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Regulacja ekspresji i funkcji astrocytarnego transportera SN1 w warunkach hiperamonemii *in vitro*

Rozprawa na stopień naukowy doktora nauk medycznych

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Obrona rozprawy doktorskiej przed Radą Naukową

Instytutu Medycyny Doświadczalnej i Klinicznej

im. Mirosława Mossakowskiego PAN

Warszawa, 2019

Badania, których wyniki zostały opisane w niniejszej rozprawie były finansowane przez:

1. Narodowe Centrum Badań i Rozwoju, projekt w ramach Programu Polsko-Norweskiej Współpracy Badawczej "CORE", Pol-Nor/196190/26/2013

2. Narodowe Centrum Nauki, projekt Preludium 11, 2016/21/N/NZ4/03759

Składam serdeczne podziękowania

Pani Dr hab. Magdalenie Zielińskiej za opiekę promotorską, merytoryczne wskazówki, wartościowe rady i dyskusje, a także za okazaną cierpliwość, życzliwość i wyrozumiałość w trakcie powstawania niniejszej rozprawy doktorskiej

Panu Prof. dr hab. Janowi Albrechtowi za cenne wskazówki w trakcie realizacji niniejszej pracy doktorskiej

Koleżankom i Kolegom z Zakładu Neurotoksykologii za przyjazną atmosferę sprzyjającą pracy naukowej

Rodzicom i Bratu za okazane wsparcie i pomoc

SPIS TREŚCI

WYKAZ SKRÓTÓW

Ala	alanina
Arg	arginina
Asp	kwas asparaginowy
Asn	asparagina
BisI	bisindolilomaleimid I, ang. *bisindolylmaleimide I*
Cys	cysteina
DAG	diacyloglicerol
EW	encefalopatia wątrobowa
GABA	kwas γ-aminomasłowy
GGC	cykl glutaminian- kwas γ-aminomasłowy/glutamina
Gln	glutamina
Glu	glutaminian
Gly	glicyna
GS	syntetaza glutaminowa
His	histydyna
Ile	izoleucyna
Leu	leucyna
MeAiB	kwas N-metyloaminoizomasłowy
OUN	ośrodkowy układ nerwowy
PAG	glutaminaza fosforanozależna
Phe	fenyloalanina
PKC	kinaza białkowa C
PMA	13-octan-12-O-tetradekanoiloforbolu, ang. *phorbol 12-myristate 13-acetate*
Pro	prolina
PS	fosfatydyloseryna
Ser	seryna
Thr	treonina
Trp	tryptofan
Tyr	tyrozyna

STRESZCZENIE

Glutamina (Gln) jest najbardziej rozpowszechnionym aminokwasem w ośrodkowym układzie nerwowym (OUN) odgrywającym istotną rolę w szeregu procesów metabolicznych. Gln jest transportowana między astrocytami i neuronami przez swoiste białka, które ze względu na cechy charakterystyczne sklasyfikowano jako układy transportowe: N, A, ASC, L czy y^+L. SN1 (SNAT3, Slc38a3) jest białkiem o cechach układu N, takich jak: zależność od jonów sodu czy wrażliwość na jony litu. Zlokalizowane jest w błonie astrocytów, komórek będących jednocześnie głównym miejscem syntezy Gln z glutaminianu (Glu) i amoniaku w OUN. SN1 odpowiedzialny jest za transport zsyntetyzowanej *de novo* Gln do sąsiednich neuronów, co wskazuje na jego istotną rolę w prawidłowym funkcjonowaniu cyklu Glu-kwas γ-aminomasłowy (GABA)/Gln, prowadzącym do syntezy głównych neuroprzekaźników aminokwasowych Glu i GABA. Mechanizmy regulacji ekspresji i aktywności SN1 są dopiero poznawane. Do tej pory wykazano m.in. że SN1 jest regulowany przez kinazę białkową C (PKC), przede wszystkim przez izoformy α, δ i γ, które obniżają jego ekspresję i aktywność w oocytach *X.laevis* oraz pierwotnej hodowli astrocytów szczurzych. Ponadto, udowodniono, że interakcje czynnika transkrypcyjnego Sp1 z promotorem *Snat3* w nerce myszy w warunkach kwasicy metabolicznej, prowadzą do wzrostu poziomu ekspresji SN1, co wskazuje na regulację SN1 na poziomie transkrypcji.

Amoniak uważany jest za główny czynnik sprawczy zaburzeń neurologicznych określanych jako encefalopatie hiperamonemiczne, wśród których najczęściej klinicznie rozpoznawane są ostra i przewlekła encefalopatia wątrobowa. Gromadzący się w nadmiernej ilości amoniak odpowiedzialny jest za wystąpienie stresu oksydacyjnego i zaburzeń energetyki komórki. Zaburzenia te wiązane są z upośledzeniem funkcji mitochondriów astrocytarnych oraz bezpośrednio, z wewnątrzkomórkowym gromadzeniem się Gln, co skutkuje obrzmieniem astrocytów, a w następstwie prowadzi do obrzęku mózgu. W zwierzęcych modelach ostrej formy encefalopatii wątrobowej wykazano obniżoną ekspresję transportera SN1, co wskazywało na fakt, iż przyczyną gromadzenia się Gln w astrocytach może być zaburzony aktywny transport Gln na zewnątrz komórki.

Wykazaliśmy, że w warunkach hiperamonemii *in vitro*, w transporcie [^{3}H]glutaminy z astrocytów kory czołowej myszy, dominującą rolę pełni układ N, z jego głównym transporterem – białkiem SN1. Ponadto pokazaliśmy, że amoniak nie wpływając na poziom ekspresji transporterów układu N obniża wyrzut [^{3}H]glutaminy z komórek i zmienia metabolizm mitochondrialny astrocytów i funkcjonowanie cyklu Krebsa.

Powyższe obserwacje stały się punktem wyjścia do badań przedstawionych w dysertacji, które dotyczą wpływu amoniaku na ekspresję i aktywność transporterów układu N w mysich hodowlach mieszanych astrocytarno-neuronalnych, a dalej, nad mechanizmami regulacji SN1 przez PKC oraz czynnik transkrypcyjny Sp1 w hodowli pierwotnej astrocytów kory czołowej myszy, w warunkach hiperamonemii *in vitro*.

W pierwszej pracy wykazano, że mieszana hodowla astrocytarno-neuronalna odwzorowuje obserwacje poczynione *in vivo*: obniżenie poziomu białka SN1 i SN2, jak i zależnego od układu N wychwytu [^{3}H]glutaminy pod

wpływem amoniaku. Przyczyną zmian mogły być wzajemne oddziaływania astrocytów i neuronów, które można tłumaczyć uwalniającymi się z komórek czynnikami troficznymi i/lub metabolitami.

W drugiej pracy wykazano obniżenie aktywności PKC w astrocytach traktowanych amoniakiem. Ponadto aktywacja PKC prowadziła do obniżenia poziomu białka SN1 na błonie komórkowej astrocytów oraz znosiła wpływ amoniaku na zależny od układu N wyrzut [^{3}H]glutaminy. Dalej udokumentowano wzmocnioną interakcję SN1 z izoformą PKCδ, której wyciszenie redukowało wpływ amoniaku na zależny od układu N wyrzut [^{3}H]glutaminy z komórek.

W trzeciej pracy pokazano udział czynnika transkrypcyjnego Sp1 w regulacji ekspresji i funkcji SN1 w warunkach ekspozycji na amoniak. Traktowanie astrocytów amoniakiem prowadziło do wzrostu poziomu ekspresji i translokacji czynnika Sp1 do jądra komórkowego astrocytów, co skutkowało wzmocnieniem wiązania Sp1 do sekwencji promotora *Snat3*. Wyciszenie Sp1 prowadziło do obniżenia ekspresji SN1 oraz zmniejszonego transportu [^{3}H]glutaminy z astrocytów w warunkach ekspozycji na amoniak, ale nie zmieniało badanych parametrów w warunkach kontrolnych. Było to prawdopodobnie związane z aktywacją czynnika Sp1 pod wpływem działania amoniaku. Co więcej, zaobserwowano, ze zarówno amoniak jak i aktywacja PKC estrami forbolu, prowadziła do defosforylacji Sp1, co w konsekwencji mogło skutkować obniżoną ekspresją mRNA SN1 pod wpływem aktywacji PKC.

Podsumowując, obniżona ekspresja i aktywność transporterów układu N w hodowlach mieszanych astrocytarno-neuronalnych wskazuje na istotną rolę interakcji astrocyt-neuron w ujawnianiu się działania amoniaku. W warunkach hiperamonemii astrocytarny transporter SN1 regulowany jest przez aktywację oraz oddziaływania z PKC, w szczególności z izoformą PKCδ. Jednocześnie, częściowa defosforylacja czynnika transkrypcyjnego Sp1 może regulować ekspresję SN1 na poziomie transkrypcji.

ABSTRACT

Glutamine (Gln) is the most abundant amino acid in the central nervous system where it plays an important role in metabolic processes. Gln is transported between astrocytes and neurons by specific proteins belonging to the transporting systems, such as system N, A, ASC, L or y^+L. SN1 (SNAT3, Slc38a3) is a protein prevailing properties of system N, such as sodium ion dependence or lithium ion sensitivity. It is located in cell membranes of astrocytes, which in the central nervous system are the main site for the Gln synthesis from glutamate (Glu) and ammonia. SN1 is responsible for the transport of synthesized *de novo* Gln to neighboring neurons what indicates its important role in the regulation of the Glu-γ-aminobutyric acid (GABA)/Gln cycle, leading to the synthesis of neurotransmitters Glu and GABA. The mechanisms regulating the expression and function of SN1 are not well elucidated. Recently, it was demonstrated that protein expression and activity of SN1 is regulated by protein kinase C (PKC), primarily by α, δ and γ isoforms, by decreasing its expression and activity in oocytes and rat astrocytes. In addition, it was documented that the interaction of the Sp1 transcription factor with the *Snat3* promoter in the mouse kidney under metabolic acidosis conditions led to an increase of SN1 expression, indicating a possible mechanism involved in the regulation of SN1 at the transcription level.

Ammonia is still considered to be the main cause of neurological symptoms defined as hyperammonemic encephalopathies, among which the most clinically diagnosed are acute and chronic hepatic encephalopathy. Excessive ammonia accumulation is associated with the occurrence of oxidative stress, disturbances in the cells energetics associated with the impairment of mitochondrial function of astrocytes, or directly with the accumulation of Gln, leading to the swelling of astrocytic cells and subsequently brain edema. In animal models of acute hepatic encephalopathy, decreased expression of the SN1 transporter was demonstrated, indicating that disturbed active outside Gln transport may be the cause of Gln accumulation in astrocytes.

We showed that in hyperammonemic conditions the system N with its main transporter - SN1 protein, plays the dominant role in the [^{3}H]glutamine export from mouse astrocytes. Moreover, we have shown that ammonia does not affect the expression of system N transporters, while decreases Gln release from the cells and disturbs Krebs cycle and mitochondria function.

The above observations prompted further studies, included in the dissertation, on the effect of ammonia on the expression and activity of system N transporters in astrocytic-neuronal co-cultures, and further on the SN1 regulation mechanisms by PKC and the Sp1 transcription factor in mouse astrocytes exposed to ammonia.

In the first study, it was shown that mouse astrocytic-neuronal co-cultures resemble the effects of ammonia observed *in vivo*: the decrease of SN1 and SN2 protein level and the system N-mediated [^{3}H]glutamine uptake. The cause of the observed changes may be the mutual interaction of astrocytes and neurons that can be explained by released from the trophic cell factors and/or metabolites.

The next study showed a decrease of PKC activity in mouse astrocytes treated with ammonia. Moreover, PKC activation led to a decrease in SN1 protein on the astrocyte cell membrane and abrogated the effect of ammonia on the system N-mediated [^{3}H]glutamine release. In addition, the enhanced interaction of SN1 with the PKCδ isoform, which silencing reduced the effect of ammonia on the system N-mediated [^{3}H]glutamine release, was observed.

The third study documented the contribution of the Sp1 transcription factor to the regulation of SN1 upon ammonia exposure. Treatment of astrocytes with ammonia resulted in the increase of the expression and translocation of Sp1 factor into the nucleus of astrocytes, what led to the enhanced Sp1 binding to the *Snat3* promoter region. Silencing of Sp1 decreased SN1 expression and transport of [^{3}H]glutamine from astrocytes upon ammonia treatment, but did not alter them in control conditions. This may be related to the activation of the Sp1 factor under the influence of ammonia. Moreover, it was observed that both ammonia and PKC activation with phorbol esters lead to Sp1 dephosphorylation, which in consequence may result in decreased expression of SN1 mRNA after PKC activation.

In summary, the decreased expression and activity of N transporters in astrocytic-neuronal co-cultures may indicate the important role of astrocyte-neuron interactions in presenting the ammonia effect. In hyperammonemic conditions, the astrocytic SN1 transporter is regulated by the activation of PKC and interaction with the PKCδ isoform. At the same time, partial dephosphorylation of the Sp1 transcription factor may be involved in the regulation of the SN1 transporter at the transcriptional level.

INNOWACYJNOŚĆ ROZPRAWY DOKTORSKIEJ

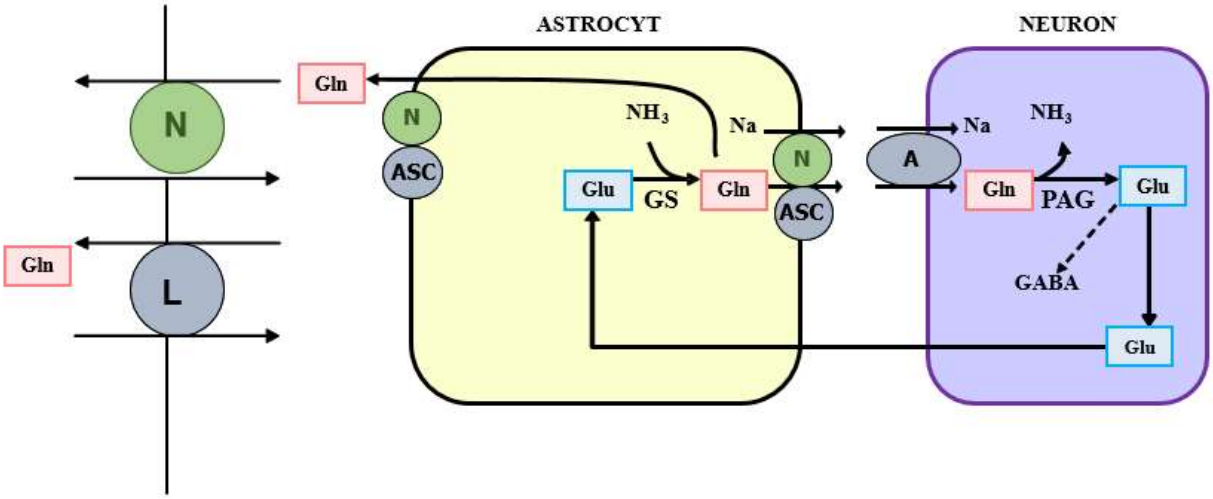
W niniejszej rozprawie doktorskiej po raz pierwszy wykazano:

1. Rolę interakcji astrocyt-neuron w ujawnianiu się wpływu amoniaku na transportery SN1 i SN2 w warunkach hiperamonemii *in vitro*.
2. Zaangażowanie PKC, a zwłaszcza izoformy PKCδ w hamowanie zależnego od układu N wyrzutu [^{3}H]glutaminy z astrocytów kory czołowej myszy w warunkach hiperamonemii *in vitro*.
3. Udział czynnika transkrypcyjnego Sp1 i jego defosforylowanej formy w regulacji ekspresji i funkcji SN1 w astrocytach kory czołowej myszy w warunkach hiperamonemii *in vitro*.

1. WSTĘP

1.1. Cykl glutaminian- kwas γ-aminomasłowy/glutamina

Glutamina (Gln) jest najbardziej rozpowszechnionym aminokwasem w ośrodkowym układzie nerwowym (OUN), a jej stężenie przewyższa o jeden rząd wielkości stężenia pozostałych aminokwasów [1-3]. Gln pełni istotną rolę w metabolizmie m.in. jako prekursor biosyntezy nukleotydów, glukozy i aminocukrów, w utrzymywaniu homeostazy glutaminianu (Glu), glutationu oraz syntezie białek [4, 5]. Przykładem reakcji kluczowych dla utrzymania metabolicznej homeostazy mózgu, w której Gln odgrywa istotną rolę, jest cykl Glu/kwas γ-aminomasłowy (GABA)-Gln (GGC). Glu jest uwalniany z zakończeń synaptycznych neuronów, a następnie transportowany do wnętrza astrocytów za pośrednictwem transporterów Glu, Glt-1 i GLAST. W astrocytach Gln syntetyzowana jest z Glu i amoniaku w reakcji katalizowanej przez syntetazę glutaminy (GS) [6]. Syntetyzowana *de novo* Gln jest transportowana do przestrzeni pozakomórkowej za pośrednictwem transporterów astrocytarnych, należących przede wszystkim do układu N, po czym transportowana jest do wnętrza neuronów za pośrednictwem transporterów neuronalnych, głównie układu A. W neuronach Gln jest przekształcana do Glu w reakcji katalizowanej przez glutaminazę fosforanozależną (PAG), a następnie Glu ulega przemianie do GABA w reakcji dekarboksylacji katalizowanej przez dekarboksylazę kwasu glutaminowego. W ten sposób Gln jest prekursorem neuroprzekaźników pobudzających, Glu i kwasu asparaginowego (Asp) [7, 8], oraz hamującego GABA [6]. Schemat opisanego wyżej cyklu przedstawiono na Ryc.1.



Ryc. 1. Schemat cyklu GGC w OUN na podstawie Albrecht i wsp. (2007) [1].

Utrzymanie homeostazy i prawidłowego neuroprzekaźnictwa warunkuje transport Gln między astrocytami i neuronami. Odbywa się on przy udziale transporterów białkowych, które ze względu na swoją lokalizację i właściwości należą do różnych układów transportowych, wśród których jako główne należy wyróżnić astrocytarny układ N oraz neuronalny układ A. Układ transportowy N, na który składają się transportery SN1 (SNAT3), SN2 (SNAT5), SNAT7 [9], jest układem sodo- oraz pH zależnym występującym głównie w astrocytach i transportującym aminokwasy, Gln,

histydynę (His) i Asp, przede wszystkim na zewnątrz komórki [10, 11]. Układ transportowy A, do którego należą transportery SAT1 (SNAT1) i SAT2 (SNAT2) jest układem zależnym od jonów sodu oraz pH, występującym głównie w neuronach i transportującym aminokwasy, takie jak Gln, alanina (Ala), prolina (Pro), glicyna (Gly) oraz kwas N-metyloaminoizomasłowy (MeAiB) do wnętrza komórki [12-17]. W mózgu Gln transportowana jest ponadto przez białka transportujące, należące do układów transportowych ASC, L, y^+L, B, B^0 i ATB^+. Charakterystyka głównych układów transportujących Gln została przedstawiona w Tabeli 1.

Tabela 1. Charakterystyka głównych układów transportujących Gln w OUN

Układ	**A**	**N**	**ASC**	**L**	**y^+L**
Substraty poza Gln	Ala, Pro, Gly, Cys, MeAiB	His, Asn	Ala, Cys, Ser, Thr	Ala, Ser, Pro, Cys, Trp, Tyr, Ile, Phe	Arg
Izoformy preferujące Gln	SAT1 (SNAT1), SAT2 (SNAT2)	SN1 (SNAT3), SN2 (SNAT5), SNAT7	ASCT2	LAT1, LAT2	y^+LAT2
Komórkowa lokalizacja *in situ*	neurony	astrocyty	astrocyty	astrocyty neurony	endotelium
Hodowle komórkowe	neurony	astrocyty neurony	astrocyty	astrocyty neurony	astrocyty neurony
Kierunek transportu	do wnętrza komórki	do i na zewnątrz komórki (preferowany kierunek na zewnątrz)	do i na zewnątrz komórki	do i na zewnątrz komórki	na zewnątrz komórki
Mechanizm	elektrogenny Gln/Na^+ kotransport	elektroobojętny Gln/Na^+ - kotransport; H+ antyport	elektroobojętny Gln/(ASCT) - wymiana	elektroobojętny Gln/(LAT) - wymiana	elektroobojętny Gln/Arg - wymiana
Zależność od pH	transport ↑ ze wzrostem pH			różna	

1.2. Transporter SN1

SN1 (SNAT3, Slc38a3) jest głównym transporterem Gln należącym do układu N, kodowanym przez gen *Snat3*. Podobnie jak pozostałe białka układu N, łączy ono transport aminokwasów z wymianą jonów H^+ - transportowi jednej cząsteczki Gln towarzyszy transport jonu Na^+ i jonu H^+ na zasadzie antyportu [18, 19]. Ponadto wykazano, że obniżone zewnątrzkomórkowe pH oraz zwiększone wewnątrzkomórkowe stężenie jonów Na^+ zmienia aktywność transportera SN1 w kierunku wyrzutu [^{3}H]glutaminy z komórek. W związku z tym oba te parametry wydają się być ważnymi regulatorami

transportu Gln przez SN1 w mózgu, wątrobie i mięśniach [18]. SN1 charakteryzuje się optymalną aktywnością w warunkach fizjologicznego stężenia Gln w komórkach (Km ~0.4 mM), niezależnością od obecności substratów po stronie *trans* (umożliwiając w ten sposób jego dominującą rolę w wyrzucie [^{3}H]glutaminy), wzrostem ekspresji w warunkach wysokiego stężenia Glu. Powyższe cechy sugerują, że jest on ważnym elementem regulującym działanie cyklu GGC, w którym odpowiedzialny jest za transport nowo zsyntetyzowanej Gln z astrocytów do sąsiednich neuronów [1, 19-21]. SN1 silniej oddziałuje z synapsami GABAergicznymi niż z synapsami glutaminianergicznymi wskazując, że egzogenna Gln zwiększa produkcję neuroprzekaźnika Glu, a nie GABA [22]. W synapsach GABAergicznych znajdują się mitochondria różniące się regulacją cyklu Krebsa, wykorzystaniem Gln i acetylokoenzymu A. W jednym z typów mitochondriów Gln wykorzystywana jest do syntezy Glu, a dopiero w dalszych jego przemianach syntetyzowany jest GABA [23]. Inkubowanie hodowanych astrocytów w warunkach, w których Glu jest w fizjologicznym stężeniu powoduje wzrost aktywności transportu przez SN1 [21]. Co ciekawe, sugeruje się, że SN1 jest wrażliwy na Glu pochodzący z neuronów [24]. Ponadto D-Asp, inny substrat transportera Glu – GLAST, wzmacnia interakcje GLAST z SN1, podczas gdy Gln wykazuje tendencję do osłabienia tej interakcji w komórkach Bergmann'a wskazując na funkcjonalne sprzężenie wychwytu Glu i wyrzutu [^{3}H]glutaminy w mózgu [24].

1.3. Hiperamonemia

Amoniak jest neurotoksyną, której zwiększone systemowe oraz mózgowe stężenie (hiperamonemia) jest jedną z podstawowych przyczyn wystąpienia zaburzeń neurologicznych w różnorodnej etiologicznie grupie schorzeń, wśród których najczęściej występujące to ostra i przewlekła encefalopatia wątrobowa (EW), ale także wrodzone zaburzenia cyklu mocznikowego, zespół Reye'a czy liczne encefalopatie metaboliczne i toksyczne [7]. W mózgu, astrocyty są głównym miejscem detoksykacji amoniaku w reakcji syntezy Gln katalizowanej przez GS [7]. Obserwowany podwyższony poziom amoniaku koreluje z podwyższonym stężeniem Gln, zarówno na obwodzie jak i wewnątrz astrocytów [25-28]. Gln jest osmolitem [29], więc jej gromadzenie się wskutek podwyższonego stężenia amoniaku zaburza równowagę osmotyczną w komórkach [30]. W konsekwencji obserwuje się wzrost objętości komórek spowodowany napływem wody do komórki, ich obrzmienie i dysfunkcję astrocytów [31]. Syntetyzowana i gromadząca się w astrocytach w warunkach hiperamonemii Gln zgodnie z hipotezą „konia trojańskiego" uważana jest za bezpośrednie źródło amoniaku w mitochondriach, który powstając w reakcji katalizowanej przez PAG, ujawnia swoje cytotoksyczne działanie [32]. Bezpośrednie działanie amoniaku, jak i ekspozycja mitochondriów na podwyższone stężenia Gln prowadzi do uprzepuszczalniania błony mitochondrialnej (tzw. zjawisko *mitochondrial permeability transition*), obrzmienia astrocytów, a w warunkach *in vivo* do obrzęku mózgu [33].

Jedną z możliwych przyczyn gromadzenia się Gln w komórkach jest jej zaburzony transport na zewnątrz astrocytów. Nieliczne doniesienia ostatnich lat wskazują na zmiany poziomu ekspresji transporterów Gln w zwierzęcych modelach EW i/lub hiperamonemii *in vivo* [30, 34-36]. Bardziej szczegółowo, ani mechanizmów regulacji ekspresji, ani

funkcji białek układu N, w warunkach hiperamonemii dotychczas nie badano. Dlatego białko SN1, dominujący przedstawiciel tego układu stał się przedmiotem badań niniejszej rozprawy.

W ramach badań dotyczących udziału poszczególnych układów transportujących Gln w astrocytach inkubowanych z amoniakiem wykazano, że ekspresja na poziomie mRNA obu transporterów nie ulega zmianie, zaś głównym transporterem tego układu w astrocytach jest SN1. Pokazano, że za wyrzut [^{3}H]glutaminy z astrocytów odpowiada przede wszystkim układ N, zaś inkubacja komórek z amoniakiem istotnie obniżała wyrzut [^{3}H]glutaminy przez ten układ. Ponadto zaobserwowano, że obniżona przez amoniak aktywność transporterów układu N może odgrywać istotną rolę w wykazanych zmianach ilości metabolitów cyklu Krebsa (na podstawie badań przemian znakowanych substratów [2-^{13}C]octanu i [1-^{13}C]glukozy) oraz metabolizmie mitochondrialnym [37; załączona jako **Publikacja „0”**].

1.4. Regulacja ekspresji i funkcji transportera SN1 przez kinazę białkową C

Aktywność transporterów białkowych zależy od ich całkowitej ilości w komórce, ale również, a może przede wszystkim od ich obecności w błonie komórkowej, co może podlegać regulacji na poziomie zarówno transkrypcji jak i translacji. Przemieszczanie się białka w obrębie komórki warunkujące również jego aktywność może zależeć od modyfikacji potranslacyjnych. Jedną z najczęstszych modyfikacji potranslacyjnych, której podlegają różne białka, w tym transportery, jest fosforylacja z udziałem kinaz białkowych, w tym kinazy białkowej C (PKC).

PKC należy do rodziny kinaz serynowo-treoninowych zależnych od wapnia i fosfolipidów [38] odgrywających istotną rolę w regulacji ekspresji i aktywności białek uczestniczących w procesach komórkowych, takich jak transdukcja sygnałów, wzrost i różnicowanie komórek czy regulacja transkrypcji genów [39]. Ze względu na właściwości strukturalne oraz sposoby aktywacji wyróżnia się: a) izoformy klasyczne (konwencjonalne) – α, βI, βII i γ, zależne od jonów wapnia (Ca^{2+}), aktywowane przez diacyloglicerol (DAG) i fosfatydyloserynę (PS); b) izoformy nowe – δ, ε, θ i η, niezależne od Ca^{2+}, aktywowane przez DAG i PS oraz c) izoformy atypowe – ζ i λ, które do swojej aktywacji nie wymagają Ca^{2+} ani DAG, jednak PS może regulować ich aktywność [39]. Poziom poszczególnych izoform PKC w hiperamonemii był badany jedynie w hodowli szczurzych neuronów móżdżkowych [40]. W badaniach tych wykazano obniżenie poziomu izoform α, βI, ε i θ, δ i ζ oraz brak zmian w ilości izoformy γ. Ponadto amoniak powodował translokację izoform γ, δ i θ z błony komórkowej do cytozolu, izoformy ζ z cytozolu do błony komórkowej, i pozostawał bez wpływu na subkomórkową lokalizację izoform α, βI i ε [40]. Jednakże znaczenie obserwowanych zmian nie zostało jeszcze zweryfikowane.

We wcześniejszych badaniach wykazano, że transporter SN1 podlega regulacji przez PKC w warunkach fizjologicznych [41-44]. Inkubacja oocytów *X.laevis* [41] oraz astrocytów szczurzych [42, 44] z aktywatorem PKC, 13-octanem-12-O-tetradekanoiloforbolu (PMA), powodowała obniżenie ilości i aktywności SN1 w błonie komórkowej, internalizację białka SN1 oraz zahamowanie wychwytu [^{3}H]glutaminy [41, 42, 44]. Efekt ten był odwracalny po zastosowaniu inhibitora PKC, bisindolilomaleimidu I (BisI) [41, 42, 44]. Metodą klonowania pokazano, że docelowy

region działania PKC nie znajduje się na N-końcu szczurzego białka SN1. Zaobserwowano obniżenie aktywności SN1 w komórkach, w których N-koniec SN1 został zastąpiony N-końcem białka SAT1. Biorąc pod uwagę powyższe dane wnioskowano, że transporter SN1 nie podlega bezpośrednio fosforylacji przez PKC, a obniżenie jego aktywności związane jest z interakcjami z białkami regulatorowymi oocytów [41]. W sekwencji szczurzego SN1 zidentyfikowano pięć potencjalnych miejsc fosforylacji przez PKC (T32, S52, S314, T458 i T466) oraz trzy miejsca fosforylacji przez kinazę kazeinową CKII (S52, T59 i T458). Natomiast w mysiej sekwencji SN1 miejsce fosforylacji zlokalizowane jest w bliskiej odległości od S52, a w miejscu Ser52 obecna jest Pro [42]. Techniką mutagenezy wykazano, że Ser52 szczurzego SN1 jest miejscem docelowym fosforylacji przez PKC, a izoformy α i γ są bezpośrednio odpowiedzialne za fosforylację SN1 [42]. Z dotychczasowych danych literaturowych wynika także, że PKC jest zaangażowana w obniżenie ekspresji i aktywności SN1 w warunkach ekspozycji szczurzych astrocytów na jony manganu (Mn^{2+}) poprzez interakcje transportera z izoformą δ [44].

Powyższe dane podkreślają rolę PKC w regulacji ekspresji i aktywności transportera SN1 w warunkach fizjologicznych, mimo że nie pokazują jego bezpośredniej fosforylacji przez PKC. Dlatego też, w niniejszej pracy postanowiono się zająć udziałem PKC w regulacji ekspresji i aktywności białka SN1 warunkach hiperamonemii.

1.5. Regulacja ekspresji transportera SN1 przez czynnik transkrypcyjny Sp1

Aktywność transporterów może być modulowana także na poziomie transkrypcji przy udziale białek wiążących DNA genu docelowego i regulujących ich transkrypcję, czyli czynników transkrypcyjnych. Jednym z nich, którego oddziaływaniem z transporterem SN1 postanowiono zająć się w rozprawie, jest czynnik transkrypcyjny Sp1. W sekwencji DNA SN1 występują dwa miejsca wiążące Sp1 w regionie 50 bp powyżej miejsca startu transkrypcji, jedno istotne dla podstawowej aktywacji promotora i drugie, wrażliwe na zmiany pH, odpowiedzialne za regulację ekspresji SN1 w kwasicy wywołanej wysokim stężeniem amoniaku [45].

Sp1 jest czynnikiem transkrypcyjnym należącym do rodziny białek Sp/XKLF [46] odpowiedzialnym za aktywację genów posiadających w promotorach potencjalne miejsca wiążące bogate w pary zasad GC [46, 47]. W jedynym dostępnym doniesieniu literaturowym wykazano, że długotrwała inkubacja (72 i 120 godz.) pierwotnej hodowli astrocytów szczurzych z 5 mM stężeniem jonów amonowych obniżała ekspresję Sp1, zaś w przypadku 24-godzinnej inkubacji prowadziła do wzrostu jego ekspresji [48]. Ponadto pokazano, że ekspozycja szczurzych astrocytów na wysokie stężenia amoniaku powoduje częściową defosforylację Sp1 co może być związane z inaktywacją p38MAPK, jednej z kinaz związanych z procesem obrzmienia astrocytów [48]. Aktywacja PKC przez PMA powoduje fosforylację Sp1 prowadząc do wzrostu ekspresji tego czynnika transkrypcyjnego w wielu typach komórek [47]. Z drugiej strony, długotrwała ekspozycja oligodendrocytów na PMA prowadzi do obniżenia poziomu białka Sp1 w tych komórkach [49].

Dostępne dane literaturowe wskazują na oddziaływanie czynnika transkrypcyjnego Sp1 z miejscami wiążącymi w promotorze transportera SN1 w wątrobie, jelitach i nerce myszy oraz w komórkach linii HepG2. W warunkach kwasicy

metabolicznej wywołanej wysokim stężeniem amoniaku w nerce obserwuje się wzrost ekspresji SN1 oraz enzymów związanych z glukoneogenezą i metabolizmem Gln. Tym samym, w warunkach kwasicy metabolicznej obserwowana jest wzmożona aktywność obu tych procesów [45, 50]. Wykazano, że SN1 zawiera w regionie 3'UTR element wrażliwy na zmiany pH, który stabilizuje mRNA w trakcie długotrwałych zmian zewnątrzkomórkowego pH oraz powoduje wzrost ekspresji SN1 w odpowiedzi na zmiany pH. Dodatkowo udokumentowano, że wzrost ekspresji SN1 w nerce, w warunkach kwasicy metabolicznej, związany jest ze wzmocnionym wiązaniem Sp1 do regionu promotora *Snat3* [45].

Na podstawie opisanych wyżej zależności, w niniejszej rozprawie postanowiono sprawdzić czy podobny mechanizm regulacji SN1 na poziomie transkrypcji obserwowany jest w astrocytach kory czołowej myszy w warunkach hiperamonemii *in vitro*.

2. CEL PRACY

Obniżony poziom białka SN1 w korze czołowej myszy z ostrą EW [36] oraz zmniejszony w warunkach traktowania komórek patofizjologicznym stężeniem amoniaku odzwierciedlającym warunki hiperamonemii *in vivo*, transport [^{3}H]glutaminy z hodowanych astrocytów, przy braku zmian w poziomie ekspresji transporterów układu N [37; załączona jako Publikacja „0"] stały się punktem wyjścia do sformułowania założeń badawczych niniejszej rozprawy doktorskiej.

Poszczególne cele badawcze:

I. Badanie wpływu amoniaku na ekspresję i aktywność transporterów SN1 i SN2 w mysich mieszanych hodowlach astrocytarno-neuronalnych w warunkach hiperamonemii *in vitro*.

II. Analiza regulacji ekspresji i aktywności SN1 przez kinazę białkową C w astrocytach kory czołowej myszy w warunkach hiperamonemii *in vitro*.

III. Zbadanie roli czynnika transkrypcyjnego Sp1 w regulacji ekspresji i aktywności transportera SN1 w astrocytach kory czołowej myszy w warunkach hiperamonemii *in vitro*.

3. METODYKA

3.1. Hodowle komórkowe

W badaniach wykorzystano pierwotne hodowle astrocytów kory czołowej myszy oraz hodowle mieszane astrocytarno-neuronalne prowadzone w temperaturze 37°C, w atmosferze zawierającej 5% CO_2. Astrocyty były hodowane przez 3 tygodnie w pożywce DMEM według opisanego wcześniej protokołu [51]. Hodowle mieszane astrocytarno-neuronalne uzyskiwano przez wysiewanie neuronów na dwutygodniową hodowlę astrocytów. Dojrzałe komórki inkubowane były z 5 mM NH_4Cl („amoniak") przez 24 godziny. W badaniach wpływu PKC komórki dodatkowo inkubowano z 200 nM aktywatorem PKC, PMA oraz 1 µM inhibitorem PKC, BisI.

Zaletą wykorzystanego modelu badawczego jest uzyskanie jednego typu komórek mózgowych (hodowle homogenne) o możliwie jednorodnym fenotypie, co jest praktyczniejsze w stosowanych technikach badawczych. W przypadku prowadzonych hodowli mieszanych starano się częściowo odwzorować sytuację *in situ*. Warto wspomnieć, że najlepszym modelem badawczym byłyby hodowle astrocytów ludzkich, aby móc bezpośrednio odnieść obserwowane zmiany do warunków klinicznych, jednakże ich hodowla jest bardzo kosztowna i dlatego też stosuje się modele zwierzęce. W badaniach zastosowano hodowlę mysich astrocytów korowych, kontynuując i odnosząc wyniki do obserwacji poczynionych wcześniej w Zakładzie Neurotoksykologii, które dotyczyły mysiego modelu EW.

3.2. Wyciszenie ekspresji genów

Wyciszanie ekspresji badanych genów przeprowadzono wykorzystując technologię siRNA z zastosowaniem odczynnika transfekującego HiPerfect Transfection Reagent. Kontrolą negatywną była transfekcja komórek sekwencjami niewyciszającymi AllStars. Ponadto, czynnik transkrypcyjny Sp1 wyciszany był poprzez godzinną inkubację astrocytów z 10 µM inhibitorem Sp1, mitramycyną A.

Ograniczeniem tej metody jest możliwość wpływu odczynnika transfekującego na ekspresję wyciszanych genów. Dlatego też, możliwy efekt działania odczynnika sprawdzono w próbach „mock" (transfekcja odczynnikiem transfekującym). Zaletą zastosowanej metody jest wysoka swoistość wyciszenia danego genu (kontrola negatywna AllStars nie powodowała wyciszenia). W jednej transfekcji stosowano mieszaninę czterech sekwencji oligonukleotydowych siRNA skierowanych w różne regiony genu, dzięki czemu możliwe było uzyskanie wysokiej efektywności wyciszenia badanych genów.

Wydaje się, że użycie astrocytów pochodzących z myszy z nokautem wybranych genów byłoby bardziej odpowiednim rozwiązaniem, gdyż ich ekspresja byłaby wyciszona w większym stopniu. Jednakże, nokaut niektórych badanych genów jest letalny, a zatem niemożliwa byłaby hodowla komórek. Dotychczas wykazano, że nokaut SN1 u myszy nie jest w 100% letalny, jednak myszy nie osiągają dojrzałości. Badania wskazują, że białko SN1 odgrywa ważną rolę w funkcjonowaniu organów, w których jest eksprymowany, takich jak mózg, wątroba czy nerka, a jego brak nie może być całkowicie kompensowany obecnością innych transporterów dla Gln. U myszy z nokautem SN1 zaburzona

była synteza Gln, proces glukoneogenezy oraz cykl mocznikowy. Powyższe obserwacje sugerują, że SN1 jest głównym i niezbędnym transporterem Gln w organizmie ssaków [52]. Z kolei nokaut Sp1 u myszy jest letalny, a embriony nieposiadające tego białka wykazują poważne opóźnienia w rozwoju i obumierają około 10 dnia życia płodowego [46, 53].

3.3. Badanie poziomu mRNA

1 µg mRNA wyizolowanego z zużyciem TRI Reagent przepisywano na cDNA stosując High Capacity cDNA Reversed Transcription Kit. Poziom ekspresji mRNA badanych genów sprawdzano przy użyciu metody real-time qPCR wykorzystując sondy Taqman i Taqman Fast Universal PCR Master Mix. Zmiany poziomu mRNA poszczególnych genów obliczano stosując metodę $2^{-\Delta\Delta Ct}$ w odniesieniu do genu referencyjnego, β-aktyny [54].

Zaletami powyższej metody, w porównaniu do klasycznej reakcji PCR, są przede wszystkim szybkość i wydajność przeprowadzanej reakcji. Czas detekcji produktu jest dużo szybszy niż w przypadku klasycznej reakcji PCR, gdyż nie stosuje się czasochłonnych technik, tj. elektroforeza w żelu agarozowym. Dzięki temu jej wydajność jest wyższa, ponieważ reagenty nie wyczerpują się, reakcja nie ustaje oraz nie dochodzi do hybrydyzacji produktów ze sobą.

