inner segment at 3w. The inner segment was less immunoreactive for UCH-L3 at 6w, 8w, and 12w, compared with 3w.

Histopathological Changes of Retinal Degeneration in the Uchl3-Deficient Mice

Microscopic examination of retinal cross-sections revealed no obvious histopathological changes during early postnatal development at P0 and P10 in the retina of *Uchl3*-deficient mice (Figure 2). At 3w of age, the mutant retina began to degenerate in the inner segment and ultimately disappeared at 12w (Figures 2B and 3D). Thickness of the outer segment, outer nuclear layer, and outer plexiform layer was also significantly decreased in the mutant mice at 6w of age (Figure 3, C, E, and F). Despite the conspicuous change in the photoreceptor cells, the thickness of the mutant inner retina up to 12w of age was not altered compared with that of the wild-type (Figure 3, G–I).

Ultrastructurally, vacuolar changes were found in the inner segment of *Uchl3*-deficient mice at 3w of age (Figure 4). Mitochondria at the inner segment of mutant mice were slightly swollen. Groups of small round-to-oval structures were observed in the degenerated inner segment (Figure 4D), and these structures were considered to be the cross-sections of cell processes. Chromatin condensation in photoreceptor nuclei was sometimes seen in the outer nuclear layer at 3w (Figure 4F). Morphometric analysis showed that the percentage of cristae area to whole area of mitochondrion in the inner segment of *Uchl3*-deficient mice was significantly lower than that of wild-type mice (Figure 4, G and H).

Altered Expressions of Apoptosis-Related Proteins in the Degenerated Retina

Apoptotic cells in the retinal cross-sections were identified using the TUNEL staining. TUNEL-positive cells were identified in the ventricular zone at P0 and inner nuclear layer at P10 of both genotypes during the developmental period (Figure 5, A and C). The number of TUNEL-positive cells slightly increased in the inner nuclear layer at P10. After 3w of age, TUNEL-positive cells of mutant retina significantly increased at the outer nuclear layer of the mutant retina at 3w, 6w, and 8w (Figure 5, A and D).

To determine which apoptotic pathway was activated in *Uchl3*-deficient mice, we examined immunoreactivities of apoptosis-related proteins. Expression of cytochrome *c*, caspase-3, and cleaved caspase-3 and caspase-1, essential molecules for the caspase-dependent pathway, were unchanged in both genotypes (Figure 6A), whereas oxidative stress markers, COX and Mn-SOD as well as AIF and Endo G, indicators of the caspase-independent pathway, were altered in the mutant retina (Figure 6B). Chronological changes in expression of markers for oxidative stress and caspase-independent apoptosis at P0, P10, 3w, 6w, 8w, and 12w are shown in Table 1. The immunoreactivity of COX was increased in the inner segment at 3w and 6w. Mn-SOD was mildly increased in the inner segment at 3w, 6w, and 8w. Although AIF was enriched in the inner segment of *Uchl3*-deficient mice at 3w and 6w, nuclear labeling of AIF was not observed. On the other hand, Endo G was localized to the nuclei of the outer nuclear layer of the mutant retina at 3w and 6w. Expression of Endo G was slightly increased in the outer plexiform layer, inner nuclear layer, and inner plexiform layer of *Uchl3*-deficient mice after 3w of age (Table 1). Thus, degeneration of photoreceptor cells in *Uchl3*-defi-

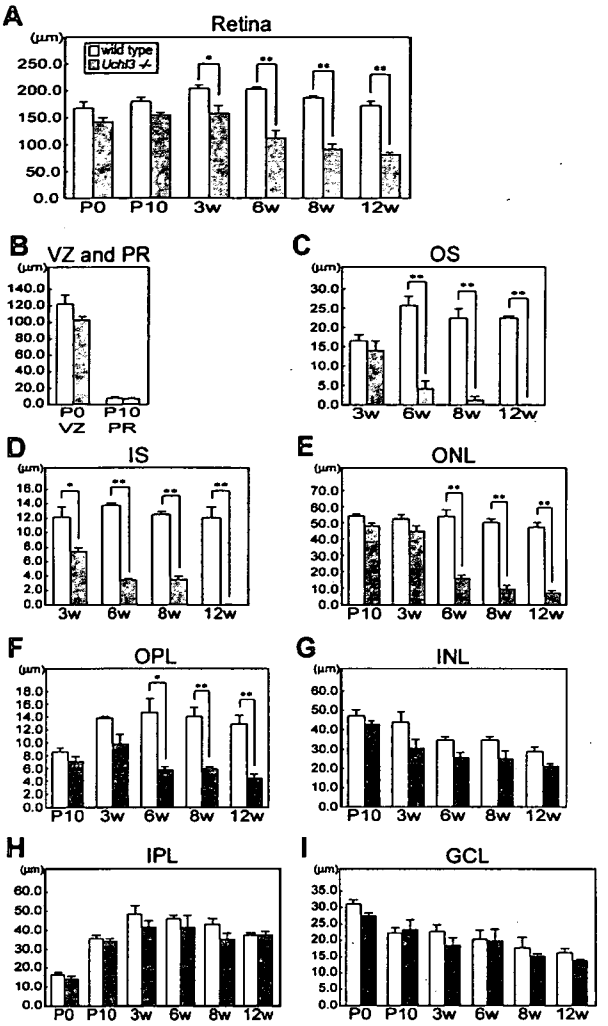

Figure 3. Chronological changes of retinal degeneration as assessed by thickness of each layer at different ages in wild-type and *Ucbl3*-deficient mice. **A:** Total retinal thickness is progressively decreased after 3w of age. **B:** Thickness of ventricular zone at P0 and photoreceptor layer at P10 shows no significant changes between both genotypes. **C–F:** Thickness of outer retinal layers in wild-type and *Ucbl3*-deficient mice at different ages. The earliest change is revealed at 3w of age in inner segment of mutant retina (**D**). Thickness of outer segment (**C**), outer nuclear layer (**E**), and outer plexiform layer (**F**) in *Ucbl3*-deficient mice is significantly decreased with age compared with that in the wild-type. **G–I:** Thickness of inner retinal layers in wild-type and *Ucbl3*-deficient mice at different ages. Thickness of inner nuclear layer (**G**), inner plexiform layer (**H**), and ganglion cell layer (**I**) are unchanged between both genotypes. Each value represents the mean ± SE ($^*P < 0.05$; $^{**}P < 0.01$). In all panels, the white bars represent the thickness in wild-type mice and the black bars represent the thickness in *Ucbl3*-deficient mice. VZ, ventricular zone; PR, photoreceptor; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.

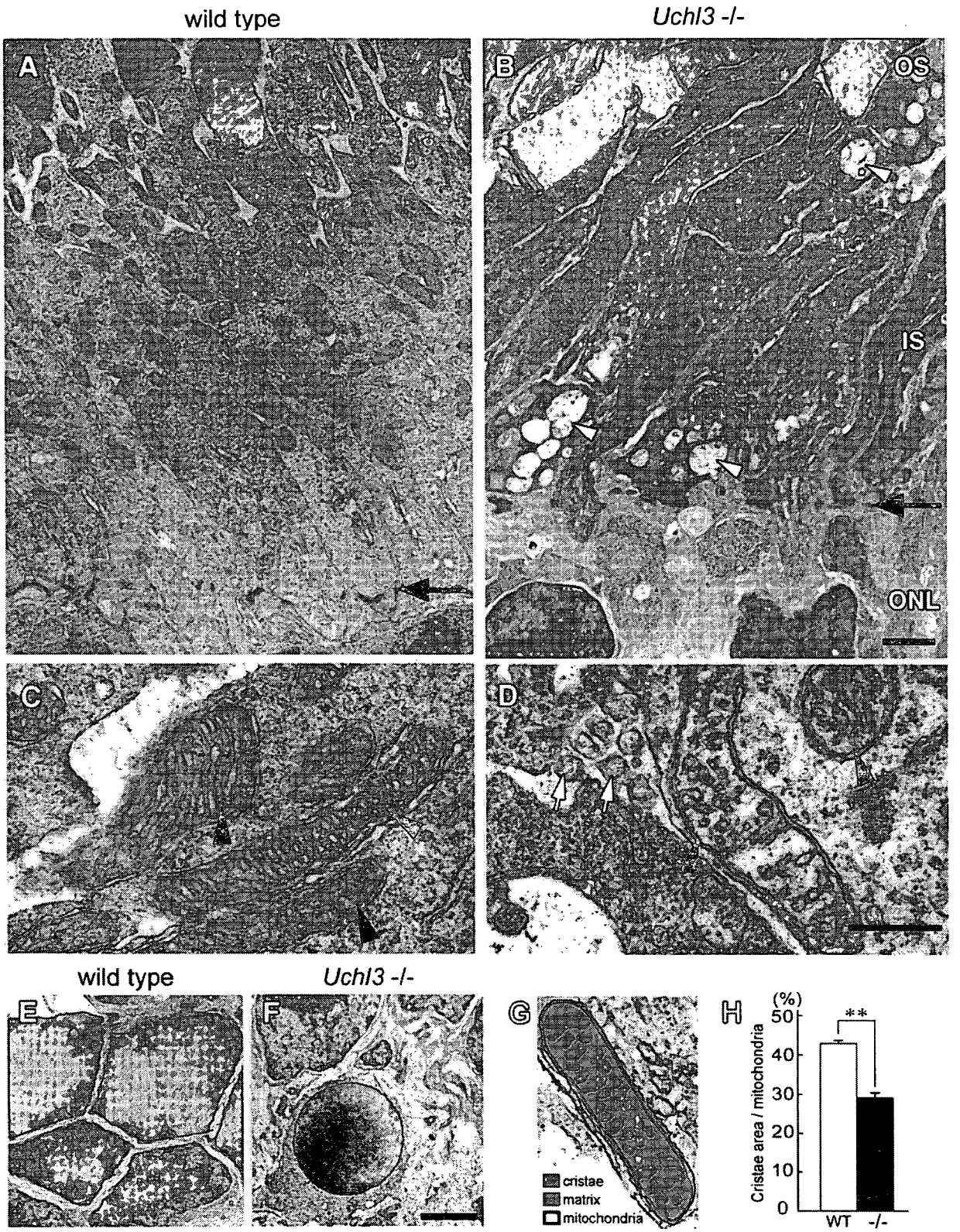

Figure 4. Ultrastructure of the outer retina in wild-type (**A**, **C**, and **E**) and *Ucbl3*-deficient mice (**B**, **D**, and **F**) at 3w of age. **A** and **B:** Inner segment of mutant retina is shrunken associated with vacuolar changes (**arrowheads** in **B**). **Arrows** in **A** and **B** indicate outer limiting membrane. **C** and **D:** Subsets of mitochondria at the inner segment in *Ucbl3*-deficient mice are swollen with decreased cristae (**arrowheads** in **D**) compared with that of wild-type (**arrowheads** in **C**). Groups of small round-to-oval shaped structures are occasionally seen in degenerated inner segment (**white arrows** in **D**). **E** and **F:** Outer nuclear layer of wild-type (**E**) and *Ucbl3*-deficient (**F**) mice. Chromatin condensation of photoreceptor cells is observed in mutant mice (**F**). **G** and **H:** Morphometric analysis of mitochondria was performed with the percentage of cristae area (**G**; **red**) against mitochondrial area ($n = 50$ for each genotype). Cristae area in the inner segment is significantly decreased in mutant retina (**H**, $-/-$, **black bar**) compared with that in wild-type (**H**, WT, **white bar**). Each value represents the mean ± SE (**$P < 0.01$). OS, outer segment; IS, inner segment; ONL, outer nuclear layer. Scale bars = 1 μm (**A** and **B**), 500 nm (**C** and **D**), and 1 μm (**E** and **F**).

