familial cases[26,42] and at 10% to 20% in idiopathic cases.[26,30,31,42] It is noted that a considerable number of patients carry a single heterozygous mutations. Clinical features of the carriers of heterozygous *parkin* mutations are more similar to those of idiopathic PD, including a significantly later age at onset and more asymmetric disease presentation, than those of carriers of two mutations.[27,42–44] Since *parkin*-associated PD is considered to be an autosomal recessively transmitted disease, the role of mutations in single heterozygous state is difficult to interpret. There are two possibilities: one is haploinsufficiency, the other dominant negative effect. In addition, a possible explanation is that still unknown factors such as mutations located elsewhere in the genome[45] and environmental factors act in combination to cause the disease. On the other hand, in a North American study of more than 300 PD patients unselected for age at onset or family history, the frequency of *parkin* mutations reported as pathogenic in homozygous or compound heterozygous individuals is essentially the same in PD patients (3.8%) and controls (3.1%).[46] Thus, whether single heterozygous mutations in *parkin* are associated with PD remains to be elucidated.

The gene product, parkin, is localized to the Golgi complex in addition to the cytoplasm in human brain, although parkin has no transmembrane domain.[47] In cultured cells and rat brain, parkin also associates with synaptic vesicles as a peripheral membrane protein.[48] Recently, parkin was implicated in the ubiquitin–proteasome system (UPS) as an E3 ubiquitin ligase, and mutations in the *parkin* gene is reported to result in loss of ligase function.[36] The UPS is involved in two tasks. One is the accurate timely regulation of the level of short-lived proteins that plays a role in processes such as cell cycle regulation, signal transduction, and metabolism. The other task is protein quality control. Polyubiquitination of the target proteins for degradation by proteasomes is catalyzed by three enzymes, E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzyme), and E3 ubiquitin ligase. Parkin has been shown to catalyze the proteasomal degradation of target proteins by interacting with E2 and target proteins through its RING domain[49–51] and by binding the Rpn10 subunit of 26S proteasomes through its Ubl domain.[52] Parkin is also reported to exhibit E3 activity by interacting with components of SCF complexes (Skp1–Cullin–F-box protein).[53] In addition, parkin forms a complex with Hsp70 and CHIP, resulting in enhancement of its E3 enzymatic activity.[54] Since *parkin*-associated PD is recessively inherited, that is, loss of function of parkin leads to development of *parkin*-associated PD, substrates for parkin (for its E3 function) would be expected to accumulate in the brain. Substrates identified to date include cell division control–related protein 1 (CDCrel-1),[50] parkin-associated endothelin receptor–like receptor (Pael-R),[51] the *O*-glycosylated form of α-synuclein (αSp22),[55] synphilin-1,[56] synaptotagmin XI,[57] SEPT5_v/CDCrel-2,[58] cyclin E,[53] the p38 subunit of the aminoacyl–tRNA synthetase complex,[59] and α/β-tubulin.[60] In addition to the substrates, several interactive molecules have been reported.[61] One may postulate that the substrates that escape from ubiquitination by parkin for degradation by the proteasome accumulate in *parkin*-associated PD brains, leading to nigral degeneration. In fact, three of the substrates (CDCrel-1,[58] Pael-R,[51] αSp22[55]) have been reported to accumulate in *parkin*-associated PD brains. However, an important caveat is that most substrates have been identified by in vitro experiments and remain to be validated.[62] On the other hand, parkin has recently been shown to catalyze lysine63 (K63)-linked ubiquitination, which is not recognized by the proteasome.[63,64] Assembly of polyubiquitination occurs through the sequential formation of an isopeptide bond between the carboxy-terminal glycine residue and specific lysine residues in ubiquitin. Substrates tagged with the polyubiquitin chain linked via K29 or K48 are recognized and degraded by the proteasome.[65] In contrast, K63-linked ubiquitination is involved in diverse cellular processes such as endocytosis[66–69] and protein sorting and trafficking.[70–72] Although the relevance of K63-linked ubiquitination by parkin in the pathogenesis of *parkin*-associated PD remains unclear, this insight might facilitate elucidation of the pathogenesis.

With regard to loss of function of parkin as a mechanism underlying *parkin*-associated PD, *parkin*-knockout animal models should help to elucidate the mechanisms of the disease (Table 3). Although three lines of *parkin*-knockout mice have been reported,[73–75] none of these develops a PD-like phenotype or PD pathology. Two exon 3–disrupted knockouts show subtle motor and cognitive deficits, inhibition of amphetamine-induced dopamine release and glutamate neurotransmission, and abnormal dopamine metabolism, but no loss of substantia nigra or locus coeruleus catecholaminergic neurons. Meanwhile, an exon 7–disrupted knockout exhibits loss of noradrenergic locus coeruleus neurons. Interestingly, none of the previously reported substrates for parkin in the UPS accumulated in the knockout brains,[74,75] suggesting that parkin might function as an E3 for K63-linked ubiquitination. Proteomic analysis of ventral midbrain from the exon 3–disrupted mice revealed decreased abundance of a number of proteins involved in mitochondrial function or oxidative stress.[76] Consistent with reduction of these proteins, the mice exhibited de-

TABLE 3. *Animal models of* parkin *and* DJ-1 *PD*

Knockout models	Phenotypes	Pathology
Parkin		
Exon 3-knockout mice[73]	Subtle motor and cognitive deficits, inhibition of amphetamine-induced dopamine release, inhibition of glutamate neurotransmission, increased dopamine level	Normal brain morphology
Exon 3-knockout mice[74]	Subtle behavioral deficits, increased extracellular dopamine concentration, reduced synaptic excitability	Normal brain morphology
Exon 3-knockout mice[75]	Reduced norepinephrine-dependent startle response	Loss of catecholaminergic neurons in the locus coeruleus
Drosophila null mutants[77]	Reduced life span, locomotor defects, male sterility	Muscle fiber degeneration, mitochondrial pathology
Drosophila null mutants[78]	Low mass, reduced life span, locomotor defects, sterility	Muscle fiber degeneration, mitochondrial defects
DJ-1		
Exon 2-knockout mice[122]	Hypoactivity in the open field, reduced evoked dopamine overflow due to increased reuptake, less sensitivity to the inhibitory effects of D2 autoreceptor stimulation	Normal brain morphology
Promoter-exons 1-5—knockout mice[123]	Age-dependent and task-dependent motoric behavioral deficits, increased dopamine reuptake rates, elevated tissue dopamine content	Normal brain morphology
Exons 3-5 with modified first coding exon[124]	Depression in amphetamine-induced locomotor activity, increased striatal denervation and dopaminergic neuron loss induced by MPTP	Normal brain morphology

creased respiratory capacity of striatal mitochondria, reduced serum antioxidant capacity, and increased protein and lipid peroxidation. A recent study in Drosophila with an inactivated orthologue of human *parkin* also suggested mitochondrial dysfunction.[77] The *parkin*-null mutants exhibited reduced longevity, locomotor defects, and sterility.[77,78] Mitochondrial pathology has been the earliest manifestation of muscular degeneration and defective spermatogenesis in the *parkin* mutants. These observations suggest a new role for parkin in the regulation of normal mitochondrial function. Parkin overexpression in cultured cells protects against mitochondria-dependent apoptosis and parkin associates with the outer mitochondrial membrane.[79] Furthermore, parkin has been shown to have cytoprotective effects against diverse cellular insults, including dopamine-mediated toxicity,[80] kainate-induced excitotoxicity,[53] overexpression of Pael-R[51] or the p38 subunit,[59] and toxicity induced by proteasomal inhibition or overexpression of mutant α-synuclein.[81] Recently, antisense knockdown of *parkin* was reported to cause apoptotic cell death of human dopaminergic cells associated with caspase activation, accompanied by accumulation of oxidative dopamine metabolites due to auto-oxidation of DOPA and dopamine.[82] These results suggest that parkin may function as a multipurpose neuroprotectant.[83] Interestingly, parkin has been shown recently to be *S*-nitrosylated in vitro, as well as in brains of patients with idiopathic PD.[84,85] Chung and colleagues[84] reported that *S*-nitrosylation of parkin impaired its E3 activity and its protective function. On the other hand, Yao and colleagues[85] showed that *S*-nitrosylation increased, rather than decreased, the E3 activity of parkin. Several technical differences may explain the discrepancy between them.[86] These findings may thus provide a molecular link between *parkin*-associated PD and sporadic PD.

PINK1 (PARK6)

Although a small number of studies have been published, the clinical manifestations of *PINK1*-associated PD are characterized by parkinsonism associated with good response to levodopa, frequent occurrence of levodopa-induced dyskinesias, and infrequent occurrence of dystonia, very similar to idiopathic PD. The only distinctive features are an earlier age of onset (18 to 51 years) and slower disease progression.[10,87–95] Hyperreflexia and sleep benefit are not common. Some patients, however, exhibit foot dystonia at onset and sleep benefit, thereby resembling *parkin*-associated PD.[88,89,2,94] Psychiatric symptoms such as hallucination, depression, and anxiety are reported in some patients.[88,89,92,94] Dementia is described in one Israeli patient.[88,89] Autopsy data are not available.

The gene responsible for *PINK1*-associated PD, *PINK1* (PTEN-induced kinase 1), was previously cloned by two groups examining gene regulation by the tumor suppressor PTEN[96] and differential gene expression in mouse cell lines that vary in their metastatic potential.[97] The frequency of *PINK1* mutations (homozygous or compound heterozygous) has been estimated at 3.3% among Italian sporadic early-onset PD.[93,94] Hatano and colleagues[88,89] have reported that 6 (~15%) of 39 early-onset autosomal recessive families, mostly Asian, had homozygous or compound heterozygous *PINK1* muta-

tions. Interestingly, despite autosomal recessive transmission, 5% of the sporadic early-onset PD have single heterozygous *PINK1* mutations.[93,94] Although only small number of reports are available so far,[87,89–91,93] the clinical features between the homozygous and heterozygous patients are generally similar, but onset age is about 10 years earlier in the former than in the latter. The role of single heterozygous *PINK1* mutations in PD remains to be clarified. *PINK1* contains eight exons spanning 18 kilobases (kb) and encodes a 581 aa protein with a 34 aa mitochondria targeting signal and a highly conserved protein kinase domain (residues 156 to 509) similar to serine/threonine kinases of the Ca^{2+}/calmodulin family.[19] Most mutations have been reported to cluster in or around the putative kinase domain,[19,89–95] suggesting that the loss of PINK1 kinase activity may cause the disease. The kinase substrates for PINK1 have not yet been identified. In cultured cells, transfected PINK1 is localized in the mitochondria and exerts a protective effect against stress induced by proteasome inhibitors, which is abrogated by the mutations,[19] although how PINK1 protects the cells and whether PINK1 kinase activity plays a role in protection is not clear. Interestingly, PINK1, particularly the N-terminally processed mature form, has been shown to exist in the cytosol as well as in mitochondria.[98] Recently, two mutations found in patients, G309D and L347P, were associated with reduced kinase activity.[98] The L347P mutation markedly decreases the stability of PINK1 and consequently reduces its kinase activity, whereas G309D has much more modest effects on these parameters in vitro. The relevance of PINK1 kinase activity for the pathogenesis of *PINK1*-associated PD thus awaits further clarification.

DJ-1 (PARK7)

The clinical features of *DJ-1*–associated PD, which cannot be fully appreciated as yet because only a few studies have been published, are similar to *parkin*-associated PD and *PINK1*-associated PD, namely, parkinsonism associated with an early age of onset (17 to 42 years), good response to levodopa, slow progression, and levodopa-induced dyskinesias.[11,99–103] Behavioral and psychic disturbances at onset or early stages in the disease course and focal dystonias, including blepharospasm, have been reported.[11,99] Brain single positron emission computed tomography with dopamine transporter tracer showed severe abnormalities consistent with presynaptic dysfunction of nigrostriatal dopaminergic systems.[11] The gene responsible for *DJ-1*–associated PD, *DJ-1*, contains eight exons spanning over 24 kb and encodes a 189 aa protein that belongs to the ThiJ/PfpI family.[20] A number of pathogenic mutations have been identified in the *DJ-1* gene, including exonic deletions, truncations, and homozygous and heterozygous missense mutations.[20,100–105] However, the frequency of the mutations appears to be low.[95,102] It has been reported that only 2% of early-onset PD have *DJ-1* mutations.[102] DJ-1 is ubiquitously expressed, including in brain, where both neurons and glia are immunopositive for the protein.[106,107] Interestingly, DJ-1 is not an essential component of Lewy bodies, but localized in glial cytoplasmic inclusions in multiple-system atrophy, and in neuronal tau inclusions of tauopathies such as Pick's disease, corticobasal degeneration, progressive supranuclear palsy, and Alzheimer's disease.[106,108,109] In addition, DJ-1 levels are markedly increased in the detergent-insoluble fraction of brains of patients with sporadic PD.[110] These findings suggest that DJ-1 might have diverse functions. Indeed, DJ-1 appears to be implicated in multiple processes, including chaperone activity,[111] protease activity,[107] and the oxidative stress response.[112–115] The crystal structure of human DJ-1 has been resolved and shows that DJ-1 exists in solution as a homodimer, and the mutation found in patients disrupts the dimerization.[116–119] Although the biological function of DJ-1 remains elusive, there is growing evidence that DJ-1 functions as an antioxidant protein and/or as a redox sensor of oxidative stress. DJ-1 has been shown to demonstrate an acidic shift in isoelectric point in cultured cells under oxidative stress owing mainly to oxidative modification of cysteine residues within the molecule.[112–115] DJ-1 eliminates hydrogen peroxide in vitro by oxidizing itself.[113] Moreover, overexpression of DJ-1 protects against oxidative injury, whereas knockdown of *DJ-1* by short interfering RNA enhances the susceptibility to oxidative stress.[113,120] DJ-1 also protects against oxidative stress through its cysteine–sulfinic acid–driven mitochondrial localization.[121] Interestingly, DJ-1 interacts with parkin under conditions of oxidative stress,[110] suggesting that these proteins may cooperate in a common neuroprotective pathway. Indeed, DJ-1 exhibits a protective effect against the toxicity induced by overexpression of Pael-R, a substrate for parkin, in addition to endoplasmic reticulum stress and proteasomal inhibition.[120] These results also suggest that DJ-1 functions as a molecular chaperone or protease for refolding or degradation of misfolded or aggregated proteins induced by endoplasmic reticulum stress and proteasomal inhibition. Very recently, three reports describe *DJ-1* knockout mice generated by targeted disruption of exon 2,[122] the first five exons and part of the promoter,[123] or exons 3–5 with modified first coding exon.[124] Although none of these models demonstrate alteration in gross brain morphology or dopamine neuron loss, two lines of

knockouts[122,123] exhibit abnormalities in striatal dopaminergic function with motor deficits. Interestingly, these two lines of mice show increased dopamine reuptake, suggesting that DJ-1 plays an essential role in normal dopaminergic physiology. The other knockout[124] shows increased striatal denervation and dopaminergic neuron loss induced by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), suggesting protective effect of DJ-1 against dopaminergic insults.

