The impact of age on the elicitation of spreading depolarization and the implication of prostanoids in the associated cerebral blood flow response

Ph.D. Thesis

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Introduction

Spreading depolarization (SD) is a wave of massive depolarization of neurons and presumably glia cells, which – together with a concomitant depression of spontaneous brain electrical activity and accompanying cerebral blood flow (CBF) changes – propagates across the cerebral grey matter at a low rate of 2-8 mm/min. Cellular depolarization appears on local field potential (LFP) or electrocorticogram (EcoG) recordings filtered in DC mode as a transient negative shift of the DC potential trace, while the concomitant depression of neuronal activity is visualized as a transient local depression of the ECoG. Since the first observation made by Leão, the pathophysiological role of SD has been recognized in several neurological diseases. SD promotes migraine aura and likely contributes to headache evolution, whereas accumulating clinical and experimental evidence suggest that SD contributes to secondary injury following both ischemic and hemorrhagic stroke, traumatic head injury (TBI) and subarachnoid hemorrhage (SAH).

Under physiological conditions extracellular K^+ concentration is kept close to 3-4 mM, independent of fluctuations in blood serum levels, but local changes in extracellular K^+ levels do occur following neuronal activity. In the injured brain, the high concentration of K^+ (10-15 mM) sufficient to induce SD is presumably determined by the balance between K^+ efflux and the efficacy of K^+ clearance. When a critical threshold of K^+ level is reached, the self-propagating SD cycle takes off and invades neighboring tissue. The mass cellular depolarization reflects then a near-complete breakdown of neuronal transmembrane potential. More specifically, at any point of the tissue involved in SD, the influx of Na^+ leads the depolarization, causing a reduction of extracellular Na^+ concentration from 140-150 to 50-70 mM, accompanied by a sudden extracellular surge of K^+ from 2.7-3.5 to 30-60 mM, a concurrent decrease of extracellular Ca^{2+} levels from 1-1.5 to 0.2- 0.8 mM and that of Cl^- from 130 to 74 mM. Intracellular ion concentrations change in the opposite direction.

Even though K^+ is considered one of the primary driving forces of SD, the channels mediating K^+ efflux during depolarization are still to be explored. However, it is reasonable to suggest that voltage-gated (Kv), large-conductance Ca^{2+}-activated (BK) or ATP-sensitive potassium channels must be involved.

In the injured brain, the limited availability of ATP reduces the efficiency of Na^+/K^+-ATPase and thus K^+ reuptake. Na^+/K^+-ATPase also plays a central role in the recovery of the tissue from

SD, which is further supported by astrocytic K^+ siphoning via inwardly rectifying K^+ channels (Kir 4.1) or water flux mediated through aquaporin-4 channels.

In addition to the ion dislocations, interstitial glutamate concentration also increases with SD. The SD-related glutamate efflux may be mediated by intracellular Ca^{2+} accumulation through P/Q type voltage-gated (Cav) channels, presynaptic N-methyl-D-aspartate (NMDA) receptor-dependent vesicular exocytosis, pannexin-1 channels or from astrocytes through excitatory amino acid transporters (EAATs) operating in reverse mode. Glutamate accumulation may overstimulate NMDA receptors, deepen the depolarization, and contribute to SD propagation by further increasing K^+ and glutamate release. Similar to K^+ clearance, glutamate buffering is an equally important factor in SD evolution.

SD is coupled with typical changes in CBF, mediated by the various vasoactive agents released during cellular depolarization. In the rat - and most probably in humans - the physiological pattern of the SD-associated CBF response includes four sequential components: (i) an initial, brief hypoperfusion; (ii) a marked, transient peak hyperemia; (iii) a less obvious late hyperemia; and (iv) a sustained hypoperfusion also known as spreading or post-SD oligemia. The share of each element in the CBF response is prone to the level of baseline cerebral perfusion and the metabolic state of the tissue. The peak hyperemic component is the most conspicuous and most extensively studied, however its underlying mechanisms are still incompletely understood. Prostanoids have been implicated in mediating vasodilatation in physiological neurovascular coupling, but their exact role in the regulation of the SD-coupled CBF response is yet to be determined.

