る。JaCALS 推進のために、日本版 ALSFRS-R 電話調査について検証を行った。

A. 研究目的

JaCALS における基礎資料として、CRC による日本語版 ALSFRS-R 電話調査の検証を行う。

B. 研究方法

CRC 業務マニュアルにおいて、電話調査における話し方、記録の仕方とレポート作成法などについて定めた。また ALSFRS-R の各項目について、点数を定めるフローチャートを作成し、これを見ながら電話をすることとした。担当 CRC に対して、ALS の臨床像、疫学、病態、治療、ALS 患者および介護者に対して行うべき配慮、JaCALS の背景、目的、関連する倫理指針および電話調査にあたっての具体的な手順に関する研修を実施した。

名古屋大学神経内科において、JaCALS の登録を行った 27 例の ALS 患者について、医師が診察室で直接本人を診察して記載した ALSFRS-R スコア（医師スコア）と CRC が電話調査で点数を定めた ALSFRS-R スコア（電話スコア）の比較検討を行った。

ALSFRS-R 総点の相関について Pearson 相関係数を算出し、各項目(Table 1)についてκ統計量による一致性の検討を行った。統計解析には Microsoft Excel 2002 SP3 および Dr.SPSS II for Windows (SPSS Japan Inc. Tokyo Japan)を用いた。

倫理面への配慮

すべての解析対象の患者から、JaCALS 参加について十分な文書でのインフォームドコンセントを得た。JaCALS 研究計画および説明書・同意書は名古屋大学医学部倫理委員会の承認を得た。

C. 研究結果

解析対象患者 27 例の性別は男性 18 人、女性 9 人、年齢は 63.7±10.6 (平均±S.D.)歳、罹病期間は 3.0±2.3 (平均±S.D.)年、電話調査に対して、本人が回答したのが 13 名、主介護者が回答したのが 14 名であった。

医師スコアと電話スコア総点の散布図と回帰直線を Figure1 に示す。相関係数は 0.967(Pearson)($p<0.001$)と良好な相関を示した。電話調査に本人が回答した例では相関係数 0.941、主介護者が回答した例では相関係数 0.961 といずれも良好な相関を示した。各項目のκ統計量を Table2 に示す。κ統計量は 1 の場合完全な一致を示し、0.75 以上で excellent agreement、0.4 以上 0.75 未満で fairly to good agreement、0.4 未満で poor agreement と判定される。今回のκ統計量は 0.59 から 0.88 の間で分布し、良好な一致を示した。

D. 考察

日本版 ALSFRS-R について、患者と直接対面した評価での検者間の一致が検討[2]されている。この報告では総点の相関係数は 0.97 であり、各項目のκ統計量は 0.53 から 1.0 の間で分布し、κの平均は 0.67 であった。今回の電話調査の妥当性検討ではκの平均は 0.73 となり、総点の

相関係数（0.965)と合わせて直接対面での検者間の一致と比較しても遜色のないレベルの一致を示した。アメリカ合衆国における ALSFRS-R 電話調査の報告 [3]における診察室でのスコア総点と電話調査でのスコア総点間の相関係数も 0.966 と類似した値であった。

これらの値からは、JaCALS における ALSFRS-R 電話調査は十分な信頼性を持つと考えられる。

E. 結論

JaCALS での手順書、マニュアル、教育講習に基づいて実施された CRC による ALSFRS-R 日本版電話調査は、十分な信頼性があるものと認められる。

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H. 知的財産権の出願・登録状況　なし

Figure 1.　ALSFRS-R　医師スコアー電話スコア　散布図

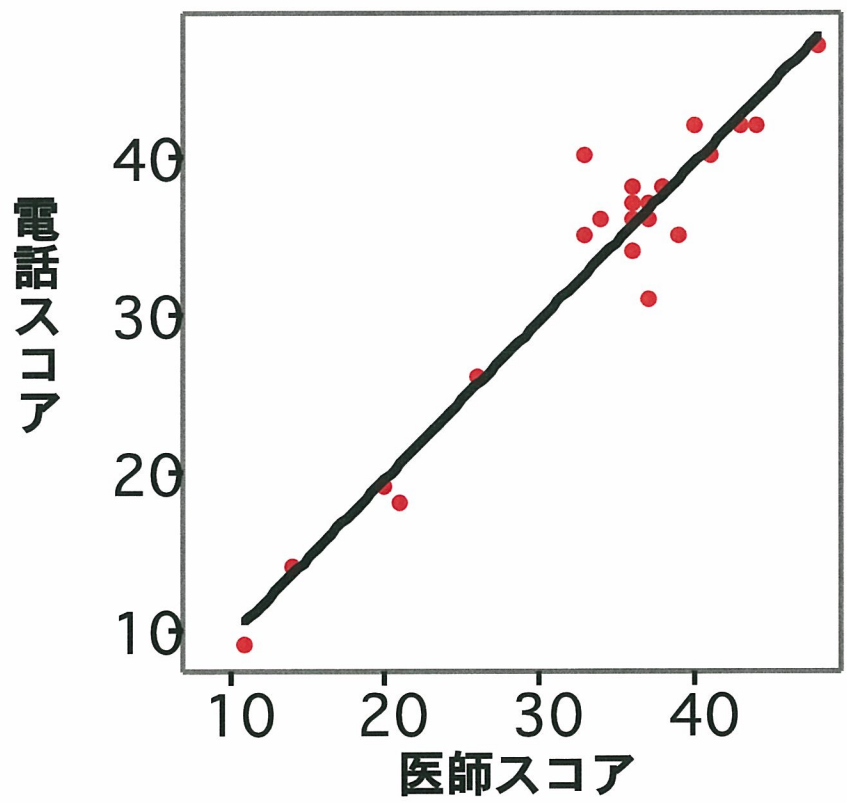

Table1.　日本語版 ALSFRS-R

言語
4　会話は正常
3　会話障害が認められる
2　繰り返し聞くと意味がわかる
1　声以外の伝達手段と会話を併用
0　実用的会話の喪失

唾液分泌
4　正常
3　口内の唾液はわずかだが、明らかに過剰（夜間はよだれが垂れることがある）
2　中等度に過剰な唾液（わずかによだれが垂れることがある）
1　顕著に過剰な唾液（よだれが垂れる）
0　著しいよだれ（絶えずティッシュやハンカチを必要とする）

嚥下
4　正常な食事習慣
3　初期の摂食障害（時に食物を喉につまらせる）
2　食物の内容が変化（継続して食べられない）
1　補助的なチューブ栄養を必要とする
0　全面的に非経口性または腸管性栄養

書字
4　正常
3　遅い、または書きなぐる（すべての単語が判読可能）
2　一部の単語が判読不可能
1　ペンは握れるが、字を書けない
0　ペンが握れない

摂食動作（胃瘻設置の有無により（1）、（2）のいずれか一方で評価する）
（1）食事用具の使い方（胃瘻設置なし）
4　正常
3　幾分遅く、ぎこちないが、他人の助けを必要としない
2　フォークは使えるが、はしは使えない
1　食物は誰かに切ってもらわなくてはならないが、何とかフォークまたはスプーンで食べる事ができる
0　誰かに食べさせてもらわなくてはいけない

（2）指先の動作（胃瘻設置患者）
4　正常
3　ぎこちないが全ての手先の作業ができる
2　ボタンやファスナーを留めるのにある程度手助けが必要
1　看護者にわずかに面倒をかける
0　全く何もできない

着衣、身のまわりの動作
4　正常
3　努力して（あるいは効率が悪いが）独りで完全にできる
2　時折手助けまたは代わりの方法が必要
1　身の周りの動作に手助けが必要
0　全面的に他人に依存

