Cinnabarinic Acid, an Endogenous Metabolite of the Kynurenine Pathway, Activates Type 4 Metabotropic Glutamate Receptors

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ABSTRACT

Cinnabarinic acid is an endogenous metabolite of the kynurenine pathway that meets the structural requirements to interact with glutamate receptors. We found that cinnabarinic acid acts as a partial agonist of type 4 metabotropic glutamate (mGlu4) receptors, with no activity at other mGlu receptor subtypes. We also tested the activity of cinnabarinic acid on native mGlu4 receptors by examining 1) the inhibition of cAMP formation in cultured cerebellar granule cells; 2) protection against excitotoxic neuronal death in mixed cultures of cortical cells; and 3) protection against 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine toxicity in mice after local infusion into the external globus pallidus. In all these models, cinnabarinic acid behaved similarly to conventional mGlu4 receptor agonists, and, at least in cultured neurons, the action of low concentrations of cinnabarinic acid was largely attenuated by genetic deletion of mGlu4 receptors. However, high concentrations of cinnabarinic acid were still active in the absence of mGlu4 receptors, suggesting that the compound may have off-target effects. Mutagenesis and molecular modeling experiments showed that cinnabarinic acid acts as an orthosteric agonist interacting with residues of the glutamate binding pocket of mGlu4. Accordingly, cinnabarinic acid did not activate truncated mGlu4 receptors lacking the N-terminal Venus-flytrap domain, as opposed to the mGlu4 receptor enhancer, *N*-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC). Finally, we could detect endogenous cinnabarinic acid in brain tissue and peripheral organs by high-performance liquid chromatography-tandem mass spectrometry analysis. Levels increased substantially during inflammation induced by lipopolysaccharide. We conclude that cinnabarinic acid is a novel endogenous orthosteric agonist of mGlu4 receptors endowed with neuroprotective activity.

Introduction

The kynurenine pathway is a major metabolic route of L-tryptophan in the liver and other tissues, including the central nervous system (CNS). The two initial enzymes of the

ABBREVIATIONS: CNS, central nervous system; NMDA, *N*-methyl-D-aspartate; mGlu, metabotropic glutamate; L-AP4, L-(+)-2-amino-4-phosphonobutyric acid; PHCCC, *N*-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide; PPG, (R,S)-4-phosphonophenylglycine; DCG-IV, (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine; ACPT-I, (1S,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid; IBMX, 3-isobutyl-1-methylxanthine; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; HEK, human embryonic kidney; InsP, inositol phosphate; DIV, days in vitro; TBS-T, Tris-buffered saline/Tween 20; LDH, lactate dehydrogenase; DA, dopamine; TH, tyrosine hydroxylase; EAE, experimental autoimmune encephalomyelitis; CSF, cerebrospinal fluid; HPLC, high-performance liquid chromatography; MS/MS, tandem mass spectrometry; LPS, lipopolysaccharide; LY379268, (1R,4R,5S,6R)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid; ANOVA, analysis of variance; PLSD, protected least significant difference; DMSO, dimethyl sulfoxide.

pathway, tryptophan-2,3-dioxygenase in the liver and indoleamine-2,3-dioxygenase in all other tissues, open the pyrrole ring of L-tryptophan leading to the synthesis of kynurenine (Wirleitner et al., 2003). Kynurenine is oxidized into 3-hydroxykynurenine by kynurenine-3-monoxygenase or, alternatively, is transaminated into kynurenic acid by three aminotransferase (kynurenine aminotransferase I and II, and mitochondrial aspartate aminotransferase). Type 2 kynurenine aminotransferase is highly expressed in brain tissue (Amori et al., 2009). 3-Hydroxykynurenine is transaminated into xanthurenic acid or, alternatively, is converted into 3-hydroxyanthranilic acid, the metabolic precursor of quinolinic acid (Nishizuka and Hayaishi, 1963). Cinnabarinic acid is a by-product of the kynurenine pathway, which derives from the condensation of two molecules of 3-hydroxyanthranilic acid (Fig. 1). Its biological function is largely unknown (Dykens et al., 1987). Indoleamine-2,3-dioxygenase is induced by proinflammatory cytokines, such as interferon-γ; therefore, the kynurenine pathway is activated in the context of neuroinflammation, as occurs in the ischemic brain or in the brain of patients with HIV infection (for review, see Stone and Darlington, 2002). Patients with AIDS-dementia complex have cerebrospinal fluid concentrations of quinolinic acid 20-fold higher than control subjects (Heyes et al., 1991). Neuroactive metabolites of the kynurenine pathway, such as quinolinic acid, kynurenic acid, and xanthurenic acid, are involved in synaptic signaling and in mechanisms of neuro-

degeneration/neuroprotection (Schwarcz and Pellicciari, 2002; Stone and Darlington, 2002; Gobaille et al., 2008). Quinolinic acid acts as an orthosteric agonist of N-methyl-D-aspartate (NMDA) receptors, whereas kynurenic acid and its synthetic derivatives are competitive antagonists at the co-agonist glycine site of NMDA receptors (Stone and Perkins, 1981; Kessler et al., 1989). Inhibitors of kynurenine-3-monoxygenase have been developed in an attempt to shunt kynurenine metabolism toward the formation of the neuroprotectant kynurenic acid (Carpenedo et al., 1994; Schwarcz and Pellicciari, 2002). There is no evidence so far that any metabolite of the kynurenine pathway interacts with metabotropic glutamate (mGlu) receptors. For example, quinolinic acid has no activity at native mGlu receptors coupled to polyphosphoinositide hydrolysis in brain tissue (Nicoletti et al., 1986b).

Cinnabarinic acid is a potential candidate because it harbors two carboxyl groups and a neutral free amino group, which is the basic requirement for an interaction with the orthosteric site of excitatory amino acid receptors. We report that cinnabarinic acid acts as a weak agonist at both recombinant and native mGlu4 metabotropic glutamate receptors, shares some of the actions of conventional mGlu4 receptor agonists, and binds to the glutamate binding pocket of mGlu4 as revealed by mutagenesis and molecular modeling data. In addition, we could detect endogenous cinnabarinic acid in the
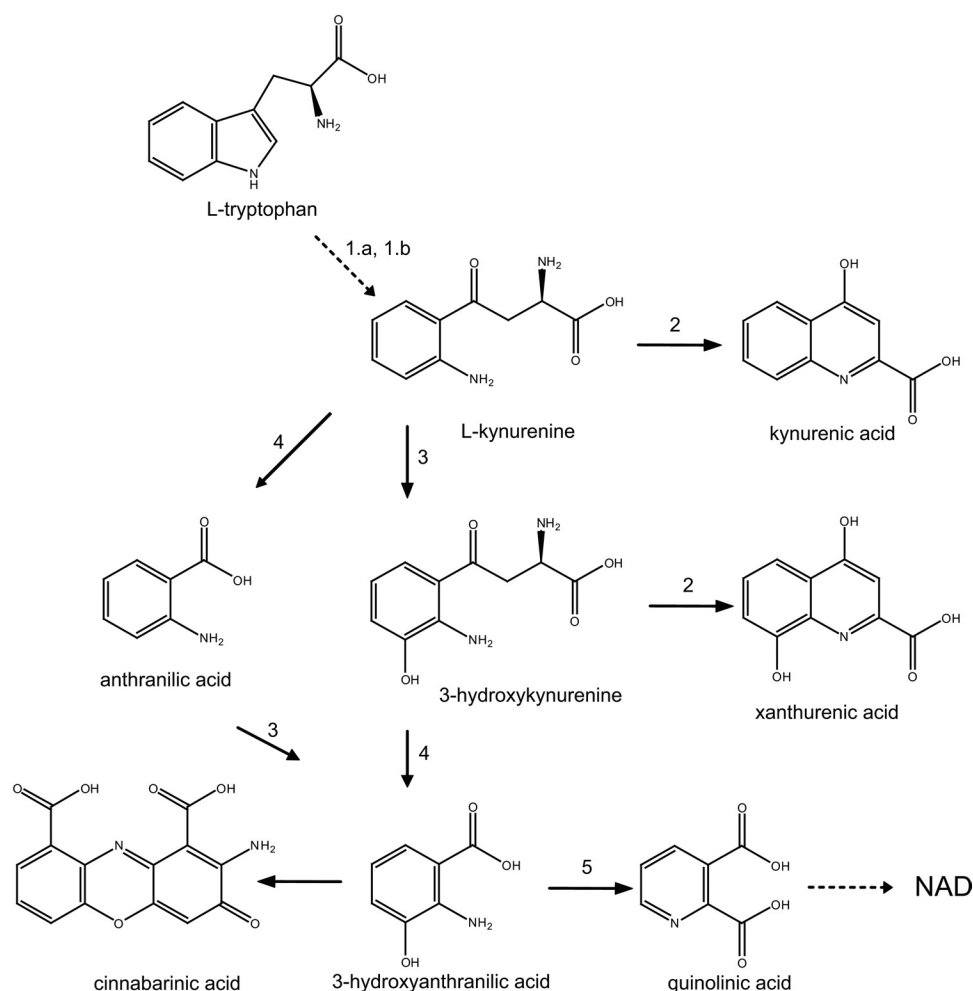

Fig. 1. The kynurenine pathway. Indoleamine-pyrrole 2,3-dioxygenase (1.a) and tryptophan 2,3-dioxygenase (1.b) convert L-tryptophan into N-formyl-kynurenine, which, in turn, is converted into L-kynurenine. L-Kynurenine and 3-hydroxykynurenine are transaminated by kynurenine aminotransferase (2) into kynurenic acid and xanthurenic acid, respectively. Kynurenine monooxygenase (3) transforms L-kynurenine into 3-hydroxykynurenine and anthranilic acid into 3-hydroxyanthranilic acid. Kynureninase (4) transforms L-kynurenine into anthranilic acid and 3-hydroxykynurenine into 3-hydroxyanthranilic acid. Quinolinic acid is formed from 3-hydroxyanthranilic acid by the action of 3-hydroxyanthranilic acid oxygenase (5). In the liver and other organs, the pathway eventually leads to the synthesis of NAD. Cinnabarinic acid is an endogenous oxidation product of 3-hydroxyanthranilic acid.

brain and peripheral organs, particularly under conditions of systemic inflammation.

