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Spreading depolarizations : the missing link between mirgraine and stroke

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SPREADING DEPOLARIZATIONS: THE MISSING LINK BETWEEN MIGRAINE AND STROKE

Cenk Ayata

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SPREADING DEPOLARIZATIONS: THE MISSING LINK BETWEEN MIGRAINE AND STROKE

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There is a fine line between elegance and arrogance.

(Cenk Ayata)

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CHAPTER 1

GENERAL INTRODUCTION

1. MIGRAINE

Migraine is a common and disabling episodic neurovascular disease[1] that affects ~15% of the world population; it is three times more common among women of reproductive age than men.[2, 3] Recurrent migraine attacks are characterized by severe headache accompanied to various degrees by nausea, vomiting, and increased sensitivity to light, sound, and smell, lasting anywhere between an hour to days (migraine *without* aura).[4] Attack frequency differs widely among patients, from one per year to several per week. Every day, millions of people are plagued by blistering migraines, which ranks among the most disabling medical conditions worldwide.[2, 5]

Migraine aura. One-third of migraine patients also experience transient focal neurological symptoms termed *aura* that usually precede or sometimes accompany the headache (migraine *with* aura).[4,6] Aura symptoms can be visual, sensory or motor, or involve language or brain stem function. Aura often shows a characteristic spread, such as a scintillating scotoma gradually expanding from a point of origin in one hemi visual field (Figure 1), or paresthesias marching up from the fingers and hand to sequentially involve the arm and then lower face. This form of spread pattern suggests an intracortical event propagating in a retinotopic or somatotopic fashion. It is widely accepted[7,8] that the symptomatology of migraine aura is caused by the electrophysiological event called *spreading depression (SD)*, an intense neuronal and glial spreading depolarization wave slowly propagating (~3 mm/min) in cerebral cortical or subcortical grey matter.[9-11] Mechanisms of migraine headache are still debated, although there is general agreement that activation and sensitization of the trigeminovascular nociceptive pathways is a critical step.[12,13] A large body of experimental evidence strongly suggests that SD is capable of activating the trigeminovascular system, and thus directly relevant for headache mechanisms.[11] The

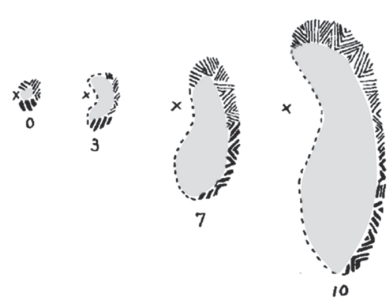


Figure 1. Scintillating scotoma of visual migraine aura. Fovea is indicated by x, and the time between each successive drawing by the numbers in minutes. The zig-zag pattern at the propagating wavefront represents scintillations (i.e., positive symptom at the wavefront) while the gray shaded area represents visual loss (i.e., scotoma, negative symptom).[15] Visual aura is caused by SD in occipital cortex.

trigeminovascular system consists of nociceptive trigeminal afferents surrounding the intracranial vessels. These perivascular trigeminal afferents project through the trigeminal ganglion to neurons in the trigeminocervical complex, and then relayed to the thalamus where all nociceptive inputs are integrated.[14]

2. SPREADING DEPRESSION

Definition. Spreading depression (SD) is an intense neuronal and glial depolarization wave that slowly propagates in brain tissue (~3 mm/min) by way of gray matter contiguity irrespective of functional divisions or vascular territories.[16] The near-complete loss of membrane potential is a result of massive transmembrane ionic and water shifts, including K^+ and glutamate efflux, and Na^+, Ca^{2+} and water influx, which last up to a minute and does not lead to injury in otherwise normal brain tissue. All these ionic and water shifts create a signature extracellular negative slow potential shift (aka DC shift) accompanied by suppression of action potentials and synaptic activity (Figure 2). As a result, electrocorticogram (ECoG) is depressed, hence the historical term ''spreading depression'' originally coined by Leao.[17] Similar spreading depolarization events also occur in ischemic brain, and are termed peri-infarct depolarizations (PIDs) or injury depolarizations (see below). Therefore, SD is a form of spreading depolarization event that occurs in otherwise normal brain.

Basic electrophysiology. SD is triggered when a sufficiently strong stimulus (e.g., topical application of concentrated KCl solution, direct cathodal electrical stimulation, seizure activity or tissue ischemia) simultaneously depolarizes a minimum critical volume of brain tissue estimated to be ~1 mm^3 in rodent cortex, *in vivo*.[18] The depolarizing stimulus overloads the extracellular K^+ ($[K^+]_e$) clearance mechanisms causing $[K^+]_e$ to exceed a critical threshold concentration of ~12 mM.[18-24] These thresholds can vary in different species and brain regions depending on neuronal and excitatory synaptic density among other factors.[25,26] The inciting event causes a sudden drop in membrane resistance via opening of non-selective large conductance cation channels, the presence and identity of which are yet incompletely understood.[27] As a result, both intracellular and extracellular ions move along their transmembrane concentration gradients. Massive K^+ efflux raises $[K^+]_e$ from ~3 mM at resting state[28-30] to about ~30-50 mM, and sometimes as high as 80 mM, in an all-or-none fashion, in most species, tissues and model systems.[19,22,29,31-34] This large K^+ efflux is reciprocated by Na^+ and Cl^- influx that pulls water, causing cell swelling.[22,35-40] Extracellular space shrinks by as much as 50%.[41, 42] Depolarization also triggers Ca^{2+} influx and a more than 10-fold drop in $[Ca^{2+}]_e$,[22,43,44] which, along with Na^+ and water influx, leads to release of many if not all neurotransmitters and neuromodulators within the depolarized tissue. Extracellular glutamate, aspartate, glycine, GABA and taurine concentrations increase during SD,[32,45,46] and similar increases have been shown for catecholamine and ascorbate levels.[47-49]

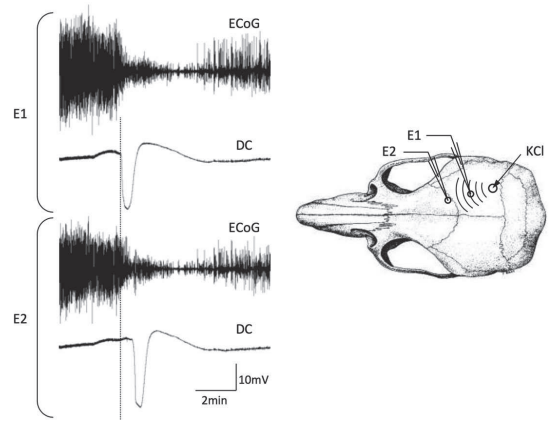


Figure 2. Representative tracings of electrocorticogram (ECoG) and extracellular DC potential during a cortical SD wave in rat brain, triggered by 1M KCl briefly applied on to the occipital cortex, and recorded by two intracortical glass microelectrodes placed in series with the KCl application site. SD is characterized by transient depression of cortical electrical activity and a slow DC shift 20-30 mV in amplitude lasting less than 1 min. When triggered by an intense stimulus that simultaneously depolarizes a minimum critical volume of brain tissue, SD spreads centrifugally to be detected first at the proximal (E1), and after a latency, at the distal (E2) microelectrode. In otherwise normal tissue, ECoG activity starts to recover a few minutes after the onset of SD, but may take up to 10 min to return to normal. The speed of propagation is calculated from the distance and the latency between the electrodes. Modified from [10].

Propagation of SD. It is believed that the rise in $[K^+]_e$ and glutamate act as chemical signals diffusing to and depolarizing adjacent cells, and in this way the depolarization slowly spreads. The massive rise in $[K^+]_e$ to levels sufficient to depolarize neighboring cells is the critical factor mediating the contiguous spread of the wave.[20,22,50] Elevated extracellular levels of the strongly depolarizing excitatory amino acids (glutamate and aspartate) further fuel SD and facilitate its propagation by activating NMDA receptors.[51,52] For the released K^+ and glutamate to reach the critical depolarization threshold of adjacent cells, high neuronal and synaptic density and low extracellular space volume are required; therefore, white matter is characteristically resistant to SD. However, SD can be triggered in subcortical grey matter structures such as striatum, thalamus and hippocampus, with the exception of brain stem, which is resistant to SD unless tested in immature animals or after pharmacological preconditioning (e.g., K^+-channel blockade).[53-57] Lastly, cortical SD can propagate into subcortical structures that have direct gray matter contiguity with the cortex.[58]

Recovery of SD. The massive redistribution of ions, water and neurotransmitters is self-limited. A number of mechanisms, including the Na^+/K^+-ATPase, intracellular buffering of $[Ca^{++}]_i$, reuptake and metabolism or spatial buffering by the astrocytic network, and quite likely vascular clearance, all help restore the homeostasis usually

within a minute. The process is in part energy-dependent and strongly stimulates O_2 and glucose consumption. Therefore, in severely hypoperfused (i.e., ischemic) tissue, restoration of homeostasis is delayed, with deleterious consequences on tissue viability.

SD and migraine. Since the discovery of SD decades ago, similarities between the electrophysiological properties of SD and the neurological signs and symptoms during migraine aura suggested a causative link between the two.[59-61] Experimental evidence also suggests that SD can trigger headache by activating the trigeminovascular system.[11,62-67] Although whether an asymptomatic SD triggers migraine headache without a perceived aura is still debated, suppression of SD susceptibility by migraine prophylactic drugs as a class effect supported this notion since these drugs have been equally efficacious in migraine with or without a perceived aura. Therefore, SD is now considered a potential therapeutic target in migraine, and experimental models of SD susceptibility are increasingly being used in migraine drug screening.

SD susceptibility. The ease with which SD can be initiated and sustained is often termed *SD susceptibility*, and can be used as an experimental surrogate for migraine (with aura) susceptibility. Indeed, modulation of SD susceptibility by drugs appears to be the basis for migraine prophylaxis.[68] There are several experimental models that can be used to measure SD susceptibility.[69] The most common attributes used to define SD susceptibility are: i) the threshold stimulus intensity that triggers an SD (i.e., electrical charge measured in Coulombs, or KCl concentration threshold) determined by sequential stimuli of stepwise escalating intensity until an SD is triggered, ii) the frequency of SDs triggered during continuous constant suprathreshold stimulus for up to an hour (i.e., topical high concentration of KCl), and iii) the propagation speed of SD.

3. ISCHEMIC STROKE

Definition of the problem. Ischemic stroke is an acute cerebrovascular catastrophe and a leading cause of death and disability worldwide.[70] It is caused by occlusion of an artery supplying the brain or spinal cord. Despite intense research into the pathophysiology and treatment of ischemic stroke, the only proven and widely adopted therapeutic modality is thrombolysis to achieve reperfusion in a timely manner. Brain tissue is highly sensitive to ischemia. Infarction ensues unless reperfusion is achieved within a few hours after cerebral arterial occlusion. Therefore, the therapeutic efficacy and safety window of thrombolysis is limited to only 4.5 hours after stroke onset.[71]

Ischemic core. Cerebral arterial occlusion typically creates a focal perfusion defect within its territory (Figure 3). Some degree of collateral blood flow reaches the focal ischemic tissue via surrounding patent arteries. However, efficacy of collateral flow drops as a function of distance, creating an inverted bell-shaped gradient of blood flow from the relatively well-perfused periphery towards the severely ischemic center. Irreversible ischemic injury (i.e., infarct) starts in the center and gradually expands over minutes to days into the periphery. Energy shortage in the severely ischemic center results in failure of Na^+/K^+ ATPase, gradual increase in extracellular potassium concentrations ($[K^+]_e$). Within a few minutes after the arterial occlusion, when $[K^+]_e$ reaches a critical threshold of ~12 mM (from a normal resting level of ~3-4 mM), neurons develop a sudden onset catastrophic loss of resting membrane potential (V_m), termed *anoxic depolarization (AD)*, which marks the *ischemic core*. The process is analogous to the triggering of SD as described above, with the critical distinction being tissue inability to restore the transmembrane ionic gradients because of ongoing ischemia. In that sense, AD is a persistent form of SD that can become permanent. It is generally believed that AD that continues longer than ~10-15 minutes results in irreversible cell injury and eventual death by necrosis and/or apoptosis.

Ischemic penumbra. Surrounding the core, there is sufficient collateral flow to maintain the resting V_m, but neural activity and synaptic transmission are suppressed via intrinsic protective mechanisms creating a ring of electrical silence (Figure 3). This 'still-viable tissue at risk for infarction' is called *ischemic penumbra*. The spatiotemporal evolution of injury in penumbra is complex. Depending on residual tissue perfusion and the intrinsic tissue sensitivity to ischemia, portions of penumbra succumb to AD, and get incorporated into the ischemic core over time. Therefore, ischemic core gradually expands into the penumbra (Figure 4). A critical factor that facilitates ischemic injury, expands the infarct and worsens the neurological outcome is the occurrence of spontaneous spreading depression waves within the peri-infarct tissue, called peri-infarct depolarizations.

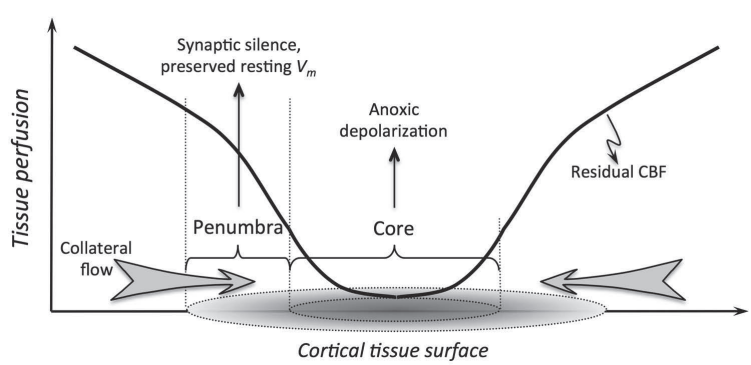


Figure 3. The hemodynamic status and membrane potential of acute focal ischemic brain tissue showing the definitions of ischemic penumbra and core. V_m, membrane potential.

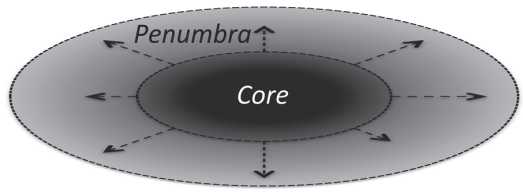


Figure 4. Ischemic core gradually expands into the penumbra because of persistent ischemia and the occurrence of peri-infarct depolarizations.

Peri-infarct depolarizations. In addition to AD, ischemic brain also develops spontaneous recurrent *peri-infarct spreading depolarization waves* (PIDs) that originate at the boundary and propagate throughout the ischemic and non-ischemic tissue. When PIDs enter the non-ischemic tissue, they are electrophysiologically indistinguishable from SD, the substrate of migraine aura, suggesting that PIDs are analogous to SDs (Figure 5). The local rise in $[K^+]_e$ and the hypoxic suppression of Na^+/K^+-ATPase in ischemic brain serve as the strongly depolarizing stimuli that trigger PIDs, analogous to the topical KCl or electrical stimulation to trigger SDs. Importantly, occurrence of PIDs have been shown in human brain as well.[72] Therefore, SDs and PIDs represent a mechanistic overlap between migraine and stroke.

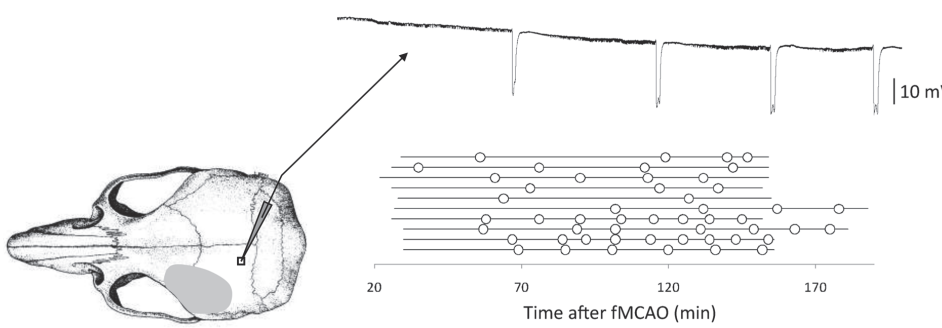


Figure 5. Recurrent peri-infarct injury depolarizations (PIDs; four negative deflections on the tracing) recorded during filament occlusion of the middle cerebral artery by an intracortical glass micropipette placed outside the ischemic tissue in a representative mouse. These PIDs are indistinguishable from SDs triggered by intense depolarization in otherwise normal cortex. Lower right panel is the summary of 10 experiments, where each horizontal line shows the beginning and end of electrophysiological recordings after the onset of ischemia, and each circle represents a PID. Grey shaded area shows the approximate location of the perfusion defect upon arterial occlusion. Recurrent PIDs appear spontaneously throughout the recording period in all mice.

Acute infarct progression on MRI. Ionic changes and cell swelling in tissue that has undergone AD cause a characteristic decrease in apparent diffusion coefficient (ADC) of water on diffusion-weighted MRI (DWI) as the *MRI signature of core* infarction (Figure 6). Perfusion-weighted MRI (PWI) outlines the total tissue at risk for infarction. Therefore, the mismatch between DWI and PWI lesion volumes is a measure of viable tissue at risk for infarction (i.e., penumbra). Ischemic penumbra is the primary target of all acute stroke rescue interventions. The volume of penumbra present at any given time is a good measure of how much tissue is available that can be rescued from ischemic infarction.

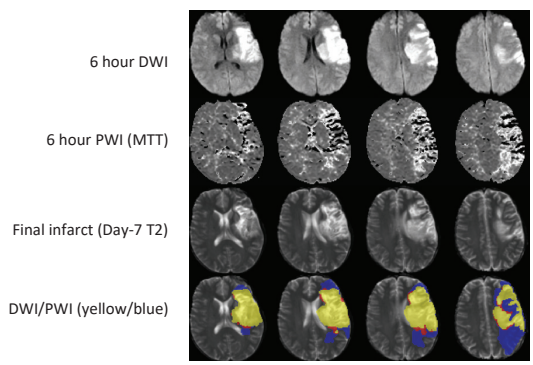


Figure 6. Acute MRI of a typical middle cerebral artery stroke. Diffusion-weighted imaging (DWI) shows the infarcted tissue (yellow), whereas perfusion-weighted imaging (PWI; measured by mean transit time, MTT, blue) delineates the hypoperfused tissue. The DWI/PWI mismatch (blue tissue outside the yellow) is a surrogate MRI measure of ischemic penumbra, defined as 'still-viable tissue at risk for infarction'. Day 7 T2 MRI shows the final infarct volume in this patient. Courtesy of Dr. Hakan Ay.

4. CLINICAL ASSOCIATION BETWEEN MIGRAINE AND STROKE

Migraine has traditionally been viewed as a benign chronic episodic condition. However, accumulating evidence suggests that migraine with aura, can be associated with increased risk for stroke and white matter lesions.[73-79]

Observational data. Abundant data from retrospective and population- or hospital-based case-control studies, as well as small and large population-based prospective studies including tens of thousands of subjects, have firmly established a link between migraine and ischemic stroke.[77,80,81] The odds ratio (OR) for ischemic stroke is ~2 among migraineurs (95% confidence interval [CI] 1.72-2.43). Important insights are:

I. The association is explained by migraine with aura alone (OR 2.5, 95% CI 1.5-4.1), and does not reach statistical significance for migraine without aura (OR 1.3, 95% CI 0.8-2.1),

II. The association is stronger in women (OR 2.9, 95% CI 2.4-3.5),[77] and in subjects younger than 45 (OR 2.7, 95% CI 1.4-5.0),
III. The risk is higher in patients who experience active migraine attacks (OR 1.9, 95% CI 1.2-3.1), and not in those with just a history of migraine without recent attacks,[82]
IV. The risk is higher in those who experience >12 attacks per year (OR 1.7, 95% CI 1.1-2.8).[83]

Neuroimaging. A number of neuroimaging studies over the past decade revealed a higher prevalence of subclinical brain lesions in migraineurs, including infarcts and white matter hyperintensities, suggesting acute or chronic ischemic disease. CAMERA (Cerebral Abnormalities in Migraine and Epidemiological Risk Analysis) was a cross-sectional, population-based MRI lesion prevalence study in subjects between ages 30 and 60 (mean age 48; 161 migraine with aura, 134 migraine without aura and 140 matched controls) randomly selected from the Genetic Epidemiology of Migraine study. Data suggested an increased risk of subclinical posterior circulation infarct-like lesions, mostly located in the cerebellum, in migraineurs compared to controls (OR 7.1, 95% CI 0.9-55).[78,84] Important insights are:

I. The risk was substantially higher in migraineurs with aura (OR 13.7, 95% CI 1.7-112),
II. The risk was also higher with frequent migraine attacks (≥1 attack/month) (OR 15.8, 95% CI 1.8-140),
III. The risk was independent of triptan use or vascular risk factors,
IV. In the 9-year follow up of the same cohort, none of the lesions disappeared, and new posterior circulation lesions were found in 5% of migraineurs compared with none in control subjects.[85]

The conclusions of CAMERA study were later independently confirmed in the Age Gene/Environment Susceptibility Reykjavik study,[86] and in the population-based Epidemiology of Vascular Aging study (780 subjects, mean age of 69).[87] Clinical contrasts between migraine and stroke make this association highly intriguing:

1. Unlike the perceived benign nature of migraine (i.e., no imminent risk of injury), stroke is: (i) an acute and often catastrophic cerebrovascular event, (ii) the leading cause of acquired physical disability in adults in the US,[88] and (iii) the second leading cause of mortality worldwide.[70]
2. Stroke is predominantly a disease of the elderly, while migraine prevalence peaks around age 40.
3. Stroke risk is higher in males than females of reproductive age, whereas migraine is higher in females of reproductive age and increases stroke risk most prominently in this group.

Investigation of the mechanisms underlying the association between migraine and stroke can impact hundreds of millions of people worldwide.

5. TRANSGENIC ANIMAL MODELS OF HUMAN MIGRAINE SYNDROMES

Modeling susceptibility to migraine. In order to elucidate the mechanisms linking migraine with aura and stroke, animal models are needed. While animal models of stroke are plenty, animal models of migraine with aura are relatively scarce. Migraine is an inherited disease.[89] Genetic determinants of migraine susceptibility, occurrence and severity range from rare monogenic inherited conditions (e.g., a single mutation is sufficient to cause disease in a patient) with large effect sizes to common polygenic influences (e.g., multiple polymorphisms are needed to cause disease in a patient) with small effect sizes. While the latter (i.e., common variants with small effects) is difficult to model experimentally, transgenic mouse models expressing human migraine mutations have been generated that recapitulate the clinical features of monogenic inherited conditions characterized by migraine with aura as well as ischemic stroke (e.g., CADASIL[90, 91]). Among these are mutant mouse models of familial hemiplegic migraine (FHM).

Familial hemiplegic migraine. Hemiplegic migraine is a rare monogenic form of migraine with aura characterized by motor weakness during the attacks sometimes accompanied by sensory, aphasic, visual and basilar symptoms, that can be sporadic, or familial (autosomal dominant), linked to genes involved in ion regulation (Table 1). Auras are often severe and prolonged, and a third of patients can experience a decrease in level of consciousness and even coma, which may be prolonged.[92] The net result of all FHM mutations identified to date is dysregulation of membrane ionic equilibrium and hyperexcitability, predicting enhanced susceptibility to SD as the overarching theme and the overlapping feature between migraine and stroke. FHM has been used as a model for more common forms of migraine with aura because of shared clinical features and trigger factors, female preponderance, and because two thirds of FHM patients and their first-degree relatives also suffer from attacks of common migraine with and without aura.

Table 1. Familial hemiplegic migraine genes identified to date. Modified from Russell and Ducros, 2011.[92]

	FHM1	**FHM2**	**FHM3**
Chromosome	19p13	1q23	2q24
Gene	*CACNA1A*	*ATP1A2*	*SCN1A*
Protein	Pore-forming α1 subunit of neuronal $Ca_V2.1$ (P/Q-type) voltage-gated Ca^{2+} channels	Catalytic α2 subunit of a glial and neuronal Na^+/K^+ ATPase	Pore-forming α1 subunit of neuronal $Na_V1.1$ voltage-gated Na^+ channels

Familial hemiplegic migraine type 1. FHM1 is caused by mutations in the pore-forming α_{1A} subunit of neuronal $Ca_V2.1$ (P/Q-type) voltage-gated Ca^{2+} channels (Figure 7). Mutations shift the voltage-current relationship of the channel to the left so that channels open at more negative membrane potentials (i.e., upon smaller membrane depolarizations), and stay open longer, increasing the Ca^{2+} influx. $Ca_V2.1$ channels are major regulators of presynaptic glutamate release. The net result is increased presynaptic Ca^{2+} entry and glutamate release resulting in enhanced cerebral excitability[93] that may be shared with more common forms of migraine.[13] Among the FHM1 mutations identified to date, the S218L missense mutation confers stronger gain of $Ca_V2.1$ channel function and a more severe clinical phenotype with prolonged auras that can progress to coma.[94,95] In contrast, the R192Q mutation is associated with modest gain of $Ca_V2.1$ channel function and pure hemiplegic auras.[96]

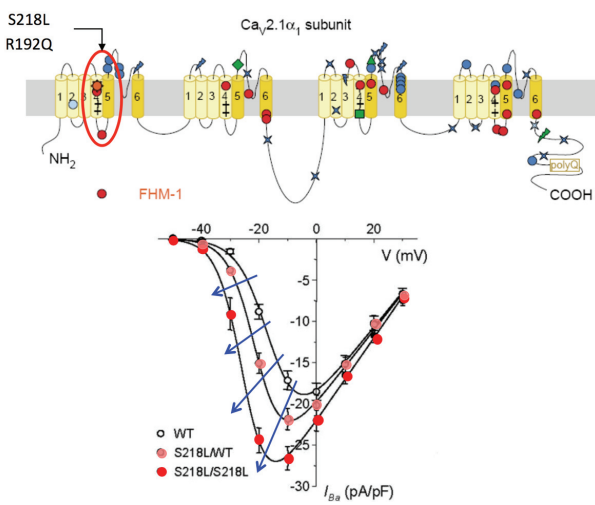


Figure 7. FHM1 mutations and the $Ca_V2.1$ gain-of-function. The two missense mutations studied as part of this thesis are also shown (S218L and R192Q). Blue arrows show the direction of change by the S218L mutation with an allele dosage effect. Modified from Pietrobon, 2005.[97]

6. OUTLINE OF THE THESIS

The overall hypothesis. The central hypothesis states that susceptibility to SD determines both the susceptibility to migraine with aura and the susceptibility to hypoxic/ischemic injury in the same direction. Therefore, factors that enhance the susceptibility to SD increase the likelihood of migraine with aura as well as ischemic stroke. Such factors that can modulate the susceptibility to SD may include genes, hormones and pharmacological agents.

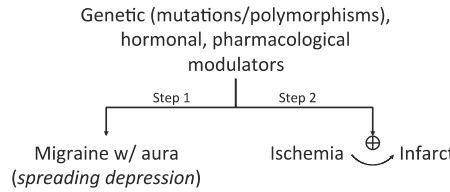


Figure 8. The central hypothesis.

The hypothesis predicts that genetic, hormonal and pharmacological modulators that enhance or suppress SD susceptibility will render the brain more or less susceptible to ischemic injury, respectively (Figure 8). As such, SD is hypothesized to be the missing link between migraine and ischemic stroke. In order to test the hypothesis in a logical sequential manner, it is first necessary to identify and characterize a set of factors that modulate the susceptibility to SD that mimic the clinical observations of susceptibility to migraine aura (Figure 8, step 1), and then study the impact of those factors on outcome of a cerebral ischemic event (Figure 8, step 2).

The aim of this thesis is to investigate how genetic, hormonal and pharmacological modulators of SD susceptibility will influence the susceptible to ischemic injury. To this end we will unravel underlying mechanisms of SD susceptibility and susceptibility to ischemic injury by making use of two transgenic mouse models of migraine that carry migraine-relevant gene mutations in voltage-gated $Ca_V2.1$ Ca^{2+} channels (**Chapters 2 and 3**) and will review all relevant clinical and experimental data from the literature (**Chapter 4**).

In **Chapter 2**, we describe the genetic and gonadal hormone modulation of cortical SD susceptibility using state-of-the-art *in vivo* electrophysiological studies under full systemic physiological monitoring and maintenance in mice. To characterize the genetic modulation, we utilize two transgenic (knock-in) mouse models of FHM1 expressing the *Cacna1a* R192Q or S218L missense mutation in $Ca_V2.1$ voltage-gated (P/Q-type) Ca^{2+} channels. As mentioned above, the magnitude of single channel gain-of-function is larger in S218L mutants compared with R192Q, and with homozygous mutants compared with heterozygotes. Therefore, the use of two mutations and three genotypes (wild-type controls, heterozygous and homozygous mutants) allows a graded modulation the SD susceptibility. **Chapter 2A** also dissects the female gonadal hormone modulation of CSD susceptibility by gonadectomy (i.e., ovariectomy) and hormone replacement, and draws parallels between the clinical symptomatology of FHM and the neurological signs displayed by FHM1 mutant mice after an SD. Because higher SD susceptibility in female mutants can in part be due to an inhibitory effect of male gonadal hormones on SD in male mice, in **Chapter 2B** we investigate the effect of

androgens on SD once again using gonadectomy (i.e., orchiectomy). Ischemic events are not limited to cortex; therefore, in **Chapter 2C** we study the SD susceptibility in subcortical structures, including striatum, thalamus and hippocampus, and its genetic and gonadal hormone modulation, with the same graded approach using different mutants, genotypes and sexes as described above. Altogether, studies in **Chapter 2** set the stage to investigate the same genetic and hormonal modulators on the outcome of cerebral ischemic events in **Chapter 3**.

In **Chapter 3**, we build upon the data obtained in **Chapter 2** and investigate how SD modulators impact the outcome of cerebral ischemic events. In **Chapter 3A**, we test the impact of genetic susceptibility to SD on susceptibility to ischemic injury by making use of FHM1 knock-in mouse models. By comparing the R192Q and S218L mutants, different genotypes (heterozygotes vs. homozygotes) and sexes, we get an opportunity to study the correspondence between SD susceptibility and ischemic sensitivity across a wide range of phenotypes. **Chapter 3B** tests the opposite effect, i.e., decreased SD susceptibility, using chronic treatment by migraine prophylactic drugs known to suppress SD. By studying two drugs and comparing the effects of chronic treatment (i.e., mimicking migraine prophylaxis) to acute effects of a single dose, we once again get an opportunity to study a wide range of effect size. And finally in **Chapter 3C**, we take the first step towards clinical translation in a retrospective case-control study, by testing whether a history of migraine, particularly migraine with aura, accelerates infarction of ischemic penumbra in acute human stroke as well.

Lastly, **Chapter 4** puts the data generated in this thesis and all relevant clinical and experimental data from the literature together into a comprehensive review to form a synthesis that can explain the clinical association between migraine and stroke. Novel hypotheses and potential experimental approaches to test them are proposed to elucidate potential mechanisms.

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CHAPTER 2

GENETIC AND HORMONAL REGULATION OF SPREADING DEPRESSION

CHAPTER 2A

GENETIC AND HORMONAL FACTORS MODULATE SPREADING DEPRESSION AND TRANSIENT HEMIPARESIS IN MOUSE MODELS OF FAMILIAL HEMIPLEGIC MIGRAINE TYPE 1

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ABSTRACT

Familial hemiplegic migraine type 1 (FHM1) is an autosomal dominant subtype of migraine with aura that is associated with hemiparesis. As with other types of migraine, it affects women more frequently than men. FHM1 is caused by mutations in the *CACNA1A* gene, which encodes the α_{1A} subunit of $Ca_v2.1$ channels; the R192Q mutation in *CACNA1A* causes a mild form of FHM1, whereas the S218L mutation causes a severe, often lethal phenotype. Spreading depression (SD), a slowly propagating neuronal and glial cell depolarization that leads to depression of neuronal activity, is the most likely cause of migraine aura. Here, we have shown that transgenic mice expressing R192Q or S218L FHM1 mutations have increased SD frequency and propagation speed; enhanced corticostriatal propagation; and, similar to the human FHM1 phenotype, more severe and prolonged post-SD neurological deficits. The susceptibility to SD and neurological deficits is affected by allele dosage and is higher in S218L than R192Q mutants. Further, female S218L and R192Q mutant mice were more susceptible to SD and neurological deficits than males. This sex difference was abrogated by ovariectomy and senescence and was partially restored by estrogen replacement, implicating ovarian hormones in the observed sex differences in humans with FHM1. These findings demonstrate that genetic and hormonal factors modulate susceptibility to SD and neurological deficits in FHM1 mutant mice, providing a potential mechanism for the phenotypic diversity of human migraine and aura.

INTRODUCTION

Spreading depression (SD) is characterized by an intense depolarization of neuronal and glial membranes that propagates in brain tissue at a rate of approximately 3 mm/min.[1,2] Evoked when local extracellular K^+ concentrations exceed a critical threshold, SD is associated with disruption of membrane ionic gradients, and massive K^+ efflux and glutamate release; both are believed to depolarize adjacent neurons and glia, thereby facilitating its spread. There is growing evidence from animal experiments, suggesting that cortical SD (CSD) is the electrophysiological event underlying migraine aura and a possible trigger of headache mechanisms.[3-5] In humans, evidence for a causal role of SD in the aura comes from functional MRI performed during migraine with aura attacks.[6,7]

SD is also implicated in the pathophysiology of familial hemiplegic migraine (FHM), an autosomal dominant subtype of migraine with aura associated with hemiparesis.[8] The aura and headache features are otherwise identical to those in the common, multi-factorial forms of migraine. This and the fact that the majority of FHM patients also experience attacks of common migraine with or without aura[9] make FHM a valid model for studying the pathogenesis of common, complex types of migraine.

Thus far, 3 FHM genes have been identified.[10] The FHM1 *CACNA1A* gene encodes the pore-forming α_{1A}-subunit of neuronal, voltage-gated $Ca_v2.1$ (previously known as P/Q-type) calcium channels.[11,12] Two knock-in FHM1 mouse models, carrying the human pathogenic R192Q or S218L missense mutation, were generated.[13,14] In patients, the R192Q mutation causes pure FHM without other associated neurological features,[12] whereas the S218L mutation causes a severe migraine phenotype with excessive and often fatal cerebral edema.[15] When expressed in transfected cultured neurons, both mutations shift channel opening toward more negative membrane potentials and delay channel inactivation; the S218L mutation causes more pronounced single-channel gain of function than R192Q.[14,16] As a result, channels open with smaller depolarization and stay open longer, allowing more Ca^{2+} to enter presynaptic terminals. FHM1 mouse models exhibit a reduced threshold for electrically evoked CSD and increased SD velocity.[13,17]

Gonadal hormones are important modulators of migraine[18] and cortical excitability.[19-21] In migraine with aura, the incidence of attacks reportedly increases during periods of high circulating estrogen.[18,22,23] Higher plasma estrogen concentrations were measured during normal menstrual cycle in migraineurs with aura.[24] Whereas the prevalence of migraine is similar in boys and girls before puberty (4%), after puberty it rises to about 3-fold higher in adult females (25%) than in males (8%).[25,26] A female preponderance is also described for familial (5:2 ratio) and sporadic (4.25:1 ratio) hemiplegic migraine.[9,27,28]

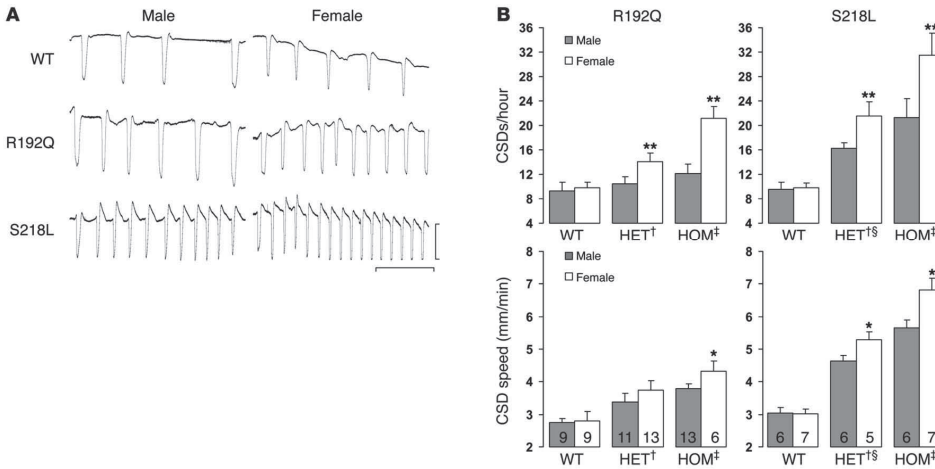


Figure 1. Enhanced CSD susceptibility in FHM1 mutant mice. (**A**) Representative electrophysiological recordings showing increased frequency of slow (DC) potential shifts during repetitive CSDs in male and female homozygous R192Q or S218L mutants compared with WT mice evoked by topical KCl application (300 mM) for 30 minutes. The number of CSDs was substantially higher in FHM1 mutant strains compared with WT controls and in female mutants versus males. Calibration bars: vertical, 20 mV; horizontal, 10 minutes. (**B**) The impact of R192Q and S218L mutations, allele dosage, and sex on CSD frequency and propagation speed (upper and lower graphs, respectively). Higher CSD frequencies and propagation speeds were found in both FHM1 mutants, with values higher in S218L than in R192Q mutants and an allele dosage relation. CSD frequency was greater and propagation faster in females compared with males in both FHM1 mutant strains. No sex difference was found in WT mice. Covariance analysis revealed that 82% of variation in CSD frequency and 93% of variation in CSD propagation speed were explained by the independent variables mutation, genotype, and sex (see Methods). Numbers of mice per group are shown within the bars. HET, heterozygous; HOM, homozygous. Data are mean ± standard deviation. **P < 0.001, *P < 0.01 versus male in CSD frequency and propagation speed; †P < 0.001 versus WT and homozygous mutant; ‡P < 0.001 versus WT and heterozygous mutant; §P < 0.001 versus corresponding R192Q genotype.

In this study, we investigated the modulating effect of different allelic mutations, gene dosage, and gonadal hormones on SD susceptibility and subsequent neurological deficits as surrogates for migraine aura in mouse models of FHM1. We found that both FHM1 mutant strains exhibited enhanced SD susceptibility, and SD induced severe and prolonged unilateral motor deficits akin to the human FHM phenotype. Furthermore, both SD susceptibility and severity of neurological deficits were modulated by: (a) the degree of gain of function caused by allelic mutations, (b) allele dosage, and (c) female gonadal hormones. Our data provide a potential mechanism for the severe and prolonged neurological deficits in FHM patients and underscore that genetic and hormonal factors contribute to the phenotypic diversity of human migraine syndromes.

RESULTS

CSD susceptibility in FHM1 mutant mice is modulated by $Ca_v2.1$ gain of function, allele dosage, and ovarian hormones. SD susceptibility was assessed by: (a) counting the number of evoked SDs during continuous topical KCl (300 mM) application and (b) measuring the propagation speed of SDs between 2 recording electrodes. The values in the different strains were compared. In WT mice, epidural KCl evoked repetitive CSDs (9.5 ± 1.0 SDs/h) with a propagation speed of 2.8 ± 0.2 mm/min. Both the frequency and the propagation speed were increased in the R192Q mutants and even more so in the S218L mutants (Figure 1). Furthermore, heterozygous mice of both FHM1 mutant strains showed SD frequencies and propagation speeds intermediate between those in WT and homozygous mutant mice, indicating an allele dosage effect. The duration or amplitude of SDs after KCl application did not differ among the groups (Table 1). The electrocorticogram did not show seizure activity in mutant mice during these SD recordings under anesthesia.

SD susceptibility (i.e., frequency and propagation speed) was strikingly higher in females than in males of both FHM1 mutant strains but not in WT mice (Figure 1). When tested in R192Q mutant mice, the sex difference was abrogated by ovariectomy, implicating effects of female gonadal hormones in adult brain, rather than a perinatal

Genotype/sex	Gonadal status	Age (mo)	*n*	Duration (s)		Amplitude (mV)	MABP	pH	pCO_2	pO_2
R192Q strain				**1st CSD**	**All CSDs**	**All CSDs**				
WT male	Normal	4.3 ± 0.8	9	42 ± 10	23 ± 11	25 ± 5	81 ± 10	7.36 ± 0.1	38 ± 9	147 ± 19
WT female	Normal	3.7 ± 0.9	9	40 ± 10	16 ± 6	28 ± 3	81 ± 6	7.35 ± 0.03	40 ± 4	133 ± 13
	Ovx	3.7 ± 0.6	10	40 ± 9	18 ± 5	27 ± 3	82 ± 7	7.35 ± 0.03	35 ± 4	139 ± 18
	Ovx + estrogen	3.3 ± 0.0	5	34 ± 6	17 ± 5	30 ± 1	68 ± 6	7.36 ± 0.05	33 ± 4	155 ± 23
	Aged	11.9 ± 1.5	7	26 ± 4	11 ± 3	25 ± 6	83 ± 8	7.38 ± 0.04	30 ± 3	141 ± 22
HET male	Normal	5.2 ± 1.4	11	41 ± 9	19 ± 6	25 ± 7	81 ± 6	7.37 ± 0.06	33 ± 6	133 ± 14
HET female	Normal	4.3 ± 1.3	13	39 ± 11	16 ± 6	24 ± 8	77 ± 6	7.34 ± 0.04	33 ± 6	124 ± 15
HOM male	Normal	4.4 ± 0.8	13	38 ± 10	15 ± 4	24 ± 5	80 ± 6	7.36 ± 0.05	33 ± 6	127 ± 20
HOM female	Normal	3.3 ± 0.4	6	35 ± 7	12 ± 2	30 ± 4	81 ± 4	7.35 ± 0.02	34 ± 2	126 ± 20
	Ovx	4.4 ± 0.9	12	31 ± 6	13 ± 2	28 ± 3	88 ± 4	7.34 ± 0.05	36 ± 9	135 ± 34
	Ovx + estrogen	6.1 ± 0.3	6	30 ± 2	14 ± 2	30 ± 1	79 ± 2	7.38 ± 0.03	31 ± 3	130 ± 22
	Aged	13.0 ± 2.7	7	29 ± 1	13 ± 2	26 ± 1	81 ± 7	7.35 ± 0.04	33 ± 4	153 ± 22
S218L strain										
WT male	Normal	2.7 ± 0.5	6	34 ± 2	14 ± 4	25 ± 2	88 ± 15	7.41 ± 0.03	27 ± 4	148 ± 17
WT female	Normal	2.9 ± 0.7	7	32 ± 7	14 ± 4	25 ± 3	96 ± 7	7.36 ± 0.05	31 ± 4	138 ± 20
HET male	Normal	3.0 ± 0.0	6	29 ± 4	11 ± 2	25 ± 6	93 ± 5	7.41 ± 0.02	29 ± 3	140 ± 9
HET female	Normal	2.6 ± 0.5	5	35 ± 6	11 ± 2	24 ± 2	95 ± 6	7.38 ± 0.01	30 ± 2	144 ± 7
HOM male	Normal	2.7 ± 0.5	6	36 ± 3	12 ± 3	21 ± 5	86 ± 9	7.37 ± 0.03	32 ± 4	126 ± 24
HOM female	Normal	2.6 ± 0.5	7	29 ± 5	10 ± 1	25 ± 4	90 ± 2	7.35 ± 0.03	31 ± 4	134 ± 21

Table 1. Electrophysiological measures of CSD and systemic physiological parameters in FHM1 mutant mice. Values are mean ± standard deviation. CSD duration was measured at half amplitude. Because the duration, but not the amplitude, gradually decreased upon repetitive SDs in all groups, both the duration of first CSD and the average of all CSDs are presented. Group differences are not statistically significant (1-way ANOVA). Estrogen: 0.075 mg/pellet 21-day release. Mean arterial blood pressure (MABP) and arterial partial pressure of oxygen (pO_2) and carbon dioxide (pCO_2) are expressed in mmHg, averaged over 1-hour recordings. n, number of mice; Ovx, ovariectomized; HET, heterozygous; HOM, homozygous.

organizational effect of gonadal hormones or chromosomal sex per se, as modulators of cortical excitability (Figure 2). Consistent with these findings, SD frequencies and propagation speeds were significantly reduced in senescent female R192Q mutant mice after putative cessation of estrous cycling and reduced gonadal hormone production (Figure 2). Chronic estrogen replacement by subcutaneous implantation of pellets containing 0.075 mg 17β-estradiol (3-week release) was associated with a small increase in SD susceptibility in ovariectomized R192Q mutant, but not in WT, mice (13 ± 1 vs. 16 ± 1 SDs/h in control and estrogen-treated homozygous R192Q mutant, $n = 5$ and 6, respectively; $P < 0.01$). A lower dose of estrogen (0.025 mg/pellet) was ineffective in both WT and homozygous R192Q mutant mice (data not shown). Neither gonadectomy nor advanced age influenced SD susceptibility in WT mice using the described protocols (Figure 2). These data suggest that female gonadal hormones modulate SD susceptibility only in mice that have a genetic predisposition.

