**Heterogeneity of spreading depolarizations**

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**Running Headline:**

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**Abstract**

Spreading depolarizations are implicated in a diverse set of neurologic diseases. They are unusual forms of nervous system activity in that they propagate very slowly and approximately concentrically, apparently not respecting the anatomic, synaptic, functional or vascular architecture of the brain. However there is evidence that spreading depolarizations are not truly concentric, isotropic, or homogeneous, either in space or in time. Here we present evidence from KCl-induced spreading depolarizations, in mouse and rat, *in vivo* and *in vitro*, showing the great variability that these depolarizations can exhibit. This variability can help inform the mechanistic understanding of spreading depolarizations, and it has implications for their phenomenology in neurologic disease.

**Key Words**

spreading depolarization, cortical spreading depression, susceptibility, velocity, anisotropy

**Introduction**

Spreading depolarizations (SD) are massive disruptions of ionic, metabolic, and vascular homeostasis that propagate slowly and approximately concentrically across the gray matter of the brain1–4. Once considered a curiosity, SD are now recognized as contributing to neurologic diseases as diverse as migraine5–7, stroke8, subarachnoid hemorrhage, and traumatic brain injury2,4.

Given their clinical significance, much still remains unclear about SD. Though to a first approximation they are uniform, disregarding synaptic, cytoarchitectural, and vascular architecture, this characterization does not bear up under scrutiny. With the possible exception of retina9, SD are not completely concentric or isotropic; however this deviation from concentricity has not been well characterized. SD also have apparent cytoarchitectonic preferences. Leão10–12 and later others13–15 showed that SD were unlikely to propagate into retrosplenial cortex. There is also an apparent tropism of SD for superficial or dendritic layers, rather than deeper layers of cortex16–18. The mechanisms of these tropisms are unclear, but myelin content, astrocytic density, and vascular disruption have all been advanced as explanations19,20. Finally, it is well known but under-reported that SD vary considerably over time14.

The spatial and temporal heterogeneity of SD are important, because the factors that influence and constrain SD have the potential to explain basic features of the phenomena, which in turn could lend insight into clinical approaches. Thus we systematically examined the characteristics of SD in different preparations that would allow us to assess its heterogeneity. We show that SD susceptibility as well as propagation varies by cortical location, and by depth within the cortex. We show that non-uniform propagation is the rule, not the exception, for SD, and that this anisotropic propagation becomes more prominent with time, likely due to different relative refractory periods. Finally, we report patterns of SD propagation that suggest both vascular and cytoarchitectonic modulation of the phenomenon, and suggest avenues for future research.

**Methods**

***SD preparation:*** Male C57Bl/6J mice or Sprague-Dawley rats, 3-6 months of age, were used to avoid the known effects of sex on SD susceptibility21,22. Animals were anesthetized with isoflurane (5% induction, 1.4-1.7% maintenance) and mounted on a stereotaxic frame (Kopf Instruments). Vital signs (oxygen saturation, heart rate, respiratory rate, body temperature) were monitored and stabilized using a physiological monitoring apparatus (MouseStat, Kent Scientific). In mice, the parietal skull was exposed between bregma and lambda, and a standardized region measuring 3.2mm × 2.4 mm centered between bregma, lambda, sagittal sinus and temporal ridge was thinned to transparency. A burr hole for SD induction was created in four different locations, one location per animal (coordinates represent distance from bregma): *Lateral* – 2.6 mm posterior and 4.2 mm lateral. *Medial* – 2.6 mm posterior, 0.5 mm lateral. *Anterior* - 0.5 mm posterior and 2.3mm lateral. *Posterior* – 4.6 mm posterior, 2.3 mm lateral. A second burr hole for local field potential recording was created 4.6 mm posterior and 0.4 mm lateral to bregma. In rats, either a craniotomy with boundaries 1mm from bregma, lambda, sagittal sinus, and temporal ridge was made, or, for depth electrode recordings, a large (1-2mm diameter) burrhole was made (2mm lateral, 4mm posterior to bregma). For the craniotomy preparation, SD was induced midway between bregma and lambda at the temporal ridge (corresponding to the *Lateral* location in mice); for the depth electrode preparation, a second burrhole (2mm lateral, 7mm posterior to bregma) was used.

***Optical imaging:*** Briefly23,24, the cortex was illuminated by a white-light LED (5500K, Phillips Lumileds) and reflected light (optical intrinsic signal; OIS) was collected with a lens system consisting of two f/0.95 lenses connected front to front focused on a high-sensitivity 8-bit charge-coupled device camera (Mightex CCE-B013-U, Pleasanton, CA). Images were acquired at 2Hz for the duration of the experiment. In all experiments SD was induced by continuous application of 1M KCl solution for 1 hour. Excess KCl was removed by the capillary action of rolled filter paper placed adjacent to the KCl burr hole. Thin but intact skull preparation restricted KCl exposure and SD induction to the burrhole (all SDs were seen emanating from the burrhole).

***Optical spectroscopy:*** A fiber optic probe (R400-7-UV/VIS, Ocean Optics; Dunedin, FL, USA) (225um diameter), was placed 3.9mm posterior and 1.4mm lateral to bregma, parfocal with the OIS camera, and used to collect reflected light for spectroscopy. A spectrometer (USB400-UV-VIS, Ocean Optics) collected probe output at 1 Hz with a 1 s integration time and 5 nm boxcar smoothing. Hemoglobin saturation was derived using the Beer-Lambert law as previously described24.

