

Table 4 Asian specific effects and influence of between-study heterogeneity

Gene ^a	SNP	Q test p value	I ² (95% CI)	Odds ratio (95% CI) by random effects	Fixed effects p value	Random effects p value
<i>STK39</i>	rs2102808	0.01	73 (0-88)	1.14 (0.85-1.52)	0.28	0.37
<i>LAMP3</i>	rs11711441	0.33	12 (0-72)	0.81 (0.67-0.97)	0.01	0.03
<i>BST1</i>	rs11724635	0.07	53 (0-81)	0.74 (0.68-0.81)	1.2 × 10 ⁻⁶	0.001
<i>HLA-DRB5</i>	rs3129882	0.81	0 (0-68)	0.98 (0.85-1.13)	0.85	0.85
<i>SNCA</i>	rs356219	0.49	0 (0-68)	1.24 (1.08-1.43)	0.002	0.002
<i>LRRK2</i>	rs1491942	0.31	16 (0-73)	1.13 (1.02-1.24)	0.005	0.01
<i>HIP1R</i>	rs10847864	0.36	6 (0-70)	1.14 (0.99-1.32)	0.05	0.06

Abbreviations: CI = confidence interval; HWE = Hardy-Weinberg equilibrium; SNP = single nucleotide polymorphism.

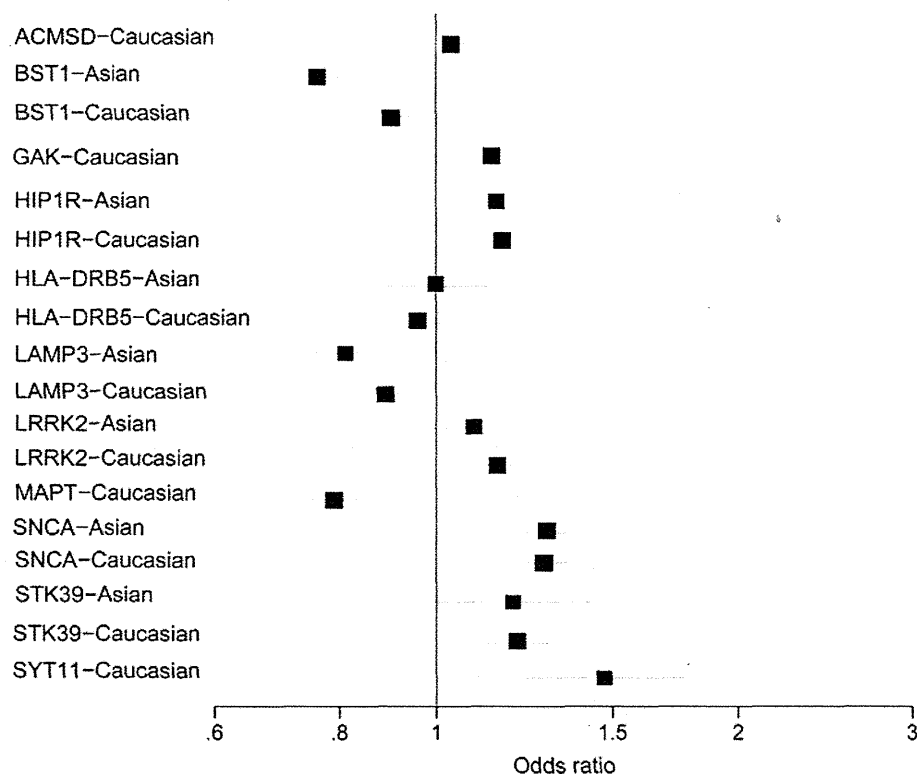
^a *ACMSD*, *SYT11*, *MAPT* SNPs were monomorphic; *GAK* SNP showed departure from HWE and thus is not included in the table.

where both Caucasians and Asian populations were represented showed no difference in effect size estimates that were different beyond chance (figure 1). Conversely for *BST1*, the effects were different beyond chance for Asian and Caucasian populations with stronger genetic effects in the former.

DISCUSSION We performed a large-scale evaluation to assess the role of recently discovered genetic risk variants in the pathogenesis of PD in different

populations. Our study confirms 9 of the 11 postulated susceptibility SNPs for PD. The confirmed SNPs include the previously well documented *LRRK2*, *SNCA*, *BST1*, *GAK*, and *MAPT* associations,^{3,6-8,10,20} and 4 of the 5 more recently proposed associations in *STK39*, *SYT11*, *LAMP3*, and *HIP1R* SNPs that might act as risk factors for PD. Conversely, we were unable to confirm the association between PD risk and SNPs rs10928513, rs3129882 in *ACMSD* and *HLA-DRB5*, respec-

Figure 1 Forest plot showing the comparison of effect of each single nucleotide polymorphism (SNP) in Caucasian and Asian population



Boxes indicate the summary effect estimate. SNPs in 3 loci (*MAPT*, *SYT11*, and *ACMSD*) were monomorphic in the Asian population and thus are not included in the graph. *GAK* SNP showed deviation from Hardy-Weinberg equilibrium in Asian series and thus excluded from the graph.

tively. Three SNPs were monomorphic in the Asian datasets.

The recently proposed *ACMSD* locus is likely to have represented a spurious association. The OR is very close to the null and 95% CI also excludes an OR larger than 1.08. Of note, even in GWAS meta-analysis, the OR estimate was only 1.07 in the replication phase, as compared with 1.38 in the discovery phase. Our results are in agreement with a recently published study.²¹ This finding suggests that large-scale replication with direct genotyping is useful even for SNPs that pass conventional genome-wide significance thresholds. It is possible that with the use of the more extended imputation platforms using the 1,000 Genomes Project, the number of comparisons made is larger than what was done in the past with more limited imputation platforms and this may thus require more stringent levels of significance to claim genome-wide significance.

As observed in a recently published study,⁹ the frequency of the *HLA-DRB5* SNP, rs3129882, varies considerably even within seemingly homogenous Caucasian populations; the frequency of risk allele is low in subjects of Northern European descent as compared to subjects from Southern European descent. Therefore, the observed lack of association for the *HLA-DRB5*-specific SNP rs3129882 should be interpreted with caution. Moreover, directionality as well as the magnitude of effect estimates obtained in our study for the *HLA-DRB5* locus specific SNP are comparable with previously published studies.^{9,22}

Our study shed light on the role of heterogeneity in PD genetics. Detection of heterogeneity could provide new insight to understand the genetic architecture of the disease.^{17,23,24} A number of factors can be attributed to the observed heterogeneity. First, clinically overlapping pathologies may lead to heterogeneity. Indeed the presence of distinct subgroups of patients during early clinical stages of PD could contribute to clinical heterogeneity.²⁵ Therefore, it is worth it to consider that genetic variants may exert different pathologic processes that eventually lead to complex clinical phenotypes.²⁶ For example, it has been shown that multiplications of *SNCA* gene lead to clinical phenotype, which clinically overlap with multiple system atrophy (MSA).²⁷ Moreover, SNPs in the 3' UTR of *SNCA* were shown to be associated with PD as well as MSA.^{3,6–8,28} The most significant SNPs in both diseases clustered around 3' UTR of *SNCA*; SNP rs11931074, that was significantly associated with MSA in contrast to rs356219 in the PD meta-analysis.^{14,28} The r^2 between these 2 markers was only 0.16 in the Caucasian population. These 2 distinct signals in different yet overlapping pathologies therefore might reflect one cause of genetic heterogeneity. Second, heterogeneity might reflect that

different tagging polymorphisms were used in previously published GWAS. Of note, a recently published GWAS from United Kingdom, France, and Netherlands provided weak (as they did not surpass genome-wide significant threshold) yet consistent association signals for the *BST1* and *GAK* gene.^{7,8,13} This probably reflects that the investigated markers were not causal variants but in linkage disequilibrium with a potential causal variant across different studies.

Our study helps to understand the role of population-specific heterogeneity in PD risk loci. Some risk variants exist only in populations of specific ancestry. For example, it is known that an ancient inversion (~900 kb) in the *MAPT* region occurred, which led to the formation of 2 nonrecombining haplotypes, H1 and H2.²⁹ The H2 haplotype is absent in East Asian populations as has been shown also by a recently published Asian GWAS that revealed no association for the *MAPT* locus.⁶ This is in contrast to previously published candidate gene studies and GWAS in Caucasian populations that have shown consistent association with the *MAPT* locus.^{3,7,8,13}

Some other variants may have a different magnitude of effect in populations of different ancestry. In our data, this is well exemplified by *BST1*, where the OR was significantly larger in populations of Asian than those of Caucasian ancestry. Conversely, we found that associations in 5 loci (*SNCA*, *LRRK2*, *LAMP3*, *HIP1R*, and *STK39*) had similar effects in these 2 ancestries.

The consistency or diversity of effect sizes in identified associations may reflect different patterns of linkage disequilibrium in these loci in diverse populations. It may also reflect differences in the susceptibility to develop PD and perhaps also differences in age at onset in different ancestry groups. The diversity in the magnitude of effects is important to take into account when considering the use of such information for personalized risk modeling.³⁰ Therefore caution should be used in extrapolating risks across different populations.

Our study provides strong and independent support for the role of 9 loci in the pathogenesis of PD in different populations. The detection and documentation of heterogeneity across different populations is useful in understanding the genetic architecture of this complex disease and in properly framing our ability to use this information in different clinical populations.

AUTHOR AFFILIATIONS

From the Department for Neurodegenerative Diseases (M.S., T.G., R.K.), Hertie Institute for Clinical Brain Research, University of Tübingen, and DZNE, German Centre for Neurodegenerative Diseases, Tübingen, Germany; Stanford Prevention Research Center (J.P.A.), Department of Medicine and Department of Health Research and Policy, Stanford University School of Medicine, Stanford, CA; Department of Neurology (J.O.A.), St Olavs Hospital and NTNU Trondheim, Trond-

heim, Norway; Institute of Neurological Sciences (G. Annesi, A.Q.), National Research Council, Mangone (CS), Italy; INSERM, UMR_S975 (A.B., S.L.), Université Pierre et Marie Curie-Paris, CNRS, UMR 7225, AP-HP, Pitié-Salpêtrière Hospital, Paris, France; Department of Genetics and Cytogenetics (A.B.), Pitié-Salpêtrière Hospital, Paris, France; Neurodegenerative Brain Diseases Group (C.V.B., D.C., J.T.), Department of Molecular Genetics, VIB, Antwerp; Institute Born-Bunge (C.V.B., D.C., J.T.), University of Antwerp, Antwerp, Belgium; Neuropsychiatric Genetics Group (L.B., C.M.L.), Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, Berlin, Germany; General Hospital of Syros (M.B.), Syros; 10. "Hygeia" Hospital (M.B.), Clinic of Neurodegenerative Disorders, Athens; 2nd Neurology Clinic (M.B., L.S.), University of Athens, "Attikon" Hospital, Athens, Greece; Department of Neurology (D.C.), Antwerp University Hospital, Antwerp, Belgium; School of Clinical and Experimental Medicine (C.C., K.E.M.), College of Medical and Dental Sciences, University of Birmingham, and City Hospital, Birmingham, UK; Centre for Biomedicine (M. Facheris, A.A.H., P.P.P., I.P.), EURAC Bolzano, Bolzano, Italy; Department of Medical Genetics (M. Farrer, C.V.-G.), University of British Columbia, Vancouver, Canada; Cyclotron Research Centre (G.G.), Department of Neurology, University of Liège, Liège, Belgium; Department of Neurology (S.G., G. Auburger), Goethe University, Frankfurt am Main, Germany; Department of Neurology (G.M.H., G.X.), Faculty of Medicine, University of Thessaly and Institute of Biomedical Research and Technology, CERETETH, Larissa, Greece; Department of Neurology (N.H., H.T.), Juntendo University School of Medicine, Tokyo, Japan; Department of Neurology (B.J., S.S.P.), Seoul National University Hospital, Seoul, South Korea; Department of Neurology (C.M.L.), Johannes Gutenberg University, Mainz, Germany; Department of Neurology (J.-J.L., K.-C.Y.), Chushang Show-Chwan Hospital, Nantou and Chung-Shan Medical University Hospital, Taichung, Taiwan; The Dublin Neurological Institute at the Mater Misericordiae University Hospital (T.L.), and Conway Institute, University College Dublin, Ireland; Helmholtz Zentrum München (P.L.), German Research Centre for Environmental Health (GmbH), Neuherberg, Germany; Movement Disorders Centre (A.E.L.), Toronto Western Hospital, University of Toronto, and Edmond J Safra Program Parkinson's Disease, Toronto, Canada; Clinical Neurology Research Centre (V.M.), Department of Medicine & Therapeutics, The Chinese University of Hong Kong, Prince of Wales Hospital, Shatin, New Territories, Hong Kong; Department of Neurology (B.J.-M., G.O.), Medical University of Silesia, Katowice, Poland; Eskitis Institute for Cell and Molecular Therapies (G.D.M.), Griffith University, Australia; Neurosciences Department (K.E.M., J.D.S.), Queen Elizabeth Hospital Birmingham, University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK; Tanz Centre for Research in Neurodegenerative Diseases (E.R.), Department of Medicine, University of Toronto, Toronto, Canada; Departments of Neuroscience (O.A.R.) and Neurology (R.J.U., Z.W.), Mayo Clinic, Jacksonville, FL; Divisions of Basic Neurosciences & Cell Biology (L. Stefanis), Biomedical Research Foundation of Academy of Athens, Athens, Greece; Division of Neurology/Molecular Brain Science (W.S., T.T.), Kobe University Graduate School of Medicine, Kobe, Japan; University of Queensland Centre for Clinical Research (P.A.S.), Herston, Australia; Department of Neurology (E.-K.T., Y.Z.), Singapore General Hospital, National Neuroscience Institute, Singapore; Department of Medical Epidemiology and Biostatistics and Department of Clinical Neuroscience (K.W.), Karolinska Institutet, Stockholm, Sweden; and Department of Neurology (D.M.), NorthShore University Health System, Chicago, IL.