CONCLUSIONS

Based on clinical features, differentiating these three forms of recessively inherited PD, sometimes even from idiopathic PD, appears to be difficult, as similar neuronal groups, notably dopaminergic neurons, are predominantly affected. Genetic analysis is therefore essential for an accurate diagnosis. Genetic testing has become commercially available for these genes (MRC-Holland, Amsterdam, The Netherlands). The kit seems to have the advantage of a relatively low running costs, dealing with large numbers of samples, and low investment for setting. A point to note is that the kit is not suitable to detect new mutations. The clinical and pathological similarities suggest that common molecular pathways may contribute to the pathogenic mechanisms of both recessive and sporadic PD. Mitochondrial dysfunction and oxidative stress are thought to play a prominent role in the pathogenesis of sporadic PD.[2–5] Furthermore, impairment of the UPS with protein mishandling has recently been suggested to be a major pathway leading to neurodegeneration in sporadic PD.[124,125] Although the mechanisms of these abnormal events in the neurons of sporadic PD are still unknown, recent advances in recessive PD have implicated parkin, PINK1, and DJ-1 in mitochondrial function, oxidative stress, and the UPS, as described above. This growing evidence suggests that loss of function of parkin, PINK1, and/or DJ-1 might contribute to the pathogenesis of sporadic PD. Further studies of recessive PD and associated gene products will help elucidate the molecular relationships between recessive and sporadic PD and hopefully lead to development of new strategies for nigral protection.

REFERENCES

1. Lang AE, Lozano AM. Parkinson's disease: first of two parts. N Engl J Med 1998;339:1044–1053.
2. Schapira AH, Cooper JM, Dexter D, Clark JB, Jenner P, Marsden CD. Mitochondrial complex I deficiency in Parkinson's disease. J Neurochem 1990;54:823–827.
3. Hattori N, Tanaka M, Ozawa T, Mizuno Y. Immunohistochemical studies on complexes I, II, III, and IV of mitochondria in Parkinson's disease. Ann Neurol 1991;30:563–571.
4. Ben-Shachar D, Riederer P, Youdim MB. Iron-melanin interaction and lipid peroxidation: implications for Parkinson's disease. J Neurochem 1991;57:1609–1614.
5. Jenner P. Oxidative stress in Parkinson's disease. Ann Neurol 2003;53(Suppl. 3):S26–S36.
6. Polymeropoulos MH, Higgins JJ, Golbe LI, et al. Mapping of a gene for Parkinson's disease to chromosome 4q21–q23. Science 1996;274:1197–1199.
7. Matsumine H, Saito M, Shimoda-Matsubayashi S, et al. Localization of a gene for an autosomal recessive form of juvenile Parkinsonism to chromosome 6q25.2-27. Am J Hum Genet 1997; 60:588–596.
8. Gasser T, Muller-Myhsok B, Wszolek ZK, et al. A susceptibility locus for Parkinson's disease maps to chromosome 2p13. Nat Genet 1998;18:262–265.
9. Leroy E, Boyer R, Auburger G, et al. The ubiquitin pathway in Parkinson's disease. Nature 1998;395:451–452.
10. Valente EM, Bentivoglio AR, Dixon PH, et al. Localization of a novel locus for autosomal recessive early-onset parkinsonism, PARK6, on human chromosome 1p35–p36. Am J Hum Genet 2001;68:895–900.
11. van Duijn CM, Dekker MC, Bonifati V, et al. Park7, a novel locus for autosomal recessive early-onset parkinsonism, on chromosome 1p36. Am J Hum Genet 2001;69:629–634.
12. Funayama M, Hasegawa K, Kowa H, Saito M, Tsuji S, Obata F. A new locus for Parkinson's disease (PARK8) maps to chromosome 12p11.2-q13.1. Ann Neurol 2002;51:296–301.
13. Hicks AA, Petursson H, Jonsson T, et al. A susceptibility gene for late-onset idiopathic Parkinson's disease. Ann Neurol 2002;52: 549–555.
14. Pankratz N, Nichols WC, Uniacke SK, et al. Significant linkage of Parkinson disease to chromosome 2q36–37. Am J Hum Genet 2003;72:1053–1057.
15. Singleton AB, Farrer M, Johnson J, et al. Alpha-synuclein locus triplication causes Parkinson's disease. Science 2003;302:841.
16. Hampshire DJ, Roberts E, Crow Y, et al. Kufor–Rakeb syndrome, pallido-pyramidal degeneration with supranuclear upgaze paresis and dementia, maps to 1p36. J Med Genet 2001;38:680–682.
17. Polymeropoulos MH, Lavedan C, Leroy E, et al. Mutation in the alpha-synuclein gene identified in families with Parkinson's disease. Science 1997;276:2045–2047.
18. Kitada T, Asakawa S, Hattori N, et al. Mutations in the parkin gene cause autosomal recessive juvenile parkinsonism. Nature 1998;392:605–608.
19. Valente EM, Abou-Sleiman PM, Caputo V, et al. Hereditary early-onset Parkinson's disease caused by mutations in PINK1. Science 2004;304:1158–1160.
20. Bonifati V, Rizzu P, van Baren MJ, et al. Mutations in the DJ-1 gene associated with autosomal recessive early-onset parkinsonism. Science 2003;299:256–259.
21. Paisan-Ruiz C, Jain S, Evans EW, et al. Cloning of the gene containing mutations that cause PARK8-linked Parkinson's disease. Neuron 2004;44:595–600.
22. Zimprich A, Biskup S, Leitner P, et al. Mutations in LRRK2 cause autosomal-dominant parkinsonism with pleomorphic pathology. Neuron 2004;44:601–607.
23. Yamamura Y, Sobue I, Ando K, Iida M, Yanagi T. Paralysis agitans of early onset with marked diurnal fluctuation of symptoms. Neurology 1973;23:239–244.
24. Abbas N, Lucking CB, Ricard S, et al. A wide variety of mutations in the parkin gene are responsible for autosomal recessive parkinsonism in Europe: French Parkinson's Disease Genetics Study Group and the European Consortium on Genetic Susceptibility in Parkinson's Disease. Hum Mol Genet 1999;8:567–574.
25. Klein C, Pramstaller PP, Kis B, et al. Parkin deletions in a family with adult-onset, tremor-dominant parkinsonism: expanding the phenotype. Ann Neurol 2000;48:65–71.
26. Lucking CB, Durr A, Bonifati V, et al. Association between early-onset Parkinson's disease and mutations in the parkin gene:

French Parkinson's Disease Genetics Study Group. N Engl J Med 2000;342:1560–1567.
27. Lohmann E, Periquet M, Bonifati V, et al. How much phenotypic variation can be attributed to parkin genotype? Ann Neurol 2003; 54:176–185.
28. van de Warrenburg BP, Lammens M, Lucking CB, et al. Clinical and pathologic abnormalities in a family with parkinsonism and parkin gene mutations. Neurology 2001;56:555–557.
29. Morales B, Martinez A, Gonzalo I, et al. Steele–Richardson–Olszewski syndrome in a patient with a single C212Y mutation in the parkin protein. Mov Disord 2002;17:1374–1380.
30. Khan NL, Graham E, Critchley P, et al. Parkin disease: a phenotypic study of a large case series. Brain 2003;126:1279–1292.
31. Periquet M, Latouche M, Lohmann E, et al. Parkin mutations are frequent in patients with isolated early-onset parkinsonism. Brain 2003;126:1271–1278.
32. Mori H, Kondo T, Yokochi M, et al. Pathologic and biochemical studies of juvenile parkinsonism linked to chromosome 6q. Neurology 1998;51:890–892.
33. Takahashi H, Ohama E, Suzuki S, et al. Familial juvenile parkinsonism: clinical and pathologic study in a family. Neurology 1994;44:437–441.
34. Hayashi S, Wakabayashi K, Ishikawa A, et al. An autopsy case of autosomal-recessive juvenile parkinsonism with a homozygous exon 4 deletion in the parkin gene. Mov Disord 2000;15:884–888.
35. Gouider-Khouja N, Larnaout A, Amouri R, et al. Autosomal recessive parkinsonism linked to parkin gene in a Tunisian family: clinical, genetic and pathological study. Parkinsonism Relat Disord 2003;9:247–251.
36. Sasaki S, Shirata A, Yamane K, Iwata M. Parkin-positive autosomal recessive juvenile Parkinsonism with alpha-synuclein–positive inclusions. Neurology 2004;63:678–682.
37. Mori H, Hattori N, Mizuno Y. Genotype–phenotype correlation: familial Parkinson disease. Neuropathology 2003;23:90–94.
38. Farrer M, Chan P, Chen R, et al. Lewy bodies and parkinsonism in families with parkin mutations. Ann Neurol 2001;50:293–300.
39. Pramstaller PP, Schlossmacher MG, Jacques TS, et al. Lewy body Parkinson's disease in a large pedigree with 77 Parkin mutation carriers. Ann Neurol 2005;58:411–422.
40. Hattori N, Kitada T, Matsumine H, et al. Molecular genetic analysis of a novel Parkin gene in Japanese families with autosomal recessive juvenile parkinsonism: evidence for variable homozygous deletions in the Parkin gene in affected individuals. Ann Neurol 1998;44:935–941.
41. Hedrich K, Eskelson C, Wilmot B, et al. Distribution, type, and origin of Parkin mutations: review and case studies. Mov Disord 2004;19:1146–1157.
42. Hedrich K, Marder K, Harris J, et al. Evaluation of 50 probands with early-onset Parkinson's disease for Parkin mutations. Neurology 2002;58:1239–1246.
43. Kann M, Jacobs H, Mohrmann K, et al. Role of parkin mutations in 111 community-based patients with early-onset parkinsonism. Ann Neurol 2002;51:621–625.
44. Foroud T, Uniacke SK, Liu L, et al. Heterozygosity for a mutation in the parkin gene leads to later onset Parkinson disease. Neurology 2003;60:796–801.
45. Pankratz N, Nichols WC, Uniacke SK, et al. Genome-wide linkage analysis and evidence of gene-by-gene interactions in a sample of 362 multiplex Parkinson disease families. Hum Mol Genet 2003;12:2599–2608.
46. Lincoln SJ, Maraganore DM, Lesnick TG, et al. Parkin variants in North American Parkinson's disease: cases and controls. Mov Disord 2003;18:1306–1311.
47. Shimura H, Hattori N, Kubo S, et al. Immunohistochemical and subcellular localization of Parkin protein: absence of protein in autosomal recessive juvenile parkinsonism patients. Ann Neurol 1999;45:668–672.
48. Kubo S, Kitami T, Noda S, et al. Parkin is associated with cellular vesicles. J Neurochem 2001;78:42–54.
49. Shimura H, Hattori N, Kubo S, et al. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein ligase. Nat Genet 2000;25:302–305.
50. Zhang Y, Gao J, Chung KK, Huang H, Dawson VL, Dawson TM. Parkin functions as an E2-dependent ubiquitin-protein ligase and promotes the degradation of the synaptic vesicle-associated protein, CDCrel-1. Proc Natl Acad Sci U S A 2000;97:13354–13359.
51. Imai Y, Soda M, Inoue H, Hattori N, Mizuno Y, Takahashi R. An unfolded putative transmembrane polypeptide, which can lead to endoplasmic reticulum stress, is a substrate of Parkin. Cell 2001; 105:891–902.
52. Sakata E, Yamaguchi Y, Kurimoto E, et al. Parkin binds the Rpn10 subunit of 26S proteasomes through its ubiquitin-like domain. EMBO Rep 2003;4:301–306.
53. Staropoli JF, McDermott C, Martinat C, Schulman B, Demireva E, Abeliovich A. Parkin is a component of an SCF-like ubiquitin ligase complex and protects postmitotic neurons from kainate excitotoxicity. Neuron 2003;37:735–749.
54. Imai Y, Soda M, Hatakeyama S, et al. CHIP is associated with Parkin, a gene responsible for familial Parkinson's disease, and enhances its ubiquitin ligase activity. Mol Cell 2002;10:55–67.
55. Shimura H, Schlossmacher MG, Hattori N, et al. Ubiquitination of a new form of alpha-synuclein by parkin from human brain: implications for Parkinson's disease. Science 2001;293:263–269.
56. Chung KK, Zhang Y, Lim KL, et al. Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. Nat Med 2001;7: 1144–1150.
57. Huynh DP, Scoles DR, Nguyen D, Pulst SM. The autosomal recessive juvenile Parkinson disease gene product, parkin, interacts with and ubiquitinates synaptotagmin XI. Hum Mol Genet 2003;12:2587–2597.
58. Choi P, Snyder H, Petrucelli L, et al. SEPT5_v2 is a parkin-binding protein. Brain Res Mol Brain Res 2003;117:179–189.
59. Corti O, Hampe C, Koutnikova H, et al. The p38 subunit of the aminoacyl-tRNA synthetase complex is a Parkin substrate: linking protein biosynthesis and neurodegeneration. Hum Mol Genet 2003;12:1427–1437.
60. Ren Y, Zhao J, Feng J. Parkin binds to alpha/beta tubulin and increases their ubiquitination and degradation. J Neurosci 2003; 23:3316–3324.
61. Hattori N, Mizuno Y. Pathogenetic mechanisms of parkin in Parkinson's disease. Lancet 2004;364:722–724.
62. Mata IF, Lockhart PJ, Farrer MJ. Parkin genetics: one model for Parkinson's disease. Hum Mol Genet 2004;13:127–133.
63. Doss-Pepe EW, Chen L, Madura K. Alpha-synuclein and parkin contribute to the assembly of ubiquitin lysine63-linked multiubiquitin chains. J Biol Chem 2005;280:16619–16624.
64. Lim KL, Chew KC, Tan JM, et al. Parkin mediates nonclassical, proteasomal-independent ubiquitination of synphilin-1: implications for Lewy body formation. J Neurosci 2005;25:2002–2009.
65. Hershko A, Ciechanover A. The ubiquitin system for protein degradation. Annu Rev Biochem 1992;61:761–807.
66. Haglund K, Sigismund S, Polo S, Szymkiewicz I, Di Fiore PP, Dikic I. Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation. Nat Cell Biol 2003;5:461–466.
67. Nakatsu F, Sakuma M, Matsuo Y, et al. A di-leucine signal in the ubiquitin moiety: possible involvement in ubiquitination-mediated endocytosis. J Biol Chem 2000;275:26213–26219.
68. Roth AF, Davis NG. Ubiquitination of the PEST-like endocytosis signal of the yeast a-factor receptor. J Biol Chem 2000;275:8143–8153.
69. Shih SC, Sloper-Mould KE, Hicke L. Monoubiquitin carries a novel internalization signal that is appended to activated receptors. EMBO J 2000;19:187–198.

