Ignatov et al., 2003b). HA had no effect on GPR99, GPR100, GalRL, GPR1, GPR7, GPR8, GPR19, GPR75 and SALPR, just to name a few. Of special interest was a sub-branch of GPCRs that regulate cellular proliferation, namely the endothelin, bombesin and neuromedin receptors. Two orphan receptors are part of this group: GPR37 and GPR37L1 (Marazziti et al., 2001). We focused our interest on GPR37 because of its prominent expression in neurons of the brain compared with a more glial location of GPR37L1 (Marazziti et al., 1997; Zeng et al., 1997). GPR37 has also been isolated and characterised as a substrate for the ubiquitin ligase parkin, hence its alternative name – parkin-associated endothelin-like receptor (Pael R) (Imai et al., 2001). GPR37 was shown to fold improperly in the absence of parkin, and its aggregation to insoluble complexes results in endoplasmic reticulum stress (Imai et al., 2001; Imai et al., 2003). This leads to preferential loss of dopaminergic neurons in the substantia nigra and contributes to neurodegeneration in Parkinson's disease (Yang et al., 2003). Accumulation of GPR37 in Lewy bodies in the brain of patients with Parkinson's disease supports this notion (Murakami et al., 2004).

To study a possible interaction of HA with GPR37, various assay systems were used that allow detection, directly or indirectly, of ligand-receptor interactions. In this paper, we present evidence that HA is a high-affinity ligand for GPR37.

Results

HA stimulates internalisation of GPR37 in COS-7 cells

We tried to express GPR37 heterologously in Chinese hamster ovary (CHO-K1) cells, in human embryonic kidney (HEK-293) cells and in green monkey kidney (COS-7) cells. Transient transfection efficiencies in HEK-293 and CHO-K1 cells were far below 5%, and cells expressing GPR37 looked sick and decreased in number at 48 hours compared with 24 hours after transfection. Transfection efficiencies in COS-7 cells were better and reached levels in the range of 15-30% (Fig. 1A). COS-7 cells were therefore suitable for experiments with individual, transfected cells. There was no difference in expression levels between GPR37 with (Fig. 1B) and without (Fig. 1C) FLAG tag at the C-terminus. This indicated that the tag did not interfere with GPR37 protein biosynthesis and localisation. GPR37 immunoreactivity was visible in the cytoplasm of transfected COS-7 cells, but also extended to cell protrusions, hinting at cell-surface expression (Fig. 1B,C). Cell-surface expression was confirmed by treating living cells before fixation with a monoclonal antibody against GPR37 (Fig. 1D) that reacts with extracellular epitopes of GPR37 (Imai et al., 2001).

HA treatment of COS-7 cells transiently transfected with GPR37-FLAG led to internalisation of the receptor. This was visible as disappearance of the GPR37-FLAG immunoreactivity from the protrusions after 10 minutes (compare Fig. 1E and F), and as translocation into the cytoplasm after 20 minutes (Fig. 1G). Protrusions started to show FLAG staining again after 30-60 minutes (Fig. 1H,I).

GPR37 aggregation is prevented by stable inducible expression in HEK-293 cells

Transient expression of GPR37 led in all cell lines assayed to aggregation of complexes with apparent molecular masses of ≥250 kDa (Fig. 2A). Surface biotinylation showed that only the monomeric receptor appeared at the outer cell membrane (Fig. 2B), indicating that most of the overproduced GPR37 was not properly folded, stayed in the cytoplasm and was probably

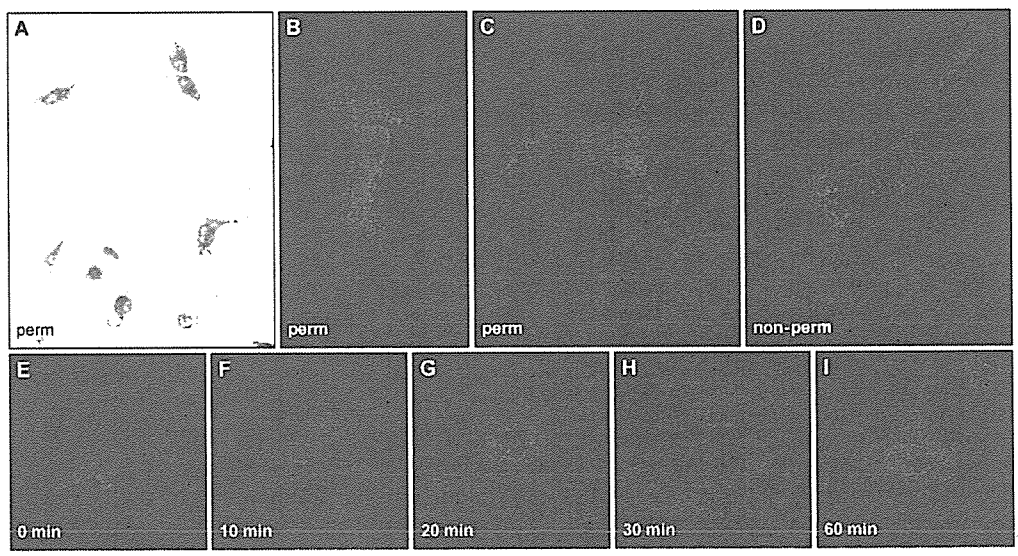

Fig. 1. GPR37 is expressed at the cell surface of COS-7 cells and internalises after HA treatment. (A-I) COS-7 cells were transfected with GPR37 with (B,D-I) or without (A,C) FLAG tag, immunostained with anti-GPR37 antibody (A,C,D) or with anti-FLAG antibody (B,E-I) and visualised with alkaline phosphatase-coupled secondary antibodies for light microscopy (A) or with Cy3-coupled antibodies for confocal analysis (B-I). Cells were permeabilised (perm) by fixation with 1% acetic acid in ethanol and by washing with Triton X-100, except in D, where living cells were incubated with the primary antibody before fixation (non-perm) to show surface staining. (E-I) COS-7 cells 48 hours after transfection with GPR37-FLAG were treated at 37°C with 2 nM HA for 0, 10, 20, 30 and 60 minutes, respectively, and immunostained with anti-FLAG antibody.

degraded (Imai et al., 2001). To prevent aggregation and subsequent degradation, we integrated GPR37 stably into HEK-T-REx cells with a construct that allowed induction by tetracycline (HEK-T-REx–GPR37). Incubation of cells with the tetracycline derivative doxycycline for 24 hours resulted in production predominantly of the monomeric form of GPR37 (Fig. 2C, first lane). Without doxycycline induction, GPR37 was not detectable (Fig. 2C, second lane). Confocal image analysis revealed that, after induction with doxycycline, GPR37 localised mainly to the outer cell membrane, both in permeabilised (Fig. 2D) and non-permeabilised cells (Fig. 2F). The non-induced cells showed no GPR37 immunoreactivity (Fig. 2E,G). To study internalisation, HEK-T-REx–GPR37 cells were incubated in defined medium for 24 hours with doxycycline to induce GPR37 expression. Subsequent treatment with HA for 10 minutes led to rapid internalisation of GPR37 (Fig. 2H,I). This internalisation was much faster in HEK than in COS-7 cells, probably as a result of differences in β-arrestin levels (Ménard et al., 1997).

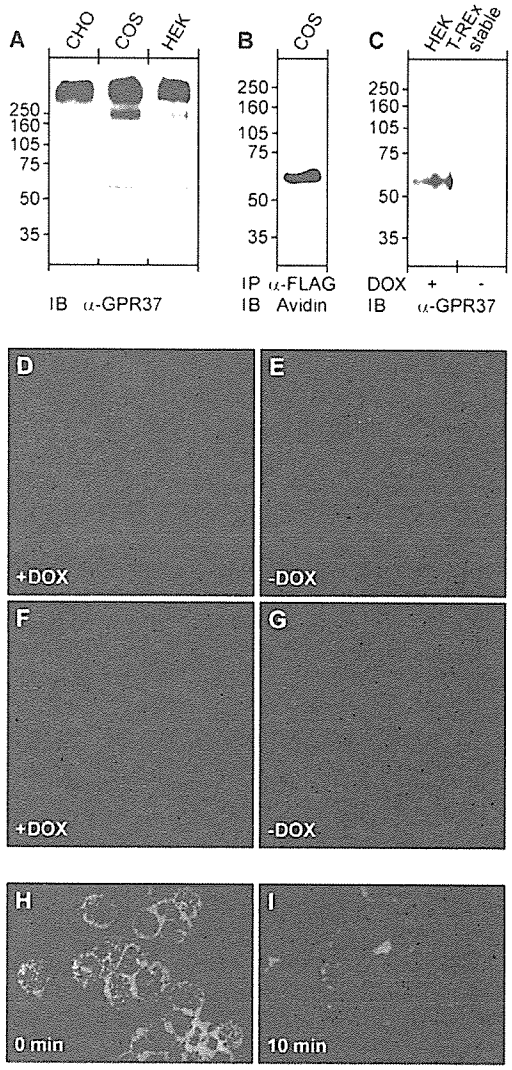

Fig. 2. Inducible, stable expression of GPR37 prevents aggregate formation. (A) CHO-K1, COS-7 and HEK-293 cells were transiently transfected with GPR37, and membrane fractions were assayed by immunoblotting (IB) with anti-GPR37 antibody (α-GPR37). (B) COS-7 cells transiently transfected with GPR37-FLAG were cell-surface biotinylated, and the solubilised membrane fraction was immunoprecipated (IP) with anti-FLAG antibody (α-FLAG) and visualised after immunoblotting with avidin. (C) GPR37 was introduced stably into the flip-in cell line HEK-T-REx, where GPR37 expression is inducible by doxycycline (DOX). Membrane fractions were subjected to western blotting with anti-GPR37 antibody (α-GPR37) with (first lane) and without (second lane) induction for 24 hours with doxycycline. (D-I) HEK-T-REx–GPR37 cells with (D,F,H,I) and without (E,G) doxycycline induction for 24 hours were immunostained with anti-GPR37(R2) antibody after permeabilisation (D,E) and with anti-GPR37 antibody without permeabilisation (F-I). (H,I) HEK-T-REx–GPR37 cells were treated with 2 nM HA for 0 and 10 minutes at 37°C, respectively, fixed with 2% formaldehyde for 10 minutes and subsequently immunostained with anti-GPR37 antibody.