AUTHOR CONTRIBUTIONS

Manu Sharma, PhD: Dr. Sharma designed the study, performed the statistical analysis, and wrote the manuscript. John P.A. Ioannidis, MD: Dr. Ioannidis performed the statistical analysis and participated in writing of the manuscript. Jan O Aasly, MD: Dr. Aasly contributed samples and collected phenotypic data. Grazia Annesi, MD: Dr. Annesi contributed samples and collected phenotypic data. Alexis Brice, MD: Dr. Brice contributed samples and collected phenotypic data. Christine Van Broeckhoven, PhD: Dr. Van Broeckhoven contributed samples and collected phenotypic data. Lars Bertram, MD: Dr. Bertram participated in writing the manuscript. Maria Bozi, MD: Dr. Bozi contributed samples and col-

lected phenotypic data. David Crosiers, MD: Dr. Crosiers contributed samples and collected phenotypic data. Carl Clarke, MD: Dr. Clarke contributed samples and collected phenotypic data. Maurizio Facheris, MD: Dr. Facheris contributed samples and collected phenotypic data. Matthew Farrer, PhD: Dr. Farrer contributed samples and collected phenotypic data. Gaetan Garraux, MD, PhD: Dr. Garraux contributed samples and collected phenotypic data. Suzana Gispert, PhD: Dr. Gispert contributed samples and collected phenotypic data. Georg Auburger, MD: Dr. Auburger contributed samples and collected phenotypic data. Carles Vilarinho-Güell, PhD: Dr. Vilarinho-Güell contributed samples and collected phenotypic data. Georgios M. Hadjigeorgiou, MD: Dr. Hadjigeorgiou contributed samples and collected phenotypic data. Andrew Hicks, PhD: Dr. Hicks contributed samples and collected phenotypic data. Nobutaka Hattori, MD: Dr. Hattori contributed samples and collected phenotypic data. Beom Jeon, MD: Dr. Jeon contributed samples and collected phenotypic data. Suzanne Lesage, MD: Dr. Lesage contributed samples and collected phenotypic data. Christina M Lill, MD: Dr. Lill participated in writing the manuscript. Juei-Jueng Lin, MD: Dr. Lin contributed samples and collected phenotypic data. Timothy Lynch, MD: Dr. Lynch contributed samples and collected phenotypic data. Peter Lichtner, PhD: Dr. Lichtner contributed samples and collected phenotypic data. Anthony E. Lang, MD: Dr. Lang contributed samples and collected phenotypic data. Vincent Mok, MD: Dr. Mok contributed samples and collected phenotypic data. Barbara Jasinska-Myga, MD, PhD: Dr. Jasinska-Myga contributed samples and collected phenotypic data. George D. Mellick, PhD: Dr. Mellick contributed samples and collected phenotypic data and critically reviewed the manuscript. Karen E Morrison, MD: Dr. Morrison contributed samples and collected phenotypic data. Grzegorz Opala, MD: Dr. Opala contributed samples and collected phenotypic data. Peter P. Pramstaller, PhD: Dr. Pramstaller contributed samples and collected phenotypic data. Irene Pichler, MD: Dr. Pichler contributed samples and collected phenotypic data. Sung Sup Park, MD: Dr. Park contributed samples and collected phenotypic data. Aldo Quattrone, MD: Dr. Quattrone contributed samples and collected phenotypic data. Ekaterina Rogacheva, PhD: Dr. Rogacheva contributed samples and collected phenotypic data and participated in writing of the manuscript. Owen A Ross, PhD: Dr. Ross contributed samples and collected phenotypic data. Leonidas Stefanis, MD: Dr. Stefanis contributed samples and collected phenotypic data. Joanne D. Stockton, PhD: Dr. Stockton contributed samples and collected phenotypic data. Wataru Satake, MD, PhD: Dr. Satake contributed samples and collected phenotypic data. Peter A. Silburn, PhD: Dr. Silburn contributed samples and collected phenotypic data. Jessie Theuns, PhD: Dr. Theuns contributed samples and collected phenotypic data. Eng-King Tan, MD: Dr. Tan contributed samples and collected phenotypic data. Tatsushi Toda, MD, PhD: Dr. Toda contributed samples and collected phenotypic data. Dr. Hiroyuki Tomiyama, MD, PhD: Dr. Tomiyama contributed samples and collected phenotypic data. Ryan J Uitti, MD: Dr. Uitti contributed samples and collected phenotypic data. Karin Wirdefeldt, MD: Dr. Wirdefeldt contributed samples and collected phenotypic data. Zbigniew Wszolek, MD: Dr. Wszolek contributed samples and collected phenotypic data. Georgios Xiromerisiou, MD: Dr. Xiromerisiou contributed samples and collected phenotypic data. Kuo-Chu Yueh, MD: Dr. Yueh contributed samples and collected phenotypic data. Yi Zhao, MD: Dr. Zhao contributed samples and collected phenotypic data. Thomas Gasser, MD: Dr. Gasser contributed samples and collected phenotypic data and participated in writing of the manuscript. Demetrius M. Maraganore, MD: Dr. Maraganore contributed samples and collected phenotypic data. Rejko Krüger, MD: Dr. Krüger contributed samples and collected phenotypic data and participated in writing of the manuscript.

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SHORT REPORT

A multi-centre clinico-genetic analysis of the VPS35 gene in Parkinson disease indicates reduced penetrance for disease-associated variants

Manu Sharma,^{1CA} John P A Ioannidis,² Jan O Aasly,³ Grazia Annesi,⁴ Alexis Brice,^{5,6,7} Lars Bertram,⁸ Maria Bozi,^{9,10,11} Maria Barcikowska,¹² David Crosiers,^{13,14,15} Carl E Clarke,¹⁶ Maurizio F Facheris,¹⁷ Matthew Farrer,¹⁸ Gaetan Garraux,¹⁹ Suzana Gispert,²⁰ Georg Auburger,¹⁹ Carles Vilariño-Güell,¹⁸ Georgios M Hadjigeorgiou,²¹ Andrew A Hicks,¹⁷ Nobutaka Hattori,²² Beom S Jeon,²³ Zygmunt Jamrozik,²⁴ Anna Krygowska-Wajs,²⁵ Suzanne Lesage,^{5,6,7} Christina M Lill,^{9,26} Juei-Jueng Lin,²⁷ Timothy Lynch,²⁸ Peter Lichtner,²⁹ Anthony E Lang,³⁰ Cecile Libioulle,¹⁸ Miho Murata,³¹ Vincent Mok,³² Barbara Jasinska-Myga,³³ George D Mellick,³⁴ Karen E Morrison,^{17,35} Thomas Meitner,^{36,37} Alexander Zimprich,³⁸ Grzegorz Opala,³⁶ Peter P Pramstaller,¹⁹ Irene Pichler,¹⁹ Sung Sup Park,²⁶ Aldo Quattrone,⁴ Ekaterina Rogaeva,³⁹ Owen A. Ross,⁴⁰ Leonidas Stefanis,^{11,41} Joanne D Stockton,³⁵ Wataru Satake,⁴² Peter A Silburn,⁴³ Tim M Strom,^{37,39} Jessie Theuns,^{14,15} Eng- King Tan,⁴⁴ Tatsushi Toda,⁴² Hiroyuki Tomiyama,²² Ryan J Uitti,⁴⁵ Christine Van Broeckhoven,^{14,15} Karin Wirdefeldt,⁴⁶ Zbigniew Wszolek,⁴⁵ Georgia Xiromerisiou,²¹ Harumi S Yomono,⁴⁷ Kuo-Chu Yueh,²⁷ Yi Zhao, Thomas Gasser,¹ Demetrius Maraganore,⁴⁸ Rejko Krüger,¹ on behalf of GEOPD consortium

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For numbered affiliations see end of article

Correspondence to

Dr Manu Sharma, Department of Neurodegenerative diseases, Hertie-Institute for Clinical Brain Research and DZNE- German Center for Neurodegenerative Diseases, Tübingen, Hoppe-Seyler-Str. 3, Tübingen 72076, Germany; manu.sharma@uni-tuebingen.de

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ABSTRACT

Background Two recent studies identified a mutation (p.Asp620Asn) in the vacuolar protein sorting 35 gene as a cause for an autosomal dominant form of Parkinson disease. Although additional missense variants were described, their pathogenic role yet remains inconclusive.

Methods and results We performed the largest multi-center study to ascertain the frequency and pathogenicity of the reported vacuolar protein sorting 35 gene variants in more than 15,000 individuals worldwide. p.Asp620Asn was detected in 5 familial and 2 sporadic PD cases and not in healthy controls, p.Leu774Met in 6 cases and 1 control, p.Gly51Ser in 3 cases and 2 controls. Overall analyses did not reveal any significant increased risk for p.Leu774Met and p.Gly51Ser in our cohort.

Conclusions Our study apart from identifying the p.Asp620Asn variant in familial cases also identified it in idiopathic Parkinson disease cases, and thus provides genetic evidence for a role of p.Asp620Asn in Parkinson disease in different populations worldwide.

INTRODUCTION

There is increasing interest to try to identify uncommon and rare genetic variants that increase the risk of common diseases and that are difficult to identify using traditional genome-wide association studies (GWAS) approaches.¹ Rare variants which are not mapped by GWAS can be identified by using next generation sequencing, that is, exome sequencing in large families with multiple affected individuals.² Exome sequencing is now

routinely used to identify rare mutations in familial forms of disease in diverse phenotypes.²

Two recent studies independently performed exome sequencing in large families of Caucasian descent, and identified a mutation in the vacuolar protein sorting 35 (VPS35) gene as a possible cause for an autosomal dominant form of Parkinson disease (PD).^{3,4} In addition, several non-synonymous base exchanges were identified, but their involvement in disease pathogenesis remains inconclusive. Furthermore, recently published studies provided conflicting results regarding the role of VPS35 in PD.⁵⁻⁸ Here, we performed a large multi-centre study to determine the frequency and pathogenicity of VPS35 variants in PD in diverse populations worldwide.

METHODS
Consortium

Investigators from the Genetic Epidemiology of Parkinson disease Consortium were invited to participate in this study. A total of 23 sites representing 19 countries from four continents agreed to contribute DNA samples and clinical data for a total of 15 383 individuals (8870 cases and 6513 controls). Control individuals underwent neurological examination and were excluded from the study whenever there was clinical evidence for any extrapyramidal disorder.

Genotyping

We selected seven non-synonymous variants exactly as they were proposed.³ In addition, we selected tag single nucleotide polymorphisms

Genotype-phenotype correlations

(SNPs) (HapMap Rel 28 phase II+III, Aug10, National Centre for Biotechnology Information. B36 dbSNP b126; <http://www.hapmap.org>) that cover the common genetic variants in the VPS35 gene using an r^2 threshold of 0.8–1.0 to select tag SNPs for VPS35 gene. Using this strategy, we were able to capture 23 SNPs in a 40 kb region, including VPS35 ('chr16:46 693 589–46 723 144 based on hg 19'). Therefore, in total, 10 SNPs located in the VPS35 were genotyped (including seven rare non-synonymous and three common variants). Genotyping was performed by a central genotyping core. Genotyping was performed using a matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry on a MassArray system (Sequenom, San Diego, California, USA). Cleaned extension products were analysed by a mass spectrometer (Bruker Daltonic, Billerica, MA, USA) and peaks were identified using the MassArray Typer 4.0.2.5 software (Sequenom). Assays were designed by the AssayDesigner software 4.0 (Sequenom) with the default parameters for the iPLEX Gold chemistry and the Human Genotyping Tools ProxSNP and PreXTEND (Sequenom). All variants were genotyped in one multiplex assay. The average call rate of the variants was >97%. The local Ethics Committee approved the study. All participants gave signed informed consent.

Statistical analysis

Logistic regression was used to test the association between VPS35 and PD in our overall cohort. For common variants (minor allele frequency >5%), we synthesised the effect estimates using fixed and random effects models. Fixed effect models assume that the genetic effect is the same in populations from different sites and that observed differences are due to chance alone. For associations showing between-study heterogeneity, fixed effect estimates yield narrower CIs and smaller p values as compared with random effects models, which incorporate between-study heterogeneity.^{9 10} Random effects models allow the genetic effects might be different due to genuine heterogeneity that may exist across different sites. Random effects calculations take into account the estimated between-study heterogeneity. Cochran's Q test of homogeneity and the I^2 metric were used to evaluate the between-site heterogeneity. The I^2 metric ranges from 0% to 100% and measures the proportion of variability that is beyond chance. Typically, estimates of I^2 <25% are considered to reflect little or no heterogeneity, 25%–50% moderate heterogeneity, 50%–75% large heterogeneity and >75% very large heterogeneity. The overall main analysis considered all sites and populations irrespective of ancestry. For variants with minor allele frequency <1%, an exact test was used to compare the frequency differences between cases and controls combining data across all 21 sites.

