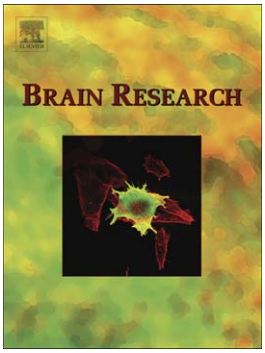

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Abstract

G-protein-coupled receptor kinase 5 (GRK5) has been reported to accumulate in Lewy bodies (LBs), a histological hallmark of Parkinson's disease. Recent findings propose that GRK5 might function in Parkinson's disease via phosphorylation of α-synuclein, a major component of LBs. In this study, the changes of the expression levels of GRK5 and its possible effects in Parkinson's disease were evaluated in cell lines and transgenic mice model of α-synuclein overexpression. Both the expression levels of cytoplasmic and nuclear distributed GRK5 were induced an increase by α-synuclein overexpression *in vivo* and *in vitro*. The observations that the levels of α-synuclein phosphorylated at Ser-129 (pS129-α-synuclein) remain unchanged despite the downregulation of GRK5 by short hairpin ribo nucleic acid (shRNA) transfection suggest that GRK5 is not the sole kinase involved in phosphorylating α-synuclein in Parkinson's disease. In addition, the findings that nuclear accumulation of GRK5 inhibits blc2 transcription and expression, at least in part by enhancing histone deacetylase (HDAC) activity, show an unexpected role for nuclear GRK5 in the regulation of an apoptosis-related gene. The present study suggests that GRK5 may be extensively involved in the mechanism of Parkinson's disease.

Keywords: α-synuclein; GRK5; histone deacetylase; bcl-2; gene regulation

Abbreviations: GRKs, G protein-coupled receptor kinases; LBs, Lewy bodies; pS129-α-synuclein, α-synuclein phosphorylated at Ser-129; shRNA, short hairpin ribo nucleic acid; HDAC, histone deacetylase; GPCRs, G protein-coupled receptors; NLS, DNA-binding nuclear localization sequence

1. Introduction

G protein-coupled receptor kinases (GRKs) are members of the serine/threonine protein kinases family and play a critical role in cellular signaling. The central role of GRKs in phosphorylation and desensitization of G protein-coupled receptors (GPCRs) is well established (Lefkowitz, 1998; Pitcher et al, 1998). Recent studies have revealed that these GRKs may perform new cellular functions by phosphorylating non-receptor substrates such as tubulin, synucleins and the ribosomal protein P2 (Carman et al., 1998; Freeman et al., 2002; Pronin et al., 2000). GRK5, a member of GRKs family, shares characteristics in common with the other family members, possessing the ability to phosphorylate GPCRs as well as non-receptor substrates. Some of the structural and functional domains contributing to the localization and function of GRK5 have been identified: an N-terminal phosphatidylinositol -4, 5-bisphosphate binding domain (Pitcher et al., 1996; Pronin et al., 1998); a phospholipid binding domain; an autoinhibitory domain; two calmodulin (CaM) binding domains (Levay et al., 1998) and a DNA-binding nuclear localization sequence (NLS) (Johnson et al., 2004).

GRK5 has been correlated with the pathogenesis of Parkinson's disease, a neurological disorder characterized by the degeneration of dopaminergic neurons in the substantia nigra and the formation of Lewy bodies (LBs). α-Synuclein forms a major component of the LBs and is phosphorylated at Ser-129 (pS129-α-synuclein) (Baba et al., 1998; Fujiwara et al., 2002; Pronin et al., 2000; Spillantini et al., 1997, 1998), rendering it toxic to the dopaminergic neurons (Chen et al., 2005; Yamada et

al., 2004). The accumulation of GRK5 in the LBs and its colocalization with α-synuclein within the neurons of patients with PD has been demonstrated (Arawaka et al., 2006). For determining the function of GRK5 in phosphorylating α-synuclein, we have down-regulated the expression of GRK5 *in vitro* through short hairpin ribo nucleic acid (shRNA) transfection and then observed the changes of pS129-α-synuclein protein levels.

