Pergamon

SCIENCE DIRECT®

Bioorganic & Medicinal Chemistry 11 (2003) 171–183

BIOORGANIC & MEDICINAL CHEMISTRY

Synthesis and Pharmacological Characterisation of 2,4-Dicarboxy-pyrroles as Selective Non-Competitive mGluR1 Antagonists

Fabrizio Micheli,* Romano Di Fabio,* Paolo Cavanni, Joseph M. Rimland, Anna Maria Capelli, Cristiano Chiamulera, Mauro Corsi, Corrado Corti, Daniele Donati, Aldo Feriani, Francesco Ferraguti,† Micaela Maffeis, Andrea Missio, Emiliangelo Ratti, Alfredo Paio, Roberta Pachera, Mauro Quartaroli, Angelo Reggiani,‡ Fabio Maria Sabbatini, David G. Trist, Annarosa Ugolini and Giovanni Vitulli

GlaxoSmithkline Medicine Research Centre, Via Fleming, 4-37135 Verona, Italy

Received 28 June 2002; accepted 22 August 2002

Abstract—Metabotropic glutamate receptors (mGluRs) are an unusual family of G-protein coupled receptor (GPCR), and are characterised by a large extracellular N-terminal domain that contains the glutamate binding site. We have identified a new class of non-competitive metabotropic glutamate receptor 1 (mGluR1) antagonists, 2,4-dicarboxy-pyrroles which are endowed with nanomolar potency. They interact within the 7 transmembrane (7TM) domain of the receptor and show antinociceptive properties when tested in a number of different animal models.

Introduction

Glutamate, the principal excitatory neurotransmitter in mammalian brain, is involved in many brain functions. Its actions are mediated through ionotropic (NMDA, AMPA and Kainate)[1–5] and metabotropic receptors (mGluRs).[6,7] These latter receptors couple to G proteins and control the activity of membrane enzymes and ion channels.[8–10] A distinctive feature of the mGluRs is the large amino-terminal domain which contains the glutamate binding site[11,12] and, together with the parathyroid calcium sensing, the γ-aminobutyric $(GABA)_B$, and the vomeronasal receptors, form a separate family of GPCRs showing very little homology with other cloned receptors.[13–15]

To date, the mGluRs family comprises eight subtypes which have been cloned and named mGluR1–8 according to the succession of the molecular cloning. The eight receptors are divided into three groups on the basis of sequence similarity, pharmacology and transduction mechanisms: Group I (mGluR1 and mGluR5), Group II (mGluR2 and mGluR3) and Group III (mGluR4, mGluR6, mGluR7 and mGluR8). This picture is made more complex by the expression of several splice variants for some of the different subtypes.[16] In the specific case of mGluR1, there are four different splice variants named 1a, 1b, 1c, and 1d. It is reported that mGluR1 mainly activates phospholipase C (PLC) to increase intracellular calcium.

The role of mGluR1 in physiological and pathological conditions is being investigated using currently available agonists and antagonists.[17] Quisqualate,[18] and (*S*)-3,5-dihydroxyphenylglycine [(*S*)-3,5-DHPG],[19] are selective group I agonists and (*S*)-4-carboxy-3-hydroxyphenyl glycine [(*S*)-4C3HPG],[20] (*S*)-(+)-2-(3′-carboxybicyclo [1.1.1]pentyl)-glycine [(*S*)-CBPG],[21] (*S*)-4-carboxy-phenyl glycine [(*S*)->4CPG],[22] 2-(3′-(1H-tetrazol-5-yl)bicyclo [1.1.1]pent-1-yl)glycine (S-TBPG),[23] LY-367366[24] are

*Corresponding authors. Tel.: +39-045-921-8515; fax: +39-045-921-8196 e-mail: fm20244@gsk.com (F. Micheli); Tel.: +39-045-921-8879; fax: +39-045-921-8196 e-mail: rdf26781@gsk.com (R. Di Fabio).
†Current address: MRC Anatomical Neuropharmacology Unit, University of Oxford, Mansfield Road, Oxford, OX1 3TH UK.
‡Current address: ScheringPlough, S. Raffaele Science Park, Milan, Italy.

0968-0896/03/$ - see front matter © 2002 Elsevier Science Ltd. All rights reserved.
PII: S0968-0896(02)00424-8

competitive antagonists. CPCCOEt [7-(hydroxyimino)-cyclopropan[*b*]-chromen-1a-carboxylic acid ethylester], the first non-competitive antagonist of mGluR1, was identified and characterised,[25] and another very potent and non competitive derivative, BAY 36-7620, was also recently disclosed.[26] Some of these derivatives are reported in Figure 1.

According to studies reported in the literature, the mGluR1 receptor seems mainly involved in therapeutic opportunities for the treatment of stroke/brain injury,[27,28] and pain.[29,30] Evidence, though discording, on the physiopathological role of mGluR1 are also available from the generation of knock-out mice.[31,32]

Results

Synthesis

Despite intense efforts both in academia and industry, to date only relatively few mGluR1 antagonists are available. Moreover, these derivatives, regardless of their relatively high selectivity, generally show poor pharmacokinetic properties. This 'relative lack' of very potent antagonists might be due either to the peculiar characteristics of this receptor or to a lack of the appropriate chemical diversity in corporate databases used as a main source for High Throughput Screening (HTS) efforts. To shed light on this problem, a theoretical structure of the mGluR1 glutamate binding site was deeply analyzed and elucidated by a number of modeling studies by Prof. Pellicciari's group in Perugia.[33,34]

As a part of our continuing efforts in the identification of New Chemical Entities (NCE) endowed with pharmacological activity, and to carry out focussed screening activities, literature data were used to bias the selection of a number of discrete compounds belonging both to the corporate Data Base and to commercially available sets. Among the different groupings obtained, a set was arranged considering (S)-4C3HPG and (S)-4CPG as primary tools and topologically matching the 'distance' existing between the aminoacidic moiety of these molecules and the remaining carboxylic group. This exercise led to the selection of compounds endowed with an averaged 'distance' from three to five bond lenght betwen the two considered moiety, as schematically depicted in Figure 2. All the derivatives fulfilling the above reported query were 'filtered' using general 'drug like' properties (MW < 600; ClogP < 5; Rotable bonds < 10; H-donors < 5; H-acceptors < 5) and the approximately four thousands derivatives obtained were screened in CHO cells expressing rat mGluR1a (r-mGluR1a-CHO) measuring accumulation of cytidine diphosphate-diacylglycerol (CDP-DAG).[35]

Further characterisation was subsequently performed by measuring intracellular calcium $[Ca^{2+}]_i$.[36] mobilization using a fluorescent imaging plate reader (FLIPR). The methods are described in the biology part of this paper.

Among the different hits identified, 2,4-carboxy-3,5-dimethyl-pyrrole ethyl ester (**1,** Fig. 3) showed micromolar potency at the receptor ($IC_{50} = 15.8$ μM; $pIC_{50} = 4.8$) at the receptor. This structure immediately elicited our interest for a number of reasons. Firstly, it was not an amino acid derivative. Secondly, it had no relationship with any known structures of agonists or antagonists. Finally, it was quite a small and simple molecule and it represented an attractive scaffold for chemical manipulation exploiting combinatorial methodologies. However, derivative **1** could also have been a false positive compound which, instead of acting at the receptor, interfered at some point in the second messenger cascade. This issue, together with selectivity versus other receptor subtypes, was immediately tackled and solved for this product and other derivatives as reported in the biology part of the paper.

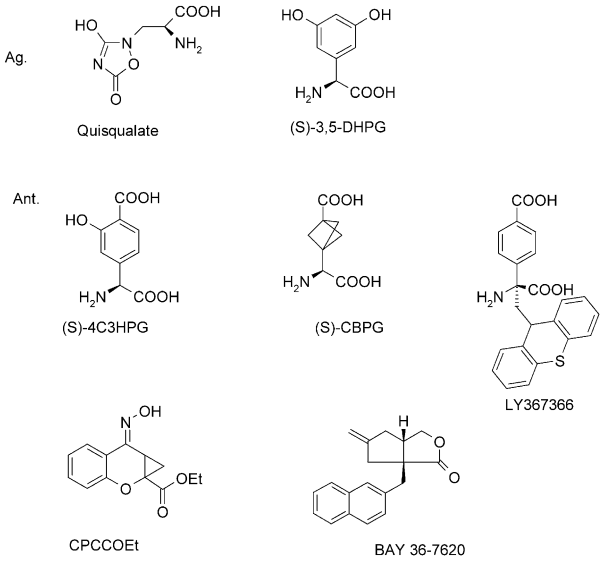


Figure 1. Some known agonist and antagonists at the mGluR1 receptor.

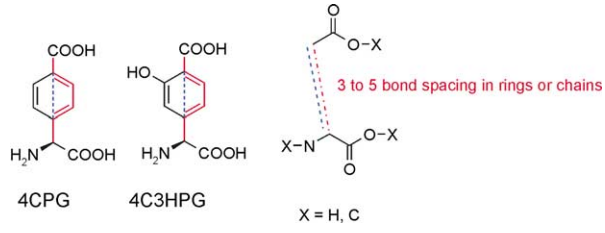


Figure 2. Schematic representation of the topological query used to select derivatives both on the corporate and commercial databases and based on (S)-4C3HPG and (S)-4CPG.