RESULTS

Characteristics of sites and overall database

Overall, 23 sites contributed a total of 8870 cases and 6513 controls. Characteristics of all participating sites are shown in table 1. Most sites contributed participants of Caucasian ancestry ($N=19$); four sites included participants of Asian ancestry. The proportion of men and women ranged from 42% to 58% across different participating sites (table 1). The median age at onset of PD in our studied population was 61 years.

Rare variants

Overall, we observed p.Asp620Asn in seven cases, p.Leu774Met in six cases and one control, p.Gly51Ser in three cases and two

controls. Details per site are shown in table 2. The controls subjects carrying p.Leu774Met (P-13) and p.Gly51Ser (P-2 and P-16) at the time of study sampling were 81, 84 and 76 years, respectively. In Caucasian populations, the number of carriers in cases and controls for the three variants were 5 versus 0 (p.Asp620Asn), 4 versus 1 (p.Leu774Met) and 3 versus 1 (p.Gly51Ser), respectively. In Asian descent populations, the respective numbers were 2 versus 0 (p.Asp620Asn), 2 versus 0 (p.Leu774Met) and 0 versus 1 (p.Gly51Ser). Most interestingly, two out of seven patients carrying the p.Asp620Asn variant presented without any family history for PD. This represents the first evidence for reduced penetrance of the respective variant initially attributed to autosomal dominant familial PD. We did not observe any carriers for one variant (p.Arg524Trp) in our cohort. Two non-synonymous variants (p.Met57Ile, p.Thr82Arg) failed genotyping. By collapsing the rare variants across different sites, we did not observe statistically significant increased risk for p.Leu774Met and p.Gly51Ser in our cohort (see online supplementary table S1).

Overall data synthesis for common variants

Out of three tag SNPs, one SNP (rs3218745) failed genotyping. We did not observe significant association for any of common variants with PD either with either fixed effect or random effect models (see online supplementary table S2). The OR ranged from 0.96 to 0.99 and tight 95% CIs excluded modest association effects. We observed no substantial heterogeneity for the two genotyped SNPs, and also the Q test was non-statistically significant for common SNPs. Moreover, examining the Caucasian or Asian populations separately did not change our results (data not shown).

Clinical features

All PD patients who carried potential pathogenic variants (p.Asp620Asn, p.Gly51Ser, p.Leu774Met) were clinically diagnosed with PD (Online supplementary clinical analysis data). A few of these (0.2%) affected individuals also have a positive family history. Affected individuals exhibited classical symptoms of PD (resting tremor, bradykinesia, rigidity) (table 2). The clinical diagnosis of PD was made by movement disorder specialists who used UK brain bank criteria for PD. Non-motor symptoms were present in the majority of PD patients carrying a pathogenic variant (table 2). Interestingly, hallucinations and dementia were also observed in one asymptomatic carrier suggesting clinical heterogeneity associated with VPS35. The identified healthy carriers have not shown any sign of PD as yet (table 2).

DISCUSSION

We performed the first multi-centre study to define the role of the VPS35 gene (PARK17) in PD by assessing the frequency of the reported non-synonymous variants in familial and sporadic PD patients from different populations worldwide. Among 15 383 subjects genotyped, we found a pathogenic relevance for p.Asp620Asn in different populations. Most interestingly, out of seven subjects who carry p.Asp620Asn, two have a negative family history. Therefore, our results provide additional evidence that VPS35 is a rare cause of familial as well as the common sporadic form of PD. In total, about 0.4% of PD cases in diverse population were due to disease-associated variant in the VPS35 gene. Our lack of supporting the role of common variants of the VPS35 gene in PD is consistent with recently published GWAS and also meta-analyses of GWAS of PD, as none of these highlighted the role of common variability in VPS35 gene as a risk factor for PD.^{11–15} The p.Asp620Asn

Genotype-phenotype correlations

Table 1 Description of datasets contributed by each study site

Site	Country	N	Case	Control	Male (%)	Female (%)	Mean AAO	Mean Age at study	Diagnostic criteria
Annesi	Italy	394	197	197	204 (51.7%)	190 (48.2%)	61.5	63.7	UKPDBB
Brice	France	505	272	233	302 (59.8%)	203 (40.1%)	47.6	57.8	UKPDBB
Bozi	Greece	222	114	108	107 (48.1%)	115 (51.8%)	69.9	74.5	UKPDBB
Wszołek	USA	1518	692	826	794 (52.3%)	724 (47.6%)	64.4	71.7	UKPDBB
Garraux	Belgium	82	68	14	45 (54.8)	37 (45.1%)	62.1	69.6	UKPDBB
Hadjigeorgiou	Greece	714	357	357	379 (53.0%)	335 (46.9%)	63.4	63.7	UKPDBB
Jeon	Korea	749	408	341	314 (41.9%)	435 (58.0%)	57.6	NA	UKPDBB
Opala	Poland	629	352	277	340 (54.0%)	288 (45.7%)	50.2	68.1	UKPDBB
Lynch	Ireland	740	368	372	340 (45.9%)	400 (54.0%)	50.5	70.7	UKPDBB
Lin	Taiwan	320	160	160	160 (50%)	160 (50%)	62.0	70.8	UKPDBB
Facheris	Italy	181	114	67	86 (47.5%)	95 (52.4%)	63.0	NA	UKPDBB
Maraganore	USA	1024	801	223	600 (58.5%)	361 (35.3%)	59	74.7	Bower
Mellick	Australia	2024	1012	1012	1042 (51.4%)	981 (48.4%)	59	72.2	Bower
Morrison	England	1120	766	354	606 (54.1%)	514 (45.8%)	66.1	NA	UKPDBB
Mok	China	436	260	176	264 (60.5%)	170 (38.9%)	NA	63.5	UKPDBB
Aasly	Norway	1278	656	622	721 (56.4%)	557 (43.5%)	58.8	72.9	UKPDBB
Wirdefeldt	Sweden	299	83	216	147 (49.1%)	152 (50.8%)	65.8	71.4	Gelb
Van Broeckhoven	Belgium	1010	501	509	500 (49.5%)	509 (50.3%)	60.5	66.3	Pals/Gelb
Rogaeva	Canada	560	387	173	303 (54.1%)	257 (45.8%)	49.7	64.2	UKPDBB
Tan	Singapore	391	194	197	244 (62.4%)	147 (37.5%)	59.7	54.0	UKPDBB
Hattori	Japan	121	121	0	62 (51.2%)	59 (48.7%)	NA	NA	UKPDBB
Gasser/Sharma	Germany	760	760	0	479 (63.3%)	281 (36.9%)	58.9	NA	UKPDBB
Toda	Japan	306	227	79	161 (52.6%)	145 (47.3%)	57.8	65.1	UKPDBB
Total		15 383	8870	6513			59.5	67.6	

AAO, Age at onset; NA: Not applicable.

variant is located in the C-terminal region of the VPS35 protein pointing that subtle structural changes might influence the disease pathogenesis.³

The spectrum of proteins involved in PD aetiology has grown considerably. This includes proteins that are related to mitochondrial quality control (Parkin, PINK1 and DJ1), proteins involved in protein aggregation (SNCA) (Synuclein, MAPT) Microtubule associated protein Tau), and proteins

which are involved in sorting and degradation within endocytic and autophagy pathways ((VDAC) Voltage dependent anion channel, (GBA) Glucocerebrosidase gene, VPS35).^{16 17} So far, very little is known about the specific role of VPS35 in PD, except that it is hypothesised that it is involved in cargo recognition as part of a retrograde complex recycling membrane proteins from endosomes to the trans-Golgi network.^{3 4} Indeed, in vitro and in vivo studies strongly implicate the role of VPS35

Table 2 Clinical description of carriers of non-synonymous variants of vacuolar protein sorting 35 gene

Id	Ethnicity	Rare variant	Age at onset	Clinical signs	Bradykinesia	Rigidity	Tremor	Postural instability	L-dopa responsive	Non-motor symptoms	Family history
P-1	Caucasian	p.Asp620Asn	59	Classical PD	+	+	+	+	+	Negative	Negative
P-2	Caucasian	p.Gly51Ser	NA	Control	—	—	—	—	—	Negative	Negative
P-3	Caucasian	p.Gly51Ser	NAV	Classical PD	+	+	+	+	+	Dementia, visual hallucinations	Negative
P-4	Caucasian	p.Gly51Ser	55	Classical PD	+	+	+	+	+	Negative	Negative
P-5	Caucasian	p.Gly51Ser	49	Classical PD	+	+	+	+	+	Negative	Negative
P-6	Caucasian	p.Asp620Asn	37	Classical PD	+	+	+	+	+	Negative	Positive
P-7	Caucasian	p.Asp620Asn	59	Classical PD	+	+	+	+	+	Negative	Positive
P-8	Caucasian	p.Asp620Asn	55	Classical PD	+	+	+	+	+	Negative	Positive
P-9	Caucasian	p.Asp620Asn	66	Classical PD	+	+	+	+	+	Negative	Positive
P-10	Caucasian	p.Leu774Met	41	Classical PD	+	+	+	+	+	Negative	Positive
P-11	Caucasian	p.Leu774Met	65	Classical PD	+	+	+	+	+	Negative	Positive
P-12	Caucasian	p.Leu774Met	65	Classical PD	+	+	+	+	+	Disturbance of gait and balance	Positive
P-13	Caucasian	p.Leu774Met	NA	Control	—	—	—	—	NA	—	Negative
P-14	Caucasian	p.Leu774Met	44	Classical PD	+	+	+ Rest 1st sx	+	+	Autonomic dysfunction	Positive
P-15	Asian	p.Asp620Asn, p.Leu774Met	52	Classical PD	+	+	+	+	+	Negative	Negative
P-16	Asian	p.Gly51Ser		Control	—	—	—	—	—	—	Negative
P-17	Asian	p.Leu774Met	75	Classical PD	+	+	+	+	+	Negative	Negative
P-18	Asian	p.Asp620Asn	43	Classical PD	+	+	+	+	+	Mild cognitive impairment	Positive

NA, not applicable; NAV, not available; PD, Parkinson disease; +positive; —negative.

Genotype-phenotype correlations

gene in neurodegeneration. For example, reduced levels of VPS35 have been found in affected brain regions of Alzheimer disease (AD) patients¹⁸ and loss of VPS35 function has been shown to increase the levels of amyloid β and cause synaptic impairment in a mouse model of AD. Furthermore, variants in another member of the VPS family and substrate of retromer complex, SORL1, have been implicated in AD.¹⁹

In this study, we have focused only on non-synonymous variants identified by Zimprich and colleagues.³ Of note, we confirmed the pathogenic relevance of the p.Asp620Asn variant which was identified by both studies for familial cases and in sporadic PD. Recently, published studies also identified p.Asp620Asn mutations in PD,^{6 20} thus providing support to the role of p.Asp620Asn in PD. In our study, clinically, the symptomatic carriers showed a broad spectrum of clinical phenotypes ranging from typical PD to (DLB) Dementia with Lewy body, so longitudinal evaluation of carriers at risk will provide unique information on the natural course of the disease caused by VPS35. Even though our data support the role of p.Asp620Asn variant in PD, given the fact that the frequency in diverse population is far below <1%, it is likely to be a rare cause of PD worldwide. Nevertheless, sequencing of families is encouraged for identifying additional missense variants which may provide mechanistic insight into the causes of PD.

Author affiliations

- ¹Department of Neurodegenerative diseases, Hertie-Institute for Clinical Brain Research and DZNE- German Center for Neurodegenerative Diseases, Tübingen
- ²Stanford Prevention Research Center, Department of Medicine and Department of Health Research and Policy, Stanford University School of Medicine, Stanford, California, USA
- ³Department of Neurology, St Olavs Hospital and NTNU Trondheim, Trondheim, Norway
- ⁴Institute of Neurology, Department of Medical Sciences, University Magna Graecia, Catanzaro; Neuroimaging Research Unit, National Research Council, Catanzaro, Italy
- ⁵INSERM, UMR_S975, Université Pierre et Marie Curie-Paris, CNRS, UMR 7225, AP-HP, Pitié-Salpêtrière Hospital
- ⁶Cnrs, UMR 7225, Paris, France
- ⁷AP-HP, Hôpital Pitié-Salpêtrière, Department of Genetics and Cytogenetics, Paris, France
- ⁸Neuropsychiatric Genetics Group, Department of Vertebrate Genomics, Max Planck Institute for Molecular Genetics, Berlin, Germany.
- ⁹General Hospital of Syros, Syros, Greece
- ¹⁰'Hygeia' Hospital, Clinic of Neurodegenerative Disorders, Athens, Greece
- ¹¹2nd Neurology Clinic, University of Athens, 'Attikon' Hospital, Athens, Greece
- ¹²Department of Neurodegenerative Disorders, Medical Research Centre, Polish Academy of Sciences, Warsaw, Poland
- ¹³Neurodegenerative Brain Diseases Group, Department of Molecular Genetics, VIB, Antwerp, Belgium
- ¹⁴Institute Born-Bunge, University of Antwerp, Antwerp, Belgium
- ¹⁵Department of Neurology, Antwerp University Hospital, Antwerp, Belgium.
- ¹⁶School of Clinical and Experimental Medicine, College of Medical and Dental Sciences, University of Birmingham, City Hospital, Birmingham, UK
- ¹⁷Centre for Biomedicine, European Academy Bozen/Bolzano, Italy. Affiliated institute of the University of Lübeck, Lübeck, Germany
- ¹⁸Department of Medical Genetics, University of British Columbia, Vancouver, British Columbia, Canada
- ¹⁹Human Genetic Centre, University Hospital of Liège, Liège, Belgium and Department of Neurology, General Central Hospital, Bolzano, Italy
- ²⁰Department of Neurology, Goethe University Frankfurt am Main, Frankfurt, Germany
- ²¹Department of Neurology, University of Thessaly and Institute of Biomedical Research and Technology, CERETETH, Larissa, Greece
- ²²Department of Neurology, Juntendo University School of Medicine, Tokyo, Japan
- ²³Department of Neurology, Seoul National University Hospital, Seoul, Korea
- ²⁴Department of Neurology, Medical University of Warsaw, Warsaw, Poland
- ²⁵Department of Neurology, Jagiellonian University, Krakow, Poland
- ²⁶Department of Neurology, Johannes Gutenberg University, Mainz, Germany
- ²⁷Department of Neurology, Chushang Show-Chwan Hospital, Nantou and Chung-Shan Medical University Hospital, Taichung, Taiwan
- ²⁸The Dublin Neurological Institute at the Mater Misericordiae University Hospital, and Conway Institute, University College Dublin, Dublin, Ireland
- ²⁹Helmholtz Zentrum München, German Research Centre for Environmental Health (GmbH), Neuherberg, Germany

