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**Author:** Shyti, Reinald  
**Title:** Modulating factors for and consequences of cortical spreading depression  
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Chapter 8
General discussion
Migraine is a common brain disorder that is characterized by attacks of severe unilateral headaches and associated neurological symptoms. In about one third of patients, attacks are accompanied by an aura that is likely caused by cortical spreading depression (CSD). In this thesis, transgenic FHM1 R192Q mice were used as a relevant migraine model to investigate mechanisms underlying the effects of CSD modulating factors, as well as the consequences of CSD (Figure 1). FHM1 mice carry an R192Q missense mutation in the $\alpha_1$ subunit of voltage-gated Ca$_{2.1}$ channels (van den Maagdenberg et al 2004), which was previously identified in patients with familial hemiplegic migraine (Ophoff et al 1996). The experimental approaches and outcomes as described in the chapters of this thesis are discussed below, also in relation to relevant literature and with a look forward to future research.

8.1 Should CSD susceptibility measurements be performed in anesthetized or in freely behaving mice?

Technical progress has made it possible to investigate CSD in relation to cortical activity and behavior in freely behaving animals. Nevertheless, for certain research questions anesthesia can be the preferred regime. Possibilities and caveats of performing experiments under anesthesia and in freely behaving animals were investigated in various chapters of this thesis and are discussed in the paragraphs below.

8.1.1 CSD studies under anesthesia

Under anesthesia, CSD induction and monitoring are technically easier to control than in the longitudinal chronic recordings lasting days-weeks in freely behaving mice. Anesthesia was used for experiments where a fixed number of CSD events was induced by topical KCl application on the dura to study modulatory factors and consequences of CSD, in Chapters 5, 6 and 7. The possibility to precisely time CSD events is a great advantage when administering drugs/modulators, as performed for stress modulators in experiments described in Chapter 4. Moreover, when drugs are administered under anesthesia, stress and discomfort of the mice is avoided to a large extent. A drawback, however, is that anesthetics affect critical parameters of animal physiological status, such as blood pressure (BP), blood gases and pH, in particular during long-duration experiments. Changes in these parameters are known to affect properties of brain tissue that are relevant to initiate and sustain CSD (Somjen 2001). The implementation of physiological monitoring in Chapter 2 yields important information on physiological parameters in relation to CSD characteristics, and makes possible the exclusion of animals in which physiological parameters are out-of-range. The use of physiological control by adjusting breathing patterns by mechanical ventilation is superior to the use of monitoring alone in the sense that it ensures a more comparable physiological status between mice; especially when experimental designs are used in which anesthesia last longer. Physiological control was performed for experiments to assess effects of gender (Chapter 2), diurnal rhythm (Chapter 3) and stress hormone (Chapter 4). Physiological control may however influence CSD characteristics and can sometimes act as a double-edged sword. By keeping physiological parameters within tight ranges, these parameters are kept comparable across experiments. However, if the experimental
readout itself depends on differences in physiological parameters, controlling them will logically interfere with the experimental outcome. This is illustrated in Chapter 2, in which the increased CSD frequency in female compared with male FHM1 R192Q mice, as reported by Eikermann-Haerter et al. (Eikermann-Haerter et al 2009b), was only revealed in the presence of physiological control. In an opposite way, in Chapter 3, physiological control appeared to mask a putative effect of a difference in CSD susceptibility between the visual and motor cortex in mutant mice; an effect that was observed only when experiments were performed in an uncontrolled and non-monitored manner (Chapter 2).

Since anesthetics suppress neuronal activity (Hertle et al 2013), CSD and its characteristics will be affected by anesthetics, and in many instances masked. Common anesthetics such as isoflurane, which is used in the CSD experiments of this thesis, in particular in combination with N₂O (used in CSD experiments under full physiological control described in Chapters 2, 3 and 4) are known to suppress CSD susceptibility (Kudo et al 2013, Takagaki et al 2014). Anesthesia may also directly interfere with the experimental intervention, e.g., when studying effects of drugs that influence GABAergic function, which is known to be modulated by many anesthetics (Hoffmann et al 2011). There seems to be no ideal anesthetic to investigate the susceptibility of CSD.

8.1.2 CSD studies in freely behaving mice

Studying CSD characteristics in freely behaving mice can overcome some of the disadvantages of studies under anesthesia. Since both autonomous and central functions are retained, the physiology and brain functions of the mouse are more likely to mimic the situation in patients. With chronic implantation of miniaturized electrodes and a counter-balanced EEG/MUA-cable with swivel, it has been possible to record both EEG and MUA activity from the cortex with minimal discomfort to the mouse for multiple days to weeks (Chapter 3). Such recordings in freely behaving mice enable correlations between CSD characteristics and changes in neuronal network activity, vigilance states and other behavioral characteristics that are not possible under anesthesia. In rats, a similar awake electrophysiology approach has been used to correlate induced events of spreading depression to migraine-relevant pain pathways (Fioravanti et al 2011, Tepe et al 2015) and epilepsy (Broberg et al 2014). Our studies in Chapter 3 show that the freely behaving approach is now also feasible in mice. Thus, we could demonstrate the occurrence of spontaneous CSD events in FHM1 R192Q mice, which were not observed in WT mice. Studies on spontaneous CSD events in freely behaving mice will allow investigating how natural triggers and modulatory factors of migraine (e.g., changes in sleep patterns, stress, and light) affect CSD susceptibility, thus enabling better translation of observations from and to the clinic. Because recordings are possible for several days to weeks, multiple trigger paradigms and modulators can be studied in a single animal. This include the possibility for intra-individual vehicle controls and assessment of repeatability.