HA binds to GPR37

To show direct interaction of HA with GPR37, COS-7 cells were analysed after incubation with 2 nM HA by fluorescence resonance energy transfer (FRET). Localisation of HA was detected with a HA-specific polyclonal antiserum and was visualised with a Cy2-coupled secondary antibody (green). To detect GPR37, monoclonal antibodies directed against the extracellular domain of human GPR37 were used in combination with a Cy3-coupled secondary antibody (red). Fig. 3A-D shows a typical example of FRET between HA and ectodomains of GPR37. After bleaching a discrete area in a GPR37-positive cell (Fig. 3A,B), an increase in HA fluorescence was observed (Fig. 3C,D). The difference in staining pattern is due to the fact that COS-7 cells, in addition to GPR37, express endogenous HA receptor(s) (Boels et al., 2001). The experiment was repeated several times on different days yielding similar results. On average, the calculated energy-transfer efficiencies were in the range of 19.4±4.5%, indicating the close association of GPR37 and HA. Non-transfected cells were negative, and no transfer of signal was obtained if an antibody against the FLAG tag at the C-terminus of GPR37 was used (data not shown).

For visualisation of HA binding to GPR37, a fluorescent derivative of HA was produced. For this purpose, the fluorophore Cy3B was coupled to the ε-amino group of Lys7 of HA. The neuroblastoma cell line NH15-CA2, which reacts with HA (Ulrich et al., 1996) and endogenously expresses GPR37 (Fig. 3E), was used as positive control. Binding of Cy3B-labelled HA to NH15-CA2 cells was observed starting from a concentration of 50 nM, with optimal binding at 150 nM, achieved after incubation for 10 minutes at 37°C (Fig. 3F). Pre-incubation with unlabelled HA for 50 minutes prevented Cy3B-HA binding (Fig. 3G). Cy3B-labelled HA did not bind to HEK-T-REx–GPR37 cells without induction of GPR37 expression by doxycycline (Fig. 3H), but reacted after induction for 24 hours with doxycycline (Fig. 3I). Pre-incubation with unlabelled HA inhibited binding (Fig. 3J), demonstrating that the two ligands compete for the same receptor and that the receptor is either occupied or, more likely, internalised after interaction with HA.

HA induces an increase in Ca^{2+} mobilisation in cells expressing GPR37

To confirm the interaction of HA with GPR37, Ca^{2+} mobilisation was measured in CHO-K1 cells stably

transfected with apoaequorin as Ca^{2+} sensor and with the promiscuous G-protein subunit Gα16 for signal enhancement. After reconstitution with the aequorin cofactor coelenterazine, agonist action was monitored as increase in bioluminescence (Stables et al., 1997). Since GPR37 was not sufficiently expressed in this cell line (CHO) by transient transfection, a stable cell line was established that, in addition to Gα16 and apoaequorin, also expressed GPR37 (CHO-GPR37). Treatment of these cells with HA dose dependently led to an increase in Ca^{2+} mobilisation with an EC_{50} value of 3.3 nM (Fig. 4A). To our surprise, an endogenous response was also observed (Fig. 4A). Northern blots were negative, but immunocytochemistry (Fig. 4B) and western blots (Fig. 4C) probed with an antiserum against the very conserved intracellular C-tail confirmed presence of GPR37 in CHO cells. The hippocampal mouse cell line HT22, expressing GPR37 endogenously, was used as a positive control (Fig. 4C). The active monomeric form of GPR37 was predominantly present both in CHO-GPR37 and HT22 cells.

HA stimulates a current increase in frog oocytes expressing GPR37

In our hands, the frog oocyte system has proven to be very reliable and robust for studying the interaction of ligands with orphan GPCRs (Ignatov et al., 2003a; Ignatov et al., 2003b). Since HA signal transduction for mitotic stimulation is coupled to an inhibitory G protein (Kayser et al., 1998; Ulrich et al., 1996), frog oocytes were injected not only with complementary RNAs (cRNAs) coding for human GPR37, but also with cRNAs coding for the G-protein-coupled inwardly rectifying K^+ channel GIRK, which is activated by βγ subunits of inhibitory G proteins (Kofuji et al., 1995). The concatemer between GIRK1 and GIRK2 (GIRK1/2) was chosen to enhance the current increase and improve the signal to noise ratio (Wischmeyer et al., 1997). Treatment with HA led to an additional increase in the basal inward current induced by changing the external bath medium to high K^+ in oocytes expressing GPR37 together with GIRK1/2 (Fig. 5A). A minute, negligible response was also obtained with medium alone (Fig. 5A). The effect of HA was concentration dependent, and a dose-response curve yielded an EC_{50} value of 5.6 nM (Fig. 5B). Since HA was diluted about twofold by addition to the oocyte bath medium, this EC_{50} value is in agreement with that obtained in the Ca^{2+}-mobilisation assay in CHO cells. Oocytes expressing GIRK1/2 without GPR37 were unresponsive to HA (Fig. 5B). Comparable dose-response curves were obtained from 30 oocytes.

HA signal transduction

One of the most prominent effects of HA is that it stimulates cells to enter mitosis (Hampe et al., 2000; Kayser et al., 1998; Ulrich et al., 1996). At the G2-mitosis transition, histone H3 is phosphorylated and is, therefore, an excellent marker for mitotic events. HEK-T-REx–GPR37 cells were incubated with and without doxycycline for 24 hours, before HA was added for 1.7 hours. Cells induced with doxycycline to express GPR37 showed an increase over uninduced cells in the percentage of mitotic cells, as visualised with the antibody against phosphorylated histone H3 (Fig. 6A). This suggested a direct role for GPR37 in mediating the action of HA as a mitogen. To monitor HA signalling mediated by GPR37, transiently transfected COS-7 cells and HEK-T-REx–GPR37 cells were subjected to electrophysiological analysis by patch clamping. Treatment of cells with HA led

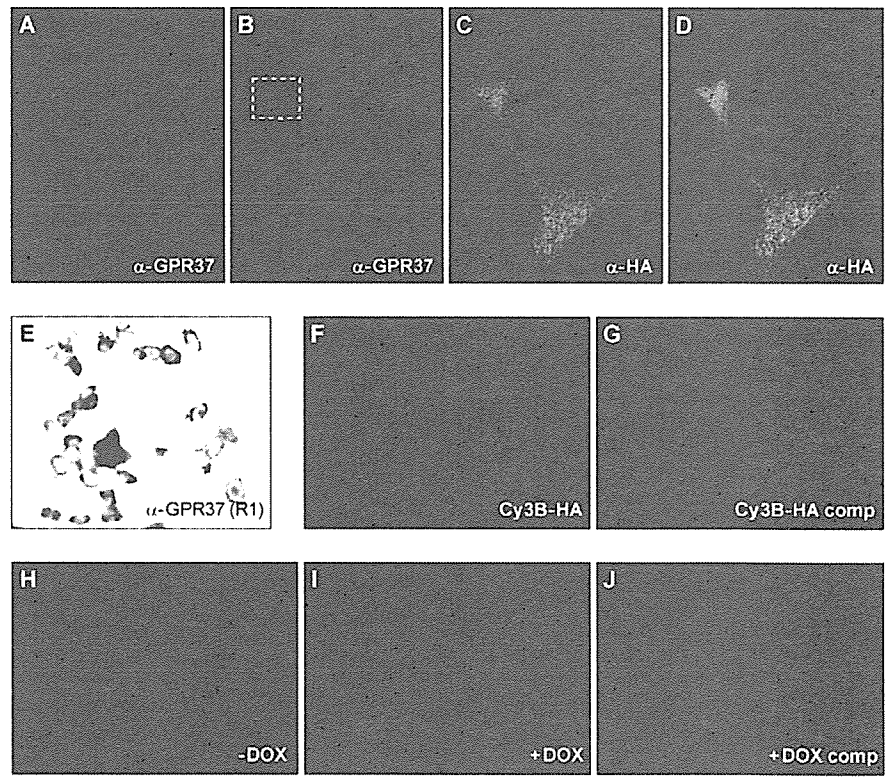

Fig. 3. HA colocalises with and binds to GPR37. (A-D) Interaction of HA and GPR37 analysed by FRET. Shown is a typical example of FRET between HA and an extracellular epitope of GPR37. COS-7 cells transiently transfected with GPR37 were treated with 2 nM HA for 20 minutes on ice to prevent internalisation, followed by incubation for 20 minute on ice with the antiserum against HA (α-HA). After fixation with 4% formaldehyde in PBS, cells were immunostained with anti-GPR37 antibody (α-GPR37). GPR37 immunoreactivity was visualised with Cy3 (A,B) and that of HA with Alexa Fluor 488 (C,D). (A) The Cy3 signal (GPR37) is shown after excitation at 568 nm. (B) A discrete area was photobleached using intense 568 nm laser. The Alexa Fluor 488 signal (HA) after excitation at 488 nm is shown before (C) and after (D) photobleaching. In this example, the Alexa Fluor 488 signal was increased by 18%. (E-G) The neuroblastoma cell line NH15-CA2 was used as a positive control to show specific Cy3B-HA binding to endogenous HA receptors. (E) NH15-CA2 cells endogenously express GPR37, as visualised with anti-GPR37(R1) antibody [α-GPR37(R1)]. (F,G) Binding is optimal at 150 nM of Cy3B-HA after incubation for 10 minutes at 37°C (Cy3B-HA) and is inhibited by pretreatment for 50 minutes at 37°C with 100 nM unlabelled, monomerised HA (Cy3B-HA comp). (H-J) HEK-T-REx–GPR37 cells bound Cy3B-HA only after GPR37 induction with doxycycline (±DOX), and binding was competed with unlabelled HA (+DOX comp).

