Valproate Reduces Delayed Brain Injury in a Rat Model of Subarachnoid Hemorrhage

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Background and Purpose—Spreading depolarizations (SDs) may contribute to delayed cerebral ischemia after subarachnoid hemorrhage (SAH). We tested whether SD-inhibitor valproate reduces brain injury in an SAH rat model with and without experimental SD induction.

Methods—Rats were randomized in a 2×2 design and pretreated with valproate (200 mg/kg) or vehicle for 4 weeks. SAH was induced by endovascular puncture of the right internal carotid bifurcation. One day post-SAH, brain tissue damage was measured with T2-weighted magnetic resonance imaging, followed by cortical application of 1 mol/L KCl (to induce SDs) or NaCl (no SDs). Magnetic resonance imaging was repeated on day 3 followed by histology to confirm neuronal death. Neurological function was measured with an inclined slope test.

Results—In the groups with KCl application, lesion growth between days 1 and 3 was 57±73 mm3 in the valproate-treated versus 237±232 mm3 in the vehicle-treated group. In the groups without SD induction, lesion growth in the valproate- and vehicle-treated groups was 8±20 mm3 versus 27±52 mm3. On fitting a 2-way analysis of variance model, we found a significant interaction effect between treatment and KCl/NaCl application of 161 mm3 (P=0.04). Number and duration of SDs, mortality, and neurological function were not statistically significantly different between groups. Lesion growth on magnetic resonance imaging correlated to histological infarct volume (Spearman’s rho =0.83; P=0.0004), with areas of lesion growth exhibiting reduced neuronal death compared with primary lesions.

Conclusions—In our rat SAH model, valproate treatment significantly reduced brain lesion growth after KCl application. Future studies are needed to confirm that this protective effect is based on SD inhibition. (Stroke. 2017;48:452-458. DOI: 10.1161/STROKEAHA.116.014738.)
of nimodipine has not been elucidated, it has shown to be effective in inhibiting SDs in animal studies. Valproate is another effective SD-inhibiting drug. Intraperitoneal injection of valproate was found to decrease lesion size after ischemic stroke in a rat model, where SDs have been shown to contribute to lesion growth. Valproate treatment has also been shown to improve the outcome in a mouse model with SAH induced by subarachnoid blood injection. However, the mechanisms through which valproate may reduce brain injury after SAH remains unknown.

We recently developed a rat model for SD-induced delayed brain injury after SAH. The aim of our study was to investigate whether valproate inhibits post-SAH lesion growth after SAH with and without experimental induction of SDs.

Methods

Study Design

This study was performed in accordance with guidelines of the European Communities Council Directive and approved by the Animal Experiments Committee of the University Medical Center Utrecht. Data reporting is in compliance with the Animal Research: Reporting of In Vivo Experiments (ARRIVE; http://www.nc3rs.org.uk/arrive-guidelines). Adult male Wistar rats of 200 to 250 g (Charles River, Sulzfeld, Germany) were housed in a 12-hour light, 12-hour dark cycle and had access to standard laboratory chow and water ad libitum. A sample size of 16 to 17 animals per group was a priori calculated based on the Chi-square test with a hypothesized valproate-induced lesion reduction from 500 (±75) mm³ to 200 (±75) mm³ and 35% mortality before day 3, based on previous research from our group. Rats were randomized in a 2×2 design with randomization through an electronically generated list by an independent person with allocation concealment. Animals were treated daily, starting 4 weeks before SAH induction, with 0.2 mL/100 g of 100 mg/mL intraperitoneally administered sodium valproate (200 mg/kg; valproate groups) or saline (with a similar osmolarity and pH as valproate; vehicle groups). Four weeks after treatment onset, SAH was induced in all animals, followed by cortical application of KCl—to induce SDs—(KCl groups) or application of saline—which does not induce SDs—(NaCl groups). The treatment was continued until 2 days after SAH. This resulted in the following groups: valproate–KCl group (N=17); vehicle–KCl group (N=16); valproate–NaCl group (N=17), and vehicle–NaCl group (N=16). Rats were excluded if their body weight dropped below 280 g after pretreatment and in case no SAH was present on postmortem investigation. Data from the vehicle groups have also been used for an earlier study.

