SN1 transporter regulation by Sp1 in ammonia-treated mouse cortical astrocytes: role of Sp1 phosphorylation status

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Abstract: The involvement of astrocytic SN1 (SNAT3) transporter in ammonia-induced L-glutamine retention was recently documented in mouse cultured astrocytes. Here we investigated the involvement of specificity protein 1 (Sp1) transcription factor in SN1 regulation in ammonium chloride ("ammonia")-treated astrocytes. Sp1 expression and its cellular localization were determined using real-time qPCR, Western blot and confocal microscopy, respectively. Sp1 binding to Snat3 promoter was analyzed by chromatin immunoprecipitation. Ammonia-induced Sp1 regulatory role in SN1-mediated [3H]glutamine transport was verified using siRNA and mithramycin A. The involvement of protein kinase C (PKC) isoforms in Sp1 level/phosphorylation status was verified using siRNA technology. Sp1 translocation to the nuclei and its enhanced binding to Snat3 promoter, along with Sp1 dependence of system N-mediated [3H]glutamine transport were observed in astrocytes upon ammonia exposure. Ammonia decreased the level of phosphorylated Sp1, and the effect was reinforced by long-term incubation with PKC modulator, phorbol 12-myristate 13-acetate, a treatment likely to dephosphorylate Sp1. Furthermore, silencing of PKCδ isoform abolished the increase of Sp1 level by ammonia. Collectively, the results demonstrate the regulatory role of Sp1 in regulation of SN1 expression and activity in ammonia-treated astrocytes and implicate altered Sp1 phosphorylation status in this capacity.

Keywords: astrocytes, glutamine, SN1 (SNAT3), Sp1, protein kinase C, ammonia, transport

1. Introduction

L-glutamine (Gln) is the most abundant amino acid in the central nervous system (CNS) where its concentration is at least one order of magnitude higher than any other amino acid [1-3]. In the brain Gln is synthesized in astrocytes from L-glutamate (Glu) and ammonia in the reaction catalyzed by glutamine synthetase [4]. The glutamate/glutamine cycle is known as a metabolite shuttle in which Gln transported from astrocytes to adjacent neurons is converted to Glu or gamma-aminobutyric acid (GABA) in enzymatic reaction catalyzed by glutaminase or glutamate decarboxylase [4-7].

Gln is considered to play an important role in the pathogenesis of neurological diseases associated with hyperammonemia, including hepatic encephalopathy (HE) due to its excessive synthesis and accumulation in astroglial cells [8-11]. Astrocytes, the main place of ammonia detoxification in the brain are considered as a target of ammonia-derived Gln excess [12, 13]. In vitro experiments documented that in astrocytes treated with pathologically relevant concentrations of ammonia, as well as in cells exposed to a high concentrations of Gln [14, 15], the induction of mitochondrial permeability transition accompanied the astrocytic swelling [16-18]. Accordingly, astrocyte swelling is considered as a primary cause of cytotoxic component of brain edema, a frequent and fatal complication of acute HE forms [19, 20]. It have been also shown that induction of mitogen-activated protein kinases (MAPK) occurs in the astroglial cells exposed to the excess of Gln [21].
addition, intracellular Gln accumulation induces the osmotic stress and activates the p38 cascade [22, 23]. Therefore an efficient transport by specific amino acid carriers guarantying proper Gln intercellular distribution in hyperammonemic astrogliopathy is of importance.

Gln transport in astrocytes is primarily mediated by Na+-coupled amino acid transport system N that consists of three carriers: SN1 (aliases: SLC38A3, SNAT3), SN2 (aliases: SLC38A5, SNAT5) and SN7 (aliases: SLC38A7, SNAT7). SN1, the most abundant system N transporter, is responsible for the release of newly synthesized Gln from astrocytes [13, 24, 25] and its expression dominates in cultured mouse cortical astrocytes [13]. It was shown that Glu, GABA and glycine synthesis is enhanced by the presence of SN1 [26]. The involvement and importance of SN1 in neurotransmitter recycling was confirmed by the lack of SN1 immunoreactivity in oligodendrocytes where presynaptic processes are absent [27]. Moreover, it was demonstrated that silencing of system N transporters causes the retention of Gln in astrocytes and that ammonia-derived Gln release from astrocytes is mediated by impairing of SN1-mediated Gln efflux [13, 25]. SN1 is abundant in neocortex, cerebellum and olfactory bulb, therefore the functional ablation of this transporter with N-ethyl-N-nitrosourea (ENU)-induced mutagenesis in vivo leads to ataxia in mice [26]. This, albeit indirectly, confirms the important role of SN1 in sustaining proper neurotransmission. Therefore, investigation of the mechanisms involved in the regulation of SN1 is an important issue for understanding the Gln metabolic fates in the brain.

