Simultaneous alterations of oligodendrocyte-specific CNP, astrocyte-specific AQP4 and neuronal NF-L demarcate ischemic tissue after experimental stroke in mice

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ABSTRACT

Ischemic stroke not only affects neurons, but also glial and vascular elements. The development of novel neuroprotective strategies thus requires an improved pathophysiological understanding of ischemia-affected cell types that comprise the ‘neurovascular unit’ (NVU). To explore spatiotemporal alterations of oligodendrocytes, astrocytes and neurons after experimental ischemic stroke, we applied a permanent middle cerebral artery occlusion model in mice for 4 and 24 h. Using fluorescence microscopy, the oligodendrocyte marker 2′,3′-cyclic nucleotide phosphodiesterase (CNP), the neuronal neurofilament light chain (NF-L) and the astroglial aquaporin-4 (AQP4) were analyzed in regional relation to one another.

Immunofluorescence intensities of CNP and NF-L were simultaneously increased in the ischemic neocortex and striatum. AQP4 immunoreactivity was decreased in the ischemic striatum, which represents the initial and potentially strongest affected site of infarction. The more distant ischemic neocortex and infarct border zones exhibited areas with alternately increased or decreased AQP4 immunoreactivity, leading to an increase of fluorescence intensity in total.

Further, deformed CNP-immunopositive processes were found around axonal spheroids, indicating a combined affection of oligodendrocytes and neurons due to ischemia. Importantly, altered AQP4 immunosignals were not limited to the ischemic core, but were also detectable in penumbral areas. This applies for CNP and NF-L also, since altered immunosignals of all three markers coincided regionally at both time points.

In conclusion, the present study provides evidence for a simultaneous affection of oligodendrocytes, astrocytes and neurons after experimental focal cerebral ischemia. Consequently, CNP, AQP4 and NF-L immunofluorescence alterations can be utilized to identify ischemia-affected tissue. The simultaneity of the described alterations further strengthens the concept of interdependent NVU components and distinguishes NF-L, CNP and AQP4 as highly ischemia-sensitive elements. Consequently, future therapeutic approaches might influence stroke evolution via strategies simultaneously addressing both neuronal and glial functions.

1. Introduction

Stroke is the second leading cause of death worldwide and a major cause of disability [1,2]. Despite the prevalence of ischemic insults, therapeutic options remain limited to a minority of patients [3]. In order to develop novel treatment strategies, stroke research not only focused on the pathophysiological understanding of neurons, but also glial cells, vascular elements and the associated extracellular matrix [4], which together comprise the ‘neurovascular unit’ (NVU) [5]. Consequently, ischemia-induced interactions between cells of the NVU were extensively addressed and oligodendrocyte affection came into focus [6–8].

Under physiological conditions, oligodendrocytes and axons are well described in regard to myelin sheaths and metabolic coupling [9,10]. Further, intercellular communication between oligodendrocytes and astrocytes is considered to allow membrane potential restoration [11], thereby highlighting glial and neuronal interdependencies. More
Specifically, the absence of the oligodendrocyte-specific protein 2',3'-cyclic nucleotide phosphodiesterase (CNP) was found to be associated with an impaired cellular transport in the axonal compartment. These problems might be indirectly caused by closure of myelin-associated channels, which consequently disrupts the metabolic coupling [10,12]. CNP has been further suggested to promote microtubule assembly and to be firmly associated with tubulins, indicating it is functionally linked to the cytoskeleton [13,14]. As neuronal cytoskeletal elements, the neurofilament light chain (NF-L) is crucial for maintaining cellular integrity and axonal transport [15]. This is supported by immunohistochemical studies showing relevant NF-L alterations after focal cerebral ischemia, which led to the conclusion that neurofilaments are critically involved in ischemia evolution due to axonal damage and cytoskeletal degeneration [16,17].