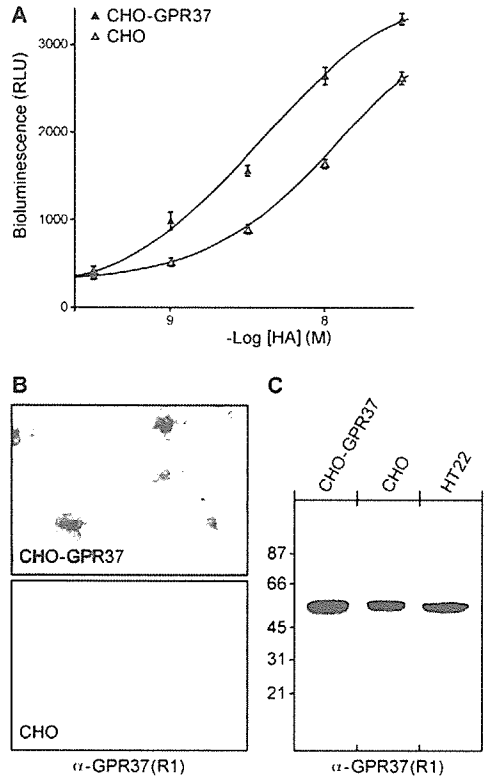

Fig. 4. HA stimulates Ca^{2+} mobilisation in CHO-K1 cells stably transfected with GPR37-FLAG, Gα16 and apoaequorin. (A) The Ca^{2+}-bioluminescence response was measured at 469 nm and is expressed in relative light units (RLU), from which the medium response was subtracted. Values are given as means ± s.d. CHO-Gα16-AEQ cells stably expressing GPR37-FLAG (CHO-GPR37) responded with an EC_{50} value of 3.3 nM; the endogenous response of CHO-Gα16-AEQ cells (CHO) resulted in an EC_{50} value of 11 nM. Data show representative results of three independent experiments. (B) CHO-GPR37 (upper panel) and CHO cells (lower panel) reacted with anti-GPR37(R1) antibody [α-GPR37(R1)], a polyclonal antiserum produced against the conserved intracellular C-tail. (C) Western blot analysis of membrane fractions confirmed an increased expression of GPR37 in transfected cells. The mouse hippocampal cell line HT22, expressing GPR37 endogenously, was used as a positive control.

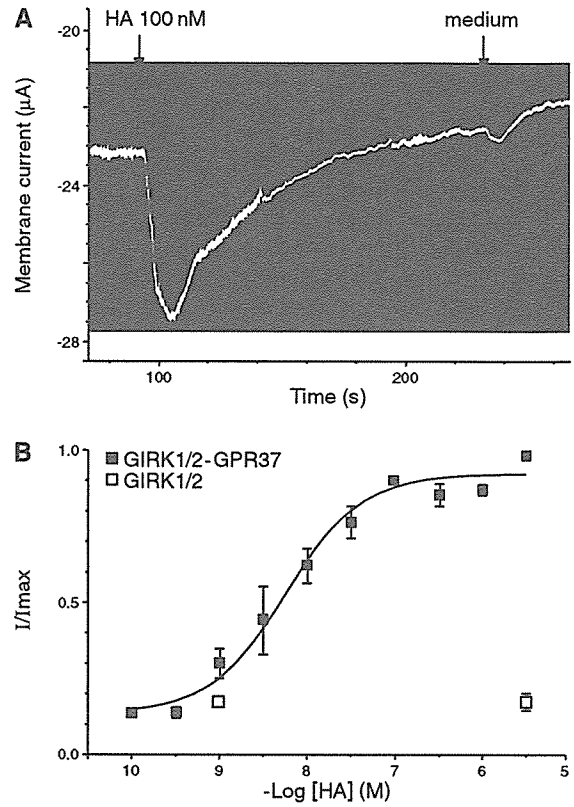

Fig. 5. HA is a high-affinity ligand for GPR37 expressed in frog oocytes. (A) Currents induced by 100 nM HA were recorded from *Xenopus* oocytes injected with cRNAs coding for GPR37 and for GIRK1/2. Stimulation with medium served as control. (B) The current increase was dependent on HA concentration. Dose-response curves for a HA-induced increase in GIRK1/2-mediated inward currents were normalised against maximal currents obtained for each oocyte. Current increases were averaged over four oocytes prepared and injected on the same day. The values represent means ± s.d. Data are representative of several independent experiments.

to an increase in membrane currents (Fig. 6B), which was blocked by La^{3+} and by SKF (Fig. 6B), both of which are known inhibitors of TRP-like Ca^{2+} channels. These Ca^{2+} channels, upon stimulation of receptors with ligands, translocate from an intracellular pool to the plasma membrane, for which activation of phosphoinositide 3-kinase (PI 3-kinase) and Ca^{2+}-dependent calmodulin (CaM) kinase is a prerequisite (Boels et al., 2001). The HA-induced increase in current was prevented by pre-incubating cells with pertussis toxin, wortmannin and KN93 (Fig. 6C), which demonstrates that an inhibitory G protein, PI 3-kinase and CaM-kinase II, respectively, are involved in the HA-GPR37 signalling cascade. A preliminary scheme of HA signalling is shown in Fig. 7.

Discussion

We present evidence that HA is a high-affinity ligand for GPR37. After heterologous expression in frog oocytes and in mammalian cells, EC_{50} values in the low nanomolar range were obtained. Electrophysiological analysis revealed that GPR37 activation by HA involved the same signalling cascade (Fig. 7) as found earlier for the endogenous HA receptor (Boels et al., 2001; Kayser et al., 1998; Ulrich et al., 1996). Interaction with HA resulted in GPR37 internalisation and stimulated entry into mitosis.

HA is bound to a carrier-like molecule both in hydra and in mammals, which improves the half-life and function of HA (Roberge et al., 1984; Schaller et al., 1996). The HA-binding protein HAB was isolated from hydra using HA-affinity chromatography, and later SorLA was discovered as an orthologue of HAB (Hampe et al., 2000). SorLA is a multi-ligand sorting receptor that, in addition to HA, binds glial-cell-derived neurotrophic factor (GDNF), PDGF and apolipoprotein E (ApoE) (Gliemann et al., 2004; Taira et al., 2001; Westergaard et al., 2004). HAB and SorLA are type I

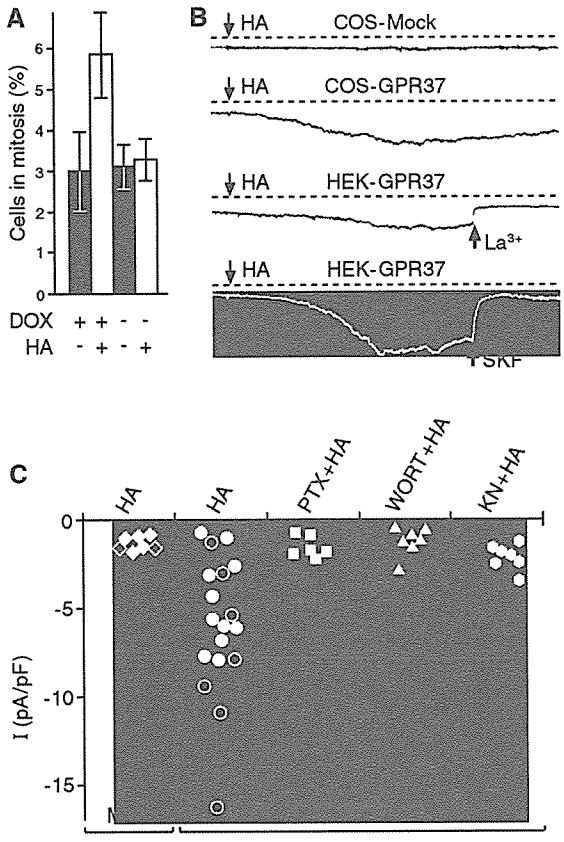

Fig. 6. GPR37 mediates HA signalling to stimulate mitosis. (A) HEK-T-REx–GPR37 cells were treated with and without doxycycline for 24 hours. Incubation with 2 nM HA for 1.7 hours led to an increase of cells in mitosis after induction of GPR37 expression. Immunostaining of cells with anti-phospho-histone H3 (1:1000) was used to determine cells in mitosis. 6×350 cells were counted, and the percentage of stained mitotic cells is given as means ± s.d. (B) Membrane currents were measured in the perforated patch configuration at a holding potential of –80 mV. Treatment with 1 nM HA induced an increase in membrane currents in COS-7 cells transiently expressing GPR37 (COS-GPR37), but not in mock-injected cells (COS-Mock). Membrane currents activated by HA in HEK-T-REx–GPR37 cells were blocked by application of 1 mM La^{3+} or 10 μM SKF. (C) Membrane-current densities were recorded from mock- and GPR37-transfected COS-7 cells. HA signal transduction was inhibited by pretreating cells for 2-3 hours with 200 ng ml^{-1} pertussis toxin (PTX), for 30-60 minutes with 100 nM wortmannin (WORT), or 30 μM KN93 (KN). Each symbol represents one cell measured in the whole-cell (filled symbols) or the perforated patch (open symbols) configuration

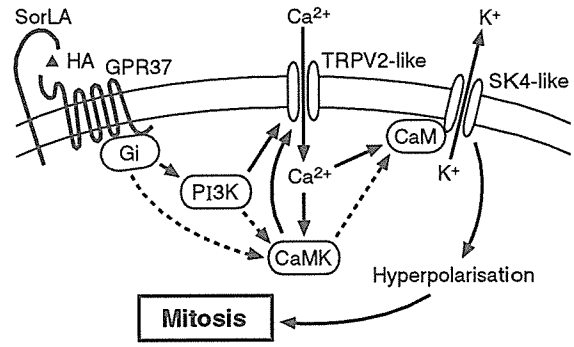

Fig. 7. Scheme of the signalling pathway from HA through GPR37 to stimulate mitosis. After binding of HA to GPR37 with or without help of the coreceptor SorLA, a pertussis-toxin-sensitive inhibitory G protein (Gi) is activated, which interacts through the phosphoinositide 3-kinase (PI3K) and the calcium-calmodulin dependent kinase II (CaMK) with a Ca^{2+} channel of the transient receptor potential family (TRPV2-like). The resulting Ca^{2+} influx activates a Ca^{2+}-dependent K^+ channel of the small and intermediate conductance family (SK4-like), leading to hyperpolarisation, which is a prerequisite for cells to enter mitosis. Dashed lines indicate hypothetical pathways.

transmembrane receptors with a large extracellular domain that can be shed by metalloprotease cleavage (Hampe et al., 2000). This represents an ideal mechanism to regulate the range of action of a morphogen like HA. GPR37 contains a relatively large extracellular domain, which is unusual for a peptide receptor. The notion that SorLA interacts with this domain of GPR37 as co-receptor to enhance HA binding (Hampe et al., 2000) is plausible and outlined in Fig. 7.

