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The pharmacological suppression of spreading depolarization in ischemic neuroprotection

Phd Thesis

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1. Introduction

1.1. Ischemic stroke and spreading depolarization

Ischemic stroke is a prominent, acute form of cerebrovascular disease, responsible for 87% of all stroke cases. The ischemic tissue is divided into two major zones: the ischemic core and the penumbra. In the ischemic core region, the perfusion is severely decreased and the nervous tissue undergoes rapid necrosis. In the surrounding penumbra region, residual blood flow persists 60-80 % below the baseline cerebral blood flow (CBF). The penumbra consists of electrophysiologically inactive but viable and, most importantly, salvageable tissue, which places it in the center of ischemic neuroprotective therapy. With the progression of time after stroke onset, the penumbra region transforms into the core region, increasing lesion size and worsening neurological outcome. The neurophysiological phenomenon that has been recognized to accelerate the conversion of the ischemic penumbra to the infarcted region is spreading depolarization (SD), a wave of mass depolarization of neurons and glia that propagates across the cerebral grey matter at a rate of 2–6 $mm \cdot min^{-1}$. The restoration of the membrane potential requires the mobilization of metabolic resources for the operation of ATP-dependent ion pumps (e.g. the Na^+/K^+ ATP-ase). This need is met by a profound hyperemic CBF response coupled to SD in otherwise non-compromised tissue. The CBF response to SD typically consists of four elements: an early hypoperfusion, a dominant hyperemia, sometimes a late hypereamia and a long-lasting oligaemia. The coupling between SD and local CBF becomes impaired under ischemia, which may create a metabolic supply-demand mismatch, a subsequent energy crisis superimposed on prevailing ischemia, and ultimately facilitate tissue injury.

1.2. Pharmacological targets to suppress SD

We first tested the promising novel dihydropyridine compound LA1011, which has been known as a co-inducer of heat shock proteins (Hsp; especially Hsp27 and Hsp70) with no affinity to calcium channels and, hence, devoid of vasodilator and anti-hypertensive action. Long-term administration of LA1011 was shown to improve learning ability, counteract neuronal loss, increase dendritic spine density, and restrain tau pathology and amyloid plaque formation in a transgenic mouse model of Alzheimer's disease, possibly by maintaining protein homeostasis. Taken that Hsp27 and Hsp70 activation has been suggested to be protective against ischemic neurodegeneration, it is of interest whether LA1011, a co-inducer of Hsp, has the potential to preserve neuronal function under cerebral ischemia. ***Here, we set out to assess the impact of***

LA1011 treatment on neuronal activation, SD and the development of the coupled CBF response in the acutely ischemic rat cerebral cortex (Aim 1) (Fig. 1).

The PGF2α – FP receptor signaling cascade has been implicated in ischemia/reperfusion-induced neurodegeneration, as well. The level of PGF2α was shown to increase in the brain tissue and the cerebrospinal fluid in animal models of cerebral ischemia. Neurological deficit and ischemic lesion volume were found reduced in FP receptor knock-out compared to wild type mice, and the pharmacological antagonism of FP receptors also resulted in smaller infarct volume and better functional outcome in a mouse model of focal cerebral ischemia.

The synthesis of the experimental results on the implication of PGF2α – FP receptor signaling in ischemic brain injury and SD prompted us to hypothesize that SD must be a central mechanism in FP receptor activation-related ischemic neuronal injury. We postulate that neuronal FP receptors are activated by PGF2α accumulating with SD, which may directly promote neurodegeneration. Simultaneously, cerebrovascular PGF2α – FP receptor signaling in response to SD may potentiate vasoconstriction, and thereby impede neuronal survival under penumbra-like ischemic conditions. ***The purpose of our work has been to evaluate the protective potential of FP receptor antagonism against SD and the related neurodegeneration (Aim 2) (Fig. 1)***.

N,N-Dimethyltryptamine (DMT) is a natural indole alkaloid found in plants (e.g. Psychotria viridis and Diplopterys cabrerana) and is known for its psychedelic effects when ingested by humans as spiritual medicine or with recreative purposes (e.g. ayahuasca brew). Importantly, DMT has also been identified as an endogenous trace amine in mammalian tissues (e.g. lung, pineal gland, brain) and body fluids (urine, blood plasma and cerebrospinal fluid). The physiological and pathophysiological role of endogenous DMT has been the subject of ongoing debate and speculations. DMT has been claimed to be produced at increased concentration in the rodent brain when exposed to environmental stress delivered by electrical shock. More importantly, the DMT content of the rat brain was found elevated after experimental cardiac arrest. Recently, DMT was shown to mitigate the deleterious consequences of hypoxia and focal cerebral ischemia.

The pharmacological mechanisms of DMT action have been extensively explored, revealing a complex interaction with numerous signaling pathways including biogenic amine receptors, uptake transporters, and trace amine-associated receptors. In addition, fundamental evidence has been gathered that DMT acts as an endogenous sigma-1 receptor (Sig-1R) agonist. Sig-1Rs are intracellular receptors localized to the segment of the endoplasmic reticulum (ER) membrane, which is apposed to the mitochondrial outer membrane (the mitochondria-

associated ER membrane, MAM). Via precise molecular machinery, Sig-1Rs are known to control Ca^{2+} trafficking between the MAM and mitochondria, regulate the expression of specific potassium channels, suppress the generation of reactive oxygen species, and ultimately promote cell survival under stress. ***We set out to explore whether DMT administration achieves neuroprotection via Sig-1R activation in the acute phase of experimental, transient, cerebral forebrain ischemia, which we aggravated by the recurrent induction of SD and a subsequent transient hypoxic episode (Aim 3) (Fig. 1).***

