Selective modulation of epileptic tissue by an adenosine A3 receptor-activating drug

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Abstract

Background and Purpose Adenosine, through the A1 receptor (A1R), is an endogenous anticonvulsant. Development of adenosine receptor agonists as antiseizure medications has been hampered by their cardiac side effects. A moderately A1R-selective agonist, MRS5474, has been reported to suppress seizures without considerable cardiac action. Hypothesizing that this drug could act through other than A1R 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, [3H]GABA uptake and GABAergic currents were recorded from rodent or human hippocampal tissue. Alterations in adenosine A3 receptor (A3R) density in human tissue were assessed by Western Blot. Key Results MRS5474 (50-500nM) was devoid of effect upon rodent excitatory synaptic signals in hippocampal slices, except when hyperexcitability was previously induced in vivo or ex vivo. This contrasted with the effect of other A1R agonists. MRS5474 inhibited GAT-1 mediated GABA uptake, an action not blocked by an A1R antagonist but blocked by an A3R antagonist and mimicked by an A3R agonist. A3R 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 hippocampal membranes prepared from epileptic hippocampal tissue but not from non-epileptic tissue, an action blocked by an A3R antagonist. Conclusion and Implications We identified a drug that activates A3R and has selective actions on epileptic hippocampal tissue. This underscores A3R as a promising target for the development of antiseizure medications.

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Running Title: Adenosine A_3R as disease-selective targets for antiseizure medication

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Bullet point summary (this is a requisite from the Journal. No more than 2 bullets per heading (headings defined by the Journal); no more than 15 words per bullet):

What is already known:

Allostatic and antiseizure action of endogenous adenosine upon synaptic signalling.

Antiseizure effect of a moderately selective agonist of inhibitory adenosine A_1 receptors (A_1R), MRS5474.

Author Contribution: AMS conceived the study; KAJ, EP, GR, MJD and JAR contributed to the discussion of the ongoing experiments; DKT synthesized MRS5474; AG and AMS performed the fEPSP recording experiments and the GABA uptake studies; GABA uptake studies also received input from NR, TPM and SHV; LR-R prepared the organotypic slices cultures, being trained in this by CAV, and performed the field potential recordings of organotypic slices; LR-R also performed Western Blot and induced the *in vivo* model of epilepsy, being trained in this by SX. LR-R and BB created the program to analyze the field potential recordings of organotypic slices. GR, AG and VA performed the experiments involving recordings of GABAergic currents from oocytes injected with human tissue samples; CD and DMR performed the EPSC recording experiments; AR-C, CB and EA were involved in the selection of human tissue samples making them available for the study. AMS, KAJ, EP, AG, LR-R, GR and CD contributed to MS writing, conceived Tables and Figures. All authors read the final version of the MS contributing to its final form.

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Abstract

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Key Results

MRS5474 (50-500nM) was devoid of effect upon rodent excitatory synaptic signals in hippocampal slices, except when hyperexcitability was previously induced *in vivo* or *ex vivo* . This contrasted with the effect of other A_1R agonists. MRS5474 inhibited GAT-1 mediated 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 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; GABAergic transmission; epilepsy; hippocampus, neuronal excitability.

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Methods

Animals

All procedures complied with 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). The animals (Wistar rats, Sprague-Dawley rats and C57BL/6J strain mice, as specified below) were housed in a strictly controlled iMM rodent facility, as authorized by the Portuguese authority for Animal Welfare (*Direção-Geral de Alimentação e Veterinária - DGAV). The work using female Xenopus laevis frogs to obtain oocytes (Sapienza University, Rome, Italy) conformed to institutional policies and guidelines of the Italian Ministry of Health (no. authorization 427/2020-PR).*