GPCRs play key physiological roles, and their dysfunction is implicated in several diseases. This might be reflected by the fact that about half of the current drugs, and certainly more in the future, are targeted to these receptors. GPR37 is of special interest for pharmacology, since it was shown to contribute to Parkinson's disease. GPR37 was characterised as a substrate for the E3 ubiquitin ligase parkin (Imai et al., 2001). Ubiquitylation marks proteins for degradation. Parkin mutations have been shown to be causative for neurodegeneration in Parkinson's disease, where dopaminergic neurons of the substantia nigra are especially affected (Imai et al., 2001; Yang et al., 2003). We found that overexpression of GPR37 resulted in complexes of molecular masses ≥250 kDa. Aggregated GPR37 did not translocate to the cell surface, as shown by cell-surface biotinylation experiments, and most probably led to preferential cell death of transfected cells. We could express GPR37 successfully in frog oocytes and in mammalian cells after stable integration into the chromosome. Since frog oocytes are cultured at room temperature, more time for proper folding may have been advantageous for GPR37 expression. Similarly, lower levels of GPR37 transcripts by stable expression may have caused less stress for the cells. The fact that insoluble GPR37 was enriched in brains of patients with juvenile Parkinson's disease (Imai et al., 2001) and its presence in Lewy bodies (Murakami et al., 2004) supports the notion that GPR37 misfolding contributes to neuronal cell death (Imai et al., 2003). This might be confirmed by the recent finding that GPR37-knockout mice showed altered dopaminergic signalling and were resistant to the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which preferentially kills dopaminergic neurons (Marazziti et al., 2004). As SorLA was found to be downregulated in the brains of patients with Alzheimer's disease (Scherzer et al., 2004), it is intriguing to speculate that a connection exists between SorLA, GPR37 and HA to improve neuronal cell survival.

Materials and Methods

Monomerisation of HA and synthesis of Cy3B-labelled HA

HA was from Bachem AG. Monomerisation was achieved by heating a 10 μM solution of HA in 0.1 N HCl for 5 minutes to 95°C. After neutralisation with NaOH to pH 7.0, samples were stored frozen at –20°C and used 2-3 times only (Bodenmuller et al., 1986). For labelling Cy3B, 150 nmoles of monomerised HA were lyophilised and dissolved in 100 μl dimethylformamide containing 0.2% *N*-methylmorpholine. Cy3B-mono-*N*-hydroxysuccinimide (NHS) ester (Amersham Biosciences) was dissolved in the same buffer (0.5 mg in 50 μl) and incubated with HA overnight in the dark. The Cy3B-labelled HA was purified by C18 reverse-phase HPLC, yielding approximately 30-40 nmoles of Cy3B-labelled HA.

Molecular biology

Human GPR37 cDNA was inserted into pcDNA 3.1 (+) and into pcDNA3-FLAG-His6C as described earlier (Imai et al., 2001). GPR37 and GPR37-FLAG were subcloned into the dual-function vector pXOON, a kind gift from T. Jespersen, optimised for expression both in frog oocytes and in mammalian cells (Jespersen et al., 2002). GPR37-FLAG was introduced into CHO-K1 cells stably expressing Gα16 and apoaequorin (CHO-Gα16-AEQ) (Stables et al., 1997) with the vector pIRES-P, a kind gift from S. Hobbs (Hobbs et al., 1998). Stable integration was monitored by immunostaining with antibodies against FLAG (Sigma-Aldrich). For inducible expression, GPR37 was transfected into HEK-293 cells using the Flp-In T-REx system of Invitrogen (Karlsruhe, Germany). The concatemeric construct between GIRK1 and GIRK2 (GIRK1/2) was kindly provided by A. Karschin (Wischmeyer et al., 1997). All constructs were confirmed by sequencing.

Expression of GPR37 in *Xenopus laevis* oocytes and electrophysiology

For functional expression in frog oocytes, the GPR37 cRNA was transcribed in vitro with T7 polymerase from the *Xba*I-linearised pXOON-GPR37-FLAG vector and co-injected at a ratio of 5:1 with cRNA of the concatemeric GIRK1/2 construct transcribed from the *Nhe*I-linearised plasmid. For recordings, oocytes were superfused with ND-96 medium (96 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, 5 mM Hepes, pH 7.5). Two-electrode voltage-clamp recordings were performed with electrodes pulled to a tip resistance of 0.5-2.0 MΩ. A Gene Clamp 500B amplifier (Axon Instruments), pClamp9 (Axon Instruments) and Origin (Microcal Software) served for data acquisition and analysis. Whole cells were clamped at –100 mV. For agonist measurements, the medium was changed to high K^+ (ND-96 with 96 mM KCl, 2 mM NaCl). After the initial inward current had reached a plateau, agonists were applied in high K^+ medium. Agonist treatment was terminated by wash-out with low K^+ to control intactness of the oocyte membrane. All recordings were performed at room temperature.

Cell culture, transfection and immunostaining

NH15-CA2, HT22 and COS-7 cells were cultured in DMEM supplemented with 10% fetal calf serum (FCS), HEK-T-REx-GPR37 cells with 10% newborn calf serum (tetracycline-free) and CHO-K1 cells in DMEM-F12 with 5% FCS. For routine culture, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin and 10 mM Hepes, pH 7, were added to these media. CHO-Gα16-AEQ cells stably expressing GPR37 required the addition of 750 μg ml^{-1} geneticin, 200 μg ml^{-1} hygromycin and 5 μg ml^{-1} puromycin. HEK-T-REx-GPR37 cells were induced to express GPR37 by incubation in 1 μg ml^{-1} doxycycline. Lipofectamine 2000 (Invitrogen), Fugene 6 (Roche Diagnostics), or electroporation were used for transfection. To assay ligands, cells were transferred overnight into serum-free defined medium consisting of the respective basal media to which 5 μg ml^{-1} insulin, 30 μg ml^{-1} transferrin, 20 μM ethanolamine, 30 nM sodium selenite, 1 μM sodium pyruvate, 1% non-essential amino acids and 2 mM glutamine were added.

For immunocytochemistry, cells were fixed either with 4% formaldehyde in PBS for 30 minutes at room temperature or with ice-cold 1% acetic acid in ethanol for 5 minutes. After washing with 0.1% Triton X-100 and pre-absorption with 1% bovine serum albumin, first and second antibodies were applied. For cell-surface staining, living cells were incubated with ligand and/or antisera for 20-30 minutes on ice, washed, fixed and visualised as indicated. No Triton X-100 was added to prevent permeabilisation. For western blotting, cells were harvested by treatment with 2 mM EDTA in PBS for 10 minutes, collected by centrifugation, and ultrasonicated for 20 seconds in Tris-HCl buffer, pH 7.4, containing 2 mM EDTA and a protease-inhibitor cocktail (Roche Diagnostics). After centrifugation at 100,000 *g*, the membrane pellets were dissolved in sample buffer and separated by SDS-PAGE. The monoclonal mouse anti-GPR37 antibody, recognising an extracellular domain of recombinant human GPR37, was used at a dilution of 1:400, the polyclonal rabbit antisera against the intracellular C-terminal domain of GPR37, anti-GPR37(R1) and anti-GPR37(R2), were diluted 1:1000 and 1:2000, respectively. All GPR37-specific antibodies were produced in the laboratory of Takahashi and have been described previously (Imai et al., 2001). The antibody against FLAG (M2) was from Sigma-Aldrich and that against phospho-histone H3 from Biomol. Cy2 or Cy3 secondary antibodies were used for confocal analysis, and alkaline phosphatase- or peroxidase-conjugated secondary antibodies were used for light microscopy and western blotting. Western blots were visualised by ECL. Biotinylated proteins were detected with an avidin-peroxidase conjugate (Bio-Rad).

FRET analysis

For fluorescence resonance energy transfer (FRET) experiments, HA was reacted with the highly specific HA antiserum 102.8, which binds to HA in the picomolar range and was described earlier (Schaller et al., 1984). It was used at a dilution of 1:3000 and visualised with Alexa Fluor 488 goat anti-rabbit as donor (Invitrogen). GPR37 was detected with anti-GPR37 antibody and visualised with Cy3 anti-mouse antibody (Amersham) as acceptor. The energy transfer was detected as increase in donor fluorescence (Alexa Fluor 488) after complete photobleaching of the acceptor molecule (Cy3). Initial images were recorded after excitation at 488 and 568 nm. A discrete area of the sample was illuminated with intense 568 nm light (laser power 100%) for a few minutes to destroy completely the acceptor fluorescence. The cell was then rescanned using excitation at 488 nm. An increase within the photobleached area was used as a measure for the amount of FRET obtained. The efficiency of energy transfer (E) was expressed as E=1-(D1/D2), where D1 is the donor fluorescence before, and D2 after, photobleaching. Data were collected for 4-5 different fields from a single coverslip; 2-3 coverslips were used for each measurement; the experiment was repeated at least three times.

Biotinylation of surface proteins

COS-7 cells were transiently transfected with GPR37-FLAG using the Fugene 6 reagent (Roche Diagnostics). 48 hours after transfection, cells were washed 2× with PBS and biotinylated for 30 minutes at room temperature with 1 mM S-NHS-biotin (Perbio Science). The reaction was stopped by addition of 0.5 M Tris-HCl, pH 7.5, for 5 minutes at room temperature, and the cells were washed with PBS to remove free biotin. Cell lysates were prepared in a buffer consisting of 5 mM EDTA, 10 mM Tris-HCl, pH 7.4, and protease-inhibitor cocktail. Samples were ultrasonicated for 20 seconds and centrifuged at 100,000 *g* for 30 minutes. Pellets were solubilised in buffer containing 1% Triton X-100, 0.5% NP40, 150 mM NaCl, 7 mM EDTA, 1 mM EGTA, 10 mM Tris-HCl, pH 7.4, and protease-inhibitor cocktail for 30 minutes on ice, followed by centrifugation at 16,000 *g* for 15 minutes at 4°C. The supernatant was used for immunoprecipitation.

Immunoprecipitation with anti-FLAG M2-agarose

Since high concentrations of NP40 inhibited binding to FLAG-agarose, the supernatant from the NP40-solubilised and biotinylated COS-7 cells was diluted fivefold with TBS (150 mM NaCl, 50 mM Tris-HCl, pH 7.4) to reduce the NP40 concentration to 0.1%. Samples were incubated with 100 μl anti-FLAG M2-agarose (Sigma-Aldrich) overnight at 4°C and then centrifuged at 1500 *g* for 5 minutes at 4°C. Pellets were resuspended in 1 ml TBS and centrifuged again at 16,000 *g* for 2 minutes at 4°C. After washing with TBS, pellets were dissolved in 50 μl sample buffer and subjected to western blotting.

