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Localization, Occurrence, and CSF Changes of SP-G, a New Surface Active Protein with Assumable Immunoregulatory Functions in the CNS

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Abstract

Conventional surfactant proteins (A, B, C, and D) are important players of the innate immunity in the central nervous system and serve as effective regulators of cerebrospinal fluid rheology, probably being involved in clearance of detrimental metabolites like beta-amyloid and phospho-tau. Recently, a novel surfactant protein, SP-G, was described in kidneys and peripheral endocrine and exocrine glands. So far, its presence and possible functions in the central nervous system are unknown. Therefore, our study aimed to elucidate the presence of SP-G in the brain and its concentration in normal and pathologic samples of cerebrospinal fluid in order to gain first insight into its regulation and possible functions. A total of 121 samples of human cerebrospinal fluid (30 controls, 60 hydrocephalus patients, 7 central nervous system infections, and 24 brain hemorrhage patients) and 21 rat brains were included in our study. CSF samples were quantified using a commercially available ELISA system. Results were analyzed statistically using SPSS 22, performing Spearman Rho correlation and ANOVA with Dunnett's post hoc analysis. Rat brains were investigated via immunofluorescence to determine SP-G presence and colocalization with common markers like aquaporin-4, glial fibrillary acidic protein, platelet endothelial adhesion molecule 1, and neuronal nuclear antigen. SP-G occurs associated with brain vessels, comparable to other conventional SPs, and is present in a set of cortical neurons. SP-G is furthermore actively produced by ependymal and choroid plexus epithelium and secreted into the cerebrospinal fluid. Its concentrations are low in control subjects and patients suffering from aqueductal stenosis, higher in normal pressure hydrocephalus ($p < 0.01$), and highest in infections of the central nervous system and brain hemorrhage ($p < 0.001$). Interestingly, SP-G did correlate with total CSF protein in patients with CNS infections and hemorrhage, but not with cell count. Based on the changes in CSF levels of SP-G in hydrocephalus, brain hemorrhage, and CNS infections as well as its abundance at CSF flow-related anatomical structures closely associated with immunological barrier systems, importance for CSF rheology, brain waste clearance, and host defense is assumable. Thus, SP-G is a potential new CSF biomarker, possibly not only reflecting aspects of CNS innate immune responses, but also rheo-dynamically relevant changes of CSF composition, associated with CSF malabsorbtion. However, further studies are warranted to validate our findings and increase insight into the physiological importance of SP-G in the CNS.

Keywords Surfactant protein G · CNS · Hydrocephalus · Brain · Cerebral surfactant system

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Introduction

Surfactant proteins (SPs) were reported to be inherent proteins of the CNS [1], They appear at different time points during development in tissue-specific clusters [2]. In the mature CNS, SP-A and SP-D show a similar distribution in the central nervous system (CNS), which is different compared with the distinct expression pattern of SP-B and SP-C. The mainly rheologically active SP-B and SP-C are predominantly present in choroid plexus and ependymal cells of brain and spinal canal, representing the major sites of CSF formation and the CSF—tissue interface, although SP-C also is characterized by a strong perivascular presence. This specific expression pattern indicates importance for CSF rheology and a possible role of SPs for brain waste clearance via the glymphatic pathway [3, 4]. The collectins SP-A and SP-D were found at the sites of the blood-brain and the blood-CSF barrier, respectively [1, 2]. The localization of both antibody-equivalent molecules [5] at the barrier sites of the CNS suggests their significance for CNS immunity. Furthermore, SP-A, SP-C, and SP-D showed significantly altered CSF profiles in different hydrocephalic conditions [6] which were closely linked to the extent of ventricular enlargement [7]. Also, their importance as molecular regulators of CSF flow has been demonstrated [4].

The novel surfactant protein SP-G (surfactant associated 2, SFTA2) has been described and detected as rheologically active protein within kidneys, lacrimal glands, testes, and salivary glands [8]. It was submitted to the NCBI Gene Bank by M.G. Walker and P. Spiro in 2002 (Accession AY102070) and described as "co-expressed with pulmonary surfactants".