Preparation of acute hippocampal slices

For fEPSP recordings and GABA uptake studies, the hippocampus (6-12 weeks-old Sprague-Dawley rats or C57BL/6 mice as specified in results section) were dissected within ice-cold artificial cerebrospinal fluid (aCSF) solution composed of (mM): NaCl 124, KCl 3, $NaHCO_3$ 25, $NaH_2PO_4.H_2O$ 1.2, $MgSO_4$ 1, $CaCl_2$ 2; and glucose monohydrate 10, 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) and allowed to recover functionally and energetically for at least 1 hour in a resting chamber filled with the same solution, at room temperature (RT) and continuously gassed. For patch clamp recordings, the slices (300 µM thick from 5-8 weeks old Wistar rats) were cut with a vibratome (Leica VT 1000S, Leica Microsystems) in the ice-cold dissecting solution containing (in mM): sucrose 110; KCl 2.5; $CaCl_2$ 0.5; $MgCl_2$ 7; $NaHCO_3$ 25; NaH_2PO_4 1.25; glucose monohydrate 7, oxygenated with 95% O_2 and 5% CO_2, pH 7.4; after dissection, the slices were incubated for 20 minutes at 35°C in aCSF and then transferred to a resting chamber at RT for at least 1 hour before use.

Field excitatory postsynaptic potentials (fEPSPs) recordings

Acute hippocampal slices were individually transferred into a submerged recording chamber over the nylon mesh and continually superfused with gassed aCSF solution at a constant flow (3 mL/min) and temperature (32 °C). Stimulation (rectangular 0.1 ms pulses, once every 15 seconds, S48 Square Pulse Stimulator, Grass Instruments) was delivered through a concentric bipolar electrode placed on Schaffer collateral-commissural fibbers, in the *stratum radiatum* near the CA3-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) and digitized BNC-2110 (National Instruments). Individual responses were monitored, and averages of 8 consecutive responses were continuously stored on a personal computer with the WinLTP software (Anderson and Collingridge, 2007). Test drugs were added to the superfusing aCSF after obtaining a stable baseline of recordings for at least 15 minutes.

Preparation of organotypic rhinal-hippocampal slice cultures

Organotypic rhinal-hippocampal slice cultures were prepared from Sprague-Dawley rats (6 to 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 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, was meticulously separated and sliced (350 μm thick) transversely using a McIlwain tissue chopper. Four slices were then transferred to porous insert membranes (0.4 μm) (PICM 03050, Millipore, Bedford, MA) placed in each well of six-well culture trays (Corning Costar, Corning, NY). 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, Massachusetts, USA), 25 mM D-(+)-glucose (45% in water, Sigma), 30 μg/mL Gentamycin solution (50 mg/mL, 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, 1mM L-glutamine, 30 μg/mL gentamycin (all from Thermo Fisher).

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 (γ-aminobutyric acid) were purchased from Sigma. Kainic acid, DPCPX (8-cyclopentyl-1,3-dipropylxanthine, A_1R selective antagonist) and CPA (N^6-cyclopentyladenosine, selective A_1R agonist), CCPA (2-chloro-N^6-cyclopentyladenosine, selective A_1R agonist) and picrotoxin ($GABA_A$R antagonist) were from Tocris (Bristol, England). Pentobarbital (Euthasol® 400 mg/mL) was from Dechra, Northwick, England). 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 NIH (Jacobson group) as reported (Tosh et al., 2012a,b, 2015). 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 deionized 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.

Statistical Analysis

All data are presented as mean ± standard error of the mean (mean ± SEM) of n experiments, where n corresponds to the number of independent observations. The statistical significance of the differences was assessed using the Student's t-test, One-way ANOVA, or Two-way ANOVA as specified in the legends of Figures and Tables. Statistical analyses were performed using GraphPad Prism 8.0 (Dotmatics, Boston, MA, USA) or the SigmaPlot 15 (Inpixon HQ, Palo Alto, CA, USA) softwares**.** Differences were considered significant when $P<0.05$ (two-tailed)**.**

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Results

MRS5474, in contrast to a canonical A_1receptor 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_1R 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_1R (Carlin et al., 2017), was virtually devoid of effect upon excitatory postsynaptic currents (EPSCs) recorded by patch clamp from CA1 pyramidal cells in the whole cell configuration (Figure**1**). At the end of the perfusion with MRS5474 (120nM), 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-B**) 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% of pre-dug 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), 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=0.4353, Figure **1C**). In contrast to the absence of effect of MRS5474, the canonical A_1R agonist, CCPA (30 nM) clearly inhibited the EPSC peak amplitude within 3–5 minutes after its application, being the maximal effect attained after about 20 minutes (70 ± 5.2% inhibition, n=4, P<0.05, Figure**1D**).