FHM1 mutant mice develop severe and prolonged motor deficits after SD that are modulated by gene dosage and sex. Prior to SD induction, both WT and FHM1 mutant strains appeared phenotypically normal and did not exhibit neurological deficits when assessed using the wire grip test and neurological examination protocols. In WT mice, a single SD induced by brief topical application of 300 mM KCl caused only mild deficits that lasted less than 10 minutes in the wire grip test and only a mild and short-lasting

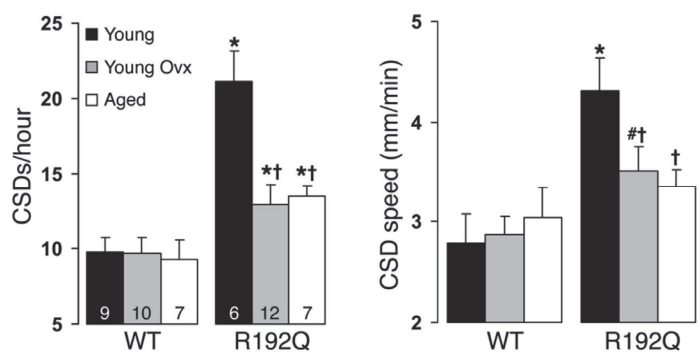


Figure 2. Gonadal hormone–mediated modulation of CSD susceptibility in female FHM1 mutant mice. The impact of ovariectomy and senescence on CSD susceptibility in WT and homozygous R192Q mutant mice. Both ovariectomy (3–4 months of age; gray) and senescence (13 months of age; white) reduced CSD frequency (left panel) and propagation speed (right panel) in homozygous female R192Q to the level of male mutants (see Figure 1B). Ovariectomy ($n = 5$) and aging ($n = 7$) had similar effects in heterozygous female R192Q mice (data not shown). In WT mice, gonadectomy or senescence did not alter CSD susceptibility. Experiments were performed 3 weeks after ovariectomy. Numbers of mice for each group are shown within the bars. Ovx, ovariectomized. Data are mean ± standard deviation. $^{*}P < 0.001$, $^{\#}P < 0.01$ versus WT; $^{\dagger}P < 0.001$ versus naive young female mice of the same genotype.

hemiparesis that completely recovered before the first neurological assessment at 5 minutes after SD induction (Figure 3A). In contrast, a single SD caused hemiplegia with leaning and circling in both R192Q and S218L mutant mice (Figure 3, Supplemental Figure 1, and Supplemental Videos 1–13, available at http://www.jci.org/articles/view/36059#sd). In the wire grip test, they were initially unable to move along the wire and fell more often than WT mice. S218L mutants were more severely impaired, and unlike R192Q mice, some remained unconscious for up to 10 minutes after a single SD. Homozygous FHM1 mutant mice were impaired more than heterozygotes, indicating an allele dosage relation (Figure 3). Interestingly, FHM1 mutants showed 1 or more transient episodes of neurological deterioration after full or partial recovery during the 60- to 80-minute monitoring period (Supplemental Figure 1E).

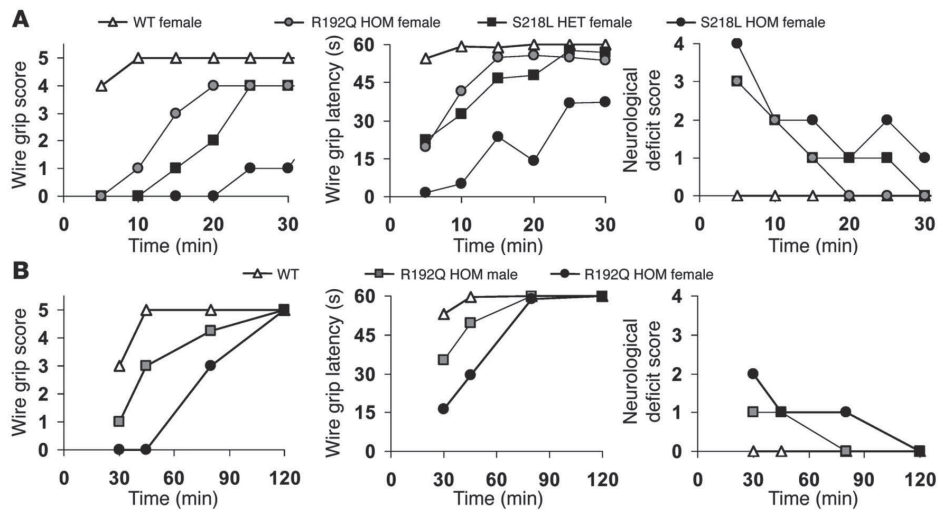


Figure 3. Prolonged and severe neurological deficits after SD in FHM1 mutant mice. Time course of neurological deficits in WT and FHM1 mutant mice after a single SD (**A**) or 9 SDs over 1 hour (**B**), as assessed by wire grip score and latency as well as neurological score. (**A**) Impact of allelic mutations and allele dosage. In WT mice, deficits were mild and short-lasting in wire grip test and undetectable by neurological scoring (triangles). FHM1 mutants developed severe and prolonged deficits ($P < 0.01$ versus WT). These deficits were markedly more severe and prolonged in homozygous S218L (black circles) than in homozygous R192Q mutants (gray circles; $P < 0.01$) or heterozygous S218L mutants (squares; $P < 0.01$ in wire grip test, $P < 0.05$ in neurological scoring). n = 13, 7, 6, and 3 WT, homozygous R192Q, heterozygous S218L, and homozygous S218L mutant mice, respectively. (**B**) Sex differences. In WT mice, deficits were mild and completely recovered within 45 minutes after 9 SDs. In contrast, R192Q mutant mice developed severe and prolonged deficits that lasted 80 minutes or more after 9 SDs ($P < 0.01$ versus WT). Importantly, female mutant mice (circles) were more severely affected than males (squares; $P < 0.01$). Deficits did not differ between WT males and females; therefore, data were pooled (triangles). n = 15, 6, and 7 WT, male R192Q, and female R192Q mutant mice, respectively. Error bars were omitted for clarity but were less than 25% of mean.

All 3 homozygous S218L mutant mice developed generalized seizures 45, 55, and 75 minutes after a single SD. Two of the mice died immediately after the seizure due to respiratory arrest; the third mouse survived the seizure and completely recovered at 80 minutes. Seizures were not observed in heterozygous S218L or homozygous R192Q mutant mice during recovery from SD. The deficits in heterozygous S218L mutant mice in the absence of overt seizure activity suggest that the clinical phenotype was most likely due to SD and not seizure activity.

Compared with a single SD, multiple SDs (9 in 1 hour) caused more severe motor deficits. In WT mice, deficits in the wire grip test were detectable for 30 minutes after the last SD (Figure 3B), whereas multiple SDs caused even more severe deficits in R192Q mutants. Moreover, female R192Q mutant mice developed more severe and longer-lasting deficits than males (Figure 3B).

Recovery of cortical evoked potentials after SD is not delayed in FHM1 mutant mice. To test whether prolonged neurological deficits were due to a slower recovery of cortical electrophysiological function, we recorded somatosensory evoked potentials in the whisker barrel cortex after a single SD. Evoked potentials were abolished after SD but reemerged within approximately 2 minutes and completely recovered to pre-SD baseline levels within 10 minutes (Figure 4A). The recovery rate was identical in WT and R192Q or S218L mutant mice (Figure 4, A and B). No seizure activity was observed in these recordings under anesthesia. These data indicated that delayed electrophysiological recovery of cortical synaptic function was not the cause of prolonged and severe unilateral motor deficits after CSD in FHM1 mutant mice.

Corticostriatal propagation of SD is facilitated in FHM1 mutant mice. CSD occasionally propagates into subcortical structures in rodent brain.[29,30] We therefore tested whether prolonged post-SD neurological deficits such as hemiplegia and circling were associated with enhanced subcortical spread into the striatum. In WT mice, CSDs did not reach the striatum (Figure 5; see Methods for experimental conditions). In contrast, CSDs readily propagated into the striatum in FHM1 mutant mice (Figure 5A). Corticostriatal SD propagation was more frequent and arose with shorter latency in S218L compared with R192Q mutant mice ($P < 0.05$), homozygotes compared with heterozygotes of both mutant strains ($P < 0.01$), and female homozygous (but not heterozygous) mutants compared with males ($P < 0.01$; Figure 5B and Table 2). SD did not propagate to thalamus and hippocampus under our experimental conditions ($n = 7$ heterozygous female and homozygous male S218L mice; $n = 2$ WT mice; data not shown). Therefore, corticostriatal SD propagation corresponded well to the severity of post-SD neurological deficits and was modulated by: (a) the degree of ion channel dysfunction; (b) allele dosage; and (c) sex. These data implicate corticostriatal SD propagation as a likely explanation for the more severe motor deficits in FHM1 mutant mice.

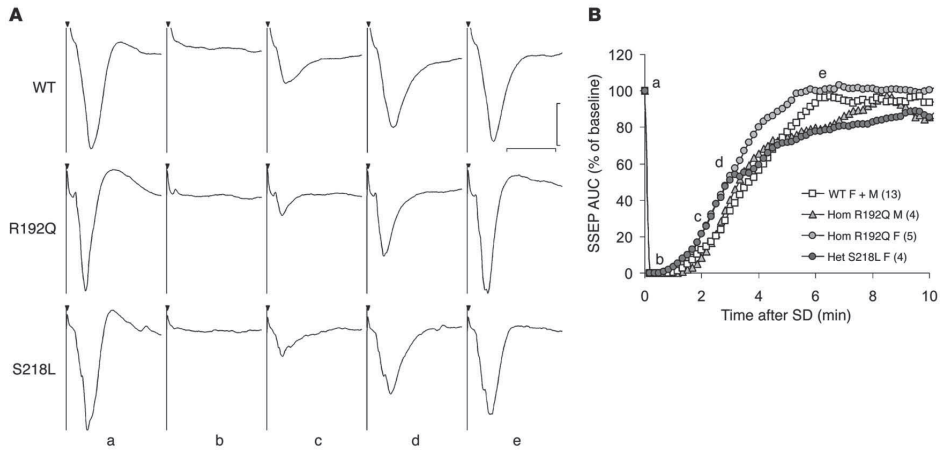


Figure 4. The recovery of whisker pad stimulation–evoked cortical field potentials. (**A**) Representative tracings showing field potentials in a female WT (top), homozygous female R192Q mutant (middle), and heterozygous female S218L mutant mouse (bottom), evoked by electrical stimulation of the whisker pad (black triangles) and recorded from the whisker barrel field using extracellular glass micropipettes (400 µm depth, layer IV). Evoked potentials ranging from 2 to 4 mV in amplitude at baseline (column a) were abolished upon arrival of SD at the recording site (column b) and gradually recovered within less than 10 minutes (columns c–e), as shown in the time course graph in B. Calibration bars: vertical, 1 mV; horizontal, 20 ms. (**B**) The time course of recovery of somatosensory evoked field potentials (SSEP) after a single SD (time 0).The AUC for each evoked field potential (mV • ms) was expressed as percent of pre-SD baseline. The rate of recovery of cortical evoked field potentials did not differ among WT and FHM1 mutant mice. Recovery of peak amplitudes also did not differ among groups (data not shown). Numbers of mice are indicated in the graph. Male and female WT mice did not differ; therefore, pooled data are shown. Error bars were omitted for clarity.

FHM1 mutant mice develop delayed recurrent SDs after a single KCl-induced SD. In order to test whether prolonged neurological deficits were caused by recurrent SDs, we recorded from cortex and striatum simultaneously for 60 minutes after one initial SD induced by brief topical KCl application that was followed by extensive saline wash. In all FHM1 knock-in mice, 1 or more recurrent SDs were observed throughout the recording period (range, 1-55 minutes after the initial SD, n = 6 heterozygous female and 2 homozygous male S218L mutants and n = 3 homozygous female R192Q mutants; Supplemental Figure 3). None of the WT mice showed SD recurrence. As suggested by their latency, recurrent SDs appeared to originate from cortex and consistently spread into striatum. In a few instances, there was evidence for cortico-striato-cortical reentrant SDs. In the absence of prior SD induction, we did not observe spontaneous SDs during 60-minute recordings in mutant or WT strains (n = 6).

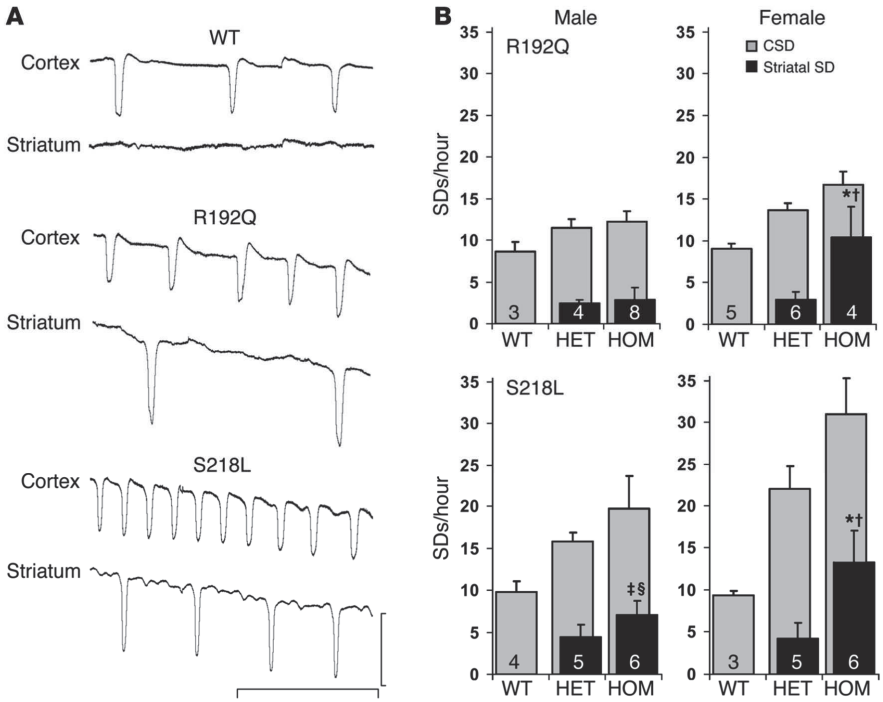


Figure 5. Facilitated corticostriatal SD propagation in FHM1 mutant mice. (**A**) Representative extracellular DC potential shifts recorded simultaneously from cortex and striatum in female WT and homozygous R192Q and S218L mutant mice. SD was not observed to propagate into the striatum in any of the WT mice. A substantial proportion of CSDs propagated into the striatum in R192Q and to a greater extent in the S218L mutant. Calibration bars: vertical, 20 mV; horizontal, 10 minutes. (**B**) The frequency of SDs during continuous topical KCl application (300 mM) to the occipital cortex. CSD frequency (gray bars) was substantially higher in S218L and to a lesser extent in R192Q mutant mice than WT. They were higher in females compared with males and homozygous FHM1 mutants compared with heterozygotes (see Figure 1). CSDs readily propagated into striatum (black bars) in both FHM1 mutant strains, but never in the WT. Striatal propagation was more frequent in S218L (lower graphs) compared with R192Q mutants (upper graphs) and in females (right) compared with males (left), with an allele dosage relation (see Table 2 for latencies between cortical and striatal SDs). Covariance analysis revealed that 83% of the variance of striatal SD frequency was explained by the independent variables mutation, genotype, and sex (see Methods). n = 3–8 mice per group as shown within each bar. Data are mean ± standard deviation. *P < 0.01 versus male; †P < 0.001, §P < 0.05 versus heterozygous mutants; ‡P < 0.01 versus R192Q males.

Strain	Genotype	Male	Female
R192Q	HET	3.7 ± 0.3	4.0 ± 0.6
	HOM	3.5 ± 0.5	1.5 ± 0.2[A,B]
S218L	HET	3.3 ± 0.8	3.6 ± 0.5
	HOM	2.0 ± 0.6[A,C]	1.5 ± 0.3[A]

Table 2. Corticostriatal propagation latency of SD. Values are mean ± standard deviation, expressed in minutes. [A]*P* < 0.01 versus heterozygote; [B]*P* < 0.01 vs. male; [C]*P* < 0.01 versus R192Q. See Figure 5 for the number of mice in each group.

DISCUSSION

Migraine susceptibility is modulated by genetic, physiological, and environmental factors. Here, we provide experimental evidence showing that genetic ($Ca_v2.1$ voltage-gated Ca^{2+} channel mutations) and physiological factors (gonadal hormones) modulating susceptibility to migraine also modulate SD susceptibility. Enhanced SD susceptibility shows a clear relation to the degree of single-channel gain of function caused by S218L and R192Q mutations and is associated with more severe and prolonged neurological deficits after SD, consistent with the clinical phenotypes observed in FHM1 patients. Both enhanced SD susceptibility (i.e., frequency and propagation speed) and neurological deficits are linked to the propensity of corticostriatal SD propagation and are modulated by the interaction of allelic mutations, gene dosage, and gonadal hormones.

The $Ca_v2.1$ calcium channels are important modulators of SD.[31,32] The first evidence that linked $Ca_v2.1$ channel mutations to a CSD phenotype was described in tottering and leaner mice. These naturally occurring mouse mutants have loss-of-function mutations in their $Ca_v2.1$ channels and showed an *increase* in SD threshold.[33] In a subsequent study, van den Maagdenberg and colleagues[13] demonstrated a *reduced* threshold for SD induced by electrical stimulation in R192Q FHM1 mutant mice. The increased SD frequency and propagation speed following epidural KCl application in R192Q mutant mice in the present study are in line with these results. Consistent with more negative opening voltages and a greater increase in the single-channel opening probability associated with S218L mutation, SD susceptibility was even higher in this mutant, suggesting incremental modulation of the phenotype by allelic mutations.[13,14,16] Because excitatory neurotransmitters promote SD via NMDA receptors,[34-38] we speculate that FHM1 mutations facilitate the initiation and propagation of SDs by increasing presynaptic Ca^{2+} influx and subsequent glutamate release. Although augmented neurotransmitter release has been shown at the neuromuscular junctions of R192Q mutant mice,[13,39] this remains to be tested in central synapses.

Sex hormones. Our data establish the importance of female gonadal hormones as modulators of genetically driven enhanced SD susceptibility. Ovarian hormones, rather than sex chromosome-related developmental differences in synaptic organization and structure, were implicated in aged female mice and after ovariectomy. These findings may be clinically relevant, since migraine improves at menopause in two-thirds of patients.[40] The frequency of migraine attacks decreases after age 50, both in patients with FHM and those with more typical migraine subtypes.[9] Estradiol did partially restore SD susceptibility when administered chronically to ovariectomized R192Q mutant mice. Clinically, estrogen reportedly increases cortical excitability in humans during transcranial magnetic stimulation,[41] and high doses increase the

incidence of aura during hormone replacement therapy.[42-44] Moreover, higher levels of estrogen are associated with an increase in seizure frequency in females.[45] Experimentally, seizure thresholds are decreased during peak estrogen levels,[46] and amygdala kindling is increased.[47] Estrogen augments excitatory glutamatergic neurotransmission by upregulating NMDA receptor expression, downregulating glutamate uptake by astrocytes, and increasing the number of dendritic spines, which are densely populated with NMDA receptors.[19-21] A large body of evidence suggests that estrogen modulates nociceptive processing as well,[48,49] so that enhanced SD susceptibility is only one mechanism by which estrogen may impact migraine.

Clinical and epidemiological data on hormonal modulation of FHM are sparse. In a population-based study, the female/male ratio was 5:2; males and females did not differ in age of onset or in symptoms of aura and headache.[9] In one case report, a 48-year-old woman with hemiplegic migraine ceased to experience any further neurological signs during migraine attacks after ovariectomy.[50] Hence, available evidence supports the notion that female gonadal hormones aggravate the hemiplegic migraine phenotype as well.

The lack of gonadal hormone modulation of SD in WT mice we observed argues against a simple additive effect between genetic and hormonal factors and suggests that gonadal hormones modulate SD susceptibility predominantly in brains made susceptible by gene mutations. The precise nature of the interactions between gonadal hormones and mutant $Ca_v2.1$ channels remains to be determined. It has been shown, however, that estrogen upregulates expression of some voltage-gated Ca^{2+} channels (e.g., L- and T-type) in hypothalamus and pituitary.[51-53] Expression levels of the α_{1A} subunit of P/Q-type channels show sexual dimorphism in anterior pituitary (higher in females than in males) and fluctuate during estrous cycle, suggesting a direct modulation by female hormones.[54] Furthermore, $Ca_v2.1$ channels undergo extensive alternative splicing that shows sexual dimorphism.[55] Therefore, female hormones may enhance the impact of genotype on SD phenotype via increased expression or by favoring alternative splicing patterns that enhance mutant channel activity.

It should be noted that Brennan et al. recently reported that SD thresholds are reduced by approximately 50% in WT female mice compared with males.[56] Using the SD frequency model, we did not detect sex differences in the WT strain, despite 95% power to detect a 25% difference between the means in our model ($\alpha = 0.05$). We also did not detect a threshold difference using direct epidural cortical electrical stimulation (140 µm tip diameter, 200 µm tip separation, 4.3 kΩ tip resistance) very similar to that used by Brennan et al.[56] (electrical SD threshold, 413 ± 168 [females] vs. 460 ± 124 [males] µC, $n = 11$ and 8, respectively; $P = 0.7$). At present, we do not have an explanation for the discrepant results. Nevertheless, consistent with our data, the propagation speed, SD duration, and number of successful SD inductions did not differ

between WT males and females in their study, and the studies agree that gonadal hormones play an important part in modulating SD susceptibility.

Motor deficits. Motor deficits (i.e., contralateral hemiplegia with leaning and circling) were significantly more severe and prolonged in FHM1 mutant mice, as compared with the WT strain, lasting 20 minutes or more after induction of a single SD. As can be expected from single-channel kinetics,[14] the deficits were more severe in S218L than in R192Q mutant mice, and often experiments were terminated by fatal generalized seizures. Delayed electrophysiological recovery of cortical function did not explain the post-SD deficits (Figure 4). Instead, we believe that the propagation of CSD into the striatum in FHM1 mutants may have been responsible. The frequency of striatal SDs (as well as their propagation speed from cortex to striatum) was greater in the homozygous mutants, in female mutant mice, and in the S218L strain, i.e., the one with the most severe calcium channel dysfunction.[14]

Striatal SDs are associated with contralateral circling and hemiparesis.[57-59] CSDs did not propagate into the striatum in WT mice, consistent with the absence of severe neurological deficits. CSD spreads into the striatum through the amygdala as SD propagation is impeded in tissues such as white matter with less than a critical density of neurons.[1,29,30,60-62] Consistent with this, direct thalamic and hippocampal electrophysiological recordings failed to detect concurrent SD propagation into these structures in either WT or FHM knock-in mouse. In rats, the proportion of CSD propagating into striatum reportedly varied between 4% and 60% in different studies, depending on the strain and the use and type of anesthesia, and subcortical propagation of CSD could be facilitated pharmacologically (e.g., with pyrrolopyrimidine BW 58271) or physiologically (e.g., in undernourished rats).[30,59,60] To our knowledge, this has not been studied in mice at this level of detail. Our data suggest that FHM1 mutations facilitate corticostriatal SD propagation. The anesthetic regimen used in our model may explain the complete lack of corticostriatal SD propagation in WT mice.[63,64]

Post-SD neurological deficits were not only more severe in the mutant strains but also lasted longer than in WT mice. Indeed, mutants showed one or more episodes of transient neurological worsening, delaying the neurological recovery after a single KCl-induced SD. Prolonged deficits and episodes of transient worsening were linked to recurrent and possibly reentrant SDs in FHM1 mutants. It should also be noted that SD causes severe and long-lasting tissue hypoperfusion[65] and hypoxia in mice.[66] It remains to be tested whether the hemodynamic and metabolic impact of SD is more severe in FHM1 mice and whether this contributes to the prolonged post-SD neurological deficits.

Seizures. Recurrent SDs observed in FHM1 mutants might have directly caused or predisposed to delayed seizures in the S218L knock-in. Homozygous female S218L

knock-in mice developed generalized seizures 45-75 minutes after a single SD. Interestingly, the incidence of premature death at a young age (i.e., <20 weeks, both males and females) was higher in mice of both knock-in strains, in some cases linked to apparently spontaneous seizures (our unpublished observations), suggesting that FHM1 mutations predispose to seizures without an experimentally triggered SD. SD may also render the cortex transiently hyperexcitable. For example, SD enhances excitatory postsynaptic field potentials as well as the repetition rate and amplitude of spontaneous rhythmic potentials 20-90 minutes after its induction; it augments long-term potentiation in human neocortical slices.[67,68] SD is followed by hyperexcitability in rat neocortex and spinal cord after transient depression of neuronal activity.[69,70] Last but not least, SD can directly precipitate seizure-like electrocorticogram activity, as first demonstrated by Leão.[2] Consistent with our findings, epilepsy is more frequent in FHM patients than in the general population, with seizures occurring either during FHM attacks[71-74] or interictally.[75-78] Epilepsy has been reported in many patients carrying FHM1 mutations.[10,71,79-82] For example, 2 patients harboring the S218L mutation have developed seizures at the onset of severe attacks associated with coma.[15,83]

In summary, data showing enhanced SD susceptibility and prolonged neurological deficits after SD in genetic mouse models of migraine strengthen the link between SD and the migraine aura. Furthermore, they provide mechanistic insight by implicating recurrent SDs and facilitated propagation of SD into subcortical tissues. The modulation of SD susceptibility, neurological deficits, and subcortical propagation by specific FHM1 mutations (i.e., R192Q vs. S218L mutation), genotype (i.e., heterozygous vs. homozygous), and gonadal hormones underscores the complex synergistic interactions between genetic and hormonal factors determining migraine susceptibility.

METHODS

Experimental groups. Experimental groups are summarized in Table 1. A total of 337 mice were used in this study. Male and female FHM1 mutant mice, homozygous or heterozygous for R192Q or S218L mutation in the mouse *Cacna1a* gene (encoding the α_{1A} pore-forming subunit of $Ca_v2.1$ channels), were compared with WT littermates. In addition, R192Q mutants were backcrossed onto C57BL6/J mice (Charles River Laboratories) for more than 8 generations. None of the measured endpoints differed between WT littermates of R192Q mutant and C57BL6/J control mice (data not shown). Therefore, data from WT littermates of R192Q mutants and C57BL6/J control mice were pooled for the analysis. The S218L mutants were compared with their littermates only.

Preparation of targeting construct and generation of FHM1 mutant mice. Transgenic knock-in $Ca_v2.1$-α_{1A} migraine mouse models were generated by a gene targeting approach in which the endogenous *Cacna1a* gene was modified. In the R192Q mice,[13] we modified the CGG triplet (arginine) of codon 192 to CAG (glutamine) using in vitro mutagenesis to introduce the human FHM1 mutation R192Q.[12] The targeting vector that was used for generating the S218L mice contained the mutant TTA (leucine) instead of the original TCA (serine) triplet of codon 218; thus creating the S218L mutation that had previously been found in patients.[15] This targeting vector also contained a PGK-driven neomycin-resistance cassette that was flanked by *loxP* sites in the endogenous *Hind*III site that is located 537 nucleotides upstream of exon 5. Chimeras were obtained by injecting correctly targeted E14 ES cells into C57BL/6J blastocysts according to standard procedures in order to generate a transgenic line of mice. In mice that were used for the experiments, the cassette was deleted by crossing these transgenic mice with mice of the EIIA-Cre deleter strain,[84] which express Cre recombinase driven by the EIIA early promoter. For both the R192Q and S218L mice, heterozygous mice (>96% C57BL/6J background) were subsequently interbred to provide litters containing all 3 possible genotypes that were used for the experiments. Litters were genotyped after weaning by PCRs specific for the respective transgenic line, essentially as described previously.[13] All experiments were carried out with the investigator blinded for genotype, and confirmatory genotyping was done after the experiment. All animal experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and performed in accordance with the NIH *Guide for the care and use of laboratory animals* (NIH publication no. 85-23. Revised 1985).

General surgical and electrophysiological procedures. Mice were housed under diurnal lighting conditions and allowed food and tap water ad libitum. To assess SD susceptibility under full systemic physiological monitoring, femoral artery was catheterized for blood sampling and measurement of mean arterial pressure and trachea intubated for mechanical ventilation (SAR-830; CWE), under isoflurane anesthesia (2.5% induction, 1% maintenance, in 70% N2O/30% O2). Arterial blood gases and pH were measured once every 20 minutes during each experiment in 25 µl samples (Corning 178 blood gas/pH analyzer; Ciba Corning Diagnostic Corp.) and maintained within normal limits by adjusting ventilation parameters (Table 1). Mice were then placed in a stereotaxic frame (David Kopf Instruments), and 3 burr holes were drilled under saline cooling at the following coordinates on both sides (mm from bregma): 3.5 mm posterior, 2 mm lateral (2 mm diameter for KCl application onto occipital cortex); 1.5 mm posterior, 2 mm lateral (1 mm diameter, recording site 1); 0.5 mm anterior, 2 mm lateral (1 mm diameter, recording site 2). The dura was kept intact to minimize trauma. Two glass capillary microelectrodes were placed to

record extracellular steady (DC) potential and electrocorticogram (ECoG) at a depth of 300 µm. For striatal recordings, the electrode at recording site 2 was lowered to a depth of 3 mm into the striatum. A third electrode was simultaneously inserted at 2 mm posterior, 1.2 mm lateral from bregma, at depths of 3 and 1.2 mm from the pial surface for thalamic and hippocampal recordings, respectively. Recording sites were later confirmed by tracking the electrode placement (Supplemental Figure 2). An Ag/AgCl reference electrode was placed subcutaneously in the neck. Recording sites were covered with mineral oil after electrode placement to prevent cortical drying. After surgical preparation, the occipital cortex was allowed to recover for 20 minutes under saline irrigation. A cotton ball (2 mm diameter) soaked with 300 mM KCl was placed on the dura and replaced every 15 minutes to maintain steady KCl concentration during stimulation. In order to test whether occipital epidural KCl application causes SD in the striatum via direct diffusion, in a subgroup of FHM1 mutant mice we evoked SD by pinprick on the occipital cortex and observed reproducible propagation of SD into striatum with the same latency as after topical KCl application (n = 3 female R192Q mutant mice; data not shown). All data were continuously recorded using a data acquisition system for off-line analysis (PowerLab; ADInstruments).

The frequency of SDs evoked by topical KCl application and their propagation speed were determined as previously described with minor modifications.[4] KCl-evoked CSDs were detected based on the characteristic slow DC potential shift and ECoG suppression. The frequency of evoked CSDs was determined over 30 minutes on each hemisphere. CSD frequencies obtained from the right and left hemispheres did not statistically differ in any experimental group and were averaged to calculate the CSD frequency in each mouse. Propagation speed was calculated from the distance between the 2 recording electrodes divided by the latency between the first CSDs recorded at these sites. The DC shift amplitude and duration at half-maximal amplitude were averaged for all CSDs in each experiment.

Epidural application of NaCl (300 mM) did not trigger SD or cause neurological deficits in sham-operated knock-in controls (n = 2 homozygous female R192Q mutant mice; data not shown). In subgroups of WT and FHM1 mutant mice, histopathological assessment of the KCl application site after the experimental recordings did not reveal any evidence of cortical injury in H&E-stained frozen sections (data not shown).

Ovariectomy and estrogen replacement. The impact of diminished gonadal hormone production on SD susceptibility was studied using ovariectomized (3–4 months old) or senescent (13 months old, after cessation of estrous cycle) homozygous R192Q mutant mice. Because the sex effect on SD phenotype appeared in both heterozygous and homozygous mutants (Figure 1), further experiments exploring post-SD neurological deficits (see below) were performed on homozygous mutants only. Ovaries were exteriorized, ligated, and removed via bilateral dorsal approach in young adult mice

(3-4 months old) under isoflurane anesthesia. In addition, subgroups of ovariectomized mice were chronically treated with estrogen via subcutaneous implantation (dorsal scapular) of 21-day slow-release pellets (Innovative Research of America) containing 17β-estradiol 3-benzoate to achieve constant plasma estrogen levels corresponding to the proestrus stage of cycle (0.025 mg/pellet) or higher-than-normal estradiol concentration (0.075 mg/pellet).[85-90] SD susceptibility was tested 3 weeks after ovariectomy with or without estrogen replacement (i.e., empty pellet implantation). The C57BL/6J background of FHM1 mutant mice has a gonadal hormone profile of aging very similar to that observed in menopausal women: prolonged cycles with delayed preovulatory rise of estrogen progress to acyclicity, lower estrogen levels, and hypergonadotrophic hypogonadism;[91,92] decreased nuclear estrogen receptors and a nuclear translocation defect of estrogen-receptor complex have also been described as in humans.[93-95]

Neurological testing. In order to minimize the confounding effect of invasive surgical procedures on neurological testing, arterial and tracheal catheterizations were not performed, and freely breathing mice were anesthetized via a face mask during the SD induction procedure. Neurological deficits were assessed either after 1 SD or after 9 SDs induced over 1 hour. In the 1-SD group, mice were briefly anesthetized, and KCl (300 mM) was topically applied (typically for less than 1 minute) on the parietooccipital cortex until an SD was recorded in the frontal cortex as described above, followed by extensive saline wash of cortical surface. In the 9-SD group, KCl (300 mM) was briefly applied in the same manner approximately every 7.5 minutes, and SD induction confirmed at the frontal recording site. Motor deficits were assessed at predefined time points after induction of the last SD in a blinded fashion. Mice fully awakened from anesthesia usually within 3 minutes in the 1-SD group; therefore, neurological assessments were started 5 minutes after SD induction and carried out every 5 minutes until full reversal of deficits. Because full awakening was delayed in the 9-SD group for up to 15-20 minutes in all groups, first neurological assessment was carried out 30 minutes after the last SD and then repeated at 45, 80, and 120 minutes. The severity of deficits and high mortality after a single SD in S218L mutant mice precluded testing of the 9-SD paradigm in this strain. There was no difference among WT and FHM1 mutant strains in the time required for full recovery of neurological function after 10 minutes of isoflurane anesthesia (data not shown).

We relied exclusively upon motor performance, since preliminary studies of sensory deficits and neglect (e.g., corner test) did not reliably detect deficits in this model (data not shown). Motor deficits were assessed using 2 independent tests. The 5-point neurological scale is most commonly used to quantify unilateral motor deficits after stroke. The deficits are scored as: 0 (no neurological deficit: normal), 1 (mild neurological deficit: failure to extend forepaw fully), 2 (moderate neurological deficit:

circling), 3 (severe neurological deficit: falling to one side), 4 (very severe neurological deficit: no spontaneous walking, depressed level of consciousness), as previously described.[96] The wire grip test is used to assess coordination and fine movement of the digits based on the ability of the mouse to remain on the wire and successfully climb down the pole and scored as: 0, unable to remain on wire more than 30 seconds; 1, holds on wire more than 30 seconds, but not with both sets of paws on wire; 2, holds on to the wire with all paws but not the tail; 3, uses the tail along with all paws but does not move on wire; 4, moves along the wire on all 4 paws plus tail; 5, moves along the wire on all 4 paws plus tail and ambulates down one of the posts used to support the wire. Besides the wire grip score, we also recorded the latency to fall off the wire; if the mouse stayed on the wire for more than 60 seconds, or climbed down the pole successfully, it was scored as 60 seconds.[97]

Somatosensory evoked potentials. Two cranial windows (1 mm diameter) were drilled under saline cooling over the right whisker barrel cortex (posterior 1.3 mm, lateral 3.7 mm from bregma) and occipital cortex (posterior 4 mm, lateral 2 mm from bregma). The dura was kept intact. A glass micropipette electrode filled with 150 mM NaCl was inserted to a depth of 400 µm. Somatosensory evoked potentials as well as the extracellular steady (DC) potential were recorded using a differential amplifier (EX-1; Dagan Corp.) and stored using a data acquisition system for off-line analysis (PowerLab 200; ADInstruments). An Ag/AgCl reference electrode was also placed subcutaneously in the neck, and the cortex was covered with a thin layer of mineral oil to prevent drying. Somatosensory potentials in the whisker barrel cortex were evoked by electrical stimulation of the entire whisker pad (single square pulse, 0.2 ms duration, 700 µA, 0.1 Hz; S48, Grass Technologies; and A395 Linear Stimulus Isolator/Constant Current Unit, WPI) via 2 needle electrodes placed in the contralateral whisker pad (5 mm electrode separation). In preliminary experiments, we determined that nitrous oxide in inhalation gas potently suppressed somatosensory evoked potentials. Therefore, in these experiments, we replaced nitrous oxide with nitrogen.

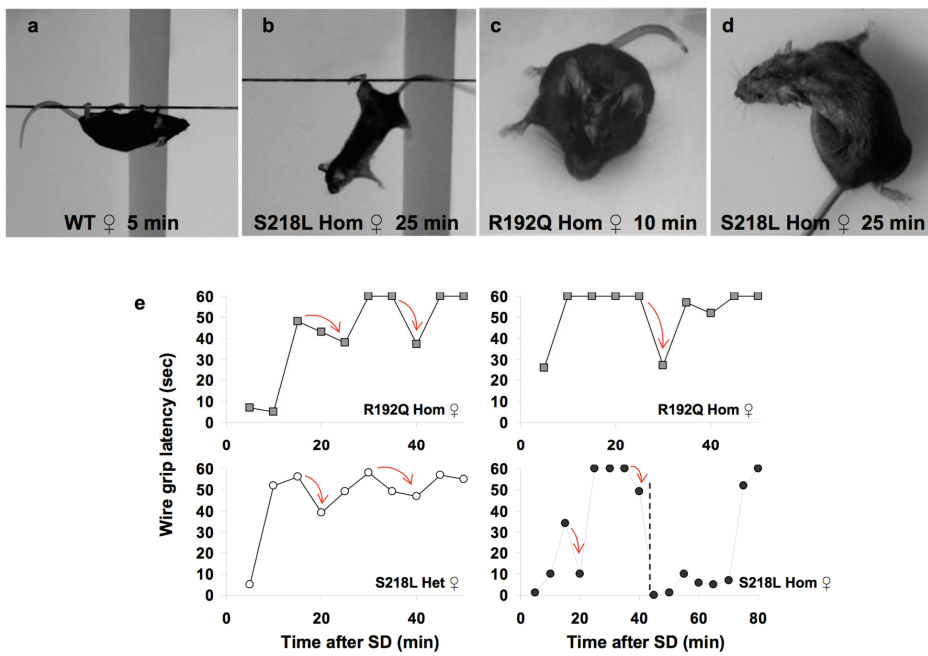
Whisker pad stimulation typically evoked a monophasic negative field potential at this recording depth (Figure 4). After acquiring 50 such evoked potentials at baseline, a CSD was triggered using brief occipital epidural KCl application and confirmed by the characteristic DC potential shift at the recording site in whisker barrel cortex. In order to avoid multiple CSDs, the KCl application site was immediately washed with saline upon detection of the SD at the recording site. Experiments with multiple SDs were excluded from analysis. Both the amplitude of negative peak and the area under the field potential curve were measured before, during, and after SD and expressed as percent of baseline.

Statistics. Data were analyzed using SPSS software package (version 11.0). The impact of independent variables allelic mutations (R192Q vs. S218L), allele dosage (WT, heterozygous, homozygous), and sex (male vs. female) on the dependent variables cortical and striatal SD frequency and propagation speed were tested using 3-way ANOVA (Figures 1 and 5). The impact of independent variables R192Q mutation (WT vs. R192Q) and gonadal status (normal, ovariectomized, and senescent) on the dependent variables CSD frequency and propagation speed were tested using 2-way ANOVA (Figure 2). The time course of neurological deficits and recovery of somatosensory evoked potentials after SD were tested among experimental groups using 2-way ANOVA for repeated measures (Figures 3 and 4). Electrophysiological measures of CSD (Table 1), systemic physiological data (Table 1), and the impact of estrogen replacement in ovariectomized FHM1 mutant mice (see Results) were compared among experimental groups using 1-way ANOVA. Corticostriatal propagation latencies were compared using 3-way ANOVA (Table 2). In addition, using pooled data from all mice and a general linear model of covariance analysis (ANACOVA), we tested for an effect of the independent variables mutation, genotype, and sex (fixed factors) on the dependent variables cortical and striatal SD frequency and propagation speed. Data are presented as mean ± standard deviation. $P < 0.05$ was considered statistically significant.

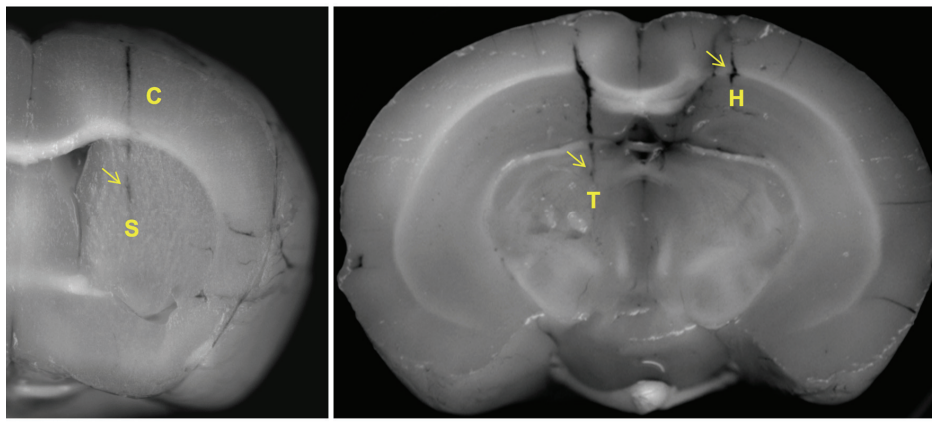
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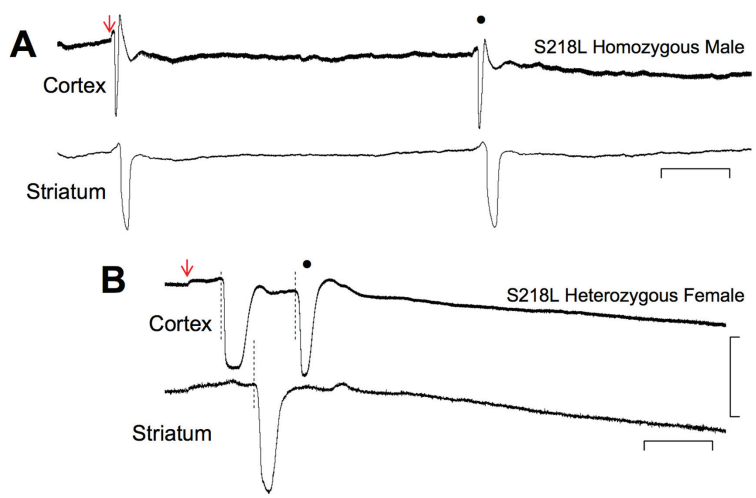
SUPPLEMENTARY MATERIAL



Supplemental Figure 1. Prolonged and more severe neurological deficits after SD in FHM1 mutant mice. Representative photographs demonstrating the wire grip and neurological deficits after a single SD in female wild type and homozygous R192Q and S218L mutant mice. In wire grip test, wild type mice showed minimal deficits soon after SD (**A**), while S218L mutant mice continued to fall off the wire for prolonged periods (**B**). Neurological examination showed leaning (**C**) and circling (**D**) in R129Q and S218L mutant mice. SD was evoked in the right hemisphere in all mice. (**E**) Graphs showing the time course of neurological deficits (only wire grip latency shown) from four representative FHM1 knockin mice. One or more episodes of transient neurological deterioration (red arrows) were observed after full or partial recovery in 7/7 homozygous female R192Q knockin, 4/6 homozygous male R192Q knockin, 3/3 homozygous female S218L knockin, and 4/5 heterozygous female S218L knockin. Vertical dashed line indicates a non-fatal generalized seizure that occurred shortly after the 40 min assessment time point.