The Snat3 gene promoter region possesses a well-conserved G-rich sequence with characteristics of a GT box, a potential sequence for the DNA-binding specificity protein (Sp) factors and related GT box-binding proteins [28, 29]. Sp1 is an ubiquitously expressed transcription factor responsible for activation of expression of many genes due to its ability to recruit TATA-binding protein and to fix the transcriptional start site at TATA-less promoters [30]. It is involved in many cellular processes such as cell cycle regulation, chromatin remodeling, propagation of methylation-free islands [31], apoptosis, angiogenesis or carcinogenesis [30]. Consequently, cells lacking Sp1 are severely retarded and die after 10 days of development [31]. Interestingly, the Sp1 transcription factor is regulated by pH changes [32]. Of note in this context, the role of SN1 in pH regulation has been investigated in gliomas in situ in which SN1 was overexpressed [33, 34]. More recently, the literature data indicated that Sp1 interacts with SN1 in mouse liver, intestine, kidney and HepG2 cell line. Balkrishna et al. (2014) investigated the mechanisms underlying SN1 expression in the liver and in the brain, where SN1 levels are relatively high. It has been documented that SN1 expressing tissues contain demethylated promotors and RNA polymerase complex occupies transcriptional start-sites, what allows activation of transcription of SN1 by Sp1 transcription factor [28]. ENCODE database indicates the enhanced activity of Snat3 promoter in HepG2 cell line, liver, cerebellum and brain cortex [35]. It was further shown that SN1 up-regulation occurs mainly due to enhanced binding of Sp1 to Snat3 promoter [28]. Moreover, it was demonstrated that SN1 expression is regulated by transcription factor binding, mRNA stability and epigenetic control mechanisms. Those mechanisms mediate tissue-specific, cell-specific and pH-specific changes of mRNA levels and further changes of protein abundance [28]. More importantly in the context of this study, an increase of Sp1 mRNA expression was previously observed in cultured rat cortical astrocytes treated for 24 h with 5 mM ammonia [36].

Sp1 is a subject of posttranslational modifications such as phosphorylation, glycosylation and acetylation. The isoforms of protein kinase C (PKC) have been suggested to phosphorylate Sp1 as the PKC modulator phorbol 12-myristate 13-acetate (PMA) upregulate Sp1 in multiple cell types [30]. In turn, long-term exposure (12 hours) of oligodendrocytes to PMA, decreases the expression of Sp1 [37]. This classical mechanism controls intracellular distribution and activity of different membrane proteins. We recently demonstrated that at the translational level SN1 transporter cells cell surface expression and activity are regulated by PKC, mainly by the PKCδ isoform in ammonia-treated astrocytes [25].

Based on above described information and the structure of the Snat3 gene regulatory region we aimed to analyze the possible contribution of Sp1 in the ammonia effect on the SN1 regulation. We hypothesized that the mechanism by which ammonia interferes SN1 expression and Gln efflux from astrocytes may be related to the activation of Sp1 and its enhanced interaction with Snat3 promotor.
The study also included the analysis of the role of PKC in Sp1 phosphorylation and its role in SN1 mRNA level upon ammonia exposure.

2. Results

2.1. Ammonia-induced Sp1 expression increase and Sp1 translocation to the nucleus

Sp1 mRNA expression level after 24-hour treatment with 5 mM ammonia and 5 mM CH₃NH₃Cl was analyzed in real-time qPCR experiments. CH₃NH₃Cl, a metabolically inert compound that induces intracellular pH changes similar to ammonia [38] was used to evaluate the effect of ammonia on the Sp1 mRNA level. In our experiments the extracellular pH was increased after both treatments (Figure 1a), whereas the upregulation of Sp1 transcription factor was observed only in the presence of ammonia (Figure 1b, c) proving a direct role of ammonium ion (NH₄⁺) in Sp1 regulation. Since our results were in line with the results of Bodega et al. (2006), the extended experiments further revealed cellular localization of Sp1 transcription factor, as was observed using confocal microscopy. In astrocytes treated with ammonia Sp1 was observed in cell nuclei, while in control astrocytes Sp1 transcription factor was observed mainly in the cytoplasm (Figure 1d).
Figure 1. (a) Extracellular pH after 24-hour incubation of astrocytes with 5 mM ammonia and CH3NH3Cl. Results are mean ± SD (n=4). (*)p<0.05 vs control, One-Way ANOVA. (b) Sp1 mRNA level in mouse cortical astrocytes after 24-hour 5 mM ammonia or 5 mM CH3NH3Cl treatment. Results are mean ± SD (n=4). (*)p<0.05 vs control; One-Way ANOVA with Dunnet post-hoc test. (c) Sp1 protein level in mouse cortical astrocytes after 24-hour 5 mM ammonia treatment. Upper panel shows densitometry analysis, lower panel shows representative immunoblot. Results are mean ± SD (n=4). (*)p<0.05 vs control, student t-test. (d) Intracellular Sp1 transcription factor localization after 24-hour 5 mM ammonia treatment.

2.2. Ammonia enhances Sp1 binding to the Snat3 promoter region

Sp1 binding activity on the Snat3 promoter was measured by using a chromatin immunoprecipitation (ChIP) assay. We observed the enrichment for the Snat3 promoter region in both, control and ammonia-treated mouse astrocytes versus the negative control IgG (Figure 2). The obtained results indicate higher enrichment for the Snat3 promoter region in astrocytes after ammonia treatment (Figure 2).

Figure 2. Sp1 transcription factor binding to the Snat3 promoter region (Sp1 Ab) in mouse astrocytes treated with 5 mM ammonia for 24 hours. Histone H3 was used as a positive control, IgG was used as a negative control of Sp1 binding to the Snat3 promoter region. Results are mean ± SD (n=4). (*)p<0.05 vs control; Two-Way ANOVA, Bonferroni post-hoc test.

