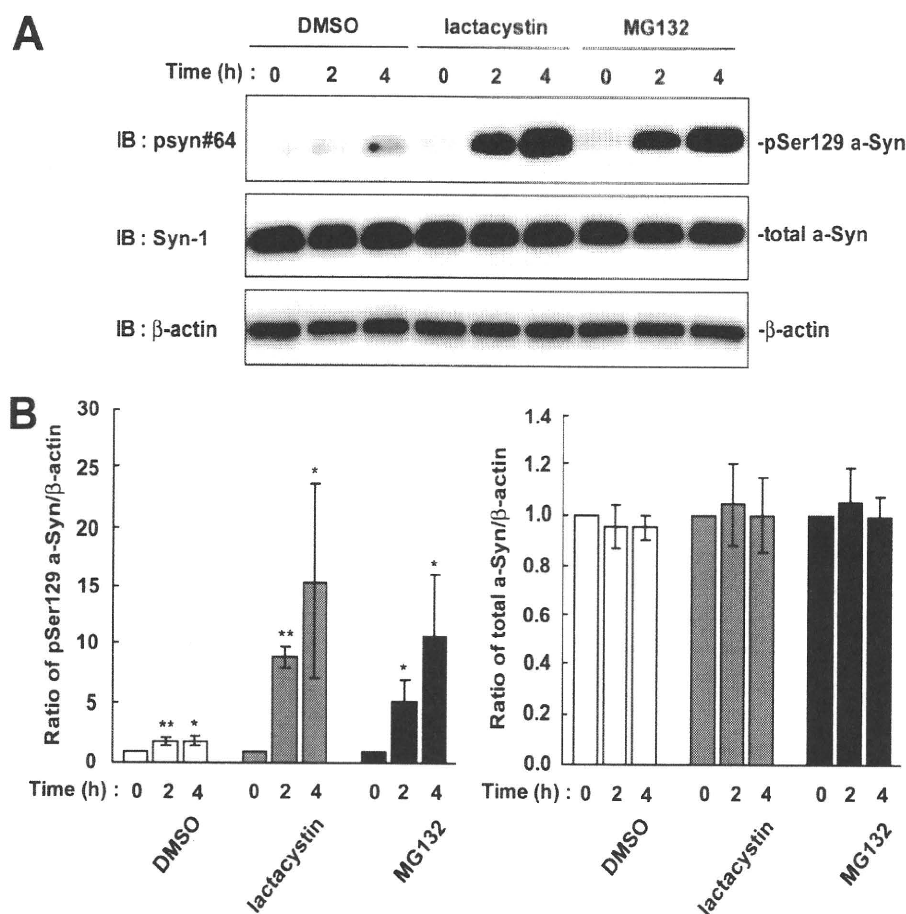

FIGURE 2. **The effect of proteasome inhibitors on the levels of Ser-129-phosphorylated a-Syn in wt-aS/SH cells.** The cells were incubated in medium containing either 0.1% DMSO, 10 μM lactacystin, or 10 μM MG132 for 4 h. *A*. The cell lysates (20 μg/lane) were analyzed by immunoblotting (*IB*) with psyn 64 and Syn-1 antibodies. For loading control, the same amounts of samples were immunoblotted with anti-β-actin antibody. Representative blots are shown. *B*, quantitative analysis of the alteration in the expression levels of Ser-129-phosphorylated a-Syn in cells in the absence or presence of proteasome inhibitors. Relative ratios of the band intensity of Ser-129-phosphorylated a-Syn to β-actin and total a-Syn to β-actin are shown. Relative ratio was normalized to the starting material. Data represent means ± S.D. and the p values (*, $p < 0.05$ and **, $p < 0.01$) are shown.

We next assessed the metabolism of phosphorylation-mimic mutants (S129E and S129D) and phosphorylation-abolished mutant (S129A) of a-Syn. Each mutant cDNA was transfected into SH-SY5Y cells, and the cells were treated with lactacystin for 4 h. The expression levels of S129A mutant a-Syn were stable at 4 h, as compared with the starting levels (Fig. 5*A*). In phosphorylation-mimic mutants, the expression levels of S129E mutant a-Syn were significantly increased 1.39 ± 0.15-fold at 2 h ($p = 0.014$, $n = 4$) and 1.55 ± 0.23-fold at 4 h ($p = 0.016$, $n = 4$), as compared with the starting levels, whereas S129D mutant a-Syn did not alter the expression levels (Fig. 5*A*). In the experiments using CHX, S129A and S129D mutants were not decreased within 4 h (Fig. 5*B*). On the other hand, S129E mutant a-Syn showed a rapid decrease (Fig. 5*B*). Treatment with MG132 suppressed the rapid decrease in the levels of S129E mutant a-Syn (Fig. 5*B*). The data of S129A and S129E mutants supported that Ser-129-phosphorylation played a role in the degradation of a-Syn by the proteasome pathway. However, S129D mutant a-Syn did not reproduce the metabolic fate of Ser-129-phosphorylated form.

To test whether the phosphorylation of other Ser residues contributed to the metabolism of Ser-129-phosphorylated a-Syn, we made S9A/S42A/S87A mutant a-Syn cDNA, whose product abolished the possible Ser-phosphorylation sites except for Ser-129. In the experiment using CHX, Ser-129-phosphorylated form of this mutant a-Syn was rapidly decreased (Fig. 6). The expression levels of total a-Syn in the mutant were stable (Fig. 6). Treatment with MG132 suppressed the rapid decrease in the levels of Ser-129-phosphorylated form of the mutant a-Syn (Fig. 6).

Effects of Dephosphorylation and the Proteasome Pathway on the Metabolism of Ser-129-phosphorylated a-Syn in Rat Primary Cortical Neurons—To assess whether or not overexpression of a-Syn artificially targeted Ser-129-phosphorylated a-Syn toward degradation by the proteasome pathway, we investigated the effect of the proteasome inhibitors on the endogenous a-Syn protein in rat primary cortical neurons. When 21 day cultured neurons were incubated in the presence of proteasome inhibitors for 4 h, the expression levels of Ser-129-phosphorylated a-Syn were increased 2.84 ± 0.59-fold in the presence of lactacystin ($p = 0.008$, $n = 4$) and 2.03 ± 0.39-fold in the presence of MG132 ($p = 0.013$, $n = 4$), as compared with starting levels (Fig. 7). The expression levels of total a-Syn were constant in the presence of lactacystin (1.04 ± 0.17-fold, $p = 0.637$, $n = 4$) and MG132 (1.07 ± 0.30-fold, $p = 0.730$, $n = 4$) (Fig. 7). Additionally, when the primary neurons were treated with OA, the expression levels of

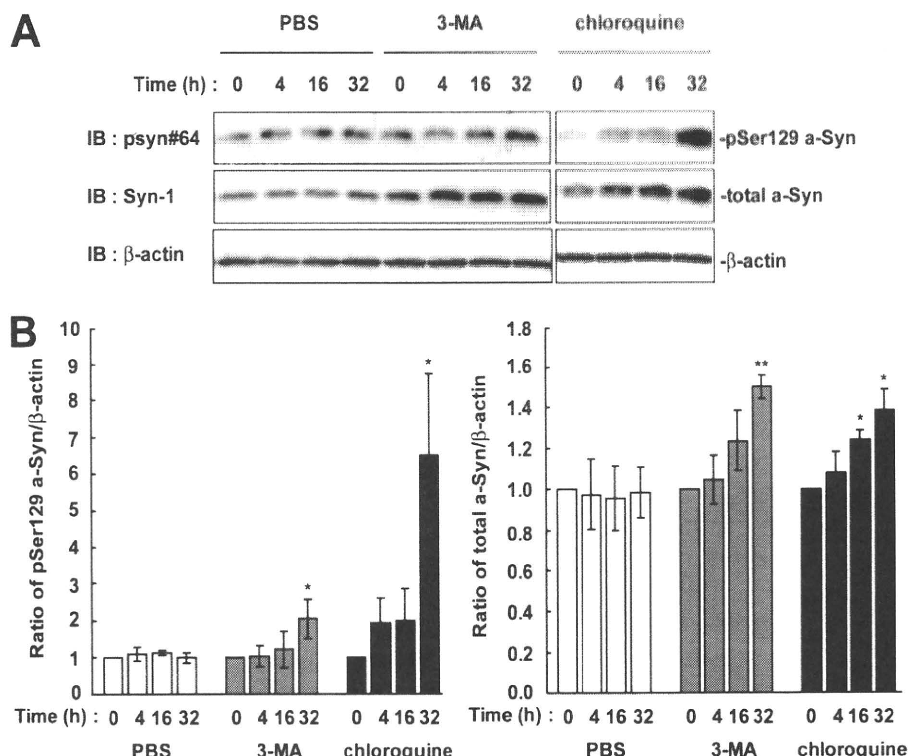

FIGURE 3. **The effect of autophagy-lysosome inhibitors on the levels of Ser-129-phosphorylated a-Syn in wt-aS/SH cells.** The cells were incubated in medium containing either 0.1% PBS, 10 mM 3-MA or 100 μM chloroquine for 32 h. *A*, cell lysates (20 μg/lane) were analyzed by immunoblotting (*IB*) with psyn 64 and Syn-1 antibodies. For loading control, the same amounts of samples were immunoblotted with anti-β-actin antibody. Experiments were performed three times. Representative blots are shown. *B*, quantitative analysis of the alteration in the expression levels of Ser-129-phosphorylated a-Syn in cells in the absence or presence of autophagy-lysosome inhibitors. Relative ratios of the band intensity of Ser-129-phosphorylated a-Syn to β-actin and total a-Syn to β-actin are shown. Relative ratio was normalized to the starting material. Data represent means ± S.D. and the *p* values (*, $p < 0.05$; **, $p < 0.01$) are shown.

Ser-129-phosphorylated a-Syn were increased 2.40 ± 0.69-fold ($p = 0.007$, $n = 4$), as compared with starting levels (Fig. 7). Although treatment with DMSO showed a tendency to elevate the levels of phosphorylated a-Syn, there was no significant difference between 0 and 4 h. The data demonstrated that Ser-129-phosphorylated a-Syn underwent degradation by the proteasome pathway and dephosphorylation at the endogenous level in neurons.

Role of Ubiquitination in the Degradation of Ser-129-phosphorylated a-Syn by the Proteasome Pathway—To assess whether polyubiquitination plays a role in targeting Ser-129-phosphorylated a-Syn to the proteasome pathway, we investigated wt-aS/SH cells and parental SH-SY5Y cells in the absence or presence of lactacystin for 16 h. Immunoblotting of post-12,000 × *g* centrifuged supernatant fractions with anti-ubiquitin antibody showed that polyubiquitin conjugates were increased in the presence of lactacystin in both wt-aS/SH and parental cells (Fig. 8*A*). In post-12,000 × *g* centrifuged pellet fractions, polyubiquitin conjugates were also increased in the presence of lactacystin in these cells (Fig. 8*A*). These findings indicated that lactacystin treatment effectively blocked protein degradation by the proteasome pathway, resulting in accumulation of polyubiquitinated conjugates in the cells. However, there was no difference in the pattern of ubiquitin-positive bands between wt-aS/SH cells and parental cells in both supernatant and pellet fractions (Fig. 8*A*). In supernatant fractions of lactacystin-treated wt-aS/SH cells, immunoblotting with anti-Ser-129-phosphorylated a-Syn antibody showed that monomeric Ser-129-phosphorylated a-Syn was increased; however, there was no appearance of the specific bands migrating at a position of higher molecular weight than its monomer (Fig. 8*A*). Although we analyzed the pellet fractions of wt-aS/SH cells, the specific bands corresponding to polyubiquitinated Ser-129-phosphorylated a-Syn were not detectable (Fig. 8*A*). We also found no specific band in immunoblotting with anti-total a-Syn antibody (Fig. 8*A*). We next investigated wt-aS/SH cells transiently overexpressing ubiquitin. Although polyubiquitinated conjugates were clearly increased in supernatant and pellet fractions by treatment with lactacystin, there was no appearance of the specific bands corresponding to polyubiquitinated a-Syn in the immunoblots of these fractions with anti-ubiquitin, anti-Ser-129 phosphorylated a-Syn or anti-total a-Syn antibody (Fig. 8*A*). Additionally, we did not find polyubiquitinated a-Syn in rat primary cortical neurons in the presence of MG132 or lactacystin, as compared with the neurons in the absence of inhibitor (Fig. 8*A*).

