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A new metabotropic glutamate receptor agonist with in vivo anti-allodynic activity

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ABSTRACT

As part of the vital search towards improved therapeutic agents for the treatment of neuropathic pain, the central nervous system glutamate receptors have become a major focus of research. Outlined herein are the syntheses of two new biologically active 3′-cycloalkyl-substituted carboxycyclopropylglycines, utilizing novel synthetic chemistry. The reaction between substituted 1,2-dioxines and an aminophosphonate furnished the cyclopropane core in a single step with all required stereochemistry of pendant groups. In vitro binding assays at metabotropic glutamate receptors revealed selective activity. In vivo testing in a rodent model of neuropathic pain indicated one amino acid significantly and dose-dependently decreased mechanical allodynia.

1. Introduction

Chronic neuropathic pain affects a significant proportion of the population worldwide and decreases quality of life. Sufferers experience increased sensations of pain, known as hyperalgesia as well as allodynia where painful sensations arise from innocuous touch or pressure. Many of the current medications used in its treatment do not give adequate and effective pain relief in all cases[1,2] thus, much research has been focused on finding alternatives. The excitatory neurotransmitter L-glutamate (**1**), plays a significant role in the modulation of pain signalling.[3] There are two main classes of glutamate receptors, the ionotropic and the metabotropic. The ionotropic glutamate receptors (iGluRs) are ligand-gated ion channels divided into three broad types: *N*-methyl-D-aspartate (NMDA), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and 2-carboxy-3-carboxymethyl-4-isopropenylpyrrolidine (kainate). In contrast, the metabotropic glutamate receptors (mGluRs) are G-protein coupled receptors (GPCRs), having eight known subtypes, mGluR1–mGluR8, divided into three groups based on sequence homology, signal transduction mechanisms and agonist/antagonist interactions.[4] It has previously been shown that drugs targeting specific subtypes of the metabotropic glutamate receptors show efficacy in various pain models including those where allodynia and hyperalgesia are present.[5,6] Over the past 10 years, many new mGluR ligands have been synthesized both as experimental probes and potential therapeutic agents.[7] One potent class of competitive mGluR2/3 ligands, the carboxycyclopropylglycines (CCGs, Fig. 1) show potential for development as a new range of therapeutic agents.[8,9] These analogues of the neurotransmitter

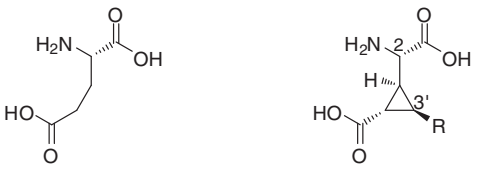

Figure 1. Structural comparison between glutamate and carboxycyclopropylglycines.

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glutamate (**1**) are conformationally constrained by a constituent cyclopropane ring that contains an additional substituent (R) that can influence receptor specificity and selectivity of one mGluR subtype over another.

Recent advances in synthetic chemistry have provided a general route for synthesis of 3′-substituted CCGs, allowing exploration of their therapeutic potential.[10] Herein, we report the synthesis and biological activity of two new cycloalkyl-substituted CCGs ((±)-**3** and (±)-**4**, Fig. 2), one of which ((±)-**3**) shows anti-allodynic activity in a rodent model of neuropathic pain. Based on known structure–activity relationships of previously tested CCGs, it was hypothesized that the activity of these compounds may switch from agonist to antagonist with increasing cycloalkyl ring size.

2. Results and discussion

2.1. Synthesis

The key step in the synthesis of the desired CCG analogues (±)-**3** and (±)-**4** involved reaction of an aminophosphonate and a *cis* γ-hydroxyenone formed by base catalysed ring-opening of a 1,2-dioxine (Scheme 1). Michael addition of the phosphonate nucleophile to the γ-hydroxyenone and intramolecular ring-closure formed the protected cyclopropane amino acids as a mixture of diastereoisomers, that were separated by flash chromatography.[10] This synthetic route is significantly shorter than other reported methods with the added advantage that the 3′-substitution can be altered simply by preparing the appropriate 1,2-dioxine.[8,11]

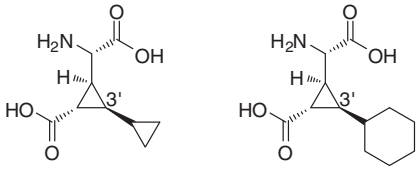
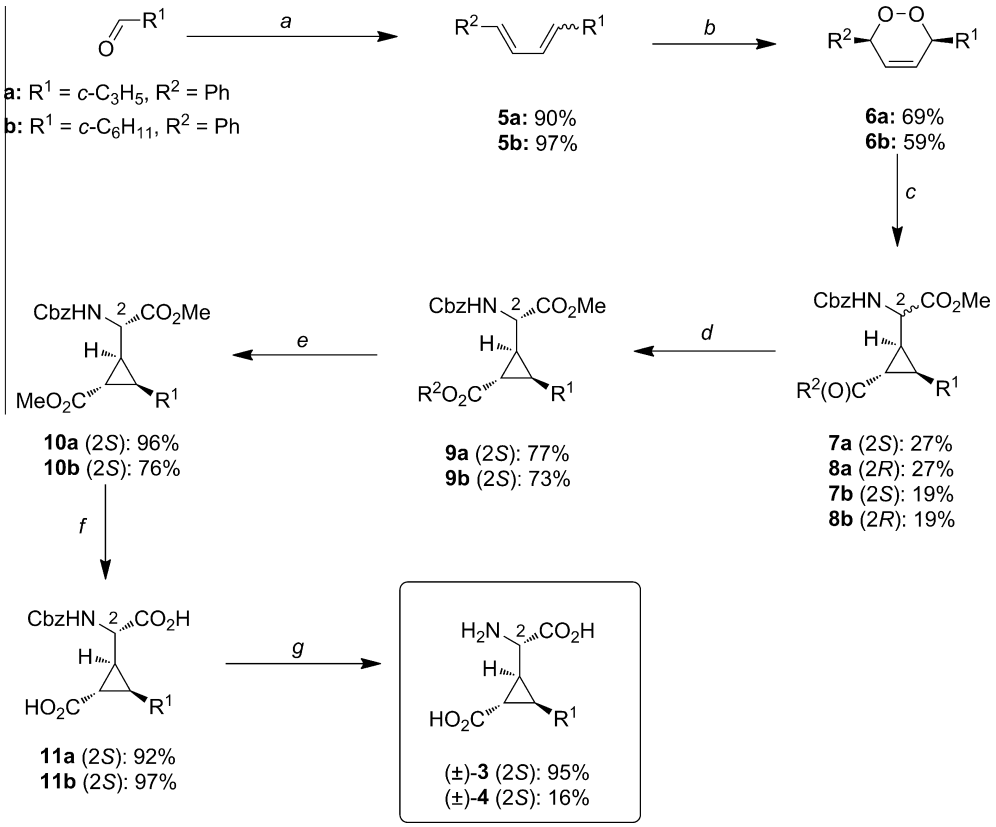


Figure 2. Two new carboxycyclopropylglycines.

The starting 1,3-butadienes (**5a**, **5b**) were prepared in excellent yields, 90% and 97%, respectively, by Wittig reaction of cycloalkylcarboxaldehydes with the ylide derived from cinnamyl triphenylphosphonium chloride on reaction with potassium *tert*-butoxide. Photolysis (dye sensitised photo-oxidation) of the 1,3-butadienes employing rose bengal bis(triethylammonium) salt in the presence of oxygen, afforded the desired 3-cycloalkyl-6-phenyl-3,6-dihydro-1,2-dioxines (**6a**, **6b**) in good yields of 69% and 59%, respectively. The protected cyclopropane amino acids were obtained by reaction with (±)-Cbz-α-phosphonoglycine trimethylester under basic conditions followed by separation of the diastereoisomers by flash chromatography to provide the pure isomers (**7a**, **7b**). The relative stereochemistry of the cyclopropane substituent could not be determined by ^{1}H NMR or 2D NMR due to overlap of the cyclopropyl and cycloalkyl signals and since identical ROESY interactions were observed for both diastereoisomers. However, the relative stereochemistry of cyclopropane **7a** (Fig. 3) and **7b** was determined by single crystal X-ray crystallography and shown to match that for the previously reported pharmacologically active CCGs.[8,9]

A Baeyer–Villiger oxidation of **7b** was performed using trifluoroperacetic acid produced in situ from trifluoroacetic anhydride and hydrogen peroxide.[34] This gave the desired phenyl ester, **9b** in 73%



Scheme 1. Reagents and conditions: (a) $PhCH_2CH{=}CHPPh_3Cl$, $KOBu^t$, rt, Et_2O; (b) O_2, *hv*, rose bengal, 0 °C, DCM; (c) (i) (±)-Cbz-α-phosphonoglycine trimethylester, LDA mono THF complex in cyclohexane, −78 °C to rt, THF; (ii) chromatographic separation of diastereoisomers; (d) for **9a**: *m*-CPBA, rt, $CHCl_3$; for **9b**: TFAA, H_2O_2, 0 °C, DCM; (e) MeOH, H_2SO_4, reflux; (f) $LiOH_{(aq)}$, rt, THF; (g) H_2, Pd/C (5 wt %), MeOH.

