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Tissue acidosis associated with ischemic stroke to guide nimodipine delivery

PhD Thesis

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1. Introduction

Nowadays cerebrovascular diseases are the second most frequent cause of death and the most common cause of long-term disability worldwide. Despite this global health problem, there are only limited therapeutic possibilities, and none of them is effective for every stroke patient. It would be suitable for the medical treatment to target the site of injury selectively, to enrich the site of ongoing injury with the protective agent, and to avoid undesirable side effects at the same time.

1.1. Mechanistic insight into cellular pathomechanisms of ischemic stroke and spreading depolarization

Focal cerebral ischemia, which accounts for 80 % of all stroke cases, is caused by the obstruction of a cerebral blood vessel by atherosclerosis or embolization. The limited supply of O_2 and nutrients creates a supply-demand mismatch, which compromises neuronal function. Most often, cerebral ischemia is incomplete, the brain is partially hypoperfused. Falling cerebral blood flow (CBF) from the normal 50 ml/100 g/min value, below 10 ml/100 g/min, causes anaerob metabolism and the disruption of cell membrane transport, leading to subsequent neuronal death within minutes. In focal ischemia, adjacent to the core of the injury, residual blood flow persists, the local CBF ranging between 15-25 ml/100 g/min or 20-40 % relative to baseline. This narrow tissue band embracing the infarcted core has been known as the ischemic penumbra. In contrast with the necrotic core, the penumbra consists of electrophysiologically inactive but viable and, most importantly, salvageable tissue, which places it in the center of ischemic neuroprotective therapy.

The shortage of metabolic substrates and O_2, a condition that characterizes cerebral ischemia leads to metabolic acidosis. The limited availability of O_2 favors anaerobic glycolysis: pyruvate is reduced to lactate at the concomitant generation of a H^+, which causes lactic acidosis. In turn, tissue pH after cerebral ischemia onset decreases following an inversely linear relationship with tissue lactate concentration. In addition, tissue pCO_2 rises to 3-4 fold, which may also contribute to tissue acidosis. Tissue pH in the ischemic core may become as low as pH 6.0, while tissue pH fluctuates around pH 6.5-6.9 in the peri-infarct penumbra, as estimated in the acute middle cerebral artery occlusion (MCAO) rodent model of focal ischemic stroke.

It is important to realize that the penumbra evolves dynamically in space and time. After the onset of cerebral ischemia, spreading depolarization (SD) events evolve spontaneously from the border of the core and penumbra regions in clusters for several days after the ischemic insult, and propagate across tissue at risk. In fact, recurrent SDs (rSDs), which are appreciated to arise

at the inner penumbra, from minutes to days after the primary impact, have been understood as a principal mechanism of lesion progression in the acutely injured human brain. SD is a synchronized wave of massive depolarization of cortical neurons and glia cells, propagating slowly (2-6 mm/min) across the cerebral grey matter. SDs occur in hypoperfused nervous tissue due to metabolic supply demand mismatch, and in turn, exacerbate the ischemia-related metabolic burden. Releasing metabolites and ions (K^+, lactate, ATP/ADP, and adenosine), the production of vasoactive substances (e.g., prostaglandins), and remarkable metabolic demand with SD events generate a hemodynamic response typical of the metabolic status of the tissue. In the optimally perfused, intact brain, the CBF response to SD consists of three subsequent elements: a brief transient hypoperfusion, a transient peak and late hyperemia, and a focal, long-lasting oligemia. In contrast, in the injured brain (e. g. ischemic or hemorrhagic stroke, traumatic brain injury), vasoconstrictive elements of the CBF response dominate at the expense of hyperemia, which is instrumented by the release of vasoconstrictive substances. Simultaneous with the negative shift of the DC (direct current) potential, an extracellular pH-change is initiated starting with a short alkaline shift, which is followed by a longer lasting transient acidosis, and thus a decrease of tissue pH by around 0.3-0.5 pH units. The duration of the SD-related acid burden corresponds to SD duration lasting for a few minutes under ischemic penumbra conditions, which is increasingly longer in tissue zones undergoing more severe metabolic crisis **(Fig. 1.)**.

