ORIGINAL RESEARCH ARTICLE

STAT3-dependent regulation of the electrogenic Na\(^+\)/HCO\(_3\)\(^-\) cotransporter 1 (NBCe1) functional expression in cortical astrocytes

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Abstract
The electrogenic Na\(^+\)/HCO\(_3\)\(^-\) cotransporter (NBCe1) in astrocytes is crucial in regulation of acid-base homeostasis in the brain. Since many pathophysiological conditions in the brain have been associated with pH shifts we exposed primary mouse cortical and hippocampal astrocytes to prolonged low or high extracellular pH (pHo) at constant extracellular bicarbonate concentration and investigated activation of astrocytes and regulation of NBCe1 by immunoblotting, biotinylation of surface proteins, and intracellular H\(^+\) recordings. High pHo at constant extracellular bicarbonate caused upregulation of NBCe1 protein, surface expression and activity via upregulation of the astrocytic activation markers signal transducer and activator of transcription 3 (STAT3) signaling and glial fibrillary acidic protein expression. High pHo-induced increased NBCe1 protein expression was prevented in astrocytes from Stat3\(^{flox/flox}\)::GfapCre/\(^+\) mice. In vitro, basal and high pHo-induced increased NBCe1 functional expression was impaired following inhibition of STAT3 phosphorylation. These results provide a novel regulation mode of NBCe1 protein and activity, highlight the importance of astrocyte reactivity on regulation of NBCe1 and implicate roles for NBCe1 in altering/modulating extracellular pH during development as well as of the microenvironment at sites of brain injuries and other pathophysiological conditions.

KEYWORDS
acidosis, astrogliosis, bicarbonate, glial cells, pH, signaling

1 | INTRODUCTION

Astrocytes are fundamental for many homeostatic processes within the central nervous system (CNS), thereby critical for its overall integrity (for review, see Verkhratsky & Nedergaard, 2018). Among astroglial homeostatic functions, ionic homeostasis is of particular importance and mediated by the plethora of transport proteins of the SLC family expressed in the astrocytic membrane. SLC4A4, the electrogenic Na\(^+\)/HCO\(_3\)\(^-\) cotransporter 1, NBCe1, is highly expressed in astrocytes and a key molecular component in modulating intracellular-, extracellular- and synaptic pH (reviewed in Deitmer & Rose, 2010). NBCe1 is sensitive to bicarbonate, and operates with a stoichiometry 1Na\(^+\):2HCO\(_3\)\(^-\) in an
inward or outward mode (Theparambil & Deitmer, 2015; Theparambil, Naoshin, Thyssen, & Deitmer, 2015; Theparambil, Ruminot, Schneider, Shull, & Deitmer, 2014; Theparambil et al., 2017). Though mostly investigated in epithelial cells, the physiological importance of NBCe1 in the brain has been demonstrated following mutations of the Slc4a4 gene (Horita et al., 2005; Igarashi et al., 1999, 2001; Suzuki et al., 2010; Toye et al., 2006). Astrocytic NBCe1 activity is shown to be regulated by several stimuli, among them acute extracellular pH changes, metabolic alkalosis, growth factors, and 4-aminopyridine (4AP; Khakipoor et al., 2017; Salameh, Ruffin, & Boron, 2014; Schrödl-Häußel, Theparambil, Deitmer, & Roussa, 2015; Theparambil et al., 2015, 2017). At the molecular level, NBCe1 associated cellular responses within or outside the CNS can be mediated either through direct phosphorylation (J. H. Hong et al., 2013; Khakipoor et al., 2019; Vachel et al., 2018; Yang et al., 2011) or/and by activation of several signaling pathways, such as mTOR, JNK, and Src/ERK signaling (Khakipoor et al., 2019; Namkoong et al., 2015; Schrödl-Häußel et al., 2015).

Signal transducer and activator of transcription (STAT) proteins are transcription factors that become activated mainly by cytokines and growth factors. Upon binding of the cytokine to its cognate receptor, Janus kinase (JAK) is activated by transphosphorylation that in turn recruits and phosphorylates STAT. Subsequently, activated STAT proteins form (hetero)dimers and translocate to the nucleus to drive transcription (reviewed by Levy & Darnell, 2002). The JAK–STAT signaling is involved in several cellular processes, among them cell growth, survival, and differentiation (Bonni et al., 1997) and it is meanwhile well established that activation of the JAK/STAT pathway by IL6-cytokines, like leukemia inhibitory factor (LIF), ciliary neurotrophic factor (CNTF) and others, promotes differentiation of cerebral cortical precursors along a glial lineage (Bonaguidi et al., 2005). Out of the six STAT family members, STAT3 is the one whose constitutive deletion results to embryonic lethality (K. Takeda et al., 1997). STAT3 mediates the gp130 signaling that induces glial differentiation and its conditional deletion results in depletion of white matter astrocytes (S. Hong & Song, 2014). The physiological significance of STAT3 has been investigated in detail in the context of pathophysiological conditions accompanied by astrogliosis, a pathological hallmark of all types of CNS injuries (see review by Burda, Bernstein, & Sofroniew, 2016). Reactive astrocytes play critical roles in maintaining neuronal excitability, in reorganization of neural circuits and in synaptic plasticity after traumatic brain injury. Activation of STAT3 pathway in astrocytes is a commonly broadly triggered signaling pathway for astrogliosis and a marker for such (Herrmann et al., 2008).

Although based on these considerations it would be reasonable to assume that NBCe1 could be an attractive and potent candidate in regulating extracellular microenvironment following brain pathologies, a putative contribution of NBCe1 has not been addressed in detail and the putative underlying molecular mechanisms are unknown.