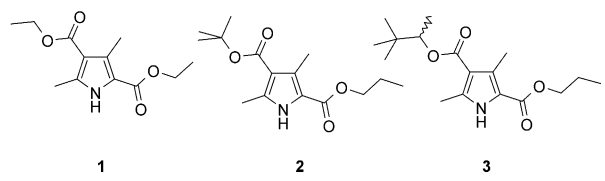
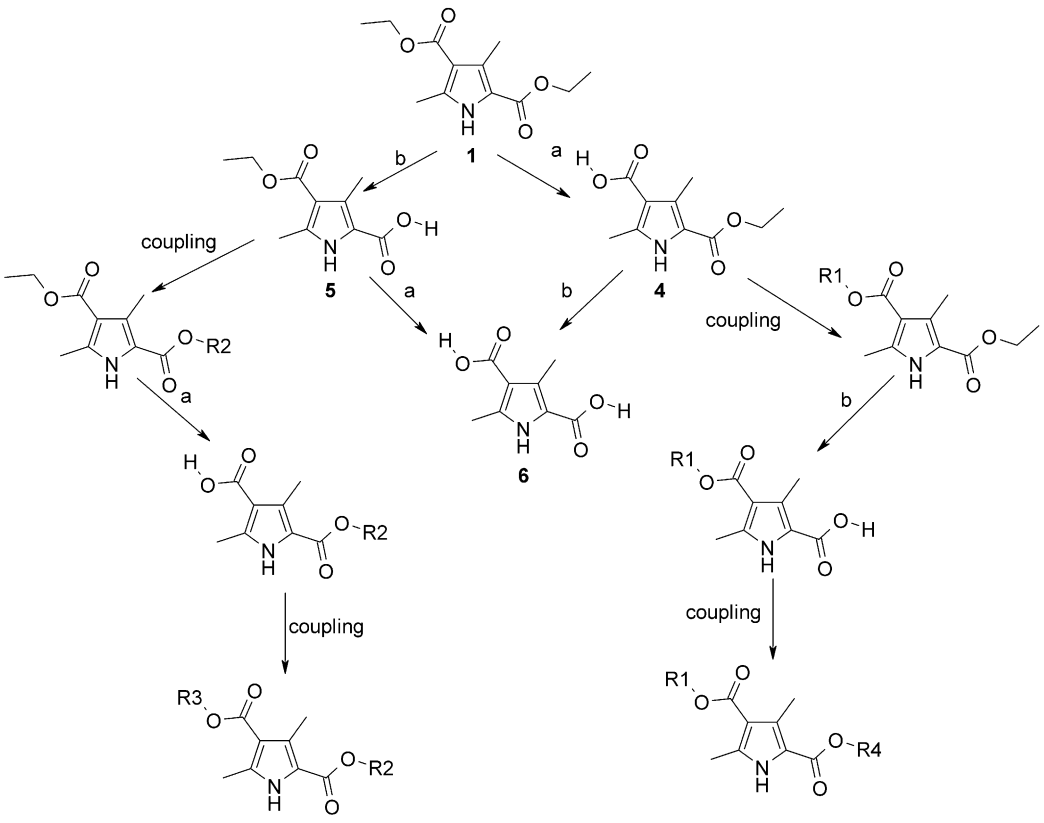


Figure 3. Some of the newly discovered mGluR1 antagonists.



Scheme 1. Exploitation of the pyrrolic template versatility: chemical exploration around derivative **1**. Reagents and conditions: (a) H_2SO_4; (b) NaOH.

Considering all the 'attractive hooks' mentioned above for the derivative **1** class, a limited investigation was initially planned to understand the key pharmacophoric features of this molecule and therefore a relatively low number of discretes was synthesized.

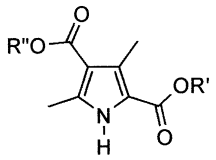
The chemical versatility of derivative **1** was known since the end of the XIXth century[37–39] and was exploited as depicted in Scheme 1.

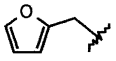
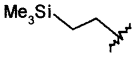
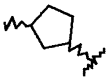
Acid hydrolysis selectively affected position C-4, while basic saponification modified only position C-2. The coupling of the selectively obtained C-2 or C-4 free acid with appropriate alcohols or amines led to a preliminary structure–activity relationship (SAR).

We noticed that the removal of the esteric moiety led to a complete loss of activity at the receptor: neither the monoacid derivatives in position C-4 (**4**, Scheme 1) nor its C-2 regioisomer (**5**, Scheme 1) showed measurable interaction with the receptor. Furthermore, the completely deprotected product (**6**, Scheme 1) was also inactive. In contrast, the increase in the bulkyness/lipophilicity of the C-4 esteric moiety led to a significant increase in potency. As depicted in Table 1, the C-2 position needed to be substituted too, but it was less stringent in terms of pharmacophoric features.

The first phase of our analysis stopped with the identification of 2-propoxy-4-*t*-butoxy-3,5-dimethyl-pyrrole dicarboxylic acid (**2**, Fig. 3) with a 100-fold increase in potency compared to the parent compound **1** (158 nM, $pIC_{50} = 6.8$). Derivative **2** was further tested to validate its activity on the mGluR1 receptor. It is worth noting that not only did the analogue behave as a non-competitive mGluR1 antagonist like **1** but it was also selective (>100-fold) versus rat-mGluR2, -4 and -5 (no activity shown up to 500 μM). Considering these extremely promising results, a combinatorial exploration of the C-2 and C-4 positions of the pyrrolic scaffold was appropriately designed to optimize the receptor potency of the compounds. According to the synthetic pathway shown in Scheme 2, commercially available primary and secondary alcohols were computationally selected and the chemically interfering groups (e.g., free carboxylic acids, un-protected amines, phenols, etc.) were removed. Two different sets were prepared: one to be reacted with position C-4 (R^5-OH) and one for modification of position C-2 (R^6OH). In order to produce a library endowed with general 'drug like' properties, both monomer sets underwent molecular weight and lipophilicity cut-offs: the R^5OH alcohols were chosen with a $M_r < 250$, while the R^6OH monomers were selected among those with a $M_r < 200$. Moreover, all the monomers selected had a ClogP <2 unless they were bearing heteroatoms such as N, O, S, Cl, Br, in which case the threshold used was ClogP <3.5. The remaining molecules were clustered according to a non-hierarchical Jarvis–Patrick method. The centroid of each cluster was then chosen and a virtual library generated. At the end of the filtration process, the

Table 1. Potency values of the pyrrolic derivatives on r-mGluR1a-CHO cells using the CDP-DAG accumulation method[35]



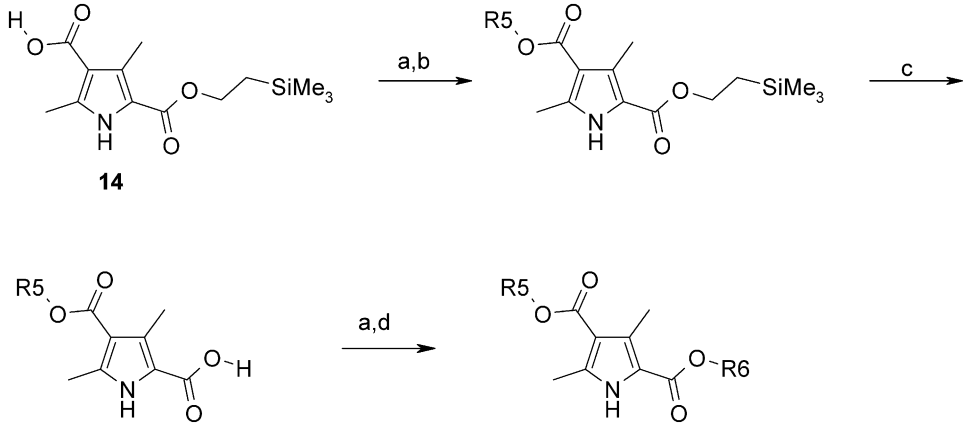
Entry	R'	R'	IC_{50} (μM)
1	Et	Et	15.8
2	*n*-Pr	*t*-Bu	0.16
3	*n*-Pr		0.0158 (15.8 nM)
4	Et	H	N.A.
5	H	Et	N.A.
6	H	H	N.A.
7	Bn	*t*-Bu	1.9
8	Allyl	*t*-Bu	0.39
9	Bn	Allyl	N.A.
10	Bn	Bn	N.A.
11		*t*-Bu	0.2
12	*n*-Octyl	*t*-Bu	6.3
13	Ph	*t*-Bu	3.0
14		H	N.A.
15	*n*-Pr		0.0158[a](15.8 nM)
16	*n*-Pr		0.040[b] (40 nM)
17	*n*-Pr		0.040 (40nM)
18	*n*-Pr		0.26
19	*t*-Bu		0.050 (50 nM)

IC_{50}s were measured from at least six-point inhibition curves and they are the geometric means of at least three independent experiments. The standard error of the mean was less than 0.05.
[a]Purified on chiral stationary phase.
[b]N.A., not active up to 100 uM.

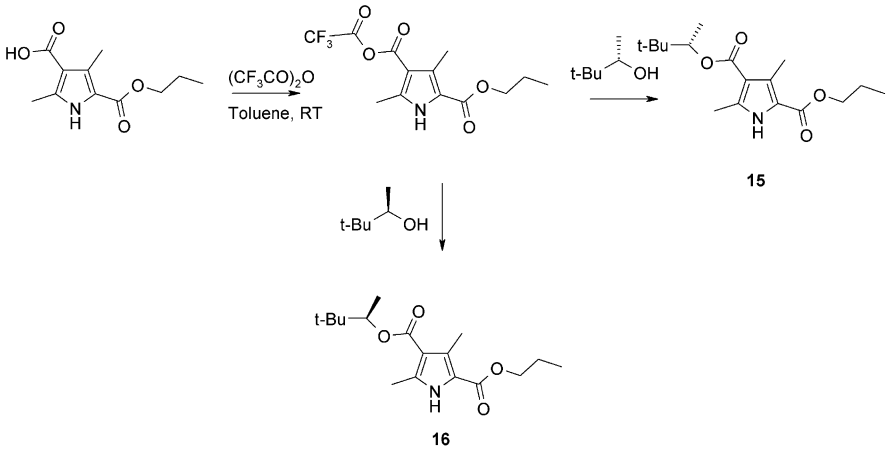
R^5OH sets were formed by 80 monomers, while the R^6OH group was reduced to only 20 derivatives. Obviously, such a library (1600 derivatives) could not be produced with the synthetic methodology shown in Scheme 1 because of the possible problems related to both acid and basic hydrolysis of the esteric moieties. The new methodology described in Scheme 2 was designed in order to use the easily obtainable and biologically inactive intermediate **14** (and therefore overcoming the possible problem of any residue of **14** left in the plates which underwent the biological test), exploiting a deprotection method which would not interfere with the subsequent esterification reaction.