Materials and Methods

Materials

Forskolin, L-(+)-2-amino-4-phosphonobutyric acid (L-AP4), N-phenyl-7-(hydroxyimino)cyclopropa[b]chromen-1a-carboxamide (PHCCC), (R,S)-4-phosphonophenylglycine (PPG), NMDA, (2S,2′R,3′R)-2-(2′,3′-dicarboxycyclopropyl)glycine (DCG-IV), and (1S,3R,4S)-1-aminocyclopentane-1,3,4-tricarboxylic acid (ACPT-I) were purchased from Tocris Cookson (Bristol, UK) and quisqualic acid was purchased from Anawa Trading SA (Zurich, Switzerland). Tissue culture reagents were from Invitrogen (Milan, Italy). Cytosine β-D-arabinofuranoside and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma-Aldrich (St. Louis, MO). Reagents for the synthesis of cinnabarinic acid were obtained from Fluka (Buchs, Switzerland), Merck KGaA (Darmstadt, Germany) or Sigma-Aldrich. 1-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) was obtained from Sigma. Cinnabarinic acid was also purchased from Santa-Cruz (Heidelberg, Germany).

Synthesis of Cinnabarinic Acid

Manganese (IV) oxide (2.591 g, 25.34 mmol) was added to a solution of 3-hydroxyanthranilic acid (1 g, 6.33 mmol) in methanol (470 ml) under magnetic stirring at room temperature. Products of the reaction were monitored by thin-layer chromatography (mobile phase, 20% dichloromethane/methanol for 3-hydroxyanthranilic acid; butanol-glacial acetic acid-H$_2$O 4:1:2 for cinnabarinic acid). After 24 h, 3-hydroxyanthranilic was no longer detectable. Triethylamine (1.942 g, 19 mmol) was added, and the mixture was stirred for a further 10 min. The reaction mixture was filtered on a Buchner funnel with Celite 521 pad (Sigma-Aldrich) and washed several times with methanol. The methanol solution was concentrated under reduced pressure, diluted with water (20 ml), and then acidified with 2 N HCl (hydrochloric acid) up to pH ~1 to 2. The dark-red solid formed was filtered on Buchner and washed with water (three times, 5 ml) and then with ether (twice, 20 ml). The dark-red solid was dried on P$_2$O$_5$ (phosphoric anhydride) under vacuum, obtaining 670 mg (yield 70%) of cinnabarinic acid, identified by NMR spectroscopy as follows: ^{1}H-NMR (400 MHz, DMSO-d_6, parts per million): δ = 6.60 (s, 1H, H-4), 7.60 (m, 1H, H-7), 7.76 (d, J = 1.2, 8.3 Hz, 1H, H-6), 7.95 (d, J = 1.2, 7.7 Hz, 1H, H-8), 8.76 (bs, 1H, COOH), and 9.72 (bs, 1H, COOH). ^{13}C-NMR (100 MHz, DMSO-d_6, parts per million): δ = 93.5, 105.7, 120.9, 126.9, 128.6, 129.5, 129.8, 143.1, 148.3, 151.2, 153.3, 167.0, 169.8, and 178.8.

Cell Transfection and Functional Assay Protocols

Human embryonic kidney (HEK) 293 cells were transiently transfected with rat clones of mGlu1, mGlu2, mGlu4, mGlu5, mGlu6, mGlu7, and mGlu8 receptors by electroporation. To avoid the influence of extracellular glutamate, the excitatory amino acid carrier 1 (a high-affinity glutamate transporter) was cotransfected with the receptor. Because group II (mGlu2, mGlu3) and group III (mGlu4, mGlu6, mGlu7, mGlu8), mGlu receptor subtypes are negatively coupled to adenylyl-cyclase through G$_i$/G$_o$ proteins, these receptors were cotransfected with a chimeric G$_q$/G$_i$-protein. This modified Gq-protein is recognized by these receptors but couples to the phospholipase-C pathway and allows the monitoring of receptor activity by measurements of inositol phospholipid hydrolysis.

Construction of the N-terminal truncated mutant of mGlu4 receptor (ΔV-mGlu4) was performed as reported by Goudet et al. (2004). In brief, ΔV-mGlu4 was generated by inserting between the MluI and XbaI sites of pRK5-NHA, the sequence between the residues Gln514 and Ala885 of the rat mGlu4 cDNA obtained by PCR. The different mutants of mGlu4 binding pocket were obtained using the QuikChange strategy (Agilent Technologies, Santa Clara, CA). All mutations were verified by sequencing.

[^{3}H]Inositol phosphate ([^{3}H]InsP) accumulation experiments were performed in 96-well microplates, as described previously (Goudet et al., 2004). In brief, transfected cells were incubated overnight with [myo-^{3}H]inositol (16 Ci/mmol; GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK). The following day, after medium removal, ambient glutamate was degraded by incubation with alanine aminotransferase in the presence of pyruvic acid (Goudet et al., 2004). Then, cells were stimulated with cinnabarinic acid or with subtype-selective mGlu receptor agonists for 30 min in the presence of 10 mM LiCl. Then, total [^{3}H]InsP accumulated during receptor stimulation was recovered by anion exchange chromatography as described previously (Goudet et al., 2004). Radioactivity was counted using a Wallac 1450 Microbeta stintillation and luminescence counter (PerkinElmer Life and Analytical Sciences, Courtaboeuf, France). Results are expressed as the ratio between [^{3}H]InsP and total radioactivity in each sample. All points are from triplicates.

Cinnabarinic acid was applied at 100 μM, quisqualic acid was used as a selective group I (mGlu1 and -5) agonist (EC$_{20}$, 10 nM; EC$_{80}$, 100 nM). DCG-IV was used as a selective group II (mGlu2 and -3) agonist (EC$_{20}$, 10 nM; EC$_{80}$, 500 nM). L-AP4 was used as a selective group III (mGlu4, -6, -7, and -8) agonist (for mGlu4: EC$_{20}$, 30 nM; EC$_{80}$, 300 nM; for mGlu6: EC$_{20}$, 1 μM; EC$_{80}$, 10 μM; for mGlu7: EC$_{20}$, 10 μM; EC$_{80}$, 300 μM; and for mGlu8: EC$_{20}$, 100 nM; EC$_{80}$, 1 μM).

Molecular Modeling: Docking of Cinnabarinic Acid in mGlu4 Amino-Terminal Domain

All calculations were performed in Discovery Studio 2.5.5 (Accelrys Software Inc., San Diego, CA). Cinnabarinic acid was docked in a homology model of mGlu4R amino terminal domain previously validated (Selvam et al., 2010). The ligand was initially positioned in the binding site using GOLD version 4.1 (http://www.ccdc.cam.ac.uk/products/life_sciences/gold/). GOLD uses a genetic algorithm for docking flexible ligands into receptor binding sites. Protein-ligand interactions were further optimized by 1-ns molecular dynamics using CHARMm. Once the trajectory was equilibrated, snapshots of the trajectory were analyzed in terms of protein-ligand contacts and the selected ones were submitted to energy minimization leading to the model presented in this article.

Studies in Primary Neuronal Cultures

Primary cultures of cerebellar granule cells or mixed cultures of cortical cells were prepared from wild-type or mGlu4 receptor knockout mice. Hybrid homozygous mGlu4 knockout mice (strain name, STOCK Gprc1d^{tm1Hpn}; stock number 003576) were obtained from The Jackson Laboratory (Bar Harbor, ME). 129Sv and CD1 mice (Charles River, Calco, Italy) were backcrossed to obtain wild-type controls (Battaglia et al., 2006). Animals were genotyped for the mGlu4 receptor gene by multiple primer polymerase chain reaction (Pekhletski and Hampson, 1996).

Preparation of Cultured Cerebellar Granule Cells

Primary cultures of mouse cerebellar granule cells were prepared using the same procedure described for rat granule cells (Nicoletti et al., 1986b). Cells obtained from the cerebella of 8-day old mice were suspended in basal Eagle's medium supplemented with 10% fetal calf serum, 25 mM KCl, 2 mM glutamine, and 100 μg/ml gentamicin and then plated (2.5 $\times$ 10^6 cells/dish) onto 35-mm Nunc dishes (Thermo Fisher Scientific, Waltham, MA) coated with poly-L-lysine (10 μg/ml). Cultures were incubated at 37°C in a humidified atmosphere of 5% CO$_2$ and 95% air. Ara-C (10 μM) was added after 16 to 18 h of culture to inhibit the replication of non-neuronal cells. Cultures were used at 6 to 8 days in vitro (DIV).

Immunoblot Analysis of mGlu4 Receptors in Cultured Cerebellar Granule Cells

We examined the expression of mGlu4 receptors in protein extracts obtained from cultured cerebellar granule cells prepared from

wild-type or mGlu4 receptor knockout mice. Cells were harvested and homogenized at 4°C in 50 mM Tris-HCl buffer, pH 7.4, containing 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml aprotinin, 1 μg/ml pepstatin, and 1 μg/ml leupeptin. After sonication, 3 μl of total extracts were used for protein determinations. One hundred milligrams of protein extracts were resuspended in SDS-bromphenol blue reducing buffer with 40 mM dithiothreitol. Western blot analysis was carried out using 8% SDS polyacrylamide gels electroblotted onto polyvinylidene difluoride membrane (Bio-Rad, Milan, Italy) for 1 h. Filters were blocked for 1 h in TBS-T buffer (100 mM Tris-HCl, 0.9% NaCl, and 0.1% Tween 20, pH 7.4) containing 5% nonfat dry milk. Blots were then incubated overnight at 4°C with a specific rabbit polyclonal anti-mGlu4 receptor antibody (Corti et al., 2007) (1:100) or mouse monoclonal anti-β-actin antibody (1:100,000; Sigma, St. Louis, MO), washed with TBS-T buffer, and then incubated for 1 h with secondary antibodies [peroxidase-coupled anti-rabbit or anti-mouse (GE Healthcare), diluted 1:10,000 or 1:5000 with TBS-T, respectively]. Immunoreactivity was revealed by enhanced chemiluminescence.

Measurement of cAMP Formation in Cultured Cerebellar Granule Cells

Cultures were washed twice with Locke's solution (154 mM NaCl, 5.6 mM glucose, 5 mM HEPES, 1.3 mM CaCl$_2$, and 3.6 mM NaHCO$_3$, pH 7.4) and preincubated with 0.5 mM IBMX for 20 min to inhibit cAMP metabolism by phosphodiesterases. Cultures were then challenged with forskolin (10 μM) for 20 min. mGlu4 receptor drugs or cinnabarinic acid were added 1 min before forskolin. Incubations were stopped by addition of 0.4 N perchloric acid. Cells were scraped from the dishes, sonicated, and centrifuged with a microfuge at maximal speed. The supernatants were stored at -20°C. On the day of the assay, the perchloric acid contained in the samples was neutralized with K$_2$CO$_3$, and cAMP levels were measured by radioimmunoassay using a commercial kit (PerkinElmer Life and Analytical Sciences, Milan, Italy).