However, experiments in awake, freely behaving mice have also disadvantages. Chronic electrode implantation can cause variation among animals with respect to electrode depth and location. Furthermore, an inflammatory response of the brain to chronic electrode implantation (as discussed
Figure 1. Overview of key experimental findings in FHM1 R192Q mice described in this thesis. (Center) FHM1 R192Q gain-of-function mutation increases neuronal Ca\(^{2+}\) influx via P/Q-type Ca\(_{\text{V}}\)\(_{2}\) Ca\(^{2+}\) channels that leads to increased release of excitatory neurotransmitters in the synaptic cleft. (A) We established an experimental platform to measure CSD susceptibility in anesthetized mice under continuous monitoring and control of critical physiological parameters, such as blood pressure and blood gases (Chapter 2). (B) In awake freely moving mice, long-term EEG recordings revealed increased cortical excitability and spontaneous CSD events in a subgroup of FHM1 R192Q mice but not in WT (Chapter 3). (C) Corticosterone injection in FHM1 R192Q mice increased CSD susceptibility compared to vehicle injection, via a GR-mediated mechanism (Chapter 4). (D) CSD induction resulted in changes of metabolite, peptide and protein distribution in the cortex and subcortical brain areas that were genotype-and CSD-specific (Chapter 6). (E) CSD induction in the cortex of FHM1 R192Q mice triggered an activation of compensatory mechanisms likely to restore inhibition/excitation misbalance that occurred following CSD and this could be measured in plasma (Chapter 7).
in **Chapter 3** may affect CSD characteristics (Sukhotinsky et al 2011). Another difficulty with experiments in awake mice is that implanted electrodes and the EEG cable may increase levels of stress hormones, which can affect CSD characteristics (**Chapter 4** and see **Section 10.2.3**). What remains challenging as well is to standardize CSD induction paradigms in freely behaving animals. This is illustrated by the KCl infusion paradigm used in **Chapter 3**, which does not induce a single CSD but multiple CSD events with high variability. Due to their larger size, and willingness to be handled, it is easier to overcome such problems in rats when an implanted cannula for KCl infusion is used (Fioravanti et al 2011, Tepe et al 2015). The strength of an experimental CSD design lies in overcoming technical disadvantages by the integration of experimental platforms for the induction and monitoring of CSD that either use or do not use anesthesia, as illustrated in **Chapter 3**. Finally, the advent of novel technologies for modulation of neuronal activity, such as optogenetics (Williams & Deisseroth 2013), is expected to overcome at least some of the disadvantages thus far associated with chronic recordings. This is well illustrated by the recent implementation of optogenetics for induction of CSD in freely behaving mice (Tolner et al 2015).

### 8.2 Factors that modulate CSD susceptibility

#### 8.2.1 Gender

It is well established that women are more susceptible to migraine than men (Finocchi & Strada 2014, Lipton et al 2001). The higher frequency of experimentally induced CSD events observed for female FHM1 R192Q mice in experiments with physiological control (**Chapter 2**) is in line with previous data (Eikermann-Haerter et al 2009b). A potential underlying mechanism for the higher CSD susceptibility in female mice (and the female preponderance of migraine) may be the response to female gonadal hormones changes (Borsook et al 2014). It is hypothesized that a sudden drop in estradiol level just before the start of menstruation (Somerville 1972a, Somerville 1972b) is involved in initiating attacks. This drop may lead to direct enhancement of glutamatergic neuronal excitability via up-regulation of NMDA receptor expression, down-regulation of glutamate uptake by astrocytes, and increasing the dendritic spine number (Kelly et al 2003, Sato et al 2003, Smith 1989, Woolley et al 1997). Vascular changes may also be involved in the effect of female hormones on CSD frequency in FHM1 R192Q mice. This is supported by the observation that a gender effect was only noticed in mutant mice when mechanical ventilation was performed and thus physiological parameters were controlled. A gender effect remained masked in physiologically monitored but not controlled mice that had a lower blood pressure (**Chapter 2**). It is known that mechanical ventilation can reduce brain blood volume and blood flow (Milan et al 2009), which in turn may have affected CSD characteristics (Ayata 2013). In addition, direct changes in vascular function may occur in relation to gender, since estradiol was shown to alter vascular responses to calcitonin gene-related peptide (CGRP) (Gupta et al 2007). In this context it is plausible that different neurovascular responses between physiologically controlled and uncontrolled experiments in female mice may be the reason that a gender effect on CSD frequency was observed only in the presence of physiological control. This gender effect in
mutant mice was detected irrespective of the phase in the estrous cycle, as mice were not investigated at a specific phase in the cycle (Chapter 2 and Eikermann-Haerter et al 2009b), which suggests that the increased CSD susceptibility in female mutant mice may in fact be due to intrinsic brain differences between females and males (Borsook et al 2014) and perhaps less to a sudden drop in estrogen level during a phase of the cycle.