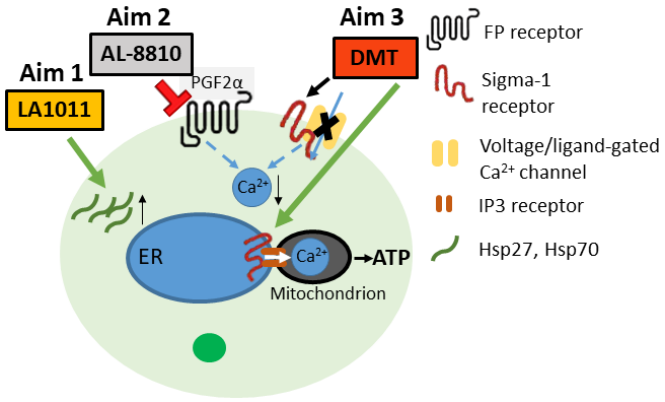

Figure 1. A schematic illustration of a cell (neuron, astrocyte or cerebrovascular smooth muscle cell) to represent the cellular targets of pharmacological interventions and the related Aims of the study. Abbreviations: ATP: adenosine triphosphate, DMT: N,N-dimethyltryptamine, ER: endoplasmic reticulum, Hsp: heat shock protein, IP_3 receptor: inositol triphospate receptor, PGF2α: prostaglandin-F2α.

2. Materials and methods

Young adult, male Sprague-Dawley rats (n = 132) were used in this study. On the day of the experiments, animals were anaesthetized with 1.5–2% isoflurane in N_2O:O_2 (3:2) except in the study with LA1011, where in Series 2, isoflurane was substituted with α-chloralose for the period of actual data acquisition. The mean arterial blood pressure (MABP) was continuously monitored via a left femoral artery catheter. The adjacent femoral vein was also cannulated for the administration of the agents (anaesthetic or drug infusion), whenever relevant. The common carotid arteries were disected for the later initiation of incomplete global forebrain ischemia. Two cranial windows were prepared over the right parietal cortex. The rostral window was later used for data acquisition (i.e., electrophysiology and CBF measurement) and topical drug administration, while the caudal window served SD elicitation. In the experiment with AL-8810, the caudal window was used for electrophysiological monitoring, while the rostral window served SD elicitation. The rest of the bone was thinned, including the medial part of the contralateral parietal bone in order to visualize CBF changes with laser speckle contrast imaging (LSCI). Experiments were terminated at the end of the experimental protocol by an overdose of the anaesthetic agent or the animals were transcardially perfused for histological analysis.

2.1. Application of the novel dihydropyridine derivative LA1011

In Series 1, LA1011 (property of Gedeon Richter Plc., kindly provided at the time of experimentation by LipidArt Ltd., Szeged, Hungary; 1 $mg \cdot kg^{-1}$ body weight in 0.4 ml saline) or its vehicle (i.e., saline) was administered i.p. in equal volume, twice a day for 2 weeks before surgical intervention. In Series 2, the rostral cranial window was incubated with LA1011 (100 μM, in aCSF) following a 5 min baseline recording. In other rats, rinsing the cranial window with vehicle served as control for pharmacological treatments. In Series 1, three SD events were triggered in each animal by placing a 1 M KCl-soaked cotton ball in the caudal craniotomy. In Series 2, after drug incubation for 30 min, both common carotid arteries were permanently occluded (two-vessel occlusion [2VO]). Sham operated rats served as control for 2VO. Fifteen minutes after 2VO onset, whisker stimulation involving the entire left whisker pad was performed mechanically. Subsequently, exactly as in Series 1, three SD events were triggered. Considering the combination of 2VO and pharmacological manipulations, six experimental groups were established.

2.2. FP receptor inhibition with AL-8810

After 10 min of baseline period, both common carotid arteries were occluded. The FP-receptor antagonist AL-8810 (1 mg/bwkg, dissolved in 0.1% dimethyl sulfoxide (DMSO) in physiological saline, Sigma- Aldrich, USA) or its vehicle were bolus administered i.v. in 1 ml volume 10 min after 2VO onset. SDs were triggered with a 1 M KCl-soaked cotton ball placed on the cortex 20 min after drug infusion. Reperfusion was initiated by releasing the carotid arteries, and the animals were kept under light sedation (1% isoflurane) for an additional 3 h in order for ischemic tissue injury to mature. The animals were transcardially perfused with ice-cold physiological saline followed by 4% paraformaldehyde (PFA) under deep chloral hydrate anesthesia (5%, i.p., 500 mg/bwkg) at the end of the experimental protocol. Slices were selected for the permanent immunocytochemical staining of cleaved caspase-3 (CC3), the immunofluorescent co-localization of CC3 with the neuron marker neuronal nuclear protein (NeuN) or the astrocyte marker glial fibrillary acidic protein (GFAP), or the immunofluorescent co-localization of FP receptors with neural, glial, as well as cerebrovascular elements. Microglial activation was detected with Iba-1 immunohistochemistry.

2.3. Putative Sig-1R agonism with DMT

All drugs were dissolved in physiological saline, and administered at a dose of 1mg/bwkg given over an hour and infused through the left femoral vein continuously throughout the experimental protocol. The following treatments were applied: DMT alone, the highly selective Sig-1R agonist PRE-084 alone, the selective Sig-1R antagonist NE-100 alone or in combination

with DMT, and the non-subtype specific serotonin receptor (5-HTR) antagonist asenapine alone or in combination with DMT. Vehicle (i.e. physiological saline) served as control for the treatments. NE-100 and DMT or asenapine and DMT were used in combination to test the receptor-specificity of DMT.

