Striatal GPR88 expression is confined to the whole projection neuron population and is regulated by dopaminergic and glutamatergic afferents

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Abstract

GPR88, an orphan G protein-coupled receptor, was designated Strg/GPR88 for striatum-specific G protein-coupled receptor (K. Mizushima et al. (2000) Genomics, 69, 314-321). In this study, we focused on striatal GPR88 protein localization using a polyclonal antibody. We established that the distribution of immunoreactivity in rat brain matched that of GPR88 transcripts and provided evidence for its exclusive neuronal expression. GPR88 protein is abundant throughout the striatum of rat and primate, with expression limited to the two subsets of striatal projection medium spiny neurons (MSNs) expressing preproenkephalin-mRNAs. Ultrastructural immunolabelling revealed the GPR88 concentration at post-synaptic sites along the somatodendritic compartments of MSNs, with pronounced preference for dendrites and dendritic spines. The GPR88-rich expression, in both striatal output pathways, designates this receptor as a potential therapeutic target for diseases involving dysfunction of the basal ganglia, such as Parkinson’s disease. Hence, we investigated changes in GPR88 expression in a model of Parkinson’s disease (unilateral 6-hydroxydopamine-lesioned rats) following repeated L-DOPA treatment. In dopamine-depleted striatum, GPR88 expression was differentially regulated, i.e. decreased in striatopallidal and increased in striatonigral MSNs. L-DOPA treatment led to a normalization of GPR88 levels through dopamine D1 and D2 receptor-mediated mechanisms in striatopallidal and striatonigral MSNs, respectively. Moreover, the removal of corticostriatal inputs, by ibotenate infusion, downregulated GPR88 in striatopallidal MSNs. These findings provide the first evidence that GPR88 is confined to striatal MSNs and indicate that L-DOPA-mediated behavioural effects in hemiparkinsonian rats may involve normalization of striatal GPR88 levels probably through dopamine receptor-mediated mechanisms and modulations of corticostriatal pathway activity.

Introduction

The basal ganglia form a subcortical richly interconnected neuronal network involved in the adaptive control of behaviour through interactions with the cerebral cortex and other brain areas (Flaherty & Graybiel, 1994). The main input structure of the basal ganglia is the striatum and its major neuronal population, the GABAergic projection medium spiny neurons (MSNs), which directly receive excitatory glutamatergic terminals from cortical and thalamic areas, and modulatory dopaminergic terminals from the midbrain (Gerfen & Wilson, 1996). Current anatomical and functional models of the basal ganglia organization indicate that two segregated MSN subpopulations give rise to the direct striatonigral and indirect striatopallidal pathways, which connect the striatum with the thalamo-cortical projection through the basal ganglia output structures. Accordingly, it is thought that changes in dopamine and glutamate neurotransmission at the level of striatonigral MSNs affect the activity of the thalamo-cortical projection pathway, thereby influencing motor behaviour (Calabresi et al., 2007). Furthermore, there is evidence for the implication of other neurotransmitters in physiological and pathophysiological striatal processes such as adenosine-mediated mechanisms via selective A2A and D2 receptor interactions in striatopallidal MSNs (Ferré et al., 1997; Richardson et al., 1997). Thus, the striatum has a critical integrative role, leading to the acquisition of motor sequences (Flaherty & Graybiel, 1994; Graybiel, 1995; White, 1996; Wickens et al., 2003).

A previous study reported GPR88 as a new striatum-specific G protein-coupled receptor (GPCR) displaying highest sequence homology with SHT1D and β3 receptors, for which GPR88 has 27 and 21% of amino acid identity, respectively; however, it lacks the DRY motif critical for receptor activation (Mizushima et al., 2000). A different sequence signature was obtained by the analysis of key residues of GPCRs, which brought GPR88 closer to class C GPCRs including metabotropic glutamate and GABA receptors (Surgand et al., 2006). Hence, the
GPR88 endogenous putative ligand as well as the cellular and subcellular GPR88 protein expression and their functional role are unknown.

Recent studies have focused particular attention on the regulation of GPR88 gene expression in rat models of psychiatric disorders. Changes in expression levels have been reported in several brain regions following various treatments with addictive drugs, antidepressants or mood regulators (Ogden et al., 2004; Brandish et al., 2005; Boehm et al., 2006; Conti et al., 2007; Befort et al., 2008). Moreover, GPR88-knockout mice display psychosis-like behaviours probably due to hypersensitive D2 receptors (Pausch et al., 2006).

In the present report, using immunohistochemical approaches with a polyclonal antiserum against a peptide corresponding to the last 13 C-terminal amino acids of the GPR88, we examined the expression pattern of GPR88 protein in the rat and monkey basal ganglia, as well as the cellular and subcellular GPR88 distribution within the rat striatum. Our findings indicate that GPR88 is highly concentrated in the somata and dendrites of all striatal MSNs, which play an important role in a wide array of psychomotor functions ascribed to basal ganglia. In order to investigate potential relationships between GPR88 and neurological disorders such as Parkinson’s disease and its treatment, we then measured GPR88 expression in unilateral 6-hydroxydopamine (6-OHDA)-lesioned rats and after treatment with L-DOPA.

Materials and methods

Immunization, antisera titration and antibody purification

Coupling, immunization and bleeding were performed by Sigma–Aldrich (Saint-Quentin Fallavier, France). The Cter peptide of GPR88 Y-QAQLGTRAAGQHW, which is homologous in human, rat and mouse, was coupled to the carrier protein keyhole limpet haemocyanin for antibody production. Two rabbits were immunized six times with the keyhole limpet haemocyanin peptide and bled (five bleeds/rabbit, including pre-bleed day 0) on days 35, 49, 63 and 77. The peptide was coupled to bovine serum albumin in order to titrate the antisera by enzyme-linked immunosorbent assay. The best antisera titre was collected at the end of the immunization procedure, precipitated with ammonium sulphate and immunopurified on a HiTrap N-hydroxy-succinimide-activated column (Amersham Pharmacia Biotech, Little Chalfont, UK) coupled to the peptide by the amino group. The purified antibody was eluted in glycine-HCl (0.1 M, pH 2.3).

Cell line cultures and transfection

The human embryonic kidney (HEK)293 and Chinese hamster ovary (CHO) cells were cultured in a cell culture medium made with a 1 : 1 mixture of Dulbecco’s modified Eagle’s medium and F-12 or OPTIMEM for the latter (Gibco, Carlsbad, CA, USA), supplemented with 10% fetal calf serum and 100 µg/mL penicillin/streptomycin in humidified atmosphere of 5% CO₂/95% air. HEK293 cells were seeded in 10 cm dishes at 50–80% confluence and cDNA encoding the human GPR88 was transfected (10 µg) and small interfering RNA (siRNA) (Dharmacon, Chicago, IL, USA) (1.5 µg), antisense 5′-PUUCAACAUGAAGGCGUUGCUU-3′, sense 5′-GCAACCGCUUAGUGUAAUU-3′ or non-specific siRNA (siGenome non-targeting siRNA) (Dharmacon) using Lipofectamine 2000 (Invitrogen) to interfere with GPR88 expression. Experiments were performed 12 or 24 h later.

Western blot analysis

Crude extracts of CHO cells or brain regions were used for gel electrophoresis. Rats were decapitated and the brains quickly removed. The sample buffer for reducing gels contained 2% sodium dodecyl sulphate and 2.5% 2-mercaptoethanol. Samples were incubated at 95°C for 5 min before loading. Western blotting was performed using polyvinylidene difluoride membranes (Immobilon; Millipore Corp., Milford, MA, USA) and chemoluminescent visualization. Proteins (25 µg) were separated by electrophoresis in 12% sodium dodecyl sulphate-polyacrylamide gel, electrophoretically transferred to polyvinylidene difluoride membranes and probed with anti-GPR88 (1 : 15 000) or monoclonal anti-lamin B2 (1 : 1000) overnight at 4°C. Blots were incubated with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG antibodies (Amersham) for 1 h at room temperature (≈22°C) and developed using the enhanced chemiluminescence procedure (ECL-Amersham Pharmacia Biotech).

Animals

Young adult male Wistar rats (180–220 g; Janvier, Le Genest-St-Isle, France) were maintained under a 12 h light/dark cycle with constant temperature and humidity. Food and water were available ad libitum. All experimental procedures were performed in strict accordance with the European Communities Council Directive of 24 November 1986 (86/609/EEC) and were approved by the Regional Ethical Committee for animal experimentation (Ile-de-France, Paris Descartes, France). All efforts were made to reduce the number of animals used in these experiments. Surgical procedures were performed under pentobarbital anaesthesia (60 mg/kg, injected i.p.). The temperature of the animals was monitored and restored using a heating carpet.