3.4. Oznaczanie poziomu białka metodą Western Blot

Izolację białka całkowitego oraz frakcji błonowej wykonywano z użyciem odpowiedniego buforu do izolacji. W pierwszym przypadku próbki wirowano przez 10 min w 4°C przy 10000g, a uzyskany supernatant stanowił białko całkowite. Z kolei w przypadku izolacji białka frakcji błonowej próbki wirowane były najpierw przez 10 min w 4°C przy 1000g, a następnie zebrany supernatant wirowano ponownie przez 20 min w 4°C przy 14000g. Otrzymany w ten sposób osad był zawieszany w buforze do izolacji i stanowił białko frakcji błonowej.

Stężenie białka mierzono metodą kolorymetryczną BCA wykorzystując komercyjnie dostępny kit. Równe ilości białka rozdzielano w 10% żelu poliakrylamidowym, przenoszono na membranę nitrocelulozową, którą następnie blokowano w 5% roztworze BSA w TBS-T. Membrany inkubowano przez noc z przeciwciałem pierwszorzędowym, po czym przez godzinę z drugorzędowym. Dodatkowo na tych samych membranach badano poziom białka referencyjnego (GAPDH w przypadku białka całkowitego, Na^+/K^+ATPazy w przypadku białka frakcji błonowej). Detekcję białek wykonywano z zastosowaniem substratu Clarity Western ECL Substrate w aparacie G-Box. Analizę densytometryczną wykonywano przy użyciu programu GeneTools (Syngene).

Zaletą stosowanej techniki jest oznaczenie ilościowe poziomu badanych białek, podczas gdy w mikroskopii konfokalnej możliwe jest ich oznaczenie jakościowe.

3.5. Transport [^{3}H]glutaminy

W badaniach transportu Gln wykonywanych według protokołów rutynowo stosowanych w Zakładzie Neurotoksykologii używano radioaktywny izotop Gln, [^{3}H]glutaminę. Transport [^{3}H]glutaminy zależny od układu N analizowano w obecności substratów pozostałych układów transportowych: Ala i Leu w stężeniach 10 mM.

Ograniczeniem doświadczeń z wykorzystaniem hodowli komórkowych jest fakt, iż w tym modelu badawczym obecne i aktywne są wszystkie układy transportujące Gln, dlatego w celu zbadania transportu przez jeden z układów należy stosować substraty innych układów, które je kompetycyjnie hamują. Idealnym rozwiązaniem tego problemu byłoby zastosowanie mutagenezy w celu wklonowania sekwencji transporterów układu N w komórki normalnie ich nie posiadające. Jednakże uzyskany w ten sposób model, byłby jeszcze mniej natywny w porównaniu do homogennej hodowli astrocytów czy też hodowli mieszanej astrocytarno-neuronalnej.

3.6. Test aktywności PKC

Aktywność PKC mierzona była spektrofotometrycznie z zastosowaniem zestawu PKC Activity Assay Kit (Abcam) zgodnie z protokołem producenta.

Jedną z zalet tej metody jest krótki czas w porównaniu z techniką opartą na analizie poziomu fosfoseryny w komórkach metodą Western Blot. Ponadto pozwala ona na bezpośrednią ocenę aktywności PKC, gdyż wyniki porównywane są do krzywej standardowej mierzonej dla określonych stężeń aktywnej PKC.

3.7. Biotynylacja

Astrocyty traktowane były odczynnikiem biotynylującym EZ-Link Sulfo-NHS-LC-Biotin, a następnie inkubowane przez noc ze złożem awidynowym (Pierce Avidin Agarose). Biotynylowane białka były wypłukiwane ze złoża przy użyciu buforu obciążającego, a następnie poddawane analizie Western Blot. Jako kontrolę stosowano białko z całkowitych lizatów komórek.

Ograniczeniem tej metody jest liczba etapów doświadczenia, które mogą częściowo prowadzić do utraty badanego białka. Dlatego też, zastosowana w niniejszej pracy technika izolacji białka frakcji błonowej pozwala zweryfikować uzyskane wyniki.

3.8. Koimmunoprecypitacja bialek

1 mg białka oczyszczano wykorzystując zestaw PANSORBIN® Cells (Callbiochem), a następnie inkubowano przez noc z przeciwciałem anty-SN1. Uzyskane kompleksy białkowe rozpuszczane były w buforze obciążającym w obecności złoża protein-G agarose (Sigma-Aldrich), a następnie poddawane analizie Western Blot.

3.9. Immunocytochemia

Komórkowa lokalizacja czynnika Sp1 w astrocytach była sprawdzana przy użyciu mikroskopu konfokalnego LSM780 Carl Zeiss w Środowiskowym Laboratorium Laserowych Technik Mikroskopowych IMDiK PAN. Komórki inkubowane były przez noc z przeciwciałem pierwszorzędowym, a następnie drugorzędowym sprzężonym z Alexa488. Z kolei jądra komórkowe znakowano barwnikiem fluorescencyjnym DAPI. Analiza wykonanych zdjęć wykonana została z użyciem oprogramowania ZEN 2012 (Zeiss).

3.10. Immunoprecypitacja chromatyny

Astrocyty utrwalano z użyciem 1% formaldehydu, sonikowano w celu fragmentacji chromatyny, a następnie inkubowano przez noc z przeciwciałem anty-Sp1, IgG lub histonami H3. Próbki inkubowano z agarozowymi kulkami opłaszczonymi białkiem A, a następnie wymywano z nich immunoprecypitaty, po czym izolowano DNA z wykorzystaniem roztworu fenol:chloroform:alkohol izoamylowy (stosunek 25:24:1). Analizę ilościową wykonano stosując reakcję real-time qPCR, a wyniki wyrażone według równania $2^{-\Delta\Delta Ct}$ [54] odniesione były do kontroli i regionu niewiążącego.

Zaletą tej metody jest możliwość wykazania interakcji między białkami a DNA innych genów. Jednakże nie wskazuje ona czy dany czynnik transkrypcyjny jest aktywatorem bądź inhibitorem transkrypcji genów docelowych. W tym celu stosuje się bioluminescencyjny test lucyferazy.

3.11. Analiza statystyczna danych

Analizę statystyczną wykonano używając oprogramowania GraphPad Prism 7.0., wykorzystując jedno- i dwuczynnikową analizę wariancji (odpowiednio ang. *One Way* i *Two Way ANOVA*) oraz odpowiednio testy post hoc: Dunnett'a i Bonferroni'ego (dla porównań więcej niż dwóch grup odpowiednio o jednej bądź dwóch zmiennych niezależnych), a także test Studenta t (t-test; dla porównań dwóch grup). Poziom ufności $p<0,05$ uznawano za istotny statystycznie.

4. OMÓWIENIE WYNIKÓW

4.1. Amoniak obniża poziom białka transporterów SN1 i SN2 oraz wychwyt [^{3}H]glutaminy w hodowlach astrocytarno-neuronalnych

Obserwowany brak zmian ekspresji transporterów układu N w astrocytach z kory mózgowej myszy oraz opisane we wstępie obniżenie poziomu białka tych transporterów w mysim modelu encefalopatii wątrobowej było punktem wyjścia do ich zweryfikowania w mysich mieszanych hodowlach astrocytarno-neuronalnych.

Hodowle astrocytarno-neuronalne inkubowano przez 24 godziny z 5 mM amoniakiem, a następnie badano poziom białka transporterów SN1 i SN2. Wykazano, że amoniak powoduje obniżenie poziomu białek obu transporterów (Ryc.3a, Publikacja I). W zgodzie z powyższymi wynikami zaobserwowano obniżony całkowity wychwyt [^{3}H]glutaminy oraz tendencję spadkową wychwytu [^{3}H]glutaminy zależnego od układu N po inkubacji komórek z amoniakiem (Ryc. 3b, Publikacja I).

Uzyskane wyniki sugerować mogą udział czynników pochodzenia neuronalnego (metabolity, czynniki troficzne) w regulacji ekspresji transporterów w warunkach hiperamonemii *in vitro*. W związku z tym postanowiono zbadać poziom białka oraz aktywność transporterów w hodowlach astrocytarnych hodowanych przez tydzień w pożywce zebranej znad hodowli neuronalnej. Amoniak dodawano na dwa sposoby: a) do pożywki neuronalnej po 7 dniach hodowli neuronów; b) do pożywki neuronalnej w pierwszym dniu hodowli astrocytów w tej pożywce. Poziom białka w komórkach traktowanych w ten sposób był obniżony (Ryc. 4a, Publikacja I), natomiast ani całkowity, ani zależny od układu N wychwyt [^{3}H]glutaminy nie był zmieniony (Ryc. 4b, Publikacja I).

4.2. PKC zaangażowana jest w regulację ekspresji i funkcji SN1 w astrocytach kory czołowej myszy traktowanych amoniakiem

W dalszych etapach pracy postanowiono zbadać wybrane mechanizmy regulacji ekspresji i funkcji transportera SN1 w mysich astrocytach w warunkach hiperamonemii *in vitro*.

4.2.1. Amoniak obniża aktywność PKC

Inkubacja astrocytów z amoniakiem obniżała aktywność PKC (Ryc. 1, Publikacja II). Jednakże tego efektu nie obserwowano w astrocytach traktowanych amoniakiem po aktywacji PKC przez zastosowanie jej aktywatora, PMA (Ryc. 1, Publikacja II). Obserwowane efekty znosiły się kiedy astrocyty inkubowano w obecności inhibitora BisI, a następnie PMA (Ryc. 1, Publikacja II).

4.2.2. Amoniak oraz aktywacja PKC obniżają poziom białka SN1 w błonie komórkowej astrocytów

W związku z tym, że zmieniona przez amoniak aktywność PKC może wpływać na poziom aktywnego transportera w błonie komórkowej, zbadano poziom białka transportera SN1 na błonach komórkowych astrocytów wykorzystując metodę biotynylacji oraz analizę Western Blot w wyizolowanej frakcji błonowej astrocytów. Amoniak

obniżał poziom SN1 we frakcji błonowej astrocytów, a także ilość białka biotynylowanego (Ryc. 2a, b, Publikacja II). Wykonane analizy wskazują też, na obniżenie ilości SN1 w astrocytach kontrolnych inkubowanych z PMA (Ryc. 2a, b, Publikacja II), a efekt był znoszony przez uprzednią inkubację komórek z inhibitorem PKC, BisI. Z kolei w astrocytach inkubowanych z amoniakiem efekt ten pozostawał niezmieniony (Ryc. 2a, b, Publikacja II). W całkowitych lizatach komórkowych poziom białka SN1 nie zmieniał się w żadnym z zastosowanych wariantów (Ryc. 2c, Publikacja II).

4.2.3. Amoniak oraz aktywacja PKC regulują transport [^{3}H]glutaminy

W kolejnym etapie postanowiono funkcjonalnie zweryfikować obserwowane zmiany ekspresji transportera SN1 pod wpływem amoniaku oraz aktywacji PKC. Dlatego też analizowano wychwyt i wyrzut [^{3}H]glutaminy z mysich astrocytów po ich inkubacji z amoniakiem oraz aktywacji PKC. Wykazano, że amoniak nie wpływał na wychwyt [^{3}H]glutaminy w żadnych z zastosowanych warunków, podczas gdy aktywacja PKC obniżała całkowity i zależny od układu N wychwyt [^{3}H]glutaminy do astrocytów (Ryc. 3a, Publikacja II). Efekt ten był odwracalny w przypadku całkowitego wychwytu [^{3}H]glutaminy po zastosowaniu inhibitora PKC, BisI. Amoniak obniżał całkowity oraz zależny od układu N wyrzut [^{3}H]glutaminy z astrocytów, natomiast aktywacja PKC przez inkubację komórek z PMA nie prowadziła do zmian uwalniania [^{3}H]glutaminy (Ryc. 3b, Publikacja II). Ponadto, zaobserwowano, że aktywacja PKC redukowała efekt obniżenia wyrzutu [^{3}H]glutaminy wywołany przez amoniak (Ryc. 3b, Publikacja II), co może wskazywać na wzajemne znoszenie się wpływu amoniaku i aktywacji PKC.

4.2.4. Wyciszenie izoform PKCα i PKCδ powoduje wzrost poziomu białka SN1 w błonie komórkowej astrocytów traktowanych amoniakiem

Przedstawione wyniki wskazują na udział PKC w regulacji ekspresji i aktywności transportera SN1 w warunkach hiperamonemii *in vitro*. W związku z tym postanowiono sprawdzić, która z wybranych izoform PKC jest zaangażowana w regulację ilości białka SN1 na błonie. Na podstawie danych literaturowych wytypowano te izoformy PKC, które bezpośrednio mogą być zaangażowane w regulację SN1, tj. α i δ. Zaobserwowano obniżony poziom białka izoformy δ oraz brak zmian poziomu izoformy α w astrocytach inkubowanych z amoniakiem (Ryc. 4a, Publikacja II). Wpływ wybranych izoform PKC na poziom SN1 sprawdzano po ich wyciszeniu z zastosowaniem technologii siRNA. Zarówno w warunkach kontrolnych, jak i po inkubacji astrocytów z amoniakiem, wyciszenie obu izoform PKC powodowało wzrost poziomu białka SN1 na błonach komórkowych (Ryc. 4b, Publikacja II).

4.2.5. Amoniak wzmacnia interakcje SN1 z izoformą PKCδ

Następnie zweryfikowano zdolność SN1 do tworzenia kompleksów białkowych z badanymi izoformami PKC w astrocytach inkubowanych z amoniakiem. Pokazano, że w warunkach kontrolnych SN1 tworzy kompleksy białkowe z obiema badanymi izoformami, potwierdzając tym samym wcześniejsze doniesienia literaturowe [42, 44]. Ponadto, zaobserwowano tendencję wzmocnienia interakcji SN1 z PKCδ po inkubacji astrocytów z amoniakiem (Ryc. 5, Publikacja II).

4.2.6. Wyciszenie izoformy PKCδ redukuje wpływ amoniaku na wyrzut [^{3}H]glutaminy w astrocytach

Funkcjonalnie zweryfikowano wzmocnioną interakcję izoformy PKCδ z SN1 w hiperamonemii, dlatego też zbadano wyrzut [^{3}H]glutaminy z astrocytów inkubowanych z amoniakiem po wyciszeniu wybranych izoform PKC (α i δ). Wykazano, że wyciszenie obu izoform prowadzi do wzmożonego wyrzutu [^{3}H]glutaminy z astrocytów zarówno kontrolnych, jak i traktowanych amoniakiem (Tabela 1, Publikacja II). Ponadto zaobserwowano, że jedynie wyciszenie PKCδ znosi wpływ amoniaku na zależny od układu N wyrzut [^{3}H]glutaminy z astrocytów (Tabela 1, Publikacja II).

4.3. Czynnik transkrypcyjny Sp1 i jego poziom fosforylacji regulują ekspresję i funkcję SN1 w astrocytach kory czołowej myszy traktowanych amoniakiem

4.3.1. Amoniak powoduje wzrost ekspresji Sp1 i jego translokację do jądra komórkowego astrocytów

Wykazano wzrost ekspresji mRNA Sp1 po inkubacji komórek z amoniakiem, co było zgodne z wcześniejszymi doniesieniami literaturowymi [48]. Ponadto zaobserwowano wzrost poziomu białka Sp1 (Ryc. 1a, b, Publikacja III) i wywołaną amoniakiem translokację Sp1 do jądra komórkowego astrocytów (Ryc. 1c, Publikacja III).

4.3.2. Amoniak wzmacnia wiązanie czynnika transkrypcyjnego Sp1 do promotora Snat3

Zbadano wiązanie czynnika transkrypcyjnego Sp1 do regionu promotora *Snat3* w warunkach kontrolnych oraz po inkubacji astrocytów z amoniakiem (Ryc. 2, Publikacja III). Wykazano, że wiązanie to jest silniejsze w komórkach inkubowanych z amoniakiem (Ryc. 2, Publikacja III).

4.3.3. Amoniak obniża ekspresję transportera SN1 po wyciszeniu Sp1

Powyższe wyniki sugerować mogą zależność ekspresji SN1 od czynnika Sp1. W związku z tym postanowiono zbadać ekspresję SN1 po wyciszeniu Sp1. Pokazano, że w warunkach kontrolnych wyciszenie Sp1 nie wpływało na ekspresję mRNA SN1, natomiast prowadziło do wzrostu poziomu białka transportera (Ryc. 3a, b, Publikacja III). Inkubacja astrocytów z amoniakiem obniżała zarówno poziom ekspresji mRNA, jak i poziom białka w komórkach z wyciszoną ekspresją czynnika transkrypcyjnego Sp1 (Ryc. 3a, b, Publikacja III).

4.3.4. Amoniak obniża transport [^{3}H]glutaminy w astrocytach z wyciszonym Sp1

W celu funkcjonalnego potwierdzenia roli czynnika transkrypcyjnego Sp1 w transporcie [^{3}H]glutaminy przez układ N (transporter SN1) analizowano wychwyt aminokwasu w astrocytach z wyciszoną ekspresją Sp1. Wykazano, że amoniak obniża całkowity oraz zależny od układu N wychwyt [^{3}H]glutaminy do komórek z wyciszoną przy pomocy technologii siRNA ekspresją Sp1 (Tabela 1, Publikacja III). Natomiast po zastosowaniu 10 μM mitramycyny A, inhibitora Sp1, amoniak powodował obniżenie jedynie całkowitego wychwytu [^{3}H]glutaminy do astrocytów (Tabela 1, Publikacja III).

4.3.5. Amoniak obniża poziom ufosforylowanej formy białka Sp1

Dane literaturowe wskazują, że czynnik Sp1 może być substratem dla izoform PKC. Dodatkowo przedstawione wcześniej badania roli PKC w regulacji SN1, skierowały uwagę na potencjalny udział PKC w regulacji poziomu białka Sp1 w astrocytach inkubowanych z amoniakiem. Wpływ amoniaku na poziom białka Sp1 nie ujawniał się po aktywacji PKC (Ryc. 4a, Publikacja III). Dodatkowo wykazano, że amoniak oraz 24-godzinna inkubacja astrocytów z PMA powoduje obniżenie poziomu ufosforylowanego Sp1 (Ryc. 4b, Publikacja III). Ponadto zaobserwowano, że inkubacja z inhibitorem BisI przed stymulacją komórek PMA nie odwracała efektu wywołanego przez inkubację z PMA (Ryc. 4a, b, Publikacja III).

4.3.6. Aktywacja PKC obniża ekspresję mRNA SN1 w obecności amoniaku

Aktywacja PKC przez PMA obniżała ekspresję mRNA SN1 w astrocytach inkubowanych z amoniakiem (Ryc. 5, Publikacja III), a efekt ten był odwracany po uprzedniej pre-inkubacji komórek z inhibitorem PKC, BisI (Ryc. 5, Publikacja III).

4.3.7. Amoniak obniża poziom białka Sp1 w astrocytach z wyciszoną izoformą PKCδ

W kolejnych badaniach weryfikowano, która z izoform PKC (α czy δ) jest odpowiedzialna za zmiany ilości ufosforylowanego Sp1. Amoniak obniżał poziom białka Sp1 w astrocytach z wyciszoną izoformą δ, nie wpływał natomiast na poziom Sp1 po wyciszeniu izoformy α (Ryc. 6, Publikacja III).

5. PODSUMOWANIE I WNIOSKI

I. Interakcje astrocyt-neuron odgrywają istotną rolę w ujawnianiu się zmian ilości i aktywności transporterów SN1 i SN2 w warunkach hiperamonemii *in vitro*

i. Amoniak obniża poziom ekspresji transporterów SN1 i SN2 w hodowlach mieszanych astrocytarno-neuronalnych oraz astrocytach hodowanych w medium zebranym znad hodowli neuronów, podczas gdy nie wpływa na ich ekspresję w homogennej hodowli astrocytów.

ii. Amoniak obniża zależny od układu N wyrzut [^{3}H]glutaminy w hodowlach mieszanych astrocytarno-neuronalnych.

II. Aktywacja PKC, w szczególności izoformy PKCδ, bierze udział w regulacji ekspresji i funkcji transportera SN1 na poziomie translacji w astrocytach kory czołowej myszy w warunkach hiperamonemii *in vitro*

i. Amoniak obniża aktywność PKC, poziom białka SN1 na błonie komórkowej astrocytów, a w konsekwencji zaburza transport [^{3}H]glutaminy z komórek.

ii. PKCδ wydaje się być odpowiedzialna za regulację ekspresji SN1 w warunkach ekspozycji na amoniak, co odzwierciedla brak wpływu amoniaku na wyrzut [^{3}H]glutaminy po wyciszeniu tej izoformy.

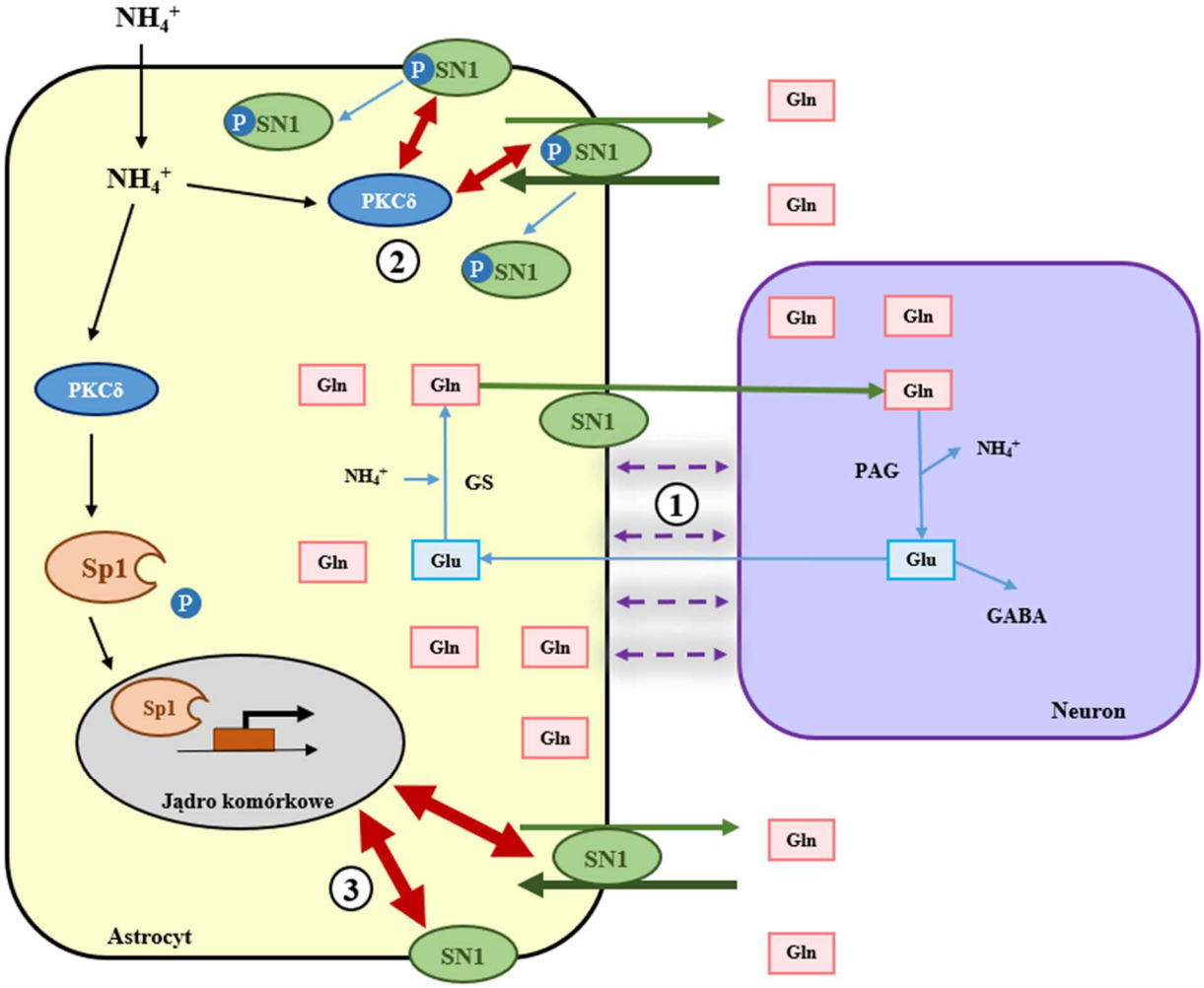
III. Czynnik transkrypcyjny Sp1 i jego defosforylacja bierze udział w regulacji ekspresji i funkcji transportera SN1 na poziomie transkrypcji w astrocytach kory czołowej myszy w warunkach hiperamonemii *in vitro*

i. Amoniak powoduje wzrost ekspresji Sp1 i prowadzi do translokacji białka Sp1 do jądra komórkowego. Amoniak wzmacnia interakcje Sp1 z promotorem *Snat3*, zaś wyciszenie Sp1 obniża poziom ekspresji SN1 oraz wychwyt [^{3}H]glutaminy do komórek inkubowanych z amoniakiem.

ii. Aktywacja PKC przez PMA prowadzi do defosforylacji Sp1, a także do obniżenia ekspresji mRNA SN1 po ekspozycji komórek na amoniak.

iii. Wydaje się, że obniżenie fosforylowanej formy czynnika transkrypcyjnego Sp1 jest odpowiedzialne za obniżenie ekspresji mRNA SN1 w astrocytach inkubowanych z amoniakiem.

Poniżej graficznie przedstawiono wnioski wynikające z badań wybranych elementów mechanizmu regulacji SN1 w warunkach hiperamonemii *in vitro* (Ryc. 2).



W warunkach ekspozycji na amoniak transporter SN1 regulowany jest przez:

1. Wzajemne oddziaływanie astrocytów i neuronów
2. Na poziomie translacji przez aktywację PKC, w szczególności izoformy PKCδ
3. Na poziomie transkrypcji czynnik transkrypcyjny Sp1 i jego defosforylację

Ryc. 2. Proponowany schemat regulacji transportera SN1 w warunkach hiperamonemii in vitro.

7. LITERATURA

1. Albrecht, J., Sonnewald, U., Waagepetersen, H.S., Schousboe, A., Glutamine in the central nervous system: function and dysfunction. *Front. Biosci* 2007, *12*, 332-343.
2. Cynober, L., Plasma amino acid levels with a note on membrane transport: characteristics, regulation, and metabolic significance. *Nutrition* 2002, *18* (9), 761-766.
3. Pithon-Curi, T. C., Trezena, A.G., Tavares-Lima, W., Curi, R., Evidence that glutamine is involved in neutrophil function. *Cell Biochem Funct* 2002, *20* (2), 81-86.
4. Bode, B. P., Recent molecular advances in mammalian glutamine transport. *J Nutr* 2001, *131*, 2475S-2485S.
5. Sidoryk-Wegrzynowicz, M., Impairment of glutamine/glutamate-γ-aminobutyric acid cycle in manganese toxicity in the central nervous system. *Folia Neuropathol* 2014, *52* (4), 377-382.
6. Albrecht, J., Sidoryk-Węgrzynowicz, M., Zielińska, M., Aschner, M., Roles of glutamine in neurotransmission. *Neuron Glia Biol* 2010a, *6*, 263-276.
7. Albrecht, J., Zielińska, M., Norenberg, M.D., Glutamine as a mediator of ammonia neurotoxicity: A critical appraisal. *Biochem Pharmacol* 2010b, *80* (9), 1303-1308.
8. Suárez, I., Bodega, G., Fernández, B., Glutamine synthetase in brain: effect of ammonia. *Neurochem Int* 2002, *41* (2-3), 123-142.
9. Pochini, L., Scalise, M., Galluccio, M., Indiveri, C., Membrane transporters for the special amino acid glutamine: structure/function relationships and relevance to human health. *Front Chem* 2014, *2*, Article 61.
10. Mackenzie, B., Erickson, J.D., Sodium-coupled neutral amino acid (System N/A) transporters of the SLC38 gene family. *Pflugers Arch* 2004, *447* (5), 784-795.
11. Palacín, M., Estévez, R., Bertran, J., Zorzano, A., Molecular biology of mammalian plasma membrane amino acid transporters. *Physiol Rev* 1998, *78* (4), 969-1054.
12. Christensen, H. N., Oxender, D.L., Liang, M., Vatz, K.A., The use of N-methylation to direct route of mediated transport of amino acids. *J Biol Chem* 1965a, *240* (9), 3609-16.
13. Christensen, H. N., Liang, M., An amino acid transport system of unassigned function in the Ehrlich ascites tumor cell. *J Biol Chem* 1965b, *240* (9), 3601-8.
14. Christensen, H. N., Organic ion transport during seven decades. The amino acids. *Biochim Biophys Acta* 1984, *779* (3), 255-269.
15. Christensen, H. N., On the strategy of kinetic discrimination of amino acid transport systems. *J Membr Biol* 1985, *84* (2), 97-103.
16. Christensen, H. N., Distinguishing amino acid transport systems of a given cell or tissue. *Methods Enzymol* 1989, *173*, 576-616.
17. Christensen, H. N., Role of amino acid transport and countertransport in nutrition and metabolism. *Physiol Rev* 1990, *70* (1), 43-77.
18. Bröer, A., Albers, A., Setiawan, I., Edwards, R.H., Chaudhry, F.A., Lang, F., Wagner, C.A., Bröer, S., Regulation of the glutamine transporter SN1 by extracellular pH and intracellular sodium ions. *J Physiol* 2002, *539* (Pt 1), 3-14.

19. Chaudhry, F. A., Krisaj, D., Larsson, P., Reimer, R.J., Wreden, C., Storm-Mathisen, J., Copenhagen, D., Kavanaugh, M., Edwards, R.H., Coupled and uncoupled proton movement by amino acid transport system N. *EMBO J* 2001, *20*, 7041-7051.

20. Boulland, J. L., Rafiki, A., Levy, L.M., Storm-Mathisen, J., Chaudhry, F.A., Highly differential expression of SN1, a bidirectional glutamine transporter, in astroglia and endothelium in the developing rat brain. *Glia* 2003, *41* (3), 260-275.

21. Bröer, A., Deitmer, J.W., Bröer, S., Astroglial glutamine transport by system N is upregulated by glutamate. *Glia* 2004, *48* (4), 298-310.

22. Boulland, J. L., Osen, K.K., Levy, L.M., Danbolt, N.C., Edwards, R.H., Storm-Mathisen, J., Chaudhry, F.A., Cell-specific expression of the glutamine transporter SN1 suggests differences in dependence on the glutamine cycle. *Eur J Neurosci* 2002, *15* (10), 1615-1631.

23. Waagepetersen, H. S., Sonnewald, U., Schousboe, A. , Compartmentation of glutamine, glutamate, and GABA metabolism in neurons and astrocytes: functional implications. *Neuroscientist* 2003, *9* (5), 398-403.

24. Martinez-Lozada, Z., Guillem, A.M., Flores-Mendez, M., Hernandez-Kelly, L.C., Vela, C., Meza, E., Zepeda, R.C., Caba, M., Rodriguez, A., Ortega, A., GLAST/EAAT1-induced glutamine release via SNAT3 in Bergmann glial cells: evidence of a functional and physical coupling. *J Neurochem* 2013, *124* (4), 545-554.

25. Butterworth, R. F., Pathophysiology of brain dysfunction in hyperammonemic syndromes: The many faces of glutamine. *Mol Genet Metab* 2014, *113* (1-2), 113-117.

26. Lavoie, J., Giguere, J.F., Layrargues, G.P., Butterworth, R.F. , Amino acid changes in autopsied brain tissue from cirrhotic patients with hepatic encephalopathy. . *J. Neurochem* 1987, *49*, 692-697.

27. Ratnakumari, L., Qureshi, I.A., Butterworth, R.F., Regional amino acid neurotransmitter changes in brains of Spf/Y mice with congenital ornithine transcarbamylase deficiency. *Metab. Brain Dis* 1994, *9*, 43-51.

28. Tofteng, F., Hauerberg, J., Hansen, B.A., Pedersen, C.B., Jørgensen, L., Larsen, F.S. , Persistent arterial hyperammonemia increases the concentration of glutamine and alanine in the brain and correlates with intracranial pressure in patients with fulminant hepatic failure. *J Cereb Blood Flow Metab* 2006, *26*, 21-27.

29. Lockwood, A. H., Yap, E.W., Rhoades, H.M., Wong, W.H., Altered cerebral blood flow and glucose metabolism in patients with liver disease and minimal encephalopathy. *J Cereb Blood Flow Metab* 1991, *11* (2), 331-336.

30. Rama Rao, K. V., Norenberg, M.D., Glutamine in the pathogenesis of hepatic encephalopathy: the trojan horse hypothesis revisited. *Neurochem Res* 2014, *39* (3), 593-598.

31. Brusilow, S. W., Hyperammonemic encephalopathy. *Medicine (Baltimore)* 2002, *81* (3), 240-249.

32. Albrecht, J., Norenberg, M.D., Glutamine: a Trojan horse in ammonia neurotoxicity. *Hepatology* 2006, *44*, 788-794.

33. Bai, G., Rama Rao, C.R.K., Norenberg, M.D., Ammonia induces the mitochondrial permeability transition in primary cultures of astrocytes. *J Neurosci Res* 2001, *66*, 981-991.

34. Desjardins, P., Du, T., Jiang, W., Peng, L., Butterworth, R.F., Pathogenesis of hepatic encephalopathy and brain edema in acute liver failure: role of glutamine redefined. *Neurochem Int.* 2012, *60* (7), 690-696.

35. Leke, R., Escobar, T.D., Rao, K.V., Silveira, T.R., Norenberg, M.D., Schousboe, A., Expression of glutamine transporter isoforms in cerebral cortex of rats with chronic hepatic encephalopathy. *Neurochem Int.* 2015, *88*, 32-37.

36. Zielińska, M., Popek, M., Albrecht, J., Roles of changes in active glutamine transport in brain edema development during hepatic encephalopathy: an emerging concept. *Neurochem Res* 2014, *39* (3), 599-604.

37. Zielińska, M., Dąbrowska, K., Hadera, M.G., Sonnewald, U., Albrecht, J., System N transporters are critical for glutamine release and modulate metabolic fluxes of glucose and acetate in cultured cortical astrocytes: changes induced by ammonia. *J Neurochem* 2016, *136* (2), 329-338.

38. Battaini, F., Mochly-Rosen, D., Happy birthday protein kinase C: past, present and future of a superfamily. *Pharmacol Res* 2007, *55* (6), 461-466.

39. Mackay, H. J., Twelves, C.J. , Targeting the protein kinase C family: are we there yet? *Nat Rev Cancer* 2007, *7* (7), 554-562.

40. Giordano, G., Sanchez-Perez, A.M., Burgal, M., Montoliu, C., Costa, L.G., Felipo, V., Chronic exposure to ammonia induces isoform-selective alterations in the intracellular distribution and NMDA receptor-mediated translocation of protein kinase C in cerebellar neurons in culture. *J Neurochem* 2005, *92* (1), 143-157.

41. Balkrishna, S., Broer, A., Kingsland, A., Broer, S., Rapid downregulation of the rat glutamine transporter SNAT3 by a caveolin-dependent trafficking mechanismin Xenopus laevis oocytes. *Am J Physiol Cell Physiol* 2010, *299*, C1047-C1057.

42. Nissen-Meyer, L. S., Popescu, M.C., Hamdani el, H., Chaudhry FA., Protein kinase C-mediated phosphorylation of a single serine residue on the rat glial glutamine transporter SN1 governs its membrane trafficking. *J Neurosci Res* 2011, *31*, 6565-6575.

43. Nissen-Meyer, L. S., Chaudhry, F.A. , Protein kinase C phosphorylates the system N glutamine transporter SN1 (Slc38a3) and regu-lates its membrane trafficking and degradation. *Front Endocrinol (Lausanne)* 2013, *4*, Article 138.

44. Sidoryk-Wegrzynowicz, M., Lee, E., Mingwei, N., Aschner, M., Disruption of astrocytic glutamine turnover by manganese is medi-ated by the protein kinase C pathway. *Glia* 2011, *59* (11), 1732-1743.

45. Balkrishna, S., Bröer, A., Welford, S.M., Hatzoglou, M., Bröer, S., Expression of glutamine transporter Slc38a3 (SNAT3) during acidosis is mediated by a different mechanism than tissue-specific expression. *Cell Physiol Biochem* 2014, *33* (5), 1591-1606.

46. Philipsen, S., Suske, G., A tale of three fingers: the family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res* 1999, *27* (15), 2991-3000.

47. Black, A. R., Black, J.D., Azizkhan-Clifford, J., Sp1 and krüppel-like factor family of transcription factors in cell growth regulation and cancer. *J Cell Physiol* 2001, *188* (2), 143-160.

48. Bodega, G., Suárez, I., Almonacid, L., Ciordia, S., Beloso, A., López-Fernández, L.A., Zaballos, A., Fernández, B., Effect of ammonia on ciliary neurotrophic factor mRNA and protein expression and its upstream signalling pathway in cultured rat astroglial cells: possible implication of c-fos, Sp1 and p38MAPK. *Neuropathol Appl Neurobiol* 2007, *33* (4), 420-430.

49. Guo, L., Eviatar-Ribak, T., Miskimins, R., Sp1 phosphorylation is involved in myelin basic protein gene transcription. *J Neurosci Res* 2010, *88* (15), 3233-3242.

50. Solbu, T. T., Boulland, J.L., Zahid, W., Lyamouri Bredahl, M.K., Amiry-Moghaddam, M., Storm-Mathisen, J., Roberg, B.A., Chaudhry, F.A., Induction and targeting of the glutamine transporter SN1 to the basolateral membranes of cortical kidney tubule cells during chronic metabolic acidosis suggest a role in pH regulation. *J Am Soc Nephrol* 2005, *16*, 869-877.

51. Hertz, L., Peng, L., Hertz, E., Juurlink, B.H., Yu, P.H. , Development of monoamine oxidase activity and monoamine effects on glutamate release in cerebellar neurons and astrocytes. *Neurochem Res* 1989, *14*, 1039-1046.

52. Chan, K., Busque, S.M., Sailer, M., Stoeger, C., Bröer, S., Daniel, H., Rubio-Aliaga, I., Wagner, C.A., Loss of function mutation of the Slc38a3 glutamine transporter reveals its critical role for amino acid metabolism in the liver, brain, and kidney. *Pflugers Arch* 2016, *468* (2), 213-227.

53. Marin, M., Karis, A., Visser, P., Grosveld, F., Philipsen, S. , Transcription factor Sp1 is essential for early embryonic development but dispensable for cellgrowth and differentiation. *Cell Biochem Funct* 1997, *89* (4), 619-628.

54. Livak, K. J., Schmittgen, T.D., Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). *Methods* 2001, *25*, 402-408.