After a baseline period (5 min), cerebral ischemia was induced by 2VO. The administration of DMT, PRE-084 or vehicle was initiated upon ischemia induction through the femoral vein, with the aid of a microinjector syringe pump. In the case of DMT co-application with NE-100 or asenapine, the infusion of NE-100 or asenapine began 10 min before ischemia induction to act on receptors before DMT application. As further metabolic challenge superimposed on ischemia, three SD events were triggered at an inter-SD interval of at least 15 min with a 1 M KCl-soaked cotton ball placed on the cortex in the caudal craniotomy, starting 10 min after 2VO onset. The ischemic insult was further aggravated by an episode of transient hypoxia (1 min) achieved by the controlled withdrawal of O_2 from the anesthetic gas mixture. The hypoxic episode was followed by reoxygenation and the prompt release of the common carotid arteries to afford cerebral reperfusion for another hour.

Naïve and vehicle- or DMT-treated rats exposed to the full experimental protocol were transcardially perfused and coronal brain slices were made. Apoptotic cell death was characterized with permanent immunocytochemical staining of CC3, ferroptotic injury by 4-hydroxinonenal (4-HNE), whereas neuronal loss was identified with the neuron marker NeuN, and astrocyte damage with the astrocyte marker GFAP on selected coronal slices. Microglial activation was detected with Iba-1 immunohistochemistry. In order to identify the cellular and subcellular localization of Sig-1Rs, the receptors were co-labelled with NeuN (neuronal marker), GFAP (astrocyte marker) and Iba-1 (microglia marker) in slices from a naïve animal.

3. Results

3.1. Application of the novel dihydropyridine derivative LA1011

3.1.1. Physiological variables and baseline variations of CBF

Statistical analysis did not reveal any significant ischemia- or treatment-related difference in physiological variables (MABP, arterial blood pH, and partial arterial pressure of O_2 and CO_2) across experimental groups.

Variation of baseline CBF was assessed at selected time points of the experimental protocol, in order to evaluate the impact of pharmacological treatments. Ischemia induction in Series 2 obviously caused a marked reduction of CBF (53±23%), which stabilized in the vehicle-treated group at 74 ± 11% prior to first SD (SD1) and at 67±15% prior to recurrent SDs (rSDs). The

occurrence of SD produced long-lasting oligaemia, the final element of the CBF response to SD, which was apparent between SD events in the intact groups, as well. Neither chronic nor acute treatment with LA1011 altered baseline CBF at any time point or condition considered.

3.1.2. Somatonsensory stimulation

For the estimation of drug effect on physiological neurovascular coupling, somatosensory evoked field potentials (EFPs) and the associated CBF response provoked by whisker stimulation were evaluated in the somatosensory barrel cortex in Series 2. The amplitude of EFPs was considerably attenuated during ischemia (in the presence of 2VO) with respect to the intact condition. The application of LA1011 exerted no effect on EFPs. The relative amplitude of the hyperemic response was notably smaller during ischemia (2VO) than in the intact brain. Treatment with LA1011 did not improve functional hyperemia significantly.

3.1.3. Spreading depolarization

Experimentally-evoked SDs were exploited for the investigation of drug effect on non-physiological neural depolarization and the associated CBF response. The SD1 was analyzed separately from rSD events, because of known dissimilarities between SD1 and rSDs. Eventually, detailed data analysis was completed on rSDs. As expected, the amplitude of SD events was not altered, while their duration was extended during ischemia with respect to the intact condition. Chronic or acute application of LA1011 consistently intensified SD amplitude, irrespective of ischemia.

The CBF response to SD consists of four elements, starting with a transient hypoperfusion, followed by a peak and then a late hyperemia, and concluded by a long-lasting oligaemia. The share of the individual elements in the CBF response to SD is variable across animal species and anaesthesia protocols and appears to change markedly according to the actual metabolic status of the tissue. The analysis of the CBF response here predominantly focused on the hyperemia phase. The kinetics of the observed CBF responses exhibited a spectrum considering the weight of late hyperemia in the signature. Further, the presence of the late hyperemic element served as the basis for CBF response classification to distinguish CBF response Type 1 characterized by peak hyperemia only, from CBF response Type 2 that included late hyperemia in addition to the peak hyperemia. A semi-quantitative approach of ours indicated that the likelihood for Type 1 and Type 2 CBF responses to evolve was near equal in the vehicle-treated, intact condition. Conversely, ischemia, or treatment with LA1011, allowed late hyperemia to emerge at a clearly higher incidence. The amplitude of peak hyperemia was conserved over

experimental groups. The duration of hyperemia (i.e., peak and late hyperemia together) was not significantly altered by ischemia or the treatments.

3.2. FP receptor inhibition with AL-8810

3.2.1. Physiological parameters

The systemic physiological variables were within the normal range throughout the experimental protocol. Ischemia increased MABP (e.g. vehicle group: 85.3±10.5 vs. 78.0±9.3 mmHg, ischemia vs. baseline) and arterial pO_2 (e.g. vehicle group: 110.9±11.8 vs. 105.4±15.1 mmHg, ischemia vs. baseline), as in other experimental models of cerebral ischemia. The treatment with AL-8810 did not exert any detectable impact on the examined variables.