Stereotaxic lesions and drug treatments

Bilateral lesions of the cortex (n = 6–9) were induced by multiple stereotaxically guided injections of ibotenic acid (63 nm; Sigma–Aldrich) dissolved in phosphate-buffered saline. The skull along the frontal and parietal cortices was drilled away and small holes were drilled through the skull to introduce a micropipette connected to a Hamilton syringe (10 µL). Injections of 0.5 µL at a rate of 0.1 µL/min were made at four sites: anteroposterior (A, +1; L, ±0.35; V, −0.2 and A, +0.4; L, ±0.35; V, −0.2) (Paxinos & Watson, 1986). After each injection, the needle was left in position for 10 min to allow absorption of the bolus and to minimize spread of the toxin along the needle tract. A second group of rats (n = 7) received only the vehicle (phosphate-buffered saline). Rats with cortical lesions and controls were killed by decapitation at 1 week post-surgery and their brains were dissected out and rapidly frozen in dry-ice-cooled isopentane (−40°C). Brains were then conserved at −75°C until sectioning. Coronal sections (10 µm), at the level of the caudate-putamen, were thaw-mounted on adhesive microscope slides (SuperFrost Plus, Menzel-Glaser). The sections were then kept at −75°C until use for immunohistochemistry and in-situ hybridization. Dopamine neurons were unilaterally lesioned by stereotaxic injection of 4 µL of the 6-OHDA toxin in either the right or left medial forebrain bundle (A, −0.34; L, ±1.5; V, −8.0). 6-OHDA HCl (Sigma–Aldrich) was dissolved in 0.02% ascorbic acid at a concentration of 2 µg/µL. Injections were performed at the rate of 0.5 µL/min for 4 min, using a 10 µL Hamilton syringe with a 26-gauge steel needle. After the infusion, the needle was kept in place for an additional 3 min to allow diffusion and then carefully removed. The efficacy of the lesion was assessed at 2 weeks after the surgery using the apomorphine-induced rotation test. The contralateral rotations induced...
by apomorphine (0.05 mg/kg s.c. injection) were monitored for 5 min subsequent to injection (Ungerstedt, 1971). Only those rats showing robust contralateral turning (>5 turns/min) were used in subsequent experiments. At 15 days post-surgery, a first group of unilaterally denervated rats (n = 10–12) received a repeated treatment of L-DOPA methyl ester (3 mg/kg/day, i.p. injection) for 14 days. The second and third hemi-parkinsonian groups (n = 7/group) received the same L-DOPA treatment, together with the concomitant administration of either the D1 antagonist SCH23390 (SCH) (0.1 mg/kg twice a day i.p.) or the D2 antagonist haloperidol (Halo) (1 mg/kg/day i.p.). In all groups, L-DOPA methyl ester was mixed with the peripheral DOPA-decarboxylase inhibitor benserazide hydrochloride (6 mg/kg). A fourth group of hemi-parkinsonian rats (n = 5) received only the vehicle (0.9% NaCl). All hemi-parkinsonian rats were killed by decapitation at 24 h after the last injection and their brains were dissected out, rapidly frozen and conserved at −75 °C until sectioning. Coronal sections (10 μm) were cut out of the brains at the level of the caudate-putamen or the substantia nigra/ventral tegmental area and then kept at −75 °C until use in in-situ hybridization.

**Immunohistochemistry**

Rats were anaesthetized deeply with pentobarbital (60 mg/kg) and then perfused transcardially with 50 mL of saline solution (0.9% NaCl warmed to 37 °C), followed by 600 mL of an ice-cooled fixative solution containing 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.5. The brains were removed, post-fixed in 4% paraformaldehyde for 2 h at 4 °C and rinsed in phosphate buffer solutions. Some brains were cut with a vibratome in coronal and sagittal sections (40 μm) that were collected, cryoprotected in phosphate buffer containing 30% sucrose and freeze-thawed (−75 °C). Other brains were transferred into ascending series of sucrose solutions (10% overnight, 15% for 24 h and 20% for 24 h), frozen and stored at −75 °C, and then cryostat sectioned at 10 μm.

In the present study, we also included coronal brain sections (12-μm-thick cryostat sections; fixed for 10 min with 4% paraformaldehyde and stored at −75 °C until use in immunohistochemistry) taken at a rostral level of the striatum, including the caudate nucleus, putamen and nucleus accumbens, of adult healthy normal female *Macaca fascicularis*. These cryostat sections were taken from brains of normal monkeys used in a previous study (Bézard et al., 2003), which received approval from the local Ethical Committee of Bordeaux II University (France). Cryostat or Vibratome sections were treated for 20 min with 1% NaBH₄ in 0.1 M Na₂HPO₄, pH 8.5, and collected in 0.05 M Tris buffer, pH 7.5, containing 150 mM NaCl (TBS). Blocking serum was then added (5% normal donkey or goat serum, 0.4% bovine serum albumin, 0.1% gelatin and 0.1% Tween 20 in TBS) for 1 h at room temperature.

Vibratome brain sections of rats and cryostat brain sections of *Macaca fascicularis* were incubated overnight or for 48 h (only for electron microscopy immunostaining) at 4 °C with the immunopurified anti-GPR88 antibody (1:15 000) or with an anti-μ-opioid receptor antibody (1:10 000) (Immunostar, WI, USA) in TBS containing 5% normal donkey serum and 0.05% Tween 20 (TBS–NDST20). Some control sections were incubated with the antibody that had been previously pre-saturated overnight with the Cter peptide (1 μg/μL). The sections were rinsed (4 × 10 min) in TBS containing 0.1% gelatin and 0.05% Tween 20 (TBS–GT20) and immersed overnight at 4 °C in biotinylated donkey anti-rabbit-g-ylacoides (Amersham) diluted 1:300 in TBS-NDST20. Sections were rinsed in TBS–GT20 and then incubated for 1 h at room temperature in avidin–biotin–horseradish peroxidase complex (ABC reagent, Vectastin-Elite; Vector Laboratories, Burlingame, CA, USA). Peroxidase activity was revealed by incubation with 3,3′-diaminobenzidine for 10–30 min (5 min for vibratome rat brain sections stained for electron microscopy) at 4 °C in the presence of hydrogen peroxide using the Sigma Fast diaminobenzidine tablets (Sigma, St Louis, MO, USA).

**Fluorescence-labelling method**

Vibratome coronal rat brain sections were incubated overnight at 4 °C in a mixture of primary immunoreagents diluted in TBS–NDST20. The anti-GPR88 antibody was used either alone (1:20 000) or in combination with mouse monoclonal antibodies directed against glial fibrillary acidic protein (1:500), myelin basic protein (1:10 000) (Chemicon, Temecula, CA, USA), calretinin (1:2000) (Sigma), parvalbumin (1:2000), somatostatin (1:100), cholino acetyltransferase (1:100) or tyrosine hydroxylase (TH) (1:10 000) (Incstar, Stillwater, MN, USA), or with guinea pig polyclonal antibodies directed against vesicular glutamate transporter 1 (1:5000) (Chemicon) or vesicular glutamate transporter 2 (1:2500) (Chemicon) for double-labelling experiments. After four washes in TBS–GT20, the sections were incubated in Alexa488 anti-mouse or anti-guinea pig (1:200) (Molecular Probes, Carlsbad, CA, USA) in TBS–NDST20. Intensification of the GPR88 fluorescent immunostaining was performed by using a horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (1:500) (Amersham) followed by incubation for 10 min in cyanine 3-tyramide signal amplification (1:300) (Perkin Elmer, Shelton, CT, USA). The sections were washed, mounted on Super Frost Plus slides and then cover-slipped using Vectashield mounting medium (Vector Laboratories) with or without 4′,6′-diamidino-2-phenylindole. Control experiments were performed to ensure that each primary antibody did not react with the non-corresponding secondary antibody conjugate.