Next, we evaluated if MRS5474 could inhibit excitatory synaptic transmission under less restrictive recording conditions – field EPSP recordings from hippocampal slices, and in the same rodent where the antiseizure action was detected, i.e., the mouse. We tested concentrations ranging from 120 nM up 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 nM, 250 nM and 500 nM: with inhibition of 11 ± 10.2%, -2.7 ± 9.9% & -9.3 ± 10.6% respectively, Figure**2A**). Under similar experimental conditions, the canonical A_1R agonist, CPA (30nM), caused the expected inhibition of fEPSPs (-50 ± 9.7% inhibition, n=2, P<0.05, Figure 2B).

MRS5474 inhibited GABA uptake through an adenosine A_3 receptor

GABA transporters (GAT), in particular the GABA transporter type-1 (GAT-1) are well-known targets for antiseizure medications (Meldrum and Chapman, 1999; Sills and Rogawski, 2020). Importantly, recent evidence suggests that GAT-1 inhibitors not only 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. Since GAT-1 is present in nerve endings and astrocytes, we tested the effect of MRS5474 in slices, which enabled us to evaluate the effect upon GABA transport independently of its specific location. In slices in the presence of MRS5474 (50 nM,) the GAT-1 mediated uptake of [^{3}H]GABA was significantly lower than under control conditions (% inhibition: 47.3±6.5%, n=15, Figure **5A and C).** This effect of MRS5474 at 50nM was not maximal since higher concentrations caused stronger inhibition (120nM: 89±5.9%, n=6; 250nM: 96±2.3%, n=4) of GAT-1 mediated [^{3}H]GABA uptake. Unexpectedly, the presence of the A_1R antagonist, DPCPX, used at a concentration (50 nM) 100 times higher than its Ki value for A_1R (Lohse et al., 1987), and added 30 minutes before MRS5474, did not prevent the action of MRS5474 (n=4, P>0.05, Figure **5D**). In contrast, the inhibition was fully prevented in experiments where MRS1523 (10 μM), an A_3R antagonist (Li et al., 1998), was 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_3R. Neither DPCPX (50nM) nor MRS1523 (10μM) significantly (P>0.05) affected GAT-1 activity as compared with controls (no drug) in the same experiments (Figure 5 E-F). To further assess the involvement of A_3R upon GAT-1 mediated GABA uptake, we tested the action of a selective A_3R agonist, MRS5698 (Ki [?]3nM in human or mouse, A_{2A}R; Tosh et al., 2012b). As shown in Figure 5 (**B**–**D**), in the presence of MRS5698 (100 nM), GAT-mediated GABA uptake was significantly lower than in its absence (% inhibition: 47.7±11.1%,

n=8, P<0.05). The mimicry of the effect of MRS5474 by a selective A_3R agonist, together with the blockade of the effect of MRS5474 by an A_3R antagonist but not by a selective A_1R antagonist, strongly suggest that the effect of MRS5474 is mediated by A_3R rather than A_1R.

Enhanced A_3R immunoreactivity ***in hippocampal tissue from epileptic patients***

The unexpected finding that an antiseizure drug activates A $_3R$, 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 $_3R$. To address this possibility, we used human hippocampal samples from 8 patients with drug-resistant epilepsy who underwent focal hippocampal resection (clinical details in Table 2). As controls, we used 6 human hippocampal samples obtained post-mortem (post-mortem delay [?] 10h, details in Table 2). Western blot analysis revealed enhanced A $_3R$ immunoreactivity in samples from epileptic patients when compared with control samples (P<0.05, Figure 6).

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

not-yet-known not-yet-known not-yet-known unknown

Parameter	Number of bursts	Frequency of spikes within burst (Hz)	Amplitude of spikes within bu
Analysis time (min)	50-60	80-90	50-60
Depolarization Conditions			
No Drug (n=11)	1.78±0.61	2.61±1.17	1.02±0.11
MRS5474 (n=11)	0.64±0.15	0.59±0.21*	0.74±0.08*
Non-depolarization Conditions			
No Drug (n=7)	2.36±0.66	1.68±0.63	1.01±0.21
MRS5474 (n=7)	1.01±0.27	0.72±0.26	1.73±0.65

