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Chapter 2

Methodology driven differences in physiological parameters influence characteristics of cortical spreading depression in wild-type and familial hemiplegic migraine type 1 mice

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In preparation
ABSTRACT

Changes in physiology and anesthesia modulate susceptibility to cortical spreading depression (CSD), the neurobiological correlate of migraine aura. Here we investigated to what extent CSD susceptibility parameters (frequency and threshold) depend on whether physiological parameters (pO$_2$, pCO$_2$, pH and blood pressure) were: i) not monitored at all, ii) only monitored (i.e., measured in blood from a femoral artery catheter; physiological monitoring), or iii) monitored and controlled (i.e., adjusted during the experiment by subtle changes in mechanical ventilation using tracheotomy; physiological control). In addition, we investigated to what extent the anesthesia gas mixture affects these CSD parameters. We studied effects of the methodologies in both wild-type (WT) mice and familial hemiplegic migraine type 1 (FHM1) transgenic mice that express the human pathogenic R192Q missense mutation in voltage-gated Ca$_{v}$.2.1 Ca$^{2+}$ channels (R192Q mice). A previous study revealed an enhanced CSD frequency in the visual cortex of mutant mice, an effect that was most pronounced in females, when experiments were performed with isoflurane-N$_2$/O$_2$ anesthesia and physiological control. In the present study, however, using isoflurane-air anesthesia without physiological monitoring and without physiological control, we observed that visual cortex CSD frequency was equally enhanced in mutant mice of both genders. In contrast, a gender difference was observed for CSD threshold which was decreased in R192Q mice compared to WT to a larger extent in males than in females. The absence of a gender effect on CSD frequency in mutant mice was not related to the use of air instead of N$_2$/O$_2$ but to the presence of mechanical ventilation and possible subtle changes in pH, pCO$_2$ or blood pressure when physiological parameters were not controlled. Genotypic effects of enhanced CSD susceptibility in R192Q compared to WT mice were identified irrespective of the tested methodologies. Comparison of CSD susceptibility in visual cortex vs motor cortex revealed a regional difference in CSD susceptibility that was influenced by gender in the absence of physiological control. Physiological control can either unmask or mask a gender effect on specific CSD parameters. This sensitivity of CSD susceptibility to methodology has important implications when comparing data from different studies. Moreover, it suggests that there is a drawback of controlling physiological status of an animal as one may miss specific characteristics of CSD susceptibility when these depend on differences in physiological parameters between R192Q mutant and WT mice.
INTRODUCTION

One-third of migraine patients experience auras in addition to headaches, characterized by neurological sensory dysfunctions that in most cases consist of visual symptoms (Goadsby et al 2002, ICHD 2004). Cortical spreading depression (CSD) is the likely cause of the migraine aura, and it is characterized by a slowly propagating wave of neuronal and glial depolarization followed by a transient suppression of neuronal activity (Lauritzen 1994).

Changes in physiological parameters such as pH, pO$_2$, pCO$_2$ and blood pressure have been shown to influence CSD susceptibility measurements in animals under anesthesia as they can change neuronal excitability and vascular function (Holland et al 2012, Kudo et al 2008, Pietrobon & Moskowitz 2014, Ruusuvuori & Kaila 2014, Sukhotinsky et al 2010). For this reason, physiological parameters are in most studies monitored via femoral artery catheterization (physiological monitoring) and controlled using mechanical ventilation by changing the ventilation settings to adjust physiological parameters as needed (physiological control). In addition to the presence or absence of physiological monitoring and control, CSD experiments also vary with respect to choice of anesthesia gas mixture, which can also affect the outcome of CSD susceptibility measurements. For instance, N$_2$O (instead of pressurized air) in the anesthesia gas mixture has a suppressive effect on CSD parameters (Kudo et al 2008). Consequently, the experimental design of CSD experiments under anesthesia that may include physiological monitoring or physiological control, or neither, is expected to result in different outcomes of CSD frequency and threshold.

The importance of understanding the consequences of methodologies for assessing CSD parameters is especially relevant when comparing results from different studies. Here we show the relevance of different methodologies for CSD assessment with respect to the investigation of transgenic knock-in mice that carry the R192Q missense mutation in the $\alpha_1$ subunit of Ca$_{V2.1}$ Ca$^{2+}$ channels (van den Maagdenberg et al 2004). In humans, this mutation causes familial hemiplegic migraine 1 (FHM1; (Ophoff et al 1996). FHM1 R192Q mice have been used in different laboratories to unravel migraine-relevant mechanisms (Ferrari et al 2015).

Different methodologies and read-out measures have been used in different laboratories to assess the susceptibility of CSD in R192Q mice. A decreased threshold for the induction of CSD, assessed with increasing electrical stimulus intensity, was reported for the visual cortex of mutant mice that were kept under urethane anesthesia (van den Maagdenberg et al 2004, van den Maagdenberg et al 2010). Physiological parameters were not monitored during these experiments, and a gender difference was not reported. In another study, an increased frequency of CSD, assessed with a 30-min application of a cotton ball soaked in 300 mM KCl, was reported for the visual cortex of mutant mice in experiments performed with isoflurane-N$_2$O/O$_2$(70%/30%) anesthesia and physiological monitoring and control (Eikermann-Haerter et al 2009a, Eikermann-Haerter et al 2009b). In those studies, female mutant mice showed a higher CSD frequency than male mutants; ovariectomy normalized CSD frequency in
mutant females to males levels. WT mice showed no gender difference and no effect of ovariectomy in females. Notably, a gender difference had been observed when CSD threshold was studied in the cortex of C57BL/6 WT mice that were kept under isoflurane-air anesthesia and physiologically monitored (Brennan et al 2007). Apparently, a gender difference is revealed when using one methodology but not another methodology. This leads to the question whether certain aspects of CSD susceptibility may only be observed when a specific combination of methodology and anesthetics is used, and whether results between laboratories can be compared.