寝床での動作
4　正常
3　幾分遅く、ぎこちないが助けを必要としない
2　独りで寝返りをうったり、寝具を整えられるが非常に苦労する
1　寝返りを始めることはできるが、独りで寝返りをうったり、寝具を整えることができない
0　自分ではどうすることもできない

歩行
4　正常
3　やや歩行が困難
2　補助歩行
1　歩行は不可能
0　脚を動かすことができない

階段登り
4　正常
3　遅い
2　軽度の不安定または疲労
1　介助が必要
0　登れない

呼吸（呼吸困難、起座呼吸、呼吸不全の３項目を評価）
（1）呼吸困難
4　なし
3　歩行中に起こる
2　日常動作（食事、入浴、着替え）のいずれかで起こる
1　座位または臥位のいずれかで起こる
0　極めて困難で呼吸補助装置を考慮する

（2）起座呼吸
4　なし
3　息切れのため夜間の睡眠がやや困難
2　眠るのに支えとする枕が必要
1　座位でないと眠れない
0　全く眠ることができない

（3）呼吸不全
4　なし
3　間欠的に呼吸補助装置（bipap)が必要
2　夜間に継続的に呼吸補助装置（bipap)が必要
1　1日中呼吸補助装置（bipap)が必要
0　挿管または気管切開による人工呼吸が必要

Table2.　ALSFRS-R 各項目別 κ 統計量

項目	言語	唾液分泌	嚥下	書字	摂食動作	着衣
κ	0.84	0.59	0.88	0.78	0.73	0.72

項目	寝床	歩行	階段のぼり	呼吸困難	起座呼吸	呼吸不全
κ	0.60	0.63	0.70	0.74	0.84	0.82

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分担研究報告書

パーキンソン病の非運動機能異常；頻度とQOLにおよぼす影響

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研究要旨　JaCALS で構築した臨床・遺伝データの収集システムを、加齢に伴い発症する代表的な神経変性疾患であるパーキンソン病にも応用し、非運動機能異常を含めたパーキンソン病250例の横断像の解析を行うとともにQOLに及ぼす影響を解析した。パーキンソン病では、夜間頻尿を 57%に認め、便秘、陰萎、鬱も 30%以上に認めた．非運動機能異常を認めない症例は 22%のみであり、多くの症例で複数の非運動機能異常を認めた．非運動機能異常を有する数が多いほど QOL スコアは悪化した($r = 0.678$、$p < 0.0001$)．また、1 年間における QOL スコアの変化は、鬱スコアの変化と強い相関を示した($r = 0.75$、$p < 0.0001$)．パーキンソン病では多彩な非運動機能異常を認め、QOL にも大きく影響を及ぼすため、包括的な把握と、その対策が必要である．

研究背景

パーキンソン病は、加齢に伴い発症する代表的な神経変性疾患であり、我が国における有病率は 1000 人に１人と神経変性疾患の中では Alzheimer 病についで頻度が高い。80 歳以上では 100 人に１人以上とも想定されており、21 世紀の高齢化社会をむかえ、さらなる症例数の増加が予想されている。パーキンソン病では L-ドーパやドパミンアゴニストをはじめとする各種補充療法の開発・発展に伴い、その予後は一般人口と変わらないレベルに近づいたとされる。しかしながら、治療効果は経過とともに減弱し、進行例では薬効が低下し、副作用が目立つようになり、精神症状や自律神経不全といった多彩な非運動機能異常に苦しむ症例が少なくない。

パーキンソン病に伴い出現する神経症状は、パーキンソニズム以外に、幻覚、妄想、鬱、認知機能障害や、排尿障害、起立性低血圧、便秘、発汗異常を初めとする自律神経不全まで多岐に渡る [1]。こうした多彩な神経症状を外来の短い時間で定期的かつ網羅的に聞き取ることは難しい一方で、非運動機能異常が QOL に及ぼす影響の高さも指摘されており [2]、効率的な診療・研究システムを構築することはきわめて重要である。

A. 研究目的

パーキンソン病の QOL、ADL、精神症状、自律神経不全、運動合併症についてアンケートを主とした病状の聞き取り調査を行うとともに、医師による客観的な評価と合わせ、パーキンソン病の臨床

像や進行の実態を包括的に把握出来るシステム(Nagoya Parkinson's disease study group: NAPS)を構築し、そこから得られた日本人における非運動機能異常の頻度、QOL の実態を検討する。

B. 研究方法

ADL として Schwab & England scale、認知機能評価として Mini-mental State Examination (MMSE)、重症度の評価として Unified Parkinson's Disease Rating Scale (UPDRS)を測定し、患者用のアンケート冊子は、QOL の指標として Japanese PDQ 39、鬱の指標として Beck depression inventory、唾液過多、嚥下障害、感覚障害の指標は、UPDRS II、排尿障害の指標は国際前立腺症状スコア、循環器系、消化器系、体温調節、呼吸器系、下肢の浮腫はそれぞれ 1 ヶ月における出現頻度を 4 段階による問診、さらにアテネ不眠尺度（ＡＩＳ） による問診を組み込んで作成した。

統計解析は、Microsoft Excel 2002 SP3 および Dr.SPSS II for Windows (SPSS Japan Inc. Tokyo Japan)を用いた。

倫理面への配慮

すべての解析対象の患者から、NAPS 参加について十分な文書でのインフォームドコンセントを得た。NAPS 研究計画および説明書・同意書は名古屋大学医学部倫理委員会の承認を得た。

C. 研究結果

非運動機能異常の頻度において、夜間頻尿は 57%に認め、便秘、陰萎、鬱は 30%以上、頻尿、蓄尿障害、嚥下障害、幻覚、下肢の浮腫、発汗過多を 20%以上で認めた。非運動機能異常を全く有しない症例は 22%で、平均 4.4 種類の非運動機能異常を有していた。QOL に対する影響は、鬱が最も強く、次いで嚥下障害、幻覚、感覚異常、夜間頻尿などであった。また、非運動機能異常を有している数は、QOL の悪化と有意な相関を示した ($r = 0.678$、$p < 0.0001$)。また、既に 2 回目のアンケートを行った 75 例の検討では、QOL の変化率は、鬱スコアの変化率と高い相関を示した（$p = 0.75$、$p < 0.0001$)。

D. 考察

PD で認められる非運動機能異常に対する医師の認識度は、確立された調査票を用いた場合に比べて低いことが指摘されている[3)]。今回のアンケートでは、50%以上に認めた夜間頻尿、3 分の 1 以上に認めた便秘、陰萎、鬱をはじめ、PD では多彩な非運動機能異常を認め、非運動機能異常を多数有するほど QOL は低下することが明らかとなった。また、平均 1 年間の間隔で 2 回 QOL を調査出来た症例では、QOL の変化は鬱の変化と強い相関を示しており、鬱を中心とした非運動機能異常は、PD の QOL 改善のために重要で、今後、多数例での介入研究が必要と考えられる。

E. 結論

PD では多彩な非運動機能異常を認め、QOL とも密接な関連がある。

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F. 健康危険情報　なし

G. 研究発表

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H. 知的財産権の出願・登録状況

特になし。

Ⅲ. 研究成果の刊行に関する一覧

研究成果の刊行に関する一覧表

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雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Ishigaki S, Niwa J, Yamada S, Takahashi M, Ito T, Sone J, Doyu M, Urano F, Sobue G	Dorfin-CHIP chimeric proteins potently ubiquitylate and degrade familial ALS-related mutant SOD1 proteins and reduce their cellular toxicity.	Neurobiol Dis	25	331-341	2007
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Ⅳ. 研究成果の刊行物・別刷

