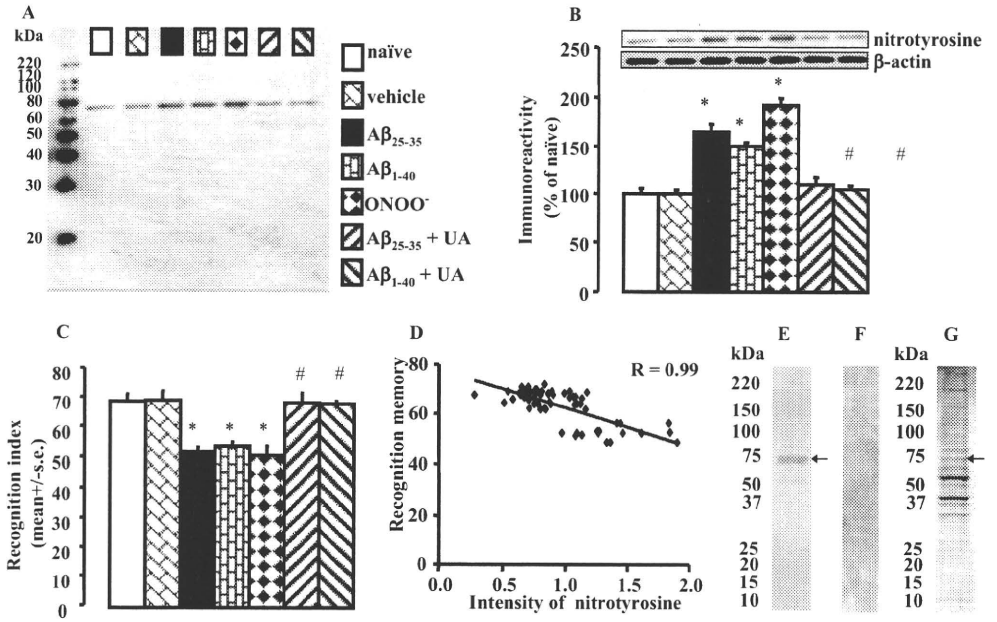

Fig. 2. The tyrosine nitration of proteins in the hippocampus and the cognitive function in mice. A and B, nitrotyrosine in the hippocampus was measured 5 days after the i.c.v. injection of Aβ peptides or $ONOO^-$. Protein samples from the hippocampus were subjected to SDS-PAGE, blotted to a PVDF membrane, and probed with a monoclonal anti-nitrotyrosine antibody. Aβ peptides induced extensive nitration of protein, which was prevented by UA, a potent scavenger of $ONOO^-$. $ONOO^-$ induced marked tyrosine nitration of proteins. The quantified intensity of the bands for nitrotyrosine was corrected by that of β-actin and expressed as a percentage of that in the naive group. Data are presented as the mean ± S.E. (n = 4). *, $p < 0.05$ versus naive and vehicle; #, $p < 0.05$ versus $A\beta_{25-35}$ or $A\beta_{1-40}$. C, the novel object recognition task was performed on days 3 to 5 after the i.c.v. injection of Aβ peptides or $ONOO^-$. Aβ peptides induced marked impairments of recognition memory, which were prevented by UA. $ONOO^-$ induced impairment of recognition memory. Data are presented as the mean ± S.E. (n = 10). *, $p < 0.05$ versus naive and vehicle; #, $p < 0.05$ versus $A\beta_{25-35}$ and $A\beta_{1-40}$. D, the panel shows the inverse association of extensive nitration of protein tyrosine in the hippocampus and the level of recognition memory in mice. E and F, protein samples from the hippocampus were subjected to 4 to 20% SDS-PAGE, blotted to PVDF membrane, and probed with a monoclonal anti-nitrotyrosine antibody before (E) and after (F) reduction of nitrotyrosine to aminotyrosine by treating the membrane with SD. G, protein bands in 4 to 20% SDS-PAGE were stained by CBB, and the band of interest was picked up for peptide analysis using LC-MS/MS.

TABLE 1
The identified protein candidates

Protein Name	gi Accession Number	Peptide Matched	% Sequence Coverage	Total Score
HSP70	gi 1661134	22	39	724
DRP-2	gi 40254595	7	20	292
NFL	gi 200038	4	9	254
ATPase, H^+transporting, V1 subunit A, isoform 1	gi 315607	9	17	184
Glycerol-3-phosphate dehydrogenase	gi 1339938	1	2	96
Ig superfamily receptor PGRL	gi 15593237	1	3	55
Solute carrier family 25 (mitochondrial carrier, aralar member12)	gi 27369581	3	8	53

gi, genInfo identifier; PGRL, prostaglandin regulatory-like protein.

nitration was observed for NFL in the $A\beta_{25-35}$ group compared with the naive or vehicle group (Fig. 3, A and B). No differences were observed in the nitration of HSP70 and DRP-2 proteins among the three groups (Fig. 3, A, C, and D). The increased nitration of NFL was inversely associated with recognition memory in mice that received $A\beta_{25-35}$ injections (Fig. 3E).

Association between Extensive Nitration of NFL and Serine Hyperphosphorylation. Hyperphosphorylation of the serine residues of NFL could lead to disruption of the subtle regulation of the NF network (Hisanaga et al., 1990; Nixon and Shea, 1992). After being nitrated in vitro, NFL is not able to form the NF assembly (Crow et al., 1997). The question of whether extensive nitration of NFL influences serine phosphorylation of the protein stimulated our interest. We immunoprecipitated NFL and blotted against nitrotyrosine and phosphoserine. Equal amounts of NFL protein were immunoprecipitated in each group (Fig. 4, A and B). The intensity of the tyrosine nitration and serine phosphorylation of NFL was greater in the $A\beta_{25-35}$ group than in the naive or vehicle group (Fig. 4, A, C, and D). The authenticity of the phosphoserine band was confirmed as indicated under *Materials and Methods*. Treatment with UA prevented the $A\beta_{25-35}$-induced intensive tyrosine nitration and serine hyperphosphorylation of NFL (Fig. 4, A, C, and D), indicating a positive association between the extensive nitration of NFL and the serine hyperphosphorylation (Fig. 4E).

Association between Extensive Nitration of NFL and Its Reduced Interaction with NUDEL. To examine whether the extensive nitration of NFL practically influences its interaction with partner proteins, we focused on the free, unassembled NFL that could be differentiated from the assembled NFL. The majority of the newly synthesized unassembled NF proteins, including NFL, are Triton X-100-solu-

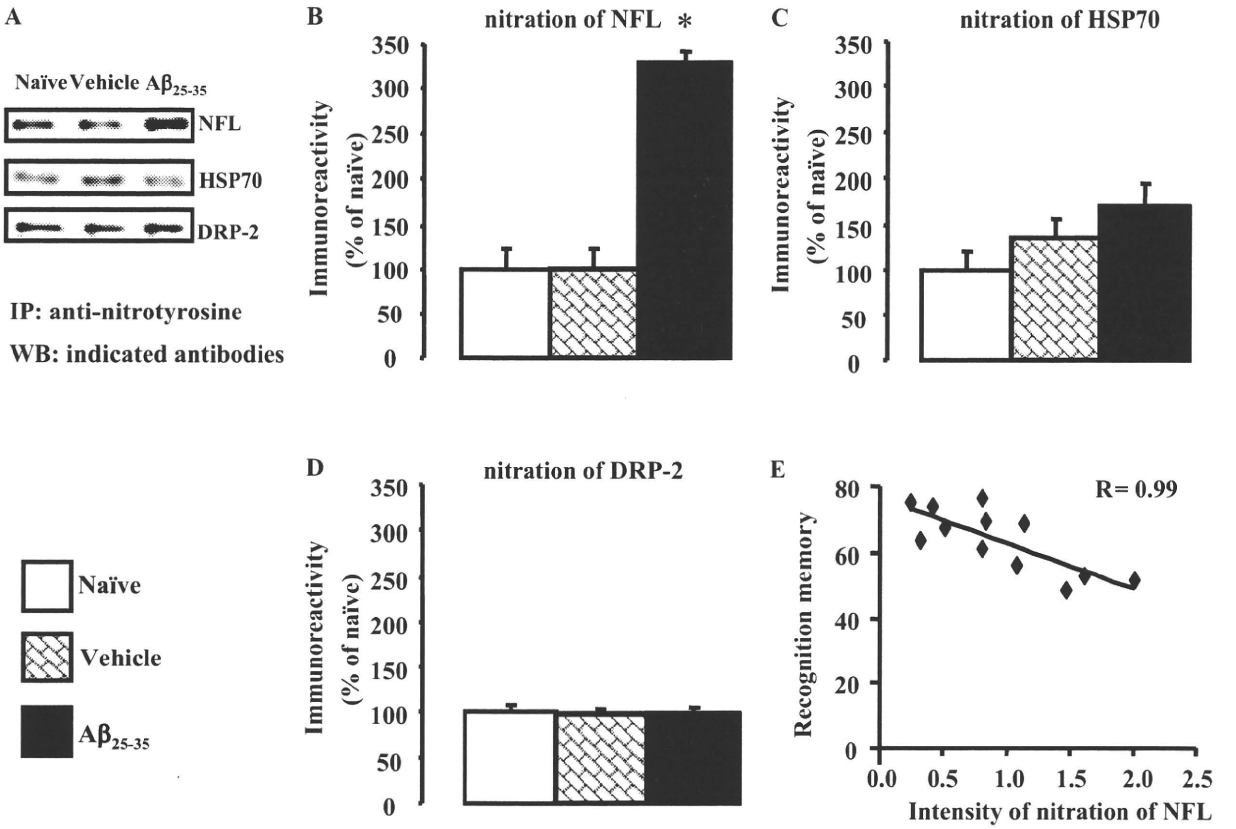

Fig. 3. Tyrosine nitration of the identified proteins. A, immunocomplexes, obtained from precleared protein samples of the hippocampus using an anti-nitrotyrosine agarose-conjugated mouse antibody, were separated by 7% SDS-PAGE, blotted onto a PVDF membrane, and probed with corresponding antibodies raised against the proteins of interest. B to D, NFL was intensely nitrated in the $A\beta_{25-35}$ group, whereas HSP70 and DRP-2 remained unchanged. E, the panel shows inverse association of the extensive nitration of NFL in the hippocampus (B) and the level of recognition memory in mice (Fig. 1B). The intensity of bands was quantified and expressed as a percentage of that in the naive group. Data are presented as the mean ± S.E. ($n = 4$). *, $p < 0.05$ versus naive and vehicle.

ble before being incorporated into the NF assembly, which is Triton X-100-insoluble (Black et al., 1986). NFL constitutes the core of the NF network, and without NFL, no filaments are formed (Zhu et al., 1997). Without binding directly with NUDEL, the Triton X-100-soluble NFL can barely lead the assembly of a stable NF network, regardless of its own abundance (Nguyen et al., 2004). We probed equal amounts of NFL immunocomplexes with antibodies raised against the nitrotyrosine and NUDEL (Fig. 5, A and B). Less NUDEL was coimmunoprecipitated in the $A\beta_{25-35}$ group that bears extensively nitrated NFL (Fig. 5, A–D). The protein expression of NUDEL did not differ among the groups (Fig. 5E). UA prevented the $A\beta_{25-35}$-induced increase of NFL nitration as well as the reduced coimmunoprecipitation of NUDEL (Fig. 5, A, C, and D). The extensive nitration of NFL was associated with its reduced interaction with NUDEL (Fig. 5F). These results suggested that the intensive nitration of NFL could disturb the normal function of the protein.