2.3. Silencing of Sp1 transcription factor affects SN1 expression in the presence of ammonia

In the next set of experiments, the expression of SN1 after silencing of Sp1 in the presence or absence of ammonia was analyzed. The knock-down of Sp1 transcription factor or its pharmacological inhibition was achieved by 1) siRNA silencing technology (2 nM, 24 h), or 2) application of Sp1 inhibitor mithramycin A (10 µM, 1 h). In the absence of ammonia, Sp1 silencing did not affect the SN1 mRNA expression (Figure 3a) while Sp1 silencing increased the SN1 protein level (Figure 3b). In turn, ammonia treatment specifically decreased both mRNA (Figure 3a) and protein level (Figure 3b) of SN1 in astrocytes with Sp1 transcription factor knock-down.
Figure 3. The effect of Sp1 transcription factor silencing (Sp1 siRNA) or inhibition (mithramycin A) on SN1 expression in mouse astrocytes treated or not with 5 mM ammonia: (a) SN1 mRNA level. (b) SN1 protein level. Results are mean ± SD (n=4). (*)p<0.05 vs control none, (@)p<0.05 vs ammonia none, (#)p<0.05 vs control Sp1 siRNA; (^)p<0.05 vs control mithramycin A; Two-Way ANOVA, Bonferroni post-hoc test.

2.4. The effect of Sp1 transcription factor silencing on system N activity in the presence or absence of ammonia

Further, the verification of Sp1 involvement in the [3H]glutamine transport via system N in astrocytes was conducted. Silencing of Sp1 by siRNA technology decreased total and system N-mediated [3H]glutamine uptake after ammonia treatment while Sp1 inhibition by mithramycin A affected only total [3H]glutamine uptake in ammonia-treated astrocytes (Table 1). Additionally, total and system N-mediated release of preloaded [3H]glutamine, expressed as the % of the total radioactivity lost, was reduced in astrocytes treated with 5 mM ammonia after silencing or pharmacological inhibition of Sp1 by used methods (Table 1). Moreover, Sp1 silencing led to the major decrease of [3H]glutamine efflux after ammonia treatment (Table 1).

Table 1. Total and system N-mediated [3H]glutamine uptake and efflux from mouse cortical astrocytes after silencing of Sp1 transcription factor by 2 nM siRNA or 10 µM mithramycin A and 5 mM ammonia exposure. Results are mean ± SD (n = 4). (*)p<0.05 vs control none (total), (#)p<0.05 vs control none (system N), (&)p<0.05 vs ammonia none (total), (+)p<0.05 vs ammonia Sp1 siRNA (total), (ˆ)p<0.05 vs ammonia none Sp1 siRNA (total), (**)p<0.05 vs ammonia system N (total), (!)p<0.05 vs ammonia none system N (total), (?)(p)0.05 vs control mithramycin A (total), (@)p<0.05 vs control ammonia mithramycin A (total), ($)p<0.05 vs control ammonia mithramycin A (system N), Two-Way ANOVA, Bonferroni post-hoc test.

<table>
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<td>total</td>
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<td>system N</td>
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<td>0.39±0.11*</td>
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<td>Sp1 siRNA</td>
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Sp1 phosphorylation is decreased in astrocytes treated with ammonia

Sp1 can be phosphorylated by different kinases such as protein kinase C (PKC) [39], protein kinase A (PKA) [40], cyclin-dependent kinase (CDK) [41], MAPK [42]. To test the involvement of PKC in Sp1 phosphorylation, the astrocytes were exposed to 200 nM PMA, a modulator of PKC activity. To verify whether the phosphorylation of Sp1 regulates the SN1 transcription in ammonia-treated astrocytes, the phosphorylation status of Sp1 was analyzed by quantification of two Sp1 forms with different molecular weight. The level of Sp1 phosphorylation status was determined by obtaining the ratio of the higher molecular weight, phosphorylated form with the total Sp1 protein level including both forms. As shown in Figure 4b in the astrocytes treated with ammonia, Sp1 phosphorylation was lower. When the cells undergo PMA treatment the phosphorylation of Sp1 was further decreased.

**Figure 4.** (a) Sp1 protein level in the astrocytes treated with 5 mM ammonia for 24 hours and 200 nM PMA and/or 1 µM BisI. Results are mean ± SD (n=4). (*)p<0.05 vs control none; (#)p<0.05 vs ammonia none; Two-Way ANOVA, Bonferroni post-hoc test. (b) The ratio of phospho-Sp1 to the total Sp1 protein level in astrocytes treated with 5 mM ammonia for 24 hours and 200 nM PMA and/or 1 µM BisI. Results are mean ± SD (n=4). (*)p<0.05 vs control none; (#)p<0.05 vs ammonia none; Two-Way ANOVA, Bonferroni post-hoc test.