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Dorfin-CHIP chimeric proteins potently ubiquitylate and degrade familial ALS-related mutant SOD1 proteins and reduce their cellular toxicity

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The ubiquitin–proteasome system (UPS) is involved in the pathogenetic mechanisms of neurodegenerative disorders, including amyotrophic lateral sclerosis (ALS). Dorfin is a ubiquitin ligase (E3) that degrades mutant SOD1 proteins, which are responsible for familial ALS. Although Dorfin has potential as an anti-ALS molecule, its life in cells is short. To improve its stability and enhance its E3 activity, we developed chimeric proteins containing the substrate-binding hydrophobic portion of Dorfin and the U-box domain of the carboxyl terminus of Hsc70-interacting protein (CHIP), which has strong E3 activity through the U-box domain. All the Dorfin-CHIP chimeric proteins were more stable in cells than was wild-type Dorfin ($Dorfin^{WT}$). One of the Dorfin-CHIP chimeric proteins, $Dorfin\text{-}CHIP^{L}$, ubiquitylated mutant SOD1 more effectively than did $Dorfin^{WT}$ and CHIP in vivo, and degraded mutant SOD1 protein more rapidly than $Dorfin^{WT}$ does. Furthermore, $Dorfin\text{-}CHIP^{L}$ rescued neuronal cells from mutant SOD1-associated toxicity and reduced the aggresome formation induced by mutant SOD1 more effectively than did $Dorfin^{WT}$.

Keywords: Dorfin; ALS; SOD1; CHIP; Neurodegeneration; Ubiquitin–proteasome system

Amyotrophic lateral sclerosis (ALS), one of the most common neurodegenerative disorders, is characterized by selective motor neuron degeneration in the spinal cord, brainstem, and cortex. About 10% of ALS cases are familial; of these, 10%–20% are caused by Cu/Zn superoxide dismutase (SOD1) gene mutations (Rosen et al., 1993; Cudkowicz et al., 1997). However, the precise mechanism that causes motor neuron death in ALS is still unknown, although many have been proposed: oxidative toxicity, glutamate receptor abnormality, ubiquitin proteasome dysfunction, inflammatory and cytokine activation, neurotrophic factor deficiency, mitochondrial damage, cytoskeletal abnormalities, and activation of the apoptosis pathway (Julien, 2001; Rowland and Shneider, 2001).

Misfolded protein accumulation, one probable cause of neurodegenerative disorders, including ALS, can cause the deterioration of various cellular functions, leading to neuronal cell death (Julien, 2001; Ciechanover and Brundin, 2003). Recent findings indicate that the ubiquitin–proteasome system (UPS), a cellular function that recognizes and catalyzes misfolded or impaired cellular proteins (Jungmann et al., 1993; Lee et al., 1996; Bercovich et al., 1997), is involved in the pathogenesis of various neurodegenerative diseases, among them ALS, Parkinson's disease (PD), Alzheimer's disease, polyglutamine disease, and prion disease (Alves-Rodrigues et al., 1998; Sherman and Goldberg, 2001; Ciechanover and Brundin, 2003). The ubiquitin ligase (E3), a key molecule for the UPS, can specifically recognize misfolded substrates and convey them to proteasomal degradation (Scheffner et al., 1995; Glickman and Ciechanover, 2002; Tanaka et al., 2004).

Dorfin, an E3 protein, contains an in-between-ring-finger (RING-IBR) domain at its N-terminus. The C-terminus of Dorfin can recognize mutant SOD1 proteins, which cause familial ALS (Niwa et al., 2001; Ishigaki et al., 2002b; Niwa et al., 2002). In cultured cells, Dorfin colocalized with aggresomes and ubiquitin-positive inclusions, which are pathological hallmarks of neurodegenerative diseases (Hishikawa et al., 2003; Ito et al., 2003). Dorfin also interacted with VCP/p97 in ubiquitin-positive inclusions in

Abbreviations: ALS, amyotrophic lateral sclerosis; CFTR, cystic fibrosis transmembrane conductance regulator; CHIP, carboxyl terminus of Hsc70-interacting protein; DMEM, Dulbecco's modified Eagle's medium; E3, ubiquitin ligase; FCS, fetal calf serum; IP, immunoprecipitation; LB, Lewy body; PD, Parkinson's disease; RING-IBR, in-between-ring-finger; SCF, Skp1-Cullin-F box complex; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; SOD1, Cu/Zn super oxide dismutase; UPS, ubiquitin–proteasome system.

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ALS and PD (Ishigaki et al., 2004). Moreover, formation of this complex was found to be necessary for the E3 activity of Dorfin against mutant SOD1. These findings suggest that Dorfin is involved in the quality-control system for the abnormal proteins that accumulate in the affected neurons in neurodegenerative disorders.

Dorfin degrades mutant SOD1s and attenuates mutant SOD1-associated toxicity in cultured cells (Niwa et al., 2002). However, in Dorfin/mutant SOD1 double transgenic mice, we found only a modest beneficial effect on mutant SOD1-induced survival and motor dysfunction (unpublished data). These findings, combined with the short half-life of Dorfin protein, led us to hypothesize that the limiting effect of the Dorfin transgene may be a consequence of autodegradation of Dorfin, since Dorfin can execute autoubiquitilation in vivo (Niwa et al., 2001).

Carboxyl terminus of Hsc70-interacting protein (CHIP) is also an E3 protein; it has a TPR domain in the N terminus and a U-box domain in the C terminus. The U-box domain of CHIP is responsible for its strong E3 activity, whereas the TPR domain recruits heat shock proteins harboring misfolded client proteins such as cystic fibrosis transmembrane conductance regulator (CFTR), denatured luciferase, and tau (Meacham et al., 2001; Murata et al., 2001, 2003; Hatakeyama et al., 2004; Shimura et al., 2004).

To prolong the protein lifetime of Dorfin and thereby obtain more potent ubiquitylation and degradation activity against mutant SOD1s than is provided by Dorfin or CHIP alone, we generated chimeric proteins containing the substrate-binding domain of Dorfin and the UPR domain of CHIP substitute for RING/IBR of Dorfin. We developed 12 candidate constructs that encode Dorfin-CHIP chimeric proteins and analyzed them for their E3 activities and degradation abilities against mutant SOD1 protein in cultured cells.

Experimental procedures

Plasmids and antibodies

We designed constructs expressing Dorfin-CHIP chimeric protein. In these constructs, different-length fragments of the C-terminus portion of Dorfin, including the hydrophobic substrate-binding domain (amino acids 333–838, 333–700, and 333–454) and the C-terminus UPR domain of CHIP with amino acids 128–303 or without amino acids 201–303, a charged region was fused in various combinations as shown in Fig. 2C. Briefly, Dorfin-CHIP$^{A, B, C, G, H,}$ and ^{I} had the C-terminus portion of Dorfin in their N-terminus and the U-box of CHIP in their C-terminus; Dorfin-CHIP$^{D, E, F, J, K}$, and ^{L} had the U-box of CHIP in their N-terminus and the C-terminus portion of Dorfin in their C-terminus.