**Preparation of tissue for electron microscopy**

After peroxidase immunostaining for GPR88 as described above, the sections were rinsed in phosphate-buffered saline (0.05 M, pH 7.4), fixed for 10 min in 1% glutaraldehyde in phosphate-buffered saline and then post-fixed for 20 min in 1% osmium tetroxide, rinsed in 50% ethanol and dehydrated in a graded series of ethanol solutions. The sections were impregnated with 1% uranyl acetate in 100% alcohol and infiltrated with 100% propylene oxide before being embedded in Epon. Ultrathin sections were then cut from the Epon-embedded specimens with a Reichert ultramicrotome. Ultrathin sections were mounted on mesh grids, stained with 0.4% lead citrate and 4.0% uranyl acetate, and finally analysed and photographed on a JEOL 100 electron microscope.

**GPR88 plasmid constructs**

The human GPR88 cDNA cloned into PCDNA3.1 (Invitrogen) was obtained from a cDNA resource (University of Missouri at Rolla, USA). Rat GPR88 cDNA was cloned by polymerase chain reaction into PCDNA3+ (Invitrogen) between the EcoRI and HindIII sites. Total RNAs (1 mg) from rat striatum were reverse transcribed with AMV reverse transcriptase (Finnzymes, Espoo, Finland). The polymerase chain reaction was performed using Phusion DNA polymerase.
(Finnzymes) with sense primer 5'-CCCAAGCTTATGACCAAACCTC-CTCC-3' and reverse primer 5'-CCGAAATTCCTACCAATGGCT-CGCC-3'. For the hybridization probe, a partial coding sequence of the rat GPR88 was amplified from striatum cDNAs using the sense primer 5'-GCGACCTACGGCTTACTCTCC-3' and antisense primer 5'-GGCACAATCTCACAACGTC-GCC-3' and subcloned into pCR 2.1 TOPO (Invitrogen).

**Lentiviral vector construction, virus production and infection**

Plasmids containing GPR88 small hairpin RNA (shRNA) or luciferase shRNA (shRNA design was based on GPR88 siRNA and enhanced green fluorescent protein as marker) were constructed by Vectalyx (Labège Cedex, France) in order to obtain rHIV-shGPR88 and rHIV-shNeg lentiviral vectors. Each shRNA was cloned into a lentiviral plasmid containing H1-RNA polymerase III promoter. At 12 h after plating neurons, the medium was replaced by 150 μL of fresh medium with an adjusted concentration of lentiviral vectors (106 TU/mL) for 12 h and then replaced by fresh medium.

**In-situ hybridization**

Radioactive [35S]-labelled or non-radioactive digoxigenin (DIG)-labelled complementary RNA (cRNA) probes were used to detect mRNA expression in tissue sections. The procedure has been described previously (Diaz et al., 1995). Briefly, paraformaldehyde-fixed cryostat sections were treated with protease K (1 mg/mL) and then with 0.25% acetic anhydride in triethanolamine buffer. Some sections were then hybridized only with the 35S-labelled cRNA probe for measuring GPR88 mRNA levels on autoradiography films. Other sections were double hybridized using the 35S-labelled cRNA probe for GPR88 mRNA with the DIG-labelled cRNA probe for preprotachykinin/substance P (SP) mRNA or with the DIG-labelled cRNA probe for preproenkephalin (ENK) mRNA to evaluate the co-localization of GPR88 transcripts with SP and ENK neuropeptides. All sections were hybridized at 55°C (14–16 h) with cRNA-labelled probes. After treatment with RNase A (200 μg/mL), sections were washed with diluted saline sodium citrate buffer, dehydrated through a graded series of ethanol, dried and exposed to Kodak-BioMax MR autoradiographic films (Sigma) for 5–10 days at 4°C.

Films were developed (4–5 min) in D-19 Kodak developer (Sigma).

For the double-radioactive (35S-labelled GPR88 probe) and non-radioactive (DIG-labelled SP and ENK probes) hybridization method, the DIG labelling was first revealed, after saline sodium citrate buffer washing, by immunohistochemistry with the alkaline phosphatase–peptides. All sections were hybridized at 55°C (14–16 h) with cRNA-labelled probes. After treatment with RNase A (200 μg/mL), sections were washed with diluted saline sodium citrate buffer, dehydrated through a graded series of ethanol, dried and exposed to Kodak-BioMax MR autoradiographic films (Sigma) for 5–10 days at 4°C. Films were developed (4–5 min) in D-19 Kodak developer (Sigma). For the double-radioactive (35S-labelled GPR88 probe) and non-radioactive (DIG-labelled SP and ENK probes) hybridization method, the DIG labelling was first revealed, after saline sodium citrate buffer washing, by immunohistochemistry with the alkaline phosphatase–bromochloro indolyl phosphate reaction for 1–3 h. After rinsing and drying at room temperature, sections were dipped in LM-1 photographic emulsion (Amerham) melted at 42°C, air-dried and stored at 4°C for 1–2 weeks. The photographic emulsion was developed at 10°C (2.5 min) in D-19 Kodak developer, rinsed in deionized water and cover-slipped.

**Quantification of film, emulsion micro-autoradiographs and immunoperoxidase-stained sections**

Relative optical density measurements of GPR88 mRNA levels in the dorsal striatum were obtained by computerized densitometry using an image analyser (Mercator autoradiographic software, Explora Nova, France). Optical grey values were converted to nCi/g of tissue equivalent by using standard curves generated with 14C standards microscales (GE Healthcare). Average levels of labelling for each area were calculated from three adjacent brain sections per animal.

The levels of GPR88 mRNA in neuronal profiles labelled or not with DIG-ENK in the dorsal striatum were quantified at the cell level on sections processed for double in-situ hybridization and emulsion autoradiography. A comparable dorsolateral striatal sector was systematically selected across experimental groups for analysis. To quantify GPR88 mRNA in both DIG-ENK-negative and DIG-ENK-positive neurons in each brain area that was analysed, we processed three images at 40x magnification: (i) a ‘cells’ image, captured under bright-field illumination to identify and count the ENK-positive and ENK-negative neurons; (ii) a ‘grains’ image of the same field, captured under dark-field illumination to count the silver grains over neuronal profiles; and (iii) a ‘background’ image under dark-field illumination in the corpus callosum bordering the dorsolateral striatum to estimate the number of silver grains generated as non-specific labeling. On the ‘grains’ image, specific grains labeling over neuronal profiles was considered only when its level was three times as high as the ‘background’ level. On average, 15–30 ENK-negative and 12–30 ENK-positive neurons per side per rat (n = 90–360 neurons) were selected and the number of silver grains in each neuron was counted on digitized photomicrographs using IMAGEJ software (National Institute of Health, Bethesda, MD, USA). A circle of constant diameter was superimposed over ENK-positive or ENK-negative neurons in each ‘cells’ and ‘grains’ image and the silver grains diameter was superimposed over ENK-positive or ENK-negative neurons in each 'cells' image. No attempt was made to quantify the intensity of the chromogen reaction for ENK-DIG and SP-DIG labelling.

The GPR88 immunostaining density was assessed on immunoperoxidase-stained sections. All brain sections analysed from animal groups were simultaneously processed under optimal conditions to obtain non-saturated GPR88 immunoperoxidase staining. To assess GPR88 protein density staining from immunoperoxidase-processed brain sections, images were digitized using identical illumination intensity and settings for both control and experimental animal groups, and converted into 8-bit grayscales images. To evaluate the overall staining density in each image, the background measured in the corpus callosum bordering the dorsal striatum was subtracted from the dorsolateral sector measurements. To obtain the overall staining density per hemisphere per rat, three sections were averaged. The density of staining was analysed using IMAGEJ software (National Institute of Health).

**Data analysis**

All data are expressed as group mean ± SEM. The raw data for the nigrostriatal lesion were analysed by two-way ANOVA with lesion and treatment as independent variables and the Bonferroni test for multiple comparisons was applied in post-hoc analysis to determine which values were significantly different. For data from the ibotenate lesion, the Student’s unpaired two-tailed t-test was used to compare drug-injected vs. saline-injected rats. The alpha level was set at 0.05.

**Results**

**Generation and characterization of anti-GPR88 antibody**

An affinity-purified polyclonal antibody was generated against a peptide corresponding to the last 13 C-terminal amino acids of the GPR88 (Fig. 1A). This sequence, which is homologous in the rat, mouse and human, does not display significant homology with other
known or predicted protein sequences. This minimizes the risk of cross-reactivity with other proteins.

Using crude extracts from CHO cells transiently expressing the rat GPR88 in western blot analysis, two bands were clearly detected at 28 and 35 kDa, which were not detected either with an extract from PCDNA3-transfected CHO control cells (Fig. 1B) or at 24 h after co-transfection with siRNA targeting GPR88 mRNA (Fig. 1B). This last result is in agreement with the marked decrease of GPR88 mRNA expression (80–90%) quantified by real time quantitative PCR at 12 or 24 h after co-transfection with the same siRNA (not shown).