3.2.2. Spreading depolarization

We explored whether the antagonism of the FP receptor of PGF2α modulates the occurrence or duration of SDs beneficially. In case of the initial, controlled elicitation, all SDs were transient with no statistical difference in the DC potential signature between the two groups (i.e. amplitude: −18.3±2.7 vs. −18.0±2.6 mV, AL-8810 vs. vehicle; duration at half amplitude: 55±25 vs. 56±19 s, AL-8810 vs. vehicle).

Subsequent to controlled SD elicitation, clusters of SDs were triggered with continuous KCl exposure. Each of these recurrent SDs was categorized as transient, prolonged or incomplete, on the basis of their DC potential signature. Then their incidence relative to the total number of recurrent SDs was expressed. The antagonism of FP receptors with AL-8810 prevented the evolution of prolonged SDs (0 vs. 15%, AL-8810 vs. vehicle), and achieved a complementary increase in the incidence of incomplete SDs (25 vs. 5%, AL-8810 vs. vehicle). At the same time, the share of regular transient SDs of all recurrent events remained comparable between the two experimental groups (75 vs. 80%, AL-8810 vs. vehicle).

Detailed, quantitative analysis of recurrent SDs focused on transient events. Similar to the controlled SDs, the amplitude of the clustered SDs was similar in the two experimental groups (−17.5±2.4 vs. −16.9±3.1 mV, AL-8810 vs. vehicle). Nevertheless, AL-8810 treatment accelerated the repolarization after SDs (0.84±0.4 vs. 0.45±0.22 mV/s, AL-8810 and vehicle). Accordingly, the average duration of an SD at half amplitude (30±10 vs. 56±14 s, AL-8810 vs. vehicle), as well as the cumulative duration of recurrent transient SDs was found shorter in the AL-8810-treated compared to the control group (304±71 vs. 577±154 s, AL-8810 vs. vehicle). In line with these data, the magnitude of individual SDs expressed as area under the curve (AUC) was reduced in the AL-8810 treated group compared to control (585±192 vs. 942±251 mV*s; AL-8810 vs. vehicle).

The spatial resolution offered by LSCI in our experiments enabled a comprehensive analysis of the CBF response to SD. We concentrated our detailed analysis on recurrent SDs, which emerged as regular, transient negative DC potential shifts (amplitude>10 mV, duration<3 min) at the electrode implanted. First, with the aid of placing multiple regions of interest (ROIs) on the CBF videos, we noted that the kinetics of the CBF response associated to a given SD may alter over the course of SD propagation. Hyperemic CBF response was most frequently encountered at the ROI positioned over the open cranial window incorporating the electrode (92 and 100%, AL-8810 and vehicle). The CBF response to the same SD often escaped detection at ROIs over the thinned parietal bone (anterior: 31 and 48%, posterior: 35 and 26%, AL-8810 and vehicle). Finally, the CBF response transformed occasionally to hypoperfusion at the anterior ROI placed over the medial fronto-parietal cortex, particularly in the control group (12 and 22%, AL-8810 and vehicle).

The detailed, quantitative analysis of the hyperemic CBF responses revealed that AL-8810-treatment elevated baseline CBF prior to SD (e.g. lateral ROI: 73.0±13.8 vs. 56.8±6.3%; AL-8810 vs. vehicle), and therefore augmented the absolute amplitude of the SD-coupled hyperemia, as well (e.g. lateral ROI: 104.1±25.0 vs. 76.0±8.6%, AL-8810 vs. vehicle). Further, AL-8810 treatment attenuated hypoemic CBF responses, which was reflected by the smaller relative amplitude of the hypoperfusion transients (−5.4±1.7 vs −14.0±1.8 pp., AL-8810 vs. vehicle). Also, progressive perfusion deficit during continuous SD elicitation was less prominent in the AL-8810 treated group compared to control, resulting in higher CBF prior to the initiation of reperfusion (68.2±14.5 vs. 58.6±9.6%, AL-8810 vs. vehicle). Finally, the rate of propagation of the SD coupled CBF response was accelerated under AL-8810 treatment compared to the control condition (3.0±0.7 vs. 2.4±0.5 mm/min, AL-8810 vs. vehicle).

3.2.3. Alpha-to-delta ratio (ADR)

We calculated ECoG power for alpha (8–13 Hz) and delta (1–4 Hz) frequency bands and expressed ADR for 4 selected time points over the experimental protocol. As expected, ADR taken prior to ischemia induction and the initiation of AL-8810 treatment was comparable for the two experimental groups (0.67±0.20 and 0.63±0.29; AL-8810 and vehicle). ADR remained similar 30 min after the induction of ischemia and the administration of AL-8810 (0.81±0.36 and 0.65±0.26; AL-8810 and vehicle). After the controlled elicitation of two SDs, ADR reduced in both experimental groups equally (0.45±0.23 and 0.36±0.22; AL-8810 and vehicle). Finally, 5 min after the initiation of reperfusion and the cessation of clusters of recurrent SDs, ADR

increased selectively in the AL-8810 treated group (0.66±0.29 vs. 0.35±0.17; AL-8810 vs. vehicle).

3.2.4. Neuroprotection achieved with AL-8810

CC3+ apoptotic cells were observed in striatal, hippocampal and cortical areas in both experimental groups, albeit to a lesser degree in the AL-8810-treated animals. The immunofluorescent colocalization of CC3 with NeuN and GFAP revealed that both neurons and astrocytes were engaged in apoptosis. With the anticipation of more extensive apoptosis in the hemisphere ipsilateral to SD elicitation, the number of CC3+ cells was normalized to the contralateral side. The quantitative approach suggested a significant neuroprotective effect of AL-8810 shown by the reduction of CC3+ cell numbers in the ipsilateral relative to the contralateral hemisphere (e.g. somatosensory and occipital cortices: 94.4±12.6% vs. 112.9±7.0%; 104.3±17.6% vs. 125,7±17,7%, respectively, AL-8810 vs. vehicle).