We here assessed cortical CSD frequency in male and female R192Q and WT mice using various methodologies. Namely, i) without monitoring and control using isoflurane-air anesthesia, ii) monitoring without control using isoflurane-air anesthesia, iii) monitoring without control using isoflurane-N₂O/O₂ anesthesia, and iv) monitoring and control using isoflurane-N₂O/O₂ anesthesia. In addition, we compared CSD frequency and threshold in visual and motor cortex, in both male and female mice, in the absence of physiological monitoring and physiological control.

**MATERIALS AND METHODS**

**Animals**

Male and female homozygous Cacna1a FHM1 R192Q knock-in (“R192Q”) and wild-type (“WT”) mice of 2-4 months were used. The R192Q mice were generated by introduction of the human FHM1 pathogenic R192Q missense mutation in the mouse Cacna1a gene using a gene targeting approach (van den Maagdenberg et al 2004). All experiments were approved by the Animal Experiment Ethics Committee of Leiden University Medical Center.

**CSD threshold and frequency recordings without physiological monitoring or physiological control**

All experiments were performed during daytime between 10.00 am–13.00 pm. Mice were anesthetized using 1.5% isoflurane in pressurized air (80% N₂ and 20% O₂) and were breathing spontaneously. Core body temperature was maintained at 37 ºC using a heating pad (Stoelting, Wood Dale, IL, USA). Mice were mounted into a stereotaxic frame (David Kopf Instruments, Tujunga, CA, USA). Subsequently, a midline incision of ~2.5 cm was made over the top of the head, the skin was retracted to expose the skull, and the periosteum was removed with cotton-tipped applicator sticks. Two craniotomy windows were drilled at the following coordinates in the right hemisphere (in mm with respect to Bregma): 3.5 posterior/2.0 lateral (visual cortex) and 1.5 anterior/2.0 lateral (motor cortex) (Figure 1A). Care was taken to keep the dura intact to minimize trauma to the underlying brain tissue. At the recording site a sharp glass capillary electrode (FHC Inc., Bowdoin, ME, USA) filled with 150 mM NaCl was advanced through the dura to a depth of 300 µm. After insertion of the electrode, a drop of mineral oil (~5 µL) was applied to the recording site to prevent drying of cortical tissue. The surgical procedure was completed within 20 min after the start of the anesthesia. DC-potential signals were measured.
with respect to an Ag/AgCl reference electrode placed subcutaneously at the neck and amplified 10x (Molecular Devices, Sunnyvale, CA, USA). The DC signal was low-pass filtered at 4 Hz and digitized at 100–200 Hz using PowerLab 16/30 hardware (AD Instruments, Inc., Colorado Springs, CO, USA). Data were recorded and analyzed off-line using LabChart Pro (AD Instruments). In each mouse, first the CSD threshold was measured either in the visual or motor cortical window. To this end a cotton pellet (Interguide Dental Supply, Burlingame, CA, USA) soaked in a solution of KCl with a specific concentration (initially 5 mM) was placed on the dura overlaying either the visual or motor cortex for 3 min. In case no CSD was induced during this time window, the cotton pellet was replaced with another pellet that contained a higher K⁺ concentration. Solutions contained increasing K⁺ concentrations with 7.5-mM increments (with total osmolarity kept at 300 mOsmol by addition of NaCl to the solution). The measurements continued until a CSD event was observed, so the CSD threshold could be determined. After the CSD threshold measurement, the induction site was rinsed with 150 mM NaCl. Subsequently, CSD frequency was determined at the same location. The location used (visual vs motor cortex) is mentioned for the respective CSD frequency result in the legend. For CSD frequency measurements, CSD events were induced by placement of a cotton pellet soaked in 1 M KCl on the dura overlaying the visual or motor cortex for 30 min, with refreshment of the pellet after 15 min. The total number of CSD events that occurred within 30 min was used to calculate the frequency per hr. For both the CSD threshold and frequency measurements, only reversible DC deflections with amplitudes larger than 5 mV were considered CSD events and included for further analysis.

**CSD frequency recordings with physiological monitoring**

Mice were maintained under 1.5% isoflurane anesthesia in 80% N₂O/20% O₂ and allowed to breathe spontaneously. Blood gas and mean arterial blood pressure values were monitored by placing a catheter in the left femoral artery. The surgical procedures were completed within 30 min after the start of the anesthesia. Blood pressure was measured continuously via a blood pressure transducer (AD instruments) connected to the femoral artery lead. To obtain information on physiological parameters (pH, pO₂, pCO₂; Table 2), 40-μL blood samples were collected before the start and at the end of the 30 min CSD frequency measurement and used for blood-gas analysis. Accepted ranges for physiological parameters were: pH=7.35-7.45; pO₂=80-140 mmHg; pCO₂=30-40 mmHg; and blood pressure=70-110 mmHg. Visual cortex CSD frequency measurements were performed upon induction of CSD by 1 M KCl application on the dura overlaying the visual cortex, as described above. In a subset of the experiments, isoflurane-air was used instead of isoflurane-N₂O/O₂ anesthesia.

**CSD frequency recordings with physiological control**

In contrast to experiments with physiological monitoring, described in the previous section, mice were now mechanically ventilated and physiological parameters adjusted when necessary. Measurement of CSD frequency with physiological control was performed as described in (Eikermann-Haerter et al 2009b), with slight modifications. In brief, mice were maintained under 1.5% isoflurane anesthesia in a gas mixture of 70-80% N₂O and 20-30% O₂. Blood gas and mean arterial blood pressure values
were monitored via a catheter in the left femoral artery, as described above. After insertion of the catheter in the femoral artery, an endotracheal tube was inserted in the trachea that allowed artificial ventilation of the mouse. The mouse received an i.p injection of 0.04 mg/kg pancuronium for muscle paralysis to suppress spontaneous breathing and was connected to a mouse ventilator (SAR-830, CWE Inc, Ardmore, PA, USA). The surgical procedures were completed within 45 min after the start of the anesthesia. Visual cortex CSD frequency was measured upon induction of CSD by 1 M KCl application on the dura overlaying the visual cortex (Figure 1A), as described in the previous section, except that physiological parameters (i.e., pH, pO$_2$, pCO$_2$ and blood pressure) were now monitored (Table 2) and, if necessary, controlled by adjustments in ventilation. Blood pressure was measured continuously, as described above. For the other parameters, 40 µL blood samples were collected before the start and at the end of the 30 min CSD frequency measurement and used for blood-gas analysis; accepted ranges for physiological parameters were: pH=7.35-7.45; pO$_2$=80-140 mmHg; pCO$_2$=30-40 mmHg; and blood pressure=70-110 mmHg. When pH and pCO$_2$ values were outside the accepted ranges, breathing rate and time were adjusted. When pO$_2$ values were outside the accepted ranges, adjustments were made to the administered O$_2$ concentration. In this way physiological parameters were controlled during the experiment. The effect of the ventilator adjustments on physiological parameters was determined by taking a blood sample, immediately after the adjustment.

