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Regular Article

G-protein coupled receptor 6 deficiency alters striatal dopamine and cAMP concentrations and reduces dyskinesia in a mouse model of Parkinson's disease

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ABSTRACT

The orphan G-protein coupled receptor 6 (GPR6) is a constitutively active receptor which is positively coupled to the formation of cyclic adenosine-3′,5′-monophosphate (cAMP). GPR6 is predominantly expressed in striatopallidal neurons. Here, we investigated neurochemical and behavioural effects of Gpr6 deficiency in mice. Gpr6 depletion decreased in vivo cAMP tissue concentrations (20%) in the striatum. An increase of striatal tissue dopamine concentrations (10%) was found in $Gpr6^{-/-}$ mice, whereas basal extracellular dopamine levels were not changed compared with $Gpr6^{+/+}$ mice, as shown by in vivo microdialysis. Western blot analyses revealed no alteration in the expression and subcellular localisation of the dopamine D2 receptor in the striatum of $Gpr6^{-/-}$ mice, and the number of tyrosine hydroxylase positive neurons in the substantia nigra was unchanged. DARPP-32 (dopamine and cAMP-regulated phosphoprotein of 32 kDa) expression in the striatum of $Gpr6^{-/-}$ mice was not altered, however, a twofold increase in the phosphorylation of DARPP-32 at Thr34 was detected in $Gpr6^{-/-}$ compared with $Gpr6^{+/+}$ mice. $Gpr6^{-/-}$ mice showed higher locomotor activity in the open field, which persisted after treatment with the dopamine D2 receptor antagonist haloperidol. They also displayed reduced abnormal involuntary movements after apomorphine and quinpirole treatment in the mouse dyskinesia model of Parkinson's disease.

In conclusion, the depletion of Gpr6 reduces cAMP concentrations in the striatum and alters the striatal dopaminergic system. Gpr6 deficiency causes an interesting behavioural phenotype in the form of enhanced motor activity combined with reduced abnormal involuntary movements. These findings could offer an opportunity for the treatment of Parkinson's disease beyond dopamine replacement.

Introduction

G-protein coupled receptors (GPCRs)[2] are a large group of transmembrane proteins involved in extra-to-intra-cellular signal transduction. In the brain, GPCRs are essential in neurotransmission by mediating the signal transduction of several neurotransmitters. For instance the dopamine (DA) D1 receptor is coupled to a stimulatory G-protein and leads to an increase of cyclic adenosine-3′,5′-monophosphate (cAMP) concentrations. In contrast, the dopamine D2 receptor decreases cAMP after activation (Pivonello et al., 2007).

Several putative GPCRs were identified by homology to known GPCRs. One of these orphan GPCRs is the G-protein coupled receptor 6 (GPR6). The constitutively active GPR6 is coupled to a stimulatory G-protein and increases cAMP (Uhlenbrock et al., 2002). There is a controversy about the endogenous ligand. Initial studies showed agonism for the lysophospholipid sphingosine-1-phosphate (S1P) but these results were not confirmed by other groups (Ignatov et al., 2003; Uhlenbrock et al., 2002; Yin et al., 2009). GPR6 is predominantly expressed in the striatum but knowledge of its function is sparse (Chenn et al., 2001; Roth et al., 2006; Tanaka et al., 2007). Lobo et al. (2007) identified GPR6 as a regulator of instrumental conditioning which is supported by a recent study in humans (Collins and Frank, 2012). Additionally, increased neurite outgrowth after the overexpression of GPR6 in vitro has been reported (Tanaka et al., 2007).

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[2] 3-MT, 3-methoxytyramine; 6-OHDA, 6-hydroxydopamine; aCSF, artificial cerebrospinal fluid; AIM, abnormal involuntary movement; cAMP, cyclic adenosine-3′,5′-monophosphate; cGMP, cyclic guanosine-3′,5′-monophosphate; D2R, dopamine D2 receptor; DA, dopamine; DOPAC, 3,4-dihydroxyphenylacetic acid; GABA, γ-amino butyric acid; DARPP-32, dopamine and cAMP-regulated phosphoprotein of 32 kDa; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GPCR, G-protein coupled receptor; GPR6, G-protein coupled receptor 6; HVA, 4-hydroxy-3-methoxyphenylacetic acid; LC–MS/MS, liquid chromatography/tandem mass spectrometry; L-DOPA, L-3,4-dihydroxyphenylalanine; MSN, medium spiny neuron; P-DARPP32, DARPP-32 phosphorylated at Thr34; PD, Parkinson's disease; PKA, proteinkinase A; S1P, sphingosine-1-phosphate; SNpc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