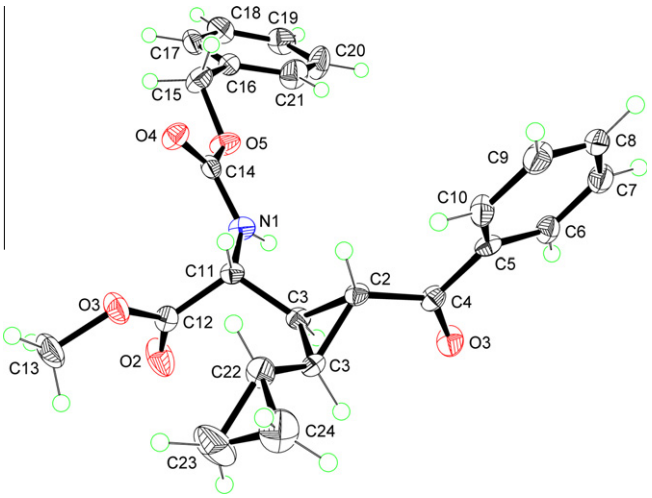


Figure 3. Molecular structure and crystallographic numbering scheme for compound **7a** ($C_{24}H_{25}NO_5$). Diagram drawn with 50% displacement ellipsoids.

yield. The use of this methodology on **7a** gave rise to some decomposition and only gave a low yield of **9a** (24%). However, the use of *m*-CPBA produced **9a** in good yield (77%). Formation of the phenyl ester was confirmed by ^{1}H NMR with an absence of aromatic and cyclopropane resonances associated with the protons adjacent to the ketone moiety and the appearance of phenyl ester resonance at ca. 7.1 ppm. After conversion to the ester, a shift in cyclopropyl carbonyl resonances was also observed in the ^{13}C NMR from approximately 198 ppm to ca. 172 ppm. Direct hydrolysis of compounds **9a** and **9b** to give carboxylic acids **11a** and **11b** was complicated by the release of phenol, making purification of the desired product very difficult. To circumvent this problem, **9a** and **9b** were first transesterified to give **10a** and **10b** in excellent (96%) and good (76%) yields, respectively, after purification by flash column chromatography. Ester hydrolysis gave almost quantitative yields of **11a** and **11b** (92% and 97%, respectively).

The final step in the synthesis involved removal of the Cbz protecting group of **11a** and **11b** by catalytic hydrogenation, to give the desired racemic amino acids **3** and **4**. These were purified by conversion to the corresponding hydrochloride salts by addition of concentrated HCl and removal of excess acid in vacuo. The desired free amino acids (±)-**3** and (±)-**4** were re-isolated by stirring with anhydrous propylene oxide followed by isolation of the precipitate by filtration.[11] It should be noted that the very low yield (16%) of (±)-**4** was thought to result from decomposition whilst converting to the hydrochloride salt. By comparison, compound (±)-**3** appeared more stable to the acidic conditions.

3. Pharmacology

3.1. Receptor binding assays

The in vitro drug screening at native rodent NMDA, kainate and AMPA ionotropic glutamate receptor subtypes showed a lack of activity for compounds (±)-**3** and (±)-**4** at any of these receptor subtypes as indicated by IC_{50} >100 μM. Compound (±)-**2** was used as a negative control as it has not previously been reported to show activity at the ionotropic receptors.

Referring to Table 1, the data obtained from functional assays at the metabotropic glutamate receptors indicates that both new compounds exhibited functional binding activity at mGluR2. The second messenger assays to measure mGluR2 activity involved the radioimmunoassay detection of forskolin-stimulated cAMP concentrations. The binding of agonists to the receptor causes a down regulation of adenylyl cyclase and a consequent decrease in cAMP levels. Antagonist activity was measured as an increase in cAMP levels in the presence of the endogenous agonist, glutamate. The methyl-substituted cyclopropyl amino acid (±)-**2** was used as a positive control and had a measured EC_{50} value of 0.01 μM at mGluR2, which is comparable to that reported previously by Collado et al.[11] Test compound (±)-**3** showed agonist activity at mGluR2 which was approximately fivefold lower than the positive control, with a measured EC_{50} of 0.05 μM. Overall, the in vitro data indicates that compound (±)-**3** is potent and selective for mGluR2 over mGluR1 and mGluR4, as well as having no activity at the ionotropic receptors. Thus cyclopropyl amino acid (±)-**3** is a good candidate for further investigation in vivo. Finally, test compound (±)-**4** showed weak antagonist activity at mGluR2 with an IC_{50} of 62 μM. The in vitro assays confirm the initial hypothesis where it was expected that cyclopropyl-substitution, as in compound (±)-**3** would create an agonist, whereas cyclohexyl substitution, as in compound (±)-**4**, would create an antagonist. Compound (±)-**3** is structurally quite similar to the positive control, however the cyclopropyl-substitution is slightly more bulky than a simple methyl group, which may account for the loss of potency. It has been shown that if the methyl group is exchanged with an ethyl group, then binding activity decreases 20-fold. However, substitution with a CH_2OH group (Fig. 1, **2a**) in place of the ethyl (Et) group increases agonist activity by more than 30-fold relative to an Et group. This suggests the region of the receptor where the cyclopropyl substituent lies is a polar region and so the presence of a hydrophobic cyclopropyl group creates a less favourable interaction. On the other hand, compound (±)-**4** is substituted with a comparatively bulky cyclohexyl group which seems to convey antagonist activity. Substitution by other bulky, hydrophobic groups, such as phenyl and xanthenyl has been reported previously to consistently produce receptor antagonists, thus this result is consistent with steric size being important for determining agonist or antagonist activity at this position.[12,13] The new compounds were only tested at three of the eight subtypes of the metabotropic glutamate receptors; mGluR1, mGluR2 and mGluR4, representing the three groups of receptors. It is possible that these compounds may have also shown some activity at the five receptor subtypes which were not investigated.

Table 1
Metabotropic glutamate receptor binding data

Compound	mGluR1 (μM)	mGluR2 (μM)	mGluR4 (μM)
(±)-**2** (R = Me)	>100	0.01[a]	>100
(±)-**3** (R = *c*-C_3H_5)	>100	0.05 [7.395 ± 0.152][a,b]	>100
(±)-**4** (R = *c*-C_6H_{11})	>100	**62**[c]	>100

Plain and bold text refers to agonist (EC_{50}) and antagonist (IC_{50}) potencies, respectively.
[a] n = 4.
[b] Values in brackets indicate pEC_{50} ± SEM.
[c] n = 3.

3.2. In vivo neuropathic pain model

The cyclopropane amino acid (±)-**3** showed potent and selective activity in the in vitro binding assays. Based on this, (±)-**3** was tested in vivo in a neuropathic pain model, the chronic constriction injury of the sciatic nerve in Sprague-Dawley rats. Von Frey filaments were employed in order to test for neuropathic pain, as observed through the presence of mechanical allodynia. During this testing procedure, fine filaments of increasing diameter and bending force are touched to the underside of the hind paw until the animal shows a withdrawal response. Illustrated in Figure 4, prior to surgery, all rats responded on the von Frey test at approximately 9.0 g, both ipsilaterally and contralaterally as indicated at baseline

(BL). Following chronic constriction injury of the sciatic nerve, subsequent von Frey testing on days 3, 10 and 14 revealed a decrease in response threshold to the minimum 0.4 g bilaterally, indicating that all rats were allodynic. On the day of drug dosing, a pre-drug baseline (PDBl) measurement was also made to ensure the reliability of inter-day test results. After 1 h an intrathecal dose of (2*R*,4*R*)-APDC (500 nmols), von Frey response thresholds were significantly increased bilaterally, compared to animals receiving vehicle. After 3 h intrathecal drug dosing, response thresholds were similar to the 1 h post-drug time point. After 1 h an equimolar intrathecal dose of compound (±)-**3**, von Frey response thresholds of drug-treated animals were significantly increased bilaterally, compared to animals receiving vehicle. After 3 h drug dosing, von Frey response thresholds of drug-treated animals were significantly increased bilaterally, compared to animals receiving vehicle. After 24 h, all animals had returned to pre-drug allodynia bilaterally, with positive control and drug-treated animals no longer significantly different from vehicle-treated controls. No other behavioural effects of either drug were noted.