**Ovariectomy**

For removal of the ovaries, female mice were anesthetized using 1.5% isoflurane in pressurized air (80% N$_2$ and 20% O$_2$). A 1 cm incision was made in the skin of the flank followed by an incision in the muscle wall. Ovaries were separated from the surrounding tissue with ligatures, and carefully removed, after which the skin was closed with sutures. The mouse was given a subcutaneous injection of 1 mL 0.9% NaCl to maintain physiological hydration. The mouse also received an intramuscular injection of 0.1 mg/kg temgesic for post-operative analgesia. After a 2-week recovery period, CSD threshold and frequency recordings were performed using isoflurane-air anesthesia in the absence of physiological monitoring or control, as described above.

**Statistical analysis**

For statistical analysis of CSD threshold, which is skewed, the Mann-Whitney U-test was used. For CSD frequency values, one-way ANOVA followed by Bonferroni correction or Student’s t-test was used. Statistical significance was set at 0.05.
Figure 1. In the absence of physiological monitoring and control under isoflurane-air anesthesia, visual cortex CSD frequency is enhanced in R192Q mice compared with WT, with no difference between genders.

(A) Schematic representation of visual cortex CSD frequency measurements in anesthetized mice. CSD is induced by placement of a cotton pellet containing 1 M KCl on the dura overlaying the visual cortex for a period of 30 min, while CSD events are measured by DC-recording via a glass-electrode placed in the motor cortex. In experiments without physiological monitoring or control, CSD frequency measurements followed CSD threshold measurements (see Methods) (B) Example CSD frequency traces illustrating enhanced visual cortex CSD frequency in male R192Q compared with WT mice in the absence of physiological control using isoflurane-air anesthesia (C) Bar diagram depicting enhanced CSD frequency in the visual cortex in both male and female R192Q compared with WT mice (†p=0.0001 and ‡p=0.0001; one-way ANOVA Bonferroni correction). No CSD frequency difference was observed for male compared with female R192Q mice in experiments without physiological control using isoflurane-air anesthesia.
RESULTS

CSD frequency in the visual cortex is enhanced in R192Q mice without revealing a gender difference, when assessed in the absence of physiological monitoring or physiological control

We determined visual cortex CSD frequency (Figure 1A, B) of male and female R192Q and WT mice using isoflurane-air anesthesia in the absence of physiological monitoring or physiological control. Both female and male R192Q mice showed an enhanced visual cortex CSD frequency compared with WT (female R192Q, 16.4±3.3 CSD/hr, vs female WT, 8.5±3.6 CSD/hr, N=9, p=0.0001; male R192Q, 17.0±2.1 CSD/hr, N=10, vs male WT, 8.9±2.8 CSD/hr, N=14, p=0.0001). For both R192Q and WT mice no gender difference was observed (female R192Q vs male p=0.6; female WT vs male p=0.7) (Figure 1C). Table 1 summarizes CSD amplitude and duration characteristics for the different groups. Except for CSD duration, which was longer for female WT compared with female R192Q mice, no statistical differences were observed. Not finding a gender difference in R192Q mice contrasts with published data that revealed that female mutant mice displayed a higher CSD frequency when isoflurane-N\textsubscript{2}O/O\textsubscript{2} was used and physiological parameters were controlled (Eikermann-Haerter et al 2009b).

CSD threshold in the visual cortex is reduced in R192Q mice, and more so in male mutants, when assessed in the absence of physiological monitoring and physiological control

A study by Brennan et al. (Brennan et al 2007) showed that CSD threshold in the visual cortex was reduced in female compared with WT mice in experiments in which physiological parameters were monitored, and not controlled. We here assessed visual cortex CSD threshold of R192Q and WT mice of both genders, using isoflurane-air anesthesia, without monitoring or controlling physiological parameters. CSD threshold, as assessed by the KCl concentration required to elicit a CSD (Figure 2A), was reduced in both female and male R192Q mice compared with WT mice of the same gender (female R192Q, median=51.8 mM KCl, N=14 vs female WT, median=68.7 mM KCl, N=9; p=0.01; male R192Q, median=38.7 mM KCl, N=11 vs female WT, median=58.7 mM KCl, N=14; p=0.001) (Figure 2B). Rather unexpectedly, male R192Q mice showed a lower CSD threshold (p=0.02) compared with female R192Q mice. CSD threshold did not statistically differ between female and male WT mice, although male WT mice showed a trend towards a lower threshold (p=0.08). There were no statistical differences in CSD amplitude or duration between female and male mice, or between genotypes for the visual cortex threshold experiments (Table 1).
Figure 2. Visual cortex CSD threshold is reduced in R192Q mice compared to WT in the absence of physiological control under isoflurane-air anesthesia, with strongest effect in males.

