Check for updates

Received: 27 March 2024 | Revised: 17 June 2024 | Accepted: 2 July 2024

DOI: 10.1111/bph.17319

RESEARCH ARTICLE

Selective modulation of epileptic tissue by an adenosine A_3 receptor-activating drug

Anwesha Ghosh[1,2] | Leonor Ribeiro-Rodrigues[1,2] | Gabriele Ruffolo[3,4] | Veronica Alfano[4] | Cátia Domingos[1,2] | Nádia Rei[1,2] | Dilip K. Tosh[5] | Diogo M. Rombo[1,2] | Tatiana P. Morais[6,7] | Cláudia A. Valente[1,2] | Sara Xapelli[1,2] | Beatriz Bordadágua[8,9,10] | Alexandre Rainha-Campos[11,12] | Carla Bentes[11,13,14] | Eleonora Aronica[15,16] | Maria José Diógenes[1,2] | Sandra H. Vaz[1,2] | Joaquim A. Ribeiro[1,2] | Eleonora Palma[3] | Kenneth A. Jacobson[5] | Ana M. Sebastião[1,2]

Correspondence
Ana M. Sebastião, Instituto de Medicina Molecular João Lobo Antunes, Universidade de Lisboa, Lisbon, Portugal.
Email: anaseb@medicina.ulisboa.pt

Present addresses
Cátia Domingos, Institute of Cellular Neurosciences, Medical Faculty, University of Bonn, Bonn, Germany; and Nádia Rei, Egas Moniz Center for Interdisciplinary Research (CiiEM), Egas Moniz School of Health & Science, Almada, Portugal.

Funding information
H2020-WIDESPREAD-05-2017-Twinning (EpiEpiNet), Grant/Award Number: 952455; NIDDK Intramural Research Program, Grant/Award Number: ZIADK031117; Fundação para a Ciência e a Tecnologia, Grant/Award Numbers: PTDC/MED-FAR/30933/2017, PTDC/MED-FAR/4834/2021, PD/BD/150344/2019; International Society for Neurochemistry; Instituto de Medicina Molecular João Lobo Antunes, Grant/Award Number: iMM-BI-4-2022; Sapienza Università di Roma, Grant/Award Numbers: RM11916B84D24429, RG12117A8697DCF1; Italian Ministry of University and Research,

Abstract

Background and Purpose: Adenosine, through the A_1 receptor (A_1R), is an endogenous anticonvulsant. The development of adenosine receptor agonists as antiseizure medications has been hampered by their cardiac side effects. A moderately A_1R-selective agonist, MRS5474, has been reported to suppress seizures without considerable cardiac action. Hypothesizing that this drug could act through other than A_1R and/or through a disease-specific mechanism, we assessed the effect of MRS5474 on the hippocampus.

Experimental Approach: Excitatory synaptic currents, field potentials, spontaneous activity, [^{3}H]GABA uptake and GABAergic currents were recorded from rodent or human hippocampal tissue. Alterations in adenosine A_3 receptor (A_3R) density in human tissue were assessed by Western blot.

Key Results: MRS5474 (50–500 nM) was devoid of effect upon rodent excitatory synaptic signals in hippocampal slices, except when hyperexcitability was previously induced in vivo or ex vivo. MRS5474 inhibited GABA transporter type 1 (GAT-1)-mediated γ-aminobutyric acid (GABA) uptake, an action not blocked by an A_1R antagonist but blocked by an A_3R antagonist and mimicked by an A_3R agonist. A_3R was overexpressed in human hippocampal tissue samples from patients with epilepsy that had focal resection from surgery. MRS5474 induced a concentration-dependent potentiation of GABA-evoked currents in oocytes micro-transplanted with human

Abbreviations: aCSF, artificial cerebrospinal fluid; CPA, N^6-cyclopentyladenosine; EE, established epilepsy; EPSC, Excitatory postsynaptic current; fEPSP, field excitatory postsynaptic potential; KA, Kainate; NBA, Neurobasal A; RIPA, radioimmunoprecipitation assay; SE, status epilepticus; TLE, temporal lobe epilepsy; VGB, Vigabatrin.

Anwesha Ghosh and Leonor Ribeiro-Rodrigues are co-first authors.

For affiliations refer to page 17

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial-NoDerivs License, which permits use and distribution in any medium, provided the original work is properly cited, the use is non-commercial and no modifications or adaptations are made.
© 2024 The Author(s). *British Journal of Pharmacology* published by John Wiley & Sons Ltd on behalf of British Pharmacological Society.

Grant/Award Numbers: PRIN 2022, PRIN-PNRR 2022; Italian Ministry of Health

hippocampal membranes prepared from epileptic hippocampal tissue but not from non-epileptic tissue, an action blocked by an A_3R antagonist.

Conclusion and Implications: We identified a drug that activates A_3R and has selective actions on epileptic hippocampal tissue. This underscores A_3R as a promising target for the development of antiseizure medications.

KEYWORDS

adenosine A_1 receptor, adenosine A_3 receptor, epilepsy, GABAergic transmission, hippocampus, neuronal excitability

1 | INTRODUCTION

Epilepsy is a common neurological disorder that affects over 1% of the global population and has substantial societal consequences, with about 30% of the patients being refractory or becoming refractory to commonly available antiseizure medications (Kalilani et al., 2018; Kwan et al., 2011; Löscher & Klein, 2021). Furthermore, most of the available drugs have considerable cognitive side effects because they act directly through key molecules in brain function. An ideal antiseizure medication would be one affecting only diseased tissue, to modulate, rather than directly block, the activity of molecules relevant to the control of brain activity. Such a drug would be expected to display fewer cognitive side effects.

Adenosine is a homeostatic/allostatic modulator of synaptic activity (Diógenes et al., 2014; Jacobson et al., 2022; Xiao et al., 2019) due to the balanced activity of enzymes and transporters that control its intracellular and extracellular concentration (Boison & Jarvis, 2021). Once in the extracellular space, adenosine activates membrane-embedded G protein-coupled receptors, with four subtypes being known: **A_1 receptor**, **A_{2A} receptor**, **A_{2B} receptor** and **A_3 receptor** (Gao et al., 2023; Sebastião & Ribeiro, 2009). Adenosine has long been considered an endogenous anticonvulsant (Dunwiddie, 1980). Dysregulation of the homeostatic/allostatic control of synaptic activity by adenosine may lead to epilepsy (Sandau et al., 2016). The antiseizure action of adenosine is mediated by inhibitory A_1 receptors present in brain tissues, namely, the cerebral cortex and hippocampus (Dunwiddie & Masino, 2001; Sebastião & Ribeiro, 2009). However, the widespread distribution of A_1 receptors throughout the body has hampered the development of A_1 receptor modulators as antiseizure medications. Indeed, the A_1 receptor is also widely expressed in the nerve endings of the autonomic nervous system and in effector organs, including the heart. Therefore, a major concern is the bradycardic and negative inotropic actions of A_1 receptor agonists (Baltos et al., 2023).

Numerous adenosine derivatives have been synthesized over the last two decades to identify molecules that could serve as prototypes of novel therapeutics. This was the case of MRS5474, a truncated *N*-methanocarba nucleoside that has selectivity for the A_1 receptor (K_i: 48 nM for human and 3.2 nM for mouse) over the A_3 receptor (K_i: 470 nM for human and 1056 nM for mouse) and has low affinity for the A_{2A} receptor (K_i: 4 μM for human and >10 μM for mouse), as assessed by standard radioligand binding approaches in Chinese hamster ovary (CHO) or human embryonic kidney (HEK) cells expressing the human or mouse adenosine receptor subtypes (Carlin et al., 2017; Tosh, Paoletta, et al., 2012). Functional data showed that MRS5474 can act as a full agonist of the A_1 receptor and a partial agonist of the A_3 receptor, having negligible activity on the A_{2B} receptor (Tosh, Paoletta, et al., 2012).

A striking characteristic of MRS5474, reported by Tosh, Paoletta, et al. (2012), was its antiseizure activity in the 6-Hz minimal clonic seizure mouse model of epilepsy. However, despite acting as a full A_1 receptor agonist, as assessed by inhibition of adenylate cyclase activity in CHO cells transfected with the human (h)A_1 receptor, it did not cause alterations in the rotarod test in mice, indicating the absence of cardiac inhibitory actions. This prompted us to hypothesize that MRS5474 could act through another receptor than the A_1 receptor

What is already known

- Endogenous adenosine affects synaptic signalling having both allostatic and antiseizure actions.
- A moderately selective agonist of inhibitory adenosine A_1receptors, MRS5474, has antiseizure action in mice.

What does this study add

- MRS5474 has selective actions in epileptic tissue (rodent and human), sparing non-epileptic tissue.
- The adenosine A_3 receptor is a target for MRS5474 actions in the hippocampus.

What is the clinical significance

- The A_3 receptor, a non-ubiquitous adenosine receptor, is a putative target for novel antiseizure medications.
- Adenosine A_3 receptor-based antiseizure medications likely induce only minimal on-target side effects.

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

and/or through a brain/disease-specific mechanism. To explore these possibilities, we tested the effects of MRS5474 in the hippocampus in a variety of models, including animal and human hippocampal tissue, addressing both excitatory and inhibitory transmission endpoints. Taken together, the data show that MRS5474, under the experimental conditions, rather than activating an A_1 receptor, activates an A_3 receptor that is likely overexpressed in epileptic tissue. Notably, the actions of MRS5474 in excitatory synaptic transmission as well as in GABAergic currents could only be detected in epileptic tissue but not in control tissue, underscoring the A_3 receptor as a promising target for the development of antiseizure medications.

2 | METHODS

2.1 | Animals

Animal studies are reported in compliance with the ARRIVE guidelines (Percie du Sert et al., 2020) and with the recommendations made by the *British Journal of Pharmacology* (Lilley et al., 2020). All procedures complied with the European rules and guidelines (2012/707/EU). The work in Lisbon also complied with the Portuguese legislative action (DL 113/2013) for the protection of animals used for scientific purposes, being authorized by the Animal Welfare Body of the Instituto de Medicina Molecular João Lobo Antunes (ORBEA-iMM) and Portuguese Authority for Animal Welfare (Direção-Geral de Alimentação e Veterinária [DGAV]). The animals (Wistar rats, Sprague–Dawley rats and C57BL/6J mice, as specified below) were randomly housed in a virus antibody-free rodent facility, receiving food (autoclaved diet pellets)and water (sterile water treated by reverse osmosis) ad libitum. Temperature (20–24°C),humidity (55 ± 10%) and light/dark cycle (14 h light:10 h dark; lights on between 7:00 AM and 9:00 PM) of the facility were controlled in a strictly controlled rodent facility at Instituto de Medicina Molecular João Lobo Antunes (iMM), as authorized by the Portuguese Authority for Animal Welfare (Direção-Geral de Alimentação e Veterinária). The work using female *Xenopus laevis* frogs to obtain oocytes (Sapienza University of Rome, Rome, Italy) conformed to institutional policies and guidelines of the Italian Ministry of Health (Authorization No. 427/2020-PR).

2.2 | Human tissue samples

Human tissue samples were used at the Lisbon and Rome institutions. Samples were provided by the Department of (Neuro)Pathology of the Amsterdam UMC and by the iMM Biobank. The use of human samples was approved by the ethics committees from Centro Hospitalar Universitário Lisboa Norte and Centro Académico de Medicina de Lisboa (No. 207/21) and met the Portuguese Law on Clinical Research (Law No. 21/2014, 16 April), amended in law (Law No. 73/2015, 27 July), as well as approved by the ethics and biobank committees of the Amsterdam UMC and Sapienza University of Rome.

The iMM Biobank provided frozen samples of the hippocampus from 11 patients submitted to temporal lobe resections due to refractory epilepsy after an extensive presurgical evaluation at 'Centro de Referência para a área da epilepsia refratária' (Centro Universitário Lisboa Norte), member of the ERN EpiCARE for complex and rare epilepsies, and under informed consent. These samples were used for the Western blot analysis shown in Figure 6; additional information on these samples can be found in Table 2.

Human tissue samples for recordings of GABAergic currents were selected from the Department of (Neuro)Pathology of the Amsterdam UMC, University of Amsterdam and the VU University Medical Center (VUMC) and sent frozen to Sapienza University of Rome. The Amsterdam UMC research code was provided by the Medical Ethics Committee. GABAergic current recordings were performed using a total of three surgical epilepsy specimens (hippocampus) from patients that underwent surgery for refractory temporal lobe epilepsy (TLE). In the text, the number of patients used in each experiment is indicated by the symbol # (additional information can be found in Table 3). A presurgical evaluation conducted with non-invasive tests was carried out in all patients, and patients were excluded from the study in cases where implantation of strip and/or grid electrodes for chronic subdural invasive monitoring before resection was necessary. All surgeries were performed at VUMC. The predominant seizure types were medically intractable focal impaired awareness seizures, and all patients were resistant to maximal doses of different anti-seizure drugs. No patients included in our series had seizures in the 24 h before surgery. All cases were reviewed independently by two neuropathologists, and the diagnosis was confirmed according to the international consensus classification (Blümcke et al., 2013).

As control tissues for both GABAergic current recordings (data in Figure 7, details in Table 3, #4) and for Western blot analysis (data in Figure 6, details in Table 2), we used brain samples from autopsies of individuals with no neurological disorders and no history of neurological disease or signs of neuroinflammation, which were also selected and provided by the Department of (Neuro)Pathology of the Amsterdam UMC.

In all cases, tissue was obtained and used in accordance with the Declaration of Helsinki as well as the Amsterdam UMC research code and protocol approved by the local (Lisbon and Rome) Ethics Committee. The samples were maintained at −80°C until further processing.

2.3 | Kainate (KA)-induced temporal lobe epilepsy (TLE) animal model

Sprague–Dawley male rats (7 weeks old) were randomly assigned for the TLE-induced model or for control. Status epilepticus (SE) was induced by intraperitoneal (i.p.) injection of 10-mg·kg^{-1} kainate (KA) (Tocris, Bristol, UK) (Sakurai et al., 2018) solubilized in saline (0.9% NaCl). The other group of animals was injected with saline at the same time and registered as the control group. Animals from the same cage were separated the day before SE induction, being housed as two animals per cage. Animals were observed directly and monitored with

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

two cameras: the GoPro 4K and the ELP night vision infrared fisheye lens wide-angle waterproof dome, along with a USB webcam (Model ELP-USBFHD05MT-DL170), positioned appropriately on either side of the cage. This allowed for further assessment of the seizure severity following KA injection. The modified Racine scale was adopted to characterize the seizures: stage 1, facial clonus; stage 2, nodding and wet dog shaking; Stage 3, unilateral forelimb clonus with lordotic posture; stage 4, lateral forelimb clonus with rearing; and stage 5, bilateral forelimb clonus with falling (Racine, 1972; Reddy & Kuruba, 2013). When a stage 5 seizure lasted for more than 5 min, animals were killed with a lethal dose (1–2 ml) of pentobarbital (Euthasol® 400 mg·ml^{-1}, Dechra, Northwick, UK) (humane endpoint). Repetitive seizures at stage 5 were allowed, providing that each one lasted no more than 5 min, and almost all animals had seizures from stage 3 to stage 5 for a period longer than 30 min. Following SE, the animals were reintroduced alongside their initial cage co-inhabitants and supervised closely for the appearance of distress symptoms. Four weeks after SE induction, animals were monitored for the appearance of spontaneous recurrent convulsive seizures, which could be considered stages 3–5 according to the Racine stage scale, fitting our observations that about 50% of the animals subjected to the same procedure attained at least stage 4. These animals were then considered to have established epilepsy (EE). The animals were then killed with isoflurane (Isoflurin® 1000 mg·g^{-1}, Hifarmax, São Domingos de Rana, Portugal), and their hippocampal slices were used in field excitatory postsynaptic potential (fEPSP) recordings. The control animals were similarly housed and grouped.

2.4 | Preparation of acute hippocampal slices

For fEPSP recordings and γ-aminobutyric acid (GABA) uptake studies, the hippocampus (6- to 12-week-old Sprague–Dawley rats or C57BL/6 mice as specified in Section 3) was dissected in ice-cold (0–4°C) artificial cerebrospinal fluid (aCSF) solution composed of the following (in mM): 124 NaCl; 3 KCl; 25 $NaHCO_3$; 1.2 $NaH_2PO_4{\cdot}H_2O$; 1 $MgSO_4$; 2 $CaCl_2$; and 10 glucose monohydrate, previously gassed with 95% O_2 and 5% CO_2, pH 7.4. Slices (400 µm thick for fEPSP recordings or 300 µm thick for GABA uptake studies) were cut perpendicularly to the long axis of the hippocampus with a McIlwain tissue chopper (Campden Instruments, Loughborough, England) and allowed to recover functionally and energetically for at least 1 h in a resting chamber filled with the same solution at room temperature (RT; ≈22°C) and continuously gassed.

