Stress-induced reduction of Na\(^+\)/H\(^+\) exchanger isoform 1 promotes maladaptation of neuroplasticity and exacerbates depressive behaviors

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Major depression disorder (MDD) is a neuropsychiatric disorder characterized by abnormal neuronal activity in specific brain regions. A factor that is crucial in maintaining normal neuronal functioning is intracellular pH (pHi) homeostasis. In this study, we show that chronic stress, which induces depression-like behaviors in animal models, down-regulates the expression of the hippocampal Na\(^+\)/H\(^+\) exchanger isoform 1, NHE1, a major determinant of pHi in neurons. Knockdown of NHE1 in CA1 hippocampal pyramidal neurons leads to intracellular acidification, promotes dendritic spine loss, lowers excitatory synaptic transmission, and enhances the susceptibility to stress exposure in rats. Moreover, E3 ubiquitin ligase culmin4A may promote ubiquitination and degradation of NHE1 to induce these effects of an unbalanced pHi on synaptic processes. Electrophysiological data further suggest that the abnormal excitability of hippocampal neurons caused by maladaptation of neuroplasticity may be involved in the pathogenesis of this disease. These findings elucidate a mechanism for pHi homeostasis alteration as related to MDD.

INTRODUCTION

With increases in societal stressors and frequencies of various high-pressure events, depression has gradually become the most widespread mood disorder (1). At present, selective serotonin reuptake inhibitors and norepinephrine reuptake inhibitors represent the main first-line antidepressant treatments in clinical practice. However, side effects and recurrence weaken the clinical efficacy of these treatments (2). These findings reveal that classical mechanisms cannot fully explain the pathogenesis of depression and suggest that multiple etiological mechanisms may underlie substantial features of depression (3). Therefore, a more comprehensive understanding of the pathological mechanisms of depression represents one of the most important issues for the development of better diagnostic and treatment strategies for this disorder.

Stressors represent one of the most prominent negative stimuli leading to mood disorders. Under normal conditions of physiological functioning, individuals will respond to external environmental stimuli through neural mechanisms, which allow them to adapt to environmental changes and thus enable their survival and development. As part of this process, neuronal plasticity regulation plays a key role. Therefore, the question of whether maladaptive plasticity in neuronal activity induces mood disorders in response to external stress represents an important issue. It has been reported that synaptic atrophy is observed in depressed patients and in animal models of depression and is positively correlated with the course and severity of depressive symptoms (4). The effectiveness of some previously unidentified antidepressants, such as ketamine, appears to function by reversing synaptic loss via promotion of neuroplasticity regulation (5), and it has been reported that the reinduction of plasticity regulation in neuronal structures is an important neuronal mechanism of antidepressant therapy (6). These findings are consistent with results from our laboratory, which have also shown that structural and functional plasticity changes in synaptic-related structures may be an important basis for the development of depression (7, 8). In this way, stress-induced maladaptive plasticity within the brain neuronal activity may serve as the foundation for abnormal adaptability of neuronal structures and functions, ultimately leading to mood disorders.

Results from previous studies have revealed many of the specific molecular pathways related to mechanisms involved with depression. However, given the possibility that chronic stress can disrupt the intracellular homeostasis of neurons, which can lead to structural and functional plasticity changes to induce depression, it is somewhat unexpected how little attention has been directed toward investigating this eventuality. In general, intracellular acid-base balance represents one of the most important processes for maintaining the homeostasis needed for functional activities, while an imbalance in this process serves as the pathophysiological basis for many diseases (9). The brain is highly sensitive to shifts in pH due to proteins involved in regulating neuronal excitability, and effective synaptic transmission is critically dependent on appropriate pH levels (10). There is growing evidence that intracellular pH (pHi) changes in the brain are associated with cognitive, emotional, and psychotic behaviors, and a significant decrease in neuronal pHi may be associated with the severity of depression (11–13). In this way, changes in homeostasis characteristics caused by intracellular acidification may be a key link between external stress and internal dysfunction. However, the processes and mechanisms controlling pHi and the means through which may contribute to normal synaptic functioning remain largely unknown. In particular, whether stress exposure leads to intracellular acidification and ultimately exacerbates depressive behaviors is only beginning to be addressed.

Na\(^+\)/H\(^+\) exchangers (NHEs) belong to a family of highly conserved transporters, whose functions are to regulate pHi by removing intracellular protons in exchange for extracellular sodium to protect cells from the acidification that occurs from metabolism (14). In mammals,
NHE is divided into three family subtypes, SLC9A1-9, SLC9B1-2 (NHA1-2), and SLC9C1-2 (NHE4) (15). The NHE1 protein is the most widely expressed subtype in the central nervous system, particularly in the cerebral cortex, hippocampus, and cerebellum (16, 17), where it is mainly localized to inhibitory and excitatory pre- and postsynaptic nerve terminals (18–21). NHE1 contributes not only to pH regulation but also to cell migration and development and serves as an anchor to interact with components of the cytoskeleton (22–24). Results from recent studies have demonstrated that disruption of the SLC9A1, the gene encoding NHE1, results in locomotor ataxia and a phenotype of slow-wave epilepsy, including a progressive neuronal degeneration (25–27). NHE1 was reported closely related to Na+ and K+-dependent ATPase (NKA) as a cotransporter system (28). Moreover, previous study showed that chronic restraint stress could decrease the activity of NKA in frontal cortex, which may impair the transport function of NHE1 and play pathophysiological roles in neuropsychiatric diseases (29). However, whether chronic stress affects NHE1 expression, to result in an imbalance of cytoplasmic pH and ultimately to maladaptive plasticity and depressive behavior, has yet to be investigated, nor is it known which factors may regulate NHE1 expression during stress exposure. Therefore, in this study, we investigated the potential mechanisms underlying the stress-induced synaptic plasticity of neurons in the pathogenesis of depression as related to the regulation of intracellular homeostasis. Previous studies have showed that the hippocampus, particularly the cornu ammonis 1 (CA1) region, is one of the important brain regions that is prone to induce synaptic plasticity (30, 31). Moreover, growing studies demonstrated that CA1 hippocampal is involved in the pathogenesis of depression to regulate mood and cognitive function (32, 33). We found that CA1 hippocampal NHE1 deficits play a critical role in maintaining pH homeostasis at this site, which can then regulate the synaptic plasticity associated with depression. These NHE1 deficits are mainly attributable to the ubiquitination and proteasomal degradation promoted by E3 ubiquitin ligase cullin4A (CUL4A). These results not only provide insight into an underlying mechanism involved with the functional regulation of neuroplasticity in depression but also suggest previously unexplored avenues of investigation for the development of potential therapeutic targets in the treatment of this condition.