8.2.2 Diurnal changes

Observations that sleep (Holland 2014) and hypothalamic function (Moulton et al 2014) are linked to migraine support the idea that attacks may be influenced by diurnal rhythm (Fox & Davis 1998). Analysis of 24-hr EEG periods in freely behaving FHM1 R192Q mice in Chapter 3 revealed an increased EEG gamma power, which had been related to increased neuronal excitability (Joho et al 1999, Lau et al 2000). This finding fits earlier data that excessive neuronal excitability underlies the increased susceptibility to CSD in these mutant animals (Tottene et al 2009). The idea of hyperexcitability has been proposed as a mechanism underlying migraine attack susceptibility in humans (Aurora & Wilkinson 2007). Notably, we could show that a subset of FHM1 R192Q, but not WT mice showed spontaneous (i.e., not experimentally-triggered) CSD events. The majority of the spontaneous CSD events occurred within 2 hr in the transition from dark-to-light or from light-to-dark phases in the animal facility. Paradoxically, results of parallel experiments, performed under anesthesia with physiological control, seem to rule out that CSD susceptibility is specifically increased at the start of the light or the dark phase. It cannot however be excluded that the anesthesia or mechanical ventilation used in the experiments may have caused changes in the animal’s physiology that mask a putative diurnal difference in CSD susceptibility. Further studies are needed to assess whether, in addition to the overall increase in EEG gamma power, other changes may be detected from EEG recordings that are present before or during these diurnal transitions that can be related to changes in cortical excitability.

We hypothesize that diurnal fluctuations in hormones and neurotransmitters that occur around these transitions may contribute to changes in neuronal excitability leading to CSD events. It is tempting to speculate that the high level of corticosterone that is present at the beginning of the dark period (Maywood et al 2007) may correlate with an enhanced CSD susceptibility at that time point. Notably, adenosine, a neuromodulator that induces sleep and decreases neuronal excitability (Nehlig et al 1992), exhibits a diurnal pattern opposite to that of corticosterone, with high levels at the beginning of the light period (and low levels at the beginning of the dark period) (Basheer et al 2004). FHM1 R192Q mice were reported to be less sensitive to exogenous modulation of adenosinergic inhibition and exhibit an increase of waking episodes during the dark period (Deboer et al 2013). One can therefore rationalize that a reduction in inhibitory adenosinergic response in mutant animals can lead to transiently enhanced neuronal excitability leading to enhanced CSD susceptibility, particularly when adenosine levels are high, i.e., at the beginning of the light period.
8.2.3 Stress

The majority of migraine patients report stress as a prominent trigger for their migraine attacks (Hauge et al. 2011), although one might argue that such self-reported information is not very reliable. As stress is a complex response of the body and involves multiple neurotransmitters and hormones with different dynamics, it is not known which aspects of the body’s stress response, if any at all, may bring about migraine attacks. In Chapter 4, we could show that administration of stress hormone corticosterone (cortisol in humans) specifically increased CSD frequency in FHM1 R192Q mice and that this occurred via a glucocorticoid receptor-mediated mechanism. Given that corticosterone had no effect in WT mice, and it is thought to increase glutamatergic neurotransmission (Popoli et al. 2011), suggest that corticosterone may have further enhanced the already present intrinsically enhanced (glutamatergic) excitability in FHM1 mice (Tottene et al. 2009). It is tempting to speculate that in freely behaving mice, external triggers such as sensory inputs (e.g., light, sound) may cause enhanced levels of corticosterone (Ishida et al. 2005, Kim et al. 2008) and thereby enhanced excitation of thalamo-cortical pathways (Noseda et al. 2010). The combined sensory input and intrinsically enhanced neuronal excitability in FHM1 R192Q mice may result in spontaneous CSD events, as described in Chapter 3.

Effects of natural stressors on CSD susceptibility seem harder to identify since exposure of FHM1 R192Q mice to mild or even severe restraint stress did not seem to affect CSD susceptibility. In Chapter 4, we provide evidence that such lack of an effect is not simply due to suppressive actions of neuromodulators such as tetrahydrodeoxycorticosterone, which like corticosterone is released during restraint stress. Although corticosterone levels rise both after exogenous administration and as a consequence of acute stress, the latter triggers a much more complex response with effects that may either enhance or suppress CSD susceptibility.