- ³⁰Movement Disorders Centre, and the Edmond J Safra Program in Parkinson's Disease, Toronto Western Hospital, University of Toronto, Toronto, Canada
- ³¹Department of Neurology, National Center Hospital of Neurology and Psychiatry, Tokyo, Japan
- ³²Department of Medicine and Therapeutics, Prince of Wales Hospital, The Chinese University of Hong Kong, Shatin, Hong Kong
- ³³Department of Neurology, Medical University of Silesia, Katowice, Poland
- ³⁴Eskitis Institute for Cell and Molecular Therapies, Griffith University, Brisbane, Australia
- ³⁵Neurosciences Department, Queen Elizabeth Hospital Birmingham, University Hospitals Birmingham NHS Foundation Trust, Birmingham, UK
- ³⁶German Research Center for Environmental Health, Institute of Human Genetics, Helmholtz Zentrum München, Neuherberg, Germany
- ³⁷Institute of Human Genetics, Technische Universität München, Munich, Germany
- ³⁸Department of Neurology, Medizinische Universität Wien, Vienna, Austria
- ³⁹Tanz Centre for Research in Neurodegenerative Diseases, Department of Medicine, University of Toronto, Toronto, Canada
- ⁴⁰Department of Neuroscience, Mayo Clinic, Jacksonville, Florida
- ⁴¹Divisions of Basic Neurosciences & Cell Biology, Biomedical Research Foundation of Academy of Athens, Athens, Greece
- ⁴²Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe, Japan
- ⁴³University of Queensland Centre for Clinical Research, Herston, Australia
- ⁴⁴Department of Neurology, Singapore General Hospital, National Neuroscience Institute, Singapore
- ⁴⁵Department of Neurology, Mayo Clinic Jacksonville, USA
- ⁴⁶Epidemiology and Biostatistics and Department of Clinical Neuroscience, Karolinska Institutet
- ⁴⁷Department of Neurology, National Hospital Organization Tokyo Hospital, Tokyo, Japan
- ⁴⁸Department of Neurology, NorthShore University HealthSystem, Chicago, USA

Acknowledgements RS Boyle, MD (Princess Alexandra Hospital, Brisbane, Site Investigator); A Sellbach, MD (Princess Alexandra Hospital, Brisbane, Site Investigator); JD O'Sullivan, MD (Royal Brisbane and Women's Hospital Brisbane, Site Investigator); GT Sutherland, PhD (Eskitis Institute for Cell and Molecular Therapies, Griffith University, Nathan, QLD, Site Investigator); GA Siebert, MD (Eskitis Institute for Cell and Molecular Therapies, Griffith University, Nathan, QLD, Site Investigator); NNW Dissanayaka, MD (Eskitis Institute for Cell and Molecular Therapies, Griffith University, Nathan, QLD, Site Investigator); Christine Van Broeckhoven, PhD DSc (Neurodegenerative Brain Diseases Group, Department of Molecular Genetics, VIB; and Laboratory of Neurogenetics, Institute Born-Bunge; and University of Antwerp); Jessie Theuns, PhD (Neurodegenerative Brain Diseases Group, Department of Molecular Genetics, VIB; and Laboratory of Neurogenetics, Institute Born-Bunge; and University of Antwerp); David Crosiers, MD (Neurodegenerative Brain Diseases Group, Department of Molecular Genetics, VIB; and Laboratory of Neurobiology, Institute Born-Bunge; and University of Antwerp; and Department of Neurology, Antwerp University Hospital, Antwerp); Barbara Pickut, MD (Department of Neurology, Antwerp University Hospital, Antwerp); Sebastiaan Engelborghs, MD, PhD (Laboratory of Neurochemistry and Behaviour, Institute Born-Bunge; and University of Antwerp; and Department of Neurology and Memory Clinic, Hospital Network Antwerp, Middelheim and Hoge Beuken, Antwerp); Aline Verstraeten (Neurodegenerative Brain Diseases Group, Department of Molecular Genetics, VIB; and Laboratory of Neurogenetics, Institute Born-Bunge; and University of Antwerp); Peter P De Deyn, MD, PhD (Laboratory of Neurochemistry and Behaviour, Institute Born-Bunge; and University of Antwerp; and Department of Neurology and Memory Clinic, Hospital Network Antwerp, Middelheim and Hoge Beuken, Antwerp; and Department of Neurology and Alzheimer Research Center, University Medical Center Groningen, The Netherlands); Patrick Cras, MD, PhD (Laboratory of Neurobiology, Institute Born-Bunge; and University of Antwerp; Department of Neurology, Antwerp University Hospital, Antwerp). Funding: The research was in part supported by the Methusalem excellence program of the Flemish Government; a Centre of Excellence grant by the Special Research Fund of the University of Antwerp; the Research Foundation Flanders (FWO); the Agency for Innovation by Science and Technology Flanders (IWT); the Interuniversity Attraction Poles program (IAP) P6/43 of the Belgian Science Policy Office; the Belgian Parkinson Foundation; and the Foundation for Alzheimer Research (SAQ/FRMA). DC is receiving a PhD fellowship of the FWO and AV of the IWT. Ekaterina Rogaeva, PhD (Tanz Centre for Research in Neurodegenerative Diseases, Department of Medicine, Division of Neurology, University of Toronto, ON, Canada, Site Investigator); Anthony E Lang, MD (Movement Disorders Centre, Toronto Western Hospital, University of Toronto, Toronto, ON, Canada, Site Investigator); Y Agid, MD (Inserm, Paris, From the French Parkinson's Disease Genetics Study Group, Site Investigator); M Anheim, MD (Inserm, Paris, From the French Parkinson's Disease Genetics Study Group, Site Investigator); A-M Bonnet, MD (Inserm, Paris, From the French Parkinson's Disease Genetics Study Group, Site Investigator); M Borg, MD (From the French Parkinson's Disease Genetics Study Group, Site Investigator); A Brice, MD (Paris, From the French Parkinson's Disease Genetics Study Group, Site Investigator); E Broussolle MD (From the French Parkinson's Disease Genetics Study Group, Site Investigator); JC Corvol MD (Inserm, Paris, From the

French Parkinson's Disease Genetics Study Group, Site Investigator); P Damier, MD (From the French Parkinson's Disease Genetics Study Group, Site Investigator); A Destée, MD (From the French Parkinson's Disease Genetics Study Group, Site Investigator); A Dürr, MD (Inserm U708, Paris, From the French Parkinson's Disease Genetics Study Group, Site Investigator); F Durif, MD (From the French Parkinson's Disease Genetics Study Group, Site Investigator); S Lesage, PhD (Inserm, Paris, From the French Parkinson's Disease Genetics Study Group, Site Investigator); E Lohmann, MD (Inserm, Paris, From the French Parkinson's Disease Genetics Study Group, Site Investigator); P Pollak, MD (From the French Parkinson's Disease Genetics Study Group, Site Investigator); O Rascol, MD (From the French Parkinson's Disease Genetics Study Group, Site Investigator); F Tison, MD (From the French Parkinson's Disease Genetics Study Group, Site Investigator); C Tranchant, MD (From the French Parkinson's Disease Genetics Study Group, Site Investigator); F Viallet, MD (From the French Parkinson's Disease Genetics Study Group, Site Investigator); M Vidali, MD (Inserm, Paris, From the French Parkinson's Disease Genetics Study Group, Site Investigator); Christophe Tzourio, MD (Inserm U708, Paris, From the French Parkinson's Disease Genetics Study Group, Site Investigator); Philippe Amouyel, MD (Inserm U744, Lille, Site Investigator); Marie-Anne Lloriot, MD (Inserm UMR5775, Paris, Site Investigator); Eugénie Mutez, MD (Inserm UMR837, Service de Neurologie et de Pathologie du Mouvement CHRU de Lille, Univ Lille Nord de France, Site Investigator); Aurélie Dufiot, MD (UMR837 Inserm-Univ Lille 2, CHRU de Lille, Site Investigator); Jean-Philippe Legendre, MD (Service de Neurologie et Pathologie du Mouvement, Clinique de Neurologie du CHU de Lille, Site Investigator); Nawal Wauquier, MD (Service de Neurologie et Pathologie du Mouvement, Clinique de Neurologie du CHU de Lille, Site Investigator); Thomas Gasser MD (Department of Neurology, University Hospital Tuebingen, Site Investigator); Olaf Riess MD (Department of Neurology, University Hospital Tuebingen, Site Investigator); Christine Klein, MD (Section of Clinical and Molecular Neurogenetics at the Department of Neurology, University of Lübeck, Site Investigator); Ana Djarmati, PhD (Department of Neurology, University of Lübeck, Site Investigator); Johann Hagenah, MD (Department of Neurology, University of Lübeck, Site Investigator); Katja Lohmann, PhD (Section of Clinical and Molecular Neurogenetics at the Department of Neurology, University of Lübeck, Site Investigator); Georg Auburger, MD (Department of Neurology, Goethe University Frankfurt am Main, Germany, Site Investigator); Rüdiger Hilker, MD (Department of Neurology, Goethe University Frankfurt am Main, Germany, Site Investigator); Simone van de Loo, MD (Department of Neurology, Goethe University Frankfurt am Main, Germany, Site Investigator); Efthimios Dardiotis, MD (Department of Neurology, Faculty of Medicine, University of Thessaly and Institute of Biomedical Research & Technology, CERETETH, Larissa; Site Investigator); Vaia Tsimouridou, MD (Department of Neurology, Faculty of Medicine, University of Thessaly, Larissa, Site Investigator); Styliani Ralli, MD (Department of Neurology, Faculty of Medicine, University of Thessaly, Larissa, Site Investigator); Persa Kountra, MD (Department of Neurology, Faculty of Medicine, University of Thessaly, Larissa, Site Investigator); Gianna Patramani, MD (Department of Neurology, Faculty of Medicine, University of Thessaly, Larissa, Site Investigator); Cristina Vogiatzi, MD (Department of Neurology, Faculty of Medicine, University of Thessaly, Larissa, Site Investigator); Nobutaka Hattori, MD, PhD (Department of Neurology, Juntendo University School of Medicine, Tokyo, Site Investigator); Hiroyuki Tomiyama, MD, PhD (Department of Neurology, Juntendo University School of Medicine, Tokyo, Site Investigator); Manabu Funayama, PhD (Department of Neurology, and Research Institute for Diseases of Old Age, Graduate School of Medicine, Juntendo University, Tokyo, Site Investigator); Hiroyo Yoshino, PhD (Research Institute for Diseases of Old Age, Graduate School of Medicine, Juntendo University, Tokyo, Site Investigator); Yuanzhe Li MD, PhD (Research Institute for Diseases of Old Age, Graduate School of Medicine, Juntendo University, Tokyo, Site Investigator); Yoko Imamichi (Department of Neurology, Juntendo University School of Medicine, Tokyo, Site Investigator); Tatsushi Toda, MD (Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe, Japan, Site Investigator); Wataru Satake, MD, PhD (Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe, Japan, Site Investigator); Tim Lynch, MD (The Dublin Neurological Institute at the Mater Misericordiae University Hospital, Clinical Investigator at the Conway Institute, University College Dublin, Ireland, Site Investigator); J Mark Gibson, MD (Department of Neurology, Royal Victoria Hospital, Belfast, Ireland, Site Investigator); Enza Maria Valente, MD, PhD (IRCCS, Casa Sollievo della Sofferenza Hospital, Mendel Institute, San Giovanni Rotondo, Site Investigator); Alessandro Ferraris, MD (IRCCS, Casa Sollievo della Sofferenza Hospital, Mendel Institute, San Giovanni Rotondo, Site Investigator); Bruno Dallapiccola, MD (Mendel Institute, Casa Sollievo della Sofferenza Hospital, Rome, Site Investigator); Tamara Ialongo, MD, PhD (Institute of Neurology, Catholic University, Rome, Site Investigator); Laura Brighina, MD, PhD (Department of Neurology, Ospedale San Gerardo, Monza, Italy, Site Investigator); Barbara Corradi, PhD (Department of Paediatrics, University of Milano-Bicocca, Monza, Site Investigator); Roberto Pioletti, MD (Department of Neurology, Ospedale San Gerardo, Monza, Italy, Site Investigator); Patrizia Tarantino, PhD (Institute of Neurological Sciences, National Research Council, Site Investigator); Ferdinando Annesi, PhD (Institute of Neurological Sciences, National Research Council, Site Investigator); Beom S Jeon, MD, PhD (Department of Neurology, Seoul National University Hospital, Site Investigator); Sung-Sup Park, MD, PhD (Department of Laboratory Medicine, Seoul National University Hospital, Site Investigator); J Aasly, MD