Since many pathophysiological conditions in the brain are associated with extracellular pH shifts, in the present study, we investigated the effect of prolonged exposure to low or high extracellular pH (pH_e) at constant extracellular bicarbonate concentration ([HCO_3^-]) as potential insults for activation of astrocytes and regulation of NBCe1 in mouse primary hippocampal and cortical astrocytes. We show that activation of STAT3 signaling and glial fibrillary protein (GFAP) expression at high pH_e at constant extracellular bicarbonate concentration is accompanied by upregulation of protein abundance and functional expression of NBCe1. Moreover, high pH_e-induced increased NBCe1 protein expression was prevented in astrocytes from Stat3^flo^/Gfrα1^−/−^ mice. In addition, basal and high pH_e-induced increased NBCe1 functional expression was impaired following inhibition of phosphorylation of STAT3.

These results provide a novel regulation mode of protein and functional expression of NBCe1 that could account for altering/modulating extracellular pH during development, as well as of the microenvironment at sites of brain injuries and other pathophysiological conditions.

## 2 MATERIAL AND METHODS

### 2.1 Antibodies

Following antibodies were used as primary antibodies: rabbit polyclonal anti-SLC4A4 (Alomone, Cat# ANT-075, RRID:AB_2341019 and Abcam, Cat# ab78326, Cat# ab38686 and Cat# ab30323, RRID:AB_777961) for western blots and immunocytochemistry; mouse monoclonal anti-GAPDH (Abcam, Cat# ab8245, RRID:AB_2107448), and anti-EA1 mouse monoclonal (Abcam, Cat# ab18175, RRID:AB_2230953), anti-GFAP mouse monoclonal (Merck Millipore, Cat# MAB360, RRID:AB_11212597), anti-STAT3 mouse monoclonal (Cell Signalling Technology, Cat# 9139, RRID:AB_331757), anti phospho-STAT3 (Tyr705) rabbit monoclonal (Cell Signalling Technology, Cat# 9145, RRID:AB_2491009), anti-Vinculin rabbit polyclonal (Cell Signalling Technology, Cat# 4650, RRID:AB_10559207), and anti-Na+/K+-ATPase mouse monoclonal (Developmental Studies Hybridoma Bank, Cat# a6F, RRID:AB_528092). Following antibodies were used as secondary antibodies for immunofluorescence: goat anti-rabbit or anti-mouse immunoglobulin G (IgG) coupled to AlexaFluor488 or AlexaFluor568 (Santa Cruz Biotechnology, Cat# sc-2005, RRID:AB_631736 or Cell Signalling Technology, Cat#704, RRID:AB_2099233). For western blots, goat-anti-mouse or anti-rabbit IgG coupled to horseradish peroxidase (Jackson ImmunoResearch Labs, Cat# 715-475-151, RRID:AB_2340840 or Thermo Fisher Scientific, Cat# A10042, RRID:AB_2534017) were used as secondary antibodies.

### 2.2 Animals

All protocols were carried out in accordance with German ethical guidelines for laboratory animals and approved by the Institutional Animal Care and Use Committee of the University of Freiburg (authorizations: X-14/16H and X-19/09C). Adult C57BL/6N mice (strain code 027) of either sex were maintained on a 12 hr dark/light cycle with food and water ad libitum. Mice were killed by cervical dislocation, and all efforts were made to minimize suffering.
2.3 | STAT3 conditional knock out (cKO) animals

STAT3loxP/loxP mice were provided by Drs. Takeda and Akira (Osaka University, Osaka, Japan; Takeda et al. (1997) and Takeda et al. (1998). Mice expressing Cre recombinase under the control of the human GFAP promoter, a generous gift from Dr. Messing (Waisman Center, Madison, WI; Zhu et al., 2001), were used to introduce Cre. GFAP-Cre/STAT3 flox/flox, and their respective controls not carrying the GFAP-Cre allele obtained by crossing STAT3loxP/loxP mice with GFAP-Cre/STAT3± mice were used in the experiments.

2.4 | Genotyping

Genotyping was performed using RedTaq Mastermix from Genaxxon (Ulm, Germany). For a 18 µl reaction 9 µl RedTaq Mastermix, 3.9 µl distilled water, 0.7 µl dimethyl sulfoxide and 0.7 µl from each primer were used and 3 µl genomic DNA was used for STAT3 and 2 µl gDNA for GFAP. For detecting the STAT3 wild type (wt) or floxed gene, the following primers were used: 5′-CCTGAAAGACGATTCACTCTGTGAC-3′ as forward primer and 5′-CACACAGCCGATCTCAAACTCTGTCCTCC-3′ as reverse primer. The expected band for the floxed gene is 350 bp, while the expected band for the wt gene is 200 bp. For detecting the GFAP-Cre gene the following primers were used. 5′-ATTACCGTCCTGACGATGCAGT-3′ as forward primer and 5′-CAGG TATCTCGACGAGATCTCA-3′ as reverse primer. If CRE present, a band at 814 bp was detectable. Polymerase chain reaction (PCR) was performed with following cycle conditions: denaturation at 93°C for 30 min, 35 cycles of PCR amplification at 93°C for 30 s and 67°C for 60 s and elongation at 74°C for 60 s were followed by 74°C for 10 min. PCR products were run on a 2% agarose gel in Tris-acetate-EDTA buffer at 100 V, and then photo documented using a UV transiluminator.

2.5 | Cell culture

2.5.1 | Mouse primary hippocampal and cortical astrocyte cultures

For primary culture of mixed glia, mouse pups aged P2/P3 were used, according to McCarthy and de Vellis (1980), as previously described (Khakipoor et al., 2019; Schrödl-Häußel et al., 2015). Hippocampi and cortex were dissected and immediately collected in cold HBSS on ice. After application of trypsin and incubation at 37°C for 30 min, probes were centrifuged for 5 min at 1,300 rpm and supernatant was removed. Pellet was resuspended with plating medium (Dulbecco’s modified Eagle’s medium) and vigorously pipetted (20–30 times) to obtain single cell suspension. Single cells were then plated on poly-o-Lysin coated 25 cm² flasks or coverslips in 24-well plate. Culture medium was changed every second day and experiments were carried out when astrocytes were confluent, at about after 15–21 days. Culture medium was replaced by medium adjusted to pH 6.8 (low pH₄), pH 7.4, or 7.8 (high pH₄) with HCl or NaOH, for 6 hr at constant extracellular bicarbonate concentration ([HCO₃⁻]). Cells were exposed to low or high pH₄ in the presence or absence of following inhibitors: PP2, an inhibitor of c-Src family kinases (10 µM; Merck Millipore, Cat# 529573), U0126, a highly selective inhibitor for MEK1 and MEK2 (10 µM; Sigma Aldrich, Cat# U120), SP600125, an inhibitor of JNK (10 µM; Sigma Aldrich, Cat# S5567) and cryptotanshinone (CTS) a selective STAT3 inhibitor (10 µM; Tocris Bioscience, Cat# 3713). Subsequently, cultures were processed for immunoblotting, surface biotinylation of proteins, immunofluorescence, or intracellular H⁺ recordings.