For patch-clamp recordings, the slices (300 µm thick from 5- to 8-week-old Wistar rats) were cut with a vibratome (Leica VT1000 S, Leica Microsystems, Aarau, Switzerland) in the ice-cold dissecting solution containing the following (in mM): 110 sucrose; 2.5 KCl; 0.5 $CaCl_2$; 7 $MgCl_2$; 25 $NaHCO_3$; 1.25 NaH_2PO_4; and 7 glucose monohydrate, oxygenated with 95% O_2 and 5% CO_2, pH 7.4. After dissection, the slices were incubated for 20 min at 35°C in aCSF (to hasten metabolic recovery) and then transferred to a resting chamber at RT for at least 1 h before use.

The whole hippocampus was chopped, but for electrophysiological recordings we routinely used slices from the middle hippocampus because these were cut at a more appropriate angle, thus having a higher probability of better signals.

2.5 | Excitatory postsynaptic current (EPSC) recordings

Acute hippocampal slices were individually fixed with a grid in a recording chamber and continuously superfused at RT by a gravitational superfusion system at 2–3 ml·min^{-1} with aCSF at RT. The aCSF was supplemented with picrotoxin (50 µM) to block the fast component of GABAergic transmission. Electrode positioning and cell selection were performed under visual guidance using a Carl Zeiss Axioskop 2 FS upright microscope (Carl Zeiss, Jena, Germany) equipped with a differential interference contrast-infrared (DIC-IR) CCD video camera (VX44, Till Photonics, Gräfelfing, Germany). EPSCs were recorded from the CA1 area upon stimulation (S48 Square Pulse Stimulator, Grass Instruments, Quincy, MA, USA) of the Schaffer collateral afferents with 0.1 ms rectangular pulses (0.1–0.5 mA) delivered once every 15 s through a concentric bipolar electrode placed in the *stratum radiatum*. Patch pipettes were filled with an intracellular solution (290–300 mOsm; pH 7.2, adjusted with 1-M KOH) containing the following (in mM): 145 K-gluconate, 20 HEPES, 10 KOH, 8 NaCl, 0.2 ethylene glycol-bis (β-amino ethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 2 MgATP and 0.3 NaGTP (4–9 MΩ). Recordings were in whole-cell voltage-clamp mode ($V_h = -70$ mV) with an Axopatch 200B amplifier (Axon Instruments, Foster City, CA, USA). The junction potential was not compensated for, and offset potentials were nulled before giga-seal formation. Small voltage steps (5 mV, 50 ms) were delivered throughout the experiment to monitor the access resistance; the holding current was also constantly monitored, and when any of these parameters varied by more than 20%, the experiment was rejected. The current signal was low-pass filtered using a 3- and 10-kHz three-pole Bessel filter, digitized at 10 kHz using a Digidata 1322A board, recorded by the pCLAMP software and analysed with Clampfit 10. EPSC recordings were started not before the first 5–10 min after going to whole-cell mode to allow diffusion of the intracellular solution. After 10 min of stable EPSC recordings, the test drugs were added to the aCSF. Averages of four consecutive individual recordings (1 min) were used for analysis.

2.6 | fEPSP recordings

Acute hippocampal slices were individually transferred into a submerged recording chamber over the nylon mesh and continuously superfused with gassed aCSF solution at a constant flow (3 ml·min^{-1}) and temperature (32°C). Stimulation (rectangular 0.1 ms pulses, once every 15 s, S48 Square Pulse Stimulator, Grass Instruments) was delivered through a concentric bipolar electrode placed on Schaffer collateral–commissural fibres in the *stratum radiatum* near the CA3–

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

CA1 border. The stimulus intensity was set to elicit nearly 50% of the maximal response and was maintained throughout the experiment. fEPSPs were recorded through a microelectrode (2-6 MΩ resistance, filled with aCSF), placed in CA1 *stratum radiatum*, coupled to an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA, USA) and digitized BNC-2110 (National Instruments, Austin, TX, USA). Individual responses were monitored, and averages of eight consecutive responses were continuously stored on a personal computer with the WinLTP software (Anderson & Collingridge, 2007). Test drugs were added to the superfusing aCSF after obtaining a stable baseline of recordings for at least 15 min.

2.7 | Preparation of organotypic rhinal–hippocampal slice cultures

Organotypic rhinal–hippocampal slice cultures were prepared from Sprague–Dawley rats (6–7 days old) as described previously (Dyhrfjeld-Johnsen et al., 2010; Magalhães et al., 2018; Valente et al., 2021) with some modifications. This method allows organotypic slices to develop spontaneous epileptiform pyramidal cell discharges. After decapitation, the brains were removed and briefly placed in a cold (≈4°C) Gey's balanced salt solution (GBSS, Biological Industries, Kibbutz Beit Haemek, Israel) supplemented with 25 mM D-(+)-glucose (Sigma, St. Louis, MO, USA) under sterile conditions. The hippocampus, together with the entorhinal cortex and perirhinal cortex (Vismer et al., 2015), was meticulously separated and sliced (350 μm thick) transversely using a McIlwain tissue chopper. Four slices were then randomly transferred to porous insert membranes (0.4 μm) (PICM 03050, Millipore, Bedford, MA, USA) placed in each well of six-well culture trays (Corning Costar, Corning, NY, USA). Each well contained 1 ml of culture media made up of 50% Opti-MEM I Reduced Serum Medium, 25% Hank's balanced salt solution (HBSS), 25% heat-inactivated horse serum (HS) (Thermo Fisher, Waltham, MA, USA), 25-mM D-(+)-glucose (45% in water, Sigma) and 30-μg·ml^{-1} gentamicin solution (50 mg·ml^{-1}, Thermo Fisher). The slices were then kept at 37°C in 5% CO_2 and 95% O_2 for 2 weeks. The culture medium was changed every 2–3 days with Opti-MEM medium pre-heated at 37°C. The day before the experiments, the culture medium was changed to serum-free Neurobasal A (NBA) medium supplemented with 2% B27, 1-mM L-glutamine and 30-μg·ml^{-1} gentamicin (all from Thermo Fisher).

2.8 | Field potential recordings from organotypic slices

Organotypic slices were individually transferred to an interface-type chamber with a humidified (5% CO_2/95% O_2) atmosphere at 37°C. Each slice was visually inspected, ensuring slice integrity and organization, and likely corresponded to middle hippocampal slices. Slices were then perfused with aCSF with an increased concentration of KCl ([KCl] = 8.5 mM, depolarizing/hyperexcitable conditions) or with NBA medium (non-depolarizing conditions), both continuously recirculating at a rate of 2 ml·min^{-1}. Recordings were done through an extracellular microelectrode (2- to 4-MΩ resistance, filled with aCSF) placed in the CA3 pyramidal cell layer. The viability of slices was initially tested by recording evoked field population spikes using the S48 Square Pulse Stimulator (Grass Instruments). Stimulation (rectangular pulses, 0.1 ms, 100–400 μA, every 15 s) was delivered through a bipolar concentric electrode placed on mossy fibres. After stimulation, spontaneous field potentials were recorded for 90 min. Drugs (MRS5474 [250 nM] or N^6-cyclopentyladenosine (CPA) [30 nM]) were added to the perfusion medium after an equilibration period of 20 min of spontaneous activity recording. No drug was added to the control slices.

Recordings were obtained with an Axoclamp 900A amplifier (Axon Instruments, Foster City, CA, USA), digitized with Digidata 1440A (Axon Instruments, Foster City, CA, USA) and stored on a computer with the pCLAMP software Version 10.7 (Molecular Devices LLC, San Jose, CA, USA) to automatically detect spontaneous spikes during a recording. All recordings were band-pass filtered (eight-pole Bessel filter at 60 Hz and Gaussian filter at 600 Hz). The baseline used to detect these spikes was specific to each recording and was settled right above the end of the noise oscillations. We quantified ictal-like discharges, which were defined as continuous discharges lasting more than 10 s (bursts) or with a minimum frequency of 2 Hz (Berdichevsky et al., 2012). The end of a burst was defined when the inter-spike interval was longer than 2 s (Noe et al., 2013). Continuous spike activity with less than 10-s duration was not accounted for as burst activity and therefore was not quantified.

To quantify the epileptiform activity, an in-house program written in C++ language was developed to automatically evaluate the number of bursts per slice, the frequency, and the positive peak amplitude (amplitude between the baseline and the peak of the spike) of spikes within a burst and its duration, according to the ictal parameters. The data were given by the mean of a specific parameter (frequency or amplitude) within the bursts per slice. To accomplish this, we analysed each burst (ictal activity lasting more than 10 s) detected in each slice in terms of both the frequency of spikes within the burst and the spike amplitude within the burst. For each slice, we derived an averaged frequency and an averaged amplitude at the defined time points. The mean values of these parameters (frequency and amplitude) per slice were then used for statistical analysis. In Section 3, the n values represent the number of slices analysed.

To allow comparisons, the values obtained between 10 and 20 min (baseline) after starting the recordings were normalized to 1 within each slice; values obtained at 50–60 min and at 80–90 min after starting recording (thus at 30–40 min and at 60–70 min after starting drug perfusion) were normalized to the baseline condition. These time intervals were selected because we anticipate that equilibrium, and therefore the full effect of the drug, would be achieved within 30 min of drug perfusion. To confirm a nearly full effect, we extended recordings up to 60–70 min. We avoided longer periods to minimize time-dependent/non-drug-dependent alterations in the parameters. Control slices were analysed at equivalent time points, further allowing to rule out that changes were solely attributable to time-dependent alterations.

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

2.9 | GABAergic current recordings from *Xenopus* oocytes micro-transplanted with human hippocampal brain tissue

The procedure of membrane extraction from human hippocampal brain tissues and preparation of *Xenopus laevis* oocytes and injection procedures have been previously described in detail (Miledi et al., 2006). Briefly, tissues were homogenized with a Teflon glass homogenizer using 2 ml of glycine buffer (composition in mM: 200 glycine, 150 NaCl, 50 EGTA, 50 ethylenediaminetetraacetic acid (EDTA), 300 sucrose, plus 20 µl protease inhibitors [P2714; Sigma], pH 9 adjusted with NaOH). Subsequently, the homogenate underwent two centrifugation cycles, the first at 9500 *g* for 15 min in a Beckmann centrifuge (C1015 rotor; Palo Alto, CA, USA). Afterwards, the supernatant was collected and subjected to a second centrifugation at 100,000 *g* for 2 h in a TL-100 rotor at 4°C. The pellet was washed, resuspended in assay buffer (glycine 5 mM) and used directly, or aliquoted and stored at −80°C for later use. From 12 to 48 h after injection, membrane currents were recorded from voltage-clamped *Xenopus* oocytes using two microelectrodes filled with 3-M KCl. The oocytes, randomly chosen, were placed in a 0.1-ml recording chamber perfused continuously (9–10 ml·min^{-1}) with the oocyte's Ringer (OR) containing the following (in mM): 82.5 NaCl; 2.5 KCl; 2.5 $CaCl_2$; 1 $MgCl_2$; and 5 HEPES, adjusted to pH 7.4 with NaOH at RT. GABA (500 µM, diluted from a 100-mM stock solution before each experiment) was applied through a gravity-driven multi-valve perfusion system controlled by a digital interface (Biologique RSC 200; Claix, France). In all the experiments, the holding potential was −60 mV.

2.10 | [^{3}H]GABA uptake

Acute hippocampal slices were used for GABA uptake experiments as previously (Chazalon et al., 2018) with modifications. After the recovery period (at least 1 h at RT), slices were randomly transferred to a 24-well plate (Corning Costar, Corning, NY, USA). Three slices were placed in each well (for triplicate measurements per condition per experiment) containing 1.2 ml non-supplemented low-glucose Dulbecco's modified Eagle's medium (DMEM) (Gibco™ 11880028, Invitrogen, Waltham, MA, USA) and the appropriate drug concentrations; incubation was for 30 min under oxygenation (5% CO_2 and 95% O_2) at RT. Slices were then gently transferred to a prefilled and prewarmed (37°C under an oxygenated atmosphere) 48-well plate (300 µl and one slice per well) for incubation with 25 µM [^{3}H]GABA (Revvity/PerkinElmer, specific activity in stock: 25–40 µCi·nmol^{-1}; specific activity in the incubation solution: approx. 22 nCi·nmol^{-1} due to addition of cold GABA) plus the same drugs as before. Zero time for incubation with [^{3}H]GABA was taken as the time of slice transfer, with temperature and oxygenation conditions being maintained until the end of the uptake period. [^{3}H]GABA uptake was allowed for 12 min (to allow equilibrium) and stopped by a quick wash in ice-cold aCSF solution (approx. 10 ml in a Petri dish), followed by immediate transfer into a 200-µl ice-cold lysis buffer solution (in mM: 100 NaOH and 0.1% sodium dodecyl sulfate. Each slice was then homogenized for posterior quantification of tritium (liquid scintillation counting) and protein (detergent compatible protein assay kit, Bio-Rad, CA, USA) content in each sample. In each step, slices were transferred sequentially at defined time intervals to allow proper incubation times for each slice. To minimize time-related bias, a complete sequence of drug conditions was run first, then (and in the same order) the duplicates were run, and then the triplicates. For each test drug condition, GABA uptake was assessed in consecutive wells without or with the GABA transporter (GAT) type 1 (**GAT-1**) selective blocker, SKF89976A (20 µM). GAT-1-mediated uptake was calculated by subtracting the amount of tritium in the samples incubated in the presence of the GAT-1 blocker (non-GAT-1-mediated) from the total tritium detected in the samples incubated in the same conditions but in the absence of the GAT-1 blocker.

2.11 | Western blotting

Human hippocampal samples were lysed in 200 µl of radioimmunoprecipitation assay (RIPA) lysis buffer (4% nonyl phenoxypolyethoxylethanol [NP-40], 40-mM Tris–HCl, 1-mM EDTA, 150-mM NaCl and 0.1% sodium dodecyl sulfate [SDS] 10%), 10-mM NaF, 5-mM Na_3VO_4 and a protease inhibitor (cOmplete™ Mini EDTA-free Protease Inhibitor Cocktail: Roche, Basel, Switzerland). Following homogenization with a sonicator (Soniprep MSS 150.CX3.5: Sanyo, Osaka, Japan), total protein was quantified (DC Protein Assay Kit, Bio-Rad, Hercules, CA, USA), and absorbance was measured at 750 nm in the Infinite M200 (Tecan Trading AG, Hombrechtikon, Switzerland). Lysates were denatured for 10 min at 95°C in sample buffer (12% SDS, 0.06% bromophenol blue, 47% glycerol, 600-mM dithiothreitol and 60-mM Tris–HCl, pH 6.8). Each sample (40 µg total protein per well) and molecular weight marker (MWM, NZYColour Protein Marker II: NZYtech, Lisbon, Portugal) were separated on 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) within a running buffer (25mM Tris base, 192-mM glycine and 10-ml SDS 10%, dissolved in Mili-Q® water, pH 8.3) at 80 V, until the marker started to separate, and then at 120 V for approximately 90 min using the Mini-Protean® Tetra System (Bio-Rad, Hercules, CA, USA). SDS-PAGE-separated proteins were transferred to a 0.2 µm polyvinylidene fluoride (PVDF) membrane (#1620177, Bio-Rad, CA, USA) inside a transfer buffer (25-mM Tris base, 192-mM glycine and 10% methanol, dissolved in Mili-Q® water, pH 8.3) using the same system at 250 mA for 1 h and 45 min. Membranes were blocked for 1 h at RT in 3% bovine serum albumin (BSA, # MB04602, NZYtech) in TBS-T (20-mM Tris base, 137-mM NaCl and 0.1% Tween 20, pH 7.6). Subsequently, membranes were incubated with the primary antibodies—adenosine A_3R rabbit polyclonal antibody (AA 85-180, 1:1000, antibodies-online GmbH, Aachen, Germany, RRID:AB_11206109) overnight at 4°C and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mouse monoclonal antibody (AM4300, 1:5000, Invitrogen, MA, USA, RRID:AB_2536381) for 40 min at RT—followed by 1 h at RT with horseradish peroxidase (HRP)-conjugated secondary antibody (goat anti-rabbit IgG-HRP, 1706515, 1:5000, Bio-Rad, RRID:AB_11125142; goat anti-

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

mouse IgG-HRP, 1706516, 1:5000, Bio-Rad, RRID:AB_2921252). All antibodies were diluted in TBS-T with 3% BSA. Primary antibody A_3R solution was used only once. Primary antibody GAPDH solution was used multiple times and stored at −20°C. Dilutions of secondary antibodies were maintained at 4°C and re-used up to three times. In between steps, membranes were rinsed in TBS-T (three times for 5 min). Immunoreactions were detected with Western Lightning ECL Pro (PerkinElmer, MA, USA), which generates a sensitive light-emitting product through HRP action, and chemiluminescence was detected using an Amersham ImageQuant 800 system (Cytiva, Marlborough, USA). Fiji software (National Institutes of Health [NIH], MD, USA) was used to quantify band intensities while maintaining the same area between bands within the same membrane. Multiple bands were observed but the 35 kDa band was the only one described here since it is the only one mentioned in the antibody supplier website. The intensity of each protein band was normalized to that of GAPDH and then to the mean of controls on the same membrane. The Immuno-related procedures used comply with the recommendations made by the *British Journal of Pharmacology* (Alexander et al., 2018).