We further examined ubiquitination of a-Syn by immunoprecipitation of wt-aS/SH and parental cells transfected with FLAG-tagged ubiquitin cDNA (Fig. 8*B*). In products immunoprecipitated with anti-total a-Syn antibody, we detected the a-Syn monomer by immunoblotting with anti-total a-Syn antibody (Fig. 8*B*). However, there was no signal showing polyubiquitination of a-Syn in the immunoprecipitated products of wt-aS/SH cells in the absence or presence of MG132 (Fig. 8*B*). Additionally, in products immunoprecipitated with anti-FLAG antibody, we

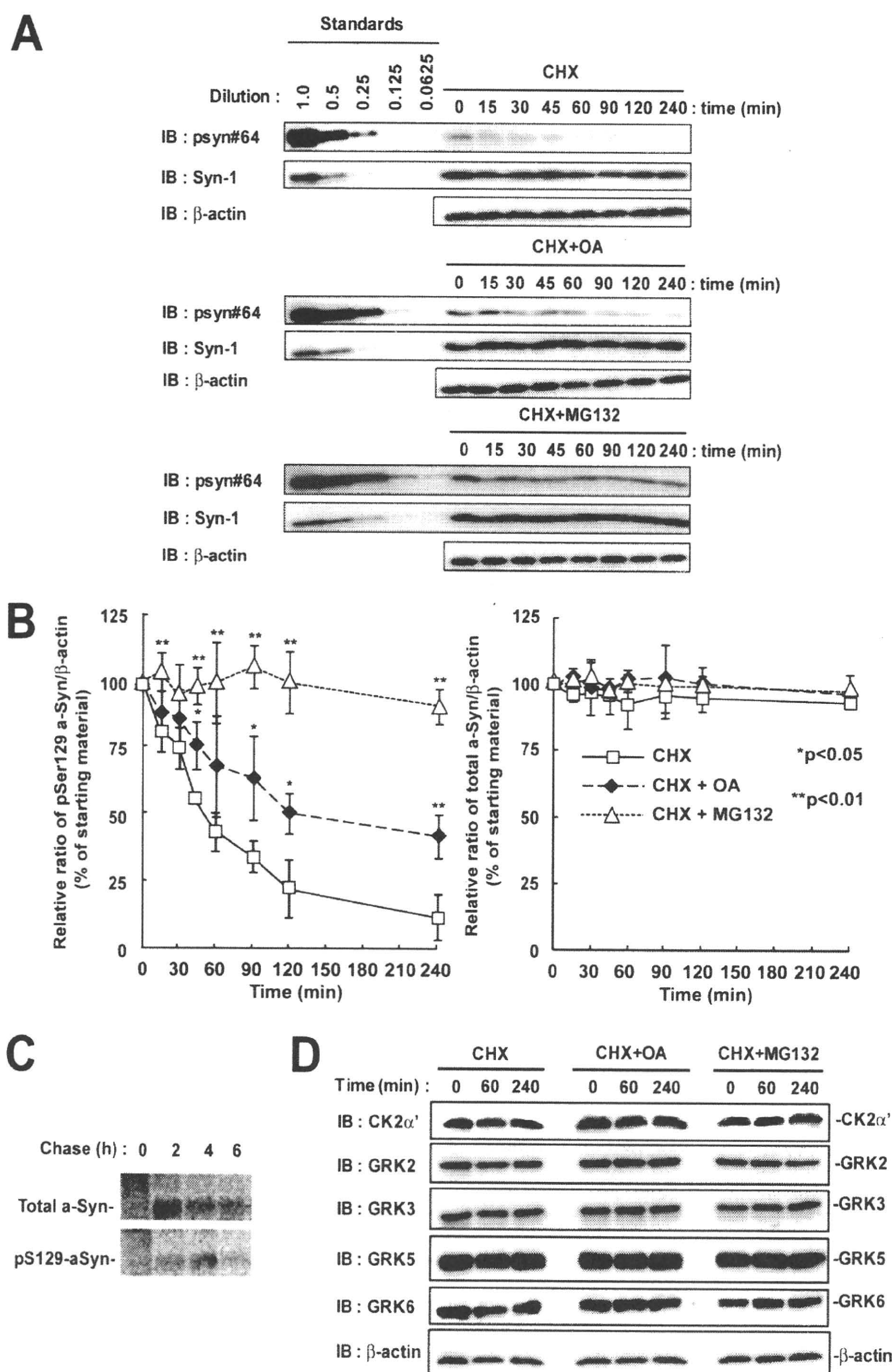

FIGURE 4. **The effect of protein phosphatase 2A/1 and proteasome inhibitors on the half-life of Ser-129-phosphorylated a-Syn in SH-SY5Y cells.** The cells were pre-incubated in medium containing either 0.1% DMSO, 10 nM OA, or 10 μM MG132 for 6 h. Then, the chase experiment was started by adding 100 μM CHX. The cells were collected at the indicated times. *A*, cell lysates (20 μg/lane) were analyzed by immunoblotting (*IB*) with psyn 64 and Syn-1. For loading control, the same amounts of samples were immunoblotted with anti-β-actin antibody. Experiments were performed three times. Representative blots are shown. *B*, quantitative analysis of the alteration in the expression levels of Ser-129-phosphorylated and total a-Syn in the chase experiments using CHX. Relative band intensities of Ser-129-phosphorylated a-Syn and total a-Syn were corrected by plotting them on the standard curves, as described in the Experimental procedures. Relative ratios of the band intensity of Ser-129-phosphorylated a-Syn to β-actin and total a-Syn to β-actin are shown by normalizing to the starting materials as percentage. Data represent means ± S.D. and the *p* values (*, $p < 0.05$; **, $p < 0.01$) are shown. *C*, cells were pulsed with 100 μCi/ml of [^{35}S]methionine/cysteine for 2 h, and subsequently chased for indicated times. Immunoprecipitation using Syn-1 antibody or anti-Ser-129-phosphorylated a-Syn antibody was carried out. *D*, expression levels of the members of G-protein-coupled receptor kinase (GRK) family and casein kinase (CK) 2 in experiments using CHX. The cell lysates (10 μg/lane) were analyzed by immunoblotting with CK2 α′ subunit antibody and antibodies against each member of GRK family (GRK2, GRK3, GRK5, and GRK6). Experiments were performed four times.

detected polyubiquitinated conjugates by immunoblotting with anti-ubiquitin antibody (Fig. 8*B*). However, there was no signal showing polyubiquitination of a-Syn in the immunoprecipitated products of wt-aS/SH cells in the absence or presence of MG132 (Fig. 8*B*).

To clarify whether the present biochemical fractionation experiments failed to detect insoluble ubiquitinated a-Syn by the aggregate formation, we performed immunocytochemical analysis using wt-aS/SH cells. The cells were transiently transfected with ubiquitin cDNA and treated with 10 μM MG132

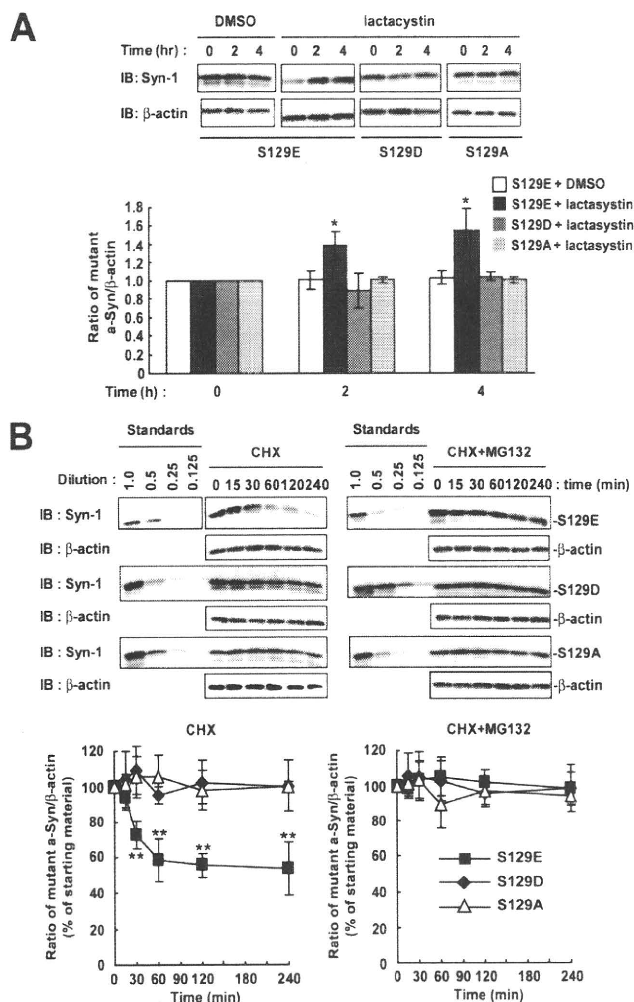

FIGURE 5. **The metabolism of phosphorylation-mimic and phosphorylation-abolished mutant a-Syn in SH-SY5Y cells.** We used S129E and S129D as phosphorylation-mimic mutants, and S129A as phosphorylation-abolished mutant of a-Syn. *A*, cells were transiently transfected with one of the a-Syn mutant cDNAs, and treated with or without 10 μM lactacystin for 4 h. The cell lysates (10 μg/lane) were analyzed by immunoblotting (*IB*) with Syn-1 antibodies or anti-β-actin antibody. The graph shows relative ratios of the band intensity of mutant a-Syn to β-actin by normalizing to the starting materials. *B*, at 42 h after transfection, the cells were pre-incubated in medium containing either 0.1% DMSO or 10 μM MG132 for 6 h. Then, 100 μM CHX was added into medium. The cells were collected at the indicated times. The cell lysates (10 μg/lane) were analyzed by immunoblotting (*IB*) with Syn-1 antibody or anti-β-actin antibody. Experiments were performed three times. Graphs show quantitative analysis of the alteration in the expression levels of mutant a-Syn. Relative ratios of the band intensity of mutant a-Syn to β-actin are shown by normalizing to the starting materials. Data represent means ± S.D. and the *p* values (*, $p < 0.05$; **, $p < 0.01$) are shown.

for 16 h. Overexpressed a-Syn and ubiquitin proteins were diffusely distributed in the cytoplasm in the absence or presence of MG132 (Fig. 9*A*). The formation of a-Syn- or ubiquitin-positive inclusions was not found in the presence of MG132 (Fig. 9*A*). These findings were consistent with the biochemical fractionation data that there were no ubiquitinated a-Syn proteins in the pellet fractions under the present condition. Also, the formation of a-Syn- or ubiquitin-positive inclusions was not detected in the presence of OA (Fig. 9*A*).