In the context of the great physiological importance of conventional SPs, the first aim of the present study was to investigate the concentrations of SP-G in human CSF of normal subjects, different hydrocephalic conditions, brain hemorrhage, and CNS infection in order to gain more insight into its physiological significance. Secondly, our study aimed to evaluate the spatial expression pattern of SP-G in rat brains and its colocalization with functionally important CNS markers. Thirdly, the study aimed to determine the temporal aspect of SP-G expression during development in fetal rat brains.

Patients and Methods

Patients

CSF specimens of 121 patients were analyzed. All patients or caregivers gave their written informed consent for the scientific use of CSF samples including the analysis of clinical and radiological information. The present study was approved by the local ethics committee (Ethikkommission Universität Leipzig Az 330-13-18112013).

The control group consisted of 30 CSF specimens of patients without conclusive evidence for neurological pathologies. Those samples were obtained during the diagnostic routine in patients that necessitated CSF examination by lumbar puncture (e.g., exclusion of subarachnoid hemorrhage, demyelinating disease, meningitis). In addition, CSF samples were obtained from 91 patients that underwent diagnostic workup and treatment of hydrocephalus. Hydrocephalus patients were categorized as follows: aqueductal stenosis (AQS, $n = 21$), acute hydrocephalus without aqueductal stenosis (acute HC w/o AQS, $n = 12$), and idiopathic normal pressure hydrocephalus (NPH, $n = 27$) [6, 9].

Additionally, 12 adult patients suffering hydrocephalus after subarachnoid hemorrhage (SAH), 12 premature newborns with intraventricular hemorrhage (IVH), and 7 pediatric patients with proven bacterial CNS infection were included.

Quantification of Surfactant Proteins in CSF

Quantification of surfactant protein concentrations was performed using enzyme-linked immunosorbent assays (ELISA) according the manufacturer's manual. Commercially available ELISA kits (USCN, Wuhan, China) were used to quantify the amount of SP-G (E90890Hu, ELISA Kit for Surfactant Associated Protein G), in CSF samples. The analysis was performed using a microplate spectrophotometer (ELISA-reader) at a wavelength of 450 nm and a reference wavelength of 405 nm for measuring the absorbance. Surfactant protein concentration in nanograms per milliliter CSF was calculated by comparison between standard series and the determined values of antigen concentration (protein concentration) according to the manufacturer's manual. CSF concentrations of SP-G ranged close to the detection limit of the ELISA kit in several specimen of various entities. The detection limit according the manufacturer's manual was 18.27 pg/ml (0.0183 ng/ml).

Further CSF Analysis

Routine CSF laboratory data (bacterial cultures, cell counts, CSF lactate and glucose concentrations, total CSF protein and protein electrophoresis) were obtained to rule out infection or other inflammatory and autoimmune diseases in controls and pathological specimen except the CNS infection group. Patients with proved bacterial CSF infection were included in the latter group.

Statistical Analysis

Statistical analysis was performed using SPSS Version 22. Data was tested for normality using Shapiro-Wilks test. Differences between groups were analyzed using analysis of variance on ranks with a Dunnett post hoc analysis.

Correlation between age and the concentration of SP-G was calculated using a Spearman Rho correlation since SP values were not normally distributed. Significance level was set to 0.01.

Immunohistochemistry

Animals, Embryos, and Tissue Preparation

Naive Sprague Dawley female rats (200–225 g, 3 months old) were housed in the local animal house facility under standard laboratory conditions, providing a standard light/dark cycle of 12 h (7 a.m.–7 p.m.), room temperature of 23 ± 2 °C and ad libitum access to pellet food and water. Timed pregnancies were set and confirmed in the dams by a 4 h pairing with breeder males followed by vaginal smear examination. For harvesting, the embryos of different embryonic age, e.g., embryonal day (ED) 10, 12, 14, 16, 18, and 20 timed pregnant females, were sacrificed on the respective days using carbon monoxide with subsequent removal of the embryos. For ED10 and ED12 animals, the whole embryo was processed, while for ED14, ED16, ED18, and ED20 ($n = 3$ each), the brains were micro-dissected from the embryos with sterilized and atraumatic instruments and fixed in TissueTec, cryoprotected with 2-methyl butane on dry ice, prior to cutting 6-μm-thick coronal sections.