In HEK293 cells stably expressing the human GPR88, intense cytoplasmic immunofluorescent patches localized in perinuclear areas and faint staining in the plasma membrane were observed (Fig. 1C). Control HEK293 cells were devoid of any immunoreactive signal (Fig. 1C). Intense immunofluorescent labelling was also found in sections of the rat basal forebrain (Fig. 1D), which was abolished when the GPR88 antibody was omitted (not shown) or pre-saturated overnight with the immunizing peptide (Fig. 1E). In order to further demonstrate the high specificity of this polyclonal antibody, we used lentiviral constructs expressing shRNA to induce specific and stable GPR88 silencing in small areas of the rat striatal complex. At 1 week after infusion into the rat nucleus accumbens of the lentivirus rHIV-shGPR88-GFP, there was complete disappearance of GPR88 immunoreactivity at a short distance surrounding the injected site (Fig. 1F), where the lentiviral-shGPR88-GFP construct reached a high concentration, as monitored by the green fluorescent protein immunostaining (Fig. 1G). The infusion of the control lentivirus rHIV-shNeg-GFP had no effect (not shown).

GPR88 immunoreactivity in the striatum of rat and monkey. Analysis of immunostained rat brain sections with the specific polyclonal GPR88 antibody revealed high levels of GPR88 immunoreactivity throughout the striatum, olfactory tubercle and nucleus accumbens, and moderate levels throughout the rostrocaudal extent of the cerebral neocortex (Fig. 2B). In addition, we detected measurable levels of GPR88 expression in the amygdala (Supporting information Fig. S1 and Fig. 1) and hypothalamus (not shown). This pattern of GPR88 protein expression perfectly matched the regional distribution of GPR88 transcripts in rat brain sections hybridized with a specific cRNA GPR88 probe (Figs 1 and 2A, and supporting Fig. S1). Western blot experiments with extracts from several rat brain areas demonstrated that GPR88 protein is more concentrated in the striatum than in the cortex but undetected in the cerebellum (Fig. 2H). Moreover, cortical GPR88 mRNA and protein expressions (Fig. 2I). Immunohistochemical experiments also demonstrated very similar densities of GPR88 protein throughout the striatal complex and cortex of the monkey M. fascicularis (Fig. 2C) and mice (not shown). Within the basal ganglia, both GPR88 protein and mRNA expressions were confined to the striatum. No immunoreactivity or hybridization signals were detected in the ventral pallidum and substantia nigra (Fig. 2A and B) or other basal ganglia structures.

Within rat and monkey striatal complexes, GPR88 immunoreactivity and transcript distributions were characterized by conspicuous heterogeneity, including a marked dorsolateral to ventromedial decreasing gradient and the presence of several small areas with high concentrations of GPR88 immunoreactivity (Fig. 2C and D). Light microscopic analysis of adjacent sections revealed GPR88-rich small areas (Fig. 2D) overlapping the striatal µ-opioid receptor-immunoreactive patches (Fig. 2E) (Gerfen & Wilson, 1996). This observation demonstrates that GPR88 is more highly expressed in striosomes than in the surrounding matrix.
Fig. 2. Pattern of GPR88 distribution in brain sections of rat (A and B) and monkey (C). In-situ hybridization (A) and immunoreactive (B and C) signals in sagittal (A and B) and coronal (C) sections. In-situ hybridization was performed with a $^{33}$P-labelled riboprobe and immunoreactivity was revealed by the ABC method using diaminobenzidine as chromogen. Inside the striatum of rat (D) and monkey (C), the densest GPR88 immunoreactivity was observed in some irregular areas (arrows), which perfectly match the patches (arrows) depicted by $\mu$-opioid receptor immunostaining on an adjacent section (E). (F and G) Double-labeling in-situ hybridization methods revealed the molecular phenotype of rat striatal neurons harbouring the GPR88. Representative images of neurons co-expressing GPR88 mRNA (labelled by silver grains) and SP mRNA (F) or ENK mRNA (G) (labelled by the DIG dark staining). Virtually all SP-positive (F) and ENK-positive (G) neurons express GPR88. (H) Immunoblots of GPR88 from total striatal, cortical and cerebellar extracts. An anti-tubulin antibody was used in the same blot and tubulin signal was used as internal standard. (I) Relative quantification of immunoreactive bands of total striatal and cortical extracts of GPR88 analysed from separated western blot, immunohistochemical or hybridization experiments (mean ± SEM; $n = 3–10$). Results are expressed as percentage of striatal levels. Cx, cortex; St, striatum; Acb, nucleus accumbens; OT, olfactory tubercle; VP, ventral pallidum; SN, substantia nigra; Cd, caudate; HIS, in situ hybridization; Pu, putamen. Scale bars: 3 mm (A–C); 250 $\mu$m (D); 30 $\mu$m (G and H).
Cellular and subcellular expression of GPR88 in striatum

In order to identify the phenotype of neural cells expressing GPR88 in the rat brain, we performed double immunostainings for GPR88 and specific neurochemical markers related to glial cells (glial fibrillary acidic protein for astrocytes and myelin basic protein for oligodendrocytes). As no GPR88 immunoreactivity was found on glial fibrillary acidic protein-positive astrocytes or myelin basic protein-positive oligodendrocytes (Fig. 3A and B), an exclusive neuronal expression of the receptor was concluded.

The phenotype of striatal GPR88-expressing neurons was investigated in rat brain sections using either double in-situ hybridization

![Fig. 3](image-url)

**Fig. 3.** Representative distribution of fluorescent signals generated in double-immunolabelled brain sections processed for GPR88 detection in the different glial cells and striatal interneurons. The intense red immunofluorescent GPR88 staining is observed in the neuropil and neuronal cell bodies of striatum (arrowheads). The green fluorescent signals in the central panels corresponds to: (A) glial fibrillary acidic protein (GFAP)-positive astrocytes, (B) myelin basic protein (MBP)-immunoreactive myelin sheath developed by oligodendrocyte on isolated and grouped axons in bundles and the different striatal interneurons immunoreactive for (C) calretinine, (D) parvalbumin, (E) somatostatin or (F) choline acetyl-transferase. The GPR88-immunoreactive signal was never detected in the other striatal interneurons or glial cells. ChAT, choline acetyl-transferase. Scale bars, 20 μm.

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for detection of two different mRNAs (GPR88 and the neuropeptides SP or ENK, respectively, expressed by striatonigral and striatopallidal MSNs) or double-fluorescent immunostaining for GPR88 and specific markers of striatal interneurons (calretinin, parvalbumin, somatostatin and choline acetyltransferase). As shown in Fig. 2F and G, GPR88 mRNA expression was detected in all of the striatal neurons co-expressing SP or ENK transcripts. Only very few parvalbumin-immunoreactive striatal interneurons displayed a faint GPR88 immunoreactive signal (Fig. 3D). No co-expression between GPR88 and other interneuron markers was observed (Fig. 3C, E and F). Therefore, striatal GPR88 is confined to virtually all MSNs that also express SP or ENK but only to a limited extent in striatal parvalbumin interneurons.

The GPR88 immunolabelling, detected in both peroxidase- (not shown) and fluorescent-immunostained sections, was extensively associated with neuropil and neuronal cell bodies throughout striatum, nucleus accumbens and olfactory tubercle (Fig. 4A–C). High magnifications revealed that this immunosignal is mainly concentrated in the somatodendritic compartments of dorsal striatum neurons (not shown). In the majority of labelled perikarya, the immunosignal was distributed along the cell surface, appearing as a dotted rim of fluorescent cytoplasm with a large unstained central region occupied by the nucleus (Fig. 4A–C). These neuronal profiles were presumed to mainly represent MSNs (Reiner & Anderson, 1990). GPR88 immunostaining was never associated with axons and axon terminals in the striatum. We did not detect any signal in the globus pallidus (supporting Fig. S1 and S2).

