Regulation of dendritic spine growth through activity-dependent recruitment of the brain-enriched Na+/H...
Regulation of dendritic spine growth through activity-dependent recruitment of the brain-enriched Na\(^+\)/H\(^+\) exchanger NHE5

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ABSTRACT Subtle changes in cellular and extracellular pH within the physiological range have profound impacts on synaptic activities. However, the molecular mechanisms underlying local pH regulation at synapses and their influence on synaptic structures have not been elucidated. Dendritic spines undergo dynamic structural changes in response to neuronal activation, which contributes to induction and long-term maintenance of synaptic plasticity. Although previous studies have indicated the importance of cytoskeletal rearrangement, vesicular trafficking, cell signaling, and adhesion in this process, much less is known about the involvement of ion transporters. In this study we demonstrate that N-methyl-d-aspartate (NMDA) receptor activation causes recruitment of the brain-enriched Na\(^+\)/H\(^+\) exchanger NHE5 from endosomes to the plasma membrane. Concomitantly, real-time imaging of green fluorescent protein–tagged NHE5 revealed that NMDA receptor activation triggers redistribution of NHE5 to the spine head. We further show that neuronal activation causes alkalination of dendritic spines following the initial acidification, and suppression of NHE5 significantly retards the activity-induced alkalization. Perturbation of NHE5 function induces spontaneous spine growth, which is reversed by inhibition of NMDA receptors. In contrast, overexpression of NHE5 inhibits spine growth in response to neuronal activity. We propose that NHE5 constrains activity-dependent dendritic spine growth via a novel, pH-based negative-feedback mechanism.

INTRODUCTION

Many components of the synaptic machinery, such as voltage-gated calcium channels (VGCCs) and N-methyl-d-aspartate (NMDA) receptors, are known to be sensitive to changes in pH within the physiological range (Traynelis and Cull-Candy, 1990; Tang et al., 1990; Klockner and Isenberg, 1994; Chen et al., 1996; Tombaugh and Somjen, 1996; Chesler, 2003; Banke et al., 2005). In addition, electrical activity in the CNS has been shown to elicit rapid pH changes in both intracellular and extracellular compartments. These localized acid–base transients in turn influence neural activity by a feedback onto pH-sensitive synaptic components (DeVries, 2001; Fedirko et al., 2007; Makani and Chesler, 2007). Significant drops in extracellular pH observed during stroke and the following seizure activity onto pH-sensitive synaptic components (DeVries, 2001; Fedirko et al., 2007; Makani and Chesler, 2007). Significant drops in extracellular pH observed during stroke and the following seizure activity have been shown to strongly inhibit synaptic transmission (Somjen, 1984; Siesjo et al., 1985; Li and Siesjo, 1997; Chesler, 2003). However, the roles of subtler, localized pH changes that occur normally in the brain have not been fully investigated. Moreover, the specific molecules responsible for regulating synaptic pH have not been identified.

Excitatory synapses undergo long-lasting changes in synaptic strength such as long-term potentiation (LTP) and long-term depression (Malenka and Bear, 2004; Kessels and Malinow, 2009). Most forms of LTP are initiated by activation of glutamatergic receptors, which elicits a transient acidification of the spine head caused by the uptake of H\(^+\) ions via NHE5, a plasma membrane exchanger. This transient acidification is efficiently counteracted by the recycling of NHE5 to the spine head. The specific functions of NHE5 in synaptic plasticity have not been fully explored.
synaptic NMDA receptors (Malenka and Bear, 2004), followed by an increase in synaptic strength due to recruitment of postsynaptic α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptors (Shepherd and Huganir, 2007; Kessels and Malinow, 2009) and an increase in the number and size of dendritic spines, small protrusions of the dendrites on which excitatory postsynaptic compartments are localized (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004; Lang et al., 2004). Due to the pH-sensitive nature of the NMDA receptor, local pH changes may influence the induction and maintenance of LTP (Velisek, 1998; Ronicek et al., 2009).

Several studies have suggested that Na+/H+ exchange is the predominant pH-regulatory mode in isolated nerve terminals (Sauvaigo et al., 1984; Nachsen and Drapeau, 1988). Na+/H+ exchange has also been implicated in the regulation of synaptic transmission at glutamatergic, GABAergic, and dopaminergic synapses (Trudeau et al., 1999; Jang et al., 2006; Rocha et al., 2008; Dietrich and Morad, 2010). Mammals have nine isoforms of Na+/H+ exchanger (NH1–9), of which NHE1 and NHE5 are highly expressed in the brain (Brett et al., 2005). NHE5 mRNA is expressed almost exclusively in the brain, showing the most-restricted distribution of any NHE isoform (Attaphitaya et al., 1999; Baird et al., 1999). The restricted expression of NHE5 suggests that it may have a unique function in the brain. However, the physiological role of NHE5 and the tissue and subcellular distribution of NHE5 protein remain unknown.

We demonstrate that enhanced neuronal activity increases the localization of NHE5 to dendritic spines and increases spine pH. Overexpression of NHE5 blocks activity-induced spine outgrowth. Conversely, knockdown of NHE5, or expression of dominant-negative NHE5 mutant, results in exuberant spine outgrowth. We propose that NHE5 acts as a negative regulator of activity-dependent spine growth by regulating pH-sensitive synaptic proteins.