Association between Extensive Nitration of NFL and the Reduced Content of NUDEL in the Cytoskeleton Fraction. A majority of NF proteins, after their synthesis in the cytoplasm, are rapidly converted to a Triton X-100-insoluble filamentous network and move down the axon using the transport machinery (Nixon and Shea, 1992). After direct and specific binding with NFL, NUDEL facilitates the assembly of a stable NF network and remains bound to the assembled filaments (Nguyen et al., 2004). Thus, the level of interaction between NFL and NUDEL in cytoplasm (Triton X-100-soluble fraction) should be reflected by their protein levels in the axonal cytoskeleton (Triton X-100-insoluble fraction). The Triton X-100-insoluble fractions from the previous step (Fig. 5) were washed twice with Triton X-100 lysis buffer before being solubilized in urea lysis buffer. Western blot analysis revealed that the level of NUDEL protein was reduced in the $A\beta_{25-35}$ group compared with the naive and vehicle groups, whereas the treatment with UA prevented the reduction (Fig. 6, A and D). This was consistent with the reduced interaction between NFL and NUDEL in the $A\beta_{25-35}$ group (Fig. 5, A and D). However, the level of NFL in the $A\beta_{25-35}$ group was surprisingly not different from that in the naive and vehicle groups (Fig. 6, A and B). Considering the increase of the intensity of the protein nitration in the $A\beta_{25-35}$ group (Fig. 6, A and C), we examined the nitration of NFL by immunoprecipitation. Intense nitration for the NFL protein in the $A\beta_{25-35}$ group was observed (Fig. 6E). Applying the multiplicative inverse (in which the inverse or reciprocal of "n" is "1/n"), a mathematical method that is useful in medical science (Silberberg, 1990), the reciprocal level of the extensively nitrated NFL in the Triton X-100-insoluble fraction was estimated (Fig. 6F). The reciprocal level of extensively nitrated NFL in the $A\beta_{25-35}$ group paralleled with that of NUDEL in the same group (Fig. 6, D and F), signifying a negative effect of the extensive nitration of NFL on NUDEL-dependent NF assembly. The increased nitration of tyrosine could modify protein function by altering the three-dimensional conformation and hydrophobicity (Dalle-Donne et al.,

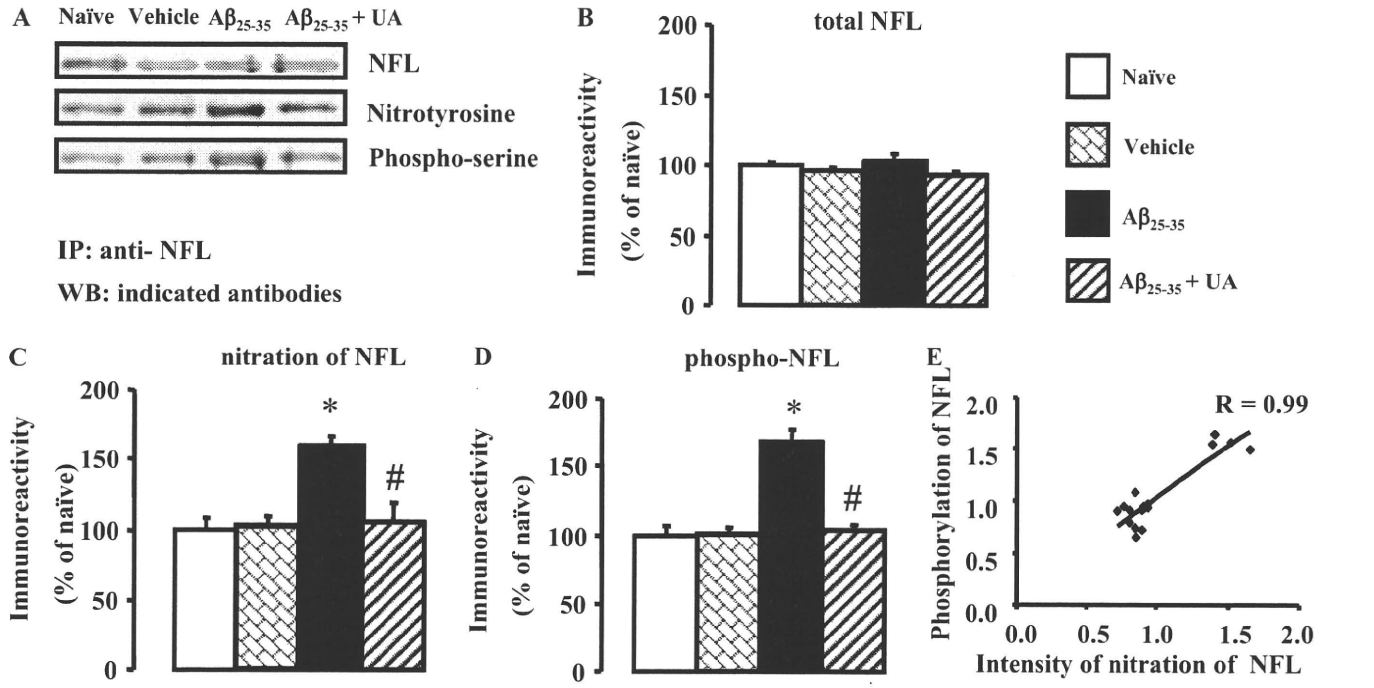

Fig. 4. The association between the increased tyrosine nitration and serine hyperphosphorylation of NFL. A, equal amounts of NFL protein immunocomplexes were obtained from precleared protein samples of the hippocampus, using anti-NFL antibody. The immunocomplexes were separated on SDS-PAGE, blotted onto a PVDF membrane, and probed with the indicated antibodies. B to D, tyrosine nitration and serine phosphorylation of NFL were increased in the $A\beta_{25-35}$ group, whereas UA prevented the increase of both. E, the increased nitration of NFL was correlated with serine hyperphosphorylation of NFL. The intensity of bands was quantified and expressed as a percentage of that in the naive group. Data are presented as the mean ± S.E. (n = 4). *, $p < 0.05$ versus naive and vehicle; #, $p < 0.05$ versus $A\beta_{25-35}$.

2005; Reynolds et al., 2007). It was therefore assumed that the overnitrated, free NFL would become less Triton X-100 soluble and, as a result, would be detected in the Triton X-100-insoluble fraction along with the assembled NF proteins. It is hardly practical to separate the unassembled extensively nitrated NFL from the assembled NFL in the Triton X-100-insoluble fraction. The majority of the cytoplasmic water-soluble proteins could be separated from the Triton X-100-soluble protein pools by using PBS lysis buffer in the first step (Aoyama and Kitajima, 1999). After the separation of the PBS-soluble and Triton X-100-soluble proteins as described under *Materials and Methods*, we examined the amount of NFL protein in these two different fractions. The majority of NFL protein in all groups was found in the PBS-soluble cytoplasmic fraction as indicated by GADPH, a cytoplasmic marker (Fig. 7A). The levels of NFL protein in both the PBS-soluble and Triton X-100-soluble fractions were increased in the $A\beta_{25-35}$ group (Fig. 7, A–C). It is interesting to note that the increase of NFL in both fractions was prevented by the treatment with of UA, a potent scavenger of $ONOO^-$, suggesting that the $A\beta_{25-35}$-induced $ONOO^-$ may increase the protein synthesis of NFL before extensively nitrating the protein (Fig. 7, A–C). The Triton X-100-soluble NFL that became insoluble in PBS in the $A\beta_{25-35}$ group was extensively nitrated (Fig. 7D), and the intensity of nitration was associated with the level of the PBS-insoluble, Triton X-100-soluble NFL (Fig. 7E). These results revealed new possibilities for Triton X-100-insolubile NFL in association with extensive nitration.

The Cell Numbers in the Hippocampus of Mice with the Impairment of Memory Induced by $A\beta_{25-35}$. On day 5 after the i.c.v. injection of $A\beta_{25-35}$, cell numbers in CA1, CA3, and the granular layer of the dentate gyrus of the hippocampal formation were examined using cresyl violet staining. The quantification of the stained cells revealed no cell loss induced by $A\beta_{25-35}$ (Table 2). These results were consistent with reports that at a dose of 3 to 5 μg, $A\beta_{25-35}$ could induce memory impairment but not cell loss within a time session of 1 month after its injection in mice (Maurice et al., 1996; Tohda et al., 2003). These results suggest that cell loss was not involved in the impairment of memory induced by $A\beta_{25-35}$ in mice.

Discussion

Neuronal oxidative damage has long been hypothesized as a critical mechanism of cellular dysfunction in neurodegenerative ailments (Perry et al., 2002). Reports showing that antioxidants delay or reduce progressive cognitive decline in both animal models and humans have emphasized the direct contribution of oxidative damage to cognitive pathology (Sano et al., 1997; Yamada et al., 1999; Lim et al., 2001). Oxidative damage is generally manifested by the increase of lipid peroxidation, DNA oxidation, protein oxidation, and peroxynitrite-mediated tyrosine nitration of proteins. The increased nitration of tyrosine could irreversibly disrupt the function of proteins (Koppal et al., 1999), and it might play a key pathogenic role in the progression of cognitive impairment (Smith et al., 1997; Keller, 2006). Until now, various proteins with tyrosine nitration have been reported in association with neurodegeneration and cognitive decline (Strong et al., 1998; Castegna et al., 2003; Tran et al., 2003; Sacksteder et al., 2006; Sultana et al., 2006). The diversity of nitrated proteins in these reports seems to depend on the species of the sources of samples (Sacksteder et al., 2006;

