Kinetic and Pharmacological Properties of Human Brain Na\(^+\)/H\(^+\) Exchanger Isoform 5 Stably Expressed in Chinese Hamster Ovary Cells*

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The recently cloned Na\(^+\)/H\(^+\) exchanger isoform 5 (NHE5) is expressed predominantly in brain, yet little is known about its functional properties. To facilitate its characterization, a full-length cDNA encoding human NHE5 was stably transfected into NHE-deficient Chinese hamster ovary AP-1 cells. Pharmacological analyses revealed that H\(^+\)-activated \(^{22}\)Na\(^+\) influx mediated by NHE5 was inhibited by several classes of drugs (amiloride compounds, 3-methylsulfonyl-4-piperidinobenzoyl guanidine methanesulfonate, cimetidine, and harmol) at half-maximal concentrations that were intermediate to those determined for the high affinity NHE1 and the low affinity NHE3 isoforms, but closer to the latter. Kinetic analyses showed that the extracellular Na\(^+\) dependence of NHE5 activity followed a simple hyperbolic relationship with an apparent affinity constant (KD) of 18.6 ± 1.6 mM. By contrast to other NHE isoforms, NHE5 also exhibited a first-order dependence on the intracellular H\(^+\) concentration, achieving half-maximal activation at pH 6.43 ± 0.08. Extracellular monovalent cations, such as H\(^+\) and Li\(^+\), but not K\(^+\), acted as effective competitive inhibitors of \(^{22}\)Na\(^+\) influx by NHE5. In addition, the transport activity of NHE5 was highly dependent on cellular ATP levels. Overall, these functional features distinguish NHE5 from other family members and closely resemble those of an amiloride-resistant NHE isoform identified in hippocampal neurons.

Transient oscillations in the extra- and intracellular pH (pHi and pHi, respectively) environments of neurons and other cell types of the nervous system can profoundly modulate neuronal membrane excitability by inhibiting various ligand receptor-mediated currents (1, 2), voltage-gated cation channels (3–6), and gap junction coupling (7), as well as by activating a depolarizing inward Na\(^+\) current (ASIC3) (8). Thus, precise control of the neuronal pH milieu is an important biological process and may fulfill a regulatory role in brain function (for review, see Refs. 9 and 10).

The ion transporters responsible for pH regulation in the nervous system are not as well characterized as in peripheral cell types, and this is particularly true for neurons, which are often difficult to isolate and/or maintain in culture in a differentiated state. Despite this limitation, accumulating evidence indicates that the acid-base transport systems in brain are heterogeneous but comparable to other organ systems. In cultured fetal or freshly isolated neonatal pyramidal CA1 neurons from rat hippocampus, restoration of steady-state pH following intracellular acidification involves two principal ion carriers: a Na\(^+\)/HCO\(_3\)/Cl\(^-\) exchanger and a novel amiloride-resistant Na\(^+\)/H\(^+\) exchanger (NHE) (11–13). Later in development, acutely dissociated hippocampal CA1 neurons from adult rats exhibit an acidifying mechanism mediated by a Na\(^+\)/HCO\(_3\)/Cl\(^-\) exchanger, most likely the AE3 isoform (14). Na\(^+\)/H\(^+\) and Na\(^+\)-dependent Cl\(^-\)/HCO\(_3\) exchangers also make distinct contributions to pH regulation in neurons of the medulla oblongata (15), superior cervical ganglion sympathetic neurons (16), cerebellar Purkinje cells (17), as well as brain synaptosomes (18). By contrast, pH regulation in primary cultures of rat astrocytes is more intricate and involves the three major acid-base transport systems mentioned above (19, 20), as well as a fourth pH-regulating mechanism, Na\(^+\)/HCO\(_3\) cotransport (20). Moreover, rat astrocytes express multiple NHE isoforms (22), which further adds to their pH-regulatory complexity.

More detailed examination of NHE mRNA expression in rat brain revealed that NHE1 is the most abundant and widely dispersed isoform, whereas other family members (i.e. NHE2–4) show a more restricted distribution (23). The importance of NHE1 in neuronal function is demonstrated convincingly by spontaneous (24) or targeted (25) null mutations in mice, which develop ataxia and epileptic-like seizures by 2 weeks of age and show significant mortality (67%) prior to weaning. These changes are associated with selective loss of neurons in the cerebellum and brainstem (24). By contrast, mice with targeted disruptions of the Nhe2 and Nhe3 loci do not display obvious neurological symptoms (26, 27); hence their particular roles in nervous system function are less apparent.

