Published in final edited form as:

Neuropharmacology. 2020 October 01; 176: 108213. doi:10.1016/j.neuropharm.2020.108213.

The adenosine A1 receptor agonist WAG 994 suppresses acute kainic acid-induced status epilepticus *in vivo*

Zin-Juan Klafft^{1,2}, Lina M. Duerrwald^{1,3}, Zoltan Gerevich^{2,*}, Chris G. Dulla^{1,*}

¹ Department of Neuroscience, Graduate School of Biomedical Science, Tufts University, Boston, MA, USA

² Institute of Neurophysiology, Charité – Universitätsmedizin Berlin, Berlin, Germany

³ Department of Neurology with Experimental Neurology, Section Brain Simulation, Charité – Universitätsmedizin Berlin, Berlin, Germany

Abstract

Status epilepticus (SE) is a neurological emergency characterized by continuous seizure activity lasting longer than five minutes, often with no recovery between seizures (Trinka et al., 2015). SE is refractory to benzodiazepine and second-line treatments in about 30% cases. Novel treatment approaches are urgently needed as refractory SE is associated with mortality rates of up to 70%. Robust adenosinergic anticonvulsant effects have been known for decades, but translation into seizure treatments was hampered by cardiovascular side effects. However, the selective adenosine A1 receptor agonist SDZ WAG 994 (WAG) displays diminished cardiovascular side effects compared to classic A1R agonists and was safely administered systemically in human clinical trials.

Here, we investigate the anticonvulsant efficacy of WAG *in vitro* and *in vivo*. WAG robustly inhibited high-K⁺-induced continuous epileptiform activity in rat hippocampal slices (IC₅₀ = 52.5 nM). Importantly, WAG acutely suppressed SE *in vivo* induced by kainic acid (20 mg/kg i.p.) in mice. After SE was established, mice received three i.p. injections of WAG or diazepam (DIA, 5 mg/kg). Interestingly, DIA did not attenuate SE while the majority of WAG-treated mice (1 mg/kg) were seizure-free after three injections. Anticonvulsant effects were retained when a lower dose of WAG (0.3 mg/kg) was used. Importantly, all WAG-treated mice survived kainic acid induced SE.

*Co-corresponding authors.

AUTHOR CONTRIBUTIONS

Zin-Juan Klafft: Conceptualization, Methodology, Validation, Formal Analysis, Investigation, Writing – Original Draft, Writing – Review & Editing, Visualization, Supervision, Funding Acquisition.

Lina M. Duerrwald: Methodology, Software, Validation, Formal Analysis, Investigation, Data Curation, Writing – Review & Editing, Visualization.

Zoltan Gerevich: Conceptualization, Methodology, Formal Analysis, Resources, Writing – Review & Editing, Visualization, Supervision, Funding Acquisition.

Chris G. Dulla: Conceptualization, Methodology, Formal Analysis, Resources, Writing – Review & Editing, Visualization, Supervision, Funding Acquisition.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

In summary, we report for the first time that an A1R agonist with an acceptable human side-effect profile can acutely suppress established SE *in vivo*. Our results suggest that WAG stops or vastly attenuates SE while DIA fails to mitigate SE in this model.

1. Introduction

Status epilepticus (SE), characterized by prolonged seizures without a return to baseline conditions, is the 2nd most common neurological emergency with an annual incidence of 10–74/100,000 (Betjemann and Lowenstein, 2015; Hesdorffer et al., 1998; Leitingner et al., 2020). Both convulsive and non-convulsive forms of SE exist, which can pose a significant diagnostic and therapeutic challenges as non-convulsive SE is often difficult to identify without EEG recording (Meierkord and Holtkamp, 2007). SE is associated with high rates of morbidity and a 30-day-mortality of ~30% which can be up to 70% in multi-morbid and elderly patient groups (Boggs, 2004; Towne et al., 1994). The duration of SE correlates with increasingly poor patient outcome and, in some cases, non-convulsive pharmacoresistant SE can last for months. SE is considered refractory to anticonvulsant treatment in about one third of cases where patients do not respond to current first- (benzodiazepine) and second-line (plus phenytoin or other antiepileptic drug) treatment (Ferlisi and Shorvon, 2012; Holtkamp et al., 2005; Rossetti and Lowenstein, 2011). SE can develop as a complication of an existing epilepsy but approximately half of all SE cases arise *de novo*, without prior epileptogenesis, in acute situations such as stroke, hyp-/anoxia, CNS trauma, intoxications and infections (Betjemann and Lowenstein, 2015; Hesdorffer et al., 1998; Trinka et al., 2015). SE prevalence in those, often drug resistant, acute situations is likely to be currently underestimated (Betjemann et al., 2015; Betjemann and Lowenstein, 2015; Holtkamp, 2016) mainly because a large proportion of SE are non-convulsive and overlooked in clinical settings where EEG monitoring does not occur. The main therapeutic principle is to stop SE as early as possible to improve patient outcome. Therefore, novel pharmacological interventions are urgently needed to reduce the burden of SE refractory to currently available drugs.

Exploiting the adenosinergic receptor system for its anticonvulsant effects has been a long-standing goal (Dunwiddie, 1980). The adenosine A1 receptor (A1R) attenuates neurotransmitter release via inhibition of voltage-gated calcium channels and reduces neuronal excitability by activating G-protein inwardly rectifying K⁺ (GIRK) channels and plays an important role in seizure termination (During and Spencer, 1992; Hill et al., 2020; Kovacs et al., 2018; Wu and Saggau, 1994). In addition, the anticonvulsant effect of hypocapnia relies on A1R activation (Dulla et al., 2005) and A1Rs have been implicated in the anticonvulsant effects of the ketogenic diet (Masino et al., 2011). In the brain, two major sources for extracellular adenosine exist: activity-dependent adenosine release by neurons and glia and extracellular metabolism of ATP and its metabolites ADP and AMP to adenosine by extracellular ectonucleotidases (Dale and Frenguelli, 2009). While A1Rs mediate robust anticonvulsant effects, activation of A2A adenosine receptors has been described to have proconvulsant effects (Moschovos et al., 2012). Therefore strategies to selectively activate A1Rs are required to harness adenosine's potential anticonvulsant properties.

While promising, adenosine interventions have failed to translate into clinically useful therapies due to cardiovascular effects of adenosine itself (Chen et al., 2013). Thus, developing adenosine-based anticonvulsant approaches requires minimization of potentially adverse cardiovascular effects. Localized adenosine delivery systems or brain specific reduction of adenosine metabolism to raise extracellular adenosine levels have been investigated in basic epilepsy research (Boison, 2012a, b). However, systemic administration of selective adenosine A1R agonists in humans has been explored in cardiology, endocrinology and pain research (Elzein and Zablocki, 2008; Jacobson et al., 2019; Shah et al., 2004). WAG has been evaluated in clinical trials against cardiological indications like heart failure, supraventricular arrhythmias and hypertension. WAG also has been explored to treat diabetes mellitus by inhibiting lipolysis in adipocytes and as analgesics, presumably by reducing sensory nerve transduction. A1R agonists with diminished cardiovascular effects have been developed and some of these compounds display anticonvulsant effects (Knutsen et al., 1999). These selective agonists may be good candidates for drug development against SE since they have been examined in humans, display blood-brain-barrier penetrance, and appear safe in clinical trials for non-neurological indications (Lerman et al., 2001; Seymour et al., 1999).

The selective A1R agonist SDZ WAG 994 (WAG) has shown safety in humans as a pain medication and for cardiological indications up to clinical phase II trials. Importantly, these clinical studies report minimal adverse effects upon systemic application of WAG (Bertolet et al., 1996; Piot et al., 1998; Seymour et al., 1999). Although treatment of epilepsies with novel A1R agonists may still require exclusion of any peripheral A1R mediated side effects for chronic long-term treatments, we propose that investigating WAGs anticonvulsant properties against SE is important in order to find novel SE treatment approaches in an acute emergency setting.

Our previous *in vitro* study in human neocortical brain slices obtained from pharmacoresistant epilepsy patients showed that low-dose WAG (1 μ M) treatment was more efficacious in suppressing carbamazepine-resistant seizure-like events than 300 μ M adenosine (Klaft et al., 2016). Here, we show that WAG attenuates epileptiform activity *in vitro* in the nanomolar range and terminates acute kainic acid induced SE *in vivo* while diazepam fails to mitigate SE.

2. Materials and Methods

2.1 *In vitro* experiments

Animal procedures were conducted in accordance with the guidelines of the European Communities Council and the institutional guidelines approved by the Berlin Animal Ethics Committee (Landesamt für Gesundheit und Soziales Berlin, T0109/10 and G0391/08). In total, 21 male Wistar rats (80 –120 days old) (Harlan, Rossdorf, Germany) were included in this study.