The above study is focused on the function of cytoplasmic distributed GRK5 in Parkinson's disease. Some studies have called our attention to quest the function of nuclear distributed GRK5. The NLS has been identified in GRK5 but its nuclear function remains largely unclear (Johnson et al., 2004). Most recently, GRK5 has been found to be a histone deacetylase (HDAC) kinase in the nucleus of cardiomyocytes (Martini et al., 2008). The NLS contained in GRK5 and the activity of HDAC kinase discovered in GRK5 prompt us to explore additional roles for GRK5 in Parkinson's disease. For this purpose, HDAC activity has been measured in stably human α-synuclein (hα-synuclein) expressing cells and control SHSY5Y cells. Then we investigate the changes of HDAC activity through downregulating GRK5 *in vitro* to confirm that GRK5 possesses nuclear HDAC kinase activity in neuroblastoma SHSY5Y cells as well. Based on the function of GRK5 in regulating HDAC activity and the controversial role of apoptosis in Parkinson's disease (Anglade et al., 1997; Hartmann et al., 2001; Hartmann et al., 2001; Jellinger, 2001; Mochizuku et al., 1996; Takai et al., 1998; Tatton et al., 2003; Tompkins et al., 1997), we examined the alteration of expression levels of some apoptosis- or cell cycle-related genes in stably

hα-synuclein expressing cells and in the SHSY5Y cells down-regulated GRK5 by shRNA transfection.

Human α-synuclein (hα-synuclein) expressing transgenic mice and stably hα-synuclein expressing cells have been developed to investigate the changes in the expression levels of GRK5 in this study. Then the α-synuclein phosphorylation function of cytoplasmic GRK5 and the effect on the activity of HDAC of nuclear GRK5 have been analyzed in cell models of Parkinson's disease. Finally, we further seek and determine the target genes regulated by GRK5.

2. Results

2. 1 Up-regulation of α-synuclein promotes GRK5 protein expression *in vivo* and *in vitro*

To analyze the role of GRK5 in Parkinson's disease, hα-synuclein expressing transgenic mice and stably hα-synuclein expressing SHSY5Y cell lines has been developed. Results from western blotting experiments revealed that GRK5 protein was significantly overexpressed in the stably hα-synuclein expressing cell lines (Fig. 1A). And increased levels of GRK5 protein expression were also detected in the brain extracts of hα-synuclein expressing transgenic mice. The expression of GRK5 was increased in an α-synuclein expression level dependent manner (Fig. 1B). The expression levels of GRK5 protein in the 3- and 6- month-old hα-synuclein expressing transgenic mice were increased 161% and 104% when compared to their age-matched non-transgenic littermates, respectively (Fig. 1C).

2. 2 GRK5 protein accumulates in the cytoplasm as well as in the nuclei.

Western blotting experiments were performed to determine whether GRK5 protein was overespressed in the nuclear or cytoplasmic extracts. Western blotting analysis showed that hα-synuclein expressing cells possessed higher protein levels of GRK5 in both cytoplasmic and nuclear extracts than the SHSY5Y control cells. A representative western blotting experiment of three independent experiments is shown (Fig. 1D). Next analysis of the expression level of GRK5 in nuclear and cytoplasmic extracts from the brain samples of 3- and 6-month-old hα-synuclein expressing transgenic mice and their age-matched non-transgenic littermates was conducted. The expression of GRK5 in the nuclear extracts from the brains of 3- and 6-month-old transgenic mice was increased 1.31 and 1.15 fold respectively, compared with their age-matched non transgenic control mice (Fig. 2A). Higher levels of GRK5 protein in the cytoplasmic extracts were shown in the transgenic mice than in the non transgenic mice (Fig. 2B). The results that increased expression is seen in nuclear distributed GRK5 as well as cytoplasmic GRK5 suggest that both cytoplasmic and nuclear GRK5 protein might participate in the pathogenesis of Parkinson's disease.

2. 3 The knockdown of endogenous GRK5 fails to suppress the phosphorylation of α-synuclein at Ser-129

GRK5 has been shown to be involved in the pathogenesis of Parkinson's disease by modification α-synuclein in the LBs (Arawaka et al., 2006). Therefore, we evaluated pS129-α-synuclein protein level in the SHSY5Y cells transiently transfected with control shRNA, shRNA1-GRK5 and shRNA2-GRK5. Under the present condition,

the expression levels of pS129-α-synuclein protein remained unchanged even when GRK5 expression was repressed (Fig. 3A).