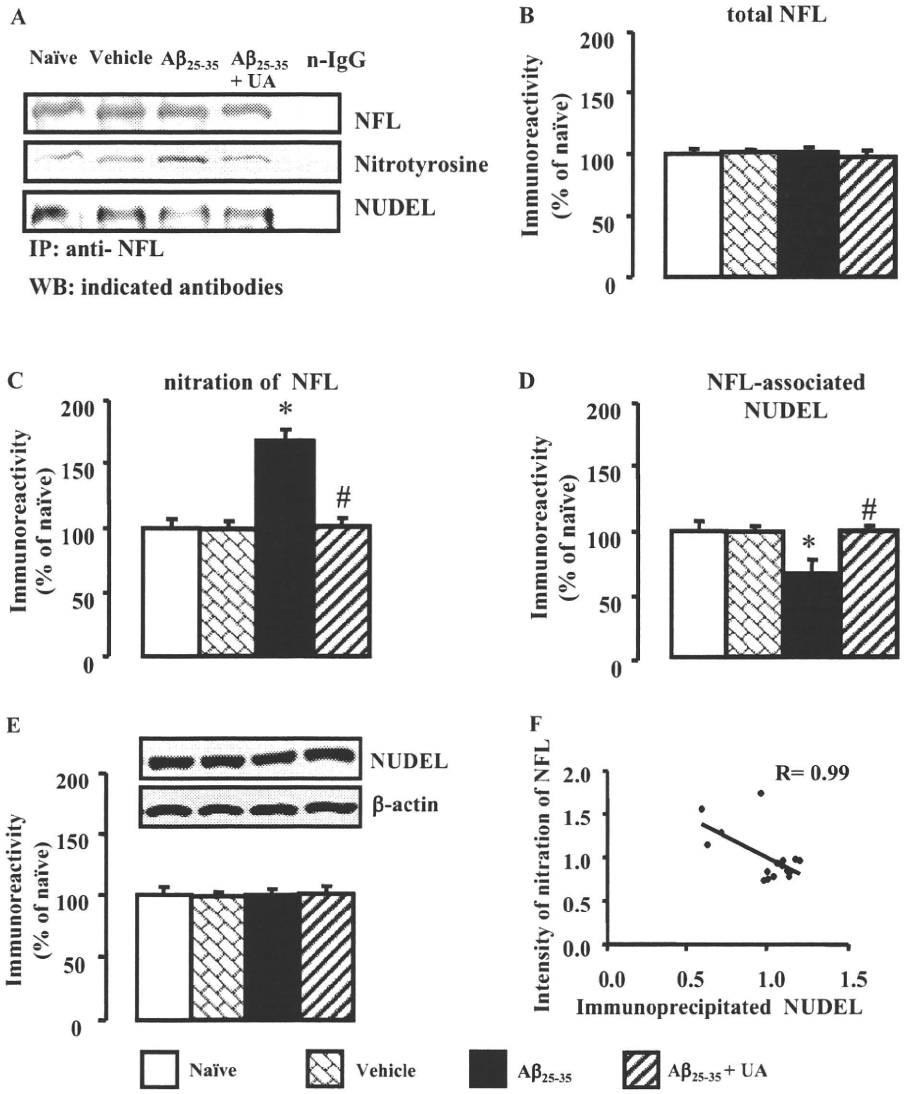

Fig. 5. Association between the extensive nitration of NFL and the reduced interaction with NUDEL in the Triton X-100-soluble fraction. A, the immunocomplexes obtained with the anti-NFL antibody, from precleared protein samples of the hippocampal homogenates, were separated by SDS-PAGE, blotted onto a PVDF membrane, and probed with the indicated antibodies. B, equal amounts of NFL protein were obtained. C, tyrosine nitration of NFL was increased in the $A\beta_{25-35}$ group, whereas UA treatment prevented the increase. D, the level of NFL-interacting NUDEL was reduced in the $A\beta_{25-35}$ group, whereas UA treatment prevented the reduction. E, no difference in NUDEL protein expression was found among the groups. F, the increased nitration of NFL was associated with reduced interaction with NUDEL. The intensity of bands was quantified and expressed as a percentage of that in the naive group. Data were presented as the mean ± S.E. ($n = 4$). *, $p < 0.05$ versus naive and vehicle; #, $p < 0.05$ versus $A\beta_{25-35}$.

Sultana et al., 2006), the proteomic detections on various conditions (Castegna et al., 2003; Sultana et al., 2006), immunodetections by means of different anti-nitrotyrosine antibodies with the diverse recognition property for nitrotyrosine (Strong et al., 1998; Tran et al., 2003), or the biological selectivity of tyrosine nitration (Ischiropoulos, 2003; Sacksteder et al., 2006). Dissimilar reports about the nitrated proteins in the brains of humans with Alzheimer's disease (AD) (Castegna et al., 2003; Sultana et al., 2006) emphasize the importance of the sources of protein, even in the same species or under the same conditions of detection during the identification process, while illustrating the diversity of nitration due to the dissimilar expression of proteins during the different stages of the disease.

In the present study, we looked for further evidence for the pathogenic role of protein nitration as one of the key contributors to the decline of cognitive function induced by Aβ. Using LC-MS/MS and immunodetection, we identified the hippocampal proteins with nitrated tyrosine residues after the i.c.v. injection of $A\beta_{25-35}$ in mice. Preferentially, in respect with currently examined proteins, intense nitration of NFL was observed, demonstrating a good correlation with the severity of cognitive impairment induced by $A\beta_{25-35}$.

NFL, one of the three subunits of NF proteins, is the indispensable core of the NF assembly (Zhu et al., 1997). Studies have reported that NFL is selectively nitrated compared with the majority of other proteins present in brain homogenates, and they suggested that newly synthesized free NFL is particularly susceptible to peroxynitrite-mediated nitration (Crow et al., 1997; Strong et al., 1998). The extensively nitrated NFL inhibits the assembly of unmodified NF subunits (Crow et al., 1997). On the other hand, the extensive serine phosphorylation of NFL could sufficiently block NF assembly (Nixon and Shea, 1992; Gibb et al., 1996). Therefore, we have evaluated the effect of tyrosine nitration on the phosphorylation of NFL at serine residues in general. The increased tyrosine nitration of NFL was associated with its serine hyperphosphorylation. Prevention of the extensive nitration of NFL by UA, a scavenger of $ONOO^-$ that nitrates proteins, restrained the serine phosphorylation of NFL at a normal level. The results indicated that the increased nitration of NFL could give rise to its serine hyperphosphorylation.

NFL requires direct binding with NUDEL, whereas NUDEL can not directly bind with other subunits of NF proteins, to initiate the assembly of NF (Nguyen et al., 2004). After the assembly of the NF network, NUDEL remains bound to the assembled Triton X-100-insoluble neurofilaments and may promote, in conjunction with molecular mo-

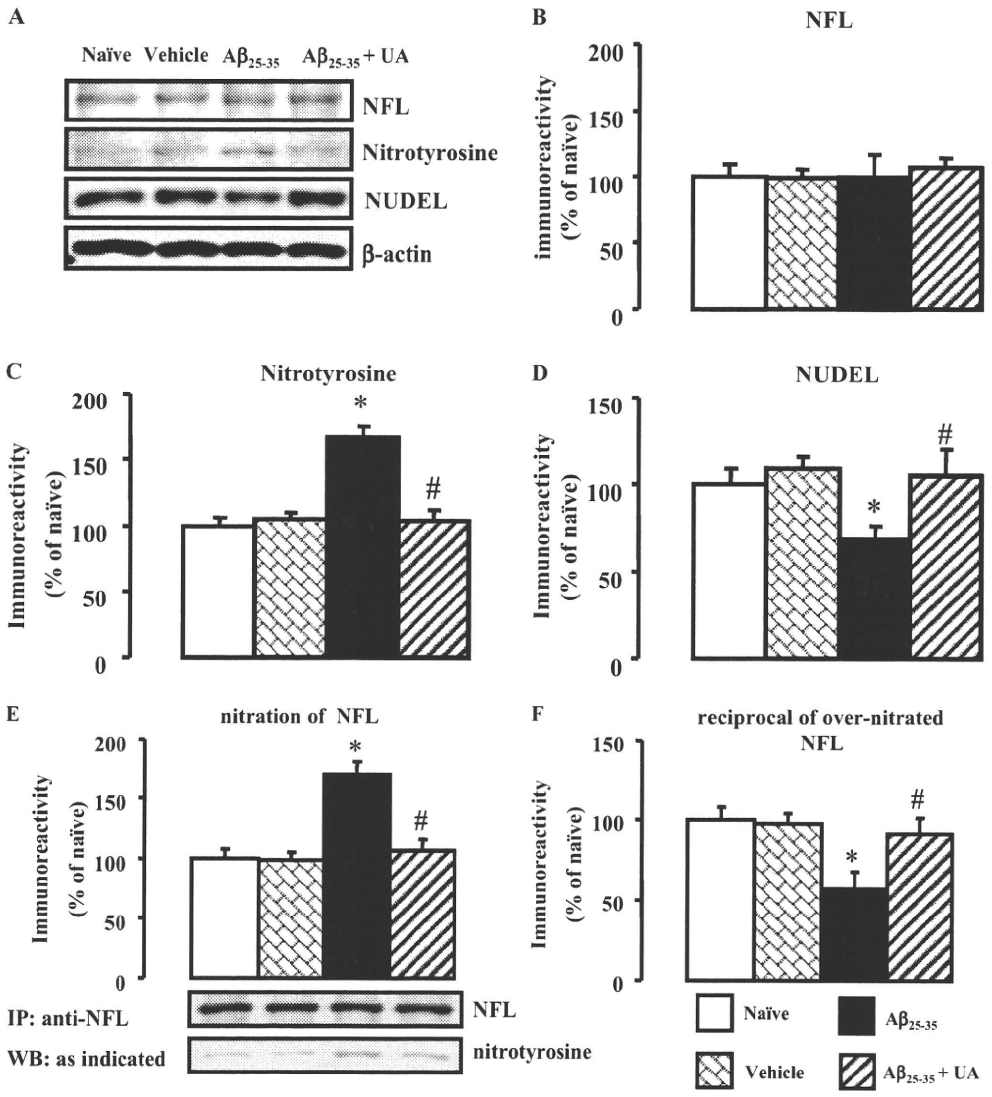

Fig. 6. The reduced content of NUDEL in the Triton X-100-insoluble cytoskeletal fraction. The Triton X-100-insoluble fraction, including cytoskeletal proteins, was solubilized in 6 M urea. A, equal amounts of protein were subjected to Western blot analysis. B, the protein levels of NFL were unchanged in all groups. C, the intensity of nitrotyrosine was increased in the $A\beta_{25-35}$ group, and the increase was prevented by UA, a scavenger of $ONOO^-$ that nitrates tyrosine. D, the protein level of NUDEL was reduced in the $A\beta_{25-35}$ group, and UA prevented this reduction. The quantified intensity of the bands was corrected by that of β-actin and expressed as a percentage of that in the naive group. E, equal amounts of NFL protein were immunoprecipitated and probed with anti-nitrotyrosine antibodies. The intensity of nitrotyrosine in NFL was increased in the $A\beta_{25-35}$ group, whereas UA prevented any increase. F, the reciprocal of the overnitrated NFL was estimated by applying multiplicative inverse (or reciprocal, in which the reciprocal of n is 1/n). The intensity of bands was quantified and expressed as a percentage of that in the naive group. Data are presented as the mean ± S.E. ($n = 4$). *, $p < 0.05$ versus naive and vehicle; #, $p < 0.05$ versus $A\beta_{25-35}$.