Supplemental Figure 2. Electrophysiological recording sites. Representative coronal gross brain sections showing the SD recording sites in cortex (C), striatum (S), thalamus (T), and hippocampus (H). The dark vertical stains (arrows) show the electrode tracks labeled using Coomassie Brilliant Blue.



Supplemental Figure 3. Recurrent SD in FHM1 mutant mice. Representative tracings from S218L knockin mice (homozygous male and heterozygous female shown) showing spontaneous recurrent SDs (black dots) after an initial SD induced by brief topical KCl (red arrows, 300 mM) application immediately followed by extensive saline wash. (**A**) The initial KCl-induced cortical SD (upper tracing) propagates into striatum (lower tracing). Approximately 50 minutes after this initial SD, a recurrent SD spontaneously appeared in the cortical electrode followed by the striatal electrode. Extreme care was taken to avoid cortical drying, mechanical stimulation or trauma during these recordings. (**B**) In some experiments, the initial SD was followed in rapid succession by a striatal and a recurrent cortical SD, suggesting a cortico-striato-cortical reentrant SD. Vertical bars indicate 20 mV. Horizontal bars indicate 10 min (**A**) or 2 min (**B**).

Supplemental Movies *(available at http://www.jci.org/articles/view/36059#sd):*

1. Representative homozygous female R192Q mutant mouse with circling behavior 5 min after a single SD.
2. Representative homozygous female R192Q mutant mouse from Supplemental Movie 1, with subsequent full recovery at 50 min.
3. Representative wild type female mouse with normal gait 5 min after a single SD.
4. Representative heterozygous female S218L mutant mouse with circling behavior 5 min after a single SD.
5. Representative heterozygous female S218L mutant mouse from Supplemental Movie 5, with subsequent recovery at 70 min.
6. Representative heterozygous female S218L mutant mouse with left hemiparesis 15 min after a single SD. Note the weakness of left forearm and paw grip. In wild type mice no weakness was demonstrated even at the earliest assessment point (5 min).
7. Representative homozygous female R192Q mutant mouse with wire grip deficits 10 min after a single SD.
8. Representative homozygous female R192Q mutant mouse from Supplemental Movie 7 with wire grip deficits 20 min after a single SD.
9. Representative homozygous female S218L mutant mouse with wire grip deficits 35 min after a single SD.
10. Representative homozygous female S218L mutant mouse from Supplemental Movie 9 with wire grip deficits 70 min after a single SD.
11. Representative homozygous female S218L mutant mouse from Supplemental Movies 9 and 10 with recovery of wire grip deficits at 100 min.
12. Representative wild type female mouse with normal wire grip performance 5 min after a single SD.
13. Representative wild type female mouse with normal wire grip performance 5 min after a single SD.

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CHAPTER 2B

ANDROGENIC SUPPRESSION OF SPREADING DEPRESSION IN FAMILIAL HEMIPLEGIC MIGRAINE TYPE 1 MUTANT MICE

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ABSTRACT

Familial hemiplegic migraine type 1 (FHM1), a severe migraine with aura variant, is caused by mutations in the *CACNA1A* gene. Mutant mice carrying the FHM1 R192Q mutation exhibit increased propensity for cortical spreading depression (CSD), a propagating wave of neuroglial depolarization implicated in migraine aura. The CSD phenotype is stronger in female R192Q mutants and diminishes after ovariectomy. Here, we show that orchiectomy reciprocally increases CSD susceptibility in R192Q mutant mice. Chronic testosterone replacement restores CSD susceptibility by an androgen receptor-dependent mechanism. Hence, androgens modulate genetically-enhanced CSD susceptibility and may provide a novel prophylactic target for migraine.

INTRODUCTION

Familial hemiplegic migraine (FHM) is an autosomal dominant subtype of migraine with aura associated with transient hemiparesis. Aura and headache features are otherwise identical to those in common forms of migraine.[1] FHM1 is caused by missense mutations in the *CACNA1A* gene, which encodes the pore-forming α_{1A}-subunit of neuronal $Ca_v2.1$ voltage-gated Ca^{2+} channels (VGCC).[2] When expressed in transfected cultured neurons, FHM1 mutations shift channel opening toward more negative membrane potentials and delay channel inactivation. Channels open with smaller depolarization and stay open longer, allowing more Ca^{2+} to enter presynaptic terminals.[3,4] Increased action potential-evoked Ca^{2+} influx has been shown to enhance excitatory neurotransmission at pyramidal cell synapses of FHM1 mutant mice.[5] Accordingly, mutant mice carrying the FHM1 R192Q mutation show enhanced susceptibility to cortical spreading depression (CSD), the electrophysiological correlate of migraine aura, and a possible trigger of migraine headache mechanisms.[4,6-8] CSD is characterized by an intense depolarization of neuronal and glial membranes propagating at a rate of ~3mm/minute. Evoked when extracellular K^+ concentrations exceed a critical threshold, CSD is associated with massive K^+ and glutamate efflux, depolarizing adjacent neurons and glia and facilitate CSD spread.

Gonadal hormones are important modulators of migraine and cortical excitability.[9,10] Incidence of common types of migraine both with or without aura is three-fold higher in females (25%) than in males (8%).[11] A female preponderance has also been described for familial (5:2) and sporadic (4.25:1) hemiplegic migraine.[1,12]

Brennan et al.[13] recently reported that KCl and electrical stimulation thresholds for CSD induction are both reduced by approximately 50% in wild-type (WT) female mice compared to males. We found a similar increase in CSD susceptibility in female FHM1 knockin mice compared to males; the sex difference was abrogated by ovariectomy and partly restored by estradiol replacement, suggesting that estrogens modulate CSD susceptibility.[7] Although the female preponderance of migraine has been largely attributed to ovarian sex steroids, anecdotal evidence suggests a role for testosterone and its synthetic derivatives in suppressing migraine in both men and women.[14-16] Here, we provide in vivo experimental evidence for androgenic suppression of CSD susceptibility, as a surrogate model for migraine aura. The data suggest that male and female gonadal hormones exert reciprocal effects on CSD susceptibility, and that androgens may contribute to the lower prevalence of FHM and common types of migraine in males.

MATERIALS AND METHODS

Experimental groups and the number of mice in each group are shown in the Table (n = 106). Adult (4-8 months) or senescent (11-13 months) male FHM1 knockin mice, homozygous for the R192Q mutation that was introduced in the mouse *Cacna1a* gene

by a gene-targeting approach,[4] were compared to WT littermates and C57BL6/J mice. All experiments were carried out with the investigator blinded for the genotype, and confirmatory genotyping was done after the experiment.

Experiments were conducted in accordance with the U.S. Public Health Service's Policy on Humane Care and Use of Laboratory Animals, and were approved by the institutional review committee. The femoral artery was catheterized for blood sampling and measurement of mean arterial pressure, and the trachea was intubated for mechanical ventilation under isoflurane anesthesia (2.5% induction, 1% maintenance, in 70% N_2O/30% O_2). Arterial blood gases and pH were measured every 20 minutes and maintained within normal limits by adjusting ventilation (Table). Mice were placed in a stereotaxic frame and burr holes were drilled at the coordinates described previously.[7] Two glass capillary microelectrodes were placed to record extracellular steady (DC) potential and electrocorticogram at a depth of 300 μm. After surgical preparation, the occipital cortex was allowed to recover for 20 minutes under saline irrigation. The frequency of CSDs evoked by epidural KCl application (300mM for 30 minutes) was determined, as previously described.[7] The propagation speed, amplitude, and duration of the first CSD were also measured.

Orchiectomy was performed under brief isoflurane anesthesia 3 weeks prior to CSD susceptibility testing. Subcutaneous testosterone pellets (0.1 mg/pellet, 21-day release; Innovative Research of America, Sarasota, FL) were implanted into the dorsal neck and shoulder region on the day of orchiectomy. The pellets restore physiological circulating levels of testosterone for at least 21 days, but may cause an early peak in plasma levels during the first week after implantation. In order to test whether testosterone replacement exerts its effects on CSD via androgen receptors, a subgroup of orchiectomized testosterone-replaced mice also received pellets containing the androgen receptor antagonist, flutamide (25 or 50 mg/pellet, 21-day release; Innovative Research of America). In addition, acute effects of testosterone propionate (1.2 mg per mouse in 0.1 ml of β-cyclodextrin injected subcutaneously; Sigma, St. Louis, MO) were tested 1 hour before electrophysiological recording in castrated mice. The effectiveness of orchiectomy, testosterone, and flutamide treatments was confirmed by measuring the prostate and seminal vesicle weights after sacrifice.

Data were analyzed using SPSS (version 11.0; SPSS, Inc., Chicago, IL). Using a general linear model of covariance analysis (ANACOVA), we tested for an effect of the independent variables genotype, age, orchiectomy, testosterone treatment (acute, chronic) and flutamide treatment (25mg, 50mg) on the dependent variables CSD frequency and propagation speed. Other electrophysiological measures of CSD and systemic physiological data were compared among groups using one-way analysis of variance (ANOVA). Data are presented as mean ± standard deviation (SD), and $p < 0.05$ was considered statistically significant.

RESULTS

Continuous epidural KCl application evoked repetitive CSDs in all mice (Figure). Both the frequency and the propagation speed of CSDs were significantly higher in R192Q mutants compared to the WT, as reported previously.[7] Orchiectomy further increased CSD frequency (by 40%) and to a lesser extent the propagation speed in R192Q mutants, but not in WT mice. Chronic testosterone replacement for 21 days completely prevented the orchiectomy-induced increase in CSD susceptibility in R192Q mutant mice (Figure; Table). In contrast, a single dose of testosterone propionate administered 1 hour before electrophysiological recordings had no effect (16 ± 2 CSDs/hour, 4.3 ± 0.3 mm/minute; $p > 0.05$ vs. orchiectomized controls). The CSD suppression by chronic testosterone replacement was prevented by cotreatment with androgen receptor antagonist flutamide (50 mg pellet); a lower dose of flutamide was ineffective (25 mg pellet; data not shown). Aging (11-13 months) had no effect on CSD frequency and propagation speed in either WT or R192Q mutant mice, consistent with the maintenance of plasma testosterone levels during aging in this WT background strain.[17] In WT mice, gonadectomy or testosterone replacement did not significantly alter CSD susceptibility, suggesting that in our model androgens modulate CSD susceptibility only if the latter is genetically enhanced. The CSD duration and amplitude, and systemic physiological parameters, did not significantly differ among groups (Table).

				CSD				Systemic Physiology			
	n	Age (months)	BW (g)	Frequency (CSD/hour)	Speed (mm/minute)	Duration (sec)	Amplitude (mV)	BP (mmHg)	pH_a	p_aCO_2 (mmHg)	p_aO_2 (mmHg)
WT											
Naive	10	5 ± 1	30 ± 4	9 ± 1	2.7 ± 0.1	43 ± 11	24 ± 5	81 ± 9	7.37 ± 0.06	35 ± 6	149 ± 28
Orx	8	5 ± 1	27 ± 3	10 ± 1	2.9 ± 0.1	36 ± 9	28 ± 4	83 ± 5	7.36 ± 0.05	35 ± 5	133 ± 19
Orx+$T_{Chronic}$	4	4 ± 0	27 ± 1	9 ± 1	2.8 ± 0.2	41 ± 7	23 ± 1	86 ± 1	7.40 ± 0.03	31 ± 3	142 ± 18
Aged	5	11 ± 0	37 ± 3	9 ± 1	2.7 ± 0.0	23 ± 9	24 ± 4	87 ± 5	7.43 ± 0.02	31 ± 3	157 ± 19
R192Q											
Naive	19	5 ± 1	27 ± 3	12 ± 1	3.8 ± 0.2	37 ± 10	23 ± 5	84 ± 5	7.38 ± 0.05	32 ± 6	133 ± 22
Orx	16	5 ± 1	27 ± 2	17 ± 2	4.1 ± 0.1	32 ± 8	25 ± 7	86 ± 5	7.37 ± 0.05	32 ± 4	140 ± 20
Orx+$T_{Chronic}$	16	6 ± 1	28 ± 2	12 ± 1	3.5 ± 0.3	38 ± 9	23 ± 3	88 ± 3	7.38 ± 0.03	30 ± 4	153 ± 22
Orx+$T_{Chronic}$+F_{25}	7	8 ± 0	30 ± 6	12 ± 0	3.7 ± 0.2	32 ± 6	18 ± 2	85 ± 7	7.42 ± 0.04	33 ± 5	161 ± 18
Orx+$T_{Chronic}$+F_{50}	8	5 ± 0	27 ± 1	17 ± 1	4.2 ± 0.4	33 ± 6	25 ± 3	91 ± 5	7.40 ± 0.06	31 ± 4	157 ± 36
Orx+T_{Acute}	6	7 ± 0	28 ± 2	16 ± 2	4.3 ± 0.3	33 ± 10	25 ± 4	90 ± 5	7.40 ± 0.03	31 ± 3	151 ± 37
Aged	7	13 ± 2	37 ± 4	12 ± 1	3.6 ± 0.3	42 ± 12	27 ± 3	80 ± 6	7.37 ± 0.05	34 ± 5	146 ± 19

Table. Electrophysiological Measures of CSD, and Systemic Physiological Parameters. Values are mean ± SD. CSD duration was measured at one-half amplitude. The duration and the amplitude of only the first CSD are shown. Systemic physiological parameters were averaged over 1 hour recording duration. CSD = cortical spreading depression; SD = standard deviation; BW = body weight; Orx = orchiectomized mice; $T_{Chronic}$ = testosterone 0.1mg pellet for 21 days; T_{Acute} = single 1.2mg dose of testosterone administered 1 hour prior to CSD testing; $F_{25/50}$ = flutamide 25 or 50mg/pellet for 21 days; BP = mean arterial blood pressure; pH_a = acidity of arterial blood; p_aCO_2 = arterial partial pressure of carbon dioxide; p_aO_2 = arterial partial pressure of oxygen; WT = wild-type; R192Q = homozygous R192Q knockin mice; Aged = 11-13 months old.

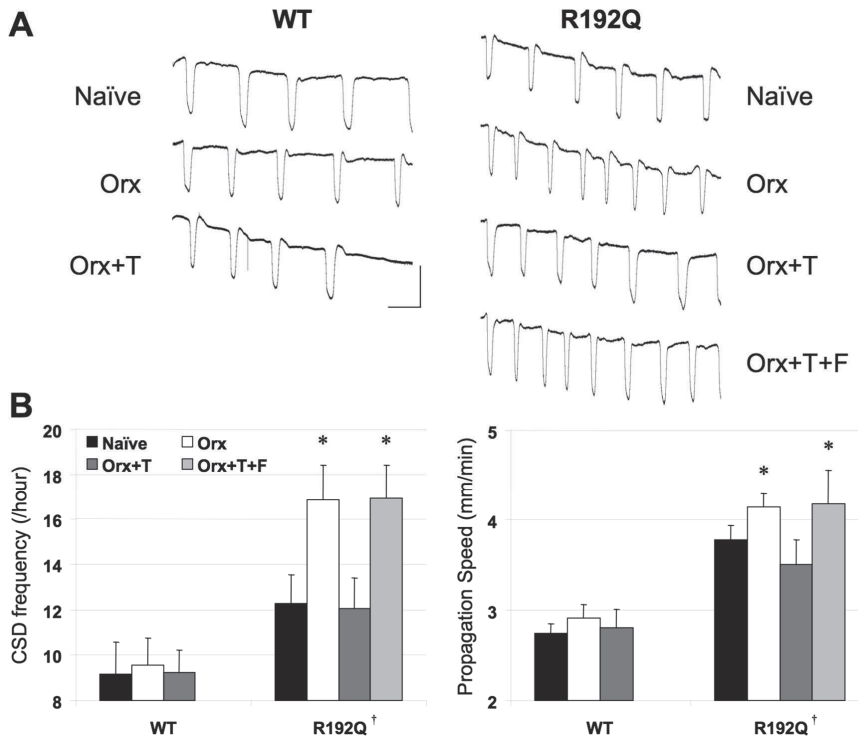


Figure. Androgenic modulation of CSD in R192Q mutant mice. (**A**) Representative electrophysiological recordings from male wild-type (WT) and homozygous R192Q mutant mice showing repetitive CSDs evoked by topical KCl application (300 mM) for 30 minutes. (**B**) Graphic representation of CSD frequency and propagation speed in WT and R192Q mutant mice. Naive R192Q mutant mice developed higher frequency of CSDs compared to WT. Orchiectomy (Orx) further increased CSD frequency in the R192Q mutant, which was restored to the level of naive R192Q mutants by chronic testosterone replacement (T). The androgen receptor blocker flutamide (F) completely abolished the effects of testosterone replacement. Vertical bar = 20 mV; horizontal bar = 4 minutes. Data are mean ± SD; $^{*}p < 0.001$ vs. naive and Orx+T R192Q mutant; $^{\dagger}p < 0.001$ vs. WT. Numbers of mice for each group are shown in the Table.

DISCUSSION

We showed that testosterone, acting via androgen receptors, suppresses genetically-enhanced CSD susceptibility. CSD suppression required chronic androgen replacement. We recently showed that estradiol augmented genetically-enhanced CSD susceptibility in FHM1 knockin mice.[7] To the extent that mice homozygous for the FHM1 allele represent the human condition, the data suggest that estrogen and androgen exert reciprocal effects on CSD susceptibility, providing a dual mechanism that may account for the female preponderance of migraine.

Observational studies suggest that methyltestosterone and danazol, a synthetic testosterone derivative, may decrease attack frequency and severity in migraineurs.[14-16,18,19] As androgens are known to downregulate estrogen receptor expression[20] and danazol also inhibits ovarian sex hormone production, it is unclear whether the clinical effects of danazol are a direct result of androgen receptor activation or secondary to suppression of estrogen actions on excitability.[10,21,22] Complete cessation of migraine with aura attacks was reported in men treated with gonadotrophins for infertility, further implicating androgens secreted by the testes.[23] In a small cohort of male-to-female transsexuals, the prevalence of migraine with aura increased during anti-androgen combined with estrogen therapy to levels similar to that seen in females.[24]

Unlike estrogens, the influence of androgens on neuronal structure and function has not been studied in detail. There are data suggesting that androgens modulate both presynaptic and postsynaptic mechanisms. For example, orchiectomy enhances spontaneous acetylcholine release (ie, increased frequency of miniature endplate potentials) at the neuromuscular junction possibly related to altered expression and function of VGCCs (eg, $Ca_v2.2$).[25] Although a specific modulation of $Ca_v2.1$ channels has not been reported, similar mechanisms may be operational at the glutamatergic central synapses. Postsynaptic glutamate receptors, particularly the *N*-methyl-D-aspartic acid (NMDA) subtype, are critical for the propagation of CSD. The nonaromatizable androgen, 5-α-dihydrotestosterone (5αDHT), modulates NMDA responses in a complex manner in hippocampal slices from orchiectomized rats: despite larger NMDA-induced currents, irreversible depolarization and cell death at high NMDA concentrations were significantly inhibited by 5αDHT.[26] The latter effect required 5αDHT exposure times of 8 hours or more, implicating transcriptional mechanisms.

There is a well-established bidirectionally increased risk of comorbidity of migraine and epilepsy, suggesting shared underlying mechanisms.[27] Interestingly, there is a clinical association between androgen deficiency and epilepsy.[28] Consistent with this, androgens possess anticonvulsant activity in rodents by acutely enhancing γ-aminobutyric acid type A ($GABA_A$) receptor activity independent of androgen receptors.[29] However, we found that acute testosterone administration did not suppress CSD, and that suppression by chronic testosterone treatment was abolished by the androgen receptor blocker flutamide. Taken together with previous data suggesting that barbiturates do not significantly suppress CSD,[30] it is unlikely that GABAergic mechanisms play a significant role in androgenic CSD suppression.

In our study, orchiectomy and testosterone modulated CSD only in FHM1 mutant mice, and not in the WT. The mechanisms of interaction between gonadal hormones and the mutant $Ca_v2.1$ channels are not known; however, the need for chronic treatment with testosterone implicates mechanisms linked to gene expression and, possibly,

ultrastructural changes. Presynaptic, postsynaptic and astrocytic mechanisms may all be involved in the interaction between gonadal hormones and FHM1 mutations. The clear female preponderance in clinical migraine strongly suggests a reciprocal modulation of yet unidentified polygenetic migraine susceptibility factors by androgen and estrogen.

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CHAPTER 2C

ENHANCED SUBCORTICAL SPREADING DEPRESSION IN FAMILIAL HEMIPLEGIC MIGRAINE TYPE 1 MUTANT MICE

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ABSTRACT

Familial hemiplegic migraine type 1, a monogenic migraine variant with aura, is linked to gain-of-function mutations in the *CACNA1A* gene encoding $Ca_V2.1$ channels. The S218L mutation causes severe channel dysfunction, and paroxysmal migraine attacks can be accompanied by seizures, coma and hemiplegia; patients expressing the R192Q mutation exhibit hemiplegia only. Familial hemiplegic migraine knock-in mice expressing the S218L or R192Q mutation are highly susceptible to cortical spreading depression, the electrophysiological surrogate for migraine aura, and develop severe and prolonged motor deficits after spreading depression. The S218L mutants also develop coma, seizures and sometimes die. To investigate underlying mechanisms for these symptoms, we used multielectrode electrophysiological recordings, diffusion-weighted MRI, and c-*fos* immunohistochemistry to trace spreading depression propagation into subcortical structures. We showed that unlike the wild type, cortical spreading depression readily propagated into subcortical structures in both familial hemiplegic migraine type 1 mutants. Whereas the facilitated subcortical spread appeared limited to the striatum in R192Q, hippocampal and thalamic spread was detected in the S218L mutants with an allele-dosage effect. Both strains exhibited increased susceptibility to subcortical spreading depression and reverberating spreading depression waves. Altogether, these data show that spreading depression propagates between cortex, basal ganglia, diencephalon, and hippocampus in genetically susceptible brains, which could explain the prolonged hemiplegia, coma, and seizure phenotype in this variant of migraine with aura.

INTRODUCTION

Familial hemiplegic migraine (FHM) is a monogenic subtype of migraine with aura characterized by transient neurological signs and symptoms including hemiparesis, aphasia, seizures, and coma. Different aura signs and symptoms can coexist, typically transforming into each other (e.g., visual followed by sensory and motor), and resolve in the order they appear.[1] FHM type 1 (FHM1) is caused by missense mutations in the *CACNA1A* gene (e.g., R192Q, S218L) encoding the poreforming α_{1A} subunit of neuronal voltage-gated $Ca_V2.1$ channels.[2,3] In cultured neurons, FHM1 mutations shift channel opening toward more negative membrane potentials and delay channel inactivation. Channels open with smaller depolarization and stay open longer,[4,5] presumably allowing more Ca^{2+} to enter presynaptic terminals, resulting in enhanced glutamate release. Consistent with this, excitatory neurotransmission is enhanced in pyramidal cell synapses of R192Q knock-in mice.[6]

Spreading depression (SD), widely viewed as the electrophysiological event underlying migraine aura, is an intense wave of neuronal depolarization that propagates (~3 mm/min) by way of gray matter contiguity, regardless of functional divisions. SD is characterized by massive K^+ efflux, Ca^{2+} influx, and glutamate release, which are believed to depolarize adjacent neurons and glia, thereby facilitating its spread. The transmembrane ionic and water shifts also lead to characteristic apparent diffusion coefficient (ADC) changes on diffusion-weighted magnetic resonance imaging (MRI), and upregulation of the immediate-early gene c-*fos*, as surrogate measures of intense neuronal depolarization. Mutant mouse models expressing the pathogenic R192Q or S218L FHM1 mutation exhibit increased susceptibility to cortical SD.[6-9] The S218L mutation shows larger gain-of-function, *in vitro*, and higher SD susceptibility, *in vivo*, compared with the R192Q variant.[7-9] In contrast to pure hemiplegic migraine associated with the R192Q mutation, attacks in patients carrying the S218L mutation are sometimes accompanied by coma or stupor and generalized seizures.[2,10] Accordingly, experimentally induced cortical SD induces pure hemiplegia in R192Q mutant mice, whereas S218L mutants additionally develop coma and often fatal seizures.[8,9]

Experimental SD produces seizures and coma when evoked in hippocampus or thalamus, respectively.[11] We investigated whether the severe clinical phenotype observed only with the S218L mutation could be caused by subcortical SD involving hippocampus and thalamus. We now provide evidence for enhanced subcortical SD susceptibility in FHM1 mutant mice that facilitates SD propagation bidirectionally between cortex, hippocampus, and thalamus in the S218L mutant strain. Our findings suggest a role for subcortical SD as a potential mechanism to explain hemiplegia, seizures, and coma in FHM1.

METHODS

Experimental groups. A total of 75 male and female wild-type (WT) or FHM1 knock-in mice [homozygous (HOM) R192Q, or HOM or heterozygous (HET) S218L][7] were used (*n* = 44 for electrophysiological recordings, *n* = 22 for c-*fos* immunostaining, *n* = 4 for MRI, and *n* = 5 for laser speckle flowmetry). R192Q mutant mice were compared with their WT littermates or C57BL/6J mice,on which the mutants were backcrossed for >10 generations. Because none of the end points differed between WT littermates and C57BL/6J mice, data from WT strains were pooled. S218L mutant mice were compared with their WT littermates. In female mice, we did not control for the estrus stage as we did not observe high variability in SD susceptibility in randomly tested female mice in our previous study.[9] To test whether enhanced glutamatergic activity contributes to subcortical spread in FHM1 mutants, a separate group of female WT and S218L HOM mice was treated with guanosine (7.5 mg/kg, i.p., 30 min before SD induction), an anticonvulsant that enhances astrocytic glutamate uptake and suppresses glutamatergic transmission.[12-15] All experiments were performed with the investigator blinded to genotype, followed by confirmatory genotyping.

General surgical preparation. Experimental procedures were approved by the institutional review committee. Mice were housed under diurnal lighting conditions and allowed food and tap water *ad libitum*. The femoral artery was catheterized for blood pressure (BP) monitoring, and the trachea was intubated for mechanical ventilation (SAR-830; CWE) under isoflurane anesthesia (2.5% induction, 1% maintenance in 70% N_2O/30% O_2). Arterial blood gases and pH were measured every 20 min and maintained within normal limits by adjusting the ventilation. Systemic physiological parameters did not differ among groups (supplemental Table 1, see Notes). There was no mortality during these experiments.

Electrophysiology. Mice were placed in a stereotaxic frame, and burr holes were drilled (Fig. 1). Up to four glass microelectrodes were placed to simultaneously record extracellular steady (DC) potential and fast neuronal activity (electrocorticogram) at the following coordinates (posterior, lateral, and ventral from bregma; in mm): cortex: 3.5, 1, 0.3; striatum: -0.5, 2, 3; hippocampus: 1.8, 1.2, 1.2; thalamus: 1.8, 1.2, 3. Table 2 shows the numbers of mice and SD recordings in each experimental group. In a subset of S218L HOM mice, simultaneous bilateral recordings from cortex (*n* = 9), hippocampus (*n* = 3), or thalamus (*n* = 4) were obtained to examine contralateral spread. In a separate group, SD induced cortical blood flow changes were measured simultaneously in both hemispheres using laser speckle imaging to detect contralateral cortical spread (*n* = 5), as described previously.[16] Because isoflurane and N_2O, but not pentobarbital, suppress SD susceptibility,[17] after surgical preparation anesthesia was switched to pentobarbital, and the ventilation gas was switched to 70% N_2/30% O_2, for all experiments. Pentobarbital

was chosen over urethane because it allowed us to better maintain a normal systemic physiological state in prolonged experiments performed in this study. After 30 min, one SD was induced through a parietal window (1.5 mm diameter, 2.5 mm posterior, and 3 mm lateral from bregma) by an epidural cotton ball (1.5 mm) soaked with 300 mM KCl. After the onset of the DC shift, KCl was removed by extensive saline wash. Fifteen minutes were allowed between each SD induction whether induced by topical KCl or mechanically during electrode insertion.[18] In a separate group of mice, we induced the cortical SD by a single pin prick and obtained similar data (not shown).

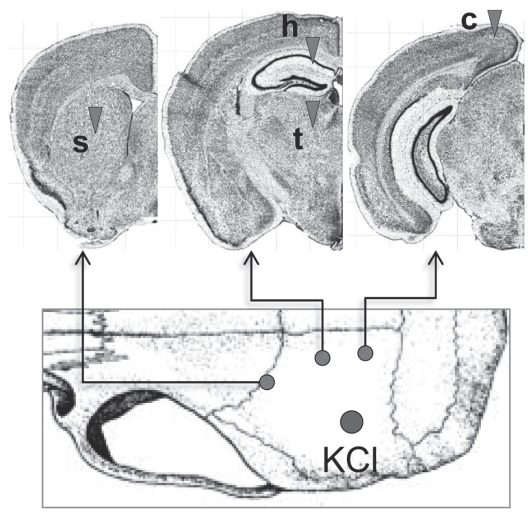


Figure 1. Electrophysiological recording sites. SD was triggered by brief topical KCl (300 mM) application onto parietal cortex and recorded with up to four glass micropipettes (arrowheads) placed into cortex (c), striatum (s), hippocampus (h), thalamus (t). [Adapted from Allen Mouse Brain Atlas (Internet), 2009. Seattle: Allen Institute for Brain Science. Available at http://mouse.brain-map.org].

Strain	Gender	Genotype	Striatum	Hippocampus	Thalamus
R192Q	Female	WT	28% (7/25 CSDs in 3/4 mice)	0% (0/11 CSDs in 0/4 mice)	0% (0/14 CSDs in 0/4 mice)
		HOM	71%* (10/14 CSDs in 4/4 mice)	0% (0/7 CSDs in 0/4 mice)	0% (0/7 CSDs in 0/4 mice)
S218L	Female	WT	22% (5/22 CSDs in 4/4 mice)	0% (0/11 CSDs in 0/5 mice)	0% (0/7 CSDs in 0/4 mice)
		HET	100%*† (51/51 CSDs in 5/5 mice)	27%*† (7/26 CSDs in 5/5 mice)	0% (0/25 CSDs in 0/5 mice)
		HOM	100%*† (35/35 CSDs in 7/7 mice)	72%*†‡ (13/18 CSDs in 7/7 mice)	39%*†‡ (15/39 CSDs in 9/10 mice)
		WT + guanosine	29% (2/7 CSDs in 2/2 mice)	NR	NR
		HOM + guanosine	95%* (42/44 CSDs in 5/5 mice)	41%*$^{+}$ (16/39 CSDs in 5/5 mice)	0%$^{+}$ (0/10 CSDs in 1/2 mice)
	Male	WT	20% (4/20 CSDs in 3/4 mice)	0% (0/10 CSDs in 0/4 mice)	0% (0/10 CSDs in 0/4 mice)
		HOM	91%*$^{\#}$ (39/43 CSDs in 5/5 mice)	70%* (14/20 CSDs in 5/5 mice)	21% (4/19 CSDs in 4/5 mice)

Table 1. Propagation rate of cortical SDs into subcortical structures. Structures were recorded in random order. Values indicate the proportion of CSDs propagating into indicated subcortical structures while recording from that structure and the proportion of mice that showed subcortical propagation into indicated structures. *$p < 0.05$ versus WT; $^{\dagger}p < 0.05$ versus R192Q HOM; $^{\ddagger}p < 0.05$ versus S218L HET; $^{+}p < 0.05$ and $^{\#}p = 0.06$ versus untreated S218L female HOM. NR, Not recorded.

Strain	Gender	Genotype	Cortex	Striatum	Hippocampus	Thalamus
R192Q	Female	WT	90 ± 30	294 ± 32		
		HOM	52 ± 3*	110 ± 19*		
S218L	Female	WT	110 ± 26	345 ± 110		
		HET	41 ± 5*	98 ± 15*	131 ± 33	
		HOM	31 ± 4*	80 ± 17*	97 ± 12†	148 ± 56
		WT + guanosine	73 ± 8	280, 280		
		HOM + guanosine	29 ± 4*	72 ± 7	94 ± 21	90
	Male	WT	99 ± 22	267 ± 112		
		HOM	37 ± 8*#	77 ± 8*	86 ± 21	165 ± 73

Table 2. Latency between KCl application and the onset of SD. Values indicate the latency (seconds) between topical cortical KCl application and SD onset in indicated structures. Only two cortical SDs were recorded in striatum in S218L female WT treated with guanosine; individual values are shown. *$p < 0.01$ versus WT; †$p < 0.05$ versus S218L HET; #$p = 0.10$ versus S218L female HOM. Values are mean ± SD.

Magnetic resonance imaging. MRI was performed in S218L HOM mice ($n = 4$) using a 9.4T horizontal bore (Magnex Scientific) scanner with a custom-made surface-radiofrequency coil, under pentobarbital anesthesia. The ADC was measured with a sequence of four diffusion-weighted echoplanar images (weighting along Z direction, $b = 0$ –1287.9 s/mm^2; echo time, 20 ms; repetition time, 5000 ms; field of view, 14 mm; slice thickness, 0.50 mm; matrix, 64 X 64). Sixteen contiguous coronal slices were acquired to cover cerebrum with a temporal resolution of 20 s. After surgical preparation and the onset of sequential MRI, cortical SD (CSD) was evoked by topical application of 300 mM KCl on the parietal cortex via a polyethylene tubing mounted above an open cranial window. Ten seconds after topical application of 300 mM KCl, cortex was washed with saline via a separate polyethylene tubing, to minimize cortical exposure to high levels of K^+. Imaging was continued for 15 min after CSD induction.

c-fos immunostaining. Under pentobarbital anesthesia, one cortical SD was induced every 15 min for a total of three cortical SDs and was electrophysiologically confirmed ($n = 6$, 4, and 12 female WT, R192Q HOM, and S218L HOM mutants, respectively). Three hours after the induction of the first cortical SD, mice were deeply anesthetized and transcardiac perfusion fixed. Sham animals underwent the same procedure, duration, and form of anesthesia, but cotton balls soaked with saline instead of KCl were placed on the cortex. Brains were postfixed in 2.5% paraformaldehyde, and 30 μm cryosections were immunostained for *c-fos*[19] and qualitatively examined at two coronal levels through (1) the striatum and septal nuclei and (2) the hippocampus, thalamus, and amygdala.

Data analysis. Data were analyzed using SPSS (version 11.0; SPSS). Using pooled data from all mice and a general linear model of variance analysis, we tested for an effect of independent-variable mutation and genotype (fixed factors) on physiological

parameters of dependent variables, latency between KCl application and occurrence of SD, and duration and amplitude of SD in the respective structures. The incidence of subcortical SD and mechanical SD induction in respective structures were compared among groups using the χ^2 test. Data are presented as mean ± SD. $p < 0.05$ was considered statistically significant. Strong statistical trends ($0.05 \leq p \leq 0.10$) were also shown.

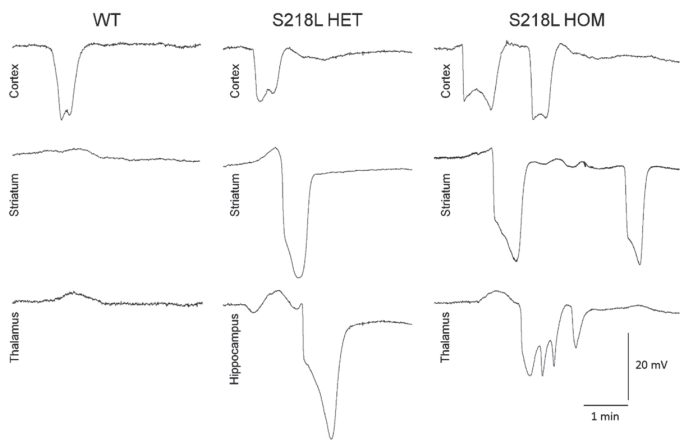


Figure 2. Subcortical propagation of cortical SD into hippocampus and thalamus in S218L mutant mice. Representative microelectrode recordings show SD propagation from cortex into striatum and hippocampus or thalamus in HET or HOM S218L mutants but not in WT mice. Thalamic or hippocampal propagation was not found in R192Q HOM mice (see Table 2). Hippocampal SDs were often prolonged compared with other structures. Thalamic SDs were often multiphasic, sometimes coalescing into a prolonged depolarization. The multiphasic waveformmaybe attributable to multiple corticothalamic propagation pathways arriving sequentially at the thalamic recording site or caused by circling within thalamic nuclei.

			SD amplitude (mV)				SD duration (sec)			
Strain	Gender	Genotype	Cortex	Striatum	Hippocampus	Thalamus	Cortex	Striatum	Hippocampus	Thalamus
R192Q	Female	WT	10 ± 4	30 ± 2			38 ± 6	41 ± 6		
		HOM	13 ± 6	31 ± 10			41 ± 6	45 ± 9		
S218L	Female	WT	9 ± 4	21 ± 7			35 ± 2	37 ± 2		
		HET	11 ± 4	35 ± 4	34 ± 7		34 ± 5	51 ± 14	67 ± 24	
		HOM	15 ± 6	28 ± 12	29 ± 8	24 ± 4	29 ± 5*	35 ± 10	53 ± 9	29 ± 9
		WT + guanosine	13 ± 2	13 ± 6			35 ± 5	27 ± 3		
		HOM + guanosine	15 ± 4	17 ± 11	24 ± 12	25	28 ± 6	34 ± 6	62 ± 12	45
	Male	WT	15 ± 4	16 ± 9			32 ± 5	33 ± 6		
		HOM	15 ± 2	18 ± 4#	28 ± 3	12 ± 4†	33 ± 11	29 ± 8	72 ± 27	32 ± 17

Table 3. Amplitudes and durations of SD. Values are mean ± SD. *$p < 0.05$ versus R192Q HOM; †$p < 0.05$ and #p = 0.10 versus female S218L HOM.

			Incidence of mechanical SD (%) (mechanical SD/electrode insertion)				Incidence of reciprocal spread (%) (reciprocal spread into cortex/mechanical subcortical SD)		
Strain	Gender	Genotype	Cortex	Striatum	Hippocampus	Thalamus	Striatum	Hippocampus	Thalamus
R192Q	Female	WT	25% (1/4 in 1/4 mice)	0% (0/4 in 0/4 mice)	25% (1/4 in 1/4 mice)	0% (0/4 in 0/4 mice)		0% (0/1 in 0/1 mouse)	
		HOM	29% (2/7 in 2/4 mice)	83%* (5/6 in 4/4 mice)	43% (3/7 in 2/4 mice)	29% (2/7 in 2/4 mice)	40% (2/5 in 2/4 mice)	0% (0/3 in 0/2 mice)	0% (0/2 in 0/2 mice)
S218L	Female	WT	0% (0/8 in 0/4 mice)	0% (0/8 in 0/4 mice)	10% (1/10 in 2/4 mice)	13% (1/8 in 1/4 mice)		0% (0/1 in 0/2 mice)	0% (0/1 in 0/1 mouse)
		HET	71%* (5/7 in 3/5 mice)	67%* (4/6 in 4/5 mice)	50% (3/6 in 3/5 mice)	38% (3/8 in 2/5 mice)	25% (1/4 in 1/4 mice)	33% (1/3 in 1/3 mice)	0% (0/3 in 0/2 mice)
		HOM	58%* (7/12 in 4/6 mice)	73%* (8/11 in 5/6 mice)	67%* (12/18 in 6/6 mice)	60%* (9/15 in 6/6 mice)	25% (2/8 in 2/5 mice)	33% (4/12 in 3/6 mice)	78%† (7/9 in 5/6 mice)
		WT + guanosine	0% (0/3 in 0/2 mice)	0% (0/3 in 0/2 mice)					
		HOM + guanosine	40%$^{\$}$ (2/5 in 2/5 mice)	75%* (3/4 in 3/5 mice)	50%$^{@}$ (4/8 in 3/5 mice)	0% (0/2 in 0/2 mice)	0% (0/3 in 0/5 mice)	50% (2/4 in 2/5 mice)	
	Male	WT	0% (0/7 in 0/4 mice)	0% (0/7 in 0/4 mice)	0% (0/7 in 0/4 mice)	0% (0/7 in 0/4 mice)			
		HOM	33%^ (3/9 in 3/5 mice)	55%* (6/11 in 4/5 mice)	8%‡ (1/12 in 1/5 mice)	28%$^{\#}$ (5/18 in 3/5 mice)	17% (1/6 in 1/5 mice)	0% (0/1 in 0/5 mice)	40% (2/5 in 2/5 mice)

Table 4. Incidence of mechanical SD and reciprocal spread of subcortical SD into cortex. Values indicate the proportion of electrode insertions and the proportion of mice that showed a mechanical SD and reciprocal spread. *$p < 0.05$, ^$p = 0.09$, $^{\$}p = 0.05$, and $^{@}p = 0.06$ versus WT; $^{\dagger}p < 0.05$ versus S218L HET and R192Q HOM; $^{\ddagger}p < 0.05$ and $^{\#}p = 0.06$ versus S218L HOM female.

RESULTS

Electrophysiology. Topical application of 300 mM KCl consistently induced cortical SD (Fig. 2). In WT mice, only 20-30% of cortical SDs propagated into the striatum, and none propagated into the hippocampus or the thalamus (Table 1). In R192Q mutant mice, over 70% of cortical SDs propagated into the striatum but not into other subcortical structures. In contrast, all cortical SDs propagated into the striatum, and a substantial proportion propagated into the hippocampus in S218L mutant mice, with an allele-dosage effect. Moreover, propagation into thalamus was found only in S218L HOM mice. The subcortical propagation rate did not significantly differ between male and female S218L HOM mice. Guanosine (7.5 mg/kg, administered intraperitoneally 30 min before SD recordings) suppressed hippocampal and thalamic spread in female S218L HOM mice. In contrast, striatal spread was not affected in either WT or S218L HOM. Consistent with the faster propagation speed reported previously,[8,9] the latency between cortical and subcortical SD was significantly shorter in both FHM1 mutants compared with WT (Table 2). Although the overall morphology of SDs differed between cortex and subcortical structures, SD amplitudes and durations were comparable among WT and FHM1 mutants within each structure (Table 3).

An SD was occasionally evoked in cortex or subcortical structures during electrode insertion. These mechanical SDs occurred with higher incidence in FHM1 mutants compared with WT (Table 4), and when evoked in cortex, they spread into subcortical structures similar to KCl-induced SDs. Moreover, when directly evoked in a subcortical structure (i.e., via electrode insertion), mechanical SDs did reciprocally spread into the cortex and other subcortical structures, suggesting that cortico-subcortical propagation can be bidirectional (Fig. 3; Table 4).

In all S218L HOM mutants ($n = 7$), we observed repetitive cortical SD waves after a single KCl application despite a vigorous saline wash (median, three SDs; interquartile range, two to five SDs) (Fig. 4). None of the other groups showed

repetitive SDs. Recurrent cortical SDs occurred after 19 and 38% of all SD inductions in male and female S218L HOM mice, respectively, and spread into the striatum (43 and 31%), hippocampus (0 and 11%), and thalamus (33 and 10%) in clusters. Inter-SD intervals were relatively regular, suggesting reverberating SD waves (average inter-SD intervals were 126 ± 20 and 118 ± 22 s in cortex, 202 ± 40 and 229 ± 54 s in striatum, 86 ± 16 and 174 s in hippocampus, and 144 ± 64 and 187 s in thalamus in male and female S218L HOM, respectively). Importantly, striatal but not hippocampal or thalamic propagation was required for recurrent SDs to occur, suggesting that they were reentry waves between cortex and striatum, as described previously.[20]

Cortical SD propagation into the contralateral cortex was not detected (n = 2 cortical SDs in two female and n = 5 cortical SDs in five male S218L HOM). These results using bilateral electrophysiological recordings were also confirmed using full-field laser speckle imaging to detect SD-evoked cortical blood flow changes (n = 5 cortical SDs in five S218L HOM mice) (supplemental Fig. 1 and supplemental Movie 1, http://www2.massgeneral.org/NCS/Supplemental_Online_Movie.mpeg).[21] Hippocampal SDs were similarly limited to the ipsilateral hemisphere (n = 3 hippocampal SDs in three S218L HOM mice). In contrast, all thalamic SDs (n = 4 thalamic SDs in four S218L HOM mice) propagated into the contralateral thalamus with a latency of 13 ± 3 s between bilateral thalamic electrodes (~2.4 mm apart).