**RESULTS**

**NHE5 is expressed in hippocampal neurons**

Previous studies from other laboratories have shown robust NHE5 mRNA expression in neuron-rich structures such as the hippocampus and cerebral cortex (Attaphitaya et al., 1999; Baird et al., 1999). However, it is not known whether NHE5 protein is present in brain for potential function. To address this question, we generated a polyclonal antibody against a peptide corresponding to the first extracellular loop of rat NHE5. The specificity of this antibody was verified by Western blot and immunofluorescence microscopy (Supplemental Figure S1). Immunolabeling of mouse coronal brain sections revealed robust NHE5 protein expression in CA1–CA3 hippocampal pyramidal neurons (Figure 1, A, A’, and B), as well as within the stratum radiatum layer where CA3 neurons synapse onto CA1 pyramidal cells (Figure 1, A and A’). This result shows for the first time that NHE5 protein is expressed in neurons.

To examine the subcellular distribution of NHE5 in hippocampal neurons, primary hippocampal cultures were labeled with the anti-NHE5 antibody. NHE5 displayed a punctate staining pattern and was expressed in neurites positive for either microtubule-associated protein 2 (MAP2) or tau protein, suggesting that NHE5 is present in both dendrites and axons (unpublished data). NHE5 puncta often overlapped with the excitatory synapse markers vesicular glutamate transporter 1 (VGluT1) and postsynaptic density protein of 95 kDa (PSD95) (Figure 1C), and occasionally with the inhibitory synapse markers vesicular γ-amino butyric acid (GABA) transporter (VGAT) and gephyrin (Figure 1D). Threshold analysis of triple colocalization between NHE5 and presynaptic and postsynaptic markers revealed that 39.8% of excitatory synapses and 23.8% of inhibitory synapses were positive for NHE5. Although NHE5 is not a global component of synapses, these results suggest that at steady state, NHE5 is targeted to a subset of synapses.

**NHE5 is recruited to the cell surface by synaptic activity**

Ronicek and colleagues previously suggested a role of pH-regulatory proteins at excitatory synapses and LTP (Ronicek et al., 2009). This finding led us to postulate that NHE5 might regulate excitatory synapse function, and therefore we primarily focus our attention on excitatory neurons in this study. In response to synaptic activity, synapses undergo extensive remodeling of membrane composition, which requires delivery of some membrane proteins from recycling endosomes in a Rab11-dependent pathway (Park et al., 2004, 2006; Wang et al., 2008). Given that NHE5 traffics between recycling endosomes and the plasma membrane via the Rab11 pathway (Diering et al., 2009), we speculated that NHE5 localization to synapses and the plasma membrane might be regulated by synaptic activity. To test this possibility, we manipulated neuronal activity in culture by treating neurons at 12 d in vitro (12DIV) for 48 h with bicuculline (20 μM), which enhances neuronal activity by blocking inhibitory GABA~A receptors (Curtis et al., 1971), or tetrodotoxin (TTX, 1 μM), which suppresses neuronal activity by inhibiting voltage-gated Na~ channels (Narahashi et al., 1964). At 14DIV surface NHE5 levels were quantified following bicuculline or TTX treatment using a cell-surface biotinylation assay. Compared to untreated control neurons, surface NHE5 levels were significantly increased or decreased in bicuculline- or TTX-treated neurons, respectively (Figure 2, A and B). Alternatively, we enhanced synaptic activity in a more acute manner by using a previously described cocktail of glycine, bicuculline, and TTX (Lu et al., 2001). This paradigm has been used in hippocampal slices to induce chemical LTP (Shahi et al., 1993; Musleh et al., 1997), and glycine is used routinely to acutely enhance activity in neuronal cultures through activation of NMDA receptors (Lu et al., 2001; Park et al., 2006). Compared to untreated cultures (14DIV), there was a marked increase in surface NHE5 in neurons treated with glycine (200 μM for 3 min). This increase was completely prevented by the NMDA-receptor inhibitor D-AP5 (100 μM) (Collingridge et al., 1983; Figure 2, C and D). Recruitment of NHE5 to the cell surface occurred in a time-dependent manner beginning within 5 min following glycine treatment (Figure 2, E and F). Notably, this increase in recruitment of NHE5 to the cell surface following glycine treatment was specific, as no change in surface or total expression of NHE1 was observed following bicuculline, TTX, or glycine treatment (Figure 2, A–F). These results suggest that surface NHE5 abundance in neurons is bidirectionally regulated by neuronal activity in both a chronic and an acute manner.

**NHE5 is delivered to synapses and dendritic spines following synaptic activity**

Next, we used immunofluorescence microscopy to determine whether NHE5 targeting to synapses was also activity dependent. 14DIV hippocampal neurons were treated with 200 μM glycine or control solution for 3 min to enhance activity. At 45 min after glycine stimulation, neurons were fixed and NHE5 localization at excitory synapses was assessed. We noted a significant increase in the number of excitatory synapses positive for NHE5 following glycine treatment (45.1 ± 4.7% control, 73.3 ± 2.2% glycine treated, p < 0.01, Student’s t test; Supplemental Figure S2).

To determine whether the NHE5 recruited to synapses was inserted into the plasma membrane, we took advantage of the fact that the anti-NHE5 antibody raised against the first extracellular loop of
NHE5 could specifically recognize surface NHE5 in fixed neurons by immunofluorescence microscopy under nonpermeabilized conditions. We found that very little NHE5 is present at the cell surface at steady state, and that surface NHE5 was almost exclusively localized to synapses, as shown by colocalization of surface NHE5 with PSD95 and VGlut1 (Figure 3A). Following glycine treatment, we noted a dramatic increase in the number and intensity of NHE5 puncta at the cell surface (Figure 3B). Similar to that observed at steady state, surface NHE5 was almost exclusively localized to synapses (Figure 3, A and B). Quantitative colocalization analysis revealed a significant increase in the number of synapses positive for surface NHE5 following glycine treatment compared with untreated cells (Figure 3C).