To elucidate whether ubiquitination of lysine residues of a-Syn contributed to the metabolism of Ser-129-phosphorylated a-Syn, we made a K12R/K21R/K23R mutant a-Syn cDNA, whose product abolished the previously reported ubiquitination sites of a-Syn (8). In the experiment using CHX, Ser-129-phosphorylated form of this mutant a-Syn was rapidly decreased (Fig. 9*B*). The expression levels of total a-Syn in the mutant were constant (Fig. 9*B*). Treatment with MG132 suppressed the rapid decrease in the levels of Ser-129-phosphorylated form of the mutant a-Syn (Fig. 9*B*).

DISCUSSION

The present data demonstrated that Ser-129-phosphorylated a-Syn underwent dephosphorylation and degradation. In SH-SY5Y cells stably expressing a-Syn, inhibition of the proteasome pathway or the autophagy-lysosome pathway resulted in the accumulation of Ser-129-phosphorylated a-Syn. However, the inhibitory effect of the proteasome pathway was different in two points from that of the autophagy-lysosome pathway. First, inhibition of the proteasome pathway did not accompany the alteration in the levels of total a-Syn. Second, inhibition of the proteasome pathway increased the levels of Ser-129-phosphorylated a-Syn faster than that of the autophagy-lysosome pathway. To elucidate these differences, we assessed the half-life of Ser-129-phosphorylated a-Syn using CHX. The result showed that the half-life of Ser-129-phosphorylated a-Syn was much shorter than that of total a-Syn. Inhibition of the proteasome pathway remarkably prolonged the short half-life of Ser-129-phosphorylated a-Syn with no alteration in that of total a-Syn. In rat primary cortical neurons, inhibition of the proteasome pathway also accumulated phosphorylated a-Syn at endogenous levels. These findings suggest that Ser-129-phosphorylated a-Syn specifically undergoes degradation by the proteasome pathway, and that the portion of Ser-129-phosphorylated a-Syn is too small to affect the levels of total a-Syn. On the other hand, contribution of the autophagy-lysosome pathway to degradation of phosphorylated a-Syn remains to be elucidated. One may speculate that the autophagy-lysosome pathway selectively degrades non-phosphorylated a-Syn. The increase in the levels of phosphorylated a-Syn may be a consequence of accumulation of non-phosphorylated proteins in the cytosol due to inhibition of the autophagy-lysosome pathway. Alternatively, the autophagy-lysosome pathway may degrade both non-phosphorylated and phosphorylated a-Syn. In this case, the portion of phosphorylated a-Syn is estimated to be very small, because this corresponds to phosphorylated a-Syn, which remains undiminished in the present experiments using CHX.

Since Bennett *et al.* (20) initially reported that inhibition of the proteasome pathway led to the accumulation of overexpressed a-Syn in SH-SY5Y cells, this finding has also been shown in more recent studies (21–24). However, other studies have failed to detect accumulation of endogenous or overexpressed a-Syn by inhibition of the proteasome pathway (25–28). In contrast, a-Syn is reported to accumulate in cells by inhibition of the autophagy-lysosome pathway (29–31). At present, the involvement of the proteasome pathway or the autophagy-lysosome pathway in degradation of a-Syn is still debated. In general, proteins with short half-lives are mostly degraded by the proteasome pathway, whereas most cytosolic proteins with long half-lives (> 10 h) are degraded by the ly-

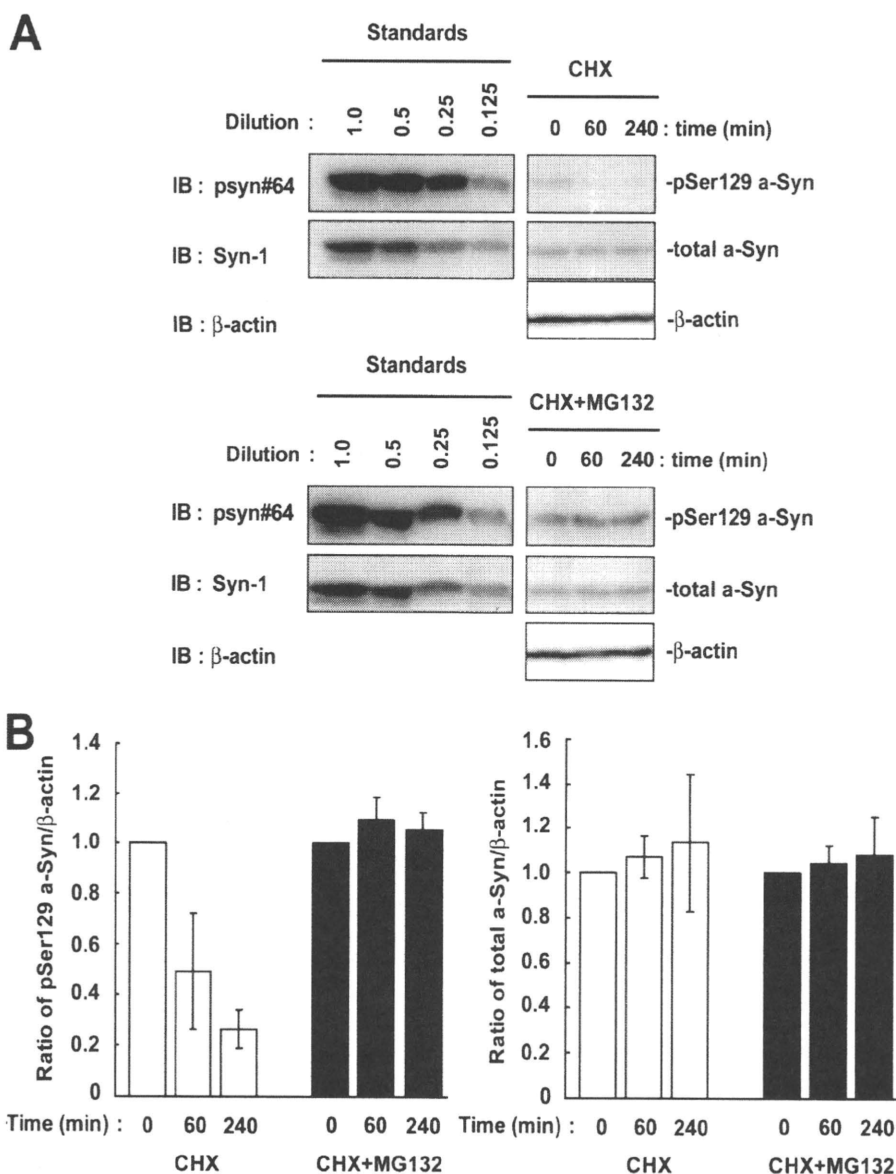

FIGURE 6. **The effect of Ser-9, Ser-42, and Ser-87 residues on the metabolism of Ser-129-phosphorylated a-Syn in SH-SY5Y cells.** The cells were transiently transfected with S9A/S42A/S87A mutant a-Syn cDNA. At 42 h after transfection, the cells were pre-incubated in medium containing either 0.1% DMSO or 10 μM MG132 for 6 h. Then, 100 μM CHX was added into medium. The cells were collected at the indicated times. *A*, cell lysates (10 μg/lane) were analyzed by immunoblotting (*IB*) with psyn 64 and Syn-1 antibodies. For loading control, the same amounts of samples were immunoblotted with anti-β-actin antibody. Experiments were performed three times. Although the blots of standards are separated from those of samples, these blots are originally derived from the same blot. *B*, quantitative analysis of the alteration in the expression levels of Ser-129-phosphorylated and total a-Syn in experiments using CHX. Relative ratios of the band intensity of Ser-129-phosphorylated a-Syn to β-actin and total a-Syn to β-actin are shown by normalizing to the starting materials. Data represent means ± S.D.

sosome pathway (30). In contrast to this principle, a previous study utilized long incubation times over 48 h to detect accumulation of a-Syn by inhibition of the proteasome pathway (21). Additionally, treatment with a selective proteasome inhibitor, epoxomicin, only showed a 2.3-h increase in the half-life of a-Syn (16.8 ± 2 h) in rat ventral midbrain cultures (30). The findings suggest that a portion of a-Syn, which is targeted to the proteasome pathway, is small. These studies may have seen accumulation of Ser-129-phosphorylated a-Syn by inhibition of the proteasome pathway. Also, the present data were consistent with the finding that a-Syn was degraded by the autophagy-lysosome pathway. Ser-129-phosphorylation may be a key for resolving whether the metabolism of a-Syn fulfills this general principle.

In the present study, we investigated the role of Ser residues in targeting a-Syn to the proteasome pathway. a-Syn has four Ser residues and is known to be phosphorylated at Ser-87, as well as at Ser-129 (15). To assess the effect of Ser residues other than Ser-129 on proteasomal degradation, we constructed the S9A/S42A/S87A mutant of a-Syn. In experiments using CHX, overexpressed mutant a-Syn demonstrated a rapid decrease in the levels of the Ser-129-phosphorylated form and the stable expression of total protein. The rapid decrease in the levels of the Ser-129-phosphorylated form was inhibited by MG132. These findings were similar to wild-type a-Syn, suggesting that Ser-129 may play a central role in targeting the protein to the proteasome pathway. However, this study could not exclude a possibility that

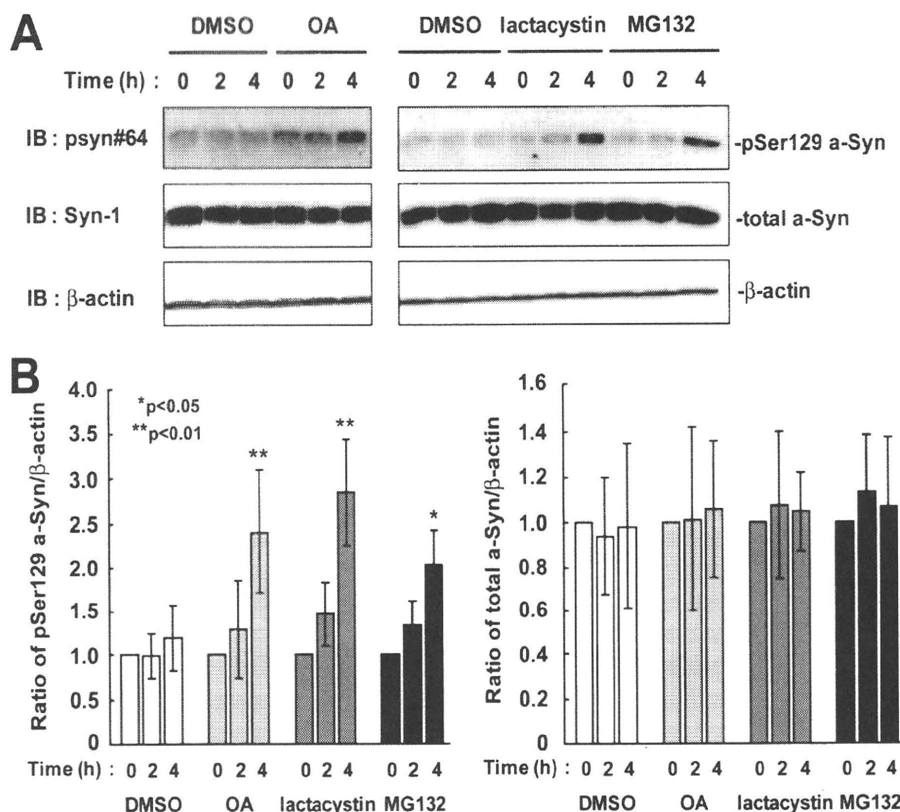

FIGURE 7. **The effects of protein phosphatase 2A/1 and proteasome inhibitors on the levels of Ser-129-phosphorylated a-Syn in rat primary cortical neurons.** *A*, 21-day cultured primary neurons were incubated in fresh medium containing either 0.1% DMSO, 10 μM MG132, 10 μM lactacystin, or 10 nM OA for 4 h. The cell lysates (50 μg/lane) were loaded on SDS-PAGE and analyzed by immunoblotting (*IB*) with psyn 64 and Syn-1 antibodies. For loading control, the same amounts of samples were immunoblotted with anti-β-actin antibody. Experiments were performed four times. Representative blots are shown. *B*, quantitative analysis of the alteration in the expression levels of Ser-129-phosphorylated a-Syn in the cells. Relative ratios of the band intensity of Ser-129-phosphorylated a-Syn to β-actin and total a-Syn to β-actin are shown by normalizing to the starting material. Data represent means $\pm$ S.D., and the *p* values (*, $p < 0.05$; **, $p < 0.01$) are shown.

targeting of a-Syn to the proteasome pathway depends on the phosphorylation of other residues, because a-Syn is also known to be phosphorylated at three tyrosine residues.