***Electrophysiology:*** In all mice and rat craniotomy experiments, local field potential was recordedwith ACSF-filled (in mM: 125 NaCl, 3 KCl, 1.25 NaH2PO4, 2 CaCl2, 1 MgCl2, 25 NaHCO3, 10 glucose) glass microelectrodes (3MΩ) advanced 500um into the LFP burrhole25. The ground electrode was placed in the cervical muscles. Local field potentials were recorded using an Axopatch 1D amplifier (Molecular Devices) (0-500Hz band pass; digitized at 1kHz) synchronized with imaging data by a LabView Virtual Instrument (National Instruments). In rat depth electrode experiments, a two or three-electrode array (ACSF-filled, 10 um diameter tips, 10 MΩ resistance, horizontal separation 200-400 um. vertical separation 100 to 1800 um) was advanced into the burrhole. Interelectrode distances were verified after each recording and confirmed with histology. An Ag/AgCl reference electrode was inserted subcutaneously in the neck. The electrical signals were amplified with an ISODAM-8A bioamplifier at a DC-10 kHz band width (WPI Inc, USA), digitized at a 200 Hz sampling rate and stored for off-line analysis using Micro1401 MKII data acquisition system and Spike2 software (CED Co., UK).

***Brain slice recordings:*** Briefly26, male C57Bl/6 mice (age: 1–3 months) were deeply anaesthetized with isoflurane and decapitated. Coronal slices of 400 µm thickness were cut in an ice-cold oxygenated sucrose solution using a vibratome (MA752 Motorised Advance Vibroslice, Campden Instruments). The slices were then incubated in an oxygenated ACSF solution at room temperature for at least one hour before the experiments. SD was induced using a custom microfluidic device with ejection and suction ports that allowed precise focal ejection. SD was induced by substituting K+ for Na+ in the ACSF in defined steps, until threshold was reached26,27.

**Data analysis:**

Optical intrinsic signal (OIS) analysis. In each experiment the field of view (480 × 480 pixels) was divided into three rectangular regions of interest (ROIs, 480 × 180 pixels) placed perpendicular to the advancing SD wave front. A plot representing the change in cortical reflectance over time was generated for each ROI. SD were identified as a propagating change in reflected light during optical imaging. A histogram representing pixel brightness distribution was generated for each ROI. Any ROI which showed saturated pixels was discarded from further analysis. “*Full*” SD were defined as CSDs that propagated concentrically across the whole imaging field. “*Partial*” SD did not propagate across the whole imaging field, and had variable patterns of propagation as described below. The SD perfusion response was measured as shown in **Figure 1d**, between the maximum and minimum in wave-associated reflectance, and then normalized by dividing each CSD amplitude by the average amplitude of all CSDs in all experiments. Amplitude, duration, and velocity were only measeured for full CSDs.

**Statistics.** All analysis was performed using Graphpad Prism v5.03. A p<0.05 was considered significant. Data were plotted as box-whisker plots showing median, 25th percentile, 75th percentile, and minimal and maximal values. Comparisons between the different induction sites were made using one-way ANOVA followed by Tukey multiple comparison test, Student’s t-test. Comparisons between different ROIs were done using a repeated measure ANOVA followed by Dunnet’s test.

**Results**

***Incidence of SD varies by location of induction***

We examined the characteristics of SD induced by identical technique (1 hour continuous 1M KCl administration) from four locations at opposite ends of a standardized 3.2\*2.4mm region of thinned skull that was centered between bregma, lambda, sagittal suture and temporal ridge. The four stimulus locations were each in different cytoarchitectonic regions: the medial stimulation site was in retrosplenial granular cortex, just medial to the medial edge of parietal association cortex (in mm from bregma: 2.6 posterior, 0.5 lateral); the lateral site was in auditory cortex, just lateral to the posterolateral edge of barrel cortex (2.6 posterior, 4.2 lateral), the anterior stimulation site was in forelimb somatosensory cortex (0.5 posterior, 2.3 lateral), and the posterior site was in primary visual cortex (4.6 posterior, 2.3 lateral)(**Figure 1a**).

There was a significant difference in number of total SD elicited per hour over the four groups (one way ANOVA F-25.75, p<0.001, n=9, 8, 12, 8 for the medial, lateral, anterior and posterior locations, respectively). This result was driven by significant differences between the posterior induction site and all other induction sites (p<0.001) (**Figure 1b**).

The spatial extent of SD also varied significantly by location. The posterior site, with the highest number of induced SD, also had the highest number of ‘incomplete’ or ‘*partial*’ SD – waves that did not propagate through the whole imaging field. In contrast, the anterior induction site had significantly fewer SD than posterior (p<0.001 for total CSDs), but also had a significantly lower proportion of ‘complete’ SD (**Figure 1b**).