2. 4 GRK5 regulates HDAC activity

The HDAC assay was performed to investigate whether GRK5 might regulate the activity of HDAC. The assay revealed that compared with control cells, hα-synuclein expressing cells with high levels of GRK5 expression showed increased HDAC activity. The HDAC activity in hα-synuclein expressing cells was 139% compared to the 100% set in SHSY5Y control cells (Fig. 3B). The HDAC activity was observed to be significantly (50–80%) reduced in the SHSY5Y cells transiently transfected with shRNA1-GRK5 and shRNA2-GRK5 (Fig. 3C), suggesting that GRK5 could regulate the HDAC activity. Specifically, an increased expression of GRK5 resulting from an overexpression of α-synuclein enhances HDAC activity.

2. 5 **Nuclear accumulation of GRK5 regulates blc-2 transcription and expression**

In an attempt to elucidate a potential regulatory role for nuclear GRK5, we selected three apoptosis- or cell cycle-related genes (p27, bcl-2 and c-fos) and TH gene, a rate limiting enzyme for the synthesis of catecholamines that play a key role in Parkinson's disease. The results of the ChIP assay showed that GRK5 could bind to bcl-2 promoter and regulate its expression (Fig. 3D). The levels of p27, c-fos and TH remained unaffected (data not shown). The presence of bcl-2, p27, c-fos and TH promoter sequences in the input DNA and in that recovered from antibody-bound chromatin segments was analyzed by real-time PCR. Then we detected the expression levels of bcl-2 in the models. In the hα-synuclein expressing cells, bcl-2 mRNA and

protein levels decreased in comparison with control cells (Fig. 4A and 4B). And the same results were observed in the transgenic mice (Fig. 4C). The expression levels of bcl-2 in the brains of 3- and 6-month-old transgenic mice were decreased 1.27 and 1.68 fold respectively, compared with their age-matched non transgenic control mice. In order to further determine whether GRK5 could indeed regulate the expression of bcl-2, we transfected SHSY5Y cells with control shRNA, shRNA1-GRK5 and shRNA2-GRK5. Under the present condition, the repression of GRK5 expression by shRNA1 and shRNA2 was observed simultaneous with an increase in bcl-2 mRNA and protein levels (Fig. 4D and 4E). Overall, these results suggest that GRK5 may regulate the expression of bcl-2 partially by modulating the activity of HDAC.

3. Discussion

Bychkov and colleagues demonstrate that GRK5 protein and mRNA levels are overexpressed in patients of Parkinson's disease with dementia. However, GRK5 expression levels do not differ between Parkinson's disease groups and control groups (Bychkov et al., 2008). Our data indicate that GRK5 protein is expressed at higher levels in stably hα-synuclein expressing cells than in the control cells and in hα-synuclein expressing transgenic mice than in the control mice. The results differ from this previous report perhaps because the cellular and mice models of Parkinson's disease used in the present study were generated by increasing the expression levels of hα-synuclein protein. We speculate that one possible explanation for the results that α-synuclein promotes the expression of GRK5 is the dopaminergic dysfunction in

Parkinson's disease. Dopamine (DA) receptors belong to the GPCRs superfamily and undergo desensitization via activation-dependent receptor phosphorylation by GRKs. The subsequent binding of arrestins shield the cytoplasmic surface of the receptors precluding further G protein coupled signaling from persistent stimulation (Krupnick et al., 1998). The dysregulation of DA receptor signaling in the basal ganglia implicated in Parkinson's disease plays a role in generating motor deficits (Guigoni et al., 2005; Joyce et al., 2001; Muriel et al., 1999; Ryoo et al., 1998). Therefore we think that dopaminergic dysfunction may determine the changes in GRK5 concentrations seen in our study. But the underlying mechanism remains largely unexplored.