A detailed understanding of the potential interaction between oligodendrocytes and neuronal cytoskeletal elements may help to conceive specific strategies to prolong neuronal survival after stroke. Therefore, this study investigated the spatiotemporal impairment of CNP in relation to NF-L after experimental focal cerebral ischemia in mice. Analyses also included aquaporin 4 (AQP4) water channels, which are located on vessel-associated astrocytic end-feet and are known to contribute to edema formation and neurovascular dysfunction under ischemic conditions [18-21].

2. Material and methods

2.1. Study design

Focal cerebral ischemia was induced by filament-based permanent right-sided middle cerebral artery occlusion in 18 adult male C57BL/6J mice with a mean weight of 25 g and an average age of 10 weeks. Mice were provided by Charles River (Sulzfeld, Germany). Neurobehavioral deficits were captured by the Menzies Score [22], which ranges from 0 (no neuronal deficit) to 4 (spontaneous contralateral circling). As a predefined study inclusion criterion, mice had to demonstrate a score of at least 2 to confirm sufficient stroke induction, which was not fulfilled in 1/18 mice (5.6%). In the applied model a peri-procedural loss of 0% was expected to be firmly associated with tubulins, indicating it is functionally linked to the cytoskeleton [13,14]. As neuronal cytoskeletal elements, the neurofilament light chain (NF-L) is crucial for maintaining cellular integrity and axonal transport [15]. This is supported by immunohistochemical studies showing relevant NF-L alterations after focal cerebral ischemia, which led to the conclusion that neurofilaments are critically involved in ischemia evolution due to axonal damage and cytoskeletal degeneration [16,17].

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2.2. Induction of focal cerebral ischemia

Ischemia was induced according to Longa et al. [24], with minor modifications as previously described [25]. In brief, mice were deeply anesthetized using 2–2.5% isoflurane (Baxter, Unterschleißheim, Germany) and a vaporizer (VIP 3000, Matrix, New York, USA) providing a mixture of 70% N₂O / 30% O₂, followed by a preparation of the cervical vessels and insertion of a standardized silicon-coated 6-0 monofilament (Doccol Corporation, Redlands, CA, USA) into the right internal carotid artery. The filament was carefully pushed forward to the origin of the right middle cerebral artery until resistance was felt or bending was observed. During surgery, the body temperature was controlled and adjusted to 37 °C with a rectal probe and a thermostatically regulated warming pad (Fine Science Tools, Heidelberg, Germany).

2.3. Tissue preparation and fluorescence labeling

At the end of the observation period mice were sacrificed and transcardially perfused with saline and 4% paraformaldehyde (PFA) in 0.1 M phosphate buffered saline, pH 7.4 (PBS). The brains were removed from the skull, post-fixed in the same fixative for 24 h and equilibrated in 30% phosphate-buffered sucrose. Next, they were cut into 30 μm-thick coronal sections using a freezing microtome (Leica SM 2000R, Leica Biosystems, Wetzlar, Germany). Brain sections were then stored at 4 °C in 0.1 M Tris-buffered saline, pH 7.4 (TBS), containing 0.2% sodium azide.

For immunofluorescence labeling, brain sections were thoroughly rinsed in TBS and incubated in TBS blocking buffer containing 5% normal donkey serum and 0.3% Triton X-100 for 1 h. Thereafter, primary antibodies (Table 1) were added for overnight incubation. Next, sections were washed with TBS followed by incubation with mixtures of appropriate fluorochrome-labeled secondary immunoreagents (Table 1) in TBS containing 2% bovine serum albumin for 1 h at room temperature. If biotinylated rabbit-anti-NF-L was used, remaining free binding sites of secondary antibodies were subsequently blocked with TBS buffered 50% normal rabbit serum for 4 h. Thereafter, biotinylated rabbit-anti-NF-L was incubated overnight and visualized with Cy3-streptavidin for 1 h. For staining with 4',6-diamidino-2-phenyldine (DAPI, Life Technologies, Carlsbad, CA, USA), an additional washing and incubation step (DAPI 1:10,000 in PBS for 7 min) was performed.