70. Hicke L. Ubiquitin-dependent internalization and down-regulation of plasma membrane proteins. FASEB J 1997;11:1215–1226.
71. Hicke L. Protein regulation by monoubiquitin. Nat Rev Mol Cell Biol 2001;2:195–201.
72. Katzmann DJ, Odorizzi G, Emr SD. Receptor downregulation and multivesicular-body sorting. Nat Rev Mol Cell Biol 2002;3: 893–905.
73. Itier JM, Ibanez P, Mena MA, et al. Parkin gene inactivation alters behaviour and dopamine neurotransmission in the mouse. Hum Mol Genet 2003;12:2277–2291.
74. Goldberg MS, Fleming SM, Palacino JJ, et al. Parkin-deficient mice exhibit nigrostriatal deficits but not loss of dopaminergic neurons. J Biol Chem 2003;278:43628–43635.
75. Von Coelln R, Thomas B, Savitt JM, et al. Loss of locus coeruleus neurons and reduced startle in parkin null mice. Proc Natl Acad Sci U S A 2004;101:10744–10749.
76. Palacino JJ, Sagi D, Goldberg MS, et al. Mitochondrial dysfunction and oxidative damage in parkin-deficient mice. J Biol Chem 2004;279:18614–18622.
77. Greene JC, Whitworth AJ, Kuo I, Andrews LA, Feany MB, Pallanck LJ. Mitochondrial pathology and apoptotic muscle degeneration in Drosophila parkin mutants. Proc Natl Acad Sci U S A 2003;100:4078–4083.
78. Pesah Y, Pham T, Burgess H, et al. Drosophila parkin mutants have decreased mass and cell size and increased sensitivity to oxygen radical stress. Development 2004;131:2183–2194.
79. Darios F, Corti O, Lucking CB, et al. Parkin prevents mitochondrial swelling and cytochrome c release in mitochondria-dependent cell death. Hum Mol Genet 2003;12:517–526.
80. Jiang H, Ren Y, Zhao J, Feng J. Parkin protects human dopaminergic neuroblastoma cells against dopamine-induced apoptosis. Hum Mol Genet 2004;13:1745–1754.
81. Petrucelli L, O'Farrell C, Lockhart PJ, et al. Parkin protects against the toxicity associated with mutant alpha-synuclein: proteasome dysfunction selectively affects catecholaminergic neurons. Neuron 2002;36:1007–1019.
82. Machida Y, Chiba T, Takayanagi A, et al. Common anti-apoptotic roles of parkin and alpha-synuclein in human dopaminergic cells. Biochem Biophys Res Commun 2005;332:233–240.
83. Feany MB, Pallanck LJ. Parkin: a multipurpose neuroprotective agent? Neuron 2003;38:13–16
84. Chung KK, Thomas B, Li X, et al. S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. Science 2004;304:1328–1331.
85. Yao D, Gu Z, Nakamura T, et al. Nitrosative stress linked to sporadic Parkinson's disease: S-nitrosylation of parkin regulates its E3 ubiquitin ligase activity. Proc Natl Acad Sci U S A 2004;101:10810–10814.
86. Lipton SA, Nakamura T, Yao D, Shi ZQ, Uehara T, Gu Z. Comment on "S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function." Science 2005; 308:1870.
87. Bentivoglio AR, Cortelli P, Valente EM, et al. Phenotypic characterisation of autosomal recessive PARK6-linked parkinsonism in three unrelated Italian families. Mov Disord 2001;16:999–1006.
88. Hatano Y, Sato K, Elibol B, et al. PARK6-linked autosomal recessive early-onset parkinsonism in Asian populations. Neurology 2004;63:1482–1485.
89. Hatano Y, Li Y, Sato K, et al. Novel PINK1 mutations in early-onset parkinsonism. Ann Neurol 2004;56:424–427.
90. Healy DG, Abou-Sleiman PM, Gibson JM, et al. PINK1 (PARK6) associated Parkinson disease in Ireland. Neurology 2004;63:1486–1488.
91. Rogaeva E, Johnson J, Lang AE, et al. Analysis of the PINK1 gene in a large cohort of cases with Parkinson disease. Arch Neurol 2004;61:1898–1904.
92. Rohe CF, Montagna P, Breedveld G, Cortelli P, Oostra BA, Bonifati V. Homozygous PINK1 C-terminus mutation causing early-onset parkinsonism. Ann Neurol 2004;56:427–431.
93. Valente EM, Salvi S, Ialongo T, et al. PINK1 mutations are associated with sporadic early-onset parkinsonism. Ann Neurol 2004;56:336–341.
94. Bonifati V, Rohe CF, Breedveld GJ, et al. Early-onset parkinsonism associated with PINK1 mutations: frequency, genotypes, and phenotypes. Neurology 2005;65:87–95.
95. Klein C, Djarmati A, Hedrich K, et al. PINK1, Parkin, and DJ-1 mutations in Italian patients with early-onset parkinsonism. Eur J Hum Genet 2005;13:1086–1093.
96. Unoki M, Nakamura Y. Growth-suppressive effects of BPOZ and EGR2, two genes involved in the PTEN signaling pathway. Oncogene 2001;20:4457–4465.
97. Nakajima A, Kataoka K, Hong M, Sakaguchi M, Huh NH. BRPK, a novel protein kinase showing increased expression in mouse cancer cell lines with higher metastatic potential. Cancer Lett 2003;201:195–201.
98. Beilina A, Van Der Brug M, Ahmad R, et al. Mutations in PTEN-induced putative kinase 1 associated with recessive parkinsonism have differential effects on protein stability. Proc Natl Acad Sci U S A 2005;102:5703–5708.
99. Bonifati V, Breedveld GJ, Squitieri F, et al. Localization of autosomal recessive early-onset parkinsonism to chromosome 1p36 (PARK7) in an independent dataset. Ann Neurol 2002;51: 253–256.
100. Hague S, Rogaeva E, Hernandez D, et al. Early-onset Parkinson's disease caused by a compound heterozygous DJ-1 mutation. Ann Neurol 2003;54:271–274.
101. Abou-Sleiman PM, Healy DG, Quinn N, Lees AJ, Wood NW. The role of pathogenic DJ-1 mutations in Parkinson's disease. Ann Neurol 2003;54:283–286.
102. Hedrich K, Djarmati A, Schafer N, et al. DJ-1 (PARK7) mutations are less frequent than Parkin (PARK2) mutations in early-onset Parkinson disease. Neurology 2004;62:389–394.
103. Hering R, Strauss KM, Tao X, et al. Novel homozygous p.E64D mutation in DJ1 in early onset Parkinson disease (PARK7). Hum Mutat 2004;24:321–329.
104. Clark LN, Afridi S, Mejia-Santana H, et al. Analysis of an early-onset Parkinson's disease cohort for DJ-1 mutations. Mov Disord 2004;19:796–800.
105. Djarmati A, Hedrich K, Svetel M, et al. Detection of Parkin (PARK2) and DJ1 (PARK7) mutations in early-onset Parkinson disease: Parkin mutation frequency depends on ethnic origin of patients. Hum Mutat 2004;23:525.
106. Bandopadhyay R, Kingsbury AE, Cookson MR, et al. The expression of DJ-1 (PARK7) in normal human CNS and idiopathic Parkinson's disease. Brain 2004;127:420–430.
107. Olzmann JA, Brown K, Wilkinson KD, et al. Familial Parkinson's disease-associated L166P mutation disrupts DJ-1 protein folding and function. J Biol Chem 2004;279:8506–8515.
108. Neumann M, Muller V, Gorner K, Kretzschmar HA, Haass C, Kahle PJ. Pathological properties of the Parkinson's disease–associated protein DJ-1 in alpha-synucleinopathies and tauopathies: relevance for multiple system atrophy and Pick's disease. Acta Neuropathol (Berl) 2004;107:489–496.
109. Rizzu P, Hinkle DA, Zhukareva V, et al. DJ-1 colocalizes with tau inclusions: a link between parkinsonism and dementia. Ann Neurol 2004;55:113–118.
110. Moore DJ, Zhang L, Troncoso J, et al. Association of DJ-1 and parkin mediated by pathogenic DJ-1 mutations and oxidative stress. Hum Mol Genet 2005;14:71–84.
111. Lee SJ, Kim SJ, Kim IK, et al. Crystal structures of human DJ-1 and *Escherichia coli* Hsp31, which share an evolutionarily conserved domain. J Biol Chem 2003;278:44552–44559.
112. Kinumi T, Kimata J, Taira T, Ariga H, Niki E. Cysteine-106 of DJ-1 is the most sensitive cysteine residue to hydrogen peroxide-

mediated oxidation in vivo in human umbilical vein endothelial cells. Biochem Biophys Res Commun 2004;317:722–728.
113. Taira T, Saito Y, Niki T, Iguchi-Ariga SM, Takahashi K, Ariga H. DJ-1 has a role in antioxidative stress to prevent cell death. EMBO Rep 2004;5:213–218.
114. Mitsumoto A, Nakagawa Y. DJ-1 is an indicator for endogenous reactive oxygen species elicited by endotoxin. Free Radic Res 2001;35:885–893.
115. Mitsumoto A, Nakagawa Y, Takeuchi A, Okawa K, Iwamatsu A, Takanezawa Y. Oxidized forms of peroxiredoxins and DJ-1 on two-dimensional gels increased in response to sublethal levels of paraquat. Free Radic Res 2001;35:301–310.
116. Tao X, Tong L. Crystal structure of human DJ-1, a protein associated with early onset Parkinson's disease. J Biol Chem 2003;278:31372–31379.
117. Honbou K, Suzuki NN, Horiuchi M, et al. The crystal structure of DJ-1, a protein related to male fertility and Parkinson's disease. J Biol Chem 2003;278:31380–31384.
118. Wilson MA, Collins JL, Hod Y, Ringe D, Petsko GA. The 1.1-A resolution crystal structure of DJ-1, the protein mutated in autosomal recessive early onset Parkinson's disease. Proc Natl Acad Sci U S A 2003;100:9256–9261.
119. Huai Q, Sun Y, Wang H, et al. Crystal structure of DJ-1/RS and implication on familial Parkinson's disease. FEBS Lett 2003;549: 171–175.
120. Yokota T, Sugawara K, Ito K, Takahashi R, Ariga H, Mizusawa H. Down regulation of DJ-1 enhances cell death by oxidative stress, ER stress, and proteasome inhibition. Biochem Biophys Res Commun 2003;312:1342–1348.
121. Canet-Aviles RM, Wilson MA, Miller DW, et al. The Parkinson's disease protein DJ-1 is neuroprotective due to cysteine-sulfinic acid-driven mitochondrial localization. Proc Natl Acad Sci U S A 2004;101:9103–9108.
122. Goldberg MS, Pisani A, Haburcak M, et al. Nigrostriatal dopaminergic deficits and hypokinesia caused by inactivation of the familial Parkinsonism-linked gene DJ-1. Neuron 2005;45:489–496.
123. Chen L, Cagniard B, Mathews T, et al. Age-dependent motor deficits and dopaminergic dysfunction in DJ-1 null mice. J Biol Chem 2005;280:21418–21426.
124. Kim RH, Smith PD, Aleyasin H, et al. Hypersensitivity of DJ-1–deficient mice to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) and oxidative stress. Proc Natl Acad Sci U S A 2005;102:5215–5220.
125. McNaught KS, Belizaire R, Jenner P, Olanow CW, Isacson O. Selective loss of 20S proteasome alpha-subunits in the substantia nigra pars compacta in Parkinson's disease. Neurosci Lett 2002; 326:155–158.
126. McNaught KS, Belizaire R, Isacson O, Jenner P, Olanow CW. Altered proteasomal function in sporadic Parkinson's disease. Exp Neurol 2003;179:38–46.