2.6 | Immunocytochemistry

Immunofluorescence of cultures has been performed as previously described (Khakipoor et al., 2017). Primary antibodies (NBCe1 1:50, GFAP 1:500, EEA1 1:200) were diluted in PBS. Cells were incubated with secondary antibodies goat anti-rabbit or anti-mouse IgG coupled to either AlexaFluor488 or AlexaFluor568, nuclei were stained with 4′,6′-diamidino-2-phenylindole dihydrochloride (DAPI) and viewed with a Leica TCS SP8 confocal microscope (Wetzlar, Germany).

2.7 | Image acquisition and analysis

Images were acquired with a Leica TCS SP8 confocal microscope using a HC PL APO CS2 63×/1.40 oil objective lens. Immunofluorescence intensity for NBCe1 was determined. Within each experiment, confocal microscope settings (laser power, detector gain, and amplifier offset) were kept the same for all scans in which protein expression was compared. Z-stacks of 15–25 optical sections with a step size of 0.5 µm were taken for at least four separate fields of view for each experimental condition. Maximum intensity projections were created from the z-stacks. To quantify protein expression LAS × software was used to measure the average intensity within the cell. Background subtraction was applied to the images. After quantification, data were normalized to the controls. Representative images for each figure were processed identically.

2.8 | Immunoblotting

Primary hippocampal and cortical astrocytes were harvested and homogenized, and protein concentration was determined according to Bradford (1976). Electrophoresis and blotting procedures were performed as described (Khakipoor et al., 2019). Primary antibodies were diluted as follows: NBCe1 1:1,000, Na⁺/K⁺-ATPase 1:1,000, GFAP 1:1,000, Vinculin 1:2,000, STAT3 1:1,000, pSTAT3 1:1,000, and GAPDH 1:10,000. Blots were developed in enhanced chemiluminescence reagents and signals were visualized on X-ray films. Subsequently, films were scanned and the signal ratio, NBCe1:Na⁺/K⁺-ATPase, GFAP:GAPDH, GFAP:Vinculin, and pSTAT3:STAT3 was quantified densitometrically (optical density). Differences in signal ratio were tested for significance using unpaired Student’s t test. For a comparison of more
than two groups, one-way analysis of variance (ANOVA) and Bonferroni post hoc test has been applied. Results with levels of *p < .05, **p < .01, and ***p < .001 were considered significant.

2.9 | Cell surface biotinylation

Primary hippocampal and cortical astrocytes were subjected to control or experimental conditions (at low or high pHo at constant [HCO3\(^{-}\)]) in the presence or absence of the inhibitors and then kept on ice. Isolation of cell surface proteins was performed using the Pierce\textsuperscript{\textregistered} cell surface protein isolation kit following the manufacturer's instructions. Proteins were then processed for immunoblotting with antibodies against NBCe1, and Na\textsuperscript{+}/K\textsuperscript{+}-ATPase, as described above.

2.10 | Intracellular H\textsuperscript{+} imaging in cortical astrocytes

To measure intracellular H\textsuperscript{+} concentration ([H\textsuperscript{+}]) changes in cultured cortical astrocytes at high pHo in the presence or absence of 10 µM CTS we used a Visitron imaging system (http://www.visitron.de/) and the aceotoxymethyl ester of a proton-sensitive dye, 2′,7′-bis-(carboxyethyl)-5-(and-6)-carboxyfluorescein—BCECF-AM (Thermo Fisher Scientific, Cat\# B8806, IL), as described previously (Theparambil et al., 2014). Cells were incubated with 3 µM BCECF-AM in bicarbonate-buffered saline solution for 15 min at room temperature. Cells were then mounted on a chamber of the Nikon ECLIPSE TE200 microscope and perfused continuously at room temperature with CO\textsubscript{2}/HCO\textsubscript{3\textsuperscript{-}}-buffered saline solutions (in mM): NaCl 116, KCl 3, NaH\textsubscript{2}PO\textsubscript{4} 0.5, α-δ-glucose 2, NaHCO\textsubscript{3} 26, MgCl\textsubscript{2} 1, and CaCl\textsubscript{2} 2, pH 7.4 or NaCl 82.1, KCl 3, NaH\textsubscript{2}PO\textsubscript{4} 0.5, α-δ-glucose 2, NaHCO\textsubscript{3} 26, MgCl\textsubscript{2} 1, and CaCl\textsubscript{2} 2, pH 7.8. BCECF was excited consecutively at 488 nm (proton-sensitive wavelength) and 440 nm (close to isosbestic point), and the changes in fluorescence emission were monitored at >505 nm (using ET540/40 m filter). Images were obtained every 10 s with a 20× objective. The fluorescence emission intensity of 488 nm excitation changes inversely with a change in [H\textsuperscript{+}], whereas the fluorescence emission intensity of 440 nm excitation is largely pH insensitive. The changes in [H\textsuperscript{+}] were monitored using the ratio F(440)/F(488). The ratio was converted into pH and absolute intracellular proton concentrations ([H\textsuperscript{+}]) by using the nigericin-based (4 µM) calibration technique (Khakipoor et al., 2017). Cells were perfused with calibration solution containing (in mM): KCl 145, NaH\textsubscript{2}PO\textsubscript{4} 0.4, Na\textsubscript{2}HPO\textsubscript{4} 1.6, Glucose 5, MgCl\textsubscript{2} 6H\textsubscript{2}O 1, Ca-DiGluconat H\textsubscript{2}O 1.3, adjusted at pH 6.5, 7.0, 7.5, and 8.0.