Because the imaging field (and the underlying cortex) was longer in the anteroposterior than the mediolateral axis, we compared the number of SD that reached a fixed, identical distance (470 pixels, or 2.3mm) distantfrom the site of induction, in order to make the comparison meaningful across all four locations. However, because anterior and posterior inductions both propagated along the long axis of the cortex, we were able to compare them along the whole length of the window. Here again significant differences were observed between the two locations, with significantly greater numbers of total, full, and partial SD for posterior inductions (Student’s t-test, p<0.001 for total, p<0.05 for full, and p<0.001 for partial SD, respectively).

With the difference in incidence of SD came differences in the timing of events. The posterior induction site had the shortest and most consistent (least variable) inter-event interval (ANOVA followed by Tukey test, p<0.05 for medial and p<0.001 for lateral and anterior sites), while the anterior site had the longest and most variable interevent interval. Medial and lateral locations were intermediate in value (**Figure 1c**).

***SD hemodynamic characteristics vary by cortical location***

Optical intrinsic signal (OIS) imaging is used to measure hemodynamic changes, and is primarily driven by hemoglobin concentration and/or saturation in each voxel28. We used spectroscopy to verify that reflectance in our wavelength range corresponded to changes in total hemoglobin rather than changes in saturation (**Figure 1d**). We then were able to characterize increases and decreases in reflectance as relative decreases and increases in blood volume, respectively. Because with multiple SD there are significant changes in baseline, we chose the distance between hypoperfusion peak and hyperperfusion peak3,23 as the most reliable metric for SD amplitude. We also used the time between these peaks as a proxy for SD duration. We used a 160x480 pixel region of interest, 160 pixels from each location, to compare the responses of each.

There were significant differences in amplitude and duration of SD perfusion correlates, with the posterior induction site showing both the highest amplitude (p<0.01) and the shortest duration of blood volume change (p<0.001 for medial and p<0.05 for anterior; ANOVA with post-hoc Dunnet test) (**Figure 1d**).

SD involves changes in hemoglobin saturation as well as blood volume3,24,29. We used a spectroscopic probe to measure hemoglobin saturation from a fixed region of interest in the posteromedial portion of the imaging window (**Figure 1e**). This region of interest was at different distances from each induction site (in mm from induction sites: posterior 1.1, medial 1.6, lateral 3.1, anterior 3.5), thus we hypothesized that hemoglobin desaturations would likely decrease with distance and thus might not reveal differences between locations. Surprisingly however, the posterior induction site, despite being closest to the spectroscopic probe, revealed significantly smaller desaturations than lateral and anterior SD (p<0.001 for lateral and anterior respectively), which were more distant.

***Significant differences between first and subsequent SD for all locations***

A consistent finding in all locations tested was that the first SD was significantly different from all following waves. This was most prominent for SD velocity, which was dramatically different between the first and subsequent events. It was also more variable, with a much wider distribution of velocities (**Figure 2a**). No significant differences in SD velocity were found between locations. For hemodynamic correlates, there was a significant decrease in the amplitude of SD blood volume change, though there was no difference in the duration of changes (**Figure 2b**). Another dramatic difference between first and subsequent SD was the extent of SD propagation – we observed no first SD that did not propagate through the entire imaging field, whereas subsequent SD could be either full or partial (examples in **Figure 5**).

***Pattern of propagation is inhomogeneous in all locations, and varies between locations***

SD induced from all four locations deviated significantly from concentric propagation, and the pattern of propagation was distinct for each location and each experiment, both initially and over time (**Figures 3,4**). While the first SD tended to be the most concentric and isotropic, even these usually contained inhomogeneities in both she shape of the wave front and in the rate of propagation across the imaging field.

Subsequent SD tended to become more irregular in shape, as well as slower in velocity and smaller in spatial extent. Examples included bi-lobed waves, double waves, and spiral waves, as well as waves that propagated across only part of the imaged field. There was also evidence of re-entrant waves, which left the imaging field only to return from another location. This was true for rat as well as mouse (**Figures 3,4**).

In order to systematize the analysis of wave patterns we divided the imaging field into an 8\*10 grid of equally sized squares and quantified the incidence of each square on the grid experiencing a SD passage, for all SD elicited from a given location. For example, if a square was affected by every SD elicited, its incidence was 100%. This gave a summary view of SD susceptibility (formally, SD occupancy) for each location. There were significant differences in the overall propagation pattern from each location. (**Figure 5a**). Other summary measures were the total area occupied by SD, and the mean percent activation of all regions, for each stimulus location. The former gives an index of how much cortex is affected by SD over time; the latter reveals how likely a given SD was to affect the whole cortex. Despite having the lowest percent activation score due to number of partial events, posterior-induced SD occupied the largest area over time by virtue of the very high frequency of events. Conversely, anterior-induced SD occupied the smallest area over time due to a small number of events, but had a very high activation score due to the low proportion of partial events **(Figure 5b,c)**.

**Factors modulating SD propagation**

*Modulation of SD by prior SD: refractory phenomena.*

The most obvious effect of prior SD on subsequent events was the decrement seen after the first SD (see above). However prior events continued to have major effects on subsequent SDs over time. A prominent phenotype was an increased likelihood of partial SD with increasing numbers of SD events (correlation of all partial SD with all total SD, Pearson r 0.78, p=3e-8, n=342). There was also an alternating pattern to full and partial SD events, especially for posterior inductions. When observing the different possible transitions from one SD to another (full to full SD, full to partial, partial to full, and partial to partial) 156/311 transitions (50%) were alternations from full to partial or partial to full SD; 109/311 (35%) were full to full; and 46/311 (15%) were partial to partial. These overall figures masked a large variability in full/partial and partial/full transitions between locations: 73% in posterior inductions, 65% in medial inductions, 33% in anterior inductions, and 21% in lateral inductions. This variability scaled with the number of partial SD (posterior > medial > anterior > lateral) and scaled approximately inversely with the interevent interval (anterior > lateral > medial > posterior).

