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Pharmacological activation of mGlu5 receptors with the positive allosteric modulator VU0360172, modulates thalamic GABAergic transmission

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HIGHLIGHTS

- VU0360172 is an mGlu5 receptor positive allosteric modulator.
- VU0360172 increases GABA uptake and reduces the tonic GABA current in the thalamus.
- These effects selectively occur in the thalamus and not in the somatosensory cortex.
- These effects may contribute to the anti-absence effects of VU0360172.

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ABSTRACT

Previous studies have shown that injection of the mGlu5 receptor positive allosteric modulator (PAM) VU0360172 into either the thalamus or somatosensory cortex markedly reduces the frequency of spike-and-wave discharges (SWDs) in the WAG/Rij model of absence epilepsy. Here we have investigated the effects of VU0360172 on GABA transport in the thalamus and somatosensory cortex, as possible modes of action underlying the suppression of SWDs. Systemic VU0360172 injections increase GABA uptake in thalamic synaptosomes from epileptic WAG/Rij rats. Consistent with this observation, VU0360172 could also enhance thalamic GAT-1 protein expression, depending on the dosing regimen. This increase in GAT-1 expression was also observed in the thalamus from non-epileptic rats (presymptomatic WAG/Rij and Wistar) and appeared to occur selectively in neurons. The tonic $GABA_A$ receptor current present in ventrobasal thalamocortical neurons was significantly reduced by VU0360172 consistent with changes in GAT-1 and GABA uptake. The *in vivo* effects of VU0360172 (reduction in tonic GABA current and increase in GAT-1 expression) could be reproduced *in vitro* by treating thalamic slices with VU0360172 for at least 1 h and appeared to be dependent on the activation of PLC. Thus, the effects of VU0360172 do not require an intact thalamocortical circuit. In the somatosensory cortex, VU0360172 reduced GABA uptake but did not cause significant changes in GAT-1 protein levels. These findings reveal a novel mechanism of regulation mediated by mGlu5 receptors, which could underlie the powerful anti-absence effect of mGlu5 receptor enhancers in animal models.

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1. Introduction

Absence epilepsy is a form of generalized epilepsy characterized by sudden and transient episodes of behavioural arrest, which are associated with bilateral symmetrical spike-and-wave discharges (SWDs) in the electroencephalogram (EEG). SWDs are generated in a cortico-thalamo-cortical network, which includes thalamo-cortical neurons, pyramidal neurons of the somatosensory cortex, and GABAergic neurons of the reticular thalamic (RT) nucleus projecting to the ventrobasal (VB) thalamus. It has been proposed that pathological oscillations develop when enhanced activity at the GABAergic synapses between RT and VB neurons prevents deactivation of T-type voltage-sensitive Ca^{2+} channels resulting in on-going generation of action potentials in thalamo-cortical (TC) neurons (Meeren et al., 2002; Blumenfeld, 2005). Drugs that are currently used for the treatment of absence epilepsy include the T-type Ca^{2+} channel blocker ethosuximide, and valproic acid, lamotrigine and clonazepam. All these drugs can be effective, but their use is limited by class-related adverse effects. Moreover, over 20–30% of patients with absence epilepsy are resistant to pharmacological treatment or experience intolerable adverse effects (Mikati and Holme, 1997; Panayiotopoulos, 1999; Ollivier et al., 2009; Tenney and Glauser, 2013). These problems have encouraged the development of novel therapeutic strategies. One such strategy is to target metabotropic glutamate (mGlu) receptors, since most of the mGlu receptor subtypes are expressed in cortico-thalamo-cortical networks. Subsequently, several subtype-selective mGlu receptor ligands have been shown to be effective in reducing the incidence of seizures in animal models (Ngomba et al., 2011, 2018; Celli et al., 2019).

Using the WAG/Rij rat model of absence epilepsy, we have previously identified mGlu5 receptors as a promising target. There is decreased mGlu5 receptor expression in the VB thalamus of both pre-symptomatic and symptomatic WAG/Rij rats, and mGlu5 receptor signalling is markedly reduced in the thalamus of symptomatic WAG/Rij rats. In contrast, mGlu5 receptor expression is enhanced in the somatosensory cortex of WAG/Rij rats, with no changes in receptor signalling (D'Amore et al., 2013). Systemic treatment with a selective positive allosteric modulator (PAM) of mGlu5 receptors (VU0360172) reduces the occurrence of SWDs without the development of tolerance (D'Amore et al., 2013, 2014). In an attempt to identify where within the cortico-thalamo-cortical circuit mGlu5 receptors control the generation of SWDs, VU0360172 was infused into either the somatosensory cortex or the thalamus of WAG/Rij rats and it was found that both sites were involved in the anti-absence activity of the compound (D'Amore et al., 2015).

Crunelli and associates have found that extrasynaptic $GABA_A$ receptor-dependent tonic inhibition is enhanced in thalamo-cortical neurons in different rodent models of absence epilepsy (Crunelli et al., 2011), and that pharmacological activation of extrasynaptic $GABA_A$ receptors in the thalamus induces SWDs in non-epileptic animals (Cope et al., 2009). The enhanced tonic GABAergic inhibition resulted from reduced GABA uptake mediated by the transporter GAT-1 (Cope et al., 2009). Mice with GAT-1 deletion showed spontaneous absence seizures that were sensitive to the anti-absence drugs, ethosuximide and valproate (Cope et al., 2009; Pirttimaki et al., 2013). To examine whether changes in GABAergic transmission were involved in the anti-absence activity of mGlu5 receptors, VU0360172 and the GAT-1 inhibitor, tiagabine, were locally infused in the VB thalamus or somatosensory cortex either alone or in combination. Intrathalamic infusion of tiagabine alone enhanced the occurrence of SWDs in WAG/Rij rats in a dose-dependent manner. Interestingly, VU0360172 lost its anti-absence activity when co-injected with subthreshold doses of tiagabine in the thalamus, and this combination even enhanced SWDs (D'Amore et al., 2015). This suggested that mGlu5 receptors require an intact and functional GABA transport system within the thalamus to suppress absence seizures. From this data it is a plausible hypothesis that mGlu5 receptors act by boosting GABA uptake in the thalamus, thereby limiting the amount of synaptic GABA available for the activation of $GABA_A$ receptors in thalamo-cortical neurons. In contrast, inhibiting GABA uptake with tiagabine in the cortex suppressed SWDs similarly to VU0360172 (D'Amore et al., 2015). This suggests that the suppressing action of VU0360172 probably has a different mechanism in the cortex compared to the thalamus.

Here we have investigated the effects of enhancing the activation of mGlu5 receptors (with the PAM VU0360172) on GABA transport and transmission. The major focus of this study has been to investigate the effects of single injections of VU0360172 (its acute effects). In some of the experiments we have also investigated the effects of multiple injections. We have found VU0360172 suppresses $GABA_A$ receptor-dependent tonic inhibition in the VB thalamus by up-regulating GAT-1 expression and enhancing GABA uptake. These findings unravel novel mGlu5 receptor-mediated regulation of inhibitory synaptic transmission that is a plausible mechanism for the effects of VU0360172 in reducing SWDs in models of absence epilepsy.

2. Materials and methods

2.1. Drugs

VU0360172 (N-cyclobutyl-6-[2–3 (fluorophenyl) ethynyl] pyridine-3-carboxamine), a selective mGlu5 receptor positive allosteric modulator (PAM), was purchased from Santa Cruz Biotechnology (Dallas, Texas, USA). VU0360172 was dissolved in 10% Tween 80 and injected subcutaneously (s.c.). Fresh drug solution was prepared daily. VU0360172 was administered at the centrally active dose of 3 mg/kg (Rodriguez et al., 2010); control animals received equal volumes of 10% Tween 80. U73122 was purchased from Sigma (Milan, Italy). For the incubation of acute thalamic slices, VU0360172 and U73122 were dissolved in dimethyl sulfoxide (DMSO) at the initial concentration of 10 mM. Aminooxyacetic acid (AOAA) was from SIGMA Aldrich (Milan, Italy). (RS)-3,5-dihydroxyphenylglycine (DHPG) was purchased from Tocris Bioscience (Bristol, United Kingdom); DHPG was dissolved in Krebs buffer at the initial concentration of 10 mM. GABAzine and kynurenic acid were obtained from Hello Bio (Bristol, UK).

2.2. Ethical approval

Procedures regarding animals were conducted in line with international and national regulations with the 3Rs concept. All animal care and experimental procedures at the University of Warwick were reviewed and approved by the institutional animal welfare and ethical review body (AWERB) and were in accordance with the Animals (Scientific Procedures) Act 1986.

2.3. Animals and drug treatments

We used three strains of rats: (i) male spontaneously epileptic WAG/Rij rats at 20–24 days and 5–6 months of age. At 20–24 days of age, WAG/Rij rats do not show SWDs, and, are considered as pre-symptomatic. In contrast, 5–6 months old WAG/Rij rats show about 16–20 SWDs per hour (van Luijtelaar and Coenen, 1988; Coenen and van Luijtelaar, 2003); (ii) age-matched Wistar rats, (as WAG/Rij rats originate from Wistar rats); and (iii) Sprague-Dawley rats. WAG/Rij and Wistar rats were used for studies of GAT-1 expression in the thalamus and somatosensory cortex; WAG/Rij rats were used for the study of [^{3}H]-GABA uptake in superfused thalamic and cortical synaptosomes and for *ex vivo* studies of extrasynaptic $GABA_A$-receptor mediated tonic inhibition in the thalamus; Sprague-Dawley rats were used for *in vitro* studies of tonic inhibition.