GRK5 has been shown to participate in Parkinson's disease by phosphorylating α-synuclein at serine 129 (Ser129) within the intracellular protein aggregates called LBs (Arawaka et al., 2006; Fujiwara et al., 2002). In the present study, we repressed the expression of GRK5 by transfecting SHSY5Y cells with shRNA-GRK5. Surprisingly, the expression levels of pS129-α-synuclein didn’t change. This is in agreement with the most recently published data (Sakamoto et al., 2009). This finding suggests the phosphorylatory action of other protein kinases. Indeed, casein kinases (CK) 1, CK2 and other GRKs have been shown to phosphorylate α-synuclein (Okochi et al., 2000; Pronin et al., 2000).

In this study, GRK5 increased not only in the cytoplasm but also in the nuclei was shown for the first time. It's worth noting that nuclear GRK5 might function in Parkinson's disease. GRK5 has been implicated in the regulation of transcription

events within the nuclei: the regulation of NFκB transcription activity and as a HDAC kinase in cardiomyocytes (Martini et al., 2008; Sorriento et al., 2008). In the present research, enhanced HDAC activity in the hα-synuclein cell lines and attenuation of HDAC activity in hunan neuroblastoma SHSY5Y cells of GRK5 knocked down by shRNA were detected. So GRK5 exerts the function as a HDAC kinase not only in cardiomyocytes but also in neuroblastoma cells. The overexpression of α-synuclein may facilitate the activity of HDAC by increasing the expression of GRK5.

From the observation that the expression levels of bcl-2 decreased in the hα-synuclein expressing models of GRK5 overexpression and increased in the cells of GRK5 genetic knock downs, we assert that GRK5 could regulate bcl-2 transcription and expression. Furthermore, based on the results that GRK5 possessed the HDAC kinase activity, we speculate that GRK5 could regulate bcl-2 partly by modulation of HDAC. In fact, the regulation of bcl-2 expression has been known to be complicated. In this study, the changes in bcl-2 expression levels were partly due to the alteration of the HDAC activity induced by GRK5 expression. Besides, GRK5 has been shown to inhibit the transcriptional activity of NFκB by inducing nuclear accumulation of IκB (Sorriento et al., 2008). Bcl-2, as a target gene of NFκB (Tamatani et al., 1999) could be regulated by GRK5. So the possibility that GRK5 regulates bcl-2 expression by affecting the transcriptional activity of NFκB still exists. Bcl-2 as an anti-apoptotic protein may play an important role in Parkinson's disease (Takai et al., 1998; Offen et al., 1997). Our results provide robust support for the assertion that the nuclear GRK5 is involved in the regulation of bcl-2 transcription and expression in Parkinson's

disease. At the same time, GRK5 probably regulates other as yet unknown genes as well. A better understanding of GRK5 regulatory function is needed.

The present study demonstrates that overexpression of α-synuclein causes increased expression of GRK5 in cytoplasm and nucleus, and also suggests a novel function for GRK5 in Parkinson's disease. New insights into the function of GRK5 involved in the molecular mechanisms of Parkinson's disease may lead to the discovery of exciting new therapeutic approach to combat Parkinson's disease.

4. Experimental procedures

The experimental protocols of this study involved animals were approved by the appropriate institutional review committees and met the guidelines of the appropriate governmental agency (authorization number SCXK(Jing)2004-0001).

4. 1 Antibodies and shRNA

The antibodies used in western blotting experiments included a mouse monoclonal antibody to human α-synuclein (ab1904) (Abcam, Cambridge, UK), a rabbit monoclonal (EP1536Y) to human pS129-α-synuclein (ab51253) (Abcam, Cambridge, UK), a rabbit polyclonal GRK5 antibody (sc-565) (Santa Cruz Biotechnology, Santa Cruz, CA), an anti-human bcl-2 antibody (ab18210) (Abcam, Cambridge, UK), an anti-human/mouse Glyceraldehyde-3-Phosphate Dehydrogenase (GAPDH) antibody (KC-5G5) (Kangcheng, Shanghai, P.R. China), an anti-human/mouse α-tubulin antibody (T6074) (Sigma-Aldrich Company; Saint Louis, USA)), a monoclonal anti-Proliferating Cell Nuclear Antigen antibody (PCNA) (P8825) (Sigma-Aldrich

