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

Arend M. Hamming, MD; Annette van der Toorn, PhD; Umesh S. Rudrapatna, PhD; Lisha Ma, MD; Hine J.A. van Os, MSc; Michel D. Ferrari, MD, PhD; Arn M.J.M. van den Maagdenberg, PhD; Erik van Zwart, PhD; Katherine Poinsette, BA; Ann M. Stowe, PhD; Rick M. Dijkhuizen, PhD; Marieke J.H. Wermer, MD, PhD

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 following 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.)

Key Words: cortical spreading depression ■ experimental models ■ MRI ■ subarachnoid hemorrhage ■ valproic acid
of nimodipine has not been elucidated, it has shown to be effective in inhibiting SDs in animal studies.\textsuperscript{7,8} Valproate is another effective SD-inhibiting drug.\textsuperscript{9,11} Intraperitoneal injection of valproate was found to decrease lesion size after ischemic stroke in a rat model,\textsuperscript{2} where SDs have been shown to contribute to lesion growth.\textsuperscript{13,14} Valproate treatment has also been shown to improve the outcome in a mouse model with SAH induced by subarachnoid blood injection.\textsuperscript{15} 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.\textsuperscript{16} The aim of our study was to investigate whether valproate inhibits post-SAH lesion growth after SAH with and without experimental induction of SDs.

\section*{Methods}

\subsection*{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 difference of nimodipine has not been elucidated, it has shown to be effective in inhibiting SDs in animal studies.\textsuperscript{7,8} Valproate is another effective SD-inhibiting drug.\textsuperscript{9,11} Intraperitoneal injection of valproate was found to decrease lesion size after ischemic stroke in a rat model,\textsuperscript{2} where SDs have been shown to contribute to lesion growth.\textsuperscript{13,14} Valproate treatment has also been shown to improve the outcome in a mouse model with SAH induced by subarachnoid blood injection.\textsuperscript{15} 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.\textsuperscript{16} The aim of our study was to investigate whether valproate inhibits post-SAH lesion growth after SAH with and without experimental induction of SDs.
historical analysis. From the remaining brains, we selected brain samples from rats (group valproate–KCl, N=6; group valproate–NaCl, N=6; 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. 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. 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 skewedness of the distribution of the lesion growth and possibly also heteroskedasticity between the 4 groups. For this, we used the robust Huber-White sandwich estimator for the variance. We computed this estimator by fitting a generalized linear model in SPSS with the robust option and independent errors. 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 skewedness of the distribution of the lesion growth and possibly also heteroskedasticity between the 4 groups. For this, we used the robust Huber-White sandwich estimator for the variance. We computed this estimator by fitting a generalized linear model in SPSS with the robust option and independent errors.

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 lesion 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).

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.
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; valproate–NaCl 36±6; vehicle–NaCl 39±6) as compared with before SAH (valproate–KCl group 46±4; vehicle–KCl 49±3; valproate–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; $P=2\times10^{-24}$). 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.$^9$ 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.
Future studies should test the potential of valproate, or other SD inhibiting drugs, to reduce delayed brain injury when administered acutely after SAH. Second, we did not detect spontaneous SDs, which may be explained by the relatively short recording time limited to 2 cortical regions. SDs were induced by cortical application of 1 mol/L KCl close to the lesion site, which may have directly affected lesion development in addition to the SD-induced pathophysiological effects. We accounted for this by excluding underlying cortical tissue from lesion volume calculations. With the current design, we cannot exclude effects of perforation-induced focal ischemia on lesion volume; the magnitude of this effect could be established in future experiments by application of KCl to the hemisphere contralateral to the perforation. Third, despite the observed difference in delayed brain injury between the valproate- and vehicle-treated groups, we did not measure significant differences in (changes in) sensorimotor function scores. This may be explained by lack of sensitivity of the inclination test to measure potential subtle effects on neurological function on top of the effect of the SAH and the limited sample size (only a subset of rats was tested). Fourth, the final time point of our study was at 3 days after SAH. 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.

Acknowledgments
We thank Wouter Mol for his biotechnical support and Christian Lanier for his contribution to the histological analyses.

Sources of Funding
Netherlands Organization for Scientific Research (Nederlandse organisatie voor gezondheidsonderzoek en zorginnovatie [ZonMW] Veni grant), the Netherlands Heart Foundation (2011T055), and the Netherlands Brain Foundation (project 2011(1)-102 [Dr Wermers]). This work was partly supported by the Utrecht University High Potential Program (Dr Dijkhuizen) and the EU Marie Curie IAPP Program “BRAINPATH” (nr 612360; Dr van den Maagdenberg) and the American Heart Association 14SDG18410020 (Dr Stowe).

Disclosures
None.

References


Valproate Reduces Delayed Brain Injury in a Rat Model of Subarachnoid Hemorrhage
Arend M. Hamming, Annette van der Toorn, Umesh S. Rudrapatna, Lisha Ma, Hine J.A. van Os, Michel D. Ferrari, Arn M.J.M. van den Maagdenberg, Erik van Zwet, Katherine Poinsatte, Ann M. Stowe, Rick M. Dijkhuizen and Marieke J.H. Wermer

Stroke. 2017;48:452-458; originally published online December 27, 2016;
doi: 10.1161/STROKEAHA.116.014738
Stroke is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2016 American Heart Association, Inc. All rights reserved.
Print ISSN: 0039-2499. Online ISSN: 1524-4628

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://stroke.ahajournals.org/content/48/2/452

Data Supplement (unedited) at:
http://stroke.ahajournals.org/content/suppl/2016/12/27/STROKEAHA.116.014738.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Stroke can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Stroke is online at:
http://stroke.ahajournals.org//subscriptions/
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>
<thead>
<tr>
<th>Methodological and Reporting Aspects</th>
<th>Description of Procedures</th>
</tr>
</thead>
</table>
| 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.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table. |
| 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.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table. |
| 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.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table. |
| 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.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table. |
| 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.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table.  
低成本的 arrival groups in a table. |

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