The resulting library was screened on r-mGluR1a-CHO cells by measuring mobilization $[Ca^{2+}]_i$ with a FLIPR. Among the positives, the most interesting compound was a close analogue of derivative **2** in which the *t*-butyl ester in position C-4 was replaced by the racemic 3,3-dimethyl-2-butanol (pinacolyl alcohol). This derivative (**3**, Fig. 1) showed a very good potency at the receptor (15.8 nM, $pIC_{50} = 7.8$) maintaining the non-competitive inhibition and showing high selectivity (>1000-fold) versus rat-mGluR2, -4 and -5. This derivative was further characterised in a number of in vitro and in vivo experiments described later. Given the chiral nature of the pinacolyl ester on derivative **3**, the two enantiomers were separated by HPLC on a chiral stationary phase and further characterised. The (*S*) enantiomer **15** showed the same activity as the racemic mixture, while the (*R*) enantiomer **16** was slightly less potent as reported in Table 1. The correct assignment of the stereogenic center chirality was performed by synthesizing each of the two pure pinacolyl alcohols according to known methodology[40] and coupling them with retention of configuration to the corresponding carboxylic acid as depicted in Scheme 3.

The affinity values obtained for the synthesized discretes, considering that their enantiomeric excess was greater than 80%, almost perfectly matched the data obtained on the products after separation using chromatography on chiral stationary phase.



Scheme 2. Combinatorial exploration of the pyrrolic scaffold. Reagents and conditions: (a) $(CF_3CO)_2O$, toluene, rt; (b) R^5OH (80 monomers), toluene; (c) TBAF, THF; (d) R^6OH (20 monomers), toluene.



Scheme 3. Synthesis of the pure enantiomers of derivative **3**. Reagents and conditions: (a) $(CF_3CO)_2O$, toluene, rt; (b) R^5OH (80 monomers), toluene; (c) TBAF, THF; (d) R^6OH (80 monomers), toluene.

Biology

Investigations of the mechanism of action of the pyrrolic class. To determine the behaviour and the site of action of our newly discovered class of mGluR1 antagonists, a number of pharmacological experiments were performed beyond the more classical ones (e.g., Schield analysis). First of all a rightward shift and depression of the glutamate concentration–response curve (CRC) was observed in the presence of increasing concentrations of the antagonists. This characteristic was observed for all the compounds of the pyrrolic class. In order to better understand the non-competitive behavior of these antagonists, a combined dose-ratio experiment was designed. The known competitive antagonist MCPG[42] (1 mM) and derivative **3** (10 μM) were tested separately and then co-incubated in the presence of the agonist glutamate (using a full glutamate CRC). The effect on CDP-DAG accumulation was measured. The combination of the two antagonists produced a rightward shift of the glutamate CRC suggesting a multiplicative effect (independent effects on two different sites of action) rather than an additive effect (action at the same site). Comparable results were obtained with two other mGluR1 agonists, (1*S*, 3*R*)ACPD [(1*S*, 3*R*)-1-amino-1,3-cyclopentanedicarboxylic acid] and quisqualate.

The reversibility of the interaction between pyrrole derivatives and mGluR1 was tested by performing wash-out experiments. Compounds at concentrations 100 times their IC_{50}s, were pre-incubated for 30 min on r-mGluR1a-CHO cells and then extensively washed with buffer. After this treatment, a glutamate CRC was applied. The EC_{50} value for glutamate on pre-treated cells was comparable to that obtained in the control experiments (buffer only) and after pre-treatment with the competitive antagonist MCPG. According to the pharmacological characterisation the pyrrole class displayed non-competitive antagonism of agonist activation of mGluR1. We also wanted to exclude the possibility that derivative **3** interfered somewhere along the second messenger cascade. In order to eliminate a non-specific effect on the phosphoinositide pathway, pyrrole derivatives were tested on a different Gq-coupled receptor, presumably coupled to the same second messenger pathway, that is CHO cells expressing the neurokinin 3 (NK-3) receptor.[41] None of the compounds reported in Table 1 was able to alter the CRC of the agonist NKB (50 nM) when tested at doses 100 times greater than their IC_{50} on the mGluR1 receptor. (*S*)-(+)-α-Methyl-4-carboxy phenyl glycine (MCPG),[42] a competitive mGluR1 antagonist, showed the same behaviour. To further validate our discovery, the pyrroles were tested by measuring second messenger signaling exploiting assays different from the ones previously used (CDP-DAG accumulation or $[Ca^{2+}]_i$ mobilisation). After pre-incubation with [^{3}H]arachidonic acid ([^{3}H]AA) and stimulation with a mGluR1 agonist, it was possible to observe an increased release of [^{3}H]AA in CHO cells expressing mGluR1a [this response should be mediated by activation of phospholipase A_2 (PLA_2) through a G-protein different from the G-protein responsible for receptor coupling to PLC]. Also in these studies, the pyrrolic derivatives gave a non-competitive inhibition of the agonist response, confirming that this behaviour was independent of the assay and coupling of the receptor, but is a characteristic of the ligand–mGluR1 receptor interaction. Finally, a number of experiments were performed on cultured rat cerebellar granule cells[43,44] in order to replicate the type of antagonism observed with the recombinant mGluR1a receptor expressed in CHO cells. Cerebellar granule cells express mGluR1 and other Gq-linked receptors, specifically muscarinic acetylcholine receptors. In this neuronal cell culture model, using the CDP-DAG assay, the non-competitive effect of the pyrrole derivatives was confirmed against the effect of several mGluR agonists such as quisqualate, (1*S*, 3*R*)ACPD and DHPG. No antagonism at the muscarinic receptor agonist carbachol was observed on the same preparations. On the basis of the results obtained in the previous experiments, we could therefore confirm the identification a new class of antagonist acting on a site of mGluR1 different from its agonist binding site. For this reason, a series of chimeric receptors containing parts of mGluR1 and mGluR2 receptors was created. The first chimera (Fig. 4) was composed of the extracellular glutamate binding domain, the first TM

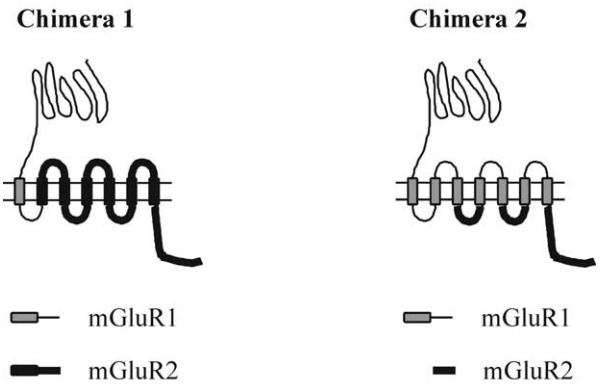


Figure 4. Schematic drawings of the chimeric receptors utilized in this study. Chimera 1 is composed of the extracellular glutamate binding domain, the first TM domain and the first intracellular loop of mGluR1 attached to the downstream portion of mGluR2. Chimera 2 has the second and third intracellular loops and carboxy terminal tail of mGluR1 substituted by the corresponding regions of mGluR2.

domain and the first intracellular loop of mGluR1 attached to the downstream portions of mGluR2. The second chimera had the second and third intracellular loops and carboxy terminal tail of mGluR1 substituted by the corresponding regions of mGluR2 (Fig. 4).

The effect of compound **3** was first tested on wild type human mGluR1α and human mGluR2 expressed in *Xenopus laevis* oocytes. 3 μM **3** significantly ($P<0.01$) reduced the current amplitude induced by 100 μM glutamate from 751 + 124 nA to 295 + 48 nA in oocytes injected with mGluR1α RNA (Fig. 5).

In contrast, the same concentration of derivative **3** did not have any significant effect on the current of mGluR2 activated by 100 μM glutamate. Thus only mGluR1α is sensitive to the compound **3**. We next examined the effect of the antagonist on the two chimeric receptors. 3 μM **3** did not inhibit the current induced by 100 μM glutamate in oocytes expressing