It is speculated that in patients the recovery from chronic stress, and not so much from acute stress, triggers a migraine attack (Lipton et al. 2014). The concept of a ‘rebound’ effect after stress refers to the emergence of a stress effect following a period of recovery after chronic exposure to a stressor or after chronic glucocorticoid administration. While acute stress has been shown to induce analgesia, thus having anti-nociceptive effects, effects of chronic stress or chronically elevated glucocorticoid levels appear less predictable (McEwen & Kalia 2010). The direct effect of chronic administration of glucocorticoids in rats was an increase in pain threshold (Pinto-Ribeiro et al. 2009). This observation is in line with studies in chronically stressed patients that suffer from chronic back pain, which reported less pain in a series of pain tests in comparison to healthy controls (Clark et al. 1986). However, children that were chronically stressed due to abdominal pain (Dufton et al. 2008) were reported to exhibit an increased reaction to a pain test (i.e., hyperalgesia). The variable results of chronic stress studies may relate to a rebound-after-stress effect, either because of fluctuations in stress levels in patients or because of different time-points after chronic stress at which readout effects were measured. In a study in which rats were measured 24 hr following exposure to chronic stress, the pain threshold
was shown to be reduced (Gamaro et al. 1998). In line with the variable reports of chronic stress effects on pain, effects of chronic stress on glutamatergic transmission are also variable with studies indicating both enhancement (Kerr et al. 1991, Joëls et al. 2004, Raudensky & Yamamoto 2007) and suppression (Moghaddam 2002, Yuen et al. 2012) of glutamatergic transmission following chronic stress. The variation in outcome may relate to differences in the studied brain regions, with e.g., the prefrontal cortex showing a reduction and hippocampal regions typically showing an enhancement of glutamatergic function (Joëls et al. 2007, Yuen et al. 2012). In these experimental studies on chronic stress, measurements were performed directly after the chronic stress paradigm, and did not provide information on glucocorticoid levels at the end of the chronic stress paradigm. Taken together, thus far, not much is known about mechanisms that could explain a possible rebound effect of (chronic) stress on triggering a migraine attack.

To gain insight into the rebound-after-stress phenomenon, we performed pilot experiments to investigate whether a time-delay after chronically elevated corticosterone levels affects CSD susceptibility (Figure 2). In brief, male WT and FHM1 R192Q mice were implanted with corticosterone or control pellets for a time period of 21 days. After the 3-week period, the pellet was removed and CSD frequency was tested either directly (on day 21) or 4 or 7 days later, at days 25 or 28 respectively (Figure 2A). High corticosterone plasma levels were shown to be maintained for 21 days when corticosterone pellets were implanted. After pellet removal at day 21, corticosterone plasma levels were decreased by day 28 in WT, but not in FHM1 R192Q mice in which corticosterone levels were found to remain high (Figure 2B). At none of the chosen time points after the 3-week corticosterone treatment however, CSD frequency was different in either WT or FHM1 mice in comparison to the respective frequency in naïve WT and FHM1 R192Q mice (Figure 2C, D). These preliminary findings suggest that a chronic elevation of systemic corticosterone levels followed by withdrawal is not a sufficient trigger to modulate CSD susceptibility. Since corticosterone levels remained elevated for several days after pellet removal in FHM1 R192Q mice, the rebound-after-stress paradigm may not have been sufficient to modulate CSD susceptibility. One may need to first determine the time point when corticosterone plasma levels show the steepest decline, and then assess CSD susceptibility around this transition point.

In this context, our findings raise an important issue regarding the translation of observations on the time-relationship between stress and migraine in humans to the time-line of stress paradigms in mice. In humans, a decline in perceived stress was recently shown to correlate with an increased probability to experience a migraine attack in the subsequent 6, 12 and 18 hr (Lipton et al. 2014). In our study, corticosterone plasma levels were still elevated several days after corticosterone pellet removal, more so in R192Q mice. Although no information was available on cortisol levels in the human study, it is possible that the dynamics of the stress response differs between humans and mice. Alternatively, it may be that recovery from chronically elevated levels of glucocorticoids is less relevant as a stress factor in migraine, if adaptation occurs in the period of chonic stress or in the period of recovery. In an animal model, after 3 weeks of daily restraint, adaptation was shown to occur as evidenced from
plasma corticosterone levels that were no longer elevated (Joëls et al 2007). Overall, it is important to emphasize the complexity of physiological responses to chronic exposure to stress and glucocorticoids (Borsook et al 2012, Maleki et al 2012, Resmini et al 2013), some of which may cause compensatory effects at the level of neuronal excitation and CSD susceptibility.