8. KOPIE PUBLIKACJI

Publikacja „0”

Zielińska M, Dąbrowska K, Hadera MG, Sonnewald U, Albrecht J.; *System N transporters are critical for glutamine release and modulate metabolic fluxes of glucose and acetate in cultured cortical astrocytes: changes induced by ammonia*; J Neurochem. 2016; 136(2):329-38. doi: 10.1111/jnc.13376 (IF 4,083)

Publikacja I

Dąbrowska K, Skowrońska K, Popek M, Obara-Michlewska M, Albrecht J, Zielińska M.; *Roles of Glutamate and Glutamine Transport in Ammonia Neurotoxicity: State of the Art and Question Mark*; Endocr Metab Immune Disord Drug Targets. 2018; 18(4):306-315. doi: 10.2174/1871520618666171219124427 (IF 2,013)

Publikacja II

Dąbrowska K, Albrecht J, Zielińska M.; *Protein kinase C-mediated impairment of glutamine outward transport and SN1 transporter distribution by ammonia in mouse cortical astrocytes*; Neurochem Int. 2018; 118:225-232. doi: 10.1016/j.neuint.2018.07.001 (IF 3,603)

Publikacja III

Dąbrowska K, Zielińska M.; *Silencing of Transcription Factor Sp1 Promotes SN1 Transporter Regulation by Ammonia in Mouse Cortical Astrocytes*; Int J Mol Sci.; 2019; 20(2):234. doi:10.3390/ijms20020234 (IF 3,687)

Journal of Neurochemistry

JOURNAL OF NEUROCHEMISTRY | 2015

doi: 10.1111/jnc.13376

ORIGINAL ARTICLE

System N transporters are critical for glutamine release and modulate metabolic fluxes of glucose and acetate in cultured cortical astrocytes: changes induced by ammonia

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Abstract

Glutamine (Gln) is synthesized in astrocytes from glutamate (Glu) and ammonia, whereupon it can be released to be transferred to neurons. This study evaluated the as yet not definitely established role of the astrocytic Gln transporters SN1 and SN2 (*Slc38a3* and *Slc38a5* respectively) in Gln release and metabolic fluxes of glucose and acetate, the canonical precursors of Glu. Cultured neocortical astrocytes were grown in the absence or presence of ammonia (5 mM NH_4Cl, 24 h), which deregulates astrocytic metabolism in hyperammonemic encephalopathies. HPLC analyses of cell extracts of SN1/SN2 siRNA-treated (SN1/SN2−) astrocytes revealed a ~ 3.5-fold increase in Gln content and doubling of glutathione, aspartate, alanine and glutamate contents, as compared to SN1/SN2+ astrocytes. Uptake and efflux of preloaded [^{3}H]Gln was likewise significantly decreased in SN1/SN2− astrocytes. The atom percent excess ^{13}C values (given as M + 1) for alanine, aspartate and glutamate were decreased when the SN1/SN2− cells were incubated with [1-^{13}C] glucose, while Gln consumption was not changed. No difference was seen in M + 1 values in SN1/SN2− cells incubated with [2-^{13}C] acetate, which were not treated with ammonia. In SN1/SN2− astrocytes, the increase in Gln content and the decrease in radiolabeled Gln release upon exposure to ammonia were found abrogated, and glutamate labeling from [2-^{13}C]acetate was decreased as compared to SN1/SN2+ astrocytes. The results underscore a profound role of SN1 and/or SN2 in Gln release from astrocytes under physiological conditions, but less so in ammonia-overexposed astrocytes, and appear to manifest dependence of astrocytic glucose metabolism to Glu/Gln on unimpaired SN1/SN2− mediated Gln release from astrocytes.

Keywords: ^{13}C isotopomers, acetate, glucose, glutamine transport, mass spectrometry, metabolism.

J. Neurochem. (2015) 10.1111/jnc.13376

In the central nervous system (CNS), glutamine (Gln) gives rise to, and is recycled from, the amino acid neurotransmitter glutamate (Glu) in a chain of metabolic reactions defined as the glutamate–glutamine cycle (GGC). The GGC is compartmented between astrocytes and neurons; Gln is synthesized from Glu and ammonia in astrocytes whereupon it is transferred to neurons to give rise to neurotransmitter Glu, which following its release from neurons is taken up by astrocytes and recycled to Gln (Hertz *et al.* 1999; Bröer and Brookes 2001; Albrecht *et al.* 2007, 2010a).

Received April 9, 2015; revised manuscript received August 25, 2015; accepted September 14, 2015.

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Abbreviations used: BSA, bovine serum albumin; DMEM, Dulbecco's modified Eagle's medium; DTT, dithiothreitol; GC-MS, gas chromatography – mass spectrometry; Gln, glutamine; Glu, glutamate; HPLC, high-performance liquid chromatography; MTBSTFA, N-methyl-N-(t-butyldimethylsilyl) trifluoroacetamide; PDH, pyruvate dehydrogenase; PMSF, phenylmethanesulfonyl fluoride; SEM, standard error of the mean; t-BDMS-Cl, t-butyldimethylchlorosilane; TCA cycle, tricarboxylic acid cycle.

Transfer of Gln from astrocytes to neurons involves Gln-transporting moieties, which are asymmetrically distributed between the two cell types (Rae *et al.* 2003). The astrocytic cell membranes abound in N system transporters SN1 and SN2 which, though bidirectional in nature, show preponderance to mediate Gln efflux (Chaudhry *et al.* 1999; Hamdani el *et al.* 2012). Neuronal cell membranes, in turn, are enriched in system A transporters (SAT1, SAT2) that exclusively mediate the inward Gln transport and as such are held responsible for neuronal Gln uptake (Chaudhry *et al.* 2002a,b, and references therein).

While the critical role of system A transporters in channeling the astroglia-derived Gln to neurons appears well established (Mackenzie *et al.* 2003), the relative contribution of the N system to Gln efflux from astrocytes has not been definitively confirmed. The picture is blurred by the presence on the astrocytic cell membrane of an array of different sodium-dependent and -independent transporters (Heckel *et al.* 2003). The alanine-serine-cysteine system (ASC system) transporter ASCT2 seems to be well suited for being involved in Gln recycling since it catalyzes bidirectional Gln transport by exchange with Glu (ASCT2) (Bröer *et al.* 2004). A related unknown is whether and to what degree, the transport-controlled efflux of Gln modulates astrocytic metabolism, especially the conversion to Glu and Gln of its two canonical substrates, glucose and acetate. In the present study, therefore, we compared (i) the amounts of glutamine, glutamate, aspartate and glutathione and the metabolism of two ^{13}C-labeled substrates [1-^{13}C]glucose and [2-^{13}C]acetate using gas chromatography mass spectrometry (Walls *et al.* 2015) and (ii) the uptake and release of preloaded radiolabeled Gln in cultured neocortical astrocytes with unmodified N system transporter repertoire (SN1/SN2+ astrocytes) with those in which SN1 and SN2 were knocked down using the siRNA silencing strategy (SN1/SN2− astrocytes). The metabolite-labeling principles are illustrated in Fig. 1.

Ammonia is a natural, direct substrate of Gln synthesis in astrocytes (Yudkoff *et al.* 1984). Accordingly, in cultured astrocytes, ammonia instantly prompts Gln synthesis and its spontaneous efflux (Waniewski and Martin 1986). However, evidence from *in vivo* and *in vitro* studies (treatment of cultured

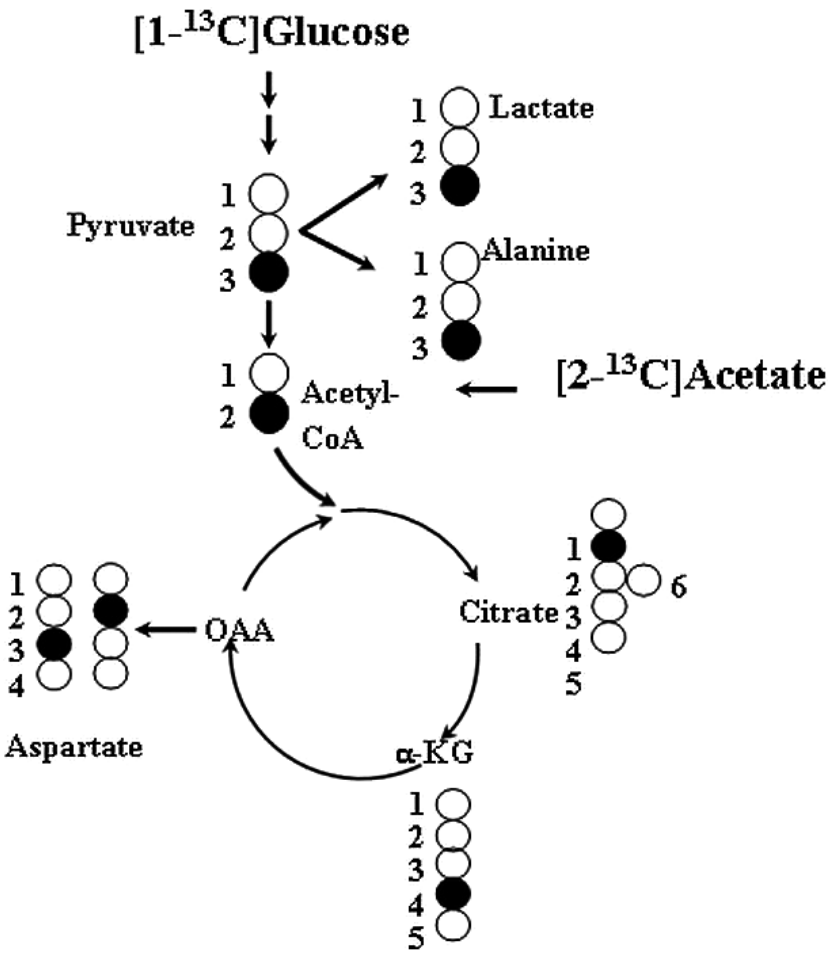

Fig. 1 Scheme of [1-^{13}C]glucose and [2-^{13}C]acetate metabolism. When metabolized *via* glycolysis, [1-^{13}C]glucose yields equal amounts of unlabeled pyruvate and [3-^{13}C]pyruvate. The labeled pyruvate can be converted to [3-^{13}C]lactate or [3-^{13}C] alanine, which may be released to the medium. Alternatively, the label from [3-^{13}C]pyruvate can be incorporated into TCA cycle intermediates following conversion to [2-^{13}C]acetyl CoA. [2-^{13}C] acetate is converted to [2-^{13}C]acetyl CoA directly. [2-^{13}C]Acetyl CoA enters the TCA cycle by condensing with oxaloacetate to form [2-^{13}C]citrate and eventually α-[4-^{13}C] ketoglutarate which can be converted to [4-^{13}C]glutamate and [4-^{13}C]glutamine. If α-[4-^{13}C]ketoglutarate remains in the TCA cycle, it is metabolized to succinate. Because of the symmetric nature of succinate, the label is scrambled, resulting in equal amounts of ^{13}C labeling in the C2and the C3 positions of succinate, and thereafter in fumarate, malate, oxaloacetate and aspartate.

astrocytes with ammonium ions) leaves no doubt that prolonged exposure of brain to ammonia, as is the case in hyperammonemic encephalopathies including hepatic encephalopathy (HE), elicits severe derangements in astrocytic metabolism and function (Sinke *et al.* 2008; Häussinger and Görg 2010; Zemtsova *et al.* 2011; Görg *et al.* 2013), some of which appear to be mediated by accumulating Gln (Albrecht and Norenberg 2006; Jayakumar *et al.* 2006). We hypothesized that alterations of astrocytic metabolism and its coupling to Gln efflux evoked by prolonged ammonia treatment, could be further modulated in cells deplete of SN1 and/or SN2. To test this hypothesis we carried out metabolic and Gln uptake/release experiments using control and SN1/SN2− astrocytes treated or not treated with ammonia.

Materials and methods

Materials

Plastic tissue culture dishes were purchased from Corning Costar (Sigma-Aldrich, St. Louis, MO, USA), culture medium and N-methyl-N-(t-butyldimethylsilyl) trifluoroacetamide + 1% t-butyldimethylchlorosilane (MTBSTFA) and *o*-phthaldialdehyde from Sigma-Aldrich (St. Louis, MO, USA), fetal bovine serum (FBS) from Gibco (Life Technologies, Grand Island, NY, USA), High Perfect Reagent (Qiagen, Germany).

[1-^{13}C]glucose (99 %) and potassium [2-^{13}C]acetate (99%) were purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA, USA). All other chemicals were of the purest grade available from regular commercial sources.

Preparation of astrocyte cultures

Cortical astrocytes were isolated from 7-day-old C57BL6/J mice of both sexes and cultured as described earlier (Hertz *et al.* 1989). 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. 87/2012). 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 and some biochemical alterations. Cells were grown at 37°C in a humidified atmosphere of 95% air and 5% CO_2, in six-well plates or in 60 mm dishes. Experiments were performed on 3-week astrocytes.

Silencing of the gene coding for SN1 transporter

To down-regulate the SN1, transfection with a mix of four types of siRNA duplexes, consisted of 21 nucleotides were used. Each type of siRNA was targeted to different gene region to obtain the most effective silencing. Sense strands used in this study: 5′-ACCGAAUAUGAUGCAUUUCAA-3′; 5′-AAGGAUAUCUUUGGAGUCGUA-3′, 5′-UUCCUUCGUCUCUUUAUUUAA-3′; 5′-CGCCUCUGUAUAUAUAUAUUA-3′.

Three-week astrocytes were washed with phosphate-buffered saline (PBS), trypsinized to detach cells from the plates and then seeded at a density of 1.8×10^5 per well in six-well plate (for efficiency test at 6×10^4 cells/well on 24-well plate) in 1.0 mL of astrocytic growth medium (Dulbecco's modified Eagle's medium 10% FBS). On the same day, cells were transfected with HiPerFect transfection reagent (Qiagen), according to fast-forward protocol, designed for adherent cells and provided by manufacturer. siRNA (2 μM) was diluted in 300 μL OptiMEM (Gibco) without serum. Nine μL of HiPerfect reagent was added to the samples. After 30 min incubation at 20–22°C, complexes between reagent and siRNA were formed and then added to the cells. Cells were cultivated with the transfection complexes under normal growth conditions for 24 h and then used for monitoring gene silencing or for metabolomic experiments.

Real time PCR analysis

Total RNA from astrocytes was isolated using TRI Reagent (Sigma-Aldrich, St. Louis, MO, USA)), then 1 μg was reverse transcribed using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Warrington, UK). Total RNA for transfection efficiency test was isolated and reverse transcribed using TaqMan Gene Expression Cells-to-CT Kit (Ambion, Austin, TX, USA, Life Technologies). Real time PCR was performed in 96-well plates with the ABI 7500 apparatus (Applied Biosystems) using the minor groove binder (MGB) Taqman probe assay. Primers and probes for SN1, SN2, ASCT2 and endogenous control β-actin (Mm0120670_m1, Mm00549967_m1, Mm00436603_m1 and Mm00607939_s1 respectively) were purchased from Applied Biosystems. Each reaction contained 5 μL Taqman Universal PCR Master Mix (Applied Biosystems) in a total volume of 10 μL, and 1.5 μL cDNA was added to the reaction. The real time PCR reactions were performed at 95°C for 10 min, followed by 40 cycles of 30 s at 95°C and 1 min at 60°C. The results of the analysis were calculated in relation to the β-actin product, and results were calculated according to, and expressed by, an equation (2-ΔΔCt) that gives the amount of target, normalized to an endogenous reference and relative to a calibrator. Ct is the threshold cycle for target amplification (Livak and Schmittgen 2001).

Protein isolation and western blot analysis

Astrocytes were washed in PBS, suspended in 1 mL PBS, scraped off and centrifuged for 5 min at 1000 *g* and 4°C. Supernatant was discarded and astrocytes were homogenized in RIPA buffer containing Protease Inhibitor Cocktail (concentration 1 : 200, Sigma-Aldrich, St. Louis, MO, USA), phosphatase inhibitor cocktail (concentration 1 : 100, Sigma-Aldrich, St. Louis, MO, USA) and 50 μM sodium fluoride 0.5 M (Fluka, Sigma-Aldrich, Switzerland) by sonication and then centrifuged for 10 min at 10 000 *g* and 4°C. The supernatants was transferred to a new Eppendorf tube and used for further investigations as a cytosolic fraction. Protein concentration was measured using bicinchoninic acid assay. Protein (30 μg) was boiled with sodium dodecyl sulfate for polyacrylamide gel sample buffer for 10 min, separated on sodium dodecyl sulfate–polyacrylamide gel electrophoresis and then transferred onto nitrocellulose membrane. Membranes were blocked with 5% non-fat dry milk in TBS-T buffer. Incubation with antibodies against SN1 (1 : 800, 14315-1-AP, ProteinTech, Manchester, UK) was

done in TBS-T buffer with 1% non-fat dry milk overnight at 4°C followed by 1 h incubation with HRP-conjugated-anti-goat IgG (1 : 2500, sc-2020, Sigma-Aldrich) for detection by Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA). The first antibody was stripped off with 0.1 M glycine, pH 2.9, and second incubation was performed with an antibody against Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) 1 h incubation at 20–22°C (1 : 7500, HRP-60004, ProteinTech). The chemiluminescent signal acquisition and densitometry analysis were conducted using the G-Box system (SynGene, Cambridge, UK) and GeneTools software (SynGene) respectively.

Incubation and extraction procedure

Control (SN1/SN2+) and system N silenced (SN1/SN2−) mice astrocytes were treated or not with 5 mM ammonium chloride (ammonia) for 24 h. After incubation, the culture medium was removed and cells were twice washed with phosphate-buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 7.3 mM Na_2HPO_4, 0.9 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4, 37°C) and incubated for 4 h in a serum-free medium containing 2.5 mM glutamine and two combinations of substrates (in the presence or absence of ammonia): (i) 3 mM [1-^{13}C] glucose and 3 mM potassium acetate; (ii) 3 mM glucose plus 3 mM potassium[2-^{13}C]acetate. Incubation was terminated by removing the medium, which was transferred to the Eppendorf tube with addition of 40 μL 250 μM α-ABA and stored at −80°C. The cells were washed with 1 mL of ice-cold PBS and the extraction procedure was performed with an addition of 1.00 mL cold 70% ethanol (contained 2.5 μM α-ABA). The cells were scrapped off and the cell suspension was transferred to a new Eppendorf tube. The extraction procedure was repeated. Tubes were centrifuged (20 000 *g*, 20 min, 4°C) and protein determination in the pellets was performed. Lyophilized cell extracts and media were stored at −20°C.

L-[^{3}H]glutamine uptake assay

For uptake experiments, cultured astrocytes were grown to a density of 3 × 10^6 per 60 mm culture. Growth medium was aspirated, and cells were washed three times with PBS, pH 7.4 then pre-incubated for 15 min in 37°C. Incubation mixtures contained PBS with 0.1 μCi/mL L-[3,4-^{3}H(N)-]glutamine (PerkinElmer, Waltham, MA, USA; specific radioactivity 37 MBq/mL) and unlabeled Gln at varying extracellular concentrations (5–2500 μM/L). The incubation was terminated after 4 min by aspirating the transport buffer followed by three washing cycles with 2 mL of PBS maintained at 4°C. Cells were lysed by addition of 0.5 mL 1 M NaOH. The radioactivity of cell lysates were measured in a Wallac 1409 Liquid Scintillation Counter (Perkin – Elmer, Finland).

L-[^{3}H]glutamine efflux assay

Cultured cortical astrocytes were incubated in PBS buffer at 37°C containing 0.25 μCi/mL 1 mM L-[3,4-^{3}H(N)]glutamine for 30 min. To initiate efflux, extracellular radioactivity was removed by washing astrocytes three times with 1 mL of PBS and finally 1 mL of PBS was added to the cells. Samples were taken from the supernatant after 0, 5, 10 and 15 min. Samples were collected and [^{3}H]glutamine radioactivity released from the cells was measured. After incubation cells were lysed by addition of 1 mL 1 M NaOH and radioactivity in an aliquot part of lysates were measured. Results were calculated and plotted as fractional efflux, that is, the percent loss in the initial intracellular radioactivity after 5, 10 and 15 min of incubation (see Fig. 2c).

Protein determination

Protein content in the cells was determined in NaOH lysates according to Bradford (1976).

High-performance liquid chromatography

Amino acids and glutathione in cell extracts and amino acids in media were quantified by HPLC on a Hewlett Packard 100 system (Agilent Technologies, Palo Alto, CA, USA) using pre-column derivatization with *o*-phthaldialdehyde (Geddes and Wood 1984) and were subsequently separated on a ZORBAX SB-C18 (4.6 × 150 mm, 3.5 μm) column from Agilent using a phosphate buffer (50 mM, pH = 5.9) and a solution of methanol (98.75%) and tetrahydrofurane (1.25%) as eluents. α-Amino butyric acid (α-ABA) was used as internal standard. The separated amino acids were detected with fluorescence and amounts calculated using a standard curve derived from standard solutions of amino acids.

Gas chromatography – mass spectrometry

The cell extracts and medium samples were lyophilized and re-dissolved in 0.05 M HCl. The pH was adjusted to < 2 and the samples were lyophilized. The amino acids were extracted in multiple steps into an organic phase of ethanol and benzene and lyophilized before derivatization with N-methyl-N-(t-butyldimethylsilyl)trifluoroacetamide + 1% t-butyldimethylchlorosilane (MTBSTFA) (Mawhinney *et al.* 1986). The samples were analyzed on an Agilent 6890 gas chromatograph connected to an Agilent 5975B mass spectrometer (Agilent Technologies).

Data analysis

The parent ion (M) and atom percent excess for one ^{13}C atom (M + 1) values for alanine, aspartate, lactate, citrate and glutamate were calculated from gas chromatography – mass spectrometry data using MassHunter software supplied by Agilent (Agilent Technologies) and correcting for the naturally abundant ^{13}C by using non-enriched standards.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 (GraphPad Software, La Jolla, CA, USA). Confirmation of normality of the data distribution was checked using Kołmogorow–Smirnow test. Statistical significance was determined by one-way analysis of variance (one-way ANOVA) followed by Dunnett's *post hoc* comparisons, two-way analysis of variance (two-way ANOVA) followed by Bonferroni post-test for more than two groups and an unpaired Student's *t*-test for two groups. A probability value of 0.05 or less was considered statistically significant.

Results

SN1 expression and silencing

The expression of SN1 transporter highly predominates in mouse cortical astrocytes, as the relative SN2 mRNA level was ~ 0.0002 compared to ~ 1.0 value for SN1 mRNA (Fig. 2a). The ASCT2 mRNA expression was ~ 0.6 com-

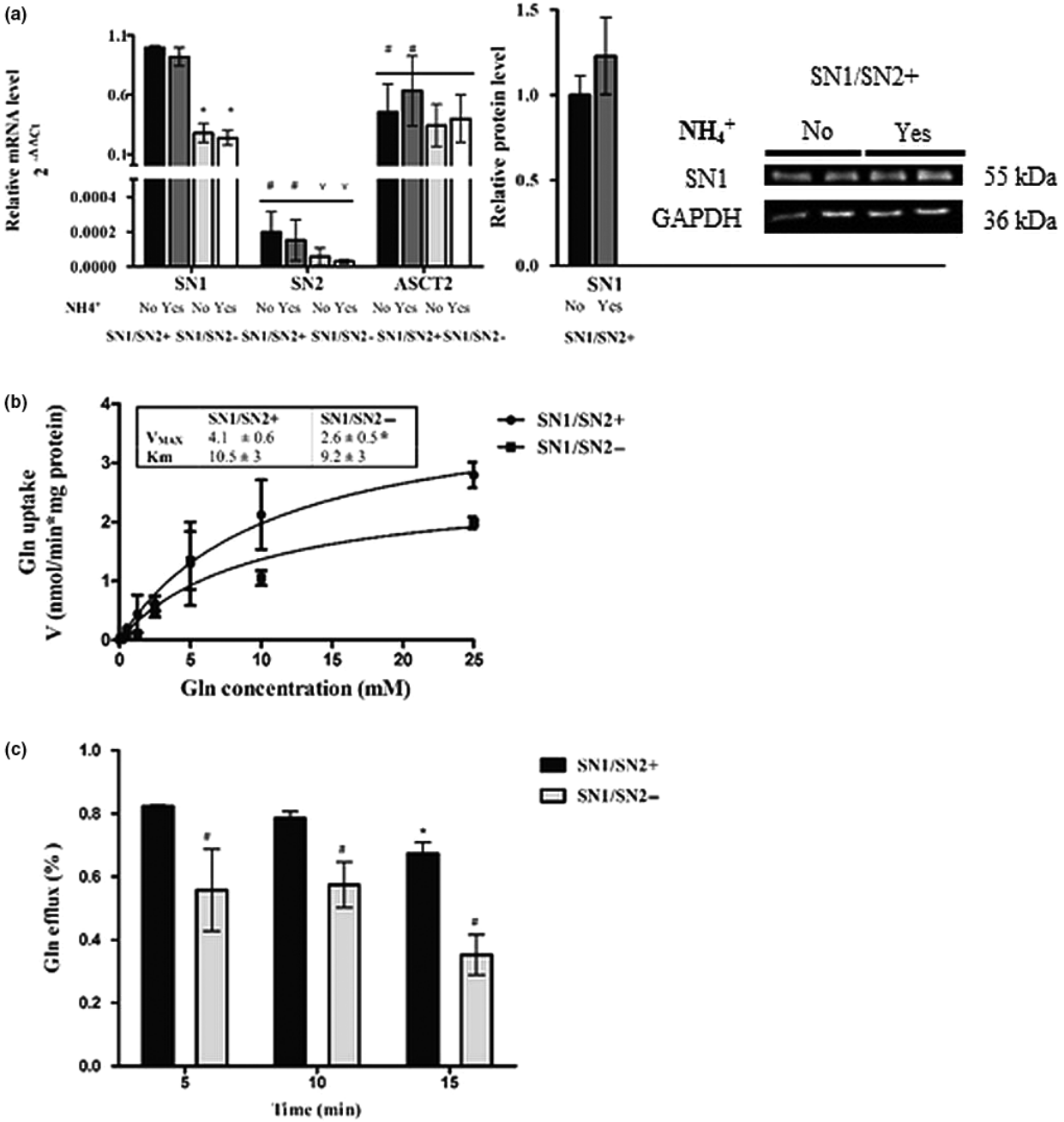

Fig. 2 (a) Expression of mRNAs coding for SN1, SN2 and ASCT2 in SN1/SN2+ and SN1/SN2− silenced astrocytes in the presence (Yes) or absence (No) of 5 mM ammonia. Results are mean ± SD (n = 3–6). (*) Significantly different from SN1/SN2+ ($p < 0.05$; two-way ANOVA). (#) Significantly different from SN1/SN2+ of SN1 mRNA expression ($p < 0.05$; two-way ANOVA). (V) Significantly different from SN1/SN2+ of SN2 mRNA expression ($p < 0.05$; two-way ANOVA). *Right panel*: Expression of SN1 protein in SN1/SN2+ cortical mice astrocytes in the presence (Yes) or absence (No) of 5 mM ammonia. Results are mean ± SD (n = 4). (b) Kinetic analysis of [^{3}H]glutamine uptake in SN1/SN2+ and SN1/SN2− silenced cortical mouse astrocytes. Results are mean ± SD (n = 3). (*) Significantly different from SN1/SN2+ ($p < 0.05$; t-test). (c) Efflux of [^{3}H]glutamine in SN1/SN2+ and SN1/SN2− silenced cortical mouse astrocytes. (*) Significantly different from SN1/SN2+ at 5 min time point; (#) Significant differences between SN1/SN2+ and SN1/SN2− at the respective time points ($p < 0.05$), two-way ANOVA).

pared to SN1 mRNA value (Fig. 2a). Treatment of cultured astrocytes for 24 h with 5 mM ammonia did not affect SN1, SN2 nor ASCT2 mRNA expression (Fig. 2a) nor SN1 protein (Fig. 2b). Silencing of SN1 mRNA by transfection with a mix of four types of siRNA duplexes resulted in SN1 mRNA expression drop by ~ 75%. At the same time the SN2

mRNA level mRNA was reduced by ~ 65% (Fig. 2a). The expression of ASCT2 mRNA was unaffected. There was no difference in SN1 mRNA level between SN1/SN2+ astrocytes and astrocytes mock-transfected with Qiagen's HiPerfect reagent alone (Fig. S1). As a negative control, siRNA construct (AllStars Negative Control siRNA, Qiagen, USA) was used.

Uptake and efflux of preloaded Gln

Kinetic analysis revealed a decrease in [^{3}H]Gln uptake in SN1/SN2− astrocytes as compared to SN1/SN2+ astrocytes (Fig. 2b). Efflux of preloaded [^{3}H]Gln, expressed as the % of total radioactivity lost, was likewise reduced in SN1/SN2− astrocytes, the reduction being observed at each time point (Fig. 2c). Collectively, the results underscore the critical role of N system in astrocytic Gln transport in both directions.

Effect of SN1/SN2 silencing on metabolism of ^{13}C-labeled glucose and acetate

In [1-^{13}C]glucose-enriched growth media derived from ammonia-treated SN1/SN2− astrocytes, the atom percent excess ^{13}C values (given as M + 1) for lactate was significantly lower than from ammonia-treated SN1/SN2+ astrocytes. The parent ion accumulation for lactate (given as M) in ammonia-treated SN1/SN2− astrocytes was increased. The M or M + 1 values for lactate were not changed in SN1/SN2− astrocytes not treated with ammonia. No manipulation altered the M or M + 1 values for alanine (Table 1).

Experiments described in Table 2 served to compare [1-^{13}C]glucose and [2-^{13}C]acetate to alanine, aspartate, glutamate and citrate. In the absence of ammonia, conversion of [1-^{13}C]glucose to alanine or glutamate (M + 1) was decreased in SN1/SN2− astrocytes as compared to SN1/SN2+ astrocytes, whereas no effect of SN1/SN2 silencing was noted with regard to aspartate or citrate. Treatment with ammonia abolished the decrease of M + 1 for alanine and glutamate in SN1/SN2− astrocytes, and caused a decrease of [1-^{13}C]glucose to aspartate. The parent ion accumulation (M) in SN1/SN2− astrocytes incubated with [1-^{13}C]glucose in the absence of ammonia was increased for alanine, aspartate and glutamate, and in the presence of ammonia returned to control level for all the compounds except aspartate. An analysis of [2-^{13}C]acetate metabolism revealed no difference in M + 1 values in the absence of ammonia and a decrease of glutamate labeling in the presence of ammonia (Table 2).

In the absence of ammonia intracellular Gln concentration in SN1/SN2− cells was 3.5-fold increased, whereas glutathione, aspartate, alanine and glutamate content were doubled. Compare to SN1/SN2+ control cells, Gln consumption in SN1/SN2− astrocytes was not changed both in the presence or absence of ammonia (Table 3).

Discussion

Synthesis of Gln from Glu and ammonia and Gln efflux from astrocytes are the principal astrocytic components of the GGC (Hertz *et al.* 1999; Bröer and Brookes 2001; Albrecht *et al.* 2007; and references therein). Glucose and acetate are the canonical metabolic precursors of Glu and Gln. Using [1-^{13}C]glucose it is possible to get information about glycolysis and mitochondrial metabolism including pyruvate dehydrogenation, whereas using [2-^{13}C]acetate it is possible to get information about compartmentation of mitochondrial metabolism since acetate and glucose are not always metabolized in the same compartment (Sonnewald *et al.* 2011).

Astrocytic Gln efflux not only enables its supply to adjacent neurons for synthesis of neurotransmitters Glu or GABA, but also facilitates clearance of excess Gln from brain to blood (O'Kane *et al.* 2004). Seminal studies by Chaudhry *et al.* (1999), Cubelos *et al.* (2005) and Bröer *et al.* (1999) proved the presence in astrocytes of the two system N transport moieties, SN1 and SN2, the characteristics of which suggested their role in astrocytic Gln efflux. However, the contribution

Table 1 Relative quantification of isotopic labeling [the parent ion (M) and atom percent excess ^{13}C (M + 1) values] for alanine and lactate of medium containing [1-^{13}C]glucose from SN1/SN2+ control or SN1/SN2− astrocytes

		Lactate		Alanine	
$NH4^+$		M	M + 1	M	M + 1
No	SN1/SN2+	75.8 ± 1.1	24.5 ± 1.1	83.3 ± 6.8	14.3 ± 6.9
No	SN1/SN2−	77.9 ± 2.7	22.3 ± 2.8	72.6 ± 12.2	27.4 ± 12.4
Yes	SN1/SN2+	73.7 ± 0.02	26.7 ± 0.07	83.9 ± 8.1	13.2 ± 7.5
Yes	SN1/SN2−	80.0 ± 3.6*	20.2 ± 3.6*	82.2 ± 2.2	15.8 ± 2.1

Neocortical astrocytes (n = 3–6 in each group) were pre-incubated with Dulbecco's modified Eagle's medium (DMEM) containing 5 or 0 mM NH_4Cl for 24 h followed by incubation in the presence of 2.5 mM Gln, 3 mM glucose, 3 mM potassium acetate plus 3 mM [1-^{13}C]glucose and those that were incubated in 5 mM NH_4Cl with 5 mM NH_4Cl (Yes) and those that were pre-incubated without NH_4Cl continued to be kept without NH_4Cl (No) for 4 h. Media were analyzed by gas chromatography – mass spectrometry (GC-MS). For more details see Materials and methods section. Results are presented as mean ± SD. Statistical significance was calculated using two-way ANOVA followed by Bonferroni *post hoc* test, *$p < 0.05$ difference from SN1/SN2+ cells.

Table 2 Relative quantification of isotopic labeling [the parent ion (M) and atom percent excess ^{13}C (M + 1) values] for alanine, aspartate, citrate and glutamate from extracts of SN1/SN2+ or SN1/SN2− astrocytes in the presence of [1-^{13}C]glucose or [2-^{13}C] acetate

		Alanine		Aspartate		Glutamate		Citrate	
$NH4^+$		M	M + 1	M	M + 1	M	M + 1	M	M + 1
[1-^{13}C]glucose									
No	SN1/SN2+	71.4 ± 8.7	28.6 ± 8.2	91.9 ± 3.9	5.1 ± 2.3	92.8 ± 1.3	7.0 ± 1.4	79.7 ± 16.6	7.7 ± 6.2
No	SN1/SN2−	89.0 ± 2.0*	12.3 ± 2.6*	101.2 ± 4.0*	2.7 ± 2.5	98.7 ± 4.4*	3.4 ± 2.4*	94.1 ± 3.6*	6.6 ± 3.7
Yes	SN1/SN2+	81.3 ± 9.4	18.9 ± 9.4	90.9 ± 2.1	7.6 ± 1.5	92.3 ± 1.7	7.4 ± 1.6	90.4 ± 3.8	9.6 ± 3.9
Yes	SN1/SN2−	70.1 ± 20.0	29.7 ± 19.4	96.6 ± 3.7*	3.5 ± 2.0*	95.2 ± 3.6	5.3 ± 2.3	92.5 ± 3.3	7.4 ± 3.1
[2-^{13}C]acetate									
No	SN1/SN2+			95.2 ± 3.1	3.7 ± 1.9	92.4 ± 4.3	7.1 ± 3.6	92.6 ± 2.8	7.0 ± 2.6
No	SN1/SN2−			96.1 ± 1.5	2.7 ± 0.9	93.5 ± 4.7	6.1 ± 3.8	92.9 ± 4.7	5.8 ± 3.8
Yes	SN1/SN2+			94.8 ± 3.5	3.3 ± 2.1	88.9 ± 5.1	9.1 ± 4.1	94.2 ± 1.2	5.1 ± 1.3
Yes	SN1/SN2−			95.7 ± 1.8	3.1 ± 1.1	94.7 ± 3.4*	5.0 ± 2.8*	95.0 ± 2.6	4.1 ± 1.6

Neocortical astrocytes (n = 7–12 in each group) were pre-incubated with Dulbecco's modified Eagle's medium (DMEM) containing 5 or 0 mM NH_4Cl for 24 h followed by incubation in the presence of 2.5 mM glutamine, 3 mM glucose, 3 mM potassium acetate plus 3 mM [1-^{13}C]glucose or [2-^{13}C]acetate plus 6 mM glucose and those that were incubated in 5 mM NH_4Cl with 5 mM NH_4Cl (Yes) and those that were pre-incubated without NH_4Cl continued to be kept without NH_4Cl (No) for 4 h. Cell extracts were analyzed by gas chromatography – mass spectrometry (GC-MS). For more details see Materials and methods section. Results are presented as mean ± SD. Statistical significance was calculated using two-way ANOVA followed by Bonferroni *post hoc* test, *$p < 0.05$ difference from SN1/SN2+ cells.

Table 3 Amounts of glutathione, aspartate, glutamate and glutamine (nmol/mg protein) and consumption of glutamine in SN1/SN2+ or SN1/SN2− astrocytes

$NH4^+$		Glutathione	Aspartate nmol/mg	Glutamate	Glutamine	Glutamine consumption nmol/mg/4 h
No	SN1/SN2+	1.1 ± 0.5	0.3 ± 0.2	1.6 ± 0.6	6.0 ± 5.2	3535 ± 2022
No	SN1/SN2−	2.5 ± 1.4*	0.7 ± 0.4*	3.8 ± 2.0*	22.4 ± 10.4*	2350 ± 853
Yes	SN1/SN2+	1.0 ± 0.4	0.2 ± 0.2	1.5 ± 0.6	3.3 ± 1.8	3516 ± 1760
Yes	SN1/SN2−	1.9 ± 1.5	0.5 ± 0.3*	2.6 ± 1.8	3.6 ± 0.7$^{\#}$	2312 ± 1020

Neocortical astrocytes (n = 12 in each group) were pre-incubated with Dulbecco's modified Eagle's medium (DMEM) containing 5 or 0 mM NH4Cl for 24 h followed by incubation in the presence of 2.5 mM glutamine, 6 mM glucose plus 3 mM acetate and those that were incubated in 5 mM NH_4Cl with 5 mM NH_4Cl (Yes) and those that were pre-incubated without NH_4Cl continued to be kept without NH_4Cl (No) for 4 h. Media and extracts were analyzed by HPLC. For more details see Materials and methods section. Results are presented as mean ± SD. Statistical significance was calculated using two-way ANOVA followed by Bonferroni *post hoc* test, *$p < 0.05$ difference from SN1/SN2+, $^{\#}p < 0.05$ difference from SN1/SN2− (No).

of system N to the efflux of Gln and its causal relation to the metabolism of, glucose and acetate, has not been analyzed in detail. The key observation of the present study is that in cultured astrocytes, a simultaneous knock down of SN1 and SN2 using the siRNA strategy, substantially increased Gln accumulation in the cells. Since Gln metabolism was not affected by silencing, the results suggested that the increased retention of Gln in the cells was because of impaired efflux. The suggestion was directly confirmed by the observed decrease in the efflux of newly loaded radiolabeled Gln from SN1/SN2− as compared to SN1/SN2+ astrocytes (Fig. 2c). Moreover, the absence of the system N transporters markedly affected the metabolic flux of one of the substrates, glucose, and relatively less so the flux of acetate. It can be speculated that acetate is entering a different compartment from glucose namely the compartment in which keto-acid catabolism is taking place as shown by Brekke *et al.* (2015) and Melø *et al.* (2006). Furthermore, the decrease of Gln efflux by system N transporters is not modulated by pathophysiological concentration of ammonia, on the output of the metabolic products of glucose and acetate.

Cultured astrocytes contain mRNAs coding for both SN1 and SN2, with SN1 appearing to predominate (Fig. 2a). Immunocytochemical studies performed in non-fragmented brain tissue revealed considerable differences in regional distribution of each of the transporters and thus its potential role in mediating Gln efflux from astrocytes *in situ* (Boulland *et al.* 2002; Chaudhry *et al.* 2002a,b; Cubelos *et al.* 2005). However, siRNA duplexes used in this study knocked down

the residual SN2 mRNA with similar efficiency as SN1 (Fig. 1a), most likely because of the considerable sequence homology of the two RNAs (~ 95%). Since the only other system N transporter detected in the brain, SNAT7, is located in neurons (Hagglund *et al.* 2011), it is plausible that knock down of SN1 and SN2 renders the cells virtually free of system N. The almost four-fold increase of Gln accumulation in the silenced astrocytes clearly bespeaks a major role of system N in catalyzing astrocytic Gln efflux. This increase in intracellular Gln was accompanied by a doubling of glutathione, aspartate, alanine and glutamate content demonstrating that astrocyte metabolism is fine-tuned to the export of Gln.

It may be noted that in contrast to absence of the ASC system in a native astrocytes (Bröer and Brookes 2001), in cultured astrocytes ASCT2 transporter belonging to ASC system, is the dominating Gln-transporting moiety engaged in Gln efflux (Bröer *et al.* 1999; Heckel *et al.* 2003; Rae *et al.* 2003). Of note in this context, in our experimental setting, ASCT2 mRNA expression was unaltered, despite its relatively high expression compare to SN1 mRNA level. The role of ASCT2 carrier in the efflux of newly loaded Gln may be secondary.