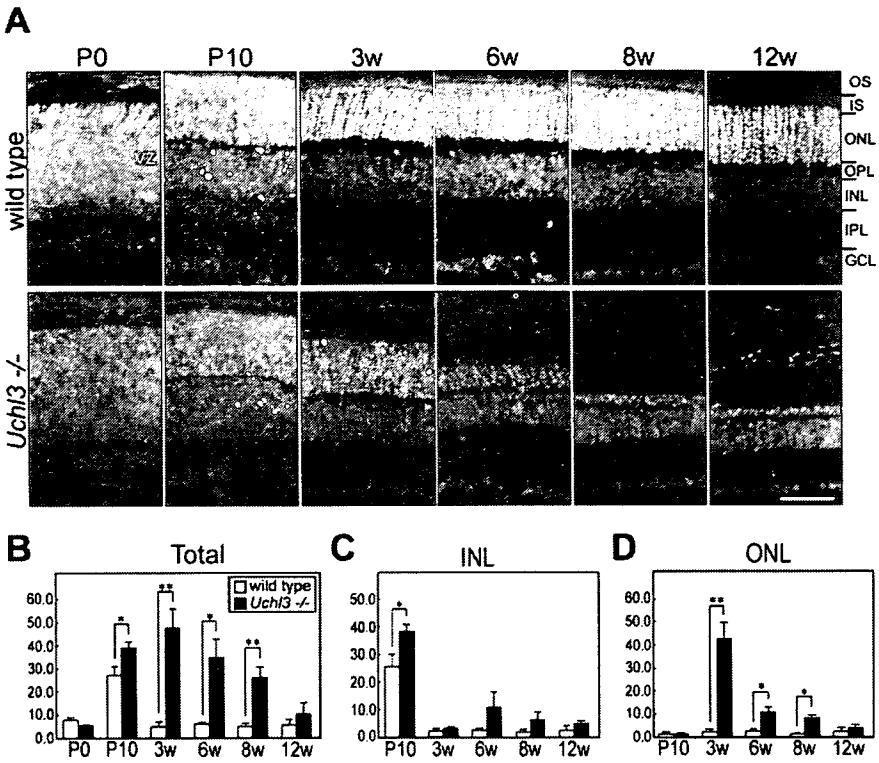

Figure 5. TUNEL analysis in wild-type and *Uchl3*-deficient mice at different ages. **A:** TUNEL staining in fluorescent microscopy shows that TUNEL-positive cells (**green**) are observed at the ventricular zone at P0 as well as at the inner nuclear layer at P10 in both genotypes. After 3w of age, TUNEL-positive cells are found in the outer nuclear layer in *Uchl3*-deficient mice. All sections are counterstained with propidium iodide (**red**). **B–D:** Number of TUNEL-positive cells in mutant mice (*Uchl3*$^{-/-}$; **black bar**) is significantly increased compared with those in wild-type (wild-type; **white bar**) at P10, 3w, 6w, and 8w (**B**). Increased number of TUNEL-positive cells in mutant mice at P10 correspond to apoptosis in the inner nuclear layer (**C**), whereas that in 3w, 6w, and 8w is reflected to apoptosis in the outer nuclear layer (**D**). VZ, ventricular zone; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bar = 20 μm (**A**). Each value in **B–D** represents the mean ± SE (*$P < 0.05$; **$P < 0.01$).

cient mice may be due to caspase-independent apoptotic pathway (Figure 7). Ubiquitin and Nedd-8, which are considered to be associated with UCH-L3 *in vitro*,[14,15] were expressed in the inner retina of both genotypes in a similar pattern as UCH-L1 (data not shown).

Discussion

This study demonstrates the unique localization of UCH-L3 to the photoreceptor inner segment that is abundantly populated with mitochondria after 3w of age in wild-type mice. The following features were found with regard to retinal degeneration in *Uchl3*-deficient mice. The retina showed no obvious morphological abnormalities during early postnatal development; however, progressive retinal degeneration was observed after 3w of age. The inner segment was originally perturbated with ultrastructural changes of mitochondria and increased expressions of markers for oxidative stress. The caspase-independent pathway was implicated during photoreceptor cell apoptosis. Thus, UCH-L3 may have a role in preventing mitochondrial oxidative stress-related apoptosis in photoreceptor cells.

Differential Localization of UCH-L1 and UCH-L3 in Murine Retina

The cellular distribution of UCH-L3 has not been studied except in the testis and epididymis, where UCH-L1 and UCH-L3 have distinct expression patterns.[25] In the present study, we found that UCH-L3 was enriched in the photoreceptor inner segment after 3w of age, whereas UCH-L1 was widely expressed in the inner retina. Photoreceptor cells are highly differentiated, and each segment has specific morphology and function; eg, inner segment contains abundant mitochondria,[27] and its oxygen consumption is considered to be high.[28] Meanwhile, expression of UCH-L1 at the inner retina was associated with that of ubiquitin and Nedd-8. Although *in vitro* studies indicate that UCH-L3 has de-neddylation activity,[14] UCH-L1 may be responsible for regulating expression level of ubiquitin and ubiquitin-like protein Nedd-8 in the retina. Because UCH-L1 expression in the retina was not altered in *Uchl3*-deficient mice, the function of UCH-L3 may not be compensated by UCH-L1. Our results indicate that UCH-L3 and UCH-L1 differ with regard to their localization and function in retina.

Mechanism of Photoreceptor Cell Death in the Uchl3-Deficient Mice

In our result, retinal apoptosis in *Uchl3*-deficient mice consisted of two different phases, during retinal development and after development. During the early postnatal development at P10, TUNEL-positive cells were observed in the inner nuclear layer of both genotypes, and the physiological apoptosis was slightly enhanced in the mutant retina. Because UCH-L3 was faintly expressed in the outer plexiform layer at P10 in wild-type mice, UCH-L3 may function during development. In the retinal development, the number of bipolar and Müller cell deaths reaches a peak at the postnatal days 8 to 11, which is associated with differentiation of the retina in

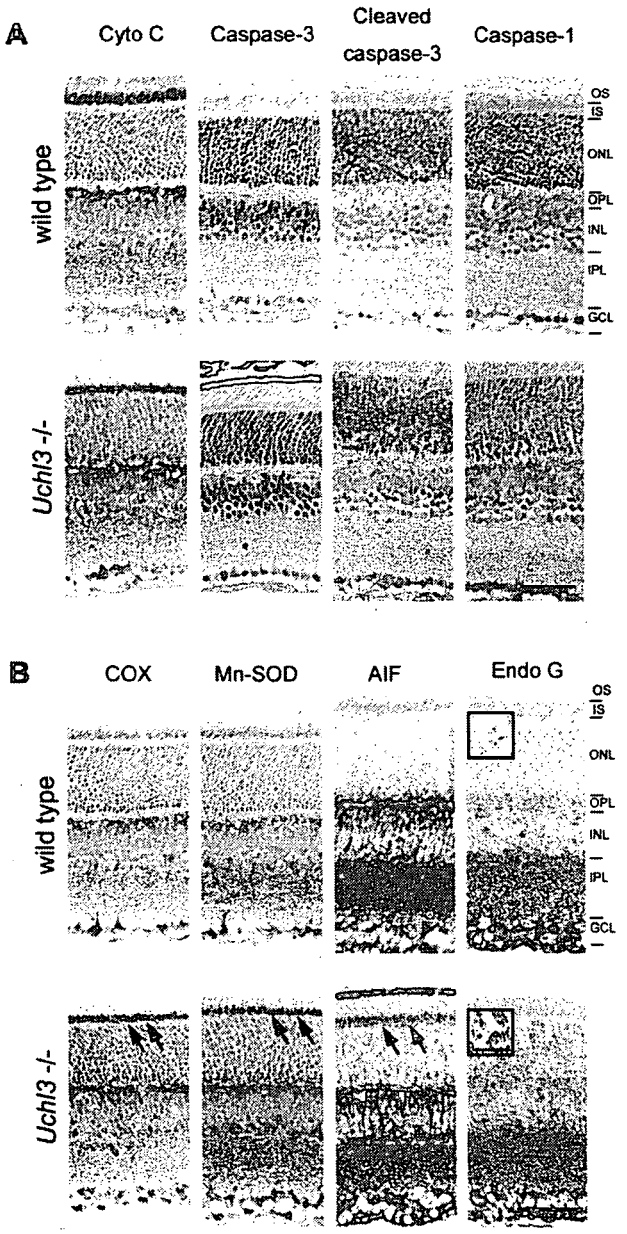

Figure 6. Immunohistochemical analysis of apoptosis- and oxidative stress-related molecules at 3w of age in wild-type and *Uchl3*-deficient mice. **A:** Expression of molecules relevant to the caspase-dependent pathway, including cytochrome *c* (Cyto C), caspase-3, cleaved caspase-3, and caspase-1, is unchanged between both genotypes. **B:** Increased immunoreactivities for oxidative stress markers, COX, Mn-SOD, and AIF, are observed in the inner segment of *Uchl3*-deficient mice (**arrows**). Translocation of Endo G to nuclei is found in the outer nuclear layer of *Uchl3*-deficient mice (**inset** in **B**). OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer. Scale bars = 50 μm (**A** and **B**); 10 μm (**inset** in **B**).

mice.[29] Therefore, loss of UCH-L3 may mildly promote the cell death of these cells.

After 3w of age, prominent and progressive photoreceptor cell apoptosis was disclosed in the outer nuclear layer of *Uchl3*-deficient mice. Under pathological conditions, several apoptotic pathways have been suggested in experimental retinal degeneration. Caspase-1 is predominantly associated with photoreceptor cell apoptosis in retinal degeneration of ischemia-reperfusion.[30] Light-induced retinal degeneration activates the parallel cascades, caspase-1[20] and caspase-independent apoptosis.[21] Oxidative stress leads to caspase-independent apoptosis in cultured cells.[31] Our results indicated that a caspase-independent pathway was activated during photoreceptor cell apoptosis in *Uchl3*-deficient mice, because immunohistochemical analysis revealed that activated caspase-3 and caspase-1 were not expressed in the degenerated retina. In addition, Endo G, a protein involved in the caspase-independent pathway, was expressed in the nuclei of the outer nuclear layer in *Uchl3*-deficient mice. Endo G is a mitochondria-specific nuclease that translocates to nuclei and serves as the DNase during a caspase-independent apoptosis.[32] Therefore, Endo G may be responsible for the DNA degradation that occurs during apoptosis in *Uchl3*-deficient mice. Expression of Endo G was slightly increased in the outer plexiform layer, inner nuclear layer, and inner plexiform layer of the *Uchl3*-deficient mice after 3w of age despite no significant UCH-L3 immunoreactivities in these layers. This result may reflect trans-synaptic secondary neuronal degeneration or glial changes of Müller cells.

AIF, another factor involved in caspase-independent apoptosis, was enriched in the inner segment; however, we did not observe translocation to nuclei for this protein. AIF is a mitochondrial flavoprotein that is a free radical scavenger of healthy cells.[33] During apoptotic induction, AIF translocates from mitochondria to nuclei.[33,34] It functions as a caspase-independent and PARP-1-dependent death effector that induces chromatin condensation and large-scale DNA fragmentation.[35] In our study, expression of AIF at the inner segment was associated with increased immunoreactivities of the oxidative stress markers, COX and Mn-SOD. Although it is unknown why AIF did not translocate to nuclei in the degenerated retina, increased immunoreactivity for AIF in the inner segment may indicate a reaction to oxidative stress. Because mouse eyes open 12 to 13 days after birth, light-induced oxidative stress may affect photoreceptor cell apoptosis in *Uchl3*-deficient mice after development. On the other hand, the retinal oxygen consumption increases under dark-adapted condition in the cat retina.[28,36] It may be interesting to study whether constant light or constant dark has any effect on the development of retinal degeneration in the *Uchl3*-deficient mice.

Uchl3-Deficient Mice as a Model of Retinal Degeneration with Mitochondrial Impairment

Apoptosis during retinal degeneration is observed in inherited diseases such as retinitis pigmentosa as well as in retinal diseases induced by a variety of stimuli, including hypoxia and oxidative stresses.[37,38] Several genetically engineered animal models of retinitis pigmentosa have been extensively investigated, including the RCS rat and *rd* mice. Retinal degeneration in the RCS rat was originally identified as an impairment of phagocytosis by pigmented epithelium due to mutation of receptor ty-

Table 1. Chronological Changes in Expression of Markers for Oxidative Stress and Caspase-Independent Apoptosis

	COX						Mn-SOD						AIF						Endo G					
	P0	P10	3w	6w	8w	12w	P0	P10	3w	6w	8w	12w	P0	P10	3w	6w	8w	12w	P0	P10	3w	6w	8w	12w
VZ*	−						−						−						−					
PR		−						−						−						−				
OS			−	−	nd	nd			−	−	nd	nd			−	−	nd	nd			−	−	nd	nd
IS			+	+	−	nd			+	+	+	nd			++	+	−	nd			−	−	−	nd
ONL		−	−	−	−	−		−	−	−	−	−		−	−	−	−	−		−	++§	+§	−	−
OPL		−	−	−	−	−		−	−	−	−	−		−	−	−	−	−		−	±	±	±	±
INL		−	−	−	−	−		−	−	−	−	−		−	−	−	−	−		−	±§	±§	−	−
IPL	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	±	±
GCL	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

*VZ, ventricular zone; PR, photoreceptor; OS, outer segment; IS, inner segment; ONL, outer nuclear layer; OPL, outer plexiform layer; INL, inner nuclear layer; IPL, inner plexiform layer; GCL, ganglion cell layer.
−, no change; ±, slight increase; +; mild increase; and ++, marked increase of immunoreactivity compared to that of wild type.
nd, not determined due to atrophic change.
§Nuclear staining.

rosine kinase (Mertk) with subsequent photoreceptor cell death occurring in a caspase-1- and -2-dependent manner.[39–42] *rd* mice have a recessive mutation in the rod cGMP phosphodiesterase β-subunit, and photoreceptor apoptosis occurs via a caspase-dependent mechanism.[43,44] Thus, these animal models of retinitis pigmentosa differ from *Uchl3*-deficient mice with regard to the mechanism of retinal degeneration.