3 | RESULTS

3.1 | Prolonged extracellular pH shifts regulate NBCe1 in astrocytes

NBCe1 transport capacity and/or direction in mouse cortical astrocytes is regulated by acute extracellular acid-base changes, such as isocapnic acidosis, hypercapnic acidosis and hypocapnia (Theparambil et al., 2017). We have first asked whether prolonged exposure of mouse cortical and hippocampal astrocytes to extracellular pH shifts at constant extracellular bicarbonate concentration might be insults that regulate NBCe1 protein.

Therefore, cortical and hippocampal astrocytes were exposed for 6 hr to either low extracellular pH (pH\textsubscript{o} 6.8) or high extracellular pH (pH\textsubscript{o} 7.8) and subsequently, double immunofluorescence for NBCe1 and GFAP was performed. As shown in Figure 1a-f', both...
hippocampal (Figure 1a) and cortical (Figure 1d) astrocytes expressed intracellular NBCe1 (asterisks), as previously shown (Khakipoor et al., 2017; Schrödl-Häußel et al., 2015) and exposure to low pHo (Figures 1b,b') had no effect on NBCe1 expression and/or localization. In contrast, exposure of astrocytes to high pHo at constant bicarbonate concentration (Figures 1c,c' and 1f,f') considerably increased NBCe1 labeling intensity. Moreover, under this experimental condition, prominent sharp NBCe1 immunoreactivity was found in the periphery of the astrocytes (arrows), implying translocation of NBCe1 from the cytosol to the astrocytic plasma membrane. Quantification of fluorescence labeling intensity confirmed these observations (Figure 1g). NBCe1 labeling intensity was significantly increased in hippocampal and cortical astrocytes following exposure to high pHo but not after exposure to low pHo (1.40 ± 0.09 fold, and 0.97 ± 0.01 fold, for high pHo, and low pHo, in hippocampal astrocytes respectively; 1.18 ± 0.06 fold, and 1.02 ± 0.04 fold, for high pHo, and low pHo, in cortical astrocytes, respectively, *p < 0.05, using the two-tailed unpaired Student's t-test). Notably, following exposure to high pHo GFAP labeling intensity was also considerably increased in astrocytes (Figure 1c' and 1f').

To determine the onset of high pHo-induced NBCe1 protein upregulation in mouse primary cortical astrocytes, cells were exposed to high pHo for 30 min to 12 hr, and subsequently NBCe1 protein abundance was determined by immunoblot analysis (Figure 2a). Using an antibody raised against NBCe1, specific immunoreactive bands at ~110 and ~120 kDa, were detected in controls, as previously reported (Khakipoor et al., 2019). Following exposure of astrocytes to high pHo for 6 hr, NBCe1 protein expression was significantly upregulated (Figure 2b; 1.15 ± 0.00 fold, *p < 0.05, using the two-tailed unpaired Student's t-test, n = 3), compared to the controls (i.e., exposure to pHo 7.4), whereas after exposure for 4 hr, although increased (1.18 ± 0.10 fold) as well, the difference did not reach statistical significance. Similarly, NBCe1 protein expression was comparable in cortical astrocytes after exposure to high pHo for either 30 min (1.01 ± 0.02 fold), 1 hr (1.00 ± 0.01 fold), 2 hr (1.09 ± 0.01 fold), or 12 hr (1.00 ± 0.02 fold), compared to the corresponding controls exposed to pHo 7.4. These results were confirmed by immunocytochemistry as shown in Figure 2c. Following double labeling of NBCe1 (red) with EEA1 (green), a specific early endosomal marker (Mu et al., 1995), colocalization of the proteins (arrow) after 6 hr exposure to high pHo was evident.

The results presented in Figure 1 imply increased NBCe1 membrane expression in hippocampal and cortical astrocytes following exposure to high pHo for 6 hr. We have next investigated NBCe1 surface expression under this experimental condition and analysed the putative impact of Src, ERK, and JNK pathways, previously described to be involved in 4AP-dependent NBCe1 increased surface expression (Schrödl-Häußel et al., 2015). Figure 3a shows surface NBCe1 expression following biotinylation of surface proteins in primary cortical astrocytes and subsequent immunoblotting with anti-NBCe1 antibody. High pHo-dependent NBCe1 membrane expression was significantly increased, compared to the control astrocytes (lane 5; Figure 3b; 1.24 ± 0.03 fold, **p < 0.01, n = 6) and was prevented in the presence of PP2 (lane 6), U0126 (lane 7) and SP600125 (lane 8; Figure 3b; 0.81 ± 0.06 fold, 0.94 ± 0.06 fold, and 1.00 ± 0.05 fold, for PP2, U0126, and SP600125, respectively, *p < 0.05 and **p < 0.01, n = 4). The high pHo-induced significant upregulation of NBCe1 was specific, since the sole presence of the inhibitors in control cultures showed no effect on NBCe1 surface expression (Figure 3a, lanes 1–4 and Figure 3b, 1.00 ± 0.03 fold, 1.00 ± 0.01 fold, and 0.90 ± 0.05 fold for PP2, U0126, and SP600125, respectively). These data suggest that the high pHo-induced increased NBCe1 membrane expression is regulated by Src, ERK, and JNK signaling pathways.