We prepared a pCMV2/FLAG-Dorfin-CHIP chimeric vector (Dorfin-CHIP) by polymerase chain reaction (PCR) using the appropriate design of PCR primers with restriction sites (ClaI, KpnI, and XbaI or EcoRI, ClaI, and KpnI). The PCR products were digested and inserted into the ClaI–KpnI site in pCMV2 vector (Sigma, St. Louis, MO). These vectors have been described previously: pFLAG-Dorfin^{WT} (Dorfin^{WT}), FLAG-Dorfin$^{C132S/C135S}$ (Dorfin$^{C132S/C135S}$), pFLAG-CHIP (CHIP), pFLAG-Mock (Mock), pcDNA3.1/Myc-SOD1^{WT} (SOD1^{WT}), pcDNA3.1/Myc-SOD1^{G93A} (SOD1^{G93A}), pcDNA3.1/Myc-SOD1^{G85R} (SOD1^{G85R}), pcDNA3.1/Myc-SOD1^{H46R} (SOD1^{H46R}), pcDNA3.1/Myc-SOD1^{G37R} (SOD1^{G37R}), pEGFP/SOD1^{WT} (SOD1^{WT}-GFP), and pEGFP/SOD1^{G85R} (SOD1^{G85R}-GFP) (Ishigaki et al., 2004). We used monoclonal anti-FLAG antibody (M2; Sigma), monoclonal anti-Myc antibody (9E10; Santa Cruz Biotechnology, Santa Cruz, CA), monoclonal anti-HA antibody (12CA5; Roche, Basel, Switzerland), and polyclonal anti-SOD1 (SOD-100; Stressgen, San Diego, CA).

Cell culture and transfection

We grew HEK293 cells and neuro2a (N2a) cells in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum (FCS), 5 U/ml penicillin, and 50 μg/ml streptomycin. At subconfluence, we transfected these cells with the indicated plasmids, using Effectene reagent (Qiagen, Valencia, CA) for HEK293 cells and Lipofectamine 2000 (Invitrogen, Carlsbad, CA) for N2a cells. After overnight posttransfection, we treated the cells with 1 μM MG132 (Z-Leu-Leu-Leu-al; Sigma) for 16 h to inhibit cellular proteasome activity. We analyzed the cells 24–48 h after transfection. To differentiate N2a cells, cells were treated for 48 h with 15 μM of retinoic acid in 2% serum medium.

Immunological analysis

At 24–48 h after transfection, we lysed cells (4×10^5 in 6-cm dishes) with 500 μl of lysis buffer consisting of 50 mM Tris–HCl, 150 mM NaCl, 1% Nonidet P-40, and 1 mM ethylenediaminetetraacetic acid (EDTA), as well as a protease inhibitor cocktail (Complete Mini, Roche). The lysate was then centrifuged at 10,000× g for 10 min at 4°C to remove debris. We used a 10% volume of the supernatants as the lysate for SDS-PAGE. When immunoprecipitated, the supernatants were precleared with protein A/G agarose (Santa-Cruz). A specific antibody, either anti-FLAG (M2) or anti-Myc (9E10), was then added. We incubated the immune complexes, first at 4°C with rotation and with protein A/G agarose (Roche) for 3 h, after which they were collected by centrifugation and washed four times with the lysis buffer. For protein analysis, immune complexes were dissociated by heating in SDS-PAGE sample buffer and loaded onto SDS-PAGE. We separated the samples by SDS-PAGE (15% gel or 5%–20% gradient gel) and transferred them onto polyvinylidene difluoride membranes. We then immunoblotted samples with specific antibodies.

Immunohistochemistry

We fixed differentiated N2a cells grown in plastic dishes in 4% paraformaldehyde in PBS for 15 min. The cells were then blocked for 30 min with 5% (vol/vol) normal goat serum in PBS, incubated overnight at 4°C with anti-FLAG antibody (M2), washed with PBS, and incubated for 30 min with Alexa 496 nm anti-mouse antibodies (Molecular Probes, Eugene, OR). We mounted the cells on slides and obtained images using a fluorescence microscope (IX71; Olympus, Tokyo, Japan) equipped with a cooled charge-coupled device camera (DP70; Olympus). Photographs were taken using DP Controller software (Olympus).

Analysis of protein stability

We assayed the stability of proteins by pulse-chase analysis using [^{35}S] followed by immunoprecipitation. Metabolic labeling was performed as described previously (Yoshida et al., 2003). Briefly, in the pulse-chase analysis of Dorfin proteins, HEK293 cells in 6-cm dishes were transiently transfected with 1 μg of

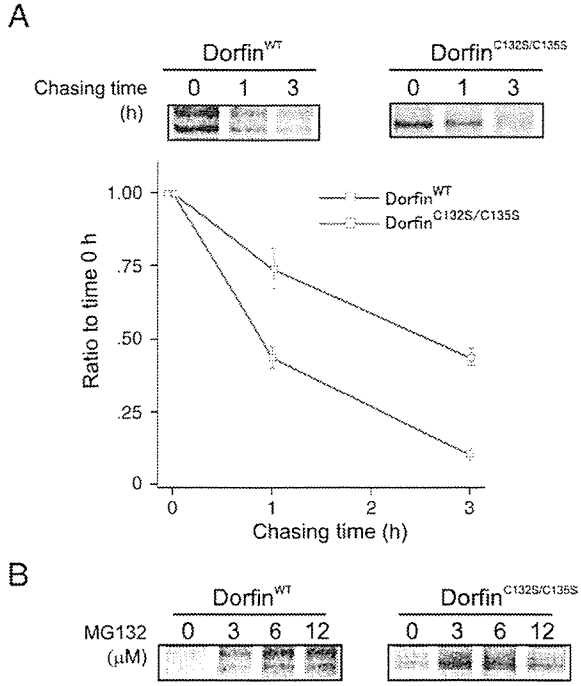

Fig. 1. Pulse-chase analysis of Dorfin^{WT} and Dorfin$^{C132S/C135S}$. (A) Dorfin^{WT} or Dorfin$^{C132S/C135S}$ was overexpressed in HEK293 cells. After overnight incubation, [^{35}S]-labeled Met/Cys pulse-chase analysis was performed. Cells were harvested and analyzed at 0, 1, or 3 h after labeling and immunoprecipitation by anti-FLAG antibody (upper panels). To determine serial changes in the amount of Dorfin^{WT} or Dorfin$^{C132S/C135S}$, four independent experiments were performed and the amounts of Dorfin^{WT} and Dorfin$^{C132S/C135S}$ were plotted. The differences between the amounts of Dorfin^{WT} and Dorfin$^{C132S/C135S}$ were significant at 1 h ($p<0.01$) and 3 h after labeling ($p<0.001$) (lower panels). Values are the means± SE, n=4. Statistics were done using an unpaired t-test. (B) Cells overexpressing Dorfin^{WT} or Dorfin$^{C132S/C135S}$ were treated with different concentrations of MG132 for 3 h after labeling.

FLAG-Dorfin^{WT} or FLAG-Dorfin$^{C132S/C135S}$. In pulse-chase experiments using SOD1^{G85R}, N2a cells in 6-cm dishes were transiently transfected with 1 μg of SOD1^{G85R}-Myc or SOD1^{G93A}-Myc and FLAG-Mock, FLAG-Dorfin, or FLAG-Dorfin-CHIP^{L}. FLAG-Mock was used as a negative control. After starving the cells for 60 min in methionine- and cysteine-free DMEM with 10% FCS, we labeled them for 60 min with 150 μCi/ml of Pro-Mix L-[^{35}S] in vitro cell-labeling mix (Amersham Biosciences). Cells were chased for different lengths of time at 37°C. In experiments with proteasomal inhibition, we added different amounts of MG132 in medium during the chase period. We performed immunoprecipitation using protein A/G agarose, mouse monoclonal anti-FLAG (M2), and anti-Myc (9E10). The intensity of the bands was quantified by ImageGauge software (Fuji Film, Tokyo, Japan).