(A) Specimen recordings of CSD threshold assessments illustrating the lower KCl concentration that is required to induce a CSD in the visual cortex of a male R192Q compared with a WT mouse (B) Box plots depicting lower visual cortex CSD threshold in male R192Q compared with female mice (*p=0.02 male R192Q vs female R192Q, Mann-Whitney U-test) and in R192Q male and female mice compared with WT (#p=0.001 male R192Q vs WT; §p=0.01 female R192Q vs WT, Mann-Whitney U-test).
Table 1. CSD amplitude and duration characteristics of CSD frequency and threshold recordings from visual and motor cortex in R192Q and WT mice for experiments performed in the absence of physiological monitoring or control, using isoflurane-air

<table>
<thead>
<tr>
<th>CSD Frequency</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Amplitude (mV)</td>
</tr>
<tr>
<td>R192Q visual</td>
<td>10</td>
<td>18.9±3.3</td>
</tr>
<tr>
<td>WT visual</td>
<td>14</td>
<td>20.9±3.0</td>
</tr>
<tr>
<td>R192Q motor</td>
<td>7</td>
<td>14.9±6.2</td>
</tr>
<tr>
<td>WT motor</td>
<td>10</td>
<td>21.1±4.1†</td>
</tr>
</tbody>
</table>

CSD Threshold

<table>
<thead>
<tr>
<th>CSD Frequency</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>Amplitude (mV)</td>
</tr>
<tr>
<td>R192Q visual</td>
<td>11</td>
<td>21.9±1.9</td>
</tr>
<tr>
<td>WT visual</td>
<td>14</td>
<td>25.0±4.3</td>
</tr>
<tr>
<td>R192Q motor</td>
<td>8</td>
<td>17.9±5.0</td>
</tr>
<tr>
<td>WT motor</td>
<td>11</td>
<td>22.7±2.7†</td>
</tr>
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</table>

Table 1. Values are shown as mean ± SD. CSD duration was measured at half-maximal amplitude. CSD frequency data: comparison of CSD characteristics in CSD frequency recordings revealed a lower CSD amplitude in female WT mice for the motor cortex compared with female WT mice for the visual cortex (p=0.0001), and compared with male WT mice in the motor cortex (p=0.038, one-way ANOVA, Bonferroni correction). CSD amplitude was also reduced in female R192Q mice for the motor cortex compared to the visual cortex (p=0.049, one-way ANOVA, Bonferroni correction). There were no differences in CSD duration except for a shorter duration in female R192Q mice for the visual cortex compared with female WT mice visual cortex (p=0.016, one-way ANOVA, Bonferroni correction). CSD threshold data: CSD amplitude was reduced in female WT mice for the motor compared to the visual cortex (p=0.0001) and compared to motor cortex in male WT mice (p=0.0001, one-way ANOVA, Bonferroni correction). There were no differences between groups in CSD duration for CSD threshold measurements.

CSD susceptibility is not affected by gender when assessed in the motor cortex in the absence of physiological monitoring and physiological control

Given reports on visual cortex hyperexcitability in migraine patients (Aurora & Wilkinson 2007), and the suggestion of CSD to initiate preferably in the visual cortex (Hadjikhani et al 2001), we next assessed whether effects of genotype and gender on measures of CSD susceptibility frequency and
threshold are specific to the visual cortex. Therefore, we investigated these CSD measurements also in the motor cortex. Experiments were carried out using isoflurane-air anesthesia without physiological monitoring or physiological control. Regarding a possible effect of genotype, for the motor cortex both male and female R192Q mice exhibited higher CSD frequencies compared with WT mice of the same gender (male R192Q, 14.0±3.6 CSD/hr, N=7 vs male WT, 7.6±1.3 CSD/hr, N=10, p=0.0001; female R192Q, 13.0±3.9 CSD/hr, N=12 vs female WT, 5.5±1.7 CSD/hr, N=7, p=0.0001) (Figure 3A). For CSD threshold however, both male and female R192Q mice showed comparable CSD thresholds as WT mice of the same gender (male R192Q, median=56.8 mM KCl, N=9 vs male WT, median=57.5 mM KCl, N=11, p=0.8; female R192Q, median=59.3 mM KCl, N=12 vs female WT, median=63.1 mM KCl, N=10, p=0.60 (Figure 3B). With respect to gender, for motor cortex CSD frequency, similar as observed for the visual cortex, no difference was observed between male and female mice for both R192Q (p=0.5) and WT mice (p=0.3) (Figure 3A). For CSD threshold measurements in the motor cortex, in contrast to the visual cortex, no difference was observed between genders in R192Q mice (p=0.7). In WT mice, as seen for the visual cortex, no gender effect was observed for motor cortex CSD threshold (p=0.8) (Figure 3B).

Figure 3. In the absence of physiological control, CSD susceptibility in the motor cortex is not influenced by gender. Experiments were performed in the absence of physiological control under isoflurane-air anesthesia (A) Bar diagrams showing an increased motor cortex CSD frequency for both male (†p=0.0001) and female R192Q mice (‡p=0.0001; one-way ANOVA, Bonferroni correction) compared with WT, with no differences between genders among mice of the same genotype (B) CSD threshold values in the motor cortex were comparable between R192Q and WT mice of both genders in experiments without monitoring or control.
Given these observations, we next assessed whether CSD susceptibility measurements of frequency and threshold may differ between the visual and motor cortex, with possible effects of genotype and gender. Between cortical regions, male R192Q mice exhibited comparable CSD frequencies for visual and motor cortex, although a trend towards higher CSD frequency was observed for the visual compared with motor cortex (p=0.05). Female R192Q mice displayed a significantly higher CSD frequency in the visual compared with motor cortex (p=0.02). CSD frequency was not different for the visual compared with motor cortex in male WT (p=0.2) and female WT mice (p=0.07) (Figures 1C and 3A). CSD threshold was lower in the visual compared with the motor cortex (p=0.009) in male R192Q mice. Female R192Q mice however showed comparable CSD thresholds for both visual and motor cortex (p=0.1). In WT mice, CSD threshold was not different between motor and visual cortex in both male (p=0.6) and female mice (p=0.7) (Figures 2B and 3B). Table 1 summarizes CSD characteristics amplitude and duration for the different groups. For CSD frequency measurements, some differences were observed regarding CSD amplitude. In particular, for female R192Q mice, lower CSD amplitude was observed in motor compared with visual cortex. Furthermore, female WT mice showed lower CSD amplitude in the motor compared with the visual cortex, and compared with male WT mice in the motor cortex. For CSD threshold measurements, no differences were observed except for lower CSD amplitude in female WT mice in the motor compared with the visual cortex.