Acute horizontal combined entorhinal cortex/hippocampus slices were prepared as described previously (Klaft et al., 2012). Briefly, rats were anesthetized with 2% isoflurane, decapitated and brains were rapidly removed and washed with ice-cold artificial

cerebrospinal fluid (ACSF) containing (in mM): NaCl, 129; KCl, 3; NaH₂PO₄, 1.25; NaHCO₃, 21; CaCl₂, 1.6; MgSO₄, 1.8; and d-glucose, 10, saturated with carbogen (95% O₂/5% CO₂). The brain was cut into 400- μ m-thick slices with a vibratome (DSK microslicer DTK-1000, Dosaka, Japan). Slices were immediately transferred to an interface recording chamber perfused with carbogenated ACSF (34°C, flow rate 1.7 ml/min, pH 7.4) and were allowed to recover for at least 2 h before experiments started (Schulz et al., 2012). Continuous epileptiform activity was induced with 8 mM-K⁺-ACSF containing (in mM): NaCl, 124; KCl, 8; NaH₂PO₄, 1.25; NaHCO₃, 21; CaCl₂, 1.6; MgSO₄, 1.8; and d-glucose, 10. After stabilization of epileptiform activity (~40 min after perfusion with 8 mM-K⁺-ACSF) pharmacological experiments were started. Recordings of extracellular field potentials (FP) were carried out in the hippocampal CA3 subregion and electrodes (resistance < 3 M Ω) were placed ~100 μ m below slice surface. FPs were amplified by a custom-made amplifier, low-pass filtered at 1 kHz and sampled at 5 kHz in DC mode with a CED 1401 interface (Cambridge Electronic Design, Cambridge, United Kingdom). FP data was analyzed using semi-automated scripts in Spike 2 recording software (Cambridge Electronic Design, Cambridge, United Kingdom) to characterize continuous epileptiform activity in regard to single event incidence, amplitudes and duration. Single events were marked by event detection with the threshold set at 5 SD of baseline amplitude and then manually confirmed to sort out recording artifacts or short fast spikes presumably representing single unit activity close to the electrode tip. Amplitude quantification represents the maximum amplitude change, including negative transients, between beginning and end of a single event. Duration of an event was determined as the time period from departure from baseline to 80 % recovery of the positive field potential amplitude towards baseline. Data from the final five minutes of control and treatment phases were analyzed and are presented as mean $\pm$ SEM. Drugs for *in vitro* experiments were pre-dissolved in DMSO with final DMSO concentration in ACSF of 0.03% v/v. SDZ WAG 994 (Cat. No. 2465) was purchased from Tocris, Bristol, United Kingdom. Phenytoin (PHR1492) and DMSO (No. 276855) were purchased from Sigma-Aldrich, St. Louis, MO, USA. Diazepam was purchased from Hospira, Lake Forest, IL, USA.

Concentration-inhibition curves of event incidences and IC₅₀ values were derived from nonlinear least-squares fits to the following equation using GraphPad Prism v7.00:

$$y = F_{min} + \frac{F_{max} - F_{min}}{1 + \left(\frac{x}{IC_{50}}\right)^{n_H}}$$

where F_{max} is the maximum event incidence in the absence of the ligand, F_{min} is the minimum equilibrium event incidence in the presence of the ligand, IC_{50} is the concentration of the ligand needed to evoke a half-maximal inhibition, x is the ligand concentration and n_H is the Hill slope.

2.2 EEG implant surgery

All animal surgeries and subsequent experiments were conducted in accordance with Tufts University's Institutional Animal Care and Use Committee guidelines and animal use

protocols. In total, 33 C57BL6/J mice (16 – 20 weeks old) of both sexes were included in the present study.

EEG implant surgery and EEG recordings were performed as previously described (Lau et al., 2017). In short, 16 – 20 week old mice were anesthetized with ketamine (100 mg/kg) and xylazine (10 mg/kg). Analgesia was achieved by buprenorphine treatment (0.1 mg/kg, SC) and additional local analgesia around the incision site by bupivacaine (4 mg/kg, SC). Mice were then placed in a stereotactic frame, the dorsal side of skull was shaved and disinfected with iodine and ethanol. Then, a scalp incision was made, and the skull surface cleaned and dried and four burr holes were drilled without puncturing the dura mater. Drill bit tip diameter for micro drill was 0.7 mm (Item No. 19007–07, Fine Science Tools, Foster City, CA, USA). Stereotaxic coordinates of the four drill holes: Anterior burr holes were –0.6 mm (bregma, AP axis) and 2 mm left (EEG) and right (ground) of the midline (ML), posterior burr holes were – 2.6 mm (bregma, AP) and 2.5 mm left (reference) and right (EEG) to ML. Four 0.1“ stainless steel screw electrodes with attached silver wires (Order # 8403, Pinnacle Technologies, Wyckoff, NJ, USA) were gently screwed into the drilled holes and fixed with dental cement and superglue to increase implant stability for chronic EEG recordings. The silver wires of the electrode screws were then soldered to separate contacts of a headmount (Order # 8402, Pinnacle Technologies, Wyckoff, NJ, USA) as quickly as possible to avoid heating of the implant. The headmount/electrode construct was then fixed to the mouse skull with dental cement taking care not to add too much weight. After surgery, animals were allowed to recover for at least seven days before chronic EEG recordings started and were given analgesia (Buprenorphine, 0.1 mg/kg, SC) if needed. 24-h-video-EEG recording signals were acquired using a preamplifier (100 x gain, 1 Hz high-pass filter, Order # 8213, Pinnacle Technologies, Wyckoff, NJ, USA) and simultaneous video monitoring, both signals were recorded using custom-made scripts running LabChart Pro software (AD instruments, Colorado Springs, CO, USA).

2.3 *In vivo* SE induction, drug treatment and EEG data analysis

Before SE induction animals were hooked up to the EEG/EMG recording setup and baseline signal was recorded for 20 minutes. Mice were briefly disconnected and injected with a single high dose of kainic acid (KA; i.p., 20 mg/kg) to induce acute SE. In total, every animal received three i.p. drug injections of either 5 mg/kg diazepam or 0.3 mg/kg WAG or 1 mg/kg WAG (single dose). Drug injections were performed 50, 70 and 90 minutes after KA injection to test for treatment efficacy against SE. Mice were continuously recorded for a total of two hours after KA injection and euthanized thereafter.

Six 10-minute windows of EEG were analyzed for each animal (see Results, Figure 3A). To quantify the percentage of time seizing, two different thresholding criteria using standard deviations (STDs) of the baseline signal were applied to 100ms sub-windows (signal_{100ms}) of the 5 later analysis windows (Equations (1),(2)).

$$Max(signal_{100ms}) > 3 \cdot STD \quad (1)$$

$$Max(Abs(signal_{100ms})) > 5 \cdot STD \quad (2)$$

Digital output of (Equ.(1),(2)) and corresponding EEG signal were examined to determine seizure on- and offset. Events lasting over 10s were counted as seizures, and counted as terminated if under threshold for at least 5s. For signals showing inter-ictal spiking, the less sensitive (Equ.(2)) was chosen to account for the qualitative difference to seizing. In ambiguous cases, original EEG traces were manually examined.

Power spectrums were calculated for each window using the Scipy python toolbox (Jones, 2001) function `scipy.signal.spectrogram` with arguments `fs=1000`, `window='hanning'`, `nperseg=4000` (specifying 4ms windows), `noverlap=2000`, `scaling='spectrum'`. For a subset of analyses power values were normalized (i.e. % KA baseline). Total power was computed as the summed power of frequencies 0.5–100 Hz.

Drugs for *in vivo* experiments were pre-dissolved in DMSO with a final DMSO concentration in sterile saline of 0.03% v/v for i.p. injections. Kainic acid (Cat. No. 0222) was purchased in bulk to avoid batch to batch variability from Tocris, Bristol, United Kingdom. DMSO (No. 276855) was purchased from Sigma-Aldrich, St. Louis, MO, USA. Diazepam was purchased from Hospira, Lake Forest, IL, USA.

2.4 Statistics

All data sets from *in vitro* and *in vivo* experiments were analyzed for normal distribution (Shapiro-Wilk) and homogeneity of variances (Brown Forsythe, Bartlett's, or Levene's for non-normally distributed data). Subsequently, either parametric or non-parametric tests to test for pharmacological effects were employed. For time-course experiments with more than two timepoints either One Way Repeated Measures ANOVA (1W RM ANOVA) (posthoc: Bonferroni) or Friedman Repeated Measures ANOVA on Ranks (F RM ANOVA oR) (posthoc: Tukey, Dunnett's) was employed according to data set distribution. Differences in before/after experiments of dependent samples were analyzed using Wilcoxon signed-rank test (Wilcoxon sR test). For comparison of differences across groups Kruskal-Wallis One Way ANOVA on Ranks (1W ANOVA oR) (posthoc: Dunn's) was used in this study. As a sensitivity analysis, we also conducted an ANOVA with Welch correction (posthoc: Games Howell) for group comparisons to address heterogenous between-group variances and reached the same conclusions as with Kruskal-Wallis unless reported otherwise. For all statistical calculations $\alpha = 0.05$. P values of ANOVAs in the result section refer to group differences in the respective test, whereas the corresponding p values in the figure legends refer to posthoc testing and indicate significance of (multiple) pairwise comparisons.

3. Results

3.1 WAG inhibits hippocampal epileptiform activity *in vitro*

High- K^+ ACSF (8 mM) was used to induce continuous epileptiform activity (CEA) (Antonio et al., 2016; Pitkänen et al., 2017) in 83 slices from 21 adult Wistar rats. Extracellular field potentials were recorded from the CA3 region of combined horizontal

hippocampus-entorhinal cortex slices. This model was chosen because in adult Wistar rats continuous epileptiform activity, reminiscent of SE, is observed rather than temporally separated seizure-like events recorded in juvenile slices with 8 mM - K⁺ ACSF (Antonio et al., 2016).