(Department of Neurology, University of Trondheim, Norway, Site Investigator); Grzegorz Opala, MD, PhD (Department of Neurology, Aging, Degenerative and Cerebrovascular Disorders, Medical University of Silesia, Katowice; Site Investigator); Barbara Jasinska-Myga, MD, PhD (Department of Neurology, Aging, Degenerative and Cerebrovascular Disorders, Medical University of Silesia, Katowice, Site Investigator); Gabriela Klodowska-Duda, MD, PhD (Department of Neurology, Aging, Degenerative and Cerebrovascular Disorders, Medical University of Silesia, Katowice, Site Investigator); Magdalena Boczarska-Jedynak, MD, PhD (Department of Neurology, Aging, Degenerative and Cerebrovascular Disorders, Medical University of Silesia, Katowice, Site Investigator); Eng King Tan, MD, PhD (National Medical and Biomedical Research Councils, and the Duke-NUS Graduate Medical School, Singapore Millenium Foundation, Site Investigator); Andrea Carmine Belin, PhD (Department of Neuroscience, Karolinska Institutet, Stockholm, Site Investigator); Lars Olson, MD (Department of Neuroscience, Karolinska Institutet, Stockholm, Site Investigator); Dagmar Galter, PhD (Department of Neuroscience, Karolinska Institutet, Stockholm; Site Investigator); Marie Westerlund, PhD (Department of Neuroscience, Karolinska Institutet, Stockholm, Site Investigator); Olof Sydow, PhD (Department of Clinical Neuroscience, Karolinska University Hospital, Stockholm; Site Investigator); Christer Nilsson, MD, PhD (Department of Geriatric Psychiatry, Lund University; Site Investigator); Andreas Puschmann, MD (Department of Neurology, Lund University Hospital, Department of Geriatric Psychiatry, Lund University, Site Investigator); JJ Lin, MD (Department of Neurology, Cushang Show-Chwan Hospital, Taiwan, Site Investigator); Demetrius M Maraganore, MD (Department of Neurology, NorthShore Health Systems, Chicago, IL, Site Investigator); J Eric Ahlskog PhD, MD (Department of Neurology, Mayo Clinic, Rochester, MN, USA, Site Investigator); Mariza de Andrade, PhD (Department of Health Sciences Research, Mayo Clinic, Rochester, MN, USA, Site Investigator); Timothy G Lesnick, MS (Department of Health Sciences Research, Mayo Clinic, Rochester, MN, USA, Site Investigator); Walter A Rocca, MD, MPH (Departments of Neurology and Health Sciences Research, Mayo Clinic, Rochester, MN, USA, Site Investigator); Harvey Checkoway, PhD (Department of Environmental and Occupational Health Sciences, University of Washington, Seattle, WA, Site Investigator); Owen A Ross PhD (Division of Neuroscience, Mayo Clinic, Jacksonville, USA, Site Investigator); Zbigniew K Wszolek, MD (Department of Neurology, Mayo Clinic, Jacksonville, FL, Site Investigator); Ryan J Uitti, MD (Department of Neurology, Mayo Clinic, Jacksonville, FL, Site Investigator).

Contributors All authors presented in this study made substantial contributions to: (1) conception and design, acquisition of data, or analysis and interpretation of data; (2) drafting the article or revising it critically for important intellectual content; and (3) final approval of the version to be published.

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Hsp40 Gene Therapy Exerts Therapeutic Effects on Polyglutamine Disease Mice via a Non-Cell Autonomous Mechanism

H. Akiko Popiel¹, Toshihide Takeuchi¹, Hiromi Fujita¹, Kazuhiro Yamamoto², Chiyomi Ito³, Hiroshi Yamane¹, Shin-ichi Muramatsu⁴, Tatsushi Toda³, Keiji Wada¹, Yoshitaka Nagai^{1,5*}

1 Department of Degenerative Neurological Diseases, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan, **2** Division of Laboratory Animals Resources, National Institute of Neuroscience, National Center of Neurology and Psychiatry, Kodaira, Tokyo, Japan, **3** Division of Neurology/Molecular Brain Science, Kobe University Graduate School of Medicine, Kobe, Hyogo, Japan, **4** Division of Neurology, Department of Medicine, Jichi Medical University, Shimotsuke, Tochigi, Japan, **5** Core Research for Evolutional Science and Technology (CREST), Japan Science and Technology Agency, Kawaguchi, Saitama, Japan

Abstract

The polyglutamine (polyQ) diseases such as Huntington's disease (HD), are neurodegenerative diseases caused by proteins with an expanded polyQ stretch, which misfold and aggregate, and eventually accumulate as inclusion bodies within neurons. Molecules that inhibit polyQ protein misfolding/aggregation, such as Polyglutamine Binding Peptide 1 (QBP1) and molecular chaperones, have been shown to exert therapeutic effects *in vivo* by crossing of transgenic animals. Towards developing a therapy using these aggregation inhibitors, we here investigated the effect of viral vector-mediated gene therapy using QBP1 and molecular chaperones on polyQ disease model mice. We found that injection of adeno-associated virus type 5 (AAV5) expressing QBP1 or Hsp40 into the striatum both dramatically suppresses inclusion body formation in the HD mouse R6/2. AAV5-Hsp40 injection also ameliorated the motor impairment and extended the lifespan of R6/2 mice. Unexpectedly, we found even in virus non-infected cells that AAV5-Hsp40 appreciably suppresses inclusion body formation, suggesting a non-cell autonomous therapeutic effect. We further show that Hsp40 inhibits secretion of the polyQ protein from cultured cells, implying that it inhibits the recently suggested cell-cell transmission of the polyQ protein. Our results demonstrate for the first time the therapeutic effect of Hsp40 gene therapy on the neurological phenotypes of polyQ disease mice.

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* E-mail: nagai@ncnp.go.jp

Introduction

The polyglutamine (polyQ) diseases are a group of inherited neurodegenerative disorders that are all caused by a common genetic mutation, namely an expansion (>40) of a polyQ-encoding CAG repeat in each unrelated disease-causing gene [1,2]. Nine polyQ diseases have been identified to date, including Huntington's disease (HD), spinocerebellar ataxia (SCA) types 1, 2, 3, 6, 7, 17, dentatorubral pallidoluysian atrophy (DRPLA), and spinobulbar muscular atrophy (SBMA). In these disorders, progressive degeneration of neurons in brain areas specific for each disorder occurs, causing various neurological and psychiatric symptoms corresponding to each affected brain area [1,2].

In the common molecular pathogenesis of the polyQ diseases, proteins with an expanded polyQ stretch become misfolded and form aggregates, and subsequently accumulate as inclusion bodies within neurons, eventually resulting in neurodegeneration [3–7]. Moreover, recent studies suggest that prion-like transmission of

aggregation-prone proteins between cells is involved in neuropathological spreading during disease progression in not only the polyQ diseases but also various other neurodegenerative diseases [8–11]. Although various therapeutic strategies against downstream targets of the pathogenic cascade have been investigated, misfolding and aggregation of the polyQ protein are ideal therapeutic targets since they are the most initial pathogenic events, and therefore their inhibition is expected to result in the suppression of a broad range of downstream pathogenic events [3,5,12,13].

In our attempt to establish a therapy for the polyQ diseases, we hypothesized that molecules that specifically bind to the expanded polyQ stretch would suppress misfolding and aggregation of the expanded polyQ protein. Accordingly, by phage display screening of combinatorial peptide libraries, we identified PolyQ Binding Peptide 1 (QBP1), and proved that QBP1 indeed inhibits polyQ protein misfolding/aggregation *in vitro* [14–16]. Furthermore, we

demonstrated that expression of QBP1 suppresses neurodegeneration *in vivo* in polyQ disease model animals [16,17]. Another approach for targeting misfolding and aggregation of the expanded polyQ protein is to utilize molecular chaperones, which are a group of biomolecules that assist the proper folding of proteins and prevent protein misfolding/aggregation [18–20]. Indeed, overexpression of molecular chaperones such as Hsp40 and Hsp70 has been shown to suppress polyQ protein aggregation and polyQ-induced neurodegeneration in various animal models of the polyQ diseases, such as flies [21–24], worms [25] and mice [26–30]. However, most studies showing the therapeutic efficacy of these aggregation inhibitors have been performed by crossing transgenic animals so far. To develop a therapy using aggregation inhibitors such as QBP1 and molecular chaperones, transgenes need to be delivered in affected individuals by administration, rather than be expressed in the next generation by crossing transgenic animals.

In this study, we employed a viral vector to deliver these transgenes into the brain and investigated their therapeutic effects on polyQ disease model mice. Among various viral vectors, we chose to use adeno-associated virus vector (AAV) because of its widespread infection throughout the brain, its long-term expression of transgenes, and its safety [31,32]. We successfully demonstrate the therapeutic effects of AAV5-QBP1 and AAV5-Hsp40 injections on a mouse model of HD. Most interestingly, we found that AAV5-Hsp40 exerts a non-cell autonomous therapeutic effect, possibly via inhibition of the recently-suggested cell-cell transmission of the polyQ protein, indicating a novel therapeutic mode of action of Hsp40.

Results

AAV5-QBP1 and AAV5-Hsp40 Inhibit Inclusion Body Formation in polyQ Disease Mouse Neurons

We employed the R6/2 HD mouse model [33] to investigate the therapeutic effect of AAV-mediated expression of QBP1 and molecular chaperones. We first tested the effect on accumulation of the pathogenic polyQ protein into inclusion bodies in the neurons of R6/2 mice by AAV5 injections. Injections were performed on mice at postnatal day 7 (P7) using an infusion pump (see Materials and Methods), which has been shown to lead to widespread delivery of the injected molecules in the brain [34], and indeed resulted in widespread expression of the transgene throughout the injected brain hemisphere with ~30% infection efficiencies (Fig. S1). R6/2 mice were injected with AAV5-GFP on one side of the striatum and AAV5-QBP1 on the other side, and htt inclusion body formation was compared between the two sides of both the striatum and the cortex. Inclusion bodies were already formed in 36.0% of AAV5-GFP infected neurons in the striatum at 4 weeks of age, which increased to 68.5% at 8 weeks and 73.8% at 14 weeks, and an age dependent increase in inclusion bodies was also observed in the cortex (Fig. S2). In contrast, AAV5-QBP1 infected neurons had significantly less inclusion bodies at most time points (Fig. S2), and the rates of neurons with inclusion bodies at 8 weeks, for example, were 68.5% for GFP vs 33.7% for QBP1 ($p < 0.001$) in the striatum, and 49.3% for GFP vs 27.4% for QBP1 ($p < 0.001$) in the cortex (Figs. 1A,B). These results demonstrate a significant inhibitory effect of AAV5-QBP1 on inclusion body formation.

We also tested the effect of AAV5-mediated expression of a molecular chaperone on inclusion body formation. Among the various molecular chaperones, we chose to use a member of the Hsp40 family, namely DNAJB1 (referred to as Hsp40 in this study), since members of the DNAJB subfamily have been

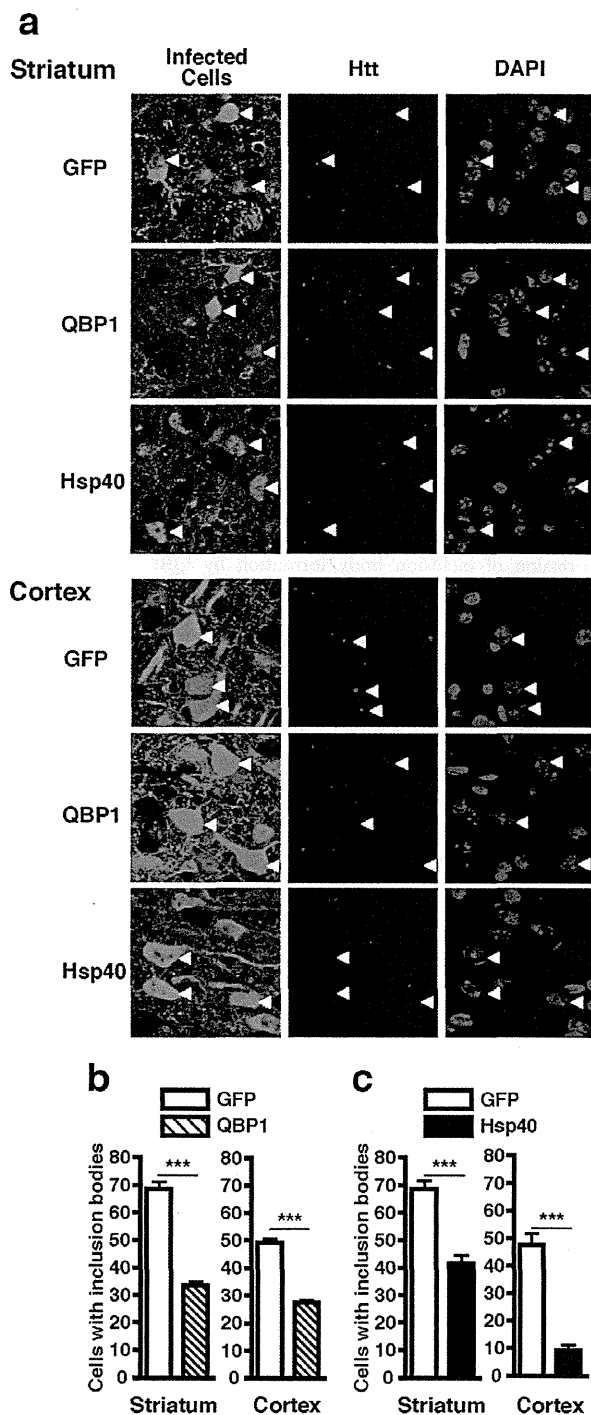


Figure 1. AAV5-QBP1 and AAV5-Hsp40 inhibit polyQ inclusion body formation in virus infected neurons of polyQ disease mice. R6/2 mice at P7 were injected with AAV5-GFP on one side of the striatum and either AAV5-QBP1 or AAV5-Hsp40 on the other side, and htt inclusion body formation in virus infected neurons was assessed at 8 weeks of age by immunohistochemistry of brain sections. (A) Representative photographs of striatal (top panels) and cortical (bottom panels) sections of R6/2 mice, injected with the indicated viruses. Green; virus infected cells, red; htt, and blue; nuclei visualized by DAPI staining. In each panel, representative virus infected cells are indicated by white