A second phenotype was the avoidance of prior SD locations, when events were closely spaced in time. Here again there was often an alternating pattern whereby SD passed through one region in one instance, and the un-affected region in the subsequent instance (**Figure 4d**). The most dramatic version of this was spiral SD, where the spiral pattern was generated by an SD propagating radially around a region it had just traversed (**Figure 4f**).

Though refractory phenomena clearly determined the course of many partial SD, there was also apparent entrainment of distinct SD patterns for each experiment. This was observed for both full and partial SD. Excluding full symmetrical SD, 27/33 (82%) of experiments showed at least one repetitive full or partial SD pattern.

*Possible vascular modulation of the SD wavefront*

Occasionally the pattern of SD propagation appeared to be constrained or stopped by vascular structures – in all cases this was due to large veins rather than arteries (**Figure 4b,d,e,g**). This was typically not seen with the first SD, or with ‘full’ SDs, although in some instances the velocity of propagation appeared to have been slowed by a vessel (**Figure 4b**). Rather it occurred later in the experiment, with ‘partial’ SDs. The frequency of these events varied by induction location: 11/75 (15%) of all SD in 6/9 experiments anteriorly, 7/102 (6.8%) of all SD in 6/7 experiments posteriorly, 3/69 (4.3%) of all SD in 3/8 experiments laterally, and 13/96 (14%) of all SD in 6/9 experiments medially. The geometry of the veins appeared to affect the likelihood of modulation. In 6/6 anterior induction experiments, 6/6 posterior inductions, 2/3 lateral inductions, and 5/6 medial inductions the vein involved was within 30 degrees of perpendicular to the direction of propagation.

It must be emphasized that vascular modulation of SD propagation was an unpredictable event. Often the same vessel that appeared to have curtailed the propagation of one SD would have no effect on the propagation of a prior or subsequent SD (**Figure 4e**). And given the varied and irregular shape of the SDs, it was not possible to determine whether an apparent conformance to venous boundaries was real or coincidental. Nevertheless it was equally difficult to substantiate the hypothesis that SD was *not* affected by vascular structures – in the cases observed it was difficult to explain the pattern of propagation without them.

*Possible cytoarchitectonic modulation*

*Avoidance of posteromedial cortex.* We were able to confirm longstanding observations that the posteromedial cortex, mostly occupied by retrosplenial cortex, was less susceptible to SD propagation. To 3/4 stimulus locations, the posteromedial region was among the least likely areas for propagation (**Figure 5a**).

*Decreased propagation across anteroposterior midline.* We also observed that there was an apparent decreased likelihood of antero-posterior or postero-anterior SD propagation, at the approximate midpoint of the imaging window (**Figure 5a**; the medial width of the window was spanned by parietal association cortex at this location). Both anterior and posterior-induced SD were less likely to propagate across this region, despite otherwise very different characteristics – this is best seen with the median occupancy contour in **Figure 5a**. The pattern of truncated propagation was not evident for SD crossing the cortex medio-laterally or latero-medially, suggesting a relative barrier to propagation in the anteroposterior axis. It was not an artifact of greater distance traveled in the anteroposterior axis, because the relative truncation occurred before such a point.

*Preferential propagation in barrel somatosensory cortex.* There was also convergent information from all four induction locations suggesting preferential propagation through somatosensory barrel cortex. Of the 53 laterally-induced SD that propagated asymmetrically (out of 69 total), 43 (81%) propagated anteriorly prior to posteriorly. This anterior propagation corresponded to the location of the whisker barrel region of primary somatosensory cortex (posterior propagation was into parietal association cortex and auditory cortex). No such bias was seen for SD induced medially. Of the 71 asymmetric propagations (out of 96 total), 39 (55%) propagated anteriorly. For medial induction, anterior propagation was into trunk and hindlimb somatosensory cortex; posterior propagation was into secondary visual cortex. There was also evidence of preferential propagation into barrel cortex from anterior and posterior induction experiments. Of 60 asymmetric anterior propagations (out of 75 total) 41 (68%) propagated laterally into barrel cortex rather than medially into trunk and hindlimb somatosensory cortex. For posterior induction, 43/67 (64%) of asymmetric propagations (out of 102 total) propagated laterally into barrel cortex rather than medially into retrosplenial cortex.

*Propagation confined to visual cortex.* The highest number of partial SDs (as well as the highest total number of SD) was observed with posterior induction (**Figure 1b**). In contrast to the other induction locations, each located near cytoarchitectonic borders, the posterior induction location was centered well within V1 visual cortex, which itself was symmetrically bordered by V2 cortex. The majority of partial SD induced posteriorly remained within the boundaries of V1 and V2 cortex – this is best seen in **Figure 5a** where the greatest SD occupancy approximates V1/2 boundaries (see also **Figure 4b**, second and fourth panels). This type of symmetric pattern of partial SD was not seen for other induction locations, suggesting it was not an artifact of the induction technique. Indeed other locations had mostly asymmetric propagation of partial SD, as described above.