The relationship between retinal degeneration and mitochondrial dysfunction has not been well studied except in Harlequin mice, which contain a mutation of AIF and exhibit progressive retinal degeneration.[45] We consider that the degeneration induced in the *Uchl3*-deficient mice is associated with mitochondrial dysfunction, because mitochondria in the inner segment of mutant retina exhibited morphological changes such as decreased cristae area. *Uchl3*-deficient mice reveal not only retinal degeneration but also muscle degeneration and mild growth retardation,[17] and thus the lack of UCH-L3 may affect general organs containing abundant mitochondria. Subtypes of mitochondrial diseases, such as chronic progressive external ophthalmoplegia and Kearns-Sayre syndrome, are caused by various mitochondrial DNA deletions and observed progressive ophthalmoplegia as well as retinitis pigmentosa.[46,47] Because UCH-L3 is predicted to be involved in the maintenance of mitochondrial function, *Uchl3*-deficient mice may be a model of disease that arises from mitochondrial impairment. Further studies are necessary to clarify the molecular mechanisms underlying retinal degeneration, as well as other organs in these animals.

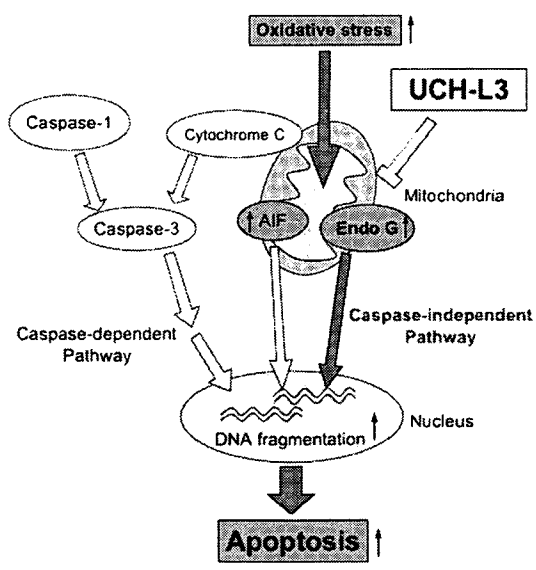

Figure 7. Function of UCH-L3 in apoptosis during retinal degeneration. Mitochondrial apoptosis is classified into caspase-dependent and caspase-independent pathways. Loss of UCH-L3 leads to oxidative stress-induced mitochondrial damage that causes translocation of Endo G from mitochondria to nuclei, resulting in caspase-independent apoptosis. **Red arrows** are considered to be activated in *Uchl3*-deficient mice.

Acknowledgments

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Parkin Potentiates ATP-Induced Currents Due to Activation of P2X Receptors in PC12 Cells

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Loss-of-function mutations of the parkin gene causes an autosomal recessive juvenile-onset form of Parkinson's disease (AR-JP). Parkin was shown to function as a RING-type E3 ubiquitin protein ligase. However, the function of parkin in neuronal cells remains elusive. Here, we show that expression of parkin-potentiated adenosine triphosphate (ATP)-induced currents that result from activation of the P2X receptors which are widely distributed in the brain and involved in neurotransmission. ATP-induced inward currents were measured in mock-, wild-type or mutant (T415N)-parkin-transfected PC12 cells under the conventional whole-cell patch clamp configuration. The amplitude of ATP-induced currents was significantly greater in wild-type parkin-transfected cells. However, the immunocytochemical study showed no apparent increase in the number of P2X receptors or in ubiquitin levels. The increased currents were attenuated by inhibition of cAMP-dependent protein kinase (PKA) but not protein kinase C (PKC) or Ca^{2+} and calmodulin-dependent protein kinase (CaMKII). ATP-induced currents were also regulated by phosphatases and cyclin-dependent protein kinase 5 (CDK5) via dopamine and cyclic AMP-regulated phosphoprotein (DARPP-32), though the phosphorylation at Thr-34 and Thr-75 were unchanged or rather attenuated. We also tried to investigate the effect of α-synuclein, a substrate of parkin and also forming Lysine 63-linked multiubiquitin chains. Expression of α-synuclein did not affect the amplitude of ATP-induced currents. Our finding provides the evidence for a relationship between parkin and a neurotransmitter receptor, suggesting that parkin may play an important role in synaptic activity. J. Cell. Physiol. 209: 172–182, 2006. © 2006 Wiley-Liss, Inc.

Recessive juvenile-onset form of Parkinson's disease (AR-JP) is the most frequent form of familial Parkinson's disease (PD). Mutations in the parkin gene were originally discovered from the linkage study of Japanese AR-JP families (Kitada et al., 1998). Thereafter its mutations have been found worldwide and parkin gene is now accepted as one of eight genes responsible for Parkinson's disease (see review by Cookson, 2005).

It has been demonstrated that parkin is associated with the ubiquitin–proteasome system. Wild-type parkin encodes for a protein-ubiquitin E3 ligase, which ubiqutinates many substrate proteins to enhance their degradation by the 26S proteasomes (Imai et al., 2000; Shimura et al., 2000; Zhang et al., 2000). As parkin mutations lose their E3 ligase activity, it is thought that accumulation of parkin substrate may lead to the selective death of catecholaminergic cell death (Ko et al., 2005) and familial-associated mutations differentially disrupt the solubility, localization, binding, and unibiquitination properties of parkin (Sriram et al., 2005).

It is reported that parkin is localized on surface of synaptic vesicle membranes (Kubo et al., 2001). As substrates of parkin, some synaptic proteins were reported, such as synaptotagmin XI (Huynh et al., 2003), septin CDCrel-1 (Zhang et al., 2000), and synphylin1 (Lim et al., 2005), suggesting that parkin may have a neuronal function. However, the nature of this function is unknown. Therefore, we have investigated the effect of parkin on one of receptor channels that affect neurotransmitter secretion.

Adenosine triphosphate (ATP) and related nucleotides induce a release of catecholamines, including dopamine, in PC12 pheochromocytoma cells, a frequently used model for sympathetic neurons (Sela et al., 1991; Nakazawa and Inoue, 1992). ATP receptors are divided into two subtypes, P2X and P2Y receptors.

Ayumi Sato and Yukiko Arimura contributed equally to this work.

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P2X receptors are ionotropic receptors and form cationic channels, while P2Y receptors are G-protein-coupled receptors. Recently, we have reported that P2X receptor-induced membrane currents were augmented by ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), presumably due to upregulation of mono-ubiquitin level (Manago et al., 2005). Therefore, the ubiquitin–proteasome pathway is also implicated in the function of ATP receptors.

In the present study, we analyzed relationships between parkin and P2X receptors by expressing parkin or a familial-linked mutant parkin (T415N-parkin) which lacks ubiquitin E3 ligase activity in PC12 cells. This is the first evidence to show the relationship between physiological function of parkin and receptor channels involved in neurotransmitter secretion. These findings may help to understand the function of parkin in the nervous system and the mechanism of Parkinson's disease caused by dysfunction of parkin.

MATERIALS AND METHODS

Chemicals

RPMI-1640 medium, ATP-2Na, H-89 (N-[2-(p-bromoocinamylamino)ethyl]-5-isoquinolinesulfonamide), H-85, chelerythrine, roscovitine (2-(R)-(1-Ethyl-2-hydroxyethylamino)-6-benzylamino-9-isopropylpurine), and PD98059 (2′-Amino-3′-methoxyflavone) were from Sigma (St. Louis, MO). Nerve growth factor (NGF) and Lipofectamine 2000 were from Invitrogen (Carlsbad, CA). KN-93 (2-[N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl))amino-N-(4-chlorocinnamyl)-N-methylbenzylamine) and okadaic acid was from Calbiochem (San Diego, CA).

Cell culture

PC12 Tet-off cells were grown in RPMI-1640 medium containing 5% fetal bovine serum (FBS) (Cell Culture Technologies, Lugano, Switzerland), 10% horse serum (HS) (Invitrogen), 100 units/ml penicillin (Life Technologies, Rockville, MD), and 100 μg/ml streptomycin (Life Technologies) in a humidified atmosphere with 10% CO_2 at 37°C. To differentiate cells, 100 ng/ml of NGF was added to the RPM 1640 medium with 0.1% HS, 0.05% FBS, 50 unit/ml penicillin, and 100 μg/ml streptomycin for 4 days.

Transfection

Plasmids used for transfection were constructed using pIRES-EYFP vector (Clontech, Nottinghamshire, UK). For electrophysiological recording, PC12 Tet-Off cells were transfected with mock, Flag-tagged wild-type or mutant (T415N) parkin cDNA, using Lipofectamine 2000. The engineered PC12 cells are constructed to have higher transfection efficiency than wild-type PC12 cells (unpublished data). After 24 h of transfection, cells were treated with NGF and differentiated for 4–5 days. More precisely, 3.0×10^5 cells were seeded in 35-mm dishes in RPMI with 10% HS and 5% FBS. Twenty-four hours after seeding, the medium was replaced with 500 μl of serum-free RPMI 1640 medium. Then, the transfection mixture containing 4 μg of cDNA and 10 μl of Lipofectamine 2000 in 500 μl of RPMI-1640 was added to each dish and incubated for 6 h in a humidified atmosphere with 10% CO_2 at 37°C. One milliliter of complete RPMI-1640 supplemented with an additional 10% HS and 5% FBS was then added to each dish. The solution for transfection was discarded 18 h later and replaced with RPMI-1640 medium for differentiation with added 100 ng/ml NGF. For transfection of α-synuclein, plasmids were constructed using pIRES-EGFP vector (Clontech) and the same protocol was used as for parkin. For protein analysis, cells (7.5×10^5/well, Clontech) were transfected in the same way. After 24 h, cells were subjected to Western blot analysis.

Western blot analysis

After 48 h of transfection of pIRES-EYFP-mock, pIRES-EYFP-Flag-wild-type parkin, or -T415N parkin with Lipofectamine 2000 (Invitrogen), cells were lysed with TBS buffer (25 mM Tris/150 mM NaCl, PH 7.4) containing 1% Triton X-100 and centrifuged at 15,000 rpm for 30 min at 4°C. Thirty micrograms of each protein was subjected to SDS–PAGE on a 15% gel and transferred to PVDF membranes (Bio Rad, CA) and immunoblotted with anti-Flag M2 (1:200, Sigma, monoclonal) or anti-Actin (1:200, Chemicon, Temecula, CA, monoclonal).

Immunocytochemical analysis

After transfection, cells were fixed with 4% paraformaldehyde. Immunocytochemistry on PC12 Tet-Off cells was performed as previously described (Osaka et al., 2003) using antibodies against parkin (5 μg/ml, Zymed, San Francisco, CA; monoclonal), $P2X_2$, $P2X_4$, or $P2X_6$ receptor (1:200, Alomone labs, Jerusalem, Israel; polyclonal), ubiquitin that is predominantly reactive to free ubiquitin in immunohistochemistry (1:100, Sigma; polyclonal), α-synuclein (1:500, BD Biosciences, San Jose, CA), and dopamine and cyclic AMP-regulated phosphoprotein (DARPP-32) (phosphor Thr-34 and phospho Thr-75) (1:500, Abcam, Cambridge, UK). For immunofluorescence studies, anti-rabbit IgG conjugated with Cy3 antibodies (1:200, Jackson Immuno Research, West Grove, PA) or Alexa Fluor 568 goat anti-mouse (1:250, Molecular Probes, Invitrogen) was used as secondary antibodies. The same strength of the laser wavelength or fluorescence was applied in the series of images, for the quantification of the fluorescence under the confocal laser microscope system (LSM510, Carl Zeiss, Oberkochen, Germany).

Electrophysiological measurements

Cells expressing EYFP were selected under the fluorescence microscope. A patch pipette was then applied to the cell to obtain a giga-ohm seal under phase-bright mode. Whole-cell membrane current recordings were made under voltage-clamp at a holding potential of −70 mV as reported previously (Noda et al., 2000; Manago et al., 2005), using an Axopatch-200B amplifier (Axon Instruments, Foster City, CA). The patch pipette was filled with a solution containing (in mM): CsCl, 120; Mg_2ATP_3, 3; HEPES, 20; $CaCl_2$, 1; $MgCl_2$, 1; EGTA, 5. The pH of the solution was adjusted to 7.2 with 1 N CsOH. The pipette resistance was 5–9 MΩ. The external solution contained (mM): NaCl, 132; KCl, 5; $CaCl_2$, 2; $MgCl_2$, 1; glucose, 10; and HEPES, 10. The pH was adjusted to 7.4 with 1 N NaOH. External ATP or drugs were applied rapidly using the 'Y tube' technique (Min et al., 1996), which allows the complete exchange of the external solution surrounding a cell within 20 msec. Temperature monitored in the recording dishes was 33–34°C.