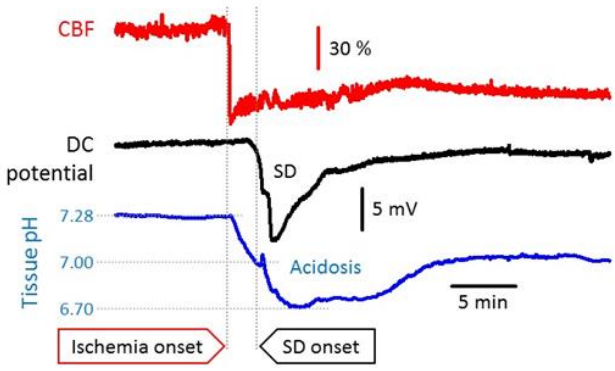

Figure 1. Representative traces demonstrate the association of tissue acidosis (blue) with ischemia onset (bilateral common carotid artery occlusion; red) and spreading depolarization (SD; black) in the rat parietal cortex. Tissue pH was measured with a pH-sensitive microelectrode implanted in the cortex, cerebral blood flow (CBF) was monitored with laser Doppler flowmetry, and the DC potential was acquired with an intracortical glass capillary microelectrode.

1.2. Ca^{2+}-channel blockade in the treatment of cerebral ischemia

In the last four decades, numerous animal experiments have indicated that administration of VGCC (voltage-gated Ca^{2+}-channel) blockers is effective in the treatment of cerebral ischemia, at the same time, it is hard to translate these data into clinical trials. One of the most potent blockers of VGCCs is nimodipine, with relatively selective pharmacological effect on cerebral vessels and neurons. This dihydropyridine derivative inhibits Ca^{2+}-influx

through vascular and neuronal L-type VGCCs, causing vasodilation and neuroprotection in the central nervous system. Due to its lipophilicity, it can cross the blood-brain barrier (BBB), augmenting its central effect. The blockade of L-type VGCCs by nimodipine reduced the elevation of intracellular [Ca^{2+}] and shortened the duration of membrane depolarization in the oxygen-glucose deprivation model of stroke in rat brain slices. This finding supports the role of L-type VGCCs in the early phase of ischemic cell damage and makes nimodipine a possible candidate for ischemic stroke therapy. Under ischemic conditions, nimodipine moderately impedes SD events, efficiently improves the hyperemic component of SD, and converts spreading ischemia to a hyperemic response. Under ischemic conditions, cerebral autoregulation may be impaired. For this reason, mean arterial blood pressure (MABP) must be monitored closely in patients suffering from ischemic stroke: cerebral perfusion pressure may decrease together with lowering blood pressure, which is accompanied by decreasing CBF due to impaired autoregulation. Although it is relatively selective to cerebrovascular VGCCs, nimodipine lowers MABP in a dose-dependent way. That is why, during the therapy of stroke patients with normo- or hypotension, the hypotensive effect of VGCC blockers should be avoided.

Obviously, the ischemic penumbra forms the central target of stroke therapy. Although recanalization is clearly intended to reperfuse and ideally save the ischemic penumbra, the delivery of pharmacological agents selectively to the penumbra zone is problematic and remains a field for exploration. Since nanomaterials have great potential of controlled and sustained drug release as well as biocompatibility and lower toxicity to human tissues, nanomedicine is having increasingly more significant impact in stroke therapy. Nanoparticles fall under structurally heterogenic groups of 1-1000 nm particles, they can potentiate the penetration of agents into the brain by prolonging their circulation time and promoting the transport of drugs through biological membranes and the BBB. Drugs can be released temporally or regionally by various internal (pH, redox, specific biomolecules, or enzymes), and external (temperature, electromagnetic radiation, ultrasound) stimuli, targeting the drug to the injured region. Intriguingly, cancer therapy has already identified low pH typical of the tumor environment to direct anticancer drug delivery selectively to a tumor to enrich the tumor tissue with anticancer agents. An analogous approach is thought to open up new possibilities in ischemic stroke therapy, and may advance the management of ischemic stroke in the future **(Fig. 2.)**.