2.12 | Materials

MRS1523 (3-propyl-6-ethyl-5-[(ethylthio)carbonyl]-2 phenyl-4-propyl-3-pyridine carboxylate, **A_3R** antagonist), **SKF89976A** hydrochloride (1-(4,4-diphenyl-3-butenyl)-3-piperidinecarboxylic acid hydrochloride, **GAT-1** blocker) and non-radioactive (cold) GABA were purchased from Sigma. KA, 8-cyclopentyl-1,3-dipropylxanthine (**DPCPX**, **A_1R** selective antagonist), **CPA** (selective A_1 receptor agonist), 2-chloro-N^6-cyclopentyladenosine (**CCPA**, selective A_1 receptor agonist), **kainate** and **picrotoxin** (**$GABA_A$** receptor antagonist) were from Tocris (Bristol, UK). **Pentobarbital** (Euthasol® 400 mg·ml^{-1}) was from Dechra (Northwick, UK). MRS5474 ((1*R*,2*R*,3*S*,4*R*,5*S*)-4-(2-chloro-6-((dicyclopropylmethyl)amino)-9*H*-purin-9-yl)bicyclo[3.1.0]hexane-2,3-diol) and **MRS5698** ((1*S*,2*R*,3*S*,4*R*,5*S*)-4-[6-[[(3-chlorophenyl)methyl]amino]-2-[2-(3,4-difluorophenyl)ethynyl]-9*H*-purin-9-yl]-2,3-dihydroxy-*N*-methylbicyclo[3.1.0]hexane-1-carboxamide, A_3R agonist) were synthesized at the NIH (Jacobson Group) as reported (Tosh et al., 2015; Tosh, Deflorian, et al., 2012; Tosh, Paoletta, et al., 2012). CPA, CCPA, DPCPX, picrotoxin, MRS5474, MRS1523 and MRS5698 were prepared as a 5-mM stock solution in dimethyl sulfoxide (DMSO), and SKF89976A was prepared as a 50-mM stock solution in DMSO. The DMSO concentration in the working solutions did not surpass 0.01%. **GABA** was prepared as a 50 mM (GABA uptake) or 100 mM (GABA currents) stock solution in Mili-Q® water water. The stock solutions were aliquoted and kept at −20°C until use. Fresh dilutions of these stock solutions to the final concentration were prepared for each experiment.

2.13 | Statistical analysis

All data are presented as mean ± standard error of the mean (SEM) of n experiments, where n corresponds to the number of independent observations, that is, values taken from different animals (for acute slice recordings), from different slices (organotypic slices) or from different oocytes (GABAergic current recordings), except when otherwise stated in Section 3 or in the figure legends. The sample size for each dataset presented in Section 3 was based on the authors' experience of each technique. Blinding was unfeasible for most experimental procedures. Data of each experiment or set of experiments were analysed by at least two authors. Statistical analysis was carried out where the number of independent experiments was 5 or more. The statistical significance of the differences was assessed using the Student's *t* test, one-way analysis of variance (ANOVA), two-way ANOVA or Friedman test, as specified in the legends of figures and tables. Post hoc tests were run only if F achieved $P < 0.05$ and there was no significant variance inhomogeneity. Statistical analyses were performed using GraphPad Prism 8.0 (Dotmatics, Boston, MA, USA) or SigmaPlot 15 (Inpixon HQ, Palo Alto, CA, USA) software. Differences were considered significant when $P < 0.05$ (two-tailed). The data and statistical analysis comply with the recommendations of the *British Journal of Pharmacology* on experimental design and analysis in pharmacology (Curtis et al., 2022).

2.14 | Nomenclature of targets and Ligands

Key protein targets and ligands in this article are hyperlinked to corresponding entries in the IUPHAR/BPS Guide to PHARMACOLOGY http://www.guidetopharmacology.org and are permanently archived in the Concise Guide to PHARMACOLOGY 2023/23 (Alexander, Christopoulos et al., 2023; Alexander, Fabbro et al., 2023; Alexander, Mathie et al., 2023).

3 | RESULTS

3.1 | MRS5474, in contrast to a canonical A_1 receptor agonist, did not affect hippocampal excitatory inputs to excitatory neurons in control conditions

We first assessed the mechanism of action of MRS5474 by evaluating its ability to affect excitatory synaptic transmission at the CA1 area of the rat hippocampus, a brain area mostly affected by seizures and where the inhibitory action of adenosine A_1 receptor agonists is well known (Sebastião et al., 1990). To our surprise, MRS5474, at a concentration (120 nM) nearly 40 times higher than its affinity for A_1 receptors (Carlin et al., 2017), was virtually devoid of effect upon EPSCs recorded by patch clamp from CA1 pyramidal cells in the whole-cell configuration (Figure 1). At the end of the perfusion with MRS5474 (120 nM), the EPSC peak amplitude was 106 ± 8.0% of the pre-drug value ($n = 6$, $P > 0.05$, Figure 1a). In two out of the six cells, there was even a tendency to an increase (Figure 1a, right panel) rather than the expected decrease. Holding current and membrane resistance were also not affected by MRS5474 (120 nM) (holding current: 2.6 ± 4.8 pA, $n = 6$, $P > 0.05$; membrane resistance: 100 ± 3.5%

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

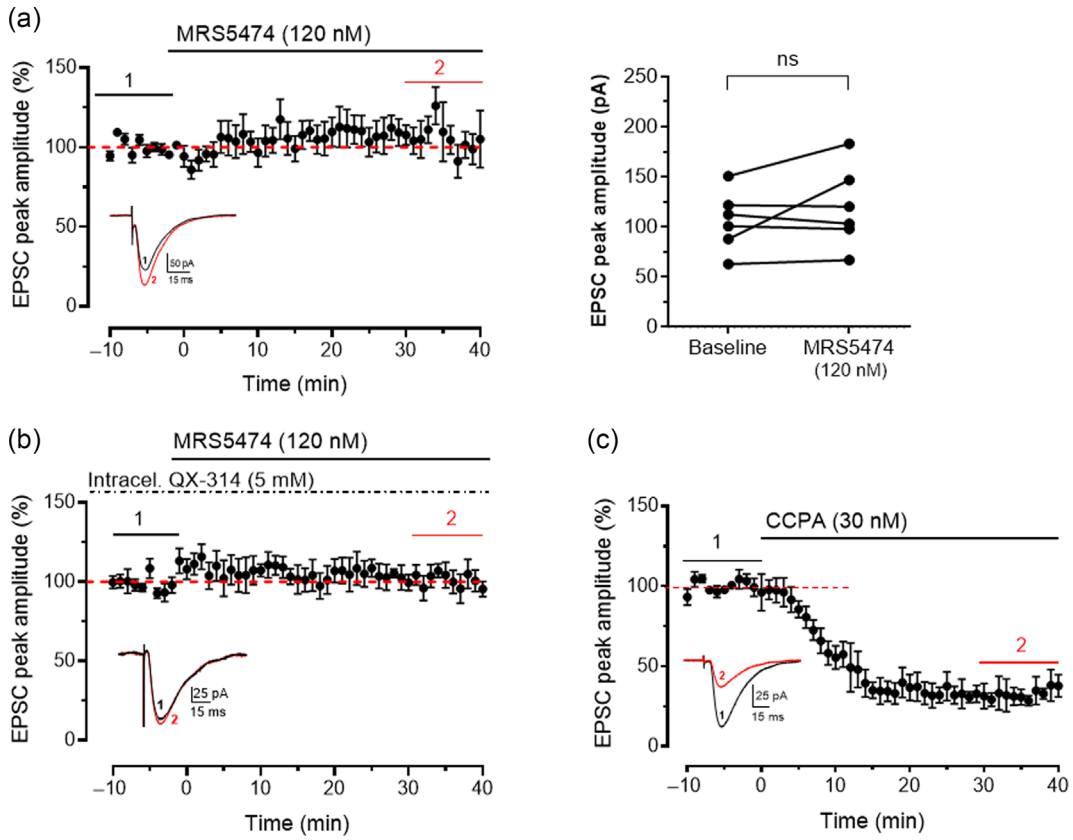

FIGURE 1 MRS5474 in a virus antibody-free rodent facility, receiving food (autoclaved diet pellets)and water (sterile water treated by reverse osmosis) RS5474 did not affect excitatory postsynaptic currents (EPSCs) in CA1 pyramidal cells in rat hippocampal slices, in clear contrast with the A_1 receptor agonist, CCPA. Time-course of changes of EPSC peak amplitude are shown in (a) n = 6 cells from five rats, (b) n = 5 cells from four rats (QX-314 in the intracellular solution to inhibit sodium channels and prevent action potential spiking independently of input strength) and (c) n = 5 cells from four rats. EPSC peak amplitude was normalized in each experiment, taking as 100% baseline the values recorded for 10 min before drug application (1); drug effects were assessed by comparing baseline values with values recorded at 30–40 min after drug application (2). Values are as mean ± SEM. The horizontal lines below drug names indicate drug presence in the perfusion solution. Insets in each panel: representative averaged superimposed EPSC traces recorded in the same cell before (1) and by the end (2) of drug application. In (a) at right is shown the paired EPSC amplitude (pA) of all cells included in the data shown in (a) at left before MRS5474 (baseline) and in the last 10 min of MRS5474 perfusion; ns: $P > 0.05$ (two-tailed paired *t* test). In all experiments, the fast component of inhibitory GABAergic transmission was blocked by adding picrotoxin (50 μM) to the aCSF. Note that the selective A_1 receptor agonist, CCPA (c), caused a marked inhibition of EPSCs in clear contrast to the absence of effect of MRS5474 (a, b).

of pre-drug values, n = 6, $P > 0.05$). The absence of effect of MRS5474 was still evident in experiments where the membrane-impermeant sodium channel blocker, QX-314 (5 mM, a supramaximal concentration), was added to the intracellular solution of the patch electrode (peak amplitude at end of application: 105 ± 6.1% of pre-drug values, n = 5, $P > 0.05$, Figure 1b). In contrast to the absence of effect of MRS5474, the canonical A_1 receptor agonist, CCPA, at a concentration (30 nM) also nearly 40 times its A_1 receptor affinity value (Klotz, 2000), clearly inhibited the EPSC peak amplitude within 3–5 min after its application, with the maximal effect attained after about 20 min (70 ± 5.2% inhibition, $P < 0.05$, n = 5, Figure 1c).

Next, we evaluated if MRS5474 could inhibit excitatory synaptic transmission under less restrictive recording conditions—fEPSP recordings from hippocampal slices—and in the same rodent where the antiseizure action was detected, that is, the mouse. fEPSP recordings had the additional advantage of allowing ruling out the remote possibility that dilution of the intracellular content (as a result of the mixing with the patch electrode filling solution) could hinder the activation of A_1 receptor-mediated signalling cascades by MRS5474. We tested a higher range of concentrations, ranging from 120 to 500 nM of MRS5474, and again, no significant effect ($P > 0.05$, n = 7) was detected (% change at the end of application of 120, 250 and 500 nM: with inhibition of 11 ± 10.2%, −2.7 ± 9.9% and −9.3 ± 10.6%, respectively, Figure 2a). Under similar experimental conditions, the canonical A_1 receptor agonist, CPA (30 nM, about 15 times higher than its A_1 receptor affinity value, Klotz, 2000), caused the expected inhibition (Figure 2b) of fEPSPs (68 ± 13% inhibition, n = 4; as n = 4 for these experiments, statistical analysis was not carried out, and results should be regarded as preliminary, though fully consistent with literature (e.g. Sebastião et al., 1990).

Because species differences (rat vs. mice) were apparently not the reason for the lack of effect of MRS5474 on excitatory

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

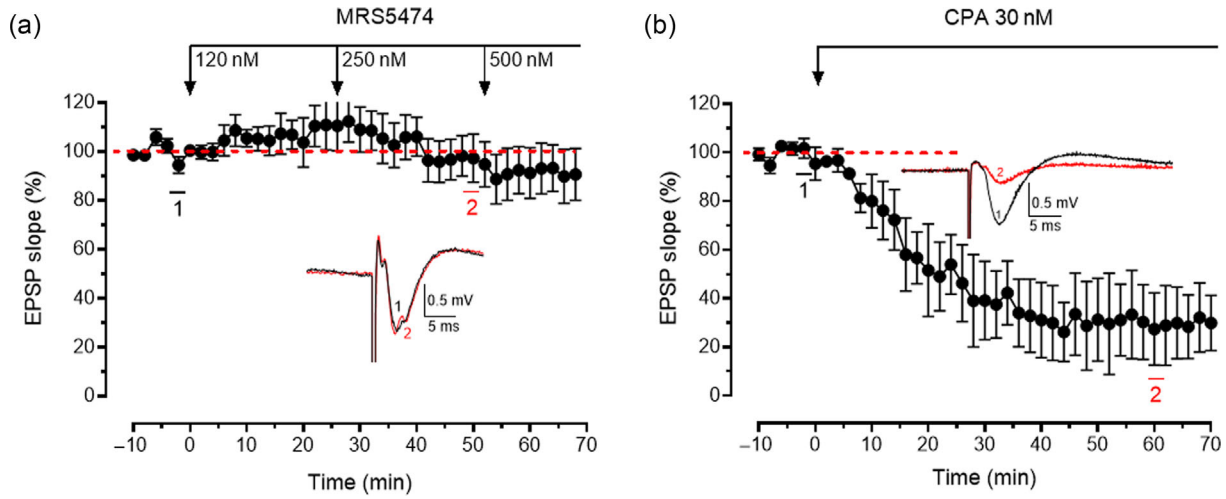

FIGURE 2 MRS5474 did not affect field excitatory postsynaptic potentials (fEPSPs) in mice hippocampal slices, in clear contrast to the A_1 receptor agonist, CPA. Recordings were performed in the CA1 area of acute hippocampal slices upon stimulation of the Schaffer collaterals. In (a) and (b), normalized averaged time-course changes in fEPSP slopes (%, mean ± SEM) taking as baseline (100%) the values recorded for 10 min before drug application, are shown. The horizontal lines below drug names indicate drug presence in the perfusion solution, and the arrows indicate the time of starting perfusion of each drug concentration. Insets: representative superimposed averaged fEPSP traces recorded from the same slice before (control) and by the end of (a) MRS5474 (250 nM) or (b) CPA (30 nM, an A_1 receptor agonist) application. (a) n = 7 slices from six mice. (b) n = 4 slices from four mice. As n=4 for these experiments, statistical analysis was not carried out, and results should be regarded as preliminary, though fully consistent with what is known from the literature (e.g. Sebastião et al., 1990). Note also that the fEPSP inhibition caused by the selective A_1 receptor agonist, CPA (b)clearly contrasts with the absence of effect of MRS5474 (a).

hippocampal synaptic transmission, the remaining experiments using non-human tissues were performed using the rat as the animal model.