In addition to NHE1–4, recent molecular cloning and tissue distribution studies in human (28) and rat (29) have identified a fifth NHE isoform that is distinguished by its predominant expression in discrete regions of the brain, including dentate gyrus, cerebral cortex, and hippocampus. Moreover, it shares high sequence similarity to the amiloride-resistant NHE3 isoform which, in brain, is detected only in cerebellar Purkinje cells (23). Based on these observations, it is reasonable to postulate that NHE5, rather than NHE3, is a likely candidate...

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†† The abbreviations used are: NHE, Na\(^+\)/H\(^+\) exchanger; EIPA, 5-(N-ethyl-N-isopropyl)amiloride; HMA, 5-(N,N-dimethylaminoethyl)-amiloride; MES, 2-(N-morpholinoethanesulfonic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; HOE694, (3-methanesulfonyl-4-piperidinobenzoxy)guanidine methanesulfonate; DIDS, 4,4′-diisothioctyanostibene-2,2′-disulfonic acid.
for the amiloride-resistant form of the NHE reported in hippocampal neurons (13) and possibly other cell types of the nervous system (19). However, its intrinsic biochemical and pharmacological properties have yet to be defined in detail. To facilitate its characterization without the complicating presence of other NHE isoforms, we stably expressed the human NHE5 cDNA in mutagenized Chinese hamster ovary cells (AP-1) devoid of endogenous NHE activity. The results reveal that the functional properties of NHE5 closely resemble those of an amiloride-resistant NHE isoform identified in hippocampal neurons.

**EXPERIMENTAL PROCEDURES**

**Materials**—Carrier-free $^{22}$NaCl (range of specific activity, 900–950 mCi/mg) was obtained from NEN Life Science Products. Amiloride, 5-(N-ethyl-N-isopropyl)amiloride (EIPA), 5-(N,N-hexamethylen)-amiloride (HMA), cimetidine, clonidine, harmaline, ouabain, bumetanide, antymycin A, 2-deoxy-o-glucose, and glucose were obtained from Sigma. HOE694 and HOE642 were kindly provided by Drs. Hans-J. Lang (Hoechst Marion Roussel, AG). Nigericin was purchased from Molecular Probes Inc. (Eugene, OR). A-Minimal essential fetal bovine serum, penicillin/streptomycin, and tryptic EDTA were from Life Technologies, Inc. All other chemicals and reagents used in these experiments were from British Drug House Inc. (St. Laurent, Quebec) or Fisher Scientific and were of the highest grade available.

**Construction and Stable Expression of Human NHE5**—A full-length cDNA encoding human NHE5 was used in this study as described previously (28), except that it did not contain a COOH-terminal influenza virus hemagglutinin epitope tag in order to preserve the native structure of the protein. The human NHE5 cDNA was inserted into a mammalian expression vector under the control of the enhancer/promoter region from the immediate early gene of human cytomegalovirus as described previously (28) and called pNHE5. The pNHE5 plasmid was stably transfected into NHE-deficient Chinese hamster ovary AP-1 cells (30) using the calcium phosphate-DNA coprecipitation method (31) and acid selection (32, 33). Several clonal cell lines were screened for their level of NHE5 expression by measuring H$^+\,$-activated $^{22}$Na$^+$ influx for these studies, the clonal cell line AP-1NHE5/C6, which had the highest level of NHE5 activity, was used between passages 2 and 10. The cells were grown to confluence in a-a-minimal essential medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, and 25 mM NaHCO$_3$, pH 7.4, and incubated in a humidified atmosphere of 95% air and 5% CO$_2$ at 37°C.