FIGURE 1: NHE5 is expressed in hippocampal neurons. (A, B) Confocal images of adult mouse brain coronal sections demonstrate NHE5 immunolabeling in CA1 pyramidal neurons of the hippocampus (A') and stratum radiatum (A'') and hippocampus CA3 (B). Scale bar, 100 μm; inset 10 μm. (C, D) Confocal images of cultured rat hippocampal neurons immunolabeled for NHE5 and the excitatory presynaptic and postsynaptic markers VGlut1 and PSD95, respectively (C), or the inhibitory presynaptic and postsynaptic markers VGAT and gephyrin (D). Scale bar, 20 μm; inset, 5 μm. Arrows indicate regions of triple colocalization between NHE5 and synapse markers suggesting synaptic localization of NHE5.
NHE5 is a negative regulator of dendritic spine growth

Dendritic spines undergo extensive remodeling in response to LTP-inducing stimuli (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004; Lang et al., 2004). Given that NHE5 is recruited to dendritic spines on glycine stimulation, we hypothesized that NHE5 might regulate activity-induced dendritic spine growth. To address this, we first used a short-hairpin RNA (shRNA) approach to knock down NHE5. Three independent shRNA constructs (shA, B, and C) were used to deplete NHE5 levels and resulted in up to 80% knockdown of endogenous NHE5 (Supplemental Figure S4). NHE5 shRNA–expressing neurons exhibited a significant increase in the density of dendritic spines and a small but significant increase in average spine head width (Figure 4, A and B). Expression of shRNA-resistant human NHE5 rescued these effects, suggesting the spine phenotypes observed in these NHE5 shRNA–expressing cells are not due to off-target effects. To further define the role of NHE5 in the dendritic spine phenotype, we explored the use of a dominant-negative mutant. Mutation of a highly conserved glutamate in human NHE1 (E266) was shown to abolish its ion transport activity, and heterologous expression of this mutant caused dominant-negative effects (Hisamitsu et al., 2006; Busco et al., 2010). Mutation of the corresponding highly conserved glutamate 209 in human NHE5 to isoleucine completely abolished the ion transport activity of this protein without affecting protein stability or trafficking, and this mutant exerted a dominant-negative effect on NHE5 transporter activity (Supplemental Figure S5). Heterologous expression of the dominant-negative NHE5 significantly enhanced dendritic spine density and head width. Moreover, the expression of neither mutant nor wild-type NHE1 rescued the spine growth effects following NHE5 knockdown by shRNA (Figure 4, A and B). Together these findings suggest that NHE5 is a negative regulator of dendritic spine growth and that this requires NHE5-specific transporter activity.

NMDA receptor activation is an important step leading to changes in dendritic spine morphology and number (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Matsuzaki et al., 2004; Lang et al., 2004; Malenka and Bear, 2004). Because NMDA receptors are inhibited by an increase in extracellular protons (Tang et al., 1990; Traynelis and Cull-Candy, 1990; Banke et al., 2005), we postulated that NHE5 recruited to the spine surface during activity may act as a local proton source to regulate NMDA receptor activity via local Na+/H+ exchange. To test this possibility, we treated transfected neurons with or without the NMDA-receptor antagonist D-AP5 (100 μM) for 48 h. Of interest, D-AP5 treatment reversed the dendritic spine phenotypes caused by shRNA or dominant-negative NHE5 (Figure 5, A and B), indicating that NHE5 typically limits exuberant spine growth by constraining NMDA-receptor activity. NMDA-receptor activity is typically low at rest due to the Mg2+ ion block (Nowak et al., 1984). However, recent studies have demonstrated signaling through NMDA receptors during spontaneous synaptic transmission (Sutton et al., 2004, 2006; Jung et al., 2008; Espinosa and Kavalali, 2009). Our findings suggest that abrogating NHE5 activity reduces a local proton...

To analyze the activity-dependent trafficking of NHE5 by live-cell imaging, cells were transfected with NHE5-GFP plus RFP as a cell fill. Although overexpressed NHE5-GFP exhibited much higher fluorescence in dendrites than endogenous NHE5 staining in dendrites, endogenous NHE5 staining showed a considerable overlap with NHE5-GFP (Supplemental Figure S3). Before glycine treatment, NHE5-GFP was distributed in the dendritic shaft, at the base of dendritic spines, in the spine neck, or in the spine head (Figure 3E). Following glycine treatment, NHE5-GFP present at the base of several spines was observed to traffic to the spine neck and then the spine head (Figure 3D). Quantification of the distribution of NHE5-GFP at spines revealed a time-dependent decrease in the proportion of spines that displayed NHE5-GFP at their base or neck and a concomitant increase in the proportion of spines that displayed NHE5-GFP in the spine head (Figure 3E). No change in the spine distribution of NHE5-GFP was seen during the same period in mock-glycine–treated neurons (Supplemental Figure S3). Together, these data suggest that NHE5 is recruited to postsynaptic spines and to the plasma membrane in an activity-dependent manner.

