the substrate uptake rate, [S] is the substrate concentration in the medium, $V_{\max 1}$ and $V_{\max 2}$ are the maximum uptakes, and K_{m1} and K_{m2} are the Michaelis–Menten constants in phases 1 and 2, respectively.

RESULTS

Construction of Differentiation Model in SH-SY5Y Cells Tau and NeuroD1 are known differentiation markers

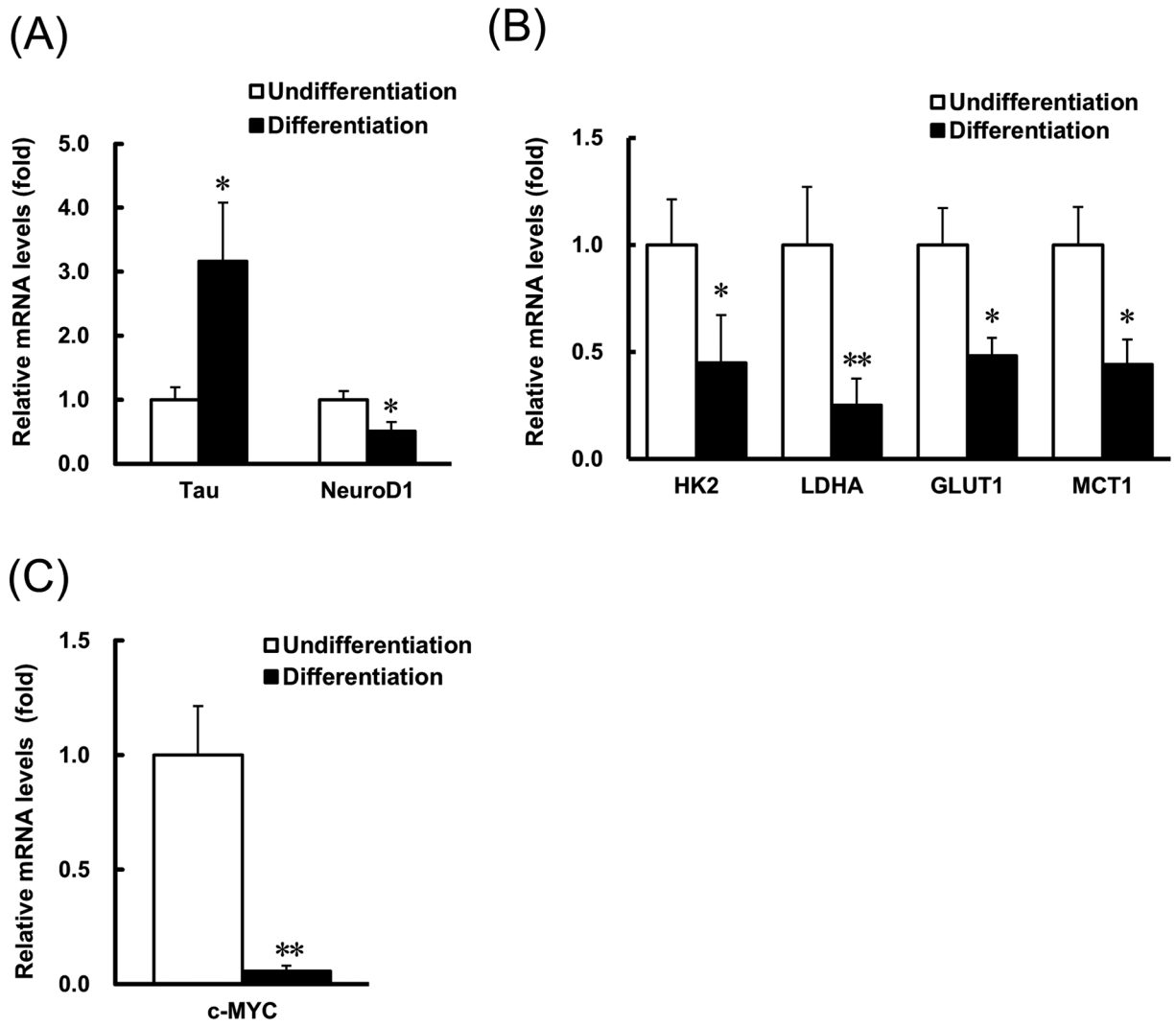

Fig. 1. Changes in mRNA Expression after Differentiation

mRNA levels were quantified *via* RT-qPCR and normalized to 18S rRNA levels. (A) Neuronal markers. (B) Glycolysis-related genes. (C) c-MYC. Data are presented as the means with standard error (S.E.) of four independent experiments. *; $p < 0.05$, compared with undifferentiated cells. **; $p < 0.01$, compared with undifferentiated cells.