Preparation of Mixed Cortical Cultures

Mixed cortical cultures containing both neurons and astrocytes were prepared from fetal wild-type or mGlu4 receptor knockout mice at 14 to 16 days of gestation, as described by Rose et al. (1992). In brief, dissociated cortical cells were plated in 15-mm multiwell vessels (Primaria; BD Falcon, Lincoln Park, NY) on a layer of confluent astrocytes, using a plating medium of minimal essential medium Eagle's salts supplemented with heat-inactivated horse serum (5%) and fetal calf serum (5%), glutamine (2 mM), glucose (21 mM), and NaHCO$_3$ (25 mM). After 3 to 5 DIV, non-neuronal cell division was halted by a 1- to 3-day exposure to Ara-C (10 μM), and cultures were shifted to a maintenance medium identical to plating medium but lacking fetal bovine serum. Subsequent partial medium replacement was performed twice a week. Cultures were used at 13 to 14 DIV.

In Vitro Exposure to Excitatory Amino Acids and Assessment of Neuronal Injury

For induction of excitotoxic death, mixed cultures were exposed to NMDA for 10 min at room temperature in a HEPES-buffered salt solution containing 120 mM NaCl, 5.4 mM KCl, 0.8 mM MgCl$_2$, 1.8 mM CaCl$_2$, 20 mM HEPES, and 15 mM glucose. Afterward, the cultures were extensively washed and incubated in minimal essential medium Eagle's (supplemented with 25 mM NaHCO$_3$ and 21 mM glucose) at 37°C. The cells were either exposed to different concentrations of NMDA for 10 min or exposed to 100 μM NMDA in the absence or presence of cinnabarinic acid (1–300 μM) and the nonselective mGlu4 receptor agonists L-AP4 (100 μM) or (R,S)-PPG (100 μM) and the mGlu4 enhancer PHCCC (30 μM). Neuronal injury was assessed by measuring the levels of lactate dehydrogenase (LDH) into the extracellular medium by using a commercially available kit (Roche Laboratories, Basel, Switzerland).

Induction of MPTP Neurotoxicity in Mice

We used the experimental animal model of parkinsonism induced by MPTP to examine whether cinnabarinic acid shared the protective activity of PHCCC (Battaglia et al., 2006). Mice were unilaterally infused in the external globus pallidus with 0.5 μl of vehicle (saline containing 50% DMSO), cinnabarinic acid (50 nmol), or PHCCC (20 nmol) by a guide cannula (Bilaney, Düsseldorf, Germany). Guide cannulas were implanted under ketamine (100 mg/kg) + xylazine (10 mg/kg) intraperitoneal anesthesia, in a David Kopf Instruments (Tujunga, CA) stereotaxic frame. The site of implantation was the left external globus pallidus (coordinates: 0.5 mm posterior to the bregma; 1.9 mm lateral to the midline, 2.8 mm ventral from the surface of skull according to the atlas of Franklin and Paxinos, 1998). After surgery, mice were housed in separate cages and allowed to recover for 3 days. On the day of the experiment, an internal cannula extending 1 mm out of the guide cannula was inserted, after removing a dummy, and injections were carried out at a flow rate of 0.25 μl/min using a microinjection pump (Bioanalytical System, West Lafayette, IN). Thirty minutes later, animals were treated with a single injection of 36 mg/kg MPTP i.p. (corresponding to 30 mg/kg free MPTP) and killed 7 days later for the assessment of nigrostriatal damage.

Measurements of Striatal Dopamine Levels

The corpus striatum was homogenized by sonication in 0.6 ml of ice-cold 0.1 M perchloric acid. Fifty microliters of the homogenate were used for protein determination (Lowry et al., 1951). The remaining aliquot was centrifuged at 8000g for 10 min, and 20 μl of the supernatant was injected into an HPLC equipped with an Autosampler 507 (Beckman Coulter, Fullerton, CA), a programmable solvent module 126 (Beckman Coulter), an analytical C-18 reverse-phase column kept at 30°C (Ultrasphere ODS 5 mm, 80 Å pore, 250 × 4.6 mm; Beckman Coulter), and a Coulochem II electrochemical detector (ESA, Chelmsford, MA).The holding potentials were set at +350 and −350 mV for DA detection. The mobile phase consisted of 80 mM sodium phosphate, 40 mM citric acid, 0.4 mM EDTA, 3 mM 1-heptanesulfonic acid, and 10% methanol, brought to pH 2.75 with phosphoric acid (run under isocratic conditions, at 1 ml/min).

Immunohistochemical Analysis

Mice ($n = 5$ for each experimental group) were killed by decapitation. Brains were dissected out and immediately fixed in a solution composed of ethyl alcohol (60%), acetic acid (10%), and chloroform (30%). Twenty hours later, brains were placed in 70% ethanol until they were included in paraffin. Twenty-micrometer sections were cut and used for stereological count analysis. Sections were soaked in 3% hydrogen peroxide to block endogenous peroxidase activity. Tissue sections were incubated overnight with anti-tyrosine hydroxylase (TH) mouse monoclonal antibodies (1:200; Sigma-Aldrich, Milano, Italy), and then for 1 h with secondary biotin-coupled anti-mouse antibodies (1:200; Vector Laboratories, Burlingame, CA). Control staining was performed without the primary antibodies. The immunoreaction was performed with 3,3-diaminobenzidine tetrachloride (ABC Elite kit; Vector Laboratories). The number of TH$^+$ cells within the substantia nigra pars compacta was assessed by stereological cell counting.

Stereological Count of TH$^+$ Cells

The number of TH-positive cells in the pars compacta of the substantia nigra was obtained by stereological technique and the optical fractionator, using a a Zeiss Axio Imager.M1 microscope equipped with a motorized stage and focus control system and a digital video camera. The software Image-Pro Plus Windows 6.2 (Media Cybernetics, Inc., Bethesda, MD) was used to control the microscope and to analyze digital images. The analysis was performed on 10 sections (20 μm) sampled every 160 μm in a rostrocaudal extension. In each stained section, the area was identified and

outlined (magnification, 2.5×). Within each delineated region, neurons were counted (magnification, 100×) according to the optical dissector method counting several boxes (250 $\mu m^2 \times 2$ μm) (Mayhew and Gundersen, 1996; Gundersen et al., 1999). The total number of TH-immunoreactive neurons per each rostrocaudal level was computed from the formula $N = \Sigma(n) \times 1/SSF \times 1/ASF \times 1/TSF$, where N is the total number of neurons counted on each dissector, SSF (fraction of sections sampled) is the number of regularly spaced sections used for counts divided by the total number of sections through the substantia nigra pars compacta (= 1/15); ASF (area sampling frequency) is the dissector area divided by the area between dissectors [= (2500 $\mu m^2 \times$ dissectors number)/region area], and TSF (thickness sampling frequency) is the dissector thickness divided by the section thickness (= 2 $\mu m/10$ μm). The total number of TH-immunoreactive neurons in the substantia nigra pars compacta is the sum of the total number of TH-immunoreactive neurons per each rostrocaudal level: $Ntot = \Sigma(Ni)$.

Microdialysis in Freely Moving Animals

Male Sprague-Dawley rats weighing 250 to 275 g were implanted stereotaxically into the left corpus striatum (0.7 mm anterior to bregma, 2.5 mm lateral to the midline, and 3.5 mm ventral) with microdialysis intracerebral guides, under pentobarbital anesthesia. After surgery, rats were allowed to recover for 5 days before the experiment. Twelve hours before the experiment, a concentric vertical probe (2 mm long and 0.5 mm o.d. having a polycarbonate membrane; molecular cut-off, 20 kDa; CMA/12, CMA/Microdialysis, Stockholm, Sweden) was inserted into the intracerebral guide cannula, and rats were transferred to a plastic bowl cage with a moving arm with free access to water and food. The probe was perfused continuously with artificial cerebrospinal fluid at a flow rate of 1.5 $\mu l/min$, using a microinjection pump. Artificial cerebrospinal fluid contained 150 mM NaCl, 3 mM KCl, 1.7 mM $CaCl_2$, and 0.9 mM $MgCl_2$. On the following day, 30 μl (20 min) of consecutive perfusate sample fractions were continuously collected by a fraction collector. After three sample fractions, used to monitor basal levels of cinnabarinic acid, veratridine (100 μM) or KCl (100 mM) was perfused through the probe for 20 min. Sample fractions of perfusate were collected for the next 2 h. In another set of experiments, rats with implanted guide cannulas were injected with LPS (0.5 mg/kg i.p.; dissolved in saline) 24 or 48 h before microdialysis experiments carried out as above. Cinnabarinic acid in the perfusate was analyzed as described below.

Cerebrospinal Fluid of Mice with Experimental Autoimmune Encephalomyelitis

Animals. C57BL/6 male mice (18–20 g body weight), 6 to 7 week old, were purchased from Charles River (Calco, Italy). Mice were kept under environmentally controlled conditions (ambient temperature, 22°C; humidity, 40%) on a 12 h light/dark cycle with food and water ad libitum. Experiments were performed following the Guidelines for Animal Care and Use promulgated by the National Institutes of Health (Institute of Laboratory Animal Resources, 1996).

Induction of Experimental Autoimmune Encephalomyelitis. Experimental autoimmune encephalomyelitis (EAE) was induced in C57BL/6 mice (6–7 weeks old) by immunization with 200 μg of MOG_{35-55} peptide s.c. emulsified in 0.1 ml of incomplete Freund's adjuvant containing 2 mg of *Mycobacterium tuberculosis*. After immunization, 200 ng of pertussis toxin in 200 μl of phosphate-buffered saline was injected i.p. on the day of immunization and 2 days later. Mice were monitored daily, and neurological impairment was scored according to the following scale: 0, no symptoms; 1, limp tail; 2, partial paralysis of hind limbs; 3, complete paralysis of hind limbs or partial hind and front limb paralysis; 4, tetraparalysis; and 5, moribund/death. In addition, body weight was monitored every day during the development of EAE. All experimental procedures were authorized by the ethical committee according to the Italian legislation

for research in animal science. Accordingly, moribund animals were euthanized by CO_2 inhalation.

Sampling of Cerebrospinal Fluid. Mice immunized with MOG_{35-55} and control mice were anesthetized with ketamine (100 mg/kg i.p.) and xylazine (10 mg/kg i.p.). During the time of anesthesia induction, mice were kept in a 37°C incubator. In brief, the skin of the neck was shaved, and the mouse was then placed prone on the stereotaxic instrument with direct contact of a heating pad. The surgical site was swabbed with 10% povidone iodine followed by 70% ethanol, and a sagittal incision of the skin was made inferior to the occiput. Under the dissection microscope, the subcutaneous tissue and muscles were separated by blunt dissection with forceps. A pair of microretractors was used to hold the muscles apart. Under the dissection microscope, the dura mater of the cisterna magna appeared as a glistening and clear reverse triangle through which the medulla oblongata and the cerebrospinal fluid (CSF) space were visible. CSF was collected from the cisterna magna through a puncture of the atlanto-occipital membrane with a 26-gauge needle (CSF visibly contaminated by red blood cells was discarded). Uncontaminated CSF was centrifuged to ensure removal of red blood cells and used for the detection of cinnabarinic acid.