Electrophysiology with mammalian cells

For electrical recordings, COS-7 cells were microinjected with 50 ng μl^{-1} GPR37-pcDNA3 and 5 ng μl^{-1} EGFP-N1-pcDNA3, the latter being used to facilitate detection of successfully transfected cells. Membrane currents were recorded in the whole-cell configuration of the patch-clamp technique (Hamill et al., 1981) or the perforated-patch configuration with nystatin (Horn and Marty, 1988). An EPC9 patch-clamp amplifier was used in conjunction with the PULSE-stimulation and data-acquisition software (HEKA Elektronik). The patch electrodes were made from 1.5 mm diameter borosilicate glass capillaries with resistances of 2.5-4 MΩ. Data were low-pass filtered at 3 kHz and compensated for both fast and slow capacity transients. Series resistance was compensated by 75-90%. All experiments were performed at room temperature (22-25°C). The pipette solution contained 140 mM KCl, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 2.5 mM EGTA, 10 mM HEPES and had a calculated free Ca^{2+} concentration of 66 nM. The pH was adjusted to 7.3 with KOH. The standard external solutions contained 140 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 5 mM KCl, 10 mM HEPES and 10 mM glucose, buffered to pH 7.3 with NaOH. Nystatin was dissolved in dimethyl sulfoxide (DMSO). Its final concentration in the standard pipette solution was 0.2 mg ml^{-1}. All chemicals for electrophysiology were purchased from Sigma-Aldrich.

Statistical analysis

The results are expressed as means of 3-6 determinations ± s.d. Curve fittings were performed with the Prism program (GraphPad). Each experiment was repeated at least three times.

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Cellular and Molecular Mechanisms of Parkinson's Disease: Neurotoxins, Causative Genes, and Inflammatory Cytokines

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SUMMARY

1. Parkinson's disease (PD) is considered to be an aging-related neurodegeneration of catecholamine (CA) systems [typically A9 dopamine (DA) neurons in the substantia nigra and A6 noradrenaline (NA) neurons in the locus coeruleus]. The main symptom is movement disorder caused by a DA deficiency at the nerve terminals of fibers that project from the substantia nigra to the striatum. Most PD is sporadic (sPD) without any hereditary history. sPD is speculated to be caused by some exogenous or endogenous substances that are neurotoxic toward CA neurons, which toxicity leads to mitochondrial dysfunction and subsequent oxidative stress resulting in the programmed cell death (apoptosis or autophagy) of DA neurons.

2. Recent studies on the causative genes of rare familial PD (fPD) cases, such as *alpha–synuclein* and *parkin,* suggest that dysfunction of the ubiquitin–proteasome system (UPS) and the resultant accumulation of misfolded proteins and endoplasmic reticulum stress may cause the death of DA neurons.

3. Activated microglia, which accompany an inflammatory process, are present in the nigro-striatum of the PD brain; and they produce protective or toxic substances, such as cytokines, neurotrophins, and reactive oxygen or nitrogen species. These activated microglia may be neuroprotective at first in the initial stage, and later may become neurotoxic owing to toxic change to promote the progression toward the death of CA neurons.

4. All of these accumulating evidences on sPD and fPD points to a hypothesis that multiple primary causes of PD may be ultimately linked to a final common signal-transduction pathway leading to programmed cell death, i.e., apoptosis or autophagy, of the CA neurons.

KEY WORDS: Parkinson's disease; dopamine; noradrenaline; neurotoxins; alpha-synuclein; parkin; inflammation; microglia; cytokines; neurotrophins; apoptosis; autophagy.

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INTRODUCTION

Parkinson's disease (PD) is assumed to be a systemic neurodegenerative disease of the catecholamine (CA) neurons. The neurons mainly affected are the A9 dopamine (DA) neurons in the substantia nigra and the A6 noradrenaline (NA) neurons in the locus coeruleus, both of which contain neuromelanin. PD is the second most common aging-related neurodegenerative disease after Alzheimer's disease (AD). The main symptom of PD is a movement disorder called parkinsonism, i.e., muscle rigidity, akinesia, and resting tremor, symptoms which are caused by a DA deficiency in the striatum due to degeneration of the A9 DA neurons in the substantia nigra. A small percentage of PD is familial (fPD), but most PD is sporadic (sPD) without any hereditary history and is related to aging. A deficiency of DA in the striatum in PD patients was predicted by Carlsson (1959) when he discovered DA as a new neurotransmitter, and this prediction was confirmed in postmortem brains by Ehringer and Hornykiewicz (1960). DA supplementation therapy by L-DOPA was also predicted by Carlsson from the results of animal experiments (1959); and after many clinical trials by several groups in the world, L-DOPA is now established as the gold standard of drug therapy for PD. sPD and some cases of fPD are characterized by the presence of intracytoplasmic eosinophilic inclusions called Lewy bodies in the neurons and glial cells of the affected individuals. Lewy bodies consist mainly of alpha-synuclein protein, which is encoded by the causative gene of fPD/PARK1 (familial Parkinson's disease 1), and are observed not only in PD but also in dementia with Lewy bodies (DLB), a disorder also called diffuse Lewy body disease (DLBD; Kosaka, 2000), and in other neurodegenerative diseases. Thus, such neurodegenerative diseases with Lewy bodies are generally referred to synucleinopathies. Lewy bodies are frequently observed in the surviving DA and NA neurons of the substantia nigra and locus coeruleus of the PD brain. Braak *et al.* (2003) recently proposed, based on anatomical investigation of Lewy bodies, that the pathological process of sPD starts from the lower brain stem and spreads throughout the brain to the midbrain, limbic brain, and cerebral cortex and that the main symptom of PD, i.e., movement disorder, appears at the late stage when the nigro-striatal DA neurons in the midbrain become involved. In this theory, movement disorder parkinsonism in PD is speculated to be a late symptom. Lewy bodies are also observed not only in CA neurons but also in non-CA neurons such as acetylcholine, amino acid, or peptide neurons in the central and peripheral nervous systems, i.e., in neurons of the anterior olfactory nucleus, dorsal nucleus of the vagus nerve, peripheral autonomic nervous system including sympathetic ganglia, adrenal medulla, and parasympathetic ganglia, and intestinal Auerbach's plexus. In relation to this wide distribution of Lewy bodies in sPD, various non-DA symptoms, e.g., REM sleep behavior disorder (RBD) (Abott, 2005), olfaction disturbance, neurocircuitry abnormalities due to cardiac sympathetic denervation (Goldstein *et al.*, 2005), or constipation, have been noticed as early signs of sPD before the appearance of parkinsonism.

The pathogenesis of sPD is still enigmatic (Foley and Riederer, 1999), but free radicals produced by mitochondrial dysfunction and oxidative stress are thought to play an important role (Youdim and Riederer, 1997; Mizuno *et al.*, 1998; Schapira *et al.*, 1998). Mitochondrial dysfunction in sPD is supported by the findings on

complex I (NADH-ubiquinone reductase complex, one of the five enzyme complexes of the inner mitochondrial membrane involved in oxidative phosphorylation) deficiency in the nigro-striatum of the postmortem brain from sPD patients and of complex I inhibition in the substantia nigra of animal PD models produced by treatment with neurotoxins such as 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP; Langston *et al.*, 1983) or the insecticide rotenone (Betarbet *et al.*, 2000). The discovery of MPTP causing PD in humans suggests the neurotoxin hypothesis, in which MPTP-like exogenous or endogenous neurotoxins acting together with presumed PD-susceptibility genes are assumed to be the cause of sPD.

On the other hand, recent molecular genetical studies on mutations of the causative genes of autosomal dominant or recessive fPD, especially *alpha-synuclein* for autosomal dominant and Lewy body-positive PARK1 (Polymeropoulos *et al.*, 1997) and *parkin* for autosomal recessive and Lewy body-negative juvenile PARK2 (Kitada *et al.*, 1998), have led to a new hypothesis, that fPD is caused by the accumulation and/or aggregation of misfolded proteins due to dysfunction of the ubiquitin–proteasome system (UPS; Table I). The discovery of the causative genes of fPD may also give important clues for elucidating the signaling pathway of cell death in sPD (for reviews, see Bonifati *et al.*, 2004; Feany, 2004; Forman *et al.*, 2004; Cookson, 2005; Krueger, 2004; Selko, 2004; Vila and Przedborski, 2004; Grandhi and Wood, 2005; Lozano and Kalia, 2005; Mizuno, 2006).

As another mechanism of neuronal death in PD, an inflammatory process in the brain called neuroinflammation, which is accompanied by changes in the levels of neuroprotective or neurotoxic cytokines and neurotrophins and the presence of activated microglia, has recently gained much attention with respect to not only PD, but also AD and other neurodegenerative diseases (for reviews, see McGeer and McGeer, 1995; Anglade *et al.*, 1997; Hirsch *et al.*, 1999; Mogi and Nagatsu, 1999; Nagatsu *et al.*, 1999, 2000a,b; Hartmann *et al.*, 2000; Jellinger, 2000; Nagatsu, 2002a; Hayley, 2005; Herrera *et al.*, 2005; Nagatsu and Sawada, 2005). The brain is considered to be an immune privileged site, i.e., one free from immune reactions, since it is protected by the blood–brain-barrier. However, accumulating findings have revealed that immune responses may occur in the brain, especially due to activation of the microglia, which cells are known to produce pro-inflammatory cytokines. This inflammatory process is now thought to be fundamental to, if not at first the initiator of, the progression of PD pathogenesis. Not only DA neurons but also other non-DA neurons may be affected by this process, whose dysfunction may negatively impact DA and non-DA pathways in the PD patients.

Herein, we will review advances in our understanding of the cellular and molecular pathogenesis of PD by separately focusing on these three groups of causative factors, i.e., neurotoxins, causative genes, and inflammatory cytokines.