For measurements of GAT-1 expression in the thalamus and somatosensory cortex, different groups of WAG/Rij (n = 3–4 or n = 4–5 per group) and age-matched Wistar rats (n = 4–5 per group) received either single or multiple s.c. injections (5 injections with a 12-h interval)

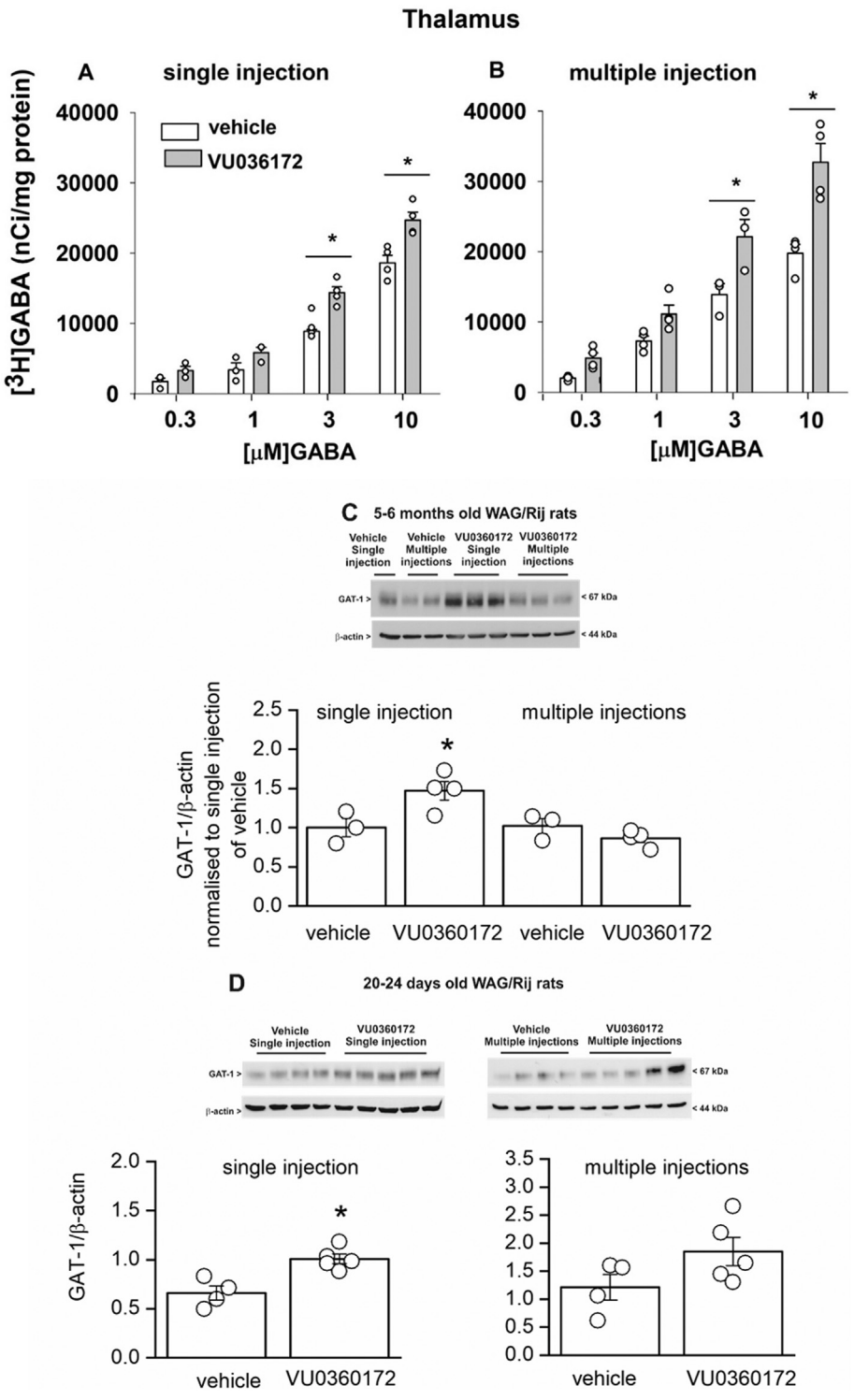

Fig. 1. Pharmacological enhancement of mGlu5 receptor activation increases thalamic GABA uptake and GAT-1 expression. **A**, The uptake of [^{3}H]-GABA by synaptosomes prepared from the thalamus of symptomatic WAG/Rij rats treated with a single injection of VU0360172 (3 mg/kg, s.c.) or vehicle. **B**, The same as (**A**) but for 5 multiple injections (interval of 12 h). Values are means + S.E.M. of radioactivity (in nCi) taken up *per* mg of synaptosomal proteins (the points are data are from individual experiments). *Statistically significant vs. the respective vehicle (**A**, 3 μM GABA two-sample t (6) = −3.82, p = 0.009, 10 μM GABA two-sample t (6) = −3.91, p = 0.008, **B**, 3 μM GABA two-sample t (4) = −2.8, p = 0.048; 10 μM GABA two-sample t (6) = −4.38, p = 0.005). **C**, Representative immunoblot and analysis of GAT-1 expression in the thalamus of symptomatic 5–6 months WAG/Rij rats. Values are means + S.E.M. with 3–4 animals in each group. Data points show data from individual experiments. *Statistically significant difference between treatment groups by one-way ANOVA [(F = 8.020, p = 0.005, df = 3) and pairwise multiple comparison procedures with Bonferroni correction (alpha = 0.050) between vehicle and single injection VU030172, with P = 0.045)] **D**, Immunoblot analysis of GAT-1 expression in presymptomatic 20–24 days old WAG/Rij rats. Values are means + S.E.M. with 4–5 animals in each group. Data points are from individual experiments. Single injection: *Statistically significant Vs respective vehicle (t = −4.089, df = 7, and 95 percent two-tailed confidence interval for difference of means: −0.549 to −0.147; Two-tailed P = 0.00464); Multiple injection: no statistically detected difference between groups (t = −1.819, df = 7, and 95 percent two-tailed confidence interval for difference of means: −1471 to 0,192; Two-tailed P = 0.112).

with VU0360172 (3 mg/kg) or its vehicle. Animals were culled 1 h after the last injection for measurements of GAT-1 mRNA and protein levels, and for immunohistochemical analysis. For *ex vivo* measurements of GAT-1 in synaptosomal preparations, WAG/Rij rats received either single or multiple administrations of VU0360172 or its vehicle, as above.

2.4. Assessment of GAT-1 expression

Western blot analysis was carried out using the thalamus and somatosensory cortex dissected from WAG/Rij and age-matched control Wistar rats, at 20–24 days or 5–6 months of age (n = 3–5 rats per group). Tissue samples were homogenized at 4 °C in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 1 mg/ml aprotinin, 1 mg/ml pepstatin, and 1 mg/ml leupeptin. Proteins were resuspended in SDS-bromophenol blue reducing buffer with 40 mM DTT. Western blot analysis were carried out with 10% SDS polyacrylamide gels, which were electroblotted onto immunoblot PVDF membranes; filters were blocked in TTBS buffer containing 0.25% non-fatty milk. Specific rabbit polyclonal antibodies for GAT-1 (AB1570W, Chemicon, Millipore Corporation, Billerica, MA, 1:1000) or mouse monoclonal antibody for β-actin (1:50,000, Sigma, St. Louis, MO) or