Last, we detected large-amplitude (1-2 mV) and rhythmic (up to 7 Hz) spike activity, reminiscent of epileptiform afterdischarges, within 1–2 min after the recovery of an SD in S218L mutants only (Figs. 2, 4). Spike activity was present in all four structures recorded, displayed an allele-dosage effect, and was more frequent in female mutants compared with males (supplemental Table 2, see Notes). Sustained seizure activity was never observed under our recording conditions.

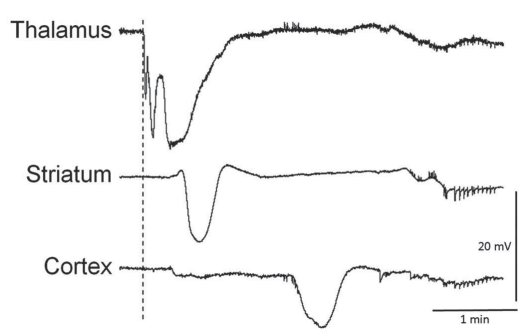


Figure 3. Reverse propagation of subcortical SD into cortex in S218L HOM mutants. A representative tracing shows a thalamic SD triggered during glass micropipette insertion in S218L HOM mice (dashed line) that preceded slow potential changes in the striatum and cortex, suggesting that SDs can propagate from thalamus to cortex as well.

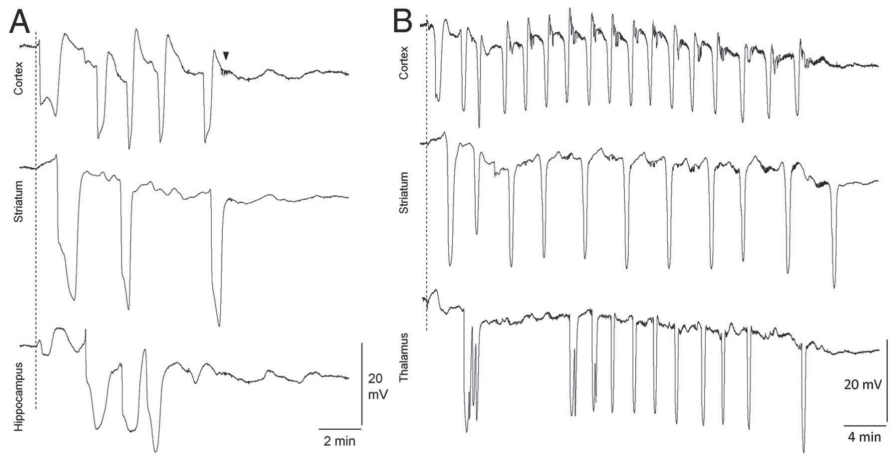


Figure 4. Recurrent cortical and subcortical SDs in S218L HOM mutants. (**A**,**B**) Representative microelectrode recordings show clusters of SD in cortex, striatum, and hippocampus (**A**) or thalamus (**B**) after a single brief epidural KCl application (dashed line) in S218L HOM mice. The arrowhead shows a typical large-amplitude spike burst observed only in the S218L HOM mutant strain. These afterdischarges usually lasted < 1 min and started within a few minutes after the recovery of slow potential shift.

MRI. We consistently observed a wave of reduced ADCs, slowly propagating into the striatum and the hippocampus after topical KCl application on to the parietal cortex in all mice (*n* = 4 female S218L HOM) (Fig. 5; supplemental Table 3, see Notes). In two animals only, we detected spread into the thalamus, which propagated into the contralateral thalamus in both cases. SD-related ADC changes consistently propagated in a lateral-to-medial direction within the striatum and in a medial-to-lateral direction within the hippocampus. It was not possible to precisely determine the propagation pathways because of the relatively rapid SD propagation in S218L HOM mice (~7 mm/min)[8,9] compared to the slow MRI acquisition time and repetition rate (once every 20 s).

c-*fos* expression. Compared with sham controls and contralateral cortex, ipsilateral cortical c-*fos* expression was increased in all mice after cortical SD (Fig. 6). c-*fos* was upregulated in the striatum in all 12 S218L HOM and 3 of 4 R192Q HOM mice but in only 1 of 6 WT mice. c-*fos* upregulation was found in hippocampus (10 of 12 mice), and in thalamus and lateral hypothalamic area (3 of 12 mice), in S218L HOM mutants only. Consistent with the electrophysiological data, cortical, striatal, and hippocampal c-*fos* upregulation was strictly unilateral, whereas thalamic c-*fos* upregulation was bilateral. In addition, all FHM1 mutants showed strong ipsilateral c-*fos* upregulation in amygdala, basal forebrain nuclei, nucleus accumbens, and the septal nuclei, as well as in regions interposed among these structures, whereas in WT there was only a slight

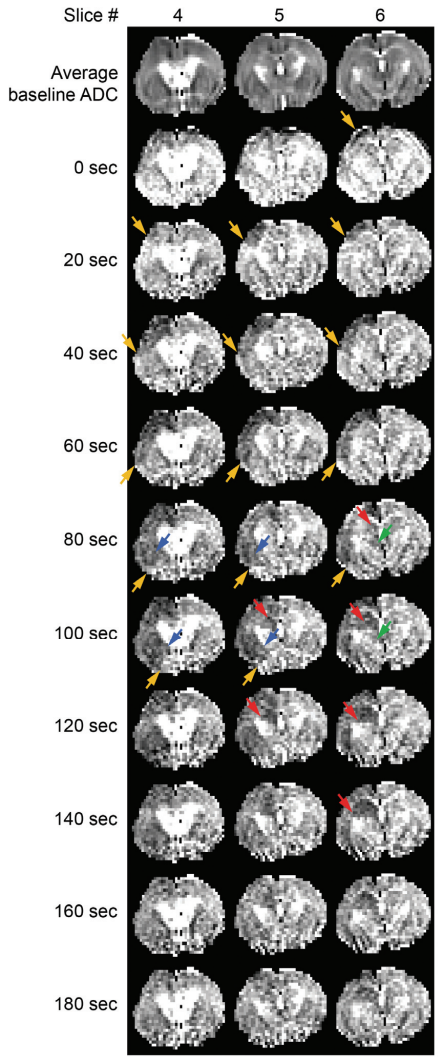


Figure 5. Diffusion-weighted MRI of SD propagation. Serial magnetic resonance images in a representative S218L HOM mouse show ADC changes during cortical SD (orange) spreading into striatum (blue), hippocampus (red), and dorsomedial thalamus (green). Three consecutive coronal slices are shown over time (acquired at 20 s interval; voxel dimensions: 0.5, 0.2, 0.2 mm). The top row shows average baseline ADC maps to serve as an anatomical reference. Arrows indicate the SD wavefront. Striatal ADC changes corresponding to SD propagation typically occurred in a lateral-to-medial direction, whereas changes in hippocampus propagated in a medial-to-lateral direction. Hippocampal ADC changes persisted longer than in all other regions.

c-*fos* staining present in three of six mice in the amygdala and in two of six in the septal nuclei only (Fig. 6). In sham controls, staining was very light and did not differ between WT and S218L HOM (n = 3 each; data not shown), thereby ruling out an effect of anesthesia.

Cardiovascular physiology. Thalamic SDs were associated with abrupt BP transients 75% of the time in all S218L HOM mice. These BP transients lasted ~1 min and were hypertensive, increasing BP > 25% without accompanying changes in heart rate (Fig. 7). Such BP fluctuations never occurred when a cortical SD failed to propagate into thalamus and thus were present only in S218L HOM mice.

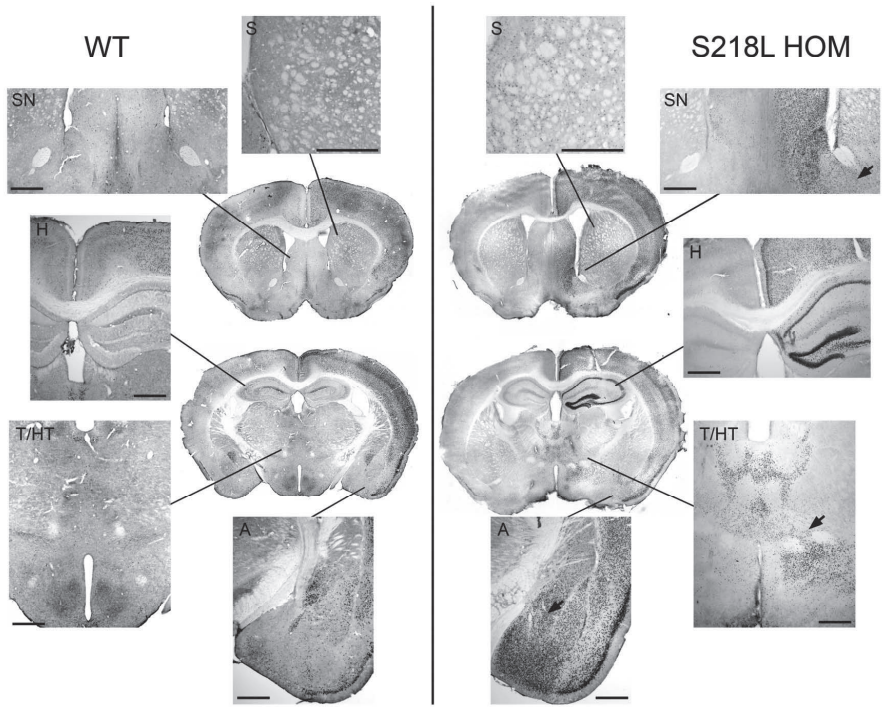


Figure 6. Enhanced c-*fos* expression in cortex and subcortical structures in S218L mutant mice. c-*fos* immunohistochemistry in coronal sections taken from representative WT and S218L HOM mice 3 h after three consecutive cortical SDs triggered 15 min apart is shown. Coronal sections were taken from two different levels (striatum and hippocampus) in whole brain. Both the number of labeled cells plus staining intensity were increased in S218L mutant brain compared with wild type. Higher-magnification images show that c-*fos* was expressed throughout cortex in both strains, although this increase appeared more prominent in the S218L HOM mutant. c-*fos* expression was upregulated in striatum (S) in all mutants (n = 12) but only one WT mouse (n = 6). c-*fos* upregulation in hippocampus (H) or thalamus (T) was observed in the mutants only. Mutants also showed c-*fos* upregulation in septal nuclei (SN; 100%), hypothalamus (HT; 50%), and amygdala (A; 100%). c-*fos* expression was observed in cells bridging contiguous structures such as striatum and amygdala, striatum and septal nuclei, and thalamus and hypothalamus (arrows). Scale bar, 500 µm.

DISCUSSION

The mechanisms underlying the signs and symptoms of severe aura in FHM patients are poorly understood. We recently reported that after an SD, S218L mutant mice exhibit neurological signs highly reminiscent of clinical attacks in FHM1 patients carrying this mutation, including hemiplegia, coma, and seizures, whereas the R192Q mutant mice developed pure hemiplegia.[9,10] Here, we present evidence that SD propagation is enhanced in subcortical structures in the S218L mutant mouse, facilitating the propagation of SD throughout the basal ganglia, hippocampus, and diencephalon and

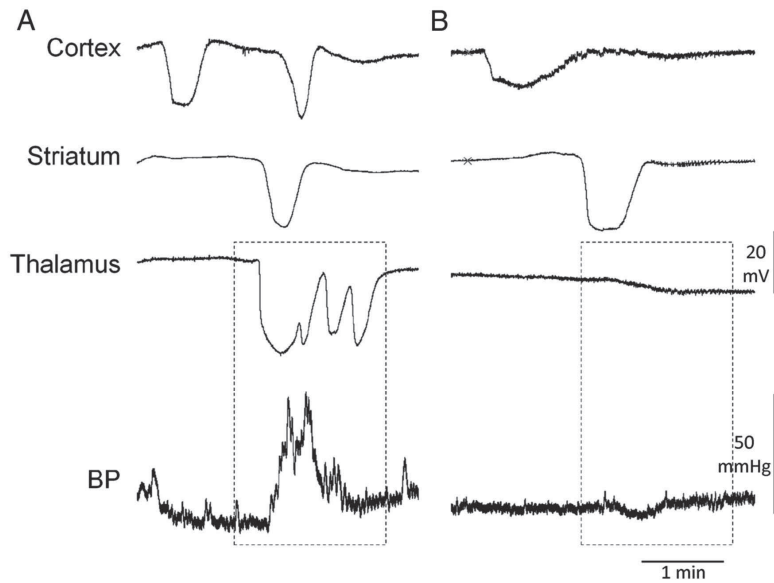


Figure 7. Thalamic SDs were associated with transient hypertension. Representative electrophysiological tracings are shown with simultaneous arterial BP recordings in an S218L HOM mutant. (**A**) When an SD propagated into thalamus, transient hypertensive episodes were observed in S218L HOM mice. (**B**) In the absence of thalamic SD, BP increases did not occur, even when SD propagated into the striatum. Hence, hypertensive episodes were never observed in R192Q mutant mice.

predisposing to reverberating SD waves. The widespread SD propagation provides a novel mechanism for the severe neurological dysfunction during FHM1 attacks. Furthermore, cortical SDs propagated into striatum more consistently in S218L than R192Q mutants, and thalamic and hippocampal SDs were observed only in S218L mutants with an allele-dosage relationship. Hence, the propensity of SD to traverse barriers with low neuronal density and high white matter content corresponds to the strength of gain of function in S218L and R192Q mutant $Ca_V2.1$ channels.[8]

Although striatal propagation of cortical SD has been reported in rats,[20,22] propagation into the hippocampus and the thalamus appears to be a manifestation of genetically enhanced SD susceptibility in FHM1 mutants. SD propagation requires high neuronal and synaptic density and is impeded by the presence of large extracellular space and an abundance of astrocytes and myelin.[23-25] Consequently, white matter and areas with lower neuronal density (e.g., subiculum) serve as natural barriers against SD propagation. Whereas thick white matter bundles such as corpus callosum are impervious to SD, the extracellular K^+ and glutamate surge may still permeate thinner white matter tracts and areas with low neuronal density to rekindle the process on the

other side of the barrier. FHM1 mutations augment $Ca_v2.1$ current density after weak depolarization and enhance presynaptic Ca^{2+} influx and glutamate release, thereby lowering the depolarization threshold for SD induction.[5,6,8,9] The S218L mutation causes larger $Ca_v2.1$ gain of function and a lower SD threshold than R192Q. The lower the SD threshold is, the more likely the ionic and neurotransmitter fluxes to evoke SD on the other side of the barrier and the more widespread the SD propagation, explaining both the allele-dosage effect and the hippocampal and thalamic spread exclusively in the S218L mutants. Also supporting a role for glutamate in subcortical spread, guanosine, which inhibits glutamatergic hyperexcitability by stimulating astrocytic glutamate uptake,[12,14] suppressed hippocampal and thalamic spread in FHM1 mutants.

In our previous work using combined isoflurane and nitrous oxide anesthesia, subcortical SD propagation rates were much lower in both WT and FHM1 mutant mice.[9] Isoflurane together with nitrous oxide is known to suppress SD susceptibility.[17] To avoid this confound, in the present study we used pentobarbital anesthesia, which has the least suppressive effect on CSD compared with other anesthetics that have been tested,[17,26] and detected widespread subcortical propagation in the FHM1 mutants. However, compared with the awake state, pentobarbital may still increase SD threshold.[27,28] Therefore, in unanesthetized FHM1 mutants, SDs might propagate even farther and exhibit longer-lasting reentrant patterns between different cortical and subcortical structures.

Results using diffusion-weighted MRI and c-*fos* immunoreactivity as surrogates of SD propagation were consistent with the electrophysiological data and reflected SD propagation into striatum, hippocampus, and thalamus of S218L mutant mice. Diffusion-weighted MRI is an established method to map SD propagation in which transient ADC reductions spatiotemporally correlate with the electrophysiological changes during SD.[29] Enhanced expression of the c-*fos* also shows spatial correspondence to SD propagation.[19,30] Although subcortical c-*fos* expression might reflect increased corticosubcortical efferent activity during a cortical SD, more typically, cortical SD leads to suppression of glucose metabolism in subcortical structures,[31] suggesting that suppression of neuronal activity (e.g., cortex) in the wake of SD has the opposite effect on efferent input to target structures.

Despite observing afterdischarges in the wake of an SD in the S218L mutant, we believe contiguous spread, rather than seizure activity, was responsible for subcortical SD occurrence. First, the SD latencies between cortical and subcortical structures were longer than expected if evoked synaptically via corticofugal axonal projections.[32] Second, ADC reductions were slow and contiguous between cortex and subcortical structures, which is not consistent with seizure activity.[33]

We observed transient BP elevations during SD in S218L HOM mice. Because the occurrence of these hypertensive episodes was tightly linked to thalamic SDs (i.e.,

never observed in the absence of a thalamic SD), and because hypothalamic nuclei showed diffuse *c-fos* upregulation, we speculate that BP transients were triggered by direct propagation of SD into the hypothalamus, rather than upstream modulation of hypothalamic autonomic output by SD in amygdala. It should be noted that although SD is limited to gray matter nuclei, the associated ionic shifts can influence conduction in white matter bundles embedded in gray matter, causing additional signs and symptoms unrelated to the involved gray matter.[34-36]

Probable subcortical propagation pathways. Amygdala is a likely path for cortico-subcortical SD propagation because of its lower proportion of myelin and direct gray matter contiguity with striatum, entorhinal and piriform cortices, and basal forebrain nuclei.[20,22,23,37-39] Subiculum may be an alternative pathway into hippocampus, despite its diminished neuronal density.[23] Human brain, of course, has a greater abundance of white matter than rodents. Nevertheless, these anatomical relationships are preserved in mammalian brains. For example, human entorhinal cortex still forms a gray matter bridge between hippocampus and neocortex. Amygdala, phylogenetically the oldest part of basal ganglia, is immediately anterior and superior to hippocampus, in contiguity with striatum, claustrum, uncus, and subiculum. Therefore, we speculate that in highly susceptible FHM1 patients (e.g., S218L carriers), cortical SD may propagate into subcortical tissues and possibly reverberate within and/or among the cortex and gray matter nuclei, leading to a prolonged and severe clinical state including encephalopathy and coma. It is equally plausible that SD may originate within the susceptible subcortical nuclei in FHM1 patients de novo. Changes in glucose metabolism and ADC have been detected in subcortical structures using diffusion-weighted MRI and ^{18}F-2-fluoro-2-deoxy-D-glucose positron emission tomography during FHM attacks.[40,41]

Speculations on subcortical SD and the clinical features of FHM. Many of the FHM1 aura symptoms and signs can be linked to dysfunction in subcortical structures. For example, hemiparesis can be caused by SD within the basal ganglia, as shown in experimental animals.[11,42,43] One-third of FHM1 patients develop severe attacks with transient impairment of consciousness ranging from stupor to sometimes fatal coma, more commonly associated with a subset of mutations including S218L.[10,40,44-49] Thalamic SD may be the mechanism for the depressed level of consciousness in FHM1 patients, because bilateral thalamic dysfunction can cause stupor and coma,[50] and we observed bilateral thalamic SD in S218L HOM mutants. Hippocampus is highly susceptible to SD,[23] and SD has been observed in human hippocampus.[51,52] Importantly, hippocampal SD can trigger seizure activity. Clinically, 20% of FHM patients carrying the S218L mutation develop seizures during attacks.[1] Similarly, we observed generalized seizures in S218L HOM mice after a cortical SD.[9] Hippocampal dysfunction may be associated

with amnesia, and limbic disturbances such as dysphoria, yawning, and fluid retention can occur during migraine attacks.[53] Amygdala may also play a role in the development of complex neuropsychiatric, autonomic, and neuroendocrine symptoms during an attack.[54]

Conclusion. Our data show that SD is a remarkable phenotype in FHM1 mutant mice. Both S218L and R192Q mutants show enhanced striatal SD as a possible cause for transient hemiplegia in FHM1. In addition, the S218L mutant shows widespread SD propagation in the forebrain, including thalamus and hippocampus, two findings that may explain coma and seizures during some attacks in patients carrying the S218L mutation. Therefore, subcortical SD, either arising *de novo* or propagating from cortex, might account for the severe attacks with hemiplegia, coma, and seizures in FHM1 patients.

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SUPPLEMENTARY MATERIAL

Strain	*Gender*	*Genotype*	*BP (mmHg)*	*pH*	*pCO_2 (mmHg)*	*pO_2 (mmHg)*
R192Q	Female	WT	87 ± 2	7.37 ± 0.03	31 ± 4	152 ± 15
		HOM	87 ± 3	7.39 ± 0.05	31 ± 5	141 ± 37
S218L	Female	WT	95 ± 5	7.35 ± 0.02	37 ± 4	141 ± 35
		HET	90 ± 3	7.38 ± 0.02	32 ± 4	134 ± 28
		HOM	95 ± 6	7.35 ± 0.03	32 ± 5	137 ± 28
		WT + guanosine	95 ± 11	7.37 ± 0.01	36 ± 2	146 ± 31
		HOM + guanosine	96 ± 5	7.36 ± 0.02	34 ± 4	151 ± 33
	Male	WT	87 ± 3	7.40 ± 0.05	33 ± 5	149 ± 24
		HOM	85 ± 7	7.38 ± 0.03	34 ± 2	137 ± 20

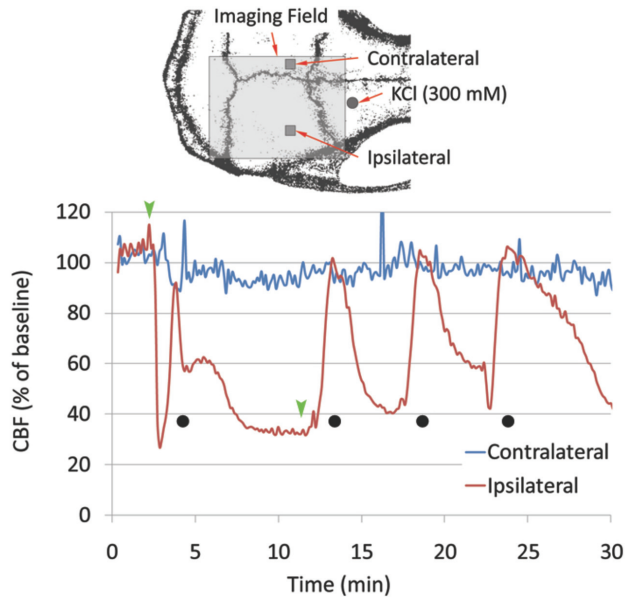
Supplemental Table 1. Systemic physiological parameters. Values are mean ± standard deviation. WT, wild type; HET, heterozygous knockin; HOM, homozygous knockin. There was no difference among groups.

Strain	Gender	Cortex	Striatum	Hippocampus	Thalamus
S218L HET	Female	25%*	16%*	21%	28%*
		(13/51 SDs)	(7/45 SDs)	(5/24 SDs)	(7/25 SDs)
S218L HOM	Female	72%	69%	42%	69%
		(60/83 SDs)	(38/55 SDs)	(8/19 SDs)	(24/35 SDs)
	Male	42%*	32%*	31%	22%*
		(22/53 SDs)	(12/38 SDs)	(4/13 SDs)	(2/9 SDs)

Supplemental Table 2. Incidence of rhythmic spike activity associated with SD. Values indicate the proportion of SDs showing rhythmic spike activity shortly after repolarization. Pooled data are shown including recurrent SDs after a single stimulus.*p<0.05 vs. S218L female HOM.

	Minimum ADC value	
	ipsilateral	contralateral
Cortex	36±2*	51±4
Striatum	35±2*	48±3
Hippocampus	35±3*	45±7
Thalamus†	32; 34	30; 36

Supplemental Table 3. Minimum ADC values measured by diffusion-weighted MRI during SD propagation. Numbers indicate minimum absolute ADC values ($x10^{-5}$ mm^2/s) in female S218L HOM (n=4) during spreading depression within the respective structures. †ADC values from the only 2 mice with thalamic SD are shown individually. *p<0.05, vs. contralateral.



Supplemental Figure 1 and Movie 1. Cortical SD-induced blood flow changes imaged using laser speckle flowmetry showing the absence of propagation into contralateral cortex. Cerebral blood flow tracings from the ipsilateral and contralateral cortices during cortical SD in a representative S218L HOM mouse. Green arrowheads indicate brief topical KCl applications (300 mM) 10 minutes apart. Each black dot indicates a cortical SD. The second KCl application triggers three consecutive SDs in this highly susceptible mutant. The first cortical SD is associated with an initial profound hypoperfusion followed by a transient normalization, as previously described (Ayata et al., 2004b). Each subsequent SD is associated with monophasic hyperemia superimposed on the oligemic baseline in the wake of the first SD. Despite multiple waves, none of the cortical SDs propagated into the contralateral cortex, evident by the absence of blood flow changes (blue tracing). Inset shows the position of the imaging field for laser speckle flowmetry and the regions of interest used to quantify the flow changes. Supplemental Movie (http://www2.massgeneral.org/ncs/Supplemental_Online_Movie.mpeg) shows a representative full field laser speckle contrast imaging of SD-induced cortical blood flow changes in an S218L HOM mutant mouse. Imaging field (6x8 mm; see Supplemental Figure 1, inset) is positioned over the entire right hemisphere (anterior to the right, posterior to the left, lateral at the bottom), including the medial segment of the left hemisphere (top). SDs, induced by brief topical KCl (300 mM) application on to the right hemisphere, are associated with characteristic blood flow changes, and do not propagate into the contralateral hemisphere (upper portion of the imaging field). Color bar indicates relative blood flow changes with respect to pre-SD baseline (red indicates an increase, blue indicates a decrease in flow). Time is indicated on top.

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CHAPTER 3

IMPACT OF SPREADING DEPRESSION SUSCEPTIBILITY ON STROKE OUTCOME

CHAPTER 3A

MIGRAINE MUTATIONS INCREASE STROKE VULNERABILITY BY FACILITATING ISCHEMIC DEPOLARIZATIONS

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ABSTRACT

Background: Migraine is an independent risk factor for stroke. Mechanisms underlying this association are unclear. Familial hemiplegic migraine (FHM), a migraine subtype that also carries an increased stroke risk, is a useful model for common migraine phenotypes because of shared aura and headache features, trigger factors, and underlying glutamatergic mechanisms.

Methods and Results: Here, we show that FHM type 1 (FHM1) mutations in $Ca_V2.1$ voltage-gated Ca^{2+} channels render the brain more vulnerable to ischemic stroke. Compared with wild-type mice, 2 FHM1 mutant mouse strains developed earlier onset of anoxic depolarization and more frequent peri-infarct depolarizations associated with rapid expansion of infarct core on diffusion-weighted magnetic resonance imaging and larger perfusion deficits on laser speckle flowmetry. Cerebral blood flow required for tissue survival was higher in the mutants, leading to infarction with milder ischemia. As a result, mutants developed larger infarcts and worse neurological outcomes after stroke, which were selectively attenuated by a glutamate receptor antagonist.

Conclusions: We propose that enhanced susceptibility to ischemic depolarizations akin to spreading depression predisposes migraineurs to infarction during mild ischemic events, thereby increasing the stroke risk.

INTRODUCTION

Migraine is the most common neurological condition affecting young to middle-age adults. Up to one third of migraineurs experience transient neurological symptoms called aura. Migraine, particularly with aura, is associated with increased stroke risk both during and between attacks, especially in women.[1-4] The biological basis for this association is unknown. Stroke risk is also increased in familial hemiplegic migraine (FHM), a monogenic migraine subtype with hemiplegic auras in addition to the common aura forms.[5] FHM is a useful model for common migraine with aura because of shared clinical features and trigger factors, female preponderance, and because two thirds of FHM patients and their first-degree relatives also have attacks of common migraine with or without aura.[6,7] Neuronal network hyperexcitability and enhanced glutamate release have been implicated in both FHM and common forms of migraine.[8]

FHM type 1 (FHM1) is caused by mutations in the *CACNA1A* gene, which encodes the pore-forming α_{1A} subunit of neuronal $Ca_V2.1$ voltage-gated Ca^{2+} channels.[9] Presynaptic $Ca_V2.1$ channels are major regulators of excitatory neurotransmitter release. FHM1 mutant channels open with smaller depolarizations and stay open longer,[10] which augments presynaptic Ca^{2+} entry and glutamate release, thereby enhancing brain excitability.[11] Transgenic mice expressing the human R192Q or S218L FHM1 mutation show an increased susceptibility to spreading depression, the electrophysiological substrate for migraine, and display characteristic clinical features of FHM such as transient hemiplegia.[12-14] Glutamatergic mechanisms and hyperexcitability also have been implicated in the pathogenesis of common forms of migraine.[15,16] Genetic support for this link was recently obtained in a genome-wide association study identifying the astrocyte elevated gene 1 (*AEG1*), encoding a regulator of glial glutamate transporter EAAT2, as the first migraine gene.[16]

Glutamate excitotoxicity also plays a pivotal role in the pathogenesis of stroke. Therefore, we hypothesized that genetic mutations conferring cerebral hyperexcitability and migraine susceptibility increase the vulnerability to ischemic stroke, as 1 mechanism to explain the migraine-stroke association. We tested this hypothesis using $Ca_V2.1$ S218L and R192Q transgenic mouse models of FHM1. The results reveal electrophysiological and hemodynamic mechanisms that accelerate hyperacute stroke evolution and worsen ischemic outcome in FHM1 mutants and suggest a pivotal role for enhanced glutamatergic transmission in increasing the vulnerability to ischemic stroke in susceptible migraineurs.

METHODS

Experimental Animals. Experimental procedures were approved by the institutional review boards. A total of 267 male and female mice were used. Transgenic knock-in *Cacna1a* migraine mouse models homozygous (HOM) or heterozygous (HET) for

R192Q or S218L FHM1 mutations were generated by a gene targeting approach.[14] The R192Q mutant strain was compared with C57BL6/J, backcrossed for 10 generations. The S218L mutants were compared with their wild-type (WT) littermates. Because stroke risk is highest in young adult migraineurs, mice were studied between 2 and 6 months of age. All experiments were carried out by blinded investigators, and confirmatory genotyping was done.

Systemic Physiological Monitoring. Arterial pH, PO_2, PCO_2, and blood pressure were measured via a femoral artery catheter under isoflurane anesthesia (2.5% induction, 1.5% maintenance, in 70% N_2O and 30% O_2; Supplemental Table 1). Rectal temperature was controlled at 37°C during ischemia, and intermittent monitoring was continued for 6 hours in a subset of mice.

Transient Filament Occlusion of the Middle Cerebral Artery. A nylon monofilament was inserted into the internal carotid artery via the external carotid artery followed by reperfusion after 30 or 60 minutes under isoflurane anesthesia (2.5% induction, 1.5% maintenance, in 70% N_2O and 30% O_2) and laser Doppler monitoring. Mice were placed in a temperature-controlled incubator with easy access to food and water. Neurological outcomes were scored 24 hours after reperfusion with a 5-point scale: 0, normal; 1, forepaw monoparesis; 2, circling to left; 3, falling to left; 4, no spontaneous walking and depressed consciousness; and 5, death. Infarct volume was calculated by integrating the infarct area in ten 1-mm-thick 2,3,5-triphenyltetrazolium chloride (TTC)-stained coronal sections. Infarct volumes were calculated by subtracting the volume of ipsilateral noninfarcted tissue from the contralateral hemisphere. Ischemic swelling volumes were calculated by subtracting the volume of contralateral hemisphere from the volume of ipsilateral hemisphere. Glutamatergic mechanisms were tested by administering MK-801 (1 mg/kg IP; Sigma, St Louis, MO) 15 minutes before filament occlusion of the middle cerebral artery (fMCAO).

Magnetic Resonance Imaging. Apparent diffusion coefficient maps were acquired under isoflurane anesthesia with a 9.4-T magnetic resonance imaging (MRI) scanner (Bruker Biospin, Inc, Billerica, MA) 30 and 60 minutes after fMCAO (repetition time/echo time, 3000/27 milliseconds; b, 154 and 1294 s/mm^2; in-plane resolution, 180 x 180 μm^2; slice thickness, 1 mm; number of averaging, 8). Means and SDs of the apparent diffusion coefficient in the cortex, striatum, hippocampus, and thalamus were extracted from the normal hemisphere, and thresholds were defined as mean minus 2 SDs to calculate lesion volumes. Normal systemic physiological parameters were confirmed under simulated MRI conditions in a separate group of mice (not shown).

Electrophysiological Recordings. After fMCAO, isoflurane-anesthetized mice were intubated and ventilated, and the femoral artery was catheterized for blood pressure

and blood gas monitoring. Two intracortical glass micropipettes were placed, and extracellular recordings (depth, 250 μm) were started within 15 minutes after the onset of ischemia and continued for ≈2 hours.

Receptor Autoradiography. The density and distribution of glutamate and GABAA receptors and glutamate reuptake sites were assessed on 10-μm frozen sections with tritium-sensitive storage phosphor screens (GE Healthcare) as described.[17]

Laser Speckle Flowmetry. Spontaneously breathing mice (S218L HOM) were anesthetized with isoflurane as above, and the femoral artery was catheterized for blood pressure and gas measurements. Mice were placed in a stereotaxic frame; a temporal burr hole (2-mm diameter) was drilled above the zygomatic arch; and the distal middle cerebral artery was occluded with a microvascular clip for 60 minutes. Cortical perfusion was imaged during distal middle cerebral artery occlusion with laser speckle flowmetry through intact skull.[18] Cerebral blood flow (CBF) changes were calculated for each pixel relative to the preischemic baseline, and the area of cortex with residual CBF ≤30% was determined by thresholding. Neurological outcomes and infarcts were assessed 48 hours later as described above. In addition, the CBF threshold for tissue viability was estimated by superimposing the images of CBF and infarct. In the R192Q HOM strain, mice were intubated and ventilated to ensure normal arterial blood gas values, precluding survival for neurological and infarct assessment in this strain.

Anatomic Analysis of the Circle of Willis and Pial Collaterals. Mice were transcardially perfused with carbon black. The diameter of cerebral arteries, patency of the posterior communicating artery, and number of pial arterial anastomoses between the anterior, posterior, and middle cerebral arteries and their distance from midline were determined.

Absolute Resting CBF. Mice were anesthetized with α-chloralose (50 mg/kg) and ventilated. The femoral artery and external jugular vein were cannulated. Arterial blood was withdrawn continuously (0.3 mL/min). *N*-isopropyl-[methyl-1,3-^{14}C]-p-iodoamphetamine (1 μCi) was injected in 0.1 mL saline over 10 seconds. Twenty seconds after injection, the animal was decapitated, and the blood withdrawal was terminated simultaneously. The brain was removed, frozen, and dissected. CBF was calculated from the radioactivity in tissue and blood measured by liquid scintillation spectrometry.

Statistical Analysis. Data were analyzed with SPSS (version 11.0) and are presented as mean ± SE or median and interquartile range. We used the χ^2 test to compare proportions, ANOVA to compare mean values of continuous measures according to genotypes, and general linear models for repeated measures to compare mean values over time according to genotypes. Comparisons of disability scores were performed

by the use of nonparametric rank tests; cumulative peri-infarct depolarization (PID) incidence was compared by the log-rank test; and PID frequency versus the area of CBF deficit was assessed by the Pearson correlation test. *P* values are 2 tailed, and values of $P<0.05$ were considered statistically significant.

RESULTS

Enhanced Susceptibility to Anoxic and Peri-Infarct Depolarizations. Anoxic depolarization is characterized by a sudden loss of membrane ionic gradients, uncontrolled glutamate release, and cell swelling, triggered by the failure of Na^+/K^+ ATPase under ischemic conditions. We found significantly earlier onset of anoxic depolarization in FHM1 mutants after fMCAO by monitoring its vasoconstrictive effect on cerebral vasculature as previously described (Figure 1A and 1B).[18,19] Importantly, the magnitude of CBF reduction in the ischemic core did not differ among groups in this fMCAO model, eliminating the possibility that faster anoxic depolarization rates were due to more severe ischemia (residual CBF, 10% to 17% of baseline in both S218L and R192Q; $P=0.634$ and 0.599, respectively; data not shown).

PIDs are recurrent propagating depolarization waves akin to spreading depression that exacerbate the metabolic mismatch in penumbra and promote infarct growth during hyperacute stroke.[18-21] We reasoned that FHM1 mutations that enhance spreading depression susceptibility[13] might also facilitate the occurrence of PIDs. Using intracortical microelectrode recordings during fMCAO, we indeed found a 2-fold increase in the frequency of PIDs in mutants over WT (5.3±1.3 versus 2.6±0.3 PIDs per hour; $P=0.028$; Figure 1C–1F). In the mutants, PIDs sometimes occurred in clusters, possibly reflecting circling around the ischemic core (see below),[22] but otherwise did not differ from WT in terms of durations and amplitudes (not shown). Together, these data suggest that genetically enhanced susceptibility to spreading depression[11-14] facilitates the occurrence of anoxic depolarization and PIDs during acute stroke as a novel mechanism to explain increased stroke vulnerability in migraineurs.

Rapid Growth of Hyperacute Ischemic Core on MRI. To assess whether enhanced susceptibility to anoxic depolarization and PIDs accelerates the hyperacute stroke evolution in FHM1 mutants, we performed serial diffusion-weighted MRI during fMCAO. Reduced apparent diffusion coefficient values on diffusion-weighted MRI reflect anoxic depolarization, loss of transmembrane ionic gradients, and cell swelling (ie, ischemic core). We found that the apparent diffusion coefficient lesion volumes expanded more rapidly in FHM1 mutant strains compared with WT controls (Figure 2). Although larger apparent diffusion coefficient lesion volumes were due primarily to more severe cortical involvement, the hyperacute lesion also encompassed the hippocampus and thalamus in S218L mutants.

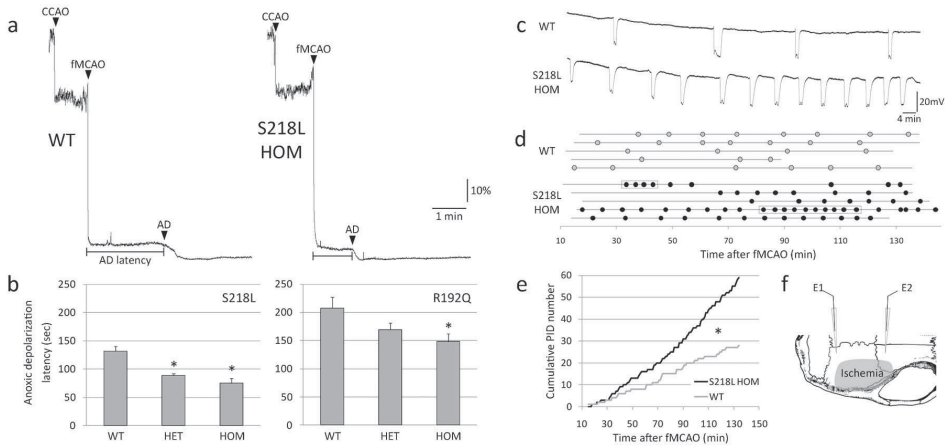


Figure 1. Faster anoxic depolarization (AD) rates and more frequent peri-infarct depolarizations (PIDs) in FHM1 mutant mice. (**A**) Representative laser Doppler tracings show cerebral blood flow (CBF) reduction on common carotid artery occlusion (CCAO) followed by filament occlusion of the middle cerebral artery (fMCAO). A further decline in CBF marks the onset of AD and is due to the vasoconstrictive effect of tissue depolarization on ischemic microvasculature.[18] Scale bars: vertical, 10%; horizontal, 1 minute. (**B**) The latency to AD was shorter in S218L and R192Q mutants ($P<0.001$ and $P=0.035$, respectively), with a trend for an allele-dosage effect. *$P<0.05$ vs wild type (WT). (**C**) Representative electrophysiological tracings show more frequent PIDs in S218L homozygous (HOM) vs WT. Scale bars: vertical, 20 mV; horizontal, 4 minutes. (**D**) PIDs (round symbols shown as a function of time) occurred in S218L HOM mice more frequently and sometimes in clusters (rectangular boxes). Horizontal lines indicate the time of onset and end of electrophysiological recordings in each mouse (n=5 each). When the average PID frequency was calculated, these minor differences in recording duration were taken into account. (**E**) Pooled cumulative PID numbers as a function of time after fMCAO was more than doubled in S218L HOM mice (*$P<0.001$; n=5 each). (**F**) Experimental setup showing 2 intracortical glass micropipettes (E1, E2) placed outside the ischemic territory to detect PIDs after fMCAO. Shaded area indicates typical distribution of CBF deficit after fMCAO. HET indicates heterozygous.

Larger Perfusion Deficit During Hyperacute Stroke. Ischemic depolarizations compromise residual CBF within the territory supplied by the occluded artery via vasoconstrictive (ie, inverse) neurovascular coupling[18,19] as a major determinant of outcome in cerebral ischemia. Using laser speckle flowmetry, we found larger cortical perfusion deficits after distal middle cerebral artery occlusion in FHM1 mutants (Figure 3A and 3B; only S218L shown), associated with an increased frequency of PIDs (0.9±0.3 versus 4.6±1.2 PIDs per hour in WT and S218L HOM, respectively; Figure 3C) that circled around the hypoperfused core in 38% of S218L mutants but not in the WT (movies I and II in the online-only Data Supplement).[22] In fact, higher PID frequencies were associated with larger cortical CBF deficits (Figure 3D), bigger infarcts (Figure 3E and 3F), and worse neurological outcomes (deficit score, 1 [interquartile range, 1–1] versus 0 [interquartile range, 0–0.25] in S218L HOM and WT mice, respectively; $P=0.019$). These data suggest that ischemic depolarizations adversely influence the

perfusion deficits and exacerbate the metabolic and O2 supply-demand mismatch in FHM1 mutants, in part via vasoconstrictive (ie, inverse) neurovascular coupling as an additional hemodynamic mechanism for infarct growth.[18,19,23]

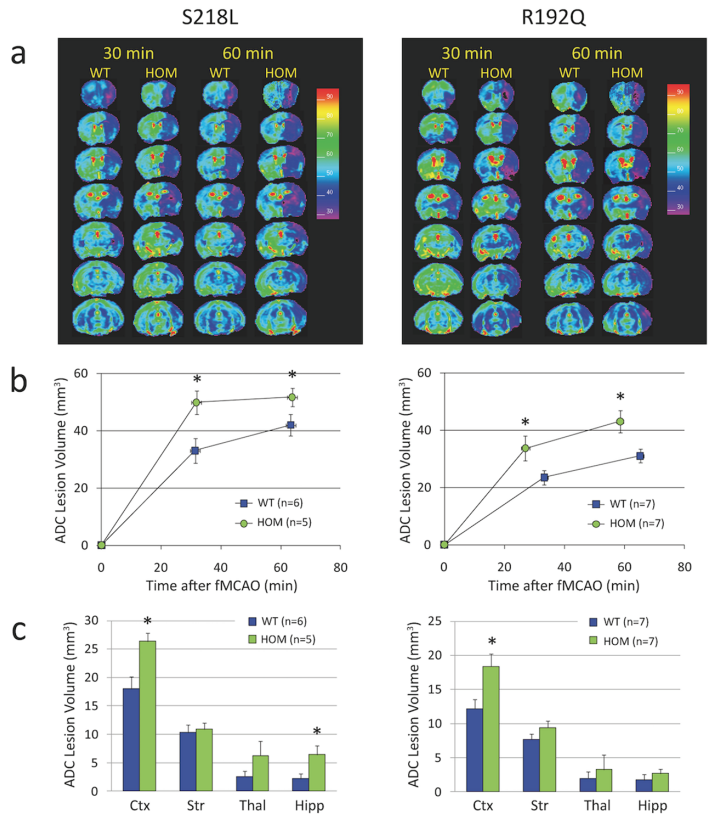


Figure 2. FHM1 mutant mice show accelerated lesion growth on magnetic resonance imaging (MRI) during hyperacute stroke. (**A**) Apparent diffusion coefficient (ADC) lesion (ie, ischemic core with restricted water diffusion, purple) was larger on diffusion-weighted MRIs in S218L (left) and R192Q (right) mutants vs wild type (WT) during the hyperacute phase after filament occlusion of the middle cerebral artery (fMCAO). (**B**) ADC lesion volumes shown as a function of time after fMCAO were 40% to 50% larger in S218L and R192Q homozygous (HOM) vs WT as early as 30 minutes after fMCAO, suggesting faster growth of the ischemic core (P=0.019 and P=0.018, respectively). The difference remained significant at 60 minutes. Vertical and horizontal error bars reflect the SEs for total ADC lesion volume and timing of MRI scans, respectively. (**C**) Thirty minutes after stroke onset, enlarged ADC lesion volumes in S218L HOM and R192Q HOM were due primarily to more severe cortical involvement (P=0.015 and P=0.023, respectively), although S218L HOM mutants also showed hyperacute ADC changes in the hippocampus and thalamus (P=0.018 and P=0.117, respectively). Of note, the average regional ADC values in the center of ischemic core did not significantly differ between FHM1 mutants and their WT controls, suggesting that cytotoxic cell swelling is complete in ischemic core in all groups (60±7% versus 56±6% in R192Q WT and HOM, and 57±5% versus 57±6% of contralateral hemisphere in S218L WT and HOM, respectively, 60 minutes after stroke onset). *P<0.05 vs WT. Ctx, cortex; Str, striatum; Thal, thalamus; Hipp, hippocampus.