Microglia activation is a rapid neuro-inflammatory response to injury, which was previously suppressed with AL-8810, assessed days after experimental traumatic brain injury. We have observed intensive Iba1 immuno-labeling in the tissue samples. While the number of labeled microglia was similar in the two hemispheres irrespective of treatment (31±7 and 28±6, contraand ipsilateral), their arborization was less dense in the ipsilateral hemisphere indicative of their increased activation (ramification index: 112.7±55.4 vs. 174.2±73.8, ipsi- vs. contralateral). The administration of AL-8810 did not exert any discernable impact on the ramification of microglia processes (ramification index: 148.6±63.8 and 138.3±80.3, control and AL-8810).

3.2.5. FP-receptor co-localization

FP receptors have been previously identified in brain synaptosomes and microvessels in newborn and adult pigs, and cultured rat astrocytes. Here we set out to visualize FP receptor presence on neuronal, glial (astrocyte and microglia) and cerebrovascular (endothelial and smooth muscle cell) compartments as potential sites of AL-8810 action. The co-localization of FP receptors with NeuN and GFAP confirmed the localization of FP receptors predominantly in the nuclear envelope of neurons, as well as in the perikaryon and perivascular endfeet of astrocytes. In addition, we observed a robust colocalization of FP receptors with Iba1-labeled microglia. The presence of FP receptors was also obvious in claudin-labeled cerebromicrovascular endothelial cells. However, we were not able to detect FP receptors unequivocally in α-actin-labeled cerebrovascular smooth muscle cells.

3.3. Putative Sig-1R agonism with DMT

3.3.1. Physiological variables

Blood gases varied in the physiological range throughout the experimental protocol, yet these physiological variables significantly shifted over the reperfusion period with respect to baseline and ischemia/hypoxia. In particular, by the end of the reperfusion period, blood pH showed a decreasing tendency (pH 7.27±0.17 vs. 7.31±0.11 and 7.3±0.12, reperfusion vs. baseline and ischemia/hypoxia; $F=2.525$, $p<0.085$), pCO_2 noticeably increased (42.3±13.6 vs. 33.2±7.6 and 34.3±10.5 mmHg, reperfusion vs. baseline and ischemia/hypoxia; $F=9.102$, $p<0.0003$**) and pO_2 decreased (99.6±24.3 vs. 111.2±17.0 and 108.4±26.6; $F=3.534$, $p<0.033$*).

MABP values confirmed sufficiently deep anesthesia. MABP slightly elevated during ischemia (83±8 vs. 77±4 mmHg, ischemia vs. baseline) and fell due to transient hypoxia (to 66±8 mmHg), at no statistical significance over the experimental protocol ($F=3.349$, $p<0.127$). DMT application had no discernable impact on MABP ($F=0.125$, $p<0.738$). Heart rate was stable throughout the experimental protocol (e.g. vehicle group: 331±27, 327±41 and 319±43 bpm, baseline, ischemia and reperfusion; $F=0.012$, $p<0.918$). DMT elevated heart rate slightly but not significantly (e.g. reperfusion: 344±19 vs. 319±43 bpm, DMT vs. vehicle; $F=1.290$, $p<0.307$).

Any consequence of DMT application on baseline CBF variation was also considered. After ischemia induction, CBF dropped sharply and then stabilized at 40.3±4.8 % prior to SD1, and at 34.2±10.1 % prior to the induction of transient hypoxia in the vehicle-treated group. With hypoxia, perfusion decreased further (to 28.3±5.4 %). Reactive hyperemia was not obvious as reperfusion was imposed; instead, CBF peaked at 70.1±17.8 %. DMT did not alter baseline CBF at any time point or condition considered.

3.3.2. The impact of DMT on the evolution of spreading depolarization

The amplitude of SD was reduced at the presence of DMT significantly (-16.5±4.1 vs. -20.1±1.3 mV, DMT vs. vehicle). Likewise, the rate of depolarization proved to be also slower in the DMT-treated group with respect to control (-2.62±1.28 vs. -3.48±0.94 mV/s, DMT vs. vehicle). Finally, the cumulative duration of SD events was shortened in the DMT treated group (140±38 vs. 191±42 s, DMT vs. vehicle).

The share of Sig-1R activation in DMT-linked SD attenuation was explored by the application of a highly selective Sig-1R agonist PRE-084, or the use of a Sig-1R antagonist NE-100 alone or in combination with DMT. At the presence of PRE-084, the DMT-related reduction of SD amplitude and slower rate of depolarization were replicated (i.e. amplitude: -16.2±5.6 vs. -20.1±1.3 mV, PRE-084 vs. vehicle; rate of depolarization: -2.26±1.06 vs. -3.48±0.94 mV/s,

PRE-084 vs. vehicle) and the cumulative duration of SDs also tended to be shorter (149±31 vs. 191±42 s, PRE-084 vs. vehicle). NE-100 alone did not cause any notable change in SD amplitude, rate of depolarization or cumulative duration (e.g. amplitude: -18.7±2.6 vs. -20.1±1.3 mV, NE-100 vs. vehicle). In contrast with DMT applied alone, the co-application of NE-100 and DMT did not achieve any meaningful reduction of SD amplitude (-19.2±4.5 vs. -20.1±1.3 mV, NE-100+DMT vs. vehicle) or depolarization rate (-3.06±1.18 vs. -3.48±0.94 mV/s, NE-100+DMT vs. vehicle) or cumulative duration (220±12 vs. 191±42 s, NE- 100+DMT vs. vehicle), either.