**Fig. 4.** Cellular and subcellular GPR88 distribution. Immunofluorescent staining for GPR88 in dorsal striatum (A), nucleus accumbens (B) and olfactory tubercle (C) of the rat. The images show widespread GPR88 immunosignal including dense neuropil staining, presumably reflecting labelling of dendritic compartments. Immunoreactive cell bodies (arrows) in striatum, nucleus accumbens and olfactory tubercle displaying intense perikaryal staining, which appears as a perimembranous fluorescent rim with an unlabelled central region largely occupied by the nucleus. Other GPR88-immunoreactive somata are indistinct within the punctated stained neuropil, as they are only labelled by a thin rim of perimembranous staining. (D–F) The subcellular GPR88 protein distribution in the dorsolateral striatum is revealed by the electron-dense immunoreactive reaction product, visible as a dotted submembranous labelling around the perikaryon (arrows in D) but mainly concentrated in dendritic shafts (d) and dendritic spines (s). These post-synaptic elements are contacted by unlabelled synaptic buttons (b) at asymmetrical (E) or symmetrical (F) synapses. Note the absence of immunolabelling in some synaptic contacts (asterisk). Scale bars: 30 μm (A–C); 1.4 μm (D); 180 nm (E and F).
Fig. 1), reticular part of the substantia nigra and ventral pallidum (Fig. 2B), innervated by striatal and nucleus accumbens projection neurons, respectively. These data indicate that GPR88 distribution is restricted to the somatodendritic compartment of striatal MSNs.

Ultrastructural localization of GPR88 immunostaining in the dorsal striatum confirmed that GPR88 immunoreactivity was essentially concentrated at discrete submembranous sites along the perikarya, dendritic shafts and dendritic spines as well as in some intracytoplasmic membrane elements (Fig. 4D–F). Labelled dendrites and dendritic spines showed clear synaptic contacts from unlabelled terminals (Fig. 4E and F). The immunoreactive electron-dense precipitate was present in the post-synaptic densities of many asymmetrical synapses but was less frequent in symmetrical synapses. Asymmetric electron densities have been shown to be indicative of synaptic contacts formed by excitatory pre-synaptic elements such as glutamate terminals (Smith et al. 1994; Okabe, 2007). We performed double-immunofluorescence labelings for GPR88 and the vesicular glutamate transporters vesicular glutamate transporter 1, vesicular glutamate transporter 2 or TH. Both glutamate transporters have been localized on synaptic vesicles in axon terminals forming asymmetrical synaptic contacts (Fremeau et al., 2004; Fujiyama et al., 2004, 2006). Confocal microscopic analysis showed that the punctate GPR88 immunostaining was frequently associated with vesicular glutamate transporter 1- but not vesicular glutamate transporter 2-immunoreactive terminals (Fig. 5A and B), confirming the post-synaptic GPR88 location in a subpopulation of asymmetrical synapses of the dorsal striatum. Dopamine and GABA terminals form symmetrical synaptic contacts with striatal projection neurons (Ribak & Roberts, 1990; Smith & Kieval, 2000). However, no associations were observed between GPR88 immunoreactivity and TH-positive axon terminals (Fig. 5C).

Effect of unilateral 6-hydroxydopamine lesion and L-DOPA treatment on striatal GPR88 expression

The extent of the lesion was assessed by the rotational behaviour induced by administration of apomorphine (0.05 mg/kg s.c.) at 15 days after 6-OHDA infusion. The loss of DA neurons induced by 6-OHDA in rats treated with L-DOPA or vehicle was assessed by in-situ hybridization of TH mRNA in the midbrain at the end of treatment. Analysis of autoradiograms confirmed that all 6-OHDA-lesioned rats included in this study had a complete loss of TH mRNA expression in the substantia nigra pars compacta of the lesioned side.
indicating a complete unilateral nigrostriatal dopaminergic deafferentation (Fig. 6A).

The effects of 6-OHDA-induced striatal dopamine depletion on the expression levels of both GPR88 protein and mRNA were evaluated by immunohistochemistry and in-situ hybridization, respectively, within the rat dorsal striatum. At 1 week after unilateral lesion by 6-OHDA, a significant decrease (~35%) in GPR88 immunolabelling was detected within the dopamine-depleted dorsal striatum (t_{8} = 4.17, P = 0.003) (Fig. 6E). At 29 days after lesion, the expression levels of both GPR88 protein (main effect of lesion, F_{1,56} = 11.16, P = 0.0015) (Bonferroni post-test, t_{20} = 2.88, P < 0.05) and mRNA (main effect of lesion, F_{1,63} = 6.59, P = 0.012) (Bonferroni post-test, t_{20} = 2.78, P < 0.05) were still decreased by 19 and 18%, respectively (Fig. 6F and G). Treatment with L-DOPA for 2 weeks (3 mg/kg/day; from day 15 to day 29 after neurotoxin infusion) completely normalized the levels of GPR88 in the dopamine-denervated striatum (Fig. 6F and G).

As GPR88 mRNA is expressed in both striatal ENK-positive and SP-positive MSNs, we then determined the subset of MSNs in which the GPR88 expression was regulated after 6-OHDA lesion and subsequent L-DOPA treatment. First, we found that the level of GPR88 mRNA expression in the dorsolateral intact striatum was twice as high in striatopallidal ENK-positive MSNs as in striatonigral SP-positive MSNs (Figs 6H and I, and 7G and H). After 6-OHDA lesion, within the dorsolateral denervated striatum, the level of GPR88 mRNA was markedly downregulated (~24%) in the subset of striatopallidal ENK-positive MSNs (main effect of lesion, F_{1,66} = 22.96, P < 0.0001) (Bonferroni post-test, t_{20} = 4.64, P < 0.001) and intact side vs. intact side of vehicle animals), whereas it was upregulated (+30%) in striatonigral SP-positive MSNs (Bonferroni post-test, t_{20} = 3.93, P < 0.001) in lesioned side vs. intact side of vehicle animals) (Fig. 6H and I). L-DOPA treatment completely reversed the variations of GPR88 expression induced by nigrostriatal dopamine denervation in both ENK-positive (Bonferroni post-test, t_{20} = 2.59, P < 0.05) and vehicle animals vs. lesioned side of L-DOPA animals) and SP-positive neurons (Fig. 6H and I).

Administration of the dopamine D1 antagonist SCH (0.1 mg/kg twice a day) partially blocked, but not significantly, the L-DOPA-induced normalization of global GPR88 protein and mRNA expression in the dorsal denervated striatum (Fig. 6F and G). In fact, SCH prevented L-DOPA-induced GPR88 normalization in ENK-positive MSNs (t_{12} = 5.26, P < 0.001) and L-DOPA-induced GPR88 normalization in SP-positive MSNs (t_{12} = 3.95, P < 0.001) vs. intact side of L-DOPA + SCH-treated animals but had no effect on L-DOPA-induced GPR88 normalization in SP-positive MSNs (main effect of treatment, F_{1,66} = 32.23, P < 0.0001; treatment \times lesion interaction, F_{1,63} = 25.52, P < 0.0001) (Bonferroni post-test, t_{16} = 6.27, P < 0.001) vs. intact side of vehicle animals and t_{12} = 4.47, P < 0.001) vs. lesioned side of L-DOPA animals) and global and striatopallidal MSN GPR88 expression levels in the denervated striatum (Bonferroni post-test, t_{12} = 4.22, P < 0.001) vs. lesioned side of L-DOPA + Halo animals vs. lesioned side of L-DOPA + Halo animals (Fig. 6F–H). However, Halo prevented a complete L-DOPA-induced normalization of GPR88 expression in striatonigral MSNs ipsilateral to the lesion and produced an obvious increase of GPR88 mRNA in striatonigral MSNs of the contralateral intact side (Bonferroni post-test, t_{12} = 7.66, P < 0.001) vs. intact side of L-DOPA + Halo animals vs. intact side of vehicle animals; t_{12} = 4.6, P < 0.001) vs. lesioned side of L-DOPA + Halo animals; t_{12} = 7.66, P < 0.001) vs. intact side of L-DOPA animals; t_{12} = 6.47, P < 0.001) vs. intact side of L-DOPA + SCH animals; t_{12} = 2.76, P < 0.05) vs. lesioned side of L-DOPA + Halo animals vs. lesioned side of L-DOPA + SCH animals (Fig. 6I).

**Effects of cortical ibotenate lesion on GPR88 expression in the striatum**

At 8 days after bilateral disruption of the corticostriatal pathway, induced by infusion of ibotenate into the somatosensory-motor cortex, a decrease in GPR88 protein (~23%, t_{11} = 4.38, P < 0.0001) (Fig. 7A, B and E) and mRNA (~23%, t_{10} = 6.82, P < 0.0001) (Fig. 7C, D and F) expression was detected in the dorsal striatum of both hemispheres. Analysis of double-labelled sections for GPR88 mRNA and ENK mRNA demonstrated that the ibotenate-induced corticostriatal pathway disruption produced a significant downregulation of GPR88 mRNA in the striatopallidal ENK-positive neurons (t_{13} = 14.48, P < 0.0001) (Fig. 7G), whereas the GPR88 mRNA was not significantly changed in SP-positive neurons (Fig. 7H).

