Cloning and functional characterization of a high-affinity Na\(^+\)/dicarboxylate cotransporter from mouse brain

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Na\(^+\)tamate. We have isolated the cDNA coding for a high-affinity Na\(^+\)/dicarboxylate cotransporter for absorption of neurotransmitter precursor substrates, such as \(\alpha\)-ketoglutarate and malate, which are subsequently metabolized to replenish pools of neurotransmitters, including glutamate. We have isolated the cDNA coding for a high-affinity Na\(^+\)/dicarboxylate cotransporter from mouse brain, called mNaDC-3. The mRNA coding for mNaDC-3 is found in brain and choroid plexus as well as in kidney and liver. The mNaDC-3 transporter has a broad substrate specificity for dicarboxylates, including succinate, \(\alpha\)-ketoglutarate, fumarate, malate, and dimethylsuccinate. The transport of citrate is relatively insensitive to pH, but the transport of succinate is inhibited by acidic pH. The Michaelis-Menten constant for succinate in mNaDC-3 is 140 \(\mu\)M in transport assays and 16 \(\mu\)M at \(-50\) mV in two-electrode voltage clamp assays. Transport is dependent on sodium, although lithium can partially substitute for sodium. In conclusion, mNaDC-3 likely codes for the high-affinity Na\(^+\)/dicarboxylate cotransporter in brain, and it has some unusual electrical properties compared with the other members of the family.

The functional properties of the brain Na\(^+\)/dicarboxylate cotransporter resemble those of the high-affinity Na\(^+\)/dicarboxylate cotransporters that have been characterized in other organs, including the basolateral membrane of kidney proximal tubule cells, the basolateral membrane of liver perivenous hepatocytes, the brush border membrane of placenta, and chick intestinal cells (10). The cDNAs coding for several high-affinity transporter orthologs, called NaDC-3, have been cloned from kidney or placenta of rat, human, and flounder (3, 5, 20, 25, 26). The NaDC-3 transporters are sodium dependent and have a high affinity for a broad range of dicarboxylate substrates, in particular \(\alpha\)-ketoglutarate and succinate. These transporters are members of a gene family, called SLC13 in the human gene nomenclature, which also contains low-affinity Na\(^+\)/dicarboxylate cotransporters, NaDC-1, and Na\(^+\)/sulfate cotransporters, NaSi-1 (10).

In this study, we report the sequence and functional characterization of the high-affinity Na\(^+\)/dicarboxylate cotransporter from mouse brain, mNaDC-3. The mRNA for NaDC-3 is found in brain, choroid plexus, liver, and kidney, similar to the tissue distribution of high-affinity Na\(^+\)/dicarboxylate transport. The mouse brain NaDC-3 is an electrogenic sodium-coupled transporter with a broad substrate selectivity and high substrate affinity. The preferred substrates of mNaDC-3 include succinate, \(\alpha\)-ketoglutarate, malate, and dimethylsuccinate. It is likely that mNaDC-3 corresponds to the Na\(^+\)/dicarboxylate cotransporter previously identified in glutamatergic synaptosomes.

METHODS

Library screening. A mouse brain cDNA library was purchased from Origene Technologies and screened by polymerase chain reaction (PCR). The PCR primers were designed on the basis of highly conserved sequences of the rat placental high-affinity Na\(^+\)/dicarboxylate cotransporter rNaDC-3 (5). The sense primer DC1 had the sequence 5'-CAC GCC TTC CAC CGC AAT GAT-3', and the antisense primer DC2 had the sequence 5'-GCA TGA AGG CGT AGG AAC AGC-3'. This primer pair amplified a cDNA fragment of \(\sim 1.1\) kb. The reactions were done using AmpliTaq enzyme (Perkin-Elmer Cetus), with the reaction buffer supplied with the enzyme, 200 nM dNTPs, and 20 pmol of each primer. The samples

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were heated for 5 min at 94°C before the enzyme was added, followed by 35 cycles at 94°C for 45 s, 52°C for 45 s, and 72°C for 2 min.