in SH-SY5Y cells.[18] The previous report showed that the mRNA level of Tau was increased while that of NeuroD1 was decreased during cell differentiation. In this study, the mRNA levels of these markers in differentiated SH-SY5Y cells were significantly altered compared to those in undifferentiated SH-SY5Y cells, as previously reported (Fig. 1A). In addition, the mRNA levels of genes involved in the glycolytic pathway were evaluated because it has been shown that during differentiation, there is a shift from the glycolytic pathway to oxidative phosphorylation.[19] The mRNA levels of hexokinase-2 (HK2), lactate dehydrogenase A (LDHA), glucose transporter 1 (GLUT1), and MCT1 significantly decreased (Fig. 1B). Furthermore, c-MYC, which is involved in the decreased expression of glycolytic pathway-related genes during differentiation,[19] was significantly downregulated (Fig. 1C). Based on these results, the SH-SY5Y cells were considered to have differentiated properly.

L-Lactate Transport Showed a pH Dependence in Differentiated SH-SY5Y Cells The time dependence of L-lactate uptake in differentiated SH-SY5Y cells was examined. The results showed that L-lactate uptake increased linearly for up to 5 min (Supplementary Fig. 2); therefore, the uptake time was set to 5 min.

Lactate transport carriers exist not only in MCTs but also in sodium-coupled monocarboxylate transporters (SMCTs), which are sodium dependent.[3] Therefore, we examined the driving force of L-lactate transport. Although L-lactate uptake without sodium tended to increase, no significant differences in L-lactate uptake in SH-SY5Y cells was observed with or without sodium (Fig. 2A). The sodium and/or proton carriers other than MCTs may be indirectly involved in L-lactate uptake in SH-SY5Y cells without sodium. In contrast, as proton concentration increased, L-lactate uptake increased (Fig. 2B). The Hill coefficient for the pH dependence returned to 1.1 ± 0.22. The data were fitted to the Henderson–Hasselbälch equation, and the pK_a was calculated to be 6.3 ± 0.14 (Fig. 2C).

Protein Expression of MCTs in Differentiated SH-SY5Y Cells We evaluated MCT expression in differentiated SH-SY5Y cells. HepG2 cells, which have been shown to express MCT1, MCT2, and MCT4,[20] were used as a positive control. Bands for MCT1 and 2, but not for MCT4, were detected compared to HepG2 cells (Fig. 3). Transfection of MCT1 siRNA reduced the signal of the larger molecular weight of the MCT1