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The striatum, the site of the most intense GPR6 expression, is a brain region important for the selection and initiation of motor movements. It consists predominantly of two types of medium spiny neurons (MSNs) that differ in expression of DA receptors. About one half of the MSNs express the D1 receptor and project into the internal globus pallidus and the substantia nigra pars reticulata (direct, striatonigral pathway). The other subtype of MSNs contains the D2 receptor and inhibits the external globus pallidus (indirect, striatopallidal pathway). Both types of MSNs release the inhibitory neurotransmitter γ-amino butyric acid (GABA) after activation (Kreitzer and Malenka, 2008). GPR6 is expressed in striatopallidal, D2 receptor-positive neurons (Heiman et al., 2008; Lobo et al., 2007). DA is released in the striatum by neurons located in the substantia nigra pars compacta (SNpc) and subsequent D1 and D2 receptor activation controls the basal ganglia motor loop and enhances movements. Degeneration of the dopaminergic neurons in the SNpc, such as occurs in patients of Parkinson's disease (PD), results in an impaired DA signalling in the striatum. The clinical symptoms of the striatal DA loss in PD are disturbances in the motoric system, i.e. bradykinesia, rigidity, tremor and postural instability (Kreitzer and Malenka, 2008).

The objective of our study was to investigate the effects of Gpr6 deficiency in a mouse model on the striatal dopaminergic system in terms of: neurochemical alterations, by measuring cAMP and dopamine concentration in the tissue and in the extracellular space; behavioural consequences, by measuring L-3,4-dihydroxyphenylalanine (L-DOPA)-induced abnormal involuntary movements (AIMs) and haloperidol-induced inhibition of motor activity.

Material and methods

Animals

Homozygous Gpr6 deficient mice (B6.129P2-Gpr6^{tm1Dgen}) that were backcrossed for at least four times to the C57BL/6 background were purchased from Deltagen, Inc. (San Mateo, USA) (Lobo et al., 2007). The single exon of the Gpr6 gene was depleted by the insertion of a LacZ-Neo construct. The Gpr6$^{-/-}$ colony was expanded by Charles River Laboratories (Sulzfeld, Germany). Male animals were used in all experiments and kept under a 12 h light/dark cycle (lights on: 06:00–18:00) in temperature (23 ± 2 °C) and humidity (55 ± 5%) controlled rooms. The mice had ad libitum access to food and water.

All animal studies were approved by the appropriate institutional governmental agency (Regierungspraesidium Tuebingen, Germany) and carried out in an AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care International)-accredited facility in accordance with the European Convention for Animal Care and Use of Laboratory Animals.

Cyclic AMP and cyclic guanosine-3′,5′-monophosphate (cGMP) measurement by liquid chromatography/tandem mass spectrometry (LC–MS/MS)

The mice were killed by cervical dislocation and the striatum was dissected out of the brain after decapitation. The striatum was homogenised in 500 μL of 0.4 M perchloric acid by sonication. After centrifugation (20,000 × *g*, 10 min, 4 °C), the pellet was resuspended in 250 μL of 1 M NaOH, centrifuged and the protein concentration measured by a commercial BCA protein assay kit (Pierce Biotechnology, Rockford, USA). The supernatant was filtered through a 0.22 μm membrane and analysed by LC–MS/MS.

LC–MS/MS analysis of cAMP and cGMP was carried out as described previously (Oeckl and Ferger, 2012) using an Agilent 1200 LC system (Agilent Technologies, Morges, Switzerland) and API4000 triple quadrupole mass spectrometer (AB Sciex, Ontario, Canada). Mobile phase A consisted of 0.1% formic acid in water and mobile phase B of 100% acetonitrile. The following gradient elution profile was applied at a flow rate of 0.4 mL/min: 0.00 min: 100% A, 0.50 min: 100% A, 1.00 min: 10% A, 2.20 min: 10% A, 2.30 min: 100% A, 3.50 min: 100% A. Cyclic AMP and cGMP were detected by multiple reaction monitoring using the following transitions: cAMP 330.08–136.10, cGMP 346.15–152.10. Stable isotope-labelled [$^{13}C_5$]cAMP and [$^{15}N_5$]cGMP were added to all samples and standards as internal standard.