In the experiments using inhibitors (except PD98059), ATP was applied twice to ensure reproducibility of the ATP-induced current in control experiments. The inhibitor solution was applied after first application of ATP for a period appropriate to the inhibitor until the end of second application of ATP. The current amplitude obtained at the second application of ATP with or without inhibitors was normalized to that of the first ATP-induced current. All values were presented as mean ± SEM. Statistical analysis was done using ANOVA. A value of $P < 0.05$ was considered to be the minimum level of significance. Curve fitting was performed using the standard Hill Equation (Igor Pro 4.07; Wavemetrics, Lake Oswego, OR).

RESULTS

Transfection of parkin in PC12 Tet-Off cells

Expression of plasmid constructs was first examined in PC12 Tet-Off cells. Western blot analysis showed immunoreactive bands by anti-Flag antibodies in cells transfected with pIRES-EYFP-wild type parkin or T415N parkin, but not with mock plasmids (Fig. 1A). The efficiency of the transfection was about 10% in PC12 Tet-Off cells. To test endogenous expression of parkin, cells were immunostained using specific antibodies for parkin. The strong expression of parkin (red) was observed in wild-type parkin-transfected cell (yellow) but not in non-transfected cells in the same field (shown

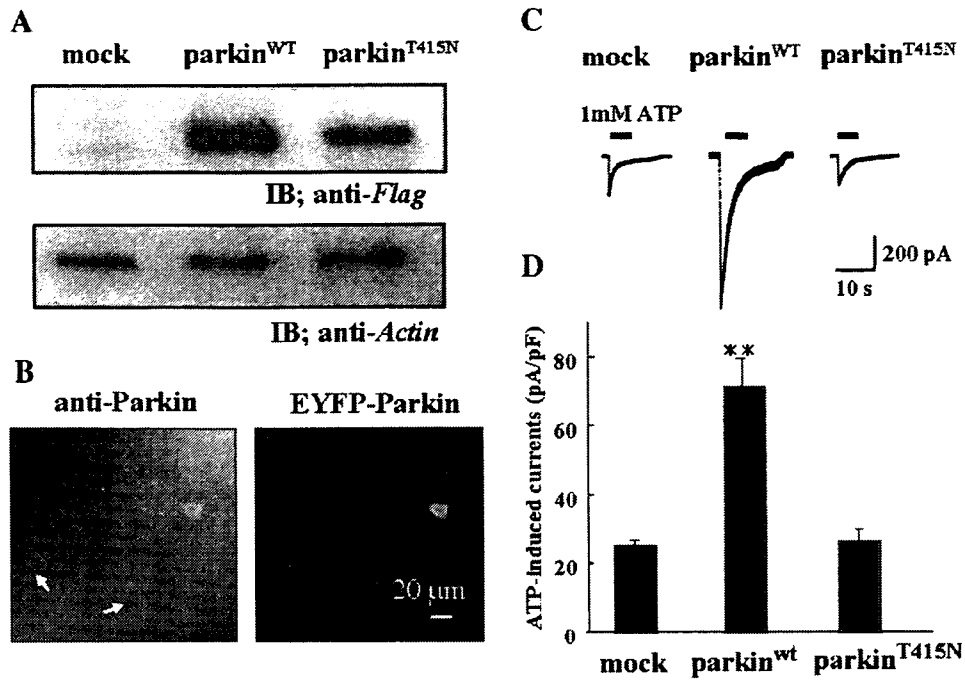

Fig. 1. Transfection of parkin and potentiation of ATP-induced currents in PC12 cells. **A**: Western blot analysis of PC12 Tet-Off cells. Cells were transfected with either pIRES-EYFP-mock, wild-type (WT) parkin, or T415N parkin. Each protein was subjected to SDS–PAGE and immunoblotted with anti-Flag or anti-Actin antibody. **B**: Confocal image of PC12 Tet-Off cells transfected with pIRES-EYFP-wild-type parkin (yellow) showed strong expression level of parkin (red) while non-transfected cells (white arrows) showed little expression of parkin. **C**: Inward membrane currents induced by 1 mM ATP at the holding potential of −70 mV in mock-, wild-type parkin-, and T415N parkin-transfected PC12 Tet-Off cells. **D**: Amplitudes of peak inward currents induced by 1 mM ATP in mock-, wild-type parkin-, and T415N parkin-transfected PC12 Tet-Off cells. The bars represent the mean ± SEM, $^{**}P < 0.01$.

with white arrows in Fig. 1B), suggesting little endogenous parkin was expressed in PC12 Tet-Off cells.

Effects of expression of parkin on ATP-induced currents

ATP-activated inward currents due to the activation of P2X receptors at negative holding potentials in PC12 cells or PC12 Tet-Off cells have been reported previously (Nakazawa et al., 1994; Manago et al., 2005). In our experiments, PC12 Tet-Off cells were voltage-clamped at −70 mV and 1 mM ATP were applied to see whether or not overexpression of parkin affected maximum inward currents. In parkin-transfected cells, ATP-induced inward currents were nearly threefold larger than those in mock- or mutant (T415N) parkin-transfected cells (Fig. 1C). The amplitudes of the peak inward currents in mock-, wild-type parkin-, and T415N parkin-transfected PC12 Tet-Off cells were 24.8 ± 1.6 pA/pF (n = 9), 71.3 ± 8.4 pA/pF (n = 5), and 26.1 ± 3.4 pA/pF (n = 7), respectively (Fig. 1D).

The current–voltage relationships of the ATP-induced inward currents were determined by applying 50 msec voltage steps in 10 mV increments between −100 mV and +50 mV at 50 msec interval from the holding potential of −70 mV before and during the application of ATP (Fig. 2A). Current traces obtained before and after application of ATP in wild-type parkin-transfected cells are shown in Figure 2B. The current levels at the end of each pulse before and during ATP application were measured in mock-, wild-type parkin-, or T415N parkin-transfected cells. The amplitudes of the ATP-induced currents at each voltage were obtained by subtracting the one before application of ATP from the one during application of ATP. The current–voltage relationships obtained at the time point after 40 msec from the beginning of each pulse were plotted as in Figure 2C. To allow for possible desensitization, the current–voltage relationships were also obtained by applying voltage steps in the opposite direction, that is, from +50 to −100 mV, but there was little change (data not shown). The reversal potential was about 0 mV, suggesting that these currents were due to non-specific cationic channels.

ATP-induced inward currents were concentration-dependent. Mock- and T415N parkin-transfected cells showed visible ATP-induced inward currents at 0.03 nM and a maximum response at 1 mM ATP (Fig. 3A). The maximum response was almost three times bigger in wild-type parkin-transfected cells (Fig. 3B). The sensitivity to ATP was not significantly changed by overexpression of either mock, wild-type, or T415N parkin. EC_{50} values (half maximum concentration) were 187 ± 45 μM, 127 ± 13 μM, and 177 ± 124 μM with Hill coefficients (n_H) of 1.05 ± 0.314, 0.97 ± 0.12, and 2.00 ± 2.26 in mock-, wild-type, and T415N parkin-transfected cells, respectively.

Expression of $P2X_2$, $P2X_4$, and $P2X_6$ receptors in parkin-transfected cells

In PC12 cells, $P2X_2$ and $P2X_4$ receptors (Hur et al., 2001) with lower level of $P2X_6$ receptor are expressed (our unpublished data). It was possible that the expression of P2X receptors was enhanced by overexpression of parkin. To define the changes in the expression level of P2X receptors semi-quantitatively, $P2X_2$, $P2X_4$, and $P2X_6$ receptors were immunostained using specific antibodies for each receptor subtype. The subcellular localization of $P2X_2$, $P2X_4$, and $P2X_6$ receptors showed no obvious difference in wild-type parkin-transfected cells compared with non-transfected cells in the same field (Fig. 4), suggesting that the potentiation of the

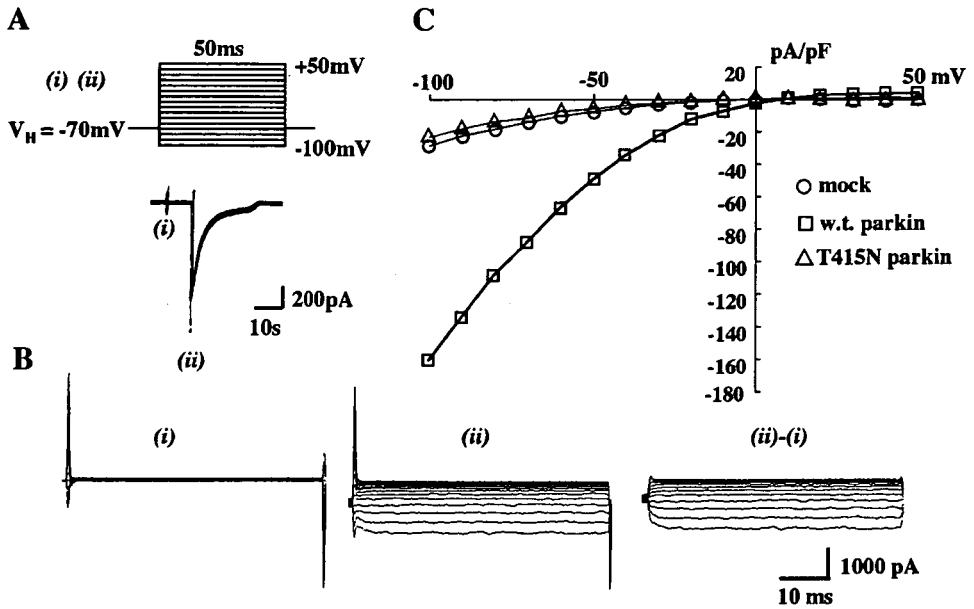

Fig. 2. Voltage-dependency of ATP-induced currents in mock-, wild-type parkin-, and T415N parkin-transfected PC12 Tet-Off cells. A: The voltage protocol shown in the upper part was applied before and during application of 1 mM ATP at the time indicated by (i) and (ii) in the lower part. B: Cumulated current traces obtained in wild-type parkin-transfected cells before (i) and during (ii) application of ATP. The subtracted currents [(ii) – (i)] show the ATP-induced currents. C: The current–voltage relationships of ATP-induced currents. The amplitudes of subtracted currents [(ii) – (i)] in (B) at the end of 50 msec pulses were plotted against the pulse potentials in mock (○)-, wild-type (w.t.) parkin (□)-, and T415N parkin (△)-transfected cells.

ATP-induced currents was not due to an increase in the total number of P2X receptors.

Expression of mono-ubiquitin in parkin-transfected cells

It has previously been reported that a de-ubiquitinating isozyme, ubiquitin carboxy-terminal hydrolase L1 (UCH-L1), also potentiated ATP-induced currents (Manago et al., 2005). However, hydrolase activity was not involved in the potentiation of ATP-induced currents because a mutant form lacking hydrolase activity also potentiated the current. Instead, UCH-L1 upregulated ubiquitin levels (Osaka et al., 2003) and overexpression of UCH-L1 in PC12 cells increased the mono-ubiquitin level (Manago et al., 2005). To test whether or not parkin also upregulate mono-ubiquitin levels, ubiquitin was stained using anti-mono-ubiquitin IgG. Unlike the effect of UCH-L1, immunoreactivity for ubiquitin in wild-type parkin-transfected cells was unchanged compared to that in mock-transfected cells or non-transfected cells in the same field (Fig. 5). These results indicated that parkin did not upregulate mono-ubiquitin.