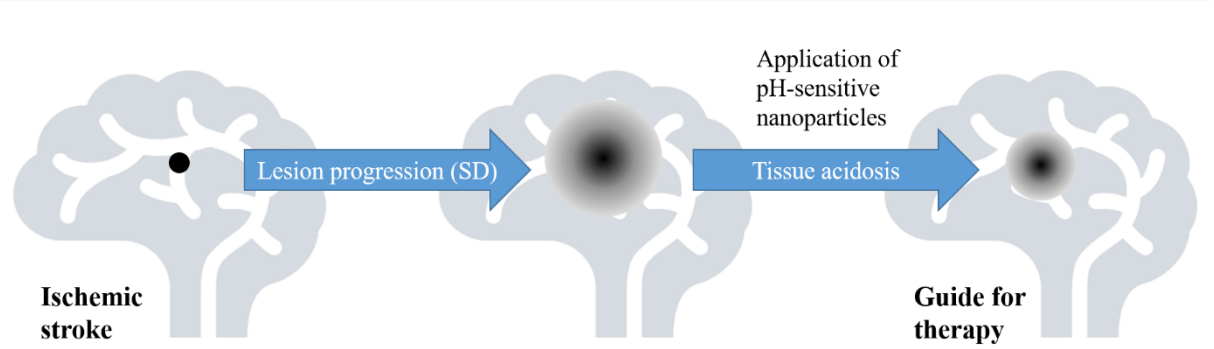

Figure 2. Application of pH-sensitive nanoparticles appears to be a feasible solution for neuroprotection in ischemic stroke therapy. Tissue acidosis linked to cerebral ischemia and spreading depolarization (SD) can be utilized as a trigger for drug release.

Based on the above, we **hypothesized,** that:

- Nanoparticles are suitable for targeting drug release to the ischemic region in the nervous tissue.
    - Tissue acidosis caused by ischemia or SD occurrence is a condition that can be harnessed to initiate drug release in the injured nervous tissue.
- Application of nimodipine is suitable for testing a novel pH-sensitive nanocarrier system in an *in vivo* global cerebral ischemia model.
    - Nimodipine is expected to re-establish neurovascular coupling that is injured under ischemia.
    - Nimodipine administered with nanoparticles is anticipated to exert vasodilator and neuroprotective effect against SD, propagating over the ischemic penumbra.

According to our hypothesis, our **aim** was:

- To investigate the impact of topically administered nimodipine in solution in the intact and ischemic brain;
- To design and test a novel treatment strategy resting on pH-sensitive nanoparticles carrying nimodipine, to be administered topically in our *in vivo* global cerebral ischemia model.

2. Materials and methods

Two sets of experiments are presented in this thesis. In *Experimental Project I*, nimodipine was applied in solution. In *Experimental Project II*, nimodipine was associated with pH-sensitive nanoparticles in suspension. Young adult, male Sprague-Dawley rats were used

in the projects. On the day of experiments, animals were anesthetized with isoflurane (1.5-2 % in N_2O:O_2 70 %:30 %), and allowed to breathe spontaneously through a head mask. In *Experimental Project I*, isoflurane was substituted by α-chloralose (intraperitoneally) for the period of actual data acquisition. In *Experimental Project II*, isoflurane anesthesia was sustained throughout the experimental protocol. The left femoral artery was cannulated to monitor MABP continuously, and to collect samples for arterial blood gas analysis. Both common carotid arteries were carefully separated from the surrounding tissue and an occluder was looped around each vessel for later induction of acute, incomplete, global forebrain ischemia. Two cranial windows were prepared on the right parietal bone with a high precision dental drill under saline cooling. The cortical surface was exposed by the careful retraction of the dura mater in each cranial window. The rostral window was later used for data acquisition (i.e., reference electrode recording local field potential (LFP) filtered in DC mode, and an adjacent Laser-Doppler probe for electrophysiology and CBF measurement in both experimental projects, and a pH sensitive microelectrode for extracellular pH measurement in *Experimental Project II*) and topical drug administration, while the caudal window served SD elicitation. The craniotomies were regularly irrigated with artificial cerebrospinal fluid (aCSF).

In *Experimental Project I,* nimodipine was applied in solution (100 μM), then global forebrain ischemia was induced in half of the animals by bilateral common carotid artery occlusion. Functional hyperemia in the somatosensory cortex was created by mechanical stimulation of the contralateral whisker pad. SD events were elicited subsequently by 1 M KCl. LFP and CBF in the parietal somatosensory cortex were monitored by electrophysiology and LDF. Considering the combination of ischemia induction and pharmacological manipulations, 4 experimental groups were established (i.e., intact vehicle, n=9; intact nimodipine-treated, n=6; 2VO vehicle, n=8; 2VO nimodipine-treated, n=6).

In *Experimental Project II*, nimodipine was associated with pH-sensitive nanoparticles in suspension. The rostral cranial window was incubated with the nanoparticle suspension including nimodipine (100 μM; n=10) or vehicle (n=8), then both common carotid arteries were occluded. SDs were elicited by 1 M KCl to deepen the ischemic insult. LFP, CBF and tissue pH were recorded from the cerebral cortex. Microglia activation (Iba1) and neuronal survival (NeuN) were evaluated in brain sections by immunocytochemistry.