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Nuclear localization of the 20S proteasome subunit in Parkinson's disease

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Received 10 March 2006; received in revised form 10 May 2006; accepted 3 July 2006

Abstract

Considering the involvement of ubiquitin–proteasome system (UPS) in Parkinson's disease (PD), the aim of the present study was to determine the distribution of proteasomes in PD brains. Immunohistochemical studies showed localization of 20S proteasome in the nuclei of neurons of the putamen and substantia nigra of PD. In contrast, no nuclear staining was observed in the same areas of brains of controls. Our results suggest that nuclear localization of 20S proteasome seems to be associated with the pathogenesis of PD.

Keywords: Parkinson's disease; 20S proteasome; Immunohistochemistry

Parkinson's disease (PD) is a neurodegenerative disorder characterized clinically by tremor, rigidity, bradykinesia and postural instability. Pathologically, PD is characterized by selective loss of neurons in the substantia nigra (SN) pars compacta, and the presence of eosinophilic inclusions, known as Lewy bodies (LBs) [12]. How and why LBs are formed in PD patients is not yet clear. However, LB components have been well investigated.

Our understanding of the PD disease process has expanded tremendously over the past few years through the discovery of gene products for monogenic familial PD although most of the patients with PD are idiopathic. Especially, the discovery of α-synuclein provided excellent information on the mechanisms of LB formation [12]. In addition, parkin and UCH-L1 are directly linked to the ubiquitin–proteasome pathway (UPP) as a component of this system [6]. Thus, the UPP seems to play an important role in the pathogenesis of PD.

With regard to the mechanisms of LB formation, identification of several components of the inclusion bodies provides valuable information. In this context, ubiquitin itself is a key protein that has been considered as a suitable marker for LBs [22]. Apart from ubiquitin, gene products, such as α-synuclein and UCH-L1 have been reported to be components of LB [7,21]. Furthermore, additional component, such as symphillin-1 has been identified also [24]. Therefore, there is growing evidence that UPP could be involved not only in the pathogenesis of the disease but also LB formation. Indeed, previous studies demonstrated that proteasome inhibitors increase the frequency of ubiquitin-positive intracellular inclusions like LBs [23].

The 20S proteasome, a multicatalytic proteinase, combines with 19S regulator complex to form the 26S proteasome, which is responsible for ubiquitin–ATP-proteolysis [14,20]. Numerous studies have recently emphasized the biological importance of the UPP, which is capable of catalyzing rapidly, timely, and unidirectionally a multitude of biological reactions including cell-cycle progression, DNA repair, cell death, signal transduction, transcription, metabolism, and immunity [5,16,25]. Several studies of proteasome activities in the SN of PD have been reported so far, though it is controversial whether the activities of the 20S proteasome decrease in PD. Proteasomes are present in the nucleus and in the cytoplasm of various types of cells [18]. In fact, immunoelectron microscopic studies of the rat central nervous system revealed that proteasomes are present not only in the cytoplasm but also in the nucleus in neurons, dendritic and axonic processes [11].

The aim of the present study was to characterize the distribution of 20S proteasome in PD patients. For this purpose, we analyzed immunohistochemically the postmortem brains of patients with idiopathic PD using antibody of proteasome subunit.

We performed immunohistochemical studies using samples from the putamen obtained from four patients with PD and four control subjects and from the SN obtained from three patients with PD and two control subjects. The clinical profiles of the patients are summarized in Table 1. We obtained informed consents from the families of all patients and control subjects.

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doi:10.1016/j.neulet.2006.07.050

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Neuroscience Letters 406 (2006) 43–48
Neuroscience Letters
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Nuclear localization of the 20S proteasome subunit in Parkinson's disease

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Received 10 March 2006; received in revised form 10 May 2006; accepted 3 July 2006

Table 1
Clinical summary of participating subjects

Case	Diagnosis	Age (years)	Sex	Duration (years)	Postmortem delay (h)	Cause of death	Sample
1	PD	51	M	11	3.5	Pneumonia	P1, N1
2	PD	77	M	7	6.5	Pneumonia	P2, N2
3	PD	65	F	4.5	2	Asthma	P3
4	PD	85	F	6	2	Pneumonia	P4
5	PD	72	F	5	<24	Lung hemorrhage	N3
6	PD	69	M	22	9	Malignant syndrome	P (Fr)
7	Control	62	M	–	11	AML	P1, N1
8	Control	66	F	–	1	Ovarian cancer	P2
9	Control	38	M	–	10	Malignant lymphoma	P3
10	Control	41	M	–	2.5	CRF	P4
11	Control	65	F	–	1.5	Pachymeningitis	N2
12	Control	99	M	–	<24	Pneumonia	Pons
13	Control	47	F	–	2	Gastric cancer	P (Fr)

AML: acute myelocytic leukemia, CRF: chronic renal failure, F: female, Fr: fresh frozen brain, M: male, N: substantia nigra, P: putamen, PD: Parkinson's disease.

The pathological diagnosis was confirmed in all patients and age-matched control subjects in the Department of Neurology, Juntendo University School of Medicine. The study protocol was approved by the Human Ethics Review Committee of Juntendo University School of Medicine.

A mouse monoclonal antibody to 20S proteasome subunit α6 (PW8100) was purchased from Affinity Research Products (Mamhead Castle, UK). The specificity of this antibody has been confirmed previously [4]. Schmidt et al [19] examined immunohistochemistry using this antibody to pontine samples obtained from control subjects and SCA3 patients. They showed strong staining in the majority of nuclei of pontine neurons in control subjects. We obtained the same results using antibody to α6 (PW8100) subunit of the 20S proteasome. We also used another mouse monoclonal antibody to the 20S proteasome subunit α6 for SN samples, which was kindly provided by Professor Keiji Tanaka, Department of Molecular Oncology, The Tokyo Metropolitan Institute of Medical Science. This antibody was used for Western blot using putamen sample of Case No. 6. The putamen sample was placed into ice-cold homogenization buffer (0.32 M sucrose and 4 mM 4-(2-hydeoxyethl)-1-piperazineethanesulgonic acid (HEPES)–NaOH, pH 7.4) and homogenized using a Potter–Elvehjem homogenizer in the presence of a mixture of protease inhibitors (Complete Mini EDTA-free, Roche Diagnostics, Penzberg, Germany). After centrifugation at 600 × *g*, the supernatant was used for analysis. Sample was separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Transferred protein was probed with the anti α6 antibody and visualized using a chemiluminescence reagent.

Immunohistochemical experiments were conducted as described previously [2]. Formalin-fixed paraffin-embedded sections (6 μm thick) were deparaffinized with xylene and hydrated in ethanol. The deparaffinized sections were microwaved in phosphate buffer (Antigen Retrival Citra, BioGenex, San Roman, CA) at high power for 3 min and low power for 5 min for antigen retrieval. Endogenous peroxidases were quenched by incubation with 3% hydrogen peroxide for 5 min. After blocking the slides with 10% normal goat serum in phosphate buffered saline (PBS) for 20 min at room temperature, the sections were treated overnight at 4 °C with primary antibody. After the treatment, the sections were incubated with biotinylated anti-mouse IgG from goat (Dako Corporation, Carpinteria, CA; diluted 1:200) as the second antibody. After incubation with streptavidin conjugated to horseradish peroxidase (HRP) (Dako; 1:200), we treated the sections with biotinyl tyramide and hydrogen peroxide. Sections were then incubated with streptavidin conjugated to HRP and visualized by 3′,3′-diaminobenidine (DAB).

Double immunofluorescence was performed using putamen sections of 2 PD brain (Case No. 1, 2) with anti-α6 subunit of proteasome (PW8100) and anti-Oct-1 (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. The anti-Oct-1 antibody was used as a marker for cell nuclei. These sections were treated with Alexa fluor goat anti-rabbit IgG (Molecular Probe, Eugene, OR; diluted 1:300) and Alexa fluor goat anti-mouse IgG (Molecular Probes, Eugene, OR; diluted 1:300). The signal was examined under Zeiss LSM 510 laser scanning confocal microscope (Zeiss, Oberkochen, Germany).

For semiquantitative analysis, microscopic photographs of the whole SN and lesions of putamen nuclei were prepared. Two independent observers blinded to the clinical information counted the number of anti-α6-positive neurons in the SN and putamen. Differences between groups were examined for statistical significance using one-way ANOVA. A P value less than 0.05 was considered significant.

Fresh frozen putamen-containing brain sections of PD (Case No. 6) and control subject (Case No. 13) were obtained for Western blot analysis. The samples were placed into ice-cold homogenization buffer (0.32 M sucrose and 4 mM HEPES–NaOH, pH 7.4) and homogenized using a Potter–Elvehjem homogenizer in the presence of a mixture of protease inhibitors (Complete Mini EDTA-free, Roche Diagnostics). After centrifugation at 600 × *g*, the pellet was collected and used for analysis.

Samples were separated by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) microporous membrane (Bio-Rad, Hercules, CA) using a transfer buffer (40 mM CAPS, 30 mM TRIS, and 15% methanol). The transferred membrane was blocked with 5% skim milk and incubated overnight with primary