Subarachnoid Hemorrhage Model

Rats were anesthetized, endotracheally intubated, and mechanically ventilated with 2% isoflurane in air/O₂ (4/1) for MRI on a 4.7-T/40 cm magnetic resonance system. The MRI protocol included T₁-weighted multiecho MRI and a flow-sensitive alternating inversion recovery (ITS-FAIR) protocol with a 2-shot gradient-echo echo planar imaging acquisition for perfusion measurement (see online-only Data Supplement). T₁ maps were calculated from a nonlinear least squares fitting routine. Images were registered to a reference T₁-weighted image using Oxford Centre for Functional MRI of the Brain (FMRIB’s) Linear Image Registration Tool (FLIRT). In this reference image, regions of interest encompassing the ipsilateral and contralateral sensorimotor cortex and striatum were drawn (by a blinded observer) for cortical and subcortical perfusion measurements. Lesion regions, characterized by clear T₁-hypointensity, were drawn using FMRIB Software Library (FSL) software by 2 independent observers who were blinded to group assignment. The intersection of both sets of lesion regions was taken for further lesion size analyses. We subdivided lesion regions in ipsilateral and contralateral cortical and subcortical regions for additional analysis. A cortical tissue volume of 3×3×2 mm³ below the burr hole was excluded from lesion volume calculation to prevent inclusion of tissue that was directly affected by KCl application. Lesion growth was calculated as the difference between lesion volumes on days 3 and 1 post-SAH. CBF was measured in the ipsilateral and contralateral cortical and subcortical regions after registering the CBF images to the T₁-weighted reference image. Cortical CBF was expressed as percentage of the CBF value in the unaffected subcortical area contralateral to the side of SAH induction.

SAH Severity Scoring

Surviving rats were euthanized on day 3 post-SAH, after MRI, by an intraperitoneal overdose of pentobarbital. Brains were perfusion-fixed with 4% paraformaldehyde and removed from the skull. Pictures were taken from the base of the brain. To score SAH severity, ventral brain images were segmented into 6 segments, followed by SAH severity scoring between 0 (no subarachnoid blood) and 18 (large SAH) according to Sugawara et al.

Histology of Tissue Damage

Extracted brains were stored in phosphate-buffered saline with 0.5 g/L sodium azide. A randomly chosen cohort of brains were used for a separate microthrombi study and was unavailable for

Induction and Recording of Spreading Depolarizations

One day after SAH (and after the first magnetic resonance imaging [MRI] session, see below), a 2-mm burr hole was drilled in the skull at 2 mm anterior to lambda and 2 mm right of the sagittal suture, and the underlying dura was opened. In addition, the skull was indented with a drill at 1 and 2 mm anterior of the burr hole. A small amount of mineral oil was placed in the indents, and a glass fiber probe was placed at each indent, connected to a laser-Doppler flowmetry (LDF) device, which continuously measured cerebral blood flow (CBF) for 1 hour. A cotton ball soaked with saline was placed in the burr hole. After 10 minutes, the ball was replaced by a cotton ball soaked in 1.0 mol/L KCl to induce SDs (KCl groups) or in saline (NaCl groups). LDF recording was continued for 50 minutes. Number of SDs, estimated from distinct, propagating, transient increases or decreases in LDF, was scored by an observer who was not present during LDF recording and blinded to group assignments. We also measured the total duration of the LDF response to SD by visually scoring the duration of all flow transients. We have previously shown that the KCl application did not induce MRI-identifiable lesions in rats without SAH.

Sensorimotor Function Test

In subgroups of rats (N=10 [group valproate–KCl], 12 [group valproate–NaCl], 10 [group vehicle–KCl], and 12 [group vehicle–NaCl]), we daily assessed functional status with an inclination test. This test assesses a rat’s ability to keep from sliding down a triplex plane with increasing horizontal angle, scored by the maximum angle before a rat slid down. The inclination test is a relatively simple and easily applicable test, which we have used in a previous study in rats after SAH.