In order to investigate any possible dose dependency of the anti-allodynic effect of (±)-**3**, two lower doses, 50 nmols and 250 nmols, were also administered. For clarity, only the data relating to the ipsilateral 3 h time point is shown in Figure 5. The data strongly indicates a dose dependency of (±)-**3** with 50 nmols causing an increase in response threshold to 5.0 g, 250 nmols causing a significant increase to 7.5 g and 500 nmols causing a significant increase in response threshold to 10.0 g compared to vehicle.

This work was carried out to ascertain the in vivo effects of the novel, selective Group II metabotropic glutamate receptor (mGluR) agonist (±)-**3** in a rodent model of neuropathic pain. The model employed in this study was chronic constriction of the sciatic nerve, where the nerve bundle undergoes loose ligation with chromic gut at the mid-thigh level. The outcome is a neuropathic pain state that develops over a period of two weeks and results in reliably measurable allodynia, resembling that reported and measured in human neuropathic pain states. Although the surgery manipulates the sciatic nerve on only one side of the animal, allodynia develops on both the operated side (ipsilateral) and the non-operated side (contralateral) as can be seen in Figure 4.[14] This phenomena is known as mirror-image allodynia and there is mounting evidence to suggest that it is caused by glial activation and the associated action of pro-inflammatory cytokines.[14] Due to a lack of test compound and the lack of pharmacokinetic data, it was opted to administer the drugs via the intrathecal route, by lumbar puncture between the L5 and L6 vertebrae. Drugs that act at the spinal level have the advantage of avoiding side effects possibly encountered when high systemic doses are required to achieve the needed

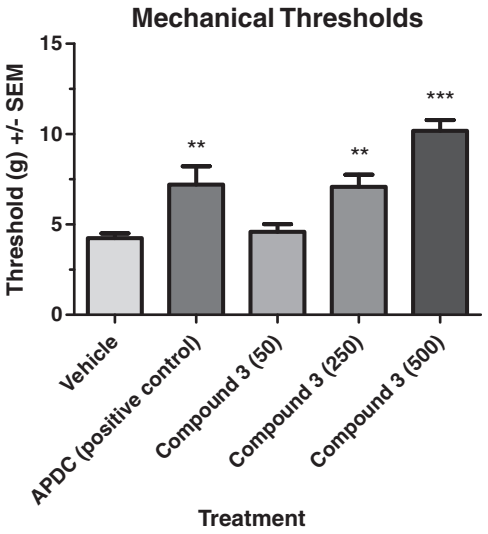


Figure 5. Comparison of the changes in ipsilateral mechanical threshold 3 h after intrathecal administration of (2*R*,4*R*)-APDC (500 nmols), (±)-**3** (50, 250 and 500 nmols) or vehicle. Data expressed in grams ± SEM, $n = 6$. $^{**}P$ <0.01, $^{***}P$ <0.001 compared to vehicle.

CNS levels. Intrathecal administration also confines drug action to the spinal cord and thus limits the possible mechanisms responsible for drug effects, thus simplifying interpretation of the data obtained. Although only in vitro receptor binding data relating to mGluR2 receptors was obtained, it is reasonable to assert that compound (±)-**3** would also activate mGluR3 receptors, given that all known ligands in this compound class are approximately equipotent at mGluR2 and R3.[8,9]

It is widely reported that agonists at these receptors can significantly decrease the behavioural signs associated with neuropathic pain, including thermal and mechanical hyperalgesia and mechanical allodynia.[15–17] Several in situ hybridization studies have identified mRNA encoding for the mGluR3 receptor in the spinal dorsal horn, however, levels of mRNA encoding for the mGluR2 receptor were found to be very low.[18,19] Jia et al. used light and electron microscopic immunocytochemistry to show the presence of mGluR2/3 in the inner part of lamina II and also the presence of these receptors pre-synaptically on GABAergic neurons within the dorsal horn.[20] These results were confirmed by Azkue and colleagues using an immunogold technique.[21] Finally, both Gerber et al. and Carlton et al. have provided evidence that mGluR2 and R3 receptors were also located pre-synaptically on glutamatergic primary afferent neurons within the dorsal horn.[18,22]

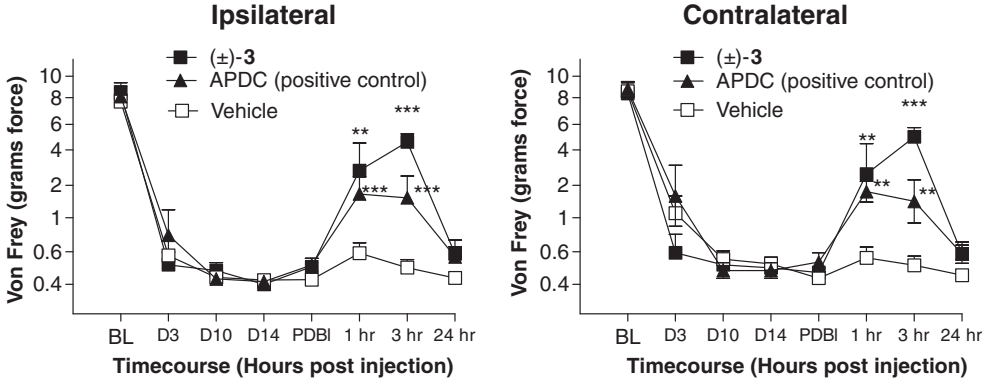


Figure 4. Effects of an acute dose of (±)-**3** (500 nmols i.t.) and a positive control, APDC (500 nmols i.t.) on mechanical allodynia following chronic constriction injury of the sciatic nerve in Sprague-Dawley rats. Data are expressed as mean ± SEM, $n = 6$. BL = baseline, PDBl = pre-drug baseline. $^{**}P$ <0.01, $^{***}P$ <0.001 compared to vehicle.

The known selective Group II mGluR agonist, (2*R*,4*R*)-APDC (aminopyrrolidine-2,4-dicarboxylate), used here as a positive control, has previously been shown by Fisher et al. to prevent the development of mechanical allodynia, following loose ligation of the sciatic nerve chronic constriction injury, and using a repeated intrathecal dosing regime.[16] Similarly, we observed that this compound is capable of producing a significant reversal of mechanical allodynia with the greatest effect apparent at the 1 h time point. There was no possibility to use receptor antagonism to verify that the anti-allodynic effects observed were indeed due to Group II mGluR activation, as receptor blockade would be pro-nociceptive, which was undesirable.[23] The test compound (±)-**3** was found to have greater in vivo anti-allodynic activity than the positive control, (2*R*,4*R*)-APDC. This result was expected given the EC_{50} value of amino acid (±)-**3** at mGluR2 is eight times higher, indicating the higher in vitro potency of (±)-**3**.[7] Interestingly, the effects on withdrawal threshold were maximal for test drug (±)-**3** when measured at the 3 h time point, compared to 1 h post drug dosing for the positive control, (2*R*,4*R*)-APDC. This observation is difficult to explain, apart from potential differences in the polarizability and lipophilicity of the two compounds, which may have affected their distribution and absorption.

Endogenous glutamate within the dorsal horn is known to act pro-nociceptively on glutamatergic neurons which project to the brain stem and higher brain centres.[24] Hence, pre-synaptic modulation of these projecting neurons is capable of attenuating pain signaling. Since mGluR2 and R3 have been identified on glutamatergic primary afferent neurons, it is reasonable to assume that mGluR2/3 ligands could modulate signaling in these projecting neurons, thus resulting in anti-nociception.[18,22] It is well known that there exists descending inhibitory pathways which serve to dampen and attenuate pain signaling at the spinal dorsal horn level.[25] Research by Zhou et al. has highlighted the action of endogenous glutamate, acting by stimulation of Group II mGluRs in the spinal dorsal horn, in causing a decrease in GABAergic neuron activity and a subsequent decrease in GABA inhibition due to nociceptive input.[26] Their data suggests that GABA inhibition in the dorsal horn is anti-nociceptive and hence is evidence for disinhibition of descending anti-nociceptive neurons which would result in analgesia. Metabotropic glutamate receptors 2 and 3 have been identified both on glutamatergic primary afferent neurons and GABAergic axon terminals. Binding to excitatory glutamatergic neurons would result in decreased ascending nerve transmission via ascending projecting neurons, whereas binding to inhibitory GABAergic neurons likely causes disinhibition of descending inhibitory neurons. Thus, it is suggested that these tandem mechanisms could partly be responsible for the observed reversal of mechanical allodynia.