**$^{22}$Na$^+$ Influx Measurements**—The cells were grown to confluence in 24-well plates. Prior to $^{22}$Na$^+$ influx measurements, the cells were acidified intracellularly using the NH$_4$Cl prepulse technique as described previously (33). The assays were initiated by incubating the cell monolayers in isotonic choline chloride solution (125 mM choline chloride, 1 mM MgCl$_2$, 2 mM CaCl$_2$, 5 mM glucose, 20 mM HEPES-Tris, pH 7.4) containing 1 mM ouabain and carrier-free $^{22}$Na$^+$ (1 μCi/ml) in the absence or presence of the NHE-specific inhibitor EIPA (0.1 mM). The Na$^+$ pump was rapidly washed with 4 volumes of ice-cold NaCl solution, and the initial rates of $^{22}$Na$^+$ influx were measured as a function of the Na$^+$ concentration, the pH, and the presence of ouabain minimized transport of Na$^+$ in the absence of inhibitor. Control cells were incubated in this same solution devoid of ouabain and antymycin A and instead contained 5 mM glucose. Data Analysis—All experiments represent the average of two to four experiments, each performed in quadruplicate. The data are presented as the mean ± S.D.

**RESULTS**

**Pharmacological Properties of Human NHE5**—The NHE isoforms characterized to date (i.e. NHE1–4) display a wide range of sensitivities to different classes of pharmacological inhibitors, including amiloride and its analogs (e.g. EIPA and HMA), benzoyl guanidine compounds (e.g. HOE694 and HOE642 (carrtide)), cimetidine, clonidine, and harmaline (33, 36–40). Many of these compounds have been shown to mediate their effects by competing with extracellular Na$^+$ at the same, or a closely associated, binding site(s) (for review, see Ref. 41). To define the sensitivity of human NHE5 to these antagonists, the rate of H$^+$-activated $^{22}$Na$^+$ influx was measured as a function of the drug concentration in a clonal isolate of NHE5-transfected AP-1 cells (i.e. AP-1NHE5/C6). As illustrated in Fig. 1 and summarized in Table I, the order of potency of these compounds was HMA > EIPA > HOE694 > amiloride > cimetidine > harmaline with apparent half-maximal inhibitory ($K_{i, 50}$) values of (in μM) 0.37, 0.42, 9.1, 21, 230, and 940, respectively. These values are intermediate between those of NHE1 and NHE3 but closer to the latter. By contrast, clonidine had little, if any, effect on NHE5 activity even though it is an effective inhibitor of other NHE isoforms (33, 40).

**Kinetic Properties of Human NHE5**—To determine the extracellular Na$^+$ ($Na^{+}$,o) affinity of NHE5, initial rates of H$^+$-activated $^{22}$Na$^+$ influx were measured as a function of the Na$^+$ concentration. The rate of $^{22}$Na$^+$ influx was saturable and conformed to simple Michaelis-Menten kinetics (Fig. 2A). Transformation of the data according to the Eadie-Hofstee algorithm (V versus V/S) yielded a linear relationship (Fig. 2B), consistent with the presence of a single Na$^+$ binding site at
translocation are usually slower than that for Na. H1, the apparent affinity constant (pKi) for Na.[22Na] influx at pH 6.0. B, the apparent affinity constant (pKi) for H+, was calculated from the linear transformation of the data according to the algorithm of Eadie-Hofstee. Values represent the average of three experiments, each performed in quadruplicate.

External protons decreased H+,-activated [22Na] influx into NHE5-transfected cells in a concentration-dependent manner (Fig. 4A), with apparent half-maximal activity reached at pHo 8.13 ± 0.15. This reduction is associated with the gradual decrease in the transmembrane H+ gradient (ΔpH) but could also partly reflect H+ competition for the Na+ binding site. To test this latter possibility, the Na+ concentration was adjusted to 1 and 10 mM, and the initial rates of influx of [22Na] were measured as a function of the H+ concentration. Transformation of the data using the Dixon algorithm (IV versus [H+]o) resulted in straight lines (Fig. 4B), with the slope of the line decreasing in the presence of higher Na+ levels. Determination of the intercept of the lines yielded an inhibitory constant (K) for H+ of approximately 17 μM. These data indicate that the effectiveness of H+ to reduce the influx of [22Na] by NHE5 is influenced by the Na+ concentration, which is consistent with the notion that H+ effectively competes with Na+ at a single site.