We also assessed the metabolism of phosphorylation-mimic (S129E and S129D) and phosphorylation-abolished (S129A) mutants of a-Syn. S129A mutant a-Syn did not show the degradation through the proteasome pathway. In contrast, S129E mutant a-Syn was degraded by the proteasome pathway. These findings were consistent with the proteasomal degradation of Ser-129-phosphorylated a-Syn. However, S129D mutant a-Syn was not targeted to the proteasome pathway. This finding indicated that substitution of aspartic acid for Ser-129 did not mimic the phosphorylated residue. The previous work reported that phosphorylation-mimic mutants of a-Syn did not reproduce the effect of the phosphorylation on the structural properties of a-Syn *in vitro* (10). One may speculate that conformation changes induced by the phosphate group, rather than negative charge, are responsible for the effect of phosphorylation on the metabolism of a-Syn. Further studies are required to elucidate the determinants that lead to the difference in the metabolism between S129E and S129D mutants of a-Syn.

It is well established that conjugation of at least four ubiquitins on a protein is necessary for ubiquitin-dependent degradation to occur (32). However, previous studies have shown that ubiquitination of unmodified a-Syn does not occur after inhibition of the proteasome in transfected cells (20, 27, 28). *In vitro* experiments using purified recombinant proteins have shown that unmodified a-Syn is degraded by the 20 S (21, 23, 24) and 26 S proteasomes (23). These findings indicate that a-Syn is degraded by the proteasome pathway in a ubiquitin-independent manner (21). Although the present data support the previous findings, we propose an idea that Ser-129-phosphorylated a-Syn is targeted to the proteasome pathway in a ubiquitin-independent manner. What is the role of Ser-129-phosphorylation in the proteasomal degradation of a-Syn? The physiological function of a-Syn is thought to require its association with lipid vesicles where it adopts an α-helical conformation from a natively unstructured one (33). Structural studies have shown that a-Syn dynamically binds to vesicles and is promoted to dissociate from the vesicle by mutation (34), oxidative stress (35) and Ser-129-phosphorylation (18). Liu *et al.* (24) reported that 20 S proteasome degraded free, unstructured a-Syn, but not vesicle-bound, α-helical a-Syn. The Ser-129-phosphorylation may trigger the dissociation of a-Syn proteins from vesicles and accumulate free, unstructured proteins in the cytosol, resulting in targeting them to the proteasome pathway.

In summary, our data suggest that Ser-129-phosphorylation plays a role in the rapid degradation of a-Syn by targeting the protein to the proteasome pathway. Bedford *et al.* reported that depletion of the 26 S proteasome in

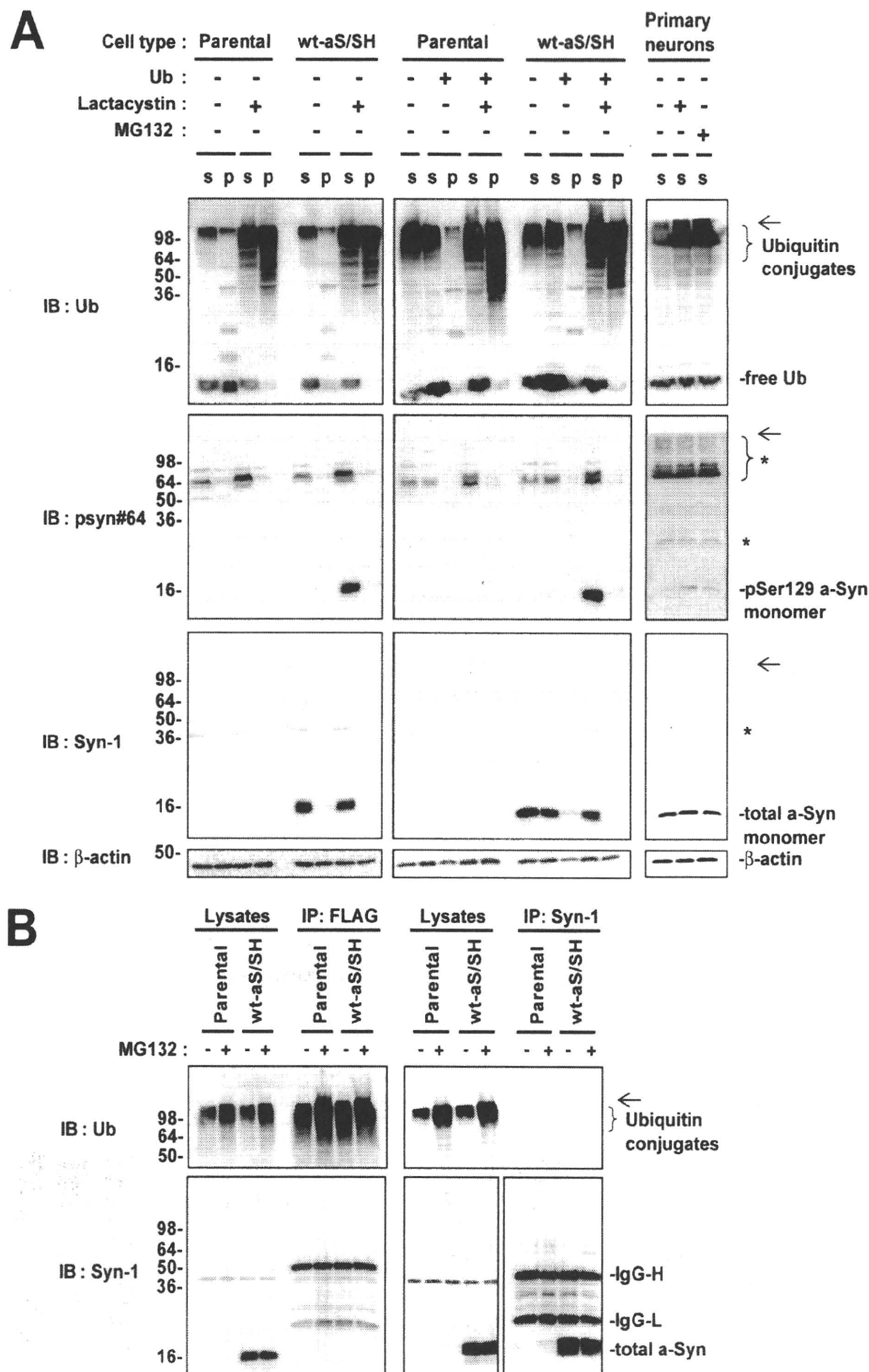

FIGURE 8. **Immunoblotting analysis of ubiquitination and proteasomal degradation of Ser-129-phosphorylated a-Syn in SH-SY5Y cells and rat primary cortical neurons.** *A*, immunoblotting (*IB*) analysis of ubiquitination of a-Syn in SH-SY5Y cells and primary cortical neurons. Parental SH-SY5Y cells and wt-aS/SH cells were transfected with or without ubiquitin cDNA and they were incubated in medium containing either 0.1% DMSO or 10 μM lactacystin for 16 h. In SH-SY5Y cells, the collected cells were fractionated to supernatant (*S*) and pellet (*P*) fractions. Rat primary cortical neurons were incubated in medium either 0.1% DMSO, 10 μM lactacystin, or 10 μM MG132 for 4 h, and collected supernatant (*S*) fractions. Samples (20 μg/lane) were loaded on SDS-PAGE and analyzed by immunoblotting with anti-ubiquitin, psyn 64 and Syn-1 antibodies. For loading control, the same amounts of samples were immunoblotted with anti-β-actin antibody. *Left* and *middle panels* show the immunoblots of SH-SY5Y cells without overexpression of ubiquitin and SH-SY5Y cells transiently overexpressing ubiquitin, respectively. *Right panels* show the immunoblots of rat primary cortical neurons. * indicates nonspecific bands. The *arrows* indicate the interface of resolving and stacking gels. *B*, immunoprecipitation (*IP*) analysis of ubiquitination of a-Syn in SH-SY5Y cells. Parental SH-SY5Y cells or wt-aS/SH cells were transfected with FLAG-tagged ubiquitin cDNA and incubated in the presence or absence of MG132 for 4 h. Cell lysates were subjected to immunoprecipitation with anti-FLAG (*left panels*) and Syn-1 antibodies (*right panels*). Equivalent amounts of immunoprecipitated products were analyzed by immunoblotting with anti-ubiquitin (*upper panels*) and Syn-1 antibodies (*lower panels*). The *arrows* indicate the interface of resolving and stacking gels.

mouse neurons caused extensive neurodegeneration in the nigrostriatal pathway and the formation of LB-like inclusions containing a-Syn (36). This study strongly indicates that 26 S proteasome dysfunction in neurons is involved in aggregation of a-Syn (36). Ser-129-phosphorylation might provide a clue for linking the degradation pathway of a-Syn with the forma-