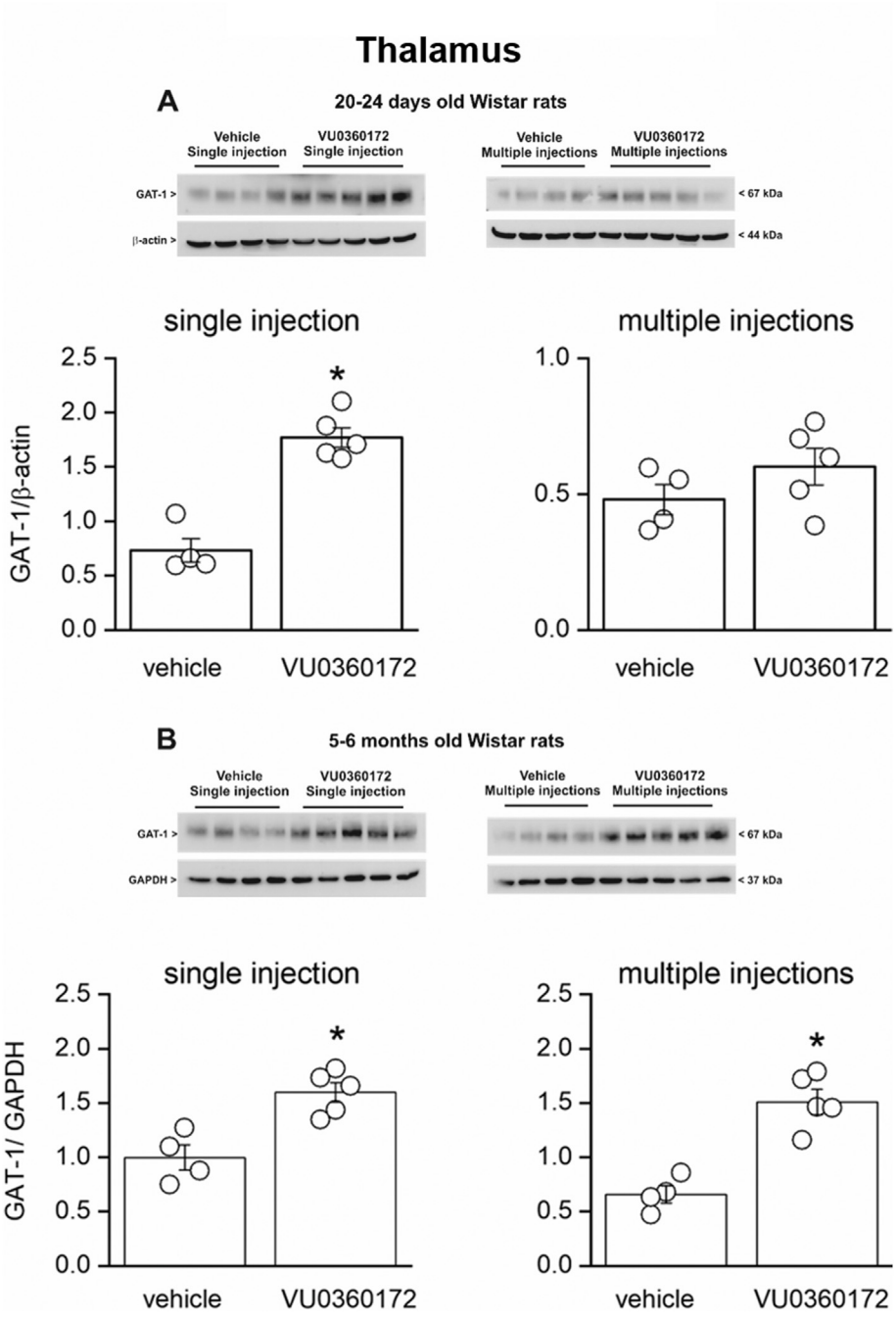

Fig. 2. Pharmacological enhancement of mGlu5 receptor activation can increases thalamic GAT-1 expression in non-epileptic rats. **A**, Immunoblot analysis of GAT-1 expression in Wistar rats age-matched to presymptomatic WAG/Rij rats (P20-24) and **B**, Wistar rats age-matched to symptomatic WAG/Rij rats (5–6 month old). Values are means + S.E.M. with 4–5 animals in each group. Data points show data from single experiments. **A**, single injection: *Statistically significant Vs respective vehicle (t = −7.119, df = 7; 95 percent two-tailed confidence interval for difference of means: −1.390 to −0.697; Two-tailed p = 0.0001); multiple injection: no statistically detected difference between groups (t = −1.314, df = 7; 95 percent two-tailed confidence interval for difference of means: −0.336 to 0.0958; Two-tailed p = 0,230); **B**, *Statistically significant Vs respective vehicle [single injection (t = −4.169, df = 7; 95 percent two-tailed confidence interval for difference of means: −0.938 to −0.259; Two-tailed p = 0.00420; multiple injection (t = −5.921 df = 7; 95 percent two-tailed confidence interval for difference of means: −1.202 to −0.516; Two-tailed p = 0.000587)].

GAPDH (1:1000, Abcam, Cambridge, UK) were used. Blots were then incubated with secondary antibodies (peroxidase-coupled anti-rabbit or anti-mouse, Amersham, Piscataway, NJ). Immunostaining was revealed by enhanced ECL (Amersham, Piscataway, NJ).

2.5. Measurements of GABA uptake

Purified cortical and thalamic synaptosomes were prepared essentially according to Dunkley et al. (1986), with minor modifications as follows. Briefly, the cortices and the thalami of control and treated rats were homogenized in 10 vol of 0.32 M sucrose, buffered to pH 7.4 with TRIS (final concentration 0.01 M) using a glass Teflon tissue grinder (clearance 0.25 mm). The homogenate was centrifuged at 1000 × *g* for 5 min, to remove nuclei and debris; the supernatant was gently stratified on a discontinuous Percoll gradient (6%, 10% and 20% v/v in Tris-buffered sucrose) and centrifuged at 33,500 × *g* for 5 min. The layer between 10% and 20% Percoll (synaptosomal fraction) was collected and washed by centrifugation. The synaptosomal pellet was then re-suspended in physiological medium (standard medium) having the following composition (mM): NaCl, 140; KCl, 3; MgSO4, 1.2; CaCl2, 1.2; NaH2PO4, 1.2; NaHCO3, 5; HEPES, 10 mM, glucose, 10; pH 7.2–7.4, AOAA 50 μM to avoid GABA metabolism. Synaptosomal protein contents were determined according to Bradford (1976).

Aliquots of purified rat cortical (2.75 ± 0.17 mg protein/ml) and thalamic (1.25 ± 0.1 mg protein/ml) synaptosomes were first pre-incubated for 2 min in standard medium at 37 °C for equilibration. Then increasing concentrations (0.1 μM–10 μM) of [^{3}H]GABA solution were added to the suspension to allow the quantification of total tritium uptake. After 10 min of incubation, the synaptosomal suspension was diluted by adding 3 ml of cold standard medium and immediately filtered under moderate vacuum using Whatman GF/B glass filters (GE Healthcare Life Sciences, Buckinghamshire, UK). Filters were rinsed

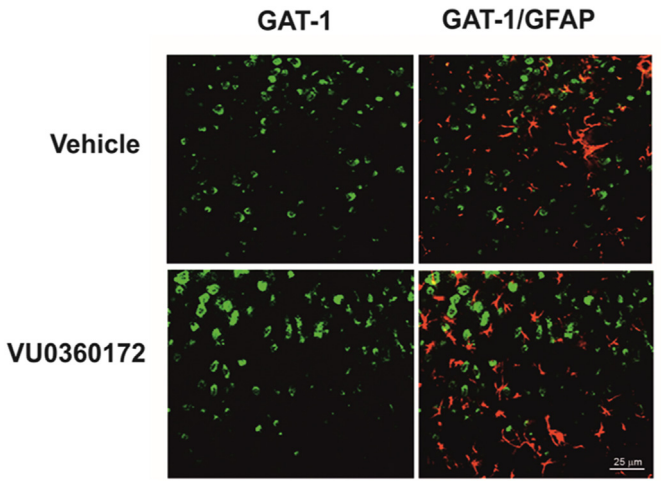

Fig. 3. The increase in GAT-1 expression occurs in thalamic neurons. Photomicrographs illustrating GAT-1 expression in the VB thalamus of WAG/Rij rats (P20-24) that had received either a single injection of VU0360172 or vehicle. GAT-1 is labelled in green with the astrocyte marker glial fibrillary acidic protein (GFAP) in red. The increase in GAT-1 expression in response to VU0360172 does not appear to co-localise with GFAP and thus presumably occurs in neurons. . (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with medium (3 ml for three times) at room temperature, collected and counted for radioactivity. Blanks were determined by incubating synaptosomes with the GABA solution at 2–4 C°. The specific activity of [^{3}H]GABA was 30 Ci/mmol (PerkinElmer Boston, MA, USA). To achieve the final concentrations (from 0.1 μM to 10 μM) of the uptake substrate, 0.285 μM [^{3}H]GABA was added to a solution containing 100 μM unlabelled GABA (SIGMA, Milano, Italy) and the GABA solution diluted to reach the expected GABA concentrations to be tested. The amount of [^{3}H]GABA taken up by the synaptosomes was calculated after subtraction of the respective blank. The amount of [^{3}H]GABA in the filter was expressed as nCi per mg of protein and it was determined by considering the dilution with the unlabelled GABA. Data handling, statistics and graph construction was carried out using SigmaPlot 10 (Systat software). The results were expressed as the amount of [^{3}H]GABA taken up in each experimental condition.

2.6. Immunohistochemical analysis of GAT-1 expression

Rats were deeply anesthetized and cardiac perfused with ice-cold 4% paraformaldehyde, brains were carefully removed and equilibrated with 30% sucrose overnight. Brain tissue was sectioned using a Leica cryostat (CM3050). For immunofluorescence analysis, 30 μm serial sections were incubated with blocking solution (5% normal serum in 0.3% Triton X-100 in PBS) and then with the following antibodies overnight at 4 °C: rabbit anti-GAT1 primary antibody (1:300, Millipore, Darmstadt, Germania) and mouse anti-GFAP primary antibody (1:250, Millipore, Darmstadt, Germania). Serial sections were then incubated with the respective secondary antibody conjugated to Alexa Fluor 488 (1:200, Molecular Probes, Eugene) and Cy3 (1:200, Jackson ImmunoResearch Labs, West Grove, PA). Finally, sections were mounted with anti-fading agent containing DAPI for nuclear staining (Vector Laboratories, Burlingame, CA) and examined with ZEISS 780 confocal laser scanning microscope (488 nm argon laser to excite Alexa 488 and 543 nm HeNe laser to excite Cy3).

2.7. Measurements of GAT-1 mRNA levels

Total RNA was extracted from the thalamus and somatosensory cortex using the trizol reagent (Invitrogen, Carlsbad, CA) according to