tors, the axonal transport of the neurofilaments (Nguyen et al., 2004). Thus, the level of interaction between NFL and NUDEL in the Triton X-100-soluble cytoplasmic fraction could be reflected by their protein levels in the Triton X-100-insoluble cytoskeletal fraction. In the current study, the increased nitration of Triton X-100-soluble NFL proteins in the $A\beta_{25-35}$ group was associated with its decreased interaction with NUDEL. In the Triton X-100-insoluble fraction, the protein level of NUDEL was reduced in the $A\beta_{25-35}$ group, and the reduction was prevented by treatment with UA. In the same fraction, the protein level of NFL surprisingly did not differ among groups, whereas the intensity of the nitration of NFL was strong in $A\beta_{25-35}$ group. Estimation by the multiplicative inverse approach indicated that the reduced level of nonextensively nitrated NFL in the $A\beta_{25-35}$ group parallels with that of NUDEL. These results required an explanation for the detection of the extensively nitrated NFL in the Triton X-100-insoluble cytoskeletal fraction, because the assembled NFL is nitration-resistant and the intensely nitrated NFL can not participate in the NF assembly (Crow et al., 1997). The alteration of the solubility of the overnitrated NFL might be involved in the detection of the extensively nitrated NFL in the Triton X-100-insoluble cytoskeletal fraction in the $A\beta_{25-35}$ group. Interpretation of the emergence of the intensely nitrated NFL in PBS-insoluble, but Triton X-100-soluble, protein pools in the $A\beta_{25-35}$ group indicates that extensive nitration would render NFL protein to have poor solubility in PBS. By this rate, it is possible that a considerable level of overnitrated NFL protein in the $A\beta_{25-35}$ group would even become Triton X-100 insoluble over a period of time, and that it would be detected along with a reduced level of NUDEL-associated assembled NFL, which is also Triton X-100 insoluble. The observation of detectable levels of nitration in NFL in the RIPA-soluble, Triton X-100-soluble, and Triton X-100-insoluble fractions in the naive and vehicle groups implies that natural nitration of tyrosine, as serine phosphorylation, might exist as a physiological property of NFL and might not be detrimental to the function of the protein, whereas extensive nitration is detrimental. The nitration-susceptible tyrosine residues of NFL are identified particularly as tyrosine 17 in the head region and tyrosines 138, 177, and 265 in the α-helical coil regions of the rod domain of the protein (Crow et al., 1997). It needs to be determined which tyrosine residue is the site for natural nitration or for extensive nitration. It has been reported that, although the exact mechanism is not clear, the newly synthesized Triton X-100-soluble NF proteins, including NFL, could separately undergo axonal transport before being in-

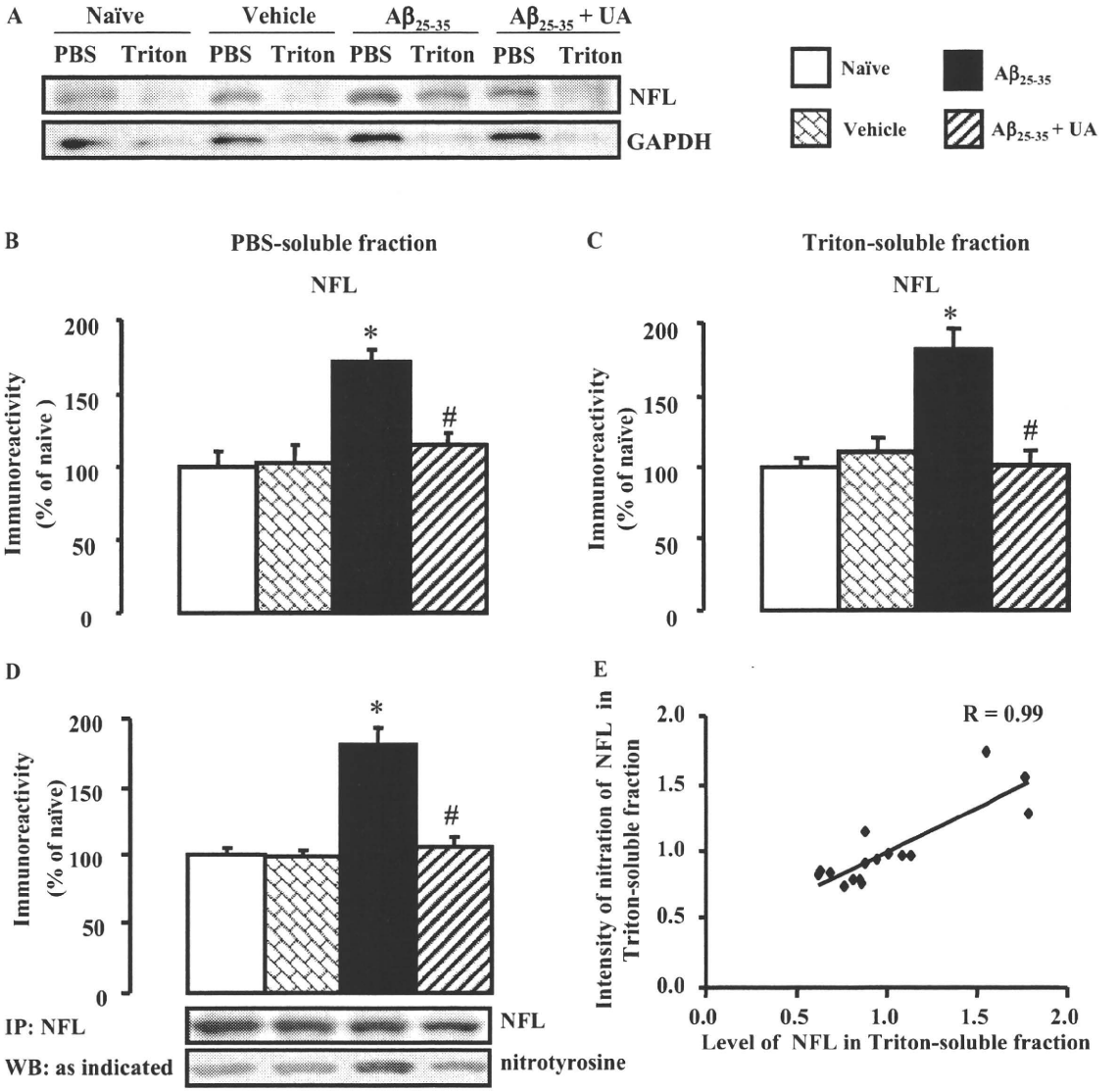

Fig. 7. The association of the extensive nitration of NFL with the alteration of solubility. The hippocampal tissues were homogenated in PBS and centrifuged at 13000*g* for 20 min, the washed pellets were solubilized in Triton X-100 as described under *Materials and Methods*, and equal amounts of protein were subjected to Western blot analysis. A to C, a majority of NFL and GAPDH proteins were soluble in PBS. NFL protein in the $A\beta_{25-35}$ group in both the PBS-soluble fraction and the Triton X-100-soluble fraction was increased, and the increase was prevented by UA, a scavenger of $ONOO^-$ that nitrates tyrosine. The quantified intensity of the bands was corrected by that of GAPDH and expressed as a percentage of that in the naive group. D, equal amounts of NFL from the Triton X-100-soluble proteins were immunoprecipitated and probed with anti-nitrotyrosine antibodies. The intensity of nitrotyrosine in NFL was increased in the $A\beta_{25-35}$ group, whereas UA prevented the increase. The intensity of bands was quantified and expressed as a percentage of that in the naive group. E, the level of NFL in the Triton X-100-soluble (PBS-insoluble) fraction was associated with the intensity of its nitration. Data are presented as the mean ± S.E. ($n = 4$). *, $p < 0.05$ versus naive and vehicle; #, $p < 0.05$ versus $A\beta_{25-35}$.

TABLE 2
The Nissl-positive cells in the hippocampus
In each group, $n = 4$.

Subfields of Hippocampus	Number of Nissl-Positive Cells			
	Naive	Vehicle	$A\beta_{25-35}$	$A\beta_{25-35}$ + UA
	counts/mm²			
CA1	10800 ± 230	10900 ± 290	10850 ± 250	10790 ± 270
CA3	6750 ± 190	6690 ± 210	6698 ± 180	6680 ± 310
GrDG	21000 ± 670	20980 ± 590	20990 ± 710	20780 ± 690

GrDG, the granular layer of the dentate gyrus.

corporated into the Triton X-100-insoluble axonal cytoskeleton (Jung et al., 1998). We do not know whether the NFL proteins with natural nitration undergo axonal transport after the NF assembly or undergo axonal transport before being incorporated into the Triton X-100-insoluble axonal cytoskeleton.

The observation of no cell loss in CA1, CA3, and the granular layer of the dentate gyrus of the hippocampus in mice

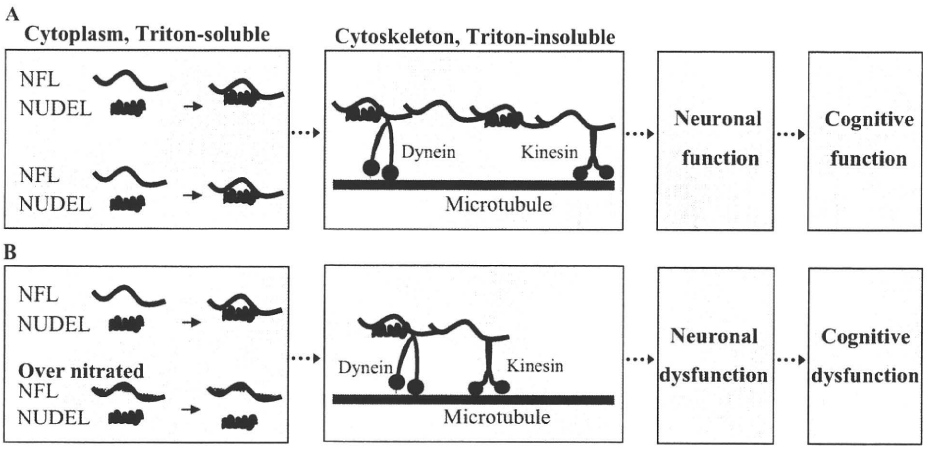

Fig. 8. The contribution of the extensive nitration of NFL to cognitive dysfunction. A, in this model, NFL interacts with NUDEL, which is essential for the incorporation of NF subunits into the network during NF assembly and elongation. A normal NF assembly and elongation favors normal neuronal and cognitive functions. B, the overnitration of NFL disrupts the interaction of NFL with NUDEL and may lead to the defective assembly of NF and abnormalities in neuronal and cognitive functions. The resized microtubules, kinesin, and dyenin have been added for clarity (modified from Nguyen et al., 2004; Holzbaur, 2004).

that received $A\beta_{25-35}$ injections favored the contribution of extensive nitration of NFL to the impairment of memory. A recent study demonstrated that rapidly formed fresh amyloid plaques cause axonal and dendritic structural changes within a minimum of 5 days after the "birth of the plaques" (Meyer-Luehmann et al., 2008). Given the time windows of Aβ neurotoxicity, $A\beta_{25-35}$ may require longer time to cause cell loss in our mouse model of cognitive impairment.

The disrupted interaction between NFL and NUDEL is regarded as the most important factor for the destabilization of the NF assembly that leads to the axonal dysfunction, which is an early event in the cognitive pathology of AD (Nguyen et al., 2004; Stokin et al., 2005). Therefore, our results suggest that disrupted interaction between NUDEL and NFL with extensive nitration could be one of the major factors that associated with the cognitive dysfunction induced by Aβ in mice (Fig. 8). However, further studies are required to investigate whether the extensive nitration of NFL and the impaired interaction with NUDEL induced by Aβ are associated with the disruption of axonal transport.