Similarly, Li+ was also a potent and competitive inhibitor of H+,-activated [22Na] influx by NHE5 (Fig. 5). The apparent half-maximal inhibition was achieved at a concentration of 318 ± 47 μM (Fig. 5A), which is an order of magnitude lower than that reported for other isoforms (33, 36). To characterize the nature of this inhibition in greater detail, the initial rates of 1 mM and 10 mM [22Na] influx were measured as a function of the Li+ concentration. Analysis of the data by Dixon plot (Fig. 5B) yielded straight lines, with the slope of the line decreasing in the presence of higher Na+ levels. This suggested that Li+ also interacted in a competitive manner with an apparent K ~ 0.63 mM. In contrast to H+ and Li+, increasing concentrations of K+ (1–100 mM) had only a minor inhibitory effect on the initial [22Na] transport rates of NHE5 (Fig. 6). A
transport as a function of Li$^+$ versus were plotted according to the Dixon algorithm (1/V versus were plotted according to the Dixon algorithm (1/V rates of transport as a function of pH 1

Values represent the average of at least two experiments, each performed in quadruplicate.

Summary of these data is presented in Table II.

Influence of extracellular K$^+$ on EIPA-inhibitable 22Na$^+$ influx in AP-1 cells expressing human NHE5. AP-1NHE5/C6 cells were preloaded with H$^+$ using the NH$_4$Cl prepulse technique. Initial rates of EIPA-inhibitable 22Na$^+$ influx were measured as a function of extracellular Na$^+$ (pH 6.0–9.5). A, the 22Na$^+$ influx medium containing carrier-free 22NaCl (1 μCi/ml) was buffered with 30 mM MES-Tris (pH 6.0–6.5), 30 mM MOPS-Tris (pH 7.0), 30 mM HEPES-Tris (pH 7.5–9.5). Data were normalized as a percentage of the maximal rate of EIPA-inhibitable 22Na$^+$ influx at pH 9.5. B, the initial rates of transport as a function of pH were measured in the presence of different extracellular Na$^+$ concentrations (1 and 10 mM), and the data were plotted according to the Dixon algorithm (1/V versus [H$^+$]). Values represent the average of at least two experiments, each performed in quadruplicate.

ATP Dependence—The transport activity of plasma membrane NHEs is driven by the relative concentration gradients of the respective cations and is not dependent on direct hydrolysis of ATP per se. Nevertheless, cellular depletion of this nucleotide has been found to reduce the activities of known NHEs dramatically (35, 45) by a mechanism that is not well understood. Thus, it was of interest to determine whether NHE5 was similarly sensitive to ATP. Under the conditions used (10-min incubation with deoxyglucose and antimycin A to block glycolysis and oxidative phosphorylation, respectively), ATP is depleted rapidly by >90% without compromising the intactness of the plasma membrane or adherence of the cells to the culture dishes (data not shown). As shown in Fig. 7, NHE5 activity was completely abolished over the pH range studied (pH 5.8 and 7.4).

**TABLE II**

| Cation | Apparent affinity constants ($K_{0.5}$) |
|-------|--------------------------------------|
|       | NHE1$^a$ | NHE3$^a$ | NHE5 |
| Na$^+$ (mM) | 10.0 ± 1.4 | 4.7 ± 0.6 | 18.6 ± 1.6 |
| H$^+$ (pK) | 6.75 ± 0.05 | 6.45 ± 0.08 | 6.43 ± 0.08 |
| H$_2$O (pK) | 7.0 ± 0.1 | 7.0 ± 0.1 | 8.13 ± 0.15 |
| Li$^+$ (mM) | 3.4 ± 0.3 | 2.5 ± 0.6 | 0.39 ± 0.04 |
| K$^+$ (mM) | 19.5 ± 1.3 | None | Slight inhibition |

* $K_{0.5}$ values for NHE1 and NHE3 were obtained from Ref. 33. Values are presented as the mean ± S.D.

**DISCUSSION**

Recent studies by us (28) and Melvin and colleagues (29) demonstrated that heterologous expression of the cloned brain cDNAs for human and rat NHE5, respectively, was capable of mediating the exchange of extracellular Na$^+$ for intracellular H$^+$, activity that could be blocked by high concentrations of amiloride or its analog EIPA. These data established that NHE5 functions as a plasma membrane NHE. The present study extends these initial observations by defining the principal biochemical and pharmacological properties of NHE5 in greater detail, thereby providing a functional basis for its identification in native tissues and cell types.