arrowheads. (B) Inclusion body formation in AAV5-QBP1 infected neurons in the striatum (left) and cortex (right). (C) Inclusion body formation in AAV5-Hsp40 infected neurons in the striatum (left) and cortex (right). In (B) and (C), data are shown as means \pm SEM of ≥ 6 fields of view, in which over 180 cells were counted (* $p < 0.05$, *** $p < 0.001$). Representative results of two mice analyzed are shown. doi:10.1371/journal.pone.0051069.g001

reported to be the most potent suppressors of expanded polyQ protein aggregation and toxicity [35]. The effect of AAV5-Hsp40 on polyQ inclusion body formation in R6/2 mice was investigated at 8 weeks, an age at which AAV5-QBP1 showed a clear inhibitory effect. AAV5-Hsp40 also exerted a robust effect on inclusion body formation, and the rates of virus infected neurons with inclusion bodies were 68.6% for GFP vs 41.7% for Hsp40 ($p < 0.001$) in the striatum, and 47.5% for GFP vs 9.2% for Hsp40 ($p < 0.001$) in the cortex (Figs. 1A,C). This difference in the effectiveness of Hsp40 between the two brain areas may be due to differences in the expression levels of its partner Hsp70. Although whether polyQ inclusion bodies themselves are cytotoxic or cytoprotective has been controversial [36], we assume that suppression of inclusion body formation by QBP1 and Hsp40 can be regarded as a therapeutic effect, since they act by preventing the initial toxic misfolding of the polyQ protein and promoting its refolding, respectively [15,16,19,20]. Therefore, these results collectively demonstrate that QBP1 and Hsp40 exert therapeutic effects in polyQ disease mouse neurons, via their widespread and long-term expression using AAV5.

AAV5-Hsp40 Improves Neurological Phenotypes of polyQ Disease Mice

Since our above results demonstrated that AAV5-QBP1 and AAV5-Hsp40 inhibit accumulation of the pathogenic polyQ protein into inclusions, we next tested whether this could also lead to amelioration of the neurological phenotypes of R6/2 mice. For this purpose we injected AAV5-GFP, AAV5-QBP1, or AAV5-Hsp40 into both sides of the striatum of P7 R6/2 mice, and analyzed their effects on various neurological phenotypes of these mice.

To evaluate motor impairments of R6/2 mice, we first tested their forced locomotor activity using the rotarod. Although the reduction in rotarod performance of R6/2 mice compared with wild-type (WT) mice becomes evident by 7 weeks of age, the performance of AAV5-QBP1 injected and AAV5-Hsp40 injected R6/2 mice did not significantly differ from AAV5-GFP injected mice (Fig. 2A). We further tested the effect of these viruses on spontaneous locomotor activity. R6/2 mice demonstrated significantly less open-field activity and rearing compared with WT mice as early as 5 weeks of age, which further decreased by 8 weeks. Although we could not detect a significant improvement in AAV5-QBP1 injected mice, AAV5-Hsp40 injected R6/2 mice exhibited significantly higher open-field activity and rearing compared with AAV5-GFP injected mice at 8 weeks of age (Activity: GFP 134.8 ± 10.2 vs Hsp40 196.4 ± 8.4 counts/min, $p < 0.001$; Rearing: GFP 2.0 ± 0.44 vs Hsp40 6.3 ± 0.67 counts/min, $p < 0.001$) (Figs. 2B,C). These results demonstrate the therapeutic effect of AAV5-Hsp40 on the decreased spontaneous locomotor activity of R6/2 mice.

We also tested the effect of each virus on the grip strength abnormality of R6/2 mice. Grip strength of R6/2 mice was significantly weaker than that of WT mice at 9 weeks of age, and further weakened by 12 weeks of age. AAV5-QBP1 injection did not have a significant effect on grip strength at either age. In contrast, AAV5-Hsp40 injected R6/2 mice exhibited significantly

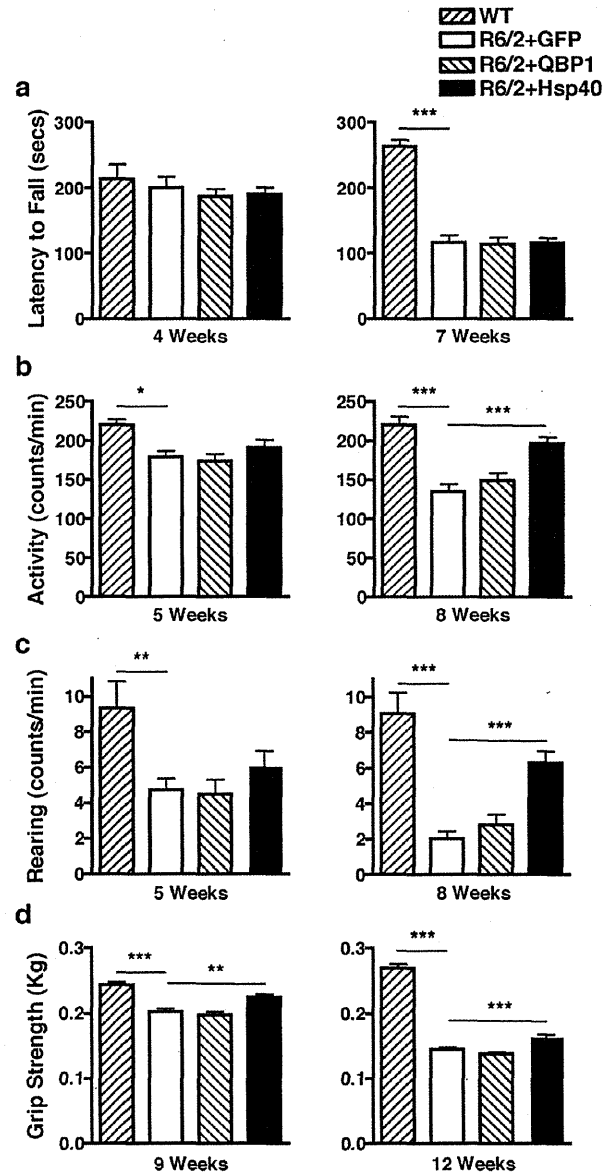


Figure 2. AAV5-Hsp40 improves spontaneous locomotor activity and grip strength abnormalities of polyQ disease mice. R6/2 mice at P7 were injected in both sides of the striatum with AAV5-GFP, AAV5-QBP1, or AAV5-Hsp40, and the following phenotypes were analyzed. (A) Rotarod performance measured at 4 (left) and 7 weeks (right) of age. (B, C) Open-field activity (B) and rearing (C) measured at 5 (left) and 8 weeks (right) of age. (D) Grip strength measured at 9 (left) and 12 weeks (right) of age. Values represent mean \pm SEM, $n \geq 9$ mice (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$). doi:10.1371/journal.pone.0051069.g002

greater grip strengths than AAV5-GFP injected mice both at 9 weeks (GFP 0.202 ± 0.005 vs Hsp40 0.225 ± 0.005 Kg, $p < 0.01$) and 12 weeks of age (GFP 0.145 ± 0.003 vs Hsp40 0.165 ± 0.003 Kg, $p < 0.001$), demonstrating the therapeutic effect of AAV5-Hsp40 (Fig. 2D).

We also evaluated the body weight loss of AAV5-injected R6/2 mice. AAV5-GFP injected R6/2 mice demonstrated significantly lower body weights than WT mice after 9 weeks of age. The

weights of AAV5-QBP1 injected R6/2 mice were almost indistinguishable from AAV5-GFP injected mice at each time point. In contrast, the weights of AAV5-Hsp40 injected R6/2 mice showed a significant improvement compared to AAV5-GFP injected mice between 9 and 13 weeks of age (Fig. 3A). These results demonstrate the therapeutic effect of AAV5-Hsp40 also against the weight loss of R6/2 mice.

We then assessed the effect of AAV5 injections on the lifespan of R6/2 mice. The survival of AAV5-QBP1 injected R6/2 mice (median lifespan 100 days) was not significantly different from AAV5-GFP injected mice (median lifespan 95 days). In contrast, AAV5-Hsp40 injection resulted in a rightward shift of the survival curve to a median lifespan of 112 days, demonstrating the therapeutic effect of AAV5-Hsp40 on the decreased survival of R6/2 mice (Fig. 3B). Taken together, these results clearly demonstrate that AAV5-Hsp40 significantly improves many neurological phenotypes of R6/2 mice.

AAV5-Hsp40 Inhibits Inclusion Body Formation also in Virus Non-infected Neurons of polyQ Disease Mice

When analyzing the effect of the viruses on inclusion body formation in R6/2 mice (Fig. 1), we suspected that on the AAV5-Hsp40 injected side, not only virus infected neurons, but even virus non-infected neurons appeared to have fewer inclusion bodies than those on the AAV5-GFP injected side. To clarify our suspicion, we focused on the virus non-infected neurons that are not stained with Hsp40 or GFP antibodies (see Materials and Methods and Fig. S3), and reanalyzed inclusion body formation in the virus non-infected neurons. On the AAV5-QBP1 injected side, virus non-infected neurons showed a similar rate of inclusion body formation as non-infected neurons on the AAV5-GFP injected side, in both the striatum and cortex, as expected (Fig. 4). Surprisingly, we found that on the AAV5-Hsp40 injected side, virus non-infected neurons had strikingly fewer inclusion bodies compared with non-infected neurons on the AAV5-GFP injected side in both the striatum (GFP side 69.0% vs Hsp40 side 49.8%, $p < 0.01$) and cortex (GFP side 59.1% vs Hsp40 side 39.6%, $p < 0.01$).

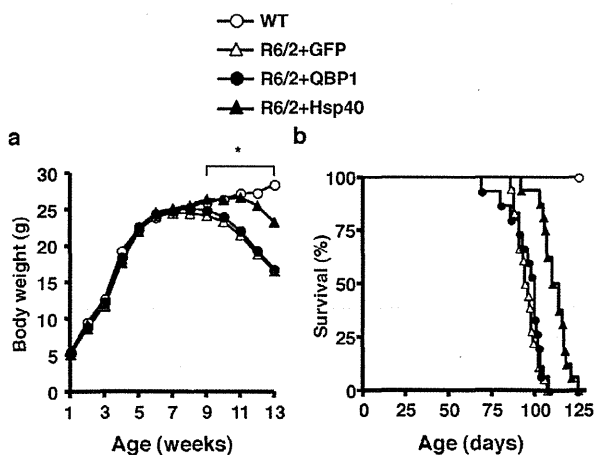


Figure 3. AAV5-Hsp40 improves body weight loss and extends the lifespan of polyQ disease mice. R6/2 mice at P7 were injected in both sides of the striatum with AAV5-GFP, AAV5-QBP1, or AAV5-Hsp40, and the following phenotypes were analyzed. (A) Body weight measured weekly. Values represent the mean ($*p < 0.05$, R6/2+GFP vs R6/2+Hsp40). (B) Survival ($p < 0.0001$, R6/2+GFP vs R6/2+Hsp40, Log-rank test). In both (A) and (B), $n \geq 9$ mice. doi:10.1371/journal.pone.0051069.g003