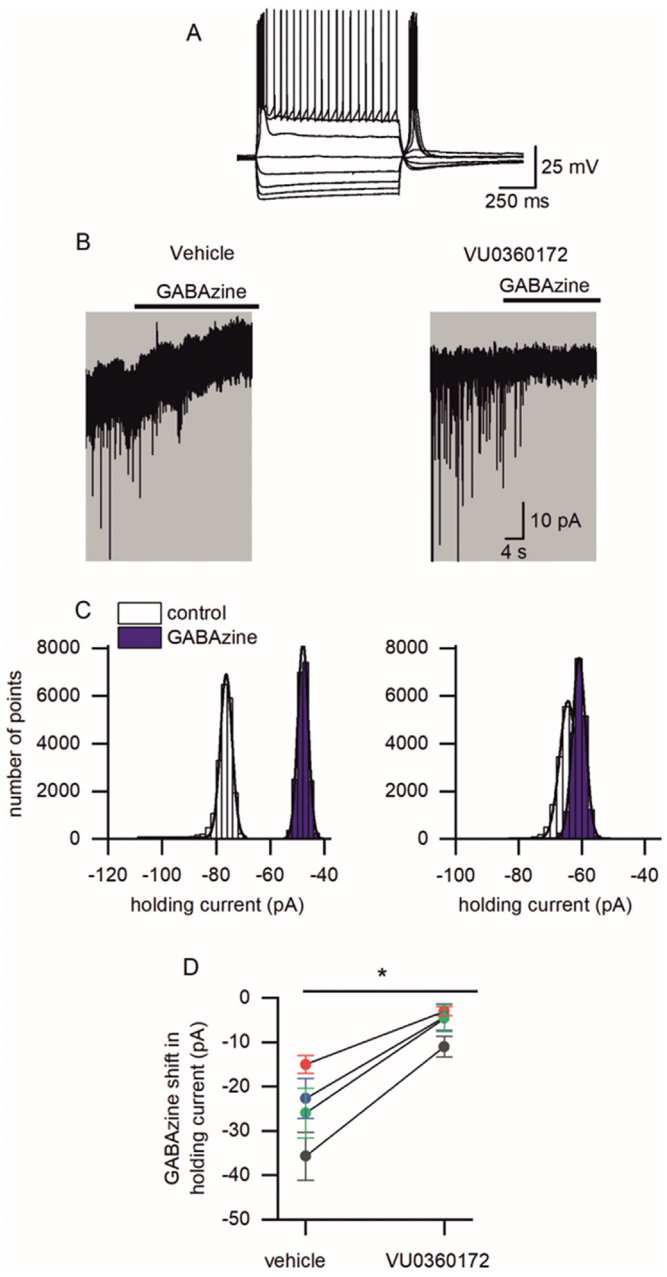

Fig. 4. Systemic treatment with the VU0360172, reduces the tonic $GABA_A$ receptor current in thalamocortical neurons. **A**, Example of voltage responses to current injections characteristic of a thalamocortical neuron (for example observe initial burst and rebound firing following hyperpolarisation steps). **B**, Example current traces (neurons held at −70 mV) recorded from thalamocortical neurons in slices from 20 to 24 day old WAG/Rij rats which had received a single injection of VU0360172 or vehicle. Application of the $GABA_A$ receptor antagonist GABAzine (100 μM) produced a much larger upward shift in holding current in the neuron from the vehicle treated WAG/Rij rat compared to the VU0360172 treated rat. **C**, All point histograms constructed from the data in (**B**) and fitted with single Gaussian distributions (mean current in vehicle treated rats: control −76.4 ± 5.1 pA, GABAzine −48.4 ± 4.06 pA, shift in current ~ 28 pA; in VU0360172 treated: control −64.4 ± 6.3, GABAzine −60.8 ± 4.88 pA, shift in current ~ 5 pA). **D**, Summary plot of data from 4 vehicles and 4 VU0360172 treated littermates. Each point is the mean of the GABAzine induced shift in holding current from 3-4 slices from each rat. The lines link the data from littermates which were recorded interleaved on the same day in slices from vehicle and VU0360172 treated animals. *There is a significant (paired t (3) = −7.08, p = 0.00579 reduction in the shift in holding current (vehicle: −24.1 ± 4.3 pA; VU03601725.7 ± 1.8 pA, n = 4 rats, 12 slices).

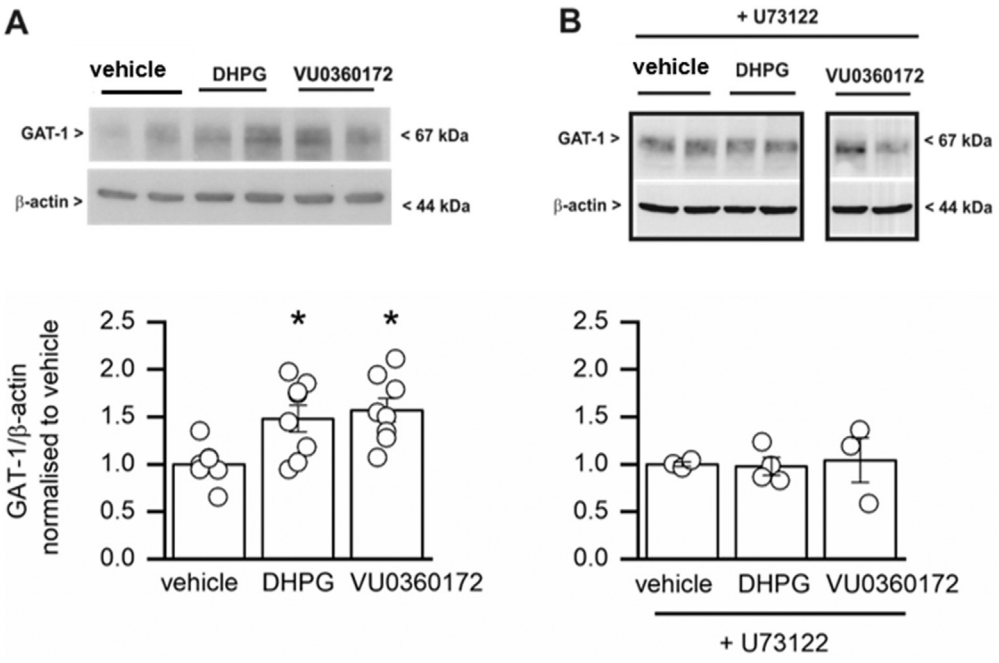

Fig. 5. Activation of mGlu5 receptors enhances GAT-1 protein expression *in vitro* via the stimulation of phospholipase C (PLC). **A**, Representative immunoblot and analysis of GAT-1 protein expression in acute thalamic slices incubated with either vehicle, DHPG (200 μM) or VU0360172 (1 μM). Both DHPG and VU0360172 significantly enhanced GAT-1 expression (*Statistically significant: P = 0.005, degrees of freedom = 2, test statistic = 10.460 and Total N = 24, by independent samples Kruskal-Wallis test). The significance values have been adjusted by the Bonferroni post-hoc test; **B,** Representative immunoblot and analysis of GAT-1 protein expression incubated with either vehicle, DHPG (200 μM) or VU0360172 (1 μM) in the presence of the PLC inhibitor, U73122 (10 μM). There was no significant effect of both DHPG and VU0360172, against vehicle in the presence of U73122 (P = 0.786, degrees of freedom = 2, test statistic = 0.482 and Total N = 10, by independent samples Kruskal-Wallis test). The test statistic is adjusted for ties. Values are means + S.E.M. Data points show data from single experiments.

the manufacturer's instructions. The RNA was further treated with DNAse (Qiagen, Hilden, Germany), and single strand cDNA was synthesized from 1.5 μg of total RNA using Superscript III (Invitrogen) and random hexamers. Real-time PCR was performed on 15 ng of cDNA by using the following primers and Power SYBR Green Master Mix (Biorad, Hercules, CA) on an Applied Biosystems Step-One instrument. Slc6a1 – GAT-1 forward: GCAATCGCCGTGAACTCTTC; reverse: AGGAAATGGA GACACACTCAAAGA and β-actin forward: GTTGACATCCGTAAAGACC; reverse: TGGAAGGTGGACAGTGAG. Thermal cycler conditions: 10 min at 95 °C, 40 cycles of denaturation (15 s at 95 °C), and combined annealing/extension (1 min at 58–60 °C). mRNA copy number was calculated from serially diluted standard curves simultaneously amplified with the samples and normalized with respect to β-actin mRNA copy number. Each sample was analyzed in duplicate together with two negative controls.

2.8. In vitro stimulation of thalamic mGlu5 receptors

Thalamic slices (350 × 350 μm) were prepared from male adult Wistar rats with a McIlwain tissue chopper. Animals were killed by decapitation, and the thalamus was dissected out and transferred in ice-cold Krebs-Henseleit buffer (NaCl 118 mM, KCl 4.7 mM, $MgSO_4$ 1.18 mM, KH_2PO4 1.18 mM, $NaHCO_3$ 24.8 mM, $CaCl_2$ 1.2 mM, D-glucose 10 mM) that had been pre-gassed with 95% O_2 and 5% CO_2 to pH 7.4. Gravity packed slices (30 μl) were incubated for 45 min in 350 μl of buffer for metabolic recovery. Slices were then incubated with U73122 (10 μM) or its vehicle for 15 min and followed by VU0360172, 1 mM and DHPG, 200 mM. One hour later, the incubation was stopped by addition of 50 μl of a lysis buffer (Tris-HCl, 100 mM; pH 7.5; containing 5 M NaCl, 0.5 M EDTA, 1% IGEPAL, 10% SDS and a cocktail of protease inhibitors). Tissue samples were homogenized by sonication and used for Western blot analysis of GAT-1.

2.9. Electrophysiological recording of tonic $GABA_A$ receptor current

In the first set of experiments tonic $GABA_A$ receptor current was measured *ex vivo* in thalamocortical slices obtained from P20-24 WAG/Rij rats injected (s.c) at least 1 h before with either vehicle or VU0360172 (3 mg/kg). In a second set of experiments the tonic $GABA_A$ receptor current was measured in thalamocortical slices obtained from P18-20 Sprague Dawley rats incubated *in vitro* (for at least 1 h) with VU0360172 or vehicle. Transverse thalamocortical slices (300–350 μm) were prepared using a Microm HM 650V microslicer in cold (2–4 °C) high Mg^{2+}, low Ca^{2+} aCSF, composed of (mM): 127 NaCl, 1.9 KCl, 8 $MgCl_2$, 0.5 $CaCl_2$, 1.2 KH_2PO_4, 26 $NaHCO_3$, 10 D-glucose (pH 7.4 when bubbled with 95% O2 and 5% CO2, 300 mOSM). Slices were stored at 34 °C for 1–6 h in aCSF (1 mM $MgCl_2$, 2 mM $CaCl_2$) before use. To measure the *in vitro* effects of VU0360172 incubation, thalamic slices were cut in half down the mid-line, and one half was kept in control conditions (vehicle, DMSO) and the other half was pre-incubated for at least 1 h in VU0360172 (5 μM). DMSO was added to the control aCSF (0.0125%) so it was the same concentration as for slices incubated in VU0360172.