3.2 | MRS5474 decreased excitability under hyperexcitable conditions

We then hypothesized that the effect of MRS5474 upon excitatory transmission could predominantly occur in hyperexcitable neurons. To test this hypothesis, we started by assessing the action of the intermediate concentration of MRS5474 (250 nM) in organotypic rhinal–hippocampal slices, which develop spontaneous activity (Valente et al., 2021). To further enhance spontaneous activity, the extracellular concentration of potassium chloride in the extracellular medium was increased to 8.5 mM during field potential recordings. The frequency of spikes within a burst did not change appreciably throughout the time of recording in control slices (no drug) (Figure 3a,c). However, the frequency of spikes within a burst was significantly decreased ($P < 0.05$) in slices perfused with MRS5474 (Table 1 and Figure 3b,d). In two out of seven slices perfused with MRS5474, the bursts were abolished within minutes after adding the drug to the perfusing medium (Figure 3b,d). In those slices where bursts remained, the frequency of spikes within the burst, at 80–90 min, was reduced to 0.65 ± 0.04 ($P < 0.05$ as compared with baseline, Table 1). When comparing values in control and MRS5474-perfused slices, using time and drug condition as independent variables, the number of bursts at 80–90 min in slices perfused with MRS5474 was significantly lower than in control slices ($P < 0.05$, two-way ANOVA, Table 1), while the effect of MRS5474 versus baseline in the frequency of bursts still holds significance ($P < 0.05$). The amplitude of spikes within the bursts was not significantly affected by MRS5474 (Table 1). Under non-depolarizing conditions (extracellular perfusing medium composed of NBA), MRS5474 was virtually devoid of effect in all parameters analysed (Table 1). In clear contrast, CPA (30 nM), an A_1 receptor agonist, fully abolished spontaneous activity within 30 min after starting its perfusion, which was zero at all parameters evaluated (n = 4, data not shown; as n=4 for these experiments, statistical analysis was not carried out, and results should be regarded as preliminary, though fully consistent with what is already known about the action of CPA upon hippocampal excitability).

To further address the effect of MRS5474 on hippocampal synaptic transmission in 'epileptic-like' tissue, we tested its effect in hippocampal slices taken from animals with EE. In hippocampal slices of animals with EE, MRS5474 (250 nM) significantly decreased fEPSP slope (Figure 4a) by 31 ± 6.0% (n = 7 slices from six rats, $P < 0.05$), while in slices from control animals, MRS5474 was virtually devoid of effect (Figure 4b) on fEPSPs (% change in slope at the end of application: −1.43 ± 8.6%, n = 6, $P > 0.05$).

3.3 | MRS5474 inhibited GABA uptake through an adenosine A_3 receptor

GATs, in particular GAT-1, are well-known targets for antiseizure medications (Meldrum & Chapman, 1999; Sills & Rogawski, 2020). Importantly, recent evidence suggests that GAT-1 inhibitors not only

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

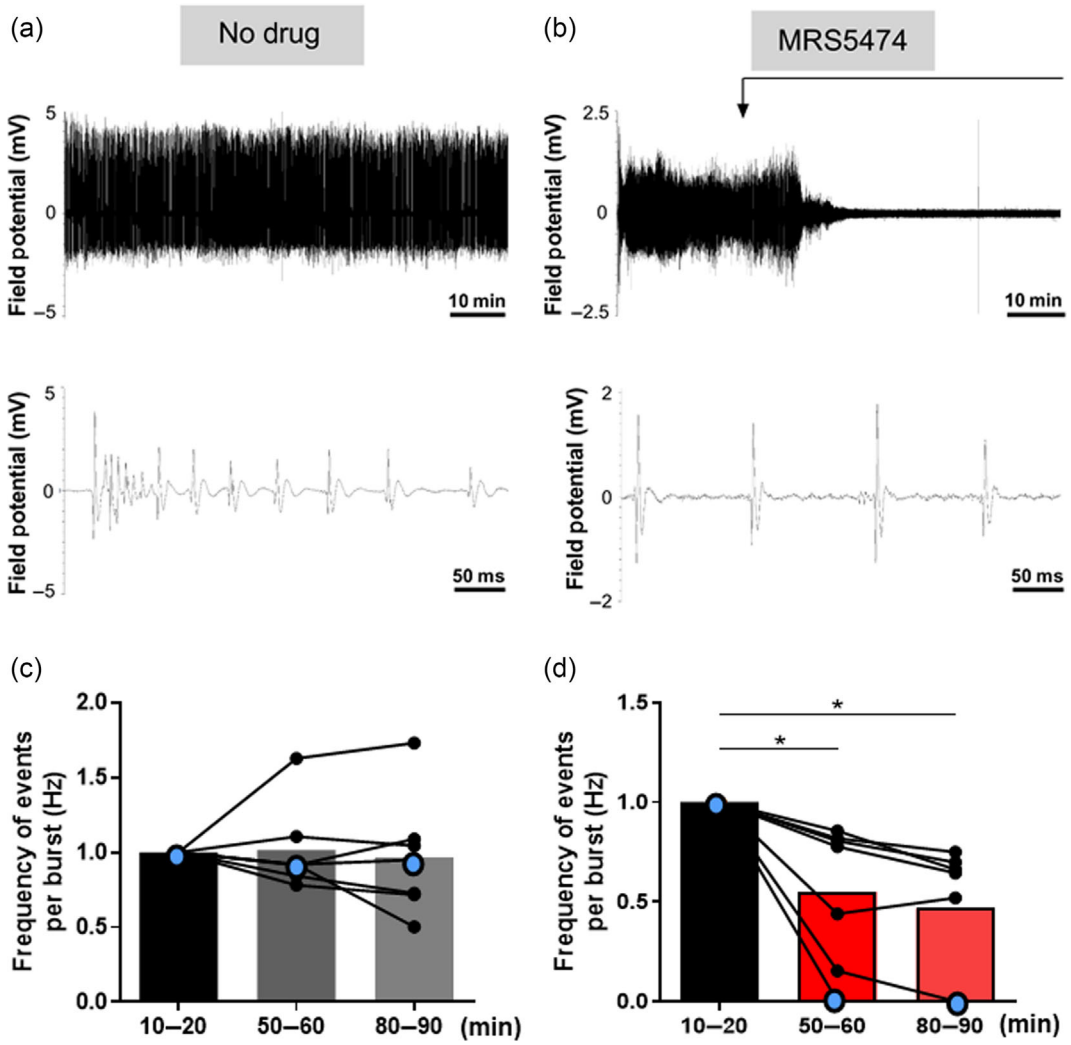

FIGURE 3 MRS5474 inhibited spontaneous activity in rat organotypic rhinal–hippocampal slices under a depolarizing condition. In (a) and (b) are shown representative field potential recordings of spontaneous activity from the CA3 area of a control slice (no drug [a]) and of a slice to which MRS5474 (250 nM) was added to the perfusion solution as indicated by the arrow (b). The upper panel in (a) and (b) shows representative recordings during the entire experimental time, while the lower panel shows representative ictal-like discharges in basal conditions in the same slices as in the corresponding upper panel; note that the timescale is different in both the upper and lower panels. In (c) and (d) are shown the mean frequency of spikes within the bursts at the times indicated below each bar; data obtained at 10–20 min were in the absence of MRS5474 (basal ictal discharge) and were normalized to 1, whereas data at 50–60 or 80–90 min were either in the absence (c) or in the presence (d) of MRS5474 (250 nM). Data are shown as mean with the dots representing individual data points. Dots from the same experiment are connected by a straight line. Dots corresponding to the experiments represented in (a) and (b) are highlighted in blue. In all experiments, the concentration of KCl in the perfusion solution was increased to 8.5 mM (depolarizing conditions). *P < 0.05. Friedman test followed by Dunnett's multiple comparisons test .

inhibit seizures but may also halt epileptogenesis (Javaid et al., 2023). We therefore hypothesized that MRS5474 could act by inhibiting GAT-1-mediated GABA transport. Because GAT-1 is present in nerve endings and astrocytes, we tested the effect of MRS5474 in rat acute hippocampal slices, which enabled us to evaluate the effect upon GABA transport independently of its specific location. We started by testing 120 nM and 250 nM MRS5474, which caused near maximal inhibition of GAT-1-mediated GABA uptake (120 nM: 89 ± 5.9%, n = 6; 250 nM: 96 ± 2.3%, n = 4; as n=4 for the experiments with 250 nM MRS5474, statistical analysis was not carried out, and the results should be regarded only as indicative of a maximal effect). In the presence of even a lower concentration of MRS5474 (50 nM), GAT-1-mediated [^{3}H]GABA uptake was already significantly lower than in control conditions (% inhibition: 47 ± 6.5%, n = 15, Figure 5a,c). Unexpectedly, the presence of the A_1 receptor antagonist, DPCPX, used at a concentration (50 nM) 100 times higher than its K_i value for A_1 receptors (Lohse et al., 1987) and added 30 min before MRS5474, did not prevent the action of MRS5474 (n = 4). Indeed, the pooled comparison between the % inhibition caused by MRS5474 in the absence and in the presence of DPCPX was not statistically significant (P > 0.05, Dunnett's test, Figure 5d). In contrast, the inhibition was fully prevented in experiments where MRS1523 (10 μM), an A_3 receptor antagonist at a supramaximal concentration (Li et al., 1998), was

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 1 Evaluation of the intrinsic parameters of the epileptiform activity depicted by rat organotypic rhinal–hippocampal slices perfused under depolarizing conditions (8.5 mM of KCl in aCSF) or non-depolarizing conditions (Neurobasal A medium) in the absence or presence of MRS5474 (250 nM).

Parameter	Number of bursts		Frequency of spikes within burst (Hz)		Amplitude of spikes within burst (mV)	
Analysis time (min)	50–60	80–90	50–60	80–90	50–60	80–90
Depolarization conditions						
No drug (n = 7)	1.78 ± 0.61	2.61 ± 1.17	1.02 ± 0.11	0.97 ± 0.15	0.96 ± 0.08	0.94 ± 0.07
MRS5474 (n = 7)	0.64 ± 0.15	0.59 ± 0.21‡	0.74 ± 0.08*	0.65 ± 0.04*	0.99 ± 0.14	0.98 ± 0.15
Non-depolarization conditions						
No drug (n = 11)	2.36 ± 0.66	1.68 ± 0.63	1.01 ± 0.21	0.86 ± 0.17	0.95 ± 0.16	0.96 ± 0.13
MRS5474 (n = 11)	1.01 ± 0.27	0.78 ± 0.26	1.73 ± 0.65	0.91 ± 0.11	1.05 ± 0.10	0.86 ± 0.05

Note: 'No drug' refers to data from slices not treated with any drug. 'MRS5474' refers to data from slices in the presence of MRS5474 (250 nM) for 30 min before first analysis time (50–60 min). In all cases and for all parameters, the analysis at time 10–20 (baseline, pre-drug condition) in each slice was normalized to 1.

Abbreviations: aCSF, artificial cerebrospinal fluid; ANOVA, analysis of variance.

*Statistically significant effects of MRS5474 when compared with the pre-drug condition in the same slices; $P < 0.05$ (one-way Friedman test followed by Dunnett's multiple comparisons test).

‡When both time and drug condition were simultaneously analysed as independent variables, an effect of MRS5474 upon the number of bursts also emerged ($P < 0.05$, comparison between the same time frame within slices in the absence or presence of the drug; two-way ANOVA followed by Šidák's multiple comparison test), while the effect of MRS5474 versus baseline in the frequency of bursts still holds significance. Note that the data for the analysis of the amplitude and frequency of spikes within the burst did not include values with zero bursts (i.e., fully blocked by MRS5474), because the frequency and the amplitude of spikes were not independent from the burst occurrence itself. Data from those experiments are included in Figure 3, where individual data points are represented.

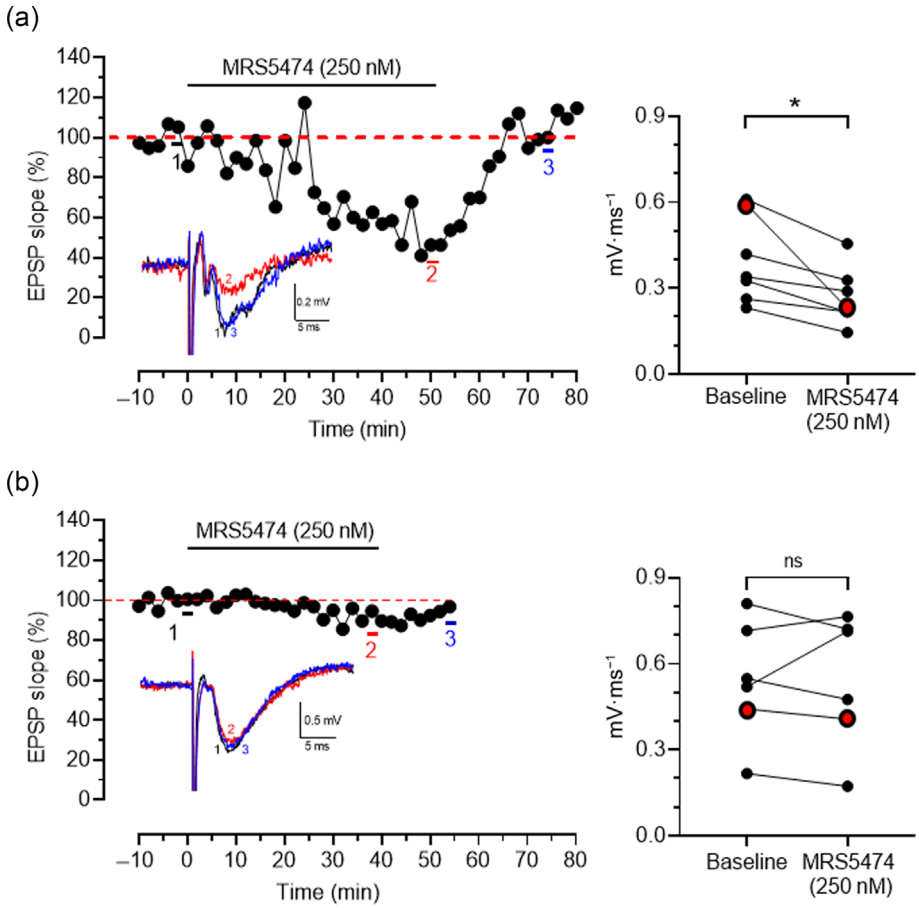

FIGURE 4 MRS5474 decreased fEPSPs in hippocampal slices taken from rats with established epilepsy (EE). Recordings were performed from the CA1 area of acute hippocampal slices upon stimulation of the Schaffer collaterals. In (a) recordings were from slices taken from a rat with EE, and in (b) from a control rat. In the left panels of both (a) and (b) are shown representative time-course changes in fEPSP slopes taking as baseline (100%) the values recorded for 10 min before drug application. The horizontal lines below drug names indicate drug presence in the perfusion solution. Insets: representative superimposed averaged fEPSPs traces recorded from the same slices as in the panel where they are inserted and at the times indicated by the numbers. In the right panels of both (a) and (b) are shown paired EPSP slope values ($mV \cdot ms^{-1}$) from all experiments included in the corresponding left panel, in baseline (Period 1 indicated on the left panel) and under MRS5474 (Period 2 indicated on the left). Dots corresponding to the experiments represented in (a) and (b) are highlighted in red. *$P < 0.05$; ns: $P > 0.05$, two-tailed paired *t* test.

added to the incubation media before MRS5474 (n = 5, $P < 0.05$, Figure 5d), indicating that the inhibitory action of MRS5474 upon GAT-1 activity is mediated by A_3 receptors. Neither DPCPX (50 nM; n = 4 therefore data should be regarded as preliminary) nor MRS1523 (10 μM; n = 5) appreciably affected GAT-1 activity as compared with controls (no drug) in the same experiments