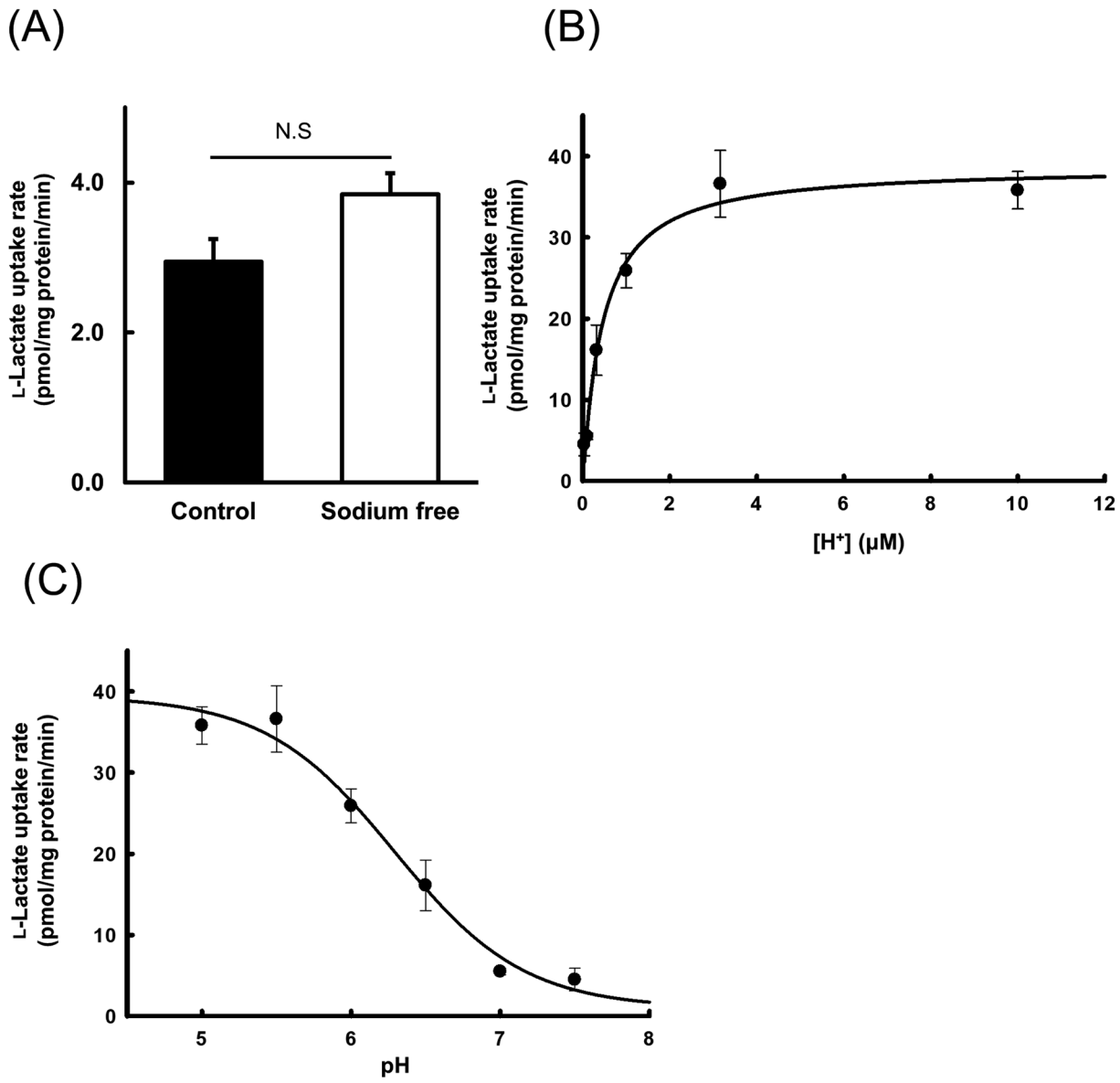

Fig. 2. Effect of Sodium and H^+ on L-Lactate Transport

(A) The uptake of L-lactate (0.64 μM) by differentiated SH-SY5Y cells was measured in the presence of 140 mM sodium and sodium-free. Data are means with S.E. of three independent experiments. No statistical significance was observed. (B, C) Uptake of L-lactate (0.64 μM) by differentiated SH-SY5Y cells was measured following 5 min incubation in the presence of increasing concentrations of H^+ (0.032–10 μM). Data are presented as means ± S.E. of three independent experiments. (B) The Hill equation. (C) The Henderson–Hasselbälch equation.

band (Supplementary Fig. 3A).

Kinetics of L-Lactate Uptake in Differentiated SH-SY5Y Cells The uptake rate increased in an L-lactate concentration-dependent manner and was saturated at high concentrations (Fig. 4). The inset in Fig. 4 shows the Eadie–Hofstee plot, which suggests that L-lactate transport was biphasic. Kinetic parameters were calculated using Eadie–Hofstee plot. K_{m1}, V_{max1} or K_{m2}, and V_{max2} were estimated to be 0.32 mM, 12 nmol/mg protein/min or 1.0 mM, and 23 nmol/mg protein/min, respectively.

MCT Inhibitors Decreased L-Lactate Transport in Differentiated SH-SY5Y Cells The effects of various MCT inhibitors on L-lactate uptake are shown in Table 1. First, L-lactate transport was inhibited by α-cyano-4-hydroxycinnamic acid (CHC), a classic non-selective inhibitor of MCTs.[21] AZD0095, an MCT4-selective inhibitor,[22] had no effect on L-lactate transport. In contrast, L-lactate uptake was strongly inhibited by 10 nM AR-C155858, a selective MCT1/2 inhibitor with K_i values (MCT1: 2.3 nM, MCT2: $<$ 10 nM).[23] AZD3965 greatly inhibited L-lactate uptake at 20 nM, which is the MCT2 K_i value, but not at 2 nM, which is close to the MCT1 K_i value.[24] In addition, incubation with pyruvate, a substrate of MCT1 and 2,[21] reduced L-lactate uptake by approximately 20% at 0.1 mM, which is close to the MCT2 K_m value, and by approximately 70% at 1 mM, which is close to that of MCT1. L-Lactate uptake is also reduced by 5-oxoproline, a selective substrate of MCT1, which is not a substrate of MCT4.[13] Furthermore, atorvastatin, which was reported to be selective for MCT2,[16] inhibited L-lactate transport at a concentration of 10 μM, which implies no inhibitory activity on MCT1.