We next asked whether high pHo at constant [HCO3–]o would differentially regulate NBCe1 protein depending to the regional origin of the astrocytes. To this end, we have used the same experimental design described above with primary mouse hippocampal astrocytes. Similar to the results obtained in cortical astrocytes, exposure of hippocampal astrocytes for 6 hr to high pHo caused a significant increase in NBCe1 surface expression as well (Figure 3c, lane 5 and Figure 3d, 1.11 ± 0.01 fold, n = 6; ***p < 0.001), which was suppressed in the presence of either the Src inhibitor PP2 (Figure 3c, lane 6 and Figure 3d; 0.90 ± 0.06 fold), the ERK inhibitor U0126 (Figure 3c, lane 7, Figure 3d; 0.92 ± 0.05 fold), or the JNK inhibitor SP600125 (Figure 3c, lane 8 and Figure 3d; 0.77 ± 0.06 fold; n = 4, *p < 0.05, and **p < 0.001). Again, these effects were a specific response of mouse hippocampal astrocytes to high pHo, since NBCe1 surface expression revealed no significant changes in control hippocampal astrocytes in the presence of PP2 (Figure 3c, lane 2 and Figure 3d; 1.06 ± 0.05 fold), U0126 (Figure 3c, lane 3 and Figure 3d, 1.01 ± 0.04 fold) or SP600125 (Figure 3c, lane 4 and Figure 3d, 1.04 ± 0.03 fold, p > 0.05, n = 3).

Taken together the results from Figure 3 suggest that cortical and hippocampal astrocytes respond similarly to high pHo and upregulate NBCe1 surface expression via Src, ERK, and JNK signaling pathways. The data also show that inhibition of an individual signaling pathway cannot be compensated by another.

### 3.2 High pHo at constant [HCO3–]o regulates NBCe1 transport activity in cortical astrocytes

We next asked whether increased NBCe1 surface expression in mouse cortical astrocytes following exposure to high pHo is associated with increased NBCe1 transport activity. Therefore, control cortical astrocytes and those exposed to high pHo for 6 hr were

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**FIGURE 1** NBCe1 localization in mouse primary hippocampal and cortical astrocytes following prolonged exposure to extracellular pH shifts at constant bicarbonate concentration. (a–f') Double immunofluorescence and subsequent confocal microscopy for NBCe1 (red) and GFAP (green) in primary hippocampal (a–c') and cortical (d–f') astrocytes. High extracellular pH (pHo) upregulated NBCe1. Arrows point to membrane NBCe1 and asterisks indicate intracellular NBCe1 distribution. Nuclei were stained with DAPI (g) Quantification of fluorescence intensity, *p < 0.05, using the two-tailed unpaired Student's t-test. ~60 cells from two to four independent experiments were analysed.
loaded with the H⁺-selective dye BCECF and subsequently intracellular [H⁺] recordings have been performed. NBCe1 activity was challenged by increasing the pH value and [HCO₃⁻] of the external perfusion solution from 7.4 and 26.0 mM to 7.8 and 60.1 mM, respectively. This activates NBCe1 to transport Na⁺–HCO₃⁻ into the cells, which results in a decrease of [H⁺]i. This decrease in [H⁺]i, was determined in control (exposed to pHo 7.4) and in astrocytes exposed to high pHo for 6 hr. Using this experimental design, the rate of alkalisation from pH 7.4 to pH 7.8 (Figures 4a, b and 4d), the rate of acidification upon returning from pH 7.8 to pH 7.4 (Figure 4a–c) and the amplitude of acidification (Figure 4e) was determined. A significant increase in all parameters was induced after exposure to increased pHo for 6 hr (36.57 ± 3.23 nM/min, –54.86 ± 4.61 nM/min and 59.26 ± 4.24 nM) compared to controls (14.71 ± 0.97 nM/min, 36.57 ± 3.23 nM/min and 59.26 ± 4.24 nM).

**FIGURE 2** NBCe1 protein expression in mouse primary cortical astrocytes after different exposure times to high extracellular pH (pHo) at constant [HCO₃⁻]o. (a) Protein abundance of NBCe1 by immunoblotting. The blots are representative for four different experiments. 50 µg protein was loaded per lane. (b) Quantification of the data, "p < .05 for significant increase after densitometric analysis of the signal ratio NBCe1: vinculin and Student’s t-test, compared to controls, n = 3. The value of control was set to 1. (c) Double immunofluorescence for NBCe1 and the early endosomal marker EEA1 in cortical astrocytes. Arrow points to colocalization of the proteins after 6 hr exposure to high pHo.
3.3 | Regulation of astrocytic activation markers by extracellular pH shifts in mouse hippocampal and cortical astrocytes

Astrocytes respond to injury and disease in the CNS through reactive astrogliosis (for review, see Sofroniew, 2009). During this process astrocytes undergo distinct molecular and morphological changes mediated by multiple signaling pathways. Thereby, activation of STAT3 signaling and upregulation of GFAP expression have been considered as hallmarks for astrocytes activation (Pekny & Nilsson, 2005).

The results of Figure 1 implicate increased expression of GFAP following exposure of astrocytes to high pHo. We have further verified these observations by immunoblot analysis in mouse hippocampal and cortical astrocytes exposed to either high or low pHo for 6 hr. Using an anti-GFAP antibody a prominent immunoreactive band at ~51 kDa could be detected in homogenates of control mouse hippocampal and cortical astrocytes (Figure 4a). GFAP protein expression was significantly upregulated in astrocytes that have been exposed to high pHo, compared to the controls (Figure 5a,b: 1.16 ± 0.12 fold and 1.11 ± 0.02 fold for hippocampal and cortical astrocytes, respectively; *p < .05 and **p < .01, using the Student’s t-test, n = 4). In contrast, GFAP protein levels were comparable...
following exposure of either hippocampal or cortical astrocytes to low $pH_o$ (Figure 5a,b; $1.15 \pm 0.17$ fold and $1.04 \pm 0.01$ fold for hippocampal and cortical astrocytes, respectively), compared to the controls.