The cDNA library was screened by a *sib*-selection approach (8). The master library plate contained ~5,000 cDNAs in each well of a 96-well plate. Twenty pooled samples were made from the library plate, consisting of samples from the 8 rows and 12 columns. The positive wells were identified by PCR, and then a subplate containing the clones from a positive well was purchased from Origene. Each of the wells in the 96-well subplate contained 50 clones as bacterial stocks. After pools from the subplate were screened by PCR, the bacteria in the positive well were plated onto LB-ampicillin plates. Individual colonies from the plates were grown in overnight liquid culture in a 96-well culture plate. These samples were again screened by *sib*-selection using PCR. The first complete screen of the library yielded clone F11, which was truncated at the 5′ end (the sequence started at nt 153). A second screen of the library (after the purchase of a new subpool) yielded clones 10f and 11b, both of which contained the start codon and 5′ untranslated region. However, clones 10f and 11b also contained an intron of 173 nt at nt 148. The 5′ end of the cDNA without the intron was amplified directly from the mouse brain cDNA library by use of the sequence-specific primer (DC2) and a vector-specific primer supplied from the mouse brain cDNA library by use of the sequence-5′9′10f and 11b also contained an intron of 173 nt at nt 148. The 5′ end of the cDNA without the intron was amplified directly from the mouse brain cDNA library by use of the sequence-specific primer (DC2) and a vector-specific primer supplied from the library (pCMV6). The final construct, called mNaDC-3, was assembled by subcloning the 5′ end PCR reaction together with the 3′ end of clone 10f into the vector pSPORT 1 for expression in *Xenopus* oocytes. Both strands of mNaDC-3 were sequenced by the University of Texas Medical Branch sequencing facility. The sequence was assembled using the Genetics Computer Group package.

**Northern blot.** A multiple-tissue Northern blot containing 2 µg of poly(A⁺) RNA in each lane was purchased from Origene. The blot was probed at high stringency, as described previously (14), with clone F11 (nt 153–3282 of mNaDC-3) excised with NotI from the pCMV6-XL4 vector.

**RT-PCR.** First-strand cDNA was reverse transcribed (RT) using a First-Strand cDNA Synthesis Kit (GIBCO BRL) and commercially purchased poly(A⁺) or total RNA as templates. The cDNA was then used in PCR with the Failsafe PCR kit (Epicenter), with premix buffer F and primers DC1/DC2. The PCR reactions followed a two-step touchdown protocol as recommended by the manufacturer, consisting of an initial 95°C denaturation step for 3 min followed by 5 cycles (94°C × 30 s, 72°C × 1.5 min), 5 cycles (94°C × 30 s, 70°C × 1.5 min), and 30 cycles (94°C × 30 s, 68°C × 1.5 min). This enzyme and protocol were used rather than the AmpliTaq polymerase to minimize mutations. The PCR products were subcloned into the pCRII vector using the TopoTA cloning kit (Invitrogen) and sequenced.

**Xenopus oocytes.** Female *Xenopus laevis* frogs were obtained from Nasco. Stage V and VI oocytes were dissected and collagenase treated as described previously (9). Oocytes were injected with 50 nl of cRNA on the following day. The control reactions contained mouse poly(A⁺) RNA that was probed at high stringency with the mNaDC-3 cDNA. A hybridization signal of ~3.3 kb was seen in both brain and kidney, although the message in kidney appears to be much more abundant (Fig. 2A). The kidney sample also contained a second hybridization signal of ~7 kb. There was no hybridization with mRNA from heart, stomach, intestine, or skeletal muscle. The tissue distribution of mNaDC-3 in brain and kidney is similar to the distribution of the rat and human NaDC-3 (3, 5, 26). The tissue distribution of mNaDC-3 message was further analyzed using RT-PCR. Because of the high sequence identity between the mouse and rat NaDC-3 sequences, RNA samples from both species were used in the RT-PCR reactions. In agreement with the results of the Northern blot, there was no amplification of message from rat heart (Fig. 2B), but a PCR product of the correct size was produced with cDNA from kidney, liver, and choroid plexus. The control reactions con-

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**RESULTS**

**Sequence of mNaDC-3.** The sequence of the cDNA coding for the mouse brain high-affinity Na⁺/dicarboxylate cotransporter mNaDC-3 contains 3,252 nucleotides and an open reading frame (Fig. 1). The sequence of mNaDC-3 contains two consensus sites for N-glycosylation at Asn-584 and Asn-594, both located at the carboxy terminus, similar to the other NaDC-3 orthologs. The alignment of mNaDC-3 with other high-affinity Na⁺/dicarboxylate cotransporters is also shown in Fig. 1. The amino acid sequence of mouse NaDC-3 is 97% identical to the NaDC-3 from rat (3, 5), 87% identical to that of human (26), and 64% identical to that of winter flounder (25). In addition, the amino acid sequence of mNaDC-3 is 49% identical to the low-affinity Na⁺/dicarboxylate cotransporter from mouse kidney, mNaDC-1 (14), and 40% identical to the mouse Na⁺/sulfate cotransporter, mNaSi-1(1).