The effect of PKC activation on SN1 mRNA expression in the presence or absence of ammonia

In the presence of ammonia, PMA treatment decreased the SN1 mRNA expression (Figure 5). The effect was reversed after incubation of astrocytes with PKC inhibitor, BisI (Figure 5). Moreover, inhibition of PKC activity by BisI treatment upregulated SN1 mRNA level (Figure 5).
Figure 5. The effect of PKC activation on the SN1 mRNA level in mouse cortical astrocytes treated with 5 mM ammonia for 24 hours. Results are mean ± SD (n=4). (*)p<0.05 vs control none; (#)p<0.05 vs ammonia none; (^)p<0.05 vs PMA control; (+)p<0.05 vs PMA ammonia; Two-Way ANOVA, Bonferroni post-hoc test.

2.7. PKCδ isoform depletion abolishes ammonia-induced increase of Sp1

According to the results described above, we aimed to identify the involvement of selected PKC isoforms in the Sp1 level. Therefore we analyzed Sp1 protein content in ammonia-treated astrocytes with silenced PKCα and δ isoforms. Ammonia decreased the Sp1 protein level in astrocytes with silenced PKCδ isoform, while in astrocytes with silenced PKCα isoform the protein level remained unaltered (Figure 6).

Figure 6. Sp1 protein level in astrocytes treated with 5 mM ammonia for 24 hours and after silencing of PKCα (PKCα-) or PKCδ (PKCδ-) isoform. Results are mean ± SD (n=4). (*)p<0.05 vs control none; (#)p<0.05 vs ammonia none; (^)p<0.05 vs control PKCδ-. Two-Way ANOVA, Bonferroni post-hoc test.

3. Discussion

The present study demonstrates that ammonia increases the Sp1 transcription factor level and its translocation to the astrocytic nucleus (Figure 1, 2), and implicates the above sequence of events in the regulation of SN1 transporter expression and activity in ammonia-exposed cultured cortical...
astrocytes (Figure 3, Table 1). The present study further documents that ammonia-induced alteration in the phosphorylation status of Sp1 transcription factor may influence SN1 transporter regulation.

It has been shown that prolonged (1, 3 or 5 days) ammonia exposure (1, 3 and 5 mM) reduced Sp1 mRNA level in rat astroglial cells [36]. The ammonia-induced downregulation of Sp1 mRNA was inversely to the rising ammonia concentration with the maximal effect at 1 mM ammonia. Interestingly, 5 mM ammonia treatment for 1 day caused an increase of Sp1 mRNA expression [36]. Sp1 activation occurring by its nuclear translocation [36, 43-46] is regulated by extracellular pH changes [32, 47]. In our study astrocytes treated with both ammonium chloride and methylammonium chloride at 5 mM concentration induced identical changes in extracellular pH value (Figure 1a), however the Sp1 mRNA expression level was affected differently (Figure 1b). The lack of Sp1 mRNA changes in the presence of methylammonium chloride indicated that in our experimental setting Sp1 activation is specifically induced by ammonium ion per se, not by ammonia-induced pH changes. Of note in this context, pathophysiological levels of ammonia do not necessarily affect the intra- or extracellular pH in astrocytes, but even a short- pH change may trigger cell signaling. The previous study documented that the effects of acute exposure to ammonia were mediated by an increase in intracellular pH, resulting in cytosolic alkalinization leading to calcium-dependent Glu release from astrocytes. The observation implicated that ammonia-induced deregulation of Glu release from astrocytes might be involved in dysfunction of the glutamatergic neurotransmission observed in acute form of HE [48].

Pathophysiological concentrations of ammonia lead to the intracellular Gln accumulation in astrocytes, which further cause osmotic stress and activate the family members of the mitogen-activated protein (MAP) kinases [21]. Stimulation of p38MAPK triggers, as have been documented, the increase of Sp1 expression [36] and its activation was further implied as a possible cause of altered expression of different genes during HE [36, 43-46].

Sp1 transcription factor is considered to be involved in the regulation of SN1 transporter in mouse kidney during ammonia-induced acidosis [28]. The mechanism of this regulation is likely to rely on the different ability of Sp1 to bind to the DNA sequences of target genes in the presence and absence of kidney acidosis. There were identified two Sp1 binding sites in the region 50 bp upstream of the SN1 transcription start site, one important for the basal activation of the promoter, and another, pH sensitive responsible for the upregulation of SN1 in the ammonia-induced acidosis [28]. According to the available data, in the physiological condition Sp1 binds to the consensus sequences further upstream transcriptional start-site than during acidosis. Therefore, it is likely that above described interaction cause the upregulation of SN1 transcription in acidosis, and Sp1 consensus sequence closest to the transcriptional start-site could be a stresses-induced transcription factor binding site [28]. Our observations are in line with the latter finding. In our study ammonia caused the decrease of the mRNA and protein level of SN1 transporter in astrocytes with silenced Sp1 transcription factor (Figure 3a, b) suggesting that SN1 expression in astrocytes exposed to ammonia may be dependent on the Sp1 transcription factor.

The ChIP analyses of the interaction between SN1 and Sp1 transcription factor showed higher enrichment for the Snat3 promoter region in the ammonia-treated astrocytes (Figure 5). Thus, the results implicate a stronger occupancy of this transcription factor on the promoter region of SN1 transporter in ammonia-exposed mouse astrocytes. Moreover, the interaction between SN1 and histone H3 remains unaltered upon ammonia exposure (Figure 2). This observation might be related to the decreased level of histone H3 by ammonia [49] and its role in the induction of SN1 transcription [28]. Taken together, the literature data and the observed lack of changes in the expression of SN1 after ammonia exposure may suggest that interaction of SN1 with histone H3 in ammonia-treated astrocytes remains unchanged compared to the control cells.