Finally, sections were rinsed with TBS, mounted onto fluorescence-free microscope slides and cover-slipped with fluorescence mounting medium (Dako North America, Inc., Carpinteria, CA, USA).

In control experiments, the omission of primary antibodies resulted in the absence of any labeling. Representative control micrographs of the ischemia-affected and contralateral hemisphere are given in Supplementary Fig. 1.

### Table 1

| Immunoreagent | Dilution | Manufacturer |
|---------------|----------|--------------|
| **Primary antibody** | | |
| guinea pig-anti-CNP1 | 1:200 | Synaptic Systems, Göttingen, Germany |
| goat anti-AQP4 | 1:1000 | Santa Cruz Biotechnology, Inc., Dallas, TX, USA |
| rabbit anti-AQP4 | 1:200 | Alomone Labs Ltd., Jerusalem, Israel |
| biotinylated rabbit anti-neurofilament L IgG | 1:500 | Synaptic Systems |
| rabbit anti-IISP70 | 1:200 | Enzo Life Sciences GmbH, Lörrach, Germany |
| guinea pig anti-NeuN | 1:200 | Synaptic Systems |
| goat anti-collagen IV | 1:200 | Merck Millipore, Billerica, MA, USA |
| **Secondary antibody / immunoreagent** | | |
| Cy2-donkey anti-guinea pig IgG | 20 μg/ml each | Dianova (Hamburg, Germany) as supplier for Jackson Immunoresearch (West Grove, PA, USA) |
| Cy3-streptavidin | 1:250 each | Thermo Fisher, Waltham, MA, USA |
| AlexaFluor647-donkey anti-guinea pig IgG | | |
| AlexaFluor568-donkey anti-rabbit IgG | | |
| AlexaFluor568-donkey anti-rabbit IgG | | |
| AlexaFluor488-donkey anti-rabbit IgG | | |

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2.4. Imaging

Micrographs were captured with a Biorevo BZ-9000 microscope (Keyence, Neu-Isenburg, Germany), with a confocal laser-scanning microscope (LSM 510 Meta or LSM 880, Carl Zeiss Microscopy, Göttingen, Germany) or with a slide scanner (Axio Scan.Z1; Carl Zeiss Microscopy). Microsoft PowerPoint (version 2015; Microsoft Corp., Redmond, WA, USA) was used for figure panel generation. If necessary, brightness and contrast were slightly adjusted without creating or deleting signals.

2.5. Quantification and statistical analyses

In general, micrographs for quantification of AQP4 and CNP immunosignals were captured at constant exposure times using the Keyence microscope. For AQP4 and CNP, six regions of interest (ROIs) were positioned within the ipsilateral, ischemia-affected hemisphere and the contralateral hemisphere as indicated in Fig. 1B for each animal. For quantification of CNP-immunopositive globular structures, three ROIs per animal were analyzed in coronal brain sections at Bregma -1.9 mm, which were positioned in the ischemic neocortex (`ischemia`), the cortical periphery of the infarct (`periphery`) and the contralateral neocortex (`control`). Images were acquired with the Axioscan.Z1 and netScope Viewer Pro software (Net-Base Software GmbH, Freiburg, Germany). Fluorescence intensities measurements and counting of CNP-positive globular structures were processed using ImageJ software (National Institutes of Health, Bethesda, MD, USA). Statistical analysis was performed with Graph Pad Prism 5.01v (GraphPad Software Inc., La Jolla, USA) using the nonparametric Kruskal-Wallis test followed by Dunn’s multiple comparison test. The Grubbs test was used to check for statistical outliers. In general, a p < 0.05 was considered statistically significant.