MRI of Brain Lesions and Perfusion

On days 1 and 3 post-SAH, rats were endotracheally intubated and mechanically ventilated with 2% isoflurane in air/O₂ (4/1) for MRI on a 4.7-T/40 cm magnetic resonance system. The MRI protocol included T₁-weighted multiecho MRI and a flow-sensitive alternating inversion recovery (ITS-FAIR) protocol with a 2-shot gradient-echo echo planar imaging acquisition for perfusion measurement (see online-only Data Supplement).
histological analysis. From the remaining brains, we selected brain samples from rats (group valproate–KCl, N=6; group valproate–NaCl, N=0; group vehicle–KCl, N=4; group vehicle–NaCl, N=5) with the following patterns of lesion development after SAH: MRI-detectable lesions on days 1 and 3; MRI-detectable lesions only on day 3; and no MRI-detectable lesions on days 1 and 3 post-SAH. Brains were cryoprotected by immersion in 15% sucrose for 48 hours followed by immersion in 30% sucrose solutions for another 48 hours. Coronal sections (30 μm) were serially cut on a freezing microtome, followed by a combined Nissl or Luxol fast blue stain.21 Images of complete serial coronal sections were acquired using digital microscopy. Severity analysis was done on 20x images of 4 or 5 selected regions of interest, characterized by (1) T2 hyperintensity on post-SAH days 1 and 3 (early lesion); (2) T2 hyperintensity only on post-SAH day 3 (delayed injury); or (3) no T2 hyperintensities. Presence of neuronal injury/death, identified by pyknotic cell staining patterns, was scored for each quadrant of each regions of interest on a scale ranging from 0 to 4 (ie, least to greatest severity) by an observer blinded to group assignment.16 Total infarct volume was determined using area annotations (μm²) in NDP view (Nanozoomer Digital Pathology software by Hamamatsu (Hamamatsu City, Japan)) by a second blinded observer.

Outcome Measures
The primary outcome measure (lesion growth) was defined as tissue with a lesion on day 3 where no lesion was seen on day 1 post-SAH. Other outcome measures were sensorimotor function score and mortality. Mortality was determined between pre-SAH and 1 day post-SAH, before SD induction, and between days 1 and 3 post-SAH, after SD induction.

Statistics
To assess the effect of SD (KCl versus NaCl) and pretreatment (valproate versus vehicle) on lesion growth, we used a 2-way analysis of variance. The interaction between medical treatment and NaCl/KCl was of particular interest to us because it captures the difference in pretreatment effect between the NaCl and KCl groups. We observed considerable skewness of the distribution of the lesion growth and possibly also heteroskedasticity between the 4 groups. To account for this, we used the robust Huber-White sandwich estimator for the standard errors. We computed this estimator by fitting a generalized estimating equations model in SPSS with the robust option and independent correlation structure.24,25 Inclination test scores were analyzed estimating equations model in SPSS with the robust option and independent correlation structure.24,25 Inclination test scores were analyzed with the same statistical model. Difference in mortality between days 1 and 3 between valproate–KCl versus vehicle–KCl and valproate–NaCl versus vehicle–NaCl groups was analyzed with a Fisher exact test. Spearman’s Rho was calculated to measure correlation between lesion sizes as scored by the 2 observers; between MRI-based and histological lesion volumes; and between T2-hyperintensity and neuronal injury severity scores. Number of SDs and total duration of the SDs were compared between treatment groups and between ipsilateral and contralateral sides with a 2-way analysis of variance model, we found a significant interaction effect between treatment and KCL/NaCl application of 161 mm³ (P=0.04). Analysis of subregions within the lesion territory revealed that statistically significant effects for treatment (P=0.004), NaCl/KCl (P<0.001), and their interaction (P=0.021) were only detectable in the ipsilateral cortical lesion subregion and not in the other lesion subregions (Table).

Cerebral Blood Flow
On day 1 after SAH, before KCl/NaCl application, cortical CBF values were 134±91% (of reference) in the ipsilateral and 131±86% in the contralateral hemisphere in the vehicle-treated animals. In valproate-treated animals, ipsilateral and contralateral CBF values were 111±39% and 96±30%, respectively. There was a significant main effect of treatment

Spreading Depolarizations
In the rats with experimental SD induction, we measured 4.1±2.9 (valproate–KCl group) and 5.0±2.7 SDs (vehicle–KCl group; P=0.54) during 50 minute recording after KCl application (Figure 1). The average total duration of LDF change was 642±571 s in the valproate–KCl group and 1158±508 s in the vehicle–KCl group (P=0.09). No spontaneous SDs were recorded after cortical saline application in the NaCl groups. Figure 1 shows typical LDF recordings after cortical KCl or NaCl application.