Importantly, we found no difference in absolute resting CBF values between WT and R192Q HOM mice in the cortex, striatum, and cerebellum using the [^{14}C] iodoamphetamine method (Supplemental Table 2), indicating that differences in

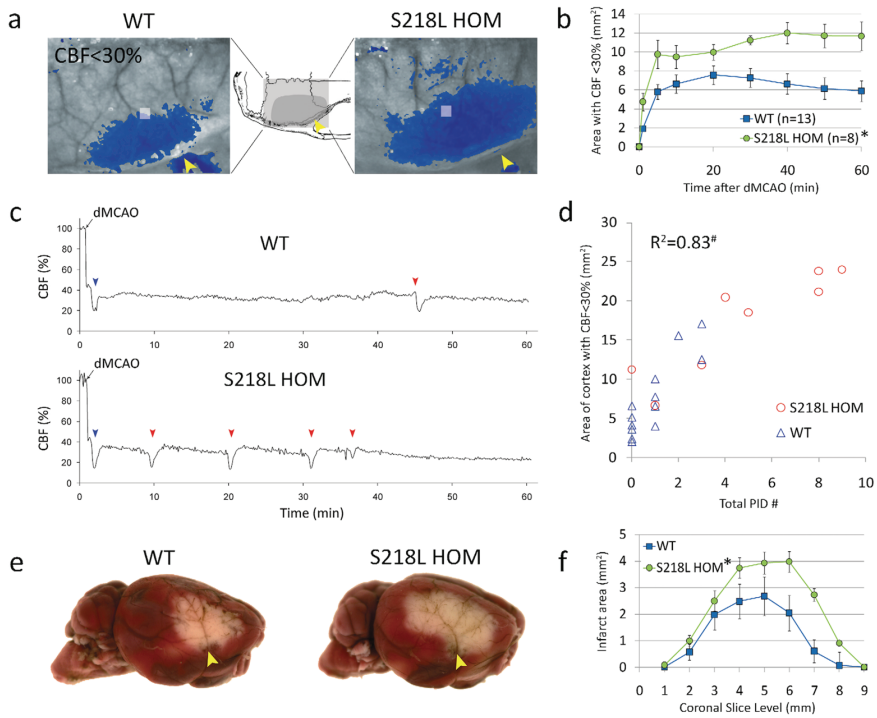


Figure 3. FHM1 mutant mice develop larger areas of cerebral blood flow (CBF) deficit during distal middle cerebral artery occlusion (dMCAO) because of increased susceptibility to ischemic depolarizations. (**A**) Representative laser speckle contrast images show the area of cortex with ≤30% residual CBF vs preischemic baseline (blue pixels) 60 minutes after dMCAO in wild-type (WT) and S218L homozygous (HOM) mice. Similar data were obtained with the R192Q strain (n=6 mutant and 6 WT; data not shown). Imaging was performed over the right hemisphere (light gray–shaded rectangle in the inset) through intact skull. Arrowheads indicate clip occlusion. (**B**) The area of CBF deficit expanded rapidly in S218L HOM throughout the 60-minute dMCAO (*P=0.004). (**C**) Representative tracings show cortical blood flow reductions in penumbra (measured within the gray squares shown in A) after dMCAO. Anoxic depolarization triggers the first peri-infarct depolarization (PID; blue arrowheads), marking a second abrupt reduction in perfusion. Each subsequent PID (red arrowheads) causes a characteristic blood flow transient. (**D**) The frequency of PIDs was higher in S218L HOM and correlated with the area of hypoperfused cortex 60 minutes after dMCAO (#P<0.001). Each symbol represents the PID frequency in individual mice. (**E**) Representative 2,3,5-triphenyltetrazolium chloride–stained whole brains show enlarged infarcts in S218L HOM 48 hours after 60 minutes of dMCAO. (**F**) The area of infarcts in 1-mm-thick coronal slices (0=anterior, 9=posterior) were larger in S218L HOM vs WT (*P=0.016, S218L HOM vs WT for infarct areas). Integrated total infarct volumes were also larger in the mutants (19±3 versus 11±2 mm^3, respectively; P=0.008).

preischemic resting CBF did not influence our measurements. We also confirmed this in WT and S218L HOM mice under isoflurane anesthesia using the correlation time values obtained by laser speckle imaging, which allow direct comparison of resting CBF among groups of mice (data not shown).[24,25] Moreover, the incidence of incomplete circle of Willis, the diameter of its major branches, and the number and location of pial arterial anastomoses did not differ between WT and S218L HOM mice, suggesting that developmental differences in cerebrovascular anatomy did not contribute to worse perfusion deficits in the mutant mice (Supplemental Figure 1 and Supplemental Table 3).

Higher CBF Threshold for Tissue Survival. To determine the critical tissue perfusion level below which infarction ensued (ie, viability threshold), we calculated the regional CBF at the infarct margin by spatially coregistering the laser speckle perfusion map during distal middle cerebral artery occlusion with the infarct that developed 48 hours later (Figure 4). We found that cortical tissue in S218L HOM mutants required a higher CBF level for survival compared with WT mice (42±3% versus 35±2% of baseline CBF, respectively; P=0.048). These data underscore the importance of parenchymal mechanisms such as neuronal hyperexcitability and ischemic depolarizations as the main cause for increased vulnerability to ischemic stroke in FHM1 mutants independently of the severity of CBF deficit.

Worse Stroke Outcomes. Enhanced susceptibility to anoxic depolarization and PID and accelerated hyperacute infarct growth with more severe CBF deficits translated into worse stroke outcomes in FHM1 mutants. Transient fMCAO for 1 hour produced larger infarcts in both S218L and R192Q mutant mice compared with their WT controls (Figure 5A). Larger infarcts reflected predominantly more severe cortical involvement in both mutants (>70% of total infarct volume); however, the incidence of hippocampal or thalamic infarction also tended to be higher in the S218L mutant (present in 33% of S218L HET mice compared with 13% of WT; P=0.1; data not shown), consistent with a higher incidence of subcortical infarction observed on MRI in this strain (see above). Functional outcomes, assessed with a combined death and neurological disability score as a clinically relevant end point, were worse in mutants compared with WT (Table 1). Indeed, the mortality rate was significantly higher in the S218L mutants, reaching 100% in the HOM within 24 hours after stroke onset (Supplemental Figure 2a). The timing of death after stroke was variable (12±3 hours after stroke onset) and was not associated with overt seizure activity. Immediate postmortem examination revealed 2-fold larger infarcts in S218L HOM compared with WT mice euthanized at the same time point of death of each mutant after 60 minutes of fMCAO (95±15 versus 44±7 mm3, respectively; n=5 and 4; P=0.031), suggesting that selection bias resulting from high mortality in the mutants diminished the strain differences in outcome.

These data were excluded from the overall comparisons among genotypes (Figure 5) because of variable time of death. Ischemic brain swelling tended to be more severe in the mutants in proportion to the actual infarct volume and might have contributed to the high mortality in the S218L mutants.

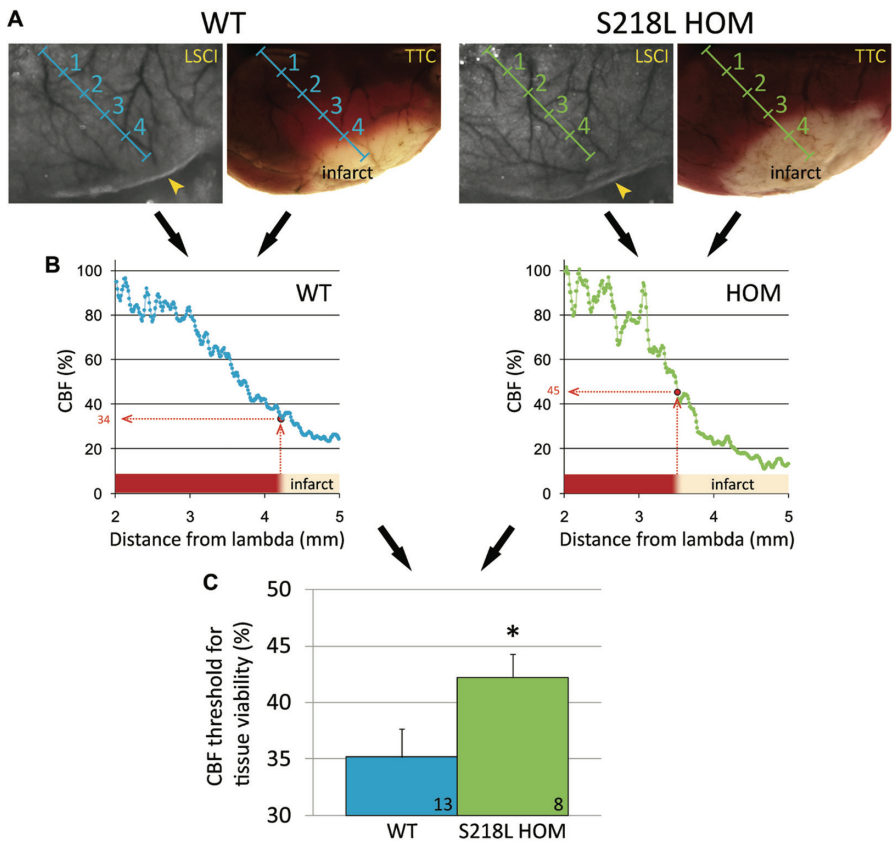


Figure 4. Elevated blood flow threshold for tissue survival in FHM1 mutant mice. (**A**) Representative laser speckle contrast images (LSCI) during distal middle cerebral artery occlusion (dMCAO; left) and 2,3,5-triphenyltetrazolium chloride (TTC)-stained brain showing the infarct 48 hours after 60 minutes of dMCAO (right) are shown for wild-type (WT) and S218L homozygous (HOM) mice. Imaging field was positioned as shown in Figure 3A. Images were spatially coregistered through the use of surface landmarks. Line profiles (blue and green oblique lines, labeled in mm) were drawn between lambda and the clip occluding the middle cerebral artery branch (yellow arrowheads). (**B**) For each animal, cortical blood flow (CBF) was plotted along these line profiles as a function of distance from lambda using laser speckle images, and the blood flow level corresponding to the infarct edge was determined (red dotted lines). This value represented the CBF threshold for viability, below which the tissue infarcted in each mouse. (**C**) The average viability threshold was significantly higher in S218L HOM mutants vs WT controls (P=0.048), indicating that FHM1 mutant brains are more vulnerable to ischemia and require higher blood flow to survive. The numbers of mice are shown on each bar. *P<0.05 vs WT.

	60-min fMCAO*			60-min fMCAO+ MK-801†‡		30-min fMCAO§				
	Male WT	Male HET	Male HOM	Male WT	Male HET	Male WT	Male HET	Male HOM	Female WT	Female HET
n	9	23	8	9	10	9	5	4	6	8
Mortality, %	11	35	100	0	10	0	0	75	0	0
Functional outcome score	2 (2–3)	3 (2–5)	5 (5–5)	2 (1–2)	2 (1–2)	1 (1–2)	2 (1–2)	5 (4–5)	1 (0.3–1)	2 (1–2)

Table 1. Death and Neurological Disability After Transient Filament Occlusion of the Middle Cerebral Artery in S218L Mutant Mice. fMCAO indicates filament occlusion of the middle cerebral artery; WT, wild type; HET, heterozygous; and HOM, homozygous. Functional outcome scores are shown as median (interquartile range); 0 best, 5 worst (see Methods for the scoring system). **P*<0.001 and *P*=0.008 for the effect of genotype on mortality and functional outcome score, respectively. †*P*=0.353 and *P*=0.757 for the effect of genotype on mortality and functional outcome score, respectively. ‡*P*=0.303 and *P*=0.315 for the effect of MK-801 on mortality and functional outcome score, respectively, in WT and *P*=0.022 and *P*=0.009, respectively, in the S218L HET compared with the untreated group. §*P*<0.001 and *P*=0.086 for the effect of genotype on mortality and functional outcome score in males, respectively, and *P*=0.02 for the effect of genotype on functional outcome score in females after 30 minutes of fMCAO. In the R192Q mutant strain, the mortality rate was 17%, 0%, and 14% in WT, HET, and HOM, respectively; neurological disability was not studied in this mutant strain.

To mimic transient ischemic attacks and to circumvent the high mortality rate in S218L HOM mice, we subjected this mutant strain to 30 minutes of fMCAO. With a shorter duration of ischemia, we did not detect overt infarcts in 27% of WT mice using TTC staining, whereas all S218L HET and HOM mutant mice developed conspicuous territorial infarcts (*P*=0.077). Selective ischemic changes in scattered neurons were nevertheless present on histological examination of brains without an overt infarct (not shown). Infarct volumes were once again larger in the S218L mutants compared with WT mice (Figure 5B). The volume of subcortical infarction, limited to the striatum in this shorter ischemia model, was also larger in the S218L HET compared with WT mice (17±4 and 5±1 mm^3, respectively, in males, *P*=0.002; 16±3 and 4±2 mm^3, respectively, in females, *P*=0.013). Despite the shorter ischemia duration, mortality was still high in the S218L HOM mutants (75%), but all HET mutants survived for at least 24 hours and showed a trend for worse functional outcome compared with WT (*P*=0.086; Table 1).

Because classic migraine, sporadic migraine, and FHM are more prevalent in women of reproductive age[5,26-29] and because susceptibility to spreading depression is higher in female FHM1 mutant mice compared with males,[13,30] we also studied female mice and found an even more striking increase in infarct volumes in S218L HET compared with WT (Figure 5B). To assess long-term tissue and neurological outcome (2 weeks) in FHM1 mutants, we subjected female S218L HET and WT mice to 30 minutes of fMCAO. However, we observed >60% mortality in the mutants predominantly between 24 and 96 hours, which precluded outcome comparisons between the mutant and WT strains at this late time point (Supplemental Figure 2b). Together, these data indicate

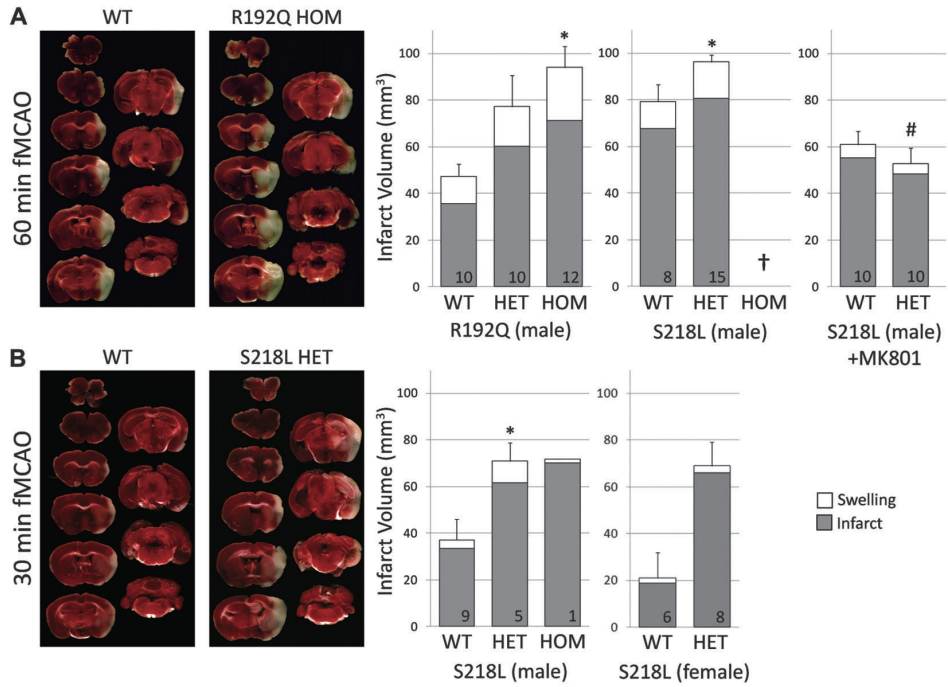


Figure 5. FHM1 mutant mice develop larger infarcts after experimental stroke selectively attenuated by the *N*-methyl-*D*-aspartate receptor antagonist MK-801. Left, Representative infarcts (unstained white tissue) 24 hours after filament occlusion of the middle cerebral artery (fMCAO). Right, Infarct and ischemic swelling volumes (gray and white bars, respectively). (**A**) After 60 minutes of fMCAO, infarcts were larger in both R192Q and S218L mutants vs wild type (WT; *P*=0.007 and *P*=0.019, respectively). One of 9 WT, 8 of 23 S218L heterozygous (HET), and all 8 S218L homozygous (HOM; †) mice died within 24 hours (Table 1) and were excluded from infarct volume analysis. Because genetic backgrounds and infarct volumes differed significantly between WT controls of the 2 mutant strains, we did not directly compare S218L and R192Q mutants in this study. MK-801 (1 mg/kg IP 15 minutes before fMCAO) significantly reduced infarct volume in the S218L HET mutants (*P*<0.001 vs untreated S218L HET shown in A) but not in the WT (*P*=0.061 vs untreated WT shown in A). Therefore, MK-801 was more efficacious in FHM1 mutants (*P*=0.026 for infarct reduction by MK-801 between WT and FHM1 mutants). As a result, after MK-801, infarct and swelling volumes were comparable between WT and S218L HET mice (*P*=0.367), as were neurological outcomes (Table 1). (**B**) Thirty minutes of fMCAO also resulted in larger infarcts in male and female S218L mutants vs WT (*P*=0.028). Despite shorter ischemia, 3 of 4 S218L HOM mutants died within 24 hours and were again excluded from infarct volume analysis; the data from the only surviving HOM mutant are shown. There was no mortality in the WT and HET groups after 30 minutes of fMCAO. The numbers of mice are shown on each bar. SE bars and *P* values refer to total volume (ie, infarct plus swelling). **P*<0.05 vs WT, #*P*<0.05 vs untreated S218L HET after 60 minutes of fMCAO.

that mice expressing FHM1 mutations are particularly susceptible to infarction when challenged by cerebral arterial occlusion.

Enhanced Neuroprotective Efficacy of Glutamate Receptor Antagonist MK-801. FHM1 mutations enhance glutamate release,[11] and glutamate plays a pivotal role in spreading depression and ischemic depolarizations, as well as in excitotoxic cell death, mainly via the *N*-methyl-*D*-aspartate (NMDA) subtype of receptors. Therefore, we tested whether enhanced glutamatergic activity in FHM1 mutants is responsible for their vulnerability to infarction. Preischemic treatment with the NMDA receptor inhibitor MK-801 abolished the differences in stroke phenotype between genotypes. MK-801 reduced infarct volume by 45% in S218L HET compared with only 23% in WT (Figure 5A), and functional outcome was improved only in S218L mutants (Table 1).

As an important control, we also examined the density of glutamate and $GABA_A$ binding sites in the FHM1 mutant and WT mice using quantitative in vitro autoradiography and did not find overt differences (Supplemental Figure 3 and Supplemental Table 4). These data are consistent with recent proteomics analysis of cortical synapses in this mutant[31] and suggest that enhanced susceptibility to ischemic depolarizations in FHM1 mutants is unlikely to reflect changes in neurotransmitter receptors and reuptake mechanisms.

DISCUSSION

Our data provide a novel mechanism to explain the higher incidence of ischemic stroke in migraineurs. Two genetic mouse models expressing FHM1 mutations were at risk of developing large infarcts and worse neurological outcomes after transient focal cerebral ischemia. Consistent with the higher stroke risk in women compared with men with migraine with aura, we found more striking increases in infarct volume in female mutants compared with males. Faster anoxic depolarization rates, more frequent PIDs, and enhanced neuroprotective efficacy of the NMDA antagonist MK-801 in the mutants implicated glutamatergic neuronal hyperexcitability as 1 mechanism, and larger perfusion defects linked to ischemic depolarizations implicated vasoconstrictive neurovascular coupling as another. Therefore, neuronal and vascular mechanisms together render migraineurs more vulnerable to cerebral infarction on ischemia.

The data have clinical implications. In susceptible migraineurs, increased sensitivity to ischemia may predispose to strokes during mild ischemic events, which remain clinically silent or manifest only as transient ischemic attacks in nonmigraineurs. Moreover, elevated CBF threshold for viability,[32] a sign of increased vulnerability to ischemia, may promote rapid infarct expansion into tissue with milder perfusion deficits, diminish salvageable tissue at risk (ie, ischemic penumbra), and shorten the therapeutic window of acute stroke interventions in migraineurs. Lastly, higher

mortality among the S218L mutants may have implications for malignant infarcts, large supratentorial strokes characterized by progressive loss of consciousness over 48 hours with up to 80% mortality if untreated.[33] To date, there has been no reliable predictor for malignant infarction, and history of migraine with aura (or its genetic determinants) may be 1 such marker increasing the risk of stroke progression and, in case of large territorial infarcts, the risk of death, as has recently been reported for hemorrhagic stroke in migraineurs.[34]

Mechanisms of Ischemic Vulnerability in FHM1 Mice. Biological mechanisms underlying the association between migraine and stroke are unknown, although in both diseases dynamic interactions among the constituents of the neurovascular unit are important to the pathophysiology. At the onset of cerebral ischemia, the gradual failure of Na^+/K^+-ATPase causes a slow rise in extracellular K^+ and loss of neuronal membrane potential until a critical threshold for initiation of anoxic depolarization is reached.[35,36] FHM1 mutations shift the $Ca_V2.1$ channel opening voltage to more negative membrane potentials so that channels open with smaller depolarizations, triggering glutamate release, which can explain faster anoxic depolarization onset in the mutants. Glutamate is also critical for PIDs, which are spreading depolarization waves triggered in ischemic penumbra. Therefore, enhanced release in ischemic penumbra can also explain higher PID frequencies in FHM1 mutants.[11] Delayed $Ca_V2.1$ channel inactivation may exacerbate the excitotoxicity by prolonging the Ca^{2+} influx and glutamate release during PIDs in penumbra. Moreover, PIDs exacerbate the metabolic mismatch in penumbra by stimulating O_2 and glucose consumption and by worsening tissue perfusion via vasoconstrictive (ie, inverse) neurovascular coupling,[18,19,23] particularly when they occur with high frequency and in clusters, as observed in FHM1 mutants.[19,37-39] With each PID, more of the penumbra is incorporated into the core, accounting for concentric infarct growth over time.[22,40-42] PIDs occur frequently in human brain after ischemic or hemorrhagic stroke and head trauma and appear to worsen patient outcomes, similar to experimental stroke.[38,43-45] Hence, migraine with aura may be a risk factor for increased occurrence of PIDs and worse outcomes in human stroke, as recently suggested in subarachnoid hemorrhage.[46]

Association Between Migraine and Stroke. Our data in FHM1 mutant mice support shared genetic risk factors enhancing susceptibility to spreading depression as a mechanism to explain the migraine-stroke association. Shared genetic factors enhancing susceptibility to migraine and stroke such as *NOTCH3* mutations in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy have been described.[47-50] Indeed, *NOTCH3* mutations, despite being exclusively expressed in vascular smooth muscle cells, augment susceptibility to spreading depression.[51] Mutations associated with FHM2 and FHM3 are also predicted to

enhance neuronal excitability and spreading depression susceptibility possibly via glutamatergic mechanisms.[52] Glutamatergic mechanisms and hyperexcitability also are implicated in common forms of migraine by recent studies linking *AEG1*, encoding a regulator of glial glutamate transporter EAAT2, and *KCNK18*, encoding the TRESK potassium channel, to migraine.[15,16] Vascular mechanisms (eg, endothelial dysfunction) have also been implicated in increasing stroke risk in migraineurs.[53,54] Indeed, functional $Ca_v2.1$ channel expression has been reported in renovascular smooth muscle cells,[55,56] but whether FHM1 mutations alter cerebrovascular physiology is not known. Together with parenchymal mechanisms that enhance vulnerability to perfusion deficits, vascular mechanisms might further augment stroke risk in migraineurs. For example, highly focal and mild ischemic vascular events such as microembolism[57] may trigger spreading depression more readily in migraineurs highly susceptible to ischemic depolarizations, providing a possible explanation for the origin of a subset of migraine auras.

Conclusions. A monogenic determinant of migraine with aura increases stroke vulnerability via glutamatergic mechanisms that enhance susceptibility to ischemic depolarizations akin to spreading depression and accelerate stroke evolution. Hence, our data put FHM1 mutations among the shared genetic determinants of migraine with aura and stroke. More work is needed to extrapolate these data to other monogenic syndromes and to the more common and genetically more complex forms of migraine with aura and to determine whether targeting hyperexcitability and spreading depression such as migraine prophylaxis[58] confers ischemic protection in susceptible mouse strains or in migraineurs.

ACKNOWLEDGMENTS

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SUPPLEMENTARY MATERIAL

		N	Age (mo)	Weight (g)	BP (mmHg)	Arterial pH	Arterial pCO_2 (mmHg)	Arterial pO_2 (mmHg)
R192Q	WT	11	3.8±0.2	27±1	84±2	7.36±0.01	33±1	147±4
	HOM	16	4.5±0.4	27±1	90±2	7.36±0.01	36±4	136±1
S218L	WT	23	4.4±0.2	27±1	89±3	7.36±0.01	37±1	129±12
	HOM	20	3.9±0.2	26±1	97±3	7.38±0.01	35±1	127±6

Supplemental Table 1. Age, body weight and systemic physiological parameters. There was no statistically significant difference between WT and HOM physiology was measured via a femoral artery catheter in non-survival experiments only, to minimize morbidity in survival groups.

	WT	R192Q HOM	p
Cortex	130 ± 28	130 ± 17	0.993
Striatum	139 ± 29	146 ± 16	0.649
Cerebellum	150 ± 24	162 ± 15	0.344

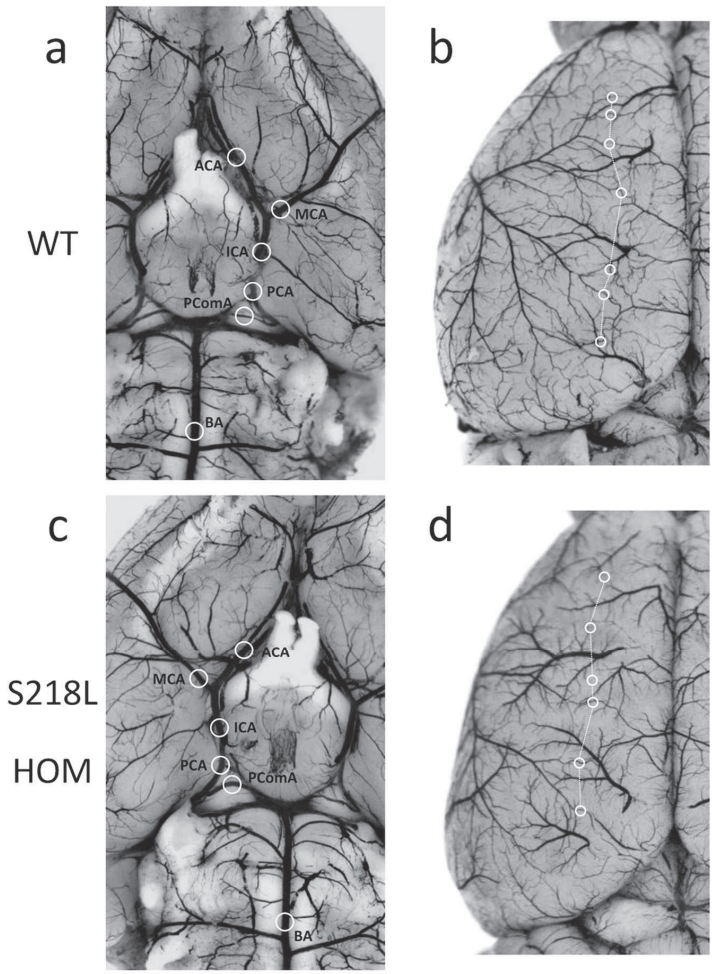
Supplemental Table 2. Resting cerebral blood flow. Values are absolute blood flow in ml/100g/min determined by [^{14}C]-iodoamphetamine radioactive tracer method. N=5 each genotype.

	WT (n=7)	S218L HOM (n=7)	p
ICA diameter (μm)	132 ± 5	128 ± 2	0.487
MCA diameter (μm)	111 ± 5	103 ± 3	0.230
ACA diameter (μm)	113 ± 5	105 ± 6	0.338
PCA diameter (μm)	115 ± 4	111 ± 2	0.391
BA diameter (μm)	163 ± 5	160 ± 7	0.707
PComA diameter (μm)	31 ± 3	29 ± 7	0.806
Number of mice with unilaterally absent PComA (n)	1	3	0.237
Number of pial anastomoses between MCA and ACA (n)	6.9 ± 0.9	6.6 ± 1.0	0.692
Anastomoses distance from midline (mm)			
Rostral 1/3 of cortex	1.6 ± 0.1	1.5 ± 0.1	0.235
Middle 1/3 of cortex	1.8 ± 0.1	1.9 ± 0.1	0.383
Caudal 1/3 of cortex	2.0 ± 0.1	2.1 ± 0.2	0.149

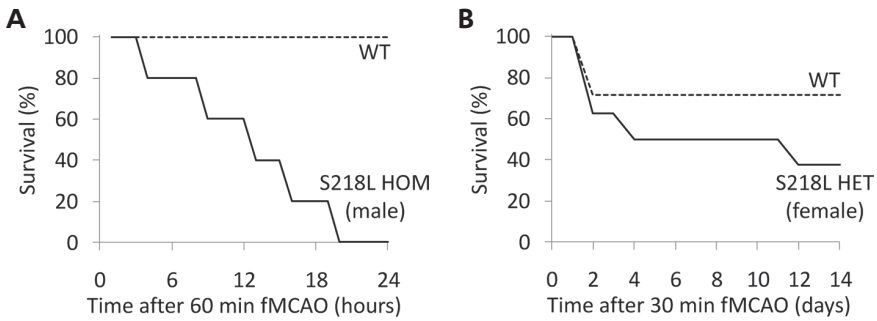
Supplemental Table 3. Cerebrovascular anatomy. Data were obtained using intracardiac carbon black infusion. Please also see Supplementary Figure 1 for representative images, abbreviations and the method of measurements.

	EAAT		AMPA		KA		NMDA		$GABA_A$	
$x10^{-3}$	WT	R192Q HOM	WT	R192Q HOM	WT	R192Q HOM	WT	R192Q HOM	WT	R192Q HOM
Cortex	85 ± 7	60 ± 5	279 ± 14	265 ± 20	157 ± 14	139 ± 7	204 ± 8	207 ± 4	145 ± 6	133 ± 9
Striatum	83 ± 6	75 ± 8	274 ± 18	271 ± 17	163 ± 11	146 ± 6	127 ± 9	128 ± 10	112 ± 6	98 ± 15
Hippocampus	134 ± 12	133 ± 13	691 ± 114	764 ± 95	94 ± 6	84 ± 5	429 ± 33	365 ± 20	127 ± 6	127 ± 10

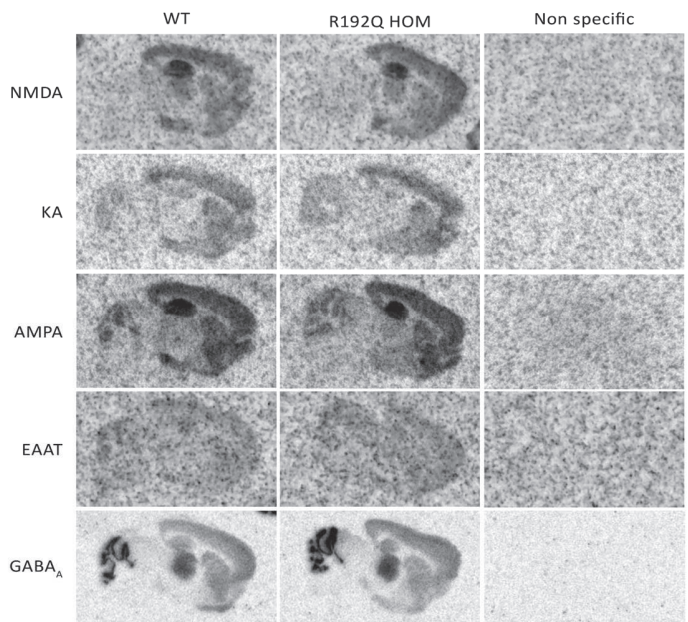
Supplemental Table 4. Quantitative receptor autoradiography. Values indicate relative optical densities ($x10^{-3}$). Although ligand binding differed among regions, we did not detect a statistically significant effect of genotype on ligand binding within cortex (visual, somatosensory, and parietal cortex), hippocampus (CA1 region) and striatum (N=8 each). The ligands were: D-[2,3-^{3}H]Aspartic acid (GE Life Sciences, Piscataway, NJ) for, excitatory amino acid transporters (EAAT); DL-[5-methyl-^{3}H]-(AMPA) (Perkin-Elmer, Boston) for α-amino-3-Hydroxy-5-methylisoxazole-4-propionic acid (AMPA) ionotropic glutamate receptors; [^{3}H]Kainic Acid for ionotropic glutamate kainic acid receptors; [^{3}H]MK-801 (American Radiolabeled Chemicals, St-Louis, MO) for the N-methyl-D-aspartate (NMDA) subtype of ionotropic glutamate receptors; [^{3}H]muscimol (Perkin-Elmer, Boston) for ionotropic $GABA_A$ receptors.



Supplemental Figure 1. Normal cerebrovascular anatomy in FHM1 mutant mice. Representative ventral (**A, C**) and dorsal (**B, D**) views of representative WT and S218L HOM brains show the circle of Willis anatomy, and pial arterial anastomoses between middle and anterior cerebral arteries, respectively, after transcardiac ink perfusion (1.4 ml, 0.5 ml/sec) under deep isoflurane anesthesia. Circles on the ventral surface (**A, C**) indicate where arterial diameters were measured, whereas circles on the dorsal surface (**B, D**) indicate the pial anatomoses (inset) that have been analyzed for their number and distance to midline. None of these endpoints significantly differed between WT and S218L HOM mice (Supplemental Table 3). Pial anastomoses were defined as the narrowest part of the vessel or half way between the nearest branch points of the anterior and the middle cerebral artery, localized by tracing the peripheral branches of these major vessels. ACA, anterior cerebral artery; MCA, middle cerebral artery; ICA, internal carotid artery; PCA, posterior cerebral artery; PComA, posterior communicating artery; BA, basilar artery.



Supplemental Figure 2. Increased mortality after stroke in FHM1 mutant mice. Survival curves show the timing of mortality after filament middle cerebral artery occlusion (fMCAO). Mortality rate was (**A**) 100% and 0% within 24h after 60 min fMCAO in male S218L HOM and WT mice, respectively (n=5 and 4), and (**B**) 62% and 30% within 2 weeks after 30 min fMCAO in female S218L HET and WT mice, respectively (n=8 and 7).



Supplemental Figure 3. Normal density of Glutamate and GABAA binding sites in FHM1 mutant mice. Representative autoradiograms on sagittal brain sections from WT and R192Q HOM mice showing specific binding for five commonly used ligands for ionotropic glutamate (N-methyl-D-aspartate, NMDA; α-amino-3-hydroxyl-5-methyl-4-isoxazole-propionate, AMPA; kainic acid, KA) and $GABA_A$ receptors and glutamate reuptake (EAAT) sites. Darker areas represent brain regions with higher levels of bound radioligand. Nonspecific binding, also shown, was not significantly different from background levels. Quantitative data are shown in Supplemental Table 4.

Supplemental Movies

(http://circ.ahajournals.org/content/suppl/2011/12/05/CIRCULATIONAHA.111.045096.DC1)

1. **Cerebral blood flow changes during distal MCAO in WT mice.** This representative movie of CBF shows the spatiotemporal hemodynamic changes upon distal MCAO by a microvascular clip through a temporal burr hole. Imaging field was positioned as shown in Figures 3 and 4 and imaging was performed through intact skull. Color bar shows CBF as % of pre-ischemic baseline. Time after imaging onset is shown on the top. MCA is clipped between 1 and 2 min (clip artifact visible in the lower right of the imaging field). Approximately 1-2 min after clipping, a wave of further decrease in perfusion originates from ischemic core and spreads throughout the ipsilateral hemisphere; this wave has previously been shown to correspond to anoxic depolarization. During the 60 min dMCAO, 1 distinct wave of spreading hypoperfusion is spontaneously triggered in the WT; this hypoperfusion transient corresponds to a peri-infarct depolarization. Reperfusion is achieved by removing the clip approximately 60 min after the onset of ischemia.
2. **Cerebral blood flow changes during distal MCAO in S218L HOM mutant mice.** This representative movie of CBF shows the spatiotemporal hemodynamic changes upon distal MCAO by a microvascular clip through a temporal burr hole, in S218L HOM mutant mice. Please see legend to Movie 1 for imaging details. During the 60 min dMCAO, 8 distinct waves of spreading hypoperfusion events are spontaneously triggered in the S218L HOM mutant; these hypoperfusion transients correspond to peri-infarct depolarizations and cause a lasting decrease in tissue perfusion in their wake.

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CHAPTER 3B

MIGRAINE PROPHYLAXIS, ISCHEMIC DEPOLARIZATIONS, AND STROKE OUTCOMES IN MICE

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ABSTRACT

Background and Purpose: Migraine with aura is an established stroke risk factor, and excitatory mechanisms such as spreading depression (SD) are implicated in the pathogenesis of both migraine and stroke. Spontaneous SD waves originate within the peri-infarct tissue and exacerbate the metabolic mismatch during focal cerebral ischemia. Genetically enhanced SD susceptibility facilitates anoxic depolarizations and peri-infarct SDs and accelerates infarct growth, suggesting that susceptibility to SD is a critical determinant of vulnerability to ischemic injury. Because chronic treatment with migraine prophylactic drugs suppresses SD susceptibility, we tested whether migraine prophylaxis can also suppress ischemic depolarizations and improve stroke outcome.

Methods: We measured the cortical susceptibility to SD and ischemic depolarizations, and determined tissue and neurological outcomes after middle cerebral artery occlusion in wild-type and familial hemiplegic migraine type 1 knock-in mice treated with vehicle, topiramate or lamotrigine daily for 7 weeks or as a single dose shortly before testing.

Results: Chronic treatment with topiramate or lamotrigine reduced the susceptibility to KCl-induced or electric stimulation-induced SDs as well as ischemic depolarizations in both wild-type and familial hemiplegic migraine type 1 mutant mice. Consequently, both tissue and neurological outcomes were improved. Notably, treatment with a single dose of either drug was ineffective.

Conclusions: These data underscore the importance of hyperexcitability as a mechanism for increased stroke risk in migraineurs, and suggest that migraine prophylaxis may not only prevent migraine attacks but also protect migraineurs against ischemic injury.

INTRODUCTION

Migraine is the most common neurological condition, affecting 10% to 20% of the population.[1] Stroke is a major cause of death and disability worldwide. An intriguing association between migraine and stroke is well established. Epidemiological studies identified migraine with aura as an independent factor increasing stroke risk by >2-fold.[2] The relative risk is particularly high in otherwise healthy young adults without cardiovascular risk factors. The prevalence of migraine is on par with that of other known stroke risk factors.

Spreading depression (SD), an intense depolarization that underlies migraine aura, also occurs in peri-infarct tissue as an overlapping mechanism between migraine and stroke. Although SD does not cause injury in the healthy brain, recurrent peri-infarct SDs and peri-infarct depolarizations (PIDs) worsen the metabolic mismatch in ischemic tissue and promote infarct growth during hyperacute stroke both in experimental animals[3-5] and in humans.[6,7]

Indirect evidence implicates enhanced cerebral excitability in common migraine,[8,9] as well as in familial hemiplegic migraine (FHM). FHM1 mutations enhance $Ca_V2.1$ channel open probability, presynaptic calcium influx and cortical glutamate release, and render the brain hyperexcitable.[10] As a result, FHM1 mutations markedly enhance SD susceptibility.[11,12] Underscoring the importance of SD in migraine and stroke, transgenic mice expressing FHM1 mutations exhibit faster onset of anoxic depolarization (AD) and rapid infarct growth linked to higher frequency of PIDs during experimentally induced focal cerebral ischemia.[13]

Chronic treatment with widely prescribed migraine prophylactic drugs of various pharmacological classes dose-dependently suppresses SD susceptibility in rats as a possible mechanism of action.[14] The majority of these drugs, however, are ineffective after a single dose, reminiscent of the delayed onset of action requiring chronic treatment in migraine prophylaxis. We, therefore, examined the efficacy of migraine prophylactic drugs on stroke outcome and its mechanisms in relation to ischemic depolarizations. We chose topiramate as a prophylactic drug because it is efficacious in migraine prophylaxis,[15] and inhibits experimental SD on chronic treatment in rats.[14] We tested lamotrigine because it also inhibits SD on chronic treatment in rats,[16] although its efficacy in migraine is not proven.[17] Both drugs have been studied previously in experimental focal ischemia models without consistent efficacy, albeit as a single dose or as short-term postischemic dosing.[18-22] Therefore, neither drug has been tested as a prophylactic intervention in stroke.

We, therefore, tested these drugs in commonly used experimental models of focal cerebral ischemia, and did this not only in wild-type (WT) but also in FHM1 mutant mice to test drug efficacy on a background of cerebral hyperexcitability modeling migraine. Here, we show that chronic daily treatment for 7 weeks with the migraine

prophylactic drugs topiramate or lamotrigine delays AD, inhibits PID occurrence and improves tissue and neurological outcomes after filament occlusion of the middle cerebral artery in both WT and FHM1 mutant mice. In contrast, single doses of each drug are ineffective, suggesting that the efficacy of migraine prophylactic drugs in stroke corresponds to their efficacy on SD, and that SD susceptibility is a critical but modifiable determinant of vulnerability to ischemic injury.

METHODS

Experimental Animals. All experimental procedures were carried out in accordance with the Guide for Care and Use of Laboratory Animals (National Health Institutes Publication No. 85-23, 1996) and were approved by the institutional review board (Massachusetts General Hospital Subcommittee on Research Animal Care [MGH SRAC]). In addition to C57BL/6J WT mice, transgenic knock-in *Cacna1a* migraine mouse models homozygous for the R192Q FHM1 mutation were used, generated by a gene targeting approach,[11,23] and backcrossed on C57BL/6J background for >10 generations. We studied mice between 2 and 6 months of age (23-30 g) because stroke risk is highest in young adult migraineurs. We studied male mice in stroke experiments to avoid the confounding effects of female hormones on outcome,[24,25] and female mice in SD experiments because of their higher SD susceptibility compared with males,[12] and because migraine is more prevalent in women.

Treatment Paradigm. In the chronic treatment group, we treated mice for 7 weeks with once a day orogastric gavage doses of migraine prophylactic drugs topiramate (80 mg kg^{-1} d^{-1}) or lamotrigine (30 mg kg^{-1} d^{-1}), and compared these with vehicle (Ora plus/Ora sweet); the last daily dose was administered 2 hours before the experiment. In a separate cohort, we tested the efficacy of a single dose of these drugs administered 2 hours before the experiment. We selected the doses based on previously reported efficacy in other experimental models in mice.[26,27] All experiments were performed with the investigators blinded, and confirmatory genotyping was done in mutant cohorts.