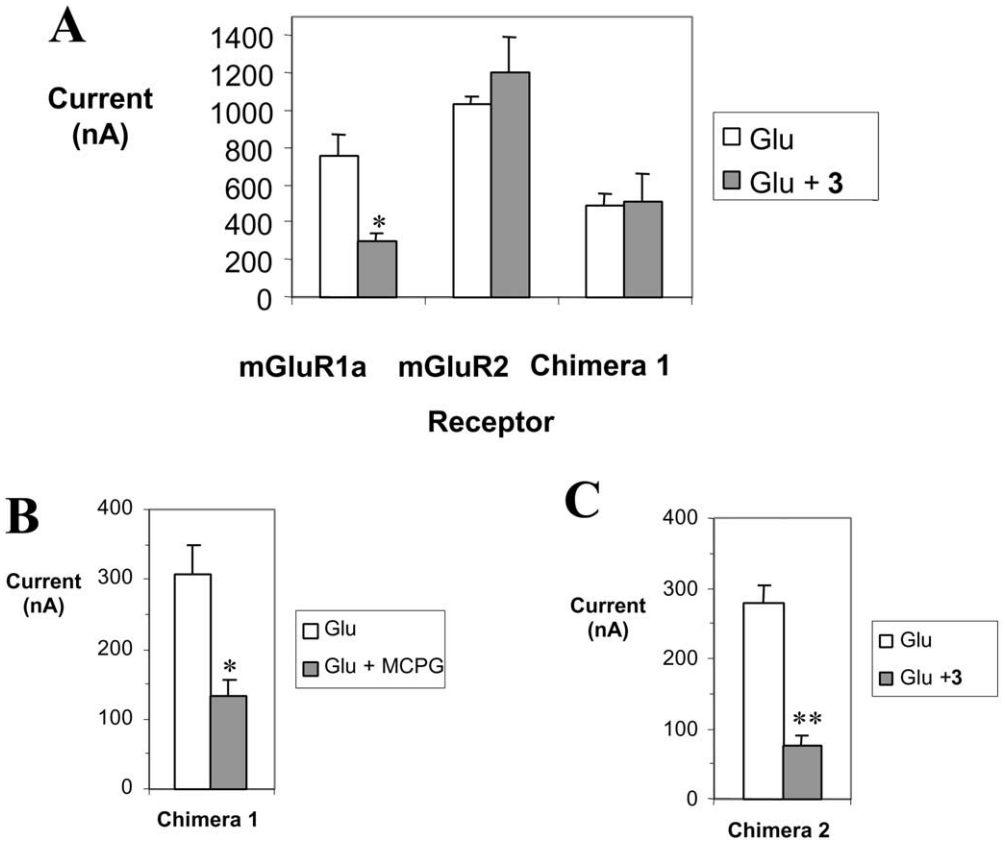


Figure 5. (A) Selective inhibition of mGluR1α by derivative 3. Oocytes were either injected with in vitro synthesized RNA coding for mGluR1α alone, with RNAs encoding chimera 1 and Kir3.1 and Kir 3.4, two subunits of a G protein-coupled inwardly-rectifying potassium channel or with RNAs coding for mR1α induced chloride currents, while they were held at −80 mV in 96 mM potassium to record mGluR2 stimulated potassium currents. Unshaded bars (Glu)- application of 100 μM glutamate. Shaded bars (Glu + 3) —oocytes were preincubated with 3 μM 3 for 20 min before applying 100 μM glutamate with 3 μM 9. Data represent the mean + sem of the following number of oocytes shown in parentheses. mGluR1a: Glu ($n=6$), Glu + 3 ($n=6$); Chimera 1: Glu ($n=3$), Glu + 3 ($n=4$); mGluR2: Glu ($n=5$), Glu + 3 ($n=3$). *, $P<0.01$ versus Glu, Student's *t*-test.). (B) MCPG inhibits chimeric receptor 1. Oocytes were coinjected with in vitro synthesized RNAs coding for chimera 1, Kir3.1 and Kir 3.4. Voltage clamp conditions were the same as in (B). Unshaded bars (Glu)—application of 1 μM glutamate. Shaded bars (Glu + MCPG)- oocytes were preincubated for 10 min with 100 μM MCPG before applying 1 μM glutamate with 100 μM of the antagonist. Data represent the mean ± SEM of the following number of oocytes shown in parentheses. Glu ($n=5$); Glu + MCPG ($n=5$). *, $P<0.01$ versus Glu, Student's *t*-test. C. Chimeric receptor 2 is blocked by derivative 3. Oocytes were coinjected with in vitro synthesized RNAs coding for chimera 2, Kir3.1 and Kir 3.4. Voltage clamp conditions were the same as in (B). Unshaded bars (Glu)—application of 100 μM glutamate. Shaded bars (Glu + **3**)- oocytes were preincubated for 20 min with 3 μM **3** before applying 100 μM glutamate with 3 μM of the antagonist. Data represent the mean + sem of the following number of oocytes shown in parentheses. Glu (n = 9); Glu + **3** ($n=8$). **, $P<0.001$ versus Glu, Student's *t*-test.

chimera 1 (Fig. 5). On the other hand, the competitive antagonist, MCPG, at 100 μM, significantly ($P<0.01$) decreased the current amplitude stimulated by 1 μM glutamate from 307±43 to 133±23 nA. In contrast to chimera 1, the stimulation of chimera 2 by 100 μM glutamate was sensitive to inhibition by compound **3** (Fig. 5). The agonist-induced current amplitude decreased from 278+25 nA to 77±13 nA after application of 3 μM **3** ($P<0.001$). These results demonstrate that the TM domains and extracellular loops of mGluR1 are required for antagonism by derivative **3,** thus confirming that the noncompetitive behaviour of the pyrroles is due to a direct interaction with the receptor.

Behavioural studies.[45] Considering the possible involvement of mGluR1 in nociceptive transmission, derivative **3** was characterized in well known animal models of pain. It was active, after oral administration, both in the early and the late phase of the formalin test[46,47] in mice (a model of both acute and inflammatory pain in the two phases respectively) with an approximately equal ED_{50} of 0.3 mg/kg. The same product showed activity in the carrageenan test[48,49] in rats (a model of inflammatory pain) when administered intraperitoneally with an ED_{50} of about 3 mg/kg. Finally, in the chronic constriction injury model (CCI)[50,51] in rats (a model of neurophatic pain), intraperitoneal administration of compound **3**, made the treated animals (blue-dotted line, Fig. 6) behave similarly to control animals (zero line) with respect to the non treated ones (red line, Fig. 6) with a relatively good duration of action (4 h).

Electrophysiological studies in vitro. The properties of derivative **3** were also tested in an in vitro functional model of the nociceptive pathway using an isolated preparation of baby rat spinal cord (BRSC).[52] This was used to assess the activity on spinal C fibres, which carry pain transmission. A single shock applied to the dorsal roots of intensity sufficient to recruit both A- and C-fibres was used as stimulus. The early component of the ventral root potential (VRP) is a short-duration A-fibre evoked wave, resistant to NMDA receptor antagonists, but sensitive to non-NMDA receptor antagonists. If repeated stimulations at low frequency of C-fibre afferents are used, a summated VRP is evoked,

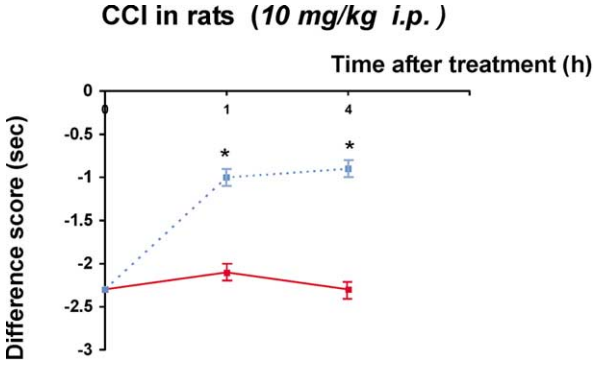


Figure 6. Chronic constriction injury (CCI) model in rats. Treated animals (blue dotted lines) showed the analgesic long-lasting effect of derivative **3** (10 mg/kg; ip) with respect to the untreated control animals (red line).

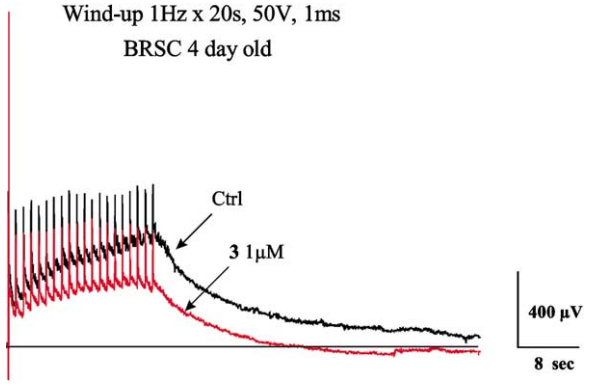


Figure 7. Wind up model. The red trace (compound **3**, 1 μM) clearly showed the ability of the pyrrolic derivative **3** to reduce the effect generated by the multiple stimulus on the C-fibre (black trace), an index of central sensitization.

which is considered to reflect the cumulative depolarization of membrane potential of ventral horn neurons. This is a complex phenomenon and is named 'wind up'. It may be used as an index of central sensitization within the spinal cord. One-hour perfusion of BRSC with 1 μM **3** reduced both the amplitude of the ventral root potential (VRP) and the cumulative VRP as depicted in Figure 7, confirming that mGluR1 antagonists can be useful in blocking the central sensitisation process.

Discussion and Conclusions

The 2,4-dicarboxy-pyrrole esters represent a new class of selective non-competitive mGluR1 antagonist. The very strict geometric and structural features required for competitive antagonism of mGluR1 described by Pellicciari[29,30] were clearly not fulfilled by our template. Since the initial identification of derivative **1**, a detailed study was designed to fully exploit the remarkable possibilities of this template. The complete inactivity of acid derivatives **4–6** immediately implied that we were probably facing something different from the known competitive antagonists. It was therefore necessary to explore the new pyrrolic scaffold to determine the pharmacophoric requirements of this new antagonist. As mentioned above, derivatives **9** and **10** described in Table 1 confirmed the need for a bulky lipophilic group in position C-4 of the pyrrolic template, while position C-2 was quite tolerant to substitution, accepting both aromatic and non-aromatic groups as demonstrated by derivatives **2**, **7**, **8**, **12** and **13.** The combinatorial exploration (1600 derivatives) of the scaffold according to the methodology described above for the selection of the alcohols, fully confirmed our preliminary findings and allowed the identification of the pinacolyl alcohol as the best substituent for the C-4 position. The potency values obtained after the enantiomeric resolution of derivative **3,** confirmed by the asymmetric synthesis of derivatives **15** and **16,** indicate that the area of the receptor mapped by the C-4 substituent not only needed bulky/lipophilic substituents, but also might have some specific stereochemical requirements, as one enantiomer was slightly more potent than the other.

In order to better understand the needs of this newly discovered region of mGluR1, an array exploration was performed, keeping the *n*-Pr substituent in position C-2 fixed. A set of different alcohols, all identified among the α-branched ones and not present in the original R^5OH set used for the original library, was selected on the basis of commercial availability and molecular weight filters. A number of interesting products were prepared and it is worth commenting on the results obtained with two of them. First of all, the pinacolyl group could be removed without great loss of potency, and the removal of a methyl group from the pinacolyl alcohol led to a slight decrease in potency (**17** vs **3**). Secondly, we obtained a further confirmation of the precise stereochemical requirements in the region of the receptor that interacted with this class of compounds. Not only did a diastereomeric mixture lead to a decrease of potency as expected, but the removal of two methyl groups (compared to the pinacolyl structure) and the occupancy of a different spatial region led to a marked decrease (**18** vs **3**).

Pharmacological characterisation of **3** demonstrated that it non-competitively blocked glutamate stimulation of recombinant mGluR1 probably by acting directly on the receptor. In the presence of **3**, the glutamate CRC was shifted rightward and depressed. In addition, when combined with the competitive antagonist, MCPG, **3**, produced a multiplicative effect in the rightward shift of the glutamate CRC suggesting that the two compounds acted at separate sites. The pyrrole derivative probably did not block mGluR1 by inhibiting the second messenger cascade, because it had no effect on two other Gq-linked receptors, NK3 expressed in CHO cells and muscarinic receptor endogenously expressed in cerebellar neurones.