Because GFAP is a STAT3 regulatory target, we have subsequently studied regulation of phosphorylated (p)STAT3 (Herrmann et al., 2008) upon exposure of mouse hippocampal and cortical astrocytes to high or low $pH_o$ (Figure 5c,d). Figure 5c illustrates immunoblot analysis of total STAT3 and of pSTAT3 in control astrocytes and in cells exposed to high or low $pH_o$ for 6 hr. Using the anti-STAT3 antibody two immunoreactive bands, a prominent band at ~86 kDa and a weaker band at ~79 kDa, were detected in homogenates of control mouse primary hippocampal and cortical astrocytes, representing Stat3$\alpha$ and Stat3$\beta$ full length proteins, respectively. STAT3 protein abundance in hippocampal and cortical astrocytes showed no significant differences in any experimental condition. pSTAT3 was also present in control astrocytes (Figure 5c) and was significantly upregulated in homogenates of both hippocampal and cortical astrocytes exposed to high $pH_o$ for 6 hr (Figure 5d; $1.16 \pm 0.06$ fold, and $1.22 \pm 0.09$ fold, *$p < .05$, for hippocampal and cortical astrocytes, respectively, using the two-tailed unpaired Student’s $t$-test). In contrast, exposure of mouse primary hippocampal astrocytes to low $pH_o$ had no effect on pSTAT3 protein (Figure 5d; $1.09 \pm 0.05$ fold, $p > .05$; $n = 3$), whereas in cortical astrocytes, exposure to low $pH_o$ significantly increased pSTAT3 protein, compared to the untreated controls (Figure 5d; $1.11 \pm 0.02$ fold, *$p < .01$, $n = 3$).

These data demonstrate cell type-dependent and context-dependent regulation of activation markers in astrocytes following extracellular pH shifts.

3.4 Loss of STAT3 prevents high $pH_o$-induced increased NBCe1 protein expression in vivo

Having shown that STAT3 signaling has been activated in astrocytes following exposure to high $pH_o$, as a next step, we have investigated a putative link between high $pH_o$-induced pSTAT3 upregulation and...
high pHö-dependent NBCe1 upregulation. Therefore, hippocampal and cortical astrocytes derived from Stat3flox/flox::GfapCre/+ mice (conditional knock out; cKO), that is, mice with conditional deletion of STAT3 from GFAP-expressing cells, were exposed to high pHö for 6 hr and subsequently, NBCe1 protein abundance was determined by immunoblot analysis. As shown in Figure 6, prolonged exposure of cortical (Figure 6a,b) and hippocampal astrocytes (Figure 6c,d) from Stat3flox+/−:GfapCre/+ (heterozygous; HET) mice revealed increased NBCe1 protein expression following exposure to high pHö (Figures 6a, lane 6 and Figure 6c, lane 6 and Figures 6b and 6d; 1.08 ± 0.04 fold and 1.19 ± 0.09 fold for hippocampal and cortical astrocytes, respectively; n = 4), the data did not reach statistical significance, compared to the respective controls.

These data imply that activation of STAT3 signaling pathway is prerequisite for high pHö-induced NBCe1 regulation in astrocytes.

We next asked whether these data can be mimicked in vitro using CTS, an inhibitor of STAT3 phosphorylation at Tyr705 (Shin et al., 2009). Figure 6e illustrates western blot analysis of cortical and hippocampal astrocytes exposed to prolonged high pHö in the presence of 10 μM CTS. The high pHö-dependent increased NBCe1 protein abundance in hippocampal (Figure 6e, lane 2) and cortical astrocytes (Figure 6e, lane 5; Figure 6f, 1.09 ± 0.02 fold, and
1.13 ± 0.04 fold, respectively, *p < .05, was prevented in the presence of 10 μM CTS, in hippocampal (Figure 6e, lane 6 and Figure 6f, 0.80 ± 0.10 fold, #p < .05, using the two-tailed unpaired Student’s t-test, n = 3) but not in cortical astrocytes (Figure 6e, lane 3 and Figure 6f, 1.08 ± 0.05 fold, not significant, n = 4).

3.5 | STAT3 signaling regulates basal and high pHo-induced increased of NBCe1 functional expression

NBCe1 functional expression is not necessarily linearly accompanied by changes of NBCe1 protein (Khakipoor et al., 2019).
We have therefore examined whether inhibition of STAT3 phosphorylation might influence NBCe1 transport capacity in cortical astrocytes, known to express high levels of NBCe1. The results are shown in Figure 7. Using the same experimental design as for Figure 4, the rate of alkalinisation from pH 7.4 to pH 7.8 of the external perfusion solution (Figures 7a,b, and 7d), the rate of acidification upon returning from pH 7.8 to pH 7.4 of the external perfusion solution (Figure 7a-c) and the amplitude of acidification (Figure 7e) was determined. Interestingly, in the presence of CTS, both the rate of acidification (9.86 ± 1.79 nM/min) and the rate of alkalinisation (~23.39 ± 2.9 nM/min) in control astrocytes was significantly reduced, compared to those in the absence of CTS (14.71 ± 0.96 and ~34.81 ± 2.06 nM/min; *p < .05 and **p < .01, using the two-tailed unpaired Student's t-test). Moreover, the high pHo-induced increased rate of alkalinisation (~54.86 ± 4.61 nM/min) was significantly decreased in the presence of CTS (~22.70 ± 2.42 nM/min) and showed no significant differences compared to the corresponding controls, that is, in controls in the presence of CTS. The rate of acidification at high pHo in the presence of CTS (21.11 ± 2.83 nM/min) although significantly decreased compared to high pHo alone (36.57 ± 3.23 nM/min), was found to be still significant increased compared to the corresponding control (control + CTS; using one-way ANOVA and Bonferroni post hoc test). The amplitude of acidification (Figure 7e) was significantly decreased in the presence of CTS at high pHo-exposed astrocytes (44.83 ± 4.32 nM and 59.26 ± 4.24 nM for the presence and absence of CTS, respectively) but comparable in controls (39.55 ± 4.74 and 41.52 ± 3.32 nM for the presence and absence of CTS, respectively), **p < .01 for significant increase, compared to controls and *p < .05, for significant decrease, compared to high pHo. In contrast, baseline intracellular pH (pHi; Figure 7f) revealed no significant differences in controls in the presence or absence of CTS (7.21 ± 0.06 and 7.14 ± 0.01, respectively). Similarly, no significant differences could be observed at high pHo in the presence or absence of CTS (7.52 ± 0.12 and 7.32 ± 0.1, respectively). However, the baseline pHi was significantly increased between control and high pHo (*p < .05, using the two-tailed unpaired Student's t-test).