It must be noted that discrepant data have been reported regarding expression patterns of system N transporters, and the specific roles of SN1 and SN2 in different animal models [hyperammonemia, HE induced by thioacetamide (TAA), *bile duct ligation* (BDL) and species (rat vs. mouse)] (Desjardins *et al.* 2012; Rama Rao and Norenberg 2014; Zielińska *et al.* 2014; Leke *et al.* 2015). Apart from differences in the techniques used (the use of different antibodies etc.), two model-related factors may be taken into account: (i) in the *in vitro* model used in the present study, astrocytes are only exposed to ammonia, whereas the *in vivo* models referred to above, reflect concerted effects of ammonia, inflammatory response and multitude of neurotoxic compounds related to liver damage, (ii) as emphasized by Rodríguez *et al.* (2014), the interspecies differences may reflect different ontogenesis patterns of SN1 and SN2 in mouse and rat. Gln consumption was not changed in the silenced astrocytes compared to control indicating that inward Gln transport was unaffected. The atom percent excess ^{13}C values (given as M + 1) for alanine, aspartate and glutamate were decreased when the cells were incubated with [1-^{13}C]glucose in the SN1/SN2− astrocytes compared to control. Considering this decrease, it should be noted, that the amounts of aspartate and glutamate were increased, thus there is no net decrease in [1-^{13}C]glucose metabolism but an accumulation of unlabeled amino acids in the SN1/SN2− astrocytes. It appears that there is no adequate feedback inhibition in the astrocytes upon Gln accumulation. This might prove to be detrimental in the event of increased Gln content in diseases such as hepatic encephalopathy (Takahashi *et al.* 1991; Albrecht and Norenberg 2006; Tofteng *et al.* 2006; Albrecht *et al.* 2010b). When [2-^{13}C]acetate was the labeled substance, no difference was seen in M + 1 values indicating that acetate metabolism was increased in the silenced astrocytes. Thus, as mentioned above, acetate must have entered a slightly different mitochondrial compartment from glucose since the two substrates for acetyl CoA synthesis revealed differential metabolic alterations. Identification of the pertinent mitochondrial compartment will be an attractive venue for further investigations.

Ammonia did not alter Gln consumption nor Gln content in either silenced or control astrocytes. It should be noted that the experiments were performed in the presence of 2.5 mM extracellular Gln, a physiological concentration which will give rise to physiological intracellular Gln levels when Gln transport is unhindered. The absence of intracellular Gln accumulation in ammonia-treated SN1/SN2− astrocytes is an intriguing observation that escapes simple interpretation. The following mutually non-exclusive hypotheses may be invoked: (i) ammonia down-regulates system N transporter expression and function, and in this way favors the action of system ASC; (ii) ammonia up-regulates system ASC, rendering system N futile regardless of its effect on system N expression. The observation that SN1/SN2 silencing does not affect ASCT2 mRNA in control- nor ammonia-treated astrocytes (Fig. 2a) appears to counter possibility 1. Clearly, more detailed analysis of the contribution of Gln-transporting systems other than system N to Gln transport in the above conditions is needed to provide clear-cut interpretation. Of note in this context, ammonia under conditions similar to those used in the present study up-regulates the heteromeric Gln/Arg exchanger in rat cortical astrocytes (Zielińska *et al.* 2012).

The presence of ammonia caused a decrease in the aspartate content in the silenced astrocytes compared to control cells, and labeling of glutamate from [2-^{13}C]acetate was decreased, indicating altered mitochondrial metabolism in the presence of ammonia. Furthermore, in the presence of ammonia a small decrease was observed in the labeling of lactate from [1-^{13}C]glucose in the silenced astrocytes suggesting that lactate and thus pyruvate was synthesized slightly more from pathways not involving glycolysis directly, such as partial pyruvate recycling from the tricarboxylic acid cycle (Morken *et al.* 2014; Sonnewald 2014).

Concluding comments

It can be concluded that the astrocytic N system transporters, SN1 and SN2, indeed, play an important role in the transport of Gln from astrocytes to the extracellular space. Moreover, their absence variably, but significantly affects [1-^{13}C]glucose and [2-^{13}C]acetate metabolism in mitochondria. The relative contributions of SN1 and SN2 in the native setting remain to be delineated using knock-down approaches *in vivo*. In general terms, impairments of system

N could have detrimental effects on amino acid neurotransmitter synthesis and, in consequence, on glutamatergic neurotransmission.

Acknowledgments and conflict of interest disclosure

We thank Dr Paweł Goryński from National Institute of Public Health – National Institute of Hygiene for expert advice on statistical evaluation of the results. The research leading to these results has received funding from the Polish-Norwegian Research Program operated by the National Centre for Research and Development under the Norwegian Financial Mechanism 2009-2014 in the frame of Project Contract No Pol-Nor/196190/23/2013. The authors have no conflict of interest to declare.

All experiments were conducted in compliance with the ARRIVE guidelines.

Supporting information

Additional supporting information may be found in the online version of this article at the publisher's web-site:

Figure S1. Efficiency of SN1 silencing in mice cortical astrocytes. Results are mean ± SD ($n = 3$). (*) Significantly different from control ($p < 0.05$; one-way ANOVA followed by Dunnett's *post hoc* test).

References

Albrecht J. and Norenberg M. D. (2006) Glutamine: a Trojan horse in ammonia neurotoxicity. *Hepatology* **44**, 788–794.

Albrecht J., Sonnewald U., Waagepetersen H. S. and Schousboe A. (2007) Glutamine in the central nervous system: function and dysfunction. *Front Biosci.* **12**, 332–343.

Albrecht J., Sidoryk-Węgrzynowicz M., Zielińska M. and Aschner M. (2010a) Roles of glutamine in neurotransmission. *Neuron. Glia Biol.* **6**, 263–276.

Albrecht J., Zielińska M. and Norenberg M. D. (2010b) Glutamine as mediator of ammonia neurotoxicity: a critical appraisal. *Biochem. Pharmacol.* **80**, 1303–1308.

Boulland J. L., Osen K. K., Levy L. M., Danbolt N. C., Edwards R. H., Storm-Mathisen J. and Chaudhry F. A. (2002) Cell-specific expression of the glutamine transporter SN1 suggests differences in dependence on the glutamine cycle. *Eur. J. Neurosci.* **15**, 1615–1631.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Brekke E., Morken T. S. and Sonnewald U. (2015) Glucose metabolism and astrocyte-neuron interactions in the neonatal brain. *Neurochem. Int.* **82**, 33–41.

Bröer S. and Brookes N. (2001) Transfer of glutamine between astrocytes and neurons. *J. Neurochem.* **77**, 705–719.

Bröer A., Brookes N., Ganapathy V., Dimmer K. S., Wagner C. A., Lang F. and Bröer S. (1999) The astroglial ASCT2 amino acid transporter as a mediator of glutamine efflux. *J. Neurochem.* **73**, 2184–2194.

Bröer A., Deitmer J. W. and Bröer S. (2004) Astroglial glutamine transport by system N is upregulated by glutamate. *Glia* **48**, 298–310.

Chaudhry F. A., Reimer R. J., Krizaj D., Barber D., Storm-Mathisen J., Copenhagen D. R. and Edwards R. H. (1999) Molecular analysis of system N suggests novel physiological roles in nitrogen metabolism and synaptic transmission. *Cell* **99**, 769–780.

Chaudhry F. A., Reimer R. J. and Edwards R. H. (2002a) The glutamine commute: take the N line and transfer to the A. *J. Cell Biol.* **157**, 349–355.

Chaudhry F. A., Schmitz D., Reimer R. J., Larsson P., Gray A. T., Nicoll R., Kavanaugh M. and Edwards R. H. (2002b) Glutamine uptake by neurons: interaction of protons with system a transporters. *J. Neurosci.* **22**, 62–72.

Cubelos B., González-González I. M., Giménez C. and Zafra F. (2005) Amino acid transporter SNAT5 localizes to glial cells in the rat brain. *Glia* **49**, 230–244.

Desjardins P., Du T., JiangW Peng. L. and Butterworth R. F. (2012) Pathogenesis of hepatic encephalopathy and brain edema in acute liver failure: role of glutamine redefined. *Neurochem. Int.* **60**, 690–696.

Geddes J. W. and Wood J. D. (1984) Changes in the amino acid content of nerve endings (synaptosomes) induced by drugs that alter the metabolism of glutamate and gamma-aminobutyric acid. *J. Neurochem.* **42**, 16–24.

Görg B., Schliess F. and Häussinger D. (2013) Osmotic and oxidative/nitrosative stress in ammonia toxicity and hepatic encephalopathy. *Arch. Biochem. Biophys.* **536**, 158–163.

Hagglund M. G., Sreedharan S., Nilsson V. C., Shaik J. H., Almkvist I. M., Backlin S., Wrange O. and Fredriksson R. (2011) Identification of SLC38A7 (SNAT7) protein as a glutamine transporter expressed in neurons. *J. Biol. Chem.* **286**, 20500–20511.

Hamdani el H., Gudbrandsen M., Bjørkmo M. and Chaudhry F. A. (2012) The system N transporter SN2 doubles as a transmitter precursor furnisher and a potential regulator of NMDA receptors. *Glia* **60**, 1671–1683.

Häussinger D. and Görg B. (2010) Interaction of oxidative stress, astrocyte swelling and cerebral ammonia toxicity. *Curr. Opin. Clin. Nutr. Metab. Care* **13**, 87–92.

Heckel T., Bröer A., Wiesinger H., Lang F. and Bröer S. (2003) Asymmetry of glutamine transporters in cultured neural cells. *Neurochem. Int.* **43**, 289–298.

Hertz L., Peng L., Hertz E., Juurlink B. H. and Yu P. H. (1989) Development of monoamine oxidase activity and monoamine effects on glutamate release in cerebellar neurons and astrocytes. *Neurochem. Res.* **14**, 1039–1046.

Hertz L., Dringen R., Schousboe A. and Robinson S. R. (1999) Astrocytes: glutamate producers for neurons. *J. Neurosci. Res.* **57**, 417–428.

Jayakumar A. R., Panickar K. S., Murthy Ch. R. and Norenberg M. D. (2006) Oxidative stress and mitogen-activated protein kinase phosphorylation mediate ammonia-induced cell swelling and glutamate uptake inhibition in cultured astrocytes. *J. Neurosci.* **26**, 4774–4784.

Leke R., Escobar T. D., Rama Rao K. V., Silveira T. R., Norenberg M. D. and Schousboe A. (2015) Expression of glutamine transporter isoforms in cerebral cortex of rats with chronic hepatic encephalopathy. *Neurochem. Int.* **S0197–0186**, 00048. doi:10.1016/j.neuint.2015.03.005.

Livak K. J. and Schmittgen T. D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). *Methods* **25**, 402–408.

Mackenzie B., Schäfer M. K., Erickson J. D., Hediger M. A., Weihe E. and Varoqui H. (2003) Functional properties and cellular distribution of the system A glutamine transporter SNAT1 support specialized roles in central neurons. *J. Biol. Chem.* **278**, 23720–23730.

Mawhinney T. P., Robinett R. S., Atalay A. and Madson M. A. (1986) Gas-liquid chromatography and mass spectral analysis of mono-, di- and tricarboxylates as their tert.-butyldimethylsilyl derivatives. *J. Chromatogr.* **361**, 117–130.

Melø T.M., Nehlig A. and Sonnewald U. (2006) Neuronal glial interactions in rats fed a ketogenic diet. *Neurochem. Int.* **48**, 498–507.

Morken T. S., Brekke E., Håberg A., Widerøe M., Brubakk A. M. and Sonnewald U. (2014) Altered astrocyte-neuronal interactions after hypoxia-ischemia in the neonatal brain in female and male rats. *Stroke* **45**, 2777–2785.

O'Kane R. L., Viña J. R., Simpson I. and Hawkins R. A. (2004) Na+ -dependent neutral amino acid transporters A, ASC, and N of the blood-brain barrier: mechanisms for neutral amino acid removal. *Am. J. Physiol. Endocrinol. Metab.* **287**, E622–E629.

Rae C., Hare N., Bubb W. A., McEwan S. R., Bröer A., McQuillan J. A., Balcar V. J., Conigrave A. D. and Bröer S. (2003) Inhibition of glutamine transport depletes glutamate and GABA neurotransmitter pools: further evidence for metabolic compartmentation. *J. Neurochem.* **85**, 503–514.

Rama Rao K. V. and Norenberg M. D. (2014) Glutamine in the pathogenesis of hepatic encephalopathy: the trojan horse hypothesis revisited. *Neurochem. Res.* **39**, 593–598.

Rodríguez A., Ortega A., Berumen L. C., García-Alcocer M. G., Giménez C. and Zafra F. (2014) Expression of the System N transporter (SNAT5/SN2) during development indicates its plausible role in glutamatergic neurotransmission. *Neurochem. Int.* **73**, 166–171.

Sinke A. P., Jayakumar A. R., Panickar K. S., Moriyama M., Reddy P. V. and Norenberg M. D. (2008) NFkappaB in the mechanism of ammonia-induced astrocyte swelling in culture. *J. Neurochem.* **106**, 2302–2311.

Sonnewald U. (2014) Glutamate synthesis has to be matched by its degradation - where do all the carbons go? *J. Neurochem.* **131**, 399–406.

Sonnewald U., Schousboe A. and Waagepetersen H. (2011) ^{13}C NMR spectroscopy and mass spectrometry analysis of intermediary metabolism in cultured neural cells. I: Neuromethods. Springer Science+Business Media B.V. 2011 ISBN 978-1-61779-076-8. s. 403-414.

Takahashi H., Koehler R. C., Brusilow S. W. and Traystman R. J. (1991) Inhibition of brain glutamine accumulation prevents cerebral edema in hyperammonemic rats. *Am. J. Physiol.* **261**, H825–H829.

Tofteng F., Hauerberg J., Hansen B. A., Pedersen C. B., Jørgensen L. and Larsen F. S. (2006) Persistent arterial hyperammonemia increases the concentration of glutamine and alanine in the brain and correlates with intracranial pressure in patients with fulminant hepatic failure. *J. Cereb. Blood Flow Metab.* **26**, 21–27.

Walls A. B., Waagepetersen H. S., Bak L. K., Schousboe A. and Sonnewald U. (2015) The glutamine-glutamate/GABA cycle: function, regional differences in glutamate and GABA production and effects of interference with GABA metabolism. *Neurochem. Res.* **40**, 402–409.

Waniewski R. A. and Martin D. L. (1986) Exogenous glutamate is metabolized to glutamine and exported by rat primary astrocyte cultures. *J. Neurochem.* **47**, 304–313.

Yudkoff M., Nissim I., Kim S. U., Pleasure D. and Segal S. (1984) Metabolism of 15NH3 in organotypic cerebellar explants and cultured astrocytes: studies with gas chromatography-mass spectrometry. *J. Neurochem.* **42**, 283–286.

Zemtsova I., Görg B., Keitel V., Bidmon H. J., Schrör K. and Häussinger D. (2011) Microglia activation in hepatic encephalopathy in rats and humans. *Hepatology* **54**, 204–215.

Zielińska M., Skowrońska M., Fręśko I. and Albrecht J. (2012) Upregulation of the heteromeric y$^+$LAT2 transporter contributes to ammonia-induced increase of arginine uptake in rat cerebral cortical astrocytes. *Neurochem. Int.* **61**, 531–535.

Zielińska M., Popek M. and Albrecht J. (2014) Roles of changes in active glutamine transport in brain edema development during hepatic encephalopathy: an emerging concept. *Neurochem. Res.* **39**, 599–604.

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Endocrine, Metabolic & Immune Disorders - Drug Targets, **2018**, *18*, **000-000**

REVIEW ARTICLE

Roles of Glutamate and Glutamine Transport in Ammonia Neurotoxicity: State of the Art and Question Marks

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ARTICLE HISTORY

Received: April 04, 2017
Revised: May 16, 2017
Accepted: September 27, 2017

DOI:
10.2174/1871520618666171219124427

Abstract: ***Background:*** Excessive accumulation of ammonia in the brain is a causative factor of an array of neurological manifestations of hyperammonemic encephalopathies ("hyperammonemias", HA) among which hepatic encephalopathy (HE) is a major epidemiologic and therapeutic challenge. While ammonia neurotoxicity is symptomatically and mechanistically very complex, there is a consensus with regard to the leading role in its pathogenesis of: i) astrocytes being the primary cellular target of ammonia toxicity; ii) alterations of glutamate (Glu)-dependent neurotransmission (over-excitation followed by inhibition of glutamatergic tone) being the cornerstone of its neurophysiological manifestations; and iii) brain edema, an often lethal consequence of astrocytic swelling, being among other factors caused by the retention of glutamine (Gln) in these cells.

Objective: This article critically evaluates the present literature attempting to relate manifestations of HA to changes in astrocytic Glu and Gln transport as observed in different *in vivo* and *in vitro* HA and/or HE models. Emphasis is put on two disproportions in the state of the art: i) the paucity of available data regarding ammonia-dependent changes in Glu transport activity vs the relative abundance of information on the expression of astrocytic Glu transporters (GLT-1/EAAT2 and GLAST/EAAT1); ii) the just emerging still not very conclusive knowledge on the response of astrocytic Gln transporters SN1 and SN2.

Conclusion: The review on the above issues is complemented by own recent data which fill some of the many gaps in the knowledge. A brief account is included on the roles of heteromeric cell membrane Glu/arginine (Arg) exchanger y^+LAT2 and on the mitochondrial Gln transport.

Keywords: Ammonia, hepatic encephalopathy, glutamate transport, glutamine transport, GLT-1, GLAST, SN1, SN2.

1. INTRODUCTION

1.1. Reasons to Consider Astrocytic Glutamate and Glutamine Transporters as Key Players in the Progression of Hyperammonemic Encephalopathies

Ammonia is neurotoxic, and its excessive accumulation in the central nervous system (CNS) is a major causative factor in a group of diseases collectively coined as hyperammonemic encephalopathies (further defined as hyperammonemia, HA) [1]. Among these, hepatic encephalopathy (HE) constitutes the most significant socio-epidemiologic, economical and therapeutic challenge [1]. While the mechanisms by which excess ammonia exerts CNS dysfunction are very complex, traits at different levels of an organization which are hallmarks of all the different HAs involve alterations in the expression and activity of astrocytic Glu or Gln transporters.

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At the cellular level, astrocytes are the primary target of ammonia. At the morphological level, this fact has been recognized in the 50s and 60s of the XXth century [2]. Over the years, impaired ability of ammonia-affected astrocytes to buffer the synaptic milieu and to modulate neurotransmission has been exhaustively documented as a major (albeit not exclusive) cause of neuro- and pathophysiological symptoms of HA or HE [3].

Neurophysiologically, progression of HA or HE is characterized by a gradual shift of balance between the excitatory and inhibitory neurotransmission in favor of the latter, whereby changes in the glutamatergic transmission contribute most to the imbalance [4]. In most simple terms, initial increase in the glutamatergic tone which is related to over-activation of ionotropic (mostly NMDA, but also AMPA) Glu receptors, is followed by downregulation of the receptors and decreased excitatory transmission [4]. Increased NMDA receptor activity stimulates nitric oxide synthesis and generates oxidative/nitrosative stress (ONS), eliciting a self-amplifying loop of astrocytic impairment. Increased Glu receptor activity is related to excessive accumulation of neurotransmitter Glu in

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the extracellular space of the CNS [5-7]. Since astrocytic Glu transporters EAAT-2/GLT-1 and EAAT-1/GLAST have long been known to play a key role in the clearance of Glu in the synaptic cleft [8] it is not surprising that the two transporters have attracted attention as potential targets of ammonia. The results of studies addressing changes of EAAT-2/GLT-1 and EAAT-1/GLAST expression and function in the different experimental settings of HE and HA and in *in vitro* systems mimicking the settings form subject of the first part of the review. Literature data are complemented by results of own preliminary experiments performed in mouse models. Mouse models, which appear superior to rat models in that they offer the possibility to genetically manipulate expression of transport moieties, have not been subjected to exhaustive investigation of Glu transport in ammonia neurotoxicity. To account for the influence of neuron-derived factors on the astrocytic response to ammonia, the experiments also measured the effects in mixed astrocyte/neuronal cultures.

At the pathophysiological level, brain edema associated with astrocytic swelling is the major complication of acute HE, leading to death in ~50-80% of cases [9]. Astrocytic swelling reflects ammonia interaction with different molecular targets, and is ultimately mediated by a vicious circle of ONS and accumulation of osmotically active metabolites [10, 11]. Overexposure of astrocytes to ammonia leads to increased conversion of Glu to Gln in a reaction catalyzed by glutamine synthetase (GS). Excessive intracellular accumulation of Gln is thought to cause astrocytic swelling in two mutually not exclusive ways: i) by increasing osmotic pressure and ii) in consequence to its intra-mitochondrial conversion back to Glu and ammonia which elicits accumulation of reactive oxygen species (ROS) and oxidative stress (OS), and promoting mitochondrial permeability transition (MPT) (the "Trojan horse" hypothesis [12, and references therein]). Retention of newly synthesized Gln in astrocytes depends upon the rate of it release to the extracellular space, a process which is primarily mediated by astroglia-specific system N transporters SN1 (SNAT3) and SN2 (SNAT5) [13, 14]. Studies reviewed in this article indicate that altered expression of SN1 and SN2 may interfere with Gln efflux and in this way affect progression of Gln accumulation in ammonia-exposed astrocytes *in vivo* and *in vitro*. As was the case with Glu transporters, this part of the review is likewise supplemented with as yet unreported own data. A few comments are also devoted to y^+LAT2, the heteromeric transporter which prevails in astrocytes and bidirectionally transports Gln in exchange for Arg [15]. Whole brain and astrocytic responses of y^+LAT2 to ammonia during the last few years have been subjected to investigate in this laboratory. With mitochondria being the potential target of Gln gliotoxicity (see above) the review would be incomplete without a brief account on mitochondrial Gln transport, albeit the knowledge of this phenomenon did not reach a conclusive stage as yet. Concluding comments are devoted to incompatibilities between the results of studies carried out in different setting (*in vivo vs in vitro*), to gaps in evidence that remain to be filled and - at last but not least - to inherent limitations of conclusions about ammonia-induced changes in Glu and Gln transport activity to be drawn from transporter expression studies, and of the role played by the transporters in impairment of ammonia-induced neurotransmission and cell volume homeostasis.

1.2. Effects of Ammonia on Glu Transport and Expression of Astroglial EAAT1/GLAST and EAAT2/GLT-1 Transporters: Studies in Human, HA/HE Models and Primary Cultures of Astrocytes

As mentioned before, increased excitatory signaling of neurons resulting from excessive extracellular Glu accumulation has been demonstrated to be critically involved in ammonia neurotoxicity [16]. Intra- and extracellular Glu homeostasis in the brain is regulated by the excitatory amino-acid (EAATs) family of transporters that display considerable homology (50–60% at the amino-acid level) [17]. In humans, the transporters are referred to as EAAT1-5, while in rats and mice they are called GLAST (Glutamate and Aspartate Transporter; homologous to EAAT1), GLT-1 (or GLT1; Glutamate Transporter One; homologous to EAAT2) and EAAC1 (excitatory amino acid carrier one; homologous to EAAT3), respectively. However, since most of Glu clearance is accomplished by astrocytic transporters EAAT1/GLAST and EAAT2/GLT-1 [18], only the two have so far been studied in the context of ammonia neurotoxicity.

As to be concluded from the collection of data illustrated in Table **1**, HA and HE deregulated the expression of both mRNA and protein level of EAAT2/GLT-1, whereas addition of ammonia to cultured astrocytes inhibited EAAT1/GLAST expression only. Reduced EAAT2, but unchanged EAAT1 expression was recently reported in autopsied brain tissue from patients with acute liver failure (ALF) resulting from viral hepatitis [19]. In this manuscript we include our own yet unpublished data on the effect of ammonia on the expression of both Glu astrocytic transporters in mouse cortical astrocytes and in mouse model of azoxymethane (AOM)-induced HE. The expression of mRNA coding for either of the two transporters was unaltered (Table **1**; Fig. **1a**). In agreement with this observation, unchanged [^{3}H]D-aspartate (D-Asp) uptake into ammonia-treated mouse astrocytes was measured (Fig. **1b**). EAAT2/GLT-1mRNA level did not decrease (Fig. **1c**) which was correlated with unchanged [^{3}H]D-Asp uptake into AOM brain slices (Fig. **1d**). No increase was observed in the level of EAAT1/GLAST protein (Fig. **1e**).

1.3. Effect of Ammonia on Expression of Glu Transporters: EAAT1/GLAST and EAAT2/GLT-1 in Mouse Astrocyte/Neuron Mixed Cultures

Experiments carried out in this laboratory demonstrated unchanged mRNA expression but an increase in protein level of both EAAT2/GLT-1 and EAAT1/GLAST in ammonia-treated mouse cortical astrocyte-neuron cultures (Fig. **2**). The mechanism(s) underlying the increase of the two transporters remains to be investigated. Discussion on the large spectrum of possibilities is beyond the scope of this review. One mechanism that may deserve attention but has not yet been addressed in the context of ammonia neurotoxicity is altered splicing of EAATs and/or altered expression of splice variants. Aberrant splicing of EAAT2 resulting in non-functional transporters has been reported in amyotrophic lateral sclerosis (ALS) patients [20], albeit later identification of similar spliced variants in control patients renders the causal relation between aberrant splicing and the ALS symptoms uncertain

Table 1. Expression of glutamate and glutamine transporters in *in vivo* models of hyperammonemia (HA) and hepatic encephalopathy (HE) and in cultured astrocytes treated with ammonia *in vitro*. n.ch. - not changed; - - not measured; ↓ - decrease; ↑- increase. Literature references in parentheses.

		GLT-1/EAAT2		GLAST/EAAT1		SN1/SNAT3		SN2/SNAT5	
		mRNA	Protein	mRNA	Protein	mRNA	Protein	mRNA	Protein
Rat	HE/HA	↓[7, 66-69]	↓[66-71]	-	-	n.ch[72]; ↓/n.ch.[34]	↓/n.ch.[34]	↑[72]; ↓/n.ch.[34]	n.ch./n.ch.[34, 72]
	Astrocytes	-	-	↓[68-69, 73, 74]; n.ch.[75]	↓[68-69, 73, 74]; n.ch.[75]	-	-	-	-
Mouse	HE/HA	↓	n.ch.	n.ch.	↑	↓	↓	↓[35]/-	n.ch.[36]/-
	Astrocytes	n.ch.	-	n.ch.	-	n.ch.[30]	n.ch.[30]	n.ch.[30]	n.ch.[35]
	Astro-neuro co-cultures	n.ch.	↑	n.ch.	↑	n.ch.	↓	n.ch.	↓
Human		↓[76]	↓[76]	n.ch.[77]	n.ch.[77]	-	-	-	-

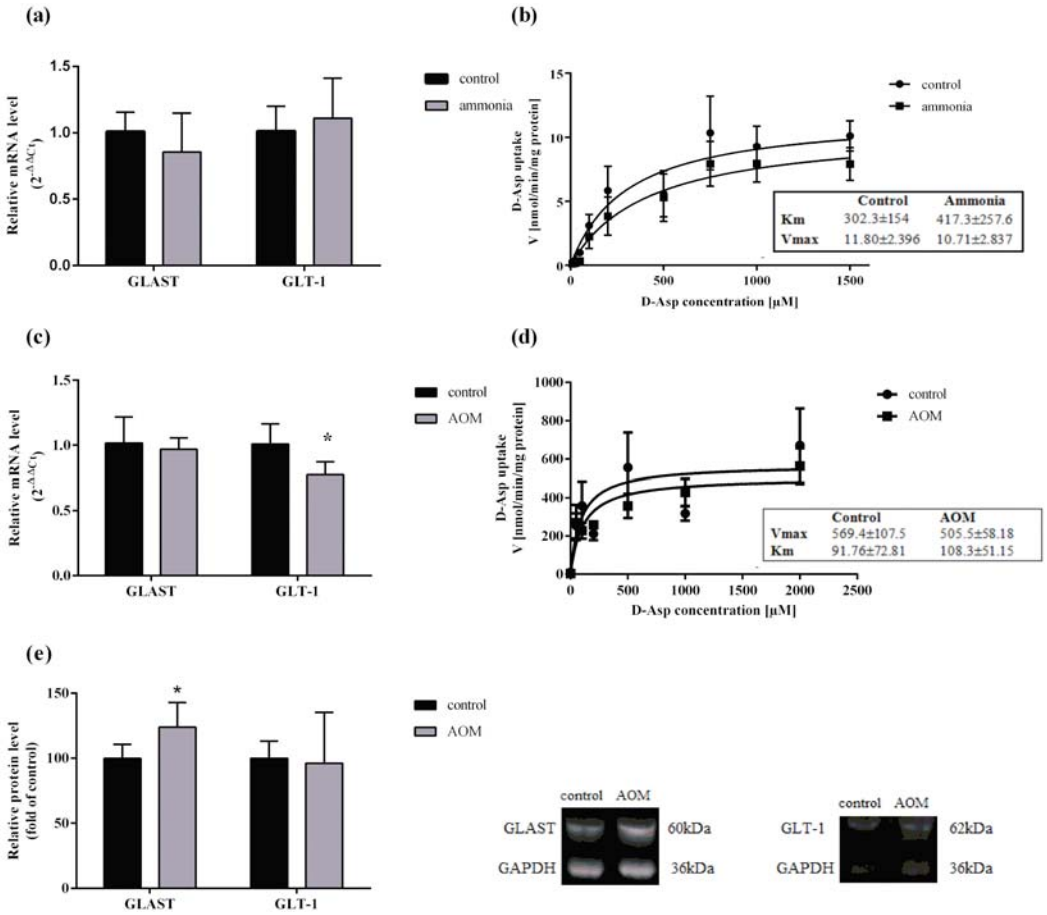

Fig. (1). Expression analysis and kinetics astrocytic Glu transporters. (**a**, **c**) EAAT2/GLT-1 and EAAT1/GLAST at the mRNA level in mouse cortical astrocytes treated for 24h with 5 mM ammonium chloride (”ammonia”) (**a**) and brain homogenates of mouse with azoxymethane (AOM)-induced HE (**c**). Relative quantification of EAAT2/GLT-1 and EAAT1/GLAST mRNA. Total RNA was isolated using TRI Reagent (Sigma-Aldrich), and reverse-transcribed using High Capacity cDNA Reverse Transcribed Kit (Life Technologies; Applied Biosystems). Probes for EAAT2/GLT-1, EAAT1/GLAST and β-actin (Mm00441457_m1, Mm00600697_m1, Mm00607939_s1, respectively) were purchased from Applied Biosystems. Further details were as described in Ref. [30]. Values in each group are mean ± SD for n = 3. (**b**, **d**) Effect of 5 mM ammonium chloride (“ammonia”; 24h) treatment on [^{3}H]D-Asp uptake in primary mouse cortical astrocytes (**b**) and AOM on [^{3}H]D-Asp uptake in mouse cortical slices (**d**). Astrocytes or slices were pre-incubated for 30 min at 37 °C and the uptake was started by adding 0.1 μCi L-[^{3}H]D-Asp (Perkin-Elmer) and D-Asp at 10-1500/2000 μmol/l final concentration and the incubation was continued for 3 min. The incubation was terminated by rapid vacuum filtration, followed by three washes with 1 ml with Krebs buffer maintained at 4 °C. The radioactivity of cell lysates was measured in a liquid scintillation spectrometer. Values in each group are mean ± SD for n = 3-9. (**e**) Quantification of EAAT2/GLT-1 and EAAT1/GLAST protein densities. The antibodies used included GLAST (Abcam; rabbit polyclonal, 1:800), GLT-1 (Abcam; rabbit polyclonal, 1:1,000) and GAPDH (Proteintech; rabbit polyclonal, 1:7,500). Representative immunoblots of GLAST, GLT-1 and GAPDH (loading control) corresponding to the immunoblots of transporters. See Ref. [30] for further experimental details. Values in each group are mean ± SD for n = 4 (*p < 0.05; T test)

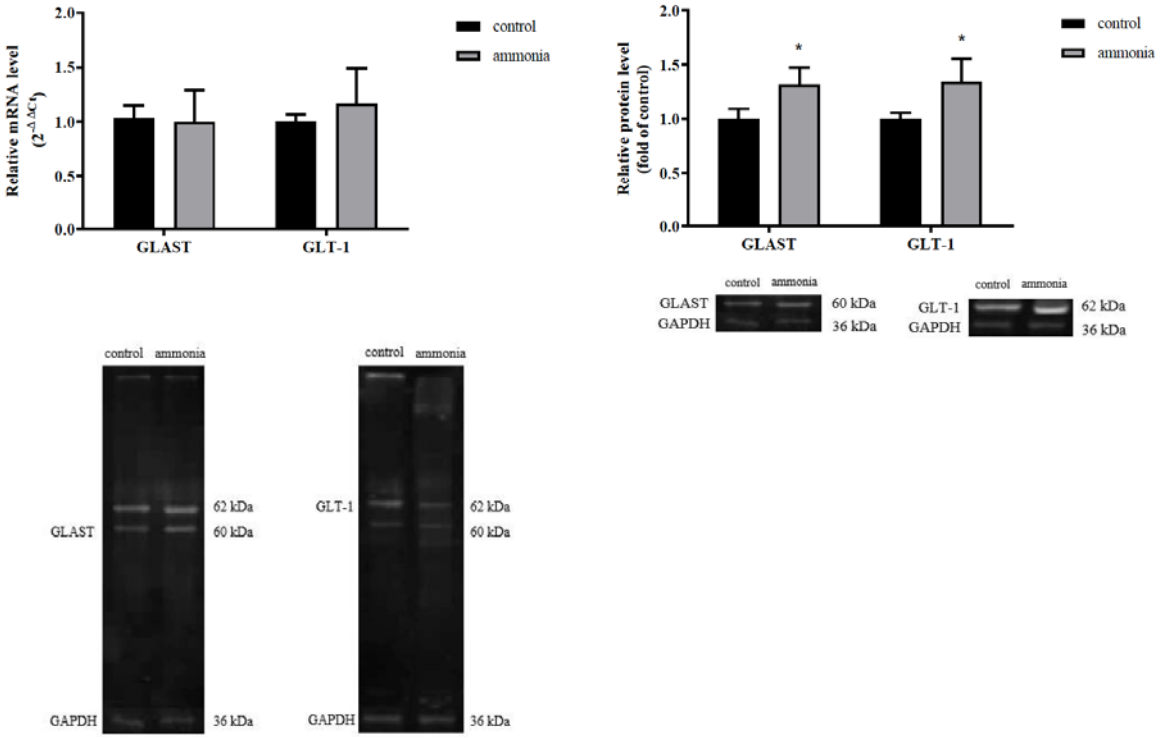

Fig. (2). Expression of astrocytic Glu transporters EAAT2/GLT-1and EAAT1/GLAST at the mRNA (*left panel*) and protein level (*right panel*) in mouse cortical astrocyte-neuron co-cultures treated for 24h with 5 mM ammonium chloride ("ammonia"). Neurons were seeded on the top of the two-week-old astrocytes in neuronal Dulbecco's modified Eagle's medium containing 10% FBS and 6 mM glucose. After 48 h of seeding cytosine arabinoside in final concentration of 20 μM was added and in 5th day of culture glucose in final concentration of 6 mM was added. Experiments were performed on 1-week co-cultures. L*eft panel*: Relative quantification of EAAT2/GLT-1 and EAAT1/GLAST mRNA. Total RNA was isolated using TRI Reagent (Sigma-Aldrich), and reverse-transcribed using High Capacity cDNA Reverse Transcribed Kit (Life Technologies; Applied Biosystems). Probes for EAAT2/GLT-1, EAAT1/GLAST and β-actin (Mm00441457_m1, Mm00600697_m1, Mm00607939_s1, respectively) were purchased from Applied Biosystems. Further details were as described in Ref. [30]. Values in each group are mean ± SD for n = 3. *Right panel*: Quantification of EAAT2/GLT-1 and EAAT1/GLAST protein densities. The antibodies used included GLAST (Abcam; rabbit polyclonal, 1:800), GLT-1 (Abcam; rabbit polyclonal, 1:1,000) and GAPDH (Proteintech; rabbit polyclonal, 1:7,500). Representative immunoblots of GLAST, GLT-1 and GAPDH (loading control) corresponding to the immunoblots of transporters. See Ref. [30] for further experimental details. *Lower panel:* The image of complete gels of representative immunoblots of GLAST, GLT-1 and GAPDH (loading control) corresponding to the immunoblots of transporters. Values in each group are mean ± SD for n = 4 (*p < 0.05; T test)

[21-24]. Interestingly, Guo *et al*. [25] implicated the presence of aberrantly spliced forms of EAAT2 mRNA as one of the causes of dysregulation of EAAT2 protein expression in human gliomas, albeit alterations of function of Glu transporters in these cells may be due to multiple causes [26]. Expression of EAAT2/GLT-1 splice variants was also found altered in the brains of mice subjected to chemical hypoxia [27]. Irrespective of the underlying mechanisms, results obtained with astrocytic/neuronal co-cultures underscore the need to delineate the neuron-derived factors involved in the regulation of the response of astrocytic Glu transport to ammonia.

1.4. Effects of Ammonia on the Expression of Astrocytic Gln Transporters SN1/SNAT3 and SN2/SNAT5: role of Astrocytic-Neuronal Communication

As mentioned in the Introduction, system N transporters SN1 and SN2 specifically mediate Gln transport in astrocytes [28, 29] and under physiological conditions, efflux is the preferred direction of Gln transport by SN1 [29, 30]. The well documented contribution of Gln accumulation to neurological symptoms associated with HA [12, 31-33 for more references see *Conclusions and Perspectives*] justified search for the link between intra-astrocytic retention of Gln and the fate of SN1 and SN2.

Decreased expression of SN1 and SN2 mRNA and of SN1 protein was observed in brain of rats with thioacetamide (TAA)-induced HE [34]. More recently, reduced expression of SN1 protein was measured in mice with azoxymethane (AOM)-induced HE (unpublished data). In turn, SN2 mRNA expression was decreased in rats with TAA-induced HE [34], and in mice induced by azoxymethane (AOM) i.p. injection [35]. However, SN2 protein level remained unaltered neither in rats [34], and mice with TAA-induced HE [36]. In contrast, simple hyperammonemia in rats (i.p. injection of ammonium acetate) did not change mRNA and protein expression of system N transporters [34]. Neither mRNA expression nor protein content of SN1 and SN2 were found altered in ammonia-treated mouse cortical astrocytes [30, 36], which was reflected by unaltered Gln uptake in these cells [30].

In mouse astrocyte/neuronal co-cultures, expression of SN1 and SN2 mRNA was not changed by ammonia, while protein level of both transporters was found decreased (Fig.

3a). Consistent with the response of the transporters, exposure to ammonia resulted in a decrease of total Gln uptake, and a tendency towards reduction of the system N-mediated Gln uptake (Fig. **3b**). To further account for the role of neuron-derived factors in the response, we analyzed SN1 and SN2 expression in cortical astrocytes which were cultured for one week in the medium collected from above the cultured neurons. Ammonia added in either of the two ways (to neuronal medium after 7 days of incubation vs to neuronal medium at the first day of astrocytes incubation in neuronal medium) decreased the SN1 and SN2 protein levels in these cultures (Fig. **4a**). However, neither total nor system N-mediated Gln uptake was changed by ammonia treatment (Fig.**4b**). While the results support the role of still unidentified neuronal factors in modulating the effect of ammonia on SN1 and/or SN2 expression, but leave open the question why decreased transporter expression is not translated to the decrease of Gln uptake.