In order to demonstrate more directly that the pyrroles interact with mGluR1, two chimeric receptors containing parts of mGluR1 and -2 were constructed. Chimera one contained the extracellular glutamate binding domain,[11] the first TM domain and the first intracellular loop of mGluR1 attached to the downstream portions of mGluR2. To create chimera two, the second and third intracellular loops and carboxy terminal tail of mGluR1 were replaced by the corresponding regions of mGluR2. The first chimera was unaffected by **3**, whereas the second chimera was inhibited. The mechanism of **3** might be similar to that of CPCCOEt, the first non-competitve mGluR1 antagonist, which was shown to interact with two amino acids on the extracellular surface of TMVII as described in the references reported above.

The in vivo activity of **3** in different pain models also confirmed the original antinociceptive properties of mGluR1 antagonists[27,28] In particular, when dosed orally in mice, the activity of **3** in the early phase of the formalin test indicated the possibility of intervening not only in chronic/neuropathic forms of pain (as shown by the results in the Carrageenan and CCI tests), but also in acute types. Accordingly, mGluR1 antagonists could be endowed with an 'opiate'-like activity profile, but devoid of dependence-inducing side effects.

The poor stability of derivative **3** to rat plasma esterases detrermined in rat 'cassette dosing' ($t_{1/2} = 12$ min versus 2.8 h in mice) prevented oral administration in rats. However, intraperitoneal administration led to significant effects in both chronic and neurogenic pain models, thus confirming that the distribution of the molecule in the body ($Vd_{ss} = 1.9$ l/kg, mean $t_{1/2} = 1.9$ h) and the possible receptor occupancy (the measured concentration of the compound both in the brain and in the plasma 5min after an iv administration led to a brain/plama concentration ratio of 20) is sufficient for a good duration of action as reported in Figure 6. To face the instability of the n-Pr ester to rat esterases, different approaches were followed. In particular, for this di-ester derivative class, the obvious substitution with more hindered C-2 groups gave interesting results. Namely, the substitution with a *t*-butyl ester led to derivative **19** which showed a very good stability ($t_{1/2} = 5.0$ h in rats and >24 h in mice) and nanomolar potency at the receptor.

Finally, further SAR on derivative **3** were performed (e.g., the replacement of the pinacolyl alcohol in position C-4, exploration of N-1, C-3 and C-5 positions) and will be the subject of future communications.

Experimental

Cell culture

Rat cerebellar granule cells. Cultured cerebellar granule cells were prepared from 8-day-old rats. Cells were seeded onto 24-well plates treated with poly-L-lysine at a density of 5×10^5 cells/well in 0.5 mL and cultured in BME (Gibco BRL) containing 10% heat-inactivated fetal bovine serum (FBS), 2 mM glutamine, 50 μg/mL gentamicin and 25 mM KCl. 10 μM cytosine arabinoside was added to the culture 16–18 h after seeding to prevent the proliferation of non-neuronal cells. Experiments were performed after 4 days of culture (4 DIV) when the response of the granule cells to the mGluR1 agonists is maximal.

CHO cell lines. CHO cells stably expressing rat-mGluR1a were produced as reported by Ferraguti et al.[35] Rat-mGluR5b, and human-mGluR4 were cloned by PCR and stably transfected in CHO cells with selection in G418. Rat-mGluR2-CHO cell line was kindly provided by Prof. S. Nakanishi and human-NK3-CHO cells were obtained from Dr S. Arkinstall (Glaxo Institute for Molecular Biology). All mGluRs CHO cell lines were cultured in DMEM (Gibco) containing 2 mM glutamine, 1% proline and 10% dialyzed FBS (Gibco). NK3-CHO cells were cultured in αMEM (Gibco) containing 2 mM glutamine and 10% FBS (Gibco).

Second messenger assays

Measurement of PLC activation through the accumulation of cytidinediphospho-diacylglycerol. The receptor-mediated activation of PLC was evaluated by measuring the accumulation of cytidinediphospho-diacylglycerol

(CDP-DAG), which is the liponucleotide precursor of phosphatidylinositol diphosphate. CDP-DAG accumulates in the presence of Li^+ as a consequence of the receptor mediated activation of PLC.[32] As described in detail by Ferraguti et al.,[35] mGluR1/5-CHO cells (plated the day before in 24-well plates, 1×10^5 cell/0.5 mL/well) or cerebellar granule cells (after 4 DIV in 24-well plates) were labelled for 1 h with 1 μCi/mL of [^{3}H]cytidine (NEN) in Locke's solution (154 mM NaCl, 5 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM $NaHCO_3$, 5 mM HEPES and 1 g/L glucose). After three washings with the same buffer, the agonist dissolved in Locke's solution containing 10 mM LiCl, with or without antagonist, was added for 1 h (500 μL/well). The reaction was stopped by replacing the buffer with ice-cold $CHCl_3$/CH_3OH/1 N HCl mixture (100:200:3 v/v). The mixture was transferred into polypropylene tubes and the well washed with other $CHCl_3$/CH_3OH/1 N HCl mixture. After phase separation, obtained by adding further water and chloroform, an aliquot of the organic phase was transferred into clean tubes and washed with 1 mL of $CHCl_3$/HCl mixture (1:1 v/v). Finally, 200 μL of the organic phase were transferred to scintillation vials, evaporated and counted in a Beta-counter. Under these conditions all the radioactivity collected in the organic phase is constituted of [^{3}H]CDP-DAG.

Intracellular calcium determination using FLIPR technology. CHO cell line expressing rat-mGluR1a receptor was seeded into 96-well plates (5×10^4 cells/well). The following day, cells were washed and pre-incubated for 30 min at 37 °C with 100 μL/well of culture medium containing 2 μM Fluo-4, 0.02% pluronic acid (Molecular Probes), 5 mM probenecid (Sigma) and 20 mM HEPES. After the labelling, cells were washed with HBSS (1.26 mM $CaCl_2$, 0.81 mM $MgSO_4$, 5.36 mM KCl, 0.44 mM KH_2PO_4, 4.17 mM $NaHCO_3$, 137 mM NaCl, 0.34 mM Na_2HPO_4, 1 g/L glucose, pH = 7.4) containing 20 mM HEPES and 2.5 mM probenecid, leaving 100μL/well of the same buffer into the cell plate. After addition of various antagonist and agonist compounds, the intracellular calcium dynamics were measured as fluorescence changes during time, for all the wells of the plate simultaneously, by using the Fluorescent Imaging Plate Reader apparatus (FLIPR, Molecular Devices, Sunnyvale, CA, USA).

Measurement of cAMP formation for selectivity assay on group II and III mGluRs. CHO cell lines expressing human-mGluR4 and rat-mGluR2 receptors were seeded in 96-well plates (5×10^4 cells/well). The following day the cells were washed and preincubated for 20 min at 37 °C in Locke's solution (154 mM NaCl, 5 mM KCl, 2.3 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM $NaCO_3$, 5 mM HEPES and 1 g/L glucose) containing 200 μM 3-isobutyl-1-methylxantine (IBMX). The reaction was started by replacing the buffer with Locke's solution containing the test agents (15 μM forskolin agonist antagonist) and 100 μM IBMX. After a 20 min incubation at 37 °C the solution was aspirated and the reaction stopped using 100μL of 'Lysis Reagent' (included into the Amersham cAMP kit) containing 200 μM IBMX. cAMP concentration in the samples was determined by means of a 'cAMP [^{125}I] SPA' kit (code RPA 538, Amersham) according to the manufacturer's instructions.

Arachidonic acid release

Cells were cultured in 24-well plates (1×10^5 cell/well) and labelled by incubation for 4–5 h with serum-free medium containing [^{3}H] arachidonic acid (0.5 μCi/mL, NEN)and supplemented with 2mg/mL of fatty acid free BSA (FAF-BSA, Sigma). Cells were then washed three times and stimulated for 25 min at 37 °C with agonists with/without antagonists, in 500 μL/well of serum-free medium containing FAF-BSA. At the end of the incubation 400 μL were transferred from wells to ice cold tubes and centriguged at 500*g* for 5 min. 350 μL of each sample were finally removed and counted in a Beta-counter.

Construction of chimeric receptors

Construction of chimeric receptor 1. A common SacI site in the cDNAs encoding human mGluR1 and human mGluR2 was used to create chimera 1. A 1.8 kb BamHI-SacI fragment coding for the ECD, 1st TM and 1st intracellular loop of human mGluR1 (...SREL^{627}) was ligated to a 0.8 kb SacI-BamHI fragment encoding TMs 2-7 and the carboxy-terminal (C-terminal) region of human mGluR2 (^{606}CYIL...).[53,54]

Construction of chimeric receptor 2. To create chimera 2 we followed the PCR overlap extension method described in Pin et al.[55] After cloning into pBluescriptII KS, the sequences of all amplified DNAs were checked in both directions by fluorescent dye terminator cycle sequencing. The regions of mGluR1α that were replaced with the corresponding portions of mGluR2 are the following: the i2 of mGluR1α ^{681}RIAR ... AWAQ^{706} was exchanged with the i2 of mGluR2 ^{656}RIAR ... PASQ^{689}; the i3 of mGluR1α ^{773}KTRN ... NEAK^{785} was replaced with the i3 of mGluR2 ^{748}KTRK ... NEAK^{760}; the C-terminus of mGluR1α ^{841}KPER ... SSTL^{1194} was substituted with the C-terminus of mGluR2 ^{820}QPQK ... TSSL^{872}.

List of primer sequences used to create chimera *2*. Oligonucleotide primers used to generate chimera 2 are presented in the 5′ to 3′ direction. The junction between mGluR1 and mGluR2 sequences are indicated by a '-'.