DISCUSSION

L-Lactate transport in the central nervous system is in-

volved in a variety of functions, such as memory formation. In rodents, it has been reported that L-lactate was released from astrocytes *via* MCT1 or MCT4 and flowed into neurons *via* MCT2.[9] However, the transporters involved in L-lactate uptake by human neuronal cells remain unclear. Therefore, we performed functional analysis of L-lactate uptake in SH-SY5Y cells, a commonly used model of human neurons. To the best of our knowledge, this is the first report of the kinetic analysis of L-lactate uptake using human neuron-like cells.

First, we measured the mRNA levels of various genes to assess neuron-like differentiation in SH-SY5Y cells. Tau is involved in microtubules stabilization and NeuroD1 is related to differentiation induction. These markers exhibit inversely correlated behavior during SH-SY5Y cells differentiation.[18] This study showed that the mRNA level of Tau was increased and that of NeuroD1 was decreased, consistent with the previous report. Moreover, c-MYC expression was reported to be downregulated during the differentiation of neural progenitor cells to neurons, and the mRNA expression of genes involved in the glycolytic pathway was found to be decreased in a study using human iPS cells.[19] In the present study, a similar decrease in the mRNA levels was observed (Figs. 1B, C). In addition, we observed morphological changes in which the cell body became shapelier, and neurites became more elongated than in undifferentiated cells (Supplementary Fig. 4). Based on the above points, we considered that differentiation proceeded adequately and that the cells were appropriate as a neuron-like model for further examination.

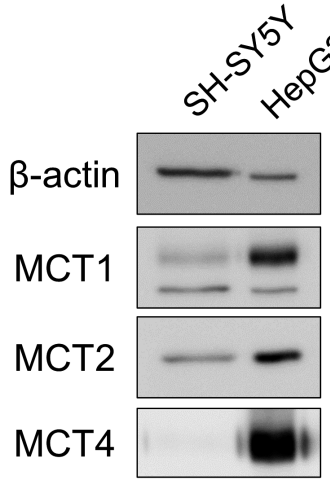

Fig. 3. Protein Expression of MCTs

Western blot of differentiated SH-SY5Y and HepG2 cells showing MCT1 (40–48 kDa), MCT2 (52 kDa), MCT4 (42–45 kDa), and *β*-actin (42 kDa).

L-Lactate transporters include MCTs and SMCTs. SMCTs have two subtypes, SMCT1 and SMCT2, which transport L-lactate and pyruvate, respectively, with sodium as the driving force. SMCT1 has been reported to be expressed in rodent neurons and to transport L-lactate.[25] SMCT2 has been reported to localize to astrocytes, Müller cells, and glial cells of the retina,[26] but there are no reports of its localization in other brain regions. In this study, L-lactate uptake did not change in the presence or absence of sodium (Fig. 2A) and SMCT1 and SMCT2 mRNA expression was not observed (data not shown), suggesting that the sodium gradient was unlikely to be involved in L-lactate uptake in SH-SY5Y cells. In contrast, L-lactate transport increased with decreasing extracellular pH. The Hill coefficient was calculated to be 1.1 ± 0.21, and the pK_a was calculated to be 6.3 ± 0.14, which is close to the calculated value in previous studies of MCT transporter characteristic.[13,27] This is consistent with the characteristics of MCT1, 2, and 4, which transport L-lactate in a pH-dependent (Figs. 2B, C).[3] MCTs are the transporters with the highest affinity in the order of MCT2, MCT1, and MCT4, with K_m values of 0.32 ± 0.02, 1.0 ± 0.1 and 3.4 ± 0.4 mM, respectively, at pH 5.5.[28–30] In this study, L-lactate transport was biphasic in pH 5.5 (Fig. 3), with K_m values calculated as 0.32 mM and 1.0 mM, which were close to previous reports.