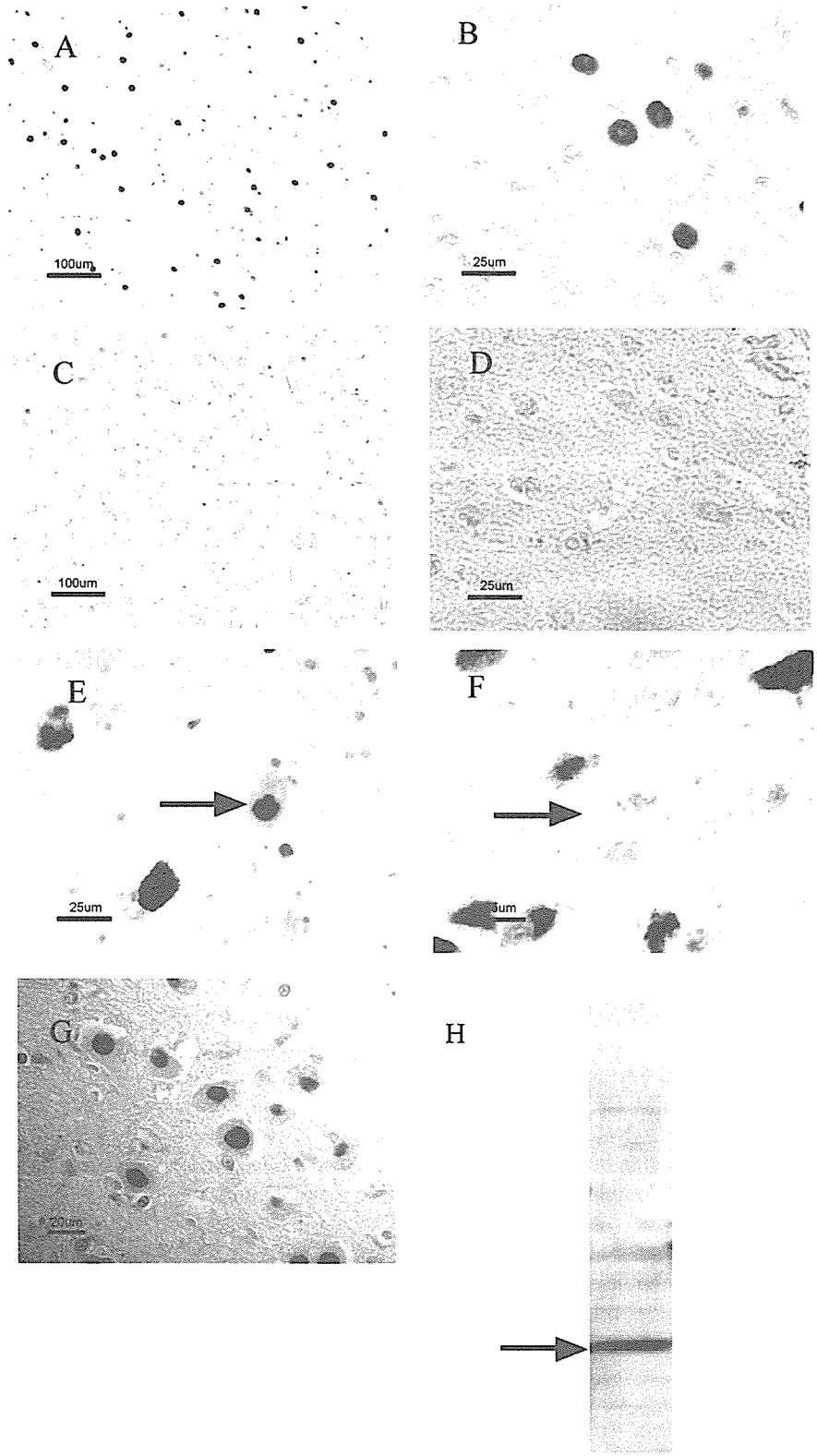

Fig. 1. Immunohistochemical staining of the striatum in a Parkinson's disease (PD) brain (A, B) and control brain (C, D) using antibodies to the α6 subunit of the 20S proteasome (PW8100) (A–D). Note the prominent nuclear staining for 20S proteasome in PD and the very weak or negative staining in the control. In addition, nuclear staining of 20S proteasome was observed in substantia nigra of PD (E) and control (F) in sections stained with antibody to the α6 subunit of the 20S proteasome (provided kindly by Professor Keiji Tanaka). Arrow indicates the body of a nigral neuron. In contrast, no staining for this subunit was observed in the control (F). Arrow indicates the body of a nigral neuron. The dark-brownish color products represent neuromelanines. (G) Staining using antibody to the α6 subunit of the 20S proteasome (PW8100) in pontine section of a control subject. (H) A single band of about 30 kDa, which is equivalent to the expected molecular weight of α6 subunit (from Professor Tanaka), was detected in PD.

antibodies at 4 °C. The primary antibodies were α6 proteasome subunit (PW8100) and β-tubulin as an internal marker (Santa Cruz Biotechnology). After incubation with HRP-conjugated secondary antibodies, the reaction was visualized using a chemiluminescence reagent.

In putamen sections of all PD brains, most nuclei of neurons showed strong staining by α6 subunit of the 20S proteasome antibody (Fig. 1A and B). In contrast, proteasome immunoreactivity was rarely observed in the nuclei of putamen neurons of the control brains (Fig. 1C and D).

Similar results were obtained when using the α6 subunit of the 20S proteasome antibody in the SN in PD and controls (Fig. 1E and F). Most nuclei of neurons showed strong staining in all three PD samples of SN. No immunoreactivity was noted in other regions of SN, locus coeruleus, dorsal vagal nucleus, or nucleus basalis of Mynert. Similar results (see Schmidt et al. [19]) were obtained when using the α6 subunit of the 20S proteasome antibody (PW8100) in pontine sections of control subjects (Fig. 1G). Anti-α6 subunit of the 20S proteasome antibody demonstrated a single band of about 30 kDa corresponding to the α6 subunit of 20S proteasome in the PD brain (Fig. 1H).

To confirm the localization of α6 subunit of the 20S proteasome in the nuclei of neurons, we performed double immunofluorescence staining with anti-α6 subunit of the 20S proteasome and Oct-1 antibodies using putamen of two PD patients. The results confirmed the localization of α6 subunit in the nuclei in PD brain (Fig. 2A–C). Semiquantitative analysis showed that the proportions of α6-positive neurons to all neurons of the SN and putamen were higher in PD that in control subjects. Repeated paired analyses with Boniferroni's correction showed significant differences between PD and control subjects ($P < 0.05$, Fig. 3A and B). Furthermore, Western blot analysis using insoluble fractions of the brain demonstrated a higher protein level of α6 subunit in PD patient compared with the control (Fig. 3C).

In the present study, we demonstrated the distribution of proteasomal subunits in idiopathic PD by immunohistochemistry. In PD brains, most nuclei of putamen neurons showed strong staining when we used antibodies against the subunits of 20S proteasome. A similar immunohistochemical distribution was observed in the SN, although only few neurons were noted in this area of PD patients. We also performed immunohistochemical studies using other subunits of 20S proteasome, such as α2, 3, 4, 5, 7, β1, 3, 5 in four patients with PD (age: 69 ± 8.6 years, disease duration: 11.03 ± 4.9 years, postmortem delay 6.5 ± 5.5 h, mean ± S.D.) and four control subjects (age: 62 ± 14.8 years, postmortem delay 4.0 ± 3.0 h, mean ± S.D.). Immunostaining for all the subunits was also observed in the nuclei, similar to the pattern seen for the α6 subunit (data not shown). In contrast,

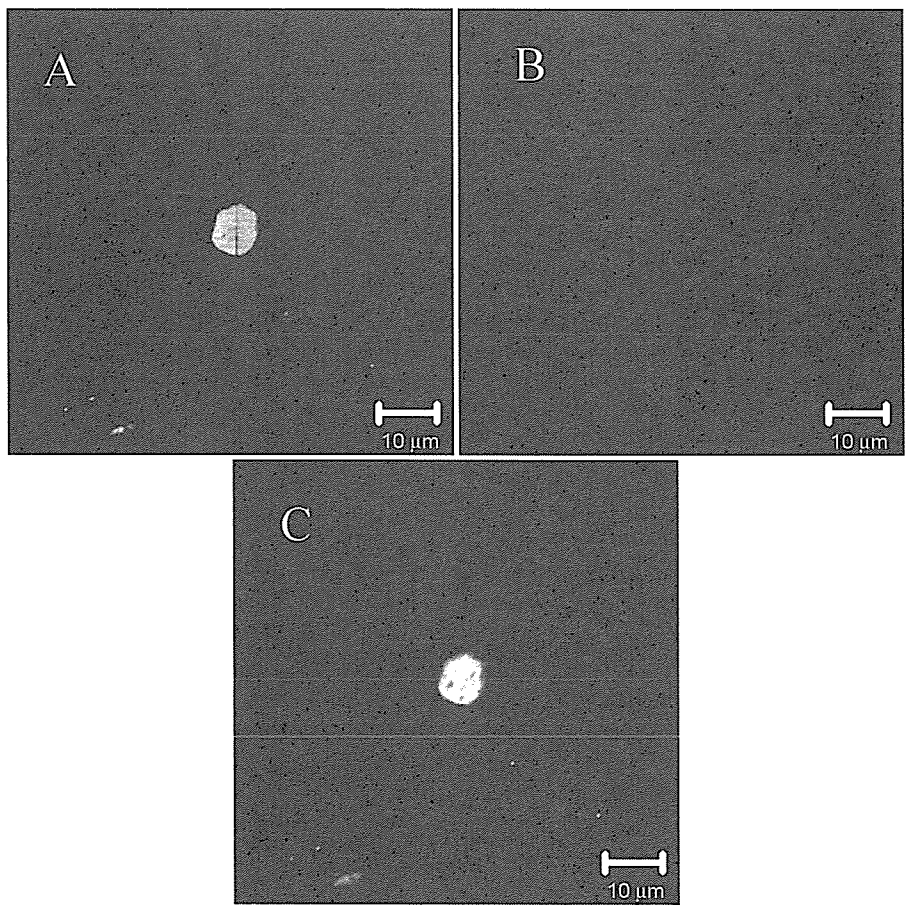

Fig. 2. Localization of α6 subunit in the nuclei of PD neurons. Double immunofluorescence staining of putamen neurons of PD with antibody for α6 subunit of the 20S proteasome, PW8100 (green in A) and Oct-1 (red in B) together with the merged image (C). Note the colocalization of α6 subunit of the 20S proteasome (green in A) and Oct-1 (red in B) in PD neurons.

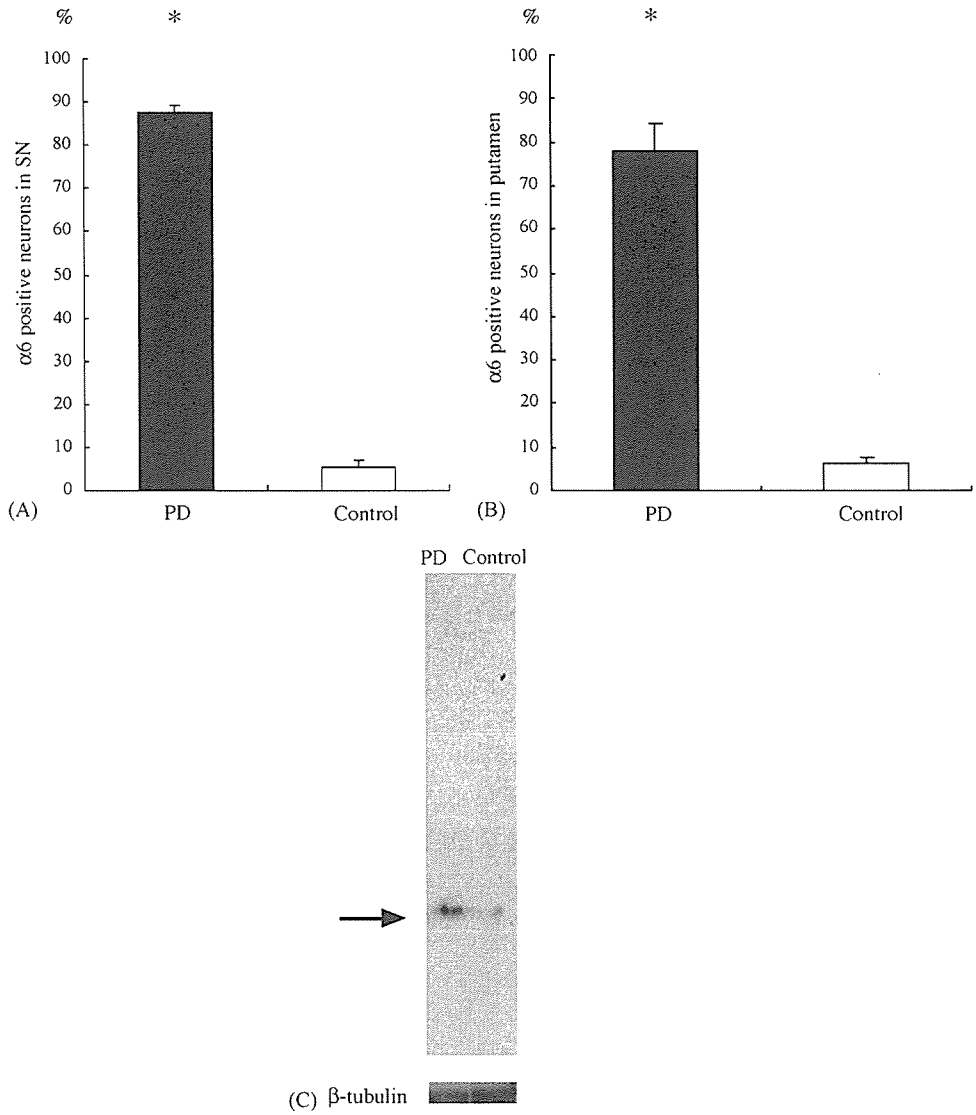

Fig. 3. Semiquantitative analysis of α6 proteasome and protein. The percentages of α6 proteasome-positive neurons in SN (A) and putamen (B) were significantly higher in SN and putamen of PD than the control (data are mean ± S.E.M., $P < 0.05$, one way ANOVA and Bonferroni's correction). (C) Immunoblotting of α6 proteasome (PW8100) in soluble fractions of putamen of PD patient and control subject. Note the higher α6 proteasome protein level in PD than control. β-tubulin is used as internal marker.

immunostaining of the frontal cortices of three patients with PD (age: 69 ± 15 years, disease duration: 10 ± 3.9 years, post-mortem delay 3.0 ± 0.5 h, mean ± S.D.) using the same antibody (PW8100), was negative for the α6 subunit (data not shown). These findings suggest that the specific nuclear localization of α6 in nigrostriatal pathway may play a role in the pathogenesis of PD or the disease process.

Several studies demonstrated altered distribution of proteasome subunits during different phases of the cell cycle [1,15], in stress conditions (e.g., glucose starvation and hypoxia) [13], and apoptosis [8,17]. Immunoelectron studies of cells arrested in mitosis showed the presence of proteasomes around the chromosomes [15], while others showed the association of proteasome with the spindle poles in metaphase [1]. Changes in proteasome distribution have also been observed in cells during apoptosis. For example, proteasomes have been detected in the apoptotic blebs in ovarian granulose cells [17] and lung cancer cell line [8]. Translocation of the proteasomes into the nucleus has also been reported [13]. Ogiso et al. [13] showed increased proteasomal distribution in the nuclei of glucose-starved cells and hypoxic cancer cells. Based on the above studies, our results could reflect certain stress conditions in PD that result in altered distribution of proteasome in the neuronal cell nuclei.

In this regard, McNaught et al. [9] used immunohistochemistry and Western blotting to describe selective reduction of α-subunits in dopaminergic neurons. Their finding is in sharp contrast to the result of the present study in SN. The reason for the different results in the two studies is not clear at present. However, it may be due to the different methods used in the analysis including the antibodies.