No Drug: refers to data from slices not treated with any drug. MRS5474: refers to data from slices in the presence of MRS5474 (250nM) for 30 minutes before first analysis time (50–60 minutes). In all cases and for all parameters, the analysis at time 10–20 (baseline, pre-drug condition) in each slice was normalised to 1. Statistically significant effects of MRS5474 when compared with pre-drug condition in the same slices are highlighted in red; *P<0.05, **P<0.01 (one-way ANOVA followed by Dunnett's multiple comparisons test). When both time and drug condition were simultaneously analyzed as independent variables, an effect of MRS5474 upon number of bursts also emerged (highlighted in blue, *P<0.05, comparison between the same time frame within slices, in the absence or presence of the drug; two-way ANOVA followed by Sidak's multiple comparison test), while the effect of MRS5474 vs baseline in the frequency of bursts still hold significance. Note that 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), since 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.

Table 2. Clinical information of tissue samples from patients who underwent hippocampal focal resection (EHS1-8) or from autopsy (CHS1-6), used for Western Blotting.

not-yet-known not-yet-known not-yet-known unknown

Sample	Age	Sex	Lesions	Laterality of resection	Primary Cause of death	Post-mortem dela
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

Sample	Age	Sex	Lesions	Laterality of resection	Primary Cause of death	Post-mortem dela
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

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(yrs)/sex	Epilepsy onset	Surgical zone, hippocampus	Seizure type	Seizures/ month	Patho
#1	41/M	20	R-T	FIAS/GS	10	HS
#2	44/M	27	R-T	FIAS	4	HS
#3	52/M	10	L-T	FIAS	1	HS
#4	63/F	n.a.	R-T	n.a.	n.a.	myoca

Abbreviations: M, male; F, female; R, right; L, left; T, temporal; HS, hippocampal sclerosis; FIAS, focal impaired awareness seizures; GS, generalized seizures; CBZ, carbamazepine; PB, phenobarbital; TPM, topiramate; VPA, valproic acid; n.a., non-applicable.

Figure Legends

Figure. 1. MRS5474 did not affect excitatory postsynaptic currents (EPSCs) in CA1 pyramidal cells at hippocampal slices . Time-course changes of EPSC peak amplitude are shown in**A** (n=6 cells, from 5 animals), **B** (n=5 cells, from 4 animals; 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 4 animals). EPSC peak amplitude was normalized in each experiment taking as 100% baseline the values recorded for 10 minutes before drug application (1); drug effects were assessed by comparing baseline values with values recorded at 30–40 minutes 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 **B** are shown paired EPSC amplitude (pA) of all cells in **A** , in baseline (period 1 indicated in **A**) and under MRS5474 (period 2 indicated**A** ; 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_1R agonist, CCPA (**C**) caused a marked inhibition of EPCs in clear contrast to the absence of effect of MRS5474 (**A, B**).

Figure 2. MRS5474 did not affect field excitatory post synaptic potentials (fEPSPs) in hippocampal slices in control conditions, in clear contrast with the A1R agonist, CPA. In panels **A** and**B** are shown normalized averaged time-course changes in fEPSP slopes (%, mean ± SEM) taking as baseline (100%) the values recorded for 10 minutes before drug application. 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 fEPSPs traces recorded from the same slice before (control) and by the end of (**A**) MRS5474 (250nM) or (**B**) CPA (30 nM) application.**A** : n=7 slices from 6 animals. **B** : n=4 slices from 4 animals. Note that the selective A1R agonist, CPA (**B**) caused a marked inhibition of fEPSPs in clear contrast to the absence of effect of MRS5474 (**A**).

Figure 3 . **MRS5474 inhibited spontaneous activity in organotypic rhinal-hippocampal slices**

under depolarizing condition. In**A** and **B** representative field potential recordings are shown of spontaneous activity from 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**). 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 time scale is different in both upper and lower panels. Panels **C** and **D** show the mean frequency of spikes within the bursts at the times indicated below each bar; data obtained at 10–20 minutes was in the absence of MRS5474 (basal ictal discharge) and was normalized to 1, whereas data at 50–60 minutes or 80–90 minutes was either in the absence (**C**) or in the presence (**D**) of MRS5474 (250nM). Data is 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; **P<0.01. One-way ANOVA followed by Dunnett's multiple comparisons test.

Figure 4. MRS5474 decreased fEPSPs in hippocampal slices taken from animals with established epilepsy (EE). MRS5474 application in A an animal with EE or in B a control animal. 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 minutes 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/ms) from all experiments with similar protocols as those shown on the left, 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).