We further investigated the effects of several MCT inhibitors on MCT isoforms that contribute to L-lactate uptake by differentiated SH-SY5Y cells (Table 1). First, L-lactate uptake was inhibited by CHC, a classic non-selective MCT

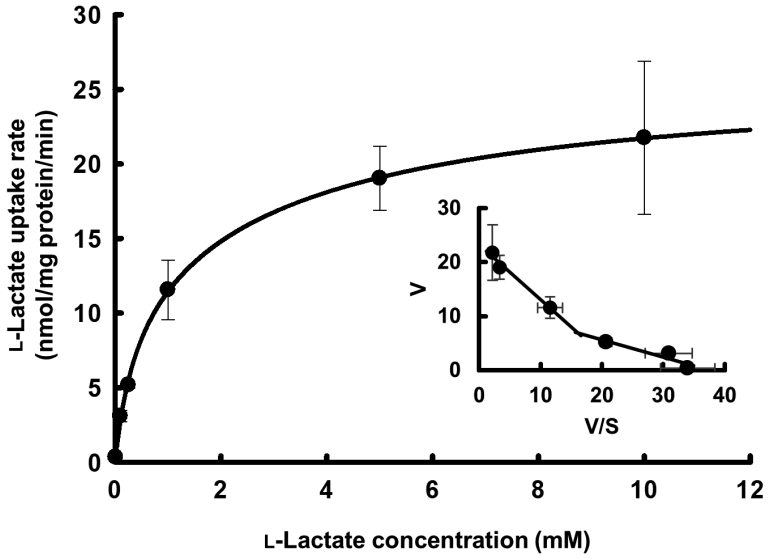

Fig. 4. Kinetics of L-Lactate Transport

L-Lactate (0.01–10 mM) uptake by differentiated SH-SY5Y cells was measured by subtracting the value in HEPES buffer (pH 7.4) at 37 °C for 5 min from the value in MES buffer (pH 5.5) at 37 °C for 5 min. The inset shows the Eadie–Hofstee plot of the data after subtraction of non-saturable L-lactate uptake. V denotes L-lactate uptake rate, while S denotes L-lactate concentration. Data are means ± S.E. of three independent experiments.

Table 1. Effect of MCT Substrates and Inhibitors on L-Lactate Transport

Treatment	% of control
CHC 30 μM	63.6 ± 6.33*
CHC 100 μM	50.0 ± 5.29*
CHC 200 μM	53.2 ± 8.74*
CHC 1 mM	28.1 ± 3.12**
AZD0095 10 μM	99.9 ± 0.09
AR-C155858 10 nM	12.0 ± 3.12**
AZD3965 2 nM	104 ± 11.9
AZD3965 20 nM	26.8 ± 3.23**
Pyruvate 0.1 mM	77.3 ± 2.20*
Pyruvate 1 mM	37.2 ± 6.38**
5-Oxoproline 10 mM	32.4 ± 2.74**
Atorvastatin 10 μM	52.6 ± 6.77**

L-Lactate uptake (0.64 μM) by differentiated SH-SY5Y cells was measured in the presence of various substrates and inhibitors (30, 100, 200 μM, and 1 mM α-Cyano-4-hydroxycinnamic acid (CHC); 10 μM AZD0095; 10 nM AR-C155858; 2 and 20 nM AZD3965; 0.1 and 1 mM Pyruvate; 10 mM 5-Oxoproline; 10 μM Atorvastatin) in MES buffer (pH 5.5) at 37 °C for 5 min. For 10 nM AR-C155858, 2 and 20 nM AZD3965, 10 min pre-incubation, for 10 μM AZD0095 and 10 μM Atorvastatin, 5 min pre-incubation was performed because it is known that it takes time to show the inhibitory effect. Data are means ± S.E. of three independent experiments. *; $p < 0.05$ compared with Control. **; $p < 0.01$ compared with Control.