After initiation of CEA, we assessed the stability of epileptiform activity upon application of 0.03% DMSO vehicle. We analyzed event incidence, duration and amplitude at three different time periods before (control), 30 and 60 min after vehicle application (Fig. 1 Aa–c). Mean event incidence and duration were stable over the course of experiments and did not significantly change over time and upon vehicle application. Average individual event amplitude slightly, but significantly, decayed between control and 60 min readout from 1.02 ± 0.21 mV to 0.87 ± 0.19 mV (Fig. 1 Ab; 1W RM ANOVA, $F(2,16) = 4.11$, $p = 0.036$). Event amplitude group means were not significantly different between control and 30 min readouts or 30 and 60 min readouts. We conclude that CEA induced with 8 mM K⁺-ACSF is sufficiently stable to conduct experiments evaluating different pharmacological treatment strategies *in vitro*.

Next, we determined the lowest WAG concentration sufficient to reliably block CEA *in vitro*. First, we bath-applied different WAG concentrations (10, 30, 100 and 300 nM) and DMSO vehicle for 40 minutes during CEA (Fig. 1B). DMSO vehicle, 10 and 30 nM WAG did not significantly change event incidence while 100 and 300 nM WAG strongly reduced or fully suppressed CEA in a concentration-dependent manner with an IC₅₀ of 52.5 nM (Fig. 1Bb). In comparison to 55.2 ± 6.9 ev/min after 40 minutes of vehicle control, 100 nM WAG treatment reduced incidence to 12.3 ± 4.8 ev/min and 300 nM WAG to 3.9 ± 3.5 ev/min (Fig. 1 Ba; 1W ANOVA oR, $H = 26.91$, $DF = 4$, $p < 0.001$). We also analyzed CEA event amplitude and duration and did not observe statistically significant changes in amplitude or duration at the lower WAG concentration (not shown).

We next examined if an intermediate concentration of WAG, 50 nM WAG, was also efficacious in reducing CEA at 30 and 60 min timepoints (Fig. 1C). CEA event incidence was significantly reduced by $75.7 \pm 7.7\%$ from 50.0 ± 5.5 ev/min before drug to 11.4 ± 3.9 ev/min after 30 min and further reduced to 2.5 ± 0.8 ev/min after 60 min resulting in a CEA attenuation by $94.5 \pm 1.7\%$ over 60 min (Fig. 1Cb, 1W RM ANOVA, $F(2,14) = 43.393$, $p < 0.001$). The reduction of CEA with 50 nM WAG was stronger than expected from the previously calculated IC₅₀ value suggesting a rather conservative IC₅₀ estimate. This group to group variability may be due to subtle differences in animal cohorts and could be amplified by the non-linearity of processes governing emergence and cessation of network phenomena like bursting. Event duration and amplitude were analyzed for all slices at 30 min and for 6 out of 8 slices at the 60 min timepoint where event incidence remained above zero. At 30 min, mean event duration and amplitude did not significantly change compared to pre-drug condition. At 60 min, event duration was reduced to 43.7 ± 6.6 ms compared to pre-drug control of 74.5 ± 6.1 ms and 79.9 ± 9.3 ms at the 30 min time point (1W RM ANOVA, $F(2,12) = 6.91$, $p = 0.01$). Event amplitude at 60 min was significantly reduced to 0.23 ± 0.05 mV compared to 0.88 ± 0.29 mV at pre-drug condition (1W RM ANOVA, $F(2,12) = 4.46$, $p = 0.036$). These results show that a low nanomolar WAG concentration can strongly inhibit CEA *in vitro*.

3.2 Efficacy of clinically-inspired SE treatment approaches *in vitro*

We next investigated whether treatments based on current clinical practice in treating SE were efficacious in reducing CEA *in vitro* (Fig. 2). In all three experimental groups we bath-applied diazepam (DIA, 200 nM) for 60 minutes as the base drug mimicking clinical first-line treatment. After 30 min of 200 nM DIA application, we added either a higher DIA concentration (1 μ M, Fig. 2A), phenytoin (PHT, 50 μ M, Fig. 2B), or WAG (50 nM, Fig. 2C). In all three groups, CEA parameters were analyzed at 30 min (200 nM DIA) and at 60 min (combined effects of 60 min 200 nM DIA plus 30 min of add-on treatment, Fig. 2Ab, Bb, Cb). DIA and PHT ACSF concentrations used in our experiments were chosen according to the CSF concentration range described in humans. 200 nM DIA represents the mid to lower end and 1 μ M DIA and 50 μ M PHT the high end of the concentration ranges found in human CSF (Engelhart and Jenkins, 2007; Rambeck et al., 2006).

200 nM DIA did not significantly alter CEA mean event incidence and duration but escalating the DIA concentration to 1 μ M for additional 30 minutes resulted in reduced mean CEA event incidence by $47.3 \pm 18.2\%$ from 47.5 ± 3.6 ev/min during pre-drug control to 28.4 ± 11.1 ev/min at 60 min (Fig 2Ab, F RM ANOVA oR, Chi-square = 7.03, DF = 2, $p = 0.03$). Moreover, CEA mean event duration was slightly, but significantly reduced after 60 min from 80.8 ± 5 ms to 66.6 ± 10 ms (1W RM ANOVA, $F(2,13) = 4.81$, $p = 0.027$). However, for both parameters a significant reduction was only present when comparing the 60 min time point with pre-drug control and not with the 30 min time point indicating no significant effects of escalating DIA on event incidence and duration. However, event amplitudes were significantly reduced at both 30 and 60 min timepoints. Average event amplitudes after 30 min with 200 nM DIA were reduced from 1.04 ± 0.13 mV pre-drug to 0.72 ± 0.14 mV and further attenuated upon escalation to 0.29 ± 0.04 mV at 60 min (1W RM ANOVA, $F(2,13) = 24.23$, $p < 0.001$). This data indicates that prolonging and escalating DIA exposure reduces epileptiform activity in this *in vitro* preparation.

Adding high-dose PHT to 200 nM DIA had variable effects on CEA event incidence, but overall resulted in a significant reduction of individual events between pre-drug control and 60 min time points by $54.6 \pm 17.2\%$ (Fig. 2Bb). As in high-dose DIA treatment, the difference in incidence between 30 and 60 min time points was not significant, likely due to high variance of combined pharmacological effects. Mean incidence during pre-drug control was 42.6 ± 3.0 ev/min and 19.8 ± 7.5 ev/min after add-on of PHT (1W RM ANOVA, $F(2,16) = 7.341$, $p = 0.005$). Mean event duration did not significantly change with any treatment. Average event amplitudes after 30 min with 200 nM DIA were reduced from 1.28 ± 0.24 mV during pre-drug control to 0.91 ± 0.27 mV and we observed further significant reduction upon application of PHT to 0.55 ± 0.23 mV after 60 minutes (1W RM ANOVA, $F(2,15) = 30.73$, $p < 0.001$).

Finally, adding 50 nM WAG to 200 nM DIA resulted in a marked and significant reduction of CEA event incidence (Fig. 2C) between 30 min and 60 min time points from 47.0 ± 5.4 ev/min to 13.4 ± 5.0 ev/min (Fig. 2Cb, 1W RM ANOVA, $F(2,16) = 25.799$, $p < 0.001$). This $71.4 \pm 9.9\%$ reduction in CEA incidence after 30 min of add-on WAG treatment almost fully accounts for the total attenuation ($72.3 \pm 10.5\%$) observed over the whole course of the experiment at 60 min (pre-drug control: 47.8 ± 4.6 ev/min). Out of the three add-on-

experiment groups, only WAG induced a significant decrease in event incidence, while the other two add-on treatments did not induce a significant change. This result suggests a more robustly discernible anticonvulsant add-on effect of WAG. CEA average individual event durations were not significantly altered at 30 or 60 min. However, CEA amplitudes were significantly lower with 0.29 ± 0.07 mV at 60 min after the addition of 50 nM WAG compared to 1.14 ± 0.19 mV during pre-drug control and compared to 0.72 ± 0.14 mV after 30 min of 200 nM DIA (1W RM ANOVA, $F(2,15)$, $p < 0.001$).

In summary, these clinically inspired *in vitro* experiments suggest that selectively targeting adenosine A1Rs by adding WAG as a second anticonvulsant on top of benzodiazepines may be at least as effective as current first- and second-line treatments available to SE patients.

3.3 Diazepam is not effective in reducing acute kainic acid (KA) induced SE

We next tested whether the potent effects of WAG seen *in vitro* would be maintained during *in vivo* SE. A single 20 mg/kg KA i.p. injection was given to induce SE in 33 C57/BL6 mice. Typically, the first seizures occurred within minutes after KA injection and consolidate into SE within the first hour after KA injection.

For *in vivo* experiments, animals were treated with three i.p. injections of diazepam (DIA, 3×5 mg/kg) 50, 70 and 90 min after KA injection while continuously recording EEG/EMG (Fig. 3A, 4A, 5A, top panel). SE severity was assayed at 1) Pre-seizure control (–10 to 0 mins before KA injection), 2) KA baseline (40 to 50 mins after KA), 3) drug injection 1 at 50 min (Inj 1: 60 to 70 mins), 4) drug injection 2 (Inj 2: 80 to 90 mins), 5) drug injection 3 (Inj 3: 100 to 110 mins) and 6) a delayed analysis window starting 20 min after injection 3 (Inj 3+20: 110 to 120 mins).