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

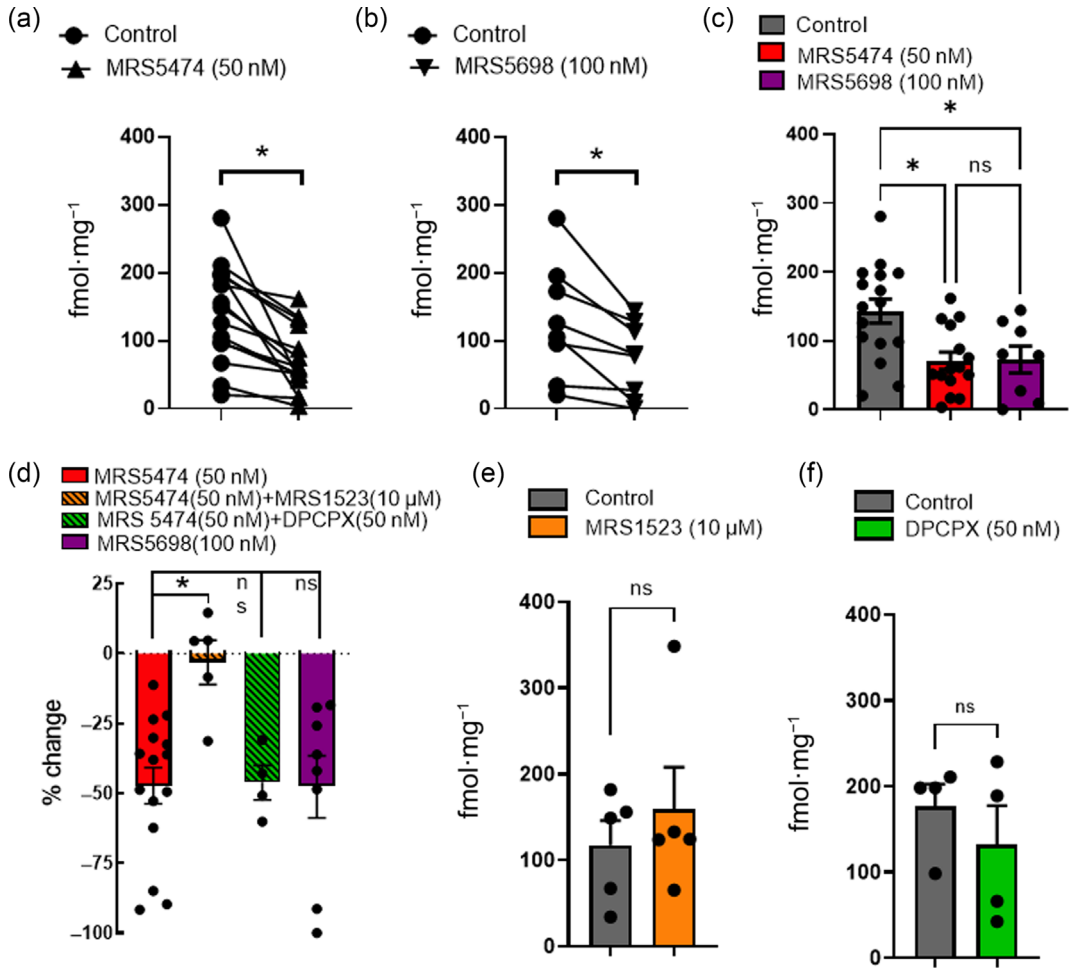

FIGURE 5 MRS5474 inhibited GAT-1-mediated GABA uptake, an action mimicked by an adenosine A_3 receptor agonist and antagonized by an A_3 receptor antagonist but not by an A_1 receptor antagonist. GABA uptake was performed in acute hippocampal slices from rats. In both (a) and (b) are shown the absolute values (fmol·mg^{-1} protein) of GAT-1-mediated GABA uptake in control conditions (no drug, ●) or in the presence of MRS5474 (50 nM) (a, ▲) or MRS5698 (100 nM) (A_3R agonist, b, ▼). Data from the same experiment are connected by a straight line. In (c) are shown the pooled data (fmol·mg^{-1} protein) in (from left to right) control conditions, in the presence of MRS5474 (50 nM), and in the presence of the A_3 receptor agonist MRS5698 (100 nM). In (d) are shown the pooled data as % change in GAT-1-mediated GABA uptake caused by (from left to right) MRS5474 (50 nM), MRS5474 (50 nM) in the presence of MRS1523 (10 μM, an A_3 receptor antagonist), MRS5474 (50 nM) in the presence of DPCPX (50 nM, an A_1 receptor antagonist) and MRS5698 (100 nM, an A_3 receptor agonist). In (e) and (f) are shown the pooled data in control conditions (no drug, left column in each panel) or in the presence of MRS1523 (10 μM, A_3 receptor antagonist) (e, right column) or DPCPX (50 nM, A_1 receptor antagonist) (f, right column). In (c–f), data are shown as mean ± SEM with the dots representing individual data points in each experiment; *P < 0.05; ns: P > 0.05, two-tailed paired t test (a, b), one-way ANOVA followed by multiple comparisons tests (c: Tukey's test, to compare each column with all other columns; d: Dunnett's test, to compare the first column with all other columns) or two-tailed unpaired Student's t test (e, f).

(Figure 5e,f). To further assess the involvement of A_3 receptors upon GAT-1-mediated GABA uptake, we tested the action of a selective A_3 receptor agonist, MRS5698 ($K_i \approx 3$ nM in human or mouse, >3000-fold selectivity over A_1 or A_{2A} receptors; Tosh, Deflorian, et al., 2012). As shown in Figure 5b–d, in the presence of MRS5698 (100 nM), GAT-mediated GABA uptake was significantly lower than in its absence (% inhibition: 48 ± 11%, n = 8, P < 0.05). The mimicry of the effect of MRS5474 by a selective A_3 receptor agonist, together with the blockade of the effect of MRS5474 by an A_3 receptor antagonist, but not by a selective A_1 receptor antagonist, strongly suggests that the effect of MRS5474 is mediated by A_3 rather than A_1 receptors.

3.4 | Enhanced A_3 receptor immunoreactivity in hippocampal tissue from epileptic patients

The unexpected finding that an antiseizure drug activates A_3 receptors, together with the finding that this drug only affects excitatory transmission under hyperexcitable conditions, led us to hypothesize that chronic hyperexcitability leads to an overexpression of A_3 receptors. To address this possibility, we used human hippocampal samples from eight patients with drug-resistant epilepsy who underwent focal hippocampal resection (clinical details in Table 2). As controls, we used six human hippocampal samples obtained post-mortem (post-mortem delay ≤10 h, details in Table 2). Western blot

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

TABLE 2 Clinical information for tissue samples from patients who underwent hippocampal focal resection (EHS 1–8) or from autopsy (CHS 1–6) used for Western blotting.

Sample	Age	Sex	Lesions	Laterality of resection	Primary cause of death	Post-mortem delay (h)
EHS 1	17	M	Hippocampal sclerosis/FCD	Right	n.a.	n.a.
EHS 2	26	F	Hippocampal sclerosis	Right	n.a.	n.a.
EHS 3	36	M	Hippocampal sclerosis/DNT	Right	n.a.	n.a.
EHS 4	40	M	Hippocampal sclerosis/FCD	Right	n.a.	n.a.
EHS 5	43	F	Hippocampal sclerosis	Right	n.a.	n.a.
EHS 6	44	F	Hippocampal sclerosis	Right	n.a.	n.a.
EHS 7	50	F	Hippocampal sclerosis	Right	n.a.	n.a.
EHS 8	59	F	Hippocampal sclerosis	Right	n.a.	n.a.
CHS 1	42	M	n.a.	n.a.	Myocarditis	10
CHS 2	46	M	n.a.	n.a.	Bronchopneumonia	9
CHS 3	53	F	n.a.	n.a.	Bronchopneumonia	7
CHS 4	57	F	n.a.	n.a.	Aorta dissection	8
CHS 5	71	M	n.a.	n.a.	Bronchopneumonia	10
CHS 6	72	M	n.a.	n.a.	Cardiorespiratory failure	9

Note: Epileptic patients 1, 4, 6 and 7 had dual pathology, as indicated.
Abbreviations: CHS, control human sample; DNT, dysembryoplastic neuroepithelial tumour; EHS, epileptic human sample; F, female; FCD, focal cortical dysplasia; M, male; n.a., not applicable.

analysis revealed enhanced A_3 receptor immunoreactivity in samples from epileptic patients when compared with control samples ($P < 0.05$, Figure 6).

3.5 | MRS5474 induced a dose-dependent potentiation of GABA-evoked currents in oocytes micro-transplanted with human hippocampal membranes

The enhanced A_3 receptor immunoreactivity in hippocampal samples from patients with epilepsy, suggesting seizure-induced overexpression of A_3 receptors, together with the finding that MRS5474 can activate A_3 receptors to affect GATs, prompted us to evaluate the action of MRS5474 upon GABAergic transmission in human tissue. Thus, we used the technique of micro-transplantation of *Xenopus* oocytes with human tissue, which allows assessing receptor-mediated neurotransmitter responses and the use of human tissue taken from autopsies or surgeries even in quantities that would not allow other kinds of functional studies (Miledi et al., 2002; Palma et al., 2003; Ruffolo et al., 2020). We recorded GABA (500 μM)-evoked currents from oocytes injected with hippocampal membranes of three epileptic patients (clinical details in Table 3). The amplitude of these currents ranged from 8.0 to 249 nA (n = 47; #1–3). Incubation with MRS5474 (5 μM) for 2 h and 30 min (to allow full activation of signalling pathways in a large oocyte—Roseti et al., 2015) induced a significant enhancement of GABA-evoked responses ($P < 0.05$, 41 ± 15% as mean of percentage changes; n = 15, #1–3; Figure 7a,b). Acute co-application of MRS5474 (5 μM) together with GABA did not exert any effect on GABA-evoked current amplitude (n = 8, not shown). As shown in Figure 7b, the effect of MRS5474 was concentration-dependent. There was a mild tendency, even though not significant, when using 50 nM of MRS5474 ($P = 0.055$; n = 8; #1 and #2), and higher concentrations produced an increasingly higher potentiation (Figure 7b).

To address the putative involvement of A_3 receptors in the effect of MRS5474 upon GABA currents from human hippocampal tissue, we tested its sensitivity to the A_3 receptor antagonist, MRS1523 (Li et al., 1998). In this set of experiments, MRS1523 (10 μM, a supramaximal concentration, Li et al., 1998) was pre-incubated for 30 min and then co-incubated for 2 h with MRS5474 (1.5 μM, chosen as an intermediate concentration in the concentration–response curve). Under such conditions, MRS5474 was virtually devoid of effect upon GABA currents (from 45 ± 5.4 nA before incubation with MRS5474 to 46 ± 4.6 nA after incubation with MRS5474; #1 and #2; Table 3), though in the same experiments but in the absence of the A_3 receptor antagonist, MRS5474 (1.5 μM) caused the usual increase ($P < 0.05$) in GABA currents (Figure 7c).

Notably, when MRS5474 (5 μM) was incubated (2 h and 30 min) with oocytes micro-transplanted with control brain tissues, it did not increase GABA-evoked currents (from 40 ± 14 nA before incubation to 37 ± 10 nA after incubation; n = 10; #4; $P > 0.05$). The comparison between the percentage change in GABA currents induced by MRS5474 (5 μM) in human epileptic versus non-epileptic tissue, ($P < 0.05$, Figure 7d), highlights a stricking difference between the responses in both tissues, thus suggesting that MRS5474 specifically affects epileptic tissue.

4 | DISCUSSION AND CONCLUSIONS

The major finding in the present work is that MRS5474 has selective actions in hippocampal epileptic tissue. Another significant discovery

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

involves the identification of the adenosine A_3 receptor as the target for MRS5474 actions within the hippocampus.

The evidence that extracellular levels of adenosine increase during seizures (Dale & Frenguelli, 2009) raised attention for the antiseizure role of adenosine and of its inhibitory A_1 receptor (Beamer et al., 2021; Rombo et al., 2018). However, the druggability of A_1 receptors is hampered by its ubiquitous distribution, a major cause of the on-target side effects that have been identified in preclinical studies focusing on A_1 receptors in epilepsy. Among the unwanted effects are bradycardia, atrioventricular block, a reduction in atrial contractility and sedation (Jacobson & Gao, 2006; Nguyen et al., 2023). The search for structurally modified A_1 receptor ligands that could achieve selectivity for some of the therapeutically relevant actions of these receptors led to the discovery of the distinguishable properties of MRS5474 (Tosh, Paoletta, et al., 2012). MRS5474 is a small molecule with favourable pharmacokinetics. In the mouse, 1 $mg \cdot kg^{-1}$ (p.o.) has a bioavailability of 97.7%, a long half-life (4.96 h), an exposure ($AUC_{0-\infty}$) of 3700 $ng \cdot h \cdot ml^{-1}$, a plasma concentration maintained at >530 nM for at least 8 h, a clearance of 4.52 $ml \cdot min^{-1} \cdot kg^{-1}$ and a distribution volume of 1.94 $L \cdot kg^{-1}$ (Tosh et al., 2019). No lethality was detected at concentrations up to 60 $mg \cdot kg^{-1}$ (i.p.) (Tosh et al., 2019), which is remarkably higher than the ED_{50} value for its antiseizure action (Tosh, Paoletta, et al., 2012). Using the StarDrop software (Segall, 2012), MRS5474 is predicted to cross the blood–brain barrier more readily than other adenosine receptor ligands, as CPA, with a predicted brain-to-blood ratio of 11.7%. It was tested in gpcrMAX and Kinome screens (Eurofins DiscoverX, Fremont, CA, USA) and found to have no major off-target interactions. Altogether, these pharmacokinetic parameters highlight the suitability of MRS5474 for drug development.

MRS5474 has A_1 receptor selectivity and agonist-like properties (Carlin et al., 2017; Tosh, Paoletta, et al., 2012). Therefore, it was highly surprising when we observed the lack of effect of MRS5474 upon synaptic potentials and excitatory synaptic currents in the hippocampus, because the inhibitory actions of A_1 receptors in the hippocampus have been known for a long time (Sebastião et al., 1990). Searching for an action upon GABAergic transmission (Sebastião & Ribeiro, 2023), we detected that MRS5474 inhibits GAT-1-mediated GABA uptake, but through an A_3 receptor rather than an A_1 receptor-mediated mechanism. Indeed, the inhibitory effect of MRS5474 upon GAT-1-mediated GABA was insensitive to A_1 receptor selective antagonism, was mimicked by a selective A_3 receptor agonist and was fully inhibited by MRS1523, an A_3 receptor antagonist. MRS1523 is selective for A_3 receptors, but at the concentrations used, it may also antagonize A_{2A} receptors (K_i 2 μM for rat $A_{2A}R$) (Li

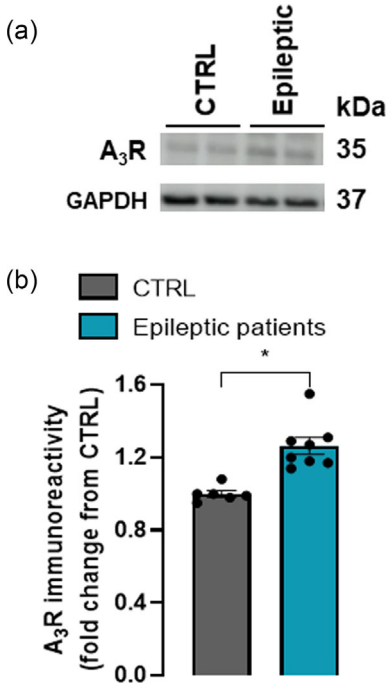

FIGURE 6 A_3 Receptor immunoreactivity was enhanced in hippocampal samples from patients with refractory epilepsy. A representative Western blot of A_3 receptor immunoreactivity in epileptic resected tissue from patients with refractory epilepsy and in post-mortem tissue from control patients (CTRL) is shown in (a). GAPDH was used as a control. (b) Pooled data from densitometric analysis of the 35 kDa band (A_3 receptor, as indicated by the antibody supplier) corrected for GAPDH (37 kDa) in the same samples. Values were normalized taking as 1 the values obtained in control samples. CTRL and epileptic samples were always analysed on the same membrane. Data in (b) are shown as mean ± SEM with the dots representing individual data points (sample details in Table 2); each data point corresponds to a sample from a different patient. $^*P < 0.05$, two-tailed unpaired *t* test with Welch's correction for unequal variances.

TABLE 3 Clinical information of tissue samples from patients who underwent focal resection (#1–3) or from autopsy (#4) used for GABAergic current recordings.

P#	Age (years)/sex	Epilepsy onset	Surgical zone, hippocampus	Seizure type	Seizures per month	Pathology	Medication
#1	41/M	20	R,T	FIAS/GS	10	HS	CBZ, TPM
#2	44/M	27	R,T	FIAS	4	HS	CBZ, TPM, VPA
#3	52/M	10	L,T	FIAS	1	HS	CBZ, PB, VGB
#4	63/F	n.a.	R,T	n.a.	n.a.	Myocardial infarction	None

Abbreviations: CBZ, carbamazepine; F, female; FIAS, focal impaired awareness seizure; GS, generalized seizure; HS, hippocampal sclerosis; L, left; M, male; n.a., not applicable; PB, phenobarbital; R, right; T, temporal; TPM, topiramate; VGB, Vigabatrin; VPA, valproic acid.