For postnatal brain tissue harvesting, timed pregnant dams were observed carefully every 2 h on the expected days of delivery to mark the day of birth as postnatal day 0 (P0). Pups were deeply anesthetized and perfusion-fixed transcardially with ice-cold saline followed by 2% PFA in 0.1 M PBS (pH 7.4). The brains were excised, post-fixed overnight with 2% PFA and subsequently cryoprotected with sucrose gradients (10, 20, 30%) prepared in 0.1 M PBS, pH 7.4.

Sections of 6 μm thickness were cut with the help of a Microm HM560 (Cryostat; Thermo Scientific, Walldorf, Germany). The sections were then stored at − 20 °C for further use. All experiments were approved by the Institutional Animal Ethics Committee and performed according to the instructions and guidelines of CPCSEA (Committee for the purpose of control and supervision on experiments on animals). Efforts were made to minimize the suffering of the animals. Due to collaboration with other study groups, animal use was kept to a minimum by using organs and tissues of animals which otherwise would have been discarded (IRB Registration no. T36/13).

Immunohistochemistry

Cryosections were washed with acetone for 10 min at − 20 °C and subsequently air-dried. Afterwards, nonspecific binding sites of the tissues were blocked by 5% goat serum (Dianova, 005-000-121) in TBS for 20 min. Commercially available antibodies for SP-G were used for overnight incubation at 4 °C in 2.5% goat serum in TBS. To reveal colocalization of SP-G with different cell types in the CNS, sections were further incubated for 1 h at similar conditions with antibodies directed against CD31, glial fibrillary acidic protein (GFAP), NeuN, and Aquaporin-4 (AQP-4). CD31, also known as platelet endothelial cell adhesion molecule 1 (PECAM-1), is widely used in clinical routine to demonstrate presence of endothelial cells representing blood vessels in histological sections [10]. GFAP is expressed by astrocytes [11] and ependymal cells [12] among a variety of other cell types and was used to visualize astrocytes. NeuN is used as a neuronal marker for postmitotic neurons and has a firm role in research and diagnosis [13]. AQP-4 is broadly expressed in choroid plexus and ependymal cells and is an important water channel associated with CSF production and regulation [14]. An overview of antibodies and dilutions is given in Table 1.

After washing in TBS, incubation was done for 30 min with Cy3-conjugated goat-anti-rabbit IgG (Dianova, 111-165-144) or Cy3-tagged goat-anti-mouse IgG (Dianova, 115-165-146) 1:200 in TBS containing rat serum (1:600). Finally, slices were embedded in DAPI-medium (Dianova, SCR 38448). Immunfluorescence analysis was performed with a Zeiss Observer A1 microscope and AxioCam ICc1 (Zeiss; Germany).

Results

Results of Human CSF Analysis and Statistical Group Comparison

CSF samples of 121 patients were analyzed for Surfactant protein G. Additionally, routine CSF examinations were performed as described above. An overview of patient demographics is given in Table 2.

Control patients with an age ranging from 14 days to 84 years (mean 43.5 years) were investigated. Under normal conditions, SP-G is present in CSF independent from gender in very low concentrations (Fig. 1). There was no association between age and SP-G levels in the control cohort ($R = 0.116$, $p = 0.54$). Furthermore, there was no association between protein concentration and SP-G CSF levels in control subjects ($R = -0.01$, $p = 0.9388$). However, SP-G showed a significant correlation with total CSF protein in patients suffering from hemorrhage and CNS infections ($R = 0.664$, $p < 0.00001$). Table 3 provides mean SP-G values of all investigated groups and their respective confidence intervals.

In patients suffering from bacterial infection, subarachnoid hemorrhage, or intraventricular hemorrhage, SP-G levels were strongly increased compared to the control group in a statistically significant manner (all $p < 0.0001$).