8.3 Insight in migraine pathophysiology from investigating modulators of CSD

Better insight in the modulating effects of e.g., female hormones, diurnal or circadian rhythm and stress on CSD susceptibility can give mechanistic insight in the interaction of various neurobiological systems relevant to migraine. Enhanced susceptibility to various migraine modulators, e.g., sleep, food intake and anxiety, which affect various neurochemical pathways, seem to converge at the level of thalamus (Noseda et al 2014). In addition, intrinsic differences in neuronal network excitability properties in specific brain regions may render the migraine brain more susceptible to attacks. As an example, neuroimaging studies have shown structural and functional alterations in the visual cortex of migraineurs (Aurora et al 1999, Granziera et al 2006), which may have relevance to explain the clinical observation that the far majority of migraine auras are visual (Eriksen et al 2005). Certain experiments in Chapter 2 seem to indicate that in FHM1 R192Q mice the visual cortex is more susceptible to CSD than the motor cortex. This difference, however, was only seen when experiments were carried out in the absence of physiological monitoring and control. Under other experimental conditions cortical regions, as tested for motor and visual cortex in the presence of physiological control in Chapter 3, appear equally susceptible to CSD induction, and cannot explain a preference for auras being visual. This finding fuels the idea that silent auras’ may exist (Ayata 2010; Denuelle et al 2008), i.e., spreading depression waves may affect (and may be initiated in) a cortical (or even non-cortical) brain region that does not lead to an abnormal visual perception (Hansen et al 2013) and could have relevance for migraine without aura patients. Relevant to this idea are also first observations made in freely behaving mice in Chapter 3 that indicate that not all spontaneous CSD events are first observed in the visual cortex (sometimes they are first seen in the motor cortex), suggesting that a CSD may start at different brain locations.

8.4 CSD-induced changes in biomolecular profiles detected by mass spectrometry imaging in the brains of mice

Mass spectrometry imaging (MSI) is an advanced bioanalytical method that allows the simultaneous detection of hundreds of biomolecules from different molecular classes directly from brain tissue (McDonnell & Heeren 2007). MSI therefore offers great potential in revealing—in an untargeted manner—biomolecular changes in the brain that are related to migraine gene mutations, CSD, or migraine-relevant triggers, while preserving spatial information of the distribution of these compounds. In Chapter 5, we demonstrated in a proof-of-principle study the applicability of MSI by the identification of changes in brain metabolite and peptide profiles upon CSD induction. In Chapter 6, we implemented the matrix-assisted laser desorption/ionization (MALDI) MSI approach
in a larger study to investigate biomolecular changes in the brain following CSD in FHM1 R192Q mice. CSD triggered specific brain changes in metabolite, peptide and protein profiles in FHM1 R192Q mice, which were not observed in WT brains that underwent the CSD procedure nor in WT and FHM1 R192Q brains that underwent a sham procedure (in which no CSDs were evoked). Metabolite \( m/z \) 146.0593, which was putatively identified as L-glutamate, appeared down-regulated in the CSD-affected hemisphere in FHM1 R192Q mice. This finding suggests an increased clearance of glutamate by the action of glial cells and glutamate transporters or an adaptation of glutamate

Figure 2. CSD susceptibility upon chronic corticosterone exposure. (A) Experimental design of chronic corticosterone experiments. At least 4 days prior to pellet implantation a blood sample (BS) was collected from a tail cut to measure corticosterone levels at baseline. Additional blood samples were collected every week to measure corticosterone plasma levels following pellet implantation. The corticosterone pellet (50 mg corticosterone/50 mg cholesterol) was implanted subcutaneously in the flank of the mouse while it was under brief isoflurane anesthesia. Control mice were implanted with a pellet containing 100 mg cholesterol. To maintain stable corticosterone release the pellet was replaced at day 10. The pellet was removed at day 21 and CSD frequency was measured at day 21, day 25, or day 28 to test for possible rebound effects following corticosterone pellet removal. (B) No significant differences in corticosterone plasma levels were detected between WT and FHM1 R192Q mice. Corticosterone plasma levels in mice implanted with the control pellet were low as expected (0, 7, 14, 21 days: WT N=5, R192Q N=4; 28 days: WT N=1, R192Q N=1). In mice implanted with corticosterone pellets, corticosterone plasma levels were strongly elevated in the first 14 days and then gradually dropped at day 21 and even more at day 28. Note the drop at day 28 in corticosterone plasma level (9.5 ng/mL) in the WT mouse below the level reported for stressed animals (i.e., 50 ng/mL). In contrast, in FHM1 R192Q mice corticosterone plasma levels remained high for 2 of 3 mice with particularly high corticosterone plasma levels at day 28 (~270 ng/mL; in line with values reported for stressed animals (Zalachoras et al 2013) (0, 7, 14, 21 days: WT N=6, R192Q N=8; 28 days: WT N=1, R192Q N=3). (C, D) Scatter plots (mean ± SD) depict CSD frequency measured at different time-points in WT (C) and FHM1 R192Q (D) mice. There was no significant differences in CSD frequency for WT (p=0.1) and FHM1 R192Q (p=0.14, one-way ANOVA Bonferroni correction) for any of the days following corticosterone compared to control pellet removal. Group sizes are shown on the x-axis.
release, in reaction to the intense metabolic and synaptic demand during repeated CSD induction. Such compensatory mechanisms in FHM1 R192Q mice might already be in place under naive conditions, since cortical synaptosomes from naive FHM1 R192Q mice showed an up-regulation of both major glutamate transporters, EAAT1 and EAAT2 (Klychnikov et al 2010). Notably, the same m/z 146.0593 compound was found down-regulated in the occluded cortical hemisphere in a rat middle cerebral artery occlusion model (Miura et al 2010). For the protein dataset, we detected a down-regulation of unidentified protein m/z 11343 following CSD in the ipsilateral hemisphere, only in FHM1 R192Q mice. The short time between the 7th CSD and sacrifice of the mouse 5 min later suggests that this mass likely represents a protein modification of an already synthesized protein. In addition, several peptides were found to be down- or up-regulated following CSD in mutant mice in cortex and several subcortical regions relevant to migraine pathophysiology (i.e., cortex, hippocampus, striatum and thalamus). The finding of robust changes in the peptide dataset seem in line with reports showing peptide concentration changes associated with both migraine attacks in humans and with CSD induction in animals. Relevant peptides include calcitonin gene related peptide (CGRP), substance P (SP) and neurokinin A (NKA), of which levels were found to be altered in the cortical extracellular space of rodents after CSD (Bolay et al 2002, Colonna et al 1994, Tozzi et al 2012, Wahl et al 1994) and in blood plasma of patients (Fusayasu et al 2007, Gallai et al 1995, Goadsby et al 1990). The observation of peptide changes in subcortical areas after cortical induction of CSD in our study is perhaps not so surprising when considering the spread of CSD waves to subcortical areas, such as striatum and hippocampus, as was shown for FHM1 R192Q mice (Eikermann-Haerter et al 2011). Future experiments are expected to reveal the identity of the compounds that were differentially regulated after CSD.