For patch clamp recording, an individual slice was transferred to the recording chamber and perfused at 3 ml min^{-1} with aCSF at 32 ± 0.5 °C containing 2.5 mM kynurenic acid (to block glutamatergic transmission). Slices were visualized using IR-DIC optics with an Olympus BX151W microscope (Scientifica) and a CCD camera (Hitachi). Whole-cell patch clamp recordings were made from thalamocortical neurons using patch pipettes (5–10 MΩ) manufactured from thick walled glass (Harvard Apparatus, Edenbridge UK) either containing (mM): 135 mM CsCl, 10 HEPES, 10 EGTA, 2 Mg-ATP pH 7.2 with TEA-OH) for voltage clamp recordings or potassium gluconate 135, NaCl 7, HEPES 10, EGTA 0.5, phosphocreatine 10, MgATP 2, NaGTP 0.3 (293 mOSM, pH 7.2) for current clamp recordings. Recordings were obtained using an Axon Multiclamp 700B amplifier (Molecular Devices, USA) and digitised at 10 KHz. Data acquisition and analysis was performed using Pclamp 10 (Molecular Devices). For voltage clamp recordings, neurons were held at −70 mV and the tonic current was measured as the difference between the holding current in control and following the application of GABAzine (100 μM). To accurately measure the shift in holding current, all point histograms were constructed from 20 s of data in control and after application of GABAzine (using Origin, Microcal). Each histogram was fitted with a single Gaussian (non-linear least-squares Levenberg-Marquadt algorithm, Origin Microcal) so the mean current at the peak could be

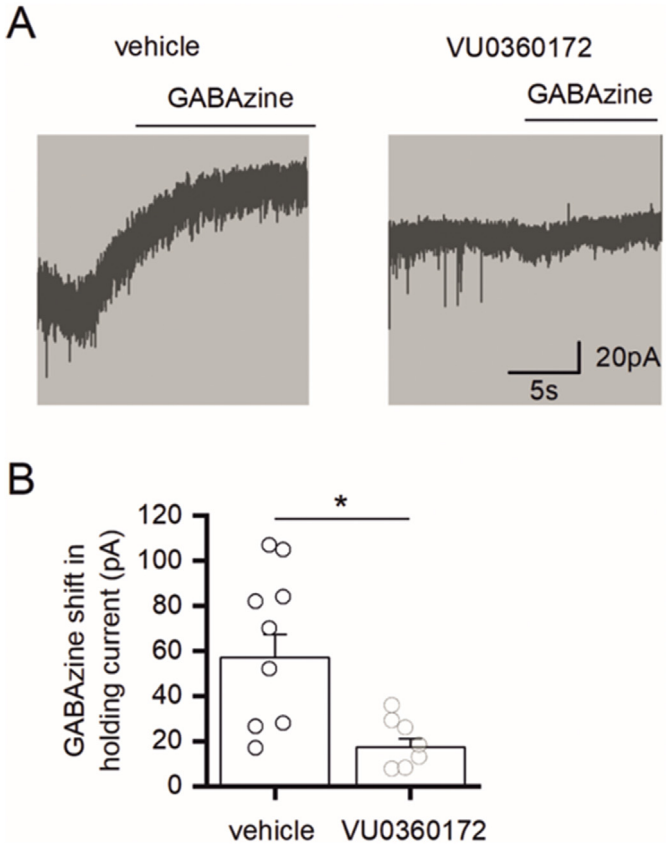

Fig. 6. The effect of VU0360172 on the tonic $GABA_A$ receptor current in thalamocortical neurons can be repeated *in vitro*. **A**, Example current traces (neurons held at −70 mV) recorded from thalamocortical neurons in acute thalamic slices incubated with either vehicle (DMSO) or VU0360172 (5 μM) for at least 1 h. The GABAA receptor antagonist GABAzine (100 μM) reduced the tonic GABA current (upward shift in holding current). GABAzine had less effect on the holding current in the neuron from the slice incubated with VU0360172 compared to the interleaved slice incubated in vehicle **B**, Graph summarizing data from 7-9 slices. The shift in holding current produced by GABAzine is significantly (two-sample t (14) = 3.23, p = 0.0071) smaller in slices pre-incubated in VU0360172 (mean shift in holding current 17.3 ± 3.8 pA) compared to those incubated in vehicle (mean shift in holding current 57.0 ± 4 pA). The points are the GABAzine-induced shift in holding current measured from individual slices.

measured (Wall and Usowicz, 1997).

2.10. Statistical analysis

All data are presented as the means ± SEM. Statistical analysis was performed using Student's t-test, Kruskal-Wallis test and one-way ANOVA + Bonferroni's post hoc test. The tests used are indicated in the figure legends. In all studies, n indicates the number of samples per group, and cases in which P-values < 0.05 were considered statistically significant.

3. Results

3.1. Selective pharmacological enhancement of mGlu5 receptor function up-regulates GABA uptake and GAT-1 protein levels in the thalamus of symptomatic and presymptomatic WAG/Rij rats

To investigate whether VU0360172 modulates thalamic GABA signalling, we first investigated whether VU0360172 could alter thalamic GABA uptake. Experiments were performed *ex vivo* with synaptosomes prepared from the thalamus of symptomatic WAG/Rij rats treated systemically with either a single injection or five multiple injections (12 h interval between injections) of VU0360172 or vehicle. Thalamic synaptosomes were prepared 1 h after the last drug injection and incubated with increasing concentrations of [H^3]GABA. The uptake of GABA was significantly enhanced after either single or multiple injections of VU0360172 (Fig. 1A and B). The increase became statistically significant with the two highest concentrations of [H^3]GABA (Fig. 1A and B).

To examine whether changes in the expression of the GABA transporter GAT-1 underlies the observed increase in GABA uptake, GAT-1 protein expression was measured in the thalamus from symptomatic WAG/Rij rats treated with either VU0360172 or vehicle. Western blot analysis showed significant increases in thalamic GAT-1 protein expression 1 h after a single injection of VU0360172 (Fig. 1C). However, GAT-1 expression returned back to control levels after multiple injections of VU0360172, although GABA uptake remained significantly enhanced (Fig. 1B). We then investigated if equivalent changes in GAT-1 could also occur in presymptomatic WAG/Rij rats (P20-24, Fig. 1D) and found similar to symptomatic WAG/Rij rats, a single injection of VU0360172 significantly enhanced GAT-1 expression, whereas after multiple injections GAT-1 expression was not significantly different from vehicle (Fig. 1D).

3.2. Selective pharmacological enhancement of mGlu5 receptor activation can increase GAT-1 expression in the thalamus of non-epileptic rats

Since a single injection of VU0360172 enhanced GAT-1 expression in the thalamus of presymptomatic WAG/Rij rats, we next investigated whether similar effects could be observed in the thalamus from age-matched non-epileptic control rats (Wistar, since WAG/Rij rats were originally derived from Wistar). A single injection of VU0360172 significantly increased thalamic GAT-1 protein levels in 20–24 day old (age matched to presymptomatic WAG/Rij rats) and in 5–6 month old Wistar rats (aged matched to symptomatic WAG/Rij rats, Fig. 2A and B). GAT-1 expression returned to control levels after multiple drug injections in the 20–24 day old Wistar rats but remained significantly elevated in 5–6 month old Wistar rats (Fig. 2A and B). The increase in GAT-1 expression produced by VU0360172 in presymptomatic WAG/Rij rats and in non-epileptic control rats (Wistar), suggests that there is not a requirement for abnormal network activity in the thalamus for VU0360172 to have its effects on GAT-1 expression.

3.3. The changes in GAT-1 expression are not associated with changes in mRNA

We next investigated whether the increase in GAT-1 expression was associated with an increase in GAT-1 mRNA. Surprisingly, the increase in GAT-1 protein expression induced by a single injection of VU0360172 in Wistar rats was not associated with significant changes in *GAT-1* gene transcription (two-sample t (4) = −0.101, p = 0.92). This suggests that there may either be changes in the rate of translation of pre-existing GAT-1 mRNA or that there are post-translational changes such as an increase in GAT-1 protein half-life.

3.4. GAT-1 expression is increased in neurons not glia

To investigate the cellular distribution of the increased GAT-1 expression, immunohistochemistry and confocal imaging was performed using thalamic slices labelled with GAT-1 and GFAP (to label glia) antibodies (Fig. 3). The analysis was performed in 20–24 day old WAG/RIJ and Wistar rats receiving either a single injection of VU0360172 or vehicle. In control conditions (vehicle) GAT-1 appeared to be expressed in neurons (as it did not co-localise with GFAP) and showed a perinuclear localization (Fig. 3). Following a single injection of VU0360172 there was increased GAT-1 labelling in both Wistar and WAG/Rij rats consistent with the western blot data (see Figs. 1 and 2). This enhanced