3. Results

3.1. Ischemia induces an increase of CNP and NF-L immunosignals and inhomogeneous alterations of AQP4 immunosignal

Triple immunofluorescence labeling was performed after 4 and 24 h of ischemia to capture alterations of astrocyte end-feet (AQP4), oligodendrocyte-specific CNP and NF-L as a constituent of the neuronal cytoskeleton (Fig. 1A). Thereby, ischemia-affected areas are delineated by an increased immunoreactivity of the ischemia-sensitive marker NF-L [17], which co-localizes with a concomitantly increased immunosignal of CNP. In contrast, the AQP4-related immunoreactivity is found to be decreased in the ischemic striatum, whereas the ischemia-affected neocortex exhibited a heterogeneous AQP4 expression pattern with circumscribed areas of either decreased or increased AQP4 immunoreactivity (Fig. 1A and B, Supplementary Fig. 2).

Further, the fluorescence intensities of AQP4 and CNP were measured in different regions of the ischemia-affected and contralateral hemisphere (Fig. 1B and C). These quantitative analyses confirmed a striatal reduction of AQP4 fluorescence intensity compared to contralateral areas, while ischemia-affected neocortical areas and adjacent border zones displayed increased fluorescence intensity levels (Fig. 1C).

As indicated by the fluorescence microscopy imaging (Fig. 1A and B, Supplementary Fig. 2), quantitative analyses demonstrated that the CNP-related immunoreactivity is significantly increased in areas affected by 24 h of ischemia, while decreased levels are only observed in border zones of the ischemic striatum after 4 h of ischemia (Fig. 1D).

3.2. Ischemia-derived alterations of oligodendroglial and vessel-associated astroglial structures

To further characterize the rather heterogeneous alterations of AQP4 immunolabeling at the cellular level, we applied high-magnification fluorescence imaging of AQP4-labeled astrocytes in ischemia-affected areas.

Astrocytes are characterized by a highly polarized expression of AQP4; this means AQP4 is restricted to astrocytic end-feet contacting the vascular basal lamina of the glia limitans [20,26,27], which can be observed within the non-affected contralateral hemisphere (Fig. 2A). In contrast, the abluminal astrocytic cell surface and cell bodies within the neuropil become AQP4-immunopositive in ischemia-affected areas (Fig. 2B). Thereby, the polarized AQP4 expression is lost [20] and AQP4 immunosignals become detectable throughout the cell body and non-vascular processes, especially close to medium- and high-caliber vessels (Fig. 2B–D).

To rule out an AQP4 expression in cells of the vasculature like pericytes or endothelial cells, we further applied double immunofluorescence labeling of AQP4 and collagen IV. Thereby, collagen IV delineates the compartment of the brain parenchyma as it outlines the glial and vascular basement membranes, which are fused at the level of microvessels [28]. Here, the AQP4 immunosignal was regularly confined to the abluminal surface of the gliovascular basement membrane (Fig. 2E). Since endothelial cells and pericytes are by definition located at the luminal side of the gliovascular basement membrane [29], an expression of AQP4 in pericytes or endothelial cells can be ruled out. Therefore, the described alterations of AQP4 immunosignal derive from astrocytes, only.

3.3. Simultaneous affection of oligodendroglial and NF-L-positive neuronal processes

Further, we investigated the relationship between ischemia-affected oligodendrocytes and neuronal structures by double immunofluorescence labeling of CNP in combination with the ischemia-sensitive neuronal marker NF-L. While CNP immunoreactivity was homogeneously distributed in the unaffected contralateral hemisphere (Fig. 3A), globular accumulations of CNP with a diameter of 2–15 μm became regularly apparent throughout the ischemia-affected neocortex (Fig. 3B). Notably, the ischemia-affected neocortex revealed significantly more globular structures immunopositive for CNP after 4 and 24 h of ischemia compared to the contralateral control area (Fig. 3C). These CNP-accumulations were found in somata of oligodendrocytes, as indicated by counter-staining with DAPI in high-power magnification images (Fig. 3D) and around NF-L-immunopositive axonal spheroids (Fig. 3E). The close morphological correlation between the axonal spheroid and its surrounding oligodendrocyte sheath indicates a simultaneous impairment of neurons and oligodendroglia, which becomes detectable by immunolabeling of NF-L and CNP.