In ischemic settings, with decreasing residual blood flow approaching the ischemic core, the CBF response to SD is increasingly dominated by vasoconstrictive elements, leading to diminished hyperemia and more prevalent hypoemia. In the most severe form, the hypoemic element completely outweighs hyperemia, and the CBF response to SD turns into spreading ischemia. This atypical SD-associated CBF variation in the injured brain aggravates metabolic supply-demand mismatch in the tissue, and can delay recovery from SD thereby increasing the risk of irreversible damage.

In neurological disorders implicating SD (i.e. migraine with aura, or acute brain injury including TBI, SAH, and malignant ischemic stroke), age is known as an independent risk factor for the incidence and prevalence of the disorders. For example, the age-dependent prevalence of migraine has been shown to be bimodal, as migraine incidence peaks at the age of 19 and 48 years in men, and at the age of 25 and 50 in women. Among the general population, TBI has a

peak incidence during childhood (falls), adolescence (motor-vehicle accidents) and geriatric age (falls). Further, SAH is one of the most common types of stroke in young adults, and younger age is an established risk factor for secondary lesion progression in SAH, caused by proximal large artery vasospasm and delayed cerebral ischemia. Finally, aging predicts poor patient outcomes after ischemic stroke. In this context, the impact of age on stroke pathophysiology has been the target of intensive research in order to understand the reason for the increased susceptibility of the aged brain to stroke-related injury, yet the potential contribution of SD has remained largely unexplored.

Based on the above, our goals were:

I. to determine the innate susceptibility of the cerebral cortex to SD, under non-ischemic and ischemic condition, over the age range from adolescence to young adulthood in rats (7 to 30 weeks);
II. to investigate the possible link between the threshold of SD elicitation and the histological organization of the cortex at this age;
III. to explore, whether the CBF response to SD is subject to any age-related modification over the age range investigated;
IV. to identify specific frequency bands of the electrocorticogram that may be selectively affected by SD or age.

SD related CBF changes are the cornerstone of SD related secondary injury, and since prostanoids have been implicated in physiological neurovascular coupling, in a separate study we aimed:

I. to explore the role of vasodilator prostanoids on the SD related CBF response.

Materials and methods

In both experimental approaches an open cranial window was mounted on the parietal bone of isoflurane-anesthetized male Sprague-Dawley rats, from which DC-potential, ECoG and laser-Doppler flow were acquired. SDs were elicited from a second craniotomy, distal to the recording one.

SD threshold was determined in animals of pre-defined age: 7-10 (n=21), 12-16 (n=12) and 30 (n=5) weeks, by stepwise incrementing cathodal stimulation. Three SDs were elicited before and three following global forebrain ischemia, which was induced via bilateral occlusion of the common carotid arteries (2VO). Dendritic spine density was assessed in 8 and 30 weeks old animals. ECoG spectral power analysis was applied for individual frequency bands. Along with

the 30-week-old group (n=5), six of the youngest animals (7/8 weeks) were selected for the analysis.

Young adult male rats (n=60) were used to dissect the role of vasodilator prostanoids in SD related CBF response. Pharmacological blockade of cyclooxigenase-1 (COX-1), cyclooxigenase-2 (COX-2) and the antagonism of the EP4 receptor of prostaglandin-E_2 (PGE_2) was achieved by superfusing SC-560, NS-398 and L161,982, respectively, upon the cortical surface. After the incubation period, 3 SDs were elicited via KCl application. SDs were examined in different animals under physiological conditions and following 2VO.

Results

Investigation of SD susceptibility

Electric threshold of SD elicitation

Initiation of SD required increasingly greater electric charge with older age during both baseline (4743±1282 vs. 3076±915 vs. 1661±649 μC, 30-week-old vs. 12-16-week-old vs. 7-10-week-old) and ischemia (8447±1763 vs. 5343±2170 vs. 2514±1032 μC, 30-week-old vs. 12-16-week-old vs. 7-10-week-old). With advancing age, the threshold during ischemia progressively departed from the threshold determined for the respective baseline (threshold difference of 4278±2352 vs. 853±839 μC, 30-week-old vs. 7-10-week-old).