Analysis of the inward and outward [3H]glutamine transport in mouse astrocytes fully confirmed functional significance of the SN1 upon mechanism the Sp1 control. Indeed, ammonia decreases total and system N-mediated [3H]glutamine uptake to astrocytes with silenced Sp1 transcription factor (Table 1). Moreover, ammonia potentiates the decrease of the total and system N-mediated [3H]glutamine release from astrocytes with silenced Sp1 transcription factor (Table 1).
It has been shown that phosphorylation of Sp1 transcription factor reversibly regulates its activity in the regenerated liver [50], where Sp1 dephosphorylation allows hepatocytes to accomplish more proliferative cell status. In turn, Sp1 up-regulation in differentiated keratinocytes [51], caused mainly by increased level of phosphorylated form of Sp1 protein was also induced by PKC [52]. Additionally, myelin basic protein transcription was also dependent on Sp1 phosphorylation in differentiated oligodendrocytes [37]. Considering the above evidence, we hypothesized that this mechanism may also contribute to the ammonia-induced Sp1-mediated changes in SN1. Towards the same end, our recent study shown that ammonia reduces PKC activity in cultured mouse cortical astrocytes [25]. In accordance with these presumptions, here we observed alterations in the Sp1 phosphorylation status in astrocytes treated with ammonia. The level of Sp1 phosphorylated form was lower in astrocytes treated with ammonia and the effect was potentiated in astrocytes exposed to PMA (Figure 5). However, co-treatment of astrocytes with the PKC inhibitor, bisindolylmaleimide I (Bis I), turned out to be ineffective in reversing PMA-evoked Sp1 phosphorylation status decrease (Figure 5). The reasons of this apparent discrepancy remains to be elucidated. One other unsolved issue of our study concerns the observed decrease of Sp1 in the absence of PKCδ. So far, the identification of the regulatory role of one selected PKC isoform has remained beyond the experimental methodology of present study. It is worth noting that, Sp1 phosphorylation could be affected not only by PKC but also by PKA [40], DNA-dependent protein kinase [53] or CDK [41]. Clearly, the specific involvement of other kinases in ammonia-exposed astrocytes needs further evaluation.

The role of the Sp1 phosphate residues in the activation of Snat3 promoter by Sp1 is obscure. It is possible that Sp1 phosphorylation may change its interaction with other transcription factors. This phenomenon has been reported for Purz, which tended to associate with phosphorylated rather than with dephosphorylated Sp1 [54]. Further, it has been shown in cultured rat cortical astrocytes that, Nrf2 transcription factor forms complexes with the transcription factor Sp1 upon the exposure to tricarbonyldichlororuthenium(II) dimer, a carbon monoxide (CO) source [55]. The complex formed, after binding to ARE1 binding site, directly affects the regulation of Sp1 and Nrf2 target genes [55].

Our results suggest that the decrease of SN1 mRNA expression in ammonia-treated astrocytes induced by PMA (Figure 6) might be mediated, at least in part, by dephosphorylation of the transcription factor Sp1. The reason why in ammonia treated astrocytes, the mRNA and protein level of SN1 remains unchanged despite the stronger association of Sp1 transcription factor with transporter remains to be elucidated. The obtained results are in contrast to those obtained by Balkrishna et al. (2014) which suggest Sp1 to be an enhancer of SN1 in kidney during ammonia-induced acidosis. Importantly, the transcriptional regulation of SN1 was reported as tissue-specific and predominantly controlled by various epigenetic factors [28]. In this context it is plausible that in contrast to kidney, in ammonia-treated astrocytes Sp1 acts as a silencer of SN1. Worth mentioning in this context is the postulated role of one other transcription factor, Nrf2. Recently it was shown that Nrf2 is involved in the regulation of SN1 upon metabolic acidosis in the kidney. However, the results obtained by Lister and colleagues documented that Nrf2 did not control basal expression of SN1 mRNA in the liver and brain, suggesting insensitivity of both organs to SN1 increase during metabolic acidosis, implicating a kidney-specific mechanism of control [56]. The role of Nrf2 to in SN1 regulation in ammonia-treated astrocytes has not been examined as yet.

In conclusion, results of this research provide substantial evidence that pathologically elevated ammonium ions activate Sp1 transcription factor and enhance its binding to the Snat3 promoter region, in this way contributing to the alteration in Gln transport in cultured mouse cortical astrocytes. Moreover, the Sp1-mediated SN1 control may in addition be exerted by Sp1 phosphorylation status. The relevance of the findings for understanding the role of Sp1 in the regulation of SN1 transporter and SN1-mediated Gln transport in the brain during hyperammonemia in vivo remains to be documented using more native systems.
4. Materials and Methods

4.1. Materials

Plastic tissue culture dishes were purchased from Corning Costar (Sigma-Aldrich, St. Louis, MO, USA), culture medium from Sigma-Aldrich (St. Louis, MO, USA), fetal bovine serum (FBS) from Biosera (Nuaillé, France), antibiotic anticyotic from Gibco (Thermofisher Scientific, USA) and HiPerfect Transfection Reagent (Qiagen, Germany). All other chemicals of the purest grade were purchased from available commercial sources.