MTS assay

We transfected N2a cells (5000 cells per well) in 96-well collagen-coated plates with 0.15 μg of SOD1^{G85R}-GFP and 0.05 μg of Dorfin, CHIP, Dorfin-CHIP^{L}, or pCMV2 vector (Mock) using Effecten reagent (Qiagen). Then we performed 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assays using Cell Titer 96 (Promega) at 48 h after incubation. This procedure has previously been described (Ishigaki et al., 2002a).

Aggregation assay

We transfected N2a cells in 6-cm dishes with 1.0 μg of SOD1^{G85R}-GFP and 1.0 μg of FLAG-Mock, FLAG-Dorfin, FLAG-CHIP, or FLAG-Dorfin-CHIP^{L}. After overnight incubation, we changed the medium to 2% FCS containing medium with 15 μM retinoic acid (RA) for differentiation. In the MG132 (+) group, 1 μM of MG132 was added after 24 h of differentiation stimuli. After 48 h of differentiation stimuli, we examined the cells in their living condition by fluorescence microscopy. The transfection ratio was equivalent (75%) among all groups. Visually observable macro aggregation-harboring cells were counted as "aggregation positive" cells (Fig. 7C). All cells were counted in fields selected at random from the four different quadrants of the culture dish. Counting was done by an investigator who was blind to the experimental condition.

Results

Dorfin degradation by the UPS in vivo

We analyzed the degradation speed of FLAG-Dorfin by the pulse-chase method using [^{35}S] labeling, finding that more than half of wild-type Dorfin (Dorfin^{WT}) was degraded within 1 h (Fig. 1A). This degradation was dose-dependently inhibited by MG132, a proteasome inhibitor (Fig. 1B). On the other hand, the RING mutant form of Dorfin (Dorfin$^{C132S/C135S}$), which lacks E3 activity (Ishigaki et al., 2004), degraded significantly more slowly than did Dorfin^{WT} (Fig. 1A and Table 1). As shown in Fig. 1A, Dorfin^{WT} showed two bands, whereas Dorfin$^{C132S/C135S}$ had a single band. This was also seen in our previous study (Ishigaki et al., 2004) and may represent posttranslational modification.

Construction of Dorfin-CHIP chimeric proteins

It is known that the C-terminus portion of Dorfin can bind to substrates such as mutant SOD1 proteins or Synphilin-1 (Niwa et al., 2002; Ito et al., 2003). We attempted to identify the domain of Dorfin that interacts with substrates. Although there was no obvious known motif in the C-terminus of Dorfin (amino acids 333–838), its first quarter contained rich hydrophobic amino acids (amino acids 333–454) (Fig. 2A). Immunoprecipitation analysis revealed that the hydrophobic region of Dorfin (amino acids 333–454) was able to bind to SOD1^{G85R}, indicating that this hydrophobic region is responsible for recruiting mutant SOD1 in Dorfin protein (Fig. 2B).

To establish more effective and more stable E3 ubiquitin ligase molecules that can recognize and degrade mutant SOD1s, we

Table 1
Serial changes in the amounts of Dorfin^{WT}, Dorfin$^{C132S/C135S}$, and Dorfin-CHIP^{L}

	0 h (%)	1 h (%)	3 h (%)
Dorfin^{WT}	100	43.7± 7.0	10.3± 4.4
Dorfin$^{C132S/C135S}$	100	73.9± 13.8	43.7± 1.9
Dorfin-CHIP^{L}	100	89.0± 5.7	47.5± 5.3

Values are the mean and SD of four independent experiments.

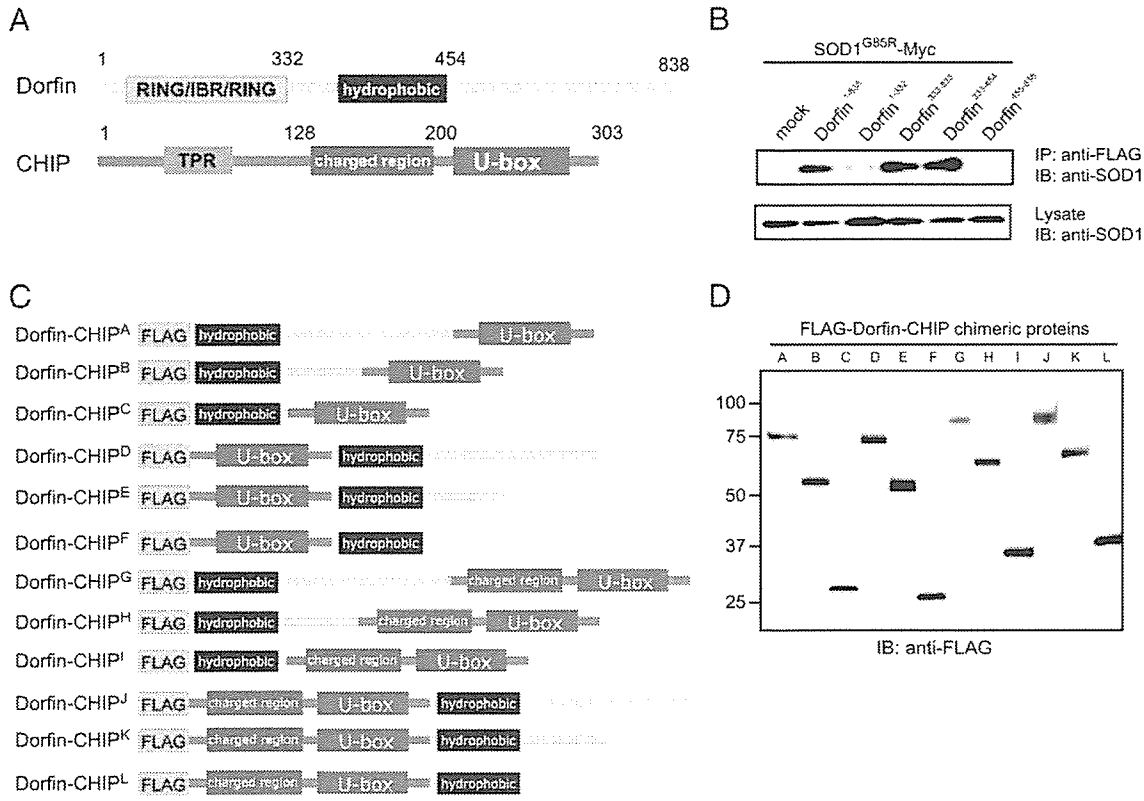

Fig. 2. Construction of Dorfin-CHIP chimeric proteins. (A) Dorfin has a RING/IBR domain in its N-terminus and a substrate-binding portion in the C-terminus. CHIP contains a TPR domain that binds to heat-shock proteins at the N-terminus; its C-terminal U-box domain has strong E3 ubiquitin ligase activity. (B) SOD1^{G85R}-Myc and FLAG-Dorfin derivatives were overexpressed in HEK 293 cells. Cell lysates were immunoprecipitated with anti-myc antibody. Immunoblotting showed that FLAG-Dorfin derivatives containing Dorfin$^{333–454}$ bound to SOD1^{G85R}-Myc, indicating that the hydrophobic region of Dorfin (Dorfin$^{333–454}$) is essential for interaction with mutant SOD1 in vivo. (C) Scheme of engineered Dorfin-CHIP chimeric proteins. Three different lengths of C-terminal Dorfin containing the hydrophobic region of Dorfin (Dorfin$^{333–454}$) and the U-box domain of CHIP with or without the charged region were fused. (D) Dorfin-CHIP chimeric proteins were overexpressed in HEK293 cells. Harvested cells were lysed and analyzed by immunoblotting using anti-FLAG antibody.