Next, we considered the possibility that DMT may activate 5-HTRs and thereby inhibit SD. To this end, we administered the broad spectrum 5-HTR antagonist asenapine alone or co-applied with DMT. The amplitude of SD was significantly greater at the presence of asenapine alone as compared with vehicle (-22.6±2.6 vs. -20.1±1.3 mV, asenapine vs. vehicle). The co-application of DMT with asenapine re-established SD amplitude to control level (-19.1±1.3 vs. -22.6±2.6 mV, asenapine+DMT vs. asenapine). These data collectively suggest that the SD-suppressing action of DMT was likely realized, at least in part, via Sig-1R activation.

Of the three subsequent elements of the CBF response to SD our analysis predominantly focused on the phase of hyperemia, which evolved reliably with each SD. We narrowed down our analysis to recurrent SDs, because the kinetics of the CBF response to the first SD in a train is substantially different from the response given to recurrent SDs.

In our experiments, the CBF response to recurrent SDs appeared to be resistant to all of the used pharmacological agents, including DMT or the selective Sig-1R agonist PRE-084. Like other parameters (i.e. amplitude or duration of hyperemia), the magnitude of hyperemia characterized by the AUC was similar across various treatment regimes.

3.3.3. Neuroprotection achieved with DMT and Sig-1R co-localization

Neuronal survival has been characterized by the surface area occupied by NeuN-labeled neurons relative to the full field of view, in the somatosensory cortex and the hippocampus. With respect to naïve animals, no significant difference in NeuN immunopositivity was noted due to ischemia/hypoxia/reperfusion (e.g. somatosensory cortex: 19.9±1.9 vs. 19.3±1.4%, vehicle vs. naive). Neuronal loss that might have been associated with SD was not found, either (e.g. somatosensory cortex: 19.5±3.1 vs. 20.2±3.2%, ipsi- vs. contralateral side to SD). Finally, DMT treatment exerted no meaningful effect on NeuN labeling (e.g. contralateral somatosensory cortex: 17.4±2.0 vs. 19.9±1.9%, DMT vs. vehicle).

As neurons in the hippocampus CA1 and dentate gyrus (DG), the most vulnerable regions to ischemia are packed with pericarya so tightly that NeuN staining may not reveal individual cell loss, we labeled cells engaged in apoptotic cell death with CC3 immunostaining. Sections from naïve animals were virtually devoid of CC3+ cells (i.e. DG: 163±14 CC3+ cells per mm^2), while CC3 labeled cell bodies were obvious in the cortex, hippocampus CA1 region, and particularly in the DG after ischemia/hypoxia/reperfusion in the vehicle group (DG: 1649±278 CC3+ cells per mm^2). The aggravation of apoptosis by recurrent SDs was anticipated in the cortex, where SDs propagated, but no lateralization of CC3+ cell count was detected (vehicle group: 106±46 vs. 104±67 CC3+ cells per mm^2, ipsi- vs. contralateral). For this reason, CC3+ cell counts obtained from the two hemispheres were averaged for each region. Importantly, significantly fewer CC3 labeled apoptotic cells were counted in the DMT-treated compared to vehicle-treated animals in the somatosensory cortex (66±33 vs. 105±56 CC3+ cells per mm^2, DMT vs. vehicle), hippocampal CA1 region (532±268 vs. 893±249 CC3+ cells per mm^2, DMT vs. vehicle) and DG (1367±311 vs. 1649±278 CC3+ cells per mm^2, DMT vs. vehicle).

In addition to apoptosis, ischemic/hypoxic stress may induce ferroptosis, an alternative pathway of programmed cell death that has been recognized recently. The typical hallmark of ferroptosis is the iron-dependent accumulation of lipid hydroperoxides, hence the cellular process can be detected by 4-HNE immunohistochemistry. Representative cases have testified that our experimental protocol induced ferroptosis in selected neurons, particularly in the hippocampus, which was suppressed by DMT treatment.

Because astrocytes have been widely known to support neuronal function under ischemia, and GFAP-positive protoplasmic astrocytes may become selectively vulnerable to ischemic conditions, we have explored whether DMT has the potential to protect astrocytes. We used GFAP immunocytochemistry to label astrocytes. The relative area occupied by GFAP immunolabeled compartments considerably decreased after ischemia/hypoxia/reperfusion compared to naive animals, which reduction was counteracted by DMT-treatment in the cortex and striatum (cortex: 3.2±1.9 vs. 1.5±0.3 vs. 4.7±0.9%, striatum: 2.3±1.1 vs. 0.9±0.2 vs. 3.2±1.6%, DMT vs. vehicle vs. naive), but not in the hippocampus (i.e. CA1 stratum oriens 10.6±4.9 vs. 11.1±5.8 vs. 15.5±2.7%, DMT vs. vehicle vs. naive).

Ischemic insults trigger microglial activation and neuroinflammatory reaction that may be modulated via Sig-1Rs. In the cerebral cortex ipsilateral to the craniotomies and SD initiation, decreasing microglial arborization was expected to denote the amplification of microglial activation. The current data confirm the previous observations by showing less dense microglia ramification in the ipsilateral cortex compared to the contralateral side (vehicle group:

106.6±50.5 vs. 194.3±95.6, ipsi- vs. contralateral). Yet, the administration of DMT did not exert any distinguishable effect on the microglial ramification index (i.e. ipsilateral cortex: 89.5±37.4 vs. 106.6±50.5 DMT vs. vehicle).