Detection of Cinnabarinic Acid in Tissue Extracts by High Performance Liquid Chromatography/Tandem Mass Spectrometry

Sample Preparation. Tissue extracts were prepared from male Sprague-Dawley rats (200–240 g) injected with either sterile saline or lipopolysaccharide (0.5 mg/kg i.p.; dissolved in saline). Animals were killed by decapitation 24 h after the injection; the brains, kidneys, lungs, livers, and spleens were rapidly removed, and tissue (150–200 mg) was sonicated in 4% trichloroacetic acid. Homogenates were centrifuged at 2200g for 30 min, and the resulting pellets were added to 300 μl of acetonitrile. After extensive vortexing (60 s), samples were centrifuged at 14,000 rpm for 5 min, and 200 μl of supernatant were transferred to clean vials and dried by vacuum centrifugation. The pellets were suspended by vortex in 150 μl of 2.5% aqueous formic acid and transferred to an autosampler for injection into the HPLC. Samples of dialysate and CSF were diluted with 2.5% aqueous formic acid (1:1) and transferred to the autosampler.

HPLC-MS/MS Analysis. For the detection and quantification of cinnabarinic acid in tissue, we used an analytical strategy based on HPLC-MS/MS technology using a multiple reaction monitoring method. The HPLC analysis was performed using a liquid chromatography system (series 1100; Agilent Technologies), which included a binary pump, an autosampler, a solvent degasser, and a column oven. Chromatographic separation was performed on a reversed-phase column (50 × 2.0 mm; Luna C18, 5 μm, 100-Å pore size; Phenomenex, Torrance, CA), equipped with a security guard precolumn (Phenomenex), containing the same packing material. The column was maintained at room temperature. The mobile phase consisted of a solution of 0.1% aqueous formic acid (eluent A) and 100% acetonitrile (eluent B); elution was performed at flow rate of 300 $\mu l/min$, using 10% solvent B for 1 min, 100% solvent B for 3 min, and afterward re-equilibrating with 90% solvent A for 6 min. The injection volume was 100 μl, and the total analysis run time was 10 min.

The mass spectrometry was performed on a 3200 triple quadrupole system (Applied Biosystems, Foster City, CA), equipped with a Turbo Ion Spray source. The detector was set in the positive ion mode. The ion spray voltage was set at 5000 V and the source temperature was 300°C. The collision activation dissociation gas was set at medium value, and nitrogen was used as collision gas. The Q1 and Q3 quadrupoles were tuned for the unit mass resolution. The transitions of the precursor ions to the product ions were monitored with a dwell time of 100 ms for each analyte. The instrument was set in the multiple reaction monitoring mode, monitoring the transitions m/z 301.2 > 264.7, 301.2 > 237.4, and 301.2 > 209.4.

Mass spectrometer parameters were optimized to maximize sensitivity for all transitions (see Fig. 8). Data were acquired and processed by Analyst 1.4.2 software. A calibration curve was established using different concentrations of cinnabarinic acid (10, 35, 100, and 150 ng/ml) dissolved in acetonitrile and processed in the same way as tissue samples. To rule out ion suppression events, the experimental set was repeated adding at each homogenized sample 20 pg of cinnabarinic acid. In each sample, an increment of $20 + 6$ pg in the amount of the calculated cinnabarinic acid was detected. Commercial cinnabarinic acid and the cinnabarinic acid we have synthesized and used in most of experiments (except in MPTP-treated mice) had the same retention time at the HPLC-MS/MS.

Results

Cinnabarinic Acid Selectively Activates Heterologously Expressed mGlu4 Receptors. We examined the potential interaction of cinnabarinic acid with mGlu receptors using HEK293 cells transiently transfected with rat mGlu1, -2, -4, -5, -6, -7, or -8 receptors. Cells expressing mGlu2, -4, -6, -7, and -8 were cotransfected with a chimeric G-protein α subunit $G\alpha_{qi}$, which allows receptor coupling to polyphosphoinositide hydrolysis. At concentrations of 100 μM, cinnabarinic acid did not activate mGlu1, mGlu2, mGlu5, mGlu6, mGlu7, and mGlu8 receptors, as shown by measurements of [^{3}H]InsP formation. In contrast, cinnabarinic acid acted as a partial agonist of mGlu4 receptors by increasing [^{3}H]InsP formation by approximately 35%. In the same set of experiments, the full agonist, $(1S,3R,4S)$-1-aminocyclopentane-1,3,4-tricarboxylic acid (ACPT-I), increased [^{3}H]InsP formation by 170% in cells expressing mGlu4 receptors. This means that at 100 μM cinnabarinic acid was 5-fold less efficacious than ACPT-I in activating mGlu4 receptors (Fig. 2A). Concentration-response curves with cinnabarinic acid, 3-hydroxyanthranilic acid on mGlu4 receptor-expressing cells are shown in Fig. 2B, where data are expressed as percentage of the maximal response to ACPT-I. Cinnabarinic acid showed a low potency in activating mGlu4 receptors, displaying a detectable agonist activity at concentrations >100 μM. 3-Hydroxyanthranilic acid was inactive at mGlu4 receptors (Fig. 2B).

We also combined cinnabarinic acid (30 or 100 μM) with the mGlu1/5 receptor agonist quisqualate, the mGlu2/3 receptor agonist DCG-IV, or the mGlu4/6/7/8 receptor agonist, L-AP4 at their EC$_{20}$ and EC$_{80}$ values for the respective mGlu receptor subtypes. Cinnabarinic acid did not affect the action of quisqualic acid, DCG-IV, or L-AP4, excluding any possible action of cinnabarinic acid as antagonist or positive allosteric modulator at any mGlu receptor subtype (not shown).

Cinnabarinic Acid Binds within the Glutamate Binding Pocket. To investigate the site of action of cinnabarinic acid, we first tested it on a truncated mGlu4 receptor deleted from its N-terminal Venus-flytrap domain (ΔV-mGlu4). Allosteric modulators of mGlu receptors bind to the receptor transmembrane domain, and we have previously shown that, in the absence of the N-terminal domain, positive allosteric modulators directly activate mGlu receptors, behaving similarly to orthosteric agonists (Goudet et al., 2004; Chen et al., 2007; Chen et al., 2008). Thus, if the agonist activity of cinnabarinic acid was due to an action in the transmembrane domain of mGlu4, this compound should have retained its activity in cells expressing ΔV-mGlu4. Concentration-response curves with PHCCC, cinnabarinic
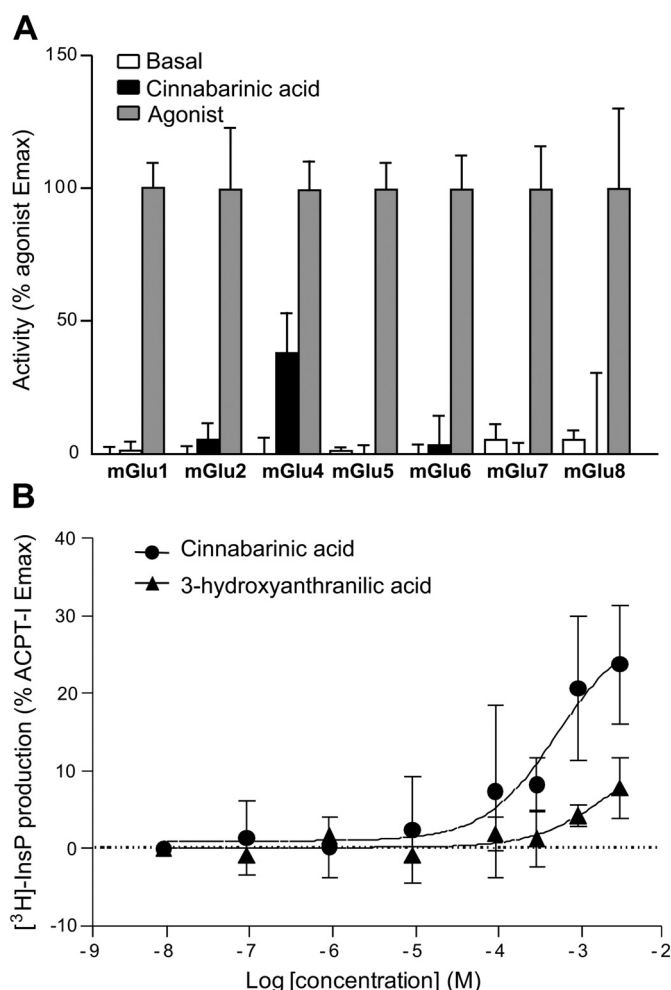

Fig. 2. Cinnabarinic acid acts as a partial agonist of mGlu4 receptors in transfected HEK293 cells. A, action of 100 μM cinnabarinic acid on HEK293 cells expressing mGlu1, -2, -4, -5, -6, -7, and -8 receptors. The action of cinnabarinic acid was compared with the following prototypical receptor agonists applied at their EC$_{80}$ concentrations (in parenthesis): quisqualate (100 nM) for mGlu1 and mGlu5 receptors; DCG-IV (500 nM) for mGlu2 receptors; and L-AP4 for mGlu4 (300 nM), mGlu6 (10 μM), mGlu7 (300 μM), and mGlu8 (1 μM) receptors. Data ([^{3}H]InsP production) are expressed as percentage of the activity displayed by the prototypical agonists. B, concentration-response curves of cinnabarinic acid and 3-hydroxyanthranilic acid at HEK293 cells expressing mGlu4 receptors; data (means + S.E.M.) are expressed as percentage of the maximal stimulation produced by the full agonist ACPT-I. Values (means + S.E.M.) are expressed as percentage of the maximal stimulation of [^{3}H]InsP production produced by PHCCC.

acid, and 3-hydroxyanthranilic acid on ΔV-mGlu4-expressing cells are shown in Fig. 3A, where data are expressed as a percentage of the maximal response to PHCCC. As expected, the mGlu4 receptor enhancer, PHCCC, activated ΔV-mGlu4 receptor in a concentration-dependent manner, whereas cinnabarinic acid and 3-hydroxyanthranilic acid were inactive (Fig. 3A). This indicates that cinnabarinic acid does not interact with the transmembrane domain of mGlu4receptor.

We then used site directed mutagenesis to further investigate whether cinnabarinic acid could bind within the glutamate binding pocket or not. To this purpose, we mutated several residues of the orthosteric binding pocket of mGlu4 that are involved in the binding and activity of glutamate and other agonists, such as L-AP4.