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**Fig. 6.** Modulation of GPR88 expression in the dorsal region of the dopamine-depleted striatum after unilateral 6-OHDA infusion into the medial forebrain bundle and L-DOPA treatment (the dopamine denervated side is marked with an asterisk in A–D). (A) The unilateral disruption of the nigrostriatal dopamine system is demonstrated by the complete loss of TH mRNA expression in the left substantia nigra pars compacta (SNc). Representative coronal brain sections processed for immunohistochemistry (B) or in-situ hybridization (C and D) exhibit decrease of GPR88 expression (B and C) and L-DOPA-induced GPR88 mRNA normalization (D) on the lesioned side. Scale bars, 1 mm. The levels of GPR88 expression in the dorsolateral striatum (areas used for quantification in immunostained and hybridized sections are outlined with dashed lines) were significantly decreased after 7 days (E) and 29 days (B, C, F and G) of unilateral lesion by 6-OHDA (n = 5). Levels of GPR88 expression were normalized in the dopamine-depleted striatum after repeated administration of L-DOPA (L-DOPA/benserazide; 3 mg/kg/6 mg/kg i.p. once a day, n = 10–12) from day 15 to day 29 after lesion (D, F and G). The repeated co-administration of L-DOPA + SCH (0.1 mg/kg i.p. for 14 days, n = 7) partially blocked the L-DOPA effect (F and G), whereas co-administration of L-DOPA + Halo (1 mg/kg/day i.p. for 14 days, n = 7) did not block the L-DOPA effect (F and G). Quantitative data are expressed as mean + SEM values of GPR88 immunostaining density (E and F) and GPR88 mRNA (G) measured in the dorsal striatum of both the dopamine-depleted and contralateral intact side of rats. \( *P < 0.01 \) (E) and \( *P < 0.1 \) (F and G) vs. intact side of vehicle-treated animals. To determine the subpopulation of nigrostriatal GPR88 expression regulated by striatal afferents 407

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Discussion

The present study, using immunohistochemistry with a highly specific GPR88 polyclonal antibody and in-situ hybridization, provides evidence that GPR88 is richly expressed in the dorsal striatum, olfactory tubercle and nucleus accumbens of the rat and non-human primate. In these brain nuclei, GPR88 expression is nearly exclusively detected in the subsets of neurons that also express ENK and SP mRNA. We found that GPR88 protein is mainly confined to the perikarya and dendrites and preferentially concentrated in post-synaptic domains contacted by asymmetrical synapses. These findings suggest a potential role for GPR88 in modulating excitatory inputs on MSNs and basal ganglia functions. We further investigated the putative regulation of striatal GPR88 expression by dopamine and cortical glutamate innervations in the rat. Unilateral dopamine neuron lesion, caused by infusion of 6-OHDA in the medial forebrain bundle, produced a decrease in GPR88 expression in the striatopallidal ENK-positive and, inversely, a rise in the SP-positive MSN of dopamine-depleted striatum. These variations were nearly completely reversed by L-DOPA treatment. Another significant finding is that D1, but not D2 receptors, are involved in the regulation of GPR88 expression.

**Fig. 7.** Expression of GPR88 immunoreactivity (A and B) and mRNA (C and D) in the dorsal striatum at 8 days after bilateral ibotenate (63 mM, n = 6–9) (B and D) or vehicle infusion (A and C) in the cerebral cortex of rats. (A) Representative images of GPR88 expression in immunoperoxidase-stained (A and B) and hybridized (C and D) coronal sections at rostral striatal level (Bregma 0.70, rat brain stereotaxic coordinates of Paxinos & Watson, 1986) from vehicle- and ibotenate-lesioned animals. Scale bars, 1 mm. Quantification of GPR88 immunoreactivity (E) and mRNA (F) in the dorsal striatal sector of controls or corticostriatal-disrupted pathway animals. Results are expressed as the mean ± SEM values (n = 6–9). (E and F) ***P < 0.001 vs. vehicle-infused animals. (G and H) GPR88 mRNA levels in ENK-positive (ENK+ cells) (G) and ENK-negative (SP+ cells) (H) neurons from the same animals, expressed as mean number of silver grains per cell (mean ± SEM). ***P < 0.001 vs. vehicle-infused animals. Prot, protein.
D2, receptor activation exerts a positive influence on GPR88 expression in the striatopallidal pathway of denervated hemisphere. On the contrary, D2 receptor stimulation controls GPR88 expression in the nigrostriatal pathway.

**Specificity of the anti-GPR88 antibody**

The use of an immunopurified antibody raised against an intracellular epitope within the GPR88 C-terminus permitted sensitive detection of native GPR88 in the brain of several species (rat and non-human primate). The specificity of the antibody was thoroughly characterized through complementary criteria. First, western blot analysis from rat striatum and cortex established GPR88-specific immunoreactive bands ranging between 28 and 40 kDa, consistent with the molecular weight predicted for mature GPR88 at 40 kDa. The two bands observed from CHO/GPR88-transfected cell extracts, slightly lower than the predicted molecular weight, suggest the detection of unprocessed or cleaved GPR88 forms. Further work is necessary to confirm the existence of such incomplete GPR88, as this may simply be an artefact of the overexpression-based system. Second, in heterologous cultured cells, in-vitro RNA interference experiments provide solid evidence of the ability of the antibody to specifically recognize the GPR88 protein. Third, the specificity of the GPR88 antibody is also supported by the pattern of immunoreactivity of the native GPR88, which remarkably matches the mRNA cerebral distribution, and the fact that local infusion of lentivirus-shRNA targeting GPR88 produced a near-total loss of GPR88 immunoreactivity in the brain infected area. Furthermore, GPR88 immunolabelling had a strikingly similar distribution in forebrain structures of rodents and non-human primates. This antibody therefore appears to have the specificity required for the reliable and precise detection of native GPR88.

**Regional, cellular and subcellular expression of GPR88 in the striatum**

No data have previously been reported on the expression of GPR88 at the protein level. The present finding, demonstrating abundant GPR88 in rat striatum, olfactory tubercle and nucleus accumbens, is consistent with real time quantitative PCR and in-situ hybridization data showing that GPR88 transcripts are highly expressed in the rat and mouse striatum (Mizushima et al., 2000; Ghate et al., 2007). Interestingly, real time quantitative PCR data from rodents suggest that GPR88 displays the highest expression levels compared with other known striatal GPCRs (Massart et al., 2007, 2008). The conspicuous dorsolateral-to-ventromedial heterogeneous striatal distribution of GPR88 protein and transcripts, with highest densities in the dorsolateral sector, provides original anatomical evidence for potential GPR88 modulation of sensorimotor-related information (Voon et al., 2004). Furthermore, their enriched expression in the patch/striosome compartment points out that GPR88 may preferentially modulate limbic cortical/basal ganglia circuits (Ragsdale & Graybiel, 1988; Gerfen & Wilson, 1996). Double-labelling experiments further demonstrated that GPR88 mRNA is expressed by all of the MSNs (SP- and ENK-positive neurons), indicating that the GPR88 immunoreactivity detected throughout the striatal neuropile probably represents labelled dendrites of both the striatonigral direct and striatopallidal indirect pathway MSNs. This assumption is supported by our electron microscopic analysis, which revealed a high number of GPR88-labelled dendrite shafts, dendritic spines and cell bodies displaying characteristic features of GABAergic projection MSNs (Somogyi et al., 1982; Bolam et al., 1983) but not axonal or terminal immunoreactive profiles in the dorsal striatum.

Likewise, the globus pallidus and substantia nigra reticulata, two regions receiving the striatopallidal and striatonigral terminals, respectively (Surmeier et al., 2007), lack GPR88 immunoreactivity. We also observed GPR88 labelling at some intracytoplasmic membrane elements of dendrites and perikarya, probably reflecting the dynamic of GPR88 expression undergoing synthesis, assembly, trafficking and degradation and/or a reserve pool of receptors (Ko et al., 2002; Bernard et al., 2006). All of these morphological data support the idea that GPR88 is addressed principally to the dendritic tree and perikaryon but not to the axonal arborization of both projection striatopallidal and striatonigral MSNs and highlight a potential post-synaptic role for GPR88 in synaptic events and their integration into MSNs.