Study Design. Study end points were defined a priori. Experiments were performed in 3 stages. First, efficacy of topiramate and lamotrigine was tested on SD susceptibility end points in WT and FHM1 mutant mice. Second, efficacy of both drugs on PID frequency and ischemic outcome was tested in 2 separate cohorts of WT mice. Finally, efficacy of both drugs on ischemic outcome was tested in FHM1 mutant mice. Animals were randomly assigned to the treatment groups for each cohort. A different experimenter blinded to the treatment performed each experimental stage. Experiments were performed according to the intention-to-treat principle; therefore, data points were excluded only if technical failures prevented reliable data collection. Because focal cerebral ischemia experiments in WT and FHM1 mutant mouse cohorts were

separated in time, and performed by different operators using different equipment and experimental setups, we could not perform comparisons of ischemic tissue and neurological outcome end points between WT and FHM1 mutant strains in this study.

Systemic Physiological Monitoring. Arterial pH, pO_2, pCO_2, and blood pressure were measured via a femoral artery catheter under isoflurane anesthesia (2.5% induction, 1.5% maintenance, in 70% N_2O and 30% O_2; Table) and maintained by endotracheal intubation and mechanical ventilation during electrophysiological recordings (ie, SD susceptibility, PID frequency). In 24-hour survival experiments, these interventions were not performed to minimize morbidity and improve survival rates. Rectal temperature was controlled at 37°C.

SD Susceptibility. As described previously,[12] 3 burr holes were drilled under saline cooling at the following coordinates (mm from bregma): 3.5 posterior, 2 lateral (2 mm diameter for electric stimulation and KCl application onto occipital cortex); 1.5 posterior, 2 lateral (1 mm diameter, recording site 1); 0.5 anterior, 2 lateral (1 mm diameter, recording site 2). The dura was kept intact to minimize trauma. Two glass capillary microelectrodes were placed to record extracellular steady (DC) potential and electrocorticogram. Electric SD threshold was determined by escalating intensity cathodal square pulses (10-8000 µC) via a bipolar electrode placed on the occipital cortex, and then a 1-mm cotton ball soaked in 300 mmol/L KCl was topically applied for 1 hour to record the frequency of evoked SDs. The protocol was then repeated on the opposite hemisphere. Data were averaged between the 2 hemispheres to yield a

Experiment	Treatment Duration	Genotype	Drug	Blood Pressure	pH	pCO_2	pO_2
SD	Chronic	WT	Control	92±7	7.41±0.04	28±4	132±18
		WT	Topiramate	98±9	7.34±0.03	30±4	140±12
		WT	Lamotrigine	89±7	7.37±0.04	30±2	113±15
		R192Q	Control	96±5	7.38±0.04	28±4	132±16
		R192Q	Topiramate	88±7	7.33±0.04	27±3	142±11
		R192Q	Lamotrigine	95±8	7.36±0.03	29±2	129±13
	Acute	WT	Control	88±6	7.31±0.04	32±4	134±11
		WT	Topiramate	81±7	7.25±0.02	34±5	153±5
		WT	Lamotrigine	82±4	7.33±0.04	34±5	137±16
PID	Chronic	WT	Control	95±16	7.38±0.03	38±5	122±30
		WT	Topiramate	88±11	7.37±0.05	37±6	131±23
		WT	Lamotrigine	94±10	7.40±0.05	33±6	136±25
	Acute	WT	Control	92±13	7.40±0.04	36±3	116±13
		WT	Topiramate	82±11	7.35±0.07	37±6	129±12
		WT	Lamotrigine	85±10	7.40±0.04	34±6	123±25

Table. Physiological Parameters. Data are displayed as mean±SD. PID indicates peri-infarct depolarization; SD, spreading depression; and WT, wild-type.

single data point per animal. SD frequency and threshold were taken as primary end points. The amplitude, propagation speed (distance/latency between the 2 recording electrodes), and duration at half-amplitude of the first SD in each hemisphere were also measured as secondary end points. There was no technical failure leading to exclusion in this cohort.

Transient Filament Occlusion of the Middle Cerebral Artery. A nylon monofilament was inserted into the internal via the external carotid artery followed by reperfusion after 60 minutes, under isoflurane anesthesia (2.5% induction, 1.5% maintenance, in 70% N_2O and 30% O_2) and laser Doppler monitoring (Perimed, Järfälla, Sweden), as described previously.[13]

PID Occurrence. To record PIDs after transient filament occlusion of the middle cerebral artery (fMCAO), mice were transferred to a stereotaxic frame and two 0.5-mm diameter burr holes were carefully drilled under saline irrigation at the following coordinates (mm from bregma): 1.5 anterior, 0.5 lateral; 3.5 posterior, 0.5 lateral. These coordinates were chosen to be reliably outside the focal ischemic cortex to allow detection of PIDs. Two intracortical glass micropipettes were inserted at a depth of 250 µm, and extracellular slow potential changes were recorded for ≈2 hours starting ≈20 minutes after the onset of fMCAO. PID frequency was taken as a primary end point. Technical failures occurred in WT cohorts only, and led to the exclusion of 1 chronic and 1 single dose vehicle, 1 chronic and 1 single dose topiramate, and 3 single dose lamotrigine-treated mice for PID assessments. Extensive surgery, intubation, mechanical ventilation, and arterial cannulation for PID monitoring precluded 24-hour survival. Therefore, infarct volumes were determined in a separate cohort.

Assessment of Tissue and Neurological Outcome After fMCAO. After reperfusion, mice were transferred to a temperature-controlled incubator with access to food and water ad libitum. Neurological outcomes were scored as a primary end point 24 hours after reperfusion, using a 5-point scale: 0, normal; 1, forepaw monoparesis; 2, circling to left; 3, falling to left; 4, no spontaneous walking and depressed consciousness; and 5, death. Premature death after ischemia was incorporated in the neurological outcome scale because of the intention-to-treat design; however, infarct volume data from these mice were not measured because of postmortem confounders. Infarct volume was calculated by integrating the infarct area in ten 1-mm-thick 2,3,5-triphenyltetrazolium chloride-stained coronal sections. Infarct volume was calculated as a primary end point by subtracting the volume of ipsilateral noninfarcted tissue from contralateral hemisphere. Ischemic swelling volume was also calculated as a secondary end point by subtracting the volume of contralateral hemisphere from the volume of ipsilateral hemisphere. Technical failures occurred in FHM1 cohorts only, and led to the exclusion of 2 chronic vehicle and 1 chronic topiramate-treated mice for tissue and neurological outcome assessments.

Measurement of AD Latency. The latency between fMCAO and AD onset was measured as a secondary end point using the characteristic secondary hypoperfusion caused by AD on laser Doppler tracings, as described in detail previously.[13] We measured this parameter in all WT mice undergoing fMCAO either for PID frequency determination or infarct and neurological outcome assessment. Absence of a detectable secondary hypoperfusion because of technical reasons was taken as an a priori exclusion criterion for this data set. Although this occurred more commonly, it resulted in the exclusion of only 16 of 112 animals in which this secondary end point was studied, distributed relatively evenly among experimental groups.

Statistical Analysis. Data were analyzed using SPSS (v11.0) and GraphPad Prism 6, and presented as whisker-box plot (whiskers, full range; box, 25% to 75% range; line, median; cross, mean) in the figures and mean±SD in the table. Statistical tests used to analyze each data set, group sizes (n) and details of statistical outcomes are provided in the figure legends. *P* values are 2-tailed, and $P<0.05$ was considered statistically significant.

RESULTS

Suppression of KCl-Induced or Electrically Triggered Cortical SD. We have previously shown in rats that migraine prophylactic drugs suppress SD susceptibility.[14] To first test whether migraine prophylactic drugs are also efficacious in mice, we treated WT and FHM1 knock-in mice with chronic daily doses of topiramate or lamotrigine for 7 weeks. Chronic treatment with topiramate or lamotrigine elevated the electrical threshold for SD induction and reduced the frequency of KCl-induced SDs (Figure 1A). Both drugs also reduced the SD propagation speed by ≈30%, albeit only in the FHM1 mutant. In addition, lamotrigine decreased SD duration, and tended to be more efficacious on all SD end points compared with topiramate. A single dose of either drug administered 2 hours before SD, tested in WT mice only, did not affect any of the SD attributes although a trend for lamotrigine to elevate the electrical threshold and reduce KCl-induced SD frequency was noted (Figure 1B).

Suppression of Cortical PIDs During Middle Cerebral Artery Occlusion. We next tested whether migraine prophylactic drugs also suppress PIDs, akin to SD. Intracortical microelectrode recordings during fMCAO showed that chronic treatment with topiramate or lamotrigine reduced PID occurrence by 50% and 80%, respectively (Figure 2A). A single dose of topiramate 2 hours before ischemia onset was ineffective, whereas lamotrigine showed a strong trend (Figure 2B). In a separate cohort of mice, we found that chronic treatment with valproate (200 mg/kg, IP for 6 weeks) also reduced the number of PIDs (3.1±0.6 PIDs/h) compared with vehicle (5.7±0.5 PIDs/h; $P<0.001$, n=5 each), consistent with its inhibitory effect on KCl or electrically induced SDs previously shown in rats,[14] and suggesting a class effect for migraine prophylactic drugs on PIDs.

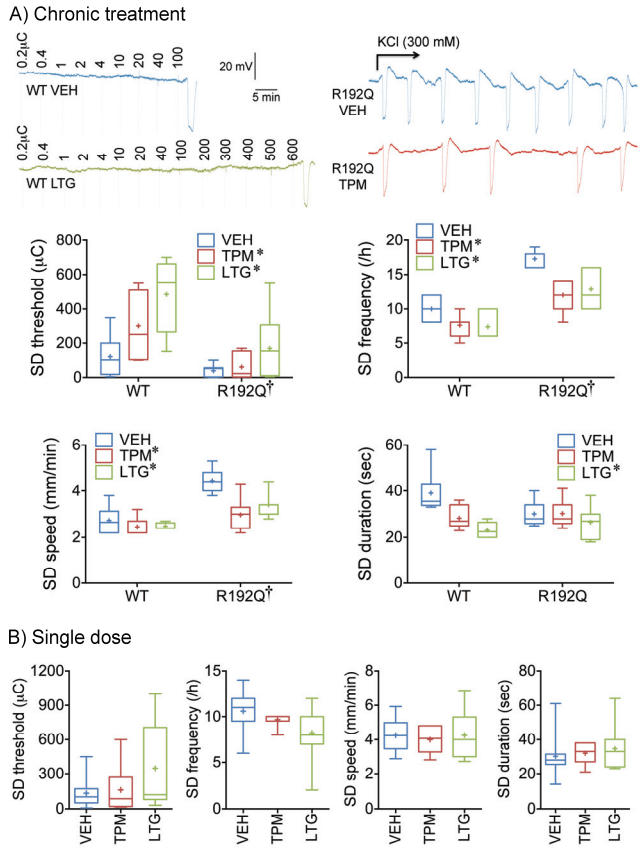


Figure 1. Chronic topiramate and lamotrigine treatment suppresses spreading depression (SD) susceptibility. (**A**) Representative electrophysiological tracings show SD triggered on stepwise escalating cortical cathodal stimulation at intensities indicated above each tracing to determine the SD threshold (**left**), and repetitive SDs triggered by continuous topical KCl application for 1 hour onto the cortex to determine SD frequency (**right**), in wild-type (WT) or familial hemiplegic migraine type 1 (R192Q) mutant mice after 7 weeks of daily treatment with vehicle (VEH, blue), topiramate (TPM, red), or lamotrigine (LTG, green). Whisker-box plots summarize the effects of chronic treatment on SD threshold, frequency, speed, and duration; n=6, 7, and 6 WT mice in vehicle, topiramate and lamotrigine groups, respectively; n=7 R192Q mice in vehicle, topiramate, and lamotrigine groups each. Two-way ANOVA followed by Sidak and Tukey multiple comparisons. SD threshold: genotype effect $F(1,34)=18.8$, $P=0.0001$; treatment effect $F(2,34)=8.4$, $P=0.0011$; interaction $F(2,34)=1.9$, $P=0.1674$. SD frequency: genotype effect $F(1,34)=83.8$, $P<0.0001$; treatment effect $F(2,34)=15.4$, $P<0.0001$; interaction $F(2,34)=1.8$, $P=0.1857$. SD speed: genotype effect $F(1,34)=42.8$, $P<0.0001$; treatment effect $F(2,34)=10.7$, $P=0.0002$; interaction $F(2,34)=4.8$, $P=0.0142$. SD duration: genotype effect $F(1,34)=0.3$, $P=0.5647$; treatment effect $F(2,34)=7.8$, $P=0.0016$; interaction $F(2,34)=3.8$, $P=0.0332$. Post hoc comparisons: $^{*}P<0.05$ vs vehicle; $^{\dagger}P<0.05$ vs WT. (**B**) Whisker-box plots summarize the effect of a single dose of each drug on SD frequency, threshold, speed, and duration in WT mice. n=10, 6, and 9 mice in vehicle, topiramate, and lamotrigine groups, respectively. One-way ANOVA followed by Holm–Sidak multiple comparisons test. Treatment effects were not statistically significant.

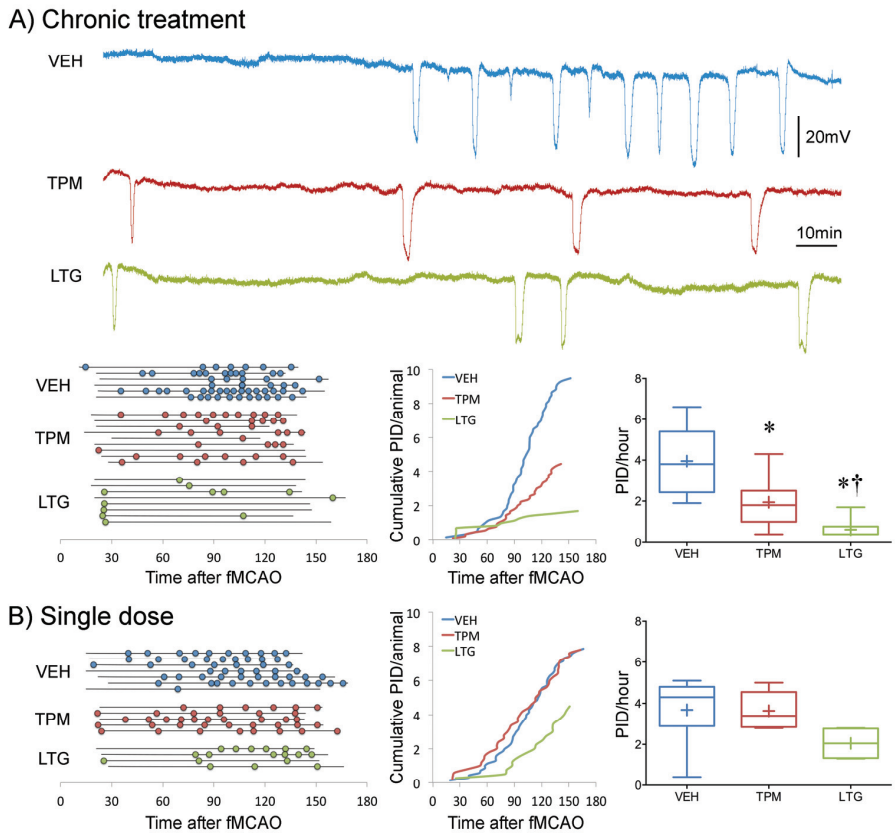


Figure 2. Chronic topiramate and lamotrigine treatment suppresses peri-infarct depolarizations (PIDs). (**A**) Upper panel shows representative electrophysiological tracings of repetitive PIDs that spontaneously arise around focal ischemic tissue during filament middle cerebral artery occlusion (fMCAO) after 7 weeks of daily treatment with vehicle (VEH, blue), topiramate (TPM, red), or lamotrigine (LTG, green) in WT mice. Lower left panel summarizes all experiments. Horizontal lines indicate the time of onset and end of electrophysiological recordings with respect to fMCAO onset in each mouse, and circles indicate PIDs. Line graph shows average cumulative PID occurrence per mouse as a function of time. When calculating the cumulative PID occurrence over time, differences in group sizes and recording durations were taken into account. Whisker-box plots show average overall PID frequency; n=6, 9, and 8 mice in vehicle, topiramate, and lamotrigine groups, respectively. One-way ANOVA followed by Holm–Sidak multiple comparisons test. Treatment effect $F(2,23)=18.1$, $P<0.0001$. Post hoc comparisons: $^{*}P<0.05$ vs VEH; $^{\dagger}P<0.05$ vs TPM. (**B**) Left panel summarizes all experiments where horizontal lines indicate the time of onset and end of electrophysiological recordings with respect to fMCAO onset in each mouse, and circles indicate PIDs. Line graph shows average cumulative PID occurrence per mouse as a function of time. When calculating the cumulative PID occurrence over time, differences in group sizes and recording durations were taken into account and corrected for. Whisker-box plots show average overall PID frequency; n=7, 5, and 4 mice in vehicle, topiramate, and lamotrigine groups, respectively. One-way ANOVA followed by Holm–Sidak multiple comparisons test. Treatment effects were not statistically significant.

Improved Stroke Outcomes After Chronic Treatment. We next tested whether suppression of PIDs translated into improved stroke outcomes in WT mice. Chronic treatment with either drug reduced the infarct size after transient fMCAO by ≈30%, and improved neurological outcomes (Figure 3A). Smaller infarcts predominantly reflected less severe cortical involvement (71±10, 50±11, and 48±9 mm^3 in vehicle, topiramate, and lamotrigine groups, respectively; $P<0.05$). Ischemic brain swelling, calculated by subtracting the contralateral from ipsilateral hemispheric volume, was

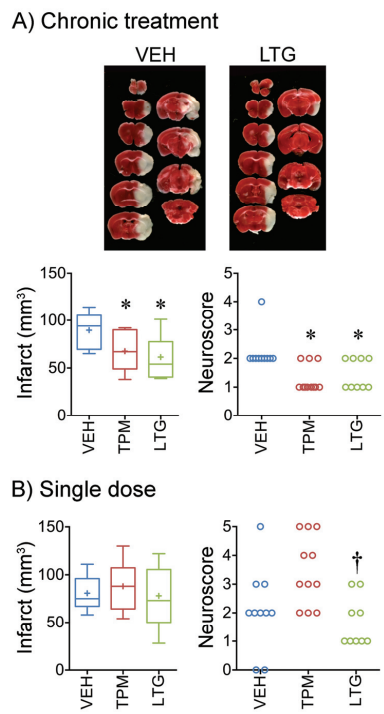


Figure 3. Chronic topiramate and lamotrigine treatment improves stroke outcomes. (**A**) Representative 2,3,5-triphenyltetrazolium chloride-stained 1-mm-thick coronal sections show the infarct 24 hours after 1-hour transient filament middle cerebral artery occlusion. Whisker-box plot summarizes the indirect infarct volumes after 7 weeks of daily treatment with vehicle (VEH, blue), topiramate (TPM, red) or lamotrigine (LTG, green) in wild-type mice. Neurological deficit scores are also shown in individual animals; n=10, 11 and 9 mice in vehicle, topiramate and lamotrigine groups, respectively. One-way ANOVA followed by Holm-Sidak multiple comparisons test for infarct volume, or Kruskal–Wallis followed by Dunn multiple comparisons test for neurological deficit score. Infarct volume: treatment effect $F(2,27)=5.5$, $P=0.01$. Neuroscore: treatment effect Kruskal–Wallis statistic 12.3, $P=0.0021$. Post hoc comparisons: $^*P<0.05$ vs vehicle. (**B**) Whisker-box plot summarizes the indirect infarct volumes after a single dose of vehicle, topiramate or lamotrigine; n=10, 11, and 9 mice in vehicle, topiramate, and lamotrigine groups, respectively. One-way ANOVA followed by Holm-Sidak multiple comparisons test. Neuroscore: treatment effect Kruskal–Wallis statistic 9.4, $P=0.009$. Post hoc comparisons: $^\dagger P<0.05$ vs topiramate.

also reduced by chronic topiramate or lamotrigine treatment compared with vehicle (8±2, 8±2, and 16±2 mm^3, respectively; $P<0.05$), possibly linked to less frequent PIDs. Neurological outcomes assessed using a combined death and disability score as a clinically relevant end point[13] were improved after chronic treatment with topiramate or lamotrigine compared with vehicle (Figure 3A). In contrast to chronic treatment, single doses of either drug did not affect any of the outcome end points compared with vehicle after transient fMCAO (Figure 3B).

Delayed AD Onset. AD represents loss of membrane ionic gradients on ischemic failure of Na^+/K^+-ATPase function. We have previously shown that migraine mutations hasten AD after focal ischemia and this correlated well with SD susceptibility and tissue outcome.[13] Therefore, we assessed whether decreased SD susceptibility after administrating migraine prophylactic drugs was associated with delayed AD onset in WT mice, detected by its cerebral vasoconstrictive effect as previously described.[4,13] Chronic treatment with lamotrigine, but not topiramate, delayed the onset of AD by ≈25% (Figure 4A). The magnitude of cerebral blood flow reduction in the ischemic core did not differ among groups (residual cerebral blood flow 12±5%, 13±5%, and 12±3% of baseline for vehicle, topiramate, and lamotrigine, respectively), eliminating the possibility that slower AD onset was because of milder ischemia. A single dose of either drug did not affect the latency to AD (Figure 4B).

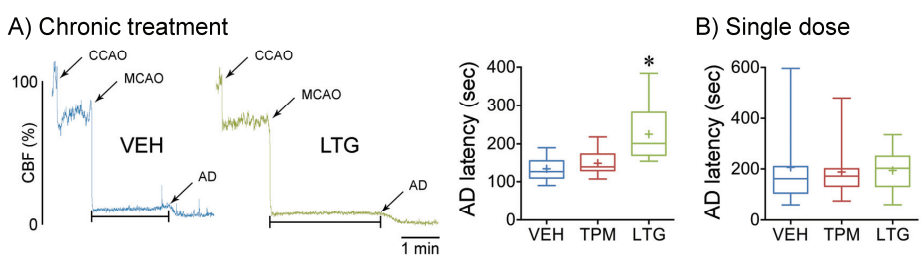


Figure 4. Chronic topiramate and lamotrigine treatment shortens anoxic depolarization (AD) latency after ischemia onset. (**A**) Left panel shows representative laser Doppler cerebral blood flow (CBF) reductions induced by occlusion of the common carotid artery and the middle cerebral artery, and the subsequent drop in CBF that marks the onset of AD. AD latency is measured as shown by the horizontal line. This secondary end point was measured in all transient filament occlusion of the middle cerebral artery experiments performed for peri-infarct depolarizations frequency and tissue and neurological outcome assessments. Whisker-box plot summarizes AD latency after 7 weeks of daily treatment with vehicle (VEH, blue), topiramate (TPM, red), or lamotrigine (LTG, green) in wild-type mice; n=17, 14, and 13 mice in vehicle, topiramate, and lamotrigine groups, respectively. One-way ANOVA followed by Holm-Sidak multiple comparisons test. Treatment effect $F(2,41)=16.0$, $P<0.0001$. Post hoc comparisons: $^*P<0.05$ vs vehicle and topiramate. (**B**) Whisker-box plot summarizes AD latency after a single dose of vehicle, topiramate or lamotrigine; n=13, 16, and 13 mice in vehicle, topiramate, and lamotrigine groups, respectively. Oneway ANOVA followed by Holm-Sidak multiple comparisons test. Treatment effects were not statistically significant.

Improved Stroke Outcomes After Chronic Treatment in FHM1 Mice. After showing that migraine prophylaxis with topiramate and lamotrigine improves stroke outcomes in WT mice, we also tested whether efficacy is sustained in migraine-susceptible FHM1 brains. Chronic treatment with either topiramate or lamotrigine reduced infarct size after transient fMCAO in FHM1 mutants by 30% to 35% (Figure 5); however, improved neurological function and the delay in the onset of AD reached statistical significance only in the lamotrigine group.

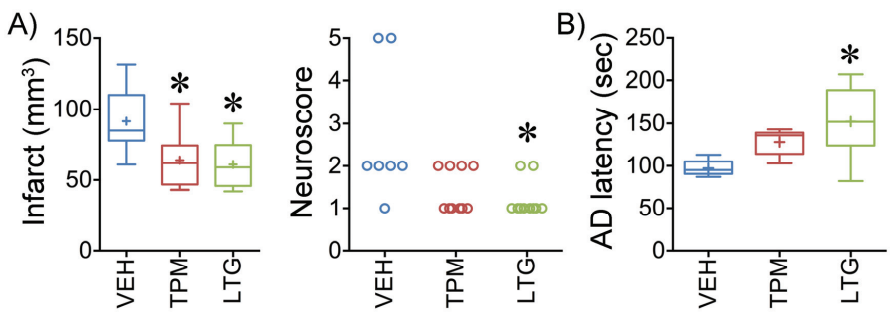


Figure 5. Chronic topiramate and lamotrigine treatment improves stroke outcomes in familial hemiplegic migraine type 1 mutant mice. (**A**) Whisker-box plot summarizes the indirect infarct volumes after 7 weeks of daily treatment with vehicle (VEH, blue), topiramate (TPM, red), or lamotrigine (LTG, green) in R192Q mutant mice. Neurological deficit scores are also shown in individual animals; n=7, 10, and 10 mice in vehicle, topiramate, and lamotrigine groups, respectively. **P*<0.05 vs vehicle. One-way ANOVA followed by Holm-Sidak multiple comparisons test for infarct volume, and Kruskal–Wallis followed by Dunn multiple comparisons test for neurological deficit score. Infarct volume: treatment effect $F(2,24)=6.0$, $P=0.0075$. Neuroscore: treatment effect Kruskal–Wallis statistic 8.6, $P=0.0136$. Post hoc comparisons: **P*<0.05 vs vehicle. (**B**) Whisker-box plot summarizes anoxic depolarization latency after a single dose of vehicle, topiramate, or lamotrigine (LTG, green) in R192Q mutant mice; n=5, 11, and 10 mice in vehicle, topiramate, and lamotrigine groups, respectively. One-way ANOVA followed by Holm-Sidak multiple comparisons test. Treatment effect $F(2,23)=6.8$, $P=0.0048$. Post hoc comparisons: **P*<0.05 vs vehicle and topiramate.

DISCUSSION

Migraine is an established risk factor for ischemic stroke. We have recently shown that genetically enhanced SD susceptibility worsens the effect of cerebral ischemia on the brain by facilitating ischemic depolarization events,[13] as a mechanism to explain the increased risk of stroke in migraineurs. Conversely, we here show that pharmacological suppression of SD susceptibility by migraine prophylactic drugs inhibits AD and PIDs and improves stroke evolution in both WT and FHM1 mutant mice. The magnitude of SD suppression by each drug corresponded well with the magnitude of AD and PID suppression, and stroke outcome. Consistent with this, genetically reduced susceptibility to SD as observed in *rolling Nagoya* and *leaner* mice, which

have spontaneously arisen mutations in the *Cacna1a* gene leading to loss of $Ca_V2.1$ function, was associated with smaller infarcts, compared with WT on experimental stroke.[28] These data strongly support intrinsic SD susceptibility of brain tissue (ie, the tissue factor) as an important determinant of stroke outcome.

Although in vitro studies of topiramate and lamotrigine have suggested a neuroprotective effect,[29] in vivo studies were generally negative in various models of focal cerebral ischemia.[18–22] All studies, however, have tested single doses or short-term treatment administered before or after ischemia onset. Our data suggest that chronic treatment is required for efficacy, as has been the case for SD suppression in rats[14,16] and for the prophylactic effect on migraine in patients. Both topiramate and lamotrigine have been shown to acutely inhibit various voltage-gated ion channels as well as glutamatergic neurotransmission.[30,31] However, whether chronic treatment simply enhances these effects by achieving higher tissue levels, or induces structural or gene expression changes, remains to be determined.

Although PIDs are generally thought to enlarge infarcts by worsening the supply demand mismatch, an alternative and possibly complementary mechanism is a further increase in cerebral excitability by SD shown in neocortical slices[32,33]; PID inhibition by migraine prophylaxis may prevent this delayed hyperexcitability and improve outcome. Of course, glial cells critically modulate SD susceptibility, and glial protective effects of topiramate and lamotrigine[34–36] may also contribute to PID suppression and infarct reduction.

It is well established that PIDs worsen stroke outcomes,[6,37] and that drugs acutely inhibiting PIDs after a single dose (eg, NMDA receptor antagonists) are protective in focal cerebral ischemia both in experimental animals and in stroke patients.[4,38–40] However, clinical translation of this neuroprotective target has been difficult because of the cognitive and sedative side effects of such potent drugs.[41–43] In this respect, migraine prophylaxis may provide a better-tolerated antiexcitatory treatment alternative targeting SD and PIDs in stroke prophylaxis. Consistent with this notion, chronic treatment with lamotrigine was reported to diminish stroke-like episodes in a migraineur with mitochondrial encephalopathy, lactic acidosis, and stroke-like episodes,[44] suggesting that the approach may be even more efficacious in hyperexcitable subsets of patients.

Summary and Conclusions. In summary, our data suggest that pharmacological suppression of SD susceptibility may protect against ischemic injury in patients at high risk for stroke, migraineurs, and nonmigraineurs alike. Whether migraine prophylaxis clinically improves stroke outcomes or reduces the stroke risk remains to be tested in large population-based studies. Although chronic treatment purely as a form of stroke prophylaxis may not be justified at this time because of potential side effects, migraine patients who are already on a migraine prophylactic regimen may indeed see a reduction in their stroke risk as an additional benefit.

ACKNOWLEDGMENTS

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CHAPTER 3C

SENSITIVITY TO ACUTE CEREBRAL ISCHEMIC INJURY IN MIGRAINEURS: A RETROSPECTIVE CASE-CONTROL STUDY

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ABSTRACT

Objective: Migraine, particularly with aura, is a risk factor for ischemic stroke. Recent data in migraine mutant mice suggest that cerebral hyperexcitability associated with migraine accelerates recruitment of ischemic penumbra into the core, resulting in faster infarct growth compared with wild type. We hypothesized that individuals with a history of migraine are more likely to exhibit increased recruitment of ischemic tissue into the infarct in acute stroke.

Methods: In this retrospective case-control study, we identified participants with reliably documented migraine history, measured lesion volumes on diffusion-weighted and perfusion-weighted MRI obtained within 72 hours of symptom onset, calculated the proportion of ischemic tissue on perfusion-weighted imaging (PWI) hyperintense on diffusion-weighted imaging (DWI), and compared the proportion of patients with no-mismatch pattern defined as DWI lesion >83% of PWI lesion.

Results: Migraineurs (n = 45) were younger, more often female, less likely to have vascular risk factors, and more often had cervical artery dissection, but otherwise did not differ from controls (n = 27). A significantly larger proportion of migraineurs had no-mismatch pattern, indicating that the entire perfusion defect was recruited into the infarct by the time of MRI (22% vs 4% of migraineurs and controls, respectively; $p = 0.044$). The difference was even more prominent in migraineurs with aura (36% vs 4%, $p = 0.019$). The association between migraine and no-mismatch pattern persisted after adjustment for time to MRI ($p = 0.041$).

Conclusions: This case-control study supports the hypothesis that a history of migraine, particularly with aura, is associated with a no-mismatch pattern during acute ischemic stroke, consistent with data obtained in migraine mutant mice.

INTRODUCTION

Recent experimental data suggest that genetic susceptibility to migraine aura may be a factor rendering the brain tissue more sensitive to ischemia.[1] Transgenic mice expressing familial hemiplegic migraine type 1 (FHM1) mutations, frequently used as animal models for migraine, develop accelerated infarct growth because of faster anoxic depolarization and more frequent peri-infarct spreading depressions during acute focal cerebral ischemia.[1] Mutant animals require higher blood flow to survive focal ischemia, and develop larger infarcts and worse neurologic outcomes compared with wild type. In addition, larger infarcts were observed in migraineurs with aura in a recent preliminary study.[2] We, therefore, sought to determine whether patients with acute ischemic stroke and a history of migraine develop faster infarct growth as a reflection of increased tissue vulnerability to ischemia.

METHODS

Study population. We retrospectively studied consecutive patients (age >18 years) with imaging-confirmed acute ischemic stroke admitted within 72 hours of symptom onset at a single tertiary care hospital (Massachusetts General Hospital) between 2003 and 2014, with approval from the Institutional Review Board. A priori inclusion criteria were (1) diffusion- and perfusion-weighted MRI within 72 hours of symptom onset and (2) patients whose records included a physician note that explicitly stated a history of migraine (with or without aura, or unspecified) and controls whose records explicitly stated that the participant had no history of migraine. Cases could not be matched with controls for age and sex because of the need to have migraine status documented in the chart.

Image analysis. Experienced neurologists blinded to case-control assignments manually outlined regions that were hyperintense on diffusion-weighted images (DWI) and hypointense on apparent diffusion coefficient (ADC) maps and hyperintense regions on mean transit time (MTT) maps using MRIcron software (University of Nottingham, UK). We determined the arterial territory involved using standard templates based on infarct distribution on DWI. We also recorded whether there was intracranial arterial occlusion on CT or magnetic resonance angiograms obtained at the same session with MRI. We considered an occlusion proximal when it was located within the first 3 segments of the intracranial arterial system.

Study endpoint. We hypothesized that migraineurs would show faster recruitment of hypoperfused but viable tissue (i.e., DWI-MTT mismatch) into the infarct (DWI lesion) during acute ischemic stroke.[3,4] We reasoned that by the time MRI was done a larger proportion of migraineurs compared to controls would develop a no-mismatch

pattern. We defined nomismatch as DWI lesion volume >83% of perfusion-weighted imaging (PWI) lesion volume.[5,6] We excluded participants if DWI and PWI volumes were both less than 10 mL because of the poor reliability of volumetric analyses in this subset.[7] None of the patients had MRI before a reperfusion intervention.

Statistical analysis. We used χ^2 or Fisher exact tests for group-wise comparisons among categorical variables and Mann-Whitney *U* test for comparisons among numerical variables. To assess the association between migraine status and tissue vulnerability to ischemia, we used a logistic regression model in which no-mismatch was the dependent variable and stroke features with a univariate $p < 0.150$ were the covariates. A *p* value of <0.05 was considered statistically significant. All analyses were performed using SPSS 16.0.

RESULTS

We identified 207 patients with reliable documentation of migraine status and an MRI within 72 hours of symptom onset confirming acute infarction (figure, A). Patients with a history of migraine (n = 142) were younger, predominantly female, more likely to have uncommon etiologies such as cervical artery dissection, and less likely to have multiple vascular risk factors as compared to those who did not have migraine (n = 65). All these associations were in the same direction in migraineurs with aura.

Of the 207 patients, 107 had both DWI and PWI. Thirty-five of these 107 patients had lesions <10 mL and were excluded from further analyses (figure, A). The remaining cohort of 45 migraineurs (11 with aura) and 27 control participants showed similar demographic, clinical, and radiologic findings noted in the excluded participants, except that the exclusion of small lesions eliminated lacunar infarcts from the etiologic subtypes (table 1). Median time from symptom onset to MRI was approximately 7 hours (figure, B).

The frequency histogram of DWI/PWI ratios displayed a bimodal distribution in migraineurs (figure, C). In contrast, the proportion of nonmigraineurs continuously decreased as DWI/PWI ratio increased. A receiver operating characteristic curve analysis revealed that the optimal operating point for DWI/PWI ratio to discriminate migraineurs from nonmigraineurs was 0.82. There was a significantly higher proportion of migraineurs with no-mismatch compared with controls (22% vs 4%, respectively; $p = 0.044$). The relationship was even stronger in migraine patients with aura (36% vs 4%, respectively; $p = 0.019$; figure, D), but did not reach significance for migraine without aura. Among all covariates (table 2), only time from symptom onset to MRI showed a borderline association with no-mismatch ($p = 0.105$). The association between DWI/PWI ratio and migraine status was significant when adjusted for time to MRI ($p = 0.041$; figure, D). There was a trend towards a similar relationship between

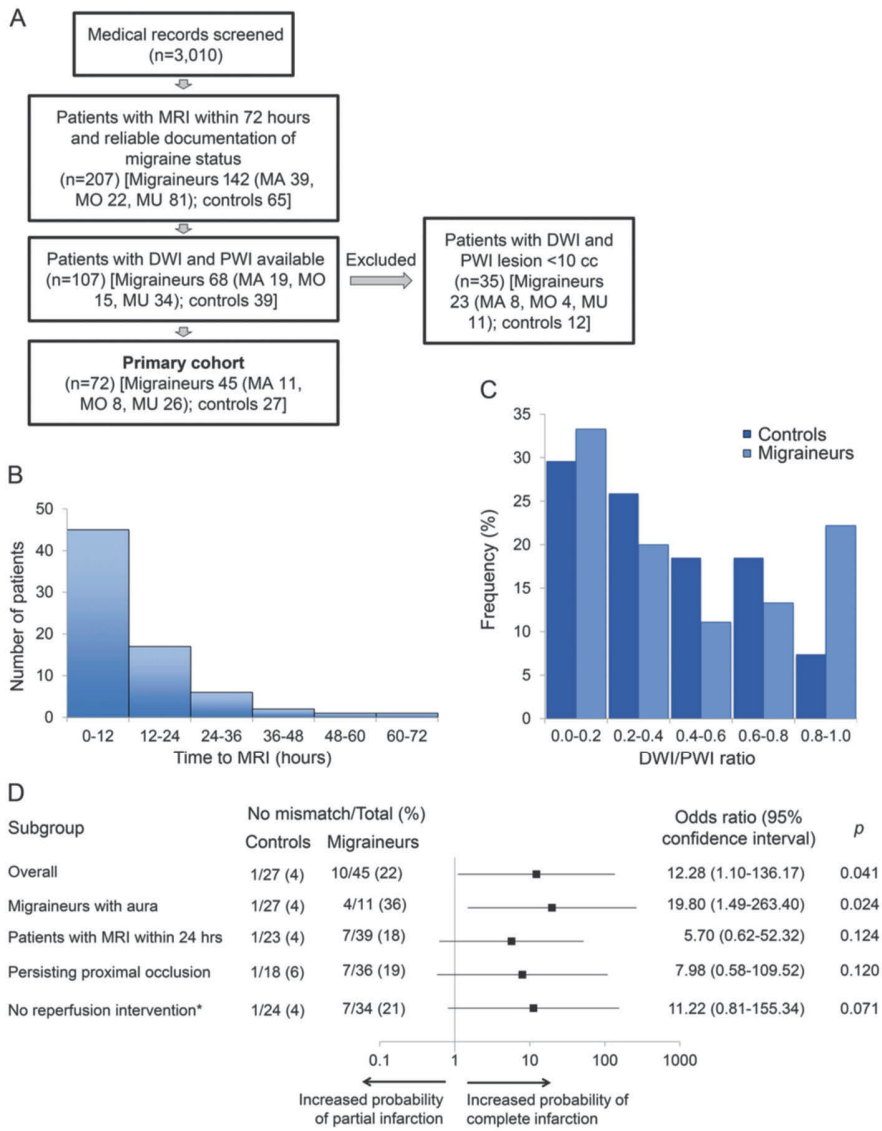


Figure. Study flowchart, time to MRI, and DWI/PWI distribution for the analysis of no-mismatch pattern. (**A**) Study flowchart. MA = migraine with aura; MO = migraine without aura; MU = migraine of unknown type. (**B**) Histogram of time from symptom onset to MRI. (**C**) Frequency histogram for diffusion-weighted imaging (DWI)/perfusion-weighted imaging (PWI) ratio in controls and migraineurs showed a bimodal distribution in migraineurs. (**D**) Subgroup analyses for no-mismatch pattern adjusted for time to MRI. Horizontal bars represent odds ratio and 95% confidence interval. *Includes IV thrombolysis and endovascular treatments.

	Controls (n = 27)	Migraineurs (n = 45)	p
Age, y, median (IQR)	68 (52-78)	53 (44-67)	0.008[a]
Female, n (%)	10 (37)	28 (62)	0.038[a]
Ethnicity, n (%)			0.822
White	21 (78)	36 (80)	
Other	6 (22)	9 (20)	
Risk factors, n (%)			
Hypertension	17 (63)	18 (40)	0.059
Diabetes mellitus	8 (30)	3 (7)	0.015[a]
Coronary artery disease	7 (26)	4 (9)	0.088
Atrial fibrillation	5 (19)	6 (13)	0.554
Hyperlipidemia	6 (22)	7 (16)	0.476
Active smoking	8 (30)	8 (18)	0.242
≥2 risk factors	15 (56)	13 (29)	0.025[a]
Stroke etiologic subtype, n (%)			0.140
Large artery atherosclerosis	7 (26)	5 (11)	
Cardio-aortic embolism	11 (41)	25 (56)	
Small artery occlusion	0 (0)	0 (0)	
Other causes	3 (11)	10 (22)	
Undetermined causes	6 (22)	5 (11)	
Admission plasma glucose, median (IQR)	108 (99-118)	114 (108-140)	0.075
IV thrombolysis or endovascular treatment, n (%)	3 (11)	11 (24)	0.225
Time from onset to MRI, median (IQR)	7.3 (5.7-16.4)	6.9 (4.0-15.2)	0.309
Infarct location, posterior, n (%)	5 (19)	8 (18)	0.937
Persistent proximal occlusion, n (%)	18 (67)	36 (80)	0.206
DWI volume, mL, median (IQR)	21 (8-40)	22 (6-104)	0.954
PWI volume, mL, median (IQR)	99 (29-212)	128 (43-201)	0.504
DWI/PWI ratio, median (IQR)	0.3 (0.2-0.6)	0.4 (0.1-0.8)	0.930

Table 1. Demographic, clinical, and radiologic characteristics of the study cohort (with PWI) stratified according to migraine history. Abbreviations: DWI = diffusion-weighted imaging; IQR = interquartile range; PWI = perfusion-weighted imaging. [a]Significant.

	No-mismatch		
	Absent (n = 61)	Present (n = 11)	*p*
Age, y, median (IQR)	56 (44–70)	64 (46–73)	0.833
Female, n (%)	32 (53)	6 (55)	0.898
Ethnicity, n (%)			1.000
White	48 (79)	9 (82)	
Other	13 (21)	2 (18)	
Risk factors, n (%)			
Hypertension	29 (48)	6 (55)	0.669
Diabetes mellitus	11 (18)	0 (0)	0.195
Coronary artery disease	10 (16)	1 (9)	1.000
Atrial fibrillation	10 (16)	1 (9)	1.000
Hyperlipidemia	11 (18)	2 (18)	1.000
Active smoking	14 (23)	2 (18)	1.000
≥2 risk factors	26 (43)	2 (18)	0.183
Stroke etiologic subtype, n (%)			0.310
Large artery atherosclerosis	12 (20)	0 (0)	
Cardio-aortic embolism	29 (48)	7 (64)	
Small artery occlusion	0 (0)	0 (0)	
Other causes	10 (16)	3 (27)	
Undetermined causes	10 (16)	1 (9)	
Plasma glucose (mg/dL), median (IQR)	111 (100–127)	114 (109–124)	0.538
IV thrombolysis or endovascular treatment, n (%)	11 (18)	3 (27)	0.438
Time from onset to MRI, median (IQR)	6.9 (4.4–15.2)	15.5 (5.0–25.0)	0.105
Infarct location, posterior, n (%)	10 (16)	3 (27)	0.405
Persistent proximal occlusion, n (%)	46 (75)	8 (73)	1.000

Table 2. Demographic, clinical, and radiologic characteristics of the study cohort (with PWI) stratified according to complete infarction status. Abbreviations: IQR = interquartile range; PWI = perfusion-weighted imaging.

migraine and tissue outcome in more homogeneous subgroups, such as in patients with MRI obtained within 24 hours of stroke onset, patients with persistent proximal occlusion, or patients who did not receive reperfusion therapies (figure, D).

DISCUSSION

Our data suggest a novel link between migraine and human stroke by showing that a history of migraine, particularly with aura, may accelerate loss of viable tissue at risk for infarction during acute cerebral ischemia. Compared to controls, a significantly higher proportion of migraineurs displayed nomismatch early after stroke onset (median 7 hours), a pattern suggestive of malignant progression in which penumbra is rapidly recruited into the ischemic core. The risk was particularly high in migraineurs with aura, which is in agreement with experimental findings in transgenic mouse models of enhanced genetic susceptibility to migraine aura.[1] Importantly, the relationship observed between migraine and no-mismatch persisted when adjusted for time from symptom onset to imaging, a wellknown determinant of the conversion of penumbra into definite infarction.