***Susceptibility of SD in superficial cortical layers***

We used arrays of three field potential electrodes to sample SD incidence and propagation at different cortical depths. Frequency of SD was consistently higher in the superficial compared to deep electrodes and depth of recording was negatively correlated with SD frequency (Spearman rank order correlation R= -0.45, p=0.02, n=22 measurements in 9 animals, 5 with triple electrode arrays, 4 with double electrode arrays) (**Figure 6a,b**).

Though we spaced KCl stimulation at least 3mm distant to the recording electrodes and in a separate burrhole, it is possible that the tropism for superficial layers in the above experients was due to superficial KCl application. To account for this possibility we used brain slice experiments where the size, location, and intensity of the stimulus, and the recording over different cortical layers, could be precisely controlled.

We used a microfluidic device with separate injection and suction ports that allowed us to control the location and shape of a plume of KCl (**Figure 6c**). The injection port center was aligned with either the upper, middle, or lower third of the cortical thickness, measured from pia to white matter. A typical SD induced from the middle location is shown in **Figure** **6c**. Despite the centered KCl perfusion, SD begins at the upper margins of the plume, and propagates preferentially in the superficial and not deep cortical layers. **Figure 6d** shows that for inductions in the middle of the slice, the majority of SD initiated in the upper layers, a minority in the middle layers, and none in the lower layers. For inductions in the upper layers, all SD were initiated in these layers. For inductions in the lower third, all SD were initiated in either the middle or upper third.

**Discussion**

The principal finding of this work is that SD are more heterogeneous – spatially, temporally, and by cortical location, than previously appreciated. This finding has implications for our understanding of SD mechanisms. It also has methodological implications for studies examining SD. Finally it has implications for the clinical disorders affected by SD.

**Preclinical implications**

From Leão’s initial observation of a lack of propagation into retrosplenial granular regions, it has been known that SD susceptibility cannot be uniform across the cortex. Yet remarkably little is known about this susceptibility. We systematically tested SD incidence and propagation in four different cortical regions - retrosplenial, auditory, forelimb somatosensory, and primary visual – and found that both incidence and propagation varied.

*SD Incidence*

The clear outlier for SD incidence was visual cortex, with a significantly higher incidence and greater frequency of SD compared to other regions. There was also a significantly higher incidence of partial events, whic often stayed within the confines of visual cortex. Hemodynamic correlates of visual cortex-induced SD were also different, with increased amplitude and decreased duration of blood volume changes associated with the event, and a smaller hemoglobin desaturation.

The mechanisms of the differential susceptibility to repetitive SD are unclear. The highest neuronal density occurs in visual cortex in mouse30 (as in human31 – by comparison, barrel cortex has the second highest neuronal density). Indeed, for both mouse and human, visual cortex is statistically separable from other regions by its distinct relationship between cellular number and cortical volume – it appears to be unique in this regard30,31. The clear outlier status in SD repetition would appear to be at least superficially consistent with the anatomical data. The cytoarchitectural separation of visual cortex might also provide an explanation for the frequent finding that SD did not propagate out of this region. The impression that emerges is that visual cortex has a different refractory period to other regions – whether because of cytoarchitectonic differences, hemodynamic response differences, or both - allowing the generation of more SD in a shorter period of time.

Note that our present results address the *number of SD* induced to a constant stimulus – they do not address absolute *threshold of SD*. While it might be intuitive to hypothesize that visual cortex would also have the lowest threshold for SD induction, we have found in separate work that this is not the case (Bogdanov et al, *under review*). When exposed to threshold concentrations of extracellular potassium, the first region to experience SD is somatosensory barrel cortex in the majority of cases. This shows that the threshold of SD is not equivalent to the ability to induce repetitive events – threshold and number are complementary and separable measures of susceptibility.

*SD Propagation*

Though SD is considered an approximately concentric and smooth phenomenon, we never observed an SD wave with either a smooth or circular wavefront. Moreover, maps of propagation showed fairly large alterations in velocity as the waves progressed over the cortex (**Figure 4**). This should not be surprising to investigators experienced with SD, but it is infrequently noted in the literature, and it has implications for SD mechanism. To our knowledge the only truly concentric and isotropic SD phenomena are generated in chick retina, which has a very uniform, and avascular, structure9,32. The evidence from chick retina suggests that the heterogeneity in SD in cortex may be due to heterogeneity in structure, both cellular and vascular.

Overall, there was a striking difference in propagation of SD induced from four different regions (**Figure 5**). Two primary patterns emerge: SD with a high likelihood of propagating broadly (forepaw and auditory cortex induction) and SD with a much more local overall propagation (visual and retrosplenial induction).

We were able to confirm prior work showing that SD was less likely to propagate into retrosplenial cortex10,13–15. This was most prominent for SD induced anteriorly. However it is noteworthy that there was no difficulty *inducing* SD in retrosplenial cortex – SD number was not significantly different from auditory and forepaw somatosensory inductions. It is possible that the *interface* between retrosplenial cortex and other regions, rather than retrosplenial cortex *per se*, constitutes the barrier to propagation. The relatively low ‘percent occupancy’ of cortex outside retrosplenial cortex, for retrosplenial-induced SD (**Figure 5a**), would be consistent with such a hypothesis. It is interesting to note the similarity with visual cortex induced SD: the high incidence of partial SD terminating within the borders of visual cortex suggesting a possible barrier at the interface of two regions.