Table 1 Overview of primary antibodies used for immunofluorescence labelling and applied dilutions

Antibody	Vendor	Catalog number	Host	Dilution
SP-G (SFTA2)	USCN	#PAD755Hu01	Rabbit	1:500
Anti-rat CD31 (TLD-3A12)	BD Pharmingen	22711D	Mouse	1:50
NeuN	Millipore	MAB377	Mouse	1:400
GFAP	BD Pharmingen	556,330	Mouse	1:400
Aquaporin 4 (AQP 4)	Abcam	Ab9512	Mouse	1:500

NPH patients also exhibited significantly increased SP-G concentrations in comparison to controls ($p = 0.0018$). Compared to the other hydrocephalic entities, mean SP-G values were highest, but SP-G concentrations were lower in comparison to CNS infections and brain hemorrhage.

Notably, AQS patients showed a tendency of decreased CSF SP-G, but the significance level of $p = 0.05$ was not achieved ($p = 0.053$).

The heterogeneous acute hydrocephalus group showed a moderate tendency of increased SP-G values, although the difference in comparison to the control group did not achieve statistical significance ($p = 0.14$).

Routine CSF parameters demonstrated typical alterations in CSF cell counts, lactate levels, and glucose concentrations in the presence of infection or hemorrhage (Table 3).

Results of Immunohistochemistry

SP-G Presence in Different Localizations of the CNS and Colocalization with CNS-Specific Molecular Markers

SP-G showed strong epithelial immunoreactivity in the CNS within the choroid plexus and ependymal lining of all ventricles (Fig. 2). SP-G presence in the choroid plexus epithelium was homogeneous throughout the cytoplasm and did not display an apicobasal gradient. In contrary, SP-G presence was associated with a very diverse morphology in the ependymal cells. More specifically, SP-G was detected in ellipsoid-shaped granula, located in the apical third of the ependymal cell layer, approximately occupying a cytoplasmic area of 10–25% of the respective individual nucleus. Marked immunoreactivity for SP-G was also detected at the Lamina glia limitans superficialis (Lgsf) and Lamina glia limitans perivascularis (Lgpv), resembling the morphological correlate of the blood-brain barrier and the outer blood-CSF barrier (Fig. 2). SP-G immunoreactivity was moderate in the subventricular and superficial cortex, exhibiting a granular cytoplasmic perinuclear pattern in large neurons and smaller neurons with pyramidal shape.

SP-G showed a clear colocalization with AQP4, CD31, and NeuN in choroid plexus epithelium, but not in the ventricular ependymal lining (Fig. 2a–h). Also, SP-G was colocalized with AQP4 and CD31 in pial and cortical vessels. More diffuse co-occurrence between SP-G and AQP4 was determinable in the cortical parenchyma. Interestingly, NeuN expression was strongest in a small perinuclear zone, directly adjacent to the SP-G positive, more peripheral neuronal cytoplasm with a small overlap of NeuN and SP-G-positive areas. No clear colocalization was observable for SP-G and GFAP.

SP-G Occurrence During Embryonic Development

Specific immunoreactivity for SP-G in the CNS was first determinable on ED20. Immunoreactivity was strong and remained at the same level, comparable to adult cryosections. Distribution patterns were as described above.

Discussion

To the best of our knowledge, this work presents the first investigation on SP-G in the mammalian CNS using a combination of human CSF samples and rat brains.

First of all, we were able to demonstrate the presence of SP-G in the CSF of healthy subjects and patients suffering from different hydrocephalic conditions, bacterial CNS infections,

Table 2 Overview of demographic data of the patient subgroups: aqueductal stenosis (AQS), acute hydrocephalus without AQS (acute HC w/o AQS), normal pressure hydrocephalus (NPH), central nervous system infections (CNS-Infections), intraventricular hemorrhage (IVH), and subarachnoid hemorrhage (SAH)

	Control	AQS	Acute HC w/o AQS	NPH	CNS infection	IVH	SAH
N	30	21	12	27	7	12	12
Age (years)	43.5 (0–84)	19.3 (0–65)	14.5 (0–75)	69.7 (31–84)	3.2 (0–45)	0	59.6 (17–82)
Sex (m/f)	15/15	8/13	5/7	18/9	2/5	6/6	7/5