We first examined whether acute KA induced SE was resistant to DIA treatment (3×5 mg/kg, 50, 70 and 90 min after KA injection; Fig. 3). KA reliably induced convulsive electrographic seizures in all mice ($n = 10$) that subsequently received DIA i.p. injections. DIA injections did not attenuate KA induced SE (representative recording Fig. 3A). In contrary, despite DIA treatment mean percent time seizing after KA baseline remained stable until after Inj 1, but then rose from $70.2 \pm 7.1\%$ at KA baseline to $96.8 \pm 2\%$ during the Inj 3 + 20 min analysis window. The observed rise in convulsant activity reflected a trend that was not statistically significant in posthoc tests (Fig. 3B, F RM ANOVA oR, Chi-square = 12.83, DF = 4, $p = 0.012$, but for posthoc Dunnett: KA baseline vs. Inj 3 or Inj 3 + 20 min $p = 0.055$ and $p = 0.066$).

Analyzing total EEG power (0.5 – 100 Hz, normalized to KA baseline) also indicated no relevant anticonvulsant effect of DIA in our experimental conditions (Fig. 3C). Average EEG power did not significantly change from KA baseline during the first two DIA injections, but EEG was increased after the third injection with a significant difference between the KA baseline and Inj 3 + 20 analysis window (Inj 3 + 20: $185.8 \pm 53.6\%$ of KA base; F RM ANOVA oR, Chi-square = 12.6, DF = 4, $p = 0.013$).

When disconnecting mice from EEG recordings all mice presented with highly rigid muscle tone and were unresponsive to gentle handling, in line with our recordings of convulsant SE.

3.4 The adenosine A1R agonist WAG attenuates KA-induced SE *in vivo*

Next, we investigated the effects of three 1 mg/kg WAG i.p. injections (50, 70 and 90 min after KA injection) on acute convulsant KA-induced SE in eleven mice implanted with EEG/EMG electrodes (Fig. 4). All animals that went on to receive WAG injections presented with a similar average seizure load at KA baseline compared to the DIA treated animals before injections started (Fig. 6Aa, 6Ba,b). The initial 2 WAG injections did not have significant effects on KA-induced SE, but a significant decrease in mean percent time seizing and mean EEG power occurred following the third injection. Mean percent time seizing at KA baseline was: $71.6 \pm 6.3\%$, after Inj 1: $87.2 \pm 4.2\%$, after Inj 2: $69.2 \pm 11.5\%$, after Inj 3: $29 \pm 12.9\%$ and after Inj 3 + 20 min: $6.7 \pm 5.3\%$ (Fig. 4B). These results show anticonvulsant effects of WAG against acute KA induced SE starting after the second WAG injection. Statistically significant reductions in percent time seizing compared to KA baseline were recorded at Inj 3 + 20 min, and when compared to Inj 1 at Inj 3 and Inj 3 + 20 min (F RM ANOVA oR, Chi-square = 25.9, df = 4, $p < 0.001$). Total absence of seizure activity was observed in 9.1% of mice after Inj 2, 63.6% of mice after Inj 3 and 81.8% of mice after Inj3 + 20 min.

Results from analyzing mean EEG power at different time windows mirrored the results obtained from analyzing percent time seizing. The first two WAG injection did not significantly decrease mean EEG power, but strongly suppressed EEG power with the third injection. Mean EEG power was normalized to the KA baseline analysis window and was consistently below KA baseline levels after Inj 2 (Inj 1: $174 \pm 47.3\%$ of KA base, Inj 2: $95.3 \pm 37.7\%$, Inj 3: $23.1 \pm 7\%$, Inj 3 + 20: $9.9 \pm 3\%$; Fig. 4C). EEG power was elevated after Inj 1, but this facilitation was not significantly different to KA baseline. Significant differences were observed when power levels of Inj 3 + 20 were compared to KA baseline, Inj1 and Inj2 in addition to a significant decline in power between Inj 1 and Inj 3 (Fig. 4 C, F RM ANOVA oR, Chi-square = 31.8, df = 4, $p < 0.001$). Latter results indicate that EEG power was not attenuated after Inj 1, but significantly decreased over time with further injections.

When disconnecting mice from EEG recordings WAG treated mice presented with relaxed muscle tone and in a sedated state. Mice could be awakened for short periods of time by gentle handling and were able to perform coordinated movements (walking away). In summary, our results demonstrate strong anticonvulsant effects of three 1 mg/kg WAG injections leading to seizure-freedom and EEG power levels similar to pre-seizure control values in the vast majority of mice after WAG injections.

3.5 The anticonvulsant properties of WAG were retained at a lower dose

Next, we tested the anticonvulsant *in vivo* efficacy of three lower dose 0.3 mg/kg WAG injections (50, 70 and 90 min after KA injection , Fig. 5). Again, KA reliably induced SE in 12 mice and average seizure load in mice of this experimental group was similar to DIA and 1 mg/kg WAG cohorts (Fig. 6Aa, 6Ba,b). Injections of low-dose WAG had no effects for the first 2 doses, but significantly attenuated percent time seizing and EEG power after three injections in this treatment group. Onset latency of WAG effect was comparable to the 1 mg/kg WAG experiments and relatively slow without any attenuating effect after the first injection. Mean percent time seizing was significantly reduced over injections with

percentages during KA baseline: $81.4 \pm 6.3\%$, after Inj 1: $82 \pm 7.5\%$, after Inj 2: $67.6 \pm 9.7\%$, after Inj 3: $44 \pm 13\%$ and after Inj 3 + 20 min: $41.7 \pm 3.9\%$ (Fig. 5B). These results demonstrate anticonvulsant effects of 0.3 mg/kg WAG against acute KA induced SE starting after the second WAG injection. Statistically significant attenuations of percent time seizing compared to KA baseline were recorded at Inj 3 and Inj 3 + 20 min and, in addition, reduction of seizure burden measured as percent time seizing was also prevalent at Inj 3 and Inj 3 + 20 min when comparing to Inj 1 analysis window (Fig. 5B, 1W RM ANOVA, $F(4,44) = 7.614$, $p < 0.001$). Both results indicate a relevant reduction of seizure load after the Inj 1 analysis window. As expected for lower doses, anticonvulsant effects after Inj 3 with 0.3 mg/kg WAG were less robust compared to the 1 mg/kg WAG cohort.

Analysis of EEG power over time confirmed our results obtained from analyzing percent time seizing (Fig. 5C). Mean EEG power was normalized to KA baseline and, similar to the 1 mg/kg WAG cohort, was consistently below KA baseline levels after Inj 2 (Inj 1: $146 \pm 34.8\%$, Inj 2: $89.6 \pm 23.6\%$, Inj 3: $40 \pm 12\%$, Inj 3 + 20: $35.3 \pm 9.1\%$; Fig. 5C). A significant attenuation compared to KA baseline (and Inj 1) was present at Inj 3 and Inj 3 + 20 analyses windows (Fig. 5C, F RM ANOVA oR, Chi-square = 26.3, $df = 4$, $p < 0.001$). Our results indicate that WAG's effect on EEG power had a slow onset, but significantly decreased power levels upon further low-dose injections.

At the end of the experiment, most mice displayed soft muscle tone and sedation and could be awakened for short time periods when they would make coordinated movements. It is important to note that no mouse died during treatment with the A1R-agonist WAG at either dose tested. Albeit lower rates of mice presenting with full seizure freedom after three 0.3 mg/kg WAG injections, these low-dose experiments confirmed our previous results that WAG is a potent anticonvulsant agent when used for treatment of diazepam-resistant SE in mice.

3.6 Comparison of DIA and WAG effects on KA-induced SE

We next compared the anticonvulsant effects of WAG to DIA (Fig. 6). Mean percent time seizing after Inj 2 was ~25% lower in both WAG treated animals than in DIA treated animals (Fig 6Ab). This difference in means of treatment groups was statistically not significant and appears to be driven by a subset of animals responding earlier to WAG treatment than others. During the subsequent analysis periods (Inj 3 and Inj 3 + 20, Fig. 6Ac,d) both 0.3 and 1 mg/kg WAG injections were significantly better at suppressing seizures compared to DIA (for Inj 3/Inj 3 + 20 analysis windows: 1W ANOVA oR, $H = 14.8/22.26$, $df = 2/2$, $p < 0.001/0.001$). In addition, the sensitivity analysis using ANOVA with Welch's correction shows a dose-dependent treatment effect comparing the different WAG doses in the last analysis window (significance bar in light grey) suggesting a possible group difference (Fig. 6Ad, ANOVA with Welch's correction, $F(2,16) = 127.33$, $p < 0.001$).

Mean EEG power analysis across groups at different analysis windows revealed the same pattern with no significant differences after Inj 2 albeit lower average EEG power in both WAG treated groups compared to DIA (Fig. 6Ca). Later, during the Inj 3 + 20 window (Fig. 6Cb), both WAG treatments displayed significantly higher anticonvulsant effects than DIA (1W ANOVA oR, $H = 19.5$, $df = 2$, $p < 0.001$). Dose-dependency of WAG effects on power

in vivo, as seen in the *in vitro* experiments and for percent time seizing *in vivo*, was not statistically evident with an alpha of 0.05. However, differences in data distribution at Inj 3 + 20 with higher variance for both parameters in the 0.3 mg/kg WAG vs. 1 mg/kg WAG group suggest dose-dependent effects of WAG *in vivo*. In conclusion, *in vivo* acute anticonvulsant effects of WAG are robust even when DIA fails to suppress SE.