Glial cells are also known to be present in the spinal dorsal horn and accumulating evidence points to the presence of mGluR3 receptors on glia.[20,21,27] Much research has demonstrated the important contribution of glial cell activation and consequent changes to glial and neuronal signaling to the development and maintenance of neuropathic pain.[14] Glial cell activation leads to increased release of pro-inflammatory cytokines such as tumour necrosis factor alpha (TNFα), interleukin 1 beta (IL-1β) and interleukin 6 (IL-6).[28] It has been shown that levels of pro-inflammatory cytokines are increased both centrally and spinally due to chronic constriction injury of the sciatic nerve.[29] These cytokines cause inflammation responses that result in changes to nerve signaling function which contributes to the allodynia and hyperalgesia which is evident in the neuropathic pain state.[30] The activation of Group II metabotropic glutamate receptors has been demonstrated to modulate glial cell activation and play a role in alterations to levels of pro-inflammatory cytokines.[31] Work by Popik et al. has revealed that the potent, selective Group II metabotropic glutamate agonist, LY354740, is able to reverse opioid tolerance in vivo; an effect which is quite feasibly due to the drug's action at glial mGlu receptors.[32]

In the present study, it is possible that the selective mGluR2/3 receptor agonist, (±)-**3**, is acting to reverse mechanical allodynia by interactions with both neurons and glia. The pharmacological action of compound (±)-**3** on metabotropic glutamate receptors 2 and 3 expressed both on glutamatergic and GABAergic neurons and on glial cells within the dorsal horn of the spinal cord, most likely mediated the reversal of mechanical allodynia observed in this study.

4. Conclusions

A concise synthesis of a new, potent and selective Group II metabotropic glutamate receptor agonist is reported which exhibits in vivo anti-allodynic efficacy in an animal model of neuropathic pain. Overall, the therapeutic potential of targeting metabotropic glutamate receptors for treatment of neuropathic pain is exemplified and the need for further research into this class of compounds highlighted.

5. Experimental section

5.1. Chemistry

Diethyl ether and dichloromethane were dried over 4 Å sieves. Methanol was dried over 3 Å sieves. THF was dried over sodium wire with benzophenone as indicator and distilled just prior to use. All organic extracts were dried over anhydrous magnesium sulfate. Thin layer chromatography was carried out using aluminium sheets coated with Silica Gel 60 F_{254} (40 × 80 mm) and visualized under 254 nm light, or developed in vanillin dip. Flash chromatography was accomplished using Silica Gel 60 (230–400 mesh). All yields reported refer to isolated material judged to be >95% homogenous by TLC and NMR spectroscopy.

^1H and ^{13}C NMR spectra were obtained using either a 300 MHz or 600 MHz instrument. NMR spectra were recorded in $CDCl_3$ solution using TMS (0 ppm) and $CDCl_3$ (77.0 ppm) as internal standards and D_2O (4.87 ppm) using *t*-butanol as external zero for ^{13}C NMR. NMR spectra collected in CD_3OD were calibrated to CD_3OD (3.31 and 49.0 ppm). Infrared spectra were recorded between solid plates (NaCl) as a nujol mull or thin film. Melting points are uncorrected. Electron impact (EI) mass spectra were recorded using a mass spectrometer operating at 70 eV. Accurate mass measurements were performed at the School of Chemistry, Monash University, Victoria, Australia using electrospray ionisation (ESI). Elemental analysis was conducted in the Department of Chemistry, University of Otago, Dunedin, New Zealand. X-ray crystallography was performed by Edward R. T. Tiekink at The University of Texas, San Antonio, USA. The following compounds were purchased and used without further purification: rose bengal bis(triethylammonium)salt, *trans* cinnamyl chloride, cyclopropanecarboxaldehyde, cyclohexanecarboxaldehyde and (±)-Cbz-α-phosphonoglycine trimethyl ester.

5.2. General procedure for the preparation of 1-cycloalkyl-4-phenyl-1,3-butadienes

Cinnamyl triphenylphosphonium chloride (36 mmol) was added to anhydrous diethyl ether or anhydrous THF (150 mL). Whilst the slurry was stirred under N_2, potassium *tert*-butoxide (39 mmol) was added in one portion. After stirring for 15 min, the cycloalkanecarboxaldehyde (30 mmol), dissolved in anhydrous ether (50 mL), was added dropwise over approximately 20 min. The reaction was stirred overnight at which point the excess base

was quenched with half-saturated $NH_4Cl_{(aq)}$ and the volatiles removed in vacuo. The residue was diluted with water (100 mL) and extracted with dichloromethane (3 × 100 mL). After drying over anhydrous $MgSO_{4(s)}$, filtration and removal of the solvent in vacuo, the residue was taken up in hexanes and filtered through a pad of silica (4 cm × 5 cm ID) to remove all triphenylphosphine oxide. Removal of the hexanes in vacuo afforded the desired crude 1,3-butadienes.

5.2.1. [(1*E*,3*E*)-4-Cyclopropylbuta-1,3-dien-1-yl]benzene (5a)

Colourless oil (17.0 g, 90%); R_f: 0.79 (35% dichloromethane in hexanes, v/v); ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.43–0.48 (m, 2H), 0.77–0.90 (m, 2H), 1.42–1.54 (m, 1H), 1.83–1.93 (m, 1H), 4.85–4.92 (m, 1H), 5.27–5.39 (m, 2H), 6.07–6.15 (m, 1H), 6.25–6.55 (m, 3H), 6.68–6.76 (m, 1H), 7.14–7.44 (m, 10H). The crude product was used without further purification.

5.2.2. [(1*E*,3*E*)-4-Cyclohexylbuta-1,3-dien-1-yl]benzene (5b)

Colourless oil (22.0 g, 97%); R_f 0.81 (35% dichloromethane in hexanes, v/v). All other physical and chemical properties were identical to those previously reported.[33] The crude product was used without further purification.

5.3. General procedure for the preparation of 1,2-dioxines

1,2-Dioxines were prepared by the rose bengal bis(triethylammonium)salt sensitized [4π+2π] cycloaddition of singlet oxygen onto the corresponding 1,3-butadiene. The 1,3-butadiene (3.0 g, 15 mmol) and rose bengal bis(triethylammonium)salt (100 mg, 0.09 mmol) were dissolved in dichloromethane (100 mL) and the reaction vessel semi-immersed in an ice bath so that the reaction mixture was maintained at a temperature of ca. 5–10 °C. A stream of oxygen was then passed through the solution, whilst irradiating with two to three tungsten halogen lamps (500 W) at a distance of 10 cm from the reaction vessel for 6–12 h. Upon consumption of the diene, as indicated by TLC (35% dichloromethane in hexanes, v/v), the volatiles were then removed in vacuo and the residue subjected to flash column chromatography (10% ethyl acetate in hexanes, v/v), furnishing the pure 1,2-dioxine.

5.3.1. (±) (3*S*,6*S*)-3-Cyclopropyl-6-phenyl-3,6-dihydro-1,2-dioxine (6a)

Recrystallization from hot hexanes gave compound **6a** as colourless needles (15.4 g, 69%); mp: 54–55 °C; R_f 0.49 (10% ethyl acetate in hexanes, v/v); IR (nujol): 2922, 2852, 2725, 2672, 1715, 1455, 1378, 1305, 1256, 1190, 1160 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 0.31–0.67 (m, 4H), 1.02–1.13 (m, 1H), 3.87–3.92 (m, 1H), 5.48 (dd, J = 1.8, 2.1 Hz, 1H), 6.09–6.20 (m, 2H), 7.30–7.43 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 1.7, 3.4, 13.2, 79.9, 82.7, 137.8; MS (EI) ($C_{13}H_{14}O_2$) *m/z* (%): (M^+ 202, <1%), 173 (10), 160 (20), 133 (10), 118 (20), 105 (100), 97 (5), 77 (40), 69 (35), 51 (10); HRMS (ESI, $[M+Na]^+$) calcd for $C_{13}H_{14}O_2Na_1$: 225.0894, found 225.0884; Anal. Calcd for $C_{13}H_{14}O_2$: C, 77.20; H, 6.98. Found: C, 77.48; H, 6.88.