NEUROTOXIC SUBSTANCES PRODUCING PD IN HUMANS AND ANIMALS

The cell death of A9 DA neurons in sPD may be caused by both genetic factors and environmental factors. As to the latter, certain neurotoxins are speculated to

Table I. Genes and Loci Linked to Familial Parkinson's Disease (fPD)

Name/locus	Chromosomal localization	Gene	Protein function	Inheritance pattern	Lowy bodies	PD features
PARK 1	4q21-q23	Alpha-synuclein	Synaptic?	AD	+	Early onset, lower prevalence of tremor
PARK 2	6q25.2-q27	Parkin	E3 ubiquitin ligase	AR	–	Juvenile onset, more frequent dystonia and L-DOPA-induced dyskinesia
PARK 3	2p13	Unknown		AD	+	Dementia, rapid progression
PARK 4	4q21-q23	Triplication (Alpha-synuclein)	Lipid binding?	AD	+	Rapid progression
PARK 5	4p14	UCHL1	Ubiquitin hydrolase/ligase	AR	Un-known	Typical PD
PARK 6	1p35-36	PINK1	Protein kinase	AR	Un-known	Early onset, slow progression
PARK 7	1p36	DJ-1	Oxidative stress response	AR		Early onset, psychiatric symptoms, slow progression
PARK 8	12p11.2-q13.1	LRRK2		AD	–	
PARK 9	1p36	Unknown		AR	Un-known	Juvenile onset, spasticity, supranuclear gaze paralysis, dementia
PARK 10	1p32	Unknown		Susceptible gene		
NR4A2	2q22-23	Nurr1		AD		

Note. AD, autosomal dominant; AR, autosomal recessive.

cause sPD. This neurotoxin hypothesis of PD started in the 1980s from the discovery of MPTP, which produces PD in humans (Davis *et al.*, 1979; Langston *et al.*, 1983). Since then, environmental or endogenous neurotoxins similar to MPTP in the brain and/or cerebrospinal fluid (CSF) of PD patients have been investigated, and two groups of amine-related compounds, i.e., isoquinolines and beta-carbolines, have been suggested as candidates of such neurotoxins (for review, see Nagatsu, 1997; Nagatsu, 2002b).

MPTP, an analogue of meperidine (a synthetic heroin) is a highly lipophilic precursor neurotoxin. After its systemic administration, it rapidly crosses the blood–brain barrier to enter the brain. Once in the brain, MPTP, which is a proneurotoxin, is metabolized to *N*-methyl-4-pheny-2,3-dihydropyridinium (MPDP+) by the enzyme monoamine oxidase B (MAO B), which is localized in the outer membrane of mitochondria within non-DA cells such as glial cells and serotonin neurons. MPDP+ is then probably spontaneously oxidized to 1-methyl-4-phenylpyridinium (MPP+), the active neurotoxin. MPP+ is taken up across the cell membrane to enter into the A9 nigro-striatal DA neurons via DA transporters, which are mostly localized at the nerve terminals in the striatum. MPP+ then passes from the cytoplasm of these neurons into synaptic vesicles by the action of vesicular monoamine transporter type 2 (VMAT2). MPP+ also accumulates within the inner mitochondrial membrane, where it inhibits complex I; interrupts electron transport; releases reactive oxygen species (ROS); and depletes ATP. Inhibition of the mitochondrial complex I also results in the opening of mitochondrial permeability transition pores, allowing the release of cytochrome *c*, which may trigger the signal transduction pathway culminating in apoptotic cell death. As a consequence of these actions, MPP+ decreases the DA level in the striatum, resulting in the appearance of PD-like symptoms. MPTP produces cell death specifically in A9 nigro-striatal DA neurons. Selegiline (L-deprenyl), which is a specific inhibitor of MAO B, completely blocks the neurotoxicity of MPTP by preventing the conversion of MPTP to MPP+ in glial cells. This may suggest that MAO B allele activity could play an important genetic component of sPD. Humans and monkeys are the most susceptible to MPTP, but mice and other non-primate animals also produce parkinsonism-like movement disorder in response to MPTP. In MPTP-induced animal models of PD, typical cytoplasmic inclusions such as the Lewy bodies found in sPD are not observed. However, chronic infusion of mice with MPTP via an osmotic minipump caused the appearance of alpha-synuclein- and ubiquitin-positive aggregates that looked like Lewy bodies (Fornai *et al.*, 2005).

As a group of MPTP-like neurotoxins, the following isoquinolines have been identified by gas chromatography–mass spectrometric analysis of postmortem brain specimens and/or CSF from PD patients and normal controls: tetrahydroisoquinoline (TIQ), 1-methyl(Me)-TIQ, 2-Me-TIQ, N-Me-6,7-$(OH)_2$-TIQ (N-Me-norsalsolinol), R-1,N-$(Me)_2$-6,7-$(OH)_2$-TIQ (R-N-Me-salsolinol), 1-phenyl-TIQ, N-Me-1-phenyl-TIQ, and 1-benzyl-TIQ (Kajita *et al.*, 2002; Kotake *et al.*, 1995; Moser and Koempf, 1992; Naoi *et al.*, 1996; Niwa *et al.*, 1987; Ohta *et al.*, 1987). TIQs are also found in various foods in small concentrations. Exogenously administered TIQ easily crosses the blood–brain barrier and passes into the brain, although TIQs are metabolized in the liver to 4-OH-TIQs by the action of

debrisoquine hydroxylase (P-450 CYP2D6). On the other hand, TIQs in the brain are also speculated to be synthesized from endogenous amines such as phenylethylamine or DA by various enzymes. However, the synthetic pathway and the enzymes for the biosynthesis of TIQs (versus beta-carbolines described below which arise from tryptophan metabolism) have not yet determined. Among these TIQs in the brain, 2-Me-TIQ (Fukuda, 1994), R-N-Me-salsolinol (Naoi *et al.*, 1996), N-Me-norsalsolinol (Moser and Koempf, 1992), and 1-Bn-TIQ (Kotake *et al.*, 1995) have gained much attention as neurotoxins that probably cause PD. R-N-Me-salsolinol, after its stereotaxic injection into the striatum of rats, induces behavioral changes similar to those seen in PD and is metabolized to 1,N-$(Me)_2$-6,7-$(OH)_2$-isoquinolinium in the substantia nigra, similar to the MPTP/MPP+ conversion (Naoi *et al.*, 1996). Among TIQs, 1-Me-TIQ is unique in that its concentration is not increased but actually decreased in the striatum of PD patients and that it prevented PD-like behavior abnormalities produced by MPTP, TIQ, and 1-Bn-TIQ (Tasaki *et al.*, 1991).

Beta-carbolines have structures similar to those of MPTP/MPP+, and may be synthesized *in vivo* from tryptophan via tryptamine (Collins and Neafsey, 2000; Matsubara, 2000). Like MPTP, beta-carbolines may be precursor neurotoxins that are N-methylated and oxidized by MAO B to form, in their case, beta-carbolinium ions, which may trigger apoptotic neuronal death and PD symptoms. Norharman, harman, 2-Me-norharmanium, and 2,9-$(Me)_2$-norharmanium were found in the human brain and/or the CSF. A neurotoxic 2,9-dimethylated beta-carbolinium ion (2,9-$(Me)_2$- norharmanium) was found in half of the PD, but not at all in non-PD, patients examined (Matsubara *et al.*, 1995; Kuhn *et al.*, 1996). These results suggest that beta-carbolinium compounds as well as isoquinolinium compounds may also be candidate neurotoxins that produce PD.

Endogenous isoquinolines and beta-carbolines in the brain, as in the case of MPTP, are speculated to be first N-methylated and then oxidized by MAO to the corresponding isoquinolinium ions or beta-carbolinium ions in glial cells. The mechanisms of the DA cell death caused by PD-producing neurotoxins, i.e., MPTP/MPP+, isoquinoline/isoquinolinium, or beta-carboline/beta-carbolinium, may be similar, acting to inhibit mitochondrial complex I, to induce ROS formation, and ATP depletion, and finally to cause apoptotic cell death of DA neurons.

Rotenone is a naturally occurring, lipophilic compound isolated from roots of certain plants (Derris species), and is used as the main component of many insecticides. Rotenone is not structurally related to amines, but is a specific inhibitor of mitochondrial complex I. Betarbet *et al.* (2000) reported that in Lewis rats, chronic, systemic inhibition of complex I by rotenone caused highly selective degeneration of the A9 nigro-striatal DA neurons with behavioral PD symptoms of hypokinesia and rigidity. Important morphological findings in these rotenone-treated rats were that the nigro-striatal DA neurons had accumulated fibrillar cytoplasmic inclusions containing ubiquitin and alpha-synuclein, similar to the Lewy bodies seen in human sPD. It was reported that the primary mechanism underlying the toxic effect of rotenone was a significant increase in O_2^- generation that causes damage to

mitochondrial complexes I and II, presumably at the level of the 4Fe–4S clusters (Panov *et al.*, 2005).

All the properties of the afore-mentioned neurotoxins indicate that inhibition of complex I, resulting in ROS formation, oxidative stress, and ATP depletion in DA neurons may trigger the pathway of apoptotic cell death in these cells. In agreement with this neurotoxin-based hypothesis, a selective defect in complex I of the mitochondria in the nigro-striatal DA neurons from the post-mortem brain of sPD patients was reported by several laboratories (Youdim and Riederer, 1997; Mizuno *et al.*, 1998; Shapira *et al.*, 1998). However, it should be noted that the development of PD has been confirmed in humans only in the case of MPTP, a synthetic neurotoxin. That is, the toxicity of the afore-mentioned endogenously identified neurotoxin candidates has been proved only in animals.

6-Hydroxydopamine (6-OHDA) is a neurotoxin specific for DA neurons *in vitro* and *in vivo* (Kostrzewa and Jacobowitz, 1974). It is a very unstable compound and is easily oxidized to produce ROS, which may kill DA neurons via apoptosis. Although 6-OHDA is formed from DA *in vitro*, it is believed not to be formed *in vivo*. 6-OHDA does not cross the blood–brain barrier. Thus, it is widely used to produce hemiparkinsonian animal models by stereotaxic injection of it directly into the nigro-striatal region.

Since DA (or NA) is easily oxidized to form DA quinones and other reactive oxygen species, toxic metabolites of DA such as DA quinones (in the case of NA, NA quinones), which are intermediates of neuromelanin biosynthesis, or 3,4-dihydroxyphenylacetaldehyde (DOPAL; in the case of noradrenaline, 3,4-dihydroxyphenylethyleneglycolaldehyde, DOPEGAL) formed by MAO are postulated to play a role as endogenous neurotoxin candidates in PD (Eisenhofer *et al*, 2002). DA quinones may be formed either non-enzymatically or by the action of tyrosinase. Tyrosinase has been implicated in DA quinone and neuromelanin formation in the brain. However, we did not detect tyrosinase protein in the A9 nigral DA neurons in the human brain by using antibodies against human tyrosinase (Ikemoto *et al.*, 1998). DOPAL was identified in the PD brain, but not in the normal brain, by gas chromatography–mass spectrometry (Mattammal *et al.*, 1993), and was suggested to be toxic for DA neurons *in vitro* (Mattammal *et al.*, 1995). However, DA depletion in DA-deficient mice did not protect against acute MPTP toxicity *in vivo*, suggesting that DA does not contribute to this toxicity *in vivo* (Hasbani *et al.*, 2005).