4 Discussion

Here, we investigated the adenosine A1R agonist SDZ WAG 994 (WAG) as a potential therapeutic intervention for pharmacoresistant status epilepticus (SE). We found that low nanomolar concentrations of WAG are sufficient to block ongoing epileptiform activity induced by 8 mM K⁺ *in vitro*. In addition, WAG acutely and potently suppressed KA-induced SE in mice *in vivo*, while DIA failed to attenuate SE. WAG has a number of unique properties that make it potentially useful to treat pharmacoresistant SE. Anticonvulsant effects of classic A1R agonists like CPA and PIA (Barker-Haliski et al., 2017; Gu et al., 2014; Longo et al., 1995) are known to occur at very low concentrations, similar to our finding for WAG's *in vitro* efficacy. These other compounds, however, come with significant cardiac adverse effect which preclude their clinical use to treat SE. Efforts to minimize the cardiovascular effects of A1R agonists have often reduced their anticonvulsant efficacy (Knutsen et al., 1999). Our results using WAG are encouraging as WAG is expected to have reduced adverse cardiac effects compared to other A1R agonists (Knutsen et al., 1999) but maintains its anticonvulsant properties even at low concentrations. In fact, WAG has already been safely used in humans (Bertolet et al., 1996; Piot et al., 1998; Seymour et al., 1999) and thus may be a promising candidate for translation into human use as a novel treatment against pharmacoresistant SE.

While many studies have demonstrated that activation of A1Rs can reduce neuronal and epileptiform activity (Barker-Haliski et al., 2017; Dunwiddie and Hoffer, 1980; Gu et al., 2014; Hargus et al., 2012; Klaft et al., 2016; Klaft et al., 2012; Longo et al., 1995; Vianna et al., 2005), our study is the first to show that a selective A1R agonist with a potentially manageable side-effect profile attenuates acute SE *in vivo*. The vast majority of previous studies investigating anticonvulsant efficacy of adenosinergic treatments addressed chronic epilepsies rather than SE. The remaining studies that have examined A1R agonists as potential treatments for SE have applied agonists prior to induction of SE (Cano-Martinez et al., 2001; MacGregor and Stone, 1993; Niglio et al., 1988; Rosim et al., 2011). This distinction is important as A1Rs inhibit seizure initiation, but also contribute to seizure termination, and both processes may rely on different adenosinergic mechanisms. Here, we investigated WAG's potency to acutely suppress established SE instead of preventing initiation of seizure activity which is more suited for investigating novel chronic anticonvulsant treatment approaches.

Importantly, our data shows that WAG can terminate SE when benzodiazepines fail. In our study, SE induced with a single i.p. 20 mg/kg kainic acid injection was resistant to three 5 mg/kg DIA injections given in 20 min intervals (15 mg cumulative DIA dose), consistent with previous reports (Fritsch et al., 2010; Reddy and Kuruba, 2013; Sivakumaran and Maguire, 2016). Benzodiazepine resistance of SE is a well-described phenomenon in

experimental and clinical settings. Mechanisms leading to diminished diazepam efficacy may include SE-induced GABA-A-R internalization (Naylor et al., 2005; Nicholson et al., 2018) and/or collapse of the chloride gradient essential to GABA-mediated inhibition (Burman et al., 2019; Moore et al., 2018). This underscores the need to have therapies available that do not target GABARs to reduce SE. In support of WAG's potential to treat human pharmacoresistant SE, we previously reported that WAG attenuates pharmacoresistant seizure-like events in human brain slices prepared from tissue resected for epilepsy surgery (Klaft et al., 2016). This suggests that WAG's ability to block epileptiform activity and pharmacoresistant SE in rodent models may be maintained in human SE. It is important to note that SE is a neurological condition that is closely related, but distinct from chronic epilepsy. SE may arise as a complication of an existing chronic epilepsy, in which case the resulting SE is usually well controlled with benzodiazepines. Approximately half the cases of SE, however, develop from diverse etiologies like intoxication, trauma, infections, etc. (Betjemann and Lowenstein, 2015; Hesdorffer et al., 1998; Trinka et al., 2015), and tend to be more benzodiazepine resistant (Leitinger et al., 2020). In addition, SE of various etiologies can represent the first neurological insult that, after a latent period, leads to the development of chronic epilepsy in those patients. Therefore, attenuating SE as soon as possible is regarded as beneficial for preventing the subsequent development of chronic epilepsies.

Our *in vivo* data suggest an initial increase in EEG power after the first WAG injection, but the apparent rise between KA baseline levels and injection 1 is not statistically significant ($p = 0.619/0.928$ for KA base vs. Injection 1 for 1/0.3 mg/kg WAG cohort). While broadband adenosinergic agonists may have proconvulsant effects by activating adenosine A_{2a} receptors (Masino et al., 2014), we have no evidence for a proconvulsive action of WAG and have not observed any initial proconvulsant effects in a previous study with WAG in human brain slices (Klaft et al., 2016). However, it is well documented that KA-induced SE has a natural evolution that includes increased severity over time (Fritsch et al., 2010; Levesque et al., 2016; Sivakumaran and Maguire, 2016), so we suspect an initial increase in seizure severity would more likely represent this natural progression of SE. An increase might have not been observed following DIA injection due to potential different pharmacokinetics of DIA and WAG. DIA has been developed as a CNS drug with excellent blood-brain-barrier (BBB) permeability and rapid effect onset which is reflected by its clinical use in emergency situations. WAG was initially developed against non-CNS indications and has not been optimized for BBB permeability. Therefore, we likely have observed a delayed onset of WAG's attenuating effect rather than a direct proconvulsive WAG effect. The quick WAG effect onset in slice experiments and the delay in *in vivo* recordings suggests that BBB crossing is robust, but relatively slow. Future translational studies will have to determine the pharmacokinetics of a single WAG dose in regards to effect onset time and duration in order to optimize dosing strategies.

Historically, cardiovascular side effects mediated by adenosine receptors have precluded development of adenosinergic anticonvulsants. In addition to its potent anti-convulsant properties, WAG appears to have reduced cardiovascular adverse effects as compared to other A1R agonists. WAG has > 40-fold higher EC₅₀ for negative inotropy in an *in vitro* assay screening for cardiological side effects compared to CPA and PIA (Knutsen et al.,

1999) supporting the clinical observation that WAG has minimal cardiological side effects when applied in humans (Bertolet et al., 1996; Piot et al., 1998; Seymour et al., 1999). Therefore, WAG is a unique compound that combines strong anticonvulsant efficacy on par with or better than the classic A1R agonists with a favorable side-effect profile. However, it is important to note that we did not perform cardiological monitoring during our *in vivo* experiments. All mice that received WAG in our experiments survived KA induced SE, but this does not exclude potential adverse cardiac effects. Clinical dose-escalation studies in humans are needed to determine cardiac safety at higher doses properly. Although WAG treatments have shown a surprising lack of cardiovascular side effects in previous clinical trials, potential cardiac complications of using WAG (bradycardia) are likely to occur and must be considered. However, a WAG treatment against refractory SE may be justifiable as pharmacoresistant SE is life-threatening and WAG would be administered in an ICU setting with cardiovascular monitoring and intervention options. In addition, the purinergic antagonist aminophylline has been in clinical use for decades and, importantly, has been previously used to rapidly reverse unwanted effects of A1R agonists in clinical trials (Lerman et al., 2001). Furthermore, drugs like 8-(sulfophenyl)theophylline, a non-blood-brain-barrier penetrant A1R antagonist, could be administered in conjunction with WAG to prevent peripheral side effects (Gouder et al., 2003).

In summary, our study shows translational potential of WAG to treat pharmacoresistant SE. WAG has been investigated in clinical trials against non-neurological indications and systemic administration was shown to be safe in humans. Nevertheless, there are potential adverse cardiac effects that must be considered, and future translational studies must carefully monitor cardiac function. In recent years, repurposing compounds, which are currently available or have at least been evaluated in clinical trials as safe for human use, for new indications is increasingly recognized as an excellent opportunity to develop novel treatments in a time- and cost-effective manner (Nosengo, 2016). Finally, WAG molecular properties (molecular weight, charge distribution) predict relevant blood-brain-barrier crossing in humans which is supported by a report describing somnolence/sedation as a side-effect in a clinical trial evaluating WAG as a novel pain medication (Seymour et al., 1999). In conclusion, the potent anticonvulsant efficacy of WAG *in vitro* and *in vivo* demonstrated in the present study in combination with the unique translational potential of WAG warrant further evaluation of WAG as a potential novel treatment approach against pharmacoresistant SE.