3.4. Glial reactions in the ischemic penumbra

To determine whether the observed glial alterations extend to the ischemic penumbra and thus involve areas of potentially salvageable tissue [30], a subset of analyses included the heat shock protein 70 (HSP70) which is known to be selectively upregulated in neurons of the penumbra [31,32]. Consequently, the expression of HSP70 in neurons can be used to identify the outer layers of the ischemia-affected penumbra [33]. For this reason, we combined the labeling of AQP4 – to illustrate glial alterations – with the immunodetection of HSP70 and neuronal nuclei (NeuN) to visualize the extent of the ischemic penumbra. In peripheral areas of the altered AQP4 immunosignal, HSP70 expression was upregulated in NeuN-positive neurons. Thus, the increased neuronal HSP70 immunoreactivity co-localized with ischemia-induced alterations of the AQP4 immunosignal (illustrated in the hippocampus and the striatum in Fig. 4). Consequently, the detected ischemia-derived alterations of the AQP4 immunosignal clearly extend to areas of the penumbra.
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A

|                  | neocortex – 24 h MCAO | striatum – 24 h MCAO | striatum – 4 h MCAO |
|------------------|-----------------------|----------------------|---------------------|
|                  | ischemia              | control              | ischemia            |
| merge            |                       |                      |                     |
| AQP4             |                       |                      |                     |
| CNP              |                       |                      |                     |
| NF-L             |                       |                      |                     |

B

regions of interest (ROIs):

ipsilateral hemisphere
1: neocortex – ischemia
2: neocortex – border zone
3: striatum – ischemia
4: striatum – border zone

contralateral hemisphere
5: neocortex – control to ROI 1 and 2
6: striatum – control to ROI 3 and 4

C

AQP4

4 h MCAO

24 h MCAO

D

CNP

4 h MCAO

24 h MCAO

(log10 fluorescence intensity (fold change of control))

1 2 3 4 1 2 3 4

neocortex striatum neocortex striatum

ischemia border zone ischemia border zone

* * **

* * **

* * *
Fig. 1. (A) Concomitant alterations of AQP4, CNP and NF-L immunoreactivities are shown at the neocortical and striatal infarct border 24 h after ischemia, as well as at the striatal infarct border 4 h after ischemia. In the ischemic tissue (i) on the left side of the dashed line, increased NF-L immunoreactivity coincides with increased CNP immunoreactivity. AQP4 immunoreactivity is abruptly diminished on the ischemic side of the infarct border, but increased in close proximity to the ischemic border compared to controls. Ischemia-derived changes of CNP and NF-L immunosignals are less distinct in the neocortex compared to the striatum. Dashed lines outline the border of altered AQP4 and CNP immunoreactivity within ischemia-affected areas (i). (B) Illustration of regions of interest (ROIs) used for quantification of AQP4 and CNP immunosignals (C and D). (C) After 4 and 24 h of ischemia, the AQP4 immunosignal is decreased in the ischemic striatum, whereas adjacent ROIs of the ipsilateral hemisphere exhibit increased AQP4 immunoreactivities compared to controls. (D) For CNP, fluorescence measurements revealed increased CNP fluorescence intensities in all but one region of the ipsilateral hemisphere and at both time points compared to controls. MCAO: middle cerebral artery occlusion; data are given as mean values; error bars indicate SEM; *p < 0.05, **p < 0.01; sample sizes: 4 h of ischemia: n = 7, 24 h of ischemia: n = 6; Scale bars: 200 μm.

Fig. 2. Immunofluorescence labeling of AQP4 in combination with CNP or Coll IV and DAPI is applied to analyze cellular alterations at high magnification using confocal laser-scanning microscopy. In control areas, astrocytic AQP4 immunoreactivity is strictly limited to the vascular surface of the glia limitans (A). In contrast, ischemic areas display a redistribution of the AQP4 immunosignal, thereby becoming detectable at the abluminal cell membrane (arrows) or throughout the entire cell body (arrow head) (B). Thus, the polarized AQP4-expression is lost in ischemic areas: astrocytic somata as well as non-vascular end-feet become AQP4-positive (C), especially in close relation to medium- and large-diameter vessels (D). Of note, the altered AQP4 immunosignal is restricted to the abluminal aspect of the gliovascular basal lamina (Coll IV), thereby ruling out an AQP4 expression in endothelial cells or pericytes. Dashed line outlines the inner border of AQP4 signal (E). Furthermore, CNP immunosignal appears increased in ischemia-affected areas (B) compared to controls (A). Nuclei were counter stained with DAPI. Scale bars: (A and B) 10 μm, (C) 20 μm, (D) 100 μm, (E) 5 μm.