2S (CGGGAGCTCTGCTACATCATCCTA)
2AS (CGAAGATGCGTGCAATGCG-ATTGGTTTT AGTCACTAAAGCAGAGTAGCAC)
3S (CGCATTGCACGCATCTTCG)
3AS (CTGTGAGGCAGGACTGATGAAGC)
4S (CTTCATCAGTCCTGCCTCACAG-GTGATCATT GCCTCAATTCTGATTA)
4AS (GTATTGCAGATAAGGTAGACTTCCTTGAT ACTTG)
5S (CCCAAGTATCAAGGAAGTCTACCTTATCTGC)
5AS (TGAAGTTTTCGGGGCACTTGCGAGTCTT-GAAGGCATAGTAGGTACAGCTCATGA)
6AS (ACAAAAGCTAGCCAGATGATACAGGTGGT GTACATGGTGAACGCGATATA-CTTGGCCTCGT TGAAGTTTTCGGGGCACTTGCG)

7S (TCCCAAGATGTACATCATTATTGCC-CAGCC GCAGAAGAACGTG)
7AS (TTACTAGTGGATCCTCAAAGCGA)

Expression of receptors in oocytes. RNA was synthesized in vitro from linearized DNA and injected into Xenopus oocytes as described in Corti et al.[56] mGluR1α RNA was injected alone, while the RNAs coding for mGluR2, chimera 1 and chimera 2 were all coinjected with RNAs coding for 2 subunits of a G protein-coupled inwardly-rectifying potassium channel, Kir 3.1[57] and Kir 3.4.[58]

Electrophysiological recordings. Recordings were made 2–4 days post injection for mGluR1α and mGluR2 and 10–11 days post injection for chimeras 1 and 2 employing glass electrodes filled with 3 M KCl and having a resistance of 0.2–2 MΩ. Oocytes were held at −60 mV to measure mGluR1α receptor activation, while they were maintained at −80 mV to follow receptor stimulation of mGluR2 and chimeras 1 and 2 in the two electrode voltage clamp mode. Drugs were applied to mGluR1α RNA injected oocytes bathed in ND96. To examine responses of mGluR2 and chimeras 1 and 2, oocytes were first clamped at −80 mV in ND96, then a high potassium solution (2 mM NaCl, 96 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES, pH 7.5) was applied. After the basal current had stabilized, drugs were applied in the high potassium solution.

Chemistry

Infrared spectra were recorded on a Bruker IFS 48 spectrometer. ^{1}H NMR spectra were recorded on a Varian Unity 400 (400 MHz); the data are reported as follows: chemical shift in ppm from the Me_4Si line as external standard, multiplicity (b = broad, s = singlet, d = doublet, t = triplet, q = quartet, m = multiplet) and coupling constants.

Chromatography was carried out by use of the Merck Silica Gel 60 (230–400 mesh). Mass spectra were performed on a Triple Quadrupole (VG-4 Fison Instrument, UK) equipped with Fast Atom Bombardment (FAB) ionization. Elemental analyses were determined by a EA 1108 Carlo Erba elemental analyzer. Melting points were determined on a Büchi 530 apparatus (scale 0–250 °C) and are uncorrected. All the reactions were carried out under a controlled atmosphere in flame dried glassware. Anhydrous solvents and reagents were purchased from Aldrich. Reactions were monitored by analytical thin-layer chromatography (TLC) using Merck Silica Gel 60 F-254 glass plates (0.25 mm).

General procedure for the synthesis of the esters

The appropriate carboxylic acid (1 mmol) was suspended in dry toluene (8 mL), trifluoroacetic anhydride (1.2 mmol) was added under a nitrogen atmosphere and the suspension stirred until complete solubilisation occurred at room temperature. The desired alcohol (1.2 mmol) was added, the solution was still stirred for 2 h at room temperature and then diluted with ethyl acetate (8 mL), washed with a 2 M NaOH solution (2×5 mL) and then with brine (2×5 mL). The organic phase was dried over Na_2SO_4 and the solvent evaporated to obtain the crude compound which was purified by column chromatography (generally cyclohexane/ethyl acetate 9:1), to give the desired compound.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 4-ethyl ester 2-ethyl ester (1). Commercially available from Aldrich. Mp 135 °C.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 4-*t*-butyl ester 2-propyl ester (2). Prepared according to the general procedure from 4-*t*-Bu-3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid as a white solid. Mp 116 °C, NMR $CDCl_3$: δ 8.79 (bs, 1H), 4.23 (t, 2H), 2.54 (s, 3H), 2.48 (s, 3H), 1.76 (m, 2H), 1.56 (s, 9H), 1.01 (t, 3H). IR ($CDCl_3$) cm^{-1}, 3400 (NH), 1680 (C=O). MS: *m*/*z* 281 $[M]^+$, 282 $[M+H]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 2-propyl ester 4-(1,2,2-trimethyl-propyl) ester (3). Prepared according to the general procedure from 2-propyl-3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid as a white solid (yield = 70%). Mp 75 °C. NMR: $CDCl_3$: δ 8.82 (bs, 1H), 4.94 (q, 1H), 4.23 (t, 2H), 2.59 (s, 3H), 2.53 (s, 3H),1.76 (m, 2H), 1.22 (d,3H), 1.01 (t,3H), 0.97 (s, 9H). IR ($CDCl_3$) cm^{-1}, 3279, 1697, 1666. MS: *m*/*z* 310 $[M+H]^+$, 309 $[M]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 2-ethyl ester (4). Commercially available from Maybridge.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 4-ethyl ester (5). Prepared according to ref 59 as a light brown solid. Mp 205 °C.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid (6). Commercially available from Chem. Star.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 2-benzyl ester 4-*t*-butyl ester (7). Prepared according to what reported in ref 60 as a light brown solid. Mp 123 °C, NMR $CDCl_3$: δ 7.43–7.3 (m, 5H), 6.78 (bs, 1H), 5.30 (s, 2H), 2.56 (s, 3H), 2.47 (s, 3H), 1.56 (s, 9H). IR ($CDCl_3$) cm^{-1}, 3308 (NH), 1691, 1661 (C=O). MS: *m*/*z* 329 $[M]^+$, 274 $[MH-t\text{Bu}]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 2-allyl ester 4-*t*-butyl ester (8). Prepared according to the general procedure from 4-*t*-Bu-3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid as a yellow solid. Mp 77 °C NMR: $CDCl_3$: δ 8.84 (bs, 1H), 6.0 (m, 1H), 5.33 (m, 2H), 4.77 (m, 2H), 2.55 (s, 3H), 2.49 (s, 3H), 1.56 (s, 9H). IR ($CDCl_3$) cm^{-1}, 3450 (NH), 1686 (C=O). MS: *m*/*z* 279 $[M]^+$, 223 $[M-t\text{Bu}+H]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 2-benzyl ester 4-allyl ester (9). Prepared according to the general procedure from 2-benzyl-3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid as a white solid. Mp 100 °C NMR: $CDCl_3$: δ 8.84 (bs, 1H), 7.45–7.3 (m, 5H), 6.02 (dd, 1H), 5.38 (dd, 1H), 5.32 (s, 2H), 5.26 (dd, 1H), 4.76 (m, 2H), 2.60 (s, 3H), 2.52 (s, 3H). IR ($CDCl_3$) cm^{-1}, 3300 (NH), 1703 (C=O),1672 (C=O). MS: *m*/*z* 314 $[M+H]^+$,313 $[M]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 2-benzyl ester 4-benzyl ester (10). Prepared according to the general procedure from 2-benzyl-3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid as a white solid. Mp 118 °C NMR: $CDCl_3$: δ 8.88 (bs, 1H), 7.44–7.3 (m, 10H), 5.30 (s, 2H), 5.28 (s, 2H), 2.57 (s, 3H), 2.48 (s, 3H). IR (nujol) cm^{-1}, 3312 (NH), 1701 (C=O),1664 (C=O). MS: *m/z* 363 $[M]^+$, 272 $[M-Bn]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 2-(2-furyl-methyl) ester 4-*t*-butyl ester (11). Prepared according to the general procedure from 4-*t*-Bu-3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid as a light yellow solid. Mp 115 °C NMR $CDCl_3$: δ 8.76 (bs, 1H), 7.44 (dd, 1H), 6.46 (d, 1H), 6.38 (dd, 1H), 5.24 (s, 2H), 2.53 (s, 3H), 2.47 (s, 3H), 1.56 (s, 9H). IR ($CDCl_3$) cm^{-1}, 3443 (NH), 1688 (C=O). MS: *m/z* 319 $[M]^+$, 320 $[M+H]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 2-octyl ester 4-*t*-butyl ester (12). Prepared according to the general procedure from 4-*t*-Bu-3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid as a white solid. Mp 75 °C, NMR: $CDCl_3$: δ 8.78 (bs, 1H), 4.25 (t, 2H), 2.54 (s, 3H), 2.48 (t, 3H), 1.73 (m, 2H), 1.56 (s, 9H), 1.40–1.24 (m, 10H), 0.88 (m, 3H). IR ($CDCl_3$) cm^{-1}, 3450 (NH), 1685 (C=O). MS: *m/z* 351 $[M]^+$, 352 $[M+H]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 2-phenyl ester 4-*t*-butyl ester (13). Prepared according to the general procedure from 4-*t*-Bu-3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid as a pink solid. Mp 125 °C, NMR: $CDCl_3$: δ 8.9 (bs, 1H), 7.41–7.17 (m, 5H), 2.63 (s, 3H), 2.53 (s, 3H), 1.58 (s, 9H). IR ($CDCl_3$) cm^{-1}, 3200 (NH), 1703 (C=O), 1691 (C=O). MS: *m/z* 316 $[M+H]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 2-(trimethyl silyl ethyl) ester (14). 3,5-Dimethyl-2,4-dicarboxylic acid 2-(trimethyl silyl ethyl) 4-benzyl ester (200 mg) was dissolved in AcOEt (10 mL) and hydrogenated at atmospheric pressure and room temperature for 5 h on 5% Pd on C. The suspension was filtered over Celite, the organic phase dried over sodium sulphate and evaporated at reduced pressure to give the title compound (yield = 75%) as a white solid. Mp > 250 °C. NMR DMSO-d_6: δ 11.91 (s, 1H), 11.71 (s, 1H), 4.27 (m, 2H), 2.47 (s, 3H), 2.37 (s, 3H), 1.05 (m, 2H), 0.03 (s, 9H). IR (Nujol) cm^{-1}, 3292 (NH), 1653 (C=O). MS: *m/z* 284 $[M+H]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 2-propyl ester 4-((*S*)-1,2,2-trimethyl-propyl) ester (15). Prepared according to the general procedure from 2-propyl-3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid as a white foam (yield = 27%). ^1H NMR ($CdCl_3$) 0.99 (S, 9H); 1.03 (t, 3H); 1.24 (d, 3H); 1.77 (m, 2H); 2.55 (s, 3H); 2.60 (s, 3H); 4.25 (t, 2H); 4.96 (q, 1H); 8.92 (bs, 1H). Configuration confirmed by experiment with $Eu(tfc)_3$ as shift reagent. IR (nujol): 3277, 2854, 1697, 1670. MS: *m/z* 309 $[M]^+$, 310 $[MH]^+$, 268, 226, 225, 250, 208. HPLC (pump: JASCO 980, detector: JASCO UV-970, λ = 275 nm, integrator: PE941A, column: Chiralpack AD, 25 cm×4.6 mm, part. size: 5 μm, temp. autosampler: 25 °C, temp. column: 25 °C, inj. vol.: 20 μL, mobile phase: Hexane/isopropanol 90/10, flux: 1 mL/min). Retention time: 6.43, e.e. = 90%.