Ovariectomy has no strong influence on visual cortex CSD susceptibility in experiments without physiological monitoring and physiological control

Although visual cortex CSD frequency was comparable between male and female R192Q mice in physiologically uncontrolled experiments, the enhanced CSD frequency that was observed for female, but not male, R192Q mice in the visual compared with the motor cortex suggests some effect of gender on CSD characteristics in the absence of physiological control. Hence we next determined, in experiments performed in the absence of physiological monitoring and control, whether ovariectomy in female R192Q mice may reduce visual cortex CSD frequency values to those observed for motor cortex. After ovariectomy in female R192Q mice, visual cortex CSD frequency did not show a difference anymore with motor cortex CSD frequency from intact female R192Q mice (p=0.2). Nevertheless, visual cortex CSD frequency itself was not significantly reduced by ovariectomy in female R192Q or WT mice (R192Q ovariectomized (Ovx), 15.2±4.0 CSD/hr, N=8 vs R192Q intact, 16.4±3.3 CSD/hr, N=14, p=0.8; WT Ovx, 7.9±2.0 CSD/hr, N=5 vs WT intact, 8.5±3.6 CSD/hr, N=9, p=0.7). Similar as for intact female R192Q compared with WT mice, ovariectomized female R192Q mice showed higher visual cortex CSD frequency compared with ovariectomized WT mice (p=0.003). Given the observed, unexpected, higher visual cortex CSD threshold observed for female compared with male R192Q mice, we next determined whether this effect may be influenced by ovariectomy. Similar as for CSD frequency, visual cortex CSD threshold was not influenced by ovariectomy for both female R192Q and WT mice (R192Q Ovx, median=57.5 mM KCl, N=7 vs R192Q intact, median=51.8 mM KCl, N=14, p=0.3; WT Ovx, median=72.5 mM KCl, N=5 vs WT intact, median=68.7 mM KCl,
N=9, p=0.4). As a consequence, ovariectomized female R192Q mice still showed a higher visual cortex CSD threshold compared with male R192Q mice (p=0.01). The genotype effect on visual cortex CSD threshold was also not influenced by ovariectomy. Ovariectomized R192Q mice showed reduced CSD threshold compared with ovariectomized WT (p=0.04). CSD amplitude and duration were not influenced by ovariectomy for both CSD frequency and threshold experiments.

_CSD frequency in the visual cortex is enhanced in R192Q mice, and more so in female mutants, when assessed in the presence of physiological monitoring and physiological control_

Although our experiments performed without physiological monitoring or physiological control show the reported genotypic effect of the R192Q mutation on CSD susceptibility, the absence of a gender effect on visual cortex CSD frequency is not in line with earlier experiments in R192Q mice from Eikermann-Haerter et al. that were performed in the presence of physiological control (Eikermann-Haerter et al 2009b). For CSD threshold in WT mice, in contrast to studies from Brennan et al. (Brennan et al 2007) we did not observe an effect of gender, and for male R192Q mice we observed an unexpected lower CSD threshold compared with female R192Q mice. Given the variable duration of a CSD threshold paradigm that may have an impact on the studies in mice without physiological control, in our next experiments we choose to use only visual cortex CSD frequency assessment. We performed visual cortex CSD frequency measurements using an experimental paradigm in which isoflurane-N\textsubscript{2}O/O\textsubscript{2} anesthesia was used, and in which mice were kept under physiological control, thus following the protocol described in Eikermann-Haerter et al. (Eikermann-Haerter et al 2009b). Physiological parameters (pO\textsubscript{2}, pCO\textsubscript{2}, pH and blood pressure) are shown in Table 2. Both female (p=0.0002) and male R192Q mice (p=0.004) exhibited a higher CSD frequency compared with WT of the respective gender. In line with published data, CSD frequency was higher in female than in male R192Q mice (female R192Q, 22.8±4.5 CSD/hr, N=7 vs male R192Q, 16.32±1.9 CSD/hr, N=6; p=0.007), while no gender difference was observed for WT mice (female WT, 10.42±1.8 CSD/hr, N=5 vs male WT, 12.0±1.7 CSD/hr, N=5; p=0.1) (Figure 4). No statistical differences were observed for CSD amplitude and duration among the different groups, except for a longer CSD duration for female WT compared with female R192Q mice and compared with male WT mice (Table 2).

_The absence of a gender effect on CSD frequency in experiments that are not physiologically controlled is related to the absence of mechanical ventilation and slight differences in physiology_

The observation of a gender effect on visual cortex CSD frequency in R192Q mice in physiologically monitored and controlled experiments raises the question whether this gender effect may also be observed for experiments that are only physiologically monitored, provided that physiological parameters are within ranges. CSD threshold was not considered as a readout measure, given the variable duration of this paradigm. Therefore, we performed visual cortex CSD frequency assessments...
Figure 4. In physiologically controlled experiments performed under isoflurane-N₂O/O₂ anesthesia, visual cortex CSD frequency is enhanced in female R192Q mice compared with males. In agreement with previous findings in which physiological parameters were controlled using mechanical ventilation and isoflurane-N₂O/O₂ anesthesia (Eikermann-Haerter et al 2009b), female R192Q mice exhibited increased visual cortex CSD frequency compared with male R192Q mice (*p=0.007 female vs male R192Q; Student’s t-test). Genotypic comparisons revealed a higher CSD frequency for both male and female R192Q mice in comparison with WT (§p=0.004 male R192Q vs male WT; †p=0.0002 female R192Q vs female WT, one-way ANOVA, Bonferroni correction).