inhibitor.[21] This suggested that MCTs are involved in L-lactate transport in differentiated SH-SY5Y cells. In addition, AZD0095, MCT4 selective inhibitor,[22] did not affect L-lactate uptake. In contrast, with MCT1, 2 selective inhibitors, L-lactate transport was greatly inhibited by exposure to sufficient doses. 2 nM AZD3965, with the K_i value of MCT1,[24] did not inhibit L-lactate transport. Since the K_i value was calculated from experiments in *Xenopus laevis* oocytes, higher concentrations than the K_i value may be required to show an inhibitory effect on L-lactate uptake in SH-SY5Y cells. It was also possible that MCT2, which has a high affinity for L-lactate transport, was preferentially utilized. Moreover, 20 nM AZD3965 has the K_i value of MCT2,[24] and L-lactate transport is inhibited by approximately 70%. In addition, 10 μM atorvastatin, a concentration that inhibits MCT2 but not MCT1,[16] inhibited L-lactate transport. The K_m value of MCT1 for 5-oxoproline is reported to be less than 10 mM,[27] and that of MCT2 for 5-oxoproline has not been reported to date. We observed that 10 mM 5-oxoproline inhibited the uptake of L-lactate in SH-SY5Y cells by more than 50%. This indicates that MCT1 at least contributes to L-lactate transport, although it is not known whether 5-oxoproline inhibits MCT2. Furthermore, 0.1 mM pyruvate, which competitively inhibits L-lactate, has a K_m value of MCT2,[21] but L-lactate transport was inhibited by only approximately 20%. Since both L-lactate and pyruvate are common substrates of MCT1 and MCT2, pyruvate at low concentrations may not have shown an inhibitory effect on MCT2. Therefore, it is difficult to conclude whether L-lactate transport is involved only in MCT2. We conducted the siRNA transfection on SH-SY5Y cells to confirm the contribution of MCT1 and MCT2 to L-lactate transport. The transfection of MCT1 siRNA markedly decreased the protein expression of MCT1 but not that of MCT2. In this condition, L-lactate uptake in SH-SY5Y cells significantly decreased (Supplementary Fig. 3). Because we could not obtain effective siRNA that decreases the expression of MCT2 in the SH-SY5Y cells, further studies of MCT2 siRNA transfection in these cells are needed. Based on the results of the kinetic analysis, inhibitory experiments, and MCT1 siRNA transfection, we concluded that both MCT1 and MCT2 are involved in L-lactate transport in SH-SY5Y cells. This is consistent with the result that MCT1 and MCT2 proteins were expressed (Fig. 3).

In this study, we used SH-SY5Y cells, which are often used as a model for neurons. Previous reports have suggested that MCT1 primarily contributes to L-lactate transport in differentiated SH-SY5Y cells because the protein expression levels of MCT1 were maintained, and those of MCT2 were decreased between pre- and post-differentiation.[31] These changes of the protein expression of MCTs are consistent with our results confirmed by Western blotting (data not shown). Other reports have indicated that MCT2 is important in the central nervous system of humans.[32] However, these reports did not verify the carriers involved in L-lactate transport by kinetic analysis. In this study, we performed the kinetic analysis of L-lactate uptake in differentiated SH-SY5Y cells and the inhibitory experiments by using atorvastatin, which has a selective inhibitory effect on MCT2. We demonstrated that both MCT1 and MCT2 contribute to L-lactate transport in differentiated human neuroblastoma SH-SY5Y cells for the first time, which is consistent with the results in rodent cells.[8] However, it remains uncertain whether both MCT1 and MCT2 contribute to L-lactate transport in actual neurons because the SH-SY5Y cells were derived from cancer cells. When further optimal neural models are established in the future, it is necessary to perform the analysis of L-lactate transport in those models. Moreover, neurons are suggested to receive L-lactate from surrounding astrocytes and microglia in the ANLS hypothesis. It is desirable to evaluate the transporters contributing to L-lactate uptake by co-culturing human neurons with astrocytes and microglia.

In conclusion, we revealed that MCT1 and MCT2 are mainly responsible for L-lactate uptake in differentiated human neuroblastoma SH-SY5Y cells. L-Lactate transport in the brain plays an important role in memory formation. Furthermore, an evaluation of the relationship between the expression of MCT1, MCT2 and memory formation capacity, and a search for factors that may modulate the expression of MCT1 and MCT2 should be conducted.

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Conflict of Interest The authors declare no conflict of interest.

Supplementary Materials This article contains supplementary materials.