5 References

- Antonio LL, Anderson ML, Angamo EA, Gabriel S, Klaft ZJ, Liotta A, Salar S, Sandow N, Heinemann U, 2016 In vitro seizure like events and changes in ionic concentration. *J Neurosci Methods* 260, 33–44. [PubMed: 26300181]
- Barker-Haliski ML, Johnson K, Billingsley P, Huff J, Handy LJ, Khaleel R, Lu Z, Mau MJ, Pruess TH, Rueda C, Saunders G, Underwood TK, Vanegas F, Smith MD, West PJ, Wilcox KS, 2017 Validation of a Preclinical Drug Screening Platform for Pharmacoresistant Epilepsy. *Neurochem Res* 42, 1904–1918. [PubMed: 28303498]
- Bertolet BD, Anand IS, Bryg RJ, Mohanty PK, Chatterjee K, Cohn JN, Khurmi NS, Pepine CJ, 1996 Effects of A1 adenosine receptor agonism using N6-cyclohexyl-2'-O-methyladenosine in patients with left ventricular dysfunction. *Circulation* 94, 1212–1215. [PubMed: 8822971]

- Betjemann JP, Josephson SA, Lowenstein DH, Burke JF, 2015 Trends in Status Epilepticus-Related Hospitalizations and Mortality: Redefined in US Practice Over Time. *JAMA Neurol* 72, 650–655. [PubMed: 25915004]
- Betjemann JP, Lowenstein DH, 2015 Status epilepticus in adults. *Lancet Neurol* 14, 615–624. [PubMed: 25908090]
- Boggs JG, 2004 Mortality Associated with Status Epilepticus. *Epilepsy Curr* 4, 25–27. [PubMed: 15346141]
- Boison D, 2012a Adenosine Augmentation Therapy In: th, Noebels JL, Avoli M, Rogawski MA, Olsen RW, Delgado-Escueta AV, (Eds), *Jasper's Basic Mechanisms of the Epilepsies*, Bethesda (MD).
- Boison D, 2012b Adenosine dysfunction in epilepsy. *Glia* 60, 1234–1243. [PubMed: 22700220]
- Burman RJ, Selfe JS, Lee JH, van den Berg M, Calin A, Codadu NK, Wright R, Newey SE, Parrish RR, Katz AA, Wilmshurst JM, Akerman CJ, Trevelyan AJ, Raimondo JV, 2019 Excitatory GABAergic signalling is associated with benzodiazepine resistance in status epilepticus. *Brain* 142, 3482–3501. [PubMed: 31553050]
- Cano-Martinez A, Villalobos-Molina R, Rocha L, 2001 Effects of chronic morphine and N(6)-cyclopentyl-adenosine administration on kainic acid-induced status epilepticus. *Epilepsy Res* 44, 89–96. [PubMed: 11325565]
- Chen JF, Eltzschig HK, Fredholm BB, 2013 Adenosine receptors as drug targets--what are the challenges? *Nat Rev Drug Discov* 12, 265–286. [PubMed: 23535933]
- Dale N, Frenguelli BG, 2009 Release of adenosine and ATP during ischemia and epilepsy. *Curr Neuropharmacol* 7, 160–179. [PubMed: 20190959]
- Dulla CG, Dobelis P, Pearson T, Frenguelli BG, Staley KJ, Masino SA, 2005 Adenosine and ATP link PCO₂ to cortical excitability via pH. *Neuron* 48, 1011–1023. [PubMed: 16364904]
- Dunwiddie TV, 1980 Endogenously released adenosine regulates excitability in the in vitro hippocampus. *Epilepsia* 21, 541–548. [PubMed: 7418669]
- Dunwiddie TV, Hoffer BJ, 1980 Adenine nucleotides and synaptic transmission in the in vitro rat hippocampus. *Br. J. Pharmacol* 69, 59–68. [PubMed: 6247005]
- During MJ, Spencer DD, 1992 Adenosine: a potential mediator of seizure arrest and postictal refractoriness. *Ann Neurol* 32, 618–624. [PubMed: 1449242]
- Elzein E, Zablocki J, 2008 A1 adenosine receptor agonists and their potential therapeutic applications. *Expert Opin Investig Drugs* 17, 1901–1910.
- Engelhart DA, Jenkins AJ, 2007 Comparison of drug concentrations in postmortem cerebrospinal fluid and blood specimens. *J Anal Toxicol* 31, 581–587. [PubMed: 18093417]
- Ferlisi M, Shorvon S, 2012 The outcome of therapies in refractory and super-refractory convulsive status epilepticus and recommendations for therapy. *Brain* 135, 2314–2328. [PubMed: 22577217]
- Fritsch B, Stott JJ, Joelle Donofrio J, Rogawski MA, 2010 Treatment of early and late kainic acid-induced status epilepticus with the noncompetitive AMPA receptor antagonist GYKI 52466. *Epilepsia* 51, 108–117. [PubMed: 19682025]
- Gouder N, Fritschy JM, Boison D, 2003 Seizure suppression by adenosine A1 receptor activation in a mouse model of pharmacoresistant epilepsy. *Epilepsia* 44, 877–885. [PubMed: 12823569]
- Gu F, Hazra A, Aulakh A, Ziburkus J, 2014 Purinergic control of hippocampal circuit hyperexcitability in Dravet syndrome. *Epilepsia* 55, 245–255. [PubMed: 24417577]
- Hargus NJ, Jennings C, Perez-Reyes E, Bertram EH, Patel MK, 2012 Enhanced actions of adenosine in medial entorhinal cortex layer II stellate neurons in temporal lobe epilepsy are mediated via A(1)-receptor activation. *Epilepsia* 53, 168–176. [PubMed: 22126400]
- Hesdorffer DC, Logroscino G, Cascino G, Annegers JF, Hauser WA, 1998 Incidence of status epilepticus in Rochester, Minnesota, 1965–1984. *Neurology* 50, 735–741. [PubMed: 9521266]
- Hill E, Hickman C, Diez R, Wall M, 2020 Role of A1 receptor-activated GIRK channels in the suppression of hippocampal seizure activity. *Neuropharmacology* 164, 107904. [PubMed: 31812775]
- Holtkamp M, 2016 Status Epilepticus Diagnosis, Management and Outcome: An Update. *J Clin Neurophysiol* 33, 1–2. [PubMed: 26840868]

- Holtkamp M, Othman J, Buchheim K, Meierkord H, 2005 Predictors and prognosis of refractory status epilepticus treated in a neurological intensive care unit. *J Neurol Neurosurg Psychiatry* 76, 534–539. [PubMed: 15774441]
- Jacobson KA, Tosh DK, Jain S, Gao ZG, 2019 Historical and Current Adenosine Receptor Agonists in Preclinical and Clinical Development. *Front Cell Neurosci* 13, 124. [PubMed: 30983976]
- Jones EO, E.; Petersen P, 2001 SciPy: Open Source Scientific Tools for Python. .
- Klaft ZJ, Hollnagel JO, Salar S, Caliskan G, Schulz SB, Schneider UC, Horn P, Koch A, Holtkamp M, Gabriel S, Gerevich Z, Heinemann U, 2016 Adenosine A1 receptor-mediated suppression of carbamazepine-resistant seizure-like events in human neocortical slices. *Epilepsia* 57, 746–756. [PubMed: 27087530]
- Klaft ZJ, Schulz SB, Maslarova A, Gabriel S, Heinemann U, Gerevich Z, 2012 Extracellular ATP differentially affects epileptiform activity via purinergic P2X7 and adenosine A1 receptors in naive and chronic epileptic rats. *Epilepsia* 53, 1978–1986. [PubMed: 23106524]
- Knutsen LJ, Lau J, Petersen H, Thomsen C, Weis JU, Shalmi M, Judge ME, Hansen AJ, Sheardown MJ, 1999 N-substituted adenosines as novel neuroprotective A(1) agonists with diminished hypotensive effects. *J Med Chem* 42, 3463–3477. [PubMed: 10479279]
- Kovacs R, Gerevich Z, Friedman A, Otahal J, Prager O, Gabriel S, Berndt N, 2018 Bioenergetic Mechanisms of Seizure Control. *Front Cell Neurosci* 12, 335. [PubMed: 30349461]
- Lau LA, Noubary F, Wang D, Dulla CG, 2017 alpha2delta-1 Signaling Drives Cell Death, Synaptogenesis, Circuit Reorganization, and Gabapentin-Mediated Neuroprotection in a Model of Insult-Induced Cortical Malformation. *eNeuro* 4.
- Leitinger M, Trinka E, Zimmermann G, Granbichler CA, Kobulashvili T, Siebert U, 2020 Epidemiology of status epilepticus in adults: Apples, pears, and oranges - A critical review. *Epilepsy Behav*, 106720.
- Lerman BB, Ellenbogen KA, Kadish A, Platia E, Stein KM, Markowitz SM, Mittal S, Slotwiner DJ, Scheiner M, Iwai S, Belardinelli L, Jerling M, Shreeniwas R, Wolff AA, 2001 Electrophysiologic effects of a novel selective adenosine A1 agonist (CVT-510) on atrioventricular nodal conduction in humans. *J Cardiovasc Pharmacol Ther* 6, 237–245. [PubMed: 11584330]
- Levesque M, Avoli M, Bernard C, 2016 Animal models of temporal lobe epilepsy following systemic chemoconvulsant administration. *J Neurosci Methods* 260, 45–52. [PubMed: 25769270]
- Longo R, Zeng YC, Sagratella S, 1995 Opposite modulation of 4-aminopyridine and hypoxic hyperexcitability by A1 and A2 adenosine receptor ligands in rat hippocampal slices. *Neurosci Lett* 200, 21–24. [PubMed: 8584257]
- MacGregor DG, Stone TW, 1993 Inhibition by the adenosine analogue, (R)-N6-phenylisopropyladenosine, of kainic acid neurotoxicity in rat hippocampus after systemic administration. *Br J Pharmacol* 109, 316–321. [PubMed: 8358536]
- Masino SA, Kawamura M Jr., Ruskin DN, 2014 Adenosine receptors and epilepsy: current evidence and future potential. *Int Rev Neurobiol* 119, 233–255. [PubMed: 25175969]
- Masino SA, Li T, Theofilas P, Sandau US, Ruskin DN, Fredholm BB, Geiger JD, Aronica E, Boison D, 2011 A ketogenic diet suppresses seizures in mice through adenosine A(1) receptors. *J Clin Invest* 121, 2679–2683. [PubMed: 21701065]
- Meierkord H, Holtkamp M, 2007 Non-convulsive status epilepticus in adults: clinical forms and treatment. *Lancet Neurol* 6, 329–339. [PubMed: 17362837]
- Moore YE, Deeb TZ, Chadchankar H, Brandon NJ, Moss SJ, 2018 Potentiating KCC2 activity is sufficient to limit the onset and severity of seizures. *Proc Natl Acad Sci U S A* 115, 10166–10171. [PubMed: 30224498]
- Moschovos C, Kostopoulos G, Papatheodoropoulos C, 2012 Endogenous adenosine induces NMDA receptor-independent persistent epileptiform discharges in dorsal and ventral hippocampus via activation of A2 receptors. *Epilepsy Res* 100, 157–167. [PubMed: 22401823]
- Naylor DE, Liu H, Wasterlain CG, 2005 Trafficking of GABA(A) receptors, loss of inhibition, and a mechanism for pharmacoresistance in status epilepticus. *J Neurosci* 25, 7724–7733. [PubMed: 16120773]