Regarding the activities of 20S proteasome, there are controversies about whether or not the activities are decreased in PD. McNaught et al. [10] reported moderately reduced peptidase activity of the proteasome in idiopathic PD. However, it is

possible that the observed reduced proteasomal activity is secondary to the severe neuronal loss in the SN. In another study, Furukawa et al. [3] found preserved proteasome peptidase activities in the striatum and cortices of PD patients, patients with multiple system atrophy, and those with progressive supranuclear palsy. Preservation of proteasome peptidase activities in the striatum and decrement in the SN suggest the presence of fragile mechanisms to systematic stresses in the SN. Indeed, the immunohistochemical findings of the proteasome subunits were similar in both the striatum and SN, suggesting vulnerability of proteasome activities in the SN of PD.

In conclusion, we have demonstrated immunohistochemically in the present study the distribution of the 20S proteasome in the nuclei of neurons in PD. Further studies are needed to understand the mechanism responsible for the localization of nuclear proteasome.

Acknowledgments

We thank the families of the patients, whose generosity made this research possible. We also thank Professor Keiji Tanaka, Department of Molecular Oncology, The Tokyo Metropolitan Institute of Medical Science, for providing antibody directed against proteasomal α6 subunit.

References

[1] A. Amsterdam, F. Pitzer, W. Baumeister, Changes in intracellular localization of proteasomes in immortalized ovarian granulosa cells during mitosis associated with a role in cycle control, Proc. Natl. Acad. Sci. USA 90 (1993) 99–103.

[2] J. Fukae, M. Takahashi, S. Kubo, K. Nishioka, Y. Nakabeppu, H. Mori, Y. Mizuno, N. Hattori, Expression of 8-oxoguanine DNA glycosylase (OGG1) in Parkinson's disease and related neurodegenerative disorders, Acta Neuropathol. 109 (2005) 256–262.

[3] Y. Furukawa, S. Vigouroux, H. Wong, M. Guttman, A.H. Rajput, L. Ang, M. Briand, S.J. Kish, Y. Briand, Brain proteasomal function in sporadic Parkinson's disease and related disorders, Ann. Neurol. 51 (2002) 779–782.

[4] K.B. Hendil, P. Kristensen, W. Uerkvitz, Human proteasomes analysed with monoclonal antibodies, Biochem. J. 305 (1995) 245–252.

[5] A. Hershko, A. Ciechanover, A. Varshavsky, The ubiquitin system, Nat. Med. 6 (2000) 1073–1081.

[6] Y. Huang, L. Cheung, D. Rowe, G. Halliday, Genetic contributions to Parkinson's disease, Brain Res. Rev. 46 (2004) 44–70.

[7] J. Lowe, H. McDermott, M. Landon, R.J. Mayer, K.D. Wilkinson, Ubiquitin carboxyl-terminal hydrolase (PGP 9.5) is selectively present in ubiquitinated inclusion bodies characteristic of human neurodegenerative diseases, J. Pathol. 161 (1990) 153–160.

[8] B.M. Machiels, M.E. Henfling, B. Schutte, M. van Engeland, J.L. Broers, F.C. Ramaekers, Subcellular localization of proteasomes in apoptotic lung tumor cells and persistence as compared to intermediate filaments, Eur. J. Cell Biol. 70 (1996) 250–259.

[9] K.S. McNaught, R. Belizaire, P. Jenner, C.W. Olanow, O. Isacson, Selective loss of 20S proteasome α subunits in the substantia nigra pars compacta in Parkinson's disease, Neurosci. Lett. 326 (2002) 155–158.

[10] K.S. McNaught, P. Jenner, Proteasomal function is impaired in substantia nigra in Parkinson's disease, Neurosci. Lett. 297 (2001) 191–194.

[11] E. Mengual, P. Arizti, J. Rodrigo, J.M. Giménez-Amaya, J.G. Castaño, Immunohistochemical distribution and electron microscopic subcellular localization of the proteasome in the rat CNS, J. Neurosci. 16 (1996) 6331–6341.

[12] R.L. Nussbaum, M.H. Polymeropoulos, Genetics of Parkinson's disease, Hum. Mol. Genet. 6 (1997) 1687–1691.

[13] Y. Ogiso, A. Tomida, H.D. Kim, T. Tsuruo, Glucose starvation and hypoxia induce nuclear accumulation of proteasome in cancer cells, Biochem. Biophys. Res. Commun. 258 (1999) 448–452.

[14] M. Orlowski, S. Wilk, Catalytic activities of the 20S proteasome, a multicatalytic proteinase complex, Arch. Biochem. Biophys. 383 (2000) 1–16.

[15] A. Palmer, G.G. Mason, J.M. Paramio, E. Knecht, A.J. Rivett, Changes in proteasome localization during the cell cycle, Eur. J. Cell Biol. 64 (1994) 163–175.

[16] C.M. Pickart, Mechanisms underlying ubiquitination, Annu. Rev. Biochem. 70 (2001) 503–533.

[17] F. Pitzer, A. Dantes, T. Fuchs, W. Baumeister, A. Amsterdam, Removal of proteasomes from the nucleus and their accumulation in apoptotic blebs during programmed cell death, FEBS Lett. 394 (1996) 47–50.

[18] A.J. Rivett, Intracellular distribution of proteasomes, Curr. Opin. Immunol. 10 (1998) 110–114.

[19] T. Schmidt, K. Lindenberg, A. Krebs, L. Schöls, F. Laccone, J. Herms, M. Rechsteiner, O. Riess, G.B. Landwehrmeyer, Protein surveillance machinery in brains with spinocerebellar ataxia type 3: Redistribution and differential recruitment of 26S proteasome subunits and chaperones to neuronal intranuclear inclusions, Ann. Neurol. 51 (2002) 302–310.

[20] A.L. Schwartz, A. Ciechanover, The ubiquitin-proteasome pathway and pathogenesis of human diseases, Annu. Rev. Med. 50 (1999) 57–74.

[21] M.G. Spillantini, M.L. Schmidt, V.M. Lee, J.Q. Trojanowski, R. Jakes, M. Goedert, α-synuclein in Lewy bodies, Nature 388 (1997) 839–840.

[22] K. Tanaka, T. Suzuki, T. Chiba, H. Shimura, N. Hattori, Y. Mizuno, Parkin is linked to the ubiquitin pathway, J. Mol. Med. 79 (2001) 482–494.

[23] K. Tanaka, T. Suzuki, N. Hattori, Y. Mizuno, Ubiquitin, proteasome and parkin, Biochim. Biophys. Acta 1695 (2004) 235–247.

[24] K. Wakabayashi, S. Engelender, M. Yoshimoto, S. Tsuji, C.A. Ross, H. Takahashi, Shinphilin-1is present in Lewy bodies in Parkinson's disease, Ann. Neurol. 47 (2000) 521–523.

[25] A.M. Weissman, Themes and variations on ubiquitylation, Nat. Rev. Mol. Cell. Biol. 2 (2001) 169–178.

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Journal of Neuroscience Research 84:1350–1357 (2006)

Decline of Striatal Dopamine Release in Parkin-Deficient Mice Shown by Ex Vivo Autoradiography

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Parkin is the causal gene of autosomal recessive juvenile parkinsonism (AR-JP). Dopamine (DA) metabolism has been linked to Parkinson's disease (PD). To understand the pathogenesis of AR-JP, we generated parkin-deficient mice to assess the status of DA signaling pathway and examine DA release and DA receptor by ex vivo autoradiography. Ex vivo autoradiography using [^{11}C]raclopride showed a clear decrease in endogenous DA release after methamphetamine challenge in parkin-deficient mice. Furthermore, parkin deficiency was associated with considerable upregulation of DA (D_1 and D_2) receptor binding in vivo in the striatum and increased DA levels in the midbrain. Our results suggest that dopaminergic neurons could behave abnormally before neuronal death.

Key words: parkin; dopamine; release; autoradiography; receptor

Parkinson's disease (PD) is the most common neurodegenerative movement disorder in the elderly and affects approximately 1% of the population >65 years of age. The major symptoms of PD are tremor, bradykinesia, cogwheel rigidity, and postural instability, which arise from the degeneration of dopaminergic (DAergic) neurons in the substantia nigra (SN). Of the many hereditary PD genes, the *parkin* gene has received special interest by researchers working in the field of PD (Kitada et al., 1998), because it displays ubiquitin-ligase activity and that early investigations showed that proteolytic dysfunction causes massive loss of DAergic neurons (Shimura et al., 2000; Chung et al., 2001; Imai et al., 2001). Several lines of parkin-null mice have been generated, however, the mechanism(s) underlying the cause of autosomal-recessive-juvenile-parkinsonism (AR-JP) are less well defined, because most (if not all) substrates for parkin reported so far remain unchanged irrespective of parkin-deficiency (our unpublished results) (Goldberg et al., 2003; Periquet et al., 2005). The aim of the present study was to uncover the pathogenesis of AR-JP using parkin-deficient mice.

Imaging of changes in neuroreceptor availability to positron emission tomography (PET) ligands can be used to indirectly measure synaptic neurotransmitter fluxes in the living human brain and several PET studies in PD have been reported (Dagher, 2001; de la Fuente-Fernandez and Stoessl, 2002). In PD, the characteristic loss of striatal dopamine (DA) terminal function, reflected by reduced dopa decarboxylase activity, can be quantified in vivo using [^{18}F]dopa PET. Although striatal [^{18}F]dopa uptake reflects the storage of DA, [^{11}C]raclopride, an in vivo marker of dopamine D_2 receptor, is useful for assessing the capacity of endogenous DA release (Piccini et al., 2003). We monitored the status of DA metabolism in the brains of parkin-null mice, and focused on DA release and DA receptor by using ex vivo autoradiography techniques.

MATERIALS AND METHODS

Generation of Parkin$^{-/-}$ Mice

For the constructed targeting vector, most of exon 2 was replaced in-frame by the coding sequence of τ-GFP to

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Received 5 February 2006; Revised 25 May 2006; Accepted 29 June 2006

Published online 29 August 2006 in Wiley InterScience (www.interscience.wiley.com). DOI: 10.1002/jnr.21032

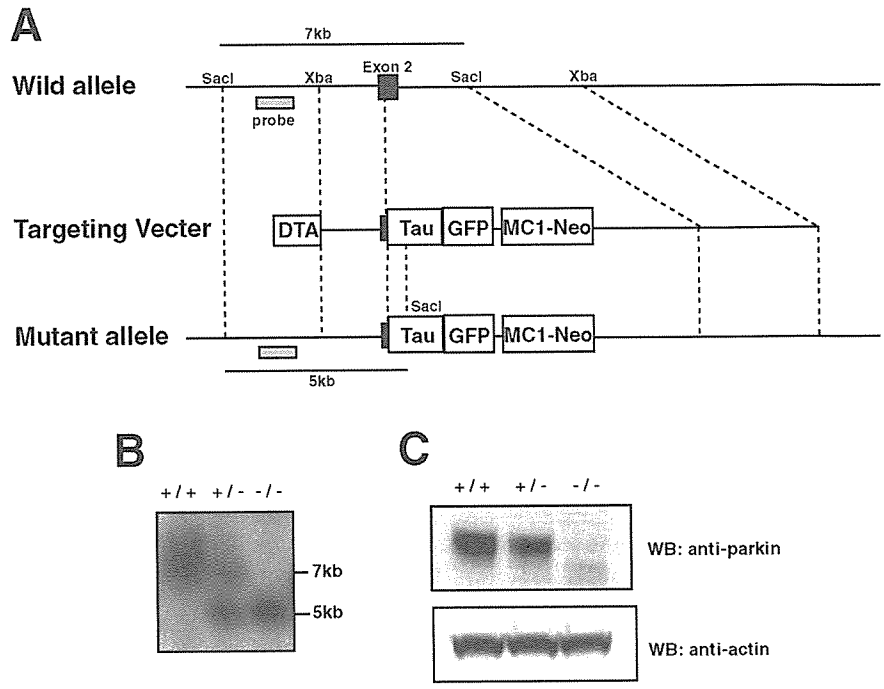


Fig. 1. Generation of parkin-deficient mice. **A:** Schematic representation of the targeting vector and the targeted allele of *parkin* gene. Exon 2 is depicted by the black box. The τ, GFP, MC1-Neo (neomycin-resistant) gene cassette is shown. The probe for Southern blot analysis is shown as a gray square. DTA, diphtheria toxin gene. **B:** Southern blot analysis of genomic DNAs extracted from tails of wild-type (+/+), heterozygous (+/−) or homozygous (−/−) mice. The genomic DNAs digested with *Sac*I were hybridized with the probe shown in (A). Wild-type and mutant alleles are detected as 7- and 5-kb bands, respectively. **C:** Western blotting of parkin in whole brain lysates. The lysates of mice indicated genotypes were immunoblotted with antibodies against parkin and actin.

use as a reporter system for axonal extension, followed by translation and transcription termination sequences and the MC1-neo cassette (Fig. 1A). A targeting vector was constructed using 1.5- and 7-kb DNA fragments as 5′ and 3′ homologous sequences, respectively. A negative selection cassette, DTA, which encodes the diphtheria toxin, was also included. The linearized targeting vector was transfected into TT2 ES cells. After selection in G418, clones were screened by Southern analysis for homologous recombination. Using the 5′ external probe and probe specific for Neo sequence, we confirmed the clones carried the desired homologous recombination. ES cells of clones were injected into C57BL/6J. Chimeric offspring were crossed with C57BL/6J mice to obtain germline transmission, which was confirmed by Southern analysis with the 5′ probe. Heterozygous mice were then interbred to obtain homozygous knockout and wild-type control mice. Mice were subsequently genotyped by PCR using primers specific for the wild-type and the targeted allele.