RESULTS
Decreased pH i was observed in hippocampus of depression animal models
The chronic unpredictable mild stress (CUMS) protocol was used to generate an animal model of depression (fig. S1A). After 5 weeks of CUMS exposure, significant depression-like behaviors were observed in rats. Results from the sucrose preference test (SPT) showed that CUMS exposure significantly decreased the percent of sucrose consumption as compared with control rats, results that are indicative of anhedonia (Fig. 1A). Forced swim test (FST) results demonstrated that rats in the CUMS group showed increased immobility times and decreased swimming times, results that are indicative of behavioral despair (Fig. 1, B and C). From the open-field test (OFT), we found that CUMS exposure did not alter the total distance traveled (Fig. 1, D and E) but did produce a decrease in the time exploring the center area (Fig. 1F). These results demonstrate that, although CUMS exposure did not affect the overall spontaneous locomotor activity of these rats, the reluctance to enter the center area suggests an increased level of anxiety in CUMS rats. In addition, elevated plus maze (EPM) results showed that CUMS exposure decreased the time in the open arms and the probability of entering the open arms of this maze as compared to controls, responses also indicative of increased anxiety (Fig. 1, G to J). Of particular significance to this study was the pHi imaging that showed that the average fluorescent intensity in CUMS groups was significantly stronger than that of control groups, which confirmed that CUMS exposure decreased the pHi in CA1 neurons (Fig. 1, J and K). These results suggest that chronic stress induces an imbalance in pHi in hippocampal pyramidal neurons, effects that may be associated with the depression- and anxiety-like behaviors observed in these rats.

CUMS induces maladaptation of hippocampal synaptic plasticity
To determine whether this CUMS treatment produced a maladaptation of synaptic plasticity, we performed a reconstruction of pyramidal neurons on the basis of Golgi-stained images within the CA1 hippocampus using Sholl analysis (Fig. 2, A and B). This CUMS exposure decreased the number of dendritic intersections against the radial distance from the soma (Fig. 2, C and D) and significantly decreased the total number of mushroom spines in CA1 pyramidal neurons (Fig. 2, E and F). Double immunofluorescent staining showed that CUMS exposure decreased the density of vesicular glutamate transporter 1 (VGLUT1)/postsynaptic density protein-95 (PSD-95) co-localization in neurons, indicating that CUMS exerts lasting effects on the density of excitatory synapses (Fig. 2, G and H), results that are consistent with that observed using electron microscopy (fig. S1, B to D). To further explore whether these structural synaptic deficits were accompanied by functional changes in CA1 pyramidal neurons, we conducted whole-cell patch-clamp recordings, with the results that CUMS decreased the frequency and amplitude of miniature excitatory postsynaptic current (EPSC) (mEPSC) and spontaneous EPSC (sEPSC) (Fig. 2, I to N). Meanwhile, the current-voltage relationship within a representative CA1 hippocampal pyramidal neuron indicated that the minimal current needed to induce action potentials (APs) was increased by CUMS exposure, and a decrease in the frequency of evoked APs at different current steps was also observed in these neurons (Fig. 2, O and P). These results suggest that CUMS exposure inhibits synaptic transmission and excitability in CA1 pyramidal neurons, which may then contribute to the depression-like behaviors of these rats.

CUMS decreases NHE1 protein expression in CA1 hippocampal neurons
To determine whether intracellular acidification is an important factor involved with inducing maladaptive synaptic plasticity and, particularly, to elucidate which molecules may be involved in the regulation of the acid-base balance within these neurons, we performed a proteomic analysis within CA1 hippocampal tissue from control and CUMS rats (fig. S2A). The heatmap diagram shows differential protein expression levels in the hippocampus (Fig. 3B), while the differentially expressed proteins between these two groups are displayed with the use of volcano plot filtering (Fig. 3C). On the basis of the Kyoto Encyclopedia of Genes and Genomes pathway analysis and Gene Ontology (GO) analysis (fig. S2, B and C), a sodium/proton exchanger isoform 1 (NHE1) was found to be significantly decreased in CA1 hippocampal regions of CUMS versus control rats (Fig. 3D). Next, to validate these proteomic analysis results, we determined the
Fig. 1. CUMS induces depression-like behaviors in rats. (A) SPT results showing that CUMS rats exhibited a decrease in percent of sucrose consumption. Control (Ctrl): 88.61 ± 1.70 versus CUMS: 54.59 ± 4.18. \( P < 0.001 \). (B and C) FST results showing that CUMS rats exhibited increased immobility times (Ctrl: 33.92 ± 5.17 versus CUMS: 126.80 ± 10.09; \( P < 0.001 \)) and decreased swimming times (Ctrl: 123.80 ± 12.06 versus CUMS: 45.08 ± 5.39; \( P < 0.001 \)). (D) Raw traces of rats in the OFT. (E) Total distance traveled in the OFT was not significantly different between control and CUMS rats. Ctrl: 7551.00 ± 597.10 versus CUMS: 6833.00 ± 553.40. \( P = 0.3869 \). (F) Time spent exploring the center area was decreased in CUMS rats. Ctrl: 7.24 ± 1.42 versus CUMS: 2.86 ± 0.52. \( P = 0.0080 \). (G) Raw traces of rats in the EPM. (H) Time spent in the open arms was decreased in CUMS rats. Ctrl: 7.69 ± 0.89 versus CUMS: 2.77 ± 0.36. \( P < 0.001 \). (I) Probability of entering open arms was decreased in CUMS rats. Ctrl: 29.24 ± 4.48 versus CUMS: 7.44 ± 1.36. \( P < 0.001 \). (J) Representative images of pHrodo Red staining in hippocampal neurons of rats. DAPI, 4′,6-diamidino-2-phenylindole. (K) Quantification analysis of the relative fluorescent intensity. Ctrl: 1.00 ± 0.06 versus CUMS: 1.33 ± 0.05. \( P = 0.0018 \). \( N = 6 \) per group. NS, not significant (\( P > 0.05 \)); ** \( P < 0.01 \) and *** \( P < 0.001 \) versus Ctrl by a two-tailed, unpaired Student’s \( t \)-test.
Fig. 2. CUMS causes maladaptation of synaptic plasticity in CA1 hippocampus of rats. (A) Representative images of Golgi-stained hippocampal CA1 neurons. (B) Reconstructions of CA1 pyramidal neurons of rats. (C and D) Sholl analysis used to trace neuronal processes and showing the number of dendritic intersections. Ctrl: 94.50 ± 4.59 versus CUMS: 54.50 ± 3.19, P < 0.01, 15 neurons from N = 6 rats per group. (E) Golgi-stained dendrites. (F) Quantification of mushroom spines in the hippocampal neurons. Ctrl: 79.32 ± 2.21 versus CUMS: 57.06 ± 4.77, P = 0.0010, 45 dendritic segments from N = 8 to 9 rats per group. (G) Immunofluorescent staining showing VGLUT1+ (green) and PSD-95+ (red) colocalization in CA1 hippocampal neurons. (H) CUMS exposure decreased the number of synapses in neurons. Ctrl: 7120.00 ± 744.20 versus CUMS: 4841.00 ± 523.60, P = 0.0277, N = 5 per group. (I) Raw traces of mEPSC. (J) CUMS exposure decreased the amplitude of mEPSC. Ctrl: 15.27 ± 0.83 versus CUMS: 12.19 ± 0.96, P = 0.0280. (K) CUMS exposure decreased the frequency of mEPSC. Ctrl: 2.97 ± 0.20 versus CUMS: 1.85 ± 0.31, P = 0.0090. (L) Raw traces of sEPSC. (M) CUMS exposure decreased the amplitude of sEPSC. Ctrl: 31.52 ± 2.15 versus CUMS: 24.26 ± 1.06, P = 0.0061. (N) CUMS exposure decreased the frequency of sEPSC. Ctrl: 5.52 ± 0.43 versus CUMS: 2.77 ± 0.23, P < 0.01. (O) Raw traces showing individual voltage responses to a series of 700-ms current pulses from 0 to 140 pA with 20-pA steps. Red traces indicate the minimal current needed to induce APs. (P) Frequencies of induced APs at different current steps. N = 8 to 10 cells from six rats per group in electrophysiological recordings. *P < 0.05, **P < 0.01, and ***P < 0.001 versus Ctrl by a two-tailed, unpaired Student’s t test.
Fig. 3. CA1 hippocampal neuronal NHE1 protein expression is decreased in CUMS rats. (A) Schematic diagram of proteomic analysis. KEGG, Kyoto Encyclopedia of Genes and Genomes; GO, Gene Ontology. (B) Heatmap diagram of differentially expressed protein levels in proteomic analysis. (C) Volcano plots indicating differentially expressed proteins. FC, fold change. (D) The bar plot of GO analysis for NHE1 showed correlation to the regulation of pH. CC, cellular component; MF, molecular function; BP, biological process. (E) Expression levels of NHE1 in hippocampal tissues was validated. Ctrl: 1.00 ± 0.03 versus CUMS: 0.70 ± 0.05, P = 0.0005, N = 6 per group. (F) Schematics of AAV vectors and bilateral injection sites in the hippocampus. Scale bar, 20 µm. eGFP, enhanced green fluorescent protein. L-ITR, left inverted terminal repeats; CaMKII, calcium/calmodulin dependent protein kinase II; CA, Cornu Ammonis. DG, dentate gyrus. (G) Validation of the efficiency by AAV-NHE1 short hairpin RNA (shRNA) injections. WT: 1.00 ± 0.08, AAV-eGFP: 0.84 ± 0.07 versus AAV-NHE1 shRNA: 0.55 ± 0.06. N = 6 per group. P = 0.0284, AAV-NHE1 shRNA versus AAV-eGFP. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. (H) Knockdown of NHE1 caused rats more liable to decrease the percent of sucrose consumption. WT: 91.18 ± 1.35, AAV-eGFP: 84.63 ± 4.31, and AAV-NHE1 shRNA: 69.66 ± 4.41. N = 7 per group. (P = 0.0123, AAV-NHE1 shRNA versus AAV-eGFP). (I) Knockdown of NHE1 caused rats more liable to increase immobility times. WT: 29.13 ± 5.89, AAV-eGFP: 19.63 ± 4.31, and AAV-NHE1 shRNA: 115.40 ± 20.88. N = 8 per group. (P < 0.001, AAV-NHE1 shRNA versus AAV-eGFP). (J) Knockdown of NHE1 caused rats more liable to decrease swimming times. WT: 109.30 ± 16.03, AAV-eGFP: 98.88 ± 10.93, and AAV-NHE1 shRNA: 53.38 ± 5.28. N = 8 per group. (P = 0.0295, AAV-NHE1 shRNA versus AAV-eGFP, NS, P > 0.05; **P < 0.05; ***P < 0.01; and ****P < 0.001 by a two-tailed, unpaired Student’s t test (F) and a one-way analysis of variance (ANOVA) with the Tukey’s post hoc correction (H to J). WT, wild type.
We assessed a potential neural mechanism through which NHE1 protein levels might be a result of increased ubiquitination, we found that CORT treatment promoted proteasomal degradation of NHE1 protein.