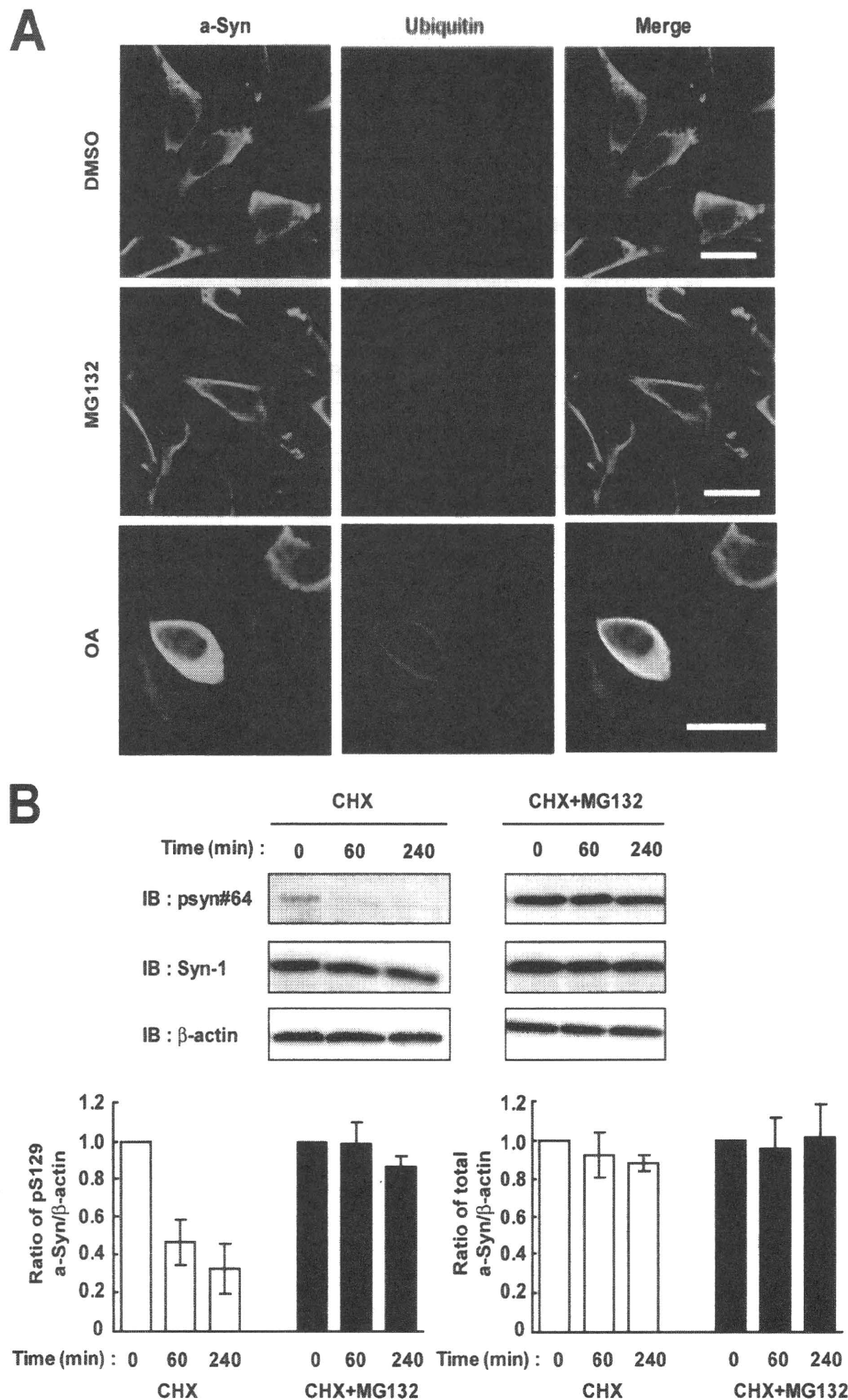

FIGURE 9. **Immunocytochemical and biochemical analysis of ubiquitination and proteasomal degradation of Ser-129-phosphorylated a-Syn in SH-SY5Y cells.** *A*, immunocytochemical analysis of a-Syn and ubiquitin in proteasomal inhibitor-treated SH-SY5Y cells. Wt-aS/SH cells were transfected with ubiquitin cDNA, and they were incubated with medium containing either 0.1% DMSO, 10 μM MG132, or 10 nM OA for 16 h. Immunostainings of a-Syn (*green, left panels*) and ubiquitin (*red, middle panels*), and merged images (*left panels*) are shown. Scale bars represent 20 μm. *B*, role of Lys-12, Lys-21, and Lys-23 residues in proteasomal degradation of Ser-129-phosphorylated a-Syn in cells. The cells were transiently transfected with K12R/K21R/K23R mutant a-Syn cDNA. At 42 h after transfection, the cells were pre-incubated in medium containing either 0.1% DMSO or 10 μM MG132 for 6 h. Then, 100 μM CHX was added into medium. The cells were collected at the indicated times. The cell lysates (10 μg/lane) were analyzed by immunoblotting (*IB*) with psyn 64 and Syn-1 antibodies. For loading control, the same amounts of samples were immunoblotted with anti-β-actin antibody. Experiments were performed three times. The graphs show the alteration in the expression levels of Ser-129-phosphorylated and total a-Syn. Relative ratios of the band intensity of Ser-129-phosphorylated a-Syn to β-actin and total a-Syn to β-actin are shown by normalizing to the starting materials. Data represent means ± S.D.

tion of LBs. However, the present study does not resolve the question why Ser-129-phosphorylated a-Syn proteins deposited in LBs are also ubiquitinated (9, 37). Our data suggest that phosphorylated a-Syn proteins undergo ubiquitination in the pathway independent of its physiological degradation. Ubiquitination might represent an unsuccessful "last-ditch

stand" of cells in their attempt to unfold and/or degrade misfolded proteins in a disease-specific pathway (37). Alternatively, ubiquitination of a-Syn might occur after polymerization of the molecule (38). Further studies are required to clarify whether the proteasome dysfunction accumulates Ser-129-phosphorylated a-Syn selectively, resulting in generation of LBs, which abundantly contain Ser-129-phosphorylated a-Syn.

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ORIGINAL COMMUNICATION

Progressive anterior operculum syndrome due to FTLD-TDP: a clinico-pathological investigation

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Abstract Pathological investigation of progressive anterior operculum syndrome has rarely been reported. We describe clinico-pathological findings in a patient with progressive anterior operculum syndrome. A 74-year-old right-handed man had noticed speech and swallowing difficulties 1 year previously. Neurological examinations showed no abnormality other than a slight limitation of upward gaze and slow tongue movement without fibrillation. We investigated the patient using neuroimaging and neuropsychological examinations and observed him for 2 years until his death, at which point we obtained pathological findings. The patient's facial and masseteric muscles seemed hypotonic with drooling, but he could laugh and yawn normally, showing automatic voluntary dissociation. Palatal and pharyngeal reflexes were normal. Magnetic resonance imaging showed cortical atrophy in the temporal lobes bilaterally. ^{123}IMP single photon emission computed tomography and positron emission tomography showed decreased blood flow and activity in the frontotemporal lobes, predominantly on the left side. Neuropsychological examinations showed no aphasia, dementia or other neuropsychological abnormality. Intubation fiberscopy, laryngoscopy and video fluorography showed no abnormality. After 6 months his anarthria and dysphagia became aggravated. He died of aspiration pneumonia 2 years after onset. Postmortem examination revealed neuronal degeneration with TDP-43-positive inclusions in the frontal, temporal and insular cortices,

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consistent with frontotemporal lobar degeneration with TDP inclusions (FTLD-TDP). However, neuronal loss with gliosis was more prominent in the inferior part of the motor cortices, bilaterally. Progressive anterior operculum syndrome could be classified as a variant of FTLD-TDP.

Keywords Progressive anterior operculum syndrome · Foix–Chavany–Marie syndrome · FTLD · TDP-43

Introduction

Anterior operculum syndrome or Foix–Chavany–Marie syndrome (FCMS), is characterized by anarthria and central voluntary paralysis of the lower cranial nerves with preserved automatic and emotional movement [7, 12]. Most of this syndrome is a result of cerebrovascular disease involving the anterior operculum, bilaterally or unilaterally [2, 3, 7, 11, 17, 19–21, 25, 26, 28, 29, 31]. However, it remains uncertain whether the causative lesion is the operculum proper or its vicinity, because other lesions are also suggested [13]. In addition to the reports of patients due to cerebrovascular disease, there are a few reports of patients with primary progressive pseudobulbar palsy showing both anarthria and dysphagia simultaneously [14, 22, 30], which can be referred to as anterior operculum syndrome or FCMS. Progressive pseudobulbar palsy has been reported as neurodegenerative disorders, including tauopathy, frontotemporal lobar degeneration (FTLD) with motor neuron disease, and dementia. However, there have been only a few detailed clinical descriptions or distinctive histologies [5, 6, 14, 15, 22, 23, 30, 32]. Histological information is necessary to clarify the causative lesion and etiology of primary progressive anterior operculum syndrome. Here, we can offer the information about a patient with primary progressive anterior operculum syndrome, in whom postmortem examination revealed marked neuronal loss with TDP-43-immunoreactive inclusions in the frontal lobes, particularly in the inferior part of the motor cortices, bilaterally. This is the first report of an autopsy-confirmed anterior operculum syndrome with FTLD and TDP-43-positive inclusions (FTLD-TDP) [18].

Case presentation

A 74-year-old right-handed man consulted our hospital with a complaint of insidious onset of speech and swallowing impairment over the previous 9 months. There was no evidence of memory impairment from the history, because he could continue his drapery job without any difficulty. At the first consultation, his facial and masseteric muscles seemed hypotonic and he held his mouth half open with a tendency to drool. His voice was hypotonic and he could manifest only laborious, syllabic phonemes or short phrases with dysprosody, whose articulation was distorted and varied depending on the situation, demonstrating anarthria. He was unable to imitate oro-bucco-facio-linguo-pharyngeal movements, such as pretending to cough, blow out a match, stick out his tongue, and click his tongue voluntarily. The most striking characteristic was the persistent dissociation between automatic (spontaneous) and voluntary movements of facial expression. For example, although he could not move his facial muscles, mouth or tongue on command, he sometimes laughed clearly at jokes or smiled at acquaintances, and he sometimes swallowed his saliva. He could occasionally lick his lips, keep candy in his mouth and sometimes drink water.

Neurological examination

He was alert, fully oriented and cooperative. He could not speak but could adequately answer every question in writing, with no difficulty in finding words or comprehending language. His cranial nerves were normal except for a slightly limited upward gaze, but he could spontaneously move his eyes in other natural situations. Motor and sensory systems were normal with normal muscle tones without muscle wasting or fasciculation and no pathological reflexes were elicited. Palatal reflex was attenuated and gag reflex was normal. His coordination, posture and gait were normal.

Neuropsychological examination

The results of neuropsychological examination are summarized in Table 1. The western aphasia battery (WAB) showed a perfect score in comprehension and in confrontation naming which was expressed by writing, because of anarthria. Reading showed a somewhat low score, because of impaired reading aloud; however, there was no difficulty in reading comprehension. Writing was not flawless, because he showed morphological impairment and a tendency toward micrographia. He could draw simple geometrical figures such as triangles and cubes, and he also demonstrated a good score (SS score 11) in the block design task from WAIS-R, which showed that he did not have constructional impairment. All of the neuropsychological examinations indicated that he preserved general intelligence and language ability without aphasia, except for speech output.

Magnetic resonance imaging of the brain

Magnetic resonance imaging (MRI) demonstrated marked cortical atrophy with multiple ischemic lesions in the

Table 1 Neuropsychological examinations

	9 M	15 M
Western aphasia battery		
Comprehension	10/10	10/10
Confrontation naming	20/20[a]	[e]
Reading	8/10[b]	–
Writing	8.4/10[c]	–
Raven's colored matrices	29/36[d]	21/36
The Wechsler adult intelligence scale-revised		
Verbal IQ	83[a]	83[a]
Performance IQ	98	–
Full IQ	89	–

M months after onset, – not administered

[a] by writing due to severe anarthria

[b] lower scores due to inability of reading aloud, but reading comprehension was perfect

[c] writing showed morphological impairment and a tendency toward micrographia

[d] this is within the age-matched normal range

[e] it was difficult for him because of his deteriorated writing ability

corona radiate, medial temporal lobe and brainstem, bilaterally. The scattered ischemic lesions seemed not to directly affect his severe impairment of speech and swallowing (Fig. 1a).