3. Results

3.1. Baseline variations of cerebral blood flow, tissue pH, and the evidence for drug release from nanoparticles

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

Variation of baseline CBF was assessed at selected time points of the experimental protocol, to evaluate the impact of pharmacological treatments. In *Experimental Project I*, ischemia induction caused a marked reduction of CBF (to 53±23 %), which stabilized in the vehicle-treated group at 74±11 % prior to SD1, and at 67±15 % prior to rSDs. The occurrence of SD produced long-lasting oligemia, the final element of the CBF response to SD, which was apparent between SD events in the intact groups, as well. Nimodipine treatment increased baseline CBF significantly by the time of ischemia induction with respect to the pre-treatment CBF level (131±43 vs. 104±12 %, nimodipine vs. vehicle). Further, nimodipine counteracted CBF reduction due to 2VO (CBF shift from baseline: 22±11 vs. -26±11 pp., nimodipine vs. vehicle), and prevented the evolution of post-SD oligemia (CBF shift from baseline: 11±24 vs. -30±12 pp., nimodipine vs. vehicle in the intact group).

In *Experimental Project II*, CBF elevation was taken as a reliable read-out of the efficacy of nimodipine treatment and was expected to confirm drug release from nanoparticles. In contrast with *Experimental Project I*, local CBF remained level during the incubation period prior to ischemia onset (99.2±2.6 vs. 99.9±3.0 %, 30 min after vs. before the application of nimodipine associated to nanoparticles) in the face of invariable tissue pH (pH 7.29±0.22 vs. 7.28±0.18, 30 min after vs. before the application of nimodipine associated to nanoparticles). Ischemia induction produced a sharp drop of CBF to 29.4±10.2 %, and an acidic tissue pH shift to 7.06±0.30). From this point on, CBF sampled prior to SD events increased in the nimodipine group, and was higher than in the vehicle group, particularly prior to rSDs (47.8±23.7 vs. 29.3±6.96 %, nimodipine vs. vehicle), which were triggered subsequent to the transient reduction of tissue pH to 6.71±0.29 with SD1).

3.2. The impact of nimodipine on neurovascular coupling

The impact of nimodipine (in solution) on neurovascular coupling under intact and ischemic conditions was measured in *Experimental Project I*. For the estimation of drug effect on physiological neurovascular coupling, somatosensory evokes field potentials (EFPs) and the

associated CBF response provoked by whisker stimulation were evaluated in the somatosensory barrel cortex. The amplitude of EFPs was considerably attenuated under ischemia with respect to the intact condition (107.7±19.5 vs. 418.9±53.5 μV, 2VO vs. intact after vehicle treatment). The application of nimodipine dramatically decreased the amplitude of evoked potentials, in the intact cortex (128.0±58.6 vs. 418.9±53.5 μV, nimodipine vs. vehicle in the intact group).

The relative amplitude of the hyperemic response was notably smaller under ischemia than in the intact brain (6.2±2.9 vs. 12.9±5.4 %, 2VO vs. intact, after vehicle treatment). Treatment with nimodipine recovered the relative amplitude of the CBF response to the intact level (15.4±6.7 vs. 6.2±2.9 % vs. 12.9±5.4 %, 2VO nimodipine vs. 2VO vehicle vs. intact vehicle). Additive to the elevation of baseline CBF achieved by nimodipine, the further improvement of the CBF response to whisker stimulation in the ischemic cortex was highly remarkable.

3.3. The impact of nimodipine on spreading depolarization

SD events were experimentally triggered in the intact brain (*Experimental Project I*) or under global forebrain ischemia (*Experimental Project I, II*) to evaluate the potential impact of nimodipine on the kinetics of SD, the associated CBF response and tissue pH variation.

3.3.1. The DC potential signature of spreading depolarization

SD occurrence was confirmed by the transient negative shift of the DC potential. In *Experimental Project I*, nimodipine in solution significantly decreased SD amplitude (-13.2±2.5 vs. -15.1±2.1 mV, nimodipine vs. vehicle in the intact condition). In addition, nimodipine shortened SD duration in the ischemic cortex to the intact level (31.1±7.4 vs. 61.4±41.9 vs. 31.5±9.5 s, 2VO nimodipine vs. 2VO vehicle vs. intact vehicle).

As expected, in *Experimental Project II*, the analysis of the DC potential signature of SDs demonstrated that nimodipine applied in the nanoparticle suspension shortened the duration of rSDs significantly with respect to control (48.07±23.29 vs. 76.25±17.2 s, nimodipine vs. vehicle). Moreover, it facilitated the rate of repolarization of rSD events (0.8±0.523 vs. 0.279±0.153 mV/s, nimodipine vs. vehicle).