FIGURE 2: Activity-dependent targeting of NHE5 to the plasma membrane. (A) Cultured hippocampal neurons were left untreated (con.) or treated for 48 h with bicuculline (20 μM, bic.) or TTX (1 μM) to enhance or suppress network activity, respectively. Neurons were then surface biotinylated, and total and surface abundance of NHE5 and NHE1 were measured by Western blot. (B) Surface NHE1 and NHE5 from bicuculline- or TTX-treated neurons were normalized to untreated control lanes. (C) Cultured hippocampal neurons were treated with or without glycine (200 μM for 3 min) or D-AP5 (100 μM, 30-min pretreatment) followed by a 45-min chase. Neurons were then cell surface biotinylated. There is a dramatic increase in biotinylated surface NHE5, but not NHE1, following glycine treatment, and this increase could be blocked by D-AP5. (D) Surface NHE1 and NHE5 from glycine/D-AP5–treated neurons were normalized to untreated control lanes. (E) Cultured hippocampal neurons were treated with glycine as in C with a 0- to 45-min chase as indicated, followed by cell surface biotinylation. There is a time-dependent increase in biotinylated surface NHE5, but not NHE1, beginning 5 min following glycine treatment. (F) Surface NHE1 and NHE5 from glycine-treated neurons were normalized to untreated (0 min) control lanes. For all biotinylation experiments, there were three independent experiments from three separate cultures. *p < 0.05 and **p < 0.01, Student’s t test.
source at the synapse, increases the spontaneous NMDA receptor signaling, and leads to the NMDA-receptor–dependent exuberant spine growth.

**NHE5 and activity-dependent spine growth**

We next investigated the role of NHE5 in activity-induced spine growth. Hippocampal neurons were transfected with wild-type NHE5, dominant-negative mutant NHE5 (NHE5 E209I), or NHE5 shRNA. At 14DIV neurons were treated with glycine or control solution, and cells were fixed and analyzed 60 min later. In control GFP-expressing neurons, glycine treatment resulted in a significant increase in the density and head width of dendritic spines (Figure 6, A and B). This activity-induced enhancement of spine density and width was abolished in cells pretreated with the NMDA receptor antagonist D-AP5 (100 μM), confirming previous reports that the glycine treatment paradigm mediates its effects through activation of NMDA receptors (Lu et al., 2001; Figure 6B). Expression of NHE5HA-E209I or NHE5 shRNA significantly increased the basal dendritic spine density and increased the width slightly but in a statistically significant manner, and no further enhancement of spine density or head width was observed following glycine treatment, whereas wild-type NHE5 abolished activity-induced enhancement of spine density (Figure 6, A and B). These findings suggest that NHE5 activity is important for limiting activity-dependent formation of dendritic spines and that abrogating NHE5 function is sufficient to trigger spontaneous dendritic spine growth irrespective of neuronal activity.

**Activity- and NHE5-dependent changes in dendritic spine pH**

Our results demonstrating activity-dependent recruitment of NHE5 to dendritic spines and to the plasma membrane suggest that there may be an associated activity-dependent change in spine pH. To test this possibility, we initially attempted to determine pH of dendritic spines using a pH-sensitive variant of GFP (de4GFP). de4GFP is a dual-emission fluorophore that emits at both 510 and 460 nm, with emission at 510 nm increasing and emission at 460 nm decreasing as pH increases. Therefore, de4GFP could theoretically be used for ratiometric pH recordings (Hanson et al., 2002; McAnaney et al., 2002; Vilas et al., 2009). However, we found that de4GFP emission at 460 nm was too faint to afford accurate pH measurement of dendritic spines. Therefore, to overcome this problem, we generated a chimera of pH-insensitive mCherry (Shaner et al., 2005) fused to de4GFP (mCherry/de4GFP) for accurate ratiometric measurement of dendritic spine pH. A similar approach was successfully used with a fusion protein of mCherry and a pH-sensitive GFP variant S Ephluorin (Koivusalo et al., 2010). In control experiments, we calibrated the fluorescence ratio of pH-insensitive mCherry and pH-sensitive de4GFP to cellular pH by expressing mCherry/de4GFP in Chinese hamster ovary cells and imaging these cells under pH-clamp conditions (Baxter and Church, 1996) using the protonophore nigericin (high...
contrast, in NHE5 knockdown cells, the spine alkalinization was greatly attenuated following glycine treatment and returned to preglycine levels after 60 min (Figure 7, C and D). In mCherry/de4GFP-expressing neurons not treated with glycine there was no change in spine pH over a 60-min imaging period (unpublished data). Altogether, these results suggest that NHE5 is recruited to the dendritic spine membrane where NHE5 affects local pH following synaptic activity.
The synapse is made up from two principal components—the presynapse, where neurotransmitter-loaded synaptic vesicles are found, and the postsynapse, where neurotransmitter receptors are clustered opposite the vesicle release sites. These two structures are separated by a narrow space of approximately 20 nm, referred to as the synaptic cleft. Many components of the synaptic machinery found in the synaptic cleft are sensitive to changes in cleft pH, including VGCCs, AMPA receptors, kainate receptors, NMDA receptors, GABA receptors, type III metabotropic glutamate receptors, and acid-sensing ion channels, ion channels directly gated by protons (Traynelis and Cull-Candy, 1990; Tombaugh and Somjen, 1996; Krishek et al., 1996; Ihle and Fatou, 2000; Mott et al., 2003; Zha et al., 2006; Levinthal et al., 2009). The NMDA receptor, which is essential for many forms of synaptic plasticity, and VGCCs, which mediate Ca²⁺-dependent release of synaptic vesicles, are particularly important to consider, as these proteins have an especially high proton sensitivity, with a pKₐ of 7.3–7.5 and 7.1–7.3, respectively (Tang et al., 1990; Traynelis and Cull-Candy, 1990; Klockner and Isenberg, 1994; Chen et al., 1996; Tombaugh and Somjen, 1996; Banke et al., 2005). Thus at resting pH close to 7.3 (Chesler, 2003), a tonic proton block exists that maintains NMDA receptor and VGCC activity to ~50%. This implies that any slight deviation in local pH should theoretically have a profound impact on synaptic transmission and synaptic plasticity. NMDA receptors have even been shown to contain a discrete proton-binding site, distinct from other ligand-binding sites (Banke et al., 2005). On the basis of the abundance of pH-sensitive proteins within the narrow confines of the synaptic cleft, it is highly likely that localized control of cleft pH will play an important role in synaptic transmission. Indeed, localized pH shifts at the synapse have been demonstrated to feed back onto nearby pH-sensitive NMDA receptors and VGCCs (DeVries, 2001; Fedirko et al., 2007; Makani and Chesler, 2007). However, the mechanisms that control local pH at the synapse and the identity of synaptic pH-regulating proteins have not been determined.