Acknowledgments

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Address correspondence to: Dr. Toshitaka Nabeshima, Department of Chemical Pharmacology, Graduate School of Pharmaceutical Science, Meijo University, Nagoya 468-8503, Japan. E-mail: tnabeshi@ccmfs.meijo-u.ac.jp

RAGE-mediated signaling contributes to intraneuronal transport of amyloid-β and neuronal dysfunction

Kazuhiro Takuma[a,b], Fang Fang[c], Wensheng Zhang[c,d], Shiqiang Yan[c,e], Emiko Fukuzaki[b], Heng Du[c], Alexander Sosunov[c], Guy McKhann[c], Yoko Funatsu[b], Noritaka Nakamichi[b,f], Taku Nagai[b,g], Hiroyuki Mizoguchi[b,g], Daisuke Ibi[b,g], Osamu Hori[h], Satoshi Ogawa[h], David M. Stern[i], Kiyofumi Yamada[b,g,1], and Shirley ShiDu Yan[c,1]

[a]Laboratory of Medicinal Pharmacology, Graduate School of Pharmaceutical Sciences, Osaka University, Osaka 565-0871, Japan; [b]Laboratory of Neuropsychopharmacology, Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa 920-1192, Japan; [c]Departments of Pathology, Surgery, Neurosurgery, and Taub Institute for Research on Alzheimer's Disease and the Aging Brain, College of Physicians and Surgeons, Columbia University, New York, NY 10032; [d]Institute of Natural Medicine and Chinese Medicine Resources, Beijing Normal University, Beijing 100875, China; [e]College of Chemistry and Chemical Engineering, Lanzhou University, Lanzhou 730000, China; [f]Laboratory of Molecular Pharmacotherapeutics, Division of Life Sciences, Graduate School of Natural Science and Technology, Kanazawa University, Kanazawa 920-1192, Japan; [g]Department of Neuropsychopharmacology and Hospital Pharmacy, Nagoya University Graduate School of Medicine, Nagoya 466-8560, Japan; [h]Department of Neuroanatomy, Kanazawa University Graduate School of Medical Science, Kanazawa 920-8641, Japan; and [i]VP and Dean's Office, University of Cincinnati College of Medicine, Cincinnati OH 45267

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Intracellular amyloid-β peptide (Aβ) has been implicated in neuronal death associated with Alzheimer's disease. Although Aβ is predominantly secreted into the extracellular space, mechanisms of Aβ transport at the level of the neuronal cell membrane remain to be fully elucidated. We demonstrate that receptor for advanced glycation end products (RAGE) contributes to transport of Aβ from the cell surface to the intracellular space. Mouse cortical neurons exposed to extracellular human Aβ subsequently showed detectable peptide intracellularly in the cytosol and mitochondria by confocal microscope and immunogold electron microscopy. Pretreatment of cultured neurons from wild-type mice with neutralizing antibody to RAGE, and neurons from RAGE knockout mice displayed decreased uptake of Aβ and protection from Aβ-mediated mitochondrial dysfunction. Aβ activated p38 MAPK, but not SAPK/JNK, and then stimulated intracellular uptake of Aβ-RAGE complex. Similar intraneuronal co-localization of Aβ and RAGE was observed in the hippocampus of transgenic mice overexpressing mutant amyloid precursor protein. These findings indicate that RAGE contributes to mechanisms involved in the translocation of Aβ from the extracellular to the intracellular space, thereby enhancing Aβ cytotoxicity.

β-amyloid | Alzheimer's disease | mitochondrial dysfunction | p38 MAPK

Alzheimer's disease (AD) is a progressive neurodegenerative process characterized by senile plaques, neurofibrillary tangles, and neuronal loss (1, 2). Deposition of amyloid-β peptide (Aβ), a 39–43-amino acid peptide derived from the transmembrane amyloid precursor protein (APP), is found in extracellular senile plaque cores and is associated with neurodegeneration in later stages of AD. In contrast, recent studies suggest that accumulation of intraneuronal Aβ may be an early event in the pathogenesis of AD (3–16). Addition of Aβ to human neuronal-like cells caused significant mitochondrial damage (17). Furthermore, our recent study revealed that binding of Aβ to Aβ-binding alcohol dehydrogenase (ABAD) or cyclophilin D (10, 11) intracellularly triggered events leading to neuronal apoptosis through a mitochondrial pathway (12, 13, 18, 19). However, mechanisms through which Aβ produced at the plasma membrane and released into the extracellular space reaches the intracellular milieu remain to be elucidated.

Receptor for advanced glycation end products (RAGE) is a multiligand receptor of the Ig superfamily of cell surface molecules (20–22). RAGE acts as a counter-receptor for several quite distinct classes of ligands, such as AGEs, S100/calgranulins, HMG1 (high mobility group 1 or amphoterin), and the family of crossed β-sheet fibrils/macromolecular assemblies, which activate receptor-mediated signal transduction pathways. These ligand-receptor interactions are believed to exert pathogenic effects through sustained cellular perturbation in a range of chronic disorders, including the secondary complications of diabetes, inflammation, and neurodegenerative processes (23, 24). RAGE, a cell surface binding site for Aβ (25), is expressed at higher levels in an Aβ-rich environment (26, 27). Targeted neuronal overexpression of a wild-type RAGE transgene in AD-type mice also expressing mutant human APP (mAPP) amplified Aβ-mediated neuronal dysfunction. The latter was shown by early abnormalities in spatial learning/memory and exaggerated neuropathologic changes not seen in single transgenics (such as transgenics expressing mAPP alone at the same ages). These data support the hypothesis that RAGE might function as a cofactor for Aβ-induced neuronal perturbation in AD (28). Interaction of Aβ with RAGE expressed on brain endothelial cells initiates cellular signaling leading to the trafficking of monocytes across the blood-brain barrier (BBB) (29). Furthermore, RAGE has been shown to mediate Aβ transport across the BBB and to contribute to pathologic accumulation of the amyloid peptide in brain (30). Herein, we demonstrate that RAGE contributes to translocation of Aβ across the cell membrane from the extracellular to the intracellular space in cortical neurons. We also present evidence that Aβ-initiated RAGE signaling, especially stimulation of p38 mitogen-activated protein kinase (MAPK), has the capacity to drive a transport system delivering Aβ as a complex with RAGE to the intraneuronal space.

Results

Extracellular Aβ Translocates into Mitochondria in Cortical Neurons. We have recently demonstrated that Aβ, endogeneously generated from a mutant APP transgene, interacts with ABAD within mitochondria and leads to apoptosis-like cell death in vivo and in vitro using a murine system (12, 13). Addition of exogenous Aβ, both 1–40 ($Aβ_{1-40}$) and 1–42 ($Aβ_{1-42}$), to culture media caused mitochondrial dysfunction and apoptotic-like cell death in cortical neurons prepared from wild-type and transgenic (Tg) ABAD mice (Fig. S1). However, evidence of Aβ-induced neuronal perturbation was significantly enhanced in the ABAD-expressing cells, indicating that an enzyme in the mitochondrial matrix (ABAD) appears to exert toxic effects in response to the exogenous Aβ. These data

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[1]To whom correspondence may be addressed. E-mail: sdy1@columbia.edu or kyamada@med.nagoya-u.ac.jp.

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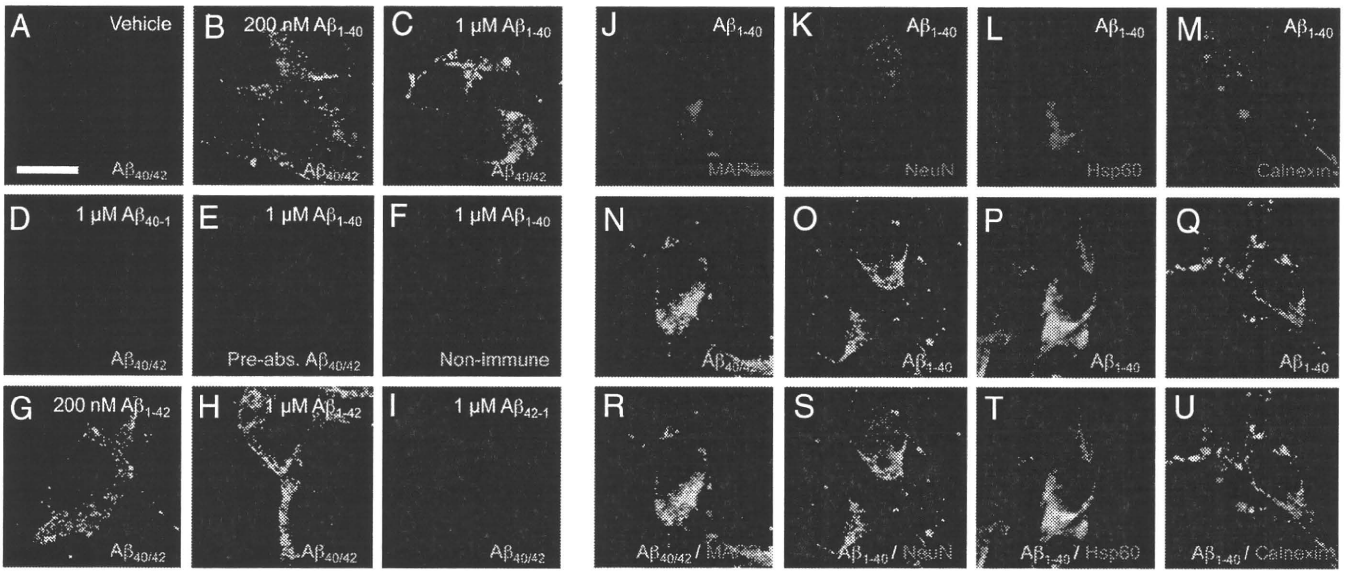

Fig. 1. Confocal images of Aβ, MAP2, NeuN, Hsp60, and calnexin in cortical neurons after exposure to Aβ-related peptides. Cells were exposed to the indicated concentration [or 1 μM (*J*–*U*)] of human $A\beta_{1-40}$, $A\beta_{1-42}$, reversed Aβ ($A\beta_{40-1}$ and $A\beta_{42-1}$), or vehicle for 60 min, fixed in 3% PFA, and stained by [anti-human Aβ (clone 4G8) (*A*–*D*, *G*–*I*, *N*, and *R*), anti-$A\beta_{1-40}$ (*O*–*P* and *S*–*U*), preabsorbed anti-Aβ (clone 4G8) (*E*) or non-immune IgG (*F*)]/Alexa Fluor 488 anti-IgG (green), anti-MAP2/Alexa Fluor 568 anti-IgG (red) (*J* and *R*) and [anti-NeuN (*K* and *S*), anti-Hsp 60 (*L* and *T*), or anti-calnexin (*M* and *U*)]/Alexa Fluor 546 anti-IgG (red). Scale bar, 10 μm. Hoechst 33342 staining and phase contrast images of the same field of cells in panels of *A*, *C*, *H*, *D*, or Fig. S4 *A*–*D* are represented in Fig. S4 *E*–*H* and *M*–*P*, respectively.

suggested the possibility that Aβ added to the extracellular milieu gained access to the intracellular space and, subsequently, interacted with its intracellular target. These findings led us to probe mechanisms through which Aβ gains access to intracellular compartments.