3.3.2. The local cerebral blood flow response to spreading depolarization

The share of the individual elements in the CBF response to SD is variable across animal species and anesthesia protocols and appears to change remarkably according to the actual metabolic status of the tissue. Considering the different anesthesia protocols during the period of actual data acquisition (i.e., α-chloralose in *Experimental Project I*, and isoflurane in

Experimental Project II), we found different kinetics of the CBF responses in *Experimental Project I* and *II*. The initial hypoperfusion proved to be detectable only occasionally, therefore the analysis focused on the peak hyperemic element of the CBF response.

In *Experimental Project I*, the CBF response to SD consisted of 4 elements, starting with a transient hypoperfusion, followed by a peak and then a late hyperemia, and concluded by a long-lasting oligemia. 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 nimodipine, allowed late hyperemia to emerge at a clearly higher incidence. The amplitude of peak hyperemia was conserved over experimental groups, except for nimodipine treatment in the ischemic condition, which augmented peak hyperemia amplitude (relative change: 185±62 vs. 131±66 %, nimodipine vs. vehicle in the 2VO group). The duration of hyperemia (i.e., peak, and late hyperemia together) was not significantly altered by ischemia or the treatment.

In contrast with the CBF response to SD under α-chloralose anesthesia, SD related CBF response in isoflurane anesthetized rats in *Experimental Protocol II*, included an initial transient hypoperfusion, followed by a peak hyperemia and a long lasting oligemia, but we did not detect the late hyperemic element. As expected, nimodipine delivered by nanoparticles significantly enhanced the amplitude (48.15±42.04 vs. 17.29±11.03 %, nimodipine vs. vehicle) and the magnitude of peak hyperemia in response to rSDs (4604.43±2572.3 vs. 2368.05±1324.71 %*s, nimodipine vs. vehicle).

3.3.3. Tissue pH variations related to spreading depolarization

In *Experimental Project II*, we measured extracellular tissue pH variations corresponding to ischemia induction and SD events. As presented above, ischemia induction causes an acidic tissue pH shift from the neutral 7.3-7.4 to 7.06±0.30. Tissue pH variations associated with SD events started with a rapid, short alkaline shift followed by a longer-lasting, dominant, transient acidosis. Tissue pH did not fully recover and remained typically mildly acidic after SD1 (pH 7.14±0.29 vs. 7.23±0.28, prior to rSDs vs. prior to SD1). Nimodipine treatment had no measurable impact on the initial alkaline shift but modified the kinetics of the subsequent transient acidosis. As such, nimodipine delivered by nanoparticles facilitated the

rate of return from the acidic shift with rSDs (0.01±0.006 vs. 0.005±0.002 pH unit/s, nimodipine vs. vehicle) and shortened the duration of acidosis with rSDs (65.46±20.2 vs. 138.3±66.07 s, nimodipine vs. vehicle). Consequently, the magnitude of acidosis expressed as AUC was substantially reduced in the nimodipine compared to vehicle group (25.75±10.69 vs. 49.46±23.38 pH unit*s, nimodipine vs. vehicle).

3.4. Histology

In order to explore whether the chitosan nanoparticles used here might trigger neuroinflammatory reactions (a potential unfavorable side effect of the drug delivery approach), we estimated microglial activation in immune-stained brain sections, at the end of *Experimental Project II*. Microglia immunolabeled for Iba1 appeared to be activated in the cerebral cortex ipsilateral to the initiation of SD events, as shown by their sparser processes and rounded, amoeboid shape. Microglia activation was quantitatively expressed by a ramification index representing the density of microglial processes. The ramification index was remarkably reduced in the ipsilateral compared to the contralateral somatosensory cortex (e.g., 398±203 vs. 1118±300, ipsi- vs. contralateral in the vehicle group). The hemisphere-specific reduction of the ramification index was attributed to SD, because it was observed in rats with bilateral craniotomy (unilateral SD induction), as well (201±102 vs. 483±244, ipsi- vs. contralateral). The application of the nanoparticle suspension alone (vehicle) or incorporating nimodipine did not reduce the ramification index any further compared to aCSF-rinsed preparations (control) (443±208 vs. 398±203 vs. 284±107, nimodipine vs. vehicle vs. control; ipsilateral). Thus, the administration of nanoparticles on the cortical surface did not produce a detectable potentiation of microglia activation.