Table 2. CSD amplitude and duration and physiological parameters of R192Q and WT mice for CSD frequency experiments performed with physiological monitoring-only, or with physiological monitoring and control

<table>
<thead>
<tr>
<th>CSD Frequency</th>
<th>N</th>
<th>pH</th>
<th>pO (mmHg)</th>
<th>pCO (mmHg)</th>
<th>MABP (mmHg)</th>
<th>Amplitude (mV)</th>
<th>Duration (sec)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male R192Q monitored-air</td>
<td>6</td>
<td>7.45±0.02</td>
<td>97.8±4.0</td>
<td>25.5±2.7</td>
<td>74.2±1.1</td>
<td>21.1±1.1</td>
<td>27.1±5.1</td>
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<tr>
<td>Female R192Q monitored-air</td>
<td>6</td>
<td>7.38±0.02</td>
<td>102.8±5.9</td>
<td>28.6±2.1</td>
<td>77.3±3.3</td>
<td>19.3±2.8</td>
<td>28.6±11.1</td>
</tr>
<tr>
<td>Male R192Q monitored-N₂O/O₂</td>
<td>5</td>
<td>7.41±0.02</td>
<td>133.7±37.7</td>
<td>29.2±3.7</td>
<td>85.0±10.8</td>
<td>14.8±2.9</td>
<td>26.9±11.1</td>
</tr>
<tr>
<td>Female R192Q monitored-N₂O/O₂</td>
<td>6</td>
<td>7.36±0.03</td>
<td>113.1±10.9</td>
<td>33.2±5.0</td>
<td>83.5±3.0</td>
<td>19.3±1.1</td>
<td>19.8±2.5</td>
</tr>
<tr>
<td>Male R192Q controlled-N₂O/O₂</td>
<td>6</td>
<td>7.40±0.03</td>
<td>123.1±13.3</td>
<td>31.1±1.9</td>
<td>95.3±9.1</td>
<td>20.3±3.9</td>
<td>28.2±5.9</td>
</tr>
<tr>
<td>Female R192Q controlled-N₂O/O₂</td>
<td>7</td>
<td>7.42±0.04</td>
<td>128.1±23.0</td>
<td>27.5±1.9</td>
<td>94.7±8.9</td>
<td>22.0±1.9</td>
<td>21.8±4.9</td>
</tr>
<tr>
<td>Male WT Controlled-N₂O/O₂</td>
<td>5</td>
<td>7.39±0.01</td>
<td>132.4±3.4</td>
<td>31.0±1.6</td>
<td>95.2±11.6</td>
<td>22.9±2.8</td>
<td>27.0±3.9</td>
</tr>
<tr>
<td>Female WT Controlled-N₂O/O₂</td>
<td>5</td>
<td>7.38±0.01</td>
<td>128.7±3.5</td>
<td>31.5±2.4</td>
<td>99.5±9.0</td>
<td>21.6±6.9</td>
<td>45.2±14.0</td>
</tr>
</tbody>
</table>
in male and female R192Q mice that were physiologically monitored (by femoral artery catheterization) but not controlled by mechanical ventilation. In these experiments mice were breathing spontaneously using isoflurane with either air or N₂/O₂. In physiologically controlled experiments, mice were both monitored and mechanically ventilated, using isoflurane-N₂/O₂ anesthesia. In physiologically controlled experiments physiological parameters were maintained within physiological range by adjusting ventilation guided by femoral artery blood measurements. For some of the experimental groups, differences in CSD amplitude, duration or physiological parameters were observed. For female R192Q and WT mice, CSD amplitude and duration were not different among groups. There were some differences in physiological parameters: pH was higher (p=0.044) whereas pCO₂ was lower (p=0.020, one-way ANOVA, Bonferroni correction) for the female R192Q controlled-N₂/O₂ compared to the female R192Q monitored-N₂/O₂ group. Furthermore, the female R192Q controlled-N₂/O₂ group exhibited higher pO₂ values (p=0.031) and higher MABP (p=0.0001, one-way ANOVA, Bonferroni correction) compared to the female R192Q monitored-air group. For male R192Q mice, CSD duration was not different among groups. With respect to amplitude, the male R192Q monitored-N₂/O₂ group showed a lower CSD amplitude compared to the male R192Q monitored-air group (p=0.009) and compared to the male R192Q controlled-N₂/O₂ group (p=0.021, one-way ANOVA, Bonferroni correction). For the male R192Q monitored-air group compared to male R192Q controlled-N₂/O₂ group, pH was slightly higher (p=0.042) and pCO₂ was lower (p=0.013, one-way ANOVA, Bonferroni correction). MABP was higher for the male R192Q controlled-N₂/O₂ group compared to both male R192Q monitored-air (p=0.0001) and male R192Q monitored-N₂/O₂ groups (p=0.010, one-way ANOVA, Bonferroni correction). Between genders, the female R192Q monitored-N₂/O₂ group showed higher CSD amplitude compared to the male R192Q monitored-N₂/O₂ group (p=0.007, Student’s t-test). In addition, the male R192Q monitored-air group exhibited higher pH values compared to the female R192Q monitored-air group (p=0.001, Student’s t-test). For the different controlled-N₂/O₂ groups, some differences were observed in CSD characteristics and physiological parameter values between WT and R192Q mice, for both males and females. Although CSD amplitude for these experiments was comparable among groups, CSD duration was longer for female R192Q compared with female WT mice (p=0.0001) and for female WT compared to male WT mice (p=0.01, one-way ANOVA, Bonferroni correction). Furthermore, of the controlled-N₂/O₂ groups female WT mice exhibited higher MABP values compared with female R192Q (p=0.002) and compared with male WT mice (p=0.005, one-way ANOVA, Bonferroni correction). Lastly, pCO₂ was lower for the controlled-N₂/O₂ group of female R192Q mice compared to female WT (p=0.038) and compared with male R192Q mice (p=0.027, one-way ANOVA, Bonferroni correction). MABP: mean arterial blood pressure.
Chapter 2

**Figure 5**

A monitored-isoflurane-air

R192Q male monitored-air (N=6)
R192Q female monitored-air (N=6)