Behavioral Test

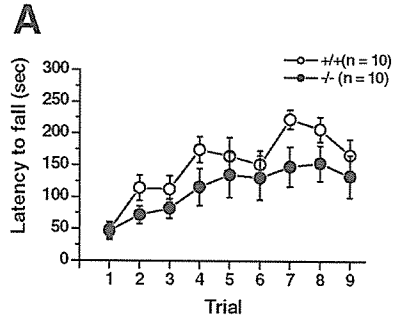
We used the rotorod test in this study, which is a test used commonly to score the severity of motor impairment in rodents. Mice were placed on an accelerating rotorod (ENV-575M, Med Associates, Inc., St. Albans, VT) and the time that a mouse stayed on the rotating drum was recorded. Two behavioral patterns noted on loss of balance on the drum were recorded: falling off the rotating rod and clinging to the rotorod with complete "passive" ride around the rod (Paylor et al., 1999). In the latter behavior, the mouse either continued to walk when it reached the top of the rod, or clung around the rod a second time. Behaviors were divided operationally into two categories. For active performing mice, they never passively clung around the rotorod, whereas mice that clung around the rod at least one time during each trial were defined as passive performing mice. For the active mice, the latency to fall was recorded for each trial. For the passive mice, the latency to fall off the rotorod, or the latency to the first cling around (latency to cling) was recorded. Passive performing mice were allowed to continue to walk on the rotorod after the first passive rotation. Thus, the data for latency to fall in the passive mice represented the whole time spent on the rotating rod with several passive rotations. Each mouse underwent three trials per a day with a 45-min intertrial interval and these tests were conducted over 3 successive days. A two-way ANOVA [genotype × trial] with repeated measures was used to analyze the latency to fall or to cling.

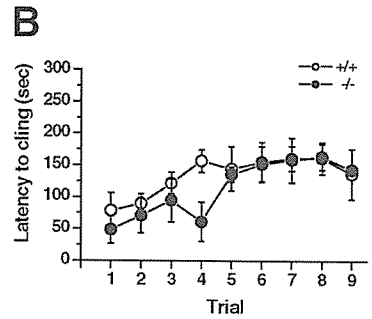
Immunohistochemistry for Tyrosine Hydroxylase

Immunolabeling using anti-tyrosine hydroxylase (TH) antibody (TH-16; dilution 1:10,000; Sigma Biosciences, St. Louis, MO) was detected by the avidin-biotinylated horseradish peroxidase complex method (Vectastain ABC elect kit, Burlingame, CA).

Neurochemical Analysis

Contents of dopamine (DA) and its metabolites 3,4-dihydroxy-phenylacetic acid (DOPAC) and homovanillic acid (HVA) were measured by high-performance liquid chromatography (HPLC) equipped with an electrochemical detection system (Kobayashi et al., 1994). Tissues were homogenized in 5 volumes of 0.2 M perchloric acid containing 0.1 M ethylenediaminetetraacetic acid (EDTA) and 100 ng/ml of isoproterenol. After centrifugation of the tissue homogenates, the pH of the supernatants was adjusted to 3.0 by adding 1 M sodium acetate. The samples were injected into the HPLC system with the mobile phase containing 0.1 M sodium citrate,





Number of mice every trial

	Passive		Active	
Trial	+/+	-/-	+/+	-/-
1	2	3	8	7
2	7	4	3	6
3	6	4	4	6
4	7	4	3	6
5	7	3	3	7
6	6	5	4	5
7	8	5	2	5
8	5	3	5	7
9	4	3	6	7

Fig. 2. Results of rotorod motor skill learning test conducted in 12-month-old parkin-deficient and wild-type mice. **A:** Latency to fall. Wild-type (+/+) (n = 10) and knockout (−/−) mice (n = 10) showed equivalent performance and motor learning. The mean ± SEM latency to fall is shown for each trial. **B:** Latency to cling to the rod in passive performing mice. Some mice rode around the rod at least once during training. Number of mice in every trial is tabulated. Active performance, mice that never passively rode around the rotorod (without clinging to the rod); passive performance, mice that rode around the rod at least once during training.

0.1 M citric acid, 0.5 mM sodium octane sulfonate, 0.15 mM EDTA, and 12% methanol (pH 3.5). The detector potential was maintained at 0.75 V vs. the Ag/AgCl electrode.

Ex Vivo Autoradiography

Chemicals. Standard compounds and precursors for labeling of (+)-α-[^{11}C]dihydrotetrabenazine ([^{11}C]DTBZ), [^{11}C]β-CFT (2β-carbomethoxy-3 β-(4-fluorophenyl)tropane: dopamine transporter probe). [^{11}C]SCH23390 (D1 receptor probe) and [^{11}C]raclopride (D_2 receptor probe) were purchased from RBI (Natick, MA). The enzymes for L-[β-^{11}C]DOPA synthesis, alanine racemase (EC 5.1.1.1.), D-amino acid oxidase (EC 1.4.3.3.) and β-tyrosinase (EC 4.1.99.2), were purchased from Ikeda Food Research Co. (Hiroshima, Japan).

Synthesis of [^{11}C]-Labeled Compounds. Carbon-11 (^{11}C) was produced by ^{14}N(p,α)^{11}C nuclear reaction using a cyclotron (HM-18, Sumitomo Heavy Industry, Tokyo) at Hamamatsu Photonics PET center and obtained as [^{11}C]CO_2. [^{11}C]β-CFT and [^{11}C]SCH23390 were labeled with ^{11}C by *N*-methylation of the corresponding nor-compounds with [^{11}C]methyl iodide prepared from [^{11}C]CO_2. [^{11}C]DTBZ and [^{11}C]raclopride were synthesized by *O*-methylation of the corresponding nor-compounds with [^{11}C]methyl iodide. The radiochemical and chemical purities used were >98% and 99%, respectively, and the specific radioactivity ranged from 182–201 GBq/μmol for [^{11}C]DTBZ, 152–181 GBq/μmol for [^{11}C]β-CFT, from 144–162 GBq/μmol for [^{11}C]SCH23390, and from 154–177 GBq/μmol for [^{11}C]raclopride, respectively. L-[β-^{11}C]DOPA (L-3,4-dihydroxyphenylalanine) was synthesized using a combination of organic synthesis and multi-enzymatic procedures using an automated synthesizer. The radiochemical and chemical purities of L-[β-^{11}C]DOPA were >98% and 99%, respectively. After analysis for identification, the solution was passed through a 0.22 μm pore size filter before intravenous (i.v.) administration.

Ex-vivo Imaging. Each labeled compound was injected i.v. at a dose of ca. 1 MBq/g body weight via the tail vein. The animals were sacrificed by decapitation under halothane anesthesia 30 min post-injection for [^{11}C]DTBZ, [^{11}C]SCH23390, and [^{11}C]raclopride, and 60 min post-injection for L-[β-^{11}C]DOPA and [^{11}C]β-CFT. The interval between injection of tracer and sacrifice for each labeled compound was determined based on data from previous reports (Inoue et al., 1991; Tsukada et al., 1994; Takamatsu et al., 2004). The brain was removed immediately, frozen by 2-methyl butane at −20°C, and 2-mm thick brain slices that included the striatal and cerebellar regions were prepared with a brain matrix (RBS-02, Neuroscience Inc., Tokyo, Japan). These slices were contacted with phospho imaging plate for 30 min, and the regional distribution of radioactivity was determined using a phospho imaging plate reader (BAS-1500 MAC, Fuji Film Co., Tokyo, Japan). The radioactivity in the cerebellum was used as the reference because of the low density of DA receptors in this region (Creese et al., 1975). Vesicular monoamine transporter (VMAT) availability, DA reuptake site availability, and DA (D_1 and D_2) receptor binding activities were expressed as follows: "Binding index" = (RIstr − RIcere)/RIcere, where RIstr was the radioactivity of each labeled ligand in the striatal regions and RIcere was the radioactivity in the cerebellum; for the quantification of dopamine synthesis, "Uptake index" was determined as (RIstr − RIcere)/RIcere, where RIstr was the radioactivity of L-[β-^{11}C]DOPA in the striatal regions, and RIcere was the radioactivity in the cerebellum.

Displacement Study of [^{11}C]raclopride With Methamphetamine

To evaluate the dopamine release from pre-synaptic neurons, saline or methamphetamine (MAP) at a dose of 0.3 mg/kg was injected i.v. 30 min before the injection of [^{11}C]raclopride. Brain slice preparation and imaging procedure were carried out as described above.

Statistical Analysis

All data were expressed as mean ± SEM. Differences between groups were examined for statistical significance using the Student's *t*-test. A *P*-value < 0.05 denoted the presence of a statistically significant difference.

RESULTS

Generation of Parkin-Deficient Mice

To investigate the in vivo roles of parkin and shed light on the pathogenic mechanisms of AR-JP, we generated a new line of mice with a targeted disruption of the *parkin* gene. We deleted exon 2 of *parkin* that encodes most of the N-terminal ubiquitin-like domain whose major role is interaction with the 26S proteasome (Sakata et al., 2003). It is noteworthy that several mutations in exon 2 have been reported in patients with AR-JP (Mata et al., 2004). Due to the lack of exon 2, RNA splicing from exons 1–3 is predicted to change the reading frame, which is capable of causing almost complete dysfunction of parkin. We therefore chose the disruption of exon 2 to generate the parkin-null mutant mouse. Deletion of exon 2 was confirmed at the *parkin* genomic locus by Southern analysis (Fig. 1B). Parkin was absent in the knockout brain based on the analysis using anti-parkin antibody (Fig. 1C). Because τ-GFP cDNA was knockin, we also carried out RT-PCR using an GFP-specific primer and confirmed the presence of GFP transcripts in parkin-deficient mice (data not shown). However, the parkin-τ-GFP fusion protein was not clearly detectable by Western and immunohistochemical analyses (data not shown).

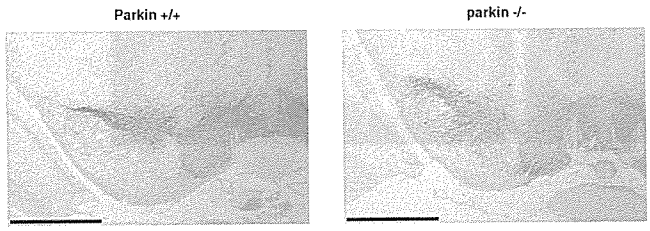


Fig. 3. Immunohistochemical localization of tyrosine hydroxylase (TH) at 3 months in the midbrain and substantia nigra (SN) of parkin-deficient mice and wild-type mice. Normal TH immunoreactivity in the SN. Scale bar = 1 mm.

Phenotype of Parkin$^{-/-}$ Mice

We carried out open field tests of *parkin*$^{-/-}$ mice and observed no significant alterations in their movement behaviors (data not shown). In addition, we eval-

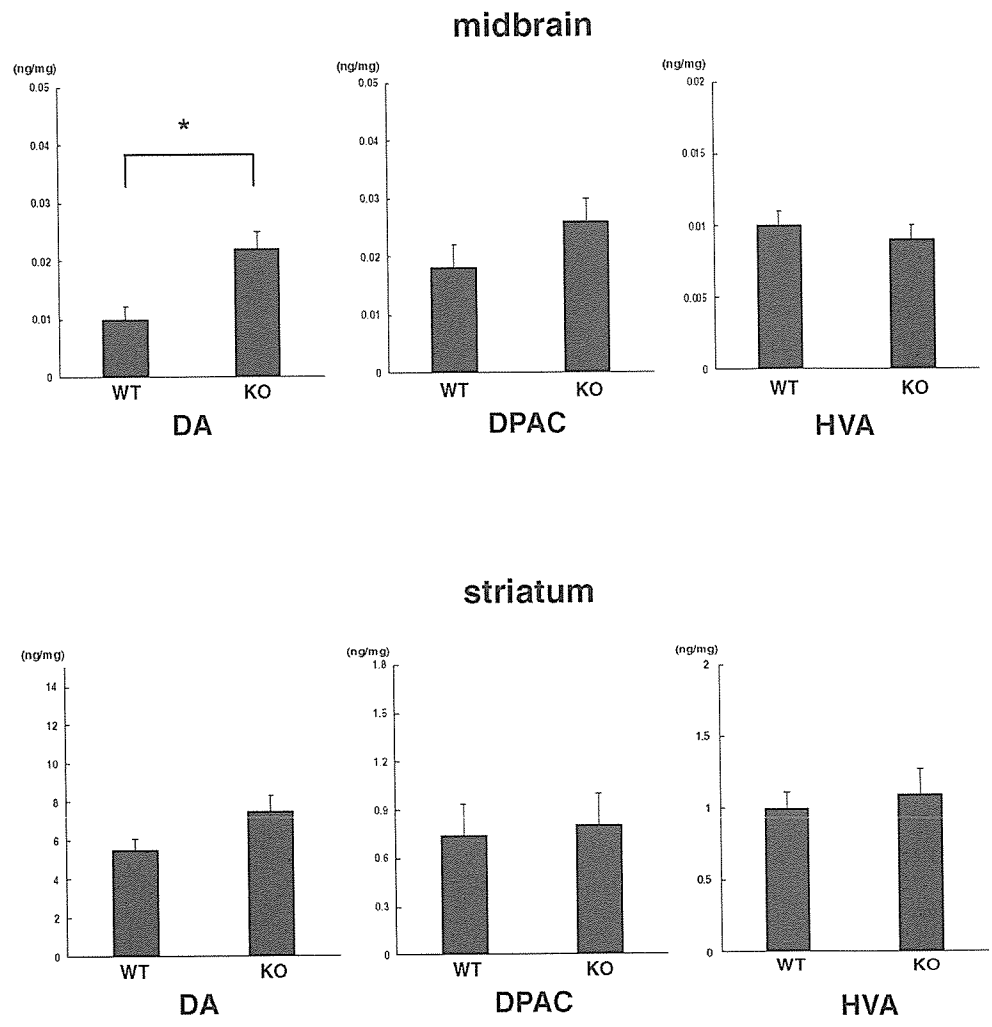


Fig. 4. The levels of DA and its metabolites (DOPAC and HVA) measured at 12 months in the midbrain and striatum in wild-type (WT) (n = 10) and parkin-deficient (KO) mice (n = 10). Concentrations of DA, DOPAC, and HVA were determined by HPLC with electrochemical detection. Data are expressed as mean ± SEM. $*P < 0.01$.