HPLC analysis of DA and DA metabolites

Striatal tissue was prepared as described for the LC–MS/MS analyses. Microdialysates were analysed directly. Isocratic separation of DA and its metabolites 3,4-dihydroxyphenylacetic acid (DOPAC), 4-hydroxy-3-methoxyphenylacetic acid (HVA) and 3-methoxytyramine (3-MT) was carried out with a reversed-phase C18 column (YMC-Pack ODS-AQ, 100 × 2.1 mm, S-3 μm). An electrochemical cell with a glassy carbon electrode and an ISAAC Ag/AgCl reference electrode (Antec VT-03, Leyden, The Netherlands) were used for detection. The mobile phase consisted of 1.7 mM 1-octanesulfonic acid sodium salt, 1.0 mM $Na_2EDTA \times 2\ H_2O$, 8.0 mM NaCl, 100 mM $NaH_2PO_4 \times 2\ H_2O$ (pH 3.80), mixed with 9.3% acetonitrile, and was delivered with a flow rate of 0.4 mL/min.

SDS-PAGE and immunoblot analysis

Total cell lysates of striatal tissue were prepared by sonication in RIPA buffer containing a protease inhibitor cocktail (both Sigma-Aldrich Chemie GmbH, Steinheim, Germany) and subsequent centrifugation (8000 × *g*, 10 min, 4 °C). For subcellular fractionation the striatal tissue was homogenised by successive passage through 22 and 26 gauge needles with a Hamilton syringe. The fractionation was carried out as described previously with all buffers containing phosphatase inhibitor cocktail 1 + 2 (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) (Xiao et al., 2009).

Proteins were separated on a NuPAGE® 4–12% or 12% Bis–Tris gel (Invitrogen GmbH, Darmstadt, Germany) by SDS-PAGE and blotted onto a nitrocellulose membrane. The following antibodies were used for immunoblot analyses: rabbit anti-tyrosine hydroxylase (TH) antibody (1:1000, Merck KGaA, Darmstadt, Germany), rabbit anti-dopamine D2 receptor antibody (1:1000, Millipore GmbH, Schwalbach, Germany), rabbit anti-DARPP-32 (dopamine and cAMP-regulated phosphoprotein of 32 kDa) antibody (1:2500, Cell Signalling Technology, Danvers, USA), rabbit anti-phospho-DARPP-32 (pThr34) antibody (1:500, AbD Serotec, Düsseldorf, Germany) and chicken anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) antibody (1:10,000, Sigma-Aldrich Chemie GmbH, Steinheim, Germany). We used IRDye®-labelled secondary antibodies to visualise the proteins of interest by the Odyssey® Infrared Imaging System (both LI-COR Biotechnology GmbH, Bad Homburg, Germany).

Immunohistochemistry and counting of TH-positive neurons

Brains were immersion-fixed in 4% buffered formalin for four days at 4 °C. After embedding in paraffin, 10 μm serial sections of the SNpc were prepared. Every 6th section was immunostained for TH. In brief, the endogenous peroxidase was blocked with 5% methanol and 0.04% H_2O_2 in PBS for 10 min. Epitope-retrieval was carried out with 10 mM citrate buffer (pH 6.0) at 90–95 °C for 10 min. After treatment with 0.5% Triton X-100 for 10 min and blocking with 2% bovine serum albumin and 2% goat serum for 1 h, the sections were incubated with rabbit anti-TH antibody (1:1000) overnight at 4 °C. TH-positive neurons were visualised with the Vectastain ABC Kit (Vector Laboratories, Inc., Burlingame, USA) and 3,3′-diaminobenzidine. Nuclei were counterstained with haematoxylin.

Immunohistochemically stained slides were scanned with a MIRAX SCAN (Carl Zeiss MicroImaging GmbH, Jena, Germany). TH-positive neurons in the SNpc were counted in every section by an investigator blind to the genotype. The neuron number of all sections belonging to