Fig.1

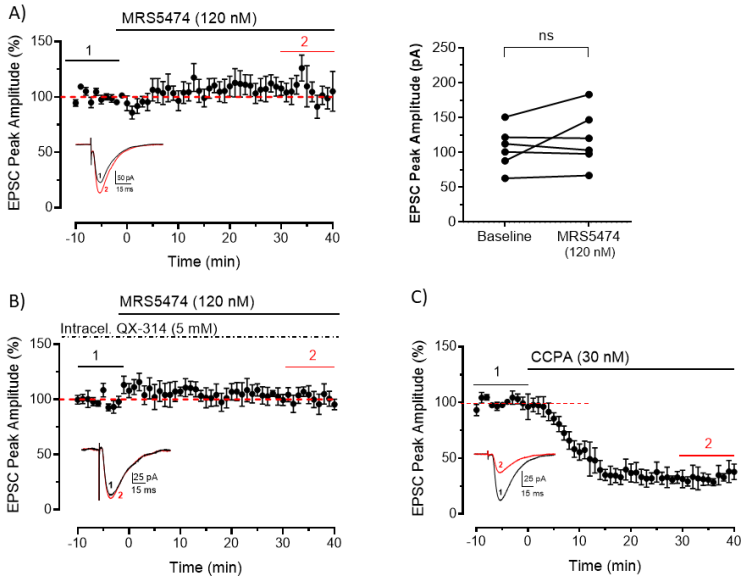


Fig.2

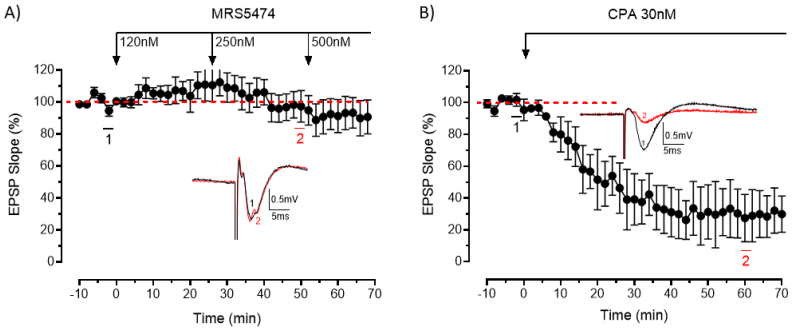


Fig. 3

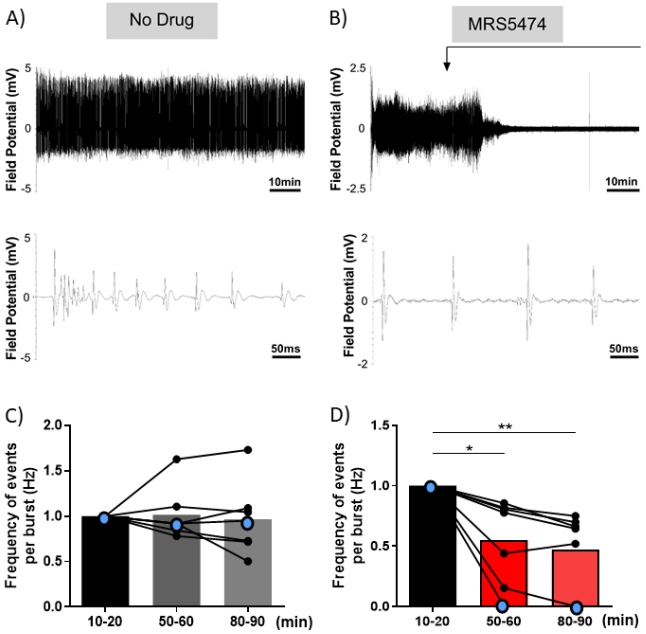


Fig. 4

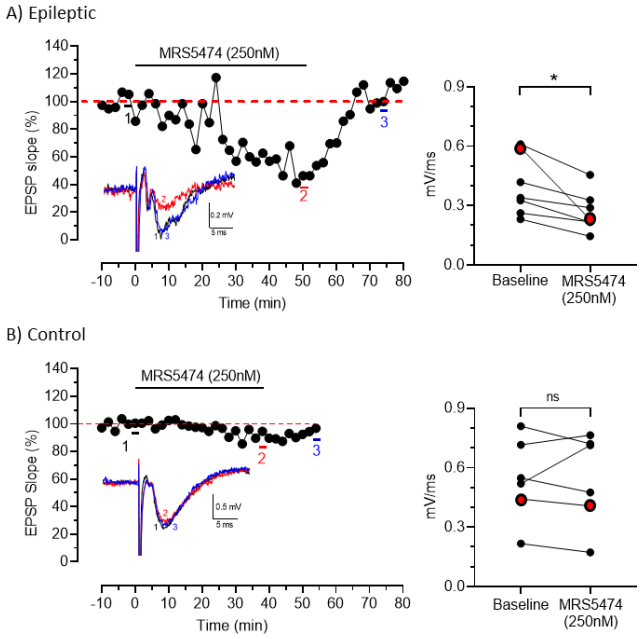