- Nicholson MW, Sweeney A, Pekle E, Alam S, Ali AB, Duchon M, Jovanovic JN, 2018 Diazepam-induced loss of inhibitory synapses mediated by PLCdelta/ Ca(2+)/calcineurin signalling downstream of GABAA receptors. *Mol Psychiatry* 23, 1851–1867. [PubMed: 29904150]
- Niglio T, Popoli P, Caporali MG, Scotti de Carolis A, 1988 Antiepileptic effects of N6-L-phenylisopropyladenosine (L-PIA) on penicillin-induced epileptogenic focus in rabbits. *Pharmacol Res Commun* 20, 561–572. [PubMed: 3174790]
- Nosengo N, 2016 Can you teach old drugs new tricks? *Nature* 534, 314–316. [PubMed: 27306171]
- Piot O, Chauvel C, Lazarus A, Pellerin D, David D, Leneveu-Ledoux L, Guize L, Le Heuzey JY, 1998 Effects of a selective A1-adenosine receptor agonist on heart rate and heart rate variability during permanent atrial fibrillation. *Pacing Clin Electrophysiol* 21, 2459–2464. [PubMed: 9825367]
- Pitkänen A, Buckmaster PS, Galanopoulou AS, Moshé SL, 2017 Models of seizures and epilepsy. Elsevier/Academic Press, London.
- Rambeck B, Jurgens UH, May TW, Pannek HW, Behne F, Ebner A, Gorji A, Straub H, Speckmann EJ, Pohlmann-Eden B, Loscher W, 2006 Comparison of brain extracellular fluid, brain tissue, cerebrospinal fluid, and serum concentrations of antiepileptic drugs measured intraoperatively in patients with intractable epilepsy. *Epilepsia* 47, 681–694. [PubMed: 16650134]
- Reddy DS, Kuruba R, 2013 Experimental models of status epilepticus and neuronal injury for evaluation of therapeutic interventions. *Int J Mol Sci* 14, 18284–18318. [PubMed: 24013377]
- Rosim FE, Persike DS, Nehlig A, Amorim RP, de Oliveira DM, Fernandes MJ, 2011 Differential neuroprotection by A(1) receptor activation and A(2A) receptor inhibition following pilocarpine-induced status epilepticus. *Epilepsy Behav* 22, 207–213. [PubMed: 21852200]
- Rossetti AO, Lowenstein DH, 2011 Management of refractory status epilepticus in adults: still more questions than answers. *Lancet Neurol* 10, 922–930. [PubMed: 21939901]
- Schulz SB, Klaft ZJ, Rosler AR, Heinemann U, Gerevich Z, 2012 Purinergic P2X, P2Y and adenosine receptors differentially modulate hippocampal gamma oscillations. *Neuropharmacology* 62, 914–924. [PubMed: 22001427]
- Seymour RA, Hawkesford JE, Hill CM, Frame J, Andrews C, 1999 The efficacy of a novel adenosine agonist (WAG 994) in postoperative dental pain. *Br J Clin Pharmacol* 47, 675–680. [PubMed: 10383546]
- Shah B, Rohatagi S, Natarajan C, Kirkesseli S, Baybutt R, Jensen BK, 2004 Pharmacokinetics, pharmacodynamics, and safety of a lipid-lowering adenosine A1 agonist, RPR749, in healthy subjects. *Am J Ther* 11, 175–189. [PubMed: 15133532]
- Sivakumaran S, Maguire J, 2016 Bumetanide reduces seizure progression and the development of pharmacoresistant status epilepticus. *Epilepsia* 57, 222–232. [PubMed: 26659482]
- Towne AR, Pellock JM, Ko D, DeLorenzo RJ, 1994 Determinants of mortality in status epilepticus. *Epilepsia* 35, 27–34. [PubMed: 8112254]
- Trinka E, Cock H, Hesdorffer D, Rossetti AO, Scheffer IE, Shinnar S, Shorvon S, Lowenstein DH, 2015 A definition and classification of status epilepticus--Report of the ILAE Task Force on Classification of Status Epilepticus. *Epilepsia* 56, 1515–1523. [PubMed: 26336950]
- Vianna EP, Ferreira AT, Dona F, Cavalheiro EA, da Silva Fernandes MJ, 2005 Modulation of seizures and synaptic plasticity by adenosinergic receptors in an experimental model of temporal lobe epilepsy induced by pilocarpine in rats. *Epilepsia* 46 Suppl 5, 166–173.
- Wu LG, Saggau P, 1994 Adenosine inhibits evoked synaptic transmission primarily by reducing presynaptic calcium influx in area CA1 of hippocampus. *Neuron* 12, 1139–1148. [PubMed: 8185949]

HIGHLIGHTS

- A1R agonist SDZ WAG 994 suppresses kainate status epilepticus (SE) *in vivo* in mice
- 0.3 and 1 mg/kg WAG, but not 5 mg/kg Diazepam, attenuate or stop established SE
- Low nanomolar concentrations of WAG vastly reduce epileptiform activity *in vitro*
- WAG IC₅₀ against high-K⁺ epileptiform activity in rat hippocampal slices: 52.5 nM

Figure 1. The A1R agonist SDZ WAG 994 (WAG) attenuates continuous epileptiform activity (CEA) *in vitro*.

A. CEA properties remain stable over time allowing for pharmacological experiments. **(a)** Illustration of horizontal combined entorhinal cortex/hippocampus slice and field potential (FP) recording from CA3 stratum pyramidale. Entorhinal cortex (EC), subiculum (Sub), gyrus dentatus (DG) and hippocampal subfields CA3 and CA1 are labelled. **(b)** FP recording showing induction of CEA in hippocampal CA3 by 8 mM K^+ and the experimental protocol. **(c)** Quantification of CEA incidence, duration, and amplitude across time. CEA is stable over time, but individual event amplitudes show slight, but significant rundown over time (1W RM ANOVA, Bonferroni posthoc, $p = 0.035$ for ctrl vs. 60 min, $n = 9$). **(d)** Representative FP traces and individual epileptiform events at different analysis windows. **B.** WAG attenuates *in vitro* epileptiform activity in a concentration-dependent manner. **(a)** CEA event incidence is significantly reduced with 100 and 300 nM WAG compared to vehicle control (1W ANOVA oR, Dunn's posthoc, $p = 0.004 < 0.001$ for 100/300 nM vs. vehicle, $n = 12/8$, $p = 0.008/0.013$ for 10/30 nM vs. 300 nM, $n = 8/10$). **(b)** Concentration-response curve for the inhibitory effect of WAG on CEA event incidence ($IC_{50} = 52.5$ nM). **(c)** Representative FP recordings at different WAG concentrations. **C.** 50 nM of WAG is sufficient to fully suppress CEA in the majority of slices. **(a)** Raw FP recording trace over the full course of the experiment. **(b)** Quantification of WAG (50 nM) effects on CEA

parameters. CEA incidence is significantly reduced after 30 min of 50 nM WAG and further reduced after 60 min of treatment. Event duration remains stable for 30 min and is significantly reduced after 60 min. Average individual event amplitude is attenuated after 60 min (1W RM ANOVA, Bonferroni posthoc, incidence: $p < 0.001$ for 30 and 60 min vs. ctrl, $n = 8$, duration: $p = 0.012/0.033$ for 30 min and ctrl vs. 60 min, $n = 8$ (ctrl, 30 min), $n = 6$ (60 min), amplitude: $p = 0.034$ for ctrl vs. 60 min, $n = 8$ (ctrl), $n = 6$ (60min)). (c) Example FP traces at different experimental time periods. Boxplots: filled black square = mean, horizontal line = median, whiskers = $1.5 * \text{IQR}$. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. Ctrl, control.

Figure 2. Comparing the effects of first- and second-line treatments against status epilepticus (SE) with WAG treatment on *in vitro* CEA.