CUL4A overexpression reduces NHE1 expression and ameliorates maladaptive neuroplasticity in CA1 hippocampus

To further evaluate the pathological significance of the CUL4A/NHE1 pathway in depression, we bilaterally injected the AAV-DIO-CUL4A-eGFP (enhanced green fluorescent protein) virus into the CA1 hippocampus of Thy1-Cre mice as a means to specifically overexpress CUL4A in pyramidal neurons (Fig. 6, A and B). Increased levels of CUL4A expression in CA1 pyramidal neurons were confirmed with Western blot assays (Fig. 6C). When measuring pHi levels of pyramidal neurons in these mice, we found that an overexpression of CUL4A decreased pHi levels (Fig. 6, D and E) and reduced the activity of GOGAT (Fig. 7A). To assess possible involvement of the CUL4A/NHE1 pathway in regulating neuroplasticity within primary cultured hippocampal neurons, we used the CORT-induced
Fig. 4. NHE1 knockdown results in decreased pHi and increased synaptic plasticity damage in CA1 hippocampal neurons. (A) Representative images of pHrodo Red staining in CA1 hippocampal neurons. (B) Quantification analysis showing the pHi in CA1 pyramidal neurons. WT: 1.00 ± 0.06, AAV-eGFP: 1.14 ± 0.11, and AAV-NHE1 shRNA: 1.58 ± 0.08. N = 6 per group. P = 0.0084, AAV-NHE1 shRNA versus AAV-eGFP. (C) Golgi-stained CA1 hippocampal neurons. (D) Reconstructions of pyramidal neurons. (E and F) Quantification of dendritic intersections. N = 15 neurons from five rats per group. WT: 87.00 ± 5.36, AAV-eGFP: 88.83 ± 2.56, and AAV-NHE1 shRNA: 60.67 ± 3.05. P = 0.0003, AAV-NHE1 shRNA versus AAV-eGFP. (G) Representative images of dendrites in pyramidal neurons. (H) Quantification of mushroom spines in neurons. WT: 78.39 ± 3.68, AAV-eGFP: 77.21 ± 4.55, and AAV-NHE1 shRNA: 54.36 ± 4.92. N = 45 dendritic segments from six rats per group. P = 0.0062, AAV-NHE1 shRNA versus AAV-eGFP. (I) Raw traces of mEPSC in neurons. (J) NHE1 knockdown decreased amplitudes of mEPSC. WT: 14.78 ± 0.45, AAV-eGFP: 14.57 ± 0.31, and AAV-NHE1 shRNA: 9.22 ± 0.39. P < 0.001, AAV-NHE1 shRNA versus AAV-eGFP. (K) NHE1 knockdown decreased frequencies of mEPSC. WT: 4.23 ± 0.34, AAV-eGFP: 3.61 ± 0.31, and AAV-NHE1 shRNA: 2.02 ± 0.11. P = 0.0024, AAV-NHE1 shRNA versus AAV-eGFP. (L) Raw traces of sEPSC. (M) NHE1 knockdown decreased amplitudes of sEPSC. WT: 31.30 ± 2.62, AAV-eGFP: 29.98 ± 2.85, and AAV-NHE1 shRNA: 18.60 ± 1.84. P = 0.0142, AAV-NHE1 shRNA versus AAV-eGFP. (N) NHE1 knockdown decreased frequencies of sEPSC. WT: 4.95 ± 0.41, AAV-eGFP: 4.39 ± 0.40, and AAV-NHE1 shRNA: 2.14 ± 0.14. P = 0.0008, AAV-NHE1 shRNA versus AAV-eGFP. N = 5 to 6 cells from five rats per group in electrophysiological recordings. *P < 0.05, **P < 0.01, and ***P < 0.001.
cell stress model and found that both CUL4A and NHE1 were present in cultured hippocampal neurons and colocalized in the ER (fig. S7, B and C). Of perhaps greater significance, the overexpression of CUL4A reduced the total number and cumulative length of neurites in these primary cultured neurons (fig. S8, A to C), thereby producing similar stress phenotypes as that resulting from CORT treatment (fig. S8, D to F). Accordingly, the frequency and amplitude of sEPSCs from whole-cell patch-clamp recordings on brain slices were decreased with the overexpression of CUL4A (Fig. 6, F to H), and the number of evoked APs were also reduced when tested with identical injected currents (Fig. 6, I and J). Last, we observed an increased susceptibility to CUMS exposure with an overexpression of CUL4A in the CA1 hippocampus, as only a 2-week exposure to CUMS was sufficient to produce a decrease in the percent of sucrose