Company; Saint Louis, USA). The shRNA for human GRK5 were purchased from Open Biosystem (Huntsville, AL, USA). The corresponding experiments were carried out by using non-silencing shRNA control plasmid as negative control and two shRNA plasmids which repressed GRK5 to a greater degree than the other three shRNA plasmids. The nucleotide sequences of the selected plasmids, shRNA1-GRK5 and shRNA2-GRK5 were as follows: 5'-CCGGACGAGATGATAG AAACAGAATCTCGAGATTCTGTTTCTATCATCTCGTTTTTT-3' and 5'-CCGGC CACCACATAAACTCAAACCACTCGAGTGGTTTGAGTTTATGTGGTGGTTTTT -3'.

4. 2 Vector construction and transgenic mice development

Human α-synuclein sequence was obtained from vector CMVP-a-Syn (constructed by Guo et al.) (Guo et al., 2008) using the restriction enzymes, HindIII and XhoI. And the promoter platelet-derived growth factor (PDGF) was obtained from the vector PsisCAT6a (gifted by doctor Tucker Collins, Harvard Medical School) using HindIII and BamHI. The α-synuclein sequence and the promoter were subcloned into the vector pCEP4 (V04450) ((Invitrogen, CA, USA) by digestion with BglII and XhoI, generating a vector including a PDGF promoter driving α- synuclein. DNA sequence of this vector was confirmed. The PDGF-hα-synuclein containing vector was linearized with Tth111I and XhoI, and the linearized DNA fragment was micro-injected into C57BL/6 fertilized mouse eggs. The founder mice were identified by PCR using the following primers: sense 5'-GAGGAAGGGTATCAAGACTACG AAC-3' and the antisense 5'-GCCGGATCATAATCAGCCATACCAC-3'. Of the four

founder mice, the one with the highest and the one with the lowest expression levels of hα-synuclein were selected for the western blotting experiments of GRK5 expression evaluation. The transgenic mice of different ages used in this study were those with the highest expression level of hα-synuclein. The off-springs were obtained by backcrossing into the C57BL/6 mouse strain. The male hα-synuclein expressing transgenic mice were selected and used for all experiments and the male non-transgenic mice were used as control.

4. 3 Cell culture and Transfection

Human neuroblastoma SHSY5Y cells were cultured in Dulbecco's modified Eagle's medium (DMEM), supplemented with 10% fetal bovine serum, 100 mg/ml streptomycin, and 100 U/ml penicillin, and were grown in humidified air with 5% CO2 at 37°C. The SHSY5Y cells were transfected with the empty vector, the PDGF-hα-synuclein vector, the control shRNA and the shRNA for human GRK5 in 24-well plates (1×10^5 cells seeded per well) using Lipofectamine™ 2000 Reagent (Invitrogen). Stably hα-synuclein expressing cell lines were established by G418 (500 μg/ml) selection.

4. 4 Tissue Collection

The transgenic mice and their age-matched normal littermates at the age of 3 and 6 months were sacrificed and the brain tissues were removed. The fresh brain tissue was immediately homogenized in Trizol（Invitrogen, Carlsbad, CA, USA）for total RNA extraction, or in RIPA buffer (Beyotime Inc., NanTong, China) for total protein sample preparation. The hα-synuclein expressing cell lines and the control cells were

similarly prepared for RNA and protein extraction.

4. 5 Nuclear and Cytoplasmic Extraction Preparation

The nuclear and cytoplasmic extraction of the brain tissue and the cell samples were performed using the NE-PER® Nuclear and Cytoplasmic Extraction Kit (Pierce, Rockford, Ill., USA) following manufacturer's instructions. The following modifications were made so as to reduce the contamination between nuclear and cytoplasmic fractions. After collection of the cytoplasmic extraction, the insoluble (pellet) fraction containing the nuclei was resuspended in Cytoplasmic Extraction Reagent I (CER-1) and centrifuged for 5 minutes at maximum speed. The supernatant was discarded, the pellet suspended in Nuclear Extraction Reagent (NER) and nuclear extracts prepared as described by the manufacturer.