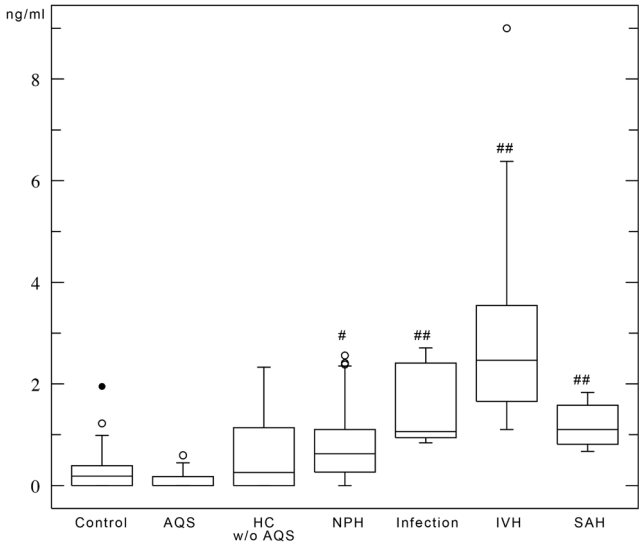

Fig. 1 Results of CSF concentrations of SP-G (SFTA-2) in human specimen of different pathological entities compared to controls revealed by ELISA. Inflammatory response is associated with elevated levels of SFTA-2 independent from causative pathology. In contrast, elevation of intracranial pressure or changes in cerebral CSF circulation do affect SP-G levels to a lesser extent, but only changes in NPH patients achieved statistical significance. #$p < 0.01$; ##$p < 0.001$. Data value outside of threefold of the respective quartile. Data value outside of 1.5-fold of the respective quartile (white circle)

and brain hemorrhage. SP-G concentrations in the CSF of healthy subjects and patients suffering from hydrocephalus secondary to aqueductal stenosis were low, whereas SP-G concentrations were higher in the heterogeneous acute hydrocephalus and NPH collective, while SP-G levels were most increased in patients suffering from CNS infections and brain hemorrhage.

More specifically, the most prominent changes of SP-G CSF levels occurred in patients who were experiencing an acute phase immune response—secondary to hemorrhage or infection. Highest levels of CSF SP-G occurred in newborn patients suffering from acute IVH, closely followed by adult patients suffering from acute SAH and pediatric patients with acute CNS infections. Interestingly, the changes in SP-G levels did not reveal a correlation with cell count, but with total protein levels, parameters, which are commonly altered in host responses of the CNS. However, SP-G concentration was neither associated with protein content in control patients nor did it show a correlation with age. Thus, SP-G seems to be elevated in acute phase states, where a multiplicity of proteins including SPs, are actively secreted into the CSF. However, a reduction of total protein concentration, which is observed in hydrocephalic states like aqueductal stenosis (AQS), was not associated with a concomitant reduction of SP-G. The observation is in concordance with Reiber's work about BBB function and CSF proteins [15].

According to previously published studies, SPs in the CSF are predominantly brain-derived proteins, and alterations of CSF levels are the result of physiologically up-regulated production and impaired CSF flow, not a consequence of malfunctioning CNS barrier mechanisms [1, 4, 6, 7, 15].

Considering this and previous reports on the regulation of conventional SPs in inflammation [1, 16], our results indicate that SP-G is involved in acute phase CNS immune response and maintenance of the CSF's rheological properties. Such conclusions are in line with the work of Mittal et al., who demonstrated the secretory nature of SP-G and its regulation in response to LPS stimulus [17]. Comparable to SP-A, it probably participates in the depletion of blood degradation products and infectious pathogens via enhanced phagocytosis [1, 18] as well as the adjustment of CSF viscosity and surface tension by interacting with CSF lipids [4, 19].