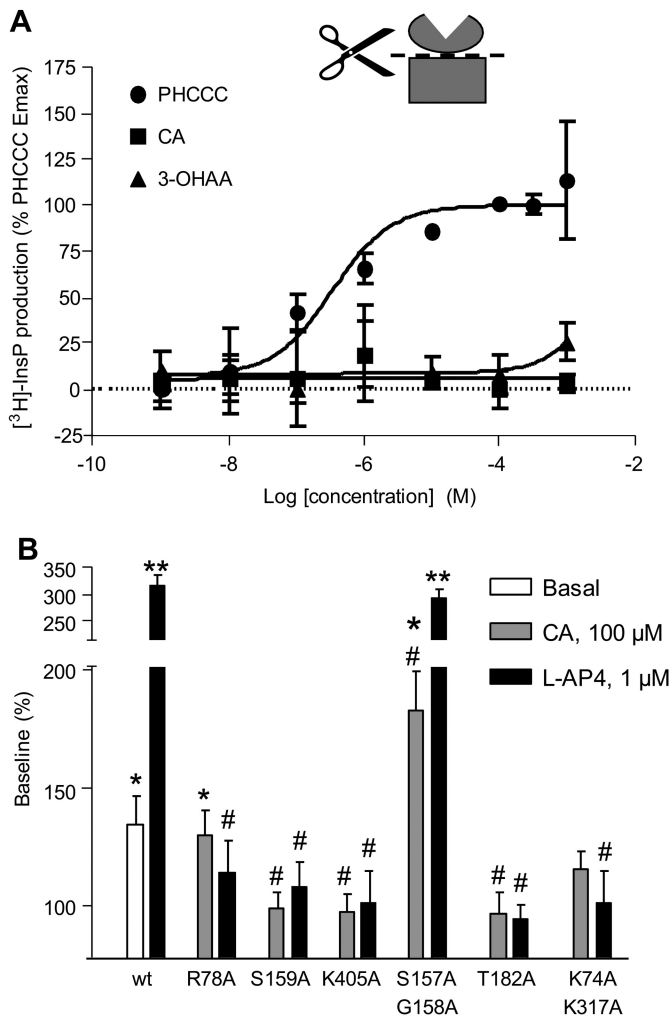Ser159 and Thr182 are involved in the binding of the α

Fig. 3. Cinnabarinic acid activity requires the extracellular domain of mGlu4 and is altered by mutations of residues of the glutamate binding pocket. A, data obtained with PHCCC, cinnabarinic acid (CA), and 3-hydroxyanthranilic acid (3-HAA) on HEK293 cells expressing a truncated mGlu4 receptor lacking the extracellular N-terminal domain. B, action of 100 μM cinnabarinic acid and 10 μM L-AP4 on HEK293 cells expressing mGlu4 wild-type or mutant receptors. Activity was assessed by determination of [^{3}H]InsP production. Data (mean ± S.E.M) are expressed as percentage of the basal activity of n = 5 experiments at least.

amino acid moiety of glutamate and L-AP4 (Hampson et al., 1999). The substitution of one of these amino acids by an Ala abolished the activity of 10 μM L-AP4 as well as the activity of 100 μM cinnabarinic acid, despite the absence of α amino acid moiety in this molecule (Fig. 3B). A cluster of four basic residues, Lys74, Arg78, Lys317, and Lys405, is involved in the binding of the distal carboxylate or phosphonate group of glutamate and L-AP4, respectively (Hampson et al., 1999; Rosemond et al., 2002). The substitution of Lys405 by an alanine abolished the stimulation of mGlu4 by cinnabarinic acid. The activity of cinnabarinic acid at 100 μM is also decreased by 50% (n = 8) in mGlu4 K74A + K317A but remains almost unchanged in mGlu4 R78A compared with the wild-type mGlu4 (Fig. 3B). Although the glutamate binding pocket is well conserved among group III mGlu receptors, two residues differ between mGlu4 and mGlu8 receptors. Ser157 and Gly158 in mGlu4 are replaced by two Ala in mGlu8 receptor. They have been shown to be responsible for mGlu4 versus mGlu8 selectivity of FP427 (Frauli et al.,

2007). The activity of cinnabarinic acid is increased in the double-mutant mGlu4 S157A + G158A (Fig. 3B), suggesting that these two residues are not the only factors involved in the mGlu4/mGlu8 selectivity of cinnabarinic acid.

Taken together, these data confirmed that cinnabarinic acid is a weak agonist of mGlu4 receptor that acts at the orthosteric binding site. Some, but not all, critical residues involved in glutamate or L-AP4 binding are also important for cinnabarinic acid activity.

Docking of Cinnabarinic Acid in the Glutamate Binding Pocket Using Molecular Modeling. Mutagenesis data confirmed that cinnabarinic acid binds to the orthosteric site of mGlu4 receptor. Cinnabarinic acid was thus docked in the mGlu4 receptor closed extracellular domain homology model that we described previously (Selvam et al., 2010). The molecule fits nicely in the cleft between the two lobes close to the hinge (Fig. 4A).

In previous articles (Bertrand et al., 2002; Selvam et al., 2010), we divided the agonist binding pocket of mGlu receptor into a proximal binding pocket and a distal binding pocket. The former binds to the α-amino acid moiety of glutamate analogs and the latter to the acidic groups of the ligand side chain. In cinnabarinic acid no α-amino acid group is present however residues of the proximal pocket are found to interact with cinnabarinic acid. It is noteworthy that Ser159 and Thr182 make hydrogen bonds with the oxygens and Asp312 with the amino group of the 2-amino 3-oxophenoxazine (Fig. 4, B and C). Five distal basic residues (Lys71, Lys74, Arg78, Lys317, and Lys405) interact with the two carboxylic functions of cinnabarinic acid. In addition, Ser110 may also interact. Gly158 protons are positioned above the planar conjugated structure of cinnabarinic acid and make CH-π interactions. Tyr230 makes the same type of interaction with H4 of cinnabarinic acid. However, the contact between cinnabarinic acid and that Tyr230 may not allow an optimal closing of the bilobate structure and may explain the partial activity of cinnabarinic acid at mGlu4 receptors. We have previously shown the critical role of interactions between agonists/antagonists and Tyr230 (Bessis et al., 2002).

Cinnabarinic Acid Inhibits cAMP Formation in Cultured Cerebellar Granule Cells. We extended the study to cultured cerebellar granule cells, which are known to express mGlu4 receptors (Santi et al., 1994). Cultured mouse cerebellar granule cells at 8 to 9 DIV were challenged with cinnabarinic acid, and activation of native mGlu4 receptors was assessed by measuring the inhibition of forskolin-stimulated cAMP formation (mGlu4 receptors are coupled to G_i/G_o proteins). Cinnabarinic acid reduced cAMP formation in a concentration-dependent manner with a potency and efficacy greater than that observed in heterologous expression systems. Cinnabarinic acid was effective at 30 μM, and substantially inhibited cAMP formation (by approximately 80%) at 100 μM (Fig. 5A). PHCCC, which behaves as a positive allosteric modulator of mGlu4 receptors (Maj et al., 2003), also reduced cAMP formation in cultured granule cells (Fig. 5A), which are known to release glutamate (Aronica et al., 1993).

To dissect the specific component mediated by the activation of native mGlu4 receptors, we also examined the action of cinnabarinic acid and PHCCC in cultured cerebellar granule cells prepared from mGlu4 receptor knockout mice. The
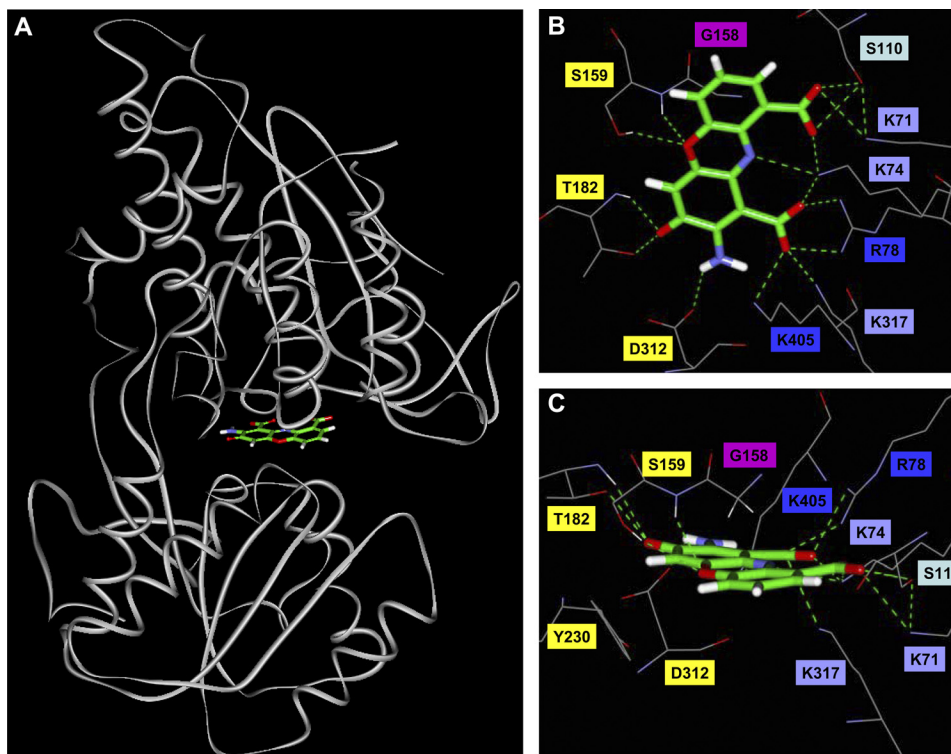
Fig. 4. Homology model of mGlu4 with cinnabarinic acid docked at the glutamate binding site. A, cinnabarinic acid binds in the cleft between the two lobes close to the hinge of the receptor extracellular domain. Expanded view of cinnabarinic acid binding site top (B) and side view (C). The agonist binding pocket of mGlu receptor is divided into a proximal binding pocket and a distal binding pocket. The former binds to the α-amino acid moiety of glutamate analogs and the latter to the acidic groups of the ligand side chain. Proximal binding residues are boxed in yellow, distal basic residues in blue (dark blue for the two conserved residues across the mGlu receptors), Gly158 in magenta (this residue varies in mGlu4/mGlu6/mGlu8), and Ser110 in light blue.

lack of mGlu4 receptors in cultures from knockout mice was confirmed by immunoblotting (Fig. 5B). As expected, PHCCC (30 μM) inhibited forskolin-stimulated cAMP formation in cultures from wild-type mice but was completely inactive in cultures from mGlu4 receptor knockout mice (Fig. 5C). At 30 μM, cinnabarinic acid reduced forskolin-stimulated cAMP formation to a greater extent in cultures from wild-type mice than in cultures from mGlu4 receptor knockout mice. In contrast, higher concentrations of cinnabarinic acid (100 μM) were equally effective in the two cultures (Fig. 5C). Thus, high concentrations of cinnabarinic acid inhibited cAMP formation by recruiting additional unknown mechanisms that can explain the difference in the efficacy between recombinant and native mGlu4 receptors.