We show here that following glycine treatment to enhance synaptic activity, NHE5 is targeted to the cell surface and recruited to synapses, where it initiates a local pH change through Na⁺/H⁺ exchange. Furthermore, our data suggest that NHE5 is a negative regulator of activity-induced dendritic spine growth and that at rest NHE5 acts to suppress spontaneous spine growth. On the basis of these findings, we propose a model in which NHE5 is recruited to the synaptic plasma membrane in dendritic spines following synaptic activity (Figure 8). Once at the plasma membrane, NHE5 Na⁺/H⁺ exchanger activity results in alkalinization of the dendritic spine and concomitant acidification of the synaptic cleft. This local acidification may serve as an autocrine feedback mechanism that regulates pH-sensitive proteins at the postsynapse such as NMDA receptors and possibly also as a paracrine mechanism to regulate presynaptic pH-sensitive NMDA receptors and VGCCs (Figure 8). The NMDA receptor is of particular interest, given its important role in dendritic spine remodeling (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Lang et al., 2004; Matsuzaki et al., 2004). Overexpression of NHE5 may enhance the tonic proton block of the NMDA receptor, preventing activity-dependent formation of new spines. Conversely, reducing the local proton source by NHE5 knockdown or expression of a dominant-negative mutant may lead to hyperactivation of the NMDA receptor. This could effectively lower the threshold needed to induce LTP, such that spontaneous network activity is sufficient to drive dendritic spine expansion to an LTP-like state. Consistent with this idea, perturbation of NHE5 function led to a significant increase in dendritic spine density and width, which was not enhanced further by glycine treatment but was completely reversed by prolonged treatment with a chemical NMDA-receptor antagonist. In addition, spine alkalinization induced by glycine treatment was also prevented by knockdown of NHE5. Therefore, in our model, NHE5 acts as part of a negative-feedback loop to stabilize dendritic spine growth following NMDA receptor activation through a local pH shift at the synapse (Figure 8). Of interest, we showed previously that NHE5 directly interacts with the signaling scaffold molecule receptor for active C-kinase (RACK1) (Onishi et al., 2007). RACK1 is known
to bind to the C-terminus of the NR2B subunit of the NMDA receptor (Yaka et al., 2002). It is unknown how NHE5 is organized at the synapse at the molecular level, but one possibility is that RACK1 tethers NHE5 adjacent to the NMDA receptor through the NR2B subunit, making NHE5-transported protons a potent NMDA receptor inhibitor.

The primary focus of this study was to investigate the role of NHE5 at the postsynapse and in dendritic spines. However, due to the confined space of the synaptic cleft, it is possible that local pH shifts by NHE5 may feed back onto presynaptic molecules such as VGCCs in a similar manner as NMDA receptors. Furthermore, our immunocytochemical analyses suggest that NHE5 is also present in presynaptic compartments. Taken together, it is possible that local pH shifts by NHE5 may feed back onto presynaptic molecules such as VGCCs in a sophisticated manner. VGCCs are best known for their role in Ca\(^{2+}\)-dependent release of synaptic vesicles (Llinas et al., 1982). Extracellular protons shift the activation voltage of VGCCs in the positive direction and reduce peak current, thereby decreasing synaptic vesicle release probability (Tombaugh and Somjen, 1996; DeVries, 2001). NHE5 inserted into the postsynapse membrane may serve to limit synaptic vesicle release from the presynapse through local acidification of the synaptic cleft. A similar proton-mediated feedback onto VGCCs has been demonstrated previously (DeVries, 2001). Thus it is interesting to speculate that reducing the local proton source by perturbation of NHE5 activity leads to higher vesicle release probability, which could act together with increased NMDA receptor activity to support the spontaneous dendritic spine growth that we observed.

Ronicke et al. (2009) showed that application of 5-(N-ethyl-N-isopropyl) amiloride (EIPA), an NHE-inhibitor drug, during tetanus induction of LTP in hippocampal CA1 neurons resulted in enhanced LTP, suggesting that NHE is a negative regulator of synapse plasticity. However, it is not clear which NHE isoform is responsible for this phenotype, as the high concentration of EIPA used in this study could inhibit all NHE activities across the plasma membrane in a nondiscriminatory manner. More recently, it was reported that NHE contributes to extracellular acidification of synapses and enhances GABAergic inhibitory signaling in cerebellar granule cells (Dietrich and Morad, 2010). Together with these studies, our current findings suggest that NHE5 is a negative regulator of synaptic activity. It will be an interesting future project to investigate targeting and function of NHE5 in inhibitory synapses.