1.5. The y^+LAT2 Transporter

As mentioned in the introductory paragraph, the y^+LAT2 is a heteromeric transporter of the y^+L class which bi-directionally transports Gln and other neutral amino acids in exchange for Arg [37]. This transporter has been found to be upregulated in the brain of rats with HA [38] and in ammonia-treated astrocytes [38, 39]. Direction of Gln or Arg transport by this carrier depends upon the actual concentrations of its substrates in the intra- and extracellular compartments, respectively, which raises some ambiguity in the interpretation of its role. An *in vivo* study has demonstrated that in a simple, ammonium acetate rat HA model, activated y^+LAT2, extracellular Gln, drives Arg out of the cells resulting in decreased NO

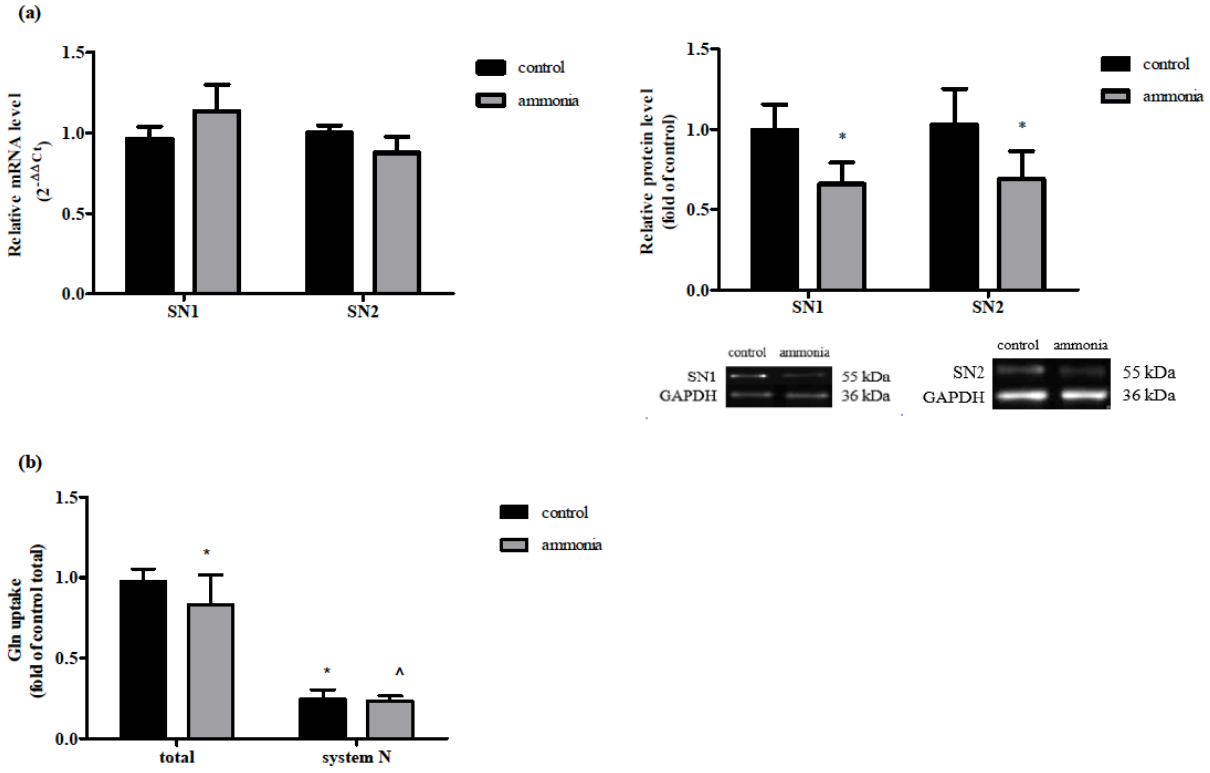

Fig. (3). Expression of astrocytic Gln transporters SN1 and SN2 at the mRNA (*left panel*) and protein level (*right panel*) in mouse cortical astrocyte-neuron co-cultures treated for 24h with 5 mM ammonium chloride ("ammonia"). Neurons were seeded on the top of the two-week-old astrocytes in neuronal Dulbecco's modified Eagle's medium containing 10% FBS and 6 mM glucose. After 48 h of seeding cytosine arabinoside in final concentration of 20 μM was added and in 5th day of culture glucose in final concentration of 6 mM was added. Experiments were performed on 1-week co-cultures. (**a**): Relative quantification of SN1 and SN2. Probes for SN1, SN2 and β-actin (Mm0120670_m1, Mm00549967_m1 and Mm00607939_s1 respectively) were purchased from Applied Biosystems. Further details were as described in Ref. [30]. Values in each group are mean ± SD for n = 4 (*left panel*). (**a**) Quantification of SN1 and SN2 protein densities. The antibodies used included SN1 (Proteintech; rabbit polyclonal, 1:800), SN2 (Santa Cruz Biotechnology; goat polyclonal, 1:1,000) and GAPDH (Proteintech; rabbit polyclonal, 1:7,500). Representative immunoblots of SN1, SN2 and GAPDH (loading control) corresponding to the immunoblots of transporters. See Ref. [30] for further experimental details. Values in each group are mean ± SD for n = 4 (*p < 0.05; T test) (*right panel*). (**b**) Effect of 5 mM ammonium chloride ("ammonia"; 24h) treatment on [^{3}H]Gln uptake in primary mouse cortical astrocytes. Astrocytes were pre-incubated for 15 min at 37 °C and the uptake was started by adding [^{3}H]Gln (Perkin-Elmer) at 0.1μCi final concentration and the incubation was continued for 4 min. The incubation was terminated by rapid vacuum filtration, followed by three washes with 1 ml with Krebs buffer maintained at 4 °C. The radioactivity of cell lysates was measured in a liquid scintillation spectrometer. The control value for [^{3}H]Gln uptake was 17.84±4.48 nmol/min/mg protein. Values in each group are mean ± SD for n = 4. (*p < 0.05 vs control total; ^p<0.05 vs ammonia total; Two-Way ANOVA followed by Bonferroni post hoc test).

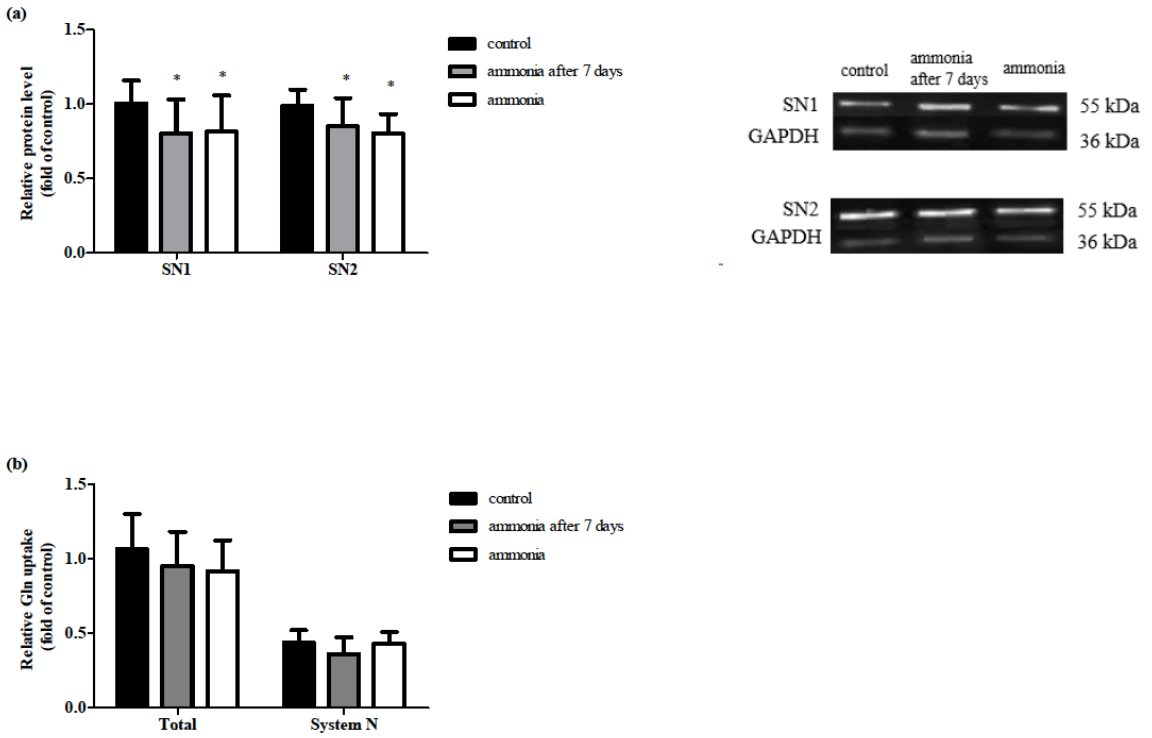

Fig. (4). (**a**) Expression of astrocytic Gln transporters SN1 and SN2 at the protein level in mouse cortical astrocytes incubated in the neuronal medium from above neuronal cell cultures treated with 5 mM ammonium chloride ("ammonia") for 24h or 7 days. The antibodies used included SN1 (Proteintech; rabbit polyclonal, 1:800), SN2 (Santa Cruz Biotechnology; goat polyclonal, 1:1,000) and GAPDH (Proteintech; rabbit polyclonal, 1:7,500). Representative immunoblots of SN1, SN2 and GAPDH (loading control) corresponding to the immunoblots of transporters. See Ref. [30] for further experimental details. Values in each group are mean ± SD for n = 4 (*$p < 0.05$; One-Way ANOVA followed by Dunnett's post hoc). (**b**) Effect of 5 mM ammonium chloride ("ammonia"; 24h) treatment on [^{3}H]Gln uptake in primary mouse cortical astrocytes incubated in the neuronal medium from above neuronal cell cultures. Astrocytes were pre-incubated for 15 min at 37 °C and the uptake was started by adding [^{3}H]Gln (Perkin-Elmer) at 0.1μCi final concentration and unlabeled the incubation was continued for 4 min. The incubation was terminated by rapid vacuum filtration, followed by three washes with 1 ml with Krebs buffer maintained at 4 °C. The radioactivity of cell lysates was measured in a liquid scintillation spectrometer. The control value for [^{3}H]Gln uptake was 11.89±5.39 nmol/min/mg protein. Values in each group are mean ± SD for n = 4.

synthesis [40]. However, this situation appeared to reflect a stage of HA at which the much of the Gln surplus egressed astrocytes and inhabited the extracellular space. In cultured astrocytes, upregulation of y^+LAT2 by ammonia elicits an increase of Arg uptake which then becomes solely dependent on this transporter. Under these conditions, the increased Arg uptake is a direct cause of increased NO synthesis, providing substrate for evolution of oxidative/nitrosative stress [39]. The role of (likely) concomitant fluctuations in the distribution of Gln between the cells and the surrounding milieu, and the relative contribution of y^+LAT2 to Gln transport remain to be established.

1.6. Mitochondrial Glutamine Transport

Excessive Gln transport to mitochondria, the cornerstone of the "Trojan horse" mode of action of Gln, has been demonstrated in ammonia - overexposed brain mitochondrial preparation [41]. Ammonia-induced Gln uptake to mitochondria [42], and swelling of cultured astrocytes [43] were both found ameliorated in the presence of His. The results are indicative of the presence in astrocytic mitochondria of a histidine (His)-sensitive Gln transporter which becomes activated by ammonia. However, no brain mitochondrial Gln transporter has been identified. The kidney mitochondrial Gln carrier, to the authors' knowledge the only one so far purified, was found to respond to asparagine (Asn) as the only amino acid tested, but its ability to interact with His was not measured by these authors [44]. Clearly, the degree of structural and functional similarity of the two transporters remains to be investigated.

CONCLUSIONS AND PERSPECTIVES

Variability of the abundance of data and effects of ammonia among species and experimental settings prompted us to provide to comment separately on Glu and Gln transporters, and to delineate the most significant gaps in the state of the art.

i) Relative abundance of information on effects of ammonia or HA/HE on Glu transporters in rat contrasts with its paucity in mouse; our own attempts to fill this gap are presented in this report. The rat data unambiguously demonstrate ammonia- induced decrease of the expression of GLAST and GLT1 both in *in vivo* and *in vitro* setting and as such, supporting the notion of Glu uptake deficiency as a possible cause of extrasynaptic Glu overflow in hyperammonemic conditions. The reasons why only GLAST but not GLT-1 absence of data on GLT-1 responded to ammonia in astrocytes *in vitro* remain to be elucidated. Literature data that cultured as-

trocytes expressing disproportionally low levels of GLT-1 and high levels of GLAST as compared to native astrocytes [45] have not been verified for the cultures used in our study. Similar to the rat model, decreased expression of GLT-1 was noted in the mouse model of HE *in situ*, but not in mouse astrocytes, which again supports the view that cultured astrocytes do not faithfully correspond to native cells (for review see [8] and references therein). Increased expression of GLAST and GLT-1 protein in astrocytic- neuronal co-cultures bespeaks the role of interaction between astrocytes and neurons in the response to ammonia, as frequently suggested in different contexts [46, 47]. As of today, no information is available whether posttranscriptional stimulation of GLT-1/GLAST also takes place in acute HA/HE models as reported in this review. Of note in this context, increased GLT-1 and GLAST protein content was observed in the cerebellum of rats with durable (6- months) portacaval shunt.

ii) Though Gln transporters as ammonia targets are "a new kid on the block", responses of SN1 are strikingly consistent: decreased expression of SN1 protein was noted across species and experimental settings. Most notably, the decrease observed in a mouse model *in vivo* was confirmed in astrocytic/neuronal co-cultures, but not in astrocytes cultured alone, again supporting the role of astrocytic-neuronal interactions in determining the response of astrocytes in the native setting.

iii) Two significant areas pertinent to the subject of the review have remained uncovered and urgently need exploration. Firstly, to the best of our knowledge, only two literature reports deal with the expression of Glu transporters in human HE, and none at all with Gln transporters. Secondly, no data are available on the effects of ammonia or HE on posttranslational modulation of the transporters. A wealth of literature reports convincingly demonstrate that structural modification and intracellular trafficking are critical determinants of transporter activity (for reviews and references see [19, 48-50]. Therefore, to render the transporter protein expression analysis fully relevant, Western blots will have to be performed in cell membrane fractions. In so far, the available literature data and own preliminary experiments have exclusively dealt with whole cell extracts. The lack of membrane fraction data may be one of the reasons of discrepancies between the results of mRNA and protein expressions often observed in one and the same experimental setting.

It must be noted however that mechanisms other than expression/activity of the Glu transporters are also responsible for the distribution of Glu between the intra- and extracellular space. Glu uptake strictly depends upon the status of membrane-located ion transporters and channels changes in their expression and activity, which are subject of modulation by ammonia and HA/HE. Indeed, decreased expression of the astroglia-specific inward-rectifying potassium channel Kir 4.1 was observed in astrocytes from hyperammonemic (ornithine transcarbamylase - deficient) mice [51], in the cerebral cortex of HE rats [52, 53], and in ammonia-treated cultures of rat cortical astrocytes [52]. Increased expression and activity of the astroglia-located sodium/potassium transporter NKCC1 was noted in ammonia-treated cultured astrocytes [16]. Electrophysiological recordings revealed that, ammonia-induced decrease of astrocytic D-Asp uptake in cultured astrocytes occurs secondary to NKCC1-dependent rise of intraastrocytic sodium and pH [54] and to Kir4.1-dependent cell membrane depolarization [55]. Decreased D-Asp uptake in ammonia-treated astrocytic cultures correlated with decreased Kir 4.1 expression in these cells [52]. One other note of caution pertains to the fact that Glu transporters form macromolecular and functional complexes with other amino acid and ion transporting moieties, such as the Gln transporter SN1 [56] or Na/K ATPase [8]. Therefore, effects of ammonia on SN1 (see this manuscript) and Na/K ATPASE [57] may induce changes in Glu transporter activities without altering their expression. Down the same valley, ammonia-induced decrease of D-Asp uptake in astrocytes is related to accumulation of ROS [58]. Accumulation of ROS exerts ONS, an event invariably accompanying overexposure of the cells to ammonia (references in Intro). However, the mechanism by which ONS affects Glu uptake may or may not be linked to the Glu transporter status; this question remains to be answered in future studies.

Extrasynaptic Glu is also regulated by exocytotic, calcium-dependent Glu release from astrocytes [59], and in cultured this process is stimulated by ammonia [60, 61]. Long-term exposure of cultured astrocytes to ammonia likewise increases Glu efflux from astrocytes [62], albeit earlier evidence to the contrary has been reported [63]. The mechanism underlying these long-term effects is not known, but transporter-mediated reversal of Glu uptake cannot be excluded [64].

In conclusion, impairment of the activity and function of astrocytic Glu and Gln transporters appear to contribute to impairment of Glu-ergic neurotransmission and brain edema, the two major manifestations of progression of hyperammonemic encephalopathies. However, the knowledge of mechanistic details and of species- specificity of the responses is far from complete. In order not to extend the number of issues touched upon beyond the scope review, some of them remained not attended enough or unattended at all. Firstly, studies of the pathomechanism of ammonia neurotoxicity has so far bypassed the existence of splice variants of Glu transporters, which do play a role in neurodegenerative diseases of the CNS [22]. One other fact neglected in the studies and, in consequence in this review is the presence of functional astrocytic Glu transporters in neurons [65]. We are aware that the latter circumstance could bear upon our results obtained with astrocytic/neuronal co-cultures, where the effects of ammonia appeared at variance from those noted in astrocytic monocultures. It is tempting to assume that in these mixed co-cultures, quantitative representation of the particular transporters does not match that noted in situ and in cultured astrocytes. This aspect is currently under study in this laboratory. On the top of that, in view of the dependence of Glu (and possibly Gln) transport on an array of different ionic and metabolic interferences, the relative role of the transporters in shaping the ammonia-induced neurotransmitter dyshomeostasis requests further detailed investigations.

LIST OF ABBREVIATIONS

ALF	=	Acute Liver Failure
ALS	=	Amyotrophic Lateral Sclerosis
AMPA	=	α-Amino-3-Hydroxy-5-Methyl-4-Isoxazolepropionic Acid
AOM	=	Azoxymethane
Arg	=	Arginine
Asn	=	Asparagine
CNS	=	Central Nervous System
D-Asp	=	D-Aspartate
EAAT1/GLAST	=	Excitatory Amino Acid Transporter 1/Glutamate Aspartate Transporter
EAAT2/GLT-1	=	Excitatory Amino Acid Transporter 2/ Glutamate Transporter 1
GAPDH	=	Glyceraldehyde-3-Phosphate Dehydrogenase
Gln	=	Glutamine
GS	=	Glutamine Synthetase
Glu	=	Glutamate
HA	=	Hyperammonemia
HE	=	Hepatic Encephalopathy
His	=	Histidine
Kir 4.1	=	The Astroglia-Specific Inward-Rectifying Potassium Channel Kir 4.1
MPT	=	Mitochondrial Permeability Transition
NKCC1	=	Na-K-Cl Cotransporter 1
NMDA	=	*N*-Methyl-D-Aspartate
NO	=	Nitric Oxide
ONS	=	Oxidative/Nitrosative Stress
OS	=	Oxidative Stress
ROS	=	Reactive Oxygen Species
SN1	=	Slc38a3, sodium-Coupled Neutral Amino Acid Transporter 3
SN2	=	Slc38a5, Sodium-Coupled Neutral Amino Acid Transporter 5
TAA	=	Thioacetamide
y^+LAT2	=	Heteromeric Amino Acid Transporter of y^+L System

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

ACKNOWLEDGEMENTS

The study was supported by the National Science Centre of the Republic of Poland (NCN), grants: 2013/08/M/NZ3/00869, 2013/09/B/NZ4/00536 and Polish-Norwegian Research Program operated by the National Centre for Research and Development under the Norwegian Financial Mechanism 2009-2014, Project Contract: Pol-Nor/196190/23/2013

REFERENCES

[1] Albrecht, J.; Sonnewald, U.; Waagepetersen, H.S.; Schousboe, A. Glutamine in the central nervous system: function and dysfunction. Front Biosci 2007, 12, 332-43.

[2] Bai, G.; Rama Rao, K.V.; Murthy, C.R.; Panickar, K.S.; Jayakumar, A.R.; Norenberg, M.D. Ammonia induces the mitochondrial permeability transition in primary cultures of rat astrocytes. J Neurosci Res 2001, 66 (5), 981-91.

[3] Norenberg, M.D. Astrocytic-ammonia interactions in hepatic encephalopathy. Semin Liver Dis 1996, 16 (3), 245-53.

[4] Albrecht, J.; Jones, E.A. Hepatic encephalopathy: molecular mechanisms underlying the clinical syndrome. J Neurol Sci 1999, 170 (2), 138-46.

[5] Bosman, D.K.; Deutz, N.E.; Maas, M.A.; van Eijk, H.M.; Smit, J.J.; de Haan, J.G.; Chamuleau, R.A. Amino acid release from cerebral cortex in experimental acute liver failure, studied by *in vivo* cerebral cortex microdialysis. J Neurochem 1992, 59 (2), 591-9.

[6] de Knegt, R.J.; Schalm, S.W.; van der Rijt, C.C.; Fekkes, D.; Dalm, E.; Hekking-Weyma, I. Extracellular brain glutamate during acute liver failure and during acute hyperammonemia simulating acute liver failure: an experimental study based on *in vivo* brain dialysis. J Hepatol 1994, 20 (1), 19-26.

[7] Vaquero, J.; Butterworth, R.F. The brain glutamate system in liver failure. J Neurochem 2006, 98 (3), 661-9.

[8] Robinson, M.B.; Jackson, J.G. Astroglial glutamate transporters coordinate excitatory signaling and brain energetics. Neurochem Int 2016, 98, 56-71.

[9] Scott, T.R.; Kronsten, V.T.; Hughes, R.D.; Shawcross, D.L. Pathophysiology of cerebral oedema in acute liver failure. World J Gastroenterol 2013, 19 (48), 9240-55.

[10] Häussinger, D.; Görg, B. Interaction of oxidative stress, astrocyte swelling and cerebral ammonia toxicity. Curr Opin Clin Nutr Metab Care 2010, 13 (1), 87-92.

[11] Skowrońska, M.; Albrecht, J. Oxidative and nitrosative stress in ammonia neurotoxicity. Neurochem Int 2013, 62 (5), 731-7.

[12] Albrecht, J.; Norenberg, M.D. Glutamine: a Trojan horse in ammonia neurotoxicity. Hepatology 2006, 44 (4), 788-94.

[13] Chaudhry, F.A.; Reimer, R.J.; Krizaj, D.; Barber, D.; Storm-Mathisen, J.; Copenhagen, D.R.; Edwards, R.H. Molecular analysis of system N suggests novel physiological roles in nitrogen metabolism and synaptic transmission. Cell 1999, 99 (7), 769-80.

[14] Chaudhry, F.A.; Reimer, R.J.; Edwards, R.H. The glutaminę commute: take the N line and transfer to the A. J. Cell Biol 2002a, 157, 349–355.

[15] Zielińska, M.; Ruszkiewicz, J.; Hilgier, W.; Fręśko, I.; Albrecht, J. Hyperammonemia increases the expression and activity of the glutamine/arginine transporter y+ LAT2 in rat cerebral cortex: implications for the nitric oxide/cGMP pathway. Neurochem Int 2011, 58 (2), 190-5.

[16] Jayakumar, A.R.; Liu, M.; Moriyama, M.; Ramakrishnan, R.; Forbush, B. 3rd; Reddy, P.V.; Norenberg, M.D. Na-K-Cl Cotransporter-1 in the mechanism of ammonia-induced astrocyte swelling. J Biol Chem 2008, 283 (49), 33874-82.

[17] Divito, C.B.; Underhill, S.M. Excitatory amino acid transporters: roles in glutamatergic neurotransmission. Neurochem Int 2014, 73, 172-80.

[18] Arriza, J.L.; Fairman, W.A.; Wadiche, J.I.; Murdoch, G.H.; Kavanaugh, M.P.; Amara, S.G. Functional comparisons of three glutamate transporter subtypes cloned from human motor cortex. J Neurosci 1994, 14 (9), 5559-69.

[19] Butterworth, R.F. Pathogenesis of hepatic encephalopathy and brain edema in acute liver failure. J Clin Exp Hepatol 2015, 5 (Suppl 1), S96-S103.

[20] Lin, C.L.; Bristol, L.A.; Jin, L.; Dykes-Hoberg, M.; Crawford, T.; Clawson, L.; Rothstein, J.D. Aberrant RNA processing in a neurodegenerative disease: the cause for absent EAAT2, a glutamate transporter, in amyotrophic lateral sclerosis. Neuron 1998, 20 (3), 589-602.

[21] Flowers, J.M.; Powell, J.F.; Leigh, P.N.; Andersen, P.; Shaw, C.E. Intron 7 retention and exon 9 skipping EAAT2 mRNA variants are

not associated with amyotrophic lateral sclerosis. Ann Neurol 2001, 49 (5), 643-9.

[22] Honig, L.S.; Chambliss, D.D.; Bigio, E.H.; Carroll, S.L.; Elliott, J.L. Glutamate transporter EAAT2 splice variants occur not only in ALS, but also in AD and controls. Neurology 2000, 55 (8), 1082-8.

[23] Meyer, T.; Fromm, A.; Münch, C.; Schwalenstöcker, B.; Fray, A.E.; Ince, P.G.; Stamm, S.; Grön, G.; Ludolph, A.C.; Shaw, P.J. The RNA of the glutamate transporter EAAT2 is variably spliced in amyotrophic lateral sclerosis and normal individuals. J Neurol Sci 1999, 170 (1), 45-50.

[24] Nagai, M.; Abe, K.; Okamoto, K.; Itoyama, Y. Identification of alternative splicing forms of GLT-1 mRNA in the spinal cord of amyotrophic lateral sclerosis patients. Neurosci Lett 1998, 244 (3), 165-8.

[25] Guo, H.; Lai, L.; Butchbach, M.E.; Lin, C.L. Human glioma cells and undifferentiated primary astrocytes that express aberrant EAAT2 mRNA inhibit normal EAAT2 protein expression and prevent cell death. Mol Cell Neurosci 2002, 21 (4), 546-60.

[26] Ye, Z.C.; Rothstein, J.D.; Sontheimer, H. Compromised glutamate transport in human glioma cells: reduction-mislocalization of sodium-dependent glutamate transporters and enhanced activity of cystine-glutamate exchange. J Neurosci 1999, 19 (24), 10767-77.

[27] Münch, C.; Zhu, B.G.; Leven, A.; Stamm, S.; Einkörn, H.; Schwalenstöcker, B.; Ludolph, A.C.; Riepe, M.W.; Meyer, T. Differential regulation of 5' splice variants of the glutamate transporter EAAT2 in an *in vivo* model of chemical hypoxia induced by 3-nitropropionic acid. J Neurosci Res 2003, 71 (6), 819-25.

[28] Bjørnsen, L.P.; Hadera, M.G.; Zhou, Y.; Danbolt, N.C.; Sonnewald, U. The GLT-1 (EAAT2; slc1a2) glutamate transporter is essential for glutamate homeostasis in the neocortex of the mouse. J Neurochem 2014, 128 (5), 641-9.

[29] Chaudhry, F.A.; Schmitz, D.; Reimer, R.J.; Larsson, P.; Gray, A.T.; Nicoll, R.; Kavanaugh, M.; Edwards, R.H. Glutamine uptake by neurons: interaction of protons with system a transporters. J. Neurosci 2002b, 22, 62–72.

[30] Zielińska, M.; Dąbrowska, K.; Hadera, M.G.; Sonnewald, U.; Albrecht, J. System N transporters are critical for glutamine release and modulate metabolic fluxes of glucose and acetate in cultured cortical astrocytes: changes induced by ammonia. J Neurochem 2016, 136 (2), 329-38.

[31] Blei, A.T.; Olafsson, S.; Therrien, G.; Butterworth, R.F. Ammonia-induced brain edema and intracranial hypertension in rats after portacaval anastomosis. Hepatology 1994, 19, 1437–1444.

[32] Takahashi, H.; Koehler, R.C.; Brusilow, S.W.; Traystman, R.J. Inhibition of brain glutamine accumulation prevents cerebral edema in hyperammonemic rats. Am J Physiol 1991, 261, H825–H829.

[33] Tanigami, H.; Rebel, A.; Martin, L.J.; Chen, T.Y.; Brusilow, S.W.; Traystman, R.J.; Koehler, R.C. Effect of glutamine synthetase inhibition on astrocyte swelling and altered astroglial protein expression during hyperammonemia in rats. Neuroscience 2005, 131 (2), 437-49.

[34] Zielińska, M.; Popek, M.; Albrecht, J. Roles of changes in active glutamine transport in brain edema development during hepatic encephalopathy: an emerging concept. Neurochem Res 2014, 39 (3), 599-604.

[35] Desjardins, P.; Du, T.; Jiang, W.; Peng, L.; Butterworth, R.F. Pathogenesis of hepatic encephalopathy and brain edema in acute liver failure: role of glutamine redefined. Neurochem Int 2012, 60 (7), 690-6.

[36] Rama Rao, K.V.; Norenberg, M.D. Glutamine in the pathogenesis of hepatic encephalopathy: the Trojan horse hypothesis revisited. Neurochem Res 2014, 39 (3), 593-8.

[37] Bröer, A.; Wagner, C.A.; Lang, F.; Bröer, S. The heterodimeric amino acid transporter 4F2hc/y+LAT2 mediates arginine efflux in exchange with glutamine. Biochem J 2000, 349 (3), 787-95.

[38] Zielińska, M.; Skowrońska, M.; Fręśko, I.; Albrecht, J. Upregulation of the heteromeric y^+LAT2 transporter contributes to ammonia-induced increase of arginine uptake in rat cerebral cortical astrocytes. Neurochem Int 2012, 61 (4), 531-5.

[39] Zielińska, M.; Milewski, K.; Skowrońska, M.; Gajos, A.; Ziemińska, E.; Beręsewicz, A.; Albrecht, J. Induction of inducible nitric oxide synthase expression in ammonia-exposed cultured astrocytes is coupled to increased arginine transport by upregulated y(+)LAT2 transporter. J Neurochem 2015, 135 (6), 1272-81.

[40] Hilgier, W.; Freśko, I.; Klemenska, E.; Beresewicz, A.; Oja, S.S.; Saransaari, P.; Albrecht, J.; Zielińska, M. Glutamine inhibits ammonia-induced accumulation of cGMP in rat striatum limiting arginine supply for NO synthesis. Neurobiol Dis 2009, 5 (1), 75-81.

[41] Dolińska, M.; Hilgier, W.; Albrecht, J. Ammonia stimulates glutamine uptake to the cerebral non-synaptic mitochondria of the rat. Neurosci Lett 1996, 213 (1), 45-8.

[42] Ziemińska, E.; Dolińska, M.; Lazarewicz, J.W.; Albrecht, J. Induction of permeability transition and swelling of rat brain mitochondria by glutamine. Neurotoxicology 2000, 21 (3), 295-300.

[43] Pichili, V.B.; Rama Rao, K.V.; Jayakumar, A.R.; Norenberg, M.D. Inhibition of glutamine transport into mitochondria protects astrocytes from ammonia toxicity. Glia 2007, 55 (8), 801-9.

[44] Indiveri, C.; Abruzzo, G.; Stipani, I.; Palmieri, F. Identification and purification of the reconstitutively active glutamine carrier from rat kidney mitochondria. Biochem J 1998, 333 (Pt 2), 285-90.

[45] Swanson, R.A.; Liu, J.; Miller, J.W.; Rothstein, J.D.; Farrell, K.; Stein, B.A.; Longuemare, M.C. Neuronal regulation of glutamate transporter subtype expression in astrocytes. J Neurosci 1997, 17, 932–40.

[46] Albrecht, J.; Faff, L. Astrocyte-neuron interactions in hyperammonaemia and hepatic encephalopathy. Adv Exp Med Biol 1994, 368, 45-54.

[47] Hertz, L.; Yu, A.C.; Kala, G.; Schousboe, A. Neuronal-astrocytic and cytosolic-mitochondrial metabolite trafficking during brain activation, hyperammonaemia and energy deprivation. Neurochem Int 2000, 37 (2-3), 83-102.

[48] Toris, G.T.; Bikis, C.N.; Tsourouflis, G.S.; Theocharis, S.E. Hepatic encephalopathy: an updated approach from pathogenesis to treatment. Med Sci Monit 2011, 17 (2), RA53-63.

[49] Nissen-Meyer, L.S.; Chaudhry, F.A. Protein Kinase C Phosphorylates the System N Glutamine Transporter SN1 (Slc38a3) and Regulates Its Membrane Trafficking and Degradation. Front Endocrinol (Lausanne) 2013, 4, Article 138.

[50] Peacey, E.; Miller, C.C.; Dunlop, J.; Rattray, M. The four major N- and C-terminal splice variants of the excitatory amino acid transporter GLT-1 form cell surface homomeric and heteromeric assemblies. Mol Pharmacol 2009, 75 (5), 1062-73.

[51] Lichter-Konecki, U.; Mangin, J.M.; Gordish-Dressman, H.; Hoffman, E.P.; Gallo, V. Gene expression profiling of astrocytes from hyperammonemic mice reveals altered pathways for water and potassium homeostasis *in vivo*. Glia 2008, 56 (4), 365-77.

[52] Obara-Michlewska, M.; Pannicke, T.; Karl, A.; Bringmann, A.; Reichenbach, A.; Szeliga, M.; Hilgier, W.; Wrzosek, A.; Szewczyk, A.; Albrecht, J. Down-regulation of Kir4.1 in the cerebral cortex of rats with liver failure and in cultured astrocytes treated with glutamine: Implications for astrocytic dysfunction in hepatic encephalopathy. J Neurosci Res 2011, 89 (12), 2018-27.

[53] Obara-Michlewska, M.; Ruszkiewicz, J.; Zielińska, M.; Verkhratsky, A.; Albrecht, J. Astroglial NMDA receptors inhibit expression of Kir4.1 channels in glutamate-overexposed astrocytes *in vitro* and in the brain of rats with acute liver failure. Neurochem Int 2015, 88, 20-5.

[54] Kelly, T.; Kafitz, K.W.; Roderigo, C.; Rose, C.R. Ammonium-evoked alterations in intracellular sodium and pH reduce glial glutamate transport activity. Glia 2009, 57 (9), 921-34.

[55] Stephan, J.; Haack, N.; Kafitz, K.W.; Durry, S.; Koch, D.; Hochstrate, P.; Seifert, G.; Steinhäuser, C.; Rose, C.R. Kir4.1 channels mediate a depolarization of hippocampal astrocytes under hyperammonemic conditions in situ. Glia 2012, 60 (6), 965-78.

[56] Martínez-Lozada, Z.; Guillem, A.M.; Flores-Méndez, M.; Hernández-Kelly, L.C.; Vela, C.; Meza, E.; Zepeda, R.C.; Caba, M.; Rodríguez, A.; Ortega, A. GLAST/EAAT1-induced glutamine release via SNAT3 in Bergmann glial cells: evidence of a functional and physical coupling. J Neurochem 2015, 125 (4), 545-54.

[57] Rose, E.M.; Koo, J.C.; Antflick, J.E.; Ahmed, S.M.; Angers, S.; Hampson, D.R. Glutamate transporter coupling to Na,K-ATPase. J Neurosci 2009, 29 (25), 8143-55.

[58] Jayakumar, A.R.; Panickar, K.S.; Murthy, Ch.R.; Norenberg, M.D. Oxidative stress and mitogen-activated protein kinase phosphorylation mediate ammonia-induced cell swelling and glutamate uptake inhibition in cultured astrocytes. J Neurosci 2006, 26 (18), 4774-84.

[59] Reyes, R.C.; Verkhratsky, A.; Parpura, V. Plasmalemmal Na+/Ca2+ exchanger modulates Ca2+-dependent exocytotic re-

lease of glutamate from rat cortical astrocytes. ASN Neuro 2012, 4 (1), pii: e00075.

[60] Görg, B.; Morwinsky, A.; Keitel, V.; Qvartskhava, N.; Schrör, K.; Häussinger, D. Ammonia triggers exocytotic release of L-glutamate from cultured rat astrocytes. Glia 2010, 58 (6), 691-705.

[61] Rose, C.; Kresse, W.; Kettenmann, H. Acute insult of ammonia leads to calcium-dependent glutamate release from cultured astrocytes, an effect of pH. J Biol Chem 2005, 280 (22), 20937-44.

[62] Ohara, K.; Aoyama, M.; Fujita, M.; Sobue, K.; Asai, K. Prolonged exposure to ammonia increases extracellular glutamate in cultured rat astrocytes. Neurosci Lett 2009, 462 (2), 109-12.

[63] Albrecht, J.; Bender, A.S.; Norenberg, M.D. Ammonia stimulates the release of taurine from cultured astrocytes. Brain Res 1994, 660 (2), 288-92.

[64] Szatkowski, M.; Barbour, B.; Attwell, D. Non-vesicular release of glutamate from glial cells by reversed electrogenic glutamate uptake. Nature 1990, 348 (6300), 443-6.

[65] Gadea, A.; López-Colomé, A.M. Glial transporters for glutamate, glycine and GABA I. Glutamate transporters. J Neurosci Res 2001, 63 (6), 453-60.

[66] Knecht, K.; Michalak, A.; Rose, C.; Rothstein, J.D.; Butterworth, R.F. Decreased glutamate transporter (GLT-1) expression in frontal cortex of rats with acute liver failure. Neurosci Lett 1997, 229 (3), 201-3.

[67] Norenberg, M.D.; Huo, Z.; Neary, J.T.; Roig-Cantesano, A. The glial glutamate transporter in hyperammonemia and hepatic encephalopathy: relation to energy metabolism and glutamatergic neurotransmission. Glia 1997, 21 (1), 124-33.

[68] Butterworth, R.F. Glutamate transporters in hyperammonemia. Neurochem Int 2002, 41 (2-3), 81-5.

[69] Butterworth, R.F. Pathophysiology of brain dysfunction in hyperammonemic syndromes: The many faces of glutamine. Mol Genet Metab 2014, 113 (1-2), 113-7.

[70] Suárez, I.; Bodega, G.; Fernández, B. Modulation of glutamate transporters (GLAST, GLT-1 and EAAC1) in the rat cerebellum following portocaval anastomosis. Brain Res 2000, 859 (2), 293-302.

[71] Cabrera-Pastor, A.; Taoro, L.; Llansola, M.; Felipo, V. Roles of the NMDA Receptor and EAAC1 Transporter in the Modulation of Extracellular Glutamate by Low and High Affinity AMPA Receptors in the Cerebellum *in vivo*: Differential Alteration in Chronic Hyperammonemia. ACS Chem Neurosci 2015, 6 (12), 1913-21.

[72] Leke, R.; Escobar, T.D.; Rao, K.V.; Silveira, T.R.; Norenberg, M.D.; Schousboe, A. Expression of glutamine transporter isoforms in cerebral cortex of rats with chronic hepatic encephalopathy. Neurochem Int 2015, 88, 32-7.

[73] Chan, H.; Hazell, A.S.; Desjardins, P.; Butterworth, R.F. Effects of ammonia on glutamate transporter (GLAST) protein and mRNA in cultured rat cortical astrocytes. Neurochem Int 2000, 37 (2-3), 243-8.

[74] Zhou, B.G.; Norenberg, M.D. Ammonia downregulates GLAST mRNA glutamate transporter in rat astrocyte cultures. Neurosci Lett 1999, 276 (3), 145-8.

[75] Bodega, G.; Suárez, I.; López-Fernández, L.A.; Almonacid, L.; Zaballos, A.; Fernández, B. Possible implication of ciliary neurotrophic factor (CNTF) and beta-synuclein in the ammonia effect on cultured rat astroglial cells: a study using DNA and protein microarrays. Neurochem Int 2006, 48 (8), 729-38.

[76] Thumburu, K.K.; Dhiman, R.K.; Vasishta, R.K.; Chakraborti, A.; Butterworth, R.F.; Beauchesne, E.; Desjardins, P.; Goyal, S.; Sharma, N.; Duseja, A.; Chawla, Y. Expression of astrocytic genes coding for proteins implicated in neural excitation and brain edema is altered after acute liver failure. J Neurochem 2014, 128 (5), 617-27.

[77] Görg, B.; Qvartskhava, N.; Bidmon, H.J.; Palomero-Gallagher, N.; Kircheis, G.; Zilles, K.; Häussinger, D. Oxidative stress markers in the brain of patients with cirrhosis and hepatic encephalopathy. Hepatology 2010, 52 (1), 256-65.

Neurochemistry International 118 (2018) 225–232

Contents lists available at ScienceDirect

Neurochemistry International

journal homepage: www.elsevier.com/locate/neuint

Protein kinase C-mediated impairment of glutamine outward transport and SN1 transporter distribution by ammonia in mouse cortical astrocytes

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ARTICLE INFO

Keywords:
Astrocytes
Glutamine
SN1 (SNAT3)
Glutamine release
Ammonia
PKC

ABSTRACT

SN1, a system N amino acid transporter specific for astrocytes, is mainly responsible for export of newly synthesized L-glutamine from the cells. Astrocytic retention of L-glutamine which plays a critical role in ammonia-induced astrocytic swelling resulting in brain edema, could be tentatively attributed to the impaired L-glutamine export from astrocytes. The present study demonstrates that treatment of cultured mouse cortical astrocytes for 24 h with 5 mM ammonium chloride ("ammonia") inhibits the system N-mediated L-glutamine transport out of the cell, and that this inhibition is related to the reduced presence of the SN1 transporter on the cell membrane. Ammonia decreased total protein kinase C (PKC) activity in the absence but not in the presence of PKC activator, phorbol 12-myristate 13-acetate (PMA), and activation of PKC by PMA reversed both the ammonia-induced decrease of system N-mediated L-glutamine release and ammonia-induced SN1 deficit in the membrane fraction. However, while ammonia did not change the protein level of PKCα isoform, it decreased the protein content of PKCδ. Moreover, ammonia treatment increased the cell surface expression of SN1 in cells with silenced PKCα and PKCδ. Silencing of PKCδ abrogated the decrease of system N (SN1)-mediated L-glutamine release by ammonia. The results implicate the involvement of PKCδ in the inhibition of SN1 membrane expression and activity by ammonia.