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

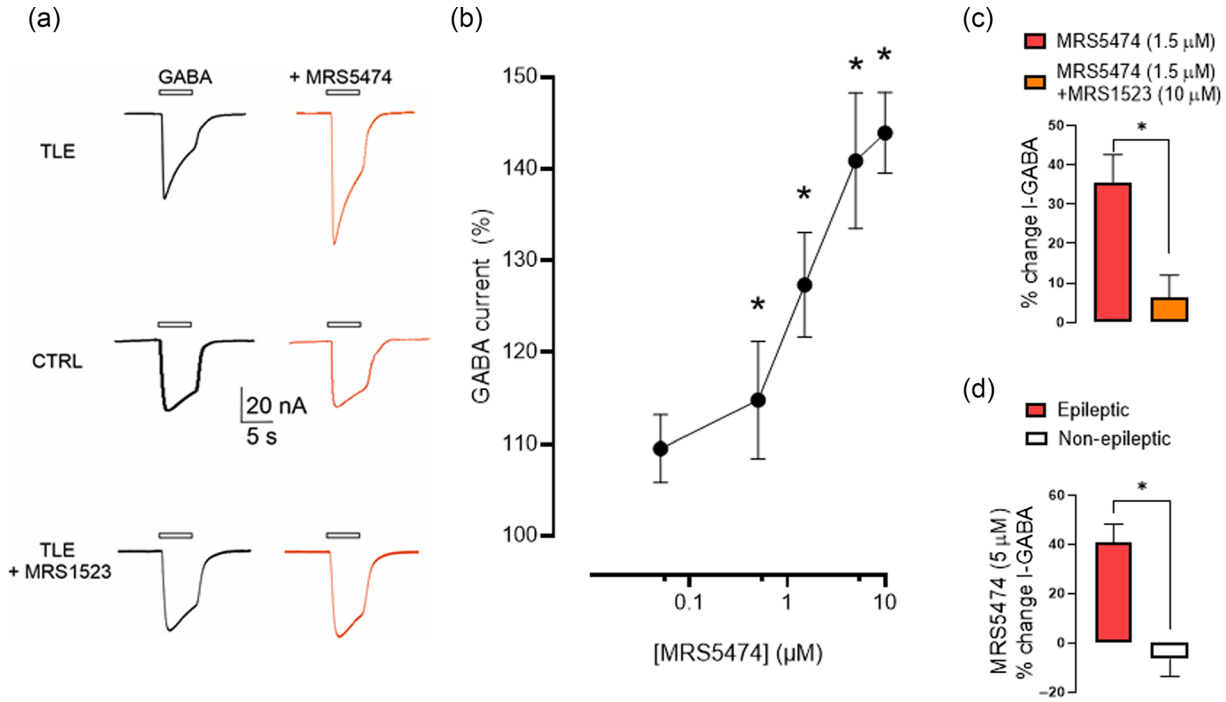

FIGURE 7 MRS5474, through A_3 receptor activation, selectively enhances GABAergic currents (I-GABA) in epileptic human hippocampal samples. (a) Representative currents evoked by GABA (500 μM, applied as indicated by the white horizontal bars) recorded before (black trace) and after (red trace) incubation with MRS5474 (5 μM, for 2 h and 30 min, first and second rows) or with MRS5474 under similar conditions but in the presence of MRS1523 (10 μM), an A_3 receptor antagonist (third row). Recordings were from oocytes injected with membranes prepared from epileptic patient tissue samples (TLE, first and third rows) or control (CTRL, second row); each row shows sample recordings from the same oocyte before and by the end of incubation with MRS5474. (b) Concentration–response curve obtained in a non-cumulative manner; thus, consecutive concentrations were tested in different oocytes. The ordinates represent the percentage change of the amplitude of GABA-evoked responses after incubation with different concentrations of MRS5474, as indicated in the abscissae; 100% represents the amplitude of currents recorded before exposure to MRS5474 in the same oocytes. The raw amplitude values before and after incubation for each concentration are the following: [0.05 μM] from 43 ± 10 nA before incubation to 47 ± 14 nA after incubation, n = 8, #1 and #2; [0.5 μM] from 68 ± 10 nA before incubation to 79 ± 16 nA after incubation, n = 8, #2 and #3; [1.5 μM] from 41 ± 4.6 nA before incubation to 53 ± 6.0 nA after incubation, n = 8, #1 and #2; [5 μM] from 47 ± 8.9 nA before incubation to 71 ± 17 nA after incubation, n = 15, #1–3; and [10 μM] from 44 ± 6.5 nA before incubation to 62 ± 6.3 nA after incubation, n = 8, #1–3. (c) Comparison (in another set of experiments) between the effect (as % change in the ordinates) in current amplitude caused by a submaximal concentration of MRS5474 (1.5 μM) in the absence (n = 8 oocytes) and in the presence (n = 11) of the A_3 receptor antagonist, MRS1523 (10 μM). (d) Comparison between the effects of a near maximal concentration of MRS5474 (5 μM) in oocytes injected with membrane samples from epileptic patients (same data as in [b], n = 15) and from oocytes injected with membranes from controls (n = 10 oocytes from different frogs, #4). In (b–d), values are expressed as mean ± SEM. The statistical analysis shown in (b) represents the comparison between current amplitude before and after MRS5474 (two-tailed paired Student's *t* test) and in (c) and (d) represents the comparison between the pooled data in the two conditions shown in each panel (two-tailed unpaired Student's *t* test). For all panels: $^*P < 0.05$.

et al., 1998). However, the involvement of A_{2A} receptors in the inhibitory action of MRS5474 upon GAT-1 is highly unlikely because the A_{2A} receptor facilitates, rather than inhibits, GAT-1 in nerve endings (Cristóvão-Ferreira et al., 2009) and astrocytes (Cristóvão-Ferreira et al., 2013).

Clinical trials with A_3 receptor agonists proved them to be well tolerated and with few side effects (Coppi et al., 2022; Jacobson et al., 2019). The A_3 receptor has a very low expression in cardiomyocytes and was even shown to be cardioprotective (Wan et al., 2019), as well as neuroprotective (Cheng et al., 2022; Von Lubitz et al., 1999). Of high importance is the neuroprotective potential of a mixed A_1 receptor/A_3 receptor agonist in both rodent and non-human primate models of stroke (Liston et al., 2020, 2022), which led to two human phase I clinical trials and a likely approaching phase II clinical trial for acute stroke and traumatic brain injury (https://www.astrocytepharma.com/portfolio/, accessed 31 May 2024). Interestingly, stroke and traumatic brain injury are two leading causes of epilepsy. Neuroinflammation is also a hallmark of epilepsy (Palumbo et al., 2023; Vezzani et al., 2023), and A_3 receptor agonists have anti-inflammatory actions (Jacobson et al., 2019).

The role of A_3 receptors in epilepsy has, however, been controversial (Beamer et al., 2021; Rombo et al., 2018; Świąder et al., 2014; Tescarollo et al., 2020). This may result from the initial lack of selective ligands for A_3 receptors (Gao et al., 2023), as well as a lack of

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

knowledge of the species differences in ligand selectivity. Importantly, we now proved the effectiveness of MRS5474 in human tissue and showed that it acts through A_3 receptors. Another issue to consider when addressing the action of A_3 receptors in epilepsy is the involvement of the GABAergic system. An A_3 receptor-mediated pro-epileptiform action has been detected under $GABA_A$ receptor blockade conditions (Laudadio & Psarropoulou, 2004), therefore precluding the observation of any modification of GABAergic transmission. As we show here, A_3 receptors may predominantly affect GABAergic rather than glutamatergic transmission. It may also be relevant to distinguish between phasic inhibition (mediated by synaptic $GABA_A$ receptors) and tonic inhibition (mediated by extrasynaptic $GABA_A$ receptors). Drugs with A_3 receptor antagonistic characteristics have been shown to decrease $GABA_A$ receptor desensitization in oocytes injected with tissue samples from a variety of human epileptic tissues (Roseti et al., 2008, 2009), which is compatible with the pro-convulsive action of A_3 receptors. In contrast, we observed that MRS5474, by activating A_3 receptors, selectively potentiates GABAergic currents in oocytes injected with human epileptic hippocampal tissue, thus having an action compatible with antiseizure activity. Whole GABAergic currents include those mediated by synaptic (fast desensitizing) and extrasynaptic $GABA_A$ (slow-desensitizing) receptors. One may thus suggest that MRS5474, by activating A_3 receptors, predominantly affects slow-desensitizing extrasynaptic GABAergic currents. Further experiments may allow elucidating this point even though it is not easy to completely isolate the extrasynaptic component in the total $GABA_A$ currents obtained with human membranes transplantation. In intact tissues, inhibition of GAT-1 by MRS5474 will even favour an extrasynaptic action, because it would facilitate GABA spillover to extrasynaptic sites. Extrasynaptic $GABA_A$ receptor activation is of high value for seizure control (Naylor, 2023; Richerson, 2004). The use of human epileptic fresh slices would allow to address the relative activity of MRS5474 and of A_3 receptors in tonic (extrasynaptic) versus phasic (synaptic) inhibition, as well as directly evaluate the action of A_3 receptors upon GAT-1 activity in humans. However, the availability of fresh human tissues is scarce and even more for control tissues. Nevertheless, we obtained evidence suggesting overexpression of A_3 receptors in human epileptic tissue, which likely acts as an amplification mechanism and may explain the lack of effect of MRS5474 in non-epileptic tissue. Importantly, the diseased tissue selectivity of MRS5474 could be detected both in humans and in rats. Therefore, it can hardly be solely attributed to variables such as medication usage, coexisting disease conditions, or other variables inherent to differences between test and control human tissue samples.

We could not detect the well-known A_1 receptor-mediated inhibition of excitatory inputs to CA1 pyramidal cells in non-epileptic rodents, even though MRS5474 binds to both A_1 and A_3 receptors, with even higher affinity for the A_1 receptor (Carlin et al., 2017; Tosh, Paoletta, et al., 2012). The reasons for this lack of A_1 receptor-mediated effects of MRS5474 may result, at least in part, from the ability of A_3 receptors to desensitize A_1 receptors in healthy tissue (Dunwiddie et al., 1997). Alternatively, one may speculate on biased agonism (McNeill et al., 2021) by MRS5474. Context-specific biased agonism, namely, differential recruitment of G_i versus G_q proteins under hyperexcitable and control conditions, might explain the epilepsy-selective actions of MRS5474 detected in the present work. Further studies are worthwhile to explore these possibilities and their putative therapeutic implications.

The present results, together with previous evidence that MRS5474 has antiseizure actions without appreciable negative side effects on cardiac function (Tosh, Paoletta, et al., 2012), strongly support the idea that targeting A_3 receptors in epilepsy may lead to fewer side effects than targeting A_1 receptors. Targeting A_3 receptors may also have fewer side effects than targeting GAT-1. MRS5474 could inhibit GAT-1 even in non-epileptic tissue, but the change in extracellular GABA concentrations is likely small because it did not affect excitatory transmission in healthy tissue. This may result from overexpression of A_3 receptors in epileptic tissue (present work), amplifying A_3 receptor-mediated actions, and/or alteration in GAT-1 in epilepsy (Medina-Ceja et al., 2012; Su et al., 2015). Inhibitors of GAT-1, such as tiagabine currently used in clinics, have sedative effects (Bauer & Cooper-Mahkorn, 2008; Kälviäinen, 2001; Masocha & Parvathy, 2016), though clearly of lower magnitude than benzodiazepines. In contrast, no sedative effects have been detected in animals administered with MRS5474 (Tosh, Paoletta, et al., 2012), which may be related to our finding that MRS5474 selectively affects hyperexcitable tissue, mostly sparing non-epileptic tissue.

The expression of A_1 receptors is known to be affected in epilepsy, and both increases and decreases have been reported (Spanoghe et al., 2020). However, the majority of studies in animal models show decreased A_1 receptor levels in chronic epilepsy (Rombo et al., 2018; Spanoghe et al., 2020), which is likely due to maladaptive modifications caused by extracellular adenosine overload (Sandau et al., 2016). It is nevertheless important to highlight that in spite of the reduced A_1 receptor levels and any maladaptations that may occur, the A_1 receptor still retains its neuroprotective role in epilepsy because its blockade clearly aggravates seizures (Beamer et al., 2021; Rombo et al., 2018; Sandau et al., 2016). In humans, decreased A_1 receptor levels were also detected in resected epileptic temporal lobe tissue from refractory TLE patients, as compared with levels in post-mortem control tissue from non-epileptic subjects (Glass et al., 1996). In contrast, when comparing similar groups but addressing A_3 rather than A_1 receptors, we obtained evidence pointing towards an increase in A_3 receptor levels in refractory TLE patients.

In conclusion, this work provided, for the first time, evidence of an A_3 receptor-mediated action upon GATs and GABAergic currents. It also provides evidence that a drug with A_3 receptor agonist properties, MRS5474, predominantly affects epileptic tissue. Therefore, we disclosed a novel mechanism of action of a drug that proved to be effective in an animal model of epilepsy and highlighted the A_3 receptor as a target for antiseizure medications. These findings pave the way for further preclinical studies to explore the therapeutic potential of MRS5474 or other A_3 receptor agonists against epilepsy, either as add-on drugs with other antiseizure drugs or even as antiepileptogenic drugs, due to their combined anti-inflammatory action.

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

AUTHOR CONTRIBUTIONS

Anwesha Ghosh: Data curation; formal analysis; investigation; visualization; methodology; writing—original draft; writing—review and editing. **Leonor Ribeiro-Rodrigues:** Data curation; formal analysis; software, investigation; visualization; methodology; writing—original draft; writing—review and editing. **Gabriele Ruffolo:** Conceptualization; data curation; formal analysis; investigation; visualization; methodology; resources; supervision; validation; writing—review and editing. **Veronica Alfano:** Investigation; methodology. **Cátia Domingos:** Data curation; formal analysis; investigation; visualization; methodology; writing—review and editing. **Nádia Rei:** Investigation; methodology; writing—review and editing. **Dilip K. Tosh:** Resources. **Diogo M. Rombo:** Formal analysis; supervision; writing—review and editing. **Tatiana P. Morais:** Methodology; writing—review and editing. **Cláudia A. Valente:** Supervision; writing—review and editing. **Sara Xapelli:** Supervision; writing—review and editing. **Beatriz Bordadágua:** Software. **Alexandre Rainha-Campos:** Resources. **Carla Bentes:** Resources; writing—review and editing. **Eleonora Aronica:** Resources; writing—review and editing. **Maria José Diógenes:** Conceptualization; writing—review and editing. **Sandra H. Vaz:** Methodology; writing—review and editing. **Joaquim A. Ribeiro:** Conceptualization; writing—review and editing. **Eleonora Palma:** Conceptualization; formal analysis; visualization; funding acquisition; supervision; writing—review and editing. **Kenneth A. Jacobson:** Conceptualization; funding acquisition; resources; supervision; writing—review and editing. **Ana M. Sebastião:** Conceptualization; data curation; formal analysis; visualization; funding acquisition; investigation; methodology; project administration; resources; supervision; validation; writing—original draft; writing—review and editing.

AFFILIATIONS

[1]Instituto de Farmacologia e Neurociências, Faculdade de Medicina, Universidade de Lisboa, Lisbon, Portugal
[2]Instituto de Medicina Molecular João Lobo Antunes, Universidade de Lisboa, Lisbon, Portugal
[3]Department of Physiology and Pharmacology, Sapienza University of Rome, Rome, Italy
[4]IRCCS San Raffaele Roma, Rome, Italy
[5]Molecular Recognition Section, Laboratory of Bioorganic Chemistry, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland, USA
[6]Neuroscience Division, School of Bioscience, Cardiff University, Cardiff, UK
[7]Department of Physiology and Biochemistry, University of Malta, Msida, Malta
[8]Heidelberg Institute for Theoretical Studies (HITS), Heidelberg, Germany
[9]Heidelberg University, Heidelberg, Germany
[10]Instituto Superior Técnico, Universidade de Lisboa, Lisbon, Portugal
[11]Centro de Referência para a área da Epilepsia Refratária (ERN EpiCARE Member), CHULN, Lisbon, Portugal
[12]Serviço de Neurologia, CHULN, Lisbon, Portugal
[13]Laboratório de EEG/Sono—Unidade de Monitorização Neurofisiológica, Serviço de Neurologia, CHULN, Lisbon, Portugal
[14]Centro de Estudos Egas Moniz, Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal
[15]Amsterdam Neuroscience, Department of (Neuro)Pathology, Amsterdam UMC, University of Amsterdam, Amsterdam, The Netherlands
[16]Stichting Epilepsie Instellingen Nederland (SEIN), Heemstede, The Netherlands

ACKNOWLEDGEMENTS

The collaborative work in Lisbon, Rome and Amsterdam was supported by H2020-WIDESPREAD-05-2017-Twinning (EpiEpiNet) under Grant Agreement No. 952455 and the NIDDK Intramural Research Program ZIADK031117. The work in Lisbon was supported by PTDC/MED-FAR/30933/2017 and PTDC/MED-FAR/4834/2021 from Fundação para a Ciência e a Tecnologia (FCT) and by the International Society for Neurochemistry (Carer Development Grant 2021 to SHV). AG received an Instituto de Medicina Molecular João Lobo Antunes (iMM) scholarship (iMM-BI-4-2022). LR-R received a fellowship from FCT (PD/BD/150344/2019). The work in Rome was supported by the Fondi Ateneo Grant Nos. RM11916B84D24429 (EP) and RG12117A8697DCF1 (EP and GR) funded by Sapienza University of Rome (Sapienza Università di Roma) and PRIN 2022 (EP and GR) and PRIN-PNRR 2022 (EP) from the Italian Ministry of University and Research. GR and VA were also supported by the funding of the Italian Ministry of Health, 'Ricerca corrente'. This study has not been preregistered.