Brain Lesion Development
MRI revealed lesions with prolonged tissue T₂ in ipsilateral and contralateral cortical and subcortical areas, which expanded between days 1 and 3 after SAH (Figure 2). Delineated lesions volumes, identified as hyperintense tissue in ipsilateral and contralateral cortical and subcortical areas on T₂-weighted magnetic resonance images, correlated between raters with Rho=0.96 (P<10⁻⁵). In the groups with SD induction, lesion growth from day 1 to day 3 post-SAH was 57±73 mm³ in the valproate group as compared with 237±232 mm³ in the vehicle-treated group (Figure 3A). In the groups without experimental SD induction, lesion growth was 8±20 mm³ in the valproate group and 27±52 mm³ in the vehicle-treated group. On fitting a 2-way analysis of variance model, we found a significant interaction effect between treatment and KCl/NaCl application of 161 mm³ (P=0.04). Analysis of subregions within the lesion territory revealed that statistically significant effects for treatment (P=0.004), NaCl/KCl (P<0.001), and their interaction (P=0.021) were only detectable in the ipsilateral cortical lesion subregion and not in the other lesion subregions (Table).

Results
Four rats were excluded because of significant loss of body weight (N=1) or absence of subarachnoid blood (N=3), resulting in the following final group sizes: valproate–KCl group (N=15); vehicle–KCl group (N=16); valproate–NaCl group (N=16), and vehicle–NaCl group (N=15). SAH severity of surviving rats at day 3 was moderate, with group scores of 11±2 (valproate–KCl), 12±3 (vehicle–KCl), 10±3 (valproate–NaCl), and 12±3 (vehicle–NaCl) and no statistically significant differences between groups.
on cortical CBF; CBF was lower in valproate-treated rats (P=0.04). There was no significant main effect of hemispheric side (P=0.51), and no significant interaction effect between treatment and hemispheric side (P=0.69).

### Sensorimotor Function
Sensorimotor function expressed by the inclination test score was significantly reduced at 1 (valproate–KCl group 33±7; vehicle–KCl 38±10; vehicle–NaCl 40±6) and 3 days after SAH (valproate–KCl group 34±6; vehicle–KCl 35±7; vehicle–NaCl 39±6) as compared with before SAH (valproate–KCl group 44±2; vehicle–KCl 46±4; vehicle–NaCl 45±6; vehicle–NaCl 46±3; P<10\(^{-4}\) in all groups). There were no significant group differences in the inclination test score change between days 1 and 3 (Figure 3B).

### Mortality
Figure 3C shows survival in the experimental groups. Between days 1 and 3 post-SAH, none of the 11 rats in the valproate-treated group died after KCL application, whereas 3 of 11 rats had died in the vehicle-treated group (P=0.21). In the NaCl groups, 4 out of 11 rats that had died in the valproate-treated group and 1 out of 14 rats in the vehicle-treated group (P=0.13).

### Histology
Histological assessment of neuronal death with Nissl staining showed that lesioned tissue, as identified with T\(_2\)-weighted MRI on day 1 post-SAH (early lesion), had pyknotic staining patterns with shrunken or absent nuclei and reduced Luxol fast blue staining, indicative of demyelination, when the rats were euthanized at day 3 post-SAH (Figure 4; neuronal injury severity score 3±1). In regions where tissue lesions were identified with T\(_2\)-weighted MRI only at day 3 (lesion growth), the histological degree of injury was generally less severe (neuronal injury severity score 2±1). Neuronal injury was absent in all regions identified as nonlesioned with T\(_2\)-weighted MRI at day 3 (neuronal injury severity score 0±0). The neuronal injury severity score was significantly correlated with absent, late, or early presence of T\(_2\) lesions (Spearman’s rho =0.91; P=2×10\(^{-23}\)). Histologically measured infarct volumes were significantly correlated with T\(_2\)-based lesion volumes (Spearman’s rho =0.83; P=0.0004), confirming infarction in MRI-identified lesion areas.