To our knowledge, NHE5 is the first example of a synaptic pH-regulating ion transporter whose synapse and cell-surface targeting is controlled in an activity-dependent manner. We present evidence that NHE5 is a novel regulatory molecule involved in synaptic plasticity and add to a growing body of evidence supporting an important role for protons in synaptic transmission. As several components of the synaptic machinery are proton sensitive, local pH regulation at the synapse and NHE5...
activity may affect multiple aspects of synapse physiology and warrants continued investigation.

MATERIALS AND METHODS

Antibodies. Rabbit polyclonal anti-NHE5 antibodies were raised against the epitope EEPTQEPGPLGEPP from rat NHE5 (amino acid residues 21–34), corresponding to the first extracellular loop of NHE5, and affinity purified (GenScript, Piscataway, NJ). The following additional primary antibodies were used: mouse monoclonal anti-Tau (Millipore, Billerica, MA), guinea pig polyclonal anti-VGlut1 (Sigma, Oakville, ON, Canada), mouse monoclonal anti-α- and β-tubulin (Sigma), rabbit polyclonal anti-actin (Sigma), guinea pig polyclonal anti-VGAT (Synaptic Systems, Göttingen, Germany), mouse monoclonal anti-gephyrin (Synaptic Systems), mouse monoclonal anti-GFP (Roche, Mississauga, ON, Canada), mouse monoclonal anti-NHE1 (BD Biosciences, San Jose, CA), mouse monoclonal anti-MAP2 (Sigma, St. Louis, MO), guinea pig polyclonal anti-VGlut1 (Millipore), mouse monoclonal anti-Tau (Millipore, Billerica, MA), guinea pig polyclonal anti-VGAT (Synaptic Systems, Göttingen, Germany), mouse monoclonal anti-hemagglutinin (HA) (Coventry, Princeton, NJ), and mouse monoclonal anti-PSD95 (Abcam, Cambridge, MA). The following secondary antibodies were used: Alexa Fluor 488– or Alexa Fluor 594–conjugated goat anti–mouse, Alexa Fluor 488– or Alexa Fluor 594–conjugated goat anti-rabbit, Alexa Fluor 594– or Alexa Fluor 647–conjugated goat anti-mouse (Molecular Probes, Eugene, OR), Cy5-conjugated donkey anti–guinea pig (Jackson ImmunoResearch, West Grove, PA), and horse-radish peroxidase–conjugated goat anti–rabbit and goat anti–mouse secondary antibodies (Jackson ImmunoResearch).

RNAi constructs and recombinant DNAs. Human NHE5 cDNA was cloned previously (Baird et al., 1999). HA-tagged human NHE5 was produced previously by introducing an artificial NotI restriction endonuclease site into the coding sequence corresponding to leucine 36 (contained in the first predicted exomembrane loop), followed by insertion of triple HA epitope (Szsasi et al., 2002). To ensure that NHE5 was produced by direct-directed mutagenesis using QuikChange Mutagenesis Kit (Stratagene, La Jolla, CA) using human NHE5 HA as a template. EGFP coding sequence flanked by NotI restriction endonuclease sites was amplified by PCR and then ligated into the artificial NotI site introduced into NHE5. The expected final protein product contains the full-length NHE5 with internal GFP located in the first exoplasmic loop. For short-hairpin RNA (shRNA A, B or C) constructs targeting rat NHE5 the following complementary synthetic oligonucleotides were used, 5′-GATCCGGTGTCTTGGTGTAATCACTCGTAAACATCTTTCAC03-3′ (sense) and 5′-AGCTTTTGGAAAACAGGATGTTAACGATATCAATAACTCAGTGTACCAAGAGACCCG-3′ (antisense, shRNA A); 5′-GATCCGGTGTCTTGGTGTAATCACTCGTAAACATCTTTCAC03-3′ (sense) and 5′-AGCTTTTGGAAAACAGGATGTTAACGATATCAATAACTCAGTGTACCAAGAGACCCG-3′ (antisense, shRNA B); 5′-GATCCGGTGTCTTGGTGTAATCACTCGTAAACATCTTTCAC03-3′ (sense) and 5′-AGCTTTTGGAAAACAGGATGTTAACGATATCAATAACTCAGTGTACCAAGAGACCCG-3′ (antisense, shRNA C). Bafoldface type indicates sequences targeting rat NHE5. When annealed, duplexes contain 5′ and 3′ overhangs, which allowed cloning into the multicloning site in pcRNA3-H1 vector (Stratagene).

Neuronal cell culture. Hippocampi from E18 rats were prepared as previously described (Xie et al., 2000) and plated at a density of 130 cells/mm² on poly-L-lysine–coated coverslips for immunohistochemistry or plated at a density of 260 cells/mm² on 35-mm, polyethyleneimine-coated, plastic culture dishes for Western analysis. For all analyses, neurons were transfected using Lipofectamine 2000 (Invitrogen Canada, Burlington, ON, Canada) at 9 DIV and imaged at 14 DIV.

Immunohistochemistry

Brain sections. Adult mice (P40–P60) were anesthetized by intraperitoneal injection of 150 mg/kg ketamine and 15 mg/kg xylazine and transcardially perfused with ice-cold phosphate-buffered saline (PBS) (pH 7.4), followed by 4% paraformaldehyde (PFA) in PBS. The brains were quickly removed and postfixed in 4% PFA for 1 h before going through a series of sucrose-PBS solutions (10–30%). Whole brains were embedded in Tissue-Tek (Sakura Finetek, Torrance, CA), frozen in dry ice, and stored at −80°C. A cryostat (Micron Instruments, San Marcos, CA) was used to cut 20-μm-thick coronal sections. For immunohistochemistry, sections were permeabilized in 0.1% Triton-X for 30 min, blocked in 4% goat serum for 1 h at room temperature, and incubated with PBS containing 1% goat serum and anti-NHE5 antibody overnight at 4°C. Following washes with PBS, sections were then incubated with goat anti–rabbit Alexa Fluor 488–conjugated secondary antibody in PBS containing 1% goat serum for 1 h at room temperature.