Cinnabarinic Acid Shares the Action of mGlu4 Receptor Agonists in Protecting Cultured Cortical Neurons against Excitotoxic Death. We moved from the evidence that mGlu4 receptor agonists/enhancers protect cultured cortical neurons against excitotoxic death (Bruno et al., 2000; Maj et al., 2003). We used mixed cultures of cortical cells in which neurons were plated over a monolayer of confluent astrocytes. Cinnabarinic acid had no effect on neuronal viability on its own when applied for 10 min or 24 h in a wide range of concentrations (1–300 μM) (not shown). For the induction of excitotoxicity, cultures were challenged with 100 μM NMDA for 10 min (paradigm of "fast" excitotoxicity), and neuronal death was assessed 24 h later. Cinnabarinic acid applied to the cultures during the NMDA pulse attenuated
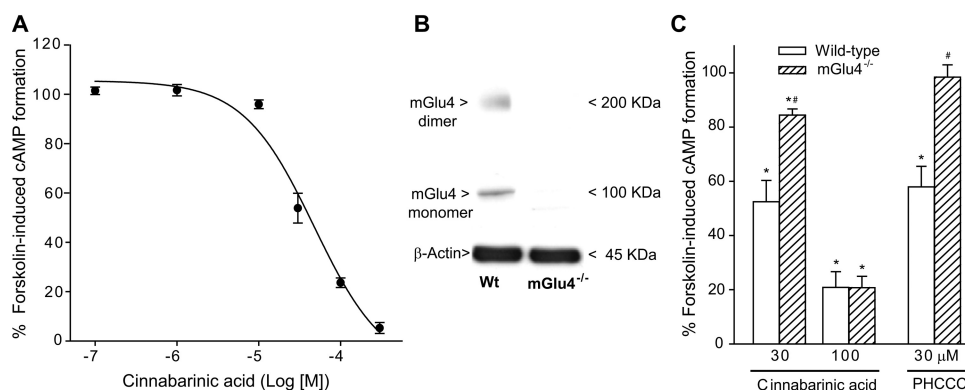

Fig. 5. Activation of native mGlu4 receptors by cinnabarinic acid in cultured cerebellar granule cells. A, concentration-dependent inhibition of forskolin-stimulated cAMP formation by cinnabarinic acid. The effect of the selective mGlu4 receptor enhancer PHCCC (30 μM) is also shown. Forskolin concentrations, 10 μM. Values (means + S.E.M.) are expressed as percentage of forskolin-stimulated cAMP formation and were calculated from 6 to 10 individual cultures from two independent experiments. B, immunoblots of mGlu4 receptors in protein extracts of cultured granule cells from wild-type or mGlu4 knockout mice. C, inhibition of forskolin-stimulated cAMP formation by cinnabarinic acid or PHCCC in cultured granule cells from wild-type or mGlu4 receptor knockout mice; values were calculated from six to eight determinations from two independent experiments. $p < 0.05$ (one-way ANOVA + Fisher's PLSD) versus values obtained with forskolin alone (∗) or versus the corresponding wild-type values (#).
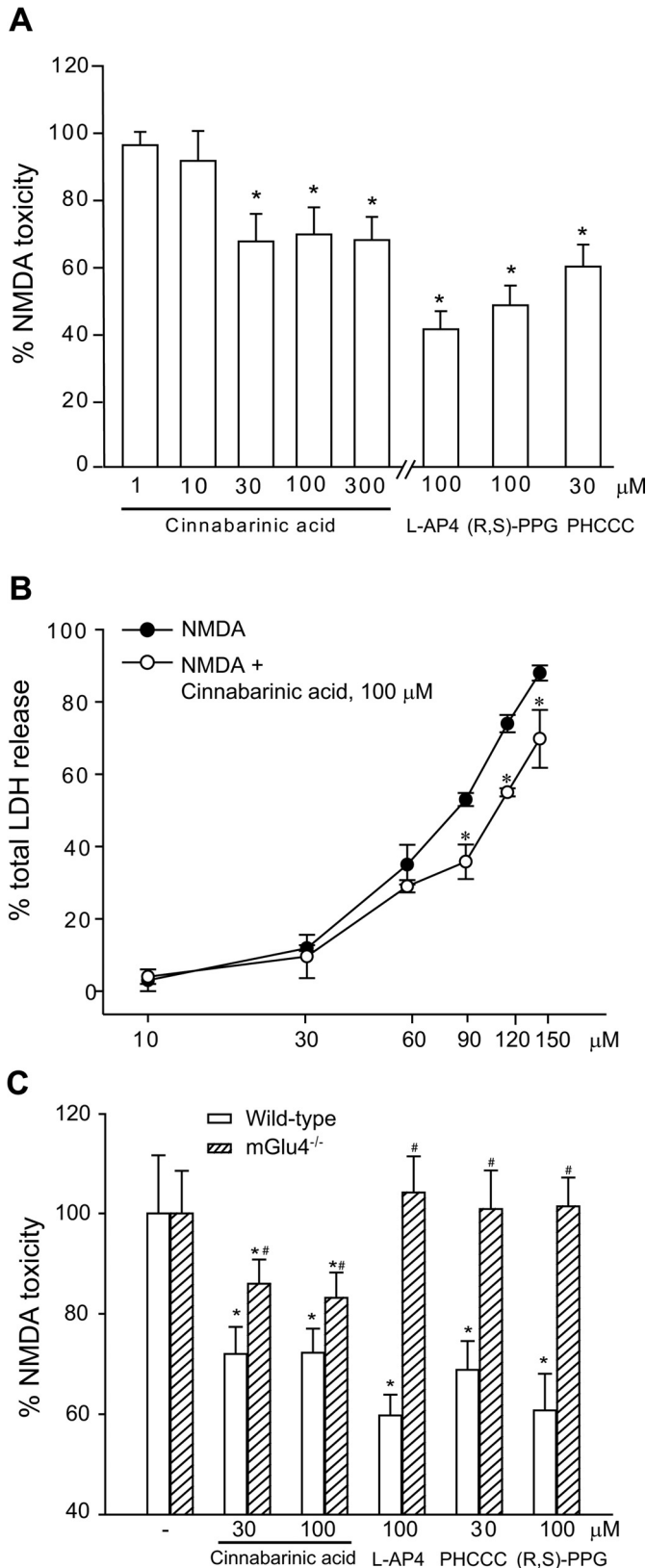
A

B

C

Fig. 6. Activation of native mGlu4 receptors by cinnabarinic acid protects cortical neurons against excitotoxic death. A, neuroprotection by different concentrations of cinnabarinic acid and selected concentrations of L-AP4, (R,S)-PPG, and PHCCC against NMDA toxicity in mixed cortical cultures. Values (means + S.E.M.) are expressed as percentage of NMDA toxicity and were calculated from four to eight individual cultures from two independent experiments. $*p < 0.05$ (One-way ANOVA + Fisher's

excitotoxic neuronal death at concentrations ≥ 30 μM. Concentrations of cinnabarinic acid of 30, 100, and 300 μM reduced NMDA toxicity to the same extent. The action of cinnabarinic acid was mimicked by drugs that activate mGlu4 receptors, including the orthosteric agonists L-AP4 and (R,S)-PPG (both at 100 μM) and the enhancer PHCCC (30 μM). L-AP4 and (R,S)-PPG showed a slightly greater efficacy than cinnabarinic acid or PHCCC as neuroprotectants (Fig. 6A). The protective activity of cinnabarinic acid was confirmed in another set of experiments in which cultures were challenged by increasing concentrations of NMDA in the absence or presence of 100 μM cinnabarinic acid. Cinnabarinic reduced excitotoxic death to a similar extent when NMDA concentrations were raised from 100 to 300 μM (Fig. 6B). Finally, we extended the study to cultured cortical cells prepared from mGlu4 receptor knockout mice. In these cultures, neuroprotection by all tested concentrations of cinnabarinic acid (30, 100, or 300 μM) was attenuated by 40 to 50% (Fig. 6C). Neuroprotection by L-AP4, (R,S)-PPG, and PHCCC was abolished in cultures prepared from mGlu4 receptor knockout mice (Fig. 6C), as expected (Bruno et al., 2000; Maj et al., 2003).

Protective Effect of Cinnabarinic Acid against MPTP-Induced Neurotoxicity. We have shown previously that PHCCC protects nigral DA-ergic neurons against MPTP toxicity by activating mGlu4 receptors in the external globus pallidus (Battaglia et al., 2006). Intrapallidal infusion of cinnabarininc acid (50 nmol/0.5 μl) was protective against nigro-striatal damage induced by MPTP (30 mg/kg i.p., single injection), as shown by measurements of striatal DA levels and stereological counts of nigral neurons after TH immunostaining. Neuroprotection by PHCCC (20 nmol/0.5 μl is also shown for comparison (Fig. 7).

Detection and Quantification of Endogenous Cinnabarinic Acid in Rat Tissue Extracts. We could detect and quantify cinnabarinic acid in rat tissue extracts by HPLC-MS/MS. In rats killed 24 h after a single injection of saline (control rats), cinnabarinic acid levels were approximately 60 pg/mg tissue in the lung, ranged from 7 to 10 pg/mg tissue in the liver, spleen, and kidney, and were below the detection limits in the brain. Systemic inflammation induced by LPS injection (0.5 mg/kg i.p.) substantially increased tissue levels of cinnabarinic acid. Under these conditions, brain levels of cinnabarinic acid were as high as 160 pg/mg tissue, and levels in the spleen and kidney were 36 and 133 pg/mg tissue, respectively (Fig. 8). We also attempted to detect extracellular cinnabarinic acid 1) in the striatal dialysate of freely