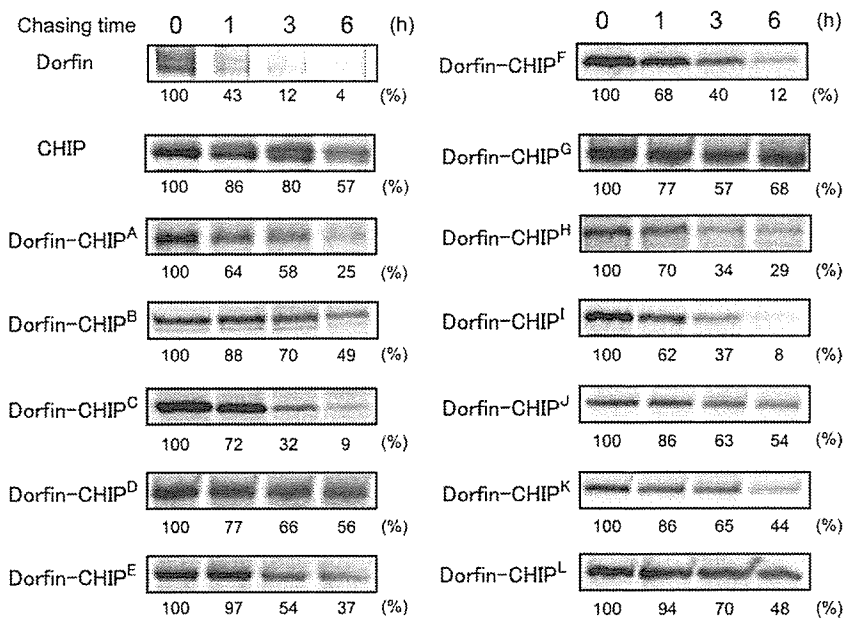

Fig. 3. The stability of Dorfin-CHIP chimeric proteins. Pulse-chase analysis using [^{35}S]-Met/Cys was performed. Dorfin, CHIP, and all the Dorfin-CHIP chimeric proteins were overexpressed in HEK293 cells and labeled with [^{35}S]-Met/Cys. **Immunoprecipitation using anti-FLAG antibody and SOD-PAGE analysis** revealed the degradation speed of FLAG-Dorfin-CHIP chimeric proteins. The amount of each Dorfin-CHIP chimeric protein was measured by quantifying the band using ImageGauge software.

designed Dorfin-CHIP chimeric proteins containing both the hydrophobic substrate-binding domain of Dorfin and the U-box domain of CHIP, which has strong E3 activity (Fig. 2C). We verified that all of the 12 candidate chimeric proteins were expressed in HEK293 cells (Fig. 2D).

Expression of Dorfin-CHIP chimeric proteins in cells

The half lives of all the Dorfin-CHIP chimeric proteins were more than 1 h. In some of these proteins, such as Dorfin-CHIP$^{D, G, J}$, and ^{L}, moderate amounts of protein still remained at 6 h after labeling, indicating that they were degraded much more slowly than was Dorfin^{WT} (Fig. 3). Repetitive experiments using Dorfin-CHIP^{L} yielded a significant difference between the amount of Dorfin^{WT} and Dorfin-CHIP^{L} at 1 h and 3 h (Table 1).

E3 activity of Dorfin-CHIP chimeric proteins against mutant SOD1

Immunoprecipitation analysis demonstrated that Dorfin and CHIP bound to mutant SOD1^{G85R} in equivalent amounts and that all of the Dorfin-CHIP chimeric proteins interacted with mutant SOD1^{G85R} in vivo. Dorfin-CHIP$^{A, D, E, F, J, K}$, and ^{L} bound to the same or greater amounts of SOD1^{G85R} than did Dorfin, whereas Dorfin-CHIP$^{B, C, G, H}$, and ^{I} did not (Fig. 4A, upper panel). None of the Dorfin-CHIP chimeric proteins bound to SOD1^{WT} in vivo