We labeled viable neurons with NeuN immunohistochemistry to estimate (i) the degree of early neurodegeneration SD might cause in the acute phase of global forebrain ischemia, and (ii) the potential neuroprotection achieved by nimodipine. We screened the somatosensory cortex (i.e., over the striatum) distant to the site of SD elicitation (i.e., over the hippocampus), with the aim to exclude areas from the analysis, in which neurodegeneration might have been caused by topical KCl application to trigger SD. In some animals, the reduced relative area covered by NeuN-immunolabeled neurons indicated early neurodegeneration in the cerebral cortex ipsilateral to the initiation of SD events, albeit the quantitative analysis did not reveal significant SD-related neuron loss (26.9±5.0 vs. 29.1±4.7 %, ipsi- vs. contralateral in the vehicle group). NeuN labeling was not enhanced in the nimodipine-treated group in a

statistically meaningful manner (29.0±4.8 vs. 26.9±5.0 %, nimodipine vs. vehicle in the ipsilateral cortex).

4. Discussion

Here we set out to explore whether nanoparticles designed to release nimodipine in response to pH decreasing below the physiological range are effective to counteract some injury markers in experimental cerebral ischemia. The model used reproduces conditions typical of the ischemic penumbra, which is indicated by tissue perfusion ranging between 20 and 40 % of baseline, and tissue pH dropping to 6.9–7.1 after ischemia induction. We hypothesized, that nanoparticles are suitable for targeting drug release to the ischemic region in the nervous tissue. Tissue acidosis caused by ischemia or SD occurrence was supposed to trigger effective drug release in the injured region. We have selected nimodipine, an L-type VGCC antagonist as the drug to be delivered, because its cerebral vasodilator, SD limiting and neuroprotective actions have been widely acknowledged to be used as a reference for the nanoparticle study. We presumed that nimodipine administered with nanoparticles should exert its expected vasodilator and neuroprotective effects and should impede SDs propagating over the penumbra.

4.1. Acidosis linked to cerebral ischemia can be employed as a trigger for targeted drug delivery

In *Experimental Project II*, nimodipine associated to pH-responsive nanoparticles did not achieve CBF elevation prior to ischemia induction, when tissue pH was physiological, which confirms that nimodipine was not dissociated from the nanoparticles at near neutral tissue pH. Subsequent to the induction of ischemia and the related transient tissue acidosis, baseline CBF was found to be higher in the nimodipine-treated compared to the vehicle group, which is consistent with the known cerebrovascular action of nimodipine, and we interpreted it as the *in vivo* verification of pH-sensitive drug release.

4.1.1. Tissue acidosis to guide neuroprotective intervention in ischemic stroke

Over the last few years, an increasing number of studies addressed the application of nanotechnology for the treatment of ischemic stroke. Nano-sized drug delivery systems (DDSs) to cross the BBB should be smaller than 100 nm, biodegradable, biocompatible, non-toxic, and stable in blood. They should be able to penetrate the BBB, to carry different types of agents, and should not trigger neuroimmune reactions. In the best scenario, nano-sized DDSs have prolonged circulation time and controlled drug release. The application of biocompatible and

biodegradable, natural, or synthetic macromolecular polymeric nanocarriers offers substantial promise in therapeutics. Among others, stimulus responsive nanoparticles present the opportunity to initiate drug release by local (patho)physiological biochemical stimuli (e.g., homeostatic, redox, enzymatic, tissue pH), which are intrinsic and restricted to the diseased tissue, and are closely related to the progression of the disease condition. These bioresponsive nanomaterials are also known as "smart" nanosystems. A negative pH shift from the neutral 7.3-7.4 to below 7.0 units, for instance, can initiate conformational or solubility changes in various smart nanosystems, including polysaccharide chitosan nanoparticles, to allow drug release.

4.1.2. Neuroimmune responses against nanoparticles

In *Experimental Project II*, we intended to examine whether chitosan nanoparticles may induce a neuroimmune response at the brain tissue in contact with the nanoparticles. Chitosan, a derivative of chitin, is a biocompatible, biodegradable, natural polysaccharide, which has been considered as immune adjuvant in cancer therapy. Microglia form the active immune defense of the brain, and their reaction to inflammatory stimuli is accompanied by their typical morphological alteration (i.e., retraction of processes, amoeboid form). We, therefore, labeled microglia to estimate their potential activation by chitosan nanoparticles in our experimental model. The application of chitosan nanoparticle suspension to the exposed cortical surface did not enhance microglia activation with respect to aCSF rinsed preparations in our experiments, which suggests that chitosan nanoparticles themselves did not trigger a detectable local immune reaction in the cerebral cortex.