Further examinations and evolution

During the 6 months after the first consultation (15 months after onset), the patient's symptoms deteriorated and he was admitted for further examinations. His facial expression seemed hypotonic, with sustained drooling, and his writing was impaired with phonological and verbal paragraphia. His penmanship was distorted and showed micrographia; however, he was courteous and well oriented as ever.

Neurological examination revealed almost the same condition as seen 6 months previously, except for additional findings of exaggerated jaw jerk (masseteric reflex). Palmomental, bilateral orbicularis oculi, orbicularis oris and snout reflexes were also easily elicited, and there was rigidity in the upper and lower extremities.

Laryngo-pharynx investigation

Intubation fiberscopy showed no abnormality. Laryngoscopy showed no vocal cord paralysis. Video fluorography revealed that the patient was unable to initiate swallowing, but reflex swallowing was preserved; therefore, once food was placed into the posterior pharynx, he could swallow it.

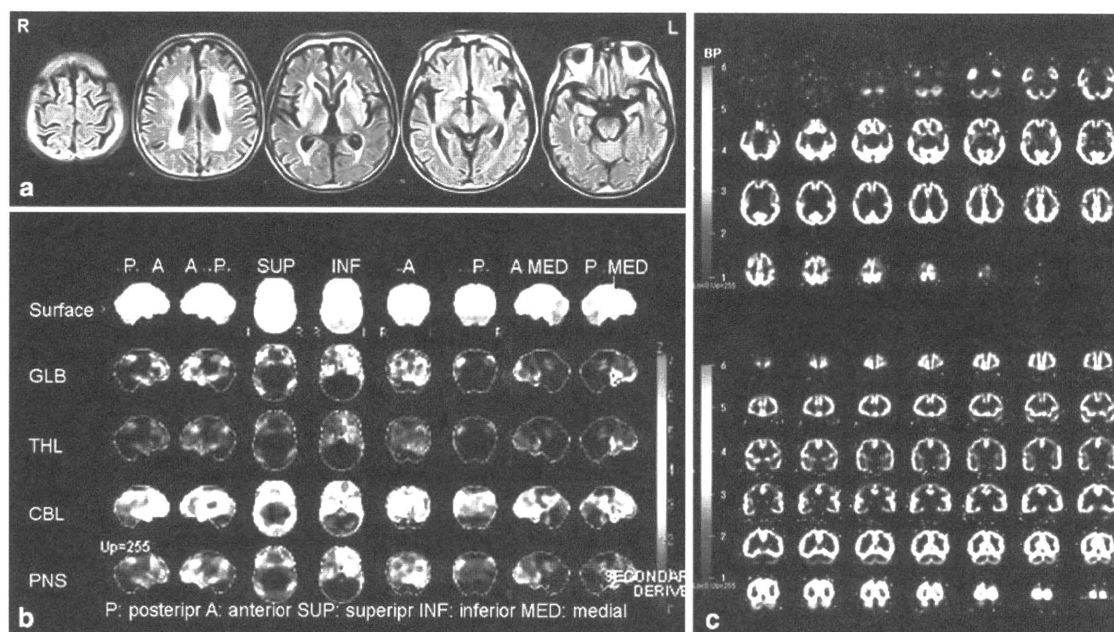

Fig. 1 Neuroimaging. **a** Magnetic resonance imaging of the patient using the fluid-attenuated inversion recovery method showed marked cortical atrophy accompanied with multiple ischemic lesions in the bilateral corona radiate, medial temporal lobe and brainstem. **b** ^{123}IMP SPECT, three-dimensional stereotaxic surface projection images. GLB (the *second row*) shows *Z* score images normalized by the whole brain count demonstrated the decrease of ^{123}IMP uptake in the frontotemporal regions bilaterally but predominantly on the left side. **c** PET Dynamic C-11-flumazenil brain positron emission computed tomography demonstrated the diffuse decrease of activities in the frontal and temporal lobes bilaterally by binding potential images

Functional neuroimaging examinations

The quantitative measurement of regional cerebral blood flow using [N-isopropyl-(iodine-123)-p-iodoamphetamine] (^{123}IMP) single photon emission computed tomography (SPECT) demonstrated that the uptake of ^{123}IMP was decreased in the frontotemporal regions bilaterally but predominantly on the left side (Fig. 1b). Dynamic C-11-flumazenil brain positron emission computed tomography (PET) demonstrated the diffuse decrease of activities in the frontal and temporal lobes bilaterally (Fig. 1c).

Neurophysiological examinations

Electromyography (EMG) of his lingual muscle, left sternomastoid muscle and left first dorsal interosseous muscle demonstrated that he could not spontaneously contract his tongue but resting lingual EMG was normal. EMG of sternomastoid and first dorsal interosseous muscles showed no abnormality.

Hematological and biochemical investigations

All hematological and biochemical investigations were normal, including thyroid function. Cerebrospinal fluid was acellular with normal total protein but there was an increase of tau protein (548 pg/ml, normal <150 pg/ml).

After these examinations, he returned home. He was re-admitted to our hospital a month later, because of dehydration. In the following 2 months, he suffered aspiration pneumonia and died 20 months after the onset of the first symptom. An autopsy was performed and neuropathological examination was limited to the brain.

Neuropathological investigation

His slightly atrophic brain weighed 1,210 g after fixation. The cerebral hemispheres were sectioned coronally and multiple samples were taken bilaterally. Histologically, microvacuolation was found in layer II of the frontal, temporal and insular cortex (Fig. 2a, b). Neuronal loss with gliosis was more prominent in the inferior part of the motor cortices, bilaterally, which was more severe on the left than the right side (Figs. 2c, d, Fig. 3). Moderate loss of neurons with gliosis was found in the amygdaloid nucleus. Gliosis without obvious neuronal loss was evident in the ventral putamen and anteromedial regions of the thalamus. In the brainstem, moderate loss of neurons with gliosis was found in the hypoglossal nucleus. The pyramidal tracts showed slight myelin pallor. TDP-43 immunohistochemistry revealed numerous neuronal cytoplasmic inclusions in the frontal and temporal cortex, amygdaloid nucleus, dentate gyrus, claustrum, neostriatum, and thalamus, and a few in the substantia nigra, facial nucleus, hypoglossal nucleus and inferior olivary nucleus (Fig. 2e–g). TDP-43-immunoreactive glial inclusions were also found in the frontal white matter and globus pallidus (Fig. 2h). Cystatin C immunohistochemistry confirmed the absence of Bunina

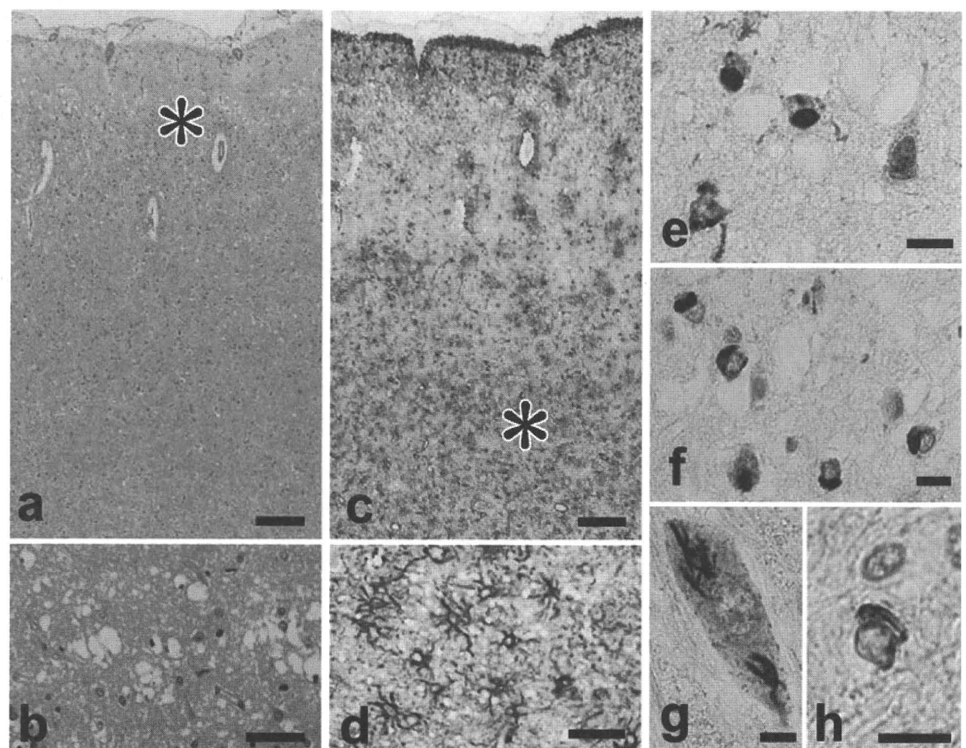

Fig. 2 **a–d** Serial sections of the inferior part of the left motor cortex. **a** Moderate to marked loss of neurons with superficial spongiosis. **b** Higher magnification view of the area indicated by *asterisk* in **a** showing marked vacuolation in the neuropils. **c** Marked gliosis in all cortical layers. **d** Higher magnification view of the area indicated by *asterisk* in **c** showing reactive astrocytes with thick processes. TDP-43-positive neuronal cytoplasmic inclusions and neurites in the frontal cortex (**e**) and amygdaloid nucleus (**f**). **g** TDP-43-positive skein-like inclusions in the facial nucleus. **h** TDP-43-positive glial inclusions in the frontal white matter. *Bars* 200 μm in **a**, **c**, 50 μm in **b**, **d** and 10 μm in **e–h**. **a**, **b** H&E; **c**, **d** GFAP immunostain; **e–h** TDP-43 immunostain

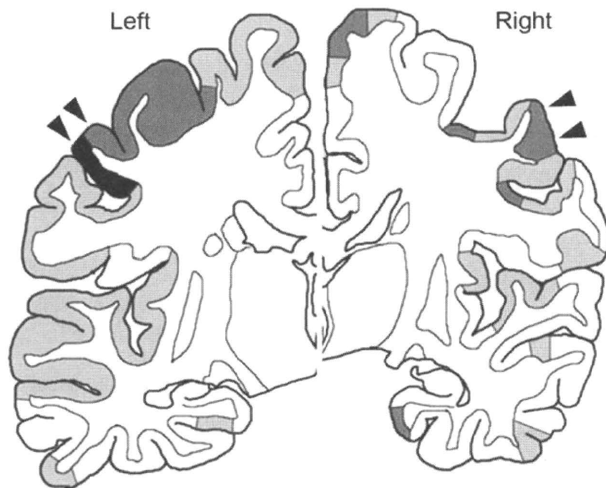

Fig. 3 Schematic representation of the distribution of neuronal loss in the patient. Shading intensity corresponds to the severity of involvement. *Arrowheads* indicate the motor cortex

bodies in the lower motor neurons. Based on the above findings, our case was diagnosed as having type 2 of FTLD-U according to Sampathu's classification [24] or FTLD-TDP using a newly established nomenclature [18]. Moreover, many neurofibrillary tangles were found in the hippocampal formation, and some in the frontal and temporal neocortex (Braak stage IV). Many senile plaques were also seen in the cerebral neocortex (Braak stage C). No Lewy bodies were found in our case.