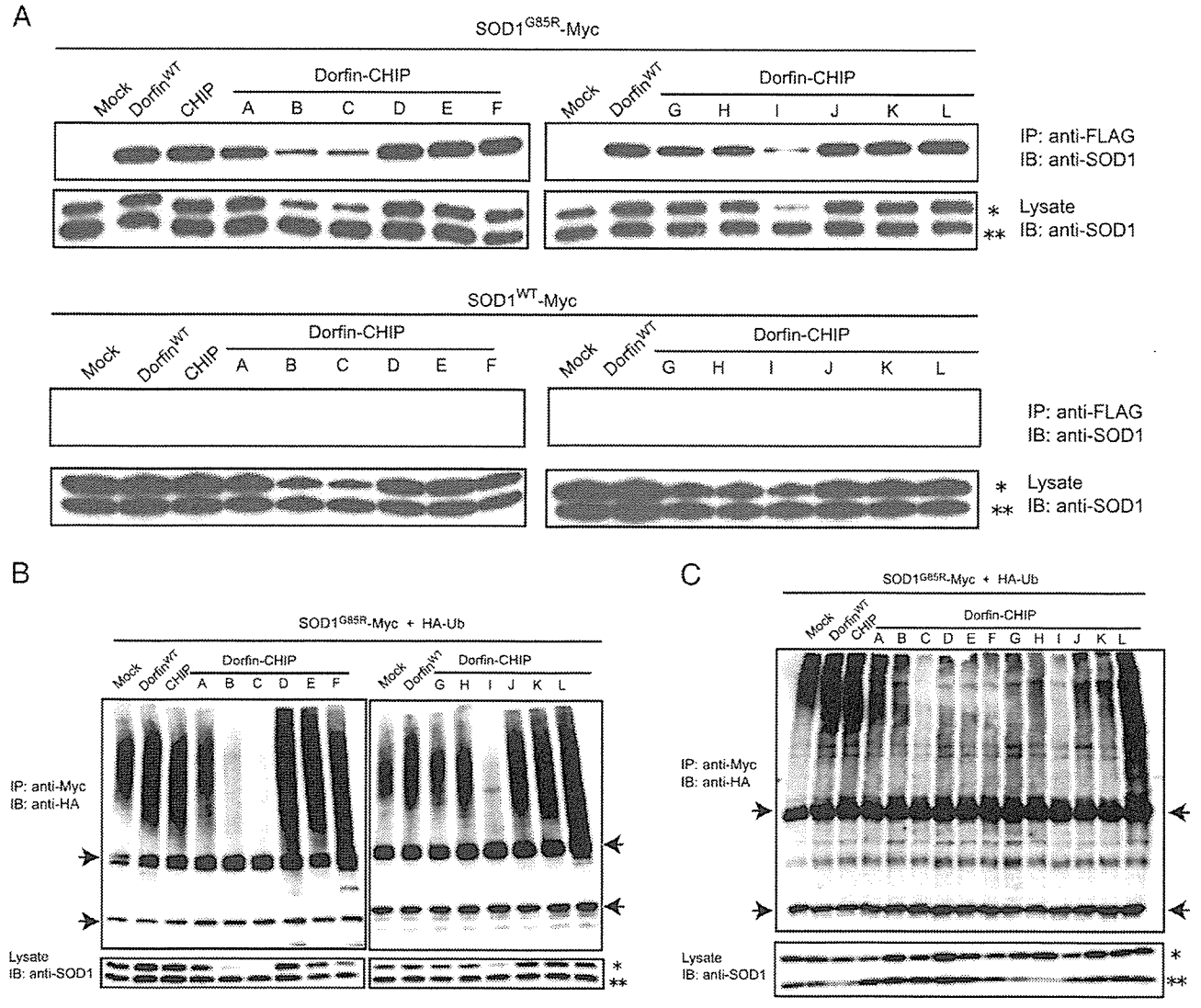

Fig. 4. The E3 activity of Dorfin-CHIP chimeric proteins on mutant SOD1 in vivo. (A) In vivo binding assay with both wild-type and mutant SOD1s. SOD1^{G85R}- or SOD1^{WT}-Myc and FLAG derivatives of Dorfin-CHIP chimeric proteins were coexpressed in HEK293 cells. Immunoprecipitation was done using anti-Myc antibody. Immunoblotting with anti-FLAG antibody revealed that all the Dorfin-CHIP chimeric proteins bound in vivo to SOD1^{G85R}-Myc but not to SOD1^{WT}-Myc. Single and double asterisks indicate overexpressed human SOD1s and mouse endogenous SOD1, respectively. (B) In vivo ubiquitylation assay in HEK293 cells. SOD1^{G85R}-Myc, HA-Ub, and FLAG derivatives of Dorfin-CHIP chimeric proteins were coexpressed in HEK293 cells. Immunoblotting with anti-HA antibody demonstrated the ubiquitylation level of SOD1^{G85R}-Myc by FLAG derivatives of Dorfin-CHIP chimeric proteins in vivo. Arrows indicate IgG light and heavy chains. Single and double asterisks indicate overexpressed SOD1 and mouse endogenous SOD1, respectively. (C) In vivo ubiquitylation assay in N2a cells. SOD1^{G85R}-Myc, HA-Ub, and FLAG derivatives of Dorfin-CHIP chimeric proteins were coexpressed in N2a cells. Arrows indicate IgG light and heavy chains. Single and double asterisks indicate overexpressed human SOD1s and mouse endogenous SOD1, respectively.

(Fig. 4A, lower panel). Some Dorfin-CHIP chimeric proteins, such as Dorfin-CHIP$^{B, C}$, and ^{I}, had lower amounts of both SOD1^{WT} and SOD1^{G85R} in the lysates. We performed quantitative RT-PCR using specific primers for SOD1-Myc, finding that coexpression of Dorfin-CHIP$^{B, C}$ or ^{I} suppressed the mRNA expression of overexpressed SOD1 gene (Supplementary Fig. 1). Considering the possibility that these Dorfin-CHIP chimeric proteins might have unpredicted toxicity for cells by affecting gene transcription via unknown mechanisms, we excluded them from further analysis. Other Dorfin-CHIP proteins did not affect SOD1-Myc gene expression, which validated the comparison among IPs and ubiquitylated mutant SOD1 in Figs. 4A–C.

To assess the effectiveness of the E3 activity of Dorfin-CHIP chimeric proteins, we did an in-vivo ubiquitylation analysis by coexpression of SOD1^{G85R}-Myc, HA-Ub, and Dorfin-CHIP chimeric proteins in HEK293 cells. We found that Dorfin and CHIP enhanced the ubiquitylation of SOD1^{G85R} protein and that the ubiquitylation levels of these two E3 ligases were almost equivalent. Moreover, Dorfin-CHIP$^{D, E, F, J, K}$, and ^{L} ubiquitylated SOD1^{G85R} more effectively than did Dorfin or CHIP (Fig. 4B).

Performing the same in-vivo ubiquitylation assay using N2a cells, we observed that the levels of ubiquitylation of SOD1^{G85R} by Dorfin and CHIP were equivalent, as they were in HEK293 cells. Among Dorfin-CHIP chimeric proteins, only Dorfin-CHIP^{L}

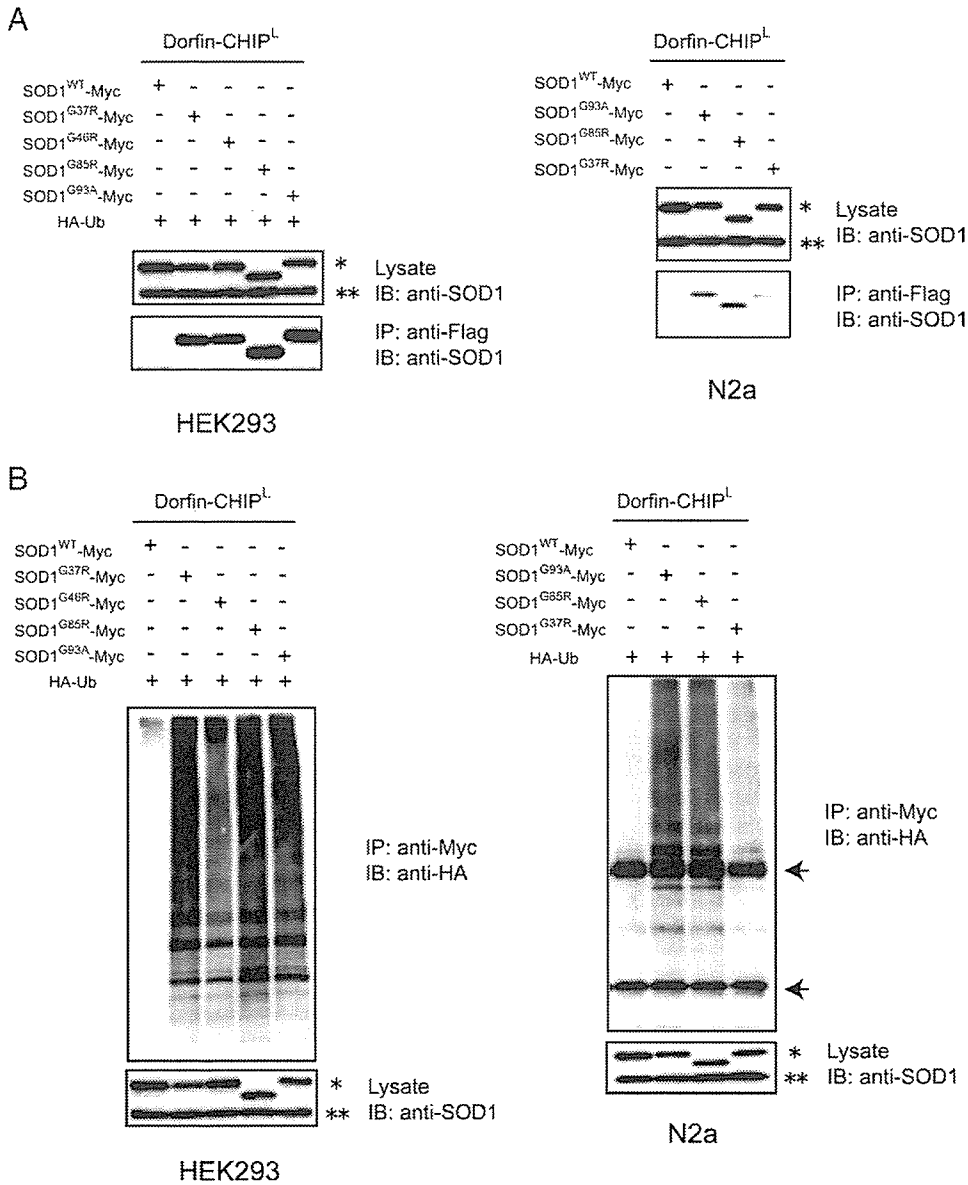

Fig. 5. Dorfin-CHIP^{L} specifically ubiquitylates mutant SOD1s in vivo. (A) In vivo binding assay with various mutant SOD1s. SOD1^{WT}-Myc, SOD1^{G93A}-Myc, SOD1^{G85R}-Myc, SOD1^{H46R}-Myc or SOD1^{G37R}-Myc, and FLAG-Dorfin-CHIP^{L} were coexpressed in HEK293 (left) and N2a cells (right). Immunoprecipitation was done using anti-Myc antibody. Immunoblotting with anti-FLAG antibody showed that both chimeric proteins specifically bound to mutant SOD1s in vivo. Single and double asterisks indicate overexpressed SOD1 and mouse endogenous SOD1, respectively. (B) In vivo ubiquitylation assay. SOD1^{WT}-Myc, SOD1^{G93A}-Myc, SOD1^{G85R}-Myc, SOD1^{H46R}-Myc or SOD1^{G37R}-Myc, as well as FLAG-Dorfin-CHIP^{L} and HA-Ub, was coexpressed in HEK293 (left) and N2a cells (right). Immunoblotting with anti-HA antibody showed the specific ubiquitylation of mutant SOD1-Myc by FLAG-Dorfin-CHIP^{L} in vivo. Arrows indicate IgG light and heavy chains. Single and double asterisks indicate overexpressed human SOD1s and mouse endogenous SOD1, respectively.