5.3.2. (±) (3*S*,6*S*)-3-Cyclohexyl-6-phenyl-3,6-dihydro-1,2-dioxine (6b)

Colourless solid (2.05 g, 59%); mp <30 °C; R_f 0.84 (30% ethyl acetate in hexanes, v/v); IR (nujol): 3082, 3054, 3032, 2932, 2862, 1491, 1453, 1337, 1316, 1292, 1272, 1254 cm^{-1}; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.30–1.90 (m, 10H), 2.18 (ddd, J = 8.7, 8.4, 8.1 Hz, 1H), 4.34–4.39 (ddd, J = 2.1, 2.1 Hz, 1H), 5.48 (dd, J = 2.4, 2.1 Hz, 1H), 6.08–6.18 (m, 2H), 7.35–7.47 (m, 5H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 25.3, 25.4, 28.4, 29.4, 42.7, 80.1, 82.3, 126.6, 128.0, 128.4, 128.6, 128.6, 137.8; MS (EI) ($C_{16}H_{20}O_2$) *m/z* (%): (M^+ 244, <1%), 198 (100), 169 (15), 156 (30), 142 (30), 129 (73), 115 (25), 105 (12), 79 (10); HRMS (ESI, $[M+4H]^+$) calcd for $C_{16}H_{24}O_2$: 248.1776, found 248.1647.

5.4. General cyclopropanation procedure

(±)-Cbz-α-phosphonoglycine trimethyl ester (15 mmol) was dissolved in anhydrous, freshly distilled THF, under N_2. The solution was cooled to ca. −78 °C and freshly prepared, titrated LDA mono-tetrahydrofuran complex in cyclohexane (13 mmol) was added dropwise. After ca. 30 min, the 1,2-dioxine (14 mmol) was added. After 15 min the reaction was warmed to ca. −15 to −10 °C. The reaction was maintained at this temperature for >4 h and then allowed to warm to rt overnight. The reaction was quenched with half-saturated $NH_4Cl_{(aq)}$, extracted with diethyl ether, dried over $MgSO_{4(s)}$, filtered and the solvents removed in vacuo to yield a 1:1 mixture of (2*RS*)-diastereoisomers. Flash column chromatography (20% ethyl acetate in hexanes, v/v) afforded the desired pure diastereoisomers.

5.4.1. (±) Methyl (2*S*)-[(1*R*,2*S*,3*S*)-3-benzoyl-1,1′-bi(cyclopropyl)-2-yl]{[(benzyloxy)carbonyl]amino}ethanoate (7a)

Colourless needles (500 mg, 27%); mp: 84–85 °C; R_f 0.25 (20% ethyl acetate in hexanes, v/v); IR (nujol): 3384, 3073, 2924, 2727, 1740, 1704, 1662, 1597, 1580, 1520, 1456, 1377, 1294, 1284, 1230 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.28–0.31 (m, 1H), 0.39–0.42 (m, 1H), 0.61–0.68 (m, 2H), 1.01–1.06 (m, 1H), 1.67 (ddd, J = 6.0, 3.6, 1.8 Hz, 1H), 2.02 (ddd, J = 6.0, 4.8, 3.0 Hz, 1H), 2.88 (dd, J = 4.8, 4.8 Hz, 1H), 3.82 (s, 3H), 4.51 (dd, J = 9.0, 1.8 Hz, 1H), 5.10 (dd, J = 12.6, 12.6 Hz, 1H), 5.57 (d, J = 8.4 Hz, 1H), 7.26–7.30 (m, 4H), 7.34–7.37 (m, 1H), 7.42–7.45 (m, 2H), 7.54–7.57 (m, 1H), 7.96–7.97 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 4.8, 8.8, 28.1, 34.2, 34.3, 52.7, 53.0, 67.1, 127.9, 128.2, 128.5, 128.6, 133.0, 136.1, 137.4, 156.1, 171.6, 198.3; MS (EI) ($C_{24}H_{25}O_5N$) *m/z* (%): (M^+ 407, <1%), 240 (6), 185 (100), 167 (6), 157 (6), 144 (12), 129 (8), 115 (9), 105 (100), 91 (9), 77 (70), 59 (4), 51 (8); HRMS (ESI, $[M+H]^+$) calcd for $C_{24}H_{26}O_5N$: 408.1811, found 408.1813; Anal. Calcd for $C_{24}H_{25}O_5N$: C, 70.74; H, 6.18; N, 3.44. Found: C, 70.74; H, 6.13; N, 3.48.

5.4.2. (±) Methyl (2*S*)-[(1*S*,2*S*,3*R*)-2-benzoyl-3-cyclohexylcyclopropyl]{[(benzyloxy)carbonyl]amino}ethanoate (7b)

White solid (642 mg, 19%); mp: 150–151 °C; R_f 0.62 (30% ethyl acetate in hexanes, v/v); IR (nujol): 3387, 2926, 2727, 2669, 1737, 1708, 1659, 1595, 1580, 1519, 1455, 1377, 1292 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 1.08–2.13 (m, 13H), 2.74–2.76 (m, 1H), 3.79 (s, 3H), 4.29–4.32 (m, 1H), 5.07 (d, J = 12.0 Hz, 1H), 5.12 (d, J = 12.0 Hz, 1H), 5.48 (d, J = 8.4 Hz, 1H), 7.26–7.57 (m, 8H), 7.98–7.99 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 25.8, 26.1, 26.2, 28.0, 32.9, 33.6, 34.3, 37.0, 39.0, 52.6, 52.9, 67.1, 128.0, 128.1, 128.2, 128.5, 128.6, 133.0, 136.0, 137.3, 156.0, 171.4, 198.6; MS (EI) ($C_{27}H_{29}O_5N$) *m/z* (%): (M^+ 450, <1%), 227 (100), 209 (20), 145 (30), 105 (90), 91 (30); HRMS (ESI, $[M+NH_4]^+$) calcd for $C_{27}H_{35}O_5N_2$: 467.2546, found 467.2548.

5.4.3. (±) Methyl (2*R*)-[(1*R*,2*S*,3*S*)-3-benzoyl-1,1′-bi(cyclopropyl)-2-yl]{[(benzyloxy)carbonyl]amino}ethanoate (8a)

Colourless needles (500 mg, 27%); mp: 104–105 °C; R_f 0.18 (20% ethyl acetate in hexanes, v/v); IR (nujol): 3342, 3181, 2924, 2727, 2671, 1749, 1725, 1716, 1648, 1596, 1577, 1531, 1462, 1377, 1340, 1270, 1202 cm^{-1}; ^{1}H NMR (600 MHz, $CDCl_3$) δ 0.22–0.26 (m, 1H), 0.29–0.33 (m, 1H), 0.53–0.57 (m, 1H), 0.59–0.63 (m, 1H), 0.81–0.82 (m, 1H), 1.54 (ddd, J = 8.4, 7.2, 4.8 Hz, 1H), 1.95 (ddd, J = 4.8, 4.2, 2.4 Hz, 1H), 2.80 (dd, J = 4.8, 4.2 Hz, 1H), 3.69 (s, 3H), 4.33–4.36 (m, 1H), 5.11 (d, J = 12.0 Hz, 1H), 5.16 (d, J = 12.0 Hz, 1H), 5.45 (d, J = 7.8 Hz, 1H), 7.31–7.59 (m, 8H), 7.98–8.00 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 4.9, 5.1, 8.3, 29.5, 31.9, 33.1, 52.5, 53.3, 67.1, 128.0, 128.1, 128.2, 128.5, 128.6, 133.1, 136.2, 137.4, 155.6, 172.1,

197.9; MS (EI) ($C_{24}H_{25}O_5N$) *m/z* (%): (M^+ 407, <1%), 240 (3), 185 (100), 167 (6), 157 (6), 144 (15), 129 (8), 115 (8), 105 (100), 91 (9), 77 (65), 59 (4), 51 (6); HRMS (ESI, $[M+H]^+$) calcd for $C_{24}H_{26}O_5N$: 408.1811, found 408.1816; Anal. Calcd for $C_{24}H_{25}O_5N$: C, 70.74; H, 6.18; N, 3.44. Found: C, 70.66; H, 6.20; N, 3.46.