We also observed an apparent preference for propagation through somatosensory barrel cortex, with directionality of propagation favoring barrel cortex for all inductions where barrel cortex was nearby (anterior, lateral, and posterior induction), and no directionality preference when barrel cortex was distant (medial induction). These data are in agreement with those of Eiselt et al13, who found that occipitally induced SD propagated laterally before moving medially. Barrel cortex is both ethologically relevant and highly developed in rodent: it has high neuronal density30 and a complex columnar structure33. We found that rises in extracellular K+ to a constant stimulus were larger in barrel cortex than surrounding regions (Bogdanov et al, *under review*), which may have accounted for earlier SD ignition in this region than others. This characteristic might also account for a relative propagation tropism.

There was an apparent modulation of SD propagation by vascular structures, with constraint or stoppage of propagation by large cortical veins, most prominently when parallel rather than perpendicular to the advancing wavefront. Given this geometry we suspect that the vessels (or surrounding structures; see below) served as physical barriers to propagation. However this putative barrier function was quite variable, occurring and not occurring in the same animal with subsequent SD.

Cytoarchitectural and vascular modulation of SD has recently been reported. Fujita et al19 observed a decrease in propagation velocity at the interface of neocortex and paleocortex. They also observed slowing or stoppage of SD at the middle cerebral artery and rhinal fissure. Modulation of SD propagation appeared to correlate with relative astrocyte density, which was greater in paleocortex, and around the middle cerebral artery and rhinal fissure. It is possible that relative differences in astrocyte density also account for the differences we observed. Though we do not know of specific astrocyte density measurements over the whole brain, Herculano-Houzel et al30 counted both neurons and non-neuronal cells (the latter are dominated by astrocytes) in all cortical regions. Interestingly, the highest density of non-neuronal cells was found in retrosplenial cortex. As neuronal density is not particularly high in this region, the non-neuron/neuron ratio is high. This might account for the resistance to propagation into retrosplenial cortex (though it does not explain why SD induction was not disfavored). It is also interesting to note that visual cortex had both very high non-neuronal and neuronal density; speculatively the high non-neuronal density might contribute to SD recovery, allowing the higher rates of SD in this region. It is likely that relative glial density is not the only contributor to different SD susceptibility and propagation – it has been demonstrated that cortical myelin suppresses SD propagation20, suggesting an effect of structural complexity beyond simple cellular density. It would also be surprising if the functional characteristics of cortex, mediated by the complement of receptors and channels in different regions, were not involved.

In addition to possible cytoarchitectonic and vascular modulation of propagation, there was a clear modulation by prior SD, suggestive of an influence of relative and absolute refractory periods. The most dramatic example of this phenomenon was the significant decrement in SD velocity between first and subsequent waves (**Figures 2,4**). This effect was long-lasting, as we observed reduced velocity SD for the duration of the experiments. As a possible mechanism, there is long-lasting tissue depolarization after SD, associated with a second direct current shift in extracellular field potential24 lasting over an hour, and membrane depolarization at least 30 minutes after the passage of the SD wave34. On a shorter time scale but also suggestive of refractory phenomena were SD that alternated location with prior SD, and spiral SD (**Figure 4d,f**). In addition, the alternation of ‘full’ and ‘partial’ SD (**Figure 4b**) may also be due to relative refractory periods: SD arising and propagating in tissue that has not fully repolarized may not have the capacity to propagate as far, or to propagate across relative barriers (cytoarchitectonic boundaries, vessels, or other).

It is worth noting that despite its lissencephalic nature, rodent cortex supported highly complex propagation patterns, including spiral waves. This kind of complex SD phenomenology has been reported in gyrencephalic cortex, and the structural nature of gyrencephalic cortex advanced as an explanation for the complexity35. While our data do not in any way rule out influences of gyration on SD propagation, they do show that it is not necessary for the generation of complex SD phenotypes. This should not be surprising, as spiral waves are observed in the even simpler retina36, and previous work in rodents has suggested such phenomenology37. From a theoretical standpoint, circling or spiral waves can be explained by refractory periods in any excitable medium38 – they do not require structural complexity.

It is also important to note that while the influences of prior SD on propagation were fairly clear cut, the putative influences of cytoarchitecture and vasculature were much more difficult to confirm. Findings were highly variable within each experiment. We suspect this is due to variably overlapping influences of architecture, vasculature, and relative and absolute refractory periods. All three parameters are highly dynamic: significant somal swelling and dendritic beading occur during SD39, along with equally large changes in vascular caliber23,40. Thus the physical structure of the cortex changes during and after the wave, coincident with massive and variable release of mediators (K+, glutamate) and the variable depolarization and repolarization of heterogeneous neuronal, glial, and vascular cells. More definitive work would evaluate SD propagation with both wide field acquisition (which is necessary to capture propagation over cytoarchitectonic and vascular territories) and cellular resolution, in order to isolate the possible contributors to variability.