4.2. The effect of nimodipine on the regulation of local cerebral blood, neuronal function, and local tissue pH

Nimodipine, a dihydropyridine derivative, inhibits Ca^{2+} influx to vascular smooth muscle cells (VSMCs) and causes vasodilation. In addition, nimodipine blocks neuronal L-type VGCCs, as well, mitigates neuronal Ca^{2+} overload, and achieves neuroprotection under ischemic stress. In the incomplete global forebrain ischemia model used, we first assessed drug effect on physiological neuronal activation (i.e., achieved by somatosensory stimulation) and the coupled functional hyperemic response. Next, we focused on the impact of the pharmacological treatments on SD and the associated CBF and pH response. SD is an ischemic preconditioning stimulus when triggered in intact tissue and represents a pathophysiological process as it occurs due to extracellular K^+ and glutamate accumulation in ischemic brain.

4.2.1. Nimodipine effectively improves neurovascular coupling, subsequently augments functional hyperemia

In *Experimental Project I*, the impact of nimodipine on the regulation of local CBF was investigated by somatosensory stimulation. In our experiments, whisker stimulation was applied to investigate functional hyperemia in the barrel cortex of anaesthetized rats. In the barrel cortex of rats somatotopy is noticeable, stimulation of a distinct whisker evokes neuronal activation, and subsequent CBF elevation, in the adherent cortical region.

Since the brain has limited capacity to store energy, it needs continuous energy supply, which is maintained by continuous perfusion through its complex web of blood vessels. Several mechanisms guarantee the continuous nutrient and O_2 transport to the brain. One of them is neurovascular coupling, which is a coordinated interaction among activated neurons, astrocytes, and contractile cells of the vessel wall. At different levels of the vascular tree, different cell types regulate local CBF. At the level of penetrating arteries and parenchymal arterioles, neurons, astrocytes and VSMCs compose the unit of local CBF regulation, called neurovascular unit. At the capillary level, the contractile cells are pericytes, that share a common basement membrane with endothelial cells. VSMCs and pericytes are covered by the endfeet of astrocytes, and all these three cell types are innervated by neurons at each level of the vascular tree. Because of their proper anatomical position, astrocytes transfer the information from activated neurons directly to contractile cells, regulating the cerebral microcirculation.

It is a novel finding of the presented work, that, in addition to baseline CBF elevation, nimodipine profoundly augmented functional hyperemia in response to somatosensory stimulation, without enhancing EFP amplitude under ischemia. Nimodipine remarkably decreased EFP amplitude in the intact cortex, while the relative magnitude of the flow response was maintained. Both observations imply that the enhancement of functional hyperemia by nimodipine is disproportionate with respect to EFP amplitude. This suggests that nimodipine augmented the amplitude of the CBF response (irrespective of the intensity of neuronal activation initiated by somatosensory stimulation), possibly by potentiating the release of vasodilator substances or the efficacy of vasodilator signaling cascades. Since vasodilator prostaglandins and epoxyeicosatrienoic acids are produced by astrocytes during neurovascular coupling, and L-type VGCCs are present in the astrocyte plasma membrane, astrocytes may be involved in the nimodipine-related enlargement of functional hyperemia, without neuronal contribution being proportionally increased in the first place.

4.2.2. Nimodipine inhibited spreading depolarization evolution and augmented hyperemia in response to SD events

Nimodipine application reduced SD size (amplitude, and duration at half amplitude), in agreement with previous reports applying nimodipine at the concentration used in our studies. Neurons express L-type VGCCs, which have been implicated in the modification of neuronal excitability and are a well-known target of nimodipine. Although pharmacologically decreased SD amplitude is often interpreted as a sign of protection, the lack of a clear-cut association between SD amplitude and histological or neurological damage imposed creates persistent controversies. Our data support earlier observations that the number of rSDs, the cumulative duration of SDs, and the inability of the tissue to recover from SD (i.e., long SD duration) correlate with injury progression. Nimodipine shortened SD duration, which may be accepted as a sign of its protective potential. SD is associated with neuronal Ca^{2+} loading, in part via ionotropic glutamate receptors, such as the N-methyl-D-aspartate receptor (NMDAR). The reduction of the SD-associated Ca^{2+} accumulation has thus emerged as a promising target to achieve SD inhibition. For example, low dose ketamine (an NMDAR blocker) applied to brain slices was shown to reduce Ca^{2+} load and to facilitate the recovery from SD. In our study, nimodipine is thought to have inhibited Ca^{2+} influx to neurons via L-type VGCCs, which also caused the more rapid recovery from SD. These results suggest that the attenuation of neuronal Ca^{2+} load (either via NMDAR blockade or L-type VGCC inhibition) shortens SD duration. To further evaluate the neuroprotective potential of the treatment, we labeled viable neurons with NeuN immunocytochemistry and quantified neuronal density in the cerebral cortex. Even at this early time point after ischemia induction, we observed in some animals less dense NeuN staining in the parietal cortex where SDs propagated, compared to the contralateral hemisphere exposed to ischemia alone, but nimodipine did not rescue neurons to a statistically meaningful degree at this endpoint (1 hour after the ischemia onset).