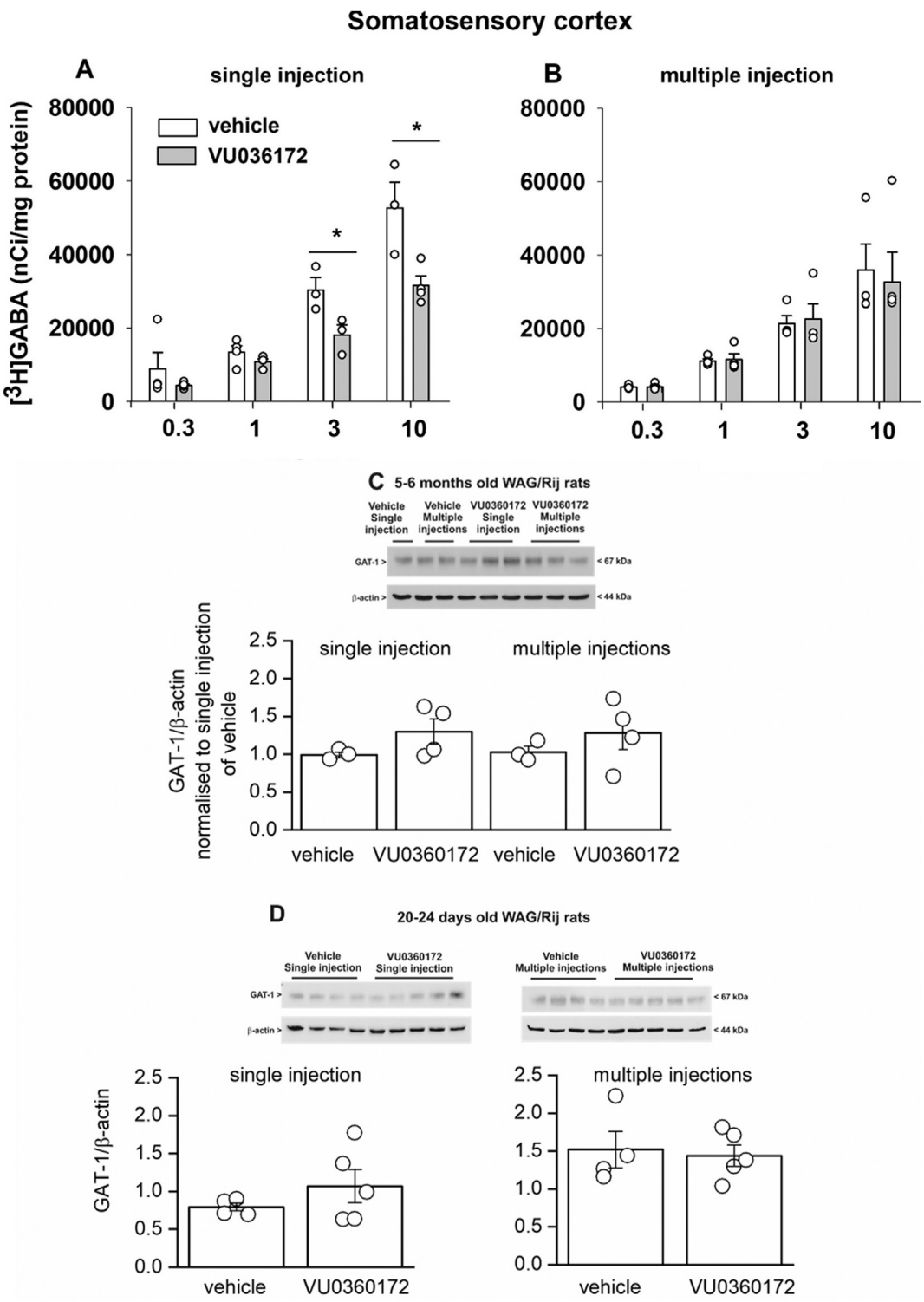

Fig. 7. Effect of systemic treatment with VU0360172 on GABA uptake and GAT-1 expression in the somatosensory cortex. **A**, The uptake of [^{3}H]-GABA by synaptosomes prepared from the neocortex of symptomatic WAG/Rij rats treated with a single injection of VU0360172 or vehicle. **B**, The same as (**A**) but for 5 multiple injections (interval of 12 h) of either VU0360172 or vehicle. Values are means + S.E.M. of radioactivity (in nCi) taken up *per* mg of synaptosomal proteins. The data points are data from individual experiments. *Statistically significant vs. the respective vehicle (**A**, 3 μM GABA two-sample t (4) = 2.74, p = 0.049, 10 μM GABA two-sample t (5) = −3.12, p = 0.025). **C**, Representative immunoblot and analysis of GAT-1 expression in the somatosensory cortex of symptomatic 5–6 months WAG/Rij rats (n = 3–4), and in (**D**) pre-symptomatic 20–24 days old WAG/Rij rats (n = 4–5), receiving either single or multiple injections of VU0360172 or vehicle. Values are means + S.E.M with (**C**) 3–4 or (**D**) 4–5 animals in each group). There were no significant differences in GAT-1 expression in the somatosensory cortex between animals pre-treated with VU0360172 and vehicle [**C,** by one-way ANOVA: F = 0.937, p = 0.458, df = 3). **D**, single injection (t = −1.124, df = 7; 95 percent two-tailed confidence interval for difference of means: −0.887 to 0.315; Two-tailed P = 0.298); multiple injection (t = 0.284, df = 7; 95 percent two-tailed confidence interval for difference of means: −0.553 to 0.704; Two-tailed P = 0.785)].

GAT-1 labelling did not co-localise with GFAP.

3.5. Enhancement of mGlu5 receptor activation decreases tonic $GABA_A$ receptor current in VB thalamocortical neurons

Thalamocortical (TC) neurons in the VB thalamus receive two forms of GABAergic transmission: (i) phasic, mediated by synaptic $GABA_A$ receptors (containing of α_{1-3}, β, and γ subunits); and, (ii) tonic, mediated by high affinity extra synaptic $GABA_A$ receptors containing the δ subunit (Belelli et al., 2005; Cope et al., 2005; Jia et al., 2005). It would be reasonable to predict that the increase in GABA uptake and GAT-1 expression produced by VU0360172 administration would reduce the tonic $GABA_A$ receptor current in TC neurons. To assess this, we made whole cell patch clamp recordings from TC neurons in thalamic slices prepared from 20 to 24 days old WAG/Rij rats which had received either a single injection of VU0360172 or vehicle. TC neurons in the VB thalamus were identified by their position in the slice and their characteristic current-voltage relationship (Fig. 4A). The upward shift in holding current produced by the $GABA_A$ receptor antagonist GABAzine (100 μM) was used to quantify the tonic $GABA_A$ receptor current (Fig. 4B and C). There was a significant ($p = 0.005776$) reduction in the amplitude of the tonic current in rats treated with VU0360172 compared to vehicle treated littermates.

3.6. The effects of VU0360172 on GAT-1 expression can be reproduced in vitro and occurs by PLC activation

Since the effects of VU0360172 occurred in non-epileptic control rats (Wistar), they do not appear to require abnormal activity to be produced. We therefore wondered if they could be reproduced *in vitro* with either a quiescent or incomplete thalamocortical circuit. Initially we investigated changes in GAT-1 expression. Experiments were performed using acute thalamic slices that had been incubated in either VU0360172 or the mGlu1/5 orthosteric agonist, DHPG or with vehicle. Incubation of thalamic slices with VU0360172 (1 μM) or DHPG (200 μM) significantly increased GAT-1 protein expression (Fig. 5A), consistent with data obtained after a single systemic injection of VU0360172.

mGlu5 receptors are coupled to $G_{q/11}$ proteins, and their activation stimulates phospholipase-Cβ (PLCβ), which converts phosphatidylinositol-l,4,5-bisphosphate into inositol-1,4,5-trisphosphate and diacylglycerol (Nicoletti et al., 2011). To examine whether this transduction mechanism was involved in the modulation of GAT-1 expression we used the PLC inhibitor, U73122 (Burgdorf et al., 2010; Shen et al., 2013). The effects of DHPG and VU0360172 on GAT-1 expression were abolished by co-incubating slices with U73122 (10 μM, applied 20 min prior to VU0360172 or DHPG incubation, Fig. 5B).

We then investigated whether the effects of mGlu5 receptor activation on tonic $GABA_A$ receptor current occur *in vitro*. Thalamic slices from four rats were cut in half, with one half incubated for at least 1 h with VU0360172 (5 μM) and the other half incubated with vehicle (DMSO). Whole cell patch clamp recordings where then made from VB TC neurons in interleaved VU0360172 (5 μM) and vehicle treated slices (Fig. 6A and B). There was a significantly smaller tonic $GABA_A$ receptor current in slices incubated in VU0360172 compared to slices incubated in vehicle. This data suggests that the effects of VU0360172 can occur when the thalamocortical circuit is not intact and must therefore be localised to the thalamus.

3.7. Effect of VU0360172 on GABA uptake and GAT-1 expression in the somatosensory cortex

It was previously shown that injecting VU0360172 into the somatosensory cortex reduced the frequency of SWDs in WAG/Rij rats (D'Amore et al., 2015). Therefore, we investigated the effects of VU0360172 on GABA signalling in the somatosensory cortex. In contrast to what was observed with thalamic synaptosomes, a significant reduction in $[H^3]$GABA uptake was observed in cortical synaptosomes prepared from 5 to 6 month old symptomatic WAG/Rij rats that had received a single injection of VU0360172 (Fig. 7A). $[H^3]$GABA uptake returned back to control levels after multiple injections of VU0360172 (Fig. 7B). No changes in GAT-1 protein expression were found in the somatosensory cortex of either symptomatic or presymptomatic WAG/Rij rats (Fig. 7C and D) or in 5–6 month old Wistar rats receiving either single or multiple injections of VU0360172 (not illustrated). There was also no significant change in GAT-1 mRNA expression in response to VU0360172 (not illustrated, GAT-1 mRNA levels were 14,300 + 2090 vs 13,400 + 1470 copy number/10^6 β-actin copy number in the cortex of Wistar rats receiving a single injection of VU0360172 or vehicle, respectively, $n = 5$ (two sample t (6) = 0.94, $p = 0.383$).