4. 6 ***Western blotting experiments***

For Western blotting analysis, the concentration of the protein was determined by utilizing the bicinchoninic acid (BCA) assay (Pierce, Rockford, Ill., USA). All protein samples were denatured, electrophoresed on SDS/polyacrylamide gels and transferred onto polyvinylidene difluoride membranes (Millipore, Billerica, MA). α-Synuclein, GRK5, bcl-2, GAPDH, α-tubulin and PCNA were detected by incubation first with the corresponding antibodies, followed by incubation with the secondary antibodies. GAPDH served as a positive control for total protein, α-tubulin and PCNA served as positive controls for cytoplasmic and nuclear proteins respectively. To test the specificity of the anti-GRK5 antibody, western blotting experiment has been performed by using the mice brain exacts, the cell lysates and the rat brain extract (sc-

2392) (Santa Cruz Biotechnology, Santa Cruz, CA) as a positive control. The bands were visualized by enhanced chemiluminescence kit (ECL, Amersham Biosciences, UK). The relative intensity of the band was scanned and quantified using UVIPhoto and UVISoft UVIBand software application V97.04 (UVI, British).

4. 7 Reverse transcriptase-Polymerase Chain Reaction (RT-PCR) and Real-time PCR

Total RNA was extracted from the cells or the mice brain with Trizol Reagent （Invitrogen, Carlsbad, CA, USA）according to the manufacturer's instructions. RNA integrity was evaluated after agarose gel electrophoresis and RNA concentration was determined by UV spectroscopy. RNA samples were treated with DNase I (Promega, Madison, WI, USA) for 30 min at 37 °C to remove DNA contamination. Reverse transcriptions of the equal amount of total RNA samples were performed using reverse transcription kit (k1622) (Fermentas, Vilnius, The Republic of Lithuania). Real-time quantitative PCR was performed using the SYBR Green I qPCR kit (TaKaRa, Shiga, Japan) in a LightCycler (Roche Applied Science, Indianapolis, IN, USA). Results were normalized to an internal control amplified with GAPDH primers included in the same run of real-time PCR. The primers used were 5'-GACCACACA GACGACGACTTC-3' and 5'- CGTTCAGCTCCTTAAAGCATTC-3' for human GRK5, 5'-TTCTTTGAGTTCGGTGGGGTC-3' and 5'-TGCATATTTGTTTGGGGC AGG-3' for human bcl-2, 5'-CATGGGTGTGAACCATGAGAGA-3' and 5'-TGTGGTCATGAGTCCTTCCA-3' for human GAPDH.

4. 8 HDAC activity

The HDAC activity of the cell lines were detected using a HDAC Assay Kit (Upstate Biotechnology, Lake Placid, NY, USA). A standard curve (0-1mM) was generated to ensure that the assay was within the linear range. 30μg cell proteins were analyzed and the absorbance read in a plate reader at 405nm.

4. 9 ***Chromatin Immunoprecipitation***

Chromatin Immunoprecipitation (ChIP) assay with stably hα-synuclein expressing cell lines, shRNA-GRK5 cells and the control cell lines was performed using the ChIP assay kit (Upstate Biotechnology, Lake Placid, NY, USA) following the manufacturer's instructions. The antibodies applied in ChIP assays for IgG (as unrelated antibody) and GRK5 have been described previously under the *Antibodies and shRNA* section. The presence of the bcl-2, p27, c-fos and tyrosine hydroxylase (TH) gene promoter sequences in both the input DNA and the recovered DNA immune-complexes were detected by real-time PCR. The primers for human bcl-2 promoter were forward, 5'- GCGACTCCTGATTCATTG -3'; reverse, 5'-AGGTGCGTTTCCCTGTA-3'. The primers for human p27 promoter were forward, 5'-GCGACTCCTGATTCATTGCTCCCGCCGCCGCAACCAAT-3'; reverse, 5'-CGAACCCAGCCGCTCTCCAAACC-3'. The primers for human c-fos promoter were forward, 5'-ATTAGGACACGCGCCAAGGC-3'; reverse, 5-ACGGTCACTGCTCGTTCGCT-3'. The primers for human TH promoter were forward, 5'-CAGATGGCACTCCTAGGAACCAC -3'; reverse, 5'- TCAGTGTGGA GGTCCGGGCT-3'. The primers used to amplify the control GAPDH promoter were forward, 5'-TACTAGCGGTTTTACGGGCG-3'; reverse, 5'-TCGAACAGGAGGAG

CAGAGAGCGA-3'. The data obtained was normalized to the corresponding DNA input control.