Since iron exists in a high concentration in the basal ganglia and interacts with neuromelanin and DA, it is suggested to play a role in the formation of ROS and in the initiation of neurodegeneration of DA neurons by oxidative stress (Mochizuki *et al.*, 1993; Gerlach *et al.*, 1994).

All the effects of exogenous or endogenous neurotoxin candidates suggest that mitochondrial dysfunction and oxidative stress are important for the pathogenesis of sPD. Inhibition of complex I results in enhanced production of ROS, which in turn inhibits complex I. Thus, the vicious cycle resulting from even partial inhibition of complex I in DA neurons may lead to excessive stress and an ATP deficit that ends in cell death (Tretter *et al.*, 2004).

CAUSATIVE GENES OF FAMILIAL PD (PARK)

A small percentage (approximately 5%) of PD cases are familial with a hereditary history (fPD; Table I). Several causative genes of fPD, which mutations produce parkinsonism, and their chromosomal localization have recently been identified: PARK1 (*alpha-synuclein*), PARK2 (*parkin*), PARK4 (triplication of *alpha-synuclein*), PARK5 (*UCHL1*), PARK6 (*PINK1*), PARK7 (*DJ-1*), and PARK8 (*LRRK2*) (for reviews, see Chiba-Falek and Nussbaum, 2003; Feany, 2004; Forman *et al.*, 2004; Selkoe, 2004; Cookson, 2005; Grandhi and Wood, 2005; Lozano and Kalia, 2005; Mizuno, 2006).

In 1997, a causative mutation of fPD was first identified in the PARK1 gene, encoding the protein alpha-synuclein in autosomal dominant Italian and Greek families (Polimeropoulos *et al.*, 1997). Alpha-synuclein is a small (144 amino acids), presynaptic protein and probably plays a role in signaling between neurons (Goedert, 2001). As a finding on the physiological role of alpha-synuclein, Chandra *et al.* (2005) reported that alpha-synuclein in conjunction with CSP-alpha (cystein-string protein alpha) has a powerful *in vivo* activity in protecting nerve terminals against injury in mice. CSP-alpha is a synaptic vesicle protein, essential for neural survival, has a cochaperon function, and may prevent the accumulation of nonnative, potentially toxic molecules during the continuous operation of a nerve terminal.

Not only mutations in alpha-synuclein such as a single amino acid substitution A30P or A53P in PARK1, but also triplication of the wild-type *alpha-synuclein* gene also causes autosomal dominant PARK4. Alpha-synuclein is a major protein component of Lewy bodies (Spillantini *et al.*, 1998), and thus may play an important role in both fPD and sPD. Alpha-synuclein is a natively unfolded soluble protein and has a central hydrophobic region and a high potency to aggregate to form oligomers or protofibrils and ultimately insoluble polymers or fibrils under certain conditions. The protofibrillar intermediates are toxic in neurons (Conway *et al.*, 2000). Alpha-synuclein fibrils cause mitochondrial complex I deficiency (Sherer *et al.*, 2003) and oxidative stress (Ischiropoulos and Beckman, 2003). Thus, alpha-synuclein may elicit a pathogenetic mechanism similar to that acting in sPD.

Drosophila (fruit fly) models based on overexpression of normal and mutant forms of the *alpha-synuclein* gene show selective loss of DA neurons and the formation of alpha-synuclein inclusions (Feany and Bender, 2000). Experiments using this model confirmed that phosphorylation at the Ser 129 residue is crucial to the toxicity of alpha-synuclein and that mutations of this serine residue abolishes the toxicity. The reduction in toxicity in this model is associated with increased inclusion body formation, which suggests that inclusion bodies may protect neurons by reducing the amount of diffusible toxic protein by sequestering it in inert bodies (Chen and Feany, 2005). All these findings on alpha-synuclein suggest that the misfolding of the protein is the key steps in mediating degeneration of DA neurons in both fPD and sPD. Alpha-synuclein transgenic mice develop neuronal mitochondrial degeneration, which also suggests a close correlation between alpha-synuclein and the pathogenesis of sPD (Martin *et al.*, 2006).

In PARK2 the gene encoding parkin protein, which was discovered in a Japanese family, is the most common mutant gene in fPD (Kitada *et al.*, 1998). The *parkin*

gene is very large, about 1.4 Mb; and mutations of it are responsible for most of the cases of autosomal recessive juvenile (young-onset) PD. The parkin protein is an E3 ubiquitin ligase with two characteristic RING finger domains separated by an IBR (in-between ring) domain (Shimura *et al.*, 2000). There is a notable absence of Lewy bodies in patients with the homozygous deletion of parkin, although these bodies are present in patients with compound heterozygous parkin mutations. These findings suggest that parkin plays a significant role in Lewy body formation and that nigral cell loss and parkinsonism can occur in the absence of Lewy bodies. The identification of mutations of the *parkin* gene in PARK2 suggests that dysfunction of the UPS due to loss of function has an important role in PD and that the *parkin* gene may play a protective role. Ubiquitin is added to proteins by the action of E3 ubiquitin ligase to target them to the proteasome, a large multiprotein complex that functions to degrade most ubiquitin-marked cellular proteins. Parkin mutations cause the accumulation of parkin substrates, which probably contributes to the death of DA neurons. Many putative parkin substrates have been identified, including synphilin-1, 22-kDa O-glycosilated form of alpha-synuclein (alphaSp22; Shimura *et al.*, 2001), Pael-R (*p*arkin-*a*ssociated *e*ndothelin receptor-*l*ike *r*eceptor; Imai *et al.*, 2001), CHIP, Cdc-Rel1A, cyclin E, and synaptotagmin XI. Overexpression of the parkin substrate Pael-R produces DA cell death *in vitro*, which cells can be rescued by parkin overexpression (Yang *et al.*, 2003).

Parkin has been shown to be S-nitrosylated *in vitro* and *in vivo* in the MPTP mouse model of PD and in brains of patients with PD or LBD, and both neuron- and microglia-derived NO contributes to the S-nitrosylation of parkin in a biphasic fashion after MPTP intoxication. S-Nitrosylation inhibits the E3 ubiquitin ligase activity of parkin and thus its protective function (Chung *et al.*, 2004).

Synphilin-1, a substrate of parkin, was shown to interact with alpha-synuclein and to promote the formation of cytosolic inclusions. Synphilin-1 also interacts with E3 ubiquitin ligase SIAH (*s*even *i*n *a*bsentia *h*omologues)-1 and SIAH-2. SIAH proteins ubiquitinate synphilin-1, promoting its degradation by UPS, and may play a role in inclusion formation, since SIAH immunoreactivity was demonstrated in Lewy bodies in PD patients (Liani *et al.*, 2004). MAO inhibitor deprenyl (selegiline) was reported to act for protection of DA neurons by binding to glyceraldehyde-3-phosphate dehydrogenase (GAPDH; Tatton *et al.*, 2003). Snyder (2005) recently reported that an apoptotic stimulus turns on inducible NO synthase with its product NO causing S-nitrosylation of GAPDH in DA neurons. This modification allows GAPDH to bind to the E3 ubiquitin ligase SIAH, which transports GAPDH to the nucleus, leading to apoptotic cell death. They also showed that MAO B inhibitor deprenyl binds to SIAH protein, thereby preventing translocation of the GAPDH–SIAH complex from the cytoplasm to the nucleus and thus preventing apoptotic cell death.

PARK5 is another autosomal dominant fPD. The gene encodes UCHL1 (*u*biquitin *C*-terminal *h*ydrolase *L1*), which generates free ubiquitin and aids the recycling of polyubiquitin chains back to monomeric ubiquitin (Leroy *et al.*, 1998). UCLH1 can also exert a ubiquitin ligase activity. The discovery of the *UCHL1* gene further supports the importance of the role of UPS in the pathogenesis of PD.

The UPS may be important not only in fPD (PARK1, PARK2, PARK4, and PARK5) but also in sPD. Postmortem brain tissues from sPD patients show functional deficits in their 20S proteasome activity (Chung *et al.*, 2001). Also, systemic administration of inhibitors of the UPS to rodents produces selective nigral cell loss and Lewy body-like inclusions, which are accompanied by clinical signs of parkisonism (McNaught *et al.*, 2004).

PARK6 is another autosomal recessive fPD, and is caused by a mutation in a mitochondrial protein kinase called PINK1 [*P*TEN (phosphatase and *ten*sin Romolog deleted on chromosome *ten*)-*in*duced *k*inase-*1*; Valente *et al.*, 2004)]. PINK1 is the first nucleus-encoded mitochondrial protein to be implicated in the pathogenesis of fPD. The *PINK1* gene encodes a serine/threonine protein kinase with significant homology to the calcium/calmodulin-dependent protein kinase. Neuroblastoma cells transiently transfected with either wild-type or mutant *PINK1* do not show any detectable alterations in viability. In contrast, when these cells are challenged with a proteasome inhibitor, MG132, overexpression of the wild-type PINK1 mitigates cell death; whereas, overexpression of mutant PINK1 neither attenuates nor enhances MG132-mediated cytotoxicity (Valente *et al.*, 2004). These results suggest that the loss of PINK1 function renders DA neurons more vulnerable to injury. Transient knockdown of PINK1 renders cells susceptible to apoptosis on exposure to taxol (MacKeigan *et al.*, 2005). This neuroprotective function and the mitochondrial localization of PINK1 suggest its probable important role in mitochondria also in sPD.

DJ-1 is the causative gene of autosomal recessive early-onset PARK7 (Bonifati *et al.*, 2003). DJ-1 is a homodimeric and multifunctional protein, ubiquitously expressed in human tissues; and it plays essential roles in tissues with higher-order biological functions such as the testis and brain. DJ-1 is related to male fertility, and its level in sperm is decreased in response to exposure to sperm toxicants. DJ-1 was discovered as a novel mitogen-dependent oncogene product involved in a Ras-related signal transduction pathway (Nagakubo *et al.*, 1997). DJ-1 is up-regulated after oxidative stress and may play a role as an antioxidant protein and a sensor for oxidative stress. The crystal structure of DJ-1 indicates that the protein is structurally similar to a cysteine protease and may induce conformational changes to acquire its catalytic activity in response to oxidative stress (Honbou *et al.*, 2003). The function of DJ-1 as an anti-oxidant protein again suggests its pathogenetic role also in sPD.