### Discussion
In our randomized, vehicle-controlled rat SAH study, treatment with valproate reduced lesion growth after KCL application to induce SDs. This protective effect was not found in absence of experimental SD induction. We previously reported that experimental SD induction with cortically applied KCl advances lesion growth after SAH in rats.16 The mechanism leading to SDs after clinical SAH is unknown, but it may be related to hemolysis products, such as potassium.26 In animal studies, many drugs have been shown to influence occurrence, frequency, or duration of SDs through modification of the induction threshold, refractory period, or even SD amplitude or length.8 We chose valproate as an SD-inhibiting treatment because, after sufficient pretreatment, this drug has proven to be an effective inhibitor of SD.9–11 One earlier study also reported beneficial effects of valproate treatment after SAH.15 In that randomized, vehicle-controlled study, valproate administration decreased the number of degenerating neurons and improved neurobehavioral outcome after SAH in mice. However, that study involved a prechiasmatic blood injection model of SAH, without development of delayed injury.27 Furthermore, a potential SD-inhibiting mechanism was not investigated.

An advantage of valproate for the translation of our results to clinical practice is the long experience with the drug as a therapy in different patient populations. Valproate is prescribed for prevention of epilepsy, migraine, manic episodes, and neuropathic pain in humans.28 Although the exact pharmacological action of valproate is unknown, it probably acts through multiple mechanisms.28 Our hypothesis was that valproate directly inhibits the effect of SDs on lesion growth.6 However, the difference in number and duration of SDs was not statistically significant between the groups. Therefore, valproate may also
have indirectly mitigated the effect of SDs through its anti-excitotoxic properties or by influencing (acute) hemodynamics—we did measure a significant treatment effect on cortical CBF before SD induction. We accounted for the effects of valproate early after SAH by selecting lesion growth after day 1 as our outcome measure. Although the effect of valproate on lesion growth was only significantly present in the KCl group, and most pronounced in the cortex ipsilateral to KCL application, an effect through CBF modulation cannot be excluded. Furthermore, one could rationalize that measurement of systemic parameters, such as blood pressure, would have been beneficial to ascertain the efficacy of valproate treatment, but because we performed survival experiments, we opted to leave out such invasive procedures.

Our study has several limitations. First, valproate was administered as pretreatment, starting 4 weeks before SAH induction.

Table. Change in Lesion Volume (mm³; Mean±Standard Deviation) in Cortical and Subcortical Subregions of the Total Lesion in the Ipsilateral and Contralateral Hemisphere, Between Days 1 and 3 After SAH in the Different Experimental Groups

<table>
<thead>
<tr>
<th></th>
<th>Valproate-KCl</th>
<th>Vehicle-KCl</th>
<th>Valproate-NaCl</th>
<th>Vehicle-NaCl</th>
<th>P Value (Treatment)</th>
<th>P Value (SD)</th>
<th>P Value (Treatment×SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cortical, ipsilateral</td>
<td>44±56</td>
<td>141±94</td>
<td>9±21</td>
<td>22±42</td>
<td>0.004</td>
<td>&lt;0.001</td>
<td>0.021</td>
</tr>
<tr>
<td>Subcortical, ipsilateral</td>
<td>8±15</td>
<td>46±74</td>
<td>0±1</td>
<td>2±4</td>
<td>0.114</td>
<td>0.063</td>
<td>0.135</td>
</tr>
<tr>
<td>Cortical, contralateral</td>
<td>8±18</td>
<td>49±98</td>
<td>1±1</td>
<td>5±12</td>
<td>0.192</td>
<td>0.152</td>
<td>0.246</td>
</tr>
<tr>
<td>Subcortical, contralateral</td>
<td>0±3</td>
<td>7±16</td>
<td>0±0</td>
<td>0±1</td>
<td>0.169</td>
<td>0.165</td>
<td>0.171</td>
</tr>
</tbody>
</table>

P values were calculated with 2-way ANOVA with factors treatment (valproate vs vehicle) and SD (KCl vs NaCl). ANOVA indicates analysis of variance; SAH, subarachnoid hemorrhage; and SD, spreading depolarization.
This may explain why a relatively large part of the lesion is present in the ipsilateral hemisphere. To assess the effects of valproate treatment on delayed brain injury, and minimize the influence of direct ischemic injury, we used lesion growth after day 1 post-SAH as our outcome measure.

Conclusions

In conclusion, we found that pretreatment with valproate, a clinically prescribed drug, decreases delayed brain injury in a rat model of SAH with experimental SD induction after KCL application. Future studies are needed to confirm that this protective effect is based on SD induction and to investigate the therapeutic potential of SD-inhibiting drugs in the prevention of DCI in humans.