REFERENCES

1) Magistretti PJ, Pellerin L. Cellular bases of brain energy metabolism and their relevance to functional brain imaging: evidence for a prominent role of astrocytes. *Cereb. Cortex*, **6**, 50–61 (1996).
2) Riske L, Thomas RK, Baker GB, Dursun SM. Lactate in the brain: an update on its relevance to brain energy, neurons, glia and panic disorder. *Ther. Adv. Psychopharmacol.*, **7**, 85–89 (2017).
3) Halestrap AP. Monocarboxylic acid transport. *Compr. Physiol.*, **3**,

1611–1643 (2013).
4) Kobayashi M, Narumi K, Furugen A, Iseki K. Transport function, regulation, and biology of human monocarboxylate transporter 1 (hMCT1) and 4 (hMCT4). *Pharmacol. Ther.*, **226**, 107862 (2021).
5) Suzuki A, Stern SA, Bozdagi O, Huntley GW, Walker RH, Magistretti PJ, Alberini CM. Astrocyte-neuron lactate transport is required for long-term memory formation. *Cell*, **144**, 810–823 (2011).
6) Dringen R, Wiesinger H, Hamprecht B. Uptake of L-lactate by cultured rat brain neurons. *Neurosci. Lett.*, **163**, 5–7 (1993).
7) McKenna MC, Tildon JT, Stevenson JH, Hopkins IB, Huang X, Couto R. Lactate transport by cortical synaptosomes from adult rat brain: characterization of kinetics and inhibitor specificity. *Dev. Neurosci.*, **20**, 300–309 (1998).
8) Cortes-Campos C, Elizondo R, Carril C, Martínez F, Boric K, Nualart F, Garcia-Robles MA. MCT2 expression and lactate influx in anorexigenic and orexigenic neurons of the arcuate nucleus. *PLOS ONE*, **8**, e62532 (2013).
9) Bergersen LH. Lactate transport and signaling in the brain: potential therapeutic targets and roles in body–brain interaction. *J. Cereb. Blood Flow Metab.*, **35**, 176–185 (2015).
10) Froberg MK, Gerhart DZ, Enerson BE, Manivel C, Guzman-Paz M, Seacotte N, Drewes LR. Expression of monocarboxylate transporter MCT1 in normal and neoplastic human CNS tissues. *Neuroreport*, **12**, 761–765 (2001).
11) Chiry O, Fishbein WN, Merezhinskaya N, Clarke S, Galuske R, Magistretti PJ, Pellerin L. Distribution of the monocarboxylate transporter MCT2 in human cerebral cortex: an immunohistochemical study. *Brain Res.*, **1226**, 61–69 (2008).
12) Murakami R, Chiba Y, Nishi N, Matsumoto K, Wakamatsu K, Yanase K, Uemura N, Nonaka W, Ueno M. Immunoreactivity of receptor and transporters for lactate located in astrocytes and epithelial cells of choroid plexus of human brain. *Neurosci. Lett.*, **741**, 135479 (2021).
13) Ideno M, Kobayashi M, Sasaki S, Futagi Y, Narumi K, Furugen A, Iseki K. Involvement of monocarboxylate transporter 1 (SLC16A1) in the uptake of L-lactate in human astrocytes. *Life Sci.*, **192**, 110–114 (2018).
14) Kovalevich J, Langford D. Considerations for the use of SH-SY5Y neuroblastoma cells in neurobiology. *Methods Mol. Biol.*, **1078**, 9–21 (2013).
15) Guan X, Rodriguez-Cruz V, Morris ME. Cellular uptake of MCT1 inhibitors AR-C155858 and AZD3965 and their effects on MCT-mediated transport of L-lactate in murine 4T1 breast tumor cancer cells. *AAPS J.*, **21**, 13 (2019).
16) Yamaguchi A, Mukai Y, Sakuma T, Furugen A, Narumi K, Kobayashi M. Atorvastatin exerts more selective inhibitory effects on hMCT2 than on hMCT1 and hMCT4. *Anticancer Res.*, **43**, 3015–3022 (2023).
17) Lin CC, Chou CH, Howng SL, Hsu CY, Hwang CC, Wang C, Hsu CM, Hong YR. GSKIP, an inhibitor of GSK3beta, mediates the N-cadherin/beta-catenin pool in the differentiation of SH-SY5Y cells. *J. Cell. Biochem.*, **108**, 1325–1336 (2009).
18) Constantinescu R, Constantinescu AT, Reichmann H, Janetzky B. Neuronal differentiation and long-term culture of the human neuroblastoma line SH-SY5Y. *J. Neural Transm. Suppl.*, **72**, 17–28 (2007).