Finally, to identify cell types that may be targeted by DMT, we co-localized Sig-1R with neurons, astrocytes and microglia with fluorescent immunocytochemistry in naïve rats. Sig-1Rs were expressed abundantly in neurons, and were also associated with astrocytes and resting microglia.

4. Discussion

4.1. Application of the novel dihydropyridine derivative LA1011

4.1.1. LA1011 exerted no effect on baseline CBF and evoked potentials

The baseline CBF remained unchanged after LA1011 administration in the anaesthetized rat in contrast with what is expected of other dihydripyridine derivatives (e.g. nifedipine, nimodipine). The current data also demonstrate that LA1011 did not alter neuronal excitability in the intact cortex and did not restore EFP amplitude or the hyperemic response compromised by ischemia, either. These results indicate that acutely administered LA1011 does not protect neuronal excitability or neurovascular coupling during the early phase of cerebral ischaemia.

4.1.2. LA1011 increased SD amplitude with no impact on the CBF response

LA1011 treatment greatly enhanced the amplitude of SD, consistent in all experimental conditions created. The amplitude of SD stands in a strong, positive correlation with the extracellular concentration of potassium, which would infer that treatment with LA1011, an Hsp co-inducer, may support cellular (possibly neuronal) potassium efflux with SD. Indeed, heat shock preconditioning preserved the diminishing potassium peak of recurrent depolarizations triggered by intermittent anoxia, and Hsp70 expression modulated potassium homeostasis throughout oxygen withdrawal in the drosophila brain. TNFα is also known to reduce the amplitude of SD in a dose-dependent manner. Interestingly, the overexpression of Hsp70 - a target of LA1011 - was shown to suppress TNFα mRNA levels in cultured, oxygen–glucose deprived (OGD) astrocytes. On the basis of these observations, it would be attractive to postulate that LA1011 increased SD amplitude possibly by the co-induction of Hsp70 to cause the downregulation of TNFα, which would normally limit the magnitude of SD.

In case of the CBF response to SD, the experimental manipulations accentuated the element of late hyperemia in the SD associated CBF response. On the basis of an original finding that L-arginine administration supported late hyperemia following SD, late hyperemia was proposed to be NO dependent. Interestingly, Hsps, particularly Hsp90, have been implicated in the

adjustment of vascular tone via modulating the production of the vasodilator NO, and a recent report has shown LA1011 to bind Hsp90 and activate the ATPase activity of Hsp90. A potential interaction between LA1011 and NO release to unravel late hyperemia in the SD related CBF response may thus be mediated via Hsp90.

4.2. FP receptor inhibition with AL-8810

4.2.1. FP-receptor antagonism improved perfusion in the ischemic cerebral cortex

Our data demonstrate that the antagonism of the FP receptor suppressed SD in the ischemic rat cerebral cortex, reduced the duration of recurrent SDs by facilitating repolarization and augmented the recovery of ECoG during reperfusion. FP receptors at the periphery have been previously implicated in the regulation of vascular tone. Since FP receptor antagonism in our experiments markedly improved the perfusion of the ischemic cerebral cortex, it is conceivable that SD duration was shortened in the AL-8810-treated animals because the tissue was metabolically less compromised.

Spreading ischemia coupled to SD that propagates across vulnerable penumbra tissue has been considered particularly harmful. The occurrence of spreading ischemia was less frequent under AL-8810 treatment, and flow reduction with the observed events in the AL-8810 group was considerably attenuated.

4.2.2. FP receptor antagonism suppressed SD and curtailed the related apoptotic cell death, but did not alter microglia activation

The pharmacological inhibition of the FP receptors in our experiments could have contributed to neuroprotection by targeting neurons directly, as well. We have observed conspicuous FP receptor labeling co-localized with neurons, which is consistent with previous work that revealed the presence of FP receptors in synaptosome preparations or cultured neurons. In support of the notion of direct neuroprotection by FP receptor antagonism, AL-8810 proved to sustain the viability of cultured neurons exposed to OGD.

The impact of AL-8810 treatment on microglia was evaluated here, as well, because microglia were found to be richly endowed with FP receptors. Previously, cultured rat microglia were shown not to express FP receptor mRNA, yet, later investigation identified functional FP receptors on transformed human brain microglial cells. In our experiments, microglia activation proved to be enhanced in the cortex ipsilateral to SD recurrence and the open cranial window, in addition to that caused by ischemia alone. Our previous data have shown that the surgical procedure itself (i.e. craniotomy) causes microglia activation, and recurrent SDs enhance it further, which amounts to the difference observed between the ipsi- and contralateral

hemispheres in our preparations. AL-8810 didn't alter microglia activation at this time point after the primary insult (i.e. 5 h after ischemia onset).

4.3. Putative Sig-1R agonism with DMT

4.3.1. DMT attenuated SD possibly through Sig-1R activation, with no effect on CBF responses

In our current experiments, SD amplitude, the rate of depolarization and cumulative SD duration were reduced under intravenous DMT administration, exhibiting the SD limiting impact of DMT. Further, the application of the selective Sig-1R agonist PRE-084 reproduced the SD hampering action of DMT, and the Sig-1R antagonist NE-100 co-applied with DMT diminished the DMT-related SD inhibition. These results collectively suggest that DMT may impede SD evolution by Sig-1R activation. The implication of Sig-1Rs is supported by previous reports demonstrating that dextromethorphan or carbetapentane – two ligands known as Sig-1R agonists – reproducibly and reliably blocked SD occurrence in live brain slice preparations. Sig-1R agonism was shown to attenuate the NMDA receptor activation-linked calcium response in cultured neurons, and to depress calcium currents with a corresponding inactivation of neuronal voltage-gated calcium channels. On the basis of these and our experimental data together, we postulate that DMT in our experiments possibly inhibited SD via Sig-1R-linked modulation of intracellular calcium homeostasis.