Little effects of α-synuclein on ATP-induced currents

Since it has recently been shown that UCH-L1, parkin, and α-synuclein form lysine 63-linked multi-ubiquitin chains (Doss-Pepe et al., 2005; Lim et al.,

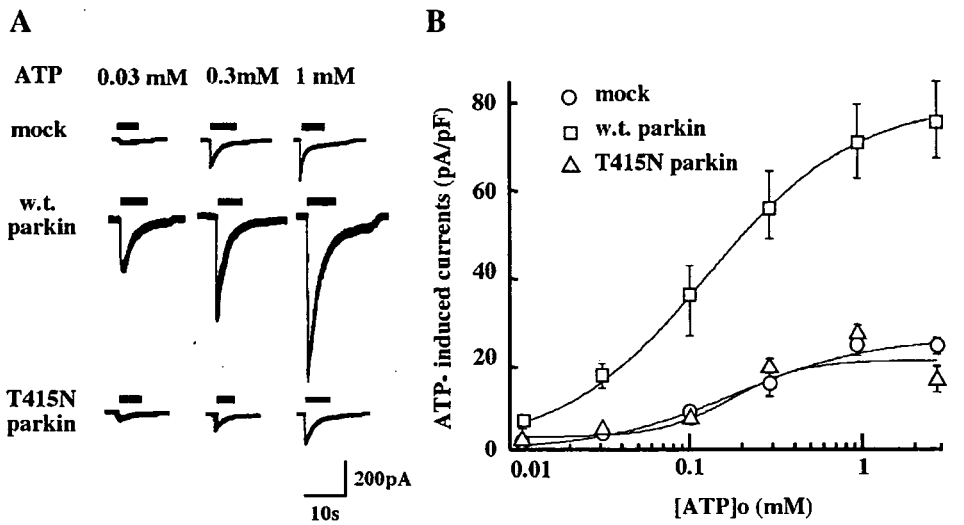

Fig. 3. Concentration-dependent curve of ATP-induced currents in mock-, wild-type parkin-, and T415N parkin-transfected PC12 Tet-Off cells. A: Inward membrane currents induced by 0.03, 0.1, and 1 mM ATP at the holding potential of −70 mV in mock-, wild-type (w.t.) parkin-, and T415N parkin-transfected PC12 Tet-Off cells. B: The peak inward current induced by ATP at the holding potential of −70 mV was plotted against the ATP concentration at several points between 0.01 and 3 mM in mock (○)-, wild-type parkin (□)-, and T415N parkin (△)-transfected PC12 Tet-Off cells. Each point represents the mean of 5–13 cells and the bar shows the mean ± SEM. The curve shows the least squares fit.

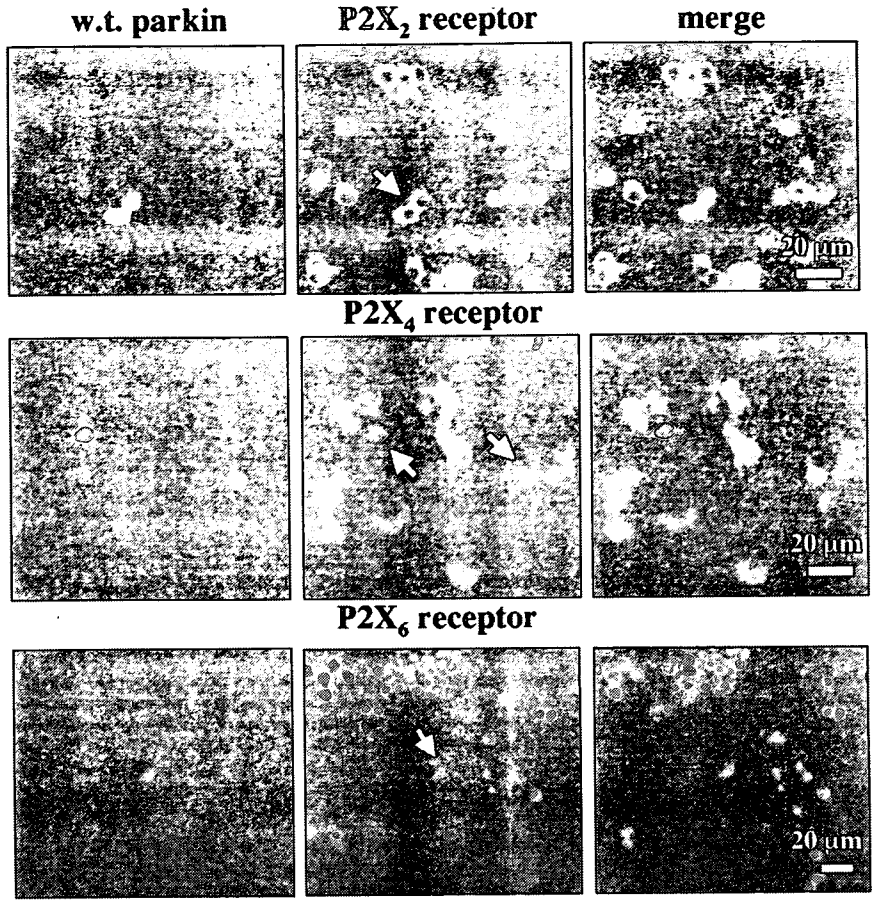

Fig. 4. Parkin has no clear effect on the expression of $P2X_2$, $P2X_4$, and $P2X_6$ receptors. Confocal images of PC12 Tet-Off cells transfected with pIRES-EYFP-wild-type (w.t.) parkin that were double stained with $P2X_2$ (**upper part**), $P2X_4$ (**middle part**), and $P2X_6$ receptors (**lower part**). EYFP (yellow)-positive cells were parkin-transfected cells, showing similar expression level of P2X receptors (red) to those in non-transfected cells.

2005), α-synuclein also might have a similar effect on P2X receptor. Transfection of α-synuclein was performed in the same way as parkin and the transfection efficiency was much greater than that of parkin (up to 30%) and the protein expression was confirmed by Western blotting (not shown). The strong expression of α-synuclein (red) was observed in transfected cell (green) but not in non-transfected cells in the same field

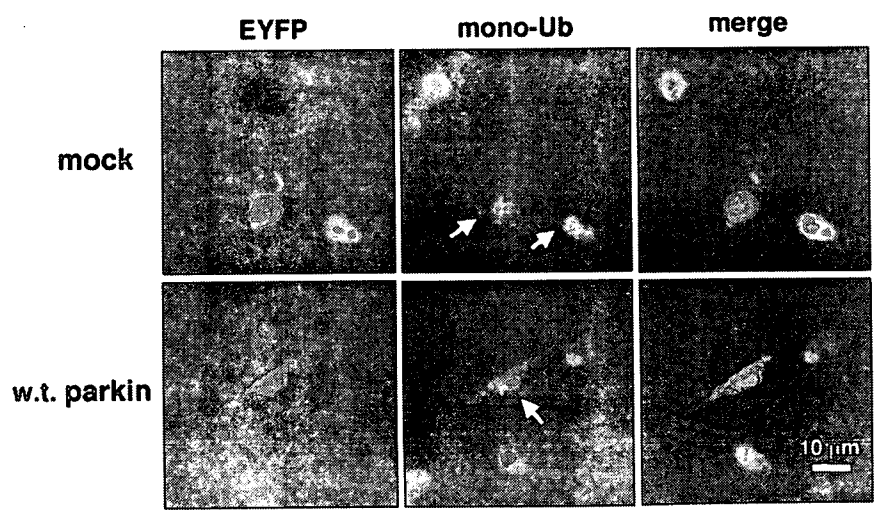

Fig. 5. Parkin had no clear effect on mono-ubiquitin expression. Confocal images of PC12 cells transfected with pIRES-mock or wild-type (w.t.) parkin that were double stained with mono-ubiquitin (red) and EYFP (yellow).

(shown with white arrows in Fig. 6A), suggesting little endogenous α-synuclein was expressed in PC12 Tet-Off cells.

ATP-induced currents in α-synuclein-transfected cells were not significantly different from those in mock-transfected cells (Fig. 6B). The relative amplitude of ATP-induced currents were 28.6 ± 4.1 pA/pF (n = 9) in mock-transfected cells and 21.5 ± 5.4 pA/pF (n = 10) in α-synuclein-transfected cells, respectively.

Effects of kinase inhibitors on ATP-induced currents in parkin-transfected cells

The mechanism by which ATP-induced currents were augmented in parkin-transfected cells was investigated. It was reported that in *Aplysia* UCH activated PKA as a result of degradation of the regulatory subunit of PKA, and that this contributed to the long-term potentiation (Hegde et al., 1997). The increase of the ATP-induced inward currents in UCH-L1-transfected cells has also been attributed to activation of PKA (Manago et al., 2005). Therefore, it was tested whether PKA might be activated in parkin-transfected cells by using H-89, a PKA inhibitor. After obtaining large ATP-induced currents in parkin-transfected cells, 10 μM H-89 was applied for 10 min. The amplitude of the ATP-induced currents in the presence of H-89 was 64.6 ± 3.5% (n = 7) of that of the first ATP-induced current in the same cell (control without H-89; 85.3 ± 4.0% (n = 4)) (Fig. 7A), implying an inhibition of about 25%. An inactive analog of H-89, H-85, did not have this inhibitory effect (current amplitude in the presence of H-85 was 84.3 ± 1.6% (n = 3) of the first current). To confirm the effect of parkin, the effect of PKA inhibitor on ATP-induced currents were tested in mock-transfected cells as well. In mock-transfected cells, application of 10 μM H-89 for 10 min had no effect on the ATP-induced inward current (H-89, 79.8 ± 1.4% (n = 3); control; 77.6 ± 5.2% (n = 3)) (Fig. 7B).

The intracellular carboxyl terminus of P2X receptor contains several consensus phosphorylation sites for protein kinase C (PKC) as well as PKA, suggesting that the function of the P2X receptors might be regulated by PKC-mediated phosphorylation (Chow and Wang, 1998). Hence, the effect of chelerythrine, a PKC inhibitor, on ATP-induced currents in parkin-transfected cells was tested. Application of 5 μM chelerythrine for 10 min had no effect on the ATP-induced inward current in wild-type parkin-transfected cells (Fig. 7A). The normalized amplitude of second ATP-induced inward currents in the presence of chelerythrine was 88.4 ± 3.3% (n = 5). The possible involvement of calmodulin-dependent protein kinase (CaMKII) was also tested by using KN-93, a CaMKII inhibitor. Application of 10 μM KN-93 for 20 min had no effect on the ATP-induced inward current in wild-type parkin-transfected cell (90.4 ± 5.1% (n = 4); control, 81.2 ± 4.6% (n = 4)) (Fig. 7C).

In PC12 cells and hippocampal neurons, activation of PKA has been reported to cause activation of extracellular signal-regulated kinase (ERK), with subsequent phosphorylation of Ca^{2+}-stimulated cAMP response element binding protein (CREB) and stimulated transcription (Impey et al., 1998). Likewise, the augmentation of ATP response in parkin-transfected cell might be due to the stimulation of transcription. To test this possibility, we examined whether mitogen-activated protein kinase (MAPK), including ERK, was activated following the activation of PKA in PC12 Tet-Off cells. However, ATP-induced currents in parkin-transfected cells were unaffected even after application of 5 μM PD98059, (one of the MAPK kinase inhibitors) for 4 days: the amplitude of the ATP-induced current after the application of PD98059 was 82.1 ± 9.9 pA/pF (n = 4) compared with 74.6 ± 3.4 pA/pF (n = 18) in controls treated with vehicle (Fig. 7D).

Involvement of DARPP-32 in parkin-transfected PC12 Tet-Off cells

It was previously reported that the dopamine and cAMP-regulated phosphoprotein with molecular weight of about 32,000 (DARPP-32) was expressed in PC12 Tet-Off cells and that the expression level tended to increase after differentiation of the cells with NGF (Manago et al., 2005). Since phosphorylation of DARPP-32 at Thr-75 by cyclin-dependent kinase 5 (CDK5) had a negative feedback regulatory effect on PKA activity (Nishi et al., 2000), the effect of roscovitine, a CDK5 inhibitor, was tested. The application of 10 μM roscovitine to wild-type parkin-expressing cells for 10 min enhanced the normalized amplitude of ATP-induced currents to the one

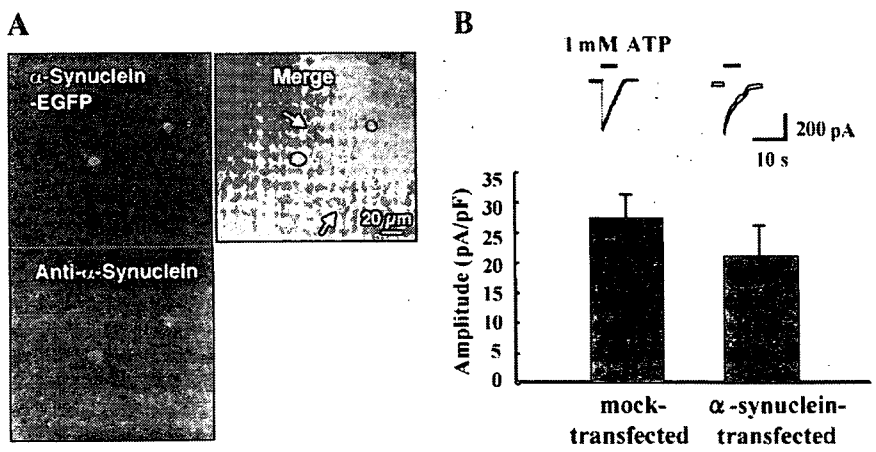

Fig. 6. The wild-type α-synuclein-transfection had no effect on ATP-induced currents. **A**: α-synuclein-transfected cells (EGFP; green) were strongly stained with anti-α-synuclein (red), while non-transfected cells (with arrows) were not. **B**: The amplitude of ATP-induced inward currents in mock and α-synuclein-transfected cells.