![Image of figure 5](image)

**Fig. 5.** CUMS activates CUL4A and ubiquitination degrades NHE1 protein within CA1 hippocampus. (A) Levels of NHE1 mRNA were validated. Ctrl: 1.00 ± 0.05 versus CUMS: 0.91 ± 0.04, \( P = 0.1859, N = 12 \) per group. (B) GO function classification annotations indicated that the differentially expressed proteins appeared to be related with the posttranslational modification pathway. (C) Heatmap diagram of differentially expressed protein levels as related with posttranslational modification. (D) Chloroquine (CQ; 40 \( \mu M \)) failed to prevent CORT-induced (10 \( \mu M \)) decreases of NHE1 expression levels. Ctrl: 1.08 ± 0.06, CORT: 0.65 ± 0.08, and CORT + CQ: 0.73 ± 0.05, \( N = 6 \) per group. (E) MG132 (20 \( \mu M \)) reversed the CORT-induced (10 \( \mu M \)) decreases of NHE1 expression levels. Ctrl: 0.92 ± 0.08, CORT: 0.45 ± 0.11, and CORT + MG132: 1.06 ± 0.07. \( N = 6 \) per group. (F) CUMS exposure increased CUL4A protein expression levels. Ctrl: 1.00 ± 0.06 and CUMS: 1.34 ± 0.05, \( P = 0.0019, N = 6 \) per group. (G) CUL4A and NHE1 were colocalized within the ER in primary cultured hippocampal neurons. **\( P < 0.01 \) and ***\( P < 0.001 \) by a two-tailed, unpaired Student’s t test (A and F) and a one-way ANOVA with the use of the Tukey’s post hoc correction (D and E).
**Fig. 6.** CUL4A overexpression produces maladaptive plasticity in CA1 hippocampal pyramidal neurons. (A) Experimental paradigm for viral construction and injection in Thy1-Cre mice. (B) Representative images of injection sites in CA1 hippocampus. Scale bar, 20 µm. (C) Expression of CUL4A (AAV-eGFP: 1.00 ± 0.05 versus AAV-CUL4A: 1.32 ± 0.08; \(P = 0.0074\)) and NHE1 (AAV-eGFP: 1.00 ± 0.09 versus AAV-CUL4A: 0.49 ± 0.10; \(P = 0.0033\)) in CA1 pyramidal neurons. (D) pHrodo Red staining in CA1 hippocampal neurons. (E) CUL4A overexpression decreased pH in neurons. AAV-eGFP: 1.00 ± 0.05 versus AAV-CUL4A: 1.50 ± 0.06. \(P < 0.001\), \(N = 6\) per group. (F) Raw traces of electrophysiological recordings. (G) CUL4A overexpression decreased amplitudes of sEPSC. AAV-eGFP: 1.00 ± 0.05 versus AAV-CUL4A: 1.50 ± 0.06. \(P = 0.0070\), \(N = 5\) to 6 cells from five rats per group. (H) CUL4A overexpression decreased the frequencies of sEPSC. AAV-eGFP: 7.52 ± 0.39 versus AAV-CUL4A: 4.12 ± 0.41. \(P = 0.0001\), \(N = 5\) to 6 cells from five rats per group. (I) Raw traces of evoked APs from injected currents (200 pA). (J) CUL4A overexpression decreased frequencies of APs. AAV-eGFP: 5.00 ± 0.84 versus AAV-CUL4A: 1.75 ± 0.48. \(P = 0.0166\), \(N = 4\) to 5 cells from four rats per group. (K) CUL4A overexpression decreased the percent of sucrose consumption. AAV-eGFP: 95.50 ± 0.32 versus AAV-CUL4A: 79.39 ± 3.14. \(P = 0.0009\), \(N = 5\) per group. (L) CUL4A overexpression increased immobility times in FST. AAV-eGFP: 26.17 ± 6.26 versus AAV-CUL4A: 109.20 ± 36.28. \(P = 0.0478\), \(N = 6\) per group. *\(P < 0.05\), **\(P < 0.01\), and ***\(P < 0.001\) by a two-tailed, unpaired Student’s t test.
consumption in these mice (Fig. 6K), a significant increase in immobility times in the FST (Fig. 6L), a decrease in the time at the center area of the OFT (fig. S9, A to C), and a decrease in the time and number of entries within the open arms of the EPM (fig. S9, D and E). All responses would normally require a 5-week regimen of CUMS treatment.

In summary, these results provide strong evidence indicating that CUL4A may prompt ubiquitination and degradation of NHE1 protein to decrease the pH value of neurons. Again, these effects would, in turn, have the potential of producing an increase in intracellular acidification, thus disturbing synaptic transmission in excitatory pyramidal neurons within the CA1 hippocampus to result in the pathogenesis of depressive symptoms induced by stress exposure.

**DREADD inhibition of CA1 hippocampal pyramidal neurons promotes depression-like behaviors**

To investigate whether the activity of pyramidal neurons in the hippocampus is required for protection from stress-induced depression or anxiety-like behaviors, we used the designer receptors exclusively activated by designer drugs (DREADD) method to inhibit CA1 pyramidal neurons by injecting an AAV-DIO-hM4Di-mCheery into Thy1-Cre mice to express the engineered Gi-coupled human M4 muscarinic designed (hM4D) receptor (Fig. 7A). Results from electrophysiological recordings indicated that clozapine-N-oxide (CNO; 5 mM) significantly increased spike thresholds and decreased spike numbers under current step injections in hM4D-expressing hippocampal CA1 pyramidal neurons. These findings suggest that the activity of these CA1 pyramidal neurons was suppressed (Fig. 7, B to D). We then injected CNO (5 mg/kg) intraperitoneally into mice to bilaterally silence the pyramidal neuronal expression of hM4Di (Fig. 7E). This CNO treatment significantly increased the immobility times of mice in the FST (Fig. 7F) and in the tail suspension test (TST) (Fig. 7G) and produced a reduction in the exploration of the center area in the OFT (Fig. 7, H and I). To further assess the activity of CA1 neurons in response to stress, we constructed an additional AAV-CaMKII-hM4Di-mCheery vector, which was bilaterally injected into the CA1 region of rats to inhibit excitatory neurons (fig. S10A). Whole-cell recordings revealed that this CNO treatment decreased CA1 neuronal activity (fig. S10, B to D). Accordingly, inhibition of CA1 excitatory neurons with CNO produced depression-like behaviors in rats (fig. S10, E and F). However, inhibition of CA1 excitatory neurons with CNO has no effects on the anxiety-like behaviors in rats (fig. S10, G to L). In addition, the pH level in CA1 excitatory neurons has no change after activation of hM4Di receptors (fig. S10, M and N). Therefore, suppressing the activity of excitatory pyramidal neurons within CA1 hippocampus may then increase the susceptibility to stress in rats.