The pharmacological data show that NHE5 has an intermediate affinity for most classes of NHE-inhibitory drugs (amiloride-based compounds, HOE694, cimetidine, and harmaline) when compared with other NHE isoforms under comparable experimental conditions, i.e. NHE1 > NHE2 > NHE5 > NHE3. By contrast, NHE5 activity is completely insensitive to clonidine within the concentration range tested, whereas other isoforms including NHE3 are effectively inhibited in the submillimolar range (33, 36). NHE4 also has an apparent low affinity for many of these antagonists, but its activity in transfected fibroblasts can only be detected under specialized conditions (i.e. prolonged intracellular acidification and exposure to hyperosmolar medium (40) or treatment with DIDS (46)). Hence, it is unclear whether these characteristics reflect the actual properties of NHE4 in native tissues, although recent indirect evidence indicates that this may be the case in rat renal cortical tubules (47). Overall, the drug profiles of NHE5 most closely resemble those of NHE3. This result agrees with earlier predictions based on their high amino acid similarity (67% identity) in the NH$_2$-terminal membrane spanning domain, a region found previously to confer drug sensitivity (37, 48).

The low sensitivity of NHE5 to inhibition by several drugs and its prominent expression in discrete regions of the brain, including the hippocampus, are consistent with our initial postulate that NHE5 is a likely candidate for the amiloride-resistant form of the NHE reported in hippocampal neurons by Raley-Susman et al. (13). However, in the latter study, NHE
activity was unaffected by 1 mM amiloride and 50 μM HMA, but inhibited by 100 μM harmaline. Conversely, NHE5 expressed in AP-1 cells is fully inhibited by amiloride and HMA but is relatively unaffected by harmaline, at the same concentrations. These apparent discrepancies in drug sensitivities are most likely caused by the different conditions used to measure transport activity in the two studies. In hippocampal neurons, NHE activity was measured as the rate of pH recovery (using the pH-sensitive fluorescent dye BCECF) after intracellular acidification in the presence of a saturating concentration (135 mM) of Na\(^+\), which competes with amiloride and its analogs for binding. By contrast, in our study, NHE5 activity was measured as acid-activated \(^{22}\text{Na}^+\) influx using trace levels of Na\(^+\), thereby allowing the drugs to bind with higher affinity and closer to their true K\(_i\). With respect to harmaline, this compound has been found by others to interfere greatly with the pH-sensitive exchange ionophore nigericin (10 μM). The osmolarity of the individual solutions was adjusted by N-methyl-D-glucammonium chloride. Because at equilibrium [K\(^+\)]/ [K\(_i\)] = [H\(^+\)]/[H\(^+\)] under these conditions the pH is determined by the imposed [K\(^+\)] gradient, and the extracellular pH (pH\(_o\) = 7.4), and can be calculated assuming K\(^+\) = 140 mM. \(^{22}\text{Na}^+\) influx measurements were initiated in the same K\(^+\)-nigericin solutions supplemented with \(^{22}\text{Na}^+\) (1 μCi/ml) and 1 mM ouabain in the absence or presence of 0.1 mM EIPA. \(^{22}\text{Na}^+\) influx was linear with time for at least 10 min under these experimental conditions. Data were normalized as a percentage of EIPA-inhibitable \(^{22}\text{Na}^+\) influx at pH 5.8. Values represent the average of two experiments, each performed in quadruplicate.

Conversely, K\(^+\) at high concentrations (i.e. 100 mM) acted as a modest competitive inhibitor of Na\(^+\) transport by NHE1 (33). Although not demonstrated in this study, Li\(^+\) and NH\(_4^+\) can also be translocated across the membrane in exchange for Na\(^+\) or H\(^+\) but usually at a slower rate (41). On the other hand, external K\(^+\) does not appear to be transported by most NHEs (33, 41); however, Chambrey and colleagues (46) have recently reported that mouse LAP1 fibroblast cells stably expressing rat NHE4 are capable of mediating K\(^+\)-dependent pH recovery upon treatment with DIDS, an effect that was not observed in untransfected cells. Again, whether this is an intrinsic property of NHE4 in vivo is uncertain.