**Tissue distribution of mNaDC-3.** A Northern blot containing mouse poly(A⁺) RNA was probed at high stringency with the mNaDC-3 cDNA. A hybridization signal of ~3.3 kb was seen in both brain and kidney, although the message in kidney appears to be much more abundant (Fig. 2A). The kidney sample also contained a second hybridization signal of ~7 kb. There was no hybridization with mRNA from heart, stomach, intestine, or skeletal muscle. The tissue distribution of mNaDC-3 in brain and kidney is similar to the distribution of the rat and human NaDC-3 (3, 5, 26). The tissue distribution of mNaDC-3 message was further analyzed using RT-PCR. Because of the high sequence identity between the mouse and rat NaDC-3 sequences, RNA samples from both species were used in the RT-PCR reactions. In agreement with the results of the Northern blot, there was no amplification of message from rat heart (Fig. 2B), but a PCR product of the correct size was produced with cDNA from kidney, liver, and choroid plexus. The control reactions con-
taining water in place of cDNA did not result in amplification of any PCR products. The PCR products were subcloned into the pCRII vector (Invitrogen) and sequenced. The sequences were found to be 100% identical to the NaDC-3 sequences from rat or mouse, depending on the origin of the cDNA. Therefore, the sequences of the NaDC-3 found in kidney, liver, and choroid plexus are identical between amino acids 145 and 521 with the mNaDC-3 isolated from brain.

Functional characterization of mNaDC-3: radioisotracer uptakes.

The expression of mNaDC-3 in Xenopus oocytes resulted in the increased transport of succinate. The substrate specificity of mNaDC-3 was determined by measuring the inhibition of succinate transport by test substrates. As shown in Fig. 3, the transport of 10 μM [3H]succinate was inhibited ~80% by 1 mM concentrations of succinate, fumarate, or 2,2-dimethylsuccinate. Malate, α-ketoglutarate, and 2,3-dimethylsuccinate were also good inhibitors of succinate transport by mNaDC-3 (Fig. 3). Citrate did not inhibit succinate transport when the experiment was done at pH 7.5, but it resulted in ~25% inhibition at pH 5.5, in agreement with the hypothesis that citrate is carried in protonated form (13). There was only a small amount of inhibition by L-glutamate and cis-aconitate. L-glutamine was tested for its interaction with mNaDC-3 because of early reports suggesting that L-glutamine stimulated Na\(^+\)/dicarboxylate co-transport in synaptosomes from mouse and rat brain (21, 22). However, there was no significant inhibition of succinate transport by L-glutamine or by sulfate, L-aspartate, or pyruvate.

The transport of succinate by mNaDC-3 is sodium dependent. Transport was abolished when sodium was replaced by cesium, but there was a small amount of transport (~3%) when sodium was replaced by choline (Fig. 4A). As seen in many of the other members of this gene family, lithium can substitute for sodium in
mNaDC-3, inducing ~20% of the transport seen in sodium. Interestingly, although lithium can partially substitute for sodium when 100 mM cation concentrations are used, there was no inhibition by 5 mM lithium in the presence of 95 mM sodium (Fig. 4B). In contrast, the rat NaDC-3 is sensitive to inhibition by 3 mM lithium (3, 5), and the low-affinity rabbit NaDC-1 has an apparent inhibitory constant ($K_i$) for lithium of ~2 mM (13).

The uptake of succinate by mNaDC-3 is saturable with an apparent $K_m$ for succinate of ~100 μM (Fig. 5A). In three experiments, the mean $K_m$ for succinate was 142 ± 23 μM, and the maximum velocity ($V_{max}$) was 6.5 ± 2.0 nmol·oocyte⁻¹·h⁻¹ (mean ± SE). The kinetics of sodium activation of succinate transport are shown in Fig. 5B. There was a sigmoid relationship between sodium concentration and succinate transport rate, with an apparent Hill coefficient of 1.77 and a sodium $K_m$ ($K_{Na}$) of 25 mM (Fig. 5B). In a second experiment, the Hill coefficient was 1.82 and $K_{Na}$ was 32 mM. The Hill coefficient is consistent with the coupling stoichiometry of three sodium ions: one substrate molecule proposed for all of the members of the SLC13 family (11).