Discussion

Our patient showed progressive anarthria and dysphagia coincidentally, with conspicuous automatico-voluntary dissociation with selective impairment of volitional facial, pharyngeal, lingual, masticatory and ocular movements but without automatic movements and reflexes, all of which are symptoms consistent with anterior operculum syndrome or FCMS [7]. The patient manifested exaggerated jaw jerk accompanied by snout, orbicularis oculi and oris, and palmomental reflexes in the presence of the gag reflex. Mao et al. [19] advocated that decreased gag reflex is a sign that can distinguish anterior operculum syndrome from other pseudobulbar palsies; however, retaining and decreasing the gag reflex does not always seem a decisive finding of significance for a diagnosis of anterior operculum syndrome, because Davies et al. [9] reported that 37% of 140 healthy people lacked a gag reflex.

Several investigators have reported progressive loss of speech output in anterior operculum syndrome [5, 6, 8, 10, 14, 15, 22, 23, 27, 30, 32]. Although progressive anarthria was common in these cases, the other clinical symptoms and the prognosis seemed to be heterogenous. Among these reports, some patients showed anarthria accompanied with pseudobulbar palsy, whereas the other patients showed advanced speech impairment for several years [6, 22, 23, 32] or more than 10 years [5, 15] before the onset of dysphagia. Our patient's speech impairment was consistent with so-called pure anarthria [16] from the viewpoint of speech and language terminology, but simultaneously accompanied with dysphasia, without time lag. Our patient must be differentiated from primary progressive anarthria [6]. Thus, we designate this syndrome as primary progressive anterior operculum syndrome. Aggravation of the symptoms in our patient was rapid as compared with other primary progressive anarthria with late-onset FCMS [5, 15, 22, 23, 32].

To our knowledge, there have been only three patients with primary pesudobulbar palsy demonstrating both anarthria and dysphagia simultaneously [14, 22, 30]. Lampl et al. [14] reported a case of primary progressive pseudobulbar palsy, in whom postmortem examination was not performed. Patient 3 reported by Tyrell et al. [30] and Patient 2 reported by Nakajima et al. [22] finally showed muscle wasting and fasciculation, indicating evidence of motor neuron disease. Neuropathological examination in Patient 2 of Nakajima et al. [22] revealed frontotemporal degeneration with superficial spongiosis, tau accumulation in the medial temporal lobe, pyramidal tract degeneration, and moderate neuronal loss in the spinal anterior horn. In our case, microvacuolation was found in the superficial layers of the frontal, temporal and insular cortex. In addition, TDP-43-immunoreactive neuronal inclusions were abundant in these areas, consistent with histopathological features of FTLD-TDP [18]. It is noteworthy that neuronal loss with gliosis was more prominent in the inferior part of the motor cortices, bilaterally. Moreover, moderate neuronal loss was found in the hypoglossal nucleus and TDP-43-positive inclusions were seen in the hypoglossal and facial nuclei, suggesting that our patient could be diagnosed as FTLD-TDP with motor neuron disease. In our patient, moderate Alzheimer pathology was also evident. Recent studies have shown that TDP-43 is also deposited in the brain of patients with neurodegenerative tauopathies, including Alzheimer's disease [1]. However, the distribution and extent of TDP-43 pathology in FTLD-TDP with or without motor neuron disease is much more severe and widespread than in Alzheimer's disease [4]. We feel that the causative lesion of anterior operculum syndrome in our patient was in the frontal lobes, especially in the inferior part of the motor cortex.

Clinico-pathological investigation of this patient will provide diagnostic and nosologic criteria for FTLD-TDP. We concluded that there is a peculiar clinical syndrome

which shows progressive anterior operculum syndrome as a variant of FTLD-TDP. Evaluation of patients with progressive speech output and dysphagia should include FTLD-TDP in the differential diagnosis.

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ORIGINAL COMMUNICATION

Writing errors as a result of frontal dysfunction in Japanese patients with amyotrophic lateral sclerosis

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Abstract Loss of communication is a critical problem for advanced amyotrophic lateral sclerosis (ALS) patients. This loss of communication is mainly caused by severe dysarthria and disability of the dominant hand. However, reports show that about 50% of ALS patients have mild cognitive dysfunction, and there are a considerable number of case reports on Japanese ALS patients with agraphia. To clarify writing disabilities in non-demented ALS patients, eighteen non-demented ALS patients and 16 controls without neurological disorders were examined for frontal cognitive function and writing ability. To assess writing errors statistically, we scored them on their composition ability with the original writing error index (WEI). The ALS and control groups did not differ significantly with regard to age, years of education, or general cognitive level. Two patients could not write a letter because of disability of the dominant hand. The WEI and results of picture arrangement tests indicated significant impairment in the ALS patients. Auditory comprehension (Western Aphasia Battery; WAB IIC) and *kanji* dictation also showed mild impairment. Patients' writing errors consisted of both syntactic and letter-writing mistakes. Omission, substitution, displacement, and inappropriate placement of the phonic marks of *kana* were observed; these features have often been reported in Japanese patients with agraphia resulted from a frontal lobe lesion. The most frequent type of error was an omission of kana, the next most common was a missing subject. Writing errors might be a specific deficit for some non-demented ALS patients.

Keywords Assessment of cognitive disorders/dementia · Amyotrophic lateral sclerosis · Agraphia · Frontal lobe dysfunction

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Introduction

Recently, considerable evidence has been gathered for cognitive impairment in amyotrophic lateral sclerosis (ALS). After the consensus criteria for frontotemporal lobar dementia (FTLD) were established, the frequency of dementia reported in ALS patients increased from 6 to 23%, and as many as 50% showed cognitive change if mild frontal dysfunction was present [17, 20, 21, 26]. One of the

most intensively investigated cognitive problems was verbal fluency [17]. Though slowness and inarticulation of speech caused by oropharyngeal motor dysfunction was present, subjects' verbal fluency for the first letter was also impaired. Functional magnetic resonance imaging revealed reduced activation of the middle and inferior frontal gyrus and anterior cingulated gyrus [1]. Many other methods of examination have been reported to effectively identify mild cognitive impairment, however, the patterns of abnormality vary so much that it is unclear which examination is most suitable for screening [32].

Some patients who seem mentally normal are unable to write well when they cannot articulate. Loss of communication is a critical problem for both patients and caregivers. In a retrospective study of Japanese ALS patients having dementia, writing errors were prominent; agraphia and anosognosia were specifically noted in the case of ALS with dementia [13]. The study also found a high prevalence of agraphia among non-demented bulbar-onset ALS patients [15]. Several cases of ALS patients with excessive spelling mistakes were reported [8, 10, 16, 21, 28]; however, the mechanism of agraphia is not well understood. Some single photon emission computed tomography studies have speculated that the cause of agraphia may be reduction in cerebral blood flow caused by a lesion in the frontal lobe, but the number of patients was too small to draw a definite conclusion.

In this study, we clarified that writing errors were frequently noted among non-demented ALS patients. Writing errors may be indicated in the early diagnosis of mild cognitive impairment. Analysis of error patterns is helpful for improving communication in patients at advanced stages of ALS.

Patients and methods

Patients

Of all the sporadic ALS patients admitted to the neurological department of Hokkaido University Hospital and Hokuyukai Hospital from May to November 2008, 18 patients agreed to participate in the study. ALS was clinically diagnosed according to the revised El Escorial Criteria (rEEC) [7]. Patients who met Neary's FTLD criteria or scored under mean − 2SD on Raven's colored progressive matrices (RCPM) were excluded [21]. Patients with respiratory symptoms [% forced vital capacity, (FVC) $<70\%$], and a history of neurological or psychiatric illness were also excluded. Among the patients examined, one was left-handed. Sixteen volunteers who did not have any neurological disease were examined as controls; they were adjusted to the patients for age and educational period. The general cognitive status was assessed by RCPM, and all 16 controls scored above the mean − 2SD. Patients and controls gave written informed consent according to the Helsinki II Declaration and the study was approved by the Ethics Committee of Hokkaido University Hospital.

Neuropsychological examination

The following neuropsychological examinations were performed: RCPM, forward and backward digit span (adopted Wechsler Adult Intelligent Scale; WAIS-R), trail making test (TMT)-A and -B, frontal assessment battery (FAB)-4 and 5, paced auditory serial addition task (PASAT)-2 and PASAT-3, imitating three kinds of hand patterns and a copy of a 2-D cube, three kinds of picture arrangements (PA) (Japanese version of WAIS-R IV 3-5), naming and pointing to 12 objects (6 pictures and 6 real ordinary objects), auditory comprehension (Western Aphasia Battery: WAB-IIC), repetition (WAB-III), reading and dictation of both kanji and kana, and composition. Reading and dictation tests were performed using 47 kanji characters and 47 pairs of kana. The kanji characters used for reading and dictation were selected from the Japanese educational kanji for the third grade in elementary schools. The kana pairs were selected such that all 50 letters were included.

Investigation of writing ability

For investigation of writing ability, the subjects were asked to give a written description of the stories for the Japanese version of WAIS-R IV (iii)–(v) and WAB-IV (a picture of a picnic). To quantify the writing error for statistic analysis, we designed the writing error index (WEI). WEI was calculated as the number of errors divided by the total number of words. The errors included not only easy mistakes such as omission, substitution, and displacement of a letter, but also complex mistakes such as writing an incomprehensible sentence and a grammatical mistake, were also checked as one error. The detail of the calculating rule is described in supplementary file 1 and 2. WEI was calculated by two examiners (one was experimentally blinded), and the mean values of their assessments were used in the analysis.

Statistical analysis

Comparative analysis between ALS patients and controls was performed by Student's t test or Wilcoxon-Mann–Whitney's test in accordance with variance, using KaleidaGraph 4.0. Correlations among batteries were calculated with Pearson's formula using StatCell 2 software, an Excel ad-on.

Table 1 The Profile of ALS patients and controls

	Patients	Control	*p* value
Number (*n*)	18	16	
Age (mean, ±SD)	65.4 ± 11.5	64.5 ± 8.4	0.8007
Female:male	9:9	11:5	
Disease duration (months, mean, ±SD)	19.4 ± 7.0	–	
Educational period (years, mean, ±SD)	10.8 ± 2.5	12.0 ± 4.1	0.1279
RPCM (mean, ±SD)	28.8 ± 5.5	31.1 ± 4.1	0.1741
WER exam (*n*)	16	16	
ALS-FRS-R (total) (mean ± SD)	34.7 ± 7.5		
rEEC at examination			
Definite	4		
Probable	8		
Laboratory-supported probable	1		
Possible	3		
Suspect	2		
Onset			
Brain stem	10		
Cervical	6		
Thoracic	0		
Lumbosacral	1		
Multiple	1		

n Number of patients, *SD* standard deviation, *RCPM* Raven's colored progressive matrices, *WEI* writing error index, *ALSFRS-R* revised ALS functional rating scale, *rEEC* revised El Escorial Criteria

Results

The profile of the patients is shown in Table 1. Patients categorized under "suspected or possible ALS" at the time of examination deteriorated to "possible or probable ALS" by August 2009. One patient who had not been diagnosed with dementia at the time of examination was suspected of behavior change 10 months later. Two patients could not speak clearly because of severe dysarthria. One patient could perform neither dictation nor composition because of disability of the dominant hand, and another patient refused to write all four compositions for the WEI because her education had been erratic and incomplete in her school-going days.

The results of the neuropsychological examination are shown in Table 2. The results of PA and WEI showed that the patients in the ALS group had significant impairment compared to the controls. Forward and reverse digit span, 2 seconds-version of PASAT (PASAT-2), auditory comprehension (WAB-IIC), and kanji dictation also showed statistically significant impairment. However, we did not label these subjects as cognitively impaired. The individual score of digit span was within normal limits. The results of the 3 seconds-version of PASAT means the PASAT-2 was influenced by motor impairment.