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Figure legends

Fig.1. Up-regulation of α-synuclein induces an increase of GRK5 protein expression *in vivo* and *in vitro,* and expression of GRK5 protein increases in the cytoplasm as well as in the nucleus of hα-synuclein expressing cells. (A) Western blotting analyses of total protein extracted from hα-synuclein expressing cells and SHSY5Y cells (control). GAPDH is used as a loading control. A representative western blotting experiment of three independent experiments is shown. (B) Western blotting analyses using total protein extracted from transgenic mice of different hα-synuclein expressing levels and non transgenic mice. The bands of α-synuclein and GRK5 are quantified and normalized to GAPDH, the data shown are the means ± SD of three independent experiments in six mice, *$p < 0.05$, **$p < 0.01$ versus the control group. (C) Western blotting experiments of GRK5 protein in brain extracts of hα-synuclein expressing transgenic mice and non transgenic mice of different ages. (D) Western blotting analyses of nuclear and cytoplasmic proteins extracted from hα-synuclein expressing cells and SHSY5Y cells (control). The α-tubulin and PCNA are used as loading controls and to reveal any crosscontamination in the cytoplasmic and nuclear fractions.

Fig.2. Western blotting experiments show the increases of nuclear GRK5 (A) and cytoplasmic GRK5 (B) in hα-synuclein expressing transgenic mice of different ages compared with their age-matched non transgenic mice (control). The bands of GRK5 are quantified and normalized to PCNA (in nuclei) and α-tubulin (in cytoplasm). The

data shown are the means ± SD of three independent experiments in six mice, *p < 0.05, **p < 0.01 versus the control group.

Fig.3. (A) Western blotting analyses GRK5, α-synuclein and pS129-α-synuclein protein of SHSY5Y cells after shRNA1-GRK5, shRNA2-GRK5 and control shRNA transfection. GAPDH is used as a loading control. (B) HDAC activity detected using the nuclear extract from hα-synuclein expressing cells and SHSY5Y cells. Results are given as a percentage of the basal control (SHSY5Y cells as control set at 100%) and shown as means ± SD compared with control extracts (n=4),*p < 0.05, **p < 0.01 versus the control cells. (C) HDAC assay with SHSY5Y cells transiently transfected with shRNA1-GRK5, shRNA2-GRK5 and control shRNA. (D) The ChIP assay indicates the interaction between GRK5 and bcl-2 promoter. ChIP experiments are performed in SHSY5Y cells, shRNA-GRK5 cells and hα-synuclein expressing cells with antibody to GRK5 and IgG (as the unrelated antibody). The purified DNA is analyzed by real-time PCR using bcl-2 promoter primers. The data are normalized to the corresponding input controls. Results are from three independent experiments, **P<0.01 vs. the corresponding control.

Fig.4. The transcription and expression of bcl-2 is regulated by GRK5. (A) Real-time PCR analyses of the mRNA level of bcl-2 in hα-synuclein expressing cells and control cells. Results are from three different experiments, each measured in triple, *p < 0.05, **p < 0.01 versus the control cells. (B) Western blotting analyses of total protein

extracted from hα-synuclein expressing cells and SHSY5Y cells (control cells), β-actin as a loading control. (C) Western blotting experiments of bcl-2 protein in brain extracts of hα-synuclein expressing transgenic mice and non transgenic mice of different ages. (D) The mRNA levels of GRK5 and bcl-2 in SHSY5Y cells transiently transfected with control shRNA, shRNA1-GRK5 and shRNA2-GRK5. All data are pooled from three independent experiments, each measured in triple, *p < 0.05, **p < 0.01 versus the control cells. (E) Western blotting analyses GRK5 and bcl-2 protein expression in SHSY5Y cells after control shRNA, shRNA1-GRK5 and shRNA2-GRK5 transfection. GAPDH is used as a loading control.