Lower CBF taken prior to SD elicitation predicted higher threshold of SD elicitation (r=-0.403), whereas the threshold of SD elicitation positively correlated with the duration of SD-related depression of the high frequency alpha and beta bands (r= 0.373 and 0.478, respectively). There was no impact of age on the features of the CBF response following SD, over either baseline or ischemia.

Density of dendritic spines

In general, dendritic spines tended to be arranged in clusters rather than individually, and appeared to be larger and more complex in shape in the 30-week-old group than in the 8-week-old group. Dendritic spine density significantly increased in the 30-week-old group with respect to the 8-week-old group (55±4 vs.51±2 spines / 50 μm dendritic segment, 30-week-old vs. 8-week-old).

Spectral analysis of the distinct ECoG frequency bands

Greater power of the alpha and theta bands in the 30-week-old group as compared with the 7/8-week-old group (e.g. theta band: 0.005±0.003 vs. 0.0025±0.001 V^2, 30-week-old vs. 7/8-week-old) was observed. Ischemia considerably extended the duration of ECoG depression on all four

frequency bands (e.g. alpha band in the 7/8-week-old: 271±126 vs. 139±40 s, ischemia vs. baseline). The lower frequency delta and theta bands started to recover from the SD-related depression sooner than the higher frequency alpha and beta bands in the 30-week-old group, in contrast with the 7/8-week-old group (e.g. duration of theta band: 123±52 vs. 132±34 s, 30-week-old vs. 7/8-week-old).

The relative peak of the SD-associated hyperemia correlated negatively with the duration of the ECoG depression on all four frequency bands (r=-0.550). At the same time, the longer duration of hyperemia was strongly linked to the longer duration of ECoG depression on all four frequency bands, being most prominent on the delta and theta bands (r=0.733 and r=0.703, respectively).

Compensation of cerebral blood flow to ischemia

In the youngest, 7-10-week-old group, CBF dropped to 18±6% immediately after 2VO onset, recovered to 37±12% before the elicitation of the first SD under ischemia, and was maintained over 30% throughout the ischemic period. In contrast, CBF fell to 11±6% after ischemia induction, recovered to 20±13% before initiation of the first ischemic SD, and remained at around only 20% in the 30-week-old group, implying significantly less efficient compensation in the 30-week-old group with respect to the 7-10-week-old group.

Pharmacological manipulations to explore potential mediators of the cerebral blood flow response to SD

Topical application of drugs was chosen in order to avoid potential systemic side effects. Measured values of mean arterial blood pressure and the outcome of blood gas analysis confirmed no difference in the systemic variables assessed in various treatment groups.

The impact of pharmacological treatments on the CBF response could be discriminated in the intact – but not in the ischemic - animals. The selective COX enzyme inhibitors NS-398 (COX-2) and SC-560 (COX-1) did not exert any clear influence on the evolution of the SD-related CBF response, as all the examined variables remained unaltered. L161,982 (EP4 receptor antagonist) selectively reduced the relative amplitude of peak hyperemia with the first SD (21±11 vs. 51±38 %), and recurrent SDs (50±21 vs. 76±37 %). Further, L161,982 augmented the relative amplitude of the post-SD oligemia after the first SD (58±13 vs. 40±14 %).

Hyperemia evolution significantly decelerated due to L161,982 treatment as demonstrated by its shallower upward slope with the first SD (0.75±0.38 vs. 1.88±1.13 %/s), and recurrent SDs

(1.05±0.43 vs. 1.60±0.70 %/s). Finally, the magnitude of hyperemia expressed as the area under the curve was also significantly decreased by L161,982 for the first SD (874±462 vs. 2885±2543 % x s), and recurrent SDs (5532±3643 vs. 7448±2072 % x s).

Pharmacological manipulation proved to be ineffective on the kinetics of SD in physiological setting. Conversely, the DC potential signature of SD was markedly and selectively elongated by SC-560 and L161,982 but not NS-398 treatment in the Ischemic group.