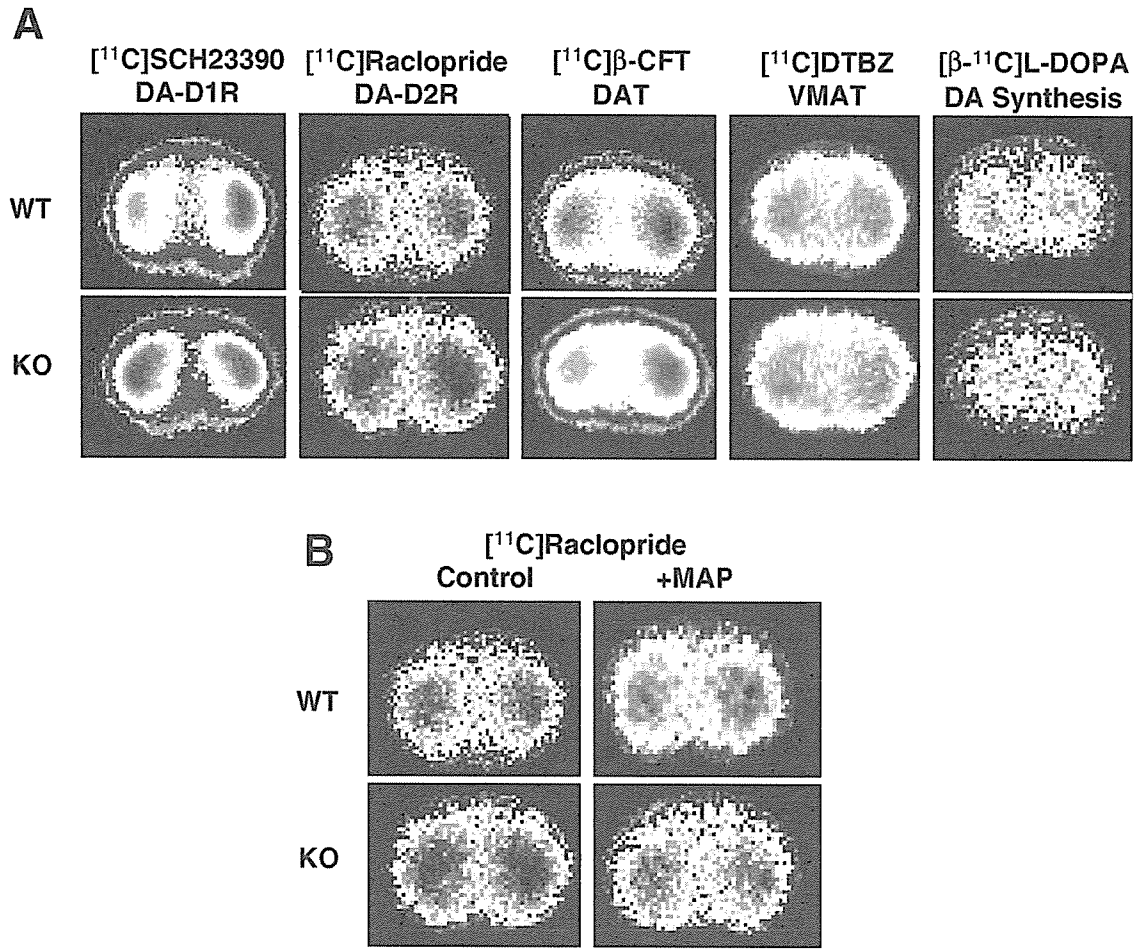


Fig. 5. Autoradiographic images. **A:** Comparison of striatal dopaminergic parameters of DA synthesis, DA receptors (D_1 and D_2), DAT, and VMAT between wild-type (WT) and parkin-deficient (KO) mice. **B:** Effects of methamphetamine (MAP) on striatal [^{11}C]raclopride binding in WT and KO mice. Saline or MAP at a dose of 0.3 mg/kg was injected i.v. 30 min before the injection of [^{11}C]raclopride. Brain slice preparation and imaging procedure were carried out as described in Materials and Methods.

uated these mice at 12 months of age using the rotorod task and found that *parkin*$^{-/-}$ (n = 10) and wild-type mice (n = 10) exhibited similar latencies for remaining on the rotating rod (Fig. 2), confirming the lack of any behavioral deficit. Furthermore, DAergic neurons of parkin-deficient mice were morphologically normal by immunohistochemical analysis with an antibody specific for TH (Fig. 3). Indeed, quantification of the number of DAergic neurons of the mutant and control mice (n = 6 each) at 3 months showed similar numbers of TH-positive neurons in SN and LC (data not shown).

Neurochemical Analysis

Next, we measured the levels of DA and its major metabolites DOPAC and HVA in *parkin*$^{-/-}$ and control mice (n = 10) at 12 months by HPLC with electrochemical detection. In the midbrain including SN, parkin-deficiency was associated with a significant increase in DA level but no change in DOPAC and HVA levels (Fig. 4). In the striatum, no changes in DA, DOPAC, and HVA levels were noted in *parkin*$^{-/-}$ mice compared to the wild-type.

Ex Vivo Autoradiography Study

Figure 5 shows representative autoradiographic images. The levels of two major DA receptors, D_1 and D_2, were measured at 12 months and expressed as the binding index by ex vivo autoradiography using receptor antagonists [^{11}C]SCH23390 and [^{11}C]raclopride, respectively. Quantitative analysis showed that the receptor binding levels of both D_1 and D_2 in the striatum of *parkin*$^{-/-}$ mice (D_1, n =7; D_2, n = 6) were higher than those of normal mice (D_1, n = 5; D_2, n = 6), although the binding index of dopamine transporter (DAT) and vesicular monoamine transporter (VMAT) using [^{11}C]β-CFT and [^{11}C]DTBZ, respectively, were similar (Fig. 6A)

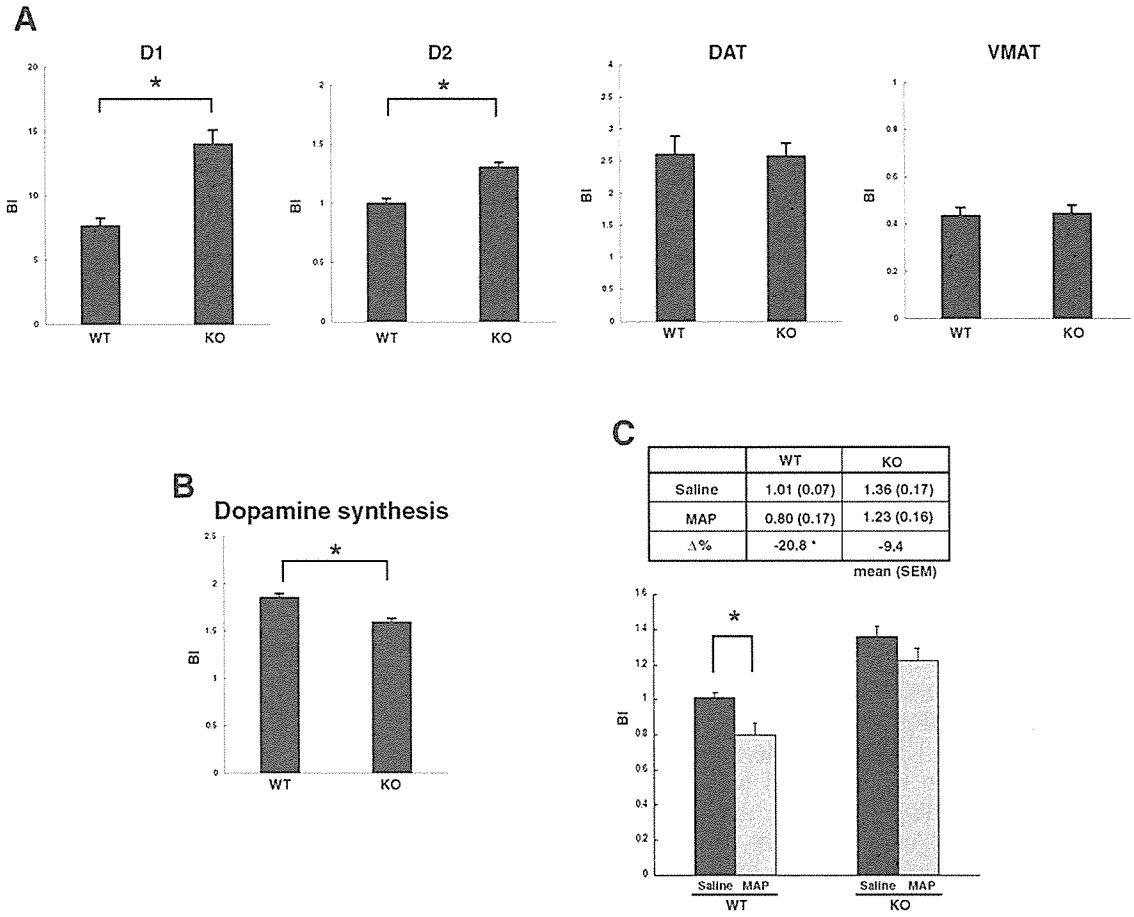


Fig. 6. Quantitative analysis of ex vivo autoradiography. **A:** Binding index for DA receptors (D_1 and D_2), DAT, and VMAT in the striatum of mice aged 12 months. **B:** DA synthesis in the striatum of mice aged 12 months. **C:** DA release of mice aged 12 months. Values of striatal [^{11}C]raclopride binding after administration of methamphetamine (MAP) or saline in wild-type and parkin-null mice are tabulated. %Δ, percentage difference [(methamphetamine binding index − saline binding index)/saline binding index] × 100. Data are expressed as mean ± SEM. $^{*}P < 0.01$.

in *parkin*$^{-/-}$ mice (DAT, n = 7; VMAT, n = 6) and normal mice (DAT, n = 5; VMAT, n = 6). Furthermore, DA synthesis determined by conversion of L-[β-^{11}C]DOPA to [β-^{11}C]DA was significantly decreased in *parkin*$^{-/-}$ mice (n = 8) compared to control mice (n = 8) (Fig. 6B).

Finally, we analyzed DA release in the striatum of *parkin*$^{-/-}$ mice using dopamine D_2 receptor antagonist [^{11}C]raclopride after treatment of methamphetamine (MAP) or saline. [^{11}C]Raclopride is considered to compete with endogenous DA at D_2 receptor sites, and competition between [^{11}C]raclopride and endogenous DA has been used recently to measure the level of DA release (Tsukada et al., 2000; Piccini et al., 2003). The binding of [^{11}C]raclopride was decreased in the presence of high levels of released endogenous DA in the synaptic clefts. The release capacity of DA can be estimated by a decrease in the binding level after methamphetamine-induced release of endogenous DA. Ex vivo autoradiography conducted at 12 months using [^{11}C]raclopride showed that the activity of DA release in *parkin*$^{-/-}$ mice (n = 8) was clearly reduced relative to that in wild-type mice (n = 9) (Fig. 6C).

DISCUSSION

Several studies have reported deletion of the mouse *parkin* gene but against expectation, the majority of such mice exhibited subtle phenotypes without causing massive loss of DAergic neurons (Goldberg et al., 2003; Itier et al., 2003; Von Coelln et al., 2004; Perez and Palmiter, 2005). Our parkin-null mice showed no obvious behavioral deficit; testing mouse mobility by using the open field test (data not shown) and rotorod test (Fig. 2) showed no reduction in locomotor activity. Histologically, no change in TH-positive nigra neurons was noted in parkin-deficient mice (Fig. 3), and no significant decrease in DAT was observed (Fig. 6A). [^{11}C]β-CFT shows a high affinity to DAT and accurately reflects a terminal density close to that of DA neurons, which could be potentially useful in tracing the drop out of DA neurons over time. These results show clearly the lack of any neurodegeneration in the cell bodies of DA neurons and nerve terminals of parkin deficient mice.