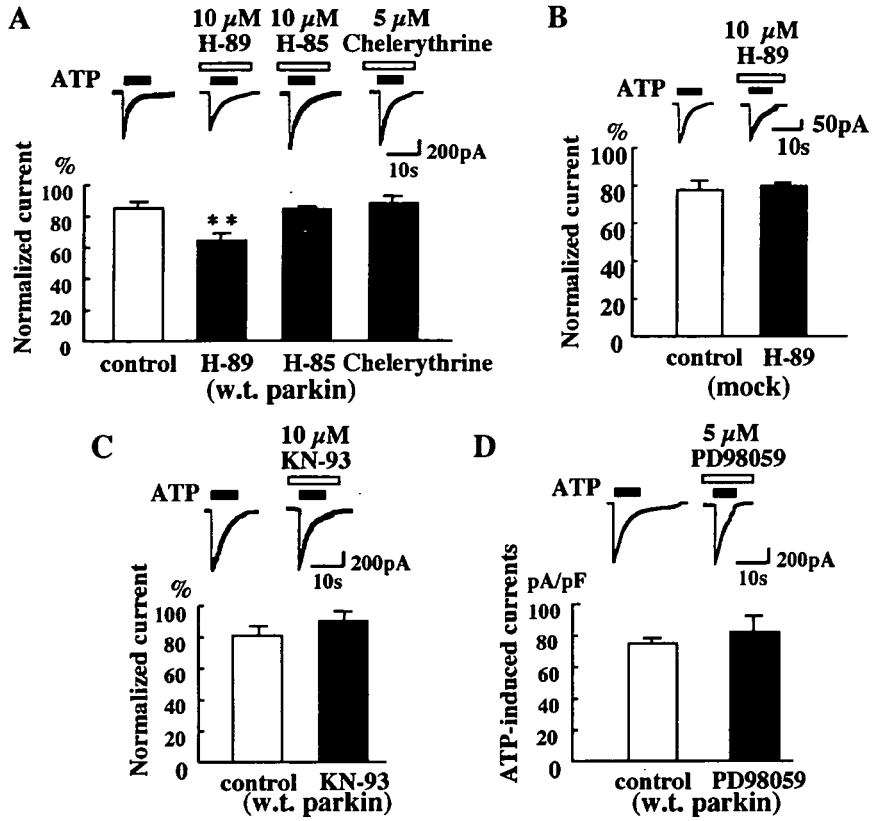

Fig. 7. Effects of kinase inhibitors on ATP-induced currents. **A**: In wild-type parkin-transfected cells, ATP-induced currents were attenuated by pre-application of 10 μM H-89, a PKA inhibitor, but not either by 10 μM H-85, an inactive analog of H-89, or 5 μM chelerythrine, a PKC inhibitor, for 10 min. **B**: H-89 had no effect on ATP-currents in control (mock-transfected) cells. **C, D**: In wild-type parkin-transfected cells, ATP-induced currents were not affected by application of 10 μM KN-93, a CaMKII inhibitor, for 20 min (C), or by treatment with 5 μM PD98059, a MAPKK inhibitor, for 4 days during differentiation (D). $^{**}P < 0.01$.

before application of roscovitine (102.1 ± 3.5% (n = 4); control without roscovitine; 85.3 ± 4.0% (n = 4)) (Fig. 8A). The result suggested that PKA activity in parkin-transfected cells was negatively regulated by the phosphorylation of DARPP-32 at Thr-75 by CDK5.

Activation of PKA also influenced on protein phosphatases relating to DARPP-32 (Nishi et al., 2000). The phosphorylation of DARPP-32 at Thr-34 has been reported to inhibit protein phosphatase-1 (PP-1), leading to an apparent increase in substrate-phosphorylation. On the other hand, PKA activates protein phosphatase-2A (PP-2A), causing dephosphorylation of DARPP-32 at Thr-75, activating PKA in turn. To investigate the role of PP-1 and PP-2A in parkin-transfected cells, we applied 100 nM okadaic acid, an inhibitor for both PP-1 and PP-2A, for 20 min. The normalized currents were augmented to 98.7 ± 4.5% (n = 5) (control without okadaic acid; 81.2 ± 4.6% (n = 4) (Fig. 8B). These results suggested that the function of PP-1 was superior to that of PP-2A in parkin-transfected cells.

The effects of CDK5 inhibitor and okadaic acid on ATP-induced currents were tested in mock-transfected cells as well. In mock-transfected cells, application of 10 μM roscovitine for 10 min had no effect on the ATP-induced inward current (roscovitine, 82.5 ± 5.2% (n = 3); control; 77.6 ± 5.2% (n = 3)) (Fig. 8C). Similarly, application of 100 nM okadaic acid for 20 min did not affect the ATP-induced currents in mock-transfected cells, (76.5 ± 3.5% (n = 3); control; 80.0 ± 4.7% (n = 3)) (Fig. 8D).

Phosphorylation of DARPP-32 in parkin-transfected PC12 Tet-Off cells

To investigate whether or not the phosphorylation of DARPP-32 at Thr-34 or Thr-75 was modified by parkin, cells were immunostained using specific antibodies for DARPP-32 (phospho Thr-34 or phospho Thr-75). The staining of phospho Thr-34 in parkin-transfected cells were not enhanced as expected from the activation of PKA (Nishi et al., 2000) but rather attenuated (Fig. 9A). While phospho Thr-75 looked similar between parkin-transfected cell and non-transfected cells in the same field (Fig. 9B).

DISCUSSION

To understand the functional role of parkin in the central nervous system (CNS), it is important to know whether parkin has any effects on ion channels and receptors that are the basic elements of neurotransmission. To test this, we used PC12 cells and overexpressed parkin protein (Fig. 1A). These show well-developed inward current response to stimulation of P2X receptors by ATP (Nakazawa et al., 1994) and we recently reported enhancement of these currents by ubiquitin C-terminal hydrolase L1 (UCH-L1) (Manago et al., 2005). In the present experiments, we have studied the effects of overexpressing of parkin on these currents.

Parkin produced a very substantial increase in the maximum ATP-induced current without significant change in sensitivity to ATP (Figs. 1 and 3). This did not appear to be due to an increased number of $P2X_2$,

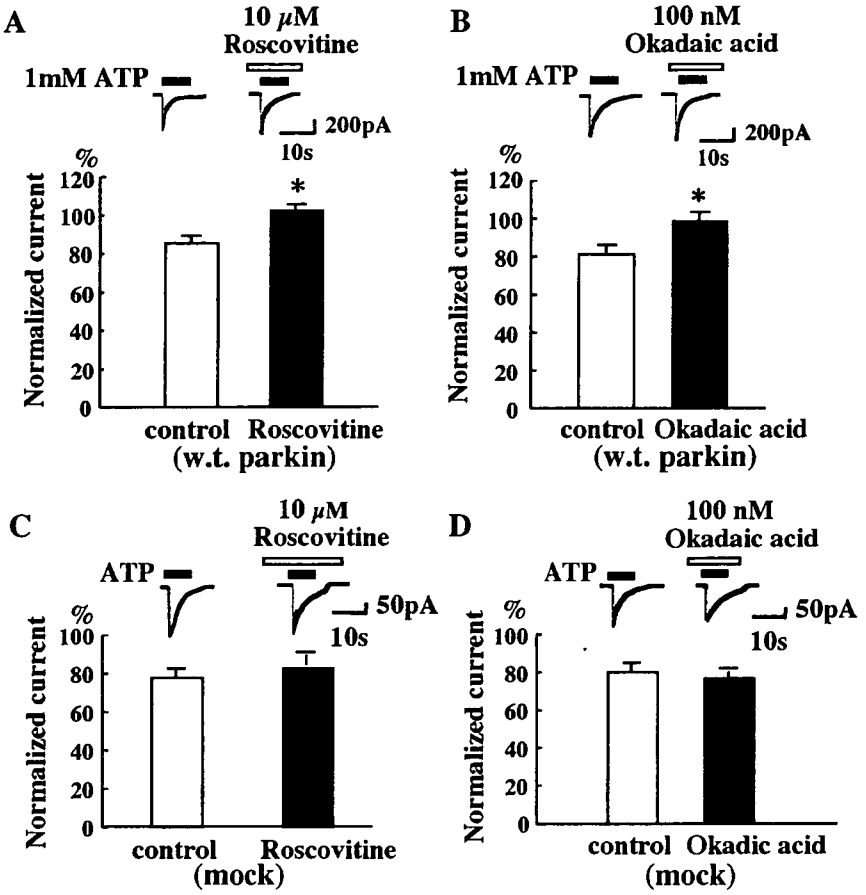

Fig. 8. Involvement of DARPP-32-relating protein kinase and protein phosphatase on ATP-induced currents. In wild-type parkin-transfected cells, ATP-induced currents were augmented by pre-application of roscovitine, a CDK5 inhibitor, for 10 min (**A**) or 100 nM okadaic acid, a protein phosphatase inhibitor, for 20 min (**B**). In mock-transfected cells, ATP-induced currents were not affected by 10 μM roscovitine (**C**) or 100 nM okadaic acid (**D**). $^{**}P < 0.05$.

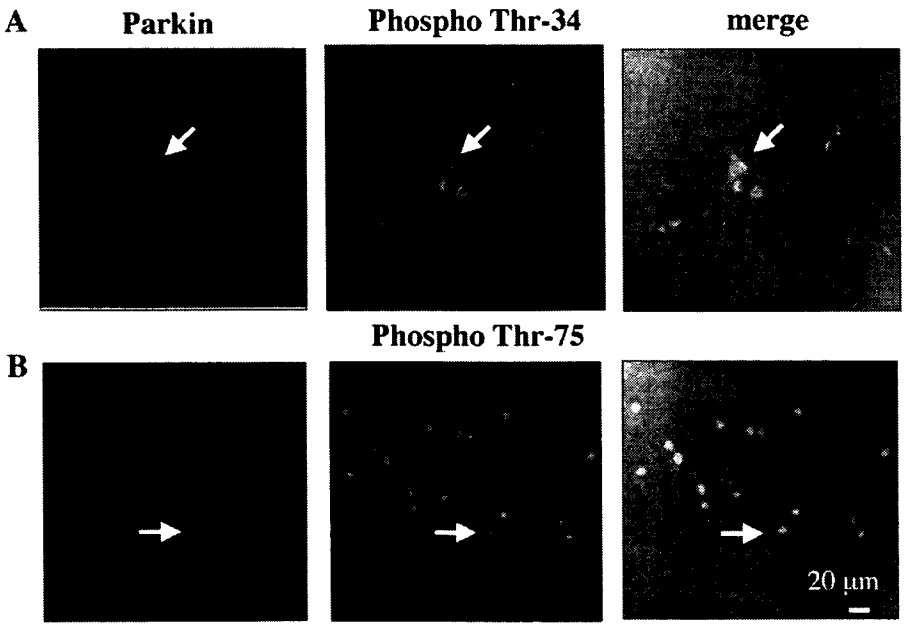

Fig. 9. Parkin did not increase the phosphorylation of DARPP-32. **A**: Immunostaining of phospho Thr-34 (red) looked rather smaller in parkin-transfected cell (yellow-green; white arrow). **B**: Immunostaining of phospho Thr-75 (red) looked similar between parkin-transfected cell (white arrow) and non-transfected cells. The merged images also include differential interference contrast images.

$P2X_4$, or $P2X_6$ receptors, as judged by immunocytochemistry (Fig. 4). Therefore, the mechanism seems to involve an increase in gating of the receptors, rather than increased affinity or receptor number. Enhancement of P2X receptor at presynaptic terminal could increase neurotransmitter release; it was reported that ischemia-induced facilitation of glutamate release was due to the activation of P2X receptors in spiny neuron in neostriatum (Zhang et al., 2006) and our preliminary result showed that ATP increased the frequency of miniature inhibitory postsynaptic potential (mIPSP) in acutely isolated neuron from substantia nigra, suggesting increased release of GABA from presynaptic terminal (unpublished data).

The enhancement of ATP-induced currents seemed to be associated with the ubiquitin ligase activity of parkin since it was not reproduced by a ligase-deficient mutant (Figs. 2C and 3). Involvement of the ubiquitin–proteasome system would accord with our previous observations with the ubiquitin hydrolase UCH-LI, though in this case hydrolase activity itself was not required since the effect of UCH-LI was replicated by a hydrolase-deficient construct. Instead, upregulation of mono-ubiquitin (Osaka et al., 2003) and ubiquitin ligase activity of UCH-L1 (Liu et al., 2002) might be responsible for the potentiation of ATP-induced currents.

Though the precise mechanism how ubiquitin ligase activity of parkin is involved is not known yet, possible signaling leading to enhancement of the ATP-induced currents is summarized in Fig. 10. It is only a part of the mechanism revealed in the present investigation, because inhibition of PKA, CDK5 or phosphatases resulted in only partial (±20%) inhibition of the parkin-potentiated currents which showed threefold increase in amplitude compared to control.