4.2.3. Nimodipine potently reduced the degree of SD-related acidosis

The use of pH-sensitive microelectrodes in our preparation offered the unique opportunity to assess the impact of nimodipine on the SD associated transient tissue acidosis, an action of nimodipine not screened before. The SD-related acidosis, which has been linked to the accumulation of lactate was previously contemplated to exacerbate ischemic injury and jeopardize the survival of penumbra tissue, therefore its inhibition is expected to be beneficial. Here we have observed that nimodipine potently reduced the degree of SD-related acidosis. Similarly, intravenously administered nimodipine was shown to moderate tissue acidosis in

experimental focal cerebral ischemia, which was attributed to the direct facilitation of metabolic lactate clearance, independent of perfusion rate. It is thus conceivable that the inhibition of Ca^{2+} entry to neurons by nimodipine may support mitochondrial function and oxidative lactate degradation, which may reduce the acid load associated with SD, as seen here. It is also reasonable to argue that the shorter duration of SD related acidosis due to nimodipine is consistent with the shorter duration of SD itself, and the primary effect of nimodipine was the inhibition of SD, causing a secondary reduction of the associated tissue acidosis.

5. Main observations and conclusion

The aim of our study was to explore whether nimodipine loaded pH-sensitive nanoparticles can be used effectively to reduce detrimental outcomes in experimental global cerebral ischemia. The work demonstrates that tissue **acidosis linked to cerebral ischemia can be employed as a trigger for targeted drug delivery**. Nimodipine associated to pH-responsive nanoparticles did not achieve CBF elevation prior to ischemia induction, when tissue pH was physiological, which confirms that nimodipine was not dissociated from the nanoparticles at physiological tissue pH (pH 7.3-7.4). After the induction of ischemia and the related transient tissue acidosis, baseline CBF was found to be higher in the nimodipine-treated compared to the vehicle group, which is interpreted as the *in vivo* verification of pH-sensitive drug release. Moreover, immunohistochemical examinations showed that the applied **chitosan nanoparticles did not activate microglia** in the brain.

Additionally, we investigated the impact of topically administered nimodipine in the intact and ischemic rat brain. By the topical application of nimodipine, we found that nimodipine **inhibited SD evolution**, possibly by blocking Ca^{2+} entry to nerve cells, and **augmented hyperemia in response to SD** events in the ischemic rat brain. Moreover, it potently **reduced the degree of SD-related acidosis**. The data generated here support the concept that L-type VGCC inhibition by **nimodipine** effectively **improves neurovascular coupling**, particularly under cerebral ischemia, **augments functional hyperemia** in response to somatosensory stimulation especially under ischemia, in addition to achieving a general, constitutive vasodilator effect. In addition, nimodipine-mediated vasodilation and neuroprotection can be achieved by pH-responsive chitosan nanoparticles applied directly to the brain surface.

Publications related to the PhD thesis:

I. Szabó Í, **M Tóth O**, Török Z, Varga DP, Menyhárt Á, Frank R, Hantosi D, Hunya Á, Bari F, Horváth I, Vigh L, Farkas E. 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. *doi: 10.1111/bph.14611*. **IF: 7.73**

II. **M Tóth O**, Menyhárt Á, Varga VÉ, Hantosi D, Iványkovits-Kiss O, Varga DP, Szabó Í, Janovák L, Dékány I, Farkas E, Bari F. Chitosan nanoparticles release nimodipine in response to tissue acidosis to attenuate spreading depolarization evoked during forebrain ischemia. Neuropharmacology. 2020 Jan 1;162:107850. *doi: 10.1016/j.neuropharm.2019.107850*. **IF: 4.431**

III. **M Tóth O**, Menyhárt Á, Frank R, Hantosi D, Farkas E, Bari F. Tissue Acidosis Associated with Ischemic Stroke to Guide Neuroprotective Drug Delivery. Biology (Basel). 2020 Dec 11;9(12):460. *doi: 10.3390/biology9120460*. **IF: 3.796**

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