This hypothesis is corroborated by the apical presence of SP-G in ependymal cells and choroid plexus epithelium—suggesting active secretion of SP-G into the CSF compartment—and expression of SP-G surrounding parenchymal microvessels allocated with AQP4, indicating further involvement in host defense and possibly CNS clearance of toxic metabolites and harmful protein aggregates via the paravascular glymphatic system [20, 21].

Table 3 Overview of mean surfactant protein G levels (ng/ml) and CSF cell counts (CC), CSF lactate (lac), CSF glucose (glu), and total CSF protein concentrations (g/l) of various types of CSF disturbances and control group and their respective 95% confidence intervals

	Control	AQS	Acute HC w/o AQS	NPH	CSF infection	IVH	SAH
n	30	21	12	27	7	12	12
SP-G	0.09 (0–0.6)	0.09 (0–0.47)	0.56 (0.06–1.06)	0.84* (0.51–1.17)	1.47*** (0.83–2.12)	3.07*** (2.55–3.59)	1.16*** (0.66–1.55)
CC	3 (2–4)	9 (4–13)	13 (9–17)	3 (2–3)	667 (40–579)	157 (016–171)	238 (14–243)
Lac	1.60 (1.53–1.67)	1.55 (1.49–1.61)	2.12 (1.89–2.35)	1.85 (1.77–1.92)	6.08 (2.51–7.96)	2.81 (2.34–3.39)	4.66 (2.89–5.34)
Glu	3.55 (3.54–3.56)	3.78 (3.41–4.15)	3.16 (2–86 – 3.46)	4.10 (3.80–4.38)	0.93 (0.11–2.67)	1.40 (0.67–1.34)	4.65 (3.59–5..37)
Total protein	0.36 (0.34–0.38)	0.11 (0.08–0.13)	0.43 (0.33–0.53)	0.38 (0.30–0.44)	5.29 (2.64–6.0)	1.96 (1.27–2.37)	2.03 (0.53–2.66)