Our electron microscopy analysis shows the presence of GPR88 mainly at dendritic spines receiving asymmetrical synaptic contacts and, to a lesser extent, at the dendritic and perikaryal submembranous domains of symmetric synapses. Although particular attention was given to limit the diffusion of the peroxidase reaction product in order to establish a precise spatial subcellular localization of GPR88 protein, these results require confirmation with immunogold electron microscopy, which provides a more precise ultrastructural detection (Chan et al., 1990). GPR88-positive symmetrical (presumably inhibitory) synapses could be supplied by terminals originating from GABAergic aspiny or cholinergic interneurons, intrastral axon collaterals from projection MSNs or dopaminergic inputs from substantia nigra. We never detected TH-immunofluorescent terminals apposed to GPR88 signals, indicating that such synaptic contacts are not dopaminergic, but further investigation is required to verify this hypothesis. A striking ultrastructural finding of the current study is the detection of GPR88 at discrete post-synaptic submembranous sites in a large proportion of asymmetrical (excitatory) synapses that generally receive glutamate as neurotransmitter (Bouyer et al., 1984; Bolam et al., 2000). The presence of GPR88-immunoreactive signal apposed to most putative vesicular glutamate transporter 1-immunoreactive terminals indicates that the receptor may be preferentially located at synapses supplied by corticostriatal inputs but not vesicular glutamate transporter 2-immunoreactive thalamostriatal inputs (Herzog et al., 2001; Kaneko & Fujiyama, 2002; Fremeau, 2004). This interpretation is consistent with the observation that GPR88 was often detected on the head of spines where corticostriatal inputs mainly contact the MSN dendritic tree. These GPR88-positive synaptic contacts may represent a site at which the receptor modulates the response of MSNs to cortical glutamatergic projections, influencing (to some degree) the flow of cortical information through the basal ganglia. Furthermore, the cortical excitatory signal is modulated by the dopaminergic signal when dopamine terminals synapse on the neck of spines (Arbuthnot et al., 2000). Thus, the presence of GPR88 at specific sites where corticostriatal and nigrostriatal afferents converge, strongly suggests a role of GPR88 in modulating both glutamatergic and dopaminergic signals in MSNs.

**Regulation of GPR88 expression in the dorsal striatum by dopamine depletion and L-DOPA**

In agreement with previous data documenting the altered expression of striatal genes following nigrostriatal dopamine disruption (Gerfen, 2000; Napolitano et al., 2002), we show that, as early as 1 week after unilateral 6-OHDA-induced nigrostriatal pathway lesion, the level of GPR88 immunoreactivity was markedly decreased in the dopamine-depleted dorsal striatum. Even at 29 days after lesion, GPR88 immunoreactivity remained downreg-
ulated, which was confirmed by a parallel decrease of GPR88 transcripts. However, the amplitude of the variation was less than initially detected, suggesting a late post-lesional partial recovery of GPR88 expression underlying the compensatory mechanisms occurring in the dopamine-depleted hemisphere. Previous reported lesion studies have shown that, after 6-OHDA infusion, about 85–95% of the dopamine neurons have been definitively lost within the first week (Schwarting & Huston, 1996). Loss of dopamine-mediated transmission leads to functional compensatory responses by the remaining dopamine neurons (elevated dopamine biosynthesis, metabolism and release) and other afferent systems, including the corticostriatal pathway, which may contribute to the recovery of intrastriatal dopamine levels and synaptic configurations (Schwarting & Huston, 1996; Meshul et al., 1999; Avila-Costa et al., 2005). It is therefore likely that such compensatory mechanisms attenuate the neurobiological consequences in the dopamine-denervated striatum, leading to reduction of the amplitude of GPR88 variations.

The sustained overall reduction of GPR88 expression in dopamine-denervated dorsal striatum suggests that dopamine transmission might exert a tonic excitatory effect on GPR88 gene expression in both striatopallidal and striatonigral projection neurons. However, the double-labelling in-situ hybridization data demonstrated that dopamine depletion induces differential effects on GPR88 expression in the two subsets of striatal MSNs. Indeed, we observed a robust decrease but a moderate increase in GPR88 mRNA levels in striatopallidal/ENK-positive and striatonigral/ENK-negative (assumed to be striatopallidal/SP-positive) neurons, respectively. As our data and also recently reported data (Heiman et al., 2008) showed that striatal GPR88 mRNA expression is twice as high in striatopallidal as in striatonigral neurons of rodents, the overall lesion-induced GPR88 downregulation is consistent with the GPR88 decrease occurring in striatopallidal MSNs, not compensated by the increase occurring in striatonigral neurons.

A large amount of evidence indicates that dopamine D1 and D2 receptors segregate in striatopallidal and striatonigral neurons, respectively (Gerfen et al., 1990; Le Moine et al., 1990; Le Moine & Bloch, 1995) and that dopamine exerts opposite effects on these subsets of striatal projection neurons. Previous studies in rodents indicated that 6-OHDA lesions induced differential effects on neuropeptide gene expression in striatal MSNs (Gerfen et al., 1991; Salin et al., 1997). In agreement with an activating role of D1 receptor stimulation on the cAMP/protein kinase A signalling pathway, SP expression is decreased by dopamine depletion in striatopallidal neurons expressing D1 receptors. Conversely, the inhibitory role of the D2 receptor stimulation on cAMP/protein kinase A signalling is in agreement with an increase in ENK expression in striatopallidal neurons that possess D2 receptors. Subchronic L-DOPA treatment in rats and non-human primates differentially reverses gene expression changes in both striatal MSN subpopulations (Zeng et al., 1995, 2000; Salin et al., 1997; Gerfen, 2000; Wang et al., 2007) and induces early gene expression in the dopamine-depleted striatum (Svenningsson et al., 2000; Carta et al., 2005).

Interestingly, we found in D2 receptor/ENK-expressing striatopallidal neurons that, in contrast to striatonigral neurons containing D1 receptors, GPR88 expression was downregulated by dopamine lesion and the reversal of this effect by L-DOPA was dependent on D1 receptor stimulation, as indicated by the blockade of this reversal by the D1 receptor-selective antagonist SCH but not by Halo, a D2 receptor-selective antagonist. In parallel, in D1 receptor/SP-expressing striatonigral neurons, GPR88 expression was upregulated by the lesion, in contrast to striatopallidal neurons containing D2 receptor and the reversal of this effect by L-DOPA was dependent on D2 receptor stimulation, as indicated by the absence of effects of SCH. Moreover, co-administration of L-DOPA and the D2 receptor antagonist Halo raised GPR88 expression in the contralateral striatopallidal MSNs. These results suggest that the effects of L-DOPA on GPR88, in each of the two MSN subsets, are not directly mediated by the respective dopamine receptor subtype that they express but indirectly by dopamine receptor transmission, which can modulate other neurotransmitter afferent inputs to the MSNs. In particular, L-DOPA and intrastratall dopaminergic transmission can act as a neuromodulator of glutamate release in the dopamine-depleted striatum (Jonkers et al., 2002; reviewed in David et al., 2005). Thus, the L-DOPA-induced differential changes in GPR88 levels on both MSN subpopulations may be mediated through altered corticostriatal glutamatergic neurotransmission mechanisms, as previously suggested for other striatal markers expressed in these neurons (Zeng et al., 1995; Salin et al., 1997; Carta et al., 2005; Wang et al., 2007).