The cation dependence of NHE5 activity was also assessed. The steady-state velocities of most NHE isoforms (i.e. NHE1, NHE2, and NHE3) show a saturating, first-order dependence on the Na\(^+\) concentration, indicative of a single binding site (33, 36). Similarly, the Na\(^+\)-dependent velocity of NHE5 follows a rectangular hyperbola, consistent with simple, saturating Michaelis-Menten kinetics. The value for half-maximal velocity (K\(_{Na} = 18.6 \text{ mM}\)) was within the range of K\(_{Na}\) values (3–50 mM) reported for other NHEs in different cell types and vesicle preparations (33, 36, 41, 45). It is noteworthy that this value is close to that reported for the amiloride-resistant NHE present in rat hippocampal neurons (i.e. ~23 mM) (13). An exception to this pattern is NHE4, which manifests either a sigmoidal (40) or hyperbolic (46) dependence on the Na\(^+\) concentration depending on whether it is expressed in hypertonic- or hypotonic-exposed PS120 fibroblasts or DIDS-treated LAP1 cells, respectively. The underlying basis for this kinetic difference is unknown.

The steady-state velocity of NHE5 also shows an apparent first-order dependence on the intracellular proton concentration, as was reported for the amiloride-resistant NHE in hippocampal neurons (13). However, this characteristic is in marked contrast to that described for the majority of other plasma membrane NHEs that exhibit a greater than first-order dependence on the H\(^+\) concentration (18, 41, 42, 50). This is suggestive of a second class of H\(^+\)-binding site, in addition to the transport site, with positive cooperative binding characteristics. This property was first described for the renal apical membrane NHE (i.e. NHE3) by Aronson and colleagues (41, 42), who proposed that this apparent allosteric H\(^+\) activation could be explained most simply by assuming the presence of one or more ionizable groups that, upon protonation, alter the conformation of the protein and enhance the rate of cation transport. However, in renal mesangial cells, the biphasic H\(^+\) -dependence of NHE activity was not strictly a function of pH\(_o\), as it could be linearized within the physiological pH range upon hormonal stimulation (51). These observations suggest that the allosteric regulation by H\(^+\) may not necessarily reflect protonation of certain ionizable residues of the transporter but instead may result from effects on cell-specific regulatory factors that modulate NHE activity in a pH- and/or hormone-sensitive manner. Nevertheless, the roles of direct protonation of NHE and of an ancillary regulator are not mutually exclusive and may in fact be complementary.

In intact cells, plasma membrane NHEs require physiological levels (i.e. millimolar) of ATP for optimal function. Acute cellular depletion of this nucleotide drastically inhibits NHE activity in native (52–54) and NHE-transfected (35, 45, 55) cells. Most notably, the activity of NHE3 is almost completely suppressed upon ATP depletion, even in the presence of a large transmembrane H\(^+\) gradient, whereas the activities of NHE1 and NHE2 are only partially reduced (35). With respect to the latter isoforms, kinetic analyses indicate that this inhibition is mainly accounted for by reductions in their affinities for H\(^+\)
However, the more drastic reduction of NHE3 activity, as well as that of NHE5, suggests a more complex mechanism, possibly reflecting alterations in both pH sensitivity and maximum velocity.

The molecular mechanisms underlying ATP regulation of the NHE isoforms remain obscure. Earlier studies of NHE1 showed that its state of phosphorylation is unaltered during acute ATP depletion (56). Hence, changes in direct phosphorylation of the exchange are unlikely to account for the effects of ATP. Functional analyses of COOH-terminal truncation mutants of NHE1 indicated that the region encompassing amino acids 516–562 is sufficient to confer sensitivity to ATP (57). More recent investigations have suggested that the ATP dependence of NHE1 involves two distinct mechanisms: one that requires hydrolysis of ATP and likely involves an energy-dependent event, and a second process that does not require the hydrolysis of the γ-phosphate of ATP but may involve its binding to an as yet unidentified ancillary factor that activates the exchanger (58, 59). Whether these mechanisms also apply to other isoforms, including NHE5, is unknown.

In summary, we have demonstrated that NHE5 has pharmacological and biochemical properties that readily distinguish it from other NHE isoforms characterized to date. Moreover, its features resemble those of an amiloride-resistant NHE isoform identified in cultured hippocampal neurons. The regulatory properties of NHE5 and its physiological role(s) in neuronal cell function remain to be elucidated.

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