Discussion

The impact of ischemia on SD elicitation

Our present data clearly demonstrate, that the electric threshold of SD elicitation markedly increases with ischemia and that the ischemia-related threshold elevation is increasingly more obvious with older age.

The ischemia-related increase of SD threshold may be the result of the metabolic state of the tissue, as indicated by the correspondence between lower CBF taken prior to evoking an SD with a higher electric threshold of SD elicitation. The supply of glucose and oxygen are crucial for the proper working of energy-dependent ion pumps, and thus the effective maintenance of resting membrane potential. However, our data on blood glucose level indicated normoglycemic conditions (blood glucose concentration around 9-11 mM) in all age groups, and revealed no difference between baseline and late ischemia. Therefore we suggest that the higher threshold of SD under ischemia was unrelated to blood glucose concentration.

It has been long appreciated that mild tissue acidosis - which typically characterizes ischemic penumbra tissue - suppresses SD. Low pH may restrict SD evolution via NMDA receptor inhibition or by the adjustment of the conductance and gating properties of Kv, Nav, and Cav channels. Therefore, we suggest that the ischemia-related fall of tissue pH could be a key factor in raising the threshold of SD elicitation as observed here. Finally, increased K^+ conductance and the gradual accumulation of extracellular K^+ that occur during ischemia generally contribute to membrane hyperpolarization and repolarization thereby depressing neuronal excitability. This ionic imbalance may effectively inhibit SD elicitation as well.

The impact of aging on SD elicitation

In the present study, we investigated the susceptibility changes to SD in young rats, corresponding to human adolescence and young adulthood. By using electric stimulation, we have precisely defined and confirmed previous results, that the threshold of SD initiation

increases with age, and we have shown that the susceptibility of the cerebral cortex to SD starts to decrease gradually already from adolescence on.

Previous studies have shown that the rate of SD propagation decelerates in the aging rodent brain and that increasingly higher concentration of KCl was required to trigger SD in middle-aged rats (*in vivo and in vitro*) than in young adults. Also, it has been noted that the same, incessant, standard trigger (1 M KCl) produced a lower number of recurrent SDs in the middle-aged with respect to the young adult cerebral cortex in anesthetized rats.

The increased threshold of SD elicitation with advancing age during early adulthood may be linked to structural changes in neuronal networks, which may alter the electrophysiological properties of the nervous tissue. Thus, it is conceivable that the stimulus applied is dissipated in the tissue before SD is ignited. The formation and retraction of dendritic spines that host the post-synaptic element of excitatory synapses dynamically changes with brain maturation and aging is heavily involved in SD propagation and is negatively affected by SD. Therefore we determined the dendritic spine density in the youngest and oldest animals. Our results exhibited an increased density of dendritic spines on the apical dendrites of cortical layer 3 pyramidal neurons at 30 weeks of age with respect to 8 weeks of age, proving that the histological organization of the cortex undergoes detectable alterations during the life span investigated.

Even though a direct link between such fine structural changes and the excitability of neurons is challenging to establish, we speculate that the threshold of SD elicitation may increase with age during early adulthood because of the histological (and connected biochemical) maturation or consolidation of cortical connections. Further on, some evidence suggest that the excitability of the aged nervous tissue is lower, because the age-specific increase in the production of reactive oxygen species modifies the operation of the redox-sensitive K^+ channels. This process may modulate the oligomer formation, permeation and gating properties of Kv channels and BK channels, but it remains to be explored whether these changes manifest at the level of SD evolution.

The impact of aging on SD evolution and propagation

As our data have revealed, that the shortening of the SD-related ECoG depression is evident first in the low frequency components (delta and theta bands) at the end of young adulthood (30 weeks), and concerns all frequency bands at old age (18 months old). These data may depict a narrower SD wave front in space, standing for a smaller volume of nervous tissue involved in SD at a given point in time.

These electrophysiological property changes may be accomplished by the age-related modification of Kv and BK channel function. P/Q type Cav channels implicated in SD evolution were shown to be affected by aging, as well.