5.4.4. (±) Methyl (2*R*)-[(1*S*,2*S*,3*R*)-2-benzoyl-3-cyclohexylcyclopropyl]{[(benzyloxy)carbonyl]amino}ethanoate (8b)

White solid (638 mg, 19%); mp: 156–158 °C; R_f 0.44 (30% ethyl acetate in hexanes, v/v); IR (nujol): 3349, 2924, 2854, 2727, 2668, 1774, 1742, 1696, 1670, 1598, 1581, 1518, 1456, 1378, 1286 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 1.07–1.28 (m, 6H), 1.06–1.76 (m, 6H), 1.93–1.97 (m, 1H), 2.70–2.72 (m, 1H), 3.65 (s, 3H), 4.17–4.21 (m, 1H), 5.08 (d, J = 12.6 Hz, 1H), 5.18 (d, J = 12.6 Hz, 1H), 5.36 (br d, J = 8.4 Hz, 1H), 7.31–7.37 (m, 5H), 7.47–7.50 (m, 2H), 7.56–7.59 (m, 1H), 8.00–8.01 (m, 2H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 25.8, 26.0, 26.1, 28.8, 32.3, 33.3, 33.6, 36.7, 37.1, 52.5, 53.2, 67.2, 128.0, 128.1, 128.2, 128.5, 128.6, 133.0, 136.2, 137.4, 155.5, 172.2, 198.3; MS (EI) ($C_{27}H_{29}O_5N$) *m/z* (%): (M^+ 450, <1%), 390 (7), 358 (5), 346 (7), 329 (8), 297 (7), 265 (5), 239 (15), 227 (75), 209 (25), 145 (30), 131 (15), 105 (100), 91 (60); HRMS (ESI, $[M+NH_4]^+$) calcd for $C_{27}H_{35}O_5N_2$: 467.2546, found 467.2547.

5.5. General Baeyer–Villiger oxidation procedures

5.5.1. Method A[34]

Trifluoroacetic anhydride (92 mmol) was added dropwise to a stirred mixture of 30% H_2O_2 (29 mmol) in dichloromethane (10 mL) and cooled to 0 °C. The temperature was maintained at 0 °C with the following dropwise addition of the phenyl ketone (0.44 mol) dissolved in dichloromethane (10 mL). The reaction was stirred under nitrogen and allowed to warm to rt overnight. The reaction was quenched by pouring into 2% $K_2CO_{3(aq)}$ and extracting with dichloromethane. The organic extracts were pooled, dried over $MgSO_4$, filtered and the volatiles removed in vacuo. Flash column chromatography (30% ethyl acetate in hexanes, v/v) afforded the pure phenyl ester.

5.5.2. Method B

The phenyl ketone (3 mmol) and *meta*-chloroperbenzoic acid (20 mmol), were dissolved in chloroform and the solution left in the dark at rt for one month. After quenching the reaction with saturated aqueous sodium thiosulfate, the resulting solution was extracted with dichloromethane. The organic extracts were washed with saturated $NaHCO_{3(aq)}$ followed by saturated $NaCl_{(aq)}$. The organic extracts were pooled, dried over $MgSO_4$, filtered and the volatiles removed in vacuo. Flash column chromatography (30% ethyl acetate in hexanes, v/v) afforded the pure phenyl ester.

5.5.3. (±) Phenyl (1*R*,2*S*,3*S*)-3-[(1*S*)-1-{[(benzyloxy)carbonyl]amino}-2-methoxy-2-oxoethyl]-1,1′-bi(cyclopropyl)-2-carboxylate (9a)

Prepared via method A: pale yellow oil (75 mg, 24%); prepared via method B: colourless oil (954 mg, 77%); R_f 0.45 (5% diethyl ether in dichloromethane, v/v); IR (nujol): 3351, 3066, 3033, 3004, 2954, 2850, 1732, 1593, 1494, 1456, 1376 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.35–0.43 (m, 2H), 0.65–0.68 (m, 2H), 0.96–0.98 (m, 1H), 1.64 (ddd, J = 5.4, 3.6, 1.5 Hz, 1H), 1.87–1.96 (m, 2H), 3.82 (s, 3H), 4.41 (t, J = 9.6 Hz, 1H), 5.15 (dd, J = 12.0, 9.9 Hz, 2H), 5.53 (d, J = 8.1 Hz, 1H), 7.04–7.07 (m, 2H), 7.19–7.39 (m, 3H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 4.6, 4.8, 8.3, 23.3, 31.1, 31.7, 52.6, 52.7, 67.2, 121.4, 125.8, 128.1, 128.2, 128.6, 129.3, 136.1, 150.6, 155.9, 171.5, 171.6; MS (EI) ($C_{24}H_{25}O_6N$) *m/z* (%): (M^+ 423, <1%), 201 (50), 162 (6), 134 (5), 119 (6), 107 (20), 91 (100), 79 (25), 65 (10), 51 (5); HRMS (ESI, $[M+NH_4]^+$) calcd for $C_{24}H_{29}O_6N_2$: 441.2026, found 441.2027.

5.5.4. (±) Phenyl (1*S*,2*S*,3*R*)-2-[(1*S*)-1-{[(benzyloxy)carbonyl]amino}-2-methoxy-2-oxoethyl]-3-cyclohexylcyclopropanecarboxylate (9b)

Prepared via method A: colourless needles (415 mg, 73%); mp: 143–145 °C; R_f 0.67 (30% ethyl acetate in petroleum spirit, v/v); IR (nujol): 3387, 3069, 2922, 2727, 2671, 1740, 1702, 1656, 1590, 1520, 1499, 1460, 1377, 1346, 1293 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.88–2.03 (m, 14H), 3.79 (s, 3H), 4.16–4.23 (m, 1H), 5.15 (dd, J = 10.2, 12.3 Hz, 2H), 5.51 (d, J = 8.4 Hz, 1H), 7.03–7.07 (m, 2H), 7.18–7.38 (m, 8H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 23.4, 25.7, 26.0, 26.1, 30.9, 32.7, 33.3, 36.1, 36.7, 52.4, 52.7, 67.2, 121.4, 125.7, 128.1, 128.3, 128.6, 129.3, 136.0, 150.6, 155.9, 171.5, 171.8; MS (EI) ($C_{27}H_{31}O_6N$) *m/z* (%): (M^+ 466, <1%), 372 (30), 344 (8), 328 (25), 310 (23), 268 (15), 251 (15), 223 (20), 91 (100); HRMS (ESI, $[M+H]^+$) calcd for $C_{27}H_{32}O_6N_1$: 466.2230, found 466.2233; Anal. Calcd for $C_{27}H_{31}O_6N_1$: C, 69.66; H, 6.71; N, 3.01. Found: C, 69.40; H, 6.98; N, 2.97.

5.6. General transesterification procedure

The phenyl ester was dissolved in anhydrous methanol and concentrated H_2SO_4 (two drops) was added. The solution was heated under reflux for 16 h and solid $NaHCO_3$ (120 mg) was added and the methanol removed in vacuo until a volume of ca. 5 mL remained. Dichloromethane was added and the solution extracted with satd aqueous $NaHCO_3$. The aqueous extract was extracted with further dichloromethane, the organic extracts pooled, dried over $MgSO_4$, filtered and the volatiles removed in vacuo. Flash column chromatography (5% diethyl ether in dichloromethane, v/v) of the residue afforded the pure methyl ester.

5.6.1. (±) Methyl (1*R*,2*S*,3*S*)-3-[(1*S*)-1-{[(benzyloxy)carbonyl]amino}-2-methoxy-2-oxoethyl]-1,1′-bi(cyclopropyl)-2-carboxylate (10a)

Colourless, viscous oil (385 mg, 96%); R_f 0.43 (5% diethyl ether in dichloromethane, v/v); IR (film): 3345, 3066, 3034, 3006, 2954, 2903, 2846, 1724, 1709, 1587, 1526, 1455, 1395 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 0.26–0.28 (m, 1H), 0.32–0.35 (m, 1H), 0.57–0.63 (m, 2H), 0.90–0.91 (m, 1H), 1.49 (ddd, J = 6.0, 3.0, 1.8 Hz, 2H), 1.70 (t, J = 4.2 Hz, 1H), 1.76 (dddd, J = 4.8, 4.8, 3.0, 1.2 Hz, 1H), 3.64 (s, 3H), 3.79 (s, 3H), 4.31 (t, J = 9.6 Hz, 1H), 5.12 (dd, J = 14.4, 12.0 Hz, 2H), 5.44 (d, J = 8.4 Hz, 1H), 7.34 (m, 5H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 0.2, 4.7, 4.8, 8.4, 23.2, 30.5, 31.0, 52.1, 52.8, 67.3, 128.3, 128.4, 128.7, 136.3, 156.1, 171.9, 173.5; MS (EI) ($C_{19}H_{23}O_6N$) *m/z* (%): (M^+ 361, <1%), 258 (5), 226 (3), 194 (3), 166 (4), 139 (70), 107 (24), 91 (100), 79 (45), 59 (10); HRMS (ESI, $[M+Na]^+$) calcd for $C_{19}H_{23}O_6N$: 384.1423, found 384.1421.