Our finding revealing a preferential concentration of GPR88 in a large proportion of dendritic spines receiving excitatory asymmetrical synaptic contacts (assumed to be glutamatergic inputs) provides an anatomical basis suggesting a tight interplay between GPR88 and corticostriatal neurotransmission, and is consistent with a modulatory role of glutamate inputs on GPR88 expression. In this respect, degeneration of nigrostriatal dopamine neurons can induce secondary disturbances affecting cortical glutamate inputs to striatal MSNs (Deutsch, 2006; Neely et al., 2007). For instance, human post-mortem studies and experimental animal models of Parkinson’s disease indicate that striatal dopamine depletion produces dystrophic changes in the dendrites, including decreased spine density, of striatopallidal neurons (Ingham et al., 1989, 1993; Stephens et al., 2005; Zaja-Milatovic et al., 2005; Day et al., 2006). Such dystrophic effects in striatopallidal MSNs seem to result from the loss of D2 receptor stimulation, which induces increased excitability to glutamate and elevated cytosolic Ca2+ concentrations at distal dendritic locations (Day et al., 2008). These observations are consistent with the idea that dopamine depletion-induced changes in GPR88 levels in striatopallidal MSNs might be mediated by altered D2 receptor/glutamatergic transmission interacting mechanisms. Accordingly, the present findings show that bilateral cortical lesion decreases, as does the dopamine depletion, the GPR88 levels in the dorsolateral striatum. Moreover, corticostriatal deafferentation induced a marked GPR88 mRNA downregulation in the striatopallidal pathway without significantly affecting GPR88 in striatopallidal SP-positive neurons. These data agree with the involvement of corticostriatal glutamatergic inputs in the effects of dopamine depletion on GPR88 changes in striatal MSNs and with a greater influence of cortical inputs on GPR88 expression in a subset of striatopallidal MSNs. Interestingly, anatomical and electrophysiological data support differential cortical innervation, including the size of terminals and glutamate release, on striatopallidal and striatonigral MSNs (Cepeda et al., 2004; Lei et al., 2004). Moreover, ENK mRNA expression is under the control of corticostriatal glutamatergic input activity (Uhl et al., 1988; Salin & Chesselet, 1992; Hajji et al., 1996).

L-DOPA effects on GPR88 expression in the striatopallidal pathway are probably regulated through D1 receptor-mediated corticostriatal input activation. For instance, it has been shown that L-DOPA induces immediate-early gene expression in the dopamine-denervated cortex and striatum of the common marmoset (Svenningsson et al., 2000) and increases glutamate release in the dopamine-depleted rat striatum (Jonkers et al., 2002). Moreover, D1 receptor stimulation by the selective agonist SKF38393 induces D3 receptor overexpression in the dopamine-depleted rat dorsal striatum (Bordet et al., 1997) via
increased corticostriatal glutamatergic input activation-mediated mechanisms (Guillin et al., 2001). One possibility is that such effects might be mediated via D1 receptor stimulation, present on the soma and dendrites of excitatory corticostriatal neurons (Huang et al., 1992). Alternatively, sensitized D1 receptor activation in the dopamine-denervated striatum may exert influences on corticostriatal glutamatergic inputs to the striatum through neuronal loops connecting the basal ganglia, thalamus and cerebral cortex (Svenningsson et al., 2000; Taymans et al., 2005). Thus, D1 receptor-mediated corticostriatal afferent activity may enhance glutamate transmission mechanisms involved in GPR88 gene expression in striatopallidal neurons. Interestingly, increasing evidence suggests that ATP is co-released with glutamate by activation of the glutamatergic terminals and converted to adenosine by ectonucleotidases (Ferre et al., 2007). Adenosine is another important modulator of striatal glutamatergic neurotransmission through its actions on adenosine receptors (Fredholm et al., 2001), which are abundant in the striatum. Striatopallidal MSNs express, in addition to the inhibitory D2 receptors, facilitatory adenosine A2A and glutamate mGlu5 receptors (Schiffmann et al., 1991; Ferre et al., 1997, 2002, 2005; Svenningsson et al., 1999). A2A receptors form heteromeric complexes with D2 and metabotropic glutamate mGlu5 receptors, which are located post-synaptically at glutamatergic synapses, in a strategic position to modulate corticostriatal input activity in striatopallidal MSNs (Hettinger et al., 2001; Ferre et al., 2005; Ciruela et al., 2006). Co-stimulation of mGlu5/A2A receptors exerts a synergistic effect, by means of interactions in the A2A/D2/mGlu5 heteromeric receptor complexes, on their ability to inhibit dopamine binding to D2 receptors and on mitogen-activated protein kinase signalling activation (Popoli et al., 2001; Ferre et al., 2002; Nishi et al., 2003). It is therefore likely that the reversal of striatal dopamine depletion effects on GPR88 levels in striatopallidal MSNs induced by L-DOPA/D1 receptor stimulation results from an increased corticostriatal input function and synergistic mGlu5/A2A receptor interaction-mediated mechanisms. However, further experiments are clearly required to clarify the precise involvement of glutamate/adenosine receptor-mediated mechanisms in the modulation of GPR88 expression in this striatal MSN subpopulation.

After dopamine disruption, GPR88 expression was moderately increased in striatonigral MSNs and L-DOPA normalized GPR88 levels in dopamine-depleted striatum. The reversal of the dopamine lesion effect depends on D2 receptor stimulation (see above). Given the expression of D2 receptors in striatopallidal neurons and striatal glutamatergic terminals (Cepeda et al., 2001; David et al., 2005), it is unlikely that D2 receptors directly mediate GPR88 gene expression in striatonigral neurons. This suggests rather an indirect L-DOPA/D2 receptor-mediated effect on GPR88 through the alteration of glutamate corticostriatal inputs. It is tempting to speculate that the L-DOPA/D2 receptor stimulation-induced GPR88 decrease in the striatonigral pathway was mediated by reduced glutamate release from corticostriatal inputs. Accordingly, data from mouse brain slices indicate that dopamine inhibits cortical glutamate release by pre-synaptic D2 receptor stimulation (Bamford et al., 2004). We show here that, like the L-DOPA-induced effect, cortical ibotenate lesion tended to reduce the basal levels of GPR88 expression in striatonigral pathway neurons. It is therefore likely that enhanced release from corticostriatal terminals by pre-synaptic D2 receptor blockade with Halo or, conversely, decreased corticostriatal input activity by L-DOPA/D2 receptor stimulation might induce upregulation and downregulation of GPR88 expression, respectively, in dopamine-deafferentated striatonigral MSNs. Metabotropic glutamate mGlu1 receptors, which activate gene induction pathways and are mainly expressed by striatonigral MSNs (Kerner et al., 1997; Mao et al., 2008), are potential candidates to mediate such effects.

Our findings show that the co-administration of L-DOPA and the D2 receptor antagonist Halo raised GPR88 expression in the contralateral striatopallidal MSNs, indicating that L-DOPA via D1 receptor stimulation induces GPR88 gene expression in both the dopamine-depleted and dopamine-intact contralateral striatum. This finding is supported by studies showing in unilateral 6-OHDA-lesioned rats that the systemic administration of SKF82958, a selective D1 receptor agonist, induces regulator of G protein signalling 2 and c-fos mRNA expression in the striatum of both the lesioned and non-lesioned hemispheres (Taymans et al., 2005). Also, unilateral D1 receptor stimulation by intrastriatal injection of SKF38393 in the normal rat induces c-fos in the contralateral nucleus accumbens via polysynaptic circuits (Blandini et al., 2003). Our results thus provide support for the hypothesis that D1 receptor activation by L-DOPA can activate gene induction pathways bilaterally in the unilateral hemiparkinsonian rat model.

Conclusions

Striatonigral and striatopallidal projection MSNs play an important role in integrating circuits of the basal ganglia/basal forebrain and findings on new GPCR proteins in these two important striatal output pathways may contribute to a better understanding of certain pathophysiological states (e.g. movement and psychiatric disorders). Hence, the rich expression of GPR88 in the two major striatal projection MSNs directly receiving dopaminergic and glutamatergic inputs provides an anatomical basis for potential therapeutic applications, particularly in the striatum where glutamatergic and dopaminergic functions have important consequences for Parkinson’s disease, schizophrenia and addiction. Our experimental findings are consistent with the idea that striatal GPR88 expression is modulated by dopamine- and glutamate-regulated mechanisms involving trans-synaptic influences on the corticostriatal pathway activity, rather than by a direct modulation of MSNs by dopamine.

Supporting information

Additional supporting information may be found in the online version of this article:

Fig. S1. GPR88 expression in the brainstem and amygdala complex of the rat brain.

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Abbreviations

CHO, Chinese hamster ovary; cRNA, complementary RNA; DIG, digoxigenin; ENK, preproenkephalin; GFP, green fluorescent protein; GPCR, G protein-coupled receptor; Halo, haloperidol; HEK, human embryonic kidney; MSN, medium spiny neuron; 6-OHDA, 6-hydroxydopamine; SCH, SCH23390; shRNA, small hairpin RNA; siRNA, small interfering RNA; SP, preprotachykinin/substance P; TBS, Tris buffer, 150 mM NaCl, pH 7.5; TBS–GT20, TBS containing 0.1% gelatin and 0.05% Tween 20; TBS–NDST20, TBS containing 5% normal donkey serum and 0.05% Tween 20; TH, tyrosine hydroxylase.

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