4. Discussion

Our data outlines a novel mechanism by which mGlu5 receptors modulate GABA transmission: enhancement of mGlu5 receptor activation increases GABA uptake in the thalamus of epileptic WAG/Rij rats. This mechanism holds promise for the treatment of absence epilepsy because defective GABA uptake has been linked to the pathophysiology of SWDs in a variety of preclinical models of absence epilepsy (Cope et. al, 2009). In addition, drugs that enhance synaptic/extrasynaptic GABA levels, such as tiagabine and vigabatrine, exacerbate absence seizures (Coenen et al., 1995; Panayiotopoulos, 1999; Errington et al., 2011), and increases in thalamic GABA levels have been linked to the expression of cortical spike and wave complexes in a patient with atypical childhood absence epilepsy (Leal et al., 2016). The enhanced GABA uptake would be expected to limit the activation of extrasynaptic $GABA_A$ receptors, which mediate tonic inhibition. Accordingly, activation of mGlu5 receptors with VU0360172 reduced tonic GABAergic inhibition. These findings further strengthen the value of mGlu5 receptors as candidate drug targets for the treatment of absence epilepsy because tonic $GABA_A$ current is increased in various experimental animal models of absence epilepsy prior to the onset of, and during absence seizures (Cope et al., 2009; Errington et al., 2011, 2014).

4.1. How does VU0360172 enhance GAT-1 expression?

A single systemic injection of VU0360172 produced a rapid increase in GAT-1 protein levels in the thalamus, but not in the somatosensory cortex. An increase in GAT-1 levels was also found *in vitro* with thalamic slices incubated with either VU0360172 or the orthosteric mGlu1/5 receptor agonist, DHPG. This suggests that the effects of mGlu5 activation on GAT-1 expression are local to the thalamus, do not require an intact thalamo-cortico-thalamic circuit, can occur when the circuit is relatively quiescent, and can occur within a short timeframe (1 h). These changes appear too rapid to be explained by a genomic effect of mGlu5 receptor activation, and indeed we found that the transcript of GAT-1 receptor was unchanged 1 h after systemic treatment with VU0360172. It is therefore possible that mGlu5 receptor activation increases the amount of GAT-1 available for GABA uptake by for example reducing GAT-1 degradation and/or enhancing translation of preformed mRNA. Stimulation of PI hydrolysis, resulting in an increase in intracellular free Ca^{2+} concentrations and activation of protein kinase C may drive these changes because, at least in thalamic slices, pharmacological blockade of PLC (with U73122) prevented the increase in GAT-1 protein levels induced by either VU0360172 or DHPG. However, since U73122 may not be entirely selective for PLC (for example see MacMillan and McCarron, 2010) additional studies with other PLC inhibitors are required to prove this conclusively.

4.2. Increased GAT-1 expression occurs in neurons not glia

A reduction in astrocytic GAT-1 activity has been associated with the epileptic phenotype of the absence model GAERS (Pirttimaki et al., 2013). However, our immunohistochemistry experiments showed that the increase in GAT-1 levels was restricted to thalamic neurons and did not occur in glia. mGlu5 receptors are expressed in both neurons and astrocytes; however, their expression is low in resting astrocytes and only becomes substantial when astrocytes become reactive, both *in vitro* (Miller et al., 1995) and *in vivo* (Aronica et al., 2000; Ulas et al., 2000; Drouin-Ouellet et al., 2011). The presence of reactive astrocytes in the thalamus of genetic animal models of absence epilepsy is controversial. While Dutuit et al. (2000) found increased GFAP expression in the thalamus of pre-symptomatic and symptomatic GAERS, no reactive astrocytes were found in extracortical regions of GAERS in a more recent study (Akin et al., 2011). In addition, we found that activation of mGlu5 receptors could also up-regulate GAT-1 activity levels in the thalamus of non-epileptic rats, in which reactive astrocytes are expected to be absent. Thus, while defective activity of glial GAT-1 might be linked to the pathophysiology of absence seizures (Pirttimaki et al., 2013), an up-regulation of neuronal GAT-1 in the thalamus may contribute to the acute anti-absence effect of drugs that activate mGlu5 receptors. Interestingly GAT-1 protein levels returned back to normal after multiple injections of VU0360172 whereas the enhanced GABA uptake found *ex vivo* in thalamic synaptosomal preparation persisted after multiple administrations of VU0360172. This suggests that an increased GAT-1 activity maintains the response to VU0360172 over time in spite of the normalization of the overall GAT-1 protein levels, thus avoiding the development of tolerance. This hypothesis is consistent with the evidence that the anti-absence activity of systemically administered VU0360172 in WAG/Rij rats persisted for at least 10 days (D'Amore et al., 2014).

4.3. Comparison with blocking GABA uptake in vivo

Our previous work has shown that GAT-1 blockade with tiagabine has opposite effects on absence seizures in WAG/Rij rats if the drug is locally injected in either the thalamus or into the somatosensory cortex. Intrathalamic infusion of tiagabine induced the expected increase in SWD incidence, whereas, surprisingly, intracortical infusion reduced absence seizure occurrence (D'Amore et al., 2015). Interestingly, the modulation of GABA uptake by mGlu5 receptors also differed between the thalamus and the somatosensory cortex. Whilst, systemic treatment with VU0360172 enhanced GABA uptake in thalamic synaptosomes, a single injection of the drug *reduced* GABA uptake in cortical synaptosomes, thus mimicking the action of intracortical tiagabine. Tolerance developed to the effect of VU0340172 in the somatosensory cortex, and no changes in cortical GABA uptake were seen after multiple injections of VU0360172. In addition, there were no changes in cortical GAT-1 expression in response to either single or multiple administrations of VU0360172. This dismisses the possibility that mGlu5 receptors are physically and uniformly linked to the molecular machinery of GABA uptake across the CNS, and rather suggests that mGlu5 receptor signalling modulates GABA uptake at multiple levels in a region- and context-dependent manner. Our findings nicely explain why, in WAG/Rij rats, intrathalamic VU0360172 loses its anti-absence activity when co-injected with sub-threshold doses of tiagabine, whereas both VU0360172 and tiagabine display anti-absence activities when co-injected in the cortex (D'Amore et al., 2015).

Even though the origin of the SWDs appears to be localised in the somatosensory cortex of WAG/Rij rats (Meeren et al., 2002), the enhancement of GABA uptake in the VB thalamus might represent a key mechanism by which mGlu5 receptor activation causes a rapid and sustained protective effect against absence seizures. That said, reduced GABA uptake in the somatosensory cortex might equally contribute to the acute (but not chronic) anti-absence effect of mGlu5 receptor activation, in the WAG/Rij rat model. Accordingly, reductions of SWD incidence were similar in terms of extent and temporal profile following VU0360172 injection in the VB thalamus and somatosensory cortex of WAG/Rij rats (D'Amore et al., 2015).

4.4. Conclusion

By definition, mGlu5 PAMs amplify receptor function in an activity-dependent manner, requiring glutamate binding to the receptor for their pharmacological activity. We therefore expect that these drugs are particularly effective when the receptors are highly activated by endogenous glutamate (as likely occurs during pathological oscillations within the cortico-thalamo-cortical network), and this might optimize the ratio between efficacy and tolerability in the treatment of absence epilepsy. mGlu5 receptor PAMs display robust antipsychotic and pro-cognitive activity, and are under development for the treatment of schizophrenia (Bruno et al., 2017; Maksymetz et al., 2017; Stansley and Conn, 2018). Some of the PAMs may cause convulsive seizures and excitotoxic neuronal death when systemically injected at high doses (Rook et al., 2013; Parmentier-Batteur et al., 2014; Conde-Ceide et al., 2016). These harmful effects may reflect the recruitment of NMDA receptors through a mechanism of mGlu5/NMDA receptor crosstalk (Awad et al., 2000; Mannaioni et al., 2001; Pisani et al., 2001). Stimulus-biased mGlu5 PAMs that do not recruit NMDA receptors, such as compound VU0409551 (Rook et al., 2015), may overcome this limitation. The evaluation of VU0409551 and other stimulus-biased mGlu5 PAMs will be an important step in translational research for the use of mGlu5 PAMs in the treatment of absence epilepsy.

Author's contributions

RC, MJW, IS, MV, LDM, GM, MC, FN, RTN, methodology and investigation; RC, MJW, GVL, AP, FC, VB, RTN, FN designed research; RC, MJW, FN, RTN wrote the paper; and all the authors read and revised the final manuscript.

Declaration of competing interest

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.neuropharm.2020.108240.

References

Akin, D., Ravizza, T., Maroso, M., et al., 2011 Dec. IL-1β is induced in reactive astrocytes in the somatosensory cortex of rats with genetic absence epilepsy at the onset of spike-and-wave discharges, and contributes to their occurrence. Neurobiol. Dis. 44 (3), 259–269.

Aronica, E., van Vliet, E.A., Mayboroda, O.A., et al., 2000 Jul. Upregulation of metabotropic glutamate receptor subtype mGluR3 and mGluR5 in reactive astrocytes in a rat model of mesial temporal lobe epilepsy. Eur. J. Neurosci. 12 (7), 2333–2344.

Awad, H., Hubert, G.W., Smith, Y., et al., 2000 Nov 1. Activation of metabotropic glutamate receptor 5 has direct excitatory effects and potentiates NMDA receptor currents in neurons of the subthalamic nucleus. J. Neurosci. 20 (21), 7871–7879.

Belelli, D., Peden, D.R., Rosahl, T.W., et al., 2005 Dec 14. Extrasynaptic GABAA receptors of thalamocortical neurons: a molecular target for hypnotics. J. Neurosci. 25 (50), 11513–11520.