5.6.2. (±) Methyl (1*S*,2*S*,3*R*)-2-[(1*S*)-1-{[(benzyloxy)carbonyl]amino}-2-methoxy-2-oxoethyl]-3-cyclohexylcyclopropanecarboxylate (10b)

Colourless solid (258 mg, 76%); mp: 129–130 °C; R_f 0.64 (30% ethyl acetate in petroleum spirit, v/v); IR (nujol): 3345, 2916, 2728, 2671, 2360, 1751, 1718, 1706, 1523, 1461, 1377, 1364, 1344, 1282, 1231 cm^{-1}; ^1H NMR (600 MHz, $CDCl_3$) δ 1.07–1.25 (m, 6H), 1.39–1.41 (m, 1H), 1.55–1.91 (m, 7H), 3.65 (s, 3H), 3.77 (s, 3H), 4.08–4.11 (m, 1H), 5.09–5.15 (m, 2H), 5.37 (d, J = 7.8 Hz, 1H), 7.32–7.38 (m, 5H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 0.0, 23.2, 25.8, 26.0, 26.2, 30.2, 32.8, 33.3, 35.2, 36.6, 51.9, 52.6, 67.2, 128.1, 128.3, 128.6, 136.1, 155.8, 171.7, 173.7; MS (EI) ($C_{21}H_{27}O_6N$) *m/z* (%): (M^+ 403, <1%), 344 (6), 300 (5), 268 (5), 236 (4), 204 (5), 181 (33), 149 (16), 133 (6), 121 (6), 108 (26), 91 (100), 79 (32), 67 (10), 55 (7); HRMS (ESI, $[M+H]^+$) calcd for $C_{22}H_{30}O_6N_1$: 404.2073, found 404.2076.

5.7. General ester hydrolysis procedure

The methyl ester (1.0 mol) was dissolved in THF (5 mL) and stirred with 2.5 M aqueous LiOH (10 mL, 25 mmol) at rt overnight. Brine (10 mL) was added followed by concentrated HCl (two drops). The solution was extracted with ether (2 × 15 mL), dried over $MgSO_4$, filtered and the solvents removed in vacuo.

5.7.1. (±) (1*R*,2*S*,3*S*)-3-[(*S*)-{[(Benzyloxy)carbonyl]amino}(carboxy)methyl]-1,1′-bi(cyclopropyl)-2-carboxylic acid (11a)

White solid (298 mg, 92%); mp: 130–132 °C; IR (nujol): 3303, 2922, 2727, 2672, 1702, 1456, 1377, 1301, 1245 cm^{-1}; ^1H NMR (600 MHz, CD_3OD) δ 0.25–0.30 (m, 2H), 0.54–0.62 (m, 2H), 0.95–0.96 (m, 1H), 1.50–1.53 (m, 2H), 1.83–1.87 (m, 1H), 4.14 (d, J = 10.2 Hz, 1H), 5.10 (dd, J = 12.6, 10.2 Hz, 2H), 7.28–7.36 (m, 5H); ^{13}C NMR (150 MHz, CD_3OD) δ 5.5, 5.6, 9.6, 24.7, 31.7, 32.7, 68.1, 129.2, 129.4, 130.0, 138.8, 159.2, 177.4, 210.3; MS (EI) ($C_{17}H_{19}O_6N$) *m/z* (%): (M^+ 333, 7%), 328 (14), 310 (10), 239 (14), 212 (27), 166 (45), 152 (14), 107 (100), 91 (55), 79 (36); HRMS (ESI, $[M+H]^+$) calcd for $C_{17}H_{20}O_6N_1$: 334.1291, found 334.1289.

5.7.2. (±) (1*S*,2*S*,3*R*)-2-[(*S*)-{[(Benzyloxy)carbonyl]amino}(carboxy)methyl]-3-cyclohexylcyclopropanecarboxylic acid (11b)

White solid (185 mg, 97%); mp: 160–167 °C; IR (nujol): 3306, 2923, 2854, 2729, 1728, 1694, 1682, 1538, 1463, 1409, 1377, 1326, 1285, 1246 cm^{-1}; ^1H NMR (600 MHz, CD_3OD) δ 1.06–1.41 (m, 8H), 1.63–2.02 (m, 6H), 3.93–3.95 (m, 1H), 5.05–5.12 (m, 2H), 7.25–7.35 (m, 5H); ^{13}C NMR (150 MHz, CD_3OD) δ 25.1, 27.4, 27.7, 27.9, 31.7, 34.4, 34.9, 37.0, 38.5, 54.6, 68.2, 129.2, 129.4, 130.0, 138.7, 159.1, 175.4, 178.1; MS (EI) ($C_{20}H_{25}O_6N$) *m/z* (%): (M^+ 375, <1%), 357 (12), 250 (15), 240 (15), 224 (16), 194 (14), 178 (32), 131 (35), 108 (47), 91 (100), 79 (70); HRMS (ESI, $[M+H]^+$) calcd for $C_{20}H_{26}O_6N_1$: 376.1760, found 376.1760.

5.8. General catalytic hydrogenation procedure

The Cbz-protected amine (0.8 mmol) was dissolved in methanol (10 mL) and 5% Pd/C (20 wt %) was added. The reaction was evacuated under vacuum and subsequently filled with $H_{2(g)}$ from a hydrogen balloon. The reaction was stirred at rt for 48 h, filtered through a pad of kenite using methanol and water and concentrated in vacuo. The crude product was dissolved in water (5 mL) and concd HCl (two drops) was added. The solvent was removed in vacuo and the residual HCl salt (0.5 mmol) dissolved in methanol (0.6 mmol). Anhydrous propylene oxide (25 mL/mmol) was then added and the solution stirred under an N_2 atmosphere at rt overnight. The precipitated solid was collected by filtration and washed with ethyl acetate to give the pure free amine.

5.8.1. (±) (1*R*,2*S*,3*S*)-3-[(*S*)-Amino(carboxy)methyl]-1,1′-bi(cyclopropyl)-2-carboxylic acid (3)

White solid (158 mg, 95%); dec: 234 °C; IR (solid): 3013, 1693, 1656, 1612, 1562, 1508, 1470, 1457, 1393, 1343, 1321, 1300, 1239 cm^{-1}; ^1H NMR (600 MHz, D_2O) δ 0.05–0.13 (m, 2H), 0.30–0.35 (m, 1H), 0.40–0.44 (m, 1H), 0.72–0.77 (m, 1H), 1.51–1.55 (m, 2H), 1.76 (ddd, J = 4.8, 4.2, 2.4 Hz, 1H), 3.82 (d, J = 11.4 Hz, 1H); ^{13}C NMR (150 MHz, D_2O) δ5.53, 9.14, 25.06, 28.72, 32.61, 53.97, 172.37, 178.03; HRMS (ESI, $[M + H]^+$) calcd for $C_9H_{14}O_4N_1$: 200.0923, found 200.0917.

5.8.2. (±) (1*S*,2*S*,3*R*)-2-[(*S*)-Amino(carboxy)methyl]-3-cyclohexylcyclopropanecarboxylic acid (4)

Off-white solid (16 mg, 16%); IR (solid): 2924, 2851, 1680, 1586, 1554, 1451, 1391, 1320, 1231 cm^{-1}; ^1H NMR (600 MHz, D_2O) δ 0.80–0.93 (m, 6H), 1.27–1.43 (m, 6H), 1.64–1.73 (m, 2H), 3.60 (d, J = 10.8, 1H); ^{13}C NMR (150 MHz, D_2O) δ25.2, 27.3, 29.1, 33.8, 34.3, 37.1, 37.2, 37.8, 53.6, 53.8, 172.2, 178.5; HRMS (ESI, $[M+H]^+$) calcd for $C_{12}H_{20}O_4N_1$: 242.1392, found 242.1387.