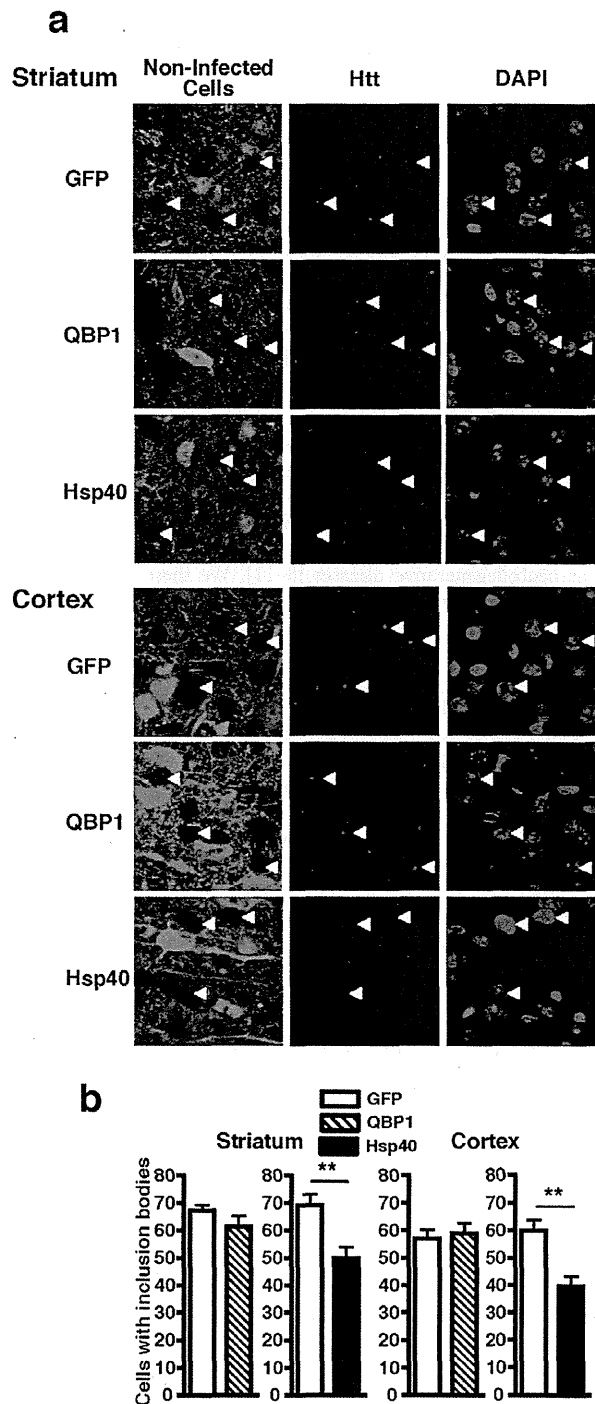


Figure 4. AAV5-Hsp40 also inhibits polyQ inclusion body formation in virus non-infected neurons of polyQ disease mouse brains. Htt inclusion body formation in 8 week old R6/2 mouse brains injected with AAV5-GFP on one side of the striatum and AAV5-QBP1 or AAV5-Hsp40 on the other side, was analyzed as in Fig. 1, but in the virus non-infected cells on each side of the brain. (A) Representative photographs of striatal (top panels) and cortical (bottom panels) sections of R6/2 mice injected with the indicated viruses. Green; virus infected cells, red; htt, and blue; nuclei visualized by DAPI staining. Representative virus non-infected cells in each panel are indicated by

white arrowheads. (B) Inclusion body formation in virus non-infected cells on the AAV5-QBP1 injected side and AAV5-Hsp40 injected side in the striatum (left) and cortex (right). Data are shown as means \pm SEM of ≥ 6 fields of view, in which over 180 cells were counted (** $p < 0.01$). Representative results of two mice analyzed are shown. doi:10.1371/journal.pone.0051069.g004

$p < 0.01$). The degree of inhibition was not as robust as in AAV5-Hsp40 infected neurons, but was still significant. These results raise a possibility that Hsp40 can exert a non-cell autonomous therapeutic effect on virus non-infected neurons in the brains of R6/2 mice, which is not observed with QBP1.

Hsp40 Inhibits Secretion of Pathogenic polyQ Proteins from Cultured Cells

We next aimed to elucidate the mechanism by which Hsp40 exerts its non-cell autonomous therapeutic effect in the brains of R6/2 mice. Recent studies suggest that prion-like cell-cell transmission of aggregation-prone proteins via their release from cells and subsequent uptake into neighboring cells, is involved in the spreading of neuropathology in the polyQ diseases as well as other neurodegenerative diseases [8–11]. We therefore hypothesized that Hsp40 may inhibit secretion of the pathogenic polyQ protein from cells to exert its non-cell autonomous therapeutic effect.

We used a cell culture model to test whether Hsp40 could inhibit secretion of a pathogenic polyQ protein from cells. An expanded polyQ stretch of 81 repeats fused with CFP and a V5 tag (Q81-CFP-V5) was co-expressed together with the GFP control, QBP1, or Hsp40 in Neuro2A cells. Twenty-four h later, the culture media were replaced with fresh media to remove all of the dead cells, and after a further 6 h of incubation, culture media were collected and concentrated using centrifugal filters, and subjected to Western blot analysis. Q81-CFP-V5 was detected in the culture medium of cells (Fig. 5A), suggesting that pathogenic polyQ proteins are secreted from cells. In cells co-expressing QBP1, the amount of Q81-CFP-V5 detected in the culture medium was similar to that in cells co-expressing GFP. In contrast, Neuro2A cells co-expressing Hsp40 showed $\sim 40\%$ less Q81-CFP-V5 in the culture medium compared with cells co-expressing GFP, suggesting that Hsp40 inhibits secretion of the pathogenic polyQ protein from cells (Figs. 5A,B). Furthermore, siRNA-mediated knockdown of endogenous Hsp40 increased the secretion of Q81-CFP-V5 by $\sim 40\%$ compared with cells treated with a control siRNA (Figs. 5C,D), indeed confirming that Hsp40 inhibits polyQ protein secretion. These results imply that inhibition of secretion of the polyQ protein from cells by Hsp40 results in a non-cell-autonomous therapeutic effect in R6/2 mouse brains, possibly via inhibition of the cell-cell transmission of the pathogenic polyQ protein.

Discussion

In this study we show for the first time that viral vector-mediated expression of a molecular chaperone, namely Hsp40 significantly improves the neurological phenotypes of a mouse model of the polyQ diseases. Although a recent study reported the effectiveness of another Hsp40 family member, HSP11a (DNAJB2a) in R6/2 mice, this study cannot be directly translated to a therapy since it was performed by the crossing of transgenic mice [30]. In addition, although lentiviral vector-mediated delivery of DNAJB2a to a polyQ disease rat model has been investigated [37], it did not demonstrate the therapeutic effect on the neurological phenotypes, perhaps because of the limited spread of lentiviruses. We

successfully overcame these above problems by using AAV, which infects a widespread area of the brain, and is already used in human patients [38].

We did not detect significant therapeutic effects of AAV5-Hsp70 on R6/2 mice unlike AAV5-Hsp40, possibly due to the very low infection rate of our AAV5-Hsp70 (data not shown), or differences in the effectiveness of Hsp40 and Hsp70 against mutant htt. Indeed, previous studies examining the effect of Hsp70 in R6/2 mice have shown no or very modest therapeutic effects [28,29]. Furthermore, a cell culture study demonstrated that Hsp40 family members are effective against the toxicity of mutant htt, while Hsp70 family members are ineffective [35]. Taken together, these studies indicate that Hsp40 family members may be more effective than Hsp70 family members against the toxic effects of mutant htt.

We surprisingly found that AAV5-Hsp40 inhibits inclusion body formation also in virus non-infected cells (Fig. 4), suggesting a non cell-autonomous therapeutic effect. Aggregation prone proteins that cause neurodegenerative diseases including pathogenic polyQ proteins, as well as α -synuclein which causes Parkinson's disease, and tau which causes the tauopathies have recently been suggested to be transmitted between cells, and this may be the mechanism leading to the progressive spread of neuropathology in these diseases [8–11]. We detected a significant amount of the pathogenic polyQ protein in the culture medium of Neuro2A cells (Fig. 5A), suggesting its cell-cell transmission, which is compatible with previous studies [39,40]. We further found that Hsp40 suppresses secretion of the pathogenic polyQ protein from cells (Fig. 5), suggesting that it may inhibit the cell-cell transmission of the pathogenic polyQ protein. The non-cell autonomous therapeutic effect of Hsp40 may also involve other mechanisms, such as (1) AAV5-Hsp40 infected neurons may create a better environment for contacting non-infected neurons [41] or (2) Hsp40 itself may be secreted to exert therapeutic effects on neighboring non-infected neurons, as is suggested for Hsp70 [42].

We were unable to detect the therapeutic effect of AAV5-QBP1 on the neurological phenotypes of R6/2 mice, although we successfully detected its inhibition of inclusion body formation. However, we and others have previously shown that expression of QBP1 exerts therapeutic effects on the neurological phenotypes of *Drosophila* and mouse models of the polyQ diseases [16,17,43]. Since the infection efficiency of all of the viruses used in this study was quite low ($\sim 30\%$), the extent of neurons expressing QBP1 in the R6/2 mouse brains was probably insufficient to exert a detectable effect on the phenotypes. On the other hand, in the case of AAV5-Hsp40, inhibition of polyQ protein secretion which should lead to an increase in the number of rescued neurons, likely contributed to its improvement of the neurological phenotypes. Other possibilities may also contribute to their varying effects, for example (1) Hsp40 is more effective than QBP1 in inhibiting polyQ protein misfolding/aggregation, and (2) Hsp40 can also support the degradation of misfolded proteins [19], while QBP1 cannot.

In this study we demonstrate a therapeutic strategy against the polyQ diseases using AAV5-Hsp40, which has great potential for clinical application, since AAVs are safe and are widely utilized in clinical trials [38]. We further suggest a novel therapeutic mode of action of Hsp40, namely suppression of pathogenic polyQ protein secretion from cells, which may consequently suppress its cell-cell transmission. Since the transmission of aggregation-prone proteins is thought to be involved also in other neurodegenerative diseases, Hsp40 may exert a non-cell autonomous therapeutic effect on these other diseases. Elucidation of how Hsp40 inhibits polyQ protein secretion should reveal new therapeutic targets and

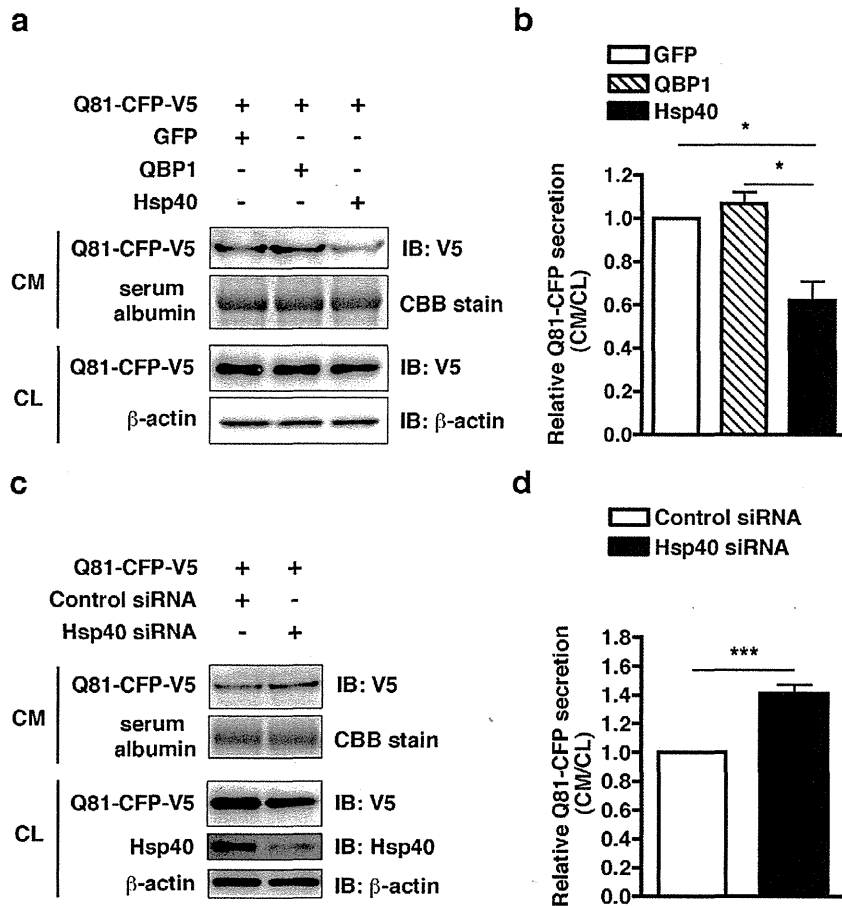


Figure 5. Hsp40 inhibits polyQ protein secretion in cultured cells. (A) Neuro2A cells were co-transfected with plasmids expressing Q81-CFP-V5 and either GFP, QBP1, or Hsp40, and the culture media (CM) and cell lysates (CL) were subjected to Western blot analysis with a V5 antibody to detect the Q81-CFP-V5 protein. (B) Relative amounts of Q81-CFP-V5 secreted into the culture media, calculated from the band intensities in (A), with the amount of Q81-CFP-V5 secreted from cells co-expressing GFP set to 1. (C) Neuro2A cells were transfected with a plasmid expressing Q81-CFP-V5 and siRNA against Hsp40 or a control siRNA, and the culture media (CM) and cell lysates (CL) were subjected to Western blot analysis with a V5 antibody to detect the Q81-CFP-V5 protein, and with an Hsp40 antibody. The loading controls are as in (A). (D) Relative amounts of Q81-CFP-V5 secreted into the culture media, calculated from the band intensities in (C). In (A) and (C), serum albumin is shown as a loading control for the culture media, and β -actin as a loading control for the cell lysates. In (B) and (D), data are shown as means \pm SEM of \geq four independent experiments (* p <0.05, *** p <0.001).

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strategies for neurodegenerative diseases caused by aggregation-prone proteins.

Materials and Methods

Viral Vectors

Adeno-associated virus type 5 (AAV5) vector plasmids contained an expression cassette with a human cytomegalovirus enhancer/chicken β actin (CAG) promoter followed by the first intron of human growth hormone, target cDNA (either a tandem repeat of QBP1 fused to GFP [14], human Hsp40 (DNAJB1) [44], or GFP), and a simian virus 40 polyadenylation signal sequence, all positioned between the inverted terminal repeats of the AAV5 genome. AAV5 vectors were produced using the AAV5 plasmid, the AAV5 helper plasmid containing the rep and cap sequences from AAV5, as well as the pHelper plasmid from the AAV Helper-Free System containing the E2A, E4, and VA RNA genes of the adenovirus genome (Stratagene, La Jolla, CA). HEK293 cells were

co-transfected with the AAV5 plasmid and two helper plasmids by the calcium phosphate method. Seventy-two h later, the cells were harvested and subjected to three rounds of freeze-thaw lysis. AAV5 vectors were then purified by two rounds of cesium chloride density gradient centrifugation. Vector titers were estimated by quantitative DNA dot-blot hybridization to be ~ 0.2 – 1.6×10^{13} genome copies/ml.