1. Introduction

L-glutamine is the most ubiquitous amino acid in all mammalian tissues and body fluids, including the central nervous system (CNS), where its concentration is at least one order of magnitude higher than of any other amino acid (Albrecht et al., 2007; Cynober, 2002; Pithon-Curi et al., 2002). In the brain L-glutamine is formed in astrocytes from L-glutamate and ammonia in an ATP-consuming reaction catalyzed by glutamine synthetase (Albrecht et al., 2010a). A significant proportion of synthesized L-glutamine exits astrocytes and enters neurons, to give rise to the excitatory neurotransmitter amino acid L-glutamate (Albrecht et al., 2010a; Suárez et al., 2002), and the inhibitory neurotransmitter γ-aminobutyric acid (Albrecht et al., 2010a), reflecting the neuronal leg of the glutamate/glutamine cycle (Albrecht et al., 2010a; Waniewski and Martin, 1986). A certain proportion of astrocyte-derived L-glutamine leaves the CNS *via* the cerebral capillary endothelial cells forming the blood-brain barrier (Albrecht et al., 2010b; Lee et al., 1998).

In CNS diseases associated with hyperammonemia including hepatic encephalopathy, excessive L-glutamine synthesis and its accumulation in astrocytes resulting from detoxification of excess of ammonia is considered to be deleterious to brain function. Astrocytes are the locus of glutamine synthetase in the brain (Suárez et al., 2002), which renders them a primary target of excess ammonia. Specifically, excess of newly synthesized L-glutamine contributes to astrocytic swelling which results from its interference with mitochondrial function and from osmotic action (Kruczek et al., 2011; Sinke et al., 2008). In turn, astrocytic swelling is the primary cause of brain edema, a frequent fatal complication of hepatic encephalopathy (Blei and Larsen, 1999; Häussinger et al., 2000).

Astrocytic L-glutamine transport is mediated by a Na^+-coupled amino acid transport system N, represented by three carriers SN1, SN2, and SN7 (SLC38A3, SLC38A5 and SLC38A7, respectively) (Pochini et al., 2014). SN1, coded by the *Snat3* gene identified and annotated on the chromosome 3p21.31 is the most abundant system N transporter in astrocytes, which is mainly responsible for export of newly synthesized L-glutamine from the cells (Bröer et al., 2004). Recently, silencing of transporters SN1 and SN2 in cultured mouse astrocytes has been reported to cause L-glutamine retention in these cells (Zielińska et al., 2016). Those observations prompted a hypothesis that ammonia may contribute to intra-astrocytic L-glutamine retention by interfering with SN1-mediated glutamine efflux. Having confirmed this hypothesis, we

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https://doi.org/10.1016/j.neuint.2018.07.001
Received 26 January 2018; Received in revised form 19 June 2018; Accepted 3 July 2018
Available online 03 July 2018
0197-0186/

asked about the mechanism by which ammonia alters the expression and activity of SN1.

Phosphorylation by protein kinase C (PKC) is the principal mechanism which controls intracellular distribution and activity of different membrane proteins (Nissen-Meyer and Chaudhry, 2013). SN1 appears to be regulated by this kinase as was documented by other groups (Balkrishna et al., 2010; Nissen-Meyer and Chaudhry, 2013; Nissen-Meyer et al., 2011; Sidoryk-Wegrzynowicz et al., 2011). PKC activation by phorbol esters (PMA) leads to internalization of SN1, a process correlated with its decreased expression and transport activity as shown in *X. laevis* oocytes and in cultured rat astrocytes *in vitro* (Balkrishna et al., 2010; Nissen-Meyer et al., 2011; Sidoryk-Wegrzynowicz et al., 2011). In turn, SN1 interactions with different PKC isozymes including α, γ and δ isoforms were shown in cultured rat astrocytes (Nissen-Meyer and Chaudhry, 2013; Nissen-Meyer et al., 2011; Sidoryk-Wegrzynowicz et al., 2011). Basing on the above considerations we hypothesized that the mechanism by which ammonia inhibits L-glutamine efflux from astrocytes during hyperammonemia may be related to altered interaction of PKC with SN1 and the ensuing modulation of its activity. Down this valley, we endeavored the role of PKCδ and α isoforms. To test this hypothesis we analyzed mutual relations between activation of total PKC or silencing the particular PKC isozymes, and the expression and cell membrane distribution of SN1 transporter in cultured mouse cortical astrocytes treated or not with ammonia. Analysis of the effect of activation of PKC by phorbol esters also included the rate of [^{3}H]glutamine release from astrocytes with silenced distinct PKC isoforms.

2. Materials and methods

2.1. 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 antimycotic from Gibco (Thermofisher Scientific, USA) and HiPerfect Transfection Reagent (Qiagen, Germany). All other chemicals of the purest grade were purchased from available commercials sources.

2.2. Astrocyte cultures

Cortical astrocytes were isolated from 7-day-old C57BL6/J mice of both sexes and cultured as described earlier (Hertz et al., 1989). The C57BL6/J mice were obtained from the animal colony of the Mossakowski Medical Research Centre, Polish Academy of Sciences in Warsaw. All 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). Seven day after birth the pups were removed from their dams, anesthetized and killed by rapid decapitation. Dissected neocortical tissues were used for the preparation of primary astrocytic cell cultures. Briefly, cortices were 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% O_2 and 5% CO_2, on 24-well, 6-well plates or on 60 mm and 100 mm dishes. Experiments were performed on 3-week-old astrocytes. Astrocytes were treated with 5 mM ammonium chloride ("ammonia") which was added into cell culture medium for 24 h (1 M stock solutions of ammonium chloride were stored at -20° C and added at indicated concentration to culture medium).

2.3. PMA and BisI treatment

Cultured astrocytes were treated with PKC activator, phorbol 12-myristate 13-acetate (PMA; 200 nM; Sigma-Aldrich, USA) and/or PKC inhibitor, bisindolylmaleimide I (BisI; 1 μM; Calbiochem, USA) for 24 h. Co-treatment with BisI and PMA was performed by adding PMA after 10–15 min incubation of cells with BisI.

2.4. 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 reversely transcribed using High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Warrington, UK). Real-time PCR was performed on 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, PKCδ, PKCα and endogenous control β-actin (Mm0120670_m1, Mm00440891_m1, Mm00440858_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 (Livak and Schmittgen, 2001).

2.5. Cell membrane isolation

Three-week-old astrocytes were washed twice with cold PBS, scrapped off and centrifuged at 2500 g for 5 min at 4 °C. Pellets were homogenized in lysis buffer (15 mM Tris-HCl, pH 7.6, 0.25 M sacharose, 1 mM DTT, 0.5 mM PMSF) containing protease (concentration 1:200, Sigma-Aldrich, St. Louis, MO, USA) and phosphatase (concentration 1:100, Sigma-Aldrich, St. Louis, MO, USA) inhibitors by sonication and subsequently centrifuged for 10 min at 1000 g at 4 °C. Supernatant was collected in the new Eppendorf tubes and the procedure was repeated. Supernatant was added to the previously collected and centrifuged for 20 min at 14000 g at 4 °C. Pellet was dissolved in 40 μl of lysis buffer and subjected to Western Blot analysis.

2.6. Protein isolation and western blot

Astrocytes were washed with PBS, scrapped off and centrifuged at 1000 g 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 10000 g 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 SDS 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), PKCδ, PKCα (1:900, ProteinTech, Manchester, UK) was done in 1% BSA in TBS-T buffer overnight at 4 °C followed by 1-h incubation with HRP-conjugated-antirabbit IgG (1:3000 for SN1 and 1:4500 for PKC isoforms; 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). In the experiments with cell membrane fractions Na^+/K^+ ATPase was used as an internal standard. Incubation with antibody against Na^+/K^+ ATPase (1:4000, Merck Millipore, USA) was done in 1% BSA in TBS-T buffer overnight at 4 °C followed by 1-h incubation with HRP-conjugated-antirabbit IgG (1:3000, Sigma-Aldrich, USA) for detection by Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA). The chemiluminescent signal acquisition and densitometry analysis were conducted using the G-Box system (SynGene, Cambridge, UK) and GeneTools software (SynGene) respectively.

2.7. PKC activity assay

PKC activity was tested using PKC kinase activity assay (Abcam, UK) according to the manufacturer protocol. Three-week-old cells were incubated with lysis buffer (20 mM MOPS, 50 mM β-glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM DTT. 1 mM benzamidine, 1 mM PMSF, 10 μg/ml leupeptin and aprotinin) for 10 min on ice and then centrifuged at 13000 g for 15 min. 0.1 μg of protein from cell lysates diluted in 30 μl of Kinase Assay Dilution Buffer were added to the presoaked wells of the PKC substrate microtiter plate. As a positive control 6 μl of purified active PKC diluted in 30 μl of Kinase Assay Dilution Buffer and as a blank 30 μl of Kinase Assay Dilution Buffer were used. The kinase reaction was initiated by adding 10 μl of ATP to each well and the samples were incubated for 30 min at room temperature with gentle shaking after 15 min. The reaction was stopped by removing the contents of each well. Samples, excluding blank ones, were incubated with 40 μl of Phosphospecific substrate antibody for 1 h at room temperature with gentle shaking every 20 min. Wells were washed four times with Wash Buffer and then incubated with 40 μl of diluted antirabbit IgG-HRP antibody for 30 min at room temperature, with gentle shaking every 10 min. Subsequently, all wells were washed four times with Wash Buffer. In order to detect PKC activity, 60 μl of TMB substrate was added to each well and the plate was incubated at room temperature for 30 min and then stopped by addition of 20 μl od Stop Solution. The PKC activity was analyzed by measuring the absorbance at OD = 450 nm.

2.8. Transport measurements

2.8.1. [^{3}H]glutamine uptake

Cultured astrocytes were washed twice with Krebs buffer (29.5 mM NaCl, 1.13 mM KCl, 0.3 mM KH_2PO_4, 0.3 mM $MgSO_4$, 11 mM glucose, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$) and then pre-incubated in this buffer for 15 min at 37 °C. Incubation mixtures containing Krebs buffer with 0.1 μCi/mL L-[$^{3,4-3}$H(N)-]glutamine (PerkinElmer, Waltham, MA, USA; specific radioactivity 37 MBq/mL), 0.1 mM unlabelled L-glutamine. The mixtures contained also 10 mM L-alanine and 10 mM L-leucine in order to block other than N, L-glutamine 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 1 M NaOH was added to lyse the cell. The radioactivity of cell lysates was measured in a Wallac 1409 Liquid Scintillation Counter (Perkin-Elmer, Finland).

2.8.2. [^{3}H]glutamine release

Cultured cortical astrocytes were incubated in Krebs buffer containing 0.25 μCi/mL 1 mM L-[$^{3,4-3}$H(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-alanine and 10 mM L-leucine was added to the cells. Samples were collected from supernatant after 10 min. The [^{3}H] 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).

2.9. Biotynylation of cell surface proteins

Three-week-old astrocytes was washed twice with cold PBS and incubated with 2 mM biotin reagent EZ-Link Sulfo-NHS-LC-Biotin (Pierce, Thermoscientific, USA) for 30 min in room temperature. To quench and remove excess biotin reagent and by-products the cells were washed twice with PBS with addition 100 mM glycine. Then, the cells were lysed with RIPA buffer containing proteinase and phosphatase inhibitor and 50 mM sodium fluoride. Cell lysates containing 400 μg of protein were added to the 100 μl of Pierce Avidin Agarose (Pierce, Thermoscientific, USA) and incubated overnight at 4 °C. Pierce Avidin Agarose was washed twice with RIPA buffer. Biotinylated proteins were solubilized using 2x concentrated SDS-PAGE loading buffer at 95 °C for 5 min and centrifuged at 12000g for 1 min. Collected supernatant was in Western Blot analysis. As a control 20 μg of protein from whole cell lysates were used.

2.10. Protein kinase C isoforms silencing

In order to down-regulate chosen PKC isoforms, PKCδ and PKCα, astrocytes were transfected with a mix of four types of siRNA duplexes consisting 21 nucleotides. Each type of siRNA sequences was targeted to a different gene region to obtain the most effective silencing. Sense strands for PKCδ silencing were: 5′-CCGATTCAAGGTTTATAACTA-3′, 5′-AGGGAAGACACTGGTACAGAA-3′, 5′-TTGAATGTAGTTATTGAA ATA-3′, 5′-CCGGGTGGACACACCACACTA-3’. Sense strands for PKCα silencing used in this study were: 5′-ATGAACTGTTTCAGTCTATAA-3′, 5′-CAGGAGCAAGCACAAGTTCAA-3′, 5′-CAGCTGGTCATTGCTAAC ATA-3′, 5′-AAGCATTATCTTAGTGGATGA-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) according to fast-forward protocol designed for adherent cells provided by manufacturer. Briefly, 4.5 μl of 2 μM 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.

2.11. Co-immunoprecipitation

Co-immunoprecipitation experiments were performed as described earlier with slight modifications (Sidoryk-Wegrzynowicz et al., 2010). Briefly, three-week-old astrocytes were lysed with RIPA buffer containing protease and phosphatase inhibitors. Cell lysates containing 1000 μg of protein were precleared by shaking with 40 μl of Pansorbin cells (Callbiochem, San Diego, CA, USA) at 4 °C for 1 h, centrifuged at 3000 g for 5 min at 4 °C and the collected supernatant was incubated overnight with SN1 antibody. Proteins were precipitated by incubation with 25 μl of protein-G agarose beads (Sigma-Aldrich) for 2 h at 4 °C and then centrifuged at 1000 g for 1 min. Immune complexes were washed three times with RIPA buffer and once with wash buffer containing 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% Nonidet P-40). Subsequently they were solubilized in 25 μl of SDS-PAGE loading buffer at 95 °C for 5 min and then used in Western blot analysis. As a positive control whole cell lysates were used and as a negative control the following samples were used: beads with antibody used for immunoprecipitation (PKC isoforms), beads with antibody used in Western blot analysis (SN1) and lysate without antibody used for immunoprecipitation (PKC isoforms).

2.12. Statistical analysis

The number 'n' indicates the number of independent experiments performed with different cell cultures. Data from at least four different experiments, with each data point in an individual experiment representing triplicate measurements, were used for statistical analysis. Statistical analysis was performed using GraphPad Prism 7 software (GraphPad Software, La Jolla, CA, USA). Confirmation of normality of the data distribution was checked using Kołmogorow–Smirnow test. Statistical significance was determined by one-way analysis of variance (One-Way ANOVA) followed by Dunnett's post-test and two-way analysis of variance (Two-Way ANOVA) followed by Bonferroni post-test for more than two groups. A probability value of 0.05 or less was considered statistically significant.

3. Results

3.1. Ammonia reduces PKC activity in cultured mouse astrocytes

PKC activity was assayed in astrocytes treated with 5 mM ammonia and/or with 200 nM PMA for 24 h. The results revealed a decrease by ~20% of PKC activity in astrocytes treated with ammonia and an increase by ~25% in cells treated with PMA. The PKC activity-reducing effect of ammonia was not significant when cells were treated simultaneously with PMA. Pre-incubation with BisI, a PKC inhibitor at concentration known to completely inhibit phosphorylation or activity of membrane proteins such as the dopamine receptor (Namkung and Sibley, 2004) and glutamate transporter EAAC1 (González et al., 2002), reversed the effect of PMA in control but not in ammonia-treated astrocytes (Fig. 1).

3.2. Ammonia decreases the amount of SN1 on the cell membrane

Previously observed decrease of L-glutamine release after ammonia treatment (Zielińska et al., 2016) and augmented PKC activity described above could suggest that the amount of SN1 in the plasma membrane would decrease after ammonia treatment. Therefore, the amount of transporter was estimated a) by measuring SN1 protein in the membrane fraction (Fig. 2a) b) by cell surface biotynylation (Fig. 2b) c) in a whole cell lysate (Fig. 2c). In contrast to the unchanged SN1 protein level in a whole cell lysate, both the SN1 membrane content and the amount of biotinylated transporter were found reduced by ammonia. In both tests, incubation with PMA reduced SN1 in control astrocytes but not in ammonia-treated astrocytes. In control astrocytes, the effect of PMA was in either test found reversed by BisI pre-treatment. In ammonia-treated astrocytes, the effect of PMA and its reversal by BisI was only observed in the biotynylation test.

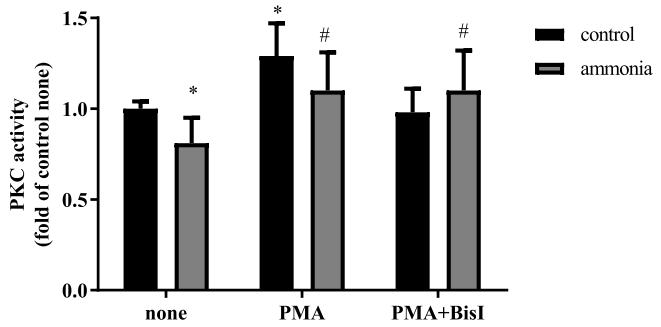

Fig. 1. PKC activity in mouse cortical astrocytes treated with 200 nM PMA and/or 1 mM BisI after 5 mM ammonia exposure. Results are mean ± SD (n = 4). (*) $p < 0.05$ vs control none; (#) $p < 0.05$ vs ammonia none; Two-Way ANOVA, Bonferroni post-hoc test.

3.3. Ammonia and phorbol ester-induced changes in system N activity

Astrocytes accumulate L-glutamine in a linear fashion for at least 10 min (data not shown), therefore the initial (5 min) transport was measured in the different conditions. As shown in Fig. 3a, pre-incubation of astrocytes with PMA results in an decrease in total and system N-mediated [^{3}H]glutamine uptake, compared to control astrocytes. This effect was not observed when cells were pre-treated with BisI (1 μM). Neither total nor system N-mediated [^{3}H]glutamine uptake was affected by 5 mM ammonia in either of the studied variants (Fig. 3a). Additionally, total and system N-mediated release of preloaded [^{3}H]glutamine, expressed as the % of the total radioactivity lost, was reduced in astrocytes treated with 5 mM ammonia, but was not changed after 200 nM PMA treatment as compared to the control conditions. PMA reduced the effect of ammonia on [^{3}H]glutamine efflux (Fig. 3b).

3.4. Silencing of PKC isoforms affects cell membrane expression of SN1 in the presence of ammonia

In this series of experiments we analysed the effect of ammonia treatment on the PKCα and PKCδ protein levels in mouse cortical astrocytes. Ammonia exposure increased PKCδ protein but not PKCα level (Fig. 4). Next, we silenced the latter two PKC isoforms using siRNA technology and confirmed the effectiveness of the procedure in real-time qPCR and WB analysis (Fig.S1a,b). In the absence of ammonia, silencing of both tested PKC isoforms increased the expression of SN1 in cell membrane fraction (Fig. 4). Treatment with ammonia increased SN1 protein expression on cell membranes of astrocytes with silenced both PKC isoforms (Fig. 4).

3.5. Ammonia affects PKCδ association with SN1

Next, we performed experiments to examine the ability of L-glutamine transporter SN1 to form complexes with PKC isoforms. We also sought to determine whether ammonia exposure can affect this interaction. The study confirmed earlier data pointing to complex formation of SN1 with both studied PKC isoforms (Nissen-Meyer et al., 2011; Sidoryk-Wegrzynowicz et al., 2011). In our hands, exposure to ammonia tended to increase the interaction between examined PKCδ isoform and SN1 (Fig. 5 *right panel* b). But the increase did not reach the level of statistical significance (Fig. 5 *left panel* a).

3.6. The effect of silencing of PKC isoforms on [^{3}H]glutamine release in the presence and absence of ammonia

In order to verify if functional deactivation of SN1 by ammonia resulted from phosphorylation of this protein, transport of L-glutamine out of the cells was analysed. Total and system N-mediated release of preloaded [^{3}H]glutamine, expressed as the % of the total radioactivity lost was compared in astrocytes with silenced PKCδ and PKCα treated or not treated with 5 mM ammonia. We documented that PKCδ silencing abrogates the inhibition of the system N-mediated [^{3}H]glutamine efflux by ammonia (Table 1).

4. Discussion

The principal findings of the present studies are that prolonged exposure of cultured cortical mouse astrocytes to ammonia treatment i) specifically inhibits the system N-mediated L-glutamine efflux and that ii), the inhibition involves PKC signalling. Both i) and ii) are novel observations. In the only related report known to us, ammonia altered the intracellular distribution of different PKC isoforms in cerebellar neurons, with implication for NMDA receptor-mediated signalling (Giordano et al., 2005). We examined whether and in which degree ammonia affects PKC signalling in SN1-mediated L-glutamine efflux and which of the preselected PKC isoforms are involved in this

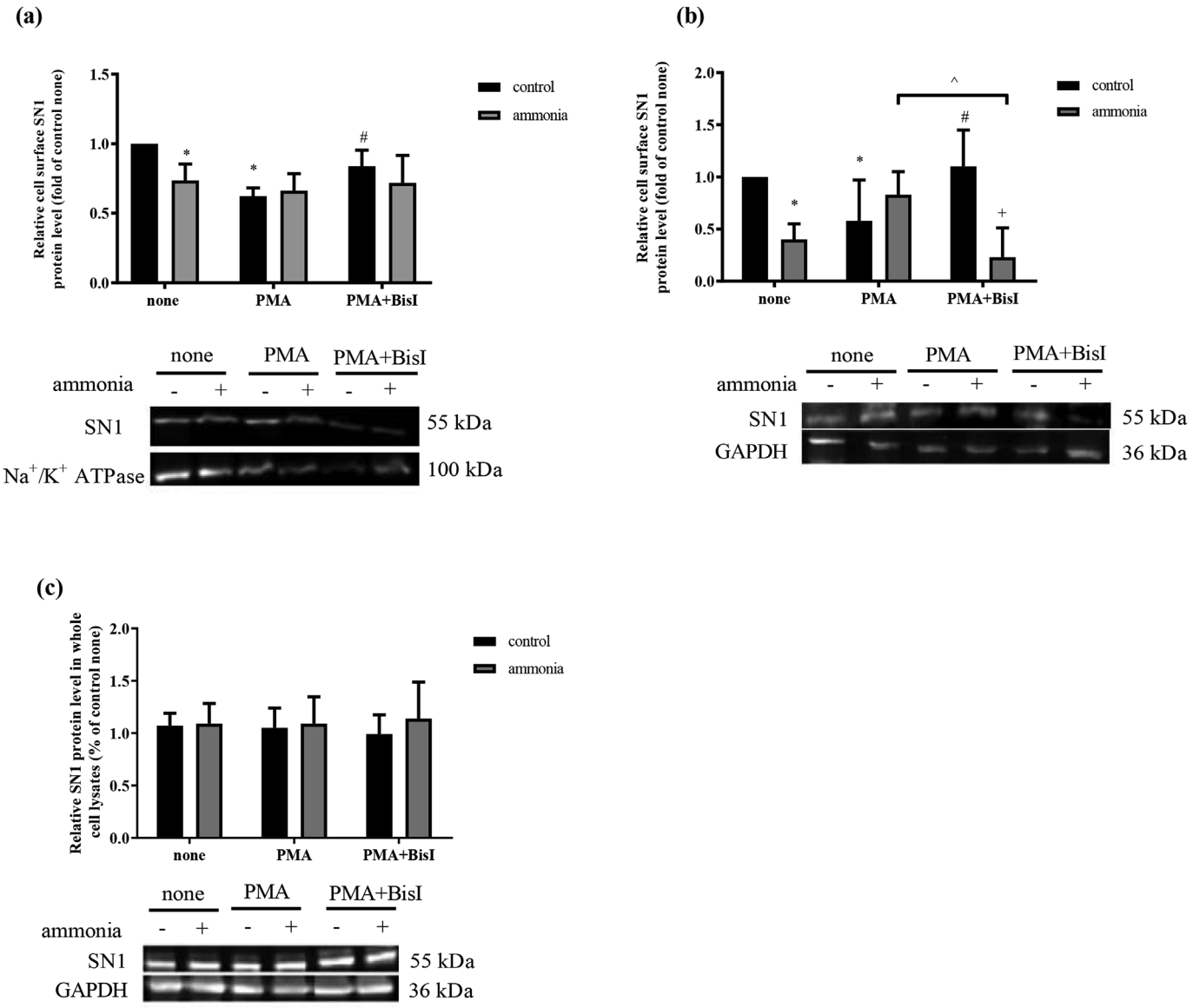

Fig. 2. (a) SN1 protein expression level in cell membranes of mouse cortical astrocytes treated with 200 nM PMA and/or 1 mM BisI in the presence or absence of 5 mM ammonia. Upper panel shows densitometry analysis of immunoblots, lower panel shows representative immunoblot. Results are mean ± SD (n = 4). (*) $p < 0.05$ vs control none; (#) $p < 0.05$ vs ammonia none; Two-Way ANOVA, Bonferroni post-hoc test. (b) SN1 protein expression level on biotynylated cell surfaces of mouse cortical astrocytes treated with 200 nM PMA and/or 1 mM BisI in the presence or absence of 5 mM ammonia. Upper panel shows densitometry analysis of immunoblots, lower panel shows representative immunoblot. Results are mean ± SD (n = 4). (*) $p < 0.05$ vs control none; (#) $p < 0.05$ vs control PMA; (+) $p < 0.05$ vs ammonia PMA; (^) $p < 0.05$ vs control PMA + BisI; Two-Way ANOVA, Bonferroni post-hoc test. (c) SN1 protein expression level in whole cell lysates of mouse cortical astrocytes treated with 200 nM PMA and/or 1 mM BisI in the presence or absence of 5 mM ammonia. Upper panel shows densitometry analysis of immunoblots, lower panel shows representative immunoblot.

response. The isoforms analysed included PKCδ, one of the novel isoforms which is widely expressed in many tissues and dominates in brain, and PKCα, a representative of the classical isoforms also expressed in many tissues and abounds in CNS astrocytes (Slepko et al., 1999). PKCγ, other representative of classical PKC isoforms which is restricted to the CNS (Barnett et al., 2007; Hughes et al., 2008), was excluded from consideration, as it turned out to be present in very low quantities in our astrocytic culture system.

The possibility that PKC may mediate ammonia-induced changes ion SN1 function was initially inferred from the observation that PKC activity is vulnerable to ammonia. Next we noted that ammonia treatment was accompanied by quantitative reduction of SN1 on the cell surface, and consistently-by a decrease of glutamine release from the cell. Further, using siRNA technique we documented that selective PKCδ isozyme silencing abolishes ammonia-induced SN1 internalization to the cell cytoplasm. The present study not only reinforces supporting earlier observations of PKC-dependence of SN1 membrane translocation and activation (Nissen-Meyer and Chaudhry, 2013; Nissen-Meyer et al., 2011), but specifies that the effects of PKC on both SN1 location on the membrane and its function are modulated by ammonia.

PKC kinase-regulated phosphorylation is a dominant mechanism of expression and function regulation of a wide spectrum of transporters (Foster et al., 2008; Jayanthi et al., 2004). SN1 contains putative PKC phosphorylation sites that are conserved in humans, rats and mice (Balkrishna et al., 2010). In the latter study, SN1 was found to be downregulated by PKC, not by the direct phosphorylation of the transporter, but in a caveolin-dependent manner (Balkrishna et al., 2010). Multiple PKC isoforms can be associated with caveolae, depending on the mechanism of activation, as was shown in fibroblasts for

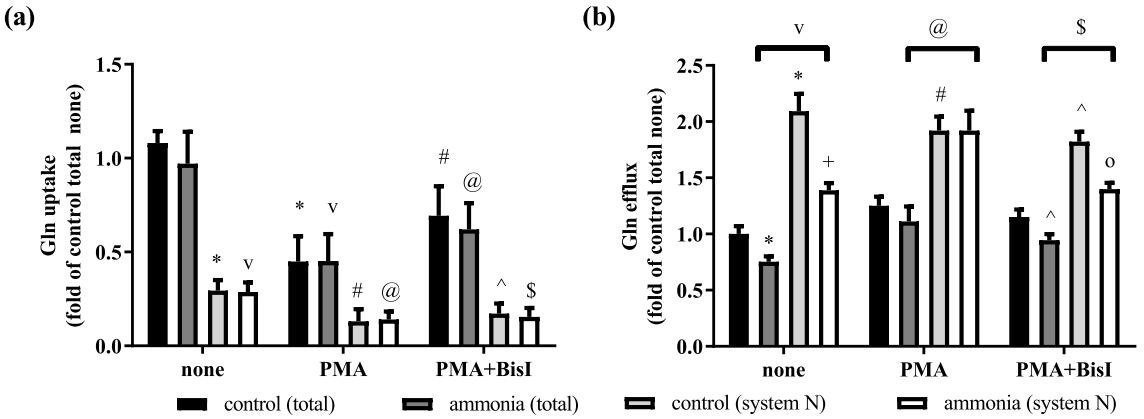

Fig. 3. (a) Total and system N-mediated [^{3}H]glutamine uptake in mouse cortical astrocytes treated with 200 nM PMA and/or 1 mM BisI after 5 mM ammonia exposure. Absolute value of [^{3}H]glutamine uptake in control cells: 21.95 ± 5.80 nmoL/min × mg protein. (b) Efflux of [^{3}H]glutamine from astrocytes treated with 200 nM PMA and/or 1 mM BisI after 5 mM ammonia exposure. Results are mean ± SD (n = 5). (*) $p < 0.05$ vs control none (total); (#) $p < 0.05$ vs control PMA (total); (^) $p < 0.05$ vs control PMA + BisI (total); (+) $p < 0.05$ vs control none (system N); (o) $p < 0.05$ vs control PMA + BisI (system N); (v) $p < 0.05$ vs ammonia none (total); (@) $p < 0.05$ vs ammonia PMA (total); ($) $p < 0.05$ vs ammonia PMA + BisI (total); Two-Way ANOVA, Bonferroni post-hoc test.

PKCα, ε and λ (Mineo et al., 1998). However, the view that caveolae play a role in signaling is based on the ability of caveolin to directly interact with PKCα and other PKC isozymes, and has recently been put under dispute (Collins et al., 2012). Current models favor the concept that cells achieve PKC signal specificity through local activation of PKC signaling pathways that are initiated at specific subcellular membranes (Steinberg, 2008). The effects of different isoforms may encompass two separate aspects of transporter regulation: for instance, in glioma cells PKCε mediated an increase the activity of the glutamate transporter EAAC1 (EAAT3), while PKCα regulated its surface expression (González et al., 2002).

An *in situ* study showed that PKC activation induces phosphorylation and internalization of SN1 transporter (Nissen-Meyer et al., 2011). Using mutagenesis approaches the authors documented that SN1 serine 52 (S52) is a target of PKC phosphorylation site in rat astrocytes. Among all tested phosphorylation sites (such as T32, T59, S51 or S53) only S52 mutated to alanine was not phosphorylated (Nissen-Meyer et al., 2011). Of note in this context, the mechanism mentioned above applies only for rat and sheep protein, whereas in mice SN1 phosphorylation site is located in a close proximity of S52 (Nissen-Meyer et al., 2011).

The possible mechanism by which ammonia affects PKC activity in

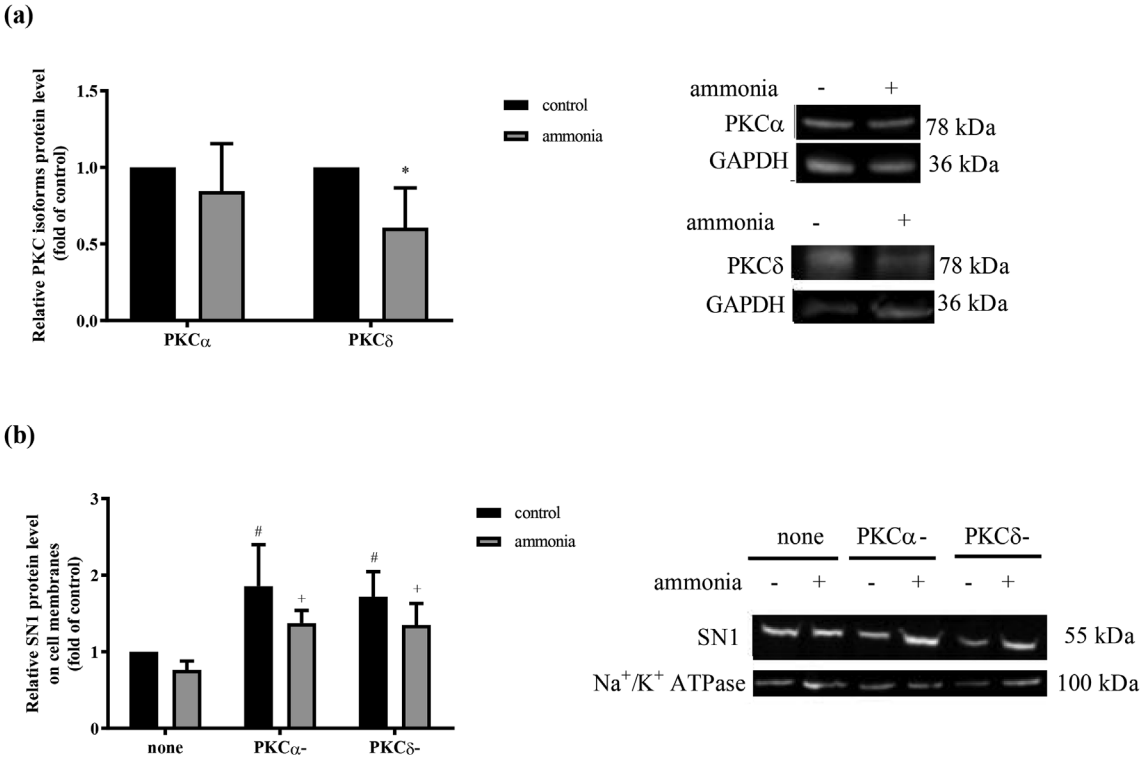

Fig. 4. (a) Protein level of PKC isoforms (δ, α) in mouse cortical astrocytes after 5 mM ammonia exposure. Left panel shows densitometry analysis of immunoblots, right panel shows representative immunoblot. Results are mean ± SD (n = 4). (*) $p < 0.05$ vs control PKC δ; Two-Way ANOVA, Bonferroni post-hoc test (b) Protein level of SN1 in cell membranes of mouse cortical astrocytes after silencing of PKC isoforms (α, δ) and 5 mM ammonia exposure. Left panel shows densitometry analysis of immunoblots, right panel shows representative immunoblot. 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.

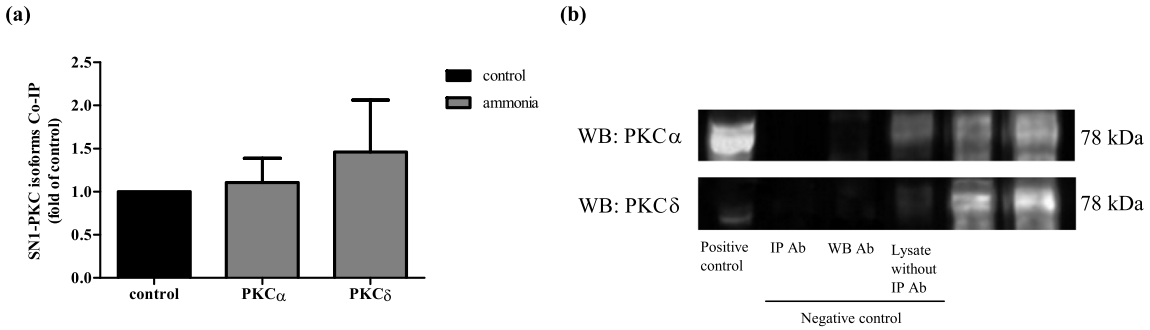

Fig. 5. Co-immunoprecipitation of PKC isoforms (α, δ) with SN1 in mouse cortical astrocytes after 5 mM ammonia treatment. (a) Summary data for mean densitometry of co-immunoprecipitation immunoblots. Results are mean ± SD (n = 4). (b) Representative co-immunoprecipitation immunoblots.

Table 1
Total and system N-mediated [^{3}H]glutamine efflux from mouse cortical astrocytes after silencing of PKC isoforms (δ, α) 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 control PKCδ- (total), (@) $p < 0.05$ vs ammonia PKCδ- (total), (+) $p < 0.05$ vs ammonia none (system N), (&) $p < 0.05$ vs control PKCα- (total), (?) $p < 0.05$ vs ammonia PKCα- (total), (!) $p < 0.05$ vs control PKCα- (system N), Two-Way ANOVA, Bonferroni post-hoc test.

		ammonia	Gln efflux (fold of control none)
none	total	–	1.00 ± 0.11
		+	0.86 ± 0.08*
	system N	–	1.40 ± 0.17*
		+	1.25 ± 0.20$^{\$,\#}$
PKCα-	total	–	2.01 ± 0.39*
		+	1.61 ± 0.36$^{\$,\wedge}$
	system N	–	2.42 ± 0.46$^{\#,\wedge}$
		+	1.89 ± 0.49$^{+,@}$
PKCδ-	total	–	1.33 ± 0.17*
		+	1.11 ± 0.14$^{\$,\&}$
	system N	–	1.58 ± 0.22$^{\#,\&}$
		+	1.49 ± 0.12$^{+,?,!}$

the astrocytic culture deserves comment. In so far, Bodega et al. (2007) showed that ammonia affects MAPK expression in cultured astrocytes. Whether the effects observed in our preparation are mediated by MAPK or other signal transduction mechanism(s) remains to be seen.

The mechanism by which the different PKC isoforms contribute to alterations of SN1 function by ammonia in mouse astrocytes has been the major focus of this study. Most of the cells express multiple PKC isoforms that may concurrently respond to the same activators by several targeting mechanisms. Literature data suggested that SN1 directly interacts with PKC isoforms δ (Sidoryk-Wegrzynowicz et al., 2011), α and γ (Balkrishna et al., 2010; Nissen-Meyer et al., 2011; Sidoryk-Wegrzynowicz et al., 2011). In our study PKCδ isoform was affected by ammonia exposure (Fig. 4). In contrast to other PKC isozymes, mostly considered as pro-survival mediators, PKCδ is ubiquitously expressed and fosters proapoptotic activity (Reyland, 2009), associated with a proteolytic cleavage mediated by caspase-3 (Kanthasamy et al., 2003).

In the present study ammonia treatment eliminated PMA-induced SN1 phosphorylation detected in biotinylated fractions, what, compared with the decreased amount of the transporter in plasma membrane after BisI pre-treatment, appeared to indicate that phosphorylation takes place in the cytoplasm, followed by SN1 transfer to the cell surface. Our results are in agreement with those of, Sidoryk-Wegrzynowicz et al. (2011), who showed that PMA reduces the SN1 content of biotinylated surface membranes and SN1 co-immunoprecipitates together with PKCδ.

Functionality of PKC isoforms is often judged on the basis of their ability to form complexes with respective target molecules (Nissen-Meyer et al., 2011; Sidoryk-Wegrzynowicz et al., 2011). Here, all two isoforms studied formed such complexes with SN1, whereby ammonia tended to specifically enhance the PKCδ-SN1 interaction. The nature of the interaction and its implications for SN1-mediated L-glutamine efflux remains subject of our future investigations.

While this study is to the authors' knowledge the first to ascribe the role of PKC signalling in SN1 transporter expression and function in the cultured mouse astrocytes affected by pathologically elevated ammonium ions, further fine-tuning of the evidence is needed to prove the relevance of these observations. In the future, the findings need to be reconciled with the fact that in the acute hepatic encephalopathy models (Desjardins et al., 2012; Zielińska et al., 2014), where ammonia is one of key (albeit not the only) pathogens, the expression of SN1 was observed to be decreased. Further studies will also have to account for astrocytic neuronal interactions which may vary in the different brain regions and physiological contexts. Of note in this context, impairment of N-system-mediated L-glutamine release was observed in astrocytic-neuronal co-cultures and astrocytes treated with ammonia cultured in neuronal medium (Dąbrowska et al., 2018).