Fig. 5

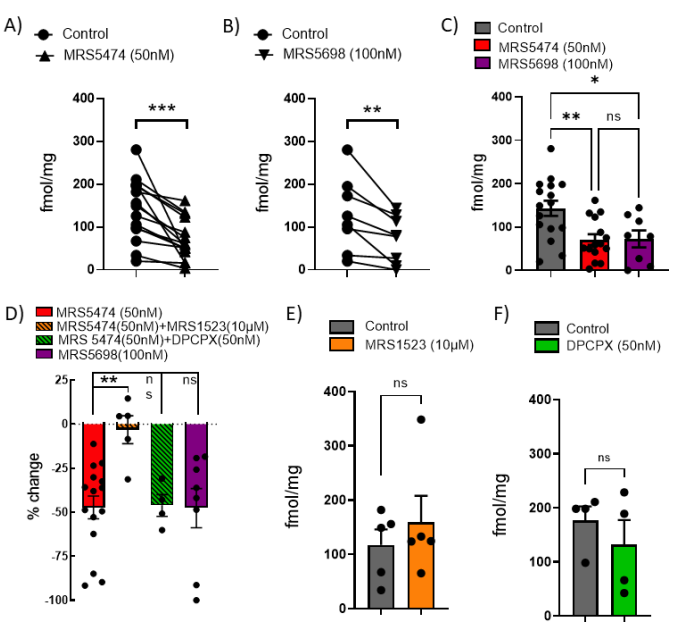


Fig. 6

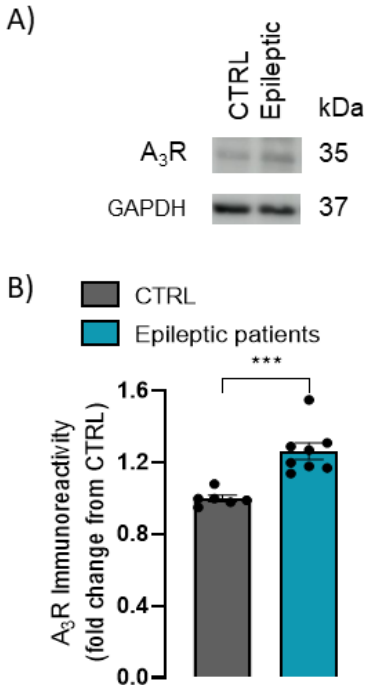
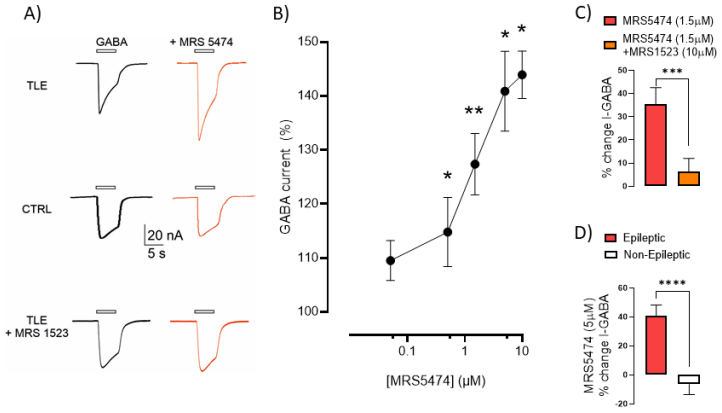


Fig. 7



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