*Differential susceptibility by cortical depth.*

Leão first showed that SD was more easily induced with electrical stimulation, and propagated faster, in the superficial cortical layers12,41. Later work with brain slices showed that SD propagates preferentially in superficial cortical layers17. However other work *in vivo* has suggested preferential propagation in *lower* cortical layers, and a relative barrier to SD propagation between superficial and deep layers42. In hippocampus, distinct propagation has been demonstrated in dendritic versus cellular layers16 that might have correlates in the cortex.

We therefore revisited the issue of SD initiation and propagation by depth, with *in vivo*  and  *in vitro* recordings. Both sets of experiments favor superficial propagation of SD: the total SD count was higher in superficial layers *in vivo*, and preferential spread in superficial layers could actually be observed *in vitro.* We also directly addressed SD susceptibility by depth *in vitro*. We were able to demonstrate that SD tended to be induced superficially even when a KCl plume was delivered to deeper layers. There are many possible mechanisms for this tropism, including neuronal, glial, and vascular density, structural complexity, and repertoire of conductances. While it is likely that many factors play a role, we suspect that the enrichment of dendritic structures, with their associated excitatory conductances, in the superficial cortical layers may be important.

**Methodological issues in SD research**

While there remains much to learn about its mechanisms, the heterogeneity of SD has clear implications for SD research. Given the different susceptibility and propagation at different cortical locations, it is important to specify (ideally with anatomical coordinates) the location of induction and recording. As susceptibility varies by depth, this applies not only to location at the cortical surface, but location within cortex.

It is also extremely important to consider the temporal order of SD in analysis and reporting. The first SD is significantly different from subsequent events, and SD can easily be induced during surgical preparation, even in experienced hands. It is likely that much analysis and reporting of SD has pooled first and subsequent SD, with a consequent ‘blurring’ of the mechanistic insight gained. We recommend that monitoring for SD commence with the beginning of the surgical preparation, and that all reporting of SD take into account the effect of prior SD as a potential modulator.

Finally, the large spatiotemporal heterogeneity of SD can clearly create ambiguity when point measurements (electrodes, biosensors) are used. Clear specification of location in x, y, and z planes is essential, and for repetitive events the possibility of eccentric waveforms generating difficult-to-interpret phenotypes must be entertained. Ideally, 2- and 3-D techniques can be combined with point measurements to better account for heterogeneity.

**Clinical/translational implications**

Clinically, SD appears to be a quite heterogeneous phenomenon. Though migraine aura can be stereotyped, it can also vary greatly even within the same subject43,44. Brain injury depolarizations recorded from humans show even greater variability2,4,8, likely owing to the diverse nature of injury, but here again there is also large intra-subject variability. It is likely that, consistent with this and prior data10,13–15,17, incidence, repetition rate, and pattern and extent of SD propagation vary by both cortical region and depth in humans as well as experimental animals. Differential susceptibility to SD can explain why the migraine aura arises where it does, and can suggest territories at risk for brain injury depolarizations. An understanding of the mechanisms that generate this differential susceptibility is a major goal for future SD research, as it offers the promise of modulating SD therapeutically.

**Acknowledgments:**

Funding: National Institutes of Health (NS 085413; KCB), Department of Defense (CDMRP PR 130373).

**Authorship Contributions**

Study conception: KCB; Experimental Design: DK, JZ, YTT, VBB, KCB; Performed experiments: DK, JZ, CAS, YTT, VBB, SM; Analysis: DK, JZ, JJT, CAS, YTT, VBB, JCC, KCB; Wrote manuscript: DK, JZ, JJT, CAS, YTT, VBB, JCC, SM, JS, YSJ, KCB.

**Conflict of Interest:**

None of the authors has any conflict to disclose.

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**Figure Legends**

**Figure 1: Susceptibility and hemodynamic characteristics of SD vary by induction site. A.** Schematic shows SD induction sites (black circles) superimposed over the mouse brain with labeled cytoarchitecture. Induction sites: medial: retrosplenial cortex; lateral: auditory cortex; anterior: forepaw somatosensory cortex; posterior: primary visual cortex. Rectangle shows boundaries of thin skull region; white circle shows location of spectroscopy probe. **B.** Box whisker plots show total, full, and partial SD counts originating from the four induction sites, measured from an equal-sized region of interest to allow comparisons between the four locations. There is a significant difference in total, full and partial SD, driven by large differences in the posterior (visual cortex) induction site (ANOVA followed by Tukey multiple comparison test, \*p<0.05, \*\*p<0.01, \*\*\*p<0.001, n(animals) = 9, 8, 9, 7, for the medial, lateral, anterior and posterior sites respectively). **C.** Total, full, and partial SD for anterior and posterior induction sites evaluated from the full 640 x 470 pixels window (Student’s t-test, \*p<0.05, \*\*\* p<0.001, n=9, 7 for the anterior and posterior sites respectively). **D.** Left trace shows equal and opposite changes in total hemoglobin (HbT) and optical intrinsic signal (OIS) during SD, showing that OIS can be used to infer blood volume changes. Right trace shows typical SD OIS profile. Amplitude of hemodynamic changes was measured from peak to trough of SD-associated reflectance change. Left graph. Normalized change in perfusion between the different induction sites. The posterior induction site had a higher normalized change in perfusion compared to the other induction sites. Right graph. Duration of perfusion between the different induction sites. The posterior site had a significantly reduced duration compared to the anterior and medial sites but not the lateral site. The lateral induction site had a significantly decreased duration of perfusion compared to the medial induction site (ANOVA followed by Tukey multiple comparison test, \*p<0.05, \*\* p<0.01, \*\*\* p<0.001, n = 6, 5, 7, and 4 for the medial, lateral anterior and posterior sites respectively). **E.** Schematic shows typical hemoglobin saturation trace during multiple SD. Hemoglobin desaturation amplitude (left graph) and half width (right graph). Hemoglobin desaturation was significantly smaller in posterior compared to anterior and lateral induction sites, despite being the closest site to the spectroscopy probe. The medial site had a significantly lower change in hemoglobin saturation compared to the lateral site. No difference between the different induction site was seen in half width duration (ANOVA followed by Tukey multiple comparison test, \*p<0.05, \*\*\*p<0.001, n = 4, 8, 4, 5, for the medial, lateral, anterior and posterior sites respectively).