19) Zheng X, Boyer L, Jin M, Mertens J, Kim Y, Ma L, Ma L, Hamm M, Gage FH, Hunter T. Metabolic reprogramming during neuronal differentiation from aerobic glycolysis to neuronal oxidative phosphorylation. *eLife*, **5**, e13374 (2016).
20) Mukai Y, Yamaguchi A, Sakuma T, Nadai T, Furugen A, Narumi K, Kobayashi M. Involvement of SLC16A1/MCT1 and SLC16A3/MCT4 in L-lactate transport in the hepatocellular carcinoma cell line. *Biopharm. Drug Dispos.*, **43**, 183–191 (2022).
21) Manning Fox JEM, Meredith D, Halestrap AP. Characterisation of human monocarboxylate transporter 4 substantiates its role in lactic acid efflux from skeletal muscle. *J. Physiol.*, **529**, 285–293 (2000).
22) Goldberg FW, Kettle JG, Lamont GM, Buttar D, Ting AKT, McGuire TM, Cook CR, Beattie D, Morentin Gutierrez P, Kavanagh SL, Komen JC, Kawatkar A, Clark R, Hopcroft L, Hughes G, Critchlow SE. Discovery of clinical candidate AZD0095, a selective inhibitor of monocarboxylate transporter 4 (MCT4) for oncology. *J. Med. Chem.*, **66**, 384–397 (2023).
23) Ovens MJ, Davies AJ, Wilson MC, Murray CM, Halestrap AP. AR-C155858 is a potent inhibitor of monocarboxylate transporters MCT1 and MCT2 that binds to an intracellular site involving transmembrane helices 7-10. *Biochem. J.*, **425**, 523–530 (2010).
24) Curtis NJ, Mooney L, Hopcroft L, Michopoulos F, Whalley N, Zhong H, Murray C, Logie A, Revill M, Byth KF, Benjamin AD, Firth MA, Green S, Smith PD, Critchlow SE. Pre-clinical pharmacology of AZD3965, a selective inhibitor of MCT1: DLBCL, NHL and Burkitt's lymphoma anti-tumor activity. *Oncotarget*, **8**, 69219–69236 (2017).
25) Martin PM, Gopal E, Ananth S, Zhuang L, Itagaki S, Prasad BM, Smith SB, Prasad PD, Ganapathy V. Identity of SMCT1 (SLC5A8) as a neuron-specific Na^+-coupled transporter for active uptake of L-lactate and ketone bodies in the brain. *J. Neurochem.*, **98**, 279–288 (2006).
26) Martin PM, Dun Y, Mysona B, Ananth S, Roon P, Smith SB, Ganapathy V. Expression of the sodium-coupled monocarboxylate transporters SMCT1 (SLC5A8) and SMCT2 (SLC5A12) in retina. *Invest. Ophthalmol. Vis. Sci.*, **48**, 3356–3363 (2007).
27) Sasaki S, Futagi Y, Kobayashi M, Ogura J, Iseki K. Functional characterization of 5-oxoproline transport *via* SLC16A1/MCT1. *J. Biol. Chem.*, **290**, 2303–2311 (2015).
28) Futagi Y, Sasaki S, Kobayashi M, Narumi K, Furugen A, Iseki K. The flexible cytoplasmic loop 3 contributes to the substrate affinity of human monocarboxylate transporters. *Biochim. Biophys. Acta Biomembr.*, **1859**, 1790–1795 (2017).
29) Yamaguchi A, Narumi K, Furugen A, Iseki K, Kobayashi M. Identification of the essential extracellular aspartic acids conserved in human monocarboxylate transporters 1, 2, and 4. *Biochem. Biophys. Res. Commun.*, **529**, 1061–1065 (2020).
30) Sasaki S, Kobayashi M, Futagi Y, Ogura J, Yamaguchi H, Takahashi N, Iseki K. Crucial residue involved in L-lactate recognition by human monocarboxylate transporter 4 (hMCT4). *PLOS ONE*, **8**, e67690 (2013).
31) Xu Y, Kusuyama J, Osana S, Matsuhashi S, Li L, Takada H, Inada H, Nagatomi R. Lactate promotes neuronal differentiation of SH-SY5Y cells by lactate-responsive gene sets through NDRG3-dependent and -independent manners. *J. Biol. Chem.*, **299**, 104802 (2023).
32) Medel V, Crossley N, Gajardo I, Muller E, Barros LF, Shine JM, Sierralta J. Whole-brain neuronal MCT2 lactate transporter expression links metabolism to human brain structure and function. *Proc. Natl. Acad. Sci. U.S.A.*, **119**, e2204619119 (2022).