CONFLICT OF INTEREST STATEMENT

There are no competing interests.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

DECLARATION OF TRANSPARENCY AND SCIENTIFIC RIGOUR

This Declaration acknowledges that this paper adheres to the principles for transparent reporting and scientific rigour of preclinical research as stated in the *BJP* guidelines for Design & Analysis, Immunoblotting and Immunochemistry, and Animal Experimentation and as recommended by funding agencies, publishers and other organizations engaged with supporting research.

ORCID

Kenneth A. Jacobson https://orcid.org/0000-0001-8104-1493

REFERENCES

Alexander, S. P. H., Christopoulos, A., Davenport, A. P., Kelly, E., Mathie, A. A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Davies, J. A., Abbracchio, M. P., Abraham, G.,

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Agoulnik, A., Alexander, W., Al-Hosaini, K., Bäck, M., Baker, J. G., Barnes, N. M., … Ye, R. D. (2023). The Concise Guide to PHARMACOLOGY 2023/24: G protein-coupled receptors. *British Journal of Pharmacology*, *180*, S23–S144. https://doi.org/10.1111/bph.16177

Alexander, S. P. H., Fabbro, D., Kelly, E., Mathie, A. A., Peters, J. A., Veale, E. L., Armstrong, J. F., Faccenda, E., Harding, S. D., Davies, J. A., Amarosi, L., Anderson, C. M. H., Beart, P. M., Broer, S., Dawson, P. A., Gyimesi, G., Hagenbuch, B., Hammond, J. R., Hancox, J. C., … Verri, T. (2023). The Concise Guide to PHARMACOLOGY 2023/24: Transporters. *British Journal of Pharmacology*, *180*, S374–S469. https://doi.org/10.1111/bph.16182

Alexander, S. P. H., Mathie, A. A., Peters, J. A., Veale, E. L., Striessnig, J., Kelly, E., Armstrong, J. F., Faccenda, E., Harding, S. D., Davies, J. A., Aldrich, R. W., Attali, B., Baggetta, A. M., Becirovic, E., Biel, M., Bill, R. M., Caceres, A. I., Catterall, W. A., Conner, A. C., … Zhu, M. (2023). The Concise Guide to PHARMACOLOGY 2023/24: Ion channels. *British Journal of Pharmacology*, *180*, S145–S222. https://doi.org/10.1111/bph.16181

Alexander, S. P. H., Roberts, R. E., Broughton, B. R. S., Sobey, C. G., George, C. H., Stanford, S. C., Cirino, G., Docherty, J. R., Giembycz, M. A., Hoyer, D., Insel, P. A., Izzo, A. A., Ji, Y., MacEwan, D. J., Mangum, J., Wonnacott, S., & Ahluwalia, A. (2018). Goals and practicalities of immunoblotting and immunohistochemistry: A guide for submission to the British Journal of Pharmacology. *British Journal of Pharmacology*, *175*, 407–411. https://doi.org/10.1111/bph.14112

Anderson, W. W., & Collingridge, G. L. (2007). Capabilities of the WinLTP data acquisition program extending beyond basic LTP experimental functions. *Journal of Neuroscience Methods*, *162*(1–2), 346–356. https://doi.org/10.1016/j.jneumeth.2006.12.018

Baltos, J. A., Casillas-Espinosa, P. M., Rollo, B., Gregory, K. J., White, P. J., Christopoulos, A., Kwan, P., O'Brien, T. J., & May, L. T. (2023). The role of the adenosine system in epilepsy and its comorbidities. *British Journal of Pharmacology*, *181*, 2143–2157. https://doi.org/10.1111/bph.16094

Bauer, J., & Cooper-Mahkorn, D. (2008). Tiagabine: Efficacy and safety in partial seizures—Current status. *Neuropsychiatric Disease and Treatment*, *4*(4), 731–736. https://doi.org/10.2147/ndt.s833

Beamer, E., Kuchukulla, M., Boison, D., & Engel, T. (2021). ATP and adenosine—Two players in the control of seizures and epilepsy development. *Progress in Neurobiology*, *204*, 102105. https://doi.org/10.1016/j.pneurobio.2021.102105

Berdichevsky, Y., Dzhala, V., Mail, M., & Staley, K. J. (2012). Interictal spikes, seizures and ictal cell death are not necessary for post-traumatic epileptogenesis in vitro. *Neurobiology of Disease*, *45*(2), 774–785. https://doi.org/10.1016/j.nbd.2011.11.001

Blümcke, I., Thom, M., Aronica, E., Armstrong, D. D., Bartolomei, F., Bernasconi, A., Bernasconi, N., Bien, C. G., Cendes, F., Coras, R., Cross, J. H., Jacques, T. S., Kahane, P., Mathern, G. W., Miyata, H., Moshé, S. L., Oz, B., Özkara, Ç., Perucca, E., … Spreafico, R. (2013). International consensus classification of hippocampal sclerosis in temporal lobe epilepsy: A Task Force report from the ILAE Commission on Diagnostic Methods. *Epilepsia*, *54*(7), 1315–1329. https://doi.org/10.1111/epi.12220

Boison, D., & Jarvis, M. F. (2021). Adenosine kinase: A key regulator of purinergic physiology. *Biochemical Pharmacology*, *187*, 114321. https://doi.org/10.1016/j.bcp.2020.114321

Carlin, J. L., Jain, S., Gizewski, E., Wan, T. C., Tosh, D. K., Xiao, C., Auchampach, J. A., Jacobson, K. A., Gavrilova, O., & Reitman, M. L. (2017). Hypothermia in mouse is caused by adenosine A_1 and A_3 receptor agonists and AMP via three distinct mechanisms. *Neuropharmacology*, *114*, 101–113. https://doi.org/10.1016/j.neuropharm.2016.11.026

Chazalon, M., Paredes-Rodriguez, E., Morin, S., Martinez, A., Cristóvão-Ferreira, S., Vaz, S., Sebastiao, A., Panatier, A., Boué-Grabot, E., Miguelez, C., & Baufreton, J. (2018). GAT-3 dysfunction generates tonic inhibition in external globus pallidus neurons in parkinsonian rodents. *Cell Reports*, *23*(6), 1678–1690. https://doi.org/10.1016/j.celrep.2018.04.014

Cheng, P., Zhang, J., Chu, Z., Liu, W., Lin, H., Wu, Y., & Zhu, J. (2022). A3 adenosine receptor agonist IB-MECA reverses chronic cerebral ischemia-induced inhibitory avoidance memory deficit. *European Journal of Pharmacology*, *921*, 174874. https://doi.org/10.1016/j.ejphar.2022.174874

Coppi, E., Cherchi, F., Venturini, M., Lucarini, E., Corradetti, R., Di Cesare Mannelli, L., Ghelardini, C., Pedata, F., & Pugliese, A. M. (2022). Therapeutic potential of highly selective A_3 adenosine receptor ligands in the central and peripheral nervous system. *Molecules (Basel, Switzerland)*, *27*(6), 1890. https://doi.org/10.3390/molecules27061890

Cristóvão-Ferreira, S., Navarro, G., Brugarolas, M., Pérez-Capote, K., Vaz, S. H., Fattorini, G., Conti, F., Lluis, C., Ribeiro, J. A., McCormick, P. J., Casadó, V., Franco, R., & Sebastião, A. M. (2013). A_1R-$A_{2A}R$ heteromers coupled to G_s and $G_{i/0}$ proteins modulate GABA transport into astrocytes. *Purinergic Signalling*, *9*(3), 433–449. https://doi.org/10.1007/s11302-013-9364-5

Cristóvão-Ferreira, S., Vaz, S. H., Ribeiro, J. A., & Sebastião, A. M. (2009). Adenosine A2A receptors enhance GABA transport into nerve terminals by restraining PKC inhibition of GAT-1. *Journal of Neurochemistry*, *109*(2), 336–347. https://doi.org/10.1111/j.1471-4159.2009.05963.x

Curtis, M. J., Alexander, S. P. H., Cirino, G., George, C. H., Kendall, D. A., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Patel, H. H., Sobey, C. G., Stanford, S. C., Stanley, P., Stefanska, B., Stephens, G. J., Teixeira, M. M., Vergnolle, N., & Ahluwalia, A. (2022). Planning experiments: Updated guidance on experimental design and analysis and their reporting III. *British Journal of Pharmacology*, *179*, 3907–3913. https://doi.org/10.1111/bph.15868

Dale, N., & Frenguelli, B. G. (2009). Release of adenosine and ATP during ischemia and epilepsy. *Current Neuropharmacology*, *7*(3), 160–179. https://doi.org/10.2174/157015909789152146

Diógenes, M. J., Neves-Tomé, R., Fucile, S., Martinello, K., Scianni, M., Theofilas, P., Lopatár, J., Ribeiro, J. A., Maggi, L., Frenguelli, B. G., Limatola, C., Boison, D., & Sebastião, A. M. (2014). Homeostatic control of synaptic activity by endogenous adenosine is mediated by adenosine kinase. *Cerebral Cortex (New York, N.Y.)*, *24*(1), 67–80. https://doi.org/10.1093/cercor/bhs284

Dunwiddie, T. V. (1980). Endogenously released adenosine regulates excitability in the in vitro hippocampus. *Epilepsia*, *21*(5), 541–548. https://doi.org/10.1111/j.1528-1157.1980.tb04305.x

Dunwiddie, T. V., Diao, L., Kim, H. O., Jiang, J. L., & Jacobson, K. A. (1997). Activation of hippocampal adenosine A_3 receptors produces a desensitization of A_1 receptor-mediated responses in rat hippocampus. *The Journal of Neuroscience*, *17*(2), 607–614. https://doi.org/10.1523/JNEUROSCI.17-02-00607.1997

Dunwiddie, T. V., & Masino, S. A. (2001). The role and regulation of adenosine in the central nervous system. *Annual Review of Neuroscience*, *24*, 31–55. https://doi.org/10.1146/annurev.neuro.24.1.31

Dyhrfjeld-Johnsen, J., Berdichevsky, Y., Swiercz, W., Sabolek, H., & Staley, K. J. (2010). Interictal spikes precede ictal discharges in an organotypic hippocampal slice culture model of epileptogenesis. *Journal of Clinical Neurophysiology*, *27*(6), 418–424. https://doi.org/10.1097/WNP.0b013e3181fe0709

Gao, Z. G., Auchampach, J. A., & Jacobson, K. A. (2023). Species dependence of A_3 adenosine receptor pharmacology and function. *Purinergic Signalling*, *19*(3), 523–550. https://doi.org/10.1007/s11302-022-09910-1

Glass, M., Faull, R. L., Bullock, J. Y., Jansen, K., Mee, E. W., Walker, E. B., Synek, B. J., & Dragunow, M. (1996). Loss of A1 adenosine receptors in human temporal lobe epilepsy. *Brain Research*, *710*(1-2), 56–68. https://doi.org/10.1016/0006-8993(95)01313-x

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Jacobson, K. A., & Gao, Z. G. (2006). Adenosine receptors as therapeutic targets. *Nature Reviews. Drug Discovery*, *5*(3), 247–264. https://doi.org/10.1038/nrd1983

Jacobson, K. A., Gao, Z. G., Matricon, P., Eddy, M. T., & Carlsson, J. (2022). Adenosine A2A receptor antagonists: From caffeine to selective non-xanthines. *British Journal of Pharmacology*, *179*(14), 3496–3511. https://doi.org/10.1111/bph.15103

Jacobson, K. A., Tosh, D. K., Jain, S., & Gao, Z. G. (2019). Historical and current adenosine receptor agonists in preclinical and clinical development. *Frontiers in Cellular Neuroscience*, *13*, 124. https://doi.org/10.3389/fncel.2019.00124

Javaid, S., Alqahtani, F., Ashraf, W., Anjum, S. M. M., Rasool, M. F., Ahmad, T., Alasmari, F., Alasmari, A. F., Alqarni, S. A., & Imran, I. (2023). Tiagabine suppresses pentylenetetrazole-induced seizures in mice and improves behavioral and cognitive parameters by modulating BDNF/TrkB expression and neuroinflammatory markers. *Biomedicine & Pharmacotherapy*, *160*, 114406. https://doi.org/10.1016/j.biopha.2023.114406

Kalilani, L., Sun, X., Pelgrims, B., Noack-Rink, M., & Villanueva, V. (2018). The epidemiology of drug-resistant epilepsy: A systematic review and meta-analysis. *Epilepsia*, *59*(12), 2179–2193. https://doi.org/10.1111/epi.14596

Kälviäinen, R. (2001). Long-term safety of tiagabine. *Epilepsia*, *42*(Suppl 3), 46–48. https://doi.org/10.1046/j.1528-1157.2001.042suppl.3046.x

Klotz, K. N. (2000). Adenosine receptors and their ligands. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *362*(4–5), 382–391. https://doi.org/10.1007/s002100000315

Kwan, P., Schachter, S. C., & Brodie, M. J. (2011). Drug-resistant epilepsy. *The New England Journal of Medicine*, *365*(10), 919–926. https://doi.org/10.1056/NEJMra1004418

Laudadio, M. A., & Psarropoulou, C. (2004). The A_3 adenosine receptor agonist 2-Cl-IB-MECA facilitates epileptiform discharges in the CA3 area of immature rat hippocampal slices. *Epilepsy Research*, *59*(2–3), 83–94. https://doi.org/10.1016/j.eplepsyres.2004.03.005

Li, A. H., Moro, S., Melman, N., Ji, X. D., & Jacobson, K. A. (1998). Structure–activity relationships and molecular modeling of 3,5-diacyl-2,4-dialkylpyridine derivatives as selective A_3 adenosine receptor antagonists. *Journal of Medicinal Chemistry*, *41*(17), 3186–3201. https://doi.org/10.1021/jm980093j

Lilley, E., Stanford, S. C., Kendall, D. E., Alexander, S. P. H., Cirino, G., Docherty, J. R., George, C. H., Insel, P. A., Izzo, A. A., Ji, Y., Panettieri, R. A., Sobey, C. G., Stefanska, B., Stephens, G., Teixeira, M., & Ahluwalia, A. (2020). ARRIVE 2.0 and the British Journal of Pharmacology: Updated guidance for 2020. *British Journal of Pharmacology*, *177*, 3611–3616. https://doi.org/10.1111/bph.15178

Liston, T. E., Hama, A., Boltze, J., Poe, R. B., Natsume, T., Hayashi, I., Takamatsu, H., Korinek, W. S., & Lechleiter, J. D. (2022). Adenosine A1R/A3R (adenosine A1 and A3 receptor) agonist AST-004 reduces brain infarction in a nonhuman primate model of stroke. *Stroke*, *53*(1), 238–248. https://doi.org/10.1161/STROKEAHA.121.036396

Liston, T. E., Hinz, S., Müller, C. E., Holstein, D. M., Wendling, J., Melton, R. J., Campbell, M., Korinek, W. S., Suresh, R. R., Sethre-Hofstad, D. A., Gao, Z. G., Tosh, D. K., Jacobson, K. A., & Lechleiter, J. D. (2020). Nucleotide $P2Y_1$ receptor agonists are in vitro and in vivo prodrugs of A_1/A_3 adenosine receptor agonists: Implications for roles of $P2Y_1$ and A_1/A_3 receptors in physiology and pathology. *Purinergic Signalling*, *16*(4), 543–559. https://doi.org/10.1007/s11302-020-09732-z