4.2. Astrocyte cultures and treatment

Cortical astrocytes were isolated from 7-day-old C57BL6/J mice of both sexes and cultured as described earlier [57]. The C57BL6/J mice were obtained from the animal colony of the Mossakowski Medical Research Centre, Polish Academy of Sciences in Warsaw. All animal experiments were performed according to institutional guidelines for animals, and all efforts were made to minimize the number of animals used (institutional approval no. 55/2015). Briefly, cortex was passed through Nitex nylon netting (80 µm pore size) into Dulbecco’s modified Eagle’s medium containing 20% (v/v) FBS. Medium was changed 2 days after plating and subsequently twice a week gradually changing to 10% FBS. In the third week of culturing, dBcAMP was added to the culture medium to promote morphological differentiation. Cells were grown at 37°C in the atmosphere of 95% O2 and 5% CO2, on 24-well, 6-well plates or on 60 mm and 100 mm dishes. Experiments were performed on 3-week-old astrocytes. Mature cells were exposed to 5 mM ammonium chloride (ammonia, Sigma-Aldrich, USA) or 5 mM methylammonium chloride (CH3NH3Cl; EMD Millipore, Bellerica, MA, USA) for 24 hours. In analysis of the role of PKC in the Sp1 expression, astrocytes were treated with 200 nM PMA and/or 1 µM BisI for 24 hours.

4.3. Sp1 transcription factor and PKC isoforms silencing

In order to down-regulate Sp1 transcription factor, PKCα and PKCδ isoforms, astrocytes were transfected with a mix of four types of siRNA duplexes consisting 21 nucleotides targeted to a different gene region to obtain the most effective silencing. Sense strands for Sp1 silencing used in this study were: 5’-CAGCACATTTGTACATCCAA-3’, 5’-CAGATTCTATATTATATAT-3’, 5’-CCAGGTGATCTAGGAACCTAAA-3’, 5’-CAGGATGGTTCTGGTCAAATA-3’; for PKCα silencing: 5’-ATGAACTGTTTCAGTCTATAA-3’, 5’-CAGGAGCAAGCACAAGTTCAA-3’, 5’-CAGCTGGTCATTGCTAACATA-3’, 5’-AAGCATTATCTTAGTGGATGA-3’ and for PKCδ silencing: 5’-CCGATTCAAGGTTTATAACTA-3’, 5’-AGGGAAGACACTGGTACAGAA-3’, 5’-TTGAATGTAGTTATTGAAATA-3’, 5’-CCGGGTGGACACACCACACTA-3’. Mature astrocytes were washed with phosphate-buffered saline (PBS), trypsinized to detach cells from the plates and then seeded at a density of 1.8x10^5 cells per well in six-well plates in 1.5 ml of astrocytic growth medium (Dulbecco’s modified Eagle’s medium with 10% FBS). Subsequently cells were transfected with siRNA duplexes and HiPerfect Transfection Reagent (Qiagen, Hilden, Germany) according to fast-forward protocol designed for adherent cells provided by manufacturer. Briefly, 4.5 µl of 2 nM siRNA duplexes an 9 µl of HiPerfect Transfection Reagent were diluted in 300 µl of OptiMEM (Gibco) without serum. After 30 min incubation at room temperature prepared complexes of reagent and siRNA were added drop-wisely to the cells. Transfected astrocytes were cultivated under normal growth conditions for 24 h and then used for monitoring gene silencing.

Sp1 transcription factor was also downregulated by 1 hour treatment of astrocytes with its inhibitor, mithramycin A in the concentration of 10 µM.

4.4. Real-time qPCR analysis

Total RNA from astrocytes was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA). RNA concentration was measured by NanoDrop1000 Spectrophotometer (ThermoFisher) and 1 µg of RNA was reverse transcribed using High-Capacity cDNA Reverse Transcriptase Kit (Applied
Biosystems, Warrington, UK). Real-time PCR was performed in 96-well plates with The Applied Biosystems 7500 Fast Real-Time PCR System using the minor groove binder (MGB) Taqman probe assay. Primers and probes for SN1, Sp1 and endogenous control β-actin (Mm0120670_m1, Mm00489039_m1 and Mm00607939_s1 respectively) were purchased from Applied Biosystems. Each reaction contained 5 µl TaqMan Fast Universal PCR Master Mix (Applied Biosystems) in a total volume of 10 µl, and 1.5 µl of cDNA. The real-time PCR reactions were performed at 95°C for 20 s followed by 45 cycles of 3 s at 95°C and 30 s at 60°C. The results of the analysis were calculated and expressed according to an equation \((2^{-\Delta\Delta Ct})\) that gives the amount of the target, normalized to an endogenous control (β-actin). Ct is a threshold cycle for target amplification.