PLSD) versus values obtained with NMDA alone. B, neurotoxicity induced by different concentrations of NMDA in the absence or presence of 100 μM cinnabarinic acid. Data (means + S.E.M.) are expressed as percentage of total LDH release into the culture medium and were calculated by four to six determinations from two independent experiments. $*$, $p < 0.05$ (Student's t test) versus the corresponding values obtained with NMDA alone. C, neuroprotection by cinnabarinic acid, L-AP4, (R,S)-PPG, and PHCCC in mixed cortical cultures prepared from wild-type or mGlu4 knockout mice; values are expressed as percentage of NMDA toxicity. NMDA (100 μM) was slightly more toxic in cultures from mGlu4 receptor knockout mice (75 + 6% of total LDH release) than in cultures from wild-type mice (61 + 5% of total LDH release), as expected (Bruno et al., 2000). Values are expressed as percentage of NMDA toxicity and were calculated from four to eight individual cultures from two independent experiments. $p < 0.05$ (one-way ANOVA + Fisher's PLSD) versus the corresponding values obtained with NMDA alone ($*$) or versus the corresponding wild-type values (#).
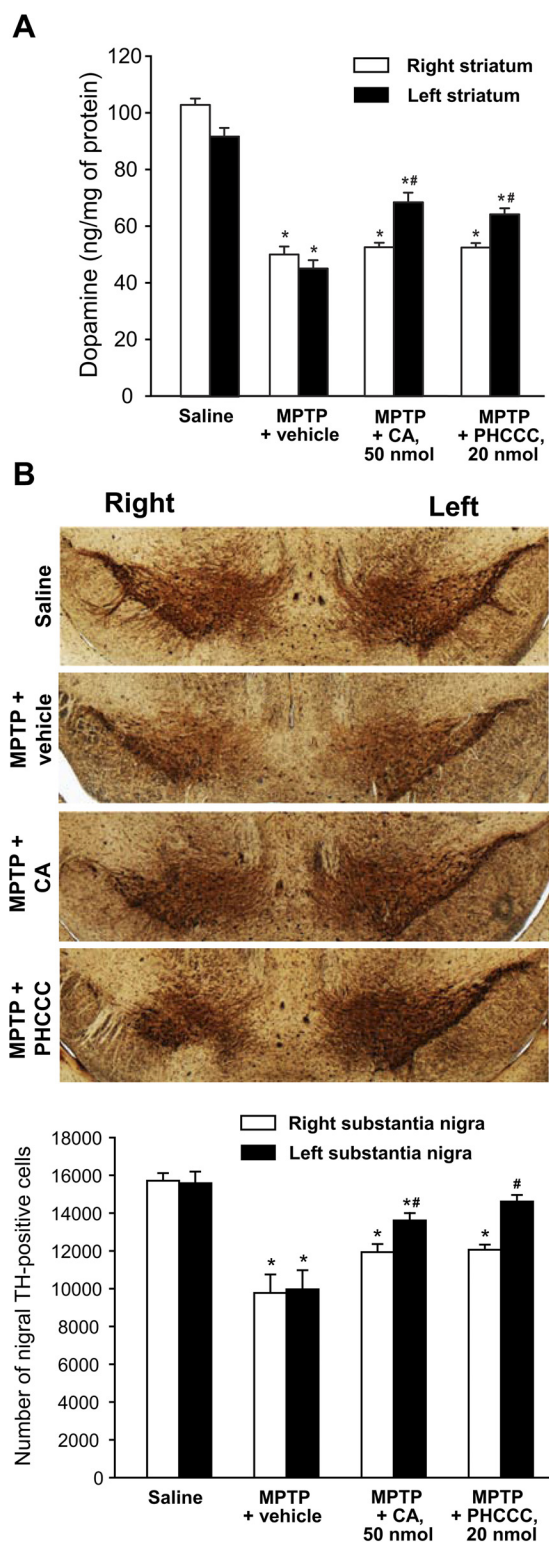
A

B

Fig. 7. Unilateral infusion of cinnabarinic acid or PHCCC into the external globus pallidus protects ipsilateral nigral dopaminergic neurons against MPTP toxicity in mice. A, striatal dopamine levels. B, immunohistochemical analysis of TH and stereological counts of TH-positive neurons in the pars compacta of the substantia nigra. Mice were injected with MPTP (30 mg/kg i.p., single injection) and unilaterally infused in the left external globus pallidus with vehicle (0.5 μl of 50% DMSO in saline), PHCCC (20 nmol/0.5 μl, dissolved in 50% DMSO), or cinnabarinic acid (CA, 50 nmol/0.5 μl, dissolved in 50% DMSO) 30 min before MPTP. Values are mean + S.E.M. of 12 determinations in A and 10 in B; $p <$ 0.05 (one-way ANOVA + Fisher's PLSD) versus the respective values

moving rats injected with saline or LPS (0.5 mg/kg i.p.) 24 or 48 h before and locally infused with depolarizing concentrations of either K^+ (100 mM) or veratridine (100 μM); and 2) in CSF samples from EAE mice 10 days after the onset of neurological symptoms (i.e., 25–30 days after immunization with MOG) and their control mice. Under all these conditions, cinnabarinic acid levels were below the detection limits.

Discussion

We have shown that cinnabarinic acid, an endogenous metabolite of the kynurenine pathway harboring two carboxyl moieties and an amino group, acts as a partial agonist of mGlu4 receptors. We wish to highlight that cinnabarinic acid is 1) the first reported kynurenine metabolite able to interact with any mGlu receptor subtype and 2) one of the few compounds that shows selectivity for mGlu4 receptors with respect to other group-III mGlu receptor subtypes. In transfected HEK293 cells, cinnabarinic acid behaved as a partial agonist of mGlu4 receptors with no activity at mGlu1, -2, -5, -6, -7, and -8 receptors. Cinnabarinic acid showed intrinsic efficacy at mGlu4 receptors under conditions that substantially lowered extracellular glutamate levels (cotransfection of HEK 293 cells with the glutamate transporter excitatory amino acid carrier 1, and enzymatic degradation of glutamate mediated by alanine amino transferase).

To elucidate the site of action of cinnabarinic acid, we tested it on a truncated mGlu4 receptor lacking the N-terminal extracellular domain where orthosteric agonists bind. As opposed to the mGlu4 receptor enhancer PHCCC, cinnabarinic acid did not activate headless mGlu4 receptors and did not potentiate the action of the agonist, L-AP4. This indicates that cinnabarinic acid does not behave as a positive allosteric modulator of mGlu4 receptor that binds within the transmembrane domain. Its action requires the presence of the N-terminal Venus Fly Trap domain that contains the primary binding site for glutamate and other orthosteric agonists. We then used modified mGlu4 receptors in which critical residues of the glutamate binding site are mutated in alanine. We showed that the two residues important for the α-amino acid functions of glutamate, Ser159 and Thr182, are also critical for cinnabarinic activity, despite the fact that this molecule is devoid of any amino acid moiety. Some basic residues involved in the binding of the distal acidic function of glutamate or L-AP4, Lys74, Lys317, and Lys405, are also involved in cinnabarinic acid activity. On the basis of these results, we docked cinnabarinic acid in a model of the extracellular domain of mGlu4 using molecular modeling. The molecule docks well in the glutamate binding pocket. Some critical residues for glutamate binding at proximal and distal sites are found to interact with cinnabarinic acid, such as Ser159 and Thr182, which make hydrogen bonds with the oxygens of the compound.

The question of the selectivity and partial agonist activity of cinnabarinic for mGlu4 receptor is more delicate to understand. Among mGluRs, mGlu4 and mGlu8 receptor are closely related. Only two residues from their orthosteric bind-

obtained in saline-treated mice (A and B) (∗) versus the right striatum (A) or substantia nigra (B) in MPTP-treated mice infused with cinnabarinic acid or PHCCC in the left external globus pallidus (#); or versus the left striatum (A) or substantia nigra (B) of MPTP-treated mice infused with vehicle in the left external globus pallidus (#).

Monitored ion transition and their parameter settings

Cinnabarinic acid precursor ion (m/z, amu)	Fragments (m/z, amu)	DP (V)	EP (V)	CE (V)	CXP (V)
301.2	264.7	35.1	5	23.7	3.4
	237.4	35.1	5	34.8	3.5
	209.4	35.1	5	42.1	3.1

DP, Declustering Potential; EP, Entrance Potential; CE, Collision Energy; CXP, Collision Cell Exit Potential.
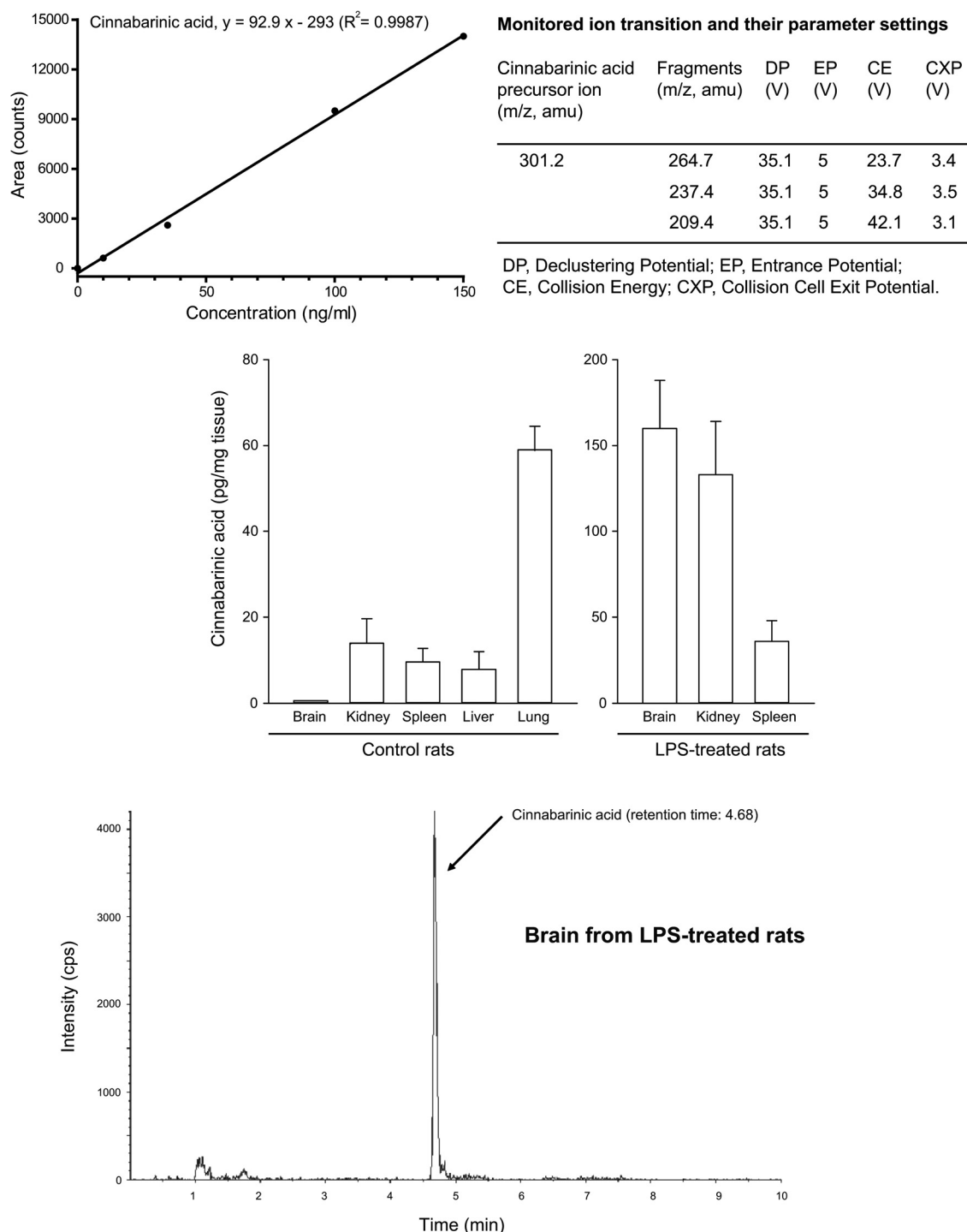
Fig. 8. HPLC-MS/MS detection of cinnabarinic acid in the brain and peripheral organs of rats injected with saline or LPS. A calibration curve with standard cinnabarinic acid and ion transition monitoring are shown on the top. Cinnabarinic acid levels in the brain, kidney, spleen, liver, and lung of rats treated with saline or 0.5 mg/kg i.p. LPS and killed 24 h later are shown on the center. Values are means + S.E.M. of three individual determination. A representative chromatogram of cinnabarinic acid in the brain of a rat treated with LPS is shown on the bottom.