These data suggest that STAT3 signaling is involved in regulation of both basal and pHo-induced increased NBCe1 transport activity.

3.6 | Proposed model for regulation of NBCe1 in astrocytes following exposure to high pHo

The main results of the present work are graphically summarized in Figure 8.

4 | DISCUSSION

Astrocytes play a pivotal role in maintaining and modulating the ionic composition of the extracellular space in the brain. Regulation mechanisms in the brain should be effective during physiological conditions, such as during neuronal activity, as well as during pathophysiological conditions, such as ischemia, stroke, traumatic brain injury, and other brain pathologies. As a common response to insults/injuries, astrocytes become reactive by changing their transcriptional profile and morphology. Interestingly, many pathophysiological conditions in the brain have been associated with acid-base shifts. One major pH regulation system in astrocytes is the NBCe1. As an insult/stimulus, in the present study, we have exposed cortical astrocytes to prolonged (6 hr) extracellular pH shifts obtained by changing extracellular pH via NaOH or HCl at constant [HCO3−]o. Our aim was neither to investigate the contribution of changes of bicarbonate or pH in the activity of NBCe1 nor to analyse the mode (inward or outward) of Na+ and bicarbonate transport. Although it is intriguing that in a previous study the response of NBCe1 after extracellular alkalosis induced by changes on [HCO3−]o had no effect on NBCe1 protein abundance (Khakipoor et al., 2019), using a different experimental approach, we aimed to provoke changes in protein synthesis of NBCe1 to investigate novel regulatory molecular mechanisms.

The novel finding of the present work is that STAT3 signaling regulates NBCe1. Several lines of evidence support this view (a) cell-type specific deletion of STAT3 from GFAP-expressing cells prevented stimulus-induced increased NBCe1 protein (Figure 6); (b) inhibition of STAT3 phosphorylation in vitro prevented stimulus-induced increased NBCe1 protein abundance (Figure 6) and (c) STAT3 signaling regulated both basal and stimulus-induced NBCe1 functional expression (Figure 7). Interestingly, although NBCe1 transport activity was impaired following inhibition of STAT3 phosphorylation, these effects were not accompanied by changes of baseline intracellular pH (Figure 7e), implicating that other molecular pathways must have been activated to compensate for impaired NBCe1 transport function, thereby ensuring pH homeostasis. These results may be particularly relevant for two astrocytic-mediated responses with (patho)physiological relevance.

First, the role of JAK/STAT signaling in astrocytic differentiation has been established (Bonni et al., 1997) and STAT3 but not STAT1 is required for this process (S. Hong & Song, 2014). With this background in mind, exposure of astrocytes to pHo shifts could have triggered differentiation of progenitors present in the culture towards glial fate. We however have not observed any morphological changes in the cells following prolonged exposure to either low or high pHo. Other studies have additionally shown that CNTF-induced STAT3 signaling is essential for the regulation of the size of the hippocampal stem cell pool in the adult dentate gyrus in the context of CNTF requirement to keep the balance between neural stem cell self-renewal and the generation of neuronal progenitors (Müller, Chakrapani, Schwegler, Hofmann, & Kirsch, 2009). With regard to NBCe1, only few studies have addressed its possible role on brain stem cell biology. NBCe1 is expressed in mouse neural progenitor cells and involved in regulation of intracellular pH (Nordström, Andersson, & Åkerman, 2019). Second, activation of JAK2/STAT3 signaling after cerebral insults, such as traumatic brain injury, ischemia or status epilepticus has been shown (Nicolas et al., 2013).
**FIGURE 7** Regulation of NBCe1 transport activity by STAT3 signaling. (a,b) Original recordings of intracellular \([H^+](\text{[H}^+\text{]})\) in cultured control cortical astrocytes and in astrocytes that have been exposed to high pHo at constant \([\text{HCO}_3^-]\) for 6 hr in the presence or absence of the inhibitor of STAT3 phosphorylation CTS (10 µM). Original recordings of \([H^+]\), during increase of external (perfusion solution) pH and \([\text{HCO}_3^-]\) from 7.4 and 26.0 mM to 7.8 and 60.1 mM, respectively, and during decrease of external (perfusion solution) pH and \([\text{HCO}_3^-]\) from 7.8 and 60.1 mM to 7.4 and 26.0 mM. (c–e) Bar plots showing the rate of acidification (c), the rate of alkalisation (d) and the amplitude of acidification (e) as measured upon changing external perfusion solution pH and \([\text{HCO}_3^-]\) to 7.8 and 60.1 mM, respectively, and back to pH 7.4 and 26.0 mM \([\text{HCO}_3^-]\). (f) Determination of baseline intracellular pH (pHi) in the experimental conditions. *\(p < .05\), **\(p < .01\), ***\(p < .001\), and ****\(p < .0001\) for significant increase, compared to the controls, using the two-tailed unpaired Student’s t-test and *\(p < .05\), **\(p < .01\), and ***\(p < .001\) for significant decrease, compared to high pHo, using one-way analysis of variance and Bonferroni post hoc test. The number of cells/cultures/animals used in the experiments is indicated in brackets. CTS, cryptotanshinone.
According to the current view, a given insult may lead to release of growth factors and cytokines that in turn cause activation of this pathway (Burda et al., 2016). Subsequently, astrocytes may become reactive and exhibit a considerably altered transcriptional profile. This is similar to our experimental setting, where exposure of cortical astrocytes to high pHo resulted in activation of STAT3 signaling and increased phosphorylation of STAT3 and expression of GFAP (Figure 5). However, STAT3 has no effect on NBCe1 protein abundance under basal conditions, whereas it is involved in the high pHo-induced NBCe1 upregulation (Figure 6a,b). Moreover, low pHo increased pSTAT3 in cortical astrocytes without an accompanied change in GFAP and NBCe1 expression (Figure 5c,d) though. Based on these observations it is reasonable to assume that regulation of NBCe1 is likely different under basal and pathological conditions and that the transformation of astrocytes to reactive ones by activation of STAT3 signaling and subsequent upregulation of GFAP expression might be the crucial molecular event that precede regulation of NBCe1 protein levels. At this point it should be noted that a crucial parameter in our study is the duration of the stimulus. Several studies have investigated short-term cellular responses and subsequent adaptation that mostly involve a change in the activity or in the number of individual transporter molecules in the cell membrane. According to the current view, a decrease in extracellular bicarbonate concentration is sensed by NBCe1 and during extracellular acid-base disturbances, intracellular pH of astrocytes primary follows the changes in the bicarbonate gradient across the cell membranes, rather than the changes in extracellular CO2 and pH (Salameh et al., 2014; Theparambil et al., 2015, 2016). The observed rapid changes can be achieved either by recruitment of transporter in the plasma membrane through altered trafficking, by posttranslational modifications, such as phosphorylation, or both. If the duration of the stimulus is short, the effect is too rapid to involve protein synthesis and is likely evoked by the above-mentioned mechanisms.