Lohse, M. J., Maurer, K., Gensheimer, H. P., & Schwabe, U. (1987). Dual actions of adenosine on rat peritoneal mast cells. *Naunyn-Schmiedeberg's Archives of Pharmacology*, *335*(5), 555–560. https://doi.org/10.1007/BF00169124

Löscher, W., & Klein, P. (2021). The pharmacology and clinical efficacy of antiseizure medications: From bromide salts to cenobamate and beyond. *CNS Drugs*, *35*(9), 935–963. https://doi.org/10.1007/s40263-021-00827-8

Magalhães, D. M., Pereira, N., Rombo, D. M., Beltrão-Cavacas, C., Sebastião, A. M., & Valente, C. A. (2018). Ex vivo model of epilepsy in organotypic slices—A new tool for drug screening. *Journal of Neuroinflammation*, *15*(1), 203. https://doi.org/10.1186/s12974-018-1225-2

Masocha, W., & Parvathy, S. S. (2016). Preventative and therapeutic effects of a GABA transporter 1 inhibitor administered systemically in a mouse model of paclitaxel-induced neuropathic pain. *PeerJ*, *4*, e2798. https://doi.org/10.7717/peerj.2798

McNeill, S. M., Baltos, J. A., White, P. J., & May, L. T. (2021). Biased agonism at adenosine receptors. *Cellular Signalling*, *82*, 109954. https://doi.org/10.1016/j.cellsig.2021.109954

Medina-Ceja, L., Sandoval-García, F., Morales-Villagrán, A., & López-Pérez, S. J. (2012). Rapid compensatory changes in the expression of EAAT-3 and GAT-1 transporters during seizures in cells of the CA1 and dentate gyrus. *Journal of Biomedical Science*, *19*(1), 78. https://doi.org/10.1186/1423-0127-19-78

Meldrum, B. S., & Chapman, A. G. (1999). Basic mechanisms of gabitril (tiagabine) and future potential developments. *Epilepsia*, *40*(Suppl 9), S2–S6. https://doi.org/10.1111/j.1528-1157.1999.tb02087.x

Miledi, R., Eusebi, F., Martínez-Torres, A., Palma, E., & Trettel, F. (2002). Expression of functional neurotransmitter receptors in *Xenopus* oocytes after injection of human brain membranes. *Proceedings of the National Academy of Sciences of the United States of America*, *99*(20), 13238–13242. https://doi.org/10.1073/pnas.192445299

Miledi, R., Palma, E., & Eusebi, F. (2006). Microtransplantation of neurotransmitter receptors from cells to *Xenopus* oocyte membranes: New procedure for ion channel studies. *Methods in Molecular Biology (Clifton, N.J.)*, *322*, 347–355. https://doi.org/10.1007/978-1-59745-000-3_24

Naylor, D. E. (2023). In the fast lane: Receptor trafficking during status epilepticus. *Epilepsia Open*, *8*(Suppl 1), S35–S65. https://doi.org/10.1002/epi4.12718

Nguyen, A. T. N., Tran, Q. L., Baltos, J. A., McNeill, S. M., Nguyen, D. T. N., & May, L. T. (2023). Small molecule allosteric modulation of the adenosine A_1 receptor. *Frontiers in Endocrinology*, *14*, 1184360. https://doi.org/10.3389/fendo.2023.1184360

Noe, F. M., Polascheck, N., Frigerio, F., Bankstahl, M., Ravizza, T., Marchini, S., Beltrame, L., Banderó, C. R., Löscher, W., & Vezzani, A. (2013). Pharmacological blockade of IL-1β/IL-1 receptor type 1 axis during epileptogenesis provides neuroprotection in two rat models of temporal lobe epilepsy. *Neurobiology of Disease*, *59*, 183–193. https://doi.org/10.1016/j.nbd.2013.07.015

Palma, E., Trettel, F., Fucile, S., Renzi, M., Miledi, R., & Eusebi, F. (2003). Microtransplantation of membranes from cultured cells to *Xenopus* oocytes: A method to study neurotransmitter receptors embedded in native lipids. *Proceedings of the National Academy of Sciences of the United States of America*, *100*(5), 2896–2900. https://doi.org/10.1073/pnas.0438006100

Palumbo, L., Carinci, M., Guarino, A., Asth, L., Zucchini, S., Missiroli, S., Rimessi, A., Pinton, P., & Giorgi, C. (2023). The NLRP3 inflammasome in neurodegenerative disorders: Insights from epileptic models. *Biomedicine*, *11*(10), 2825. https://doi.org/10.3390/biomedicines11102825

Percie du Sert, N., Hurst, V., Ahluwalia, A., Alam, S., Avey, M. T., Baker, M., Browne, W. J., Clark, A., Cuthill, I. C., Dirnagl, U., Emerson, M., Garner, P., Holgate, S. T., Howells, D. W., Karp, N. A., Lazic, S. E., Lidster, K., MacCallum, C. J., Macleod, M., … Würbel, H. (2020). The ARRIVE guidelines 2.0: Updated guidelines for reporting animal research. *PLoS Biology*, *18*(7), e3000410. https://doi.org/10.1371/journal.pbio.3000410

Racine, R. J. (1972). Modification of seizure activity by electrical stimulation: II. Motor seizure. *Electroencephalography and Clinical*

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Neurophysiology, *32*(3), 281–294. https://doi.org/10.1016/0013-4694(72)90177-0

Reddy, D. S., & Kuruba, R. (2013). Experimental models of status epilepticus and neuronal injury for evaluation of therapeutic interventions. *International Journal of Molecular Sciences*, *14*(9), 18284–18318. https://doi.org/10.3390/ijms140918284

Richerson, G. B. (2004). Looking for GABA in all the wrong places: The relevance of extrasynaptic $GABA_A$ receptors to epilepsy. *Epilepsy Currents*, *4*(6), 239–242. https://doi.org/10.1111/j.1535-7597.2004.46008.x

Rombo, D. M., Ribeiro, J. A., & Sebastião, A. M. (2018). Role of adenosine receptors in epileptic seizures. In P. Borea, K. Varani, S. Gessi, S. Merighi, & F. Vincenzi (Eds.), *The adenosine receptors* (Vol. 34). Humana Press. https://doi.org/10.1007/978-3-319-90808-3_13

Roseti, C., Martinello, K., Fucile, S., Piccari, V., Mascia, A., Di Gennaro, G., Quarato, P. P., Manfredi, M., Esposito, V., Cantore, G., Arcella, A., Simonato, M., Fredholm, B. B., Limatola, C., Miledi, R., & Eusebi, F. (2008). Adenosine receptor antagonists alter the stability of human epileptic $GABA_A$ receptors. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(39), 15118–15123. https://doi.org/10.1073/pnas.0807277105

Roseti, C., Palma, E., Martinello, K., Fucile, S., Morace, R., Esposito, V., Cantore, G., Arcella, A., Giangaspero, F., Aronica, E., Mascia, A., Di Gennaro, G., Quarato, P. P., Manfredi, M., Cristalli, G., Lambertucci, C., Marucci, G., Volpini, R., Limatola, C., & Eusebi, F. (2009). Blockage of A_{2A} and A_3 adenosine receptors decreases the desensitization of human $GABA_A$ receptors microtransplanted to *Xenopus* oocytes. *Proceedings of the National Academy of Sciences of the United States of America*, *106*(37), 15927–15931. https://doi.org/10.1073/pnas.0907324106

Roseti, C., van Vliet, E. A., Cifelli, P., Ruffolo, G., Baayen, J. C., Di Castro, M. A., Bertollini, C., Limatola, C., Aronica, E., Vezzani, A., & Palma, E. (2015). $GABA_A$ currents are decreased by IL-1β in epileptogenic tissue of patients with temporal lobe epilepsy: Implications for ictogenesis. *Neurobiology of Disease*, *82*, 311–320. https://doi.org/10.1016/j.nbd.2015.07.003

Ruffolo, G., Cifelli, P., Miranda-Lourenço, C., De Felice, E., Limatola, C., Sebastião, A. M., Diógenes, M. J., Aronica, E., & Palma, E. (2020). Rare diseases of neurodevelopment: Maintain the mystery or use a dazzling tool for investigation? The case of Rett syndrome. *Neuroscience*, *439*, 146–152. https://doi.org/10.1016/j.neuroscience.2019.06.015

Sakurai, M., Suzuki, H., Tomita, N., Sunden, Y., Shimada, A., Miyata, H., & Morita, T. (2018). Enhanced neurogenesis and possible synaptic reorganization in the piriform cortex of adult rat following kainic acid-induced status epilepticus. *Neuropathology*, *38*(2), 135–143. https://doi.org/10.1111/neup.12445

Sandau, U. S., Colino-Oliveira, M., Jones, A., Saleumvong, B., Coffman, S. Q., Liu, L., Miranda-Lourenço, C., Palminha, C., Batalha, V. L., Xu, Y., Huo, Y., Diógenes, M. J., Sebastião, A. M., & Boison, D. (2016). Adenosine kinase deficiency in the brain results in maladaptive synaptic plasticity. *The Journal of Neuroscience*, *36*(48), 12117–12128. https://doi.org/10.1523/JNEUROSCI.2146-16.2016

Sebastião, A. M., & Ribeiro, J. A. (2009). Adenosine receptors and the central nervous system. *Handbook of Experimental Pharmacology*, *193*, 471–534. https://doi.org/10.1007/978-3-540-89615-9_16

Sebastião, A. M., & Ribeiro, J. A. (2023). Adjusting the brakes to adjust neuronal activity: Adenosinergic modulation of GABAergic transmission. *Neuropharmacology*, *236*, 109600. https://doi.org/10.1016/j.neuropharm.2023.109600

Sebastião, A. M., Stone, T. W., & Ribeiro, J. A. (1990). The inhibitory adenosine receptor at the neuromuscular junction and hippocampus of the rat: Antagonism by 1,3,8-substituted xanthines. *British Journal of Pharmacology*, *101*(2), 453–459. https://doi.org/10.1111/j.1476-5381.1990.tb12729.x

Segall, M. D. (2012). Multi-parameter optimization: Identifying high quality compounds with a balance of properties. *Current Pharmaceutical Design*, *18*(9), 1292–1310. https://doi.org/10.2174/138161212799436430

Sills, G. J., & Rogawski, M. A. (2020). Mechanisms of action of currently used antiseizure drugs. *Neuropharmacology*, *168*, 107966. https://doi.org/10.1016/j.neuropharm.2020.107966

Spanoghe, J., Larsen, L. E., Craey, E., Manzella, S., Van Dycke, A., Boon, P., & Raedt, R. (2020). The signaling pathways involved in the anticonvulsive effects of the adenosine A_1 receptor. *International Journal of Molecular Sciences*, *22*(1), 320. https://doi.org/10.3390/ijms22010320

Su, J., Yin, J., Qin, W., Sha, S., Xu, J., & Jiang, C. (2015). Role for pro-inflammatory cytokines in regulating expression of GABA transporter type 1 and 3 in specific brain regions of kainic acid-induced status epilepticus. *Neurochemical Research*, *40*(3), 621–627. https://doi.org/10.1007/s11064-014-1504-y

Świąder, M. J., Kotowski, J., & Łuszczki, J. J. (2014). Modulation of adenosinergic system and its application for the treatment of epilepsy. *Pharmacological Reports: PR*, *66*(3), 335–342. https://doi.org/10.1016/j.pharep.2013.10.005

Tescarollo, F. C., Rombo, D. M., DeLiberto, L. K., Fedele, D. E., Alharfoush, E., Tomé, Â. R., Cunha, R. A., Sebastião, A. M., & Boison, D. (2020). Role of adenosine in epilepsy and seizures. *Journal of Caffeine and Adenosine Research*, *10*(2), 45–60. https://doi.org/10.1089/caff.2019.0022

Tosh, D. K., Crane, S., Chen, Z., Paoletta, S., Gao, Z. G., Gizewski, E., Auchampach, J. A., Salvemini, D., & Jacobson, K. A. (2015). Rigidified A3 adenosine receptor agonists: 1-deazaadenine modification maintains high in vivo efficacy. *ACS Medicinal Chemistry Letters*, *6*(7), 804–808. https://doi.org/10.1021/acsmedchemlett.5b00150

Tosh, D. K., Deflorian, F., Phan, K., Gao, Z. G., Wan, T. C., Gizewski, E., Auchampach, J. A., & Jacobson, K. A. (2012). Structure-guided design of A_3 adenosine receptor-selective nucleosides: Combination of 2-arylethynyl and bicyclo[3.1.0]hexane substitutions. *Journal of Medicinal Chemistry*, *55*(10), 4847–4860. https://doi.org/10.1021/jm300396n

Tosh, D. K., Paoletta, S., Deflorian, F., Phan, K., Moss, S. M., Gao, Z. G., Jiang, X., & Jacobson, K. A. (2012). Structural sweet spot for A_1 adenosine receptor activation by truncated (N)-methanocarba nucleosides: Receptor docking and potent anticonvulsant activity. *Journal of Medicinal Chemistry*, *55*(18), 8075–8090. https://doi.org/10.1021/jm300965a

Tosh, D. K., Rao, H., Bitant, A., Salmaso, V., Mannes, P., Lieberman, D. I., Vaughan, K. L., Mattison, J. A., Rothwell, A. C., Auchampach, J. A., Ciancetta, A., Liu, N., Cui, Z., Gao, Z. G., Reitman, M. L., Gavrilova, O., & Jacobson, K. A. (2019). Design and in vivo characterization of A_1 adenosine receptor agonists in the native ribose and conformationally constrained (N)-methanocarba series. *Journal of Medicinal Chemistry*, *62*(3), 1502–1522. https://doi.org/10.1021/acs.jmedchem.8b01662

Valente, C. A., Meda, F. J., Carvalho, M., & Sebastião, A. M. (2021). A model of epileptogenesis in rhinal cortex-hippocampus organotypic slice cultures. *Journal of Visualized Experiments: JoVE*, *169*. https://doi.org/10.3791/61330

Vezzani, A., Di Sapia, R., Kebede, V., Balosso, S., & Ravizza, T. (2023). Neuroimmunology of status epilepticus. *Epilepsy & Behavior: E&B*, *140*, 109095. https://doi.org/10.1016/j.yebeh.2023.109095

Vismer, M. S., Forcelli, P. A., Skopin, M. D., Gale, K., & Koubeissi, M. Z. (2015). The piriform, perirhinal, and entorhinal cortex in seizure generation. *Frontiers in Neural Circuits*, *9*, 27. https://doi.org/10.3389/fncir.2015.00027

Von Lubitz, D. K., Lin, R. C., Boyd, M., Bischofberger, N., & Jacobson, K. A. (1999). Chronic administration of adenosine A_3 receptor agonist and cerebral ischemia: Neuronal and glial effects. *European Journal of*

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License

Pharmacology, *367*(2–3), 157–163. https://doi.org/10.1016/s0014-2999(98)00977-7

Wan, T. C., Tampo, A., Kwok, W. M., & Auchampach, J. A. (2019). Ability of CP-532,903 to protect mouse hearts from ischemia/reperfusion injury is dependent on expression of A_3 adenosine receptors in cardiomyocytes. *Biochemical Pharmacology*, *163*, 21–31. https://doi.org/10.1016/j.bcp.2019.01.022

Xiao, C., Liu, N., Jacobson, K. A., Gavrilova, O., & Reitman, M. L. (2019). Physiology and effects of nucleosides in mice lacking all four adenosine receptors. *PLoS Biology*, *17*(3), e3000161. https://doi.org/10.1371/journal.pbio.3000161

How to cite this article: Ghosh, A., Ribeiro-Rodrigues, L., Ruffolo, G., Alfano, V., Domingos, C., Rei, N., Tosh, D. K., Rombo, D. M., Morais, T. P., Valente, C. A., Xapelli, S., Bordadágua, B., Rainha-Campos, A., Bentes, C., Aronica, E., Diógenes, M. J., Vaz, S. H., Ribeiro, J. A., Palma, E., … Sebastião, A. M. (2024). Selective modulation of epileptic tissue by an adenosine A_3 receptor-activating drug. *British Journal of Pharmacology*, 1–21. https://doi.org/10.1111/bph.17319

14765381, 0, Downloaded from https://bpspubs.onlinelibrary.wiley.com/doi/10.1111/bph.17319 by CochraneItalia, Wiley Online Library on [20/09/2024]. See the Terms and Conditions (https://onlinelibrary.wiley.com/terms-and-conditions) on Wiley Online Library for rules of use; OA articles are governed by the applicable Creative Commons License