ing site are different in these two receptors; Ser157 and Gly158 in mGlu4 are each replaced by alanine in the mGlu8 receptor. Because cinnabarinic acid is not active in mGlu8 receptors, it could be expected that making the glutamate binding site of mGlu4 receptor more mGlu8-like (i.e., by mutating Ser157 and Gly158 of mGlu4 to alanine as in mGlu8) would result in a decrease of cinnabarinic acid activity. However, the activity induced by cinnabarinic acid is

increased in the mGlu4 S157A + S158A double mutant, thus suggesting that these two residues are not the only factors involved in the mGlu4/mGlu8 selectivity of cinnabarinic acid. In the three-dimensional model, the mutation G158A shows a hydrophobic contact between the alanine methyl group and the aromatic ring of cinnabarinic acid, explaining an increased activity. Looking within the cleft between the two lobes, it can be observed that the loop between $\beta1$ and $\alpha1$ that

is located beside the binding pocket concentrates most of the differences between mGlu4 and mGlu8 extracellular domains. Indeed, 7 of the 19 amino acids that constitute this loop are different in the two receptors. Moreover, in the hinge between the two lobes, a serine in mGlu4 is replaced by a proline in mGlu8. Thus, because both the upper surface of the cleft and the hinge are different in mGlu4 and mGlu8, it can be speculated that the angle of closure is also different in these two receptors. Because cinnabarinic acid is bound close to the hinge connecting the two lobes, the planar and rigid structure of its phenoxazine core may impede the complete closing of the domain and explain the partial activity of this molecule on mGlu4 receptors. In the case of mGlu8 receptor, it could be inactive because of a different surrounding of the glutamate binding pocket and a different angle of closure. Further experiments will be needed to fully understand cinnabarinic acid binding mode and selectivity.

We examine whether cinnabarinic acid could activate native mGlu4 receptors using both in vitro and in vivo models. Cinnabarinic acid reduced cAMP formation in cultured cerebellar granule cells, which are known to express high levels of mGlu4 receptors (Santi et al., 1994). In an attempt to dissect the involvement of native mGlu4 receptors in the action of cinnabarinic acid, we used cultured granule cells prepared from mGlu4 receptor knockout mice. We adopted this strategy because no high-affinity, selective, and reliable mGlu4 receptor antagonists are available. Inhibition of cAMP formation by 30 μM cinnabarinic acid was largely attenuated (albeit not abolished) in granule cells lacking mGlu4 receptors. In contrast, the action of 100 μM cinnabarinic acid was independent of mGlu4 receptors. Thus, high concentrations of cinnabarinic acid might recruit additional mechanisms to inhibit cAMP formation. Because experiments were carried out in the presence of IBMX we can exclude that high concentrations of cinnabarinic acid enhance cAMP degradation by activating phosphodiesterases. The most likely explanation is that high concentrations of cinnabarinic acid activate additional membrane receptors coupled to G_i proteins. One potential candidate is the mGlu3 receptor, which is highly expressed in cultured granule cells (Santi et al., 1994) and was not tested in HEK293 cells. However, it should be highlighted that mGlu2 and mGlu3 receptors share the same agonist pharmacology, making the possibility that cinnabarinic acid activates mGlu3 but not mGlu2 receptors unlikely. Alternatively, high concentrations of cinnabarinic acid might act intracellularly and directly inhibit adenylyl cyclase activity. Intracellular actions have been reported for L-AP4 and L-serine-O-phosphate, which are the prototypical agonists of group-III mGlu receptors (Battaglia et al., 2000).

A different scenario was seen in mixed cultures of cortical cells challenged with a brief NMDA pulse. This model of excitotoxic neuronal death has been used for the demonstration of the neuroprotective activity of drugs that activate mGlu4 receptors (Bruno et al., 2000; Maj et al., 2003; see also present data). Cinnabarinic acid afforded significant protection against NMDA toxicity, showing no effect at 1 and 10 μM and an apparent plateau at 30 to 300 μM. Thus, in this particular model, we did not find the difference between 30 and 100 μM cinnabarinic acid we have seen when we measured cAMP formation in cultured granule cells. Neuroprotection by all effective concentrations of cinnabarinic acid

was attenuated, but not abolished, in cultures prepared from mGlu4 knockout mice. In contrast, neuroprotection by L-AP4, (R,S)-PPG, and PHCCC was abolished in the absence of mGlu4 receptors. If one dissects the component mediated by mGlu4 receptors, the efficacy of cinnabarinic acid as neuroprotectant is less than 50% with respect to the efficacy of L-AP4, (R,S)-PPG, and PHCCC, confirming that cinnabarinic acid acts as a partial agonist of mGlu4 receptors. The presence of an additional component in the neuroprotective activity of cinnabarinic acid was unexpected because the compound has been shown to induce apoptotic cell death in thymocytes through the generation of reactive oxygen species and caspase activation (Hiramatsu et al., 2008). However, in our cortical cultures cinnabarinic acid did not affect neuronal viability on its own even if applied to the culture medium for 24 h. The nature of the mGlu4 receptor-independent component in the protective activity of cinnabarinic acid against excitotoxic neuronal death remains to be determined.

We were surprised that the minimal effective concentration of cinnabarinic acid able to activate mGlu4 receptors was lower in cultured neurons (30 μM) than in recombinant HEK293 cells (100 μM). This is unexpected with mGlu receptor agonists. For example, L-AP4 and (R,S)-PPG activate recombinant and native mGlu4 receptors with similar potency (Bruno et al., 2000; see also Schoepp et al., 1999), whereas last-generation mGlu2/3 receptor agonists, such as eglumegad (LY354740) and (1R,4R,5S,6R)-4-amino-2-oxabicyclo[3.1.0]hexane-4,6-dicarboxylic acid (LY379268), are less potent in protecting cortical neurons against excitotoxic death (Corti et al., 2007) than in activating mGlu2 or mGlu3 receptors in recombinant cells (see Schoepp et al., 1999). The greater affinity of cinnabarinic acid for native mGlu4 receptors might depend on the presence of a coreceptor or a scaffolding protein that is absent in recombinant cells. Alternatively, mGlu4 receptors in neurons could form homo- or heteromultimeric complexes that favor the interaction with cinnabarinic acid.

An action of cinnabarinic acid at native mGlu4 receptors is supported by in vivo data obtained with the acute MPTP model of toxicological parkinsonism. It is known that microinfusion of the mGlu4 receptor enhancer PHCCC in the external globus pallidus protects nigral DA-ergic neurons against MPTP toxicity and that protection is lost in mice lacking mGlu4 receptors (Battaglia et al., 2006). In the external globus pallidus, mGlu4 receptors are presynaptically localized in nerve endings originating from striatal projection neurons of the "indirect pathway" (for review, see Conn et al., 2005). Pharmacological activation of mGlu4 receptors protects nigral DA-ergic neurons against MPTP toxicity by inhibiting GABA release in the external globus pallidus, thereby reducing the activity of glutamatergic neurons of the subthalamic neurons projecting to the pars compacta of the substantia nigra (Conn et al., 2005; Battaglia et al., 2006). Cinnabarinic acid shared the protective activity of PHCCC (used as a positive control) against MPTP toxicity when microinfused into the external globus pallidus. This evidence supports the hypothesis that cinnabarinic acid is able to activate native mGlu4 receptors.

We could detect picogram amounts of cinnabarinic acid in peripheral organs of control rats, whereas brain levels were not detectable. We also measured cinnabarinic acid levels in rats injected intraperitoneally with a bacterial endotoxin, LPS. Peripheral injection of LPS causes systemic inflammation involv-

ing the CNS (Choi et al., 2003; Qin et al., 2007; Henry et al., 2009), and proinflammatory cytokines are known to activate the kynurenine pathway (Stone and Darlington, 2002), which represents the metabolic source of cinnabarinic acid. In rats treated with LPS, cinnabarinic acid levels were as high as 160 pg/mg tissue in the brain and also increased in spleen and kidney. Thus, inflammation leads to an increased formation of cinnabarinic acid in various organs, including the brain, which is consistent with the evidence that proinflammatory cytokines activates the kynurenine pathway (for review, see Stone and Darlington, 2002). Whether cinnabarinic acid levels under control or inflammatory conditions are sufficient to activate mGlu4 receptors is unclear because so far we were unable to detect the compound in the brain dialysate or CSF of rats or mice under control or inflammatory conditions. Whether this is due to a limited amount of cinnabarinic acid released extracellularly or to a rapid clearance of the compound remains to be determined.

Our finding that cinnabarinic acid activates mGlu4 receptors has a number of potential implications. An enhanced formation of cinnabarinic acid during neuroinflammation might protect neurons against the harmful effect of proinflammatory cytokines or endogenous excitotoxins. For example, cinnabarinic acid might limit neurotoxicity caused by the high levels of quinolinic acid in the CNS of patients infected with HIV (Heyes et al., 1991). Recent evidence indicates that mGlu4 receptors are also expressed and functional in peripheral cells (Julio-Pieper et al., 2010). Activation of mGlu4 receptors in antigen-presenting cells drives the differentiation of naive T lymphocytes into T-regulatory cells (responsible for immune tolerance), thus protecting against experimental autoimmune encephalomyelitis (Fallarino et al., 2010). Activation of the kynurenine pathway has an established role in immune tolerance (Grohmann et al., 2002); therefore, activation of mGlu4 receptors by cinnabarinic acid might be one of the missing links in mechanisms of cell-to-cell communication within the immune system. These interesting aspects warrant further investigation.

Acknowledgments

Authorship Contributions

Participated in research design: Fazio, Molinaro, Bertrand, Acher, Ngomba, Curini, Di Marco, Bruno, Simmaco, Nicoletti, and Goudet.

Conducted experiments: Fazio, Lionetto, Molinaro, Bertrand, Notartomaso, Rosati, Scarselli, and Goudet.

Performed data analysis: Acher, Battaglia, and Goudet.

Wrote or contributed to the writing of the manuscript: Fazio, Acher, Pin, Nicoletti, and Goudet.

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