B monitored-isoflurane-N2O/O2

R192Q male monitored-N2O/O2 (N=5)
R192Q female monitored-N2O/O2 (N=6)

C All experimental conditions

R192Q male uncontrolled-air (N=10)
R192Q male monitored-N2O/O2 (N=5)
R192Q female uncontrolled-air (N=14)
R192Q female monitored-N2O/O2 (N=6)
R192Q female controlled-N2O/O2 (N=7)
Next, we carried out a direct comparison of the visual cortex CSD frequency data from male and female R192Q mice from the 4 different experimental methodologies used in the present study, i.e., i) uncontrolled-air, ii) monitored-air, iii) monitored-N\textsubscript{2}O/O\textsubscript{2}, and iv) monitored & controlled-N\textsubscript{2}O/O\textsubscript{2}. This comparison revealed that for male R192Q mice, CSD frequency was comparable for all 4 conditions. For female R192Q mice however, CSD frequency was significantly higher for the physiologically controlled group in which mice were both monitored and mechanically ventilated using isoflurane-N\textsubscript{2}O/O\textsubscript{2} anesthesia (p=0.001; controlled-N\textsubscript{2}O/O\textsubscript{2} vs uncontrolled-air; p=0.006; controlled-N\textsubscript{2}O/O\textsubscript{2} vs monitored-N\textsubscript{2}O; p=0.005; one-way ANOVA, Bonferroni correction) (Figure 5C). Comparison of CSD duration revealed no differences among groups. CSD amplitude however was lower in the male R192Q monitored-N\textsubscript{2}O/O\textsubscript{2} group compared to the male R192Q monitored-air and compared to the male R192Q controlled-N\textsubscript{2}O/O\textsubscript{2} group. In addition, the female R192Q monitored-N\textsubscript{2}O/O\textsubscript{2} group showed higher CSD amplitude compared to the R192Q male monitored-N\textsubscript{2}O/O\textsubscript{2} group (Table 2).

Finally, we assessed whether the absence of higher CSD frequency in R192Q female mice that were not physiologically controlled may be associated with differences in physiological parameters (pH, pO\textsubscript{2}, pCO\textsubscript{2} and blood pressure) in comparison to experiments under physiological control. For female R192Q mice in the monitoring-only-N\textsubscript{2}O/O\textsubscript{2} group, compared to the physiologically controlled-N\textsubscript{2}O/O\textsubscript{2} group, pCO\textsubscript{2} values were slightly higher (p=0.02) and pH was slightly lower (p=0.04). Blood pressure was lower in both the monitoring-only-air and the monitoring-only-N\textsubscript{2}O/O\textsubscript{2} groups, compared to the physiologically controlled-N\textsubscript{2}O/O\textsubscript{2} group (controlled vs monitoring-only-air p=0.0001; controlled vs monitoring-only-N\textsubscript{2}O/O\textsubscript{2} p=0.01) (Table 2). Physiological parameters were comparable between experiments performed in male R192Q mice with physiological control (using N\textsubscript{2}O/O\textsubscript{2}) and male R192Q mice that were monitored-only using N\textsubscript{2}O/O\textsubscript{2} except for a lower blood pressure in R192Q monitored-N\textsubscript{2}O/O\textsubscript{2} compared to R192Q controlled-N\textsubscript{2}O/O\textsubscript{2} mice (p=0.01).
DISCUSSION

Using CSD frequency as readout for CSD susceptibility, we here showed that in the absence of physiological monitoring and control, R192Q mice exhibit an increased visual cortex CSD frequency compared to WT. In contrast to earlier studies, performed in the presence of physiological control (Eikermann-Haerter et al 2009b), the effect was not more pronounced in females. The absence of a gender effect was not explained by the CSD readout parameter: also with threshold measurements visual cortex CSD susceptibility was not enhanced, and was even increased, for female compared to male R192Q mice. In addition, comparison between visual and motor cortex as sites of CSD induction revealed that also for the motor cortex, CSD frequency was specifically enhanced in R192Q compared with WT mice. CSD threshold however was not different for R192Q compared with WT mice in the motor cortex. Gender had no influence on CSD frequency or threshold in the motor cortex. When comparing the two cortical regions, CSD susceptibility was enhanced in visual compared to motor cortex for CSD threshold in male R192Q mice, and for CSD frequency in female R192Q mice, indicating an effect of gender. Ovariectomy however had no effect on either visual cortex CSD frequency or threshold in female WT and R192Q mice in the absence of physiological control. Experiments performed with physiological monitoring, but without physiological control, revealed that the lack of a gender effect on visual cortex CSD frequency in the absence of control was not related to the use of air instead of N₂/O₂ but to the absence of mechanical ventilation. In these monitored experiments, slight differences in pH, pCO₂ and blood pressure were observed that may contribute to the lack of enhanced CSD frequency in female compared to male R192Q mice when physiological parameters are not controlled.

Our data indicate that parameters of CSD susceptibility can be masked or unmasked depending on the experimental paradigm used. This has important implications for the interpretation and comparison of experimental CSD studies across laboratories, since it is plausible that certain effects on CSD characteristics are related to the used methodology. Below, we discuss possible implications of certain methodologies with respect to specific CSD characteristics.

The effect of enhanced visual cortex CSD frequency in R192Q mice in the present study for experiments performed using isoflurane-air anesthesia, is in line with findings from earlier work performed in the presence of physiological control and isoflurane-N₂/O₂ anesthesia (Eikermann-Haerter et al 2009a, Eikermann-Haerter et al 2009b). This indicates that the genotypic effect on CSD frequency is not influenced by the presence or absence of physiological monitoring or control, or by the used anesthesia gas mixture. Our data extend the genotypic effect on CSD frequency also to the motor cortex. The reduced visual cortex CSD threshold in R192Q mice which we observed for both genders in the absence of physiological control is for male mice in line with earlier uncontrolled CSD studies. In those studies urethane was used instead of isoflurane anesthesia, and for threshold assessment electrical stimulation was used (van den Maagdenberg et al 2004, van den Maagdenberg et al 2010) instead of topical KCl application. Gender effects were not studied before in FHM1
mice for CSD threshold. We observed an opposite gender effect in the absence of physiological control, with visual cortex CSD threshold being lower in male compared to female R192Q mice. In addition, contradictory effects were observed for possible regional differences in CSD susceptibility among genders. Furthermore, ovariectomy had no effect on visual cortex CSD frequency or threshold in R192Q mice for physiologically uncontrolled experiments. For CSD frequency this contrasts reported effects of ovariectomy for experiments performed under physiological control (Eikermann-Haerter et al 2009b). It is plausible that in the absence of physiological control, variations occur in physiological parameters that influence CSD susceptibility characteristics, in particular during CSD threshold paradigms with variable durations.