The suggestion that DMT predominantly achieved SD inhibition by Sig-1R activation – rather than through 5-HTR binding – is also substantiated by our finding that DMT counteracted the SD potentiating effect of asenapine, an antagonist of multiple 5-HTRs. In other words, when 5-HTRs were occupied by asenapine, DMT still reduced SD amplitude with respect to the condition when asenapine was given alone. Of note, our results that 5-HTR antagonism increased SD amplitude are complementary to previous reports showing that serotonergic activity appears to restrain SD, and, conversely, serotonergic deficiency may augment SD.

Finally, we evaluated the impact of the pharmacological treatments on the CBF response to SD, especially because it is unknown whether and how DMT might alter neurovascular coupling. DMT treatment in our experiments did not alter the SD-related hyperemia. Furthermore, none of the other agents used (the Sig-1R agonist PRE-084, the Sig-1R antagonist NE-100 or the 5-HTR antagonist asenapine) proved to be effective at modulating the SD-associated CBF response in any meaningful way. The condition that SDs occurred here in ischemic tissue could have possibly overridden physiological blood flow regulation with SD – indeed, ischemia is known to impair neurovascular coupling.

4.3.3. DMT promoted cell survival via Sig-1Rs indentified in various CNS cell types

Several groups have reported independently that Sig-1R agonism successfully reduced infarct size estimated 24 hours or a number of days after an episode of experimental focal cerebral ischemia. Therefore, we proceeded to investigate the neuroprotective potential of DMT at histological level as early as 2 hours after ischemia induction and 1 hour after reperfusion initiation. In our experiments, NeuN staining did not reveal significant loss of neurons, possibly because the end point was too early to detect massive neurodegeneration. However, the network of GFAP-positive astrocytes became remarkably sparser after ischemia/reperfusion as compared to naïve samples. This is consistent with the loss of the GFAP signal and swelling of protoplasmic astrocytes in the first hours after the onset of experimental focal cerebral ischemia, suggestive of astrocyte dysfunction. Importantly, the GFAP signal has been partially restored in our experiments when DMT was administered, and Sig-1R co-localized with astrocytes, which is complementary to a previous report demonstrating that Sig-1R agonism augmented astrocyte survival screened 96 hours after ischemia onset. These results indicate that DMT preserved the integrity of the astrocyte network – and presumably function – by Sig-1R receptor activation.

The labeling of microglia replicated our previous results that microglia activation appeared more pronounced in the cortex ipsilateral to SD elicitation. DMT has emerged to curtail inflammatory responses, substantiated by the reduced production of pro-inflammatory cytokines and the lower pro-inflammatory cytokine serum levels. Yet, in the current experiments, DMT had no discernable impact on microglia activation. Likewise, Sig-1R activation achieved no change in Iba-1 immuno-labeling and cerebral cytokine production in a focal cerebral ischemia model. Taken together, we propose that DMT treatment may suppress the activation of dendritic cells or microglia provoked with direct inflammatory stimuli in culture, but may not be as potent to inhibit microglia activation caused by ischemia or SD in anesthetized rodents.

Apoptotic cell death has emerged as an especially relevant target to investigate, because Sig-1R agonism has been revealed to suppress ER stress-related apoptosis. DMT treatment reduced here the number of CC3+ apoptotic cells, especially in the hippocampus. In addition, our qualitative approach showed that DMT reduced ferroptosis, as well. It is a novel observation, consistent with the finding that Sig-1R activation or overexpression inhibits the generation of reactive oxygen species.

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Articles to serve as the basis of the thesis

1. ***Írisz Szabó****, Orsolya M. Tóth, Zsolt Török, Dániel Péter Varga, Ákos Menyhárt, Rita Frank, Dóra Hantosi, Ákos Hunya, Ferenc Bari, Ibolya Horváth, László Vigh, Eszter Farkas* The impact of dihydropyridine derivatives on the cerebral blood flow response to somatosensory stimulation and spreading depolarization. *Br J Pharmacol* 2019 May;176(9):1222-1234. **IF: 7.73**

2. *Dániel P. Varga*, **Írisz Szabó****, *Viktória É. Varga, Ákos Menhyárt, Orsolya M. Tóth, Mihály Kozma, Armand R. Bálint, István A. Krizbai, Ferenc Bari, Eszter Farkas* The antagonism of prostaglandin FP receptors inhibits the evolution of spreading depolarization in an experimental model of global forebrain ischemia. *Neurobiol Dis* 2020 Apr;137:104780. **IF: 5.33**

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3.***Írisz Szabó****, Viktória É. Varga, Szabolcs Dvorácskó, Attila E. Farkas, Tímea Körmöczi, Róbert Berkecz, Ákos Menyhárt, Rita Frank, Dóra Hantosi, Ede Frecska, Csaba Tömböly, István A. Krizbai, Ferenc Bari, Eszter Farkas* N,N-dimethyltryptamine attenuates spreading depolarization and restrains neurodegeneration by sigma-1 receptor activation in the ischemic rat brain. Submitted: *Sci Rep* **IF: 3.99**