Interestingly, in hippocampal astrocytes, exposure to low pHo had no effect on STAT3 activation, pointing to putative regional differences (Figure 5). We have previously proposed a model in which pathological stimuli, such as 4AP, can activate extracellular TGF-β which then acting in an autocrine manner and operating via the canonical pathway can directly upregulate NBCe1 transcript, protein and functional expression in cortical astrocytes (Khakipoor et al., 2017). In the context of the present work, this model might be of particular interest since TGF-β is a known molecular player in many pathophysiological conditions and additionally, Smad-dependent and Smad independent interaction modes and crosstalk between Smad and STAT3 can either enhance or antagonize STAT3-mediated effects and sustained STAT3 activation may result in inhibition of TGF-β signaling (Jenkins et al., 2005; Shi et al., 2017; Tang et al., 2017; Wang et al., 2016). In vivo, using different mouse disease models, the question whether upregulation of STAT3 is a consequence of neuronal damage rather than a response of astrocytes has been controversially discussed as well (O’Callaghan, Kelly, VanGilder, Sofroniew, & Miller, 2014). Since our results are obtained in isolated astrocytes in vitro, neuron-astrocyte interactions cannot be validated. Nevertheless, a hypothetical axis insult/TGF-β/JAK2/STAT3/NBCe1 is indeed attractive. Further studies, importantly in vivo approaches will be necessary to validate this model.

Our results also demonstrate that Src, ERK, and JNK pathways are involved in insult-induced NBCe1 membrane expression (Figure 3) and complement our previous observations where 4AP-dependent increased membrane and functional expression of NBCe1 is regulated by Src, ERK and JNK signaling pathways (Schrödl-Häußel et al., 2015). In addition, these data suggest that activation of these signaling pathways is not specific for 4AP but rather a common astrocytic response after insults. Furthermore, hippocampal and cortical astrocytes exhibited comparable response following prolonged exposure to high but not to low pHo. Whereas cortical astrocytes apparently recruited the same molecular mechanisms to cope with either low or high pHo, hippocampal astrocytes did not.

With regard to NBCe1, in vivo, expression of NBC was increased in astrocytes in the CA1 region but no NBC immunoreactivity was observed in the ischemic CA3 region of the gerbil hippocampus at 3-4 days after ischemia/reperfusion (Sohn et al., 2011). Increased expression of electrogenic NBC was also observed in ischemic penumbra already 3 hr following focal cerebral ischemia in rats (Jung, Choi, & Kwon, 2007), but the identity of the NBC-expressing cells is not clear. When human-induced pluripotent stem cells-derived

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**FIGURE 8** Proposed model for NBCe1 regulation in astrocytes upon to insult exposure (here prolonged exposure to high extracellular pH (pHₒ) at constant extracellular bicarbonate concentration). Following an insult (1.), NBCe1 in astrocytes can be regulated either via Src-, ERK-, and JNK- signaling pathways (2a.) and/or following activation of astrocytes through upregulation of pSTAT3 and GFAP (2b.), resulting to upregulation of NBCe1 protein (3.), trafficking (dashed arrow), surface expression, and activity (4.)
astrocytes were exposed to an ischemic solution that mimics the ischemic penumbral environment, NBCe1 transcript and protein expression were significantly increased (Yao et al., 2016). In the present study, exposure of astrocytes to low pH has not affected NBCe1 abundance. Either the 6 hr exposure for this context was likely too short to evoke NBCe1 protein changes in astrocytes or increased NBCe1 transport activity could have occurred without alterations of NBCe1 protein abundance. While most studies support the view that increased NBCe1 expression is protective against ischemia, the answer to the question whether activation of NBCe1 is beneficial or detrimental for a given pathophysiological condition is not conclusive. Increased NBCe1 activity may be beneficial for astrocytic intracellular pH regulation and at the same time altering the ionic extracellular composition in that favouring neuronal excitability and seizures. Definitively, the impact of NBCe1 in pathophysiological conditions, its possible correlation with astrogliosis and the underlying molecular mechanisms are totally unknown. A correlation between STAT3 and NBCe1 may therefore be a starting point for further studies in this field.

Although in the present study the detailed molecular pathway underlying STAT3-dependent NBCe1 regulation in astrocytes is not yet complete, our results introduce STAT3 as a novel molecular determinant involved in NBCe1 regulation and implicate putative important roles of NBCe1 in modulating pH and pH during development and in the context of insult-induced activation of astrocytes after brain injury.

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CONFLICT OF INTERESTS

The authors declare that there are no conflict of interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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