### 4.5. Protein isolation and western blot

Astrocytes were washed with PBS, scrapped off and centrifuged at 1000g for 5 min at 4°C. Pellets were homogenized in RIPA buffer containing protease (concentration 1:200, Sigma-Aldrich, St. Louis, MO, USA) and phosphatase (concentration 1:100, Sigma-Aldrich, St. Louis, MO, USA) inhibitors and 50 mM sodium fluoride (Fluka, Sigma-Aldrich, Switzerland) by sonication and subsequently centrifuged for 10 min at 10000g at 4°C. Supernatant was collected and subjected to Western blot analysis. Protein concentration was measured using Pierce BCA Protein Assay Kit (Thermo Scientific, Thermofischer). Cell lysates containing 30 µg of protein were denatured by boiling in SDS-Page loading buffer for 10 min at 95°C, separated on polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Membranes were blocked in 5% BSA in TBS-T buffer. Incubation with antibodies against SN1 (1:800, ProteinTech, Manchester, UK), Sp1 (1:500, Abcam, Cambridge, UK) was done in 1% BSA in TBS-T buffer overnight at 4°C followed by 1-hour incubation with HRP-conjugated-antirabbit IgG (1:3000 for SN1 and 1:5000 for Sp1; Sigma-Aldrich, USA) for detection by Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA). The antibodies were stripped of with 0.1 M glycine, pH 2.9, and the membranes were incubated with HRP-conjugated antibody against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) for 1 h at room temperature (1:7500, Proteintech, Manchester, UK). The chemiluminescent signal acquisition and densitometry analysis were conducted using the G-Box system (SynGene, Cambridge, UK) and GeneTools software (SynGene) respectively.

### 4.6. Gln uptake

Cultured astrocytes were washed twice with Krebs buffer (29.5 mM NaCl, 1.13 mM KCl, 0.3 mM KH2PO4, 0.3 mM MgSO4, 11 mM glucose, 25 mM NaHCO3, 2.5 mM CaCl2) and then pre-incubated in this buffer for 15 min at 37°C. Incubation mixtures containing Krebs buffer with 0.1 µCi/mL L-[3,4-3H(N)]glutamine (PerkinElmer, Waltham, MA, USA; specific radioactivity 37 MBq/mL), 0.1 mM unlabelled Gln. The mixtures contained also 10 mM L-Ala and 10 mM L-Leu in order to block other than N, Gln transporter systems. The incubation was terminated after 4 min by adding cold Krebs buffer followed by two washing cycles with 1 ml of cold Krebs buffer. 0.5 ml of 1M NaOH was added to lyse the cell. The radioactivity of cell lysates was measured in a Wallac 1409 Liquid Scintillation Counter (Perkin-Elmer, Finland).

### 4.7. Gln efflux

Cultured critical astrocytes were incubated in Krebs buffer containing 0.25 µCi/mL 1 mM L-[3,4-3H(N)]glutamine for 30 min at 37°C. The efflux was initiated by removing extracellular radioactivity by washing cells twice with cold Krebs buffer. 1 ml of Krebs buffer or a mixture of Krebs buffer and 10 mM L-Ala and 10 mM L-Leu was added to the cells. Samples were collected from supernatant after 10 min. The [3H]glutamine radioactivity released from the cells was measured. Cells were lysed in 1 ml of 1 M NaOH and the radioactivity of cell lysates were measured in a Wallac 1409 Liquid Scintillation Counter (Perkin-Elmer, Finland).
4.8. Chromatin immunoprecipitation

10^7 cells were used for each experiment. Astrocytes were cross-linked in 1% formaldehyde for 10 min at room temperature (RT). The reaction was stopped by addition of glycine in final concentration of 0.125M for 5 min. The cells were centrifuged for 3 min at 1500 rpm at 4°C and then washed twice in cold PBS. The cell suspension was centrifuged for 5 min at 1500 rpm at 4°C. The cell was dissolved in sonication buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA; 0.5 mM EGTA) containing complete protease inhibitors (Roche, Mannheim, Germany), sonicated and then centrifuged for 2 min at maximum speed at 4°C. Supernatant was diluted in ChIP dilution buffer (0.01% SDS; 1.1% Triton-X-100; 1.2 mM EDTA; 17 mM Tris-HCl, pH 8.1; 167 mM NaCl) with an addition of protease inhibitors. The samples were incubated with salmon sperm agarose beads (Merck Millipore, Temecula, CA, USA) for 30 min at 4°C and centrifuged (30 s, 1500 rpm, 4°C). 50 µl of collected supernatant was taken as an input and the rest of supernatant was treated overnight at 4°C either with 5 µg of Sp1 antibody (Abcam, Cambridge, UK), histone H3 (Cell Signaling, Leiden, The Netherlands) or control IgG (Cell Signaling, Leiden, The Netherlands). After 1-hour incubation at 4°C with salmon sperm agarose beads, the samples were centrifuged (1 min, 100 rpm, 4°C) and the pellet was washed for 3 min in the following buffers: low salt (0.1% SDS; 1% Triton-X-100; 2 mM EDTA; 20 mM Tris-HCl, pH 8.1; 150 mM NaCl) and high salt (0.1% SDS; 1% Triton-X-100; 2 mM EDTA; 20 mM Tris-HCl, pH 8.1; 0.5 M NaCl) immune complex washing buffers, LiCl buffer (252 mM LiCl; 1% Np-40; 1% deoxycholic acid; 1 mM EDTA; 10 mM Tris-HCl, pH 8.1) and twice in TE buffer (10 mM Tris-HCl, pH 8; 1 mM EDTA). The samples were de-crosslinked by dissolving the washed beads in the elution buffer (0.1 M NaHCO₃, 1% SDS) and addition of 5M NaCl and overnight incubation at 65°C with shaking (950 rpm). The input samples were also prepared in the same way. The samples were incubated for 1 hour at 45°C with the mix of 0.5 M EDTA, 1 M Tris-HCl (pH 6.5) and proteinase K and then DNA from each sample was purified using phenol:chloroform:isoamylalcohol solution (Sigma-Aldrich, St. Louis, MO, USA) with an addition of glycogen (Roche, Mannheim, Germany). The quantitative analysis of the performed experiment was checked in the real-time qPCR reactions performed using Platinum Taq DNA Polymerase kit (Invitrogen, Carlsbad, CA, USA), 10 mM dNTPs (Invitrogen, Carlsbad, CA, USA) and SYBR Green (solution 1:2000; Invitrogen, Eugene, OR, USA). The enrichment at Snat3 promoter region was normalized versus input as calculated and normalized versus amylase (non-binding region). The primers used in this study are as follow: Snat3 promoter region (sense strand: 5’-AAACACTTGGAGGGGCTTCT-3’, antisense strand: 5’-CCTCGAAATCGGTGAAGTGT-3’), amylase (sense strand: 5’-CTCCGAAATCGGTGAAGTGT-3’, antisense strand: 5’-AATGATGTGCACAGCTGA-3’).