One characteristic difference between the low- and high-affinity Na⁺/dicarboxylate cotransporters is their response to pH (11). In the low-affinity NaDC-1 orthologs, the preferred substrates are thought to be divalent, and possibly also monovalent, anions. In NaDC-1, for example, there is no effect of pH on succinate transport but a strong effect of pH on citrate transport in accordance with the concentration of divalent citrate in the medium (9). The high-affinity NaDC-3 transporters probably transport dicarboxylates as divalent anions much better than in protonated form (3, 5). In mNaDC-3 there was a stimulation of succinate transport at alkaline pH values and an inhibition at acidic pH values (Fig. 6A). In contrast, there was little effect of pH on citrate transport by mNaDC-3 between pH 5.5 and 7.5, but there was a decrease in citrate transport at pH 8 (Fig. 6B).

Two-electrode voltage clamp studies. The coupled transport of succinate and sodium by mNaDC-3 is electrogenic, which supports the proposed coupling stoichiometry of three sodium ions for each divalent anion substrate. As shown in Fig. 7, inward currents were seen in the presence of succinate and sodium at all voltages tested. However, the current-voltage relationship ($I$-$V$) in mNaDC-3 was different from that of the other members of the family. Although the inward currents in mNaDC-3 increased as the membrane voltage was made more negative, at very negative voltages the currents appeared to decrease (Fig. 7). The

Fig. 2. Tissue distribution of NaDC-3. A: Northern blot of mouse poly(A⁺)-RNA probed at high stringency with mNaDC-3 cDNA. Hybridization signals are evident in brain and kidney. The positions of size standards are shown at left. B: ethidium bromide-stained agarose gel showing RT-PCR products with the use of DC1/DC2 primers. Samples from rat (R) and mouse (M) are shown. Positive reactions (1.1 kb product) were seen with kidney, liver, and choroid plexus, but not with heart or water control (no cDNA).

Fig. 3. Substrate specificity of mNaDC-3 expressed in Xenopus oocytes. Uptake of 10 μM [³H]succinate was measured in the presence or absence of 1 mM test inhibitors during a 15-min time period. αKG, α-ketoglutarate; DMS, dimethylsuccinate. Each data point represents the mean ± SE of experiments with 3 different frogs. Data are presented as %control transport measured in the absence of inhibitor. The experiment in the presence and absence of citrate was done at pH 5.5 and 7.5, and the data are expressed as %succinate transport at the same pH.
cation specificity of substrate-dependent currents in mNaDC-3 is also shown in Fig. 7. No substrate-dependent currents were seen when the sodium buffer was replaced with equimolar concentrations of choline, potassium, or cesium. However, large outward currents were seen in the presence of lithium at membrane voltages ($V_m$) more negative than $-50 \text{ mV}$, and inward currents were seen at $V_m$ more positive than $-50 \text{ mV}$. The outward currents in Li$^+$ and the curved $I-V$ relationship in Na$^+$ were not seen in control, uninjected oocytes, or oocytes from the same frog injected with other NaDC clones (results not shown).

The succinate-induced currents were examined to identify the substrate concentration at half-maximal current ($K_{0.5}$) for succinate under voltage clamp conditions by using more rapid time points (100 ms) than in the uptake experiments (5 min). Because of the unusual $I-V$ curves at membrane potentials more negative than $-50 \text{ mV}$, the kinetic parameters were calculated for membrane voltages more positive than $-50 \text{ mV}$. In the single experiment shown in Fig. 8A, the succinate-induced $K_{0.5}$ ($K_{0.5}^\text{succinate}$) was $16 \pm 5 \text{ mM}$ (mean $\pm$ SE, $n = 4$ oocytes from 3 frogs). The effect of voltage on the $K_{0.5}^\text{succinate}$ in mNaDC-3 is similar to that in the other members of the family, with large increases in $K_{0.5}^\text{succinate}$ at membrane potentials more positive than $0 \text{ mV}$ but only a small change in $K_{0.5}^\text{succinate}$ at negative membrane voltages (Fig. 8B). The succinate-induced maximum current at saturation concentration ($I_{\text{max}}^\text{succinate}$) became larger as the membrane voltage was made more negative (Fig. 8C).