3,5-Dimethy-pyrrole-2,4-dicarboxylic acid 2-propyl ester 4-((*R*)-1,2,2-trimethyl-propyl) ester (16). Prepared according to the general procedure from 2-Propyl-3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid as a a light yellowish foam (yield = 25%). ^1H NMR: ($CdCl_3$) 0.99 (S, 9H); 1.03 (t, 3H); 1.24 (d, 3H); 1.77 (m, 2H); 2.55 (s, 3H); 2.60 (s, 3H); 4.25 (t, 2H); 4.96 (q, 1H); 8.92 (bs, 1H). Configuration confirmed by experiment with $Eu(tfc)_3$ as shift reagent. IR (nujol): 3277, 2854, 1697, 1670. MS: *m/z* 309 $[M]^+$, 310 $[MH]^+$, 268, 226, 225, 250, 208. HPLC (pump: JASCO 980, detector: JASCO UV-970, λ = 275 nm, integrator: PE941A, column: Chiralpack AD, 25 cm×4.6 mm, part. size: 5 μm, temp autosampler: 25 °C, temp column: 25 °C, inj. vol.: 20 μL, mobile phase: Hexane/isopropanol 90/10, flux: 1 mL/min). Retention Time: 7.29, e.e. = 84%.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic-acid 4-(2, 3-dimethyl propyl) ester 2-propyl ester (17). Prepared according to the general procedure from 2-propyl-3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid as a light brown glassy foam NMR $CDCl_3$: δ 8.88 (bs, 1H), 4.98 (m, 1H), 4.23 (t, 2H), 2.58 (s, 3H), 2.52 (s, 3H), 1.90 (m, 1H), 1.76 (m, 2H), 1.25 (d, 3H), 1.01 (t, 3H), 0.98 (d, 3H), 0.96 (t, 3H). IR ($CDCl_3$) cm^{-1}, 3210 (NH), 1721 (C=O) . MS: *m/z* 296 $[M+H]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic-acid 4-(2-methyl cyclopentyl) 2-propyl ester (18). Prepared according to the general procedure from 2-propyl-3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid as a light brown glassy foam NMR $CDCl_3$: δ 8.77 (bs, 1H), 5.38 and 5.30 (m, 1H), 4.23 (t, 2H), 2.56 and 2.55 (s, 3H), 2.51 and 2.50 (s, 3H), 1.78 (m, 2H), 1.08 and 1.04 (d, 3H), 1.01 (t, 3H), 2.04–0.8 (m, 7H). IR ($CDCl_3$) cm^{-1}, 3150 (NH), 1690 (C=O). MS: *m/z* 308 $[M+H]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic-acid 2-*t*-butyl ester- 4-[1,2,2-trimethylpropyloxycarbonyl]-ester (19). Prepared according to the general procedure from 2-*t*-Bu-3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid as a pink solid (yield = 75%). Mp 133 °C NMR: DMSO-d_6: δ 11.6 (bs, 1H), 4.77 (q, 1H), 2.43 (s, 3H), 2.40 (s, 3H), 1.5(s, 9H), 1.12 (d, 3H), 0.91 (s, 9H). IR (nujol) cm^{-1}, 3310, 1699,1657. MS: *m/z* 324 $[M+H]^+$, 323 $[M]^+$.

Other esters reported in the Experimental and used as intermediates.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic-acid 2-propyl ester. 2 (182 mg) was dissolved in trifluoroacetic acid (3 mL) at rt. After stirring for 30 min, the reaction mixture was poured into water (7 mL) and the resulting white precipitate was filtered off to give the title compound (110 mg) as a white solid. Mp > 250 °C. NMR: DMSO-d_6: δ 11.92 (bs, 1H), 11.71 (bs, 1H), 4.14 (t, 2H), 2.45 (s, 3H), 2.39 (d, 3H), 1.067 (m, 2H), 0.95 (t, 3H). IR (nujol) cm^{-1}, 3296, 2600,1657. MS: *m/z* 225 $[M]^+$.

3,5-Dimethylpyrrole-2,4-dicarboxylic-acid 2-benzyl ester. Derivative **7** (150 mg) was dissolved in trifluoroacetic acid (3 mL) at rt. After stirring for 30 min, the reaction mixture was poured into water (10 mL) and the resulting white precipitate was filtered off to give the title compound (yield = 93%) as a white solid. Mp > 250 °C.

NMR: DMSO-d_6: δ 11.8 (bs, 1H), 7.42 (m, 5H), 5.28 (s, 2H), 2.45 (s, 3H), 2.39 (s, 3H). IR (nujol) cm^{-1}, 3290 (NH), 1657 (C=O). MS: m/z 274 $[M+H]^+$, 273 $[M]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic acid 2-(trimethyl silyl ethyl) 4-benzyl ester. Prepared from the commercially available 3,5-dimethyl-pyrrole-2,4-dicarboxylic-acid 4-benzyl ester according to the general procedure as a yellow solid. Mp 138 °C NMR $CDCl_3$: δ 8.9 (bs, 1H), 7.42–7.32 (m, 5H), 5.29 (s, 2H), 4.35 (m, 2H), 2.57 (s, 3H), 2.50 (s, 3H), 1.1 (m, 2H), 0.06 (s, 9H). IR ($CDCl_3$) cm^{-1}, 3300 (NH), 1697 (C=O), 1659 (C=O). MS: m/z 374 $[M+H]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic-acid 4-*t*-Bu-ester. Derivative **7** (200 mg) was dissolved in AcOEt (10 mL) and hydrogenated at atmospheric pressure and room temperature for 5 h on 5% Pd on C. The suspension was filtered over Celite, the organic phase dried over sodium sulphate and evaporated at reduced pressure to give the title compound as a light brown solid (yield = 95%). = Mp > 120 °C NMR: DMSO-d_6: δ 12.35 (bs, 1H), 11.65 (s, 1H), 2.40 (s, 3H), 2.34 (s, 3H), 1.47 (s, 9H). IR (nujol) cm^{-1}, 3500 (OH), 3200 (NH), 1680 (C=O). MS: m/z 239 $[M]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic-acid 2-*tert*-butyl ester 4-benzyl ester. To a solution of *t*-butylacetoacetate (20.7 mL) in acetic acid (35 mL) kept at costant temperature of 10 °C, sodium nitrite (9.5 g) was slowly added under vigorous stirring. The mixture was left at 10 °C for 24 h and then added to a suspension formed by Zn (24.25 g), benzylacetoacetate (21.6 mL) and ammonium acetate (19.25 g) in acetic acid (62.5 mL) kept at 65 °C. The temperature was raised suddenly to 90 °C and stirred at the same temperature for 5 h. The mixture was then cooled to 50 °C and poured onto ice and the crude material was crystallised from aq methanol to give the title compound (14.7 g) as a white solid.

NMR: $CDCl_3$: δ 8.78 (bs, 1H), 7.49 (d, 2H), 7.37 (t, 2H), 7.31 (m, 1H), 5.29 (s, 2H), 2.53 (s, 3H), 2.49 (s, 3H), 1.57(s, 9H).IR (nujol) cm^{-1}, 3317, 3296, 1655. MS: m/z 236 $[M+H]^+$, 235 $[M]^+$.

3,5-Dimethyl-pyrrole-2,4-dicarboxylic-acid 2-*t*-butyl ester. 3,5-Dimethyl pyrrole-2,4-dicarboxylic-acid 2-*tert*-butyl ester-4-benzyl ester (9.6 g) was dissolved in THF (250 mL) and hydrogenated over 10% Pd on C (1 g) for 8 h. The solution was filtered and the solvent evaporated to dryness. The crude product was dissolved in ethyl acetate and petroleum ether (300 mL) was added. The resulting precipitate was filtered off to give the title compound (6.7 g) as a white solid. NMR: DMSO-d_6: δ 11.86 (bs, 1H),11.51 (bs, 1H), 2.40 (s, 3H), 2.37 (s, 3H), 1.49 (s, 9H). IR (nujol) cm^{-1}, 3317, 3296, 1655. MS: m/z 236 $[M+H]^+$, 235 $[M]^+$.