**DISCUSSION**

It is well known that neural circuits and connections are in a continuous state of reorganization and modification throughout life, which enables for adaptability and remodeling of the structure and function of the brain (34, 35). In this regard, neuronal plasticity serves as the neural basis for the body to adapt to internal and external environmental changes (36). Although there exists considerable evidence demonstrating maladaptive neuroplasticity in the brain of depressed individuals, the significance of this process in the pathogenesis and treatment of depression remains unclear (37). In particular, critical questions that remain unanswered include the mechanisms through which stress exposure leads to maladaptive neuroplasticity, the structural and functional abnormalities resulting from maladaptive neuroplasticity, and identification of previously unidentified strategies for early intervention or later treatment of this process. In the present study, we found that chronic stress induced a degradation of NHE1 by activating E3 ubiquitin ligase CUL4A, resulting in intracellular acidification of pyramidal neurons within the CA1 hippocampus. This disturbance in acid-base homeostasis may, in turn, disrupt processes involved with normal synaptic plasticity through multiple pathways and thus contribute to the pathogenesis of depression.

Neuronal synaptic-related sites are key targets for plasticity regulation. Synaptic plasticity involves a variety of processes including complex changes in dendritic branching, dynamic changes of dendritic spines, alterations in the number and structure of synapses, synthesis and release of presynaptic transmitters, and functional expressions of receptors (30, 38). Therefore, we speculated that there might exist a critical trigger that could regulate synaptic plasticity through multiple downstream mechanisms. If so, then this initiating trigger should be at the cellular level, with one possible cellular response being a change in the homeostatic characteristics of the neuron. A fundamental aspect for the maintenance of normal cell functioning is the stability of the pH, and it has been reported that acidosis induces a variety of neuronal injuries in cultured primary neurons (39). In the present study, we found that chronic stress leads to structural plasticity changes as indicated by a reduction in dendritic complexity, mature dendritic spines, and synaptic densities within the CA1 hippocampal area of the rat brain. We found that associated with these structural changes was a significant reduction in pH levels, indicating a mechanistic link and potentially important risk factor for the synaptic plasticity changes observed in response to chronic stress.

NHE1 is a major determinant of pH in mammalian central neurons (40). The findings that a selective destruction of the CHPl-mediated NHE1 biosynthetic maturation leads to axonal degeneration suggest that pH homeostasis regulated by NHE1 is required for Purkinje cell axonal survival (41). Results from pharmacological studies have shown that inhibition of NHE1 reduces excitatory synaptic transmission and plays a role in epilepsy and developmental brain disorders (42), and SLC9A1 mutations produce cerebellar ataxia in mice (26). Moreover, results from recent studies have shown that genetic mutations of the human NHE1 can lead to severe functional defects such as impaired neurodevelopment in the brain (43). Together, the findings from these studies indicate that NHE1 can target multiple cellular and molecular pathways involved in neuronal development and synaptic plasticity and may play a key role in the pathogenesis and treatment of neurological and psychiatric diseases. In the present study, we found that the expression of the NHE1 protein was significantly decreased in the CA1 hippocampus of CUMS rats as demonstrated using proteomic analysis. These results suggest that the intracellular acidification resulting from a decrease in NHE1 may be a significant underlying mechanism leading to depression. Furthermore, NHE1 knockout in hippocampal pyramidal neurons resulted in a reduction of dendritic spines and synapses along with other structural abnormalities, changes that were accompanied with a decrease in the frequency and amplitude of mEPSC and frequency of APs. Accordingly, synaptic transmission is markedly impaired with NHE1 knockout. Moreover, results from our behavioral tests revealed that these NHE1 knockout rats were more susceptible to
stress, as they more readily exhibited core symptoms of depression, such as anhedonia and behavioral despair, in response to a relatively limited CUMS exposure. These above results provide strong evidence that NHE1 is probably related to neuroplasticity damage and the induction of depressive behaviors. More specifically, the cascade of events in this process would consist of a stress exposure that can lead to an acidic intracellular environment through the down-regulation of NHE1 protein expression to then produce the CA1 hippocampal neuroplasticity damage involved with the pathogenesis of depression.

To elucidate the mechanisms leading to the down-regulation of NHE1 protein levels in response to stress exposure, we coanalyzed proteomics and RNA sequencing. Results from these analyses showed...
that the NHE1 protein, but not mRNA expression, was significantly reduced in the CA1 hippocampus of depressed rats. These results suggest that these changes in NHE1 protein expression levels may result from a posttranscriptional or posttranslational process. Ubiquitination, which is an important posttranslational modifier of proteins, plays an important role in the progression of many diseases, and a variety of E3 ubiquitin ligases have been found to regulate the pathogenesis of disease. However, there are few reports on ubiquitin modification in the pathogenesis of depression. A gene that plays crucial roles in the posttranslational modification of cellular proteins involving ubiquitin is the cullin gene, which is from a family of evolutionarily conserved genes whose cullin proteins are molecular scaffolds (44). CUL4A acts as a core member of E3 ubiquitin ligase and has been reported to recognize, ubiquitinate, and degrade proteins through the UPS, an important pathway for intracellular protein degradation, to regulate multiple physiological processes (45, 46). Results from our proteomic analysis revealed that the expression of CUL4A was significantly increased in the CA1 hippocampus of depressed rats. Most prior reports on CUL4A have focused on tumor genome stability (46), cell cycle regulation (47), occurrence and development of malignant tumors (48), embryonic development (49), hematopoietic stem cells (50), and glioma invasion (51). However, the role of CUL4A in the pathogenesis of neurological diseases has received increasing attention lately. For example, CUL4A ubiquitin ligase complexes have been reported to degrade Ca2+ voltage-activated K+ channels by ubiquitination and reduce their expression in cell membranes, thereby reducing neuronal excitability and preventing epileptogenesis (52). In addition, the CUL4 E3 ubiquitin ligase can ubiquitinate the Chloride channel 2 (CLC-2) voltage-gated chloride channel through the proteasome pathway, with deficiencies in these channels contributing to vacuoles in neuronal myelin sheaths and white matter dystrophy (53, 54). These studies suggest that CUL4A may regulate the expression or activity of functional proteins such as ion channels through posttranslational modification in neurological diseases. However, whether ubiquitination degrades NHE1 and is involved in depression remains unclear. In the present study, we provide evidence that CUL4A and NHE1 were colocalized in the ER and that CUL4A was found to ubiquitinate and proteasomally degrade NHE1 protein, effects that can induce maladaptive neuroplasticity and significantly increase susceptibility to negative stress. These results suggest that stress may induce maladaptation of synaptic plasticity in CA1 hippocampus by activating CUL4A, which then targets the degradation of NHE1 via ubiquitination modification.