The substrate specificity of mNaDC-3 was tested by measuring currents induced by different test substrates at a holding potential of $-50 \text{ mV}$ (Fig. 9). The data are expressed as a percentage of the succinate-induced currents in the same oocytes. The substrate concentration used was $0.5 \text{ mM}$, which should produce the $I_{\text{max}}$, at least in the case of succinate. Succinate, malate, and $\alpha$-ketoglutarate all produced similar currents, and in some experiments the currents produced by malate and $\alpha$-ketoglutarate were greater than the succinate-induced currents. Both 2,3-dimethylsuccinate and fumarate induced currents that were $80\%$ as large as those induced by succinate, whereas the aspartate-induced currents were $60\%$ of those in-
duced by succinate. Currents <20% of control were seen in the presence of citrate, L-glutamate, and sulfate. No currents were measured with L-glutamine.

**DISCUSSION**

This study describes the cloning and functional characterization of a high-affinity Na\(^+\)/dicarboxylate cotransporter from mouse brain, mNaDC-3. Based on its functional properties, mNaDC-3 probably corresponds to the high-affinity Na\(^+\)/dicarboxylate cotransporter previously identified in brain synaptosomes and neurons. The mNaDC-3 transporter is a high-affinity Na\(^+\)-dependent transporter with a proposed coupling stoichiometry of three sodium ions for each substrate molecule, similar to the other members of the SLC13 family (11). A wide range of dicarboxylates, including succinate and \(\alpha\)-ketoglutarate, are substrates of mNaDC-3. However, despite a high sequence identity with other high-affinity Na\(^+\)/dicarboxylate cotransporters, mNaDC-3 exhibits differences in its electrical properties and interaction with lithium.

The primary function of mNaDC-3 in the brain is likely to be the uptake of neurotransmitter precursors into neurons. The intracellular pools of neurotransmitters in neurons, particularly glutamate, are maintained by the metabolism of dicarboxylates such as \(\alpha\)-ketoglutarate and malate (23). Neurons rely on the production and subsequent release of these neurotransmitter precursor molecules from astrocytes (24). The dicarboxylates released by astrocytes are then taken up across the plasma membrane of neurons by a Na\(^+\)-dependent transporter. Crude synaptosomal preparations from the brain and retina exhibit high-affinity Na\(^+\)/dicarboxylate cotransport of \(\alpha\)-ketoglutarate and malate, with estimated \(K_m\)s between 2 and 100 \(\mu\)M (6, 22). This transport activity has also been observed in glutamatergic neurons and astrocytes in primary culture (4, 16).

The message coding for mNaDC-3 was also found in choroid plexus. Na\(^+\)-dependent glutarate transport has been identified in brush-border membrane vesicles prepared from bovine choroid plexus (18). These vesicles also contain an organic anion transporter. Therefore, it is likely that the function of mNaDC-3 in choroid plexus is to participate in organic anion secretion by accumulating dicarboxylates, particularly \(\alpha\)-ketoglutarate, in the cells. The organic anion transporter then exchanges the intracellular dicarboxylates for organic anions from the cerebrospinal fluid (CSF). A similar function has been proposed for the high-affinity Na\(^+\)/dicarboxylate cotransporter in the basolateral membrane of renal proximal tubule cells (17).

The \(K_m\) for succinate in oocytes expressing mNaDC-3 was found to be 1.40 \(\mu\)M in radiotracer transport assays and between 16 (−50 mV) and 51 \(\mu\)M (+50 mV) in two-electrode voltage clamp measurements. The trans-
port of succinate in non-voltage-clamped oocytes results in the depolarization of the membrane potential, which could account for the larger $K_m$ in the radio-tracer transport assays. Other factors, such as trans effects of substrate and sodium that accumulate during the transport assay, could also affect the kinetic values. Previous kinetic studies with crude preparations of brain synaptosomes suggested that there were at least two transport pathways for $\alpha$-ketoglutarate or malate: a low-capacity high-affinity pathway with a $K_m$ between 3 and 20 $\mu$M and a high-capacity lower-affinity pathway with a $K_m$ of $\sim$100–200 $\mu$M (4, 6, 24). However, this conclusion was based on curve fits of Eadie-Hofstee plots, which can be somewhat misleading without additional evidence for multiple pathways. For example, the conditions used in those early vesicle studies probably did not represent true initial rates, because the time points were between 2 and 4 min and the experiments were done without voltage clamping. By comparison, typical kinetic experiments with renal brush-border membrane vesicles were done using 3-s uptakes in voltage-clamped vesicles (29). Therefore, the results of the early kinetic experiments do not necessarily rule out the possibility that there is only one high-affinity pathway for $\text{Na}^+/\text{dicarboxylate}$ transport in neuronal membranes.