At least, part of the increase in ATP-induced currents appeared to result from activation of PKA directly or indirectly, because PKA inhibitor partially attenuated parkin-induced potentiaion of ATP-currents (Fig. 7A). In a reverse way, activation of PKA by forskolin augmented the ATP-induced currents in mock-transfected cells (Manago et al., 2005). One possible mechanism would be phosphorylation of P2X receptors by PKA. It was reported that activation of PKA potentiated ATP-evoked current in $P2X_4$-transfected HEK293 cells (Brown et al., 2004), while there was an opposite result in $P2X_2$-transfected HEK293 cells (Chow and Wang, 1998). CaMKII could be activated by PKA indirectly via an inhibition of PP-1 (Winder and Sweatt, 2001), but KN-93 did not have any effect on ATP-induced currents in parkin-transfected cells (Fig. 7C), suggesting that CaMKII was not significantly activated by parkin.

Since the enhancement was not completely reversed by inhibition of PKA, other mechanisms must be involved. One such mechanism might be through modification of DARPP-32. In rat striatum, it has been suggested that there is positive and negative feedback regulation of DARPP-32 via activation of PKA and CDK5, respectively (Nishi et al., 2000). Since DARPP-32 was expressed in PC12 cells (Manago et al., 2005), its possible involvement was tested using roscovitine, a CDK5 inhibitor, and okadaic acid, a protein phosphatase (PP-1 and PP-2A) inhibitor. Roscovitine further enhanced the ATP-induced currents in parkin-transfected cells (Fig. 8A), suggesting a negative-feedback role for CDK5. It seemed likely that parkin stimulated CDK5 since roscovitine did not have significant effect on mock-transfected cells (Fig. 8C). On the other hand, a role for phosphatases was suggested by the fact that okadaic acid further enhanced the ATP-induced

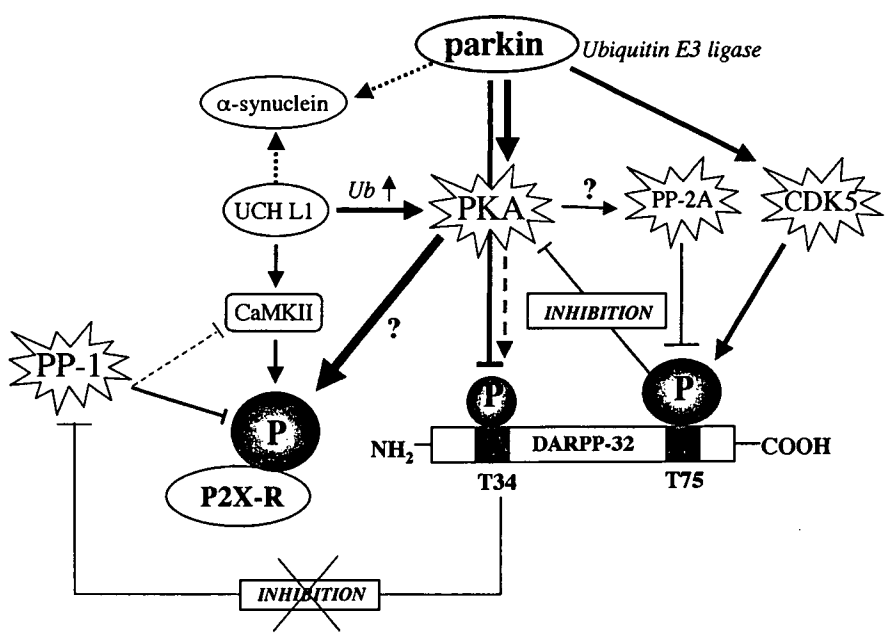

Fig. 10. Predicted signaling induced by expression of parkin. Parkin may activate PKA, subsequently phosphorylating P2X receptors. Parkin also may activate protein phosphatase-1 (PP-1) via inhibiting phosphorylation of DARPP-32 at Thr-34. On the other hand, phosphorylation and dephosphorylation of DARPP-32 at Thr-75 by CDK5 and PP-2A could compete with each other, keeping the level of phospho Thr-75 unchanged. As additional information, ubiquitin C-terminal hydrolase (UCH-L1) also activates PKA, which is independent on hydrolase activity but presumably due to the increase in mono-ubiquitin level (Manago et al., 2005) or ubiquitin ligase activity (Liu et al., 2002). Unlike parkin, UCH-L1 activates Ca^{2+} and calmodulin-dependent protein kinase (CaMKII), which could be indirectly activated by PKA and dephosphorylated by PP-1 (Winder and Sweatt, 2001). The effect of α-synuclein, a substrate for both parkin and UCH-L1 (dotted line), was not clear, because transfection of α-synuclein did not affect ATP-induced currents. Reported signaling but not confirmed in the present study was shown by dotted line.

currents in parkin-transfected cells (Fig. 8B). Since inhibition of PP-2A was supposed to inhibit PKA activity (Nishi et al., 2000; Manago et al., 2005), it seemed likely that this enhancement resulted mainly from inhibition of PP-1. In mock-transfected cells, okadaic acid did not have significant effect (Fig. 8D).

As for the phosphorylation of DARPP-32, activation of PKA would phosphorylate DARPP-32 at Thr-34 (Nishi et al., 2000). However, the staining of phospho Thr-34 was rather attenuated in parkin-transfected cells (Fig. 9A), suggesting that parkin may have inhibitory effect on the phosphorylation site at Thr-34. Therefore, parkin might indirectly activate PP-1, canceling the negative feedback from phospho Thr-34. Concerning the phosphorylation of DARPP-32 at Thr-75, CDK5, and PP-2A were supposed to have opposite effects, keeping the same level of phospho Thr-75 (Fig. 9B).

Both UCH-L1 and parkin can operate via α-synuclein as a target substrate (Shimura et al., 2001; Snyder and Wolozin, 2004). It has recently been shown that UCH-L1, parkin, and α-synuclein form lysine 63-linked multiubiquitin chains, which induce proteasomal-independent ubiquitination (Doss-Pepe et al., 2005; Lim et al., 2005). Therefore, it was possible that α-synuclein also had potentiating effect on P2X receptors if lysine 63-linked multiubiquitin was involved. However, α-synuclein did not have such effect (Fig. 6). It will be great interest to investigate the relationship between these three proteins and it may help to understand why parkin deficient-mice are not a robust model of parkinsonism (Perez and Palmiter, 2005), though there were alterations in energy metabolism, protein handling, and synaptic function (Periquet et al., 2005).

Another interesting point is that the signaling between activation of PKA and potentiation of P2X receptors induced by either UCH-L1 or parkin was not the same. For example, UCH-L1 but not parkin activated CaMKII and PP-2A whereas parkin but not UCH-L1 seemed to activate CDK5, producing a negative feedback effect on PKA (Fig. 10). In addition, we found that DARPP-32 (phospho Thr-34) was rather attenuated in spite of the report that activation of PKA increased the phosphorylation at Thr-34 (Nishi et al., 2000). The difference between UCH-L1 and parkin might due to the different substrate specificity as ubiquitin ligases.

Unfortunately, the low transfection efficiency precluded direct biochemical studies on the phosphorylation or dephosphorylation of specific proteins by parkin or UCH-L1. As a result, we have been restricted to pharmacological and immunocytochemical analyses. Nevertheless, the important point we have established is that enzymes working in the ubiquitin–proteasome system have clear and substantial effects on a neurotrasmitter receptor and hence subsequently may affect neurotransmission in vivo. It is widely accepted that there are number of diseases related to aberrations in the ubiquitin system (Ciechanover and Schwartz, 2004), but how aberrations in the ubiquitin system cause neurodegenrative diseases such as Parkinson's disease (PD) is largely unknown. In the present study, one of the ubiquitin ligases, parkin, potentiated the function of P2X receptors, as well as another enzyme working in the ubiquitin–proteasome system, UCH-L1. Presynaptic P2X receptors triggers Ca^{2+}-dependent glutamate release in the brainstem (Shigetomi and Kato, 2004), though ATP-mediated inhibition of dopamine release was reported in rat neostriatum (Trendelenburg and Bultmann, 2000). It is of great interest how endogenous parkin or UCH-L1 modulates neurotransmitter release by stimulating P2X receptors in vivo, which is now under investigation.

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Degradation of Amyotrophic Lateral Sclerosis-linked Mutant Cu,Zn-Superoxide Dismutase Proteins by Macroautophagy and the Proteasome*[S]

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Mutations in the Cu,Zn-superoxide dismutase (SOD1) gene cause ~20% of familial cases of amyotrophic lateral sclerosis (fALS). Accumulating evidence indicates that a gain of toxic function of mutant SOD1 proteins is the cause of the disease. It has also been shown that the ubiquitin-proteasome pathway plays a role in the clearance and toxicity of mutant SOD1. In this study, we investigated the degradation pathways of wild-type and mutant SOD1 in neuronal and nonneuronal cells. We provide here the first evidence that wild-type and mutant SOD1 are degraded by macroautophagy as well as by the proteasome. Based on experiments with inhibitors of these degradation pathways, the contribution of macroautophagy to mutant SOD1 clearance is comparable with that of the proteasome pathway. Using assays that measure cell viability and cell death, we observed that under conditions where expression of mutant SOD1 alone does not induce toxicity, macroautophagy inhibition induced mutant SOD1-mediated cell death, indicating that macroautophagy reduces the toxicity of mutant SOD1 proteins. We therefore propose that both macroautophagy and the proteasome are important for the reduction of mutant SOD1-mediated neurotoxicity in fALS. Inhibition of macroautophagy also increased SOD1 levels in detergent-soluble and -insoluble fractions, suggesting that both detergent-soluble and -insoluble SOD1 are degraded by macroautophagy. These findings may provide further insights into the mechanisms of pathogenesis of fALS.

Amyotrophic lateral sclerosis (ALS)[2] is a neurodegenerative disease caused by selective loss of motor neurons (1, 2). Although most cases of ALS are sporadic, ~10% of ALS cases run in families. Dominant missense mutations in the gene that encodes the Cu,Zn-superoxide dismutase (SOD1) are responsible for 20% of familial ALS (fALS) cases (3). Mice overexpressing mutant SOD1 develop an ALS-like phenotype comparable with human ALS, whereas mice lacking SOD1 do not (4, 5). These findings have led to the conclusion that SOD1 mutants cause motor neuron degeneration by a toxic gain of function. Thus, studies of the degradation process of mutant SOD1 proteins could provide important insights into understanding the mechanisms that underlie the pathology of fALS, and possibly sporadic ALS, and into developing novel therapies for fALS by removing toxic species of mutant SOD1.

Cytoplasmic proteins are mainly degraded by two pathways, the ubiquitin-26 S proteasome pathway (6) and autophagy (7). Previous studies have shown that mutant SOD1 proteins are turned over more rapidly than wild-type SOD1, and a proteasome inhibitor increases the level of mutant SOD1 proteins (8, 9). Dorfin and NEDL1, two distinct ubiquitin ligases, ubiquitinate mutant but not wild-type SOD1 (10, 11). These observations suggest that mutant SOD1 is degraded by the ubiquitin-26 S proteasome pathway and that the increased turnover of mutant SOD1 is mediated in part by this pathway. On the other hand, the 20 S proteasome, a component of the 26 S proteasome, can degrade proteins without a requirement for ubiquitination (12, 13). A recent study has found that metal-free forms of wild-type and mutant SOD1 are degraded by the 20 S proteasome *in vitro* (14).

Autophagy is an intracellular process that results in the degradation of cytoplasmic components inside lysosomes. At least three forms of autophagy have been described in mammalian cells: macroautophagy, microautophagy, and chaperone-mediated autophagy (7). Macroautophagy is the major and the most well studied form of autophagy; this process begins with a sequestration step, in which cytosolic components are engulfed by a membrane sac called the isolation membrane. This membrane results in a double membrane structure called the autophagosome, which fuses with the lysosome. The inner membrane of the autophagosome and its protein and organelle contents are degraded by lysosomal hydrolases. Recent reports have demonstrated that macroautophagy plays an important role in preventing neurodegeneration in mice (15, 16). Although macroautophagy can be induced by starvation, this

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[2] The abbreviations used are: ALS, amyotrophic lateral sclerosis; fALS, familial ALS; SOD1, Cu,Zn-superoxide dismutase(s); 3-MA, 3-methyladenine; siRNA, short interfering RNA; EGFP, enhanced green fluorescent protein; HA, hemagglutinin; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium.

pathway may take place constitutively in mammals (17). In cultured cells, inhibition of macroautophagy does not alter enhanced green fluorescent protein (EGFP) levels (18) or glyceraldehyde-3-phosphate dehydrogenase protein levels,[3] suggesting that not all cytosolic proteins are degraded by macroautophagy. To date, however, there have been no reports of macroautophagy in mutant SOD1 clearance.