To evaluate cellular uptake of Aβ, we first measured levels of intracellular Aβ in neurons treated with the synthetic human Aβ peptides by ELISA using an antibody specific for the human form of Aβ to differentiate it from endogenous mouse Aβ. To remove Aβ bound to the cell surface, cells were treated with trypsin for 5 min before harvest for measurement of the intracellular human Aβ. As shown in Figs. S2 and S3, intracellular human Aβ content was at background levels in vehicle-treated neurons, whereas levels of intracellular human Aβ were significantly increased in mouse cortical neurons incubated with human $A\beta_{1-40}$ and $A\beta_{1-42}$. The accumulation of both $A\beta_{1-40}$ and $A\beta_{1-42}$ peptides occurred in a time- (Figs. S2 *A* and *B* and S3 *A* and *C*) and dose-dependent (Figs. S2*C* and S3 *A* and *C*) manner. Biochemical subcellular fractionation further revealed that the majority of the intracellular Aβ was detected in the mitochondria-enriched fractions (Fig. S3*F*) as compared with plasma membrane (Fig. S3*E*) and cytosolic fractions (Fig. S3*G*). As a complementary approach, we performed confocal microscopy using double immunofluorescence with antibodies to Aβ and intracellular markers, such as MAP-2 (neuronal marker), NeuN (neuronal marker), Hsp60 (mitochondrial marker), and calnexin (endoplasmic reticulum marker). After exposure (60 min) to human $A\beta_{1-40}$ and $A\beta_{1-42}$, but not $A\beta_{40-1}$ (Fig. 1*H*) and $A\beta_{42-1}$ (Fig. 1*I*), neurons displayed immunoreactivity to anti-human Aβ antibody (clone 4G8) in a cytosolic-like distribution, in addition to a cell surface-like staining pattern (Fig. 1 *B*, *C*, *G*, and *H*; double staining images with Hoechst 33342, Fig. S4 *J* and *K*). In contrast, immunoreactivity to anti-human Aβ antibody (clone 4G8) preabsorbed with $A\beta_{1-40}$ (Fig. 1*E*) or to non-immune serum (Fig. 1*F*), was background level in the cells exposed to human $A\beta_{1-40}$. Cells without Aβ treatment also showed no specific staining patterns (Fig. 1*A*). Intracellular $A\beta_{1-40}$ was observed in cells stained positively for two neuronal markers, MAP2 (Fig. 1*R*) and NeuN (Fig. 1*S*). Further analysis using the mitochondrial marker Hsp60 demonstrated extensive colocalization with Aβ epitopes (Fig. 1*T*), although to a lesser extent with the endoplasmic reticulum marker calnexin (Fig. 1*U*). To confirm localization of Aβ to the intracellular space, we performed immunogold electron microscopy on cultured neurons. Immunogold particles labeled Aβ and were present in the intracellular space, such as the cytosolic compartment and mitochondria, after exposure of neurons to $A\beta_{1-42}$. In contrast, the number of immunogold particles was significantly diminished in RAGE-deficient ($RAGE^{-/-}$) neurons (Fig. 2*C*), as compared with wild-type (WT) neurons (Fig. 2 *A* and *B*). Gold particles were virtually absent when cells were treated with vehicle alone (without treatment of Aβ, Fig. S5 *A* and *B*) or $A\beta_{1-42}$ antibody was replaced by non-immune IgG (Fig. S5 *C* and *D*). Quantification of the total number of gold particles per field, based on analysis of multiple images, confirmed a significant decrease Aβ-immunogold particles in $RAGE^{-/-}$ neurons as compared with WT neurons (Fig. 2*D*). These data suggest that exogenous Aβ gains access to intracellular compartments, such as mitochondria, and that absence of RAGE reduces Aβ transport to the intracellular compartment. Neurons exposed to 1 μM $A\beta_{1-42}$ (Fig. S6, part 1) and lower concentration (200 nM) of $A\beta_{1-40}$ (Fig. S6, part 2) showed a similar intracellular distribution of the peptide.

Blockade of RAGE Diminishes Aβ Uptake and Aβ-Induced Mitochondrial Dysfunction. To determine the potential role of RAGE in neuronal Aβ transport, the effect of a blocking antibody to the receptor on Aβ uptake and neurotoxicity was examined in mouse cortical neuron cultures. Pretreatment of neuronal cultures with anti-RAGE IgG (N-16) for 2 h attenuated uptake of human $A\beta_{1-40}$ (Fig. 3*A*) and $A\beta_{1-40}$-induced mitochondrial dysfunction, at the level of MTT reduction (Fig. S7*A*). In contrast, non-immune IgG had no effect on either uptake of Aβ or MTT reduction. To further examine RAGE-dependent neuronal Aβ transport, neurons prepared from $RAGE^{-/-}$ mice were used. Neurons lacking RAGE showed a marked decrease in uptake of $A\beta_{1-40}$ (Fig. 3*B*) and complete preservation of MTT reduction in the presence of $A\beta_{1-40}$ (Fig. S7*B*). To examine the effect of RAGE on Aβ-induced mitochondrial dysfunction, we measured mitochondrial respiratory

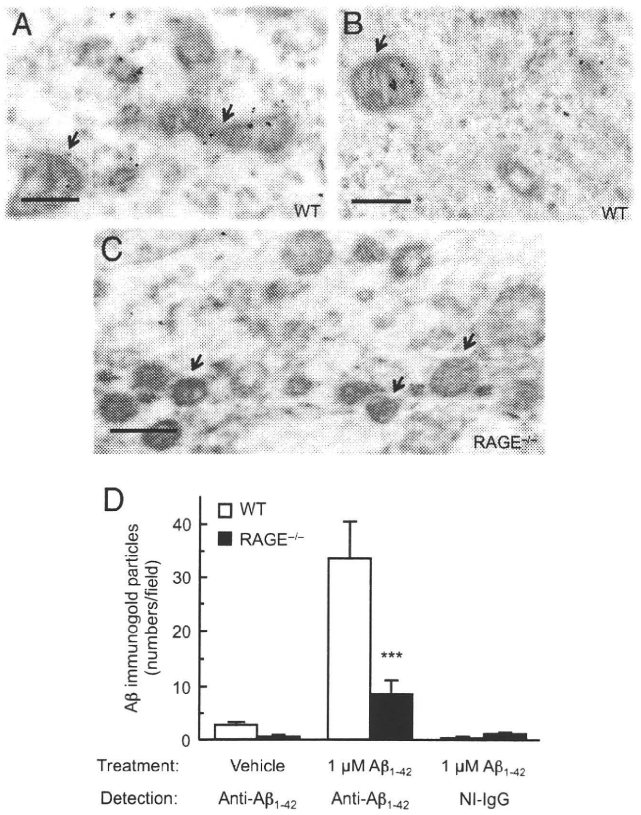

Fig. 2. Immunoelectron microscopy of Aβ in cortical neurons after exposure to Aβ. Cells were prepared from wild-type (WT) (*A* and *B*) and RAGE$^{-/-}$ mice (*C*), exposed to human 1 μM $A\beta_{1-42}$ for 60 min, fixed in 4% PFA and 0.1% glutaraldehyde, and the ultra-thin sections were stained with rabbit anti-$A\beta_{1-42}$/donkey anti-rabbit IgG conjugated to colloidal gold (18 nm particle). Arrows denote mitochondria. (Scale bar, 200 nm.) Two negative controls, in which cells were treated with vehicle or stained with non-immune IgG (NI-IgG), are represented in Fig. S5. (*D*) Quantification of Aβ immunogold particles in WT and RAGE$^{-/-}$ neurons after exposure to Aβ. Numbers of gold particles were counted per field of each microscopic image including two negative controls and expressed as mean ± SEM; ***, $P < 0.001$, versus WT; Unpaired *t*-test.

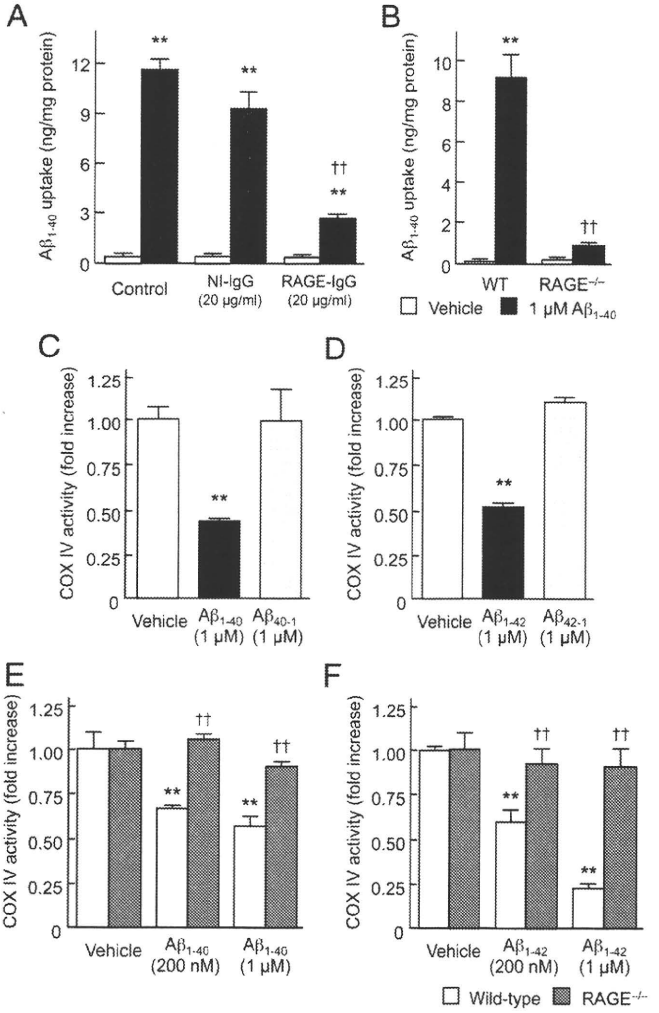

Fig. 3. Blocking RAGE or genetic deletion of the receptor suppresses Aβ uptake and minimizes Aβ-induced mitochondrial dysfunction in cortical neurons. Intracellular levels of human $A\beta_{1-40}$ (*A* and *B*) and COX IV activity (*C–F*) were assayed 60 min (*A* and *B*) and 24 h (*C–F*) after exposure to the indicated Aβ peptides. (*A*) Effect of a neutralizing antibody to RAGE. Cells were pretreated with 20 μg/mL of anti-RAGE (N-16) IgG or NI-IgG for 2 h, and then exposed to 1 μM human $A\beta_{1-40}$. (*B*, *E*, and *F*) Effect of genetic deletion of RAGE. Cells prepared from WT or RAGE$^{-/-}$ mice were exposed to the indicated concentrations of human $A\beta_{1-40}$ (*B* and *E*) or $A\beta_{1-42}$ (*F*). (*C* and *D*) Aβ-related peptides with the reverse sequence have no effect on mitochondrial function in cortical neurons. Cells prepared from wild-type mice were exposed to 1 μM human $A\beta_{1-40}$ or $A\beta_{40-1}$ (*C*), and 1 μM human $A\beta_{1-42}$ or $A\beta_{42-1}$ (*D*). Data represent mean ± SEM; **, $P < 0.01$, versus vehicle- and reversed Aβ-treated cells (*A–D*), or Aβ-treated RAGE$^{-/-}$ neurons (*E* and *F*); ††, $P < 0.01$, versus control (*A* and *B*) or WT (*E* and *F*).

key enzyme cytochrome *c* oxidase (COX IV) activity in RAGE-deficient neurons as compared with COX IV activity in WT neurons. After exposure (24 h) to human $A\beta_{1-40}$ (Fig. 3 *C* and *E*) and $A\beta_{1-42}$ (Fig. 3 *D* and *F*), but not their reversed sequence peptides, neurons displayed a significant dose-dependent reduction in COX IV activity. Notably, RAGE deficiency completely reversed the $A\beta_{1-40}$- and $A\beta_{1-42}$-induced reduction in COX IV activity (Fig. 3 *E* and *F*), which is in agreement with the results of MTT reduction activity. These data indicate that RAGE contributes to transport of Aβ from the cell membrane to the intracellular space, and subsequent induction of mitochondrial dysfunction.