The ionic movements underlying SD are also accompanied by the release of glutamate, which sustains SD by binding to and activating NMDA receptors. The decay of NMDA receptor-based signaling with aging has been repeatedly demonstrated. Thus it is plausible that NMDA receptor-based signaling is compromised due to oxidative-stress, mounting to levels relevant for functional deterioration in the aged brain. The expression of distinct NMDA receptor subunits was found to be subject to age-related changes, as well. Importantly, mRNA and protein expression of the modulatory NR2A and the NR2B subunits, both implicated in SD evolution, appeared to be downregulated in the aged brain.

Susceptibility to SD and neurological diseases

The age of the animals investigated in the present study (i.e.: 7-10 vs. 30 weeks) corresponds well with human adolescence and young adulthood, an age representing an increased risk of developing migraine. SD has been accepted as the pathophysiological phenomenon behind migraine aura. It is believed that the concomitant transient depression of cerebral activity is likely responsible for the transient neurological symptoms. In addition to triggering aura, SD could also be responsible for the evolution of the headache itself. Migraine with aura (MA) is increasingly accepted as a definitive risk factor for ischemic stroke, especially in young adults without classical stroke risk factors. Also, MA patients have increased stroke mortality and accelerated ischemic lesion consolidation. In addition to raising stroke risk, MA patients are at increased risk to develop white matter lesions in the posterior circulatory and infratentorial region. Increased susceptibility to SD might thus explain the increased stroke risk in migraine patients. Young adults are usually not exposed to classical cardiovascular risk factors, and even though SD threshold may be low, an SD igniting stimulus is not present. The most common cause of cerebral ischemia in young adults are (i) patent foramen ovale, (ii) connective tissue abnormalities and (iii) inherited/acquired hypercoagulable state. In a subset of MA patients, one of these conditions might lead to transient regional hypoperfusions or silent microlesions. In combination with increased neuronal excitability and SD susceptibility at young age, these small infarcts may provoke SD, and thus migraine like symptoms. In contrast, the relatively higher SD threshold in the elderly is probably counteracted by increased cardiovascular risk, vascular rarefication, decrease of collateral circulation and other comorbidity leading to worse stroke outcomes. MA patients however may retain their SD susceptibility throughout their life,

leading to worse stroke outcomes than the rest of the population, due to the role of SD in secondary injury.

SAH, especially due to aneurysm rupture, has the lowest prevalence out of all the major stroke subtypes, but still poses considerable threat considering the potential years lost, which are comparable with that of ischemic stroke or intracerebral hemorrhage. SAH is one of the most common types of stroke in young adults, and has a high mortality and permanent disability rate. Following the initial injury, focal neurological deteriorations and new ischemic lesions might arise unpredictably, termed delayed cerebral infarctions (DCI), significantly impairing clinical outcome. DCI has been traditionally attributed to vasospasm in the proximal large vessels. Younger age is an established risk factor for proximal large artery vasospasm and a probable risk factor for DCI. However, SD clusters, which might provoke inverse hemodynamic responses and concomitant tissue hypoxia, have been associated with DCI in SAH patients, independently from proximal vasoconstriction. In the present study, we have found that the inherent susceptibility of the nervous tissue to SD appears to be the lowest in young adults. Taken the evidence provided in the present section, the decreased threshold of SD in young patients may put them at a higher risk to develop DCI.

Traumatic brain injury (TBI) is the leading cause of permanent disability and death in the pediatric and adolescent population and is a major cause of symptomatic epilepsy in general. It has been speculated that SDs are triggered by high extracellular K^+, low CBF or increased intracranial pressure, complying with the association between SD and decreased cerebral perfusion pressure. In TBI patients, SDs are often observed for at least 7 days post-injury, and the recurrence of SDs has been associated with the decrease of mean arterial pressure or the increase in core temperature. Among the SDs recorded, the prolonged events may predict significantly poorer long-term outcome and SDs coupled with inverse neurovascular responses were shown to coincide with the failure of cerebral autoregulation and the extension of secondary injury. TBI has a peak incidence during adolescence due to motor-vehicle accidents and incidence of SD was found to be higher in younger patients following TBI (or ICH). In view of these reports and our present data showing that the lowest threshold of SD is at adolescence or young adult age underscores the importance of SDs in young TBI patients.