The gene for the LRRK2 (*l*eucine-*r*ich *r*epeat *k*inase *2*) was identified as the PARK8 locus (Paisan-Ruiz *et al.*, 2004; Zimprich *et al.*, 2004). Mutations in *LRRK2* cause autosomal dominant PD with a broad spectrum of neuropathological features, such as neuronal loss in the substantia nigra either in the absence or in the widespread presence of Lewy bodies or in the presence of neurofibrillary tangles. The affected families originated from Italy, Portugal, and Brazil, indicating the presence of this mutation in different populations. The associated phenotype is broad, including early and late disease onset (Di Fonzo *et al.*, 2005). The *LRRK2* gene encodes a 286-kDa protein that is a member of a novel family of protein kinases called "dardarin" (meaning "tremor" in the Basque region,

from where some of the affected patients came). Dardarin contains leucine-rich repeats and a Ras/small GTPase superfamily domain, a tyrosine kinase-like domain, and the WD40 domains with sequence similarity to both tyrosine and serine/threonine kinases (Shen, 2004). The presence of these novel domains in dardarin suggests a unique and new function for this kinase in the survival of nigral DA neurons.

Mutations in *NR4A2* have also been found to be significantly associated with fPD (Le *et al.*, 2003). The *NR4A2* gene (also called *Nurr1*; i.e., *nu*clear *r*eceptor-*r*elated 1) encodes a transcription factor that belongs to the steroid/thyroid hormone receptor superfamily. Alternative splicing and selective use of transcription initiation sites control the expression of the human *Nurr1* gene (Ichinose *et al.*, 1999a,b). Interestingly, *Nurr1* is essential for the differentiation of the nigral DA neurons and is closely related to the expression and function of the DA system. The *Nurr1* gene activates expression of tyrosine hydroxylase (TH; Iwawaki *et al.*, 2000), and also enhances transcription of the DA transporter (Sacchetti *et al.*, 2001). NR4A2 (+/–) mice have a parkinsonian-like phenotype and are more susceptible than the wild type to MPTP (Warbt *et al.*, 2003). Although the mechanism is not yet clear, NR4A2 is thought to be a susceptibility gene for sPD.

DA deficiency due to mutations of the genes of the enzymes involved in DA biosynthesis, i.e., DA-synthesizing-enzyme TH or its cofactor tetrahydrobiopt erin (BH4)-synthesizing enzyme GTP cyclohydrolase I (GCH), causes DOPA-responsive dystonia, parkinsonism in infancy or progressive infantile encephalopathy with L-DOPA-nonresponsive dystonia, depending upon the degree of DA deficiency (Hoffmann *et al.*, 2003; Segawa *et al.*, 2003; Kobayashi and Nagatsu, 2005). Autosomal dominant GCH deficiency, which was first described by Segawa and thus called Segawa's disease, is a DOPA-responsive dystonia caused by a partial decrease of the activity of GCH due to a mutation of one of its alleles (Ichinose *et al.*, 1994, 1995, 1999; Nagatsu and Ichinose, 1999; Segawa *et al.*, 2003). Segawa's disease is a partial DA deficiency without any DA cell death and the symptom is completely controllable by L-DOPA administration. In contrast, PARK2 or autosomal recessive juvenile PD is initially similar to DOPA-responsive dystonia but progresses to parkinsonism, and is accompanied by DA cell death.

fPDs indicate the importance of the dysfunction of UPS and protein misfolding in the pathogenesis of PD. Overexpression of alpha-synuclein in mice and rats leads to the development of mitochondrial degeneration and produces DA cell death similarly as in sPD (Yamada *et al.*, 2004; Martin *et al.*, 2006). On the other hand, mitochondrial dysfunction in sPD also causes the dysfunction of UPS due to ATP deficiency. The above genes involved in fPD may be susceptibility genes for sPD. It has been reported that DA covalen1tly modifies and functionally inactivates parkin, suggesting a vulnerability of parkin to modification by DA and a mechanism for the progressive loss of the neuroprotective parkin function in DA neurons during aging and sPD (LaVoie *et al.*, 2005). Thus, fPD and sPD are different in their primary causes, but may ultimately produce nigral DA cell death by a final common pathway.

CHANGES IN CYTOKINES PRODUCED BY ACTIVATED MICROGLIA DURING THE NEUROINFLAMMATORY PROCESS IN PD

Elevated Pro-Inflammatory Cytokine Expression in the Presence of Activated Microglia in the Nigro-Striatal Region in sPD

We and others have reported increases and decreases in the levels of pro-inflammatory cytokines and neurotrophins, along with the appearance of activated microglia, in the brain of sPD patients, thus suggesting the presence of an inflammatory process (McGeer and McGeer, 1995; Anglade *et al.*, 1997; Hirsch *et al.*, 1999; Mogi and Nagatsu, 1999; Nagatsu *et al.*, 1999; Jellinger, 2000; Hartmann *et al.*, 2000; Nagatsu *et al.*, 2000a,b; Nagatsu, 2002a; Hayley, 2005; Herrera *et al.*, 2005; Nagatsu and Sawada, 2005). Based on the results of enzyme-linked immunosolvent assay (ELISAs; for a review, see Nagatsu, 2002a), we reported the changes in the levels of the following cytokines and neurotrophins in the postmortem brain (striatum) and/or ventricular or lumbar cerebrospinal fluid (CSF) in sPD patients as compared with their normal levels: (1) increased levels of TNF-alpha (Mogi *et al.*, 1994a), IL-1beta, IL-6 (Mogi *et al.*, 1994b), IL-2, IL-4, EGF, TGF-alpha, TGF-beta1, TGF-beta2, Bcl-2 (Mogi *et al.*, 1996), soluble FAS, TNF R1 (p55), caspase 1, and caspase 3 (Mogi *et al.*, 2000); and (2) decreased levels of neurotrophins BDNT and NGF. These data on changes in the levels of cytokines in human PD brains were also supported by the results obtained from animal models of PD. For example, MPTP-treated mice show an increased level of IL-1beta and a decreased level of NGF specifically in their striatum (Mogi *et al.*, 1998). As another model of PD, in hemiparkinsonian rats produced by injecting 6-OHDA into one side of the ventrotegmental bundle without or with L-DOPA treatment, the levels of TNF-alpha were significantly increased only in the substantia nigra and striatum of the injected side. L-DOPA administration did not produce any significant changes in TNF-alpha levels in either 6-OHDA-treated or control side of any of the brains (Mogi and Nagatsu, 1999). These results agree with the changes seen in the TNF-alpha levels in the striatum and lumbar CSF in PD patients and also suggest that the increased cytokine levels may not be due to the secondary effects of L-DOPA therapy in PD patients.

The increased levels of pro-inflammatory cytokines such as TNF-alpha, IL-6, and IL-1beta and the decreased levels of neurotrophins such as BDNF and NGF, which changes are known to trigger the process of apoptosis, strongly suggest a pro-apoptotic environment in the striatum in PD. In fact, the levels of apoptosis-related factors such as Bcl-2 (Mogi *et al.*, 1996), soluble FAS, TNF R1 (p55), caspase 1 (IL-1-beta converting enzyme), and caspase 3 are increased in the PD brain (Mogi *et al.*, 2000). Fas antigen and 2 TNF receptors, p55 and p75, are implicated in triggering cell death upon stimulation by their natural ligands, i.e., TNF-alpha and Fas ligands (Nagata and Goldstein, 1995). Since TNF R1 and caspases 1 and 3 have been implicated as mediators of apoptotic cell death (Kumar, 1995), their increased levels support the presence of pro-apoptotic environment in the striatum in the PD brain. The increased levels of Bcl-2 (Mogi *et al.*, 1996) and sFAS, which are anti-apoptotic

factors, may suggest their compensatory production to cope with apoptosis. Marshall *et al.* (1997) also reported up-regulation of Bcl-2 in the basal ganglia in PD patients. We also found that the levels of two other factors trophic toward DA neurons, i.e., GDNF (*g*lial cell line-*d*erived *n*eurotrophic *f*actor) and bFGF (*b*asic *f*ibroblast *g*rowth *f*actor) were not decreased, although their concentrations were high in the striatum in control or PD brains. This is in contrast to the markedly reduced levels of BDNF or NGF specifically in that region in PD. The unchanged level of GDNF in PD could be due to compensatory production in glial cells, which occurs with neither BDNF nor NGF. In agreement with our results obtained by ELISA, Boka *et al.* (1994) found TNF-alpha immunoreactive glial cells in the substantia nigra in the PD brain, and other workers also reported increased cytokine levels in *de novo* PD without L-DOPA treatment: IL-1beta and IL-6 in lumbar CSF (Blum-Degan *et al.*, 1995) and TGF-beta1 and TGF-beta2 in ventricular CSF (Vawter *et al.*, 1996). Activated caspase 3 was also detected immunohistochemically and was proposed to be the final effector in the apoptotic cell death of DA neurons in PD (Hartmann *et al.*, 2002).

Pro-Inflammatory Cytokines in the PD Brain are Produced from Activated Microglia

The origin of pro-inflammatory cytokines in the PD brain is speculated to be activated microglia. McGeer *et al.* (1988) were the first to report an increase in the number of major histocompatibility complex class II antigen [human leukocyte antigen-DR (HLA-DR)]-positive reactive microglia in the substantia nigra in PD patients. We also speculated that activated microglia are present in the PD brain to produce pro-apoptotic cytokines and neuroinflammation, ultimately promoting death of DA neurons in the substantia nigra. Imamura *et al.* (2003) proved that increased amounts of cytokines are produced by activated microglia in the putamen of sPD patients. Mogi *et al.* (1994a,b) had previously shown, by enzyme immunoassay, increased levels of TNF-alpha and IL-6 in the striatum in sPD. Imamura *et al.* (2003) identified by Western blot analysis TNF-alpha protein and IL-6 protein, along with MHC class II (CR3/43) protein, in homogenates of the putamen from sPD patients; and they further proved by an immunofluorescence technique the coexistence of TNF-alpha and IL-6 proteins in ICAM-I and LFA-1-positive MHC class II-bearing activated microglia in the putamen from sPD patients. These results confirmed that TNF-alpha and IL-6 proteins are produced from activated microglia in the putamen in sPD.

Activated Microglia may be Initially Non-Toxic/Neuroprotective and Then by a Toxic Change Become Neurotoxic to Cause Progression of PD

Activated microglia are known to produce either neuroprotective or neurotoxic factors. The question is whether these activated microglia are neuroprotective or neurotoxic toward the nigro-striatal DA neurons.

We aimed at elucidating the role of activated microglia in the postmortem brain of sPD at the cellular level. Activated microglia have multiple roles: (1) MHC class