Apart from CSD threshold and frequency, amplitude and duration of CSD events may also be influenced by alterations in physiology. A longer CSD duration for example has been associated with impaired tissue perfusion and recovery in rats (Sukhotinsky et al 2010). In previous studies under physiologically controlled conditions, CSD duration and amplitude were not different for visual cortex CSD frequency recordings between male and female mice for both WT and R192Q mutants (Eikermann-Haerter et al 2009b). In our study, no differences in CSD duration were observed that could contribute to the enhanced CSD frequency for female R192Q mice in the physiologically controlled group. CSD amplitude differences that were observed among some of the experimental groups were not related to differences in CSD frequency. In general, it should be noted that CSD amplitude may not be considered a reliable readout for CSD susceptibility given its dependence on recorded cortical depth.

Enhanced CSD susceptibility of female FHM1 mice is clinically relevant in the context of the higher propensity of women for migraine (Fettes 1999). Underlying mechanisms could involve enhancement of glutamatergic neuronal excitability by estradiol (Kelly et al 2003, Sato et al 2003, Smith 1989, Woolley et al 1997). A direct effect of estradiol on glutamatergic neurotransmission fits the observation of a gender effect only in FHM1 and not in WT mice. The finding that a gender effect in physiologically controlled experiments was observed regardless of estrous cycle, as the phase was not determined, suggests that intrinsic brain differences between males and females (Borsook et al 2014) may contribute to enhanced CSD susceptibility in female FHM1 mice. Interestingly, expression of the subunit of P/Q-type Ca\(^{2+}\) channels was shown to be enhanced in the pituitary of female compared to male rodents, and fluctuate during the estrous cycle (Fiordelisio et al. 2007), suggesting a modulation of CSD frequency by female hormones at the level of the mutant Ca\(^{2+}\) channels. The observation that a gender effect on CSD frequency was not observed in physiologically uncontrolled experiments suggests that mechanisms underlying the gender effect are influenced by changes in physiology. Although in all monitored experiments physiological parameters were within normal ranges for anesthetized rodents, monitored-only female R192Q mice displayed slightly higher pCO\(_2\) and lower blood pressure values compared to female R192Q mice from the physiologically controlled group. Changes in pCO\(_2\) and related changes in brain pH can modulate neuronal excitability by affecting ion
channels and transporters (Ruusuvuori & Kaila 2014), whereby high pCO$_2$ and low pH are expected to lower CSD susceptibility (Holland et al 2012). It is thus possible that the absence of a gender effect in monitored-only female R192Q mice relates to the slightly higher pCO$_2$ for this group. Changes in blood pressure can also affect CSD characteristics (Sukhotinsky et al 2010). Lower blood pressure of monitored-only female R192Q mice, compared to physiologically controlled mice, may thereby have contributed to a lower CSD frequency, thus masking a gender effect in the absence of control. Alternatively, it is possible that the use of mechanical ventilation for physiological control causes certain changes in physiology that are not monitored, but which may influence CSD characteristics. It has been described that mechanical ventilation can affect cerebral blood flow (Milan et al 2009), which has a bi-directional relationship with CSD initiation and propagation (Ayata 2013). Since estradiol can alter the brain’s vascular responses to CGRP (Gupta et al 2007), it is possible that vascular effects of mechanical ventilation influence modulation of CSD susceptibility by estradiol.

Our CSD experiments performed in the absence of physiological control suggested that the visual cortex may be more susceptible to CSD than the motor cortex in R192Q, but not in WT mice. If true, this would be in line with neurophysiological studies reporting visual cortex hyperexcitability in migraine patients (Aurora et al 1998, Aurora et al 2003, Aurora & Wilkinson 2007). Further clinical relevance comes from imaging studies in migraine patients suggesting CSD initiation to occur preferably in the visual cortex (Hadjikhani et al 2001). Our data however showed that an effect of cortical region on CSD susceptibility was evident for CSD threshold only in male, and not in female R192Q mice. For CSD frequency, a regional difference was observed only in female, and not in male R192Q mice. Since these observations were made in the absence of physiological monitoring and control it is difficult to assess whether these findings may be confounded by possible changes in physiology during recordings. Studies in WT mice under halothane anesthesia, in the absence of physiological monitoring or control, showed no difference in CSD susceptibility between the occipital and frontal cortex (Godukhin & Obrenovitch 2001), similar to our observations in WT mice. For insight in a putative effect of cortical region on CSD susceptibility, additional experiments with physiological monitoring, and possibly mechanical ventilation for control, would be useful, as well as CSD studies in freely behaving mice that allow for exclusion of possible effects of anesthesia, catheterization or ventilation.

In conclusion, we showed that in experimental CSD studies in mice, control of physiological parameters can influence CSD susceptibility characteristics. Although the mechanisms remain to be unraveled, the occurrence of a gender effect on visual cortex CSD frequency in R192Q mice appeared sensitive to the use of mechanical ventilation and to possible changes in systemic pH, pCO$_2$ or blood pressure levels. Effects of other factors on CSD susceptibility, such as a putative effect of cortical region, may become apparent in the absence of control if such effects are sensitive to changes in physiology that are influenced by mechanical ventilation. Comparison among CSD studies need to take into account influences of the used methodologies and, ideally, should include studies in awake, unanesthetized animals.
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