6. Pharmacology

6.1. In vitro methods

6.1.1. Binding assays at native iGlu receptors

All binding assays were performed using rat brain synaptic membranes of cortex and the central hemispheres from adult male Sprague-Dawley rats with tissue preparation as earlier described.[35] Affinities for native AMPA, KAIN and NMDA receptors were determined using 5 nM [^3H]AMPA,[36] 5 nM [^3H]KAIN,[37] and 2 nM [^3H]CGP39653.[38] On the day of experiments, frozen membranes were quickly thawed and homogenised in 40 volumes of ice-cold buffer (pH 7.4) (30 mM Tris–HCl containing 2.5 mM $CaCl_2$, 50 mM Tris–HCl, or 50 mM Tris–HCl containing 2.5 mM $CaCl_2$, for [^3H]AMPA, [^3H]KAIN, or [^3H]CGP39653 binding, respectively), and centrifuged (48,000 *g* for 10 min). This step was repeated four times. In [^3H]AMPA binding experiments, 100 mM KSCN was added to the buffer during the final wash and during incubation. The final pellet was re-suspended in ice-cold buffer, corresponding to approx. 0.4–0.5 mg protein/mL. [^3H]AMPA, [^3H]KAIN, and [^3H]CGP39653 binding were carried out in aliquots consisting of 25 μL [^3H]ligand, 25 μL test solution, and 200 μL membrane suspension and incubated for 30 min, 60 min, and 60 min, respectively. Binding was terminated by filtration through GF/B filters using a 96-well Packard Filter-Mate Cell Harvester and washing with 3 × 250 μL buffer. After drying, 25 μL Microscint 0 (Perkin–Elmer) per well was added and the plate was counted on a Topcounter (Perkin–Elmer). Non-specific binding was determined using 1 mM (*S*)-Glu. The Bradford[39] protein assay was used for protein determination using bovine serum albumin as a standard, according to the protocol of the supplier (Bio-Rad, Milan, Italy).

6.1.2. Binding assays at recombinant mGlu receptors

All cell culture reagents were from Gibco unless stated otherwise.

6.1.3. Inhibition of forskolin-stimulated cAMP production

CHO cells expressing the mGluR2 or mGluR4 (previously described by Tanabe et al.[40,41]) were cultured in DMEM with GlutaMAX-I with 10% dialyzed FBS, 1% Penicillin–Streptomycin, and 2.5 g/L L-proline at 5% CO_2 at 37 °C. Cells were seeded at 26,000 cells/96-well 24 h before assaying. Prior to compound addition cells were washed with DPBS. For agonist testing cells were first incubated with cAMP-ground buffer (DPBS with 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 1 mM IBMX) for 20 min at 37 °C. The buffer was then replaced with test compounds diluted in cAMP-ground buffer supplemented with 25 μM forskolin and incubated at 37 °C for 10 min. For antagonist testing cells were first incubated with test compounds diluted in cAMP-ground buffer for 20 min at 37 °C, and then replaced with cAMP-ground buffer containing test compounds, 25 μM forskolin, and L-glutamate corresponding to the EC_{80} (20 μM for mGluR2, 30 μM for mGluR4) for 10 min at 37 °C. All reactions were terminated by aspiration and addition of ice-cold sodium acetate buffer pH 6.2 supplemented with 0.1% triton X-100 and 0.1 mM IBMX. cAMP levels were quantified using the Adenylyl Cyclase Activation FlashPlate® Assay (Perkin Elmer) and interpolated from a cAMP standard curve. All experiments were performed in triplicate and the results are given as mean ± SEM of at least two independent experiments.

6.1.4. Inositol phosphate (IP) turnover assay

CHO cells expressing the mGluR1 (previously described by Aramori and Nakanishi[42]) were cultured as described in the cAMP assay above. Cells were seeded at 26,000 cells/96-well and labeled

with myo-[2-^{3}H]Inositol (4 μCi/mL, TRK911, GE Healthcare) 24 h before assaying. Prior to compound addition cells were washed with DPBS. For agonist testing cells were first incubated with IP-ground buffer (HBSS containing 20 mM HEPES, 1 mM $CaCl_2$, 1 mM $MgCl_2$ and 0.85 mg/mL LiCl, pH 7.4) for 30 min at 37 °C. The buffer was then replaced with test compounds diluted in IP-ground buffer and incubated at 37 °C for 30 min. For antagonist testing cells were first incubated with test compounds diluted in IP-ground buffer for 30 min at 37 °C, and then replaced with IP-ground buffer containing test compounds and 30 μM L-glutamate corresponding to the EC_{80} for 30 min at 37 °C. All reactions were terminated by aspiration and addition of ice-cold 10 mM formic acid and incubation for 30 min at 4 °C. Yttrium silicate scintillation proximity assay beads (RPNQ0010, GE Healthcare) were used for measuring radioactivity from generated [^{3}H]IP as previously described.[43] Radioactivity was quantified in a Packard TopCount microplate scintillation counter and responses read as counts per minute (CPM).

6.2. In vivo methods

6.2.1. Animals

Experiments were carried out on 12 male, pathogen-free Sprague-Dawley rats (325–500 g) housed in groups of three per cage. Rats were given free access to food and water and maintained on a controlled 12/12 h light/dark cycle with lights on at 0700 h.

6.2.2. Ethics

Ethical approval was obtained from the University of Adelaide Animal Ethics Committee for all animal tests and manipulations and care was taken to minimize the extent of suffering and duration of pain, where doing so would not interfere with the project. All experimental work involving animals abided by the guidelines found in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes.

6.2.3. Drugs

The test compound (±) (1*R*,2*S*,3*S*)-3-[(*S*)-amino(carboxy)-methyl]-1,1′-bi(cyclopropyl)-2-carboxylic acid ((±)-**3**) was synthesized in the Discipline of Chemistry, University of Adelaide and the positive control (2*R*,4*R*)-aminopyrrolidine-2,4-dicarboxylate (APDC) was purchased from Tocris Bioscience (Bristol, UK). Both drugs were administered intrathecally, compound (±)-**3** as a suspension (500 nmols) or solution (50 and 250 nmols) and APDC as a solution in Milli-Q water containing 12 mM HCl. The method of acute intrathecal drug administration was based on that described previously.[44] Briefly, intrathecal operations were conducted under isoflurane anaesthesia (Phoenix Pharmaceuticals, St. Joseph, MO, USA) by threading sterile polyethylene-10 tubing (PE-10 Intramedic Tubing; Becton Dickinson Primary Care Diagnostics, Sparks, MD, USA) guided by an 18-gauge needle between the L5 and L6 vertebrae. The catheter was inserted such that the proximal catheter tip lay over the lumbosacral enlargement. The catheters were pre-loaded with drugs at the distal end in a total volume of no greater than 25 μL and delivered over 20–30 s once the catheter was in position. The catheters were 17 cm in length, and were attached to a pre-loaded Hamilton syringe.

6.2.4. Chronic constriction injury (CCI)

Neuropathic pain was induced using the chronic constriction injury (CCI) model of partial sciatic nerve injury. CCI was performed at the mid-thigh level of the left hind leg as previously described.[45] Animals were anesthetized with isoflurane. The shaved skin was treated with ethanol to cleanse and the surgery was aseptically performed. Animals were monitored post-operatively until fully ambulatory prior to return to their home cage, and checked daily for any sign of infection. No such cases occurred in this study. In brief, four sterile chromic gut sutures (cuticular 4–0 chromic gut, FS-2; Ethicon, Somerville, NJ, USA) were loosely tied around the gently isolated sciatic nerve. Drug testing was delayed until 16 days after surgery to ensure that neuropathic pain was well established prior to the initiation of drug delivery.

6.2.5. Von Frey testing

All testing was conducted blind with respect to group assignment. Rats received at least three 60-min habituations to the test environment prior to behavioural testing. The von Frey test was performed within the sciatic innervation region of the hind paws as previously described.[46] Assessments were made prior to (baseline) and at 1 and 3 h after intrathecal drug dosing. A logarithmic series of 10 calibrated Semmes-Weinstein monofilaments (von Frey hairs; Stoelting, Wood Dale, IL, USA) was applied randomly to the left and right hind paws to define the threshold stimulus intensity required to elicit a paw withdrawal response. Log stiffness of the hairs was determined by $\log_{10}$ (milligrams × 10) and ranged from 3.61 (4.07 g) to 5.18 (15.136 g). The behavioural responses were used to calculate absolute threshold (the 50% paw withdrawal threshold) by fitting a Gaussian integral psychometric function using a maximum likelihood fitting method, as described previously.[46] This fitting method allows parametric analyses that otherwise would not be appropriate.

6.2.6. Data analysis

All data is reported as mean ± SEM. Von Frey data was analysed as the interpolated 50% thresholds (absolute threshold) in log base 10 of stimulus intensity (monofilament stiffness in milligrams × 10). Pre-drug baseline measures were analysed by one-way ANOVA. Post drug time course measures were analysed by repeated measures two-way ANOVAs followed by Bonferroni post hoc tests. The 3 h time point data was compared to vehicle by one-way ANOVA followed by Dunnett's *t* test post hoc. Where appropriate, $P < 0.05$ was considered statistically significant.

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Supplementary data

Supplementary data (proton and carbon NMR spectra of compounds **3**, **4**, **6a**, **6b**, **7a**, **7b**, **8a**, **8b**, **9a**, **9b**, **10a**, **10b**, **11a** and **11b** as well as details of X-ray crystallography for compounds **7a** and **7b**) associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.06.051.

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