References and Notes

1. Wheal, H.V., Thomson, A.M., Eds. *Excitatory Amino Acids Synaptic Transmission*, 1995, Academic press, London
2. Meldrum, B. S., Ed. *Excitatory Amino Acids Antagonists*. Blackwell: Oxford, 1991.
3. Di Fabio, R.; Capelli, A. M.; Conti, N.; Cugola, A.; Donati, D.; Feriani, A.; Gastaldi, P.; Gaviraghi, G.; Hewkin, C. T.; Micheli, F.; Missio, A.; Mugnaini, M.; Pecunioso, A.; Quaglia, A. M.; Ratti, E.; Rossi, L.; Tedesco, G.; Trist, D. G.; Reggiani, A. *J. Med. Chem.* **1997**, *40*, 841.
4. Micheli, F.; Di Fabio, R.; Capelli, A. M.; Cugola, A.; Cucuruto, O.; Feriani, A.; Gastaldi, P.; Gaviraghi, G.; Marchioro, C.; Orlandi, A.; Pozzan, A.; Quaglia, A. M.; Reggiani, A.; van Amsterdam, F. *Arch. Pharm. Pharm. Med. Chem.* **1999**, *332*, 73.
5. Donati, D.; Micheli, F. *Exp. Opin. Ther. Pat.* **2000**, *10*, 667.
6. Nakanishi, S.; Masu, M. *Annu. Rev. Biophys. Biomol. Struct.* **1994**, *23*, 319.
7. Hollmann, M.; Heinemann, S. *Annu. Rev. Neurosci.* **1994**, *17*, 31.
8. Pin, J. P.; Duvoisin, R. *Neuropharmacology* **1995**, *34*, 1.
9. Knoepfel, T.; Kuhn, R.; Allgeier, H. *J. Med. Chem.* **1995**, *38*, 1417.
10. Conn, P. J.; Pin, J. P. *Annu. Rev. Pharmacol. Toxicol.* **1997**, *37*, 205.
11. O'Hara, P. J.; Sheppard, P. O.; Thogersen, H.; Venezia, D.; Haldeman, B. A.; McGrane, V.; Houamed, K. M.; Thomsen, C.; Gilbert, T. L.; Mulvihill, E. R. *Neuron* **1993**, *11*, 41.
12. Kunishima, N.; Shimada, Y.; Tsuji, Y.; Sato, T.; Yamamoto, M.; Kumasaka, T.; Nakanishi, S.; Jingami, H.; Morikawa, K. *Nature (London)* **2000**, *407*, 971.
13. Brown, E. M.; Gamba, G.; Riccardi, D.; Lombardi, M.; Butters, R.; Kifor, O.; Sun, A.; Hediger, M. A.; Lytton, J.; Herbert, S. C. *Nature (London)* **1993**, *366*, 575.
14. Kaupmann, K.; Huggel, K.; Heid, J.; Flor, P. J.; Bischoff, S.; Mickel, S. J.; McMaster, G.; Angst, C.; Bittiger, H.; Froestl, W.; Bettler, B. *Nature (London)* **1997**, *386*, 239.
15. Bargmann, C. *Cell* **1997**, *90*, 585.
16. Bräuner-Osborne, H.; Egebjerg, J.; Nielsen, E.Ø.; Madsen, U.; Krogsgaard-Larsen, P. *J. Med. Chem.* **2000**, *43*, 2609.
17. Schoepp, D. D.; Jane, D. E.; Monn, J. A. *Neuropharmacology* **1999**, *38*, 1431.
18. Bräuner-Osborne, H.; Krogsgaard-Larsen, P. *British J. Pharmacol.* **1998**, *123*, 269.
19. Brabet, I.; Mary, S.; Bockaert, J.; Pin, J. P. *Neuropharmacology* **1995**, *34*, 895.
20. Hayashi, Y.; Sekiyama, M.; Nakanishi, S.; Jane, D. E.; Sunter, D. C.; Birse, E. F.; Udvarhelyi, P. M.; Watkins, J. C. *J. Neurosci.* **1994**, *14*, 3370.
21. Pellicciari, R.; Raimondo, M.; Marinozzi, M.; Natalini, B.; Costantino, G.; Thomsen, C. *J. Med. Chem.* **1996**, *39*, 2874.
22. Eaton, S. A.; Jane, D. E.; Jones, P. L. S. J.; Porter, R. H. P.; Pook, P. C. K.; Sunter, D. C.; Udvarhelyi, P. M.; Roberts, P. J.; Salt, T. E.; Watkins, J. C. *Eur. J. Pharmacol., Mol. Pharmacol. Sect.* **1993**, *244*, 195.
23. Costantino, G.; Maltoni, K.; Marinozzi, M.; Camaioni, E.; Prezeau, L.; Pin, J.-P.; Pellicciari, R. *Bioorg. Med. Chem.* **2001**, *9*, 221.
24. Clark, B. P.; Harris, J. R.; Kingston, A. E.; McManus, D. *XVth Eur. Int. Symp. Med. Chem* **1998**, 183.
25. Litschig, S.; Gasparini, F.; Rueegg, D.; Stoehr, N.; Flor, P. J.; Vranesic, I.; Prezeau, L.; Pin, J. P.; Thomsen, C.; Khun, R. *CPCCOEt. Mol. Pharmacol.* **1999**, *55*, 453.
26. De Vry, J.; Horvath, E.; Schreiber, R. *Eur. J. Pharmacol.* **2001**, *428*, 203.

27. Nicoletti, F.; Bruno, V.; Copani, A.; Casabona, G.; Knöpfel, T. *Trends Neurosci*. **1996**, *19*, 267.
28. Pellegrini-Giampietro, D. E.; Cozzi, A.; Peruginelli, F.; Leonardi, P.; Meli, E.; Pellicciari, R.; Moroni, F., 1 *Eur. J. Neurosci*. **1999**, *11*, 3637.
29. Young, M. R.; Fleetwood-Walker, S. M.; Mitchell, R.; Munro, F. E. *Neuropharmacology* **1994**, *33*, 141.
30. Valerio, A.; Paterlini, M.; Biofava, M.; Memo, M.; Spano, P. *Neuroreport* **1997**, *8*, 2695.
31. Mao, L.; Conquet, F.; Wang, J. Q. *Neuroscience* **2001**, *106*, 303.
32. Fundytus, M. E.; Yashpal, K.; Chabot, J.-G.; Osborne, M. G.; Lefebvre, C. D.; Dray, A.; Henry, J. L.; Coderre, T. J. *British Journal of Pharmacology* **2001**, *132*, 354.
33. Costantino, G.; Macchiarulo, A.; Pellicciari, R. *J. Med. Chem*. **1999**, *42*, 2816.
34. Costantino, G.; Pellicciari, R. *J. Med. Chem*. **1999**, *32*, 5390.
35. Ferraguti, F.; Cavanni, P.; Eistetter, H.; Salvagno, C.; Ratti, E.; Trist, D. G. *Mol. Cell. Neurosci*. **1994**, *5*, 269.
36. Jensen, A. M.; Chiu, S. Y. *J.Neurosci*. **1990**, *10*, 1165.
37. Chu, E. J.-H.; Chu, T. C. *J. Org. Chem*. **1954**, *19*, 266.
38. Fisher, H.; Walach, B. *Chem. Ber*. **1925**, *58*, 2818.
39. Knorr, L. *Justus Liebig Ann. Chem*. **1886**, *236*, 290.
40. Di Simone, B.; Savoia, D.; Tagliavini, E.; Umani-Ronchi, A. *Tetrahedron: Asymmetry* **1995**, *6*, 301.
41. Helke, C. J.; Krause, J. E.; Mantyh, P. W.; Couture, R.; Bannon, M. J. *FASEB Journal* **1990**, *4*, 1606 Ref: 69.
42. Eaton, S. A.; Jane, D. E.; Jones, P. L.; Porter, R. H.; Pook, P. C.; Sunter, D. C.; Udvarhely, P. M.; Roberts, P. J.; Salt, T. E.; Watkins, J. C. Eur. *J. Pharmacol*. **1993**, *244*, 195.
43. Pizzi, M.; Galli, P.; Consolandi, O.; Arrighi, V.; Memo, M.; Spano, P. F. *Mol Pharmacol*. **1996**, *49*, 586.
44. Toms, N. J.; Jane, D. E.; Tse, H. W.; Roberts, P. J. *British J Pharmacol*. **1995**, *116*, 2824.
45. The research complied with national legislation and with company policy on the Care and Use of Animals and with related codes of practice.
46. Hunskaar, S.; Fasmer, O. B.; Hole, K. *J. Neurosci. Methods* **1985**, *14*, 69.
47. Hunskaar, S.; Hole, K. *Pain* **1987**, *30*, 103.
48. Arrigonimartelli, E.; Conti, I. *Farmaco, Edizione pratica Mar* **1964**, 19134.
49. Perrot, S.; Guilbaud, G.; Kayser, V. *Pain* **1999**, *83*, 249.
50. Mao, J.; Price, D. D.; Hayes, R. L.; Lu, J.; Mayer, D. J. *Brain. Res*. **1992**, *598*, 271.
51. Bordi, F.; Quartaroli, M. *Pain* **2000**, *84*, 213.
52. Seebach, B. S.; Mendell, L. M. *J. Neurophysiol*. **1996**, *76*, 3875.
53. Takahashi, K.; Tsuchida, K.; Tanabe, Y.; Masu, M.; Nakanishi, S. *J. Biol. Chem*. **1993**, *268*, 19341.
54. Okamoto, T.; Sekiyama, N.; Otsu, M.; Shimada, Y.; Sato, A.; Nakanishi, S.; Jingami, H. *J. Biol. Chem*. **1998**, *273*, 13089.
55. Pin, J.-P.; Joly, C.; Heinemann, S. F.; Bockaert, J. *EMBO J*. **1994**, *13*, 342.
56. Corti, C.; Restituito, S.; Rimland, J. M.; Brabet, I.; Corsi, M.; Pin, J.-P.; Ferraguti, F. *Eur. J. Neurosci* **1998**, *10*, 3629.
57. Kubo, Y.; Reuveny, E.; Slesinger, P. A.; Jan, Y. N.; Jan, L. Y. *Nature* **1993**, *364*, 802.
58. Ashford, M. L. J.; Bond, C. T.; Blair, T. A.; Adelman, J. P. *Nature* **1994**, *370*, 456.
59. Cordell, G. *A J. Org. Chem*. **1975**, *40*, 3161.
60. Smith, M. K.; Bisset, G. M. F. *J. Org. Chem*. **1979**, *44*, 2077.