*$p < 0.05$; ***$p < 0.001$; all vs. control

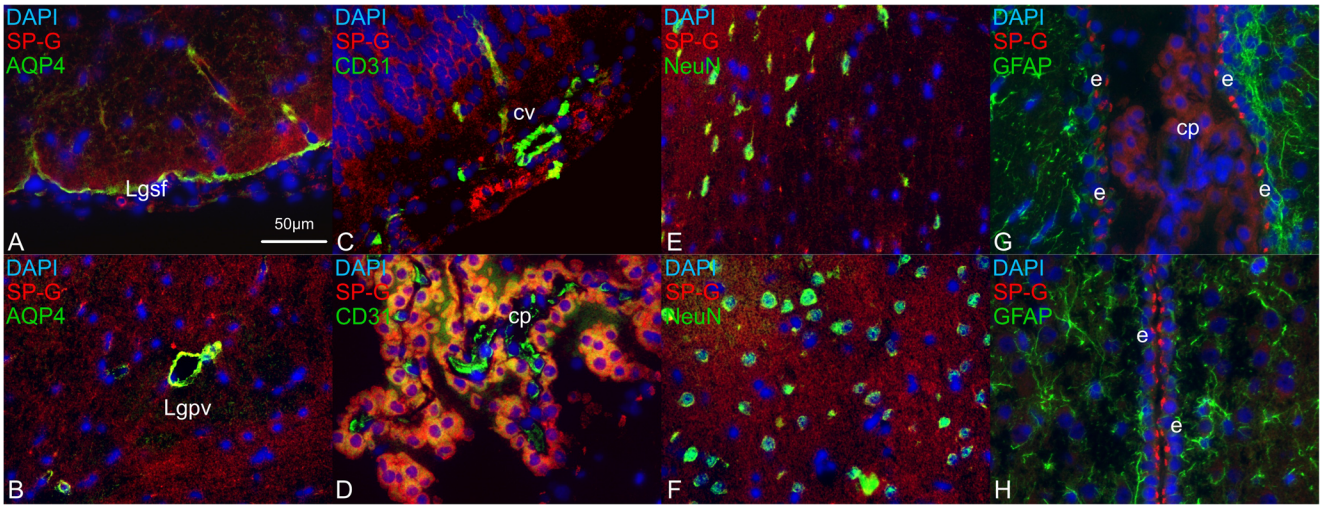

Fig. 2 Presence of SP-G (red fluorescence) in the CNS and colocalization (all green) with the water channel aquaporin 4 AQP4 (**a**, **b**), endothelial marker CD31 (**c**, **d**), neuronal marker NeuN (**e**, **f**), and astroglial marker GFAP. DAPI was used for imaging the nuclei. Marked coexpression of AQP4 and SP-G is observable at the Lamina glia superficialis (Lgsf) and along cerebral vessels (cv), resembling the Lamina glia perivascularis (Lgpv), a morphological component of the blood brain barrier. CD31 and SP-G are also colocalized along cerebral vessels. Interestingly, both proteins are coexpressed by choroid plexus (cp) epithelia. Note the absence of SP-G along the stromal vessels within the cp. Colocalization of NeuN and SP-G is determinable in a small perinuclear area in large subventricular neurons and pyramid-shaped cortical neurons. The scale provided in panel **a** is representative for all images

Although the indicative momentum of our findings for SP-G's rheological significance is notable, additional functional studies examining CSF flow phenomena in the context of SP-G CSF profiles are necessary to further elucidate SP-G function. Regarding its molecular architecture, SP-G does not exhibit significant similarity to the rheologically important SPs (B and C) [8]. However, it is an amphiphilic, small protein with the ability to switch between a hydrophilic and a hydrophobic state and is subject to a number of posttranslational modifications. As a result, SP-G possesses similar physicochemical properties like SP-B and SP-C and thus certainly participates in the regulation of CSF rheology [8].

However, the presence of SP-G in the described subset of cortical neurons is surprising, and the physiological importance of SP-G for neuronal processes cannot be inferred based upon our results. Thus, further studies are necessary to elucidate the function of SP-G in the context of neurons.

Our study suffers from a number of limitations. Firstly, the number of patients in each subgroup is relatively small, and control samples, especially from pediatric individuals without neurological disease, are lacking, since it is ethically not justifiable to perform lumbar puncture in this patient group without conclusive evidence of a suspected neurological disease. Furthermore, colocalization studies have been performed only using histological samples from rat brains, while human specimens are lacking. Therefore, confirmation of our findings in human brain tissue remains necessary. Also, the physiological role of SP-G can only be assumed based upon our results, and more functional investigations are warranted to confirm our findings and more specifically define the functional importance of SP-G in the human CNS.

Conclusions

SP-G, a novel surfactant protein, is present in human CSF and exhibits specific expression patterns in rat brains. Based on alterations of SP-G levels in CSF samples from patients with acute brain hemorrhage or CNS infections, predominant importance for host defense is assumable. This is underlined by its abundance at the morphological correlate of the blood-brain barrier and the blood-cerebrospinal fluid barrier. Furthermore, considering SP-G changes in the CSF under hydrocephalic conditions and SP-G's secretory expression pattern in choroid plexus epithelia and ependymal cells, involvement in CSF rheology seems probable. SP-G even seems to contribute to brain waste clearance via the glymphatic pathway, as perivascular abundance and association with AQP4 suggest.

In summary, SP-G is a potential new CSF biomarker, not only reflecting aspects of CNS innate immune responses, but also rheo-dynamically relevant changes of CSF composition, associated with CSF malabsorption. Nevertheless, further studies have to validate and extend our findings to provide additional insights into the physiological importance of SP-G in the context of the CNS.

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Author's Contribution Statement All named authors contributed by collecting samples, preparing and analyzing samples, and collecting clinical data. ELISA investigations were done by SS and MK. Manuscript was drafted by MK, WH, and SS; final approval and reviewing were provided by all authors.

Compliance with Ethical Standards

All patients or caregivers gave their written informed consent for the scientific use of CSF samples including the analysis of clinical and radiological information. The present study was approved by the local ethics committee (Ethikkommission Universität Leipzig Az 330-13-18112013).

Conflict of Interest The authors declare no conflict of interest for the presented study.

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