Aβ/RAGE-Mediated Signaling Contributes to Aβ Transport and Internalization. In many contexts, RAGE appears to function as a signal transduction receptor, activating multiple downstream intracellular pathways (22, 31). Thus, we sought to determine if RAGE-mediated cellular activation of such intracellular mechanisms might impact on neuronal Aβ transport. We started by examining the effect of Aβ treatment on phosphorylation of SAPK/JNK and p38 MAPK. Exposure of neurons to $A\beta_{1-40}$ for 10 min did not affect levels of total or phosphorylated forms of SAPK/JNK (Fig. S8*A*). In contrast, neurons exposed to $A\beta_{1-40}$ displayed a dose-dependent increase in phosphorylated p38 MAPK as compared to vehicle-treated controls (Fig. 4 *A* and *D*), although $A\beta_{1-40}$ did not affect total protein levels of p38 MAPK (Fig. 4*A*). $A\beta_{1-42}$ also stimulated p38 MAPK phosphorylation in a similar dose-dependent manner (Fig. S8*B*). Activation of p38 MAPK was observed immediately after $A\beta_{1-40}$ treatment and for up to 30 min (Fig. 4*E*). Pretreatment of neuronal cultures with the p38 MAPK inhibitor SB203580 blocked $A\beta_{1-40}$-stimulated p38 MAPK phosphorylation (Fig. 4*B*). Consistent with these data, neurons pretreated with SB203580, but not a SAPK/JNK inhibitor (SP600125), showed strong inhibition of $A\beta_{1-40}$ uptake (Fig. 4*F*) and MTT reduction in response to $A\beta_{1-40}$ (Fig. S8*C*). A role for RAGE in Aβ-mediated activation of p38 MAPK was indicated by inhibition of p38 phosphorylation in cortical neurons from wild-type mice exposed to Aβ in the presence of anti-RAGE IgG (N-16) (Fig. 4*B*) and in RAGE-deficient cortical neurons derived from RAGE$^{-/-}$ mice (Fig. 4*C*).

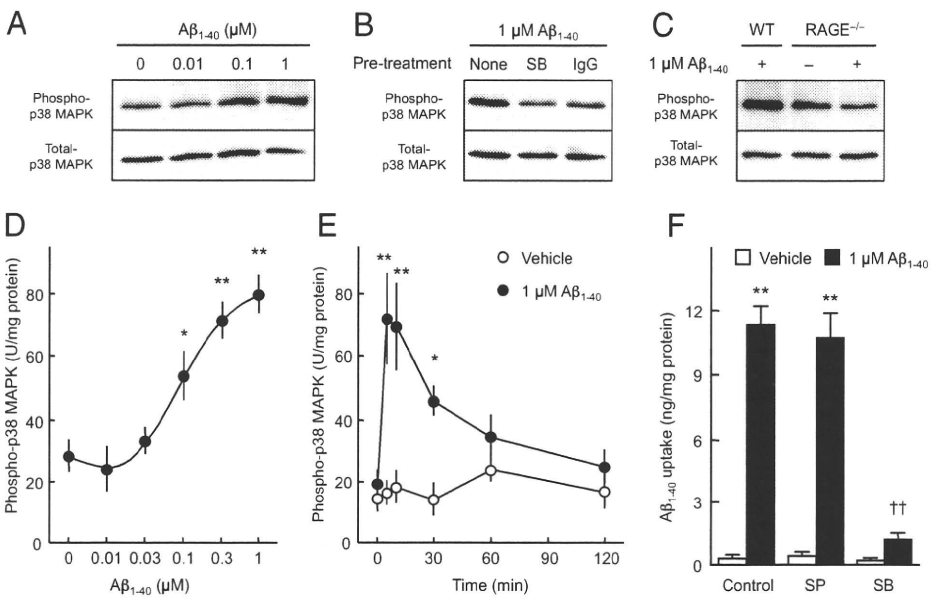

Fig. 4. Aβ-stimulated p38 MAPK activation is required for Aβ uptake in cortical neurons. (*A–C*) Immunoblot analyses of phospho-p38 MAPK in cortical neurons treated with $A\beta_{1-40}$. Cells were exposed to the indicated concentrations of human $A\beta_{1-40}$ for 10 min, lysed, and subjected to SDS/PAGE. Typical immunoblot images detected by antibodies against phospho-p38 MAPK (*A–C*, upper) and total-p38 MAPK (*A–C*, lower) are shown from 3–6 independent experiments. (*A*) Dose-dependency. (*B*) The p38 MAPK inhibitor SB 203580 (1 μM; SB) and anti-RAGE (N-16) IgG (20 μg/mL; IgG) were added 30 min and 2 h before exposure to $A\beta_{1-40}$, respectively. (*C*) Phospho-p38 MAPK levels in neurons from $RAGE^{-/-}$ mice after exposure to $A\beta_{1-40}$. WT, wild-type. (*D* and *E*) Phospho-p38 MAPK levels were determined by ELISA. (*D*) Dose-dependency. Cells were exposed to the indicated concentration of human $A\beta_{1-40}$ for 10 min. (*E*) Time course. Cortical neurons were exposed to 1 μM of human $A\beta_{1-40}$ for the indicated time. (*G*) Effects of JNK and p38 MAPK inhibitors on intracellular levels of human $A\beta_{1-40}$ in cultured neurons exposed to Aβ. Cells were pretreated with the JNK inhibitor SP 600125 (SP, 20 μM), or SB 203580 (SB, 1 μM), for 30 min, and then exposed to 1 μM human $A\beta_{1-40}$ for 60 min. Intracellular $A\beta_{1-40}$ concentrations were determined by ELISA. Data represent mean ± SEM; *, $P < 0.05$, **, $P < 0.01$, versus none (0 μM $A\beta_{1-40}$) (*D* and *F*) and 0-time (*E*); ††, $P < 0.01$, versus control (no inhibitor).

Membrane RAGE Acts as an Aβ Carrier and Co-Internalizes with Aβ. To determine molecular mechanisms underlying neuronal Aβ transport, we biotinylated neuronal cell surface proteins, incubated the labeled cells with $A\beta_{1-40}$, and then analyzed internalized biotinylated proteins. First, we assessed the distribution of biotin in labeled cells before Aβ treatment. Cells fixed immediately after biotinylation and permeabilized with detergent displayed a cell surface and focal [the latter were probably surface accumulations of biotin since they were removed by sodium 2-mercaptoethanesulfonate (MesNa) treatment; see below] distribution of the biotin (Fig. S9*D*) and, as expected, the absence of Aβ (Fig. S9 *A* and *G*). Next, we examined the intracellular distribution of biotin and Aβ in the cells after Aβ treatment. After biotinylation, cells were incubated with vehicle or $A\beta_{1-40}$ for 60 min, treated with MesNa (the latter to remove biotin remaining on the cell surface), fixed and permeabilized with detergent. Cells exposed to $A\beta_{1-40}$ displayed an overlapping intracellular distribution of Aβ (Fig. S9 *C* and *I*) and biotinylated-proteins (Fig. S9 *F* and *I*), while control cells treated with vehicle alone showed no specific signal (Fig. S9 *B*, *E*, and *H*), suggesting that Aβ is able to interact with cell surface proteins.

To analyze internalized proteins in cells exposed to Aβ, we performed Western blotting. After biotinylation of surface proteins, cells were incubated with vehicle or $A\beta_{1-40}$ for 60 min, treated with MesNa, and then whole cell lysates were collected and subjected to immunoprecipitation. Cell lysates contained same amount of total protein, in each case and from both groups, and were reacted with streptavidin followed by SDS/PAGE. Silver staining of gels revealed a broad array of protein bands, especially in cells exposed to $A\beta_{1-40}$, compared with controls (Fig. S10*A*). Interestingly, immunoblotting with anti-RAGE IgG demonstrated >8-fold more RAGE antigen had been immunoprecipitated from cells exposed to $A\beta_{1-40}$, compared with non-treated control (Fig. S10*B*). To determine whether RAGE and Aβ were in the cytosol, we performed immunoprecipitation with anti-Aβ IgG-conjugated beads using the cytosolic fraction from neurons exposed to Aβ. Such cytosolic fractions were obtained by ultracentrifugation (13, 32) and showed virtually undetectable levels of the membrane marker Na^+/K^+-ATPase, compared with presence of the latter in whole cell lysates or membrane-enriched fractions (Fig. S10*C*). Immunoprecipitation analysis was also applied to cytosolic fractions using anti-Aβ IgG-conjugated beads or non-IgG-conjugated beads as a control for nonspecific binding. SDS/PAGE of these immunoprecipitates was followed by immunoblotting with anti-RAGE IgG. While there was only a weak signal with immune precipitates prepared in the presence of non-IgG beads, the immune precipitates prepared with anti-Aβ IgG beads demonstrated a strong immunoreactive RAGE band (Fig. S10*D*). Based on image analysis, there was >4-fold more RAGE antigen detected in the immune precipitates with anti-Aβ IgG beads compared with non-IgG beads. These data are consistent with the hypothesis that Aβ stimulates internalization of RAGE, and that during this process, RAGE and Aβ interact closely.

To further assess possible colocalization of RAGE and Aβ, and the spatial topography of these two molecules after internalization of Aβ, we performed dual fluorescence confocal microscopy. Incubation of $A\beta_{1-40}$ with neurons for 60 min demonstrated extensive colocalization of epitopes visualized with anti-RAGE and anti-Aβ antibodies (Fig. S11).

Aβ Colocalizes with RAGE in Hippocampus of Aged Tg-mAPP Mice. To extrapolate these findings to the in vivo setting, we turned to a mouse model of AD-like pathology, transgenic mice overexpressing the human APP isoforms (APP695 and APP751/770) with the familial Alzheimer's dementia mutation (Tg mAPP) and Aβ. Immunohistochemical studies were performed to colocalize intracellular Aβ and RAGE in brains from 9- to 10-month-old mice after permeabilizing the cell membrane with detergent. Compared with wild-type controls (Fig. S12 *A* and *C*), low power immunofluorescence images of brain sections from aged Tg mAPP mice displayed increased staining for Aβ (Fig. S12*B*) and RAGE (Fig. S12*D*) antigens in the hippocampus, especially in the pyramidal cell layer. Plaques in Tg mAPP mice displayed strong staining for Aβ (Fig. S12*B*). High power confocal immunofluorescence images of the hippocampal CA3 region in Tg mAPP mice further demonstrated that Aβ and RAGE co-localized in an apparently intracellular distribution in pyramidal cells (Fig. S12 *F*, *H*, and *J*).