4. Discussion

Although the knowledge of the pathophysiology of ischemic stroke has substantially improved during the last decades, the need for the development of novel therapeutic options is still evident. This is exemplified by the fact that only one fourth of stroke patients are currently eligible for modern recanalizing therapies [3]. However, the roadblock to successfully translate experimental treatment strategies into the clinical setting is enormous [34]. To overcome this roadblock, an adapted perspective beyond the initial neuro-centric view was
introduced, considering several cell types according to the NVU concept [4,5]. Therefore, fundamental knowledge of cellular interdependencies and their response in the setting of ischemia is essential [35].

By applying immunohistochemical techniques, which allow detection of simultaneous cellular alterations, this study focused on oligodendrocytic CNP, astroglial AQP4 and neuronal NF-L. Thereby, this study demonstrates neuronal as well as glial alterations in a spatial pattern at clinically relevant time points after cerebral ischemia. In detail, an early time window of 4 h after ischemia onset was chosen as it allows intravenous administration of tissue plasminogen activator in the clinical setting [36], added by a later time window of 24 h, in which numerous stroke patients continue to exhibit a relevant proportion of the penumbral ‘tissue at risk’ [37].

For this purpose, we took advantage of the established ischemia-sensitive marker NF-L, which can be utilized to demarcate ischemia-affected tissue by the strong increase of immunofluorescence intensity in diverse stroke models and human brain tissue [17,38]. Importantly, the AQP4- and CNP-related alterations clearly co-localized with ischemia-affected areas as outlined by an increased NF-L immunoreactivity (Fig. 1). In line with NF-L, CNP exhibited a significantly increased immunoreactivity. The immunosignal of AQP4 was reduced in the ischemic striatum but not in the neocortex, where astrocytes might suffer to a lesser extent because leptomeningeal collateral vessels might still alleviate ischemic damage within the cortical middle cerebral artery territory [39].

In addition to the quantitative changes of AQP4 immunoreactivity, we also observed a striking redistribution of AQP4 from the juxtavascular processes of the glia limitans towards the entire cell body [Fig. 2C], which indicates a loss of the polarized AQP4 expression [20].

Further, high-resolution confocal laser-scanning microscopy

Fig. 3. Ischemia-derived morphologic alterations of oligodendrocytes and axons are visualized by immunofluorescence labeling of CNP in combination with NF-L and DAPI. CNP immunoreactivity appears to be homogeneously distributed in control regions (A), whereas the ischemia-affected neocortex exhibits CNP-immunopositive globular structures (B), which are significantly increased in the ischemic cortex after 24 h of ischemia compared to the contralateral hemisphere (C). These CNP-positive globular structures represent somata of oligodendrocytes, as revealed by counter-staining with DAPI (D). Double immunofluorescence labeling of NF-L and CNP demonstrates that NF-L-immunopositive axonal spheroids (arrows) are wrapped by deformed CNP-positive myelin sheaths (E). MCAO: middle cerebral artery occlusion; data are given as mean values; error bars indicate SEM; *p < 0.05, **p < 0.01; sample sizes: 4 h of ischemia: n = 7, 24 h of ischemia: n = 6; Scale bars: (A and B) 100 μm; (D and E) 10 μm.
demonstrated altered CNP-positive oligodendrocytic processes enwrapping NF-L-positive axonal spheroids (Fig. 3D), which are known as a sign of axonal damage [44,45]. As shown previously, the strong NF-L immunoreactivity is caused by an increase of NF-L degradation products within ischemic tissue, likely representing neuronal degeneration [17]. In general, ischemia-associated pathological changes of oligodendrocytes appear early and are concomitant with respective alterations in myelinated axons [46,47]. Therefore, the formation of axonal spheroids may result from an impaired metabolic coupling between axons and oligodendrocytes, which depends on proper CNP function [12]. Another way of interpretation is that the morphological oligodendrocytic transformation derives only secondarily from the formation of axonal spheroids.