However, even though the longer cumulative duration of recurrent SDs was associated with the expansion of tissue damage in focal ischemia, this does not directly infer that the longer cumulative duration is the outcome of a higher number of events— indeed, the duration of individual SD events can vary considerably. Further, we have previously demonstrated that

although only a few SDs occur spontaneously in the aged ischemic brain, these events cause more damage than a higher number of SDs in the young brain, as indicated by a remarkable delay of repolarization after SD and the associated evolution of perfusion deficit. Taken together, the low threshold of SD elicitation indicates the brain's high susceptibility for SD generation at young age, but it may not directly put the tissue at higher risk for SD-related injury.

Role of prostanoids in the cerebral blood flow response to SD

We have investigated the role of vasodilator prostanoids in connection with the CBF response to SD, because their central role in the mediation of functional hyperemia. In physiological neurovascular coupling, the major pathway leading to vasodilator prostanoid synthesis involves COX-2, expressed in cortical pyramidal neurons, and located in perivascular nerve terminals along intraparenchymal penetrating arterioles and capillaries. COX-2 derived vasoactive products have emerged as mediators of functional hyperemia to somatosensory stimulation. In contrast with the COX-2 route, the role of the constitutive COX-1 enzyme (which, in the context of physiological neurovascular coupling, is argued to be expressed in astrocytes) in shaping the CBF response to neuronal activity has remained controversial.

Of the subsequent phases of the CBF response to SD, we focused on the peak hyperemic and post-SD oligemic elements. It is important to note that our pharmacological manipulations had no significant effect on pre-SD baseline CBF levels, indicating that the results were not affected by cerebral perfusion changes. The first significant observation of the current study is that EP4 receptor antagonism reduced peak hyperemia, and augmented post-SD oligemia of the CBF response to SD in the intact brain, indicating that EP4 receptor activation contributes to vasodilation during the CBF response to SD. It is a novel finding, as the mediation of the hyperemic element of the CBF response by prostanoids was previously thought unlikely, and COX-derived metabolites were attributed a vasoconstrictive rather than a vasodilatory role in the CBF response to SD. Since a number of previous reports recognized the vasodilatory action of EP4 receptor activation by PGE_2 in physiological neurovascular coupling, our data indicate that EP4 receptor activation achieves CBF elevation in response to SD, as well.

The reason for the absence of the COX-enzyme related vasodilation may well be that during SD, the activity of COX enzymes also leads to the marked production of vasoconstrictive prostanoids. Thus functional hyperemia to neuronal activation seems to be COX-2 dependent, while COX-2 inhibition has no clear impact on the CBF response to SD. These data foster the

assumption that the CBF response to SD is driven by mechanisms different from physiological neurovascular coupling.

In the ischemic cortex, our study revealed a considerable reduction of the distinct elements of the CBF response to SD, with no detectable impact of COX enzyme inhibition or EP4 receptor blockade. Ischemia is known to impair physiological neurovascular coupling and in the acute phase of ischemia, the abundant release of metabolic mediators of vascular tone, or the dramatically elevated concentration of extracellular potassium must obscure potentially still effective, finer signals of vasoregulation.

Conclusion

In the present study we have provided strong evidence, that the susceptibility of the nervous tissue to SD is highest during young adulthood, and that the increase of SD threshold with age coincides with the maturation of the cerebral cortex and the narrowing of the SD wave. Further on, we have shown for the first time, that in contrast to physiological neurovascular coupling, prostanoid synthesis during the SD-related CBF response is not directly COX-2 dependent, and that PGE_2 has a central role in mediating peak hyperemia and diminishing post-SD oligemia.

The accepted connection between SD and migraine with aura is further emphasized with the decreased threshold of SD during adolescence, when migraine has a known prevalence peak. Given the increasingly accepted role of SD in mediating secondary injury, SD might provide an explanation on the increased stroke risk of migraine patients. Similarly, since TBI and DCI following SAH also have a peak incidence during adolescence young TBI or SAH patients may benefit greatly from future therapeutic options, targeting SDs specifically. Further research, especially SD monitoring in patients is needed to verify these data in humans, and to direct future potential drug developments.

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