**Figure 2: Velocity and hemodynamic amplitude are larger for first vs. subsequent SD. A.** SD velocity, though not significantly different between cortical induction sites (data not shown), shows significant decreases (as well as decreases in variability) when comparing first to all subsequent SD. **B.** Left graph. Amplitude of SD-associated blood volume transient is larger in first compared to subsequent SD. Right graph. There was no significant difference in duration of blood volume transients (\*\*\* p<0.001, \*p<0.05, Student’s t-test, n=20 mice).

**Figure 3: CSD propagation patterns for all experiments.** Each square represents a CSD propagation pattern. Total number of incidences is given above each pattern.

**Figure 4: Heterogeneous SD patterns.** All contours are plotted at 4 second intervals. **A.** All 8 SD from a medial induction experiment. Last panel is superimposed over a standard deviation map of the experiment, which shows veins as dark, arteries as bright (because they change shape during SD) and cortex as intermediate gray. SD propagation varies considerably between each event. Note that first SD propagates significantly faster than all subsequent events (also seen in B, C, E, and F). **B.** Alternating full and partial SD in a posterior induction experiment. Possible modulation of wave shape by cortical veins (first, third, and fourth panels; also see D, second and third panels of E, and G). **C.** Prominent example of difference between first and second SD velocity (see also A, B, E, and F). **D.** Eccentric propagation that avoids prior locations, avoids retrosplenial cortex (see also E). **E.** Increasing avoidance of retrosplenial cortex. Possible vascular modulation of wavefront. **F.** Partial SD followed by spiral SD that circles the location of prior partial (see also D). **G.** Heterogeneous SD propagation in rat. **H.** Varying propagation rate, possibly modulated by midline vein. Map at right plots derived SD velocity over each pixel, highlighting heterogeneity.

**Figure 5: Cumulative SD propagation characteristics. A.** Panels summarize all experiments for each induction location. Each panel is an 8\*10 grid rendering of the imaged region. Each square shows percentage occupancy by SD over all experiments for that induction location. For example, a square showing 50% occupancy was occupied by 50% of all SD waves induced. Dashed contour shows median occupancy value, giving a measure of central tendency. Each induction location has very different occupancy patterns. Also note that for all but posterior induction, there is a relative avoidance of the posteromedial (top left) squares, corresponding to retrosplenial cortex. **B.** Mean percent occupancy for the four induction sites. Lowest percent occupancy was for the posterior site (primary visual cortex), highest was for lateral site (auditory cortex), corresponding with the highest and lowest proportion of partial SD, respectively. **C.** Cumulative area exposed to SD over all experiments. Despite the large number of partial events, the high number of SD induced from the posterior site led to the largest area exposed.

**Figure 6: SD susceptibility and propagation vary by cortical depth. A.** Schematic shows depth electrode arrays and location of electrode and KCl stimulus placement in rat. **B.** Example SD traces at 450, 1000, and 1500 μm below the cortical surface (all >3 mm away from stimulus) showing decreased incidence of SD at depth. Plot at right shows quantifies lower SD incidence by depth. Box plots show number of SD/hour at 1-800 μm and 800-1600 μm depth (p=0.04, Student’s t-test); scatter plot shows all measurements. (Spearman rank order correlation R= -0.45, p=0.02, n=22 measurements in 9 animals, 5 with triple electrode arrays, 4 with double electrode arrays). **C.** Schematic shows microfluidic device with application and suction ports that allow delivery of a precisely sized plume of KCl to a brain slice. Images show SD induction and propagation in the superficial and middle but not deep layers, to a plume centered between pia and white matter. **D.** Preferential induction of SD in superficial layers. Location of plume was either in inner, middle, or outer third of cortex (schematics). SD could theoreticalliy start in either inner, middle, or outer third of cortex for each experimental paradigm (dotted circle, solid circle, filled circle, respectively – each circle represents a single experiment). For plumes located in inner cortex, there was no induction in the inner layers; 50% of inductions were in middle (solid circle), 50% were in outer cortex (filled circle). For plumes located in middle cortex, 56% were in outer cortex (the remainder were in middle cortex). For plumes located in outer cortex, 100% of SD were induced in outer cortex.