The mouse brain NaDC-3 has a substrate specificity similar to that of the rat placental and renal NaDC-3. The preferred substrates are succinate, malate, $\alpha$-ketoglutarate, and fumarate (3, 5). The high-affinity transporters from mouse and rat differ from the human NaDC-3 in their handling of citrate. No citrate-dependent currents were detected in oocytes expressing mNaDC-3. A; inward currents ($I_{\text{succinate}}$) measured at a holding potential of $-50$ mV were plotted against succinate concentration (between 2 and 1,000 $\mu$M). The succinate-induced substrate concentration at half-maximal current ($K_{0.5}\text{succinate}$) is $15.5 \pm 2.7 \mu$M, and the succinate-induced maximal current at saturating substrate concentration ($I_{\text{max succinate}}$) is $-174.3 \pm 6.6$ nA (mean $\pm$ SE of regression). B: effect of membrane potential on the $K_{0.5}\text{succinate}$. C: effect of membrane potential on the $I_{\text{max succinate}}$. 

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Fig. 8. Steady-state succinate-dependent currents as a function of succinate concentration in an oocyte expressing mNaDC-3. A; inward currents ($I_{\text{succinate}}$) measured at a holding potential of $-50$ mV were plotted against succinate concentration (between 2 and 1,000 $\mu$M). The succinate-induced substrate concentration at half-maximal current ($K_{0.5}\text{succinate}$) is $15.5 \pm 2.7 \mu$M, and the succinate-induced maximal current at saturating substrate concentration ($I_{\text{max succinate}}$) is $-174.3 \pm 6.6$ nA (mean $\pm$ SE of regression). B: effect of membrane potential on the $K_{0.5}\text{succinate}$. C: effect of membrane potential on the $I_{\text{max succinate}}$.

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Fig. 9. Inward currents induced by 0.5 mM test substrates in oocytes expressing mNaDC-3. Data are presented as %current produced by succinate ($I_{\text{succinate}}$) in the same batch of oocytes. Data shown are mean $\pm$ SE; n = 3–4 frogs. Holding potential was $-50$ mV.
ing the human NaDC-3, whereas citrate-induced currents are ~30% of the succinate-induced currents in the mouse and rat NaDC-3 (3, 26). Interestingly, the flounder NaDC-3 also differs from the mammalian NaDC-3 transporters in substrate specificity, in that it is not inhibited by malate, one of the preferred substrates of mNaDC-3 (25). The high-affinity Na⁺/dicarboxylate cotransporters (NaDC-3) and low-affinity transporters (NaDC-1) exhibit differences in their relative affinity for substrate and in the range of preferred substrates. For example, α-ketoglutarate is an important physiological substrate of the NaDC-3 transporters, whereas the NaDC-1 transporters have low affinities for α-ketoglutarate or glutarate (13, 14). In contrast, the most important physiological substrate of the NaDC-1 transporters is probably citrate, which is not a preferred substrate of the NaDC-3 transporters.

The results of this study show that mNaDC-3 transports citrate but at a lower rate than substrates such as succinate and α-ketoglutarate. Previous reports suggesting that citrate transport was low or nonexistent in brain may have underestimated the transport activity by using suboptimal assay conditions, particularly in view of the high citrate concentrations (0.4 mM) in CSF (2). For example, one study with synaptosomes used citrate concentrations of 8 μM in the presence of 4 mM divalent cations (Ca²⁺ and Mg²⁺) (23). Because the preferred species of citrate for transport is uncomplexed, protonated citrate, the low substrate concentration and the presence of divalent cations would result in very little transportable substrate. However, there is indirect evidence in support of a citrate transport pathway in brain, since neurons and astrocytes exposed to 25 μM [¹⁴C]citrate in the medium produce ¹⁴CO₂ (28). In the kidney, citrate has an important role as a calcium chelator, which prevents the formation of kidney stones (15). A similar function for citrate as a divalent cation chelator in CSF has also been proposed. The concentrations of divalent cations such as calcium, magnesium, and zinc are important in regulating the activity of some receptors (27). Our studies show that mNaDC-3 may potentially transport citrate, but the physiological importance of this pathway in regulating the CSF concentrations of citrate is not known.