Conflicts of interest

The authors declare no conflict of interest, financial or otherwise.

Acknowledgements

The study was supported by the Polish-Norwegian Research Program operated by the National Centre for Research and Development under the Norwegian Financial Mechanism 2009–2014, Project Contract No: Pol-Nor/196190/23/2013.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.neuint.2018.07.001.

References

Albrecht, J., Sidoryk-Węgrzynowicz, M., Zielińska, M., Aschner, M., 2010a. Roles of glutamine in neurotransmission. Neuron Glia Biol. 6 (4), 263–276.
Albrecht, J., Sonnewald, U., Waagepetersen, H.S., Schousboe, A., 2007. Glutamine in the central nervous system: function and dysfunction. Front. Biosci. 12, 332–343.
Albrecht, J., Zielińska, M., Norenberg, M.D., 2010b. Glutamine as a mediator of ammonia neurotoxicity: a critical appraisal. Biochem. Pharmacol. 80 (9), 1303–1308.
Balkrishna, S., Bröer, A., Kingsland, A., Bröer, S., 2010. Rapid downregulation of the rat glutamine transporter SNAT3 by a caveolin-dependent trafficking mechanism in Xenopus laevis oocytes. Am. J. Physiol. Cell Physiol. 299 (5), C1047–C1057.
Barnett, M.E., Madgwick, D.K., Takemoto, D.J., 2007. Protein kinase C as a stress sensor. Cell. Signal. 19 (9), 1820–1829.
Blei, A.T., Larsen, F.S., 1999. Pathophysiology of cerebral edema in fulminant hepatic failure. J. Hepatol. 31, 771–776.
Bodega, G., Suárez, I., Almonacid, L., Ciordia, S., Beloso, A., López-Fernández, L.A., Zaballos, A., Fernández, B., 2007. Effect of ammonia on ciliary neurotrophic factor mRNA and protein expression and its upstream signalling pathway in cultured rat

astroglial cells: possible implication of c-fos, Sp1 and p38MAPK. Neuropathol. Appl. Neurobiol. 33 (4), 420–430.
Bröer, A., Deitmer, J.W., Bröer, S., 2004. Astroglial glutamine transport by system N is upregulated by glutamate. Glia 48 (4), 298–310.
Collins, B.M., Davis, M.J., Hancock, J.F., Parton, R.G., 2012. Structure-based reassessment of the caveolin signaling model: do caveolae regulate signaling through caveolin-protein interactions? Dev. Cell 23 (1), 11–20.
Cynober, L., 2002. Plasma amino acid levels with a note on membrane transport: characteristics, regulation, and metabolic significance. Nutrition 18 (9), 761–766.
Dąbrowska, K., Skowrońska, K., Popek, M., Obara-Michlewska, M., Albrecht, J., Zielińska, M., 2018. Roles of glutamate and glutamine transport in ammonia neurotoxicity: state of the art and question marks. Endocr. Metab. Immune Disord. - Drug Targets. http://dx.doi.org/10.2174/1871520618666171219124427.
Desjardins, P., Du, T., Jiang, W., Peng, L., Butterworth, R.F., 2012. Pathogenesis of hepatic encephalopathy and brain edema in acute liver failure: role of glutamine redefined. Neurochem. Int. 60 (7), 690–696.
Foster, J.D., Adkins, S.D., Lever, J.R., Vaughan, R.A., 2008. Phorbol ester induced trafficking-independent regulation and enhanced phosphorylation of the dopamine transporter associated with membrane rafts and cholesterol. J. Neurochem. 105 (5), 1683–1699.
Giordano, G., Sanchez-Perez, A.M., Burgal, M., Montoliu, C., Costa, L.G., Felipo, V., 2005. Chronic exposure to ammonia induces isoform-selective alterations in the intracellular distribution and NMDA receptor-mediated translocation of protein kinase C in cerebellar neurons in culture. J. Neurochem. 92 (1), 143–157.
González, M.I., Kazanietz, M.G., Robinson, M.B., 2002. Regulation of the neuronal glutamate transporter excitatory amino acid Carrier-1 (EAAC1) by different protein kinase C subtypes. Mol. Pharmacol. 62 (4), 901–910.
Häussinger, D., Kircheis, G., Fischer, R., Schliess, F., vom Dahl, S., 2000. Hepatic encephalopathy in chronic liver disease: a clinical manifestation of astrocyte swelling and low-grade cerebral edema? J. Hepatol. 32 (6), 1035–1038.
Hertz, L., Peng, L., Hertz, E., Juurlink, B.H., Yu, P.H., 1989. Development of monoamine oxidase activity and monoamine effects on glutamate release in cerebellar neurons and astrocytes. Neurochem. Res. 14, 1039–1046.
Hughes, A.S., Averill, S., King, V.R., Molander, C., Shortland, P.J., 2008. Neurochemical characterization of neuronal populations expressing protein kinase C gamma isoform in the spinal cord and gracile nucleus of the rat. Neuroscience 153 (2), 507–517.
Jayanthi, L.D., Samuvel, D.J., Ramamoorthy, S., 2004. Regulated internalization and phosphorylation of the native norepinephrine transporter in response to phorbol esters. Evidence for localization in lipid rafts and lipid raft-mediated internalization. J. Biol. Chem. 279 (18), 19315–19326.
Kanthasamy, A.G., Kitazawa, M., Kanthasamy, A., Anantharam, V., 2003. Role of proteolytic activation of protein kinase Cdelta in oxidative stress-induced apoptosis. Antioxidants Redox Signal. 5 (5), 609–620.
Kruczek, C., Görg, B., Keitel, V., Bidmon, H.J., Schliess, F., Häussinger, D., 2011. Ammonia increases nitric oxide, free Zn(2+), and metallothionein mRNA expression in cultured rat astrocytes. Biol. Chem. 392 (12), 1155–1165.
Lee, W.J., Hawkins, R.A., Viña, J.R., Peterson, D.R., 1998. Glutamine transport by the blood-brain barrier: a possible mechanism for nitrogen removal. Am. J. Physiol. 274, C1101–C1107.
Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)). Methods 25, 402–408.
Mineo, C., Ying, Y.S., Chapline, C., Jaken, S., Anderson, R.G., 1998. Targeting of protein kinase Calpha to caveolae. J. Cell Biol. 141 (3), 601–610.
Namkung, Y., Sibley, D.R., 2004. Protein kinase C mediates phosphorylation, desensitization, and trafficking of the D2 dopamine receptor. J. Biol. Chem. 279 (47), 49533–49541.
Nissen-Meyer, L.S., Chaudhry, F.A., 2013. Protein kinase C phosphorylates the system N glutamine transporter SN1 (Slc38a3) and regulates its membrane trafficking and degradation. Front. Endocrinol. 4 Article 138.
Nissen-Meyer, L.S., Popescu, M.C., Hamdani el, H., Chaudhry, F.A., 2011. Protein kinase C-mediated phosphorylation of a single serine residue on the rat glial glutamine transporter SN1 governs its membrane trafficking. J. Neurosci. 31 (17), 6565–6575.
Pithon-Curi, T.C., Trezena, A.G., Tavares-Lima, W., Curi, R., 2002. Evidence that glutamine is involved in neutrophil function. Cell Biochem. Funct. 20 (2), 81–86.
Pochini, L., Scalise, M., Galluccio, M., Indiveri, C., 2014. Membrane transporters for the special amino acid glutamine: structure/function relationships and relevance to human health. Front Chem 2 Article 61.
Reyland, M.E., 2009. Protein kinase C isoforms: multi-functional regulators of cell life and death. Front. Biosci. 14, 2386–2399.
Sidoryk-Wegrzynowicz, M., Lee, E., Mingwei, N., Aschner, M., 2011. Disruption of astrocytic glutamine turnover by manganese is mediated by the protein kinase C pathway. Glia 59 (11), 1732–1743.
Sidoryk-Wegrzynowicz, M., Lee, E.S., Ni, M., Aschner, M., 2010. Manganese-induced downregulation of astroglial glutamine transporter SNAT3 involves ubiquitin-mediated proteolytic system. Glia 58 (16), 1905–1912.
Sinke, A.P., Jayakumar, A.R., Panickar, K.S., Moriyama, M., Reddy, P.V., Norenberg, M.D., 2008. NFkappaB in the mechanism of ammonia-induced astrocyte swelling in culture. J. Neurochem. 106 (6), 2302–2311.
Slepko, N., Patrizio, M., Levi, G., 1999. Expression and translocation of protein kinase C isoforms in rat microglial and astroglial cultures. J. Neurosci. Res. 57 (1), 33–38.
Steinberg, S.F., 2008. Structural basis of protein kinase C isoform function. Physiol. Rev. 88 (4), 1341–1378.
Suárez, I., Bodega, G., Fernández, B., 2002. Glutamine synthetase in brain: effect of ammonia. Neurochem. Int. 41 (2–3), 123–142.
Waniewski, R.A., Martin, D.L., 1986. Exogenous glutamate is metabolized to glutamine and exported by rat primary astrocyte cultures. J. Neurochem. 47 (1), 304–313.
Zielińska, M., Dąbrowska, K., Hadera, M.G., Sonnewald, U., Albrecht, J., 2016. System N transporters are critical for glutamine release and modulate metabolic fluxes of glucose and acetate in cultured cortical astrocytes: changes induced by ammonia. J. Neurochem. 136 (2), 329–338.
Zielińska, M., Popek, M., Albrecht, J., 2014. Roles of changes in active glutamine transport in brain edema development during hepatic encephalopathy: an emerging concept. Neurochem. Res. 39 (3), 599–604.

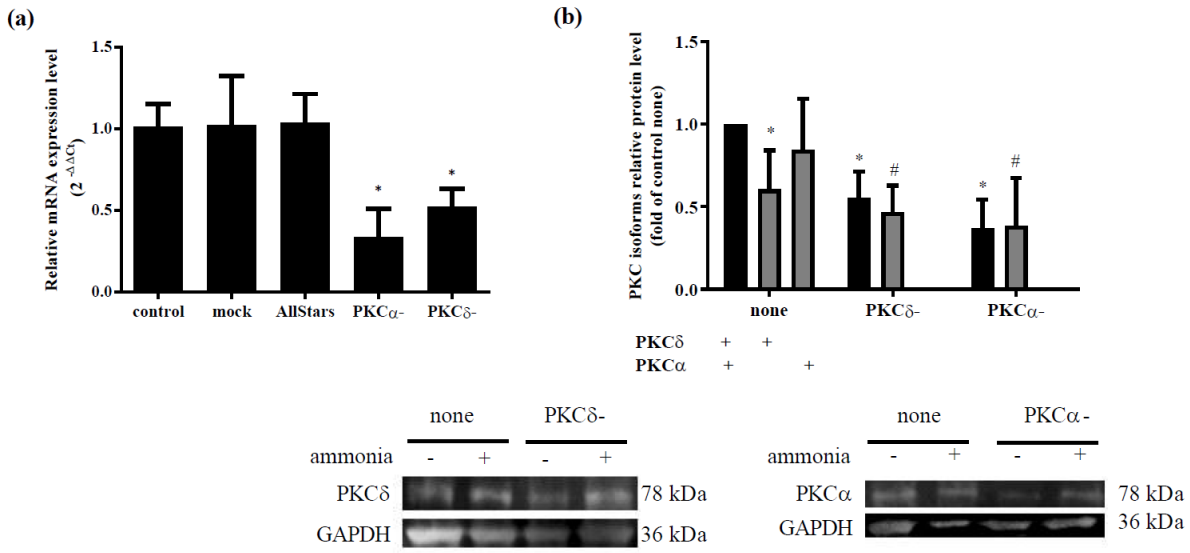

Fig. S1. (a) Efficiency of silencing of PKC isoforms in mouse cortical astrocytes. Results are mean ± SD (n = 4). (*) $p < 0.05$ vs control, One-Way ANOVA, Dunnett's post-hoc test. (b) Protein level of PKC isoforms (α, δ) in mouse cortical astrocytes after their silencing and 5 mM ammonia exposure. Lower panel shows representative immunoblots. Results are mean ± SD (n = 4). (*) $p < 0.05$ vs control none, (#) $p < 0.05$ vs ammonia none One-Way ANOVA, Dunnett's post-hoc test.

International Journal of
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Silencing of Transcription Factor Sp1 Promotes SN1 Transporter Regulation by Ammonia in Mouse Cortical Astrocytes

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Received: 9 November 2018; Accepted: 27 December 2018; Published: 9 January 2019

Abstract: The involvement of the 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. Sp1 binding to *Snat3* promoter was analyzed by chromatin immunoprecipitation. The role of Sp1 in SN1 expression and SN1-mediated [^{3}H]glutamine uptake in ammonia-treated astrocytes 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 the *Snat3* promoter, along with Sp1 dependence of system N-mediated [^{3}H]glutamine uptake, 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, which is a treatment likely to dephosphorylate Sp1. Furthermore, silencing of the PKCδ isoform appears to enhance the ammonia effect on the Sp1 level. 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

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 an 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], create the induction of a 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 has also been shown that induction of mitogen-activated protein kinases (MAPK) occurs in the astroglial cells exposed to the excess of Gln [21]. In addition, intracellular Gln accumulation induces the osmotic stress and activates the p38 cascade [22,23]. Therefore, an efficient transport via specific amino acid carriers guaranteeing proper Gln inter-cellular 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 the system N transporters causes the retention of Gln in astrocytes, and that ammonia-derived Gln release from astrocytes is mediated by impairing of the SN1-mediated Gln efflux [13,25]. SN1 is abundant in the 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 a ubiquitously expressed transcription factor responsible for activation of the 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]. More recently, the literature data indicated that Sp1 interacts with SN1 in mouse liver, intestine, kidney, and HepG2 cell line. Balkrishna and colleagues [28] 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 de-methylated promoters and RNA polymerase complex occupies transcriptional start-sites, which 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 [32]. 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 [33].

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 h) of oligodendrocytes to PMA, decreases the expression of Sp1 [34]. 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 the above described information and the structure of the *Snat3* gene regulatory region, we aimed to analyze the possible contribution of Sp1 on the SN1 regulation under hyperammonemic condition. We hypothesized that the mechanism by which ammonia interferes with SN1 expression

may be related to the activation of Sp1 and its enhanced interaction with a *Snat3* promoter. The study also included the analysis of the role of PKC in Sp1 phosphorylation.

2. Results

2.1. Ammonia-Induced Sp1 Expression Increase and Sp1 Translocation to the Nucleus

The Sp1 mRNA expression level after 24-h treatment with 5 mM ammonia was analyzed in real-time qPCR experiments. Ammonia upregulated both Sp1 mRNA and protein levels (Figure 1a,b). Since our results were in line with the results of Bodega et al. [33], the extended experiments further revealed cellular localization of the Sp1 transcription factor, as was observed using confocal microscopy. In astrocytes treated with ammonia, Sp1 was observed in cell nuclei, while in control astrocytes, the Sp1 transcription factor was observed mainly in the cytoplasm (Figure 1c).

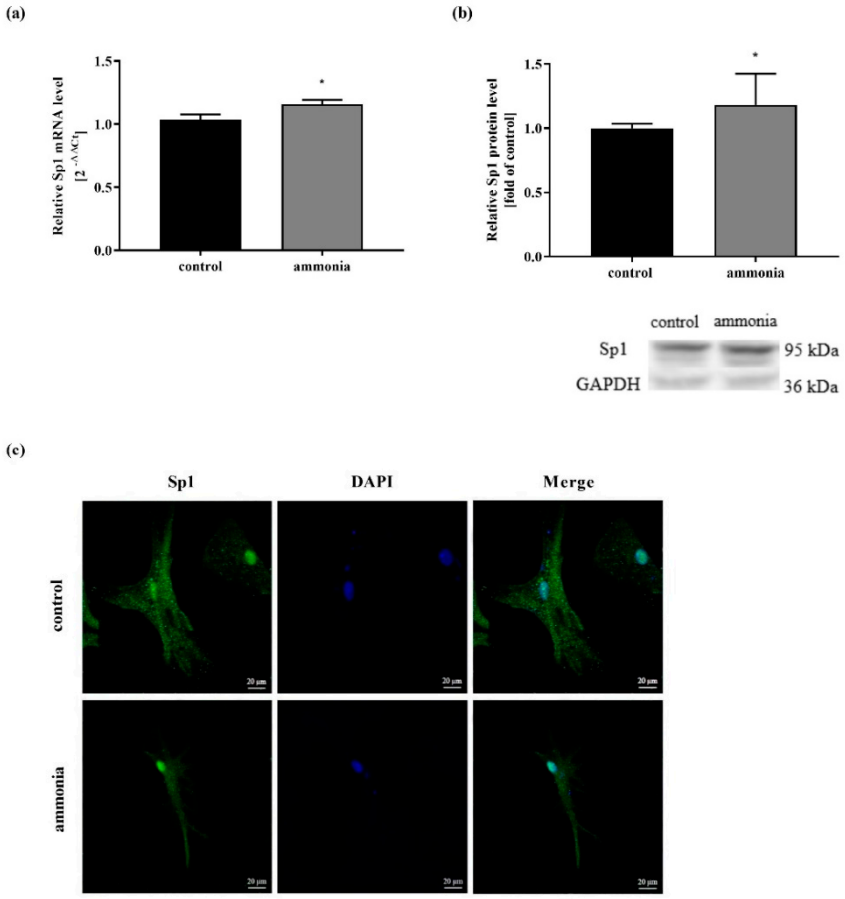

Figure 1. The effect of ammonia treatment on Sp1 expression and cellular localization. (**a**) Sp1 mRNA level in mouse cortical astrocytes after 24-h 5 mM ammonia treatment. Results are mean ± SD (n = 4). (*) $p < 0.05$ vs. control; Student t-test. (**b**) Sp1 protein level in mouse cortical astrocytes after 24-h 5 mM ammonia treatment. Upper panel shows densitometry analysis, lower panel shows representative immunoblot. Results are mean ± SD (n = 4). (**c**) Intracellular Sp1 transcription factor localization after 24-h 5 mM ammonia treatment (n = 4).

2.2. Ammonia Enhances Sp1 Binding to the Snat3 Promoter Region

Sp1 binding activity on the *Snat3* promoter was measured 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).

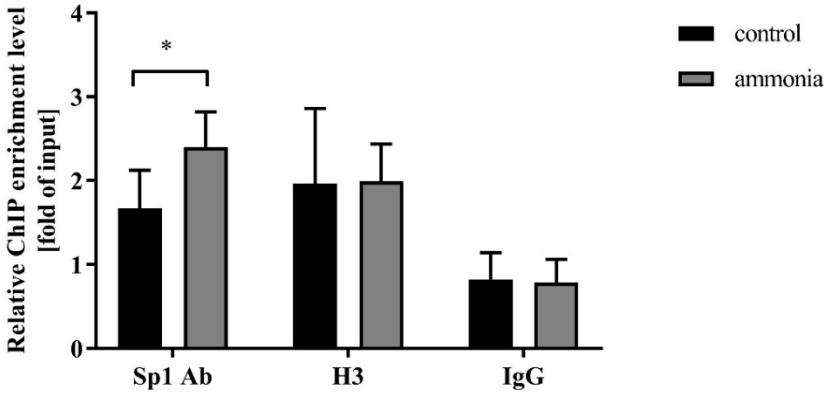

Figure 2. Sp1 transcription factor binding to the *Snat3* promoter region (Sp1 Ab) in mouse astrocytes treated with 5 mM ammonia for 24 h. Histone H3 was used as a positive control and IgG was used as a negative control of Sp1 binding to the *Snat3* promoter region. Results are mean ± SD (n = 4). (*) $p < 0.05$ vs. control; Two-way ANOVA and 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 via: (1) siRNA silencing technology (5 nM, 24 h), or (2) application of Sp1 inhibitor mithramycin A (10 μM, 1 h). The effectiveness of Sp1 silencing was checked in real-time qPCR and Western blot analyses (Figure S1). 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.

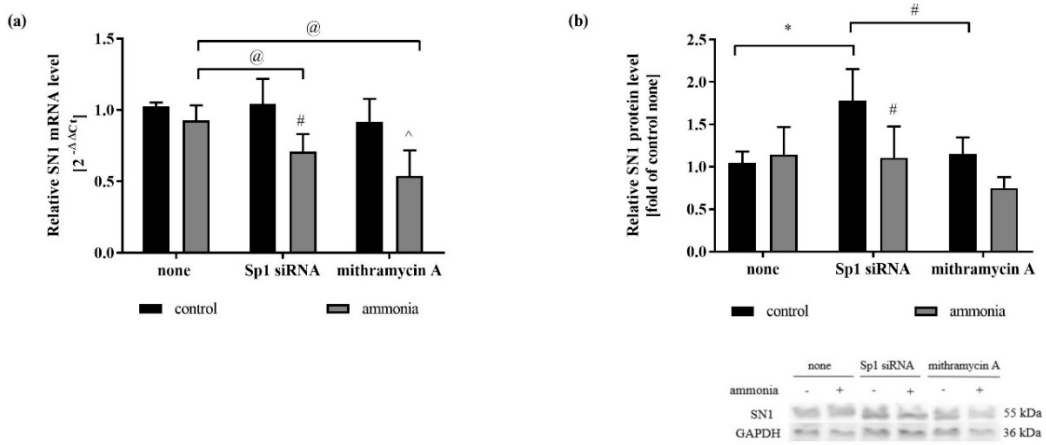

Figure 3. The effect of Sp1 transcription factor silencing (Sp1 siRNA) or inhibition (mithramycin A) on SN1 expression in mouse astrocytes treated or not with 5 mM ammonia for 24 h. (**a**) SN1 mRNA level. (**b**) SN1 protein level. Results are mean ± SD (n = 4). * $p < 0.05$ vs. control none, (@) $p < 0.05$ vs. ammonia none, (#) $p < 0.05$ vs. control Sp1 siRNA; (^) $p < 0.05$ vs. control mithramycin A; Two-Way ANOVA and 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 [^{3}H]glutamine uptake via system N in astrocytes was conducted. Silencing of Sp1 using siRNA technology decreased the total and system N-mediated [^{3}H]glutamine uptake after ammonia treatment, while Sp1 inhibition via mithramycin A affected only

the total [^{3}H]glutamine uptake in ammonia-treated astrocytes compared to control astrocytes after mithramycin A treatment (Table 1).

Table 1. Total and system N-mediated [^{3}H]glutamine uptake in mouse cortical astrocytes after 5 mM ammonia exposure for 24 h and silencing of Sp1 transcription factor by 5 nM siRNA or its inhibition by 10 μM mithramycin A. Basal [^{3}H]glutamine uptake for control cells was 8.02 ± 1.43 nmol/mg of protein/min. Control—ammonia−; 5 mM ammonia treatment—ammonia+. 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), ($^{\wedge}$) $p < 0.05$ vs. control Sp1 siRNA (total), ($^{!}$) $p < 0.05$ vs. ammonia Sp1 siRNA (total), ($^{+}$) $p < 0.05$ vs. control Sp1 siRNA (system N), ($^{?}$) $p < 0.05$ vs. ammonia none (system N), ($^{\$}$) $p < 0.05$ vs. control mithramycin A (system N); Two-Way ANOVA and Bonferroni post-hoc test.

Treatment	[^{3}H]glutamine Uptake	Ammonia	Value (Fold of Control None)
none	total	−	0.98 ± 0.09
		+	1.01 ± 0.17
	system N	−	0.39 ± 0.11 *
		+	0.36 ± 0.13 $^{\&}$
Sp1 siRNA	total	−	1.04 ± 0.19
		+	0.73 ± 0.12 $^{\wedge,\&}$
	system N	−	0.42 ± 0.06 $^{\wedge}$
		+	0.29 ± 0.06 $^{+,!}$
mithramycin A	total	−	1.28 ± 0.24 *
		+	1.10 ± 0.14 $^{\$}$
	system N	−	0.24 ± 0.10 $^{\#}$
		+	0.15 ± 0.04 $^{?}$

2.5. Sp1 Phosphorylation is Decreased in Astrocytes Treated with Ammonia

Sp1 can be phosphorylated by different kinases such as protein kinase C (PKC) [35], protein kinase A (PKA) [36], cyclin-dependent kinase (CDK) [37], and MAPK [38]. To test the involvement of PKC in Sp1 phosphorylation, the astrocytes were exposed to 200 nM PMA, an activator of PKC [25]. To verify whether the phosphorylation of Sp1 regulates the SN1 transcription in ammonia-treated astrocytes, the Sp1 protein was analyzed via quantification of two Sp1 forms with different molecular weight. The level of Sp1 protein 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. What is more, pre-treatment of astrocytes with a PKC inhibitor, bisindolylmaleimide I (BisI), did not reverse the effect induced by PMA, neither in Sp1 protein level (Figure 4a), nor in the Sp1 phosphorylation status (Figure 4b).

2.6. The Effect of PKC Activation on SN1 mRNA Expression in the Presence or Absence of Ammonia

The incubation of astrocytes with PMA in the presence of ammonia upregulates PKCδ, but not the PKCα isoform (Figure S2). In the presence of ammonia, PMA treatment decreased the SN1 mRNA expression (Figure 5). The effect was reversed after incubation of astrocytes with a PKC inhibitor, BisI (Figure 5). Moreover, inhibition of PKC activity by BisI treatment upregulated the SN1 mRNA level (Figure 5).

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).

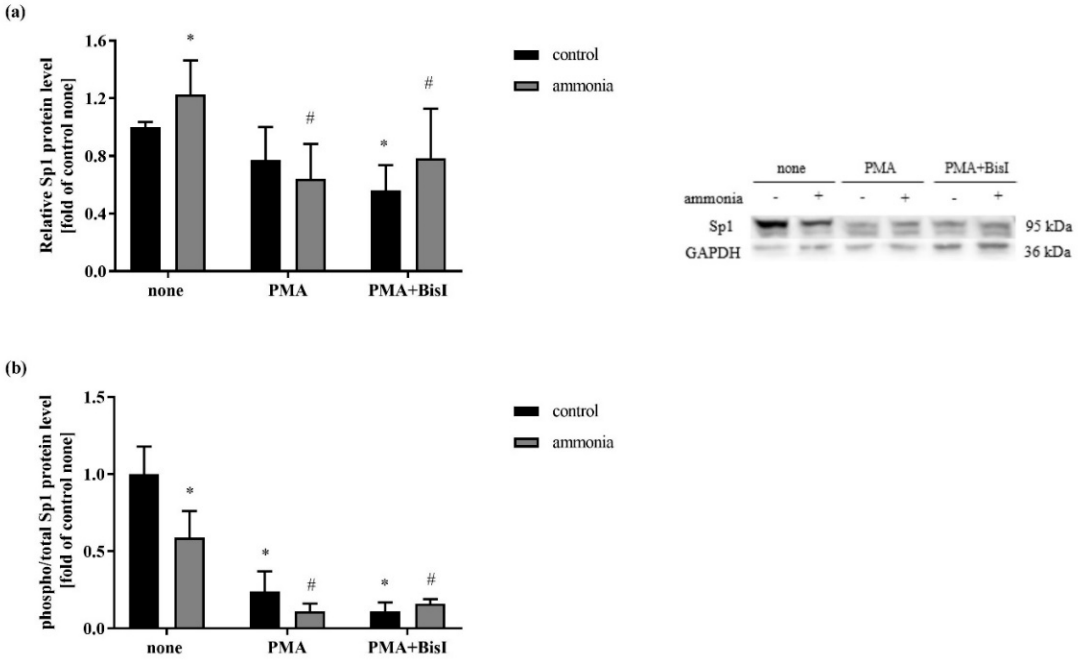

Figure 4. (**a**) Sp1 protein level in the astrocytes treated with 5 mM ammonia for 24 h and 200 nM PMA and/or 1 μM BisI. Results are mean ± SD (n = 4). (*) $p < 0.05$ vs. control none; (#) $p < 0.05$ vs. ammonia none; Two-Way ANOVA and Bonferroni post-hoc test. (**b**) The ratio of phospho-Sp1 to the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

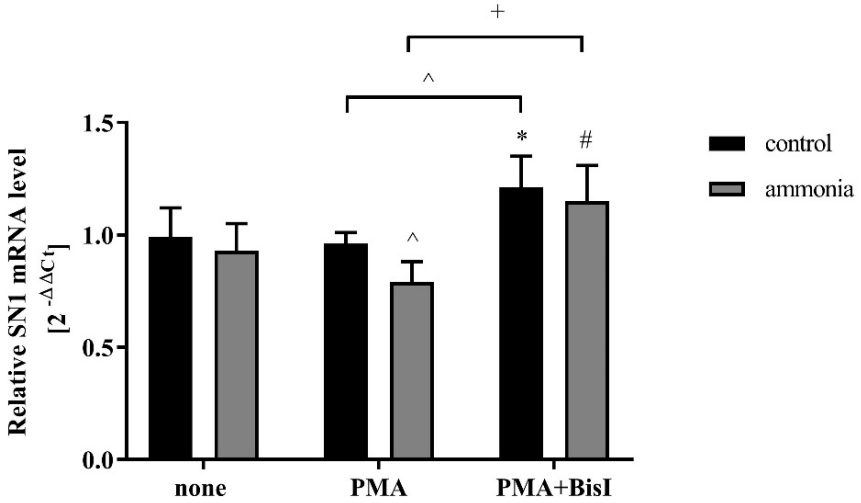

Figure 5. The effect of PKC activation on the SN1 mRNA level in mouse cortical astrocytes treated with 5 mM ammonia for 24 h. Results are mean ± SD (n = 4). (*) $p < 0.05$ vs. control none; (#) $p < 0.05$ vs. ammonia none; (^) $p < 0.05$ vs. PMA control; (+) $p < 0.05$ vs. PMA ammonia; Two-Way ANOVA and Bonferroni post-hoc test.

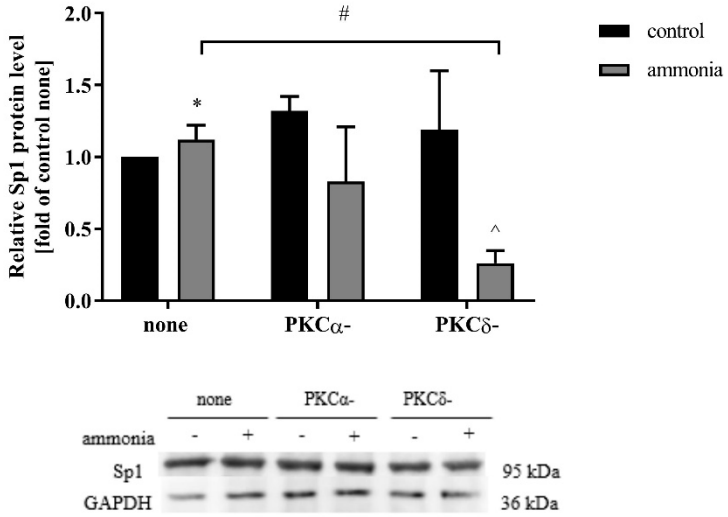

Figure 6. Sp1 protein level in astrocytes treated with 5 mM ammonia for 24 h and after silencing of PKCα (PKCα-) or PKCδ (PKCδ-) isoforms. Results are mean ± SD (n = 4). (*) $p < 0.05$ vs. control none; (#) $p < 0.05$ vs. ammonia none; (^) $p < 0.05$ vs. control PKCδ-. Two-Way ANOVA and Bonferroni post-hoc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

3. Discussion

The present study demonstrates that ammonia increases the Sp1 transcription factor level and its translocation to the astrocytic nucleus (Figure 1), 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 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-day) ammonia exposure (1, 3, and 5 mM) reduced Sp1 mRNA level in rat astroglial cells [33]. The ammonia-induced downregulation of Sp1 mRNA was inversed 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 [33]; however, its cellular localization has not been shown. Since Sp1 activation occurs via its nuclear translocation [33,39–42], the study was extended by the demonstration of ammonia-evoked Sp1 intracellular shift.

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 the increase of Sp1 expression, as has been documented [33], and its activation was further implied as a possible cause of the altered expression of different genes during HE [33,39–42].

Sp1 transcription factor is considered to be involved in the regulation of SN1 transporter in mouse kidneys 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 [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 causes the upregulation of SN1 transcription in acidosis, and Sp1 consensus sequence closest to the transcriptional start-site could be a stress-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 2). 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 [43] 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.

Although the observed changes in mRNA expression were not pronounced, it was plausible that they exert a biologically functionally significant, as it was shown previously [25,44]. Analysis of the inward [^{3}H]glutamine transport in mouse astrocytes fully confirmed functional significance of the SN1 upon mechanism the Sp1 control. Indeed, ammonia decreased the total and system N-mediated [^{3}H]glutamine uptake to 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 [45], where Sp1 dephosphorylation allows hepatocytes to accomplish a more proliferative cell status. In turn, Sp1 up-regulation in differentiated keratinocytes [46], caused mainly by increased level of phosphorylated form of Sp1 protein, was also induced by PKC [47]. Additionally, myelin basic protein transcription was also dependent on Sp1 phosphorylation in differentiated oligodendrocytes [34]. Considering the above evidence, we hypothesized that this mechanism may also contribute to the ammonia-induced Sp1-mediated changes in SN1. Toward the same end, our recent study showed that ammonia reduces PKC activity in cultured mouse cortical astrocytes [25]. In accordance with these findings, 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 4). However, co-treatment of astrocytes with the PKC inhibitor Bis I, turned out to be ineffective in reversing PMA-evoked Sp1 phosphorylation status decrease (Figure 4). The reasons for this apparent discrepancy remain to be elucidated. One other unsolved issue of our study concerns the observed decrease of Sp1 in the absence of PKCδ in ammonia-treated astrocytes. So far, the identification of the regulatory role of one selected PKC isoform has remained beyond the experimental methodology of the present study. It is worth noting that Sp1 phosphorylation could be affected not only by PKC, but also by PKA [36], DNA-dependent protein kinase [48], or CDK [37]. Clearly, the specific involvement of other kinases in ammonia-exposed astrocytes needs further evaluation. Of note in this context, Sp1 transcription factor is also regulated by other posttranslational modifications such as glycosylation and acetylation. Ubiquitous glycosylation by O-linked N-acetylglucosamine (O-GlcNAc) is considered to be analogous to phosphorylation signaling modification [49]. Sp1 glycosylation by O-GlcNAc occurs at Ser484 and translocates it to the cell nuclei leading to its activation [50,51]. However, Sp1 possess a Gln-rich trans-activating domain that contains a O-GlcNAc epitope and such modification inhibits the interactions of Sp1 with other proteins [50,52]. Moreover, acetylated on Lys703 form of Sp1 is suggested to be involved in the gene regulation by decreasing the DNA binding activity or protein interactions [53,54]. It was shown that incubation of human epidermoid carcinoma cells with PMA for more than 3 h leads to the deacetylation of Sp1 transcription factor [53]. Acetylation is related to the histone activity, which is downregulated by ammonia [43] and also by PMA treatment [53]. Since the level of histone in the cells upon such treatments is low, the acetylation of Sp1 is less likely to occur in our experimental setting than phosphorylation.

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 Purα, which tended to associate with phosphorylated rather than with dephosphorylated Sp1 [55]. 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 [56]. The complex formed, after binding to an ARE1 binding site, directly affects the regulation of Sp1 and Nrf2 target genes [56].

Our results suggest that the decrease of SN1 mRNA expression in ammonia-treated astrocytes induced by PMA (Figure 5) 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. It is worth noting that recently it was demonstrated that Sp1 is a silencer of megakaryocyte-specific αIIb gene expression and mir-20b in different commercial cell lines [57,58]. 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 [59]. The role of Nrf2 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 uptake in cultured mouse cortical astrocytes. Moreover, the Sp1 silencing promotes SN1 regulation by ammonia. The relevance of the findings for understanding the role of Sp1 in the regulation of SN1 transporter and SN1-mediated Gln uptake in the brain during hyperammonemia in vivo remains to be documented using more native systems.

4. Materials and Methods

4.1. Materials

Plastic Corning Costar cell culture plates and cell culture medium were provided by Sigma-Aldrich (St. Louis, MO, USA), fetal bovine serum (FBS) from Biosera (Nuaillé, France), and antibiotic antimycotic from Gibco (Thermofisher Scientific, Grand Island, NY, USA). All other chemicals used in this study were of the purest grade and available from the commercial sources.

4.2. Astrocyte Cultures and Treatment

Primary cultures of cortical astrocytes were prepared from the 7-day-old C57BL6/J mice obtained from the animal colony of the Mossakowski Medical Research Centre, Polish Academy of Sciences in Warsaw, according to the previously described method [51]. Briefly, isolated cerebral cortex was passed through 80 µM Nitex nylon netting and then 40 µM Nitex nylon netting into Dulbecco's Modified Eagle's Medium (DMEM) containing 20% FBS. The medium was changed twice a week, gradually reducing to 10% FBS. To promote the morpohological differentiation of the cells, dBcAMP was added to the culture medium in the third week of culturing. The cells were grown in the atmosphere of 95% O_2 and 5% CO_2 at 37 °C. All experiments of this study were performed on 3-week-old cells. Astrocytes were treated with 5 mM ammonium chloride ("ammonia", Sigma-Aldrich) [60,61] for 24 h. Moreover, in the experiments analyzing the role of PKC in the Sp1 expression, mature cells were exposed to 200 nM PMA and/or 1 µM BisI for 24 h.

4.3. Sp1 Transcription Factor and PKC Isoforms Silencing

The down-regulation of the Sp1 transcription factor, PKCα and PKCδ isoforms was performed via transfection of astrocytes with a mix of four types of siRNA duplexes. Each siRNA consisted of 21 nucleotides was targeted to a different gene region in order to obtain the most effective silencing. Sense strands used in this study were: (a) for Sp1 silencing: 5′-CAGCACATTTGTCACATCCAA-3′, 5′-CAGATTCTATATTATATATAT-3′, 5′-CCAGGTGATCATGGAACTCAA-3′, 5′-CAGGATGGTTC TGGTCAAATA-3′; (b) for PKCα silencing: 5′-ATGAACTGTTTCAGTCTATAA-3′, 5′-CAGGAGC AAGCACAAGTTCAA-3′, 5′-CAGCTGGTCATTGCTAACATA-3′, 5′-AAGCATTATCTTAGTG

GATGA-3′; and (c) for PKCδ silencing: 5′-CCGATTCAAGGTTTATAACTA-3′, 5′-AGGGAAGACACT GGTACAGAA-3′, 5′-TTGAATGTAGTTATTGAAATA-3′, 5′-CCGGGTGGACACACCACACTA-3′. After washing with phosphate-buffered saline (PBS), three-week-old astrocytes were detached from the plates by trypsinization and then plated at a density of 1.8×10^5 cells per well in six-well plates in 1.5 mL of cell culture medium (DMEM with 10% FBS). Subsequently, astrocytes were transfected with the transfection mixture consisting of 9 µL HiPerfect Transfection Reagent (Qiagen, Hilden, Germany), 4.5 µL siRNA duplexes (2 µM), and 286.5 µL OptiMEM (medium without serum; Gibco, Thermofisher Scientific, Paisley, UK) according to the fast-forward protocol designed for adherent cells provided by the manufacturer. Before drop-wise addition of the transfection mixture to the cells, it was incubated for 30 min in the room temperature to form the complexes between the reagent and siRNA. Transfected cells were cultured in the normal growth conditions for 24 h. The final concentration of siRNA in each well was 5 nM. The specificity of the silencing was checked using "mock" samples (only transfection reagent added) and negative control siRNA "AllStars" (Qiagen, Hilden, Germany) that does not silence any gene. Sp1 transcription factor was also downregulated by 1 h treatment of astrocytes with its inhibitor, mithramycin A, in the concentration of 10 µM.