The hippocampus is composed of a heterogeneous group of neurons, including glutamatergic excitatory neurons and γ-aminobutyric acid inhibitory (GABAergic) interneurons, which together maintain a delicate balance between excitation and inhibition to prevent dysfunction (55). This excitation/inhibition balance can be disturbed with decreased glutamate production, as can occur in response to intracellular acidification induced by reductions in the activity of GOGAT (10). In this study, we found that a specific overexpression of CUL4A in CA1 hippocampal pyramidal neurons resulted in decreased GOGAT activity and glutamate synthesis. These chemical/genetic results then demonstrate that inhibition of CA1 hippocampal pyramidal neurons can exacerbate depression-like behavior in animals, suggesting an association between the excitability of hippocampal neurons and induction of depressive behavioral phenotypes. Therefore, we hypothesized that a reduction in glutamate synthesis may also be one of the consequences resulting from NHE1 deficits, effects that can then weaken the synaptic transmission efficiency of pyramidal neurons. Thus, the regulation of CUL4A and NHE1 activity may provide a means to modulate the destructive effects of stress exposure on neuronal morphology and transmission to effectively ameliorate depressive behavior.

However, it should be noted that NHE1 has also been considered to serve as an anchor for the actin cytoskeleton and as a scaffold for the assembly of macromolecular signaling complexes (23, 24, 56). NHE1 can act as an upstream regulator of rho guanosine triphosphatases to promote neurite outgrowth (57). Accordingly, although our present results demonstrate that intracellular acidification is undoubtedly an important risk factor responsible for maladaptive neuroplasticity, it is also possible that NHE1 may affect neuroplasticity by regulating the intracellular signaling pathway. In this study, we did not overexpress NHE1 in CA1 hippocampal neurons of depressed rats to observe whether this treatment would ameliorate depression-like behaviors as it currently remains difficult to maintain the activity of NHE1 at appropriate levels by exogenous intervention. As shown in previous studies, an elevated activity and expression of NHE1 leads to a higher influx of sodium, which then becomes coupled with a reverse mode activity of the Na+/Ca2+ exchanger. These effects result in a detrimental influx of calcium that affects various cell signaling pathways and promotes cell death (58). We hope that previously unexplored and improved activation methods will become available in the future for the treatment of diseases in which NHE1 is inactive.

In conclusion, in this report, we provide evidence for elucidating the mechanisms leading to maladaptive neuroplasticity and depression in response to stress exposure and provide concepts for the discovery and screening of previously unidentified antidepressant targets as treatment strategies for depression. In specific, we demonstrate here that stress exposure may regulate synaptic plasticity involved in the pathogenesis of depression by activating CUL4A and thus prompting ubiquitination and degradation of NHE1. This follows from our results demonstrating that knockdown of NHE1 promotes an instability and loss of the structure and function of dendritic spines in CA1 hippocampal pyramidal neurons, a crucial neurological trigger for induction of depression-like behaviors. These effects appear to, in part, be due to an increase in intracellular acidification from NHE1 deficits, resulting from the ubiquitin modification of CUL4A. In this way, maintenance of pH i homeostasis by targeting the regulation of expression or function of NHE1 may provide a unique therapeutic approach to improve the efficacy of existing antidepressant therapies.

MATERIALS AND METHODS

Animals
Male Wistar rats (120 to 140 g; purchased from Charles River Laboratories) and male Thy1-Cre mice (6 to 8 weeks of age, purchased from OBiO Technology) were used in this study. Animals were group-housed five per cage for mice and four per cage for rats under a 12-hour light/12-hour dark cycle (lights on from 07:00 to 19:00) and had ad libitum access to food and water. All experimental procedures were approved by the Ethics Committee of Shandong University and strictly abided by the Institutional Animal Care and Use Committee.

CUMS model
The CUMS-induced animal model of depression was used as previously described (7). Male Wistar rats were exposed to a variable sequence of chronic unpredictable mild stressors, including food or water...
deprivation (24 hours), overnight illumination, cold on ice (5 min), wet bedding (24 h), titled cage (24 hours), tail clip (1 min), physical restraint (2 hours), foot shock (30 min), noise (2 hours), and shaking (1 hour). One stressor was applied daily in a random sequence for 5 weeks. Behavioral tests were then used to assess depression-like behaviors in these animals.

**Behavioral tests**
All behavioral tests were performed during the dark phase of the light/dark cycle (18:00 to 23:00 for mice and 19:00 to 24:00 for rats).

**Sucrose preference test**
The SPT was performed as previously described with minor modifications (7, 59). Animals (mice or rats) were first individually housed and then habituated with two bottles of sucrose (1% sucrose for mice and 2% sucrose for rats) for 24 hours followed by one bottle of sucrose and one bottle of water for 24 hours. After 24 hours of water deprivation, animals were exposed to two bottles filled with either sucrose or water for 24 hours (mice) or 3 hours (rats). The bottle position was reversed after 12 hours (mice) or 1.5 hours (rats). Sucrose preference was defined as sucrose consumption/[water consumption + sucrose consumption] × 100%.

**Forced swim test**
The FST was conducted as previously described with some modifications (59, 60). Animals were individually placed in a glass cylinder (12 cm in diameter and 25 cm in height for mice; 20 cm in diameter and 40 cm in height for rats) filled with water (25°C ± 2°C) for 6 min. The depth of water was set to prevent animals from their hindlimbs or tail touching the bottom. Immobility was defined as time when animals remained floating or motionless, only exposing their head above the water surface, and the immobility times were recorded during the last 4 min of 6-min test period. For rats, the procedure for this test consisted of an additional 15-min swim period on the day before testing.

**Open-field test**
The OFT was based on a previously described procedure (60). In the OFT, animals were placed in the center of the arena (40 cm by 40 cm by 40.5 cm for mice and 100 cm by 100 cm by 40 cm for rats) for 5 min. A video camera positioned directly above the arena was used to track their movements. The time spent in the central area, the number of central-zone entries crossed, and the total distance traveled were recorded.

**Elevated plus maze**
The EPM test was conducted as previously described (61). For rats, the crossed maze was elevated 50 cm above the floor, with two open arms and two closed arms. Each arm was 50 cm in length and 10 cm in width, and the arms were interconnected by a central platform. For mice, the maze was placed 40 cm above the floor, with two open arms, two closed arms, and a central platform. The length and width of each arm were 30 and 5 cm, respectively. Animals were individually placed in the central platform and allowed to explore the maze for 5 min. A video camera was positioned over the maze and recorded their movements via a computerized video tracking system (EthoVision XT11.5, Noldus, The Netherlands). To prevent any effects of interference from the testing of previous animals, the maze was cleaned with 75% ethanol after each animal was tested. The time spent in the open arms and the number of open arm entries were recorded.

**Tail suspension test**
The TST was conducted as previously described (62). Mice were individually suspended at 20 cm above the floor with tape placed 15 cm from the distal end of their tail. Immobility times, defined as an absence of body movement, was recorded for the last 4 min of 6-min test.