Animals

All animal experiments were performed in accordance with the guidelines of the Animal Ethics Committee of the National Institute of Neuroscience, National Center of Neurology and Psychiatry, Japan, and performed in accordance with the guidelines. Mice transgenic for human *huntingtin* exon 1 with approximately 150 CAG repeats (strain R6/2) [33] were obtained from the Jackson Laboratory (Bar Harbor, ME), and maintained on a B6CBAF1 background. Genotypes were analyzed and CAG repeat numbers of the transgenic mice were confirmed to be

similar by PCR as previously described [33]. Mice were housed on a 12-hour light/dark cycle, with food and water provided *ad libitum*. At least nine male R6/2 mice per group and wild-type littermate (WT) controls were used for the phenotype analyses, and two R6/2 mice were used for the inclusion body analyses.

AAV Injections

P7 old R6/2 mice were stereotactically injected with 1 μ l of virus solution (AAV5-GFP, AAV5-QBP1, or AAV5-Hsp40) into the striatum (coordinates 1 mm anterior to bregma, 2.25 mm lateral to the midline, and 3 mm ventral to the skull surface) at a rate of 0.1 μ l/min using a 10 μ l Hamilton syringe (Hamilton Company, Reno, NV) and an infusion pump (KD Scientific, Holliston, MA). For inclusion body analyses, AAV5-GFP was injected into one side of the striatum and AAV5-QBP1 or AAV5-Hsp40 into the other side. For phenotype analyses, the same virus was injected into both sides of the striatum.

Mouse Phenotype Analyses

For rotarod analysis, mice were tested at 4 and 7 weeks of age on an accelerating rotarod apparatus (Ugo Basile, Comerio, Italy) set to accelerate from 4 to 40 rpm over a period of 300 seconds. The time it took for each mouse to either fall off the rod or cling onto the rod for one full rotation was recorded. Mice were tested on the rod for three consecutive days, with three trials per day. The first day was regarded as training, and only the data from the second and third day were used. The highest and lowest values were excluded, and the middle four values were averaged. Grip strength analysis was performed at 9 and 12 weeks of age using a grip strength meter (Muromachi Kikai, Tokyo, Japan). Mice were placed gently by their tail on the metal grid of the meter so that they grip the grid with both forelimbs and hindlimbs, at which point they were pulled back gently with their tails, exerting a tension that is measured by the meter. Five trials were performed for each mouse, and the highest and lowest values were excluded and the middle three values were averaged. To measure open-field activity and rearing, mice at 5 and 8 weeks of age were tested using a spontaneous locomotor activity monitor (Supermex, Muromachi Kikai, Tokyo, Japan), consisting of an acrylic box of dimensions 40 cm \times 28 cm \times 31 cm with an activity sensor placed at the top and rearing sensors placed at the sides of the box. Mice were left in the box for a total of 15 min, and their activity count and rearing frequency were measured. For body weight analysis mice were weighed weekly. Mice were followed until their deaths in order to calculate their lifespans, a widely accepted and valuable parameter to assess therapeutic effects in these mice, which was approved by the institution's Animal Ethics Committee.

Tissue Preparation and Immunohistochemical Analyses

Mice were deeply anesthetized with sodium pentobarbital (100 mg/kg), and then perfused intercardially with saline followed by 4% paraformaldehyde (PFA) in phosphate-buffered saline (PBS). Brains were removed, post-fixed in 4% PFA in PBS at 4°C overnight, and then cryoprotected in 30% sucrose in PBS at 4°C for 24 h. Frozen 10 μ m sections were cut using a cryostat. For immunohistochemical analysis, sections were blocked in PBS containing 5% goat serum and 0.1% Triton X-100 for 1 h at room temperature. The sections were then incubated with a mouse anti-huntingtin antibody (1:250; MAB5374, Millipore, Billerica, MA), and either a rabbit anti-Hsp40 antibody (1:500; SPA-400, Enzo Life Sciences, Farmingdale, NY) or a rabbit anti-GFP antibody (1:250; A11122, Invitrogen, Carlsbad, CA) at 4°C overnight, followed by an Alexa Fluor 596-conjugated goat anti-mouse IgG antibody and an Alexa Fluor 488 goat anti-rabbit IgG antibody

(1:1000, Invitrogen) for 1 h at room temperature. Sections were mounted with Slowfade Gold antifade reagent with DAPI (Invitrogen) and examined using a confocal laser scanning microscope (FV1000; Olympus, Tokyo, Japan). The average fluorescence intensity of representative cells that were regarded as either "infected" or "non-infected" in each sample were measured using the ImageJ software, confirming that the two population of cells can readily be distinguished in the images (Fig. S3).

Cell Culture, Transfection and Western Blot Analysis

Neuro2A cells (obtained from ATCC) were grown and maintained in DMEM supplemented with 10% (v/v) FBS. Cells were plated on a 35-mm dish at a density of 4×10^5 cells per dish on the day before transfection. For the overexpression experiment, a plasmid vector encoding a tandem repeat of QBP1 fused to GFP, human Hsp40 [44], or GFP was transiently co-transfected with the Q81-CFP-V5 vector encoding an expanded polyQ stretch of 81 repeats fused with CFP and a V5 tag, using Lipofectamine LTX with PLUS reagent (Invitrogen). For the knockdown experiment, siRNA targeted against mouse Hsp40 (Santa Cruz Biotechnology, Santa Cruz, CA) or a control siRNA (RNAi Inc, Tokyo, Japan) was cotransfected with the Q81-CFP-V5 vector using Lipofectamine 2000 reagent (Invitrogen). At 24 h after transfection, culture media were replaced with fresh media, and after a further 6 h of incubation, culture media were collected and whole cell lysates were prepared with 1% Triton X in tris-buffered saline. For Western blot analysis, the culture media were concentrated using 30 kDa cutoff Amicon Ultra filters (Millipore). Q81-CFP-V5 in the concentrated culture media and whole cell lysates was separated using 10% SDS-PAGE gels and transferred onto PVDF membranes (Bio-Rad, Hercules, CA). The membranes were incubated overnight with an HRP-conjugated anti-V5 antibody (1:2500, Invitrogen) at 4°C. The HRP signal was visualized with SuperSignal West Femto Chemiluminescent Substrate (Thermo Fisher Scientific Inc, Rockford, IL), captured with a LAS-3000 mini CCD imaging system (Fujifilm, Tokyo, Japan), and band intensities were quantified using the ImageJ software. For the siRNA experiments, the membranes were then stripped and incubated with a rabbit anti-Hsp40 antibody (1:5000, SPA-400, Enzo Life Sciences Inc, Farmingdale, NY) at 4°C followed by an HRP-conjugated anti-rabbit IgG secondary antibody (1:10000, Thermo Fisher Scientific), and visualized as above.

Statistical Analyses

For the rotarod, open-field activity, rearing, grip strength and weight data, statistical analyses were performed by using one-way analysis of variance followed by Tukey's multiple comparison test to assess for significant differences between individual groups. The survival data was analyzed using the Log-rank test. For the inclusion body formation and Western blot analyses, Student's *t*-test was used. For all analyses, $p < 0.05$ was considered as significant.

Supporting Information

Figure S1 AAV5 injection into the mouse striatum at P7 results in widespread expression of the transgene. R6/2 mice at P7 were injected in the right striatum with 1 μ l of AAV5-QBP1, and 2 weeks later the expression of QBP1 was analyzed by immunohistochemistry. This widespread expression of QBP1 throughout the brain lasts for at least 13 weeks (data not shown). (PDF)

Figure S2 AAV5-QBP1 inhibits polyQ inclusion body formation in virus infected neurons of polyQ disease

mice. R6/2 mice at P7 were injected with AAV5-GFP on one side of the striatum and AAV5-QBP1 on the other side, and at 4, 8, and 14 weeks of age htt inclusion body formation in virus infected neurons of the striatum (left) and cortex (right) was assessed by immunohistochemistry. Data are shown as means \pm SEM of ≥ 6 fields of view, in which over 180 cells were counted ($*p<0.05$, $***p<0.001$). Representative results of two mice analyzed are shown.

(PDF)

Figure S3 AAV5 “infected” and “non-infected” cells can be clearly distinguished from their fluorescence intensity. The fluorescence intensity of representative cells that were regarded as either “infected” or “non-infected” in photographs of immunostained brain sections of R6/2 mice injected with either AAV5-GFP (left), AAV5-QBP1 (middle) or AAV5-

Hsp40 (right). For each sample, a total of over 100 representative cells were analyzed from 5–6 fields of view.

(PDF)

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Author Contributions

Conceived and designed the experiments: HAP T. Takeuchi SM T. Toda KW YN. Performed the experiments: HAP T. Takeuchi HF KY CI HY. Analyzed the data: HAP T. Takeuchi T. Toda KW YN. Contributed reagents/materials/analysis tools: SM. Wrote the paper: HAP T. Takeuchi YN.

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Two Cohort and Three Independent Anonymous Twin Projects at the Keio Twin Research Center (KoTReC)

Juko Ando,¹ Keiko K. Fujisawa,¹ Chizuru Shikishima,² Kai Hiraishi,³ Mari Nozaki,⁴ Shinji Yamagata,⁵ Yusuke Takahashi,⁶ Koken Ozaki,⁷ Kunitake Suzuki,⁸ Minako Deno,⁹ Shoko Sasaki,⁴ Tatsushi Toda,¹⁰ Kazuhiro Kobayashi,¹⁰ Yutaro Sugimoto,¹ Mitsuhiro Okada,¹ Nobuhiko Kijima,¹¹ Yutaka Ono,¹² Kimio Yoshimura,¹³ Shinichiro Kakihana,¹⁴ Hiroko Maekawa,¹⁵ Toshimitsu Kamakura,¹⁶ Koichi Nonaka,¹⁷ Noriko Kato,¹⁸ and Syuichi Ooki¹⁹

¹Faculty of Letters, Keio University, Tokyo, Japan

²Keio Advanced Research Centers, Keio University, Tokyo, Japan

³Department of Psychology, Yasuda Women's University, Hiroshima, Japan

⁴Graduate School of Arts and Sciences, The University of Tokyo, Tokyo, Japan

⁵National Center for University Entrance Examinations, Tokyo, Japan

⁶Center for the Promotion of Excellence in Higher Education, Kyoto University, Kyoto, Japan

⁷Department of Data Science, The Institute of Statistical Mathematics, Tokyo, Japan

⁸Osaka University of Human Sciences, Osaka, Japan

⁹Correspondence Division, Musashino University, Tokyo, Japan

¹⁰Graduate School of Medicine, Kobe University, Kobe, Japan

¹¹Faculty of Business and Commerce, Keio University, Tokyo, Japan

¹²National Center for Cognitive Behavior Therapy and Research, National Center for Neurology and Psychiatry, Tokyo, Japan

¹³School of Medicine, Keio University, Tokyo, Japan

¹⁴Department of Home Economics, Koriyama Women's University, Fukushima, Japan

¹⁵Department of Intercultural Studies, Faculty of Literature, Kanazawa Gakuin, Ishikawa University, Ishikawa, Japan

¹⁶Faculty of Letters, Aichi University, Aichi, Japan

¹⁷Faculty of Human Sciences, Wako University, Tokyo, Japan

¹⁸National Institute of Public Health, Saitama, Japan

¹⁹Ishikawa Prefectural Nursing University, Ishikawa, Japan

The Keio Twin Research Center has conducted two longitudinal twin cohort projects and has collected three independent and anonymous twin data sets for studies of phenotypes related to psychological, socio-economic, and mental health factors. The Keio Twin Study has examined adolescent and adult cohorts, with a total of over 2,400 pairs of twins and their parents. DNA samples are available for approximately 600 of these twin pairs. The Tokyo Twin Cohort Project has followed a total of 1,600 twin pairs from infancy to early childhood. The large-scale cross-sectional twin study (CROSS) has collected data from over 4,000 twin pairs, from 3 to 26 years of age, and from two high school twin cohorts containing a total of 1,000 pairs of twins. These data sets of anonymous twin studies have mainly targeted academic performance, attitude, and social environment. The present article introduces the research designs and major findings of our center, such as genetic structures of cognitive abilities, personality traits, and academic performances, developmental effects of genes and environment on attitude, socio-cognitive ability and parenting, genes x environment interaction on attitude and conduct problem, and statistical methodological challenges and so on. We discuss the challenges in conducting twin research in Japan.

■ **Keywords:** twin cohort, longitudinal, infancy, childhood, adolescence, adulthood

The Keio Twin Research Center (KoTReC) was established in 2009 as an integrated organization of two twin cohort projects at Keio University; the Keio Twin Study (KTS) for adolescence and adulthood, and the Tokyo Twin Cohort Project (ToTCOP) for infancy and childhood. These two

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ADDRESS FOR CORRESPONDENCE: Juko Ando, Faculty of Letters, Keio University, Tokyo, Japan. E-mail: juko@msa.biglobe.ne.jp