This study was subject to selection and documentation bias; a history of migraine is more likely to be inquired in young patients with no known risk factors. Migraine status is also more likely to be documented in medical records if it is present than absent. Nevertheless, the present migraine cohort was similar to previously reported larger stroke cohorts with migraine in age and sex, risk factor profile, presumed stroke etiology, and involved vascular territory, suggesting that retrospective design did not introduce an important bias towards selection of a particular risk population.[8,9] It was not possible to retrieve data on migraine features (e.g., active vs remote history of migraine, attack frequency or severity, aura with each attack vs only occasional auras, aura without headache) that might influence the rate of progression to infarction. Small sample size and retrospective design also precluded reliable assessment of the effect of different modes of migraine prophylaxis. Future prospective studies will address these hypotheses.

Brain regions abnormal on MTT maps indicate both critically and noncritically hypoperfused tissue. More frequent no-mismatch based on the MTT criterion suggests that even moderately oligemic tissue is at risk of infarction in migraineurs, further supporting the notion of increased tissue sensitivity to ischemia. Although statistically significant, the association between tissue outcome and migraine status was not absolute; 78% of the patients with migraine did not exhibit a no-mismatch, suggesting that increased risk of no-mismatch originated from a subset of highly susceptible patients, perhaps those with a genetic predisposition to develop anoxic and spreading depolarizations. Importantly, although our a priori time to MRI cutoff was <72 hours of symptom onset, the vast majority of patients in our final cohort had times to MRI <36 hours. Indeed, secondary analyses using 48 hours time to MRI cutoff

yielded the same conclusion (odds ratio 18.2 for no-mismatch in migraineurs with aura compared with controls; $p = 0.025$). Therefore, long inclusion times toMRI did not confound our conclusions in this hypothesis-generating study.

This study raises several important hypotheses[10] to be tested in subsequent studies. First, our findings support a shorter therapeutic window for reperfusion in migraineurs due to rapid loss of viable and salvageable tissue at risk. Second, higher sensitivity to ischemia may require more stringent monitoring and management of stroke risk factors and possibly antithrombotic prophylaxis in migraineurs. Third, migraine prophylaxis may reduce tissue vulnerability to ischemic injury by suppressing the susceptibility to spreading depolarizations.[11]

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CHAPTER 4

MIGRAINE AND STROKE: IN SEARCH OF SHARED MECHANISMS

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ABSTRACT

Background: Migraine, particularly with aura, increases the risk for ischemic stroke, at least in a subset of patients. The underlying mechanisms are poorly understood and probably multifactorial.

Methods: We carried out an extended literature review of experimental and clinical evidence supporting the association between migraine and ischemic stroke to identify potential mechanisms that can explain the association.

Results: Observational, imaging and genetic evidence support a link between migraine and ischemic stroke. Based on clinical and experimental data, we propose mechanistic hypotheses to explain the link, such as microembolic triggers of migraine and enhanced sensitivity to ischemic injury in migraineurs.

Discussion: We discuss the possible practical implications of clinical and experimental data, such as aggressive risk factor screening and management, stroke prophylaxis and specific acute stroke management in migraineurs. However, evidence from prospective clinical trials is required before modifying the practice in this patient population.

INTRODUCTION

Migraine is the most common neurological disorder and a major cause of disability in the western world, with a prevalence of approximately 13% (9% among men, 18% among women in the United States (US)).[1] An aura is present in up to 30% of migraineurs, usually during the hour preceding the headache.[2,3] Spreading depression (SD), an intense neuronal and glial depolarization wave that slowly propagates in brain tissue at a rate of around 3mm/min, is widely accepted as the electrophysiological substrate of migraine aura.[4,5]

Migraine has traditionally been viewed as a benign, chronic episodic condition. However, accumulating evidence suggests that migraine, particularly with aura, can be associated with increased risk for stroke and white matter lesions.[6-12] The association is even more striking considering the clinical contrasts between migraine and stroke. Unlike migraine, stroke is an acute and often catastrophic cerebrovascular event. In contrast to the perceived benign nature of migraine (i.e. no imminent risk of injury), stroke is the leading cause of acquired physical disability in adults in the US,[13] and the second leading cause of mortality worldwide.[14] The prevalence of both ischemic (85% of all strokes) and hemorrhagic stroke in the US is 2.9% in individuals 18 years or older,[13] much lower than the prevalence of migraine. And lastly, stroke is predominantly a disease of the elderly, while migraine prevalence peaks around age 40. As per International Headache Society (IHS) criteria, migraine headache may be termed secondary when it is present as part of an underlying disease process such as patent foramen ovale (PFO) or antiphospholipid antibody syndrome; many of these disorders are also associated with increased risk of cardiovascular and cerebrovascular events, discussed in detail below.

In this paper, we briefly summarize the evidence supporting a clinical association between migraine and stroke, propose mechanistic hypotheses that may explain the association, and review the experimental data supporting or refuting some of these hypotheses. Inevitably, in the absence of robust evidence the proposed mechanisms remain speculative. Our overarching aim is to stimulate translational investigations on the mechanisms linking migraine and stroke toward improved patient care. Of note, migraine also appears to increase the risk of hemorrhagic stroke.[15] However, available data are less robust, and mechanistic insight is lacking particularly in the experimental setting; therefore, we will limit the discussion to ischemic stroke.

CLINICAL EVIDENCE LINKING MIGRAINE AND STROKE

Observational data. Although limited by the lack of biomarkers to identify migraine with certainty and quantitative data on intensity, duration and frequency of attacks, abundant observational data from retrospective or population- or hospital-based case-control studies as well as small and large population-based prospective studies

including tens of thousands of individuals have firmly established a link between migraine and ischemic stroke, which have been the subject of three meta-analyses.[10,16,17] The most recent meta-analysis[10] of 13 case-control and eight cohort studies with a total of 622,381 participants also showed a link between migraine and ischemic stroke with an odds ratio (OR) of 2.04 (95% confidence interval (CI) 1.72-2.43).

The association relied on migraine with aura (OR 2.51, 95% CI 1.52-4.14), and was not significant in migraine without aura (OR 1.29, 95% CI 0.81-2.06). Further subgroup analyses revealed a stronger association in women (OR 2.89, 95% CI 2.43-3.45),[10] as well as in patients younger than 45 (OR 2.65, 95% CI 1.41-4.97), in smokers (OR 9.03, 95% CI 4.22-19.34) and in women using oral contraceptives (OR 7.02, 95% CI 1.51-32.68).[17] Moreover, in the Women's Health Study (WHS), increased risk appeared to be mainly in those who experienced active migraine attacks within the year before completing the baseline questionnaire, and not in those with just a history of migraine without recent attacks (OR, 1.91 95% CI 1.17-3.10).[18] The risk was also higher in those who experienced >12 attacks per year (OR, 1.7 95% CI 1.1-2.8) in the Stroke Prevention in Young Women study.[19] Interestingly, most ischemic events appeared to be transient, or strokes with good clinical outcomes (modified Rankin Scale 0 to 1) in the WHS.[20]

Much less is known about the stroke subtype in association with migraine. A tendency for strokes of undetermined cause (OR 1.4, 95% CI 0.9-2.0) or lacunar strokes has been suggested (OR 1.5, 95% CI 0.7-3.3),[19] and in the Italian Project on Stroke in Young adults Study[21] the frequency of right to left shunts was higher in stroke in migraineurs with aura (OR 2.41, 95% CI 1.47-3.95). Based on clinical presentation, a predilection for anterior or posterior circulation has not been demonstrated.[19,21]

Lastly, there may be an association between migraine and systemic cardiovascular event risk (e.g. myocardial ischemia and infarction, cardiovascular mortality, peripheral vascular disease). Although an earlier meta-analysis of eight studies did not show an increased risk of myocardial infarction among migraineurs (OR 1.12, 95% CI 0.95-13.2),[17] a more recent large case-control study suggested a higher risk (OR 2.16, 95% CI 1.7-2.76).[22] Most recently, a population-based prospective study showed an increased risk of ischemic heart disease (hazard ratio (HR) 2.5, 95% CI 1.8-3.5) in participants between the ages of 18 and 45.[23] Likewise, a large prospective cohort with a median follow-up of 26 years suggested increased cardiac mortality among migraineurs with aura;[24] however, the association was not significant in a meta-analysis.[25]

Neuroimaging. A number of neuroimaging studies over the past decade revealed a higher prevalence of subclinical brain abnormalities in migraineurs, including infarcts and white matter hyperintensities, suggesting acute or chronic ischemic disease.

Infarcts. Cerebral Abnormalities in Migraine and Epidemiological Risk Analysis (CAMERA) was a cross-sectional, population-based magnetic resonance imaging

(MRI) lesion prevalence study in patients between the ages of 30 and 60 (mean age 48; 161 migraine with aura, 134 migraine without aura and 140 matched controls) randomly selected from the Genetic Epidemiology of Migraine study. Results suggest increased risk of subclinical posterior circulation infarct-like lesions, mostly located in the cerebellum, in migraineurs compared to controls (OR 7.1, 95% CI 0.9-55).[11,26] The risk was substantially higher in migraineurs with aura (OR 13.7, 95% CI 1.7-112), especially with frequent migraine attacks (≥1 attack/month) (OR 15.8, 95% CI 1.8-140), and independent of triptan use or vascular risk factors, although the study was not powered to test the latter. There was no difference in the frequency of such lesions in the anterior circulation. In the nine-year follow-up of the same cohort, none of the lesions disappeared, and new posterior circulation infarct-like lesions were found in 5% of migraineurs compared with none in control subjects.[27]

The overall conclusions were later independently confirmed in the Age Gene/Environment Susceptibility Reykjavik study,[28] albeit using similar criteria. In this population-based cohort study, 689 patients with a mean age of 60 years (i.e. mid-life) were interviewed for migraine status, and MRI was performed at a mean age of 76 years. This study revealed that women (but not men) who reported active migraine with aura in mid-life had an increased risk of late-life infarct-like lesions compared to non-migraineurs (OR 1.9, 95% CI 1.4-2.6), independently from vascular risk factors. These lesions were also mostly in the cerebellum.

The population-based Epidemiology of Vascular Aging study (780 participants, mean age of 69) also found an increased risk of cerebral infarcts in migraineurs with aura only.[29] Although the interpretation was limited by the relatively small number of patients with migraine with aura (17 out of 116 migraineurs), the definition of infarct was stricter, and therefore, data were more specific for ischemic mechanisms. The lesions were mostly located outside the cerebellum and the brain stem.

There have been conflicting data as well, albeit from smaller datasets or from studies designed to test other associations. For example, the Helsinki Young Stroke Registry of 669 patients with first-ever stroke between 15 and 49 years of age did not find any association between the presence of silent brain infarcts and migraine status, despite an overall high frequency of silent brain infarcts (13%).[30] All patients with a lesion had at least two vascular risk factors. Similarly, a cohort of 100 consecutive women with chronic migraine with[51] or without[49] aura (mean age 44) revealed a much lower frequency of infarct-like lesions on MRI than expected by the high frequency of migraine attacks (6%, all with associated vascular risk factors); however, the absence of a control group precluded firm conclusions.[31]

White matter hyperintensities. A meta-analysis of retrospective case-control studies (312 subjects, 317 controls) also suggested an increased risk for white matter MRI hyperintensities in migraineurs (OR 3.9, 95% CI 2.26-6.72), even in younger individuals

and when controlled for comorbid vascular risk factors.[32] Most recent meta-analysis of this association suggested an increased risk in migraine with aura (OR 1.7, 95% CI 1.1-2.7), but not in migraine without aura (OR 1.3, 95% CI 0.96-1.87).[12]

Although the CAMERA study did not show a difference for periventricular or deep white matter lesions between migraineurs and controls, subgroup analysis revealed higher risk for deep white matter lesions only in women with migraine (OR 2.1 95% CI 1.0-4.1); there was no effect of aura status and the risk increased with increasing attack frequency.[11] A subsequent analysis also suggested an increased prevalence of infratentorial (mostly pontine) hyperintensities in migraineurs with and without aura.[33] The nine-year CAMERA follow-up revealed a higher incidence of deep white matter lesions in women with migraine (OR 2.1 95% CI 1.0-4.1), highest in the migraine without aura group.[27] There was no association between lesion progression and attack type, duration, frequency and cumulative number, or specific migraine treatments.

In the Epidemiology of Vascular Aging study, individuals with lifetime history of severe headaches (116 migraineurs and 47 non-migraine headache) were more likely to be in the highest tertile of total white matter hyperintensity volume (OR 2.0, 95% CI 1.3-3.1), preferentially located in deep white matter regions in migraineurs with aura.[29] More recently, the prospective Atherosclerosis Risk in Communities cohort of 1028 patients with a mean age of 60 showed an increased risk of moderate to severe white matter MRI hyperintensities (defined as a score 3 on a visual rating scale from 0 with no white matter hyperintensity to 9 with confluent and extensive white matter hyperintensities) in migraineurs without aura compared to participants without headache (OR 1.87, 95% CI 1.04-3.37).[34] However, follow-up between eight to 15 years did not reveal a difference in progression of white matter hyperintensities between migraineurs and controls.

In summary, structural brain imaging strongly suggests an increased risk of infarcts and white matter hyperintensities in migraineurs, and perhaps all severe headaches. The conclusions, however, have not been unanimous. The vascular origin of infarcts and white matter hyperintensities has not been confirmed, in part because of imprecise definition of lesions on MRI and lack of neuropathological correlation.[35] Furthermore, the association between the severity or duration of migraine and the severity of structural brain abnormalities is inconsistent. An association may be absent, or may be obscured by low power of individual studies and differences in the definition of lesion severity. It should be remembered, however, that migraineurs suffer countless attacks during their lifetime, but develop only a small number of neuropathological lesions. Therefore, it is unlikely that individual attacks directly cause injury.

Monogenic and other rare diseases. Albeit rare, monogenic diseases may help understand the pathophysiology of more common polygenic or multifactorial conditions. An association between migraine and stroke is further supported by

frequent co-existence of the two in monogenic diseases. For example, migraine with aura is often the first symptom in cerebral autosomal dominant arteriopathy with subcortical infarcts and leukoencephalopathy (CADASIL), present in 20%-40% of patients. CADASIL is the most common monogenic inherited cerebral small-vessel disease in middle-aged adults. It is caused by mutations in the *NOTCH3* gene that are exclusively expressed in vascular smooth muscle cells in the adult brain. Besides migraine with aura, CADASIL patients progressively develop subcortical infarcts, mood disorders and cognitive impairment culminating in frank dementia.[36] Most migraine attacks are typical, but 50% of patients also experience attacks with atypical aura including basilar or hemiplegic migraines, or prolonged migraine auras.[37]

Hereditary infantile hemiparesis, retinal arteriolar tortuosity and leukoencephalopathy (HIHRATL) is another autosomal dominant cerebral small-artery disease associated with migraine with or without aura. Mutations in the *COL4A1* gene encoding type IV collagen α1 chain destabilize the triple helix domain of collagen IV found in the basement membranes. First described in mutant mice with porencephaly generated by random mutagenesis,[38] *COL4A1* mutations have now been detected in many human families, in whom newborns and adults may be affected.[39,40] The phenotype is variable, and besides migraine, may include infantile hemiparesis, porencephaly, seizures, white matter hyperintensities, hemorrhagic more than ischemic stroke, renal and ocular vessel tortuosity, and intracranial asymptomatic aneurysms.[41]

Retinal vasculopathy with cerebral leukodystrophy is an autosomal dominant small-vessel disease of middle-age onset, previously known as cerebro-retinal vasculopathy, hereditary vascular retinopathy and hereditary endotheliopathy, retinopathy, nephropathy and stroke. All entities have been linked to mutations in the *TREX1* gene,[42] which encodes a DNA-specific exonuclease implicated in DNA repair under conditions of oxidative stress. The clinical presentation includes progressive loss of visual acuity related to retinal vasculopathy, small cerebral infarcts and white matter hyperintensities that can coalesce to form pseudotumors. Although migraine or ''migraine-like'' headache has been described in all phenotypes, migraine, equally with or without aura, seems to be more frequent in hereditary vascular retinopathy,[43] where it is found in 70% of patients and often associated with Raynaud phenomenon.

Mitochondrial encephalomyopathy, lactic acidosis and stroke-like episodes (MELAS) is caused by mutations in the mitochondrial genome with a maternal pattern of inheritance. It mostly affects children and young adults, with 90% of patients having symptoms before 30 years of age. Attacks of migraine are present in 70% of cases and may be isolated, or more frequently accompany seizures and/or focal or global neurological deficits related to post-ictal symptoms or ischemic stroke. Remarkably, strokes occur early in life, do not match a particular vascular territory, often progress

slowly (over days or weeks) and can often be partially reversible, which justifies the term ''stroke-like episodes.'' However, residual deficits often accumulate leading to motor, visual or cognitive disability. Systemic features include short stature, sensorineural hearing loss, diabetes mellitus, cardiac disease, myopathy, and gastrointestinal and renal involvement.[44-46]

Hereditary hemorrhagic telangiectasia is an autosomal dominant disorder characterized by mucocutaneous telangiectasia and arteriovenous malformations of the brain, lung, gastrointestinal tract and liver due to mutations of endoglin or activin receptor-like kinase 1.[47] Migraine is present in up to 40% of patients, in addition to the classical epistaxis and other sites of hemorrhage that start almost always before 40 years of age. Although migraine and stroke, mostly hemorrhagic, may be related to intracerebral arteriovenous malformations,[48] migraine, mostly with aura, occurs typically independent of cerebral vascular malformations and is associated with an increased probability of pulmonary arteriovenous malformation, which is also associated with an increased risk of cerebral ischemic events.[49-51] Interestingly, in hereditary hemorrhagic telangiectasia patients without a pulmonary arteriovenous malformation, prevalence of migraine without aura was also higher in one study.[52] Outside of hereditary hemorrhagic telangiectasia, an association between migraine and occipital arteriovenous malformations has been noted,[53] and migraine is a recognized accompaniment of Sturge-Weber syndrome,[54] and has been noted in association with moyamoya disease,[55] underscoring the link between vascular structural abnormalities and migraine.

Potential caveats in interpretation of clinical evidence. Heterogeneity of migraine and inconsistent use of diagnostic criteria, referral bias, difficulty differentiating between a migraine attack and a transient ischemic attack (TIA) in the absence of biomarkers, presence of comorbidities and concurrent medications, and overrepresentation of younger patients can all complicate and confound the interpretation of clinical data. It is highly unlikely that increased stroke risk in migraine with aura can be explained entirely by secondary migraine in the setting of other diseases such as CADASIL or hereditary hemorrhagic telangiectasia. These potential caveats notwithstanding, the evidence firmly supports an association, and in many cases provides clues to the pathogenesis.

MECHANISMS OF ASSOCIATION

Observational, genetic and neuroimaging data suggest that migraineurs with aura have an increased risk of ischemic stroke. The nature and mechanisms of elevated risk and whether the risk is modifiable are unclear, and the risk likely applies only to a subset of migraineurs. For obvious reasons, we cannot perform experiments to

dissect the mechanisms linking migraine and stroke in human subjects, and one has to employ experimental animal models to test hypotheses.

Based on available clinical and experimental data, one can postulate mechanisms to explain the association between migraine and ischemic stroke. The conceptual framework (Figure 1) is undeniably an oversimplification, but nevertheless allows one to generate hypotheses. These mechanisms are not mutually exclusive; they may be operational in different patients, or overlap in the same patient and interact to synergize. Some of the potential mechanisms are supported by clinical and experimental data, which we will discuss individually and elaborate on in specific subcontexts.

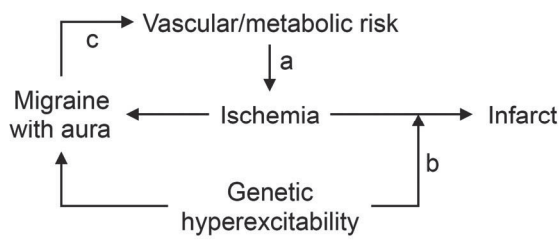


Figure 1. A conceptual framework for possible mechanisms linking migraine and stroke based on available direct or indirect clinical and experimental evidence. (**a**) Upstream vascular and metabolic risk factors for cerebral ischemia (e.g. endothelial dysfunction, cervical artery dissection, hypercoagulable states, paradoxical embolism, adverse lifestyle) can trigger migraine with aura when mild, and cause ischemic stroke when severe. (**b**) Upstream genetic risk factors that enhance susceptibility to spreading depression (SD) as a migraine trigger may sensitize the tissue to ischemia, so that infarction ensues with a milder degree of ischemia or ischemic injury progresses more rapidly. (**c**) Migraine with aura may increase vascular and metabolic risk of ischemia (e.g. migrainous infarction, vasoconstrictive complications of migraine medications, adverse lifestyle, endothelial dysfunction or platelet aggregability caused by migraine with aura).

Migrainous infarction. A first report of migrainous infarction goes back to the 19th century.[56] As defined by IHS, migrainous infarct is an ischemic infarct demonstrated by neuroimaging that develops during a typical migraine with aura attack with abnormally prolonged aura symptoms (>60 min), in a patient with a history of migraine with aura, in the absence of an alternative cause for the infarct.[2] Implicit in this definition is the fact that migrainous infarct is *caused by* the migraine attack. Therefore, migrainous infarction is by definition a diagnosis of exclusion, and depends on the depth of work-up for an alternative etiology.

The rarity (~0.5% of all ischemic strokes) of true migrainous infarction is further underscored in clinical series. A retrospective multicenter study spanning more than

20 years identified only 33 patients who fit the IHS criteria for migrainous infarct, almost half of the patients had no other vascular work-up than Doppler ultrasonography, and more than a third did not have transoesophageal echocardiography.[57] Moreover, 40% of patients indeed had a PFO. In a prospective study spanning 11 years, migrainous infarcts made up only 0.2% of all etiologic diagnoses; a third of migrainous infarcts was a first-ever aura attack (i.e. did not fulfill the IHS criteria), two-thirds had a PFO, and almost all had other vascular risk factors.[58] Moreover, detailed investigations revealed rare causes of stroke in almost a quarter of those originally diagnosed as migrainous infarction.

The diagnosis has become even more problematic after clinical and experimental evidence clearly showed that ischemic events can trigger symptomatic migraine attacks, as well as a SD as the aura surrogate.[59-62] Hence, a primary ischemic event can cause prolonged neurological signs and symptoms that may be perceived as aura; therefore, migrainous infarct should not be diagnosed without an extensive work-up to exclude other causes. The condition is even more likely to be over-diagnosed in young patients, as 40% of ischemic strokes in the young remain cryptogenic.

Nevertheless, there may be a mechanistic basis for migraine aura culminating in infarction. SD (i.e. aura) has profound metabolic and hemodynamic effects on the brain tissue. While adenosine triphosphate (ATP), O_2 and glucose consumption increase dramatically during and after SD, substrate delivery via blood flow may not be sufficient, thereby creating supply-demand mismatch.[63-65] Although SD by itself is not injurious in otherwise healthy brain tissue,[66] under certain conditions, SD may be associated with abnormal neurovascular coupling, causing marked and prolonged vasoconstriction (i.e. inverse coupling) and severely limiting tissue O_2 delivery.[67,68] For example, reduced nitric oxide levels when combined with mildly elevated extracellular K^+ or low glucose leads to constriction and laminar cortical necrosis during SD.[69,70] In theory, such ionic disturbances may even be precipitated by endothelial ion channel or pump dysfunction.[71] However, whether genetic, hormonal or environmental factors can modulate the hemodynamic and metabolic response and create severe supply-demand mismatch during SD to precipitate infarction in otherwise healthy brain remains to be proven. At least in MELAS, such a mechanism is possible (see below).

Migraine drugs predisposing to ischemic events. Another potential mechanism one can postulate to explain increased stroke risk in migraineurs is exposure to high-risk external factors linked to migraine. For example, drugs frequently used in the management of migraine may increase stroke risk. Indeed, triptans and ergotamine have vasoconstrictive properties, and are not recommended in patients with complicated migraine with aura due to the associated oligemia. Moreover, these drugs, as well as serotonin reuptake inhibitor antidepressants, can precipitate reversible cerebral vasoconstriction syndrome (RCVS), a clinical and radiological

syndrome characterized by severe unusual headache and transient multifocal cerebral vasoconstriction that can lead to stroke, subarachnoid hemorrhage and/or brain edema.[72] Interestingly, migraine is more common in RCVS patients even in the absence of an offending drug treatment,[72] suggesting that other factors also play a role.

However, large studies have not substantiated a link between triptan use and the risk of stroke in migraineurs (e.g. General Practice Research database, HR 1.13, 95% CI 0.78-1.65; United Healthcare database, OR 0.90, 95% CI 0.64-1.26).[73,74] A large, nested case-control study of cardiovascular events in triptan overuse also did not show a significant increase in the risk of hospitalization for ischemic events (OR 1.43, 95% CI 0.82-2.49).[75] And lastly, MR angiography has recently failed to show vasoconstriction in intracranial arteries upon triptan administration, despite vasoconstriction of extracranial vessels.[76] Ergotamine did increase the risk of hospitalization for cardiovascular and cerebral ischemic events (OR 2.55, 95% CI 1.22-5.36); however, this occurred only in patients with other cardiovascular comorbidities who overused the drug,[75] and was not confirmed in other studies (OR 1.49, 95% CI 0.93-2.41).[74] Moreover, ergotamine has not been a commonly used drug clinically. It should also be noted that the association between migraine and stroke is mainly driven by migraine with aura, while migraine drugs are used by all migraineurs regardless of aura status. For these reasons, migraine drug use is unlikely to explain the increased stroke risk in migraine, unless there is a specific interaction with aura.

Migraine predisposing to ischemic events indirectly through lifestyle changes. Migraine can have profound effects on a patient's mood and lifestyle, and may indirectly augment behavior patterns posing a vascular risk. For example, obesity has been associated both with episodic and chronic migraine, mostly in patients with high attack frequency,[77-79] although the direction of causality is not clear. Migraine could predispose to obesity, as women with a history of childhood migraine have a higher risk of weight gain later in life.[80] In theory, obesity may be a side effect of migraine treatments, or due to reduced physical activity to avoid triggering or exacerbating migraine attacks, although regular exercise could also reduce migraine attacks.[81] Indeed, migraineurs also exercise less, as yet another factor that can increase cardiovascular risk.[82] Conversely, obesity may predispose to migraine through hormones released from the adipose tissue, such as adiponectin.[83] Finally, migraine and obesity may also be linked by a shared underlying disorder, such as abnormalities in hypothalamic function or serotonergic transmission.[84] Regardless of the causal relationship, obesity and associated metabolic syndrome are well-known stroke risk factors, and may also link migraine with coronary and peripheral artery disease.[17,22,85]

Indeed, the Genetic Epidemiology in Migraine study, a population-based, cross-sectional study including 620 migraineurs and 5135 non-migraineurs, showed that patients with migraine, in particular with aura, are more likely to smoke and have

unfavorable cholesterol profiles and higher Framingham risk scores than non-migraineurs.[86] This was corroborated by the American Migraine Prevalence and Prevention study, a case-control study including 6102 patients and 5243 controls, which showed that migraineurs were more likely to have vascular risk factors and higher Framingham scores.[22] The lower consumption of alcohol by migraineurs,[82,87] probably avoidance due to its ability to induce attacks, could also increase stroke risk in migraineurs by the loss of its vascular protective effect when consumed in moderate amounts. Moreover, migraineurs are more likely to smoke,[82,86] or use hormone therapy after menopause,[29] as additional risk factors. Lastly, migraine and depression are linked, and although bidirectional causality has been suggested,[88] recent data from the WHS suggest that migraine (as well as non-migraine headache) increases the risk of incident depression in middle-aged women (relative risk 1.48, 95% CI 1.37-1.60), particularly in individuals with high attack frequency.[89] Depression is a risk factor for stroke,[90] and may contribute to increased stroke risk in migraineurs. Based on these data, one might speculate that often-modifiable lifestyle factors may add to increased stroke risk in migraineurs.

However, large-scale epidemiological studies have also shown that stroke risk in migraineurs is independent of vascular risk factors.[10,16,17] For example, the WHS revealed that the association between active migraine with aura and ischemic stroke was apparent only among women in the lowest Framingham risk score group.[87] Likewise, the Italian Project on Stroke in Young adults Study showed lower vascular risk factor profile in migraineurs with stroke, and the Stroke Prevention in Young Woman study showed that women without a history of hypertension, diabetes or myocardial infarct were at greatest risk of migraine-associated stroke.[19,21] Moreover, large-artery atherosclerosis as a stroke mechanism did not differ between migraineurs and controls. Lastly, no correlation was found between migraine status and atherosclerosis markers (e.g. intima-media thickness, pulse wave velocity and ankle-brachial index) in a large case-control study including 617 controls and 360 migraineurs,[91] further arguing against a role for atherosclerosis as the link between migraine and stroke. Unfortunately, there are no experimental data to support or refute these potential mechanisms.

Migraine and cervical artery dissection. Cervical artery dissection is a well-known cause of stroke, particularly in the young population. A meta-analysis of case-control studies has shown that migraineurs have an increased risk of cervical artery dissection with an OR of 2.06 (95% CI 1.33-3.19),[92] later confirmed in a larger international multicenter study consisting of 968 stroke patients with dissection and 653 stroke patients without dissection (Cervical Artery Dissection and Ischemic Stroke Patients study; OR of 1.51, 95% CI 1.15-1.99).[93] In a recent series, 60% of patients who presented with concurrent cervical artery dissection and reversible cerebral vasoconstriction syndrome were migraineurs.[94] The mechanisms responsible for cervical artery

dissection are poorly understood,[95] as is the causal relationship with migraine. Of course, ischemia due to embolism or hemodynamic insufficiency precipitated by a dissection can directly trigger a migraine attack,[59] as well as stroke. In this context, migraine attacks can be considered TIAs. It may be speculated that endothelial injury and microdissections are not infrequent in everyday life and most go unnoticed. A small hemodynamically insignificant dissection invisible by routine imaging tools can still activate platelet aggregation and serotonin release, which may increase the likelihood of a migraine attack without causing stroke.[96] However, absence of a predilection for migraine with aura is not fully congruous with an ischemic trigger for migraine in this setting. Conversely, migraine may increase the risk for cervical artery dissection, for example, by inducing matrix metalloproteinase (MMP) expression,[97] the levels of which are elevated in migraineurs both ictally and interictally,[98] and in patients with cervical artery dissection.[99] Indeed, a significant association has been demonstrated between migraine, especially with aura, and interictal serum elastase activity.[100] Therefore, chronic (i.e. ictal and interictal) elevation of MMP and elastase levels in migraineurs may perhaps cumulatively weaken the arterial wall to predispose to dissections upon mild traumatic insults. Although unrelated to dissections, a curious relationship has been reported between anatomical variations in the posterior circle of Willis (i.e. fetal posterior cerebral artery origin or basilar hypoplasia) and migraine with or without aura.[101] Although this is an interesting concept, the data have been inconsistent among studies and remain to be replicated.[102,103]

Hypercoagulability and endothelial dysfunction. Endothelial dysfunction has also been suggested as a possible link between migraine and stroke.[104] Studies of vascular reactivity as a surrogate for endothelial function in migraineurs have revealed conflicting results. In peripheral arteries vascular reactivity was reported decreased[105-107] or unchanged[108-111] in different studies. Similarly, cerebrovascular reactivity was either increased[112-114] or decreased.[115-117] Further complicating the issue, cerebral and peripheral vascular reactivity do not always correlate,[109] anterior and posterior circulation often differ in reactivity readouts,[118] and subject selection (e.g. aura status, exclusion of vascular comorbidities) and the method used to assess reactivity are highly variable among studies. Biomarker studies also suggest endothelial dysfunction. Migraineurs have higher interictal levels of circulating t-PA, high-sensitivity C-reactive protein, von Willebrand factor, vascular endothelial growth factor and nitric oxide metabolites, some only in migraineurs with aura.[110,119] Likewise, the number of circulating endothelial progenitors cells, believed to repair injured endothelium, is lower in migraineurs.[110,120] It is not known whether migraine and stroke are both facilitated by the underlying endothelial dysfunction, or whether migraine can facilitate endothelial dysfunction as a stroke risk factor. Overall, the clinical relevance of endothelial dysfunction and its role linking migraine and ischemic stroke, if any, remain to be tested. The association

between migraine and livedo reticularis (e.g. Sneddon's syndrome) is interesting to note in the context of vascular endothelial dysfunction.[121]

Data on acquired or inheritable hypercoagulable states in migraineurs have also been conflicting and not always replicated. In a small early study consisting of 35 migraineurs and 24 controls, elevated prothrombin fragment 1.2 levels in migraineurs with aura suggested activation of clotting cascade.[122] Some studies suggested a hypercoagulable state in migraineurs,[123,124] while others failed to show a difference from controls.[125-129] Low sample size, varying from 20 to 276 participants, has been a major limitation. Studies on specific markers of acquired or inherited hypercoaguble states have been more conclusive. A recent study in 1456 women (mean age 34) with a personal or familial history of venous thrombosis has shown an increased risk of migraine with aura in carriers of factor V Leiden or factor II G20210A mutations (OR 1.76, 95% CI 1.02-3.06), strongly supporting an association between thrombophilia and migraine.[130] Moreover, a higher prevalence of hypercoaguble state was found in stroke patients with migraine when compared to non-migraineurs, providing further support to the association.[21,131] Other causes of hypercoagulable states, such as antiphospholipid syndrome, systemic lupus erythematosus and polycythemia, have also been linked to migraine with aura in case-control studies, small case series and anecdotal reports; however, in the absence of robust datasets, some of these associations may be spurious.[132-134] Lastly, data from two studies consistently showed that methylenetetrahydrofolate reductase (*MTHFR*) 677 TT genotype combined with migraine aura confers an increased risk of ischemic stroke (OR 1.81, 95% CI 1.02-3.22, and HR 4.19, 95% CI 1.38-12.74).[135,136] The *MTHFR* TT genotype appears to increase the risk of migraine with aura, whereas angiotensin-converting enzyme II genotype decreases the risk of any migraine,[137] as other potential shared genetic modulators of stroke risk.

Paradoxical embolism predisposing to migraine and ischemic events. PFO is an incomplete closure of the fetal communication between right and left atrium that can serve as a conduit for circulating particulate and chemical substances to bypass pulmonary circulation, and reach the brain unfiltered. Classical epidemiological data have linked migraine and PFO. A meta-analysis of case-control studies have demonstrated an increased risk for migraine in patients with PFO (OR 5.13, 95% CI 4.67-5.59) and an increased risk for PFO in migraineurs (2.54, 95% CI 2.01-3.08); the association is even stronger in migraine with aura.[138] Furthermore, a study in 20 families with PFO and migraine with aura has suggested an autosomal pattern of inheritance of atrial shunts that in some families could be linked to inheritance of migraine with aura.[139] Highly promising anecdotal data from retrospective open-label studies on the efficacy of PFO closure in reducing migraine attack frequency, such as headache resolution in up to 80% of patients,[138] particularly in patients who experience a migraine attack during PFO closure,[140] paved the way for the Migraine Intervention

with STARFlex Technology trial (MIST). MIST randomized 147 migraineurs with aura and a PFO with moderate to large right-to-left interatrial shunt, to PFO closure or sham procedure in a double-blind fashion. The primary and most secondary outcome measures did not differ between treatment and sham arms, effectively MISTifying the field, and hampering forward progress. However, the MIST trial included only patients with highly frequent attacks (~5 attacks and five to 23 headache days/month) who had previously failed at least two prophylactic treatments.[141] Hence, most patients had intractable migraines unresponsive to multiple therapeutic trials; therefore, inclusion criteria were chosen not as an indication but a justification for an invasive procedure. Although this was probably not the best cohort in which to test the efficacy of an experimental intervention, the results of a recent meta-analysis of well-designed, unbiased studies also questioned the existence and strength of an association between migraine and PFO.[142]

Nevertheless, PFO is clearly linked to cryptogenic stroke,[143] and may act as a source of arterial micro-emboli or chemical offenders. For example, injection of sclerosing agents to treat varicose veins triggered a migraine with aura attack in a subset of individuals, almost all of whom harbor a PFO.[144,145] Moreover, intravenous injection of agitated saline with air microbubbles has been reported as a migraine trigger in patients with a PFO and large right-to-left shunt.[146,147] Besides triggering an attack, microbubbles can also alter cerebral electrical activity in migraineurs with aura and a PFO, but not in migraineurs with aura without a PFO or in patients with a PFO but no migraine history,[148] perhaps suggesting that microvascular hypoxia/ischemia may induce cortical SD in a susceptible set of patients.

Indeed, the principle that microemboli can trigger SD as an aura surrogate without causing ischemic injury has been tested and proven in the experimental setting. Intracarotid infusion of particulate material of various size and compositions (e.g. cholesterol particles of <70 μm, polystyrene microspheres of 5-20 μm), as well as air microbubbles (10 μl), reliably evoked cortical SD in mice.[60] The mechanism involved transient cerebral hypoperfusion as shown by real-time, fullfield blood flow imaging using laser speckle flowmetry. The probability of SD induction related to the duration and severity of hypoperfusion, which in turn was determined by the size and composition of the emboli. When microembolic hypoperfusion reached a threshold severity and duration, an SD occurred. In more than half the animals that developed an SD, no ischemic injury was detected by a meticulous examination of serial histological sections throughout the brain, as well as by MRI. This study suggests that in a subset of migraineurs, transient cerebral ischemia induced by microemboli may be responsible for triggering a migraine attack, and when ischemia is severe enough, triggering an ischemic stroke. In humans, PFO, as well as pulmonary arteriovenous malformation such as in hereditary hemorrhagic telangiectasia, and atrial myxoma,[149] may serve as a

source of microembolism. Of course, microembolism is unlikely to be the trigger for every attack in all migraineurs with aura and a right-to-left shunt.

Right-to-left intracardiac shunts may also allow neuroactive and/or vasoactive chemicals to access the brain. One such chemical is endothelin, plasma levels of which are elevated during a migraine attack.[150-153] Interestingly, endothelin A receptor polymorphisms have been shown to modulate migraine risk.[154] Although air microembolism has been hypothesized as a mechanism for attacks of migraine often with aura after foam sclerotherapy of varicosities,[144,145] attacks were also triggered after liquid sclerotherapy, which does not predispose to air embolism. An alternative hypothesis is endothelin release from the irritated endothelium during sclerotherapy into the venous circulation gaining access to the brain through a right to left shunt.[155] This is supported by elevated plasma endothelin concentrations after sclerotherapy in rats, regardless of whether liquid or foam sclerosing agent was used.[155,156] Similar plasma endothelin elevations have also been shown in humans after foam sclerotherapy.[156] Endothelin 1 indeed potently triggers SD in rats through endothelin A receptors,[157,158] and the mechanism involves severe vasoconstriction and hypoperfusion, leading to infarction, providing a direct link between migraine and ischemic stroke.[159] Altogether, these data suggest that right-to-left shunts are associated with migraine and can explain a subset of ischemic strokes in migraineurs.

Increased sensitivity to ischemic injury. An alternative hypothesis to explain a migraine-stroke association is that cerebral hyperexcitability phenotype associated with migraine sensitizes the tissue to ischemia. Recent experimental data in mice expressing familial hemiplegic migraine (FHM) type 1 mutations provided direct support for such a mechanism. FHM is an autosomal-dominant subtype of migraine with often severe and prolonged auras associated with motor deficits, sometimes accompanied by sensory, aphasic, visual and basilar symptoms. A third of patients can experience a decrease in level of consciousness and even coma, which may be prolonged.[160] FHM has been used as a model for more common forms of migraine with aura because of shared clinical features and trigger factors, female preponderance, and because two-thirds of FHM patients and their first-degree relatives also suffer from attacks of common migraine with and without aura.

FHM type 1 is caused by mutations in the pore-forming α_{1A} subunit of $Ca_v2.1$ voltage-gated Ca^{2+} channels, which are critical for presynaptic glutamate release. Mutant channels open on smaller membrane depolarizations and stay open longer. The net result is increased presynaptic Ca^{2+} entry and glutamate release, resulting in enhanced cerebral excitability,[161] a mechanism likely shared with more common forms of migraine.[162] Indeed, FHM1 mutants are highly susceptible to SD and frequently develop subcortical and re-entrant SDs with prolonged neurological deficits mimicking those observed in FHM patients.[163,164]

Availability of transgenic mouse models expressing FHM1 mutations has recently allowed testing of the hypothesis that the cerebral hyperexcitability phenotype associated with migraine sensitizes the brain tissue to ischemia. In support of the hypothesis, two FHM1 mutant mouse strains developed larger infarcts compared with wild type after transient focal cerebral ischemia.[165] The phenotype correlated well with the strength of gain-of-function of each mutation (S218L mutant more severe than R192Q), and showed an allele-dosage effect whereby homozygous mutants fared worse than the heterozygotes. The mechanism was indeed linked to hyperexcitability, because anoxic depolarization developed faster and diffusion-weighted MRI lesions grew rapidly after stroke onset in the mutants. Perhaps more important, mutants developed many more peri-infarct depolarizations (PIDs) during acute stroke. PIDs are SD waves spontaneously triggered within the ischemic penumbra, and expand the infarct by worsening the supply-demand mismatch, thereby explaining the accelerated infarct growth in migraine mutants, as well as in humans.[61,166] The data also showed that migraine mutants had an elevated minimum cerebral blood flow threshold required for tissue survival, directly supporting the hypothesis that brain tissue in migraineurs is more susceptible to ischemic injury. The study also showed that female mutants, which are even more hyperexcitable and susceptible to SD than males, developed larger infarcts and worse outcomes after ischemic stroke than wild-type controls, consistent with the clinical observations in women with migraine. Lastly, ischemic brain swelling appeared to be more severe in mutants and could explain the higher mortality in the S218L mutant strain.[165]

Altogether, these data suggested that familial migraine mutations (e.g. FHM1) that are known to augment cerebral excitability also facilitate infarction if and when the tissue becomes ischemic, by predisposing to frequent ischemic depolarizations. Of course, whether the mechanism is valid in other monogenic migraine disorders or in sporadic migraine remains to be tested. The hyperexcitability phenotype in migraine appears to also enhance susceptibility to SD, which is likely the final common mechanism for tissue sensitization to ischemia. In this context, it is notable that CADASIL mutant mice (Notch3^{R90C}) as well as Notch3 null mutants show markedly enhanced SD susceptibility.[167] Indeed, both Notch3 null mice and transgenic mice expressing CADASIL mutations develop larger infarcts, and at least in the null mutants, this is associated with an increased frequency of PIDs.[168,169] Although it is not clear how the mutations in the *NOTCH3* gene, which is exclusively expressed in vascular smooth muscle cells in the adult brain, lead to cerebral hyperexcitability, the data strongly support the notion that enhanced SD susceptibility translates into susceptibility to ischemic infarction, which is consistent with the stronger epidemiologic association observed between stroke and migraine with aura compared with without aura. It is important to note that aside from the hyperexcitability, CADASIL mutations clearly

lead to cerebral small-vessel disease and vascular dysfunction,[36] and increase the risk of occurrence of ischemic events and chronic cerebral hypoperfusion (e.g., lacunar infarcts, white matter disease), in addition to the risk of developing infarction during those events. In other words, mechanisms leading to lacunar infarcts and white matter disease may be different from those leading to hyperexcitability. This is further supported by the observation that the onset of migraine with aura attacks often precedes the emergence of clinical and radiological evidence of ischemic vascular disease in CADASIL, and in fact, attacks usually diminish in frequency and disappear as the ischemic lesion load increases. It remains to be tested whether other mutations clinically associated with small-vessel disease and migraine, such as *TREX1* and *COL4A1*, also augment SD susceptibility and sensitize the brain tissue to ischemic injury. Transgenic mice expressing mutations in these genes have been developed, and at least in case of *COL4A1*, mutants develop ultrastructural changes in cerebral vasculature, endothelial and smooth muscle cell dysfunction, and blood pressure regulation.[170]

And lastly, MELAS is another disease in which migraine and stroke-like episodes coexist on a spectrum. Impaired mitochondrial oxidative phosphorylation in MELAS creates a chronic state of energy shortage and inability to match increased demand, proposed as a potential mechanism linking hyperexcitability and sensitivity to ischemic injury.[171,172] Other mechanisms may also be involved, such as accumulation of dysfunctional mitochondria in endothelium and smooth muscle cells of small cerebral vessels leading to endothelial dysfunction and increased capillary permeability.[173,174] In the absence of representative animal models, whether the hyperexcitability is in any way related to SD remains to be tested. One can perhaps speculate that inadequate mitochondrial response to increased energy demand during SD impairs SD recovery, and markedly prolongs the depolarization predisposing to tissue injury.