**Drugs/reagents**
For in vivo silencing of hippocampal neurons in animals, CNO (5 mg/kg for mice and 3 mg/kg for rats) was injected intraperitoneally into animals expressing AAV2/9-hSyn-DIO-hM4D(Gi)-mCherry or AAV2/9-CaMKIIα-mCherry, and behaviors were tested at 30 min after the CNO injection (63). For in vitro experiments, 24 hours before CORT (10 μM; MedChemExpress, MCE) or chloroquin (40 μM; Sigma-Aldrich) treatment, primary hippocampal neurons from rats were treated with MG132 (10 μM; MCE) for 6 hours (52). Lysates were then assayed using co-immunoprecipitation and Western blot.

**Stereotaxic surgery and virus injection**
The procedure for stereotaxic surgery and virus injection was adopted from that as described in a previous study (7). Briefly, rats or mice were deeply anesthetized with pentobarbital sodium and then positioned in a stereotaxic apparatus (Stoelting, USA). Viruses were bilaterally injected into CA1 hippocampal regions for rats: anterior-posterior (AP), −3.24 mm from bregma; medial-lateral (ML), ±1.8 mm; dorsal-ventral (DV), −3.2 mm; and for mice: AP, −2 mm from bregma; ML, ± 1.5 mm; DV, −1.5 mm) with the use of an electric microinjection pump (Stoelting, USA) at a flow rate of 0.05 μl/min. After injections, the pipette remained at the insertion site for an additional 10 min and then was slowly retracted to prevent virus backflow upon retraction. The incision was closed, and animals were allowed to recover from anesthesia while under a heat pad and housed for 3 days post-operatively to further study. Injection sites were verified by immunofluorescence assays after completion of all behavioral tests, and only data from animals with correct injection site placements were used for subsequent experiments.

The following vectors were used: AAV2/9-CaMKIIα-NHE1 shRNA-eGFP [1.32 × 10^{13} viral genomes (v.g.)/ml], AAV2/9-CMV-DIO-CUL4A-eGFP (1.14 × 10^{13} v.g./ml), AAV2/9-CaMKIIα-NHE1-hm4D (Gi)-mCherry (2.07 × 10^{13} v.g./ml), and AAV2/9-hSyn-DIO-hm4D (Gi)-mCherry (3.21 × 10^{12} v.g./ml). All vectors used in this study were purchased from OBIO Technology and stored at −80°C until use.

**Cytosolic pH imaging**
PHi was determined with use of the pHrodo Red AM Intracellular pH Indicator (P35372, Thermo Fisher Scientific) according to the manufacturer’s protocol and previous study (64). The reagent is weakly fluorescent at neutral pH but increases its fluorescence intensity with a drop in pH value. The reagent is sensitive to pH change in the range of pH 4.0 to 9.0. The lower the pH value, the higher the intensity of red fluorescence. Modification of pHrodo Red with acetoxy-methyl (AM) ester groups results in an uncharged molecule that can permeate cell membranes. Once inside the cell, the lipophilic blocking groups are cleaved by nonspecific esterases, resulting in a compound that is retained with the intracellular space. With the help of confocal microscopy, the pH change can be detected.

For pHi determinations in the brain, acute preparations of hippocampus slices were generated as described in the procedure of electrophysiological recordings. Briefly, brains were rapidly sectioned in the cutting solution (pH 7.40) continuously infused with 95% O_{2}/5% CO_{2}
using a vibratome (Leica VT1200S, Germany). Slices were then quickly transferred to recovery solution (10 ml) continuously infused with 95% O₂/5% CO₂. The pHrodo Red dye (10 μl) was added to PowerLoad concentrate (100 μl), which facilitates the cellular loading of AM esters. The mixed dye solution was diluted in the recovery solution containing brain slices for staining at 37°C for 30 min. Then, the brain slices were washed with 1× phosphate-buffered saline (PBS), and each slice was sealed with cover slides. Fluorescent images were captured using excitation of 560 nm/emission of 585 nm with the use of a high-speed confocal platform (Dragonfly 200, Andor). The fluorescence intensity in cells was quantified using the ImageJ software.

**Golgi staining**

Rat brains were collected and processed for the Golgi-Cox staining according to the protocol provided from the FD Rapid Golgi Stain Kit (PK401, FD NeuroTechnologies). Tissues were sectioned into 150-μm coronal slices using a vibratome (Leica VT1200S, Germany), then collected on gelatin-coated microscope slides, dehydrated with a gradient series of alcohols, cleared with xylene, and cover-slipped. Stained pyramidal neurons from the hippocampal region were examined, and images were captured using an Olympus microscope (VS1200, Japan). Dendritic spine densities were calculated as the number of dendritic spines per 10 μm of dendrite length. All image processing was performed using Fiji (ImageJ, National Institutes of Health).

**Sholl analysis and dendritic spine counting**

The complexity of dendritic arbors from Golgi staining–labeled neurons of the CA1 hippocampus was assessed using the Sholl analysis, as previously described (65). Images were captured with the use of SLIDEVIEW VS200 (VS200, Olympus). The images were imported into the Fiji software (win64). Dendritic complexity was analyzed from 8-bit images according to the procedure in the website of Fiji software. On average, three to four pyramidal neurons per animal from six rats were randomly selected for Sholl analysis.

**Electron microscopy**

Samples of hippocampal CA1 tissues (1 mm by 1 mm by 1 mm) were quickly deposited in a fixative solution (2.5% glutaraldehyde) in cacodylate buffer overnight. Tissues were dehydrated in graded ethanol solutions, followed by immersion in propylene oxide overnight, and embedded with 100% Epon. Ultrathin sections (60 nm) were cut using an ultramicrotome (Leica), and tissues slices were stained with 4% uranyl acetate for 20 min and 0.5% lead citrate for 5 min with periodic agitation. For immunoprecipitation, 1 to 2 μg of specific antibodies were added to the 1 mg of protein to form an immune complex at 4°C overnight, which was then added to the Protein A/G PLUS-Agarose (26146, Thermo Fisher Scientific). The complex was washed to remove nonbound material, and 1× conditioning buffer was used to dissociate the bound immune complex from the Protein A/G. The immunoprecipitates were separated with SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and assayed using Western blot.