Hippocampal neurons. Neurons were fixed by incubation with 4% PFA in PBS for 10 min at room temperature and then permeabilized with 0.1% Triton X-100 in PBS for 10 min at room temperature. Alternatively, cells were fixed/permeabilized by incubation with prechilled methanol at −20°C for 5 min. Cells were blocked by incubation with 10% goat serum in PBS for 1 h at room temperature, followed by incubation with primary antibodies in PBS containing 1% goat serum overnight at 4°C and fluorescently labeled secondary antibodies for 1 h at room temperature.

For experiments examining NHE5 surface expression, cells were fixed with 4% PFA blocked by incubation with 10% goat serum in PBS for 30 min followed by incubation with rabbit anti-NHE5 antibody in PBS containing 1% goat serum for 2 h at room temperature. Cells were then permeabilized with 0.1% Triton X-100, incubated overnight at 4°C with mouse anti-PSD95 and guinea pig anti-VGlut1 in PBS containing 1% goat serum, followed by incubation with Alexa 488–conjugated goat anti–rabbit, Alexa 568–conjugated goat anti–mouse, and Cy5-conjugated donkey anti–guinea pig secondary antibodies.

Glycine and drug treatment. Cultured hippocampal neurons were incubated in chemical LTP buffer (140 mM NaCl, 5 mM KCl, 1.5 mM CaCl₂, 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid [HEPES], pH 7.4, 33 mM glucose, 500 nM TTX, and 20 μM bicuculline) for 30 min at 37°C. Neurons were then treated with chemical LTP buffer containing 200 μM glycine for 3 min at 37°C, followed by a return to buffer without glycine for the remainder of the experiment. Control cells were left in TTX/bicuculline solution without glycine. In some experiments, D-AP5 (100 μM) was added to the chemical LTP buffer during the 30-min pretreatment and 3-min glycine treatment steps. For chronic activity manipulations bicuculline (20 μM) or TTX (1 μM) was added to the culture medium for 48 h.

Biotin labeling and Western blot analysis. Neurons treated with glycine, bicuculline, or TTX for the indicated times were rinsed with ice-cold PBS/CM (PBS containing 1 mM MgCl₂ and 0.1 mM CaCl₂, pH 8.0) and then incubated with a protein-reactive
biotinylation reagent (NH-SS-Biotin [Thermo Scientific, Hudson, NH], 0.5 mg/ml in PBS/CM) for 30 min at 4°C to label surface proteins, and excess biotinylation reagent was quenched by incubation with PBS/CM containing 20 mM glycine. Equal amounts of cell lysates from each sample were incubated overnight at 4°C with NeutrAvidin agarose beads (Thermo Scientific). Proteins eluted from the beads were resolved by SDS–PAGE and detected by Western blot using anti-NHE5 or anti-NHE1 antibodies. Ten percent of the total whole-cell lysate not subjected to NeutrAvidin beads was also visualized by Western blot.

Image analysis and quantification
Quantification of synapse localization. Cells were immunolabeled using NHE5, VGlut1 and PSD95, or VGAT and gephyrin antibodies. Images were analyzed using ImageJ software (National Institutes of Health, Bethesda, MD) and a colocalization plug-in from the ImageJ website (http://rsb.info.nih.gov/ji/plugins/colocalization.html). Synapses were first identified as regions of colocalization greater than 10 pixels in size between presynaptic and postsynaptic markers: Vglut1 (threshold 1165) and PSD95 (threshold 1290) for excitatory synapses, or VGAT (threshold 765) and gephyrin (threshold 790) for inhibitory synapses. Thresholding levels were held constant between samples and experiments, randomly selected fields were imaged, and all the puncta were examined in a field. These regions of colocalization between presynaptic and postsynaptic markers were used to create a “mask,” and regions greater than five pixels where NHE5 puncta overlapped with the mask were considered synapses positive for NHE5. Numbers of synapses positive for NHE5 were expressed as a percentage of the total number of synapses identified, quantified using the same threshold and size criteria. The mean numbers of colocalized synaptic puncta quantified from 30 or more fields in each treatment group were analyzed for statistical significance using Student’s t test. On average, >130 excitatory synapses were scored per field (4000–7000 total excitatory synapses analyzed), and 70 inhibitory synapses were scored per field (2200 total).

Dendritic spine measurement. For all experiments examining spine number and morphology a GFP cell fill was used. Serial Z-section confocal images were compiled into a single image. Regions of dendrite within 100 μm from the cell body where dendritic spines were clearly visible (without excessive crossing of multiple dendrites or axons) were imaged. All spines on selected dendrite segments were measured and included in our analysis. Spines were identified as protrusions from the dendrite shaft <3 μm in length with or without a head or a discernible neck, that is, stubby spines. The density and width of spines were measured using ImageJ software. Statistical analysis and graph plotting were performed using Excel software (Microsoft, Redmond, WA). For the quantification of Figures 4B, 5B, and 6B, 3500–8000 μm of dendrite from 17–46 neurons per condition was analyzed. The n values correspond to the number of neurons analyzed.