8.5 CSD-induced biomolecular changes captured in blood plasma of mice

Changes in metabolite composition perhaps best reflect the response of an organism to a biological change. Relevant biomolecule changes that occur in brain may also be captured in cerebrospinal fluid (CSF), and even blood. Experiments in Chapter 7 revealed specific changes, obtained by capillary electrophoresis-mass spectrometry (CE-MS), in plasma metabolite profiles of FHM1 R192Q mice following CSD. Such changes were not observed in WT mice that underwent the same procedure. In particular, a decreased plasma level of lysine and an increased level of pipecolic acid (a by-product of lysine catabolism) were found. Given the involvement of pipecolic acid in GABA-ergic neurotransmission (Gutierrez & Delgado-Coello 1989, Kase et al 1980), the observed changes in plasma, if reflecting similar metabolite changes in the brain, may indicate a compensatory response to effects of neuronal hyperexcitability in FHM1 R192Q mice. An inhibitory compensatory reaction seems in line with the observed down-regulation of L-glutamate following the same CSD induction paradigm in the MSI study from Chapter 6. When paralleled with microdialysis studies in freely behaving animals (Rogers et al 2013) and studies of plasma (Guldiken et al 2009), urine (Jacobsen et al 2013) or CSF (Fonteh et al 2011) obtained from patients, the analysis of CSD-induced changes in
plasma or other peripheral body fluids from migraine mice has great potential for migraine biomarker identification.

### 8.6 New knowledge with respect to migraine-relevant pathways

Events such as CSD trigger intense neurometabolic activity in the brain and are likely to affect the body in various ways. Our findings show that CSD induction causes specific changes of biomolecular distribution and gene expression in the brain, as well as specific changes in levels of metabolites in peripheral body fluid (i.e., plasma). Notably, some of the observed changes after CSD were different and/or only seen in FHM1 R192Q mice compared with WT mice, which suggest that these changes reflect specific CSD-induced changes relevant to migraine pathophysiology.

Changes in brain biomolecular distribution, as revealed by MSI, and profiling of metabolites in plasma using CE-MS, pointed towards the activation of compensatory mechanisms in FHM1 R192Q mice following CSD induction. A reduced $m/z$ value (likely L-glutamate) in the CSD-affected hemisphere and an increased plasma level of pipecolic acid (with a presumed function in GABA-ergic neurotransmission) in FHM1 R192Q mice suggests that the induction of CSD triggers a body defense mechanism in order to restore the inhibition/excitation misbalance following CSD events. Our experiments cannot determine whether the observed reduction in glutamate is caused by an increased clearance of glutamate from glial cells by glutamate transporters, such as EAAT1 and EAAT2. The increased expression of EAAT1 and EAAT2 that was seen in the naïve brain of FHM1 R192Q mice using a proteomics approach on synaptosome preparations (Klychnikov et al 2010), provides some support for this scenario. Notably, previous studies in brain slices proposed increased glutamatergic neurotransmission as a key underlying mechanism of increased CSD susceptibility in FHM1 R192Q mice, whereas GABAergic neurotransmission was considered unaffected by the gene mutation (Tottene et al 2009).

Our results seem to indicate that the body is coping or counteracting the excess glutamatergic neurotransmission by: (i) increasing GABAergic neurotransmission, perhaps best reflected by the increased plasma level of pipecolic acid, and (ii) removal of excess glutamate from the synaptic cleft possibly by glutamate transporters as shown in Chapter 6. A recent *in vitro* study on cortical tissue from FHM1 R192Q mice showed that the enhanced glutamatergic transmission caused enhanced recruitment of inhibitory neuronal networks (Vecchia et al 2014). This is in line with reports on a possible compensatory inhibitory response in the cortex of migraine patients (Cosentino et al 2014).
Table 1.