4.9. Immunocytochemistry

In order to investigate the cellular localization of Sp1 transcription factor in the astrocytes, the cells were cultured on poly-L-lysine coated glass coverslips in 24-well plates. The cells were washed with PBS and fixed with 4% paraformaldehyde for 20 min at RT and then permeabilized with 0.25% Triton X-100 for 15 min at RT. The cells were blocked in 3% BSA and 3% NGS (normal goat serum, Sigma-Aldrich, St. Louis, MO, USA) for 1 hour. Incubation with antibody against Sp1 (1:100; ProteinTech, Manchester, UK) was done in 3% BSA and 3% NGS in PBS buffer overnight at 4°C and followed by 1-hour incubation with goat anti-rabbit IgG Alexa Fluor 488 (1:500, Invitrogen, Waltham, MA, USA), for 1 hour at RT in the dark. The cells were placed on the microscope slides using VectaShield mounting medium containing DAPI stain (Vector Laboratories, Burlingame, CA, USA) that labelled the cell nuclei. To obtain the detailed images of the labeled cells, a confocal laser scanning microscope LSM 780 (Zeiss) was used. An argon laser (488 nm) was used for the excitation of Alexa Fluor 488 and diode 405 nm for the excitation of DAPI. Following the acquisition, the images were processed using the ZEN 2012 (Zeiss, Jena, Germany). Immunocytochemistry studies were performed in the Laboratory of Advanced Microscopy Techniques, Mossakowski Medical Research Centre, Polish Academy of Sciences.
4.10. Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, La Jolla, CA, USA). Confirmation of normality of the data distribution was checked using Kolmogorov–Smirnov test. Statistical significance was determined by t-test, one-way analysis of variance (One-Way ANOVA) followed by Dunnet post-hoc test and two-way analysis of variance (Two-Way ANOVA) followed by Bonferroni post-hoc test. A probability value of 0.05 or less was considered statistically significant.

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Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>BisI</td>
<td>bisindolylmaleimide I</td>
</tr>
<tr>
<td>CDK</td>
<td>cyclin-dependent kinase</td>
</tr>
<tr>
<td>ChIP</td>
<td>chromatin immunoprecipitation</td>
</tr>
<tr>
<td>CH3NH3Cl</td>
<td>methylammonium chloride</td>
</tr>
<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>dBcAMP</td>
<td>dibutyryl cyclic adenosine monophosphate</td>
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<tr>
<td>ENU</td>
<td>N-ethyl-N-nitrosourea</td>
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<tr>
<td>ERK</td>
<td>extracellular signal-regulated kinases</td>
</tr>
<tr>
<td>GABA</td>
<td>γ-aminobutyric acid</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde 3-phosphate dehydrogenase</td>
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<tr>
<td>Gln</td>
<td>L-glutamine</td>
</tr>
<tr>
<td>Glu</td>
<td>L-glutamate</td>
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<td>HE</td>
<td>hepatic encephalopathy</td>
</tr>
<tr>
<td>MAPK</td>
<td>mitogen-activated protein kinases</td>
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<td>pHe</td>
<td>extracellular pH</td>
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<tr>
<td>PKA</td>
<td>protein kinase A</td>
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<tr>
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<td>protein kinase C</td>
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<tr>
<td>PKCa-</td>
<td>silenced expression of protein kinase C isoform α</td>
</tr>
<tr>
<td>PKCd-</td>
<td>silenced expression of protein kinase C isoform δ</td>
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<tr>
<td>PMA</td>
<td>phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>SN1</td>
<td>SNAT3, solute carrier family 3 member 3</td>
</tr>
<tr>
<td>SN2</td>
<td>SNAT5, solute carrier family 3 member 5</td>
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<tr>
<td>Sp1</td>
<td>specificity protein 1</td>
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References