**Hippocampal slice preparation and electrophysiological recordings**

Hippocampal slices from the animals used for electrophysiology recordings were obtained at 3 to 4 weeks after viral injection. Animals were decapitated and brains rapidly removed. Samples of brain slices (300 μm) were sectioned in the following cutting solution: 119 mM choline chloride, 30 mM glucose, 26 mM NaHCO₃, 7 mM MgSO₄, 2.5 mM KCl, 1 mM Na₂HPO₄, 1 mM CaCl₂, 3 mM sodium pyruvate, 1.3 mM sodium 1-ascorbate, and 1 mM kynurenicacid on a vibratome (VT1200s, Leica, Germany) vibrating at a velocity of 0.18 mm/s. Brain slices were maintained for at least 30 min at 33°C in a recovery solution containing 85 mM NaCl, 24 mM NaHCO₃, 4 mM MgCl₂, 2.5 mM KCl, 1.25 mM Na₂HPO₄, 0.5 mM CaCl₂, 25 mM glucose, and 50 mM sucrose and then at room temperature until use. All solutions were continuously infused with 95% O₂/5% CO₂. Whole-cell recordings were obtained using glass pipettes (4 to 6 meqhosm) filled with an internal solution (130 mM CsMeSO₄, 10 mM CsCl, 4 mM NaCl, 1 mM MgCl₂, 5 mM Mg-ATP (adenosine 5′-triphosphate), 5 mM EGTA, 10 mM Hepes, 0.5 mM Na₃GTP (guanosine 5′-triphosphate), 10 mM phosphocreatine, and 4 mM QX-314 with a pH of 7.35)
and an MultiClamp 700B amplifier. For mEPSC recordings, neurons were clamped at −60 mV in the presence of tetrodotoxin (1 μM) and picrotoxin (100 μM) in the artificial cerebrospinal fluid (ACSF). The intracellular solution contained 115 mM CsMeSO4, 20 mM CsCl, 10 mM Hepes, 2.5 mM MgCl2, 4 mM Na2-ATP, 0.4 mM Na-GTP, 10 mM Na-phosphocreatine, and 0.6 mM EGTA. Data were filtered at 2 kHz and sampled at 10 kHz using Digidata 1440A. The sEPSCs were recorded at a holding potential in the ASCF. For CNO treatments, after applying currents in steps of 20 pA, ranging from −100 to 300 pA, neurons were rested for 5 min before the brain slices were perfused with ACSF containing 5 μM CNO. The same current–clamp procedure was performed at 10 min after CNO perfusion. Data were analyzed with the use of the Mini Analysis Program (Synaptosoft).

Immunofluorescence
Immunofluorescent staining was performed according to previously established protocols (8). Briefly, animals were deeply anesthetized (mice, 8 ml/kg, 1% pentobarbital sodium; rats, 4 ml/kg, 2% pentobarbital sodium) and perfused intracardially with 0.9% saline followed by 4% paraformaldehyde in PBS. Brains were removed and post-fixed in 4% paraformaldehyde overnight, followed by transfer to a 10 to 30% sucrose gradient for 3 days at 4°C. Frozen coronal hippocampal sections were cut at a thickness of 30 μm with the use of a freezing microtome (CM1860, Leica) and stored at −20°C in cryoprotectant solution. Slices were washed in PBS for further immunofluorescent staining involving fixing of the cells in 4% paraformaldehyde for 15 min, followed by rinsing in PBS for further use. Antibodies used included rabbit anti-NHE1 (1:200; Affinity), rabbit anti-CUL4A (1:200; Proteintech), mouse anti-NHE1 (1:200; Santa Cruz Biotechnology), rabbit anti-microtubule-associated protein-2 (MAP2) (1:100; Cell Signaling Technology), mouse anti-MAP2 (1:500; Abcam), rabbit anti-VGLUT1 (1:500; Abcam), mouse anti-PSD-95 (1:500; Santa Cruz Biotechnology), Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG) (1:200; Proteintech), and Alexa Fluor 594 donkey anti-rabbit IgG (1:1000; Invitrogen). All slices or cells were counterstained with Hoechst in the final step of incubation. Fluorescent images were captured using a high-speed confocal platform (Dragonfly 200, Andor). Data were excluded from samples with incorrect infusion locations. For live-cell imaging, primary neuronal cells were washed with Hanks’ balanced salt solution and ER-Tracker Blue-White DPX (E12353, Thermo Fisher Scientific) was added to the reaction data, and quantified mRNA data were normalized to GAPDH.

Western blotting
Hippocampus CA1 regions were rapidly dissected, and tissue samples were immediately frozen in liquid nitrogen and stored at −80°C. Proteins were isolated from fresh cell and frozen brain tissue by sonication with lysis buffer containing protease and phosphatase inhibitors. Protein samples were quantified with the use of the bicinchoninic acid assay (Cwbio, China) and heated at 100°C for 5 min in loading buffer. Protein samples (20 μg) were loaded on each lane and electrophoretically separated on SDS-PAGE gels followed by transfer to polyvinylidene difluoride membranes for Western blot assay. Antibodies used included anti-NHE1 (1:500; Santa Cruz Biotechnology), anti-NHE1 (1:1000; Affinity), anti-CUL4A (1:1000; Proteintech), anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000; Proteintech), and anti–horseradish peroxidase antibodies (1:5000; BioWorld). The Enhanced Chemiluminescence Kit (Thermo Fisher Scientific) was used to detect bands on the membranes. ImageJ software was used to quantify the optical densities of detected bands, and results were presented as the percent of control after normalization.

RNA preparation and quantitative real-time polymerase chain reaction
In this study, the total RNA of tissues was isolated from hippocampal tissues using the mirNeasy Micro Kit (QIAGEN, 217084) according to the manufacturer’s protocol and then reverse-transcribed into complementary DNA (cDNA) using the HiScript II First Strand cDNA Synthesis Kit (Vazyme, Nanjing). Specific primer sequences were used for the amplification of NHE1 (forward primers: 5’-CTGCAGTCTG-GACGTCTTCTT-3’; reverse primer: 5’- GTTCTCCGTGAACTG-CCTCA-3’), CUL4A (forward primer: 5’- AAAATGGGCA CCGGCATAGA-3’; reverse primer: 5’- CACCTCTCTCCCTTGG-GGAC-3’), and GAPDH (forward primer: 5’-TCTGCTGCTCT-CCTGTC-3’; reverse primer: 5’- ACACCGACCTCACCATCT-3’). Bio-Rad’s iQ5 software was used to analyze the polymerase chain reaction data, and quantified mRNA data were normalized to GAPDH.

Statistical analysis
All data were expressed as the means ± SEMs, and statistical analyses were performed using GraphPad Prism software (version 8.0), with statistical significance set at P < 0.05. Comparisons between two independent groups were analyzed using independent group Student’s t tests (two tailed). Comparisons between three or more groups were analyzed using one-way or two-way analysis of variance (ANOVA), followed by Tukey’s post hoc test for multiple comparisons.

SUPPLEMENTARY MATERIALS
Supplementary material for this article is available at https://science.org/doi/10.1126/sciadv.add7063

View/request a protocol for this paper from Bio-protocol.

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Acknowledgments: We thank the Translational Medicine Core Facility of Shandong University for consultation and instrument availability that supported this work. Funding: This study was supported by grants to S.Y.Y. from the National Natural Science Foundation of China (NSFC81873796; 82071513) and the Natural Science Foundation of Shandong Province of China (ZR2021MH151). Author contributions: Conceptualization: S.Y.Y., Y.L., and C.F. Methodology: Y.L., C.F., C.W., L.W., and X.C. Investigation: Y.L., C.F., C.W., L.W., Y.Y., X.C., T.L., and W.W. Funding acquisition: S.Y.Y. and X.M. Writing: S.Y.Y., Y.L., and C.F. Supervision: S.Y.Y. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials.

Submitted 28 June 2022
Accepted 23 September 2022
Published 11 November 2022
10.1126/sciadv.add7063