4.4. Real-Time qPCR Analysis

One microgram of total RNA isolated from astrocytes using TRI Reagent (Sigma-Aldrich) was reverse transcribed using the High-Capacity cDNA Reverse Transcriptase Kit (Applied Biosystems, Warrington, UK). Real-time qPCR analyses were performed on 96-well plates in The Applied Biosystems 7500 Fast Real-Time PCR System using minor groove binder (MGB) Taqman probe assay and purchased from Applied Biosystems primers and probes for SN1, Sp1 and endogenous control β-actin (Mm01230670_m1, Mm00489039_m1 and Mm00607939_s1 respectively). Each reaction of total volume of 10 µL contained 5 µL TaqMan Fast Universal PCR Master Mix (Applied Biosystems), 1.5 µL of cDNA. The real-time PCR reactions were performed in the following conditions: 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 analyses were calculated and expressed according to an equation ($2^{-\Delta\Delta Ct}$) which provides the amount of the target, normalized to an endogenous reference. Ct is a threshold cycle for target amplification [52].

4.5. Protein Isolation and Western Blot

Astrocytes were scraped off from the plates, suspended in 1 mL of PBS, and centrifuged at 1000× *g* for 5 min at 4 °C. Then, pellets were homogenized in a RIPA buffer containing protease inhibitor cocktail (concentration 1:200, Sigma-Aldrich), phosphatase inhibitor cocktail (concentration 1:100, Sigma-Aldrich), and sodium fluoride (50 mM, Fluka, Sigma-Aldrich, Buchs, Switzerland), and centrifuged at 10,000× *g* for 10 min at 4 °C. The collected supernatant was subjected to Western blot analysis. Protein concentration was measured using a Pierce BCA Protein Assay Kit (Thermo Scientific, Thermofischer). Equal amounts of protein (30 µg) were denaturated by boiling in SDS-Page loading buffer for 10 min at 95 °C, separated on a SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membrane. Membranes were then blocked for 1 h in 5% BSA in TBS-T buffer and incubated overnight at 4 °C in 1% BSA in TBS-T buffer with antibodies against SN1 (1:800, ProteinTech, Manchester, U.K.), Sp1 (1:500, Abcam, Cambridge, U.K.), PKCα (1:850, ProteinTech, Manchester, U.K.), PKCδ (1:850, Proteintech, Manchester, U.K.), followed by the 1-h incubation with HRP-conjugated antirabbit IgG (1:3000 for SN1, 1:5000 for Sp1 and 1:4500 for PKC isoforms; Sigma-Aldrich) for detection by Clarity Western ECL Substrate (Bio-Rad Laboratories, Hercules, CA, USA). The first antibodies were stripped off with 0.1 M glycine at pH 2.9, and membranes were incubated for 1 h at room temperature with HRP-conjugated antibody against glycerylaldehyde 3-phosphate dehydrogenase (GAPDH; 1:7500, ProteinTech, Manchester, UK). The chemiluminescent signal acquisition and densitometry analysis were performed using 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 KH_2PO_4, 0.3 mM $MgSO_4$, 11 mM glucose, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$), and pre-incubated for 15 min at 37 °C. Subsequently, the cells were incubated for 4 min at room temperature in the mixture of Krebs buffer containing 0.1 µCi/mL L-[3,4-^{3}H(N)-]glutamine (PerkinElmer, Waltham, MA, USA; specific radioactivity 37 MBq/mL), 0.1 mM unlabelled Gln. In the experiments analyzing system N-mediated Gln uptake the mixture contained also 10 mM L-Ala and 10 mM L-Leu to block other Gln transporter systems [24]. The incubation was terminated by adding cold Krebs buffer with subsequent two washes using this buffer. To lyse the cells, 0.5 mL of 1 M NaOH was added and the radioactivity of cell lysates was measured in a Wallac 1409 Liquid Scintillation Counter (Perkin-Elmer, Turku, Finland).

4.7. Chromatin Immunoprecipitation

A total of 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 via the addition of glycine in a final concentration of 0.125 M 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 pellet was dissolved in a 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). Fifty microliters 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, U.K.), histone H3 (Cell Signaling, Leiden, The Netherlands), or control IgG (Cell Signaling, Leiden, The Netherlands). After 1-h 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 $NaHCO_3$, 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 h 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 a phenol:chloroform:isoamylalcohol solution (Sigma-Aldrich) 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 s 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 the *Snat3* promoter region was normalized versus input as calculated and normalized versus amylase (non-binding region). The primers used in this study were as follows: *Snat3* promoter region (sense strand: 5′-AAACACTTGGAGGGGCTTCT-3′, antisense strand: 5′-CCTCGAAATCGGTGAAGTGT-3′), amylase (sense strand: 5′-CTCCTTGTACGGGTTGTT-3′, antisense strand: 5′-AATGATGTGCACAGCTGAA-3′).

4.8. 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) for 1 h. 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-h incubation with goat anti-rabbit IgG Alexa Fluor 488 (1:500, Invitrogen, Waltham, MA, USA), for 1-h at RT in the dark. The cells were placed on the microscope slides using a 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.

4.9. 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 the Kołmogorow–Smirnow test. Statistical significance was determined using an unpaired Student's *t*-test for two groups, one-way analysis of variance (one-way ANOVA), followed by a Dunnet post-hoc test and two-way analysis of variance (two-way ANOVA), followed by a Bonferroni post-hoc test. A probability value of 0.05 or less was considered statistically significant.

Supplementary Materials: It can be found at http://www.mdpi.com/1422-0067/20/2/234/s1.

Author Contributions: M.Z. and K.D. conceived and designed the experiments; K.D. performed the experiments and analyzed the data; K.D. contributed reagents/materials/analysis tools; K.D. and M.Z. wrote the paper.

Funding: This study was supported by the National Science Centre grant: 2016/21/N/NZ4/03759.

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

Abbreviations

BisI	bisindolylmaleimide I
CDK	cyclin-dependent kinase
ChIP	chromatin immunoprecipitation
CNS	central nervous system
dBcAMP	dibutyryl cyclic adenosine monophosphate
ENU	N-ethyl-N-nitrosourea
ERK	extracellular signal-regulated kinases
GABA	γ-aminobutyric acid
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
Gln	L-glutamine
Glu	L-glutamate
HE	hepatic encephalopathy
MAPK	mitogen-activated protein kinases
O-GlcNAc	O-linked N-acetylglucosamine
PKA	protein kinase A
PKC	protein kinase C
PKCα-	silenced expression of protein kinase C isoform α
PKCδ-	silenced expression of protein kinase C isoform δ
PMA	phorbol 12-myristate 13-acetate
SN1	SNAT3, solute carrier family 38 member 3
SN2	SNAT5, solute carrier family 38 member 5
Sp1	specificity protein 1

References

1. Albrecht, J.; Sonnewald, U.; Waagepetersen, H.S.; Schousboe, A. Glutamine in the central nervous system: Function and dysfunction. *Front. Biosci.* **2007**, *12*, 332–343. [CrossRef] [PubMed]
2. Cynober, L. Plasma amino acid levels with a note on membrane transport: Characteristics, regulation, and metabolic significance. *Nutrition* **2002**, *18*, 761–766. [CrossRef]
3. Pithon-Curi, T.C.; Trezena, A.G.; Tavares-Lima, W.; Curi, R. Evidence that glutamine is involved in neutrophil function. *Cell Biochem. Funct.* **2002**, *20*, 81–86. [CrossRef] [PubMed]
4. Albrecht, J.; Sidoryk-Węgrzynowicz, M.; Zielińska, M.; Aschner, M. Roles of glutamine in neurotransmission. *Neuron Glia Biol.* **2010**, *6*, 263–276. [CrossRef] [PubMed]
5. Laake, J.H.; Slyngstad, T.A.; Haug, F.M.; Ottersen, O.P. Glutamine from glial cells is essential for the maintenance of the nerve terminal pool of glutamate: Immunogold evidence from hippocampal slice cultures. *Neuroscience* **1999**, *88*, 1137–1151. [CrossRef]
6. Suárez, I.; Bodega, G.; Fernández, B. Glutamine synthetase in brain: Effect of ammonia. *Neurochem. Int.* **2002**, *41*, 123–142. [CrossRef]
7. Waniewski, R.A.; Martin, D.L. Exogenous glutamate is metabolized to glutamine and exported by rat primary astrocyte cultures. *J. Neurochem.* **1986**, *47*, 304–313. [CrossRef]
8. Lavoie, J.; Giguere, J.F.; Layrargues, G.P.; Butterworth, R.F. Amino acid changes in autopsied brain tissue from cirrhotic patients with hepatic encephalopathy. *J. Neurochem.* **1987**, *49*, 692–697. [CrossRef]
9. Ratnakumari, L.; Qureshi, I.A.; Butterworth, R.F. Regional amino acid neurotransmitter changes in brains of Spf/Y mice with congenital ornithine transcarbamylase deficiency. *Metab. Brain Dis.* **1994**, *9*, 43–51. [CrossRef]
10. Butterworth, R.F. Pathophysiology of brain dysfunction in hyperammonemic syndromes: The many faces of glutamine. *Mol. Genet. Metab.* **2014**, *113*, 113–117. [CrossRef]
11. Tofteng, F.; Hauerberg, J.; Hansen, B.A.; Pedersen, C.B.; Jørgensen, L.; Larsen, F.S. Persistent arterial hyperammonemia increases the concentration of glutamine and alanine in the brain and correlates with intracranial pressure in patients with fulminant hepatic failure. *J. Cereb. Blood Flow Metab.* **2006**, *26*, 21–27. [CrossRef] [PubMed]
12. Albrecht, J.; Zielińska, M.; Norenberg, M.D. Glutamine as a mediator of ammonia neurotoxicity: A critical appraisal. *Biochem. Pharmacol.* **2010**, *80*, 1303–1308. [CrossRef] [PubMed]
13. Zielińska, M.; Dąbrowska, K.; Hadera, M.G.; Sonnewald, U.; Albrecht, J. System N transporters are critical for glutamine release and modulate metabolic fluxes of glucose and acetate in cultured cortical astrocytes: Changes induced by ammonia. *J. Neurochem.* **2016**, *2*, 329–338. [CrossRef] [PubMed]
14. Pichili, V.B.; Rao, K.V.; Jayakumar, A.R.; Norenberg, M.D. Inhibition of glutamine ransport into mitochondria protects astrocytes from ammonia toxicity. *Glia* **2007**, *55*, 801–809. [CrossRef] [PubMed]
15. Ziemińska, E.; Dolińska, M.; Lazarewicz, J.W.; Albrecht, J. Induction of permeability transition and swelling of rat brain mitochondria by glutamine. *Neurotoxicology* **2000**, *21*, 295–300. [PubMed]
16. Bai, G.; Rama Rao, C.R.K.; Norenberg, M.D. Ammonia induces the mitochondrial permeability transition in primary cultures of astrocytes. *J. Neurosci. Res.* **2001**, *66*, 981–991. [CrossRef] [PubMed]
17. Kruczek, C.; Görg, B.; Keitel, V.; Bidmon, H.J.; Schliess, F.; Häussinger, D. Ammonia increases nitric oxide, free Zn^{2+}, and metallothionein mRNA expression in cultured rat astrocytes. *Biol. Chem.* **2011**, *392*, 1155–1165. [CrossRef]
18. Sinke, A.P.; Jayakumar, A.R.; Panickar, K.S.; Moriyama, M.; Reddy, P.V.; Norenberg, M.D. NFkappaB in the mechanism of ammonia-induced astrocyte swelling in culture. *J. Neurochem.* **2008**, *106*, 2302–2311. [CrossRef]
19. Blei, A.T.; Larsen, F.S. Pathophysiology of cerebral edema in fulminant hepatic failure. *J. Hepatol.* **1999**, *31*, 771–776. [CrossRef]
20. Häussinger, D.; Kircheis, G.; Fischer, R.; Schliess, F.; vom Dahl, S. Hepatic encephalopathy in chronic liver disease: A clinical manifestation of astrocyte swelling and low-grade cerebral edema? *J. Hepatol.* **2000**, *32*, 1035–1038. [CrossRef]
21. Jayakumar, A.R.; Panickar, K.S.; Murthy, Ch.R.K.; Norenberg, M.D. Oxidative stress and MAPK phosphorylation mediate ammonia-induced cell swelling and glutamate uptake inhibition in cultured astrocytes. *J. Neurosci.* **2006**, *26*, 4774–4784. [CrossRef] [PubMed]

22. Roux, P.P.; Blenis, J. ERK and p38 MAPK-activated protein kinases: A family of protein kinases with diverse biological functions. *Microbiol. Mol. Biol. Rev.* **2004**, *68*, 320–344. [CrossRef] [PubMed]
23. Pearson, G.; Robinson, F.; Beers Gibson, T.; Xu, B.E.; Karandikar, M.; Berman, K.; Cobb, M.H. Mitogen-activated protein (MAP) kinase pathways: Regulation and physiological functions. *Endocrinol. Rev.* **2001**, *22*, 153–183. [CrossRef]
24. Bröer, A.; Deitmer, J.W.; Bröer, S. Astroglial glutamine transport by system N is upregulated by glutamate. *GLIA* **2004**, *48*, 298–310. [CrossRef] [PubMed]
25. Dąbrowska, K.; Albrecht, J.; Zielińska, M. Protein kinase C-mediated impairment of glutamine outward transport and SN1 transporter distribution by ammonia in mouse cortical astrocytes. *Neurochem. Int.* **2018**, *118*, 225–232. [CrossRef] [PubMed]
26. Chan, K.; Busque, S.M.; Sailer, M.; Stoeger, C.; Bröer, S.; Daniel, H.; Rubio-Aliaga, I.; Wagner, C.A. Loss of function mutation of the Slc38a3 glutamine transporter reveals its critical role for amino acid metabolism in the liver, brain, and kidney. *Pflugers Arch.* **2016**, *468*, 213–227. [CrossRef] [PubMed]
27. Boulland, J.L.; Osen, K.K.; Levy, L.M.; Danbolt, N.C.; Edwards, R.H.; Storm-Mathisen, J.; Chaudhry, F.A. Cell-specific expression of the glutamine transporter SN1 suggests differences in dependence on the glutamine cycle. *Eur. J. Neurosci.* **2002**, *15*, 1615–1631. [CrossRef] [PubMed]
28. Balkrishna, S.; Bröer, A.; Welford, S.M.; Hatzoglou, M.; Bröer, S. Expression of glutamine transporter Slc38a3 (SNAT3) during acidosis is mediated by a different mechanism than tissue-specific expression. *Cell. Physiol. Biochem.* **2014**, *33*, 1591–1606. [CrossRef] [PubMed]
29. Solbu, T.T.; Boulland, J.L.; Zahid, W.; Lyamouri Bredahl, M.K.; Amiry-Moghaddam, M.; Storm-Mathisen, J.; Roberg, B.A.; Chaudhry, F.A. Induction and targeting of the glutamine transporter SN1 to the basolateral membranes of cortical kidney tubule cells during chronic metabolic acidosis suggest a role in pH regulation. *J. Am. Soc. Nephrol.* **2005**, *16*, 869–877. [CrossRef] [PubMed]
30. Black, A.R.; Black, J.D.; Azizkhan-Clifford, J. Sp1 and krüppel-like factor family of transcription factors in cell growth regulation and cancer. *J. Cell. Physiol.* **2001**, *188*, 143–160. [CrossRef]
31. Philipsen, S.; Suske, G. A tale of three fingers: The family of mammalian Sp/XKLF transcription factors. *Nucleic Acids Res.* **1999**, *27*, 2991–3000. [CrossRef] [PubMed]
32. ENCODE Project Consortium. A user's guide to the encyclopedia of DNA elements (ENCODE). *PLoS Biol.* **2011**, *9*, e1001046.
33. Bodega, G.; Suarez, I.; Lopez-Fernandez, L.A.; Almonacid, L.; Zaballos, A.; Fernandez, B. Possible implication of ciliary neurotrophic factor (CNTF) and b-synuclein in the ammonia effect on cultured rat astroglial cells: A study using DNA and protein microarrays. *Neurochem. Int.* **2006**, *48*, 729–738. [CrossRef] [PubMed]
34. Guo, L.; Eviatar-Ribak, T.; Miskimins, R. Sp1 phosphorylation is involved in myelin basic protein gene transcription. *J. Neurosci. Res.* **2010**, *88*, 3233–3242. [CrossRef] [PubMed]
35. Tan, N.Y.; Midgley, V.C.; Kavurma, M.M.; Santiago, F.S.; Luo, X.; Peden, R.; Fahmy, R.G.; Berndt, M.C.; Molloy, M.P.; Khachigian, L.M. Angiotensin II-inducible platelet-derived growth factor-D transcription requires specific Ser/Thr residues in the second zinc finger region of Sp1. *Circ. Res.* **2008**, *102*, e38–e51. [CrossRef] [PubMed]
36. Rohlff, C.; Ahmad, S.; Borellini, F.; Lei, J.; Glazer, R.I. Modulation of transcription factor Sp1 by cAMP-dependent protein kinase. *J. Biol. Chem.* **1997**, *272*, 21137–21141. [CrossRef] [PubMed]
37. Fojas de Borja, P.; Collins, N.K.; Du, P.; Azizkhan-Clifford, J.; Mudryj, M. Cyclin A-CDK phosphorylates Sp1 and enhances Sp1-mediated transcription. *EMBO J.* **2001**, *20*, 5737–5747. [CrossRef]
38. Milanini-Mongiat, J.; Pouysségur, J.; Pagès, G. Identification of two Sp1 phosphorylation sites for p42/p44 mitogen-activated protein kinases: Their implication in vascular endothelial growth factor gene transcription. *J. Biol. Chem.* **2002**, *277*, 20631–20639. [CrossRef]
39. Lichter-Konecki, U.; Mangin, J.M.; Gordish-Dressman, H.; Hoffman, E.P.; Gallo, V. Gene expression profiling of astrocytes from hyperammonemic mice reveals altered pathways for water and potassium homeostasis in vivo. *GLIA* **2008**, *56*, 365–377. [CrossRef]
40. Song, G.; Dhodda, V.K.; Blei, A.T.; Dempsey, R.J.; Rao, V.L.R. GeneChip analysis shows altered mRNA expression of transcripts of neurotransmitter and signal transduction pathways in the cerebral cortex of portacaval shunted rats. *J. Neurosci. Res.* **2002**, *68*, 730–737. [CrossRef]

41. Warskulat, U.; Görg, B.; Bidmon, H.J.; Müller, H.W.; Schliess, F.; Häussinger, D. Ammonia-induced heme oxygenase 1 expression in cultured rat astrocytes and in rat brain in vivo. *GLIA* **2002**, *40*, 324–336. [CrossRef] [PubMed]
42. Warskulat, U.; Kreuels, S.; Müller, H.W.; Häussinger, D. Identification of osmosensitive and ammonia-regulated genes in rat astrocytes by northern blotting and differential display reverse transcriptase-polymerase chain reaction. *J. Hepatol.* **2001**, *35*, 358–366. [CrossRef]
43. Bodega, G.; Segura, B.; Ciordia, S.; Mena Mdel, C.; López-Fernández, L.A.; García, M.I.; Trabado, I.; Suárez, I. Ammonia Affects Astroglial Proliferation in Culture. *PLoS ONE* **2015**, *10*, e0139619. [CrossRef] [PubMed]
44. Sidoryk-Wegrzynowicz, M.; Lee, E.; Mingwei, N.; Aschner, M. Disruption of astrocytic glutamine turnover by manganese is mediated by the protein kinase C pathway. *GLIA* **2011**, *59*, 1732–1743. [CrossRef] [PubMed]
45. Leggett, R.W.; Armstrong, S.A.; Barry, D.; Mueller, C.R. Sp1 is phosphorylated and its DNA binding activity down-regulated upon terminal differentiation of the liver. *J. Biol. Chem.* **1995**, *270*, 25879–25884. [CrossRef] [PubMed]
46. Apt, D.; Watts, R.M.; Suske, G.; Bernard, H.U. High Sp1/Sp3 ratios in epithelial cells during epithelial differentiation and cellular transformation correlate with the activation of the HPV-16 promoter. *Virology* **1996**, *224*, 281–291. [CrossRef] [PubMed]
47. You, H.L.; Eng, H.L.; Hsu, S.F.; Chen, C.M.; Ye, T.C.; Liao, W.T.; Huang, M.Y.; Baer, R.; Cheng, J.T. A PKC-Sp1 signaling pathway induces early differentiation of human keratinocytes through upregulation of TSG101. *Cell. Signal.* **2007**, *19*, 1201–1211. [CrossRef]
48. Gottlieb, T.M.; Jackson, S.P. The DNA-dependent protein kinase: Requirement for DNA ends and association with Ku antigen. *Cell* **1993**, *72*, 131–142. [CrossRef]
49. Suh, H.N.; Lee, Y.J.; Kim, M.O.; Ryu, J.M.; Han, H.J. Glucosamine-induced Sp1 O-GlcNAcylation ameliorates hypoxia-induced SGLT dysfunction in primary cultured renal proximal tubule cells. *J. Cell. Physiol.* **2014**, *229*, 1557–1568. [CrossRef] [PubMed]
50. Banerjee, S.; Sangwan, V.; McGinn, O.; Chugh, R.; Dudeja, V.; Vickers, S.M.; Saluja, A.K. Triptolide-induced cell death in pancreatic cancer is mediated by O-GlcNAc modification of transcription factor Sp1. *J. Biol. Chem.* **2013**, *288*, 33927–33938. [CrossRef]
51. Du, X.L.; Edelstein, D.; Rossetti, L.; Fantus, I.G.; Goldberg, H.; Ziyadeh, F.; Wu, J.; Brownlee, M. Hyperglycemia-induced mitochondrial superoxide overproduction activates the hexosamine pathway and induces plasminogen activator inhibitor-1 expression by increasing Sp1 glycosylation. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 12222–12226. [CrossRef] [PubMed]
52. Roos, M.D.; Su, K.; Baker, J.R.; Kudlow, J.E. O glycosylation of an Sp1-derived peptide blocks known Sp1 protein interactions. *Mol. Cell. Biol.* **1997**, *17*, 6472–6480. [CrossRef] [PubMed]
53. Hung, J.J.; Wang, Y.T.; Chang, W.C. Sp1 deacetylation induced by phorbol ester recruits p300 to activate 12(S)-lipoxygenase gene transcription. *Mol. Cell. Biol.* **2006**, *26*, 1770–1785. [CrossRef]
54. Waby, J.S.; Chirakkal, H.; Yu, C.; Griffiths, G.J.; Benson, R.S.; Bingle, C.D.; Corfe, B.M. Sp1 acetylation is associated with loss of DNA binding at promoters associated with cell cycle arrest and cell death in a colon cell line. *Mol. Cancer* **2010**, *9*, 275. [CrossRef] [PubMed]
55. Tretiakova, A.; Steplewski, A.; Johnson, E.M.; Khalili, K.; Amini, S. Regulation of myelin basic protein gene transcription by Sp1 and Puralpha: Evidence for association of Sp1 and Puralpha in brain. *J. Cell. Physiol.* **1999**, *181*, 160–168. [CrossRef]
56. Chi, P.L.; Lin, C.C.; Chen, Y.W.; Hsiao, L.D.; Yang, C.M. CO Induces Nrf2-Dependent Heme Oxygenase-1 Transcription by Cooperating with Sp1 and c-Jun in Rat Brain Astrocytes. *Mol. Neurobiol.* **2015**, *52*, 277–292. [CrossRef]
57. Guo, S.; Wang, H.; Luo, Y.; He, J.; Ma, H.; Shen, L.; Tang, J.; Fang, Q.; Wang, H.; Qian, Z.; et al. Sp-1 Negatively Regulates miR-20b Expression in Macrophages. *Ann. Clin. Lab. Sci.* **2017**, *47*, 706–712. [PubMed]
58. Shou, Y.; Baron, S.; Poncz, M. An Sp1-binding Silencer Element Is a Critical Negative Regulator of the Megakaryocyte-specific αIIb Gene. *J. Biol. Chem.* **1998**, *273*, 5716–5726. [CrossRef] [PubMed]
59. Lister, A.; Bourgeois, S.; Imenez Silva, P.H.; Rubio-Aliaga, I.; Marbet, P.; Walsh, J.; Shelton, L.M.; Keller, B.; Verrey, F.; Devuyst, O.; et al. NRF2 regulates the glutamine transporter Slc38a3 (SNAT3) in kidney in response to metabolic acidosis. *Sci. Rep.* **2018**, *8*, 5629. [CrossRef]

60. Butterworth, R.F. Pathophysiology of hepatic encephalopathy: A new look at ammonia. *Metab. Brain Dis.* **2002**, *17*, 221–227. [CrossRef]
61. Norenberg, M.D.; Baker, L.; Norenberg, L.O.; Blicharska, J.; Bruce-Gregorios, J.H.; Neary, J.T. Ammonia-induced astrocyte swelling in primary culture. *Neurochem. Res.* **1991**, *16*, 833–836. [CrossRef] [PubMed]

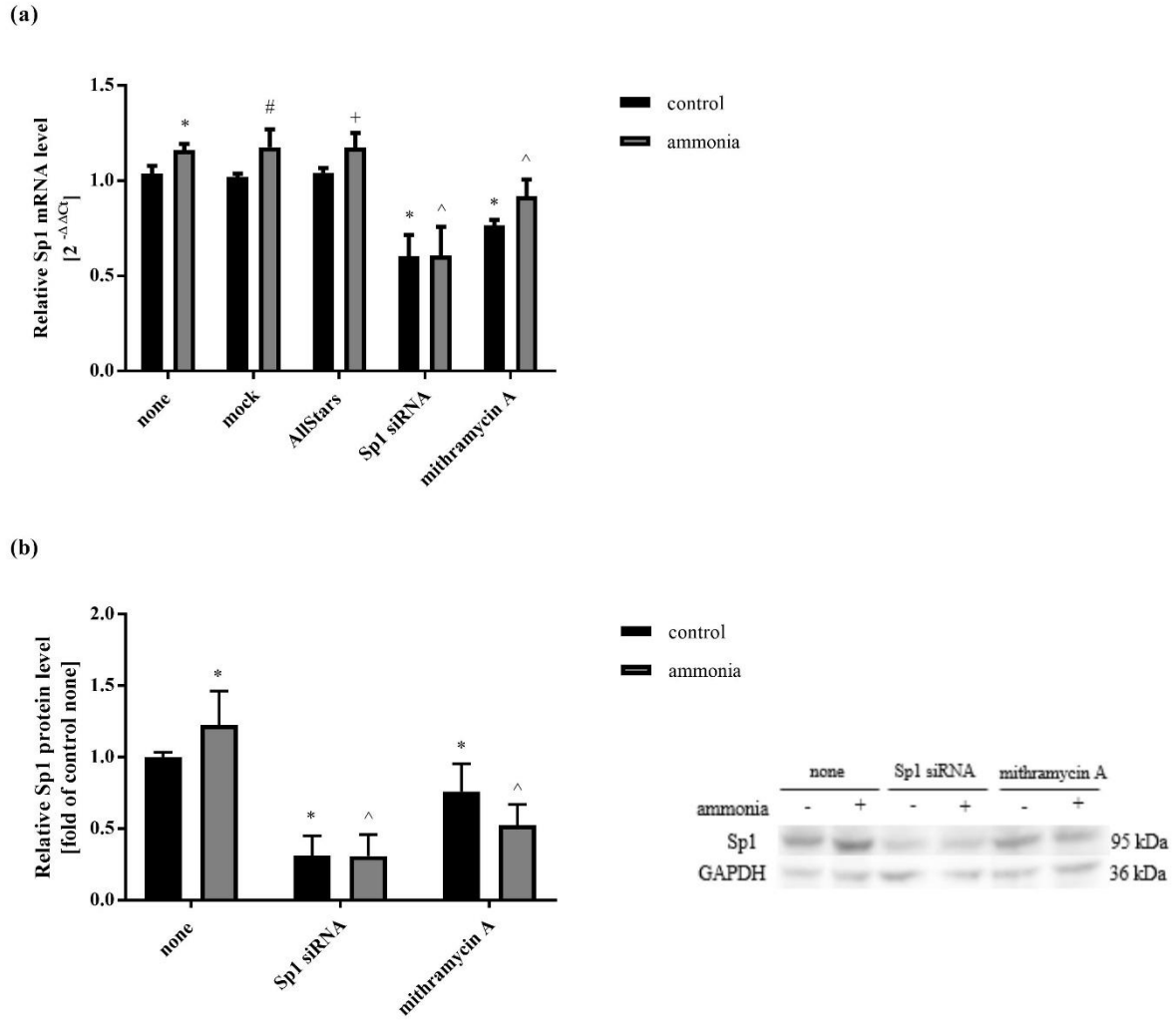

Figure S1. The effectiveness of Sp1 silencing by 5 nM siRNA and 10 mM mithramycin A. (**a**) Sp1 mRNA level. Mock is the transfection only with the transfection reagent; All Stars is a transfection with siRNA that does not silence any gene. Results are mean ± SD (n = 4). (*) $p < 0.05$ vs control none, (^) $p < 0.05$ vs ammonia none, (#) $p < 0.05$ vs control mock, (+) $p < 0.05$ vs control AllStars negative control; Two-Way ANOVA, Bonferroni post-hoc test; (**b**) Sp1 protein level. Left panel shows densitometry, right panel shows the representative immunoblots. 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.

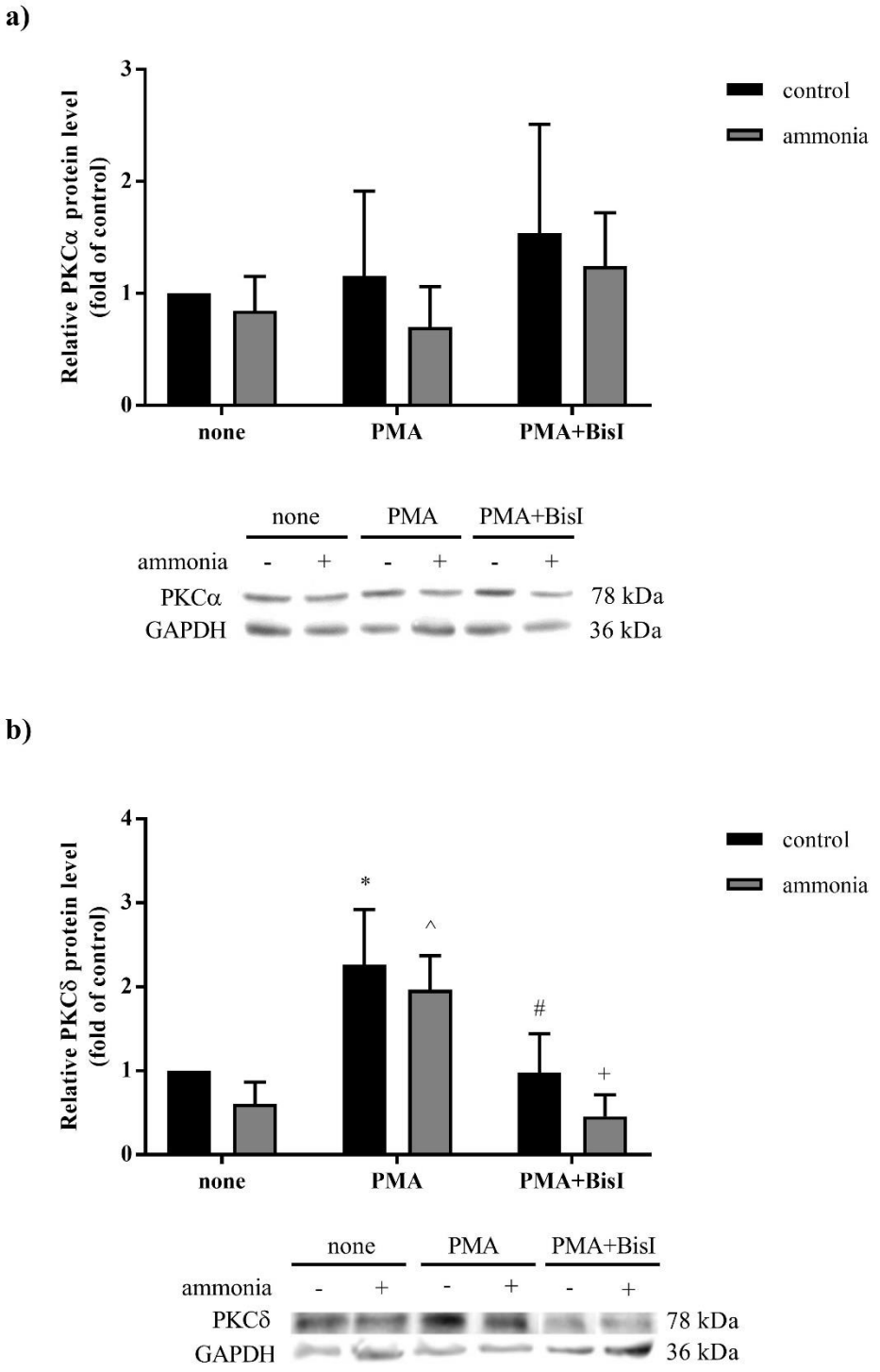

Figure S2. PKC isoforms α (**a**) and δ (**b**) protein level after 24-hour treatment with 200 nM PMA and pre-treatment with 1 mM BisI followed by 24-hour treatment with 200 nM PMA. Upper panel shows densitometry, lower panel shows the representative immunoblots Results are mean ± SD (n = 4). (*) $p < 0.05$ vs control none; (^) $p < 0.05$ vs ammonia none; (#) $p < 0.05$ vs control PMA; (+) $p < 0.05$ vs ammonia PMA; Two-Way ANOVA, Bonferroni post-hoc test.

9. OŚWIADCZENIA

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IMDiK PAN

Warszawa, 30.11.2018

OŚWIADCZENIE

Oświadczam, że mój wkład w powstanie publikacji wchodzących w skład rozprawy doktorskiej:

1. Dąbrowska K, Skowrońska K, Popek M, Obara-Michlewska M, Albrecht J, Zielińska M.; *Roles of Glutamate and Glutamine Transport in Ammonia Neurotoxicity: State of the Art and Question Mark*; Endocr Metab Immune Disord Drug Targets. 2018; 18(4):306-315. doi: 10.2174/1871520618666171219124427
2. Dąbrowska K, Albrecht J, Zielińska M.; *Protein kinase C-mediated impairment of glutamine outward transport and SN1 transporter distribution by ammonia in mouse cortical astrocytes*; Neurochem Int. 2018; 118:225-232. doi: 10.1016/j.neuint.2018.07.001
3. Dąbrowska K, Zielińska M.; *Silencing of Transcription Factor Sp1 Promotes SN1 Transporter Regulation by Ammonia in Mouse Cortical Astrocytes*; Int J Mol Sci. 2019; 20(2):234. doi:10.3390/ijms20020234

polegał na wykonaniu badań, ich analizy statystycznej, przygotowaniu 8tekstów manuskryptów opisujących metodykę i uzyskane wyniki oraz opracowaniu części wstępów i dyskusji.

dr hab. Magdalena Zielińska

Kierownik Zakładu Neurotoksykologii

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Warszawa, 30.11.2018

OŚWIADCZENIE

Oświadczam, że jestem współautorem poniższych publikacji wchodzących w skład rozprawy doktorskiej mgr inż. Katarzyny Anny Dąbrowskiej:

1. Dąbrowska K, Skowrońska K, Popek M, Obara-Michlewska M, Albrecht J, Zielińska M.; *Roles of Glutamate and Glutamine Transport in Ammonia Neurotoxicity: State of the Art and Question Mark*; Endocr Metab Immune Disord Drug Targets. 2018; 18(4):306-315. doi: 10.2174/1871520618666171219124427
2. Dąbrowska K, Albrecht J, Zielińska M.; *Protein kinase C-mediated impairment of glutamine outward transport and SN1 transporter distribution by ammonia in mouse cortical astrocytes*; Neurochem Int. 2018; 118:225-232. doi: 10.1016/j.neuint.2018.07.001
3. Dąbrowska K, Zielińska M.; *Silencing of Transcription Factor Sp1 Promotes SN1 Transporter Regulation by Ammonia in Mouse Cortical Astrocytes;* Int J Mol Sci.; 2019; 20(2):234. doi:10.3390/ijms20020234

Mój wkład w ich powstanie polegał na udziale w tworzeniu koncepcji badań i założeń merytorycznych publikacji, nadzorowaniu wykonania badań oraz opracowaniu części dyskusji.

Wyrażam zgodę na wykorzystanie wyżej wymienionych publikacji w przewodzie doktorskim mgr inż. Katarzyny Anny Dąbrowskiej.

Z poważaniem,

Zielińska

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Warszawa, 30.11.2018

OŚWIADCZENIE

Oświadczam, że jestem współautorem poniższych publikacji wchodzących w skład rozprawy doktorskiej mgr inż. Katarzyny Anny Dąbrowskiej:

1. Dąbrowska K, Skowrońska K, Popek M, Obara-Michlewska M, Albrecht J, Zielińska M.; *Roles of Glutamate and Glutamine Transport in Ammonia Neurotoxicity: State of the Art and Question Mark*; Endocr Metab Immune Disord Drug Targets. 2018; 18(4):306-315. doi: 10.2174/1871520618666171219124427
2. Dąbrowska K, Albrecht J, Zielińska M.; *Protein kinase C-mediated impairment of glutamine outward transport and SN1 transporter distribution by ammonia in mouse cortical astrocytes*; Neurochem Int. 2018; 118:225-232. doi: 10.1016/j.neuint.2018.07.001

Mój wkład w ich powstanie polegał na udziale w opracowaniu części dyskusji oraz pomocy merytorycznej w przygotowaniu powyższych prac.

Wyrażam zgodę na wykorzystanie wyżej wymienionych publikacji w przewodzie doktorskim mgr inż. Katarzyny Anny Dąbrowskiej.

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Warszawa, 30.11.2018

OŚWIADCZENIE

Oświadczam, że jestem współautorem publikacji, Dąbrowska K, Skowrońska K, Popek M, Obara-Michlewska M, Albrecht J, Zielińska M.; *Roles of Glutamate and Glutamine Transport in Ammonia Neurotoxicity: State of the Art and Question Mark*; Endocr Metab Immune Disord Drug Targets. 2018; 18(4):306-315. doi: 10.2174/1871520618666171219124427, wchodzącej w skład rozprawy doktorskiej mgr inż. Katarzyny Anny Dąbrowskiej, a mój udział polegał na wykonaniu doświadczeń i analizie statystycznej ich wyników przedstawionych na Ryc.1d.

Wyrażam zgodę na wykorzystanie wyżej wymienionej publikacji w przewodzie doktorskim mgr inż. Katarzyny Anny Dąbrowskiej.

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OŚWIADCZENIE

Oświadczam, że jestem współautorem publikacji: Dąbrowska K, Skowrońska K, Popek M, Obara-Michlewska M, Albrecht J, Zielińska M.; *Roles of Glutamate and Glutamine Transport in Ammonia Neurotoxicity: State of the Art and Question Mark*; Endocr Metab Immune Disord Drug Targets. 2018; 18(4):306-315. doi: 10.2174/1871520618666171219124427, wchodzącej w skład rozprawy doktorskiej mgr inż. Katarzyny Anny Dąbrowskiej, a mój udział polegał na wykonaniu doświadczeń i analizie statystycznej ich wyników przedstawionych na Ryc.1e.

Wyrażam zgodę na wykorzystanie wyżej wymienionej publikacji w przewodzie doktorskim mgr inż. Katarzyny Anny Dąbrowskiej.

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Warszawa, 30.11.2018

OŚWIADCZENIE

Oświadczam, że jestem współautorem publikacji: Dąbrowska K, Skowrońska K, Popek M, Obara-Michlewska M, Albrecht J, Zielińska M.; *Roles of Glutamate and Glutamine Transport in Ammonia Neurotoxicity: State of the Art and Question Mark*; Endocr Metab Immune Disord Drug Targets. 2018; 18(4):306-315. doi: 10.2174/1871520618666171219124427, wchodzącej w skład rozprawy doktorskiej mgr inż. Katarzyny Anny Dąbrowskiej, a mój udział polegał na wykonaniu doświadczeń i analizie statystycznej ich wyników przedstawionych na Ryc.1a,b.

Wyrażam zgodę na wykorzystanie wyżej wymienionej publikacji w przewodzie doktorskim mgr inż. Katarzyny Anny Dąbrowskiej.

Skowrońska Katarzyna

Grafika na okładce: na podstawie Cancer Research UK / Wikimedia Commons

(https://commons.wikimedia.org/wiki/File:Diagram_of_an_astrocyte_-_a_type_of_glial_cell_CRUK_029.svg)