ubiquitylated SOD1^{G85R} more effectively than did Dorfin or CHIP, while Dorfin-CHIP$^{D, E, F, J}$, and ^{K} did not (Fig. 4C). Thus, Dorfin-CHIP^{L} was the most potent candidate of the chimeric proteins.

Ubiquitylation of mutant SOD1 by Dorfin-CHIP^{L}

Dorfin specifically ubiquitylated mutant SOD1 proteins, but not SOD1^{WT} protein (Niwa et al., 2002; Ishigaki et al., 2004). Similarly, Dorfin-CHIP^{L} interacted with SOD1^{G93A}, SOD1^{G85R}, SOD1^{H46R}, and SOD1^{G37R}, but not SOD1^{WT}, in HEK293 cells. This was confirmed in N2a cells (Fig. 5A). In both HEK293 and N2a cells, Dorfin-CHIP^{L} also ubiquitylated mutant SOD1 proteins but not SOD1^{WT} (Fig. 5B).

Degradation of mutant SOD1 by Dorfin-CHIP chimeric proteins

To assess the degradation activity of Dorfin-CHIP^{L} against mutant SOD1s, we performed the pulse-chase analysis on N2a

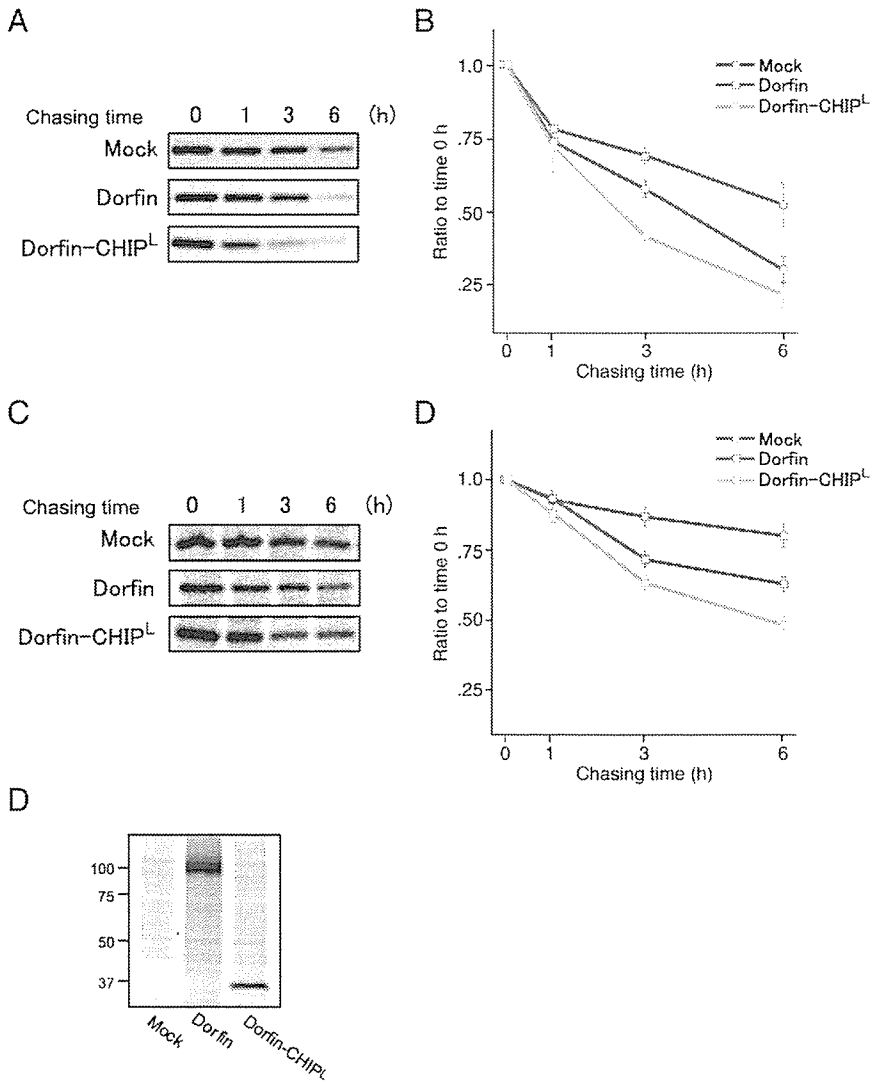

Fig. 6. Degradation of mutant SOD1 proteins with Dorfin-CHIP^{L}. (A) Pulse-chase analysis of SOD1^{G85R} with Dorfin-CHIP^{L}. N2a cells were coexpressed with SOD1^{G85R}-Myc and Mock, Dorfin, and Dorfin-CHIP^{L}. Pulse-chase experiments using [^{35}S]-Met/Cys were done. Immunoprecipitation using anti-Myc antibody and SOD-PAGE analysis revealed the degradation speed of SOD1^{G85R}-Myc. (B) Serial changes in the amount of SOD1^{G85R} coexpressed with Mock, Dorfin, or Dorfin-CHIP^{L}. Four independent experiments were performed and the amounts of SOD1^{G85R} were plotted. There were significant differences between Mock and Dorfin ($p<0.005$), Mock and Dorfin-CHIP^{L} ($p<0.005$), and Dorfin and Dorfin-CHIP^{L} ($p<0.05$) at 3 h, as well as between Mock and Dorfin ($p<0.05$), and Mock and Dorfin-CHIP^{L} ($p<0.05$) at 6 h after labeling. Values are the means ± SE, n=4. Statistical analysis was done by one-way ANOVA. (C) Pulse-chase analysis of SOD1^{G93A} with Dorfin-CHIP^{L}. N2a cells were coexpressed with SOD1^{G93A}-Myc and Mock, Dorfin, and Dorfin-CHIP^{L} as in panel A. (D) Serial changes in the amount of SOD1^{G93A} coexpressed with Mock, Dorfin, or Dorfin-CHIP^{L}. Four independent experiments were performed and the amounts of SOD1^{G93A} were plotted. There were significant differences between Mock and Dorfin ($p<0.05$) and Mock and Dorfin-CHIP^{L} ($p<0.01$) at 3 h, as well as between Mock and Dorfin ($p<0.05$), Mock and Dorfin-CHIP^{L} ($p<0.01$), and Dorfin and Dorfin-CHIP^{L} ($p<0.05$) at 6 h after labeling. Values are the means ± SE, n=4. Statistics were done by one-way ANOVA. (E) The equivalent protein expression levels of Dorfin and Dorfin-CHIP^{L}. Half of the volume of samples used in the pulse-chase analysis of panel C at 0 h was used for immunoprecipitation using anti-Flag M2 antibody. The following SOD-PAGE analysis revealed the amounts of Dorfin and Dorfin-CHIP^{L} in the experiment shown in panel C.