Quantification of NHE5-GFP localization. Individual spines were first identified from the RFP cell fill and then manually scored for the presence of NHE5-GFP either contained within the spine or within 1 μm of the spine base. NHE5-GFP location relative to spines was categorized according to the presence of NHE5-GFP at the base of the spine (in the dendritic shaft within 1 μm of the spine base), in the spine neck (between the spine base and spine head), or in the spine head (at the spine tip) as illustrated in Figure 3E. Spines containing NHE5-GFP in the head or neck and extending to the spine base were counted as positive for spine head or neck and not as spine base.

Monitoring dendritic spine pH using the ratiometric mCherry/de4GFP fusion. The cDNA encoding mCherry was obtained from AddGene (Cambridge, MA) and used as a template to for PCR. HindIII and BamHI sites were introduced to the 5’ and 3’ ends of the coding region of mCherry, respectively, by PCR and ligated into the corresponding sites of the de4GFP-N1 vector (Vilas et al., 2009) in frame with de4GFP. Hippocampal neurons, grown on gridded glass-bottom dishes (MatTek, Ashland, MA), were transfected with mCherry/de4GFP fusion alone or together with NHE5 shRNA A. The de4GFP (pH-sensitive) and mCherry (pH-insensitive) fluorescence was captured using an Olympus (Center Valley, PA) Fluoview confocal microscope by excitation with a 488- or 543-nm laser, respectively. To calibrate mCherry/de4GFP fluorescence ratio to pH, the high–[K+]/nigericin technique was used (Chaillet and Boron, 1985; Baxter and Church, 1996). In the calibration experiments, fluorescence intensity of de4GFP displayed a sigmoidal relationship to pH between pH 5.0 and 9.0, and pKₐ was calculated to be 7.45, similar to the previously reported value (Hanson et al., 2002). Between pH 6.75 and 8.0, the relationship between pH and mCherry/de4GFP fluorescence ratio was linear (Vilas et al., 2009). Experimentally measured ratios for each spine were converted to pH using the linear relationship between fluorescence ratio and pH determined in mCherry/de4GFP–transfected Chinese hamster ovary cells under pH clamp.

Supplementary materials and methods
Cell lines. Chinese hamster ovary, AP-1, and AP-1–based cell lines were maintained in α-MEM with 10% fetal bovine serum (FBS), and PC12 and PC12 cells stably expressing NHE5 shRNA were maintained in DMEM supplemented with 5% FBS and 10% horse serum. AP-1 cells are an NHE-deficient cell line derived from Chinese hamster ovary cells produced previously by chemical mutagenesis (Rotin and Grinstein, 1989). AP-1 cells stably expressing HA-tagged NHE5 or NHE1 were produced previously (Szaszi et al., 2002). AP-1 cells expressing HA-tagged NHE5 were produced previously by transfecting parental AP-1 cells with pcDNA3 containing NHE5(K209I), using the conventional calcium phosphate method. Stable transfectants were selected in growth media containing 418 (200 μg/ml), and individual clones were screened by Western blot and immunofluorescence microscopy using mouse anti-HA antibody. PC12 cells stably expressing NHE5 shRNA (A, B, or C) were established in the same way.

NHE activity assay. Cells were loaded with the pH-sensitive dye 2′,7′-bis(2-carboxyethyl)-5′-(and-6)-carboxyfluorescein (BCECF) by incubation with 2 μM BCECF acetoxymethyl ester in NaCl-saline solution (130 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 2 mM CaCl₂, 5 mM glucose, and 20 mM HEPES-NaOH, pH 7.4) for 10 min at room temperature. Coverslips with cells attached were then mounted in a temperature-controlled recording chamber filled with NaCl-saline solution, placed on the microscope stage, and superfused at 2 ml/min with NaCl-saline solution (without dye) at 34°C for the remainder of the experiment. The dual excitation ratio method was used to estimate pHι with a fluorescence ratio imaging system (Atto Biosciences, Rockville, MD) as described previously (Baxter and Church, 1996). The high–[K+]/nigericin technique was used to convert background-corrected BCECF emission intensity ratios into pH values (Rotin and Grinstein, 1989; Baxter and Church, 1996). Intracellular acid loads were imposed by exposing the cells for 2 min
to NaNH5-choline solution containing 50 mM NaNHCl followed by washout with NaN-free solution containing equimolar choline chloride. Na+/H+ exchange was induced by the return to NaCl-saline solution. The recovery of pH following an NaNH4 prepulse was fitted to a single-exponential function, and the first derivative of this function was used to determine the rate of change of pH (d pH/dt) at 0.05 pH unit increments from the point of maximum acidification (Baxter and Church, 1996). Proton efflux was calculated by multiplying the measured d pH/dt at a given pH value by the intrinsic intracellular buffering capacity (B) at the same pH value as determined previously (Diering et al., 2009).

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REFERENCES


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**A**

<table>
<thead>
<tr>
<th>Transmembrane Domain 6</th>
<th>Species</th>
</tr>
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<tr>
<td>E209</td>
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<tr>
<td>NHE5 - FIIVF8ESLLNDATVVL</td>
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<td><em>A. thaliana</em></td>
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**B**

- **WB**: HA
- E209: 130KDa, 100KDa, 72KDa

**C**

- Tf
- NHE5 WT
- Merge
- NHE5 E209I
- Merge
- NHE1
- Merge

**D**

- pH range from 6.2 to 7.6
- NHE5 WT: Nat⁺-free, 2 minutes
- NHE5 E209I: Nat⁺-free, 2 minutes

**E**

- Graph showing Proton Eflux (μM/sec) vs. pHₙ
- NHE5 WT
- NHE5 WT/E209I