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Disclosures

None.

References


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Supplemental material

MRI protocol

4.7T/40 cm MR system (Varian Inc., Palo Alto, CA, USA). A 90-mm Helmholtz volume coil and an inductively coupled surface coil (2.5 cm diameter) were used for excitation and detection of radio frequency signals, respectively. The MRI protocol included T2-weighted multi-echo MRI (repetition time (TR) = 3000 ms; echo times (TE) = 12-144 ms in twelve 12-ms steps; field-of-view (FOV) = 32x32 mm²; data matrix = 256x128; 19 slices of 1 mm; number of acquisitions (NA) = 2 and a flow-sensitive alternating inversion recovery (ITS-FAIR) protocol with a 2-shot gradient-echo EPI acquisition (TR = 10000 ms; TE = 4.8 ms; delay between the 46 images in the inversion curve = 150 ms; flip angle = 10°; FOV = 32x32 mm²; data matrix = 64x64; slice thickness = 2 mm; selective inversion slab = 10 mm; NA = 16) for perfusion measurement.

Manufacturer information

Laser-Doppler flowmetry (LDF) device: type moorVMS-LDF, Moor Instruments, Devon, UK
FSL software: 3.1.8, Flitney et al., University of Oxford, Oxford, UK
Pentobarbital: Alfasan, Woerden, The Netherlands
Sodium azide: Sigma-Aldrich, St. Louis, MO, USA
Digital microscopy: Nanozoomer 2.0HT, Hamamatsu Photonics, Hamamatsu-shi, Japan

### Table I. Checklist of Methodological and Reporting Aspects for Articles Submitted to *Stroke* Involving Preclinical Experimentation

<table>
<thead>
<tr>
<th>Methodological and Reporting Aspects</th>
<th>Description of Procedures</th>
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| **Experimental groups and study timeline** | ☑️ The experimental group(s) have been clearly defined in the article, including number of animals in each experimental arm of the study.  
☑️ An account of the control group is provided, and number of animals in the control group has been reported. If no controls were used, the rationale has been stated.  
☑️ An overall study timeline is provided. |
| **Inclusion and exclusion criteria** | ☑️ A priori inclusion and exclusion criteria for tested animals were defined and have been reported in the article. |
| **Randomization** | ☑️ Animals were randomly assigned to the experimental groups. If the work being submitted does not contain multiple experimental groups, or if random assignment was not used, adequate explanations have been provided.  
☑️ Type and methods of randomization have been described.  
☑️ Methods used for allocation concealment have been reported. |
| **Blinding** | ☑️ Blinding procedures have been described with regard to masking of group/treatment assignment from the experimenter. The rationale for nonblinding of the experimenter has been provided, if such was not feasible.  
☑️ Blinding procedures have been described with regard to masking of group assignment during outcome assessment. |
| **Sample size and power calculations** | ☑️ Formal sample size and power calculations were conducted based on a priori determined outcome(s) and treatment effect, and the data have been reported. A formal size assessment was not conducted and a rationale has been provided. |
| **Data reporting and statistical methods** | ☑️ Number of animals in each group: randomized, tested, lost to follow-up, or died have been reported. If the experimentation involves repeated measurements, the number of animals assessed at each time point is provided, for all experimental groups.  
☑️ Baseline data on assessed outcome(s) for all experimental groups have been reported.  
☑️ Details on important adverse events and death of animals during the course of experimentation have been provided, for all experimental arms.  
☑️ Statistical methods used have been reported.  
☑️ Numeric data on outcomes have been provided in text, or in a tabular format with the main article or as supplementary tables, in addition to the figures. |
| **Experimental details, ethics, and funding statements** | ☑️ Details on experimentation including stroke model, formulation and dosage of therapeutic agent, site and route of administration, use of anesthesia and analgesia, temperature control during experimentation, and postprocedural monitoring have been described.  
☑️ Different sex animals have been used. If not, the reason/justification is provided.  
☑️ Statements on approval by ethics boards and ethical conduct of studies have been provided.  
☑️ Statements on funding and conflicts of interests have been provided. |

Fluctuations in female hormones can affect the susceptibility for spreading depolarizations.