To provide a functional perspective of the observed alterations, a subset of analyses included labeling of HSP70, which allows regions of differently affected tissue to be distinguished according to the penumbra concept [30]. Here, we found alterations of AQP4 immunoreactivity in ischemic regions showing a HSP70 expression in neurons, representing penumbral areas (Fig. 4). This is of particular interest, as our previous work has demonstrated ischemia-mediated NF-L in penumbral areas, as well [17]. Since all the described alterations of NF-L, AQP4 and CNP coincide regionally, the ischemia-induced alterations of CNP also extend to the penumbra. Consequently, there might be potential for a restoration of oligodendrocytes following remyelination after ischemia [48].

Although the present study is rather descriptive, our data provide evidence for a simultaneous impairment of the oligodendrocyte marker CNP, astrocytic AQP4 and neuronal NF-L in ischemia-affected areas. Further, their concomitant visualization using immunofluorescence labeling illustrates the capacity of each of the applied markers to precisely identify ischemia-affected tissue. Consequently, this method – in contrast to the classical visualization with triphenyltetrazolium chloride (TTC) staining [49] – can be combined with a variety of other fluo-rescence markers to further explore ischemia-induced cellular alterations.

Since rodent models can only mirror a subset of pathologically relevant characteristics observed in stroke patients, the inclusion of different time points is recommended for preclinical stroke research [50]. Therefore, the present study comprised animals which underwent 4 and 24 h of ischemia. Importantly, the described alterations of AQP4 and CNP are not restricted to a single time point. This is of particular interest, since a subgroup of patients still profit from recanalizing strategies within a period of 24 h after ischemia onset [37] and individual characteristics that allow recanalization beyond the 24-h time window are sought-after [51]. Since young male, genetically identical and healthy mice do not represent the classical cohort of human stroke patients, future studies addressing potentially neuroprotective effects of drugs impacting on CNP activity should include aged, multi-morbid mice of both sexes [52]. For CNP, especially age-related alterations of myelin-associated lipid rafts and mitochondrial function have previously been demonstrated, all of which can be linked to axonal and myelin pathologies [53,54]. Since the hormone status was shown to impact on the infarct size and outcome [55,56], the present study only included male animals in order to exclude effects of the estrous cycle. However, as women exhibit a higher rate of stroke and related mortality, especially after reaching the menopause [57], aged female mice might show stronger alterations of AQP4, CNP or NF-L, which should be addressed in future studies.

5. Conclusion

We here provide evidence for a concomitant and region-specific ischemic response of oligodendrocytes, astrocytes and the neuronal cytoskeleton. These simultaneously occurring alterations implicate cellular interdependencies within the NVU and highlight CNP, NF-L and AQP4 as highly ischemia-sensitive elements. Because CNP was considered to be functionally involved in maintaining cellular integrity [14,15], our findings suggest a critical role of CNP during ischemia formation toward long-lasting tissue damage. As the described alterations also involved potentially salvageable tissue of the ischemic penumbra, future neuroprotective approaches may focus on cytoskeletal elements and support glial function to facilitate neuronal survival in the clinical setting of stroke.
Author contributions

Study design: DM, WH. Regulatory affairs concerning animal experiments: DM, MK. Animal experiments: DM, BM. Tissue preparation: BM, SA, AB. Immunofluorescence labeling, microscopy analyses, figure generation: BM. Manuscript preparation: BM, DM. Critical revision: WH, MK.

Declaration of Competing Interest

None.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1172/JNeuol.2019.134405.

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