<table>
<thead>
<tr>
<th>Migraine-related features</th>
<th>FHM1 R192Q</th>
<th>Reference</th>
<th>Remarks</th>
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<tbody>
<tr>
<td>Photophobia</td>
<td>Present</td>
<td>Chanda et al 2013</td>
<td>Modified elevated plus maze was used for behavioural studies; mutant mice spent more time in open arms compared with brightly illuminated closed safe arms.</td>
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<tr>
<td>Hemiplegia</td>
<td>Present</td>
<td>Eikermann-Haerter et al 2009</td>
<td>Hemiplegia was observed and lasted 20 min or more after recovery from a single CSD event induced under anesthesia. S218 mutants were more severely affected.</td>
</tr>
<tr>
<td>CSD susceptibility</td>
<td>Increased</td>
<td>van den Maagdenberg et al 2004, 2010, Eikermann-Haerter et al 2009b, Chapters 2 &amp; 3</td>
<td>In Chapters 2 &amp; 3 CSD susceptibility (frequency and threshold) was increased compared to WT both for experiments with and without physiological monitoring or control. S218L mice display enhanced CSD frequency compared to R192Q mutants (Eikermann-Haerter et al 2009b, van den Maagdenberg et al 2010).</td>
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<tr>
<td>Female preponderance</td>
<td>Present</td>
<td>Eikermann-Haerter et al 2009, Chapter 2</td>
<td>In S218L mice CSD frequency is also enhanced for female compared to male mice. In Chapter 2, gender effect on CSD frequency in R192Q mice is only observed for experiments with physiological monitoring and control.</td>
</tr>
<tr>
<td>Neuronal hyperexcitability</td>
<td>Present</td>
<td>Tottene et al 2009, Hullugundi et al 2014, Vecchia et al 2014, Vecchia et al 2015, Chapter 3</td>
<td>In Tottene et al. and Vecchia et al. (2014) cortical slices and neuronal cultures were analysed in vitro; Vecchia et al. (2015) shows most severe effects in S218L homozygous mice; in Hullugundi et al. cultured trigeminal ganglia neurons in vitro, and in Chapter 3 EEG activity was analysed in vivo.</td>
</tr>
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In Chanda et al. signs of headache were associated with novelty stress; S218L mutants showed stronger blink responses compared to R192Q mice.

In Franceschini et al. trigeminal ganglia were analyzed.

R192Q mice exhibited enhanced excitatory transmission and LTP in the hippocampus but impaired learning and memory.

Acute administration of corticosterone, but not a 3h restraint stress, results in enhanced CSD frequency within 3 hrs.

R192Q mice showed enhanced phase resetting to 6-hr advance shifts of the light/dark cycle in freely behaving electrophysiology studies of EEG and SCN activity; no differences between mutants and WT mice were observed in *in vitro* recordings of the suprachiasmatic nucleus.

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<thead>
<tr>
<th>Migraine triggers</th>
<th>Effective</th>
<th>Chapter 4</th>
<th>CSD frequency within 3 hrs.</th>
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<tbody>
<tr>
<td>Stress</td>
<td>Effective</td>
<td>Chapter 4</td>
<td>Enhanced CSD frequency</td>
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<tr>
<td>Circadian rhythm shift</td>
<td>Effective</td>
<td>van Oosterhout et al 2008</td>
<td>Enhanced phase resetting</td>
</tr>
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</table>

Table 1. The FHM1 mouse model is a relevant animal model for migraine

Summary of experimental findings that highlight the relevance of FHM1 R192Q mice as a useful animal model for FHM, and perhaps also for the common forms of migraine. FHM1 R192Q mice exhibit key migraine-related features, such as signs of headache, photophobia and increased susceptibility to CSD including occurrence of spontaneous CSD. In addition, the FHM1 R192Q mice phenotype can be modulated by relevant migraine triggers such as stress hormones or circadian phase shifts. For several readouts, as indicated in the remarks, the phenotype was more severe or effects were stronger in FHM1 S218L compared to FHM1 R192Q mice.

8.7 Relevance of FHM1 R192Q mice as a useful animal model to study migraine pathophysiology

Migraine is a brain disorder with symptoms varying considerably between individuals (Goadsby et
al 2002), but there are core features of the disease. A good animal model of a disorder should ideally replicate (and allow the investigation) of such core features. The FHM1 R192Q mouse model used in this thesis displays various features that seem not only relevant to FHM but also to the common forms of migraine (Table 1).

In brief, FHM1 R192Q mice exhibit:

- *signs of photophobia and unilateral headache* (Chanda et al 2013), which are prominent symptoms in migraine patients (ICHD 2004).

- *transient hemiplegia following induction of CSD* (Eikermann-Haerter et al 2009b), thus mimicking the characteristic motor problems in patients with FHM.