WEI was significantly higher in ALS patients than control groups, and a high WEI index was also found in some patients with good dictation performance. Among 16 patients examined for dictation, three patients apparently showed agraphia both in kana and kanji dictations. Four patients had low scores only in the kanji dictation. The pathological implication of an isolated kanji dictation was unclear because a kanji dictation is easily influenced by education. In the kanji-limited impaired group, one patient refused to write the compositions (as mentioned before). The WEI of the other three patients were 14.4, 12.6, and 12.9, respectively, which were much higher than the average WEI of controls. Furthermore, among nine patients who scored well on the dictation, three patients showed WEI indexes higher than 10.

On composition, writing errors were of two types—syntactic and non-syntactic. Non-syntactic errors included both kana and kanji, and the number of errors of kanji showed a smaller difference between patients and controls (Fig. 1a). Kana errors included omissions, substitutions, displacements, incorrect phonetic marks (a pair of dots that convert an unvoiced consonant to a voiced consonant when it is added in the right upper corner); and imperfect characters (Fig. 2). Among 122 total kana errors, 60 errors were omissions. Omission of a kana-letter was a characteristic of ALS patients, but it was sometimes observed in the controls, too; however, most omissions were a single letter in controls. On the other hand, the patients had a higher number of total omissions. Sometimes, sequences of letters were deleted within a word or extended to the next word. Postpositional particles, represented by a single kana letter and corresponding to the preposition in English, were frequently omitted. Other frequent errors in the patients were phonetic marks and phonologically plausible substitutions of kana letters at postpositions. Syntactic errors were classified into four major

Table 2 Results of neuropsychological examinations

	Number of patients	Full score	ALS (mean ±SD)	Control (mean ±SD)	*p* value
Digit span (forward)*	18	12	6.9 ± 2.2	8.9 ± 2.5	0.018
Digit span (reverse)*	18	12	5.6 ± 2.9	7.7 ± 1.6	0.015
TMT B/A	16		2.9 ± 1.3	3.0 ± 2.2	0.885
TMT B-A	16		110.3 ± 69.8	80.3 ± 77.5	0.258
RCPM	18	37	28.8 ± 5.5	31.1 ± 4.1	0.174
FAB-4	18	3	2.4 ± 1.1	2.9 ± 0.3	0.062
FAB-5	18	3	1.7 ± 1.3	1.9 ± 1.3	0.635
PASAT-3	16	60	29.5 ± 15.8	38.6 ± 16.1	0.133
PASAT-2*	16	60	22.5 ± 10.9	32.2 ± 11.2	0.026
Picture arrangements*	18	3	1.8 ± 1.2	2.9 ± 0.4	0.009
Parietal lobe function	15	4	3.7 ± 0.6	3.8 ± 0.5	0.645
Naming	18	12	11.9 ± 0.3	12 ± 0.0	0.333
Pointing	18	12	12	12	–
Audiotory comprehension* (WABII-C)	18	80	72.0 ± 8.0	77.6 ± 3.6	0.016
Repetition (WABIII)	16	100	91.5 ± 22.1	99.5 ± 1.2	0.169
Dictation (kana)	16	47	44.5 ± 5.7	46.8 ± 0.8	0.140
Dictation (kanji)*	16	47	36.5 ± 15.6	46.5 ± 0.6	0.022
Writing error index (WEI)*	16		12.7 ± 8.2	2.9 ± 3.0	<0.001
Non-syntactic error (index)*	16		9.6 ± 8.6	2.3 ± 2.3	0.004
Syntactic error (index)*	16		2.4 ± 2.8	0.5 ± 0.9	0.028

SD standard deviation, *n* number of patients, *TMT* trail making test, *RCPM* Raven's colored progressive matrices, *FAB* frontal assessment batteries, *PASAT* paced auditory serial addition task, *WAB* Western aphasia battery, *WEI* writing error index

patterns: (1) missing subject, (2) unfinished sentence (loss of one or more phrases including verb), (3) mismatch between a subject and its verb, and (4) inappropriate use of conjunctions (Fig. 1b). Missing a subject is a common feature of spoken Japanese, and some controls omitted subjects when writing Japanese. Therefore, it was difficult to determine whether this was an actual mistake. However, as shown in Fig. 1b, the total number of missing subjects in ALS cases was much higher than that in the control cases. When two or more people were present in a story, subjectless sentences were often unclear if the reader was not shown the pictures.

To investigate the implication of disease duration, severity, and general cognitive status in writing errors, we calculated Pearson's correlation between WEI and the parameters. As shown in Table 3, WEI was not associated with the total, bulbar, and upper limb parts of the revised ALS functional rating scale (ALSFRS-R), while it was mildly associated with disease duration and the RCPM score [9]. Indeed, the patient having the worst ALSFRS-R with moderate bulbar symptoms performed best both in writing and other cognitive examinations.

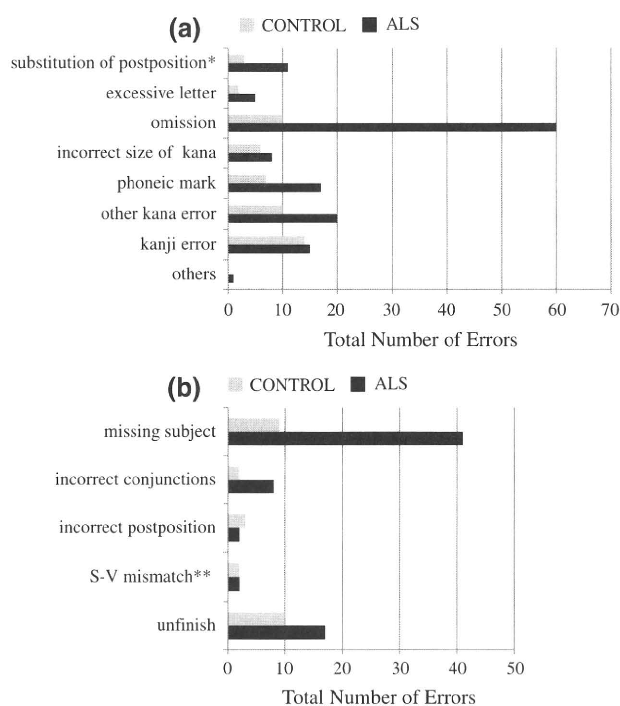

Fig. 1 Comparing error patterns between ALS and controls. **a** Classification of non-syntactic errors. Increase of omission was prominent. *Substitution of postposition means a phonologically plausible error of postposition. **b** Missing subject was **S–V mismatch means mismatch between subject and verb

Discussion

We demonstrated that ALS patients had mild disability with regard to spontaneous writing and PA. Spontaneous

writing, loss of subject, letter omissions, substitutions and displacements were frequently exhibited by ALS patients. Some patients experienced difficulties in articulation and writing because of motor complications. However, none of the patients in this study had any difficulty in both naming and pointing, and none exhibited paraphasia. Thus, we concluded that our subjects did not have aphasia. None of them had dementia, aphasia, or lexical problems at the time of the examination. Results of auditory comprehension, syntactic error at composition and frequent omission of postpositions might show agrammatism. However, to understand a sentence, several elements are necessary in addition to grammatical ability, such as concentration, short-term memory, and working memory. Hence, agrammatism should be carefully determined.

Subjects also did not show constructional apraxia, limb apraxia, ideomotor apraxia, or visuospatial agnosia. The patients who performed writing tasks could hold and manage a pen. Hence, we concluded that they had agraphia. Agraphia is classified into several types—pure agraphia, aphasic agraphia, agraphia with alexia, apraxic agraphia, and spatial agraphia [11]. Our patients were classified as having pure agraphia. Especially in ALS patients, agraphia should not be diagnosed solely on the basis of the dictation scores because even patients who get a 100% score on a dictation sometimes have difficulty in writing sentences or a composition. Therefore, we think that performance in the dictation of short words is not a proper indicator of mild agraphia in non-demented ALS.

Pure agraphia can result from several lesions. The posterior middle frontal gyrus of the dominant side (including Exner's area) is classically considered as the writing center, as proven by the patients with stroke of the region [3, 27, 29, 33]. Pure agraphia is often characterized by substitution, displacement, addition, repetition, and omission. Written Japanese has two types of symbols—kana (phonogram) and kanji (morphogram). A kana alone does not mean anything; it represents only a syllable. The dictation of kana is similar to that of regular words in European languages. Tohgi reported that a patient who had difficulty in kana writing had lesion involvement of the middle frontal gyrus and the upper part of the inferior frontal gyrus [33]. He had greater disability for spontaneous writing than for dictation. He frequently made mistakes in phonic marks, contracted sounds (e.g., kya and sho), and postpositional letters. In particular, presence of the lesions created problems in the selection and placement of letters. The features reported were similar to those of our ALS patients. The presence of lesions in the left superior parietal lobule is also known to indicate pure agraphia. Basso et al. [5] described two pure agraphic patients who had these lesions, but according to several case reports, the characteristics were different from those of pure agraphia of the frontal

	correct word			error sample	
(a)	まんが ma n ga	(comic)		まが ma ga	(no meaning)
(b)	いぬ i nu	(dog)	→	いむ i mu	(no meaning)
(c)	つり tsu ri	(fishing)	→	りつ ri tsu	(no meaning)
(d)	どらいぶ do ra i bu	(drive)	→	とらいふ to ra i fu	(no meaning)
(e)	を wo	(postposition)		お o	(ordinary use)
(f)	お	(correct kana-letter)	→	[illegible]	(imperfect kana-letter)
(g)	遊	(correct kanji)	→	[illegible]	(morphometric error)
(h)	拾う hiro u	(pick up)	→	広う hiro u	(wide)

Fig. 2 Samples of non-syntactic errors. **a–f** were samples of kana error, and g, h were kanji. **a** Omission of kana letter. **b** Substitution of a kana letter. **c** Displacement of kana letters. **d** Phonetic marks were omitted in the case of 2 kana letters. Marks consisted of a pair of dots written in the right upper corner of a kana letter when it is read as b, d, g, z. **e** Phonologically plausible substitution of kana. All postpositional particles with the pronunciation "wo" must be written as "を" instead of "お". The letter "お" is for ordinary use and not used as a postposition. The pronunciation of these letters is not distinguishable in spoken Japanese. **f** An imperfect kana letter. **g** Morphometric error of kanji. **h** Phonologically plausible substitution of kanji. Two kanji are pronounced in the same way but differ in their meanings

Table 3 Correlation between WEI and patient-profiles

	Pearson's correlation
Disease duration	0.573
ALSFRS-R (total)	−0.193
ALSFRS-R1-3 (bulbar)	−0.170
ALSFRS-R4-5 (upper limbs)	−0.326
RCPM	−0.418

WEI writing error index, *ALSFRS-R* ALS functional rating score revised, *RCPM* Raven's colored progressive matrices

lesions. The main problem associated with this lesion is clumsiness in forming the shape of the letter; this is called apraxic (or ideational) agraphia [6, 24]. It is attributed to neither apraxia nor movement disorder of the hand. People who have this lesion experience difficulty in writing letters in the correct sequence and forget the order of strokes [24]. In the present study, some patients wrote ill-shaped letters, but we did not conclude that our patients had apraxic agraphia because they showed motor disability of the dominant hand, and written symbols other than letters were