In this study, we investigated the pathway by which human wild-type SOD1 and the A4V, G85R, and G93A SOD1 mutants are degraded in neuronal and nonneuronal cells. We show that wild-type and mutant SOD1 proteins are degraded by both the proteasomal pathway and macroautophagy. The experiments with inhibitors of these degradation pathways suggested that mutant SOD1 are degraded more rapidly than wild-type SOD1 in part by macroautophagy and that the contribution of macroautophagy to mutant SOD1 clearance is approximately equal to that of the proteasome pathway. Macroautophagy decreases mutant SOD1 protein levels in both nonionic detergent-soluble and -insoluble fractions. In addition, we provide data indicating that macroautophagy has a role in mutant SOD1-mediated cell death.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The expression plasmids pcDNA3-hSOD1 containing wild-type, A4V, G85R, and G93A mutant SOD1 were kindly donated by Ryosuke Takahashi (Kyoto University, Kyoto, Japan) and Makoto Urushitani (Laval University, Quebec, Canada) (19). To construct a plasmid expressing human wild-type SOD1 with the HA tag at the carboxyl terminus of SOD1, HA-tagged SOD1 fragments were amplified by PCR using wild-type SOD1 cDNA (Open Biosystems, Huntsville, AL) as the template. The PCR products were digested with XhoI and NotI and cloned into an XhoI-NotI-digested pCI-neo vector (Promega, Madison, WI). The primers used were 5′-AAAACTCGAGCCGCCAAGATGGCGACGAAGGCCGTGTGCG-3′ and 5′-AAAAGCGGCCGCTTAAGCGTAATCTGGAACATCGTATGGGTATTGGGCGATCCCAATTACACCACA-3′. A plasmid expressing HA-tagged G93A SOD1 was generated using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. To construct a plasmid expressing fusion protein of green fluorescent protein and LC3, LC3 fragments were amplified by PCR using rat LC3 cDNA (Open Biosystems) as the template. The PCR products were digested with BglII and EcoRI and cloned into a BglII-EcoRI-digested pEGFP-C1 vector (Clontech). The primers used were 5′-ACTCAGATCTATGCCGTCCGAGAAGACCTTCAAA-3′ and 5′-TGCAGAATTCTTACACAGCCAGTGCTGTCCCGAA-3′. After construction, the DNA sequences of the plasmids were confirmed by DNA sequence analysis.

Cell Culture and Transfection—The mouse neuroblastoma cell line Neuro2a, the human neuroblastoma cell line SH-SY5Y, and the monkey kidney-derived cell line COS-7 were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum (JRH Biosciences, Lenexa, KS). Transient expression of each vector in Neuro2a and COS-7 cells was performed using the FuGENE 6 transfection reagent (Roche Applied Science). For experiments with differentiated Neuro2a cells, the medium was changed to differentiation medium (Dulbecco's modified Eagle's medium supplemented with 1% fetal calf serum and 20 μM retinoic acid) 24 h after transfection. Approximately 90% of cells in dishes (wells) were transfected in our experimental conditions (data not shown), and there was no notable differences in the transfection efficiency among the wells (supplemental Fig. S1).

Treatment of Cells with Epoxomicin, 3-Methyladenine, Cycloheximide, Rapamycin, or NH_4Cl—Cells grown in 12- or 6-well plates to 50–80% confluence were transfected with expression plasmids containing wild-type, A4V, G85R, or G93A mutant SOD1. 24 h after transfection, cells were incubated with epoxomicin (10 nM, 1 μM, 5 μM, or 10 μM; Sigma), 3-methyladenine (3-MA) (10, 20, or 30 mM; Sigma), rapamycin (100 or 200 nM; Sigma), 20 mM NH_4Cl, and/or carrier (Me_2SO or water) as a control. In some experiments, 10 μg/ml cycloheximide (Sigma) was added to the cells to avoid the confounding effects of ongoing protein synthesis. Epoxomicin, cycloheximide, and rapamycin were dissolved in Me_2SO, NH_4Cl in water. 3-MA was freshly dissolved in culture medium 30 min before use.

Cell Fractionation—For preparation of nonionic detergent-soluble and -insoluble fractions, adherent cells were harvested and lysed on ice for 15 min in 1% Triton X-100 lysis buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and protease inhibitors (Complete, EDTA-free; Roche Applied Science). Lysates were centrifuged at 20,000 × *g* for 10 min at 4 °C, and the supernatants were pooled and designated as the detergent-soluble fractions. After the pellets were washed with 1% Triton X-100 lysis buffer, they were solubilized with SDS buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 3% SDS, 1% Triton X-100, and protease inhibitors) and sonicated. The resulting solution was used as the detergent-insoluble fraction. For preparation of total cell lysates containing both detergent-soluble and -insoluble fractions, cells were lysed in SDS buffer and sonicated. Protein concentrations were determined with the protein assay kit (Bio-Rad) or the DC protein assay kit (Bio-Rad).

Western Blot Analysis—Western blotting was performed using standard procedures as described previously (20). The primary antibodies used were as follows: anti-SOD1 rabbit polyclonal antibody (1:4000; Stressgen Bioreagents, Victoria, Canada), anti-α-tubulin mouse monoclonal antibody (1:4000; Sigma), anti-β-actin mouse monoclonal antibody (1:5000; Sigma), anti-HA mouse monoclonal antibody (1:4000; Sigma), anti-Beclin 1 mouse monoclonal antibody (1:500; BD Transduction Laboratories, San Diego, CA), anti-Apg7/Atg7 rabbit polyclonal antibody (1:500; Rockland, Gilbertsville, PA). After overnight incubation with primary antibodies at 4 °C, each blot was probed with horseradish peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (1:20,000; Pierce). Immunoreactive signals were visualized with SuperSignal West Dura extended duration substrate (Pierce) or SuperSignal West Femto maximum sensitivity substrate (Pierce) and detected with a chemiluminescence imaging system (FluorChem; Alpha Innotech, San Leandro, CA). The signal intensity was quantified by densitometry using FluorChem software (Alpha Innotech).

Short Interfering RNA (siRNA) Preparation and Transfection—Double-stranded siRNA targeting mouse Beclin 1, mouse Atg7 and EGFP were purchased from RNAi Co., Ltd.

[3] T. Kabuta, Y. Suzuki, and K. Wada, unpublished data.

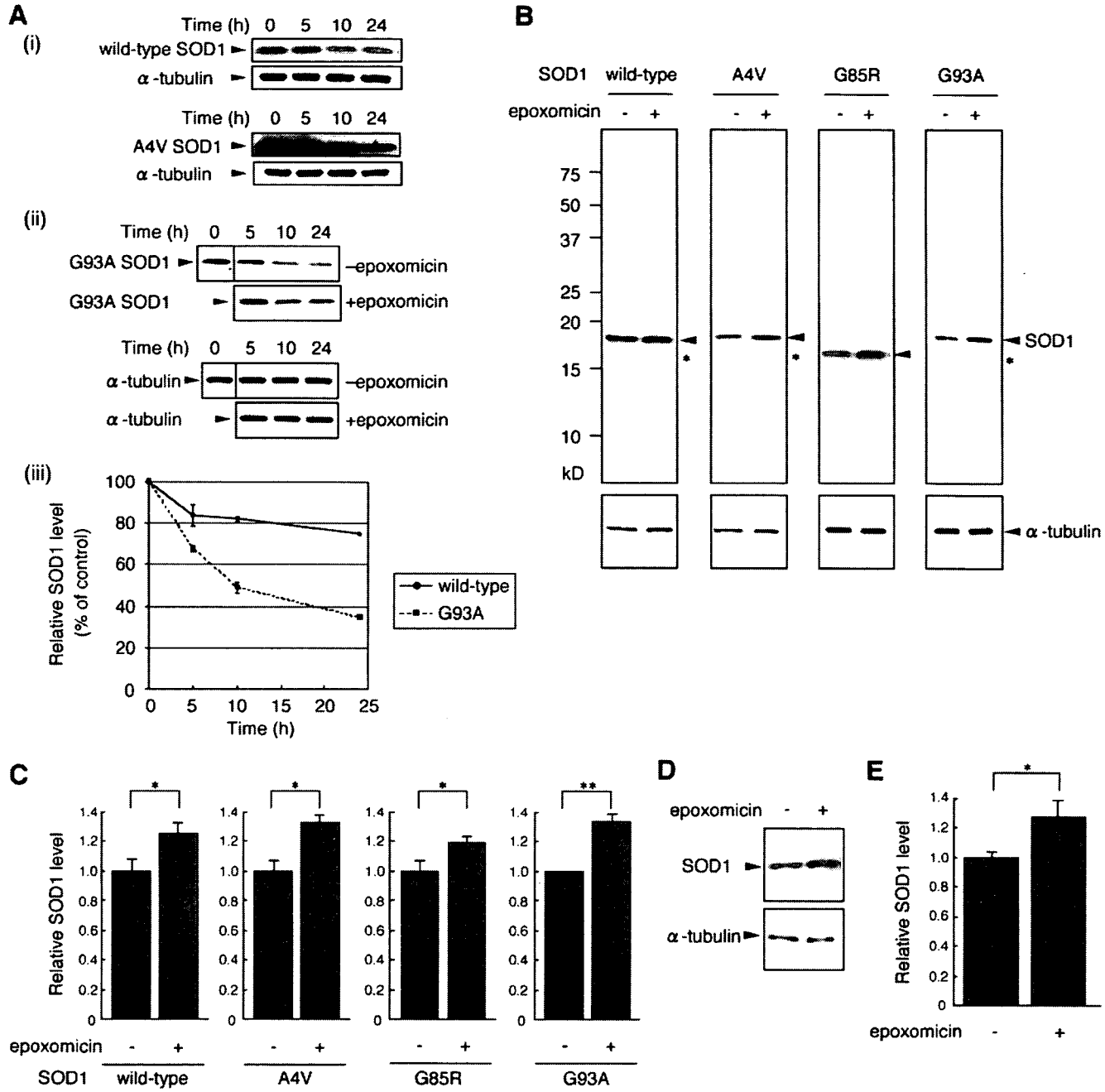

FIGURE 1. **Both mutant and wild-type SOD1 are degraded by the proteasome.** *A, i,* Neuro2a cells were transiently transfected with wild-type or mutant A4V human SOD1. 24 h after transfection, cells were treated with 10 μg/ml cycloheximide for the indicated time and lysed. Total cell lysates were analyzed by immunoblotting using anti-SOD1 or anti-α-tubulin antibody. *ii,* Neuro2a cells transfected with G93A SOD1 were incubated with or without 10 nM epoxomicin in the presence of 10 μg/ml cycloheximide for the indicated time and lysed. Total cell lysates were analyzed by immunoblotting using anti-SOD1 or anti-α-tubulin antibody. *iii,* the relative levels of wild-type or G93A SOD1 (percentage of 0-h control) were quantified by densitometry. Mean values are shown with S.E. ($n = 3$). *B* and *C*, Neuro2a cells were transiently transfected with wild-type or mutant A4V, G85R, or G93A human SOD1. 24 h after transfection, cells were incubated with or without 10 nM epoxomicin in the presence of 10 μg/ml cycloheximide for 24 h. Total cell lysates were analyzed by immunoblotting using anti-SOD1 antibody. The electrophoretic mobility of G85R SOD1 was greater than that of wild-type SOD1. α-Tubulin was used as a loading control. *Asterisks* indicate endogenous mouse SOD1 (*B*). The relative level of wild-type or mutant SOD1 was quantified by densitometry. Mean values are shown with S.E. ($n = 3$). *, $p < 0.05$; **, $p < 0.01$ (*C*). *D* and *E*, human SH-SY5Y cells were incubated with or without 10 nM epoxomicin in the presence of cycloheximide for 24 h. Total cell lysates were analyzed by immunoblotting with anti-SOD1 antibody (*D*). The relative level of human endogenous SOD1 was quantified by densitometry. Data are expressed as the means ± S.E. ($n = 3$). *, $p < 0.05$ (*E*).

(Tokyo, Japan). Sequences targeted by siRNA were selected using siDirect (RNAi Co., Ltd.): mouse Beclin 1 siRNA, sense (5′-GUCUACAGAAAGUGCUAAUAG-3′) and antisense (5′-AUUAGCACUUUCUGUAGACAU-3′); mouse Atg7 siRNA, sense (5′-GAGCGGCGGCUGGUAAGAACA-3′) and antisense (5′-UUCUUACCAGCCGCCGCUCAA-3′); EGFP siRNA, sense (5′-GCCACAACGUCUAUAUCAUGG-3′) and antisense (5′-AUGAUAUAGACGUUGUGGCUG-3′). EGFP siRNA was used as a control. Cells (3×10^5) were cotransfected with 1 μg of DNA and 3 μg of siRNA using Lipofectamine PLUS reagent (Invitrogen).