Slices in all experimental groups were exposed to diazepam (DIA, 200 nM) treatment for 60 minutes, different add-on treatments were added after 30 minutes in each group. **A.**

Escalating DIA concentration to a total of 1 μ M significantly reduces CEA in hippocampal CA3 when comparing pre-drug control with 60 min readout. **(a)** FP trace and experimental protocol. **(b)** Quantification of DIA effects after 30 min (200 nM) and 60 minutes (add on DIA (1 μ M) present for 30 min) (F RM ANOVA oR, Tukey posthoc, incidence: $p = 0.046$ for ctrl vs. 60 min, $n = 8$; 1W RM ANOVA, Bonferroni posthoc, duration: $p = 0.029$ for ctrl vs. 60 min, $n = 8$ (ctrl) and 7 (60 min), amplitude: $p = 0.043 < 0.001$ for 30/60 min vs. ctrl, $p = 0.003$ for 30 vs. 60 min, $n = 7-8$) **(c)** Representative FP traces at indicated time periods. **B.**

Base treatment with DIA for 60 min plus add-on of 50 μ M phenytoin (PHT) for 30 min significantly reduces CEA incidence between control and 60 min. **(a)** FP trace with the experimental protocol. **(b)** Quantification of PHT effects (1W RM ANOVA, Bonferroni posthoc, incidence: $p = 0.004$ for ctrl vs. 60 min, $n = 9$, amplitude: $p = 0.004 < 0.001$ for 30/60 min vs. ctrl, $p = 0.003$ for 30 vs. 60 min, $n = 9$ (ctrl, 30 min) and 8 (60 min); F RM ANOVA oR, Tukey posthoc, duration: $p = 0.029$ for ctrl vs. 60 min, $n = 8$ (ctrl) and 7 (60 min), Amplitude: $p = 0.043 < 0.001$ for 30/60 min vs. ctrl, $p = 0.003$ for 30 vs. 60 min, $n = 7-8$) **(c)** Representative FP traces. **C.** Only 30 min of add-on treatment with 50 nM of WAG

in presence of DIA significantly reduces CEA incidence between 30 and 60 min. **(a)** FP trace with experimental protocol. **(b)** Quantification of WAG effects (1W RM ANOVA, Bonferroni posthoc, Incidence: $p < 0.001$ for ctrl and 30 min vs. 60 min, $n = 9$, Amplitude: $p < 0.001/ = 0.047$ for ctrl and 30 min vs. 60 min, $n = 9$ (ctrl, 30 min) and 8 (60 min) **(c)** Representative FP traces. Boxplots: filled black square = mean, horizontal line = median, whiskers = $1.5 * \text{IQR}$. *: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$. Ctrl, control.

Figure 3. Diazepam does not attenuate *in vivo* status epilepticus (SE) induced by kainic acid (KA).

DIA injections (20 min interval) started 50 min after KA injection allowing seizure activity to progress to SE. **A.** Experimental protocol and representative recording in a mouse receiving DIA 50 minutes after KA injection. Experimental timeline and corresponding analysis windows (top). Representative EEG and EMG traces over the full course of the experiment (middle) and zoomed in EEG traces representative for respective analysis windows (bottom). **B.** Quantification of DIA effects on percent time seizing. DIA treatment did not significantly attenuate percent time seizing at any time point. **C.** Quantification of DIA effects on FP power (0.5 – 100 Hz). Despite three high-dose DIA injections power levels did not significantly attenuate below KA baseline values but were significantly elevated compared to KA base in the last analysis window (F RM ANOVA oR, Tukey posthoc, $p = 0.016$ for KA base vs. Inj 3 + 20, $n = 10$). Boxplots: open square = mean, horizontal line = median, whiskers = $1.5 \times \text{IQR}$. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Inj, injection.

Figure 4. WAG (1 mg/kg) attenuates *in vivo* status epilepticus induced by kainic acid (KA). WAG injections began 50 min after KA injection allowing for seizure activity to progress to SE before treatment began. All animals treated with WAG survived the experiment. *: p < 0.05, **: p < 0.01, ***: p < 0.001. **A.** Experimental protocol and representative recording in a mouse receiving WAG after repetitive seizures were induced with KA. Experimental timeline and corresponding analysis windows (top). Representative EEG and EMG traces over the full course of the experiment (middle) and zoomed in EEG traces representative for respective analysis windows (bottom). **B.** Quantification of WAG effects on percent time seizing reveals that large majority of mice become seizure free. WAG treatment significantly reduced percent time seizing after the second and third injection compared to seizure baseline and Inj 1 (F RM ANOVA oR, Tukey posthoc, p = 0.017 for KA base vs. Inj 3 + 20, p = 0.031/< 0.001 for Inj 1 vs. Inj 3/Inj 3 + 20, p = 0.85 for KA base vs. Inj 1, n = 11). **C.** Quantification of WAG effects on FP power (0.5 – 100 Hz). After the second WAG injection power levels consistently stayed below KA baseline values indicating strong anticonvulsant effects after Inj 2. Power levels at Inj 3+ 20 were significantly lower compared to KA baseline, Inj 1 and Inj 2 while a significant attenuation was also observed between Inj 1 and Inj 3, the apparent power facilitation between KA base and Inj 1 was not significant (F RM ANOVA oR, Tukey posthoc, p = 0.002 for KA base vs. Inj 3 + 20, p = < 0.001 for Inj 1 vs. Inj 3 + 20, p = 0.01 for Inj 2 vs. Inj 3 + 20, p = 0.002 for Inj 1 vs. Inj 3, p = 0.619 for KA base vs. Inj 1, n = 11). Boxplots: open square = mean, horizontal line = median, whiskers = 1.5 * IQR. 0.05, **: p < 0.01, ***: p < 0.001. Inj, injection.

Figure 5. Low-dose WAG (0.3 mg/kg) attenuates *in vivo* status epilepticus induced by kainic acid (KA).

WAG injections (20 min interval) started 50 min after KA injection allowing for seizure activity to progress to SE before experimental treatment. All animals treated with WAG survived the experiment. **A.** Experimental protocol and representative recording in a mouse receiving WAG after repetitive seizures were induced with KA. Experimental timeline and corresponding analysis windows (top). Representative EEG and EMG traces over the full course of the experiment (middle) and zoomed in EEG traces representative for respective analysis windows (bottom). **B.** Quantification of WAG effects on percent time seizing. WAG treatment significantly reduced percent time seizing after the third dose of low-dose WAG treatment with a subset of mice being seizure-free (1W RM ANOVA, Bonferroni posthoc, $p = 0.006/0.003$ for KA base vs. Inj 3/Inj 3 + 20, $p = 0.005/0.002$ for Inj 1 vs. Inj 3/Inj 3 + 20, $n = 12$). **C.** Quantification of WAG effects on FP power (0.5 – 100 Hz). After the second WAG injection power levels consistently stayed below KA baseline values indicating strong anticonvulsant effects of WAG. In addition, power levels after Inj 3 were significantly different lower compared to KA baseline and Inj 1 (F RM ANOVA oR, Tukey posthoc, $p = 0.037/0.007$ for KA base vs. Inj 3/Inj 3 + 20, $p = 0.003/< 0.001$ for Inj 1 vs. Inj 3/Inj 3 + 20, $p = 0.928$ for KA base vs. Inj 1, $n = 11$). Boxplots: open square = mean, horizontal line = median, whiskers = $1.5 \times \text{IQR}$. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$. Inj, injection.

Figure 6. WAG treatment is more efficient than DIA in suppressing KA-induced SE *in vivo*.

Comparison of percent time seizing and EEG power (0.5 – 100 Hz) across treatment groups.

A. Comparison of percent time seizing across treatment groups at different time points during the experiment. **(a)** KA-induced baseline seizure activity is similar across different treatment groups. **(b)** Individual mice respond to WAG treatment after the second injection. **(c)** After the third injection, percent time seizing is significantly lower in WAG treated animals compared to DIA treated (1W ANOVA oR, Dunn's posthoc, $p = 0.009/0.001$ for DIA vs. 0.3/1 mg/kg WAG, $n = 10-12$). **(d)** Recordings during delayed analysis window after third injection shows increasing anticonvulsant efficacy in 1 mg/kg WAG cohort suggesting dose-dependency (black significance bars: 1W ANOVA oR, Dunn's posthoc, $p = 0.017/< 0.001$ for DIA vs. 0.3/1 mg/kg WAG, $n = 10-12$; grey significance bar: ANOVA with Welch's correction, sensitivity analysis suggests possible group difference for 0.3 vs 1 mg/kg WAG, posthoc Games Howell, $p = 0.0325$ for 0.3 vs. 1 mg/kg WAG, $n = 10-12$). **B.** Quantification of absolute EEG power (0.5 – 100 Hz) across different treatment groups and analysis windows (top) and averaged power spectra (bottom). **(a)** Pre-seizure control and **(b)** kainate baseline EEG power is of similar magnitude in all treatment groups. **C.** Comparison of power levels normalized to KA baseline across different treatment groups and analysis windows. **(a)** After Injection 2, power levels of most WAG treated animals are below KA baseline, but not significantly different to DIA treated animals. **(b)** Group average power levels are significantly different from DIA treatment for both 1 and 0.3 mg/kg WAG cohorts 20 minutes after the third dose (1W ANOVA oR, posthoc Dunn's, $p = 0.016/< 0.001$ for DIA vs. 0.3/1 mg/kg WAG, $n = 10-11$). Boxplots: open square = mean, horizontal line = median, whiskers = $1.5 \times \text{IQR}$. *: $p < 0.05$, **: $p < 0.01$, ***: $p < 0.001$.