CLINICAL IMPLICATIONS

Migraine as a symptom of TIA: Angina cephalis? As noted above, clinical and experimental evidence suggests that migraine attacks can be triggered by transient cerebral ischemic events (i.e. symptomatic migraine), and as such, they may carry a similarly elevated risk of impending stroke and should perhaps be treated as a TIA.

Is antithrombotic stroke prophylaxis indicated in migraineurs? If migraine with aura is a TIA, antithrombotic use may not only diminish the stroke risk but also reduce the frequency of migraine attacks. A similar argument can be made for PFO closure, or for inhibitors of potential vasoactive or neuroactive mediators presumed to pass through a PFO into arterial circulation unfiltered by the pulmonary circulation and predispose to migraine attacks (e.g. endothelin). Although, failure to meet the primary efficacy

endpoints in the MIST trial rendered PFO closure in migraine highly controversial, for reasons discussed above (i.e. not the best cohort in which to test an investigational intervention, primary endpoints difficult to meet), we believe the evidence is not sufficient to support or refute the hypothesis at this time. The critical challenge will be to identify the best closure candidates in whom the PFO is symptomatic.

Is aggressive avoidance of exacerbating factors (e.g. oral contraceptive use, smoking, risk of traumatic dissection) indicated in migraineurs? Along the same lines of reasoning as above, one might argue that given the geometrically increased risk of stroke by the presence of aggravating factors in highly susceptible migraineurs, such factors should be a part of aggressive risk management. For example, the World Health Organization recommends women with migraine with aura avoid combination oral contraceptives. Perhaps the warning against chiropractic cervical manipulations[175,176] should be particularly strong in migraineurs. Once again, reduced risk for ischemic events as a migraine trigger may also decrease the frequency of migraine attacks.

Is complete screening for vascular risk factors (e.g. hypercoaguble state, PFO, genetic mutations) indicated in migraineurs in the absence of a stroke? Migraine with aura as a potential TIA, particularly when the diagnosis of migraine does not fulfill IHS criteria (e.g. first attack, atypical aura, abnormal neurological exam), when the aura is always on the same side, and in late-onset migraines or when the attack frequency or characteristics changes, might also justify searching for common stroke etiologies as part of the clinical work-up. The approach may also affect migraine classification.

Migraine as a hyperexcitable state that sensitizes the brain to ischemic injury: The tissue factor. The clinical and experimental evidence reviewed above suggests that migraine with aura reflects a hyperexcitable state that enhances susceptibility to SD as a final common mechanism. Experimental evidence also suggests that susceptibility to develop SD is related to the susceptibility to develop injury if and when the brain tissue becomes ischemic. Because aura (perceived or not) is the relevant risk biomarker, the frequency of attacks with aura, rather than the total frequency of attacks only rarely accompanied by aura, appears to be the critical determinant. It should be noted, however, that data supporting a direct link between SD susceptibility and injury susceptibility come exclusively from transgenic animal models of monogenic, familial forms of human migraine, and whether the principle applies to non-familial forms of migraine is not known.

Is therapeutic time window for revascularization in ischemic stroke shorter in migraineurs? In FHM1 mutant mice, the diffusion-weighted MRI lesion (i.e. ischemic core) expanded rapidly because of enhanced susceptibility to anoxic depolarization and PIDs.[165] If hyperexcitable brain tissue in migraineurs readily develops ischemic

depolarizations, then the core expands into the perfusion defect leading to rapid loss of viable tissue at risk (i.e. penumbra). This would effectively shorten the therapeutic time window of efficacy for stroke therapies in migraineurs.

Does migraine prophylaxis diminish the stroke or white matter lesion risk or severity in migraineurs? Migraine prophylaxis suppresses SD susceptibility,[177] and therefore, may also diminish susceptibility to ischemic injury by rendering the tissue more resistant to ischemic depolarizations. This approach to reduce tissue sensitivity to ischemia could also be efficacious in non-migraineurs as well.

Is there a clinicopathological disconnect during acute stroke in migraineurs? PIDs are indistinguishable from SD waves when they propagate into the non-ischemic brain tissue, but they nevertheless cause transient electrophysiological silence in the tissue they invade, and can cause neurological deficits much like the aura symptoms during migraine attacks. If migraineurs develop more frequent PIDs, they may exhibit more severe and perhaps fluctuating neurological deficits than what would be expected based on the size and location of the ischemic lesion as seen on MRI. The concept may be true in other brain injury states as well, such as subarachnoid or intracerebral hemorrhage, and trauma, in which injury depolarizations akin to SD are known to occur in the human brain.

Is malignant brain edema more frequent in migraineurs? It has been shown that SD disrupts the blood-brain barrier.[97] Hence, frequent PIDs may be associated with more severe blood-brain barrier breakdown and ischemic edema formation after stroke, which can even become life threatening in case of large middle cerebral artery or cerebellar infarcts. If so, early decompressive craniectomy may be indicated in migraineurs with aura.

CONCLUDING REMARKS

Migraine is a stroke risk factor with an effect size and prevalence comparable to other risk factors. Its recognition as an important and perhaps modifiable vascular risk factor, sought and documented as part of the patient's medical history, will certainly be facilitated by better definition of the mechanisms of this association and the causal relationships. Unfortunately, current diagnostic criteria do not distinguish patients with occasional vs. frequent auras,[2] a distinction that is relevant for stroke risk and should be documented. With increased awareness, management of migraine will become more than management of a headache disorder, and more holistic as with other vascular risk factors. Of course, enough evidence to change practice can be achieved only in large prospective clinical studies targeting mechanisms and in therapeutic trials.

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CHAPTER 5

GENERAL DISCUSSION

Altogether, the data presented in this thesis suggest that familial hemiplegic migraine mutations (e.g., FHM1) augment cerebral excitability and cortical and subcortical SD susceptibility, modulated by gonadal hormones. By this way genetic susceptibility to migraine facilitates cerebral infarction by predisposing to ischemic depolarizations, if and when the tissue becomes ischemic. Moreover, pharmacological suppression of SD susceptibility has the opposite effect. Therefore, the hyperexcitability and enhanced SD susceptibility phenotype may be the final common determinant of tissue sensitization to ischemia in migraineurs. Retrospective clinical data suggest that the mechanism is not exclusive to FHM1, and that migraineurs in general population are also more sensitive to ischemic injury.

GENETIC CONTROL OF SD SUSCEPTIBILITY

In vivo and *in vitro* studies strongly suggest that cerebral hyperexcitability in FHM1 stems from augmented release of excitatory neurotransmitters causing an imbalance between the excitatory and inhibitory synaptic transmission.[1] In the context of migraine with aura, cerebral hyperexcitability also includes enhanced SD susceptibility. Other monogenic conditions associated with a migraine with aura phenotype also enhance SD susceptibility. For example, FHM2 mutations in the *ATP1A2* gene encoding the α2 subunit of the Na,K-ATPase have been shown to lower the electrical threshold for cortical SD induction, and increase the SD propagation speed.[2] It is known that the α2 subunit is exclusively expressed in astrocytes,[3] at least in adults, and that enhanced SD susceptibility is probably a result of impaired K^+ and/or glutamate uptake by astrocytes.[2] Interestingly, however, the duration of SD was not prolonged in FHM2 mutant mice, a shared phenotype with the FHM1 mutants,[4-9] suggesting that K^+ uptake is not impaired in the mutants, and that enhanced synaptic glutamate concentrations may be the final common mechanism for both FHM models, due to increased release in FHM1 and impaired clearance in FHM2.[2] Glutamatergic mechanisms and hyperexcitability also are implicated in common forms of migraine by recent studies linking *AEG1*, encoding a regulator of glial glutamate transporter EAAT2, and *KCNK18*, encoding the TRESK potassium channel, to migraine.[10-12]

Increased SD susceptibility has been demonstrated in two other diseases characterized by migraine with aura. Cerebral autosomal dominant arteriopathy, subcortical infarcts and leukoencephalopathy (CADASIL) is the most common cause of monogenic inherited small vessel disease, and although the clinical features are dominated by lacunar infarcts, white matter degeneration and progressive subcortical dementia, migraine with often severe atypical aura (reminiscent of FHM) is the earliest symptom in the disease for which patients seek medical attention in their late teens and twenties.[13,14] CADASIL is caused by mutations in the *NOTCH3* gene, exclusively expressed in vascular smooth muscle cells in the adult brain.[15,16] Despite this, CADASIL

mutations, when introduced in a transgenic overexpressor mouse model, also enhance SD susceptibility; electrical threshold is lowered and the frequency of KCl-induced SDs as well as the propagation speed are increased.[17,18] Interestingly, SD susceptibility did not differ between male and female CADASIL mutants, suggesting that mutation-sex interaction is gene-specific. Therefore, CADASIL as a vascular disease provides an interesting overlap between migraine and stroke as proof-of-concept for SD being the final common determinant of sensitivity to ischemic injury in migraineurs.

More recently, another monogenic condition, familial advanced sleep phase syndrome (FASP), was shown to enhance SD susceptibility. Patients with FASP show severe disruption of the sleep-wake-cycle and other circadian rhythms. The disease is caused by missense mutations in *CSNK1D*, encoding casein kinase Iδ (CK1δ) which is involved in the phosphorylation of the circadian clock protein Per2.[19] A pathogenic *CSNK1D* mutation co-segregated in nine of 11 carriers with FASP and migraine with aura. In a transgenic overexpressor mouse model expressing a FASP mutation, the threshold volume of topically applied concentrated KCl to trigger an SD was lower, and the frequency of KCl-induced SDs was higher compared with the wild-type.[20] Altogether these data complement ours by showing that genetic modulation of SD susceptibility is a key determinant for a migraine with aura phenotype.

INCREASED SENSITIVITY TO ISCHEMIC INJURY AFFORDED BY MIGRAINE MUTATIONS

To explain the migraine-stroke association, we hypothesized that cerebral hyperexcitability (i.e., enhanced SD susceptibility) in migraineurs sensitizes the tissue to ischemia. In support of the hypothesis, two FHM1 mutant mouse strains developed larger infarcts compared with wild-type after transient focal cerebral ischemia[21] (**Chapter 3A**). The phenotype correlated well with the strength of gain-of-function of each mutation (S218L mutant being more severe than R192Q), and showed an allele-dosage effect where homozygous mutants fared worse than the heterozygotes. The mechanism was indeed linked to hyperexcitability, because anoxic depolarization developed faster and diffusion-weighted MRI lesions grew rapidly after stroke onset in the mutants, compared with the wild-type. Perhaps more importantly, mutants developed many more PIDs during acute stroke. As described in the General Introduction of this thesis, PIDs are SD waves spontaneously triggered within the ischemic penumbra, and expand the infarct by worsening the supply-demand mismatch,[22-24] thereby explaining the accelerated infarct growth[25] in migraine mutants, as well as in humans.[26] As a result of the SD susceptibility phenotype, migraine mutants required a higher level of minimum cerebral blood flow for tissue survival (i.e., the viability threshold),[27] directly supporting the hypothesis that brain tissue in migraineurs is more susceptible to ischemic injury (**Chapter 3A**). The data

also showed that female mutants, which are even more hyperexcitable and susceptible to SD than males (**Chapter 3A**), developed even larger infarcts and worse outcomes after ischemic stroke compared with wild-type females, consistent with the clinical observations of higher stroke risk in women with migraine.[28]

MIGRAINE AS A HYPEREXCITABLE STATE THAT SENSITIZES THE BRAIN TO ISCHEMIC INJURY: "THE TISSUE FACTOR"

Clinical and experimental evidence suggests that migraine with aura reflects a hyperexcitable brain state that enhances susceptibility to SD as a final common mechanism. Experimental evidence presented in this thesis indicates that susceptibility to develop SD is related to the susceptibility to develop injury if and when the brain tissue becomes ischemic. Because aura (perceived or not) is the relevant risk biomarker, the frequency of attacks with aura, rather than the total frequency of attacks only rarely accompanied by aura, is likely to be the critical determinant. In the field of stroke, we often focus on the severity and duration of perfusion defect as the final determinant of tissue outcome. In other words, we assume that given identical perfusion defects the outcome of ischemia will be the same between two brains; therefore, we ignore the intrinsic sensitivity of the brain to ischemia. Data presented in this thesis directly challenges the concept and underscores tissue sensitivity to ischemic injury as a genetic and modifiable stroke risk factor.

CAN WE EXTRAPOLATE THESE FINDINGS TO OTHER MONOGENIC CONDITIONS?

The data supporting a direct link between SD susceptibility and injury susceptibility in this thesis are exclusively obtained from a transgenic mouse model of a monogenic, familial form of human migraine (FHM1). Therefore, whether the principle applies to other monogenic conditions is not known. CADASIL (Cerebral Autosomal Dominant Arteriopathy with Subcortical Infarcts and Leukoencephalopathy) is another monogenic disease (mutations in the *NOTCH3* gene) associated with migraine with often severe and prolonged auras. It is notable that transgenic overexpressor CADASIL mutant mice ($Notch3^{R90C}$) as well as *Notch3* null mutants show markedly enhanced SD susceptibility,[17] and both *Notch3* null mice and the transgenic overexpressor mice develop larger infarcts, and at least in the null mutants, this is associated with an increased frequency of PIDs,[29,30] strongly supporting the notion that enhanced SD susceptibility translates into enhanced susceptibility to ischemic infarction. Although it is not clear how the mutations in the *NOTCH3* gene, which is exclusively expressed in vascular smooth muscle cells and pericytes in adult brain, lead to cerebral hyperexcitability, the data are consistent with the stronger epidemiologic association observed between stroke and migraine with aura compared to without aura. It is

important to keep in mind that CADASIL mutations lead to cerebral small vessel disease and vascular dysfunction,[13] and increase the risk of occurrence of ischemic events and chronic cerebral hypoperfusion (e.g., lacunar infarcts, white matter disease), and that mechanisms leading to lacunar infarcts and white matter disease may be different than those leading to hyperexcitability. This is further supported by the observation that the onset of migraine with aura attacks often precedes the emergence of clinical and radiological evidence of ischemic vascular disease in CADASIL, and in fact, migraine with aura attacks usually diminish in frequency and disappear as the ischemic lesion load increases. The latter may be explained by progressive neurodegeneration and gliosis in CADASIL,[13] since both of these factors are expected to decrease SD susceptibility.

Last but not the least, future studies using mutant mouse models of a number of monogenic diseases in which migraine and stroke frequently co-exist may shed light into other aspects of the migraine-stroke association:

I. HIHRATL (Hereditary Infantile Hemiparesis, Retinal Arteriolar Tortuosity and Leukoencephalopathy) caused by mutations in the *COL4A1* gene encoding type IV collagen α1 chain destabilize the triple helix domain of collagen IV found in the basement membranes.[31,32]
II. RVCL (Retinal Vasculopathy with Cerebral Leukodystrophy) linked to mutations in *TREX1* gene,[33] which encodes a DNA-specific exonuclease implicated in DNA repair under conditions of oxidative stress.[34]
III. MELAS (Mitochondrial Encephalomyopathy, Lactic Acidosis and Stroke-like episodes) is caused by mutations in mitochondrial genome with maternal pattern of inheritance.[35-37]
IV. HHT (Hereditary Hemorrhagic Telangiectasia) caused by mutations of endoglin or activin receptor-like kinase 1.[38]

CAN WE EXTRAPOLATE THE FINDINGS TO NON-MONOGENIC MIGRAINE?

It is also not known to what extent the results obtained in this thesis apply to common (i.e., polygenic) migraine. However, the fact that clinical association between migraine with aura and stroke is demonstrated in the general population[28,39-46] without the contribution of monogenic conditions suggests that the mechanism is active in non-monogenic migraine as well. Indeed, data obtained in our retrospective clinical study of acute infarct growth in migraineurs (**Chapter 3C**) directly support this notion, and underscore the clinical relevance of our findings.[47] Unfortunately, because of the retrospective nature of our clinical study and relatively small sample sizes, we were unable to test the effect of modifiers such as active versus a remote history of migraine, and current use of migraine prophylactic drugs or antithrombotic prophylaxis in our cohort; this will

be the subject of future studies. However, because the average age in migraineurs and controls was ~60 years in our cohort, it is unlikely that active migraine was a significant determinant of ischemic injury progression, suggesting that the genetic propensity for migraine rather than whether the patient is suffering from active migraines was more important. Regardless, if brain tissue is more sensitive to ischemic injury in migraineurs with aura due to enhanced susceptibility to SD-like ischemic depolarization events, then the mechanism is operational in a very large population segment and may ultimately modify the clinical practice as discussed below.

IS THERAPEUTIC TIME WINDOW FOR REVASCULARIZATION IN ISCHEMIC STROKE SHORTER IN MIGRAINEURS?

In FHM1 mutant mice, diffusion-weighted MRI lesion (i.e., ischemic core) expanded rapidly because of enhanced susceptibility to anoxic depolarization and PIDs (**Chapter 3A**). This was indeed confirmed in our retrospective clinical study (**Chapter 3C**). If hyperexcitable brain tissue in migraineurs readily develops ischemic depolarizations, the core expands into the perfusion defect leading to rapid loss of viable tissue at risk (i.e., penumbra). This is a significant finding that would effectively shorten the therapeutic time window of efficacy for stroke therapies in migraineurs.

IS AGGRESSIVE STROKE PROPHYLAXIS INDICATED IN MIGRAINEURS?

Our data of **Chapters 3A, 3B and 3C** suggest that migraineurs are more susceptible to developing ischemic injury if and when they suffer from cerebral ischemic events. Although this does not mean they are at higher risk to develop cerebral ischemic events, their propensity to developing ischemic injury means that antithrombotic prophylaxis (e.g., acetyl salicylic acid)[48-51] and aggressive avoidance of risk factors (e.g. oral contraceptive use, smoking, risk of traumatic dissection)[48-51] may decrease the overall lesion burden by preventing the ischemic event in the first place. This can be tested clinically in different ways. For example, the relative risk of ischemic infarcts can be compared between patients on stroke prophylaxis and patients who are not in a migraine cohort. Alternatively, the efficacy of stroke prophylaxis can be tested in a prospective clinical trial comparing migraineurs with non-migraineurs. However, because the effect size is relatively small, in both cases very large cohorts will likely be needed to reach statistical power to demonstrate a differential effect in migraineurs. Instead, demonstration of faster acute infarct progression in migraineurs in a prospective study may provide sufficient justification to alter the practice and recommend antithrombotic prophylaxis in migraineurs. Along the same lines, a complete screening for vascular risk factors (e.g., hypercoaguble state, PFO, genetic

mutations)[48-51] may be indicated in migraineurs in the absence of a prior cerebral ischemic event, so that aggressive stroke prophylaxis can be instituted preemptively.

DOES MIGRAINE PROPHYLAXIS DIMINISH THE STROKE OR WHITE MATTER LESION RISK OR SEVERITY IN MIGRAINEURS?

Migraine prophylaxis suppresses SD susceptibility.[52] If susceptibility to SD (i.e., aura) is a critical biomarker of susceptibility to ischemic injury, then migraine prophylaxis can reduce susceptibility to ischemic injury by rendering the tissue more resistant to ischemic depolarizations. This was indeed the case in our experimental model (**Chapter 3B**). If so, migraine prophylaxis may be beneficial in reducing ischemic lesion burden in migraineurs, and be indicated even if the frequency and severity of migraine attacks are not severe enough to justify chronic prophylaxis. In other words, clinicians' threshold to start migraine prophylaxis may be lowered. This approach to reduce tissue sensitivity to ischemia could also be efficacious in non-migraineurs as well, as was the case in wild type mice in our study (**Chapter 3B**).

IS THERE A CLINICOPATHOLOGICAL DISCONNECT DURING ACUTE STROKE IN MIGRAINEURS?

PIDs are indistinguishable from SD waves when they propagate into the non-ischemic brain tissue[53-55], and as such, they cause transient electrophysiological silence in the tissue they invade. Consequently, PIDs cause neurological deficits much like the aura symptoms during migraine attacks, and worsen both the neurological examination and the clinical outcome.[56,57] Therefore, more frequent PIDs in migraineurs can lead to more severe and perhaps fluctuating neurological deficits than would be expected based on lesion size and location on MRI. We know from the work by COSBID collaboration using the standard subdural electrode strips placed around the lesion that numerous PIDs occur both in the acute stroke stage as well as many days after stroke onset.[58]

IS MALIGNANT BRAIN EDEMA MORE FREQUENT IN MIGRAINEURS?

In our experiments, ischemic brain swelling appeared to be more severe in mutants and could explain the higher mortality in the S218L mutant strain (**Chapter 3A**).[21] It has been shown that SD disrupts blood brain barrier[59]. Hence, frequent PIDs may be associated with more severe blood brain barrier breakdown and ischemic edema formation after stroke,[60] which can even become life threatening in case of large middle cerebral artery or cerebellar infarcts.[60,61] If so, early decompressive craniectomy may be indicated in migraineurs with aura with large hemispheric stroke.[62,63]

IMPLICATIONS FOR OTHER BRAIN INJURY STATES WHERE SDS OCCUR

Indeed, SD-like injury depolarizations are not limited to ischemic stroke,[55] but occur in other human brain injury states as well, such as subarachnoid or intracerebral hemorrhage,[56,64-67] and head trauma,[68-72] expanding the clinical relevance of transient or long-lasting neurological deficits caused by injury depolarizations (Figure 1). In this context it will be critical to test whether migraineurs with these brain injury states develop more frequent injury depolarizations, using subdural electrode strips. Of note, worse clinical outcomes have been reported in migraineurs after subarachnoid hemorrhage,[73] although this has not yet been linked to frequent injury depolarizations via direct electrophysiological monitoring.

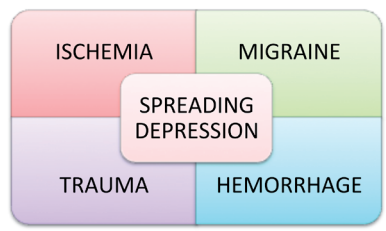


Figure 1. Spreading depression, and closely related injury depolarizations constitute a pathophysiological overlap between migraine, stroke, subarachnoid and intracerebral hemorrhage, and traumatic brain injury.

FUTURE RESEARCH

The data presented in this thesis provide proof-of-concept and pave the way for future work to be built upon them. Among many important aspects to be explored in the future are: 1) test whether the principle of genetically-enhanced SD susceptibility as a critical determinant of stroke outcome holds true in other genetic conditions as well; 2) test whether enhanced sensitivity to focal ischemic insults in FHM1 holds true for pure excitotoxic, hypoxic or global ischemic insults; 3) test whether repetitive SDs can lead to neuronal injury in the absence of energy shortage in genetically susceptible brains; and 4) clinical translation in prospective studies of acute stroke and stroke prophylaxis.

1) Is genetically-enhanced SD susceptibility a critical determinant of stroke outcome in other monogenic conditions? As noted above, there are several transgenic mouse models of monogenic conditions characterized by migraine with or without aura. These include FHM1,[21] CADASIL,[18] FHM2[2] and FASPS mutants.[20] Among these, larger

infarct volumes and worse neurological outcomes after stroke have already been shown in some CADASIL mutant mouse models (*Notch3* C455R and R1031C), albeit not in the same mutant (*Notch3* R90C) in which enhanced SD susceptibility was demonstrated.[17,18,29,30,74] Therefore, it will be important to study stroke outcomes in the *Notch3* R90C mutant, and test whether PID frequency and AD latency are increased, as is the case with FHM1 mutants. In the *Notch3* R169C mutant one could characterize both the SD and the PID phenotypes in this novel animal model.

2) Do FHM1 mutations sensitize against excitotoxicity, hypoxia or global forebrain ischemia? The data presented in this thesis clearly indicate that migrainous brain is more susceptible to *focal ischemic* injury. However, the pathophysiology of focal ischemic injury (i.e., ischemic stroke) differs from other forms insults such as direct excitotoxicity (e.g., glutamate, neurodegenerative conditions), hypoxia (e.g., drowning, suffocation) and global cerebral ischemia (e.g., cardiac arrest), in that the former involves PIDs. Therefore, we should test whether FHM1 mutations sensitize primary cortical neuronal cultures obtained from the mutants to glutamate excitotoxicity, high $[K^+]$ exposure or oxygen-glucose deprivation (OGD), *in vitro*.[75] It will be interesting to test whether FHM1 mutants develop more severe neurological signs and neuronal death after moderate to severe hypoxic challenge (e.g., 7% O_2 for 6 hours in a hypoxic chamber). And lastly, we should test whether bilateral common carotid occlusion for 20 minutes causes more severe neurological deficits and hippocampal cell death in FHM1 mutants. As with focal cerebral ischemia, we will also seek an effect of genotype (R192Q vs. S218L), allele-dosage (heterozygotes vs. homozygotes), gonadal hormones (male vs. female, and the effect of gonadectomy), as well as the age.

3) Does exposure to repetitive SDs cause cortical injury in FHM1 mutants? It is well known that SD, regardless of its numbers, is not injurious unless it occurs in the presence of energy shortage (i.e., hypoxia, hypoglycemia, ischemia).[76] However, FHM1 mutations are predicted to augment pre and postsynaptic Ca^{2+} influx and glutamate release,[77] and may therefore exert a stronger ionic and metabolic stress on neurons. Therefore, we should directly study intra-neuronal Ca^{2+} dynamics in FHM1 neurons using *in vivo* two-photon microscopy coupled with an intracellular Ca^{2+} indicator. The same set of studies will also allow morphological changes during SD to test whether dendritic beading or cell swelling differ between FHM1 mutants and wild-type controls. Furthermore, we should test whether chronic repetitive SDs cause injury in FHM1 brains, which has important clinical implications for FHM1 patients and possibly in classical migraine with aura as well.

4) Does acute stroke injury progress faster in migraineurs? The clinical data presented in this thesis (**Chapter 3C**) strongly suggest faster infract growth in migraineurs. However, retrospective design has severe limitations, particularly in documentation

of migraine status and medication use, and timing of the MRI scans after stroke onset. These weaknesses can only be addressed through a prospective design. Therefore, we should undertake a prospective study in acute stroke, where migraine status, type and features, migraine prophylactic medication use, family history of migraine, and many other relevant clinical data will be collected, and infarct growth will be studied using MRI scans performed within fixed time intervals. We can then confirm the data from the retrospective study (**Chapter 3C**), and get additional insight, for example, on whether migraine prophylaxis normalizes the accelerated infarct growth in migraineurs.

5) Do migraine prophylaxis and antithrombotic prophylaxis reduce the stroke occurrence in migraineurs and diminish the life-long cumulative lesion burden? This is the ultimate clinical study that will answer burning questions in the field: Do migraineurs need antithrombotic prophylaxis? Does migraine prophylaxis reduce the elevated stroke risk in migraineurs? However, such a study is also difficult to conclude, as large number of migraineurs will have to be randomized into control and prophylaxis arms, and followed for many years, possibly decades. Nevertheless, a collective multicenter effort can be effective.

CONCLUDING REMARKS

Migraine is a stroke risk factor with an effect size and prevalence comparable to other risk factors. Its recognition as an important and perhaps modifiable stroke risk factor, sought and documented as part of patient's medical history, will certainly be facilitated by better definition of the mechanisms of this association and the causal relationships. With increased awareness, management of migraine will become more than management of a headache disorder, and more holistic as with other vascular risk factors. Of course, enough evidence to change practice can only be achieved in large prospective clinical studies targeting mechanisms and in therapeutic trials.

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SUMMARY

The research in this thesis was aimed at investigating the central hypothesis that susceptibility to SD determines both the susceptibility to migraine with aura and the susceptibility to hypoxic/ischemic injury in the same direction. We envisage that factors that enhance the susceptibility to SD increase the likelihood of migraine with aura as well as ischemic stroke. To this end we assess to what extent genetic, hormonal and pharmacological modulators of SD susceptibility will influence the susceptible to ischemic injury. Thus we will unravel underlying mechanisms of SD susceptibility and susceptibility to ischemic injury. Central to this research is the use of two transgenic mouse models of migraine that carry migraine-relevant FHM1 gene mutations in voltage-gated $Ca_V2.1$ Ca^{2+} channels.

Chapter 1 provides an overview of the pathophysiology of migraine and aura, acute stroke and its progression, and the clinical association between migraine and stroke. The concepts of spreading depression, spreading depolarization, peri-infarct and injury depolarizations are introduced to put the data presented in **Chapters 2-4** in proper context.

In **Chapter 2A**, we show that two FHM1 missense mutations in the *Cacna1a* gene (R192Q and S218L) enhance CSD susceptibility, and that the S218L mutation, which is associated with a more severe clinical phenotype than the R192Q mutation, enhances CSD susceptibility more than the R192Q mutation. We also show an allele-dosage effect where heterozygous mutants have an intermediate phenotype between homozygous mutants and wild-type controls. We demonstrate that the neurological signs (hemiparesis and circling behavior) precipitated by CSD induced within the cortex in R192Q and S218L knock-in mice closely mimic the clinical signs in FHM patients expressing the respective mutations. Furthermore, we dissect the modulatory role of female gonadal hormones by showing that female FHM1 mutants have higher CSD susceptibility than males, that ovariectomy diminishes and estrogen replacement restores the sex difference in CSD phenotype. These data are congruent with higher migraine susceptibility in women of reproductive age, and suggest an interaction between estrogen and the genetic determinants of migraine susceptibility.

An alternative explanation for the female susceptibility to migraine is a protective effect by androgens. Therefore, in **Chapter 2B**, we investigate male gonadal hormone modulation of SD susceptibility. Using well-controlled electrophysiological methods, *in vivo*, we show that orchiectomy enhances and chronic testosterone replacement, but not a single dose, restores SD susceptibility, in an androgen receptor-dependent manner in FHM1 mutants. These data suggest that male and female gonadal hormones have opposite effects on SD susceptibility. As with the female mice, male gonadal hormones did not modulate SD susceptibility in wild-type animals, once again suggesting an interaction between hormonal status and genetic susceptibility factors.

In **Chapter 2C** we turn our attention to the severe and prolonged neurological deficits (e.g., coma and hemiparesis) that FHM1 mutant mice develop after a cortical SD (shown in **Chapter 2A**). Because cortical somatosensory evoked potentials recovered at about the same rate in wild-type and FHM1 mutant mice, delayed functional recovery of cortex did not appear to be the culprit in creating the severe and prolonged neurological deficits. Therefore, in **Chapter 2C** we investigate the alternative hypothesis that subcortical rather than cortical dysfunction is responsible for the severe and prolonged neurological deficits in FHM1. We employ multifocal intracerebral electrophysiological recordings in FHM1 mutants to show that SD susceptibility is significantly enhanced in striatum, thalamus and hippocampus, and that SD triggered in cortex is capable of propagating into all of these structures via direct gray matter connections, with the same genetic and sex-related modulation patterns described in **Chapters 2A and 2B**. Moreover, a subcortical SD can propagate back into the cortex, creating re-entrant and reverberating cortico-subcortical SDs in the highly susceptible FHM1 mutants, to explain the neurological deficits mimicking those observed in FHM patients.

In **Chapter 3A**, we test the central hypothesis that genetic and hormonal determinants of SD susceptibility sensitize the brain to ischemia, using *in vivo* electrophysiology, MRI, optical imaging of cerebral blood flow, combined with standard models of focal cerebral ischemia, all of which demonstrate that FHM1 mutations worsen the tissue and neurological outcome after focal cerebral ischemia. We show sex differences in ischemic outcome (female mice fare worse than males), which are consistent with the gonadal hormone influences on SD susceptibility (shown in **Chapters 2A and B**), but in the opposite direction compared with wild-type animals. We also demonstrate an allele-dosage effect where the homozygous FHM1 mutants fare worse than heterozygotes. Moreover, we identify enhanced AD and PIDs as the mechanism accelerating infarct growth in FHM1 mutants, akin to enhanced SD susceptibility explaining migraine aura. Therefore, we show data supporting the hypothesis that enhanced SD susceptibility worsens ischemic outcome, and form a direct mechanistic link between migraine with aura and ischemic stroke.

It is known that migraine prophylactic drugs suppress SD susceptibility as one mechanism of action in migraine. Therefore, migraine prophylaxis presents an opportunity to modulate SD susceptibility in the opposite direct compared with migraine mutations. We, therefore, show in **Chapter 3B** that, conversely, suppression of SD susceptibility by chronic (>4 weeks) treatment with migraine prophylactic drugs topiramate and lamotrigine renders the brain resistant to ischemic injury. We show that the effect is present not only in wild-type mice but also in FHM1 mutants. In this chapter, we also confirm the efficacy of migraine prophylactic drugs on SD in mice, which has only been shown in rats in previous studies. Moreover, we show that

treatment with a single dose of these drugs shortly before testing is ineffective, once again demonstrating the need for chronic treatment with these drugs. Lastly, we show that smaller ischemic infarcts are also associated with more favorable functional neurological outcomes, thus underscoring the clinical implications of our findings.

Chapter 4 discusses current knowledge on shared mechanisms of migraine and stroke.

Finally in **Chapter 5**, we put all our experimental findings, and others from the literature, together with the clinical observations on the association between migraine and stroke, in a comprehensive review focusing on potential mechanisms to explain the association, including the ones investigated in this thesis. We hope that the manuscript will spur thought and discussion and lead to new investigations on the association between migraine and cerebrovascular disease, discovery of new mechanisms or confirmation of the proposed ones, and increase awareness among the practicing physicians.

SAMENVATTING

Het onderzoek in dit proefschrift had als doel om de centrale hypothese te testen of de gevoeligheid voor 'spreading depolarization' (SD) op gelijksoortige wijze de gevoeligheid beïnvloedt op migraine met aura en hypoxie/ischemie. Uitgangspunt hierbij is dat factoren die de gevoeligheid voor SD vergroten mogelijk ook de kans op het ontwikkelen van migraine en een ischemische beroerte vergroten. Om die reden werd onderzocht in hoe verre genetische, hormonale en farmacologische modulatoren de gevoeligheid voor ischemische schade beïnvloeden. Dit geeft inzicht in onderliggende mechanismen van de gevoeligheid van zowel SD als ischemische schade. Centraal in het onderzoek staat het gebruik van twee transgene migraine muismodellen met migraine-relevante FHM1 genmutaties in voltage-afhankelijke $Ca_V2.1$ Ca^{2+} kanalen.

Hoofdstuk 1 geeft een overzicht van de pathofysiologie van migraine met aura, acute beroerte en diens beloop, en de klinische associatie tussen migraine en beroerte. De concepten 'spreading depression', 'spreading depolarization', 'peri-infarct depolarizations' en 'injury depolarizations' worden geïntroduceerd om data die gepresenteerd worden in de **Hoofdstukken 2-4** in de juiste context te kunnen plaatsen.

In **Hoofdstuk 2A** wordt aangetoond dat twee FHM1 missense mutaties in het *Cacna1a* gen (R192Q en S218L) de gevoeligheid voor CSD vergroten, en dat de S218L mutatie, die geassocieerd is met een ernstiger klinisch fenotype dan de R192Q mutatie, de gevoeligheid voor CSD in meer vergroot dan de R192Q mutatie. Er werd ook een allel-dosis effect gevonden, waarbij heterozygote mutante dieren een intermediair fenotype hebben, dat ligt tussen dat van homozygote mutante en wild-type controle dieren. Neurologische uitingen (zoals hemiparese en het draaien van rondjes om de as) als gevolg van CSD, die geïnduceerd is in de hersenschors van R192Q en S218L knock-in muizen, blijken een grote gelijkenis te vertonen met de klinische verschijnselen die FHM patiënten met de respectievelijke mutaties laten zien. Daarnaast hebben we de modulatoire rol van vrouwelijke geslachtshormonen vast kunnen stellen door te laten zien, dat i) vrouwtjes FHM mutante dieren een grotere gevoeligheid voor CSD hebben dan mannetjes, ii) ovariectomie die gevoeligheid vermindert, en iii) toediening van estrogeen het geslachtsverschil in het CSD fenotype herstelt. Deze data passen goed bij de hogere gevoeligheid van vrouwen van reproductieve leeftijd om migraine te krijgen en geeft aan dat er een interactie bestaat tussen estrogeen en genetische factoren die gevoeligheid voor het hebben van migraine bepalen.

Een alternatieve verklaring voor de verhoogde gevoeligheid van vrouwen om migraine te krijgen zou een mogelijk beschermend effect van androgenen kunnen zijn. Om die reden hebben we in **Hoofdstuk 2B** de modulatoire rol van mannelijke geslachtshormonen bestudeerd. Met goed gecontroleerde elektrofysiologische

methoden werd aangetoond dat, *in vivo*, castratie (orchiectomie) de CSD gevoeligheid verhoogt en dat chronische toediening, maar niet een enkele dosis, van testosteron die gevoeligheid herstelt via een androgen-receptor afhankelijk mechanisme in FHM1 mutante dieren. Deze data suggereren dat mannelijke en vrouwelijke geslachtshormonen tegengestelde effecten hebben op de gevoeligheid voor CSD. Net als in vrouwelijke muizen, werd geen effect van mannelijke hormonen op de gevoeligheid van SD in wild-type dieren gevonden, wat opnieuw duidt op een interactie van de hormonale status en genetische factoren die de migraine gevoeligheid bepalen.

In **Hoofdstuk 2C** gaat de aandacht naar het vaststellen van ernstige en langdurige neurologische gebreken (zoals coma en hemiparese) die FHM1 mutante dieren ontwikkelen na een corticale SD (als beschreven in **Hoofdstuk 2A**). Gezien het feit dat potentialen opgewekt in de corticale somatosensore hersenschors op ongeveer dezelfde wijze herstellen in wild-type en FHM1 mutante dieren, lijkt een vertraagd functioneel herstel van de hersenschors niet de boosdoener van de ernstige langdurige neurologische gebreken. Om die reden bestudeerden we in **Hoofdstuk 2C** de alternatieve hypothese namelijk dat een ontregeling in hersengebieden die zich bevinden onder de hersenschors ("subcortical") verantwoordelijk zijn voor de ernstige langdurige neurologische gebreken in FHM. We maakten daarvoor gebruik van multifocale intracerebrale elektrofysiologische metingen in FHM mutante dieren en toonden aan dat de gevoeligheid voor SD significant is verhoogd in striatum, thalamus en hippocampus en dat SD golven, die opgewekt werden in de hersenschors, deze hersengebieden konden bereiken via grijze stofverbindingen met dezelfde genetische en geslachts-gerelateerde patronen van modulatie als beschreven in de **Hoofdstukken 2A** en **2B**. Het is zelfs zo dat een 'subcortical' SD de hersenschors weer kan bereiken met herintredende terugkaatsende cortico-subcorticale SD golven in extra gevoelige FHM1 mutante dieren tot gevolg, die een verklaring kunnen zijn voor de neurologische gebreken die grote gelijking vertonen met die in FHM patiënten.

In **Hoofdstuk 3A** testten we de centrale hypothese dat genetische en hormonale determinanten van de gevoeligheid voor CSD de hersenen gevoeliger maken voor ischemie, door in vivo elektrofysiologie, MRI, optische beeldvorming van de cerebrale doorbloeding te combineren met standaard modellen voor de bestudering van focale cerebrale ischemie. Wij toonden aan dat FHM1 mutaties nadelige gevolgen hebben voor het klinische beeld en het hersenweefsel na een focale cerebrale ischemie. Er werden geslachtsverschillen gevonden voor de gevolgen van ischemie (vrouwtjes muizen doen het slechter dan mannetjes muizen), wat goed past bij de gevonden effecten van geslachtshormonen op de gevoeligheid van SD (als beschreven in **Hoofdstukken 2A** en **2B**), maar met tegenovergesteld effect als waargenomen in wild-type dieren. We hebben ook een allel-dosis effect gevonden, waarbij homozygote FHM1 mutante dieren

het slechter deden dan heterozygote dieren. 'Anoxic depolarisation' en 'peri-infarct depolarization' werden als mechanisme geïdentificeerd voor de versnelde groei van de infarctgrootte in FHM1 mutante muizen, net als de verhoogde gevoeligheid voor SD die de migraine aura verschijnselen kan verklaren. Kortom, de data ondersteunen de hypothese dat een verhoogde gevoeligheid voor SD leidt tot een verslechtering van de gevolgen van ischemie, die hiermee een directe mechanistische link geeft tussen migraine met aura en ischemische beroerte.

Het is bekend dat profylactische migraine drugs als werkingsmechanisme het onderdrukken van de gevoeligheid voor SD kunnen hebben. Migraine profylaxe geeft de mogelijkheid om de gevoeligheid voor SD te moduleren in de exact tegenovergestelde richting als gebeurt met migraine mutaties. In **Hoofdstuk 3B** laten we, paradoxaal, zien dat de onderdrukking van de gevoeligheid van SD met een chronische (>4 weken durende) behandeling met de migraine profylactische drugs topiramaat en lamotrigine de hersenen resistent maakt tegen ischemische schade. We lieten zien dat het effect aanwezig is zowel in wild-type muizen als FHM mutante dieren. In dat hoofdstuk bevestigen we ook de effectiviteit van profylactische migraine drugs op SD gevoeligheid, die tot dan toe alleen in ratten was aangetoond. Bovendien werd gevonden dat een behandeling met een enkele dosis van deze drugs vlak voor de test ineffectief is, waarmee nogmaals wordt aangetoond dat een chronische behandeling met de drugs noodzakelijk is. Tenslotte geven we bewijs dat kleinere ischemische infarcten leiden tot meer gewenste functionele neurologische uitkomstmaten, waarmee de klinische implicaties van onze bevindingen goed is geïllustreerd.

In **Hoofdstuk 4** wordt bestaande kennis van gedeelde mechanismen van migraine en beroerte bediscussieerd.

Tenslotte, worden in **Hoofdstuk 5** alle experimentele bevindingen en bevindingen uit de literatuur besproken in relatie tot klinische observaties over de associatie tussen migraine en beroerte, waarbij we ons richten op potentiële mechanismen voor die associatie, en andere die we hebben bestudeerd in dit proefschrift. We hopen dat dit proefschrift de weg zal vrijmaken voor nieuwe gedachtevorming en discussie omtrent additioneel onderzoek naar de associatie tussen migraine en cerebrovasculaire ziekten, de ontdekking van nieuwe ziektemechanismen, of de bevestiging van reeds voorgestelde mechanismen, en zal leiden tot een groter begrip van het klinisch belang bij praktiserend artsen.

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CURRICULUM VITAE

Cenk Ayata was born in 1968 in Ankara, Turkey. He started his medical training at the Hacettepe University Faculty of Medicine, the top medical school in Turkey, in 1985 by successfully passing a national university entrance and ranking examination. After obtaining his MD degree in 1991, he enrolled as a graduate student in the Department of Pharmacology of the same university. In 1994, he was offered a research fellowship position at the Massachusetts General Hospital, Harvard Medical School, under the mentorship of Professor Michael A. Moskowitz. After three years of research fellowship, he decided to return to his medical training, successfully passed the board examinations and started his specialty training in 1997 as a Resident in Neurology at the Tufts University program under the mentorship of Professor Alan H. Ropper. After completing his residency in 2001, he was accepted to a Clinical Fellowship in Vascular and Critical Care Neurology, at the Massachusetts General Hospital, Harvard Medical School, and by this way returned to his previous institution. As part of his fellowship, he spent part of his time in the research laboratory of Dr. Moskowitz. After completing his fellowship in 2003, he launched two new lines of investigation, focusing of optical imaging of cerebral blood flow and metabolism, and physiology and pharmacology of spreading depression. Since 2009 he has worked together with Professor Michel D. Ferrari and Professor Arn M.J.M. van den Maagdenberg to examine their transgenic knockin mouse models with human pathogenic gene mutations in order to dissect neurobiological mechanisms related to the susceptibility to spreading depression and ischemic injury relevant to the pathophysiology of migraine and stroke, and their interrelation. The work performed on these transgenic mouse models of migraine was the basis of this thesis. He was appointed as an Instructor at the Massachusetts General Hospital, Harvard Medical School in 2001, promoted to Assistant Professor in 2006, and then Associate Professor in 2010.