The preferred substrates of mNaDC-3 are likely to be divalent anions, similar to the other Na⁺/dicarboxylate cotransporters (13). The sodium activation curves of mNaDC-3 are sigmoidal with Hill coefficients of ~1.8, and inward currents were seen in the presence of sodium and substrate, both of which are consistent with a coupling stoichiometry of three Na⁺ for each divalent anion substrate. Because inward currents were also observed with citrate and the inhibition by citrate was greater at pH 5.5, it is likely that citrate is also transported as a divalent anion. The effect of pH on succinate transport in mNaDC-3 suggests that divalent succinate is preferred over protonated succinate. In contrast, succinate transport in the low-affinity NaDC-1 orthologs is not affected by pH; therefore, both monovalent and divalent succinate are potential substrates (9, 14). The effect of pH on citrate transport by Na⁺/dicarboxylate cotransporters is quite variable. In the NaDC-1 orthologs, citrate transport is highest at pH 5.5 and lowest at pH 8.5, similar to the concentration of protonated citrate in the medium. However, citrate transport in mNaDC-3 is relatively insensitive to pH values between 5.5 and 7.5 but shows inhibition at pH 8.5. The rat NaDC-3 expressed in oocytes shows a steady decrease in citrate transport as the pH is changed from 5.5 to 8.5 (3), but the same protein expressed in mammalian cells has a peak in citrate transport at pH 7 and low citrate transport at pH 5.5 and 8 (5). The variability between experiments indicates that some cell-specific factors may affect the transport of citrate. Overall, the results suggest that the high-affinity transporters are likely to prefer protonated citrate as a substrate, but the proteins also contain pH-sensitive residues that influence substrate binding or translocation.

The mouse NaDC-3 is a Na⁺-dependent transporter, but, similar to the other Na⁺/dicarboxylate cotransporters, its cation binding sites also interact with lithium (11). Lithium can substitute for sodium in mNaDC-3, although the transport rate in the presence of lithium is much lower than in sodium. In the low-affinity transporter NaDC-1, lithium binds with high affinity to one of the three cation binding sites, which results in transport inhibition (12). At higher concentrations, lithium can also substitute for sodium in NaDC-1, but the Kₘ for succinate in lithium is ~10-fold larger than in sodium (12). The other members of the SLC13 family exhibit different sensitivities to inhibition or substitution by lithium, probably related to the structure of the cation binding sites (11). Interestingly, although the two amino acid sequences are 97% identical, the mouse NaDC-3 is not sensitive to inhibition by lithium, whereas the rat NaDC-3 shows between 40 and 60% inhibition by concentrations of lithium as low as 2.5 mM (3, 5).

The electrical properties of mNaDC-3 were unusual compared with other Na⁺/dicarboxylate cotransporters of the SLC13 family (3, 12, 26, 30). The I-V plot in mNaDC-3 was curved upward at very negative potentials, suggesting that an outward current was activated or the inward current was decreased. These I-V curves were not seen in control, uninjected oocytes, or in other clones expressed in the same batches of oocytes as mNaDC-3. Therefore, either the currents are a property of mNaDC-3 itself or the expression of mNaDC-3 affects an endogenous current in the oocytes. The substrate-dependent currents measured in lithium in oocytes expressing mNaDC-3 were also unlike those seen in the other members of the family (12, 30). At potentials more negative than −50 mV, large outward currents were observed in the presence of lithium. At present, we have no explanation for the results. One possibility is that extracellular lithium is blocking inward currents of succinate and cations, which allows an outward current to be visible. In any case, because lithium is used for the treatment of...
bipolar disorder in human patients, effects of lithium on the transport of dicarboxylates in neurons may potentially affect intracellular pools of neurotransmitters.

In conclusion, we find that mNaDC-3 is a high-affinity \( \text{Na}^+ / \text{dicarboxylate} \) cotransporter from mouse brain. The mRNA for mNaDC-3 is found in brain, choroid plexus, liver, and kidney, similar to the tissue distribution of high-affinity dicarboxylate transport. The mouse brain NaDC-3 is an electrogenic sodium-coupled transporter with a broad substrate selectivity. It has unusual electrophysiological properties and interaction with lithium compared with other members of the same gene family. It is likely that mNaDC-3 corresponds to the \( \text{Na}^+ / \text{dicarboxylate} \) cotransporter previously identified in glumatergic synaptosomes.

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