Blumenfeld, H., 2005. Cellular and network mechanisms of spike-wave seizures. Epilepsia 46 (Suppl. 9), 21–33.
Bradford, M.M., 1976 May 7. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.
Bruno, V., Caraci, F., Copani, A., et al., 2017 Mar 15. The impact of metabotropic glutamate receptors into active neurodegenerative processes: a "dark side" in the development of new symptomatic treatments for neurologic and psychiatric disorders. Neuropharmacology 115, 180–192.
Burgdorf, C., Schäfer, U., Richardt, G., et al., 2010 Jun. U73122, an aminosteroid phospholipase C inhibitor, is a potent inhibitor of cardiac phospholipase D by a PIP2-dependent mechanism. J. Cardiovasc. Pharmacol. 55 (6), 555–559.
Celli, R., Santolini, I., Van Luijtelaar, G., et al., 2019 Apr. Targeting metabotropic glutamate receptors in the treatment of epilepsy: rationale and current status. Expert Opin. Ther. Targets 23 (4), 341–351.
Coenen, A.M., Blezer, E.H., van Luijtelaar, E.L., 1995 Jun. Effects of the GABA-uptake inhibitor tiagabine on electroencephalogram, spike-wave discharges and behaviour of rats. Epilepsy Res. 21 (2), 89–94.
Coenen, A.M., Van Luijtelaar, E.L., 2003 Nov. Genetic animal models for absence epilepsy: a review of the WAG/Rij strain of rats. Behav. Genet. 33 (6), 635–655.
Conde-Ceide, S., Alcázar, J., Alonso de Diego, S.A., et al., 2016 Jan 15. Preliminary investigation of 6,7-dihydropyrazolo[1,5-a]pyrazin-4-one derivatives as a novel series of mGlu5 receptor positive allosteric modulators with efficacy in preclinical models of schizophrenia. Bioorg. Med. Chem. Lett 26 (2), 429–434.
Cope, D.W., Hughes, S.W., Crunelli, V., 2005 Dec 14. GABAA receptor-mediated tonic inhibition in thalamic neurons. J. Neurosci. 25 (50), 11553–11563.
Cope, D.W., Di Giovanni, G., Fyson, S.J., et al., 2009 Dec. Enhanced tonic GABAA inhibition in typical absence epilepsy. Nat. Med. 15 (12), 1392–1398.
Crunelli, V., Cope, D.W., Terry, J.R., 2011 Dec. Transition to absence seizures and the role of GABA(A) receptors. Epilepsy Res. 97 (3), 283–289.
D'Amore, V., Santolini, I., van Rijn, C.M., et al., 2013 Mar. Potentiation of mGlu5 receptors with the novel enhancer, VU0360172, reduces spontaneous absence seizures in WAG/Rij rats. Neuropharmacology 66, 330–338.
D'Amore, V., Santolini, I., Celli, R., et al., 2014 Oct. Head-to head comparison of mGlu1 and mGlu5 receptor activation in chronic treatment of absence epilepsy in WAG/Rij rats. Neuropharmacology 85, 91–103.
D'Amore, V., von Randow, C., Nicoletti, F., et al., 2015 Jul. Anti-absence activity of mGlu1 and mGlu5 receptor enhancers and their interaction with a GABA reuptake inhibitor: effect of local infusions in the somatosensory cortex and thalamus. Epilepsia 56 (7), 1141–1151.
Drouin-Ouellet, J., Brownell, A.L., Saint-Pierre, M., et al., 2011 Feb. Neuroinflammation is associated with changes in glial mGluR5 expression and the development of neonatal excitotoxic lesions. Glia 59 (2), 188–199.
Dunkley, P.R., Jarvie, P.E., Heath, J.W., Kidd, G.J., Rostas, J.A., 1986 Apr 30. A rapid method for isolation of synaptosomes on Percoll gradients. Brain Res. 372 (1), 115–129.
Dutuit, M., Didier-Bazès, M., Vergnes, M., et al., 2000 Oct. Specific alteration in the expression of glial fibrillary acidic protein, glutamate dehydrogenase, and glutamine synthetase in rats with genetic absence epilepsy. Glia 32 (1), 15–24.
Errington, A.C., Gibson, K.M., Crunelli, V., et al., 2011 Apr 19. Aberrant GABA(A) receptor-mediated inhibition in cortico-thalamic networks of succinic semialdehyde dehydrogenase deficient mice. PloS One 6 (4), e19021.
Errington, A.C., Di Giovanni, G., Crunelli, V. (Eds.), 2014. Extrasynapitic GABAA ReCeptors. Springer, New York.
Jia, F., Pignataro, L., Schofield, C.M., et al., 2005 Dec. An extrasynaptic GABAA receptor mediates tonic inhibition in thalamic VB neurons. J. Neurophysiol. 94 (6), 4491–4501 Epub 2005 Sep. 14.
Leal, A., Vieira, J.P., Lopes, R., et al., 2016 Apr 6. Dynamics of epileptic activity in a peculiar case of childhood absence epilepsy and correlation with thalamic levels of GABA. Epilepsy Behav Case Rep 5, 57–65.
Maksymetz, J., Moran, S.P., Conn, P.J., 2017 Apr 26. Targeting metabotropic glutamate receptors for novel treatments of schizophrenia. Mol. Brain 10 (1), 15.
Mannaioni, G., Marino, M.J., Valenti, O., et al., 2001 Aug 15. Metabotropic glutamate receptors 1 and 5 differentially regulate CA1 pyramidal cell function. J. Neurosci. 21 (16), 5925–5934.
MacMillan, D., McCarron, J.G., 2010 Jul. The phospholipase C inhibitor U-73122 inhibits Ca^{2+} release from the intracellular sarcoplasmic reticulum Ca^{2+} store by inhibiting Ca^{2+} pumps in smooth muscle. Br. J. Pharmacol. 160 (6), 1295–1301.
Meeren, H.K., Pijn, J.P., Van Luijtelaar, E.L., et al., 2002 Feb 15. Cortical focus drives widespread corticothalamic networks during spontaneous absence seizures in rats. J. Neurosci. 22 (4), 1480–1495.
Mikati, M.A., Holmes, G.L., 1997 Nov. Lamotrigine in absence and primary generalized epilepsies. J. Child Neurol. 12 (Suppl. 1), S29–S37.
Miller, S., Romano, C., Cotman, C.W., 1995 Sep. Growth factor upregulation of a phosphoinositide-coupled metabotropic glutamate receptor in cortical astrocytes. J. Neurosci. 15 (9), 6103–6109.
Ngomba, R.T., Santolini, I., Salt, T.E., et al., 2011 Jul. Metabotropic glutamate receptors in the thalamocortical network: strategic targets for the treatment of absence epilepsy. Epilepsia 52 (7), 1211–1222.
Ngomba, R.T., van Luijtelaar, G., 2018 Feb. Metabotropic glutamate receptors as drug targets for the treatment of absence epilepsy. Curr. Opin. Pharmacol. 38, 43–50.
Nicoletti, F., Bockaert, J., Collingridge, G.L., et al., 2011 Jun. Metabotropic glutamate receptors: from the workbench to the bedside. Neuropharmacology 60 (7–8), 1017–1041.
Ollivier, M.L., Dubois, M.F., Krajinovic, M., et al., 2009 Dec. Risk factors for valproic acid resistance in childhood absence epilepsy. Seizure 18 (10), 690–694.
Panayiotopoulos, C.P., 1999 Oct. Typical absence seizures and their treatment. Arch. Dis. Child. 81 (4), 351–355.
Parmentier-Batteur, S., Hutson, P.H., Menzel, K., et al., 2014 Jul. Mechanism based neurotoxicity of mGlu5 positive allosteric modulators–development challenges for a promising novel antipsychotic target. Neuropharmacology 82, 161–173.
Pirttimaki, T., Parri, H.R., Crunelli, V., 2013 Feb 15. Astrocytic GABA transporter GAT-1 dysfunction in experimental absence seizures. J. Physiol. 591 (4), 823–833.
Pisani, A., Gubellini, P., Bonsi, P., et al., 2001. Metabotropic glutamate receptor 5 mediates the potentiation of N-methyl-D-aspartate responses in medium spiny striatal neurons. Neuroscience 106 (3), 579–587.
Rodriguez, A.L., Grier, M.D., Jones, C.K., et al., 2010 Dec. Discovery of novel allosteric modulators of metabotropic glutamate receptor subtype 5 reveals chemical and functional diversity and in vivo activity in rat behavioral models of anxiolytic and antipsychotic activity. Mol. Pharmacol. 78 (6), 1105–1123.
Rook, J.M., Noetzel, M.J., Pouliot, W.A., et al., 2013 Mar 15. Unique signaling profiles of positive allosteric modulators of metabotropic glutamate receptor subtype 5 determine differences in in vivo activity. Biol. Psychiatr. 73 (6), 501–509.
Rook, J.M., Xiang, Z., Lv, X., et al., 2015 May 20. Biased mGlu5-positive allosteric modulators provide in vivo efficacy without potentiating mGlu5 modulation of NMDAR currents. Neuron 86 (4), 1029–1040.
Shen, W., Martinez, K., Chuang, C.C., et al., 2013 May. The phospholipase C inhibitor U73122 attenuates trans-10, cis-12 conjugated linoleic acid-mediated inflammatory signaling and insulin resistance in human adipocytes. J. Nutr. 143 (5), 584–590.
Stansley, B.J., Conn, P.J., 2018 Feb. The therapeutic potential of metabotropic glutamate receptor modulation for schizophrenia. Curr. Opin. Pharmacol. 38, 31–36.
Tenney, J.R., Glauser, T.A., 2013 May. The current state of absence epilepsy: can we have your attention? Epilepsy Current 13 (3), 135–140.
Ulas, J., Satou, T., Ivins, K.J., et al., 2000 Jun Jun. Expression of metabotropic glutamate receptor 5 is increased in astrocytes after kainate-induced epileptic seizures. Glia 30 (4), 352–361.
van Luijtelaar, E.L., Coenen, A.M., 1988 Sep-Oct. Circadian rhythmicity in absence epilepsy in rats. Epilepsy Res. 2 (5), 331–336.
Wall, M.J., Usowicz, M.M., 1997 Mar Mar. Development of action potential-dependent and independent spontaneous GABAA receptor-mediated currents in granule cells of postnatal rat cerebellum. Eur. J. Neurosci. 9 (3), 533–548.