- *enhanced CSD susceptibility and spontaneous CSD events*, as both FHM1 transgenic mouse models exhibit enhanced CSD susceptibility compared to WT. Furthermore, as recorded, in this thesis for the first time, in freely behaving mutant mice (Chapter 3), which forms a reassuring translational paradigm to mimic the episodic nature of migraine.

- *a CSD phenotype in FHM1 R192Q mice that is more pronounced in females*, in line with the higher propensity of migraine in women; this can be explained by modulation by gender hormones and gonadectomy as shown by effects of ovariectomy in females (Eikermann-Haerter et al 2009b, Chapter 2) and orchiectomy in males (Eikermann-Haerter et al 2009a).

- *neuronal hyperexcitability* as identified in cortical brain slices (Tottene et al 2009), cortical neuronal cultures (Vecchia et al 2014; Vecchia et al 2015) and, in this thesis, at the neuronal network level in freely behaving mice (Chapter 3), which is in agreement with the concept that hyperexcitability (Aurora & Wilkinson 2007), and possibly dynamic changes in neuronal excitability (Cosentino et al 2014) underlies migraine.

- *signs of inflammation*, as shown for trigeminal ganglia (Franceschini et al 2013)

- *signs of impaired learning and memory*, which may explain cognitive changes associated with FHM and, possibly, common forms of migraine (Dilekkoz et al 2015).

- *enhancement of migraine-relevant readouts in response to triggers of migraine*, such as stress (and stress hormones) and sudden shifts in circadian rhythms, as shown by increased CSD susceptibility to acute corticosterone administration (Chapter 4) and an enhanced circadian adaptation (van Oosterhout et al 2008).

Several of the functional readouts are impacted by allele dosage, and, most importantly, by the type of FHM1 mutation: strongest effects of the mutations are observed for S218L homozygous mice in comparison to homozygous R192Q mice (Eikermann-Haerter et al 2009b, van den Maagdenberg et al...
The observation of a more severe phenotype in S218L compared to R192Q mice is in line with the clinical presentation of symptoms in patients (Haan et al 2005, Stam et al 2009), thus underscoring the usefulness of FHM1 mice for studying mechanisms of migraine pathophysiology. The finding that effects of FHM1 mutations can differ among neurons of specific brain regions (Fioretti et al 2011, Inchauspe et al 2010) and, as shown for cortex, have strong effects on excitatory but not inhibitory neurons (Tottene et al 2009, Vecchia et al 2014, Vecchia et al 2015) provides a mechanistic basis for dissecting the role of various neuronal networks in migraine pathophysiology.

8.8 Future perspectives

An important finding in this thesis is the identification of spontaneous CSD events occurring in FHM1 R192Q mice but not WT mice. Future experiments should focus on revealing changes in neuronal firing properties and changes of neuronal network properties that precede spontaneous CSD events. Revealing such changes will be instrumental for a better understanding of neurobiological mechanisms that explain characteristics of CSD and migraine headache, and in combination with the identification of biomolecules and disease mechanisms, e.g., using the molecular tools used in this thesis, can be exploited to design novel therapies for migraine.

Whereas our data revealed an increased susceptibility to CSD in FHM1 R192Q mice upon an acute, strong elevation in corticosterone plasma levels, we did not see an effect on CSD susceptibility when mutant mice were subjected to a single restraint stress (that also increased corticosterone levels) or chronically elevated corticosterone levels. These findings seem to indicate corticosterone exerts multiple effects on biological systems and that the administration of corticosterone does not faithfully mimic the exact consequence to a physiological stressor. In humans, stress has a strong subjective component and often consists of small every day stressors, instead of a single major stressor. Perhaps only their cumulative effects may sufficiently modulate the threshold for CSD. Clinical data indicate that the “let-down” of stress can increase the likelihood of a migraine attack (Lipton et al 2014). Future research should focus on identifying every day (milder) stressors, which presumably affect factors in addition to cortisol, to better understand how stress affects migraine. From a clinical point of view it may still be relevant to identify the time point of corticosterone reduction following chronically high corticosterone levels (glucocorticoid withdrawal) and assess whether CSD susceptibility is changed at that transition. In addition, to stress and corticosterone, future experiments may also address effects of other known migraine-relevant trigger factors, such as drugs overuse (e.g., triptans overuse), changes in sleep patterns or specific foods, in modulating characteristics of CSD and other migraine-relevant outcomes.

Finally, the identification of reliable disease biomarkers is important; not only for diagnosis of the disease but also for pinpointing potential novel drug targets. Such biomarkers do not exist for migraine, yet. The identification, in this thesis, of specific compounds that are differentially regulated following CSD induction in an animal model of migraine in peripheral body fluid and brain tissue, may have relevance to migraine biomarker discovery. Future research may use these compounds as
a starting point to perform a systematic targeted analysis and identify whether similar compounds are abnormally regulated during and between migraine attacks in patients. Such targeted approach may further our understanding of migraine pathophysiology and may aid in the development of more effective treatments for migraine.
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