Discussion

Our studies address a paradigm in which Aβ binding to cell surface RAGE translocates the ligand into the cytosolic compartment. Our in vitro studies show that: (*i*) exogenous Aβ translocates from the cell surface to the cytosol, with at least

some of the peptide eventually localizing in mitochondria; (*ii*) such translocation is dependent on RAGE, as it is prevented by blocking antibodies to the receptor and does not occur to an appreciable extent in neurons devoid of RAGE (from $RAGE^{-/-}$ mice); (*iii*) RAGE-mediated cellular activation at the level of p38 MAPK has a central role in internalization of the receptor-ligand complex; and, (*iv*) the presence of Aβ within the cytosol and mitochondria is associated with functional consequences, including mitochondrial dysfunction. Immunoprecipitation of cytosolic fractions after Aβ treatment showed that RAGE itself interacts closely with Aβ, consistent with the concept that the receptor may be the actual Aβ transporter/carrier. As a counterpart to these observations in cell culture, immunohistochemical studies showed colocalization of Aβ and RAGE in an apparently intracellular distribution in hippocampal pyramidal cells in the brains of AD-type transgenic mice expressing mAPP/Aβ.

Increasing evidence points to a role for intraneuronal Aβ in the pathogenesis of early neural dysfunction and AD pathology. Several observations have indicated that APP localizes not only to the plasma membrane, but also to the trans-Golgi network, endoplasmic reticulum, and endosomal, lysosomal, and mitochondrial membranes (5, 7, 33). Thus, two possible pathways could underlie the accumulation of intraneuronal Aβ: (*i*) Aβ secreted into extracellular space is subsequently taken up by neurons (and/or other cells); and, (*ii*) Aβ produced intracellularly remains within the neuron. Our results provide insight into the former pathway, which involves neuronal internalization of both $Aβ_{1-40}$ and $Aβ_{1-42}$. Initially, based on in vitro studies, it was thought that $Aβ_{1-42}$ was more neurotoxic than $Aβ_{1-40}$, in part because of the propensity of $Aβ_{1-42}$ to form large aggregates and fibrils. However, more recently, it has been appreciated that oligomeric and prefibrillar $Aβ_{1-40}$ and $Aβ_{1-42}$ have similar cytotoxic effects (34) and such soluble forms of Aβ are believed to play a critical role in the pathogenesis of AD. Recent work has demonstrated that oligomeric $Aβ_{1-42}$, at a concentration of 200 nM, is capable of blocking long-term potentiation at cortical synapses in the hippocampus and entorhinal cortex (10, 28, 35, 36). Taken together, our findings suggest that via RAGE, neuronal transmembrane transport of $Aβ_{1-40}$ and $Aβ_{1-42}$ carries soluble assemblies of amyloid peptide into the cell.

The present study revealed that intraneuronal accumulation of Aβ could be sustained during exposure to the peptide, especially in mitochondria, as previously reported (10, 12, 37–40). Considerable studies over the past decade have emerged indicating that some intracellular enzymes, insulin-degrading enzyme, endothelin-converting enzyme (ECE)-1b and ECE-2, as well as membrane enzymes, such as neprilysin, ECE-1a, ECE-1c, ECE-1d, matrix metalloproteinase (MMP)-2, MMP-3, and MMP-9, can cleave Aβ at either a single or multiple sites and cleavage products of Aβ resulting from such catabolism are less likely to aggregate and are less neurotoxic than Aβ itself (41). Moreover, a mitochondrial peptidase, PreP peptidasome, has been recently shown to be capable of degrading Aβ (42). As these various amyloid-degrading enzymes have distinct subcellular localization, Aβ metabolism may influence the subcellular accumulation of Aβ and its neurotoxicity. The mechanism through which intraneuronal Aβ is metabolized will require further study to elucidate.

Recent studies demonstrate that several plasma membrane receptors, such as *N*-methyl-D-aspartate receptors (14), α7 nicotinic acetylcholine receptors (15), and low-density lipoprotein receptor-related proteins (LRP) (16), have the capacity to bind to Aβ and, potentially, promote intracellular accumulation of Aβ. Previous studies have shown that RAGE binds monomeric, oligomeric, and even fibrillar forms of Aβ at the neuronal cell surface (22, 27, 43). Moreover, RAGE promotes Aβ-induced neuronal dysfunction in a mouse model of AD-type pathology (28). Subsequent to Aβ binding to RAGE on the cell surface, we have found that the amyloid peptide is internalized in a RAGE-dependent manner; blocking RAGE or deletion of the receptor attenuates Aβ internalization and Aβ-induced mitochondrial dysfunction in cortical neurons. These findings strongly suggest a role for RAGE as a cell surface-binding site and a potential transporter for Aβ which facilitates intracellular transfer of the peptide.

RAGE-ligand interaction has been shown to activate multiple intracellular signaling pathways including the MAPKs (ERK1/2, p38 MAPK and SAPK/JNK), rho-GTPases, phosphoinositol-3-kinase, and the JAK/STAT pathway in various cells (23, 43). In addition, the RAGE-ligand interaction has been shown to directly cause generation of reactive oxygen species via NADPH oxidases (44). As a consequence of Aβ–RAGE interaction, activation of p38 MAPK, SAPK/JNK, and NF-κB was observed in sporadic AD cybrids (45). In addition, Arancio et al. (28) reported increased phosphorylation of CREB, ERK1/2, p38 MAPK, and CaMKII in hippocampal extracts from Tg mice overexpressing RAGE and mAPP. RAGE-dependent activation of p38 signal transduction also plays an important role in Aβ-mediated synaptic failure (35, 36). However, direct links between RAGE-mediated signaling pathways and Aβ neurotoxicity remain to be fully elucidated. The present study indicates that the Aβ-RAGE interaction rapidly activates p38 MAPK, but not SAPK/JNK, and further demonstrates a link between activation of p38, intracellular Aβ accumulation, and Aβ-induced cytotoxicity in cortical neurons.

In the BBB endothelial cells, RAGE and LRP1 have shown to be critical for regulation of Aβ homeostasis in the central nervous system (46). RAGE binds soluble Aβ at the apical side of human BBB, and promotes transport of soluble Aβ from blood to brain via endocytosis and transcytosis. These events promote Aβ accumulation in brain parenchyma (29, 47). Our biotinylation study revealed that Aβ stimulated internalization of neuronal plasma membrane proteins, including RAGE, and that RAGE–Aβ complex was present intracellularly. These finding suggest that the interaction of Aβ with RAGE activates an endocytosis-like pathway that causes rapid internalization of Aβ–RAGE complex. Consistent with these in vitro results, recent studies in brains of AD patients (48) and another mouse AD model (49) displayed striking accumulation of Aβ in hippocampal pyramidal cells.

In conclusion, our study demonstrates that Aβ induces a RAGE-dependent pathway that involves activation of p38 MAPK, resulting in internalization of Aβ and leading to mitochondrial dysfunction in cultured cortical neurons. We propose that Aβ internalization may be associated with RAGE-mediated endocytosis and that RAGE itself may act as a carrier in transmembrane Aβ transport. The mechanism through which Aβ gains access to the cytosol and enters mitochondria will require further study to elucidate. Cytosolic Aβ may enter mitochondria through the TOM pathway as recently reported (39) leading to mitochondrial stress. The results of our studies contribute to a growing body of evidence demonstrating that RAGE can act as a receptor magnifying intraneuronal Aβ cytotoxicity. Blockade of RAGE may have a beneficial effect by limiting intracellular accumulation of amyloid in AD brain and serves a potential therapeutic target for AD.

Materials and Methods

For full description of this study's materials and methods, see *SI Materials and Methods*.

Animals. RAGE knockout ($RAGE^{-/-}$) mice have been described previously (35, 50).

Cell Culture. Cortical neurons were prepared from embryos at 17 days of gestation of C57BL/6J mice, transgenic mice overexpressing the human full-length ABAD (Tg-ABAD mice) and homozygous $RAGE^{-/-}$ mutant mice.

Biochemical Determination of Neuronal Perturbation. Neuronal perturbation after Aβ treatment was determined by generation of reactive oxygen species (ROS), mitochondrial membrane potential, caspase activity, DNA fragmentation, MTT reduction, and cytochrome *c* oxidase (COX IV) activity assays.

Determination of Membrane Aβ Transport. Transport of Aβ into cytosol through the plasma membrane was measured by ELISA and detected by confocal immunofluorescence and immunoelectron microscopies using anti-Aβ IgG.

Measurement of Phospho-MAPKs. Aβ-stimulated phosphorylation of SAPK/JNK and p38 MAPK was detected by Western blot analysis or measured by ELISA.

Analysis of Internalization of Membrane Surface Proteins. Internalization of membrane surface proteins after Aβ treatment was detected by Western blot analysis using biotinylation and immunoprecipitation.

Immunohistochemistry. Immunohistochemistry was executed in hippocampal sections from Tg mAPP mice (9- to 10-month-old) and age- and strain-matched wild-type mice using anti-Aβ IgG and anti-RAGE IgG.

Statistics. Statistical analysis of the experimental data were carried out using GraphPad Prism 4 for Macintosh (GraphPad Software). The significance of differences was determined by a one-way ANOVA, followed by the Dunnett's or Tukey's multiple comparison test for multigroup comparisons. Unpaired *t*-test was used for two-group comparisons. The criterion for statistical significance was $P < 0.05$.

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特集

精神神経疾患の分子的理解と創薬のアプローチ

統合失調症における発症脆弱性遺伝子と創薬

永井 拓* 鍋島俊隆§ 山田清文*

KEY WORDS

・統合失調症
・遺伝子
・遺伝子多型
・モデル動物

SUMMARY

統合失調症は重篤な精神障害を呈する精神疾患であり，複数の遺伝子と環境因子が発症に関与している．これまでに統合失調症の発症に関与すると考えられる十数種類の脆弱性遺伝子が同定され，統合失調症の病態解明に向けた研究が盛んに行われている．一方，既存の抗精神病薬とは異なる作用メカニズムを有する新規治療薬の開発も行われており，臨床研究において有効性を示す数種類の候補化合物がすでに報告されている．本稿では，統合失調症の発症に関与していることが示唆されている発症脆弱性遺伝子と新規抗精神病薬の開発状況について概説する．

はじめに

統合失調症は重篤な精神障害を呈する精神疾患であり，生涯発病率は1%と高い．統合失調症の主症状は陽性症状（妄想・幻覚，連合弛緩，緊張病症状），陰性症状（感情の平板化，思考の貧困，意欲の低下）および認知障害（注意集中困難，記憶の問題，遂行機能障害）に分類される．これらさまざまな精神症状は長期にわたって学業面でも就労面でも患者の社会的機能を低下させ，患者の約10%は自殺既遂に至る．疫学的研究によって統合失調症に遺伝的因子が強く関与することが実証されている[1]．また，連鎖解析および関連解析に代表される遺伝学的研究によれば，統合失調症は単一遺伝子の異常というよりは複数の遺伝子が関与して起こる複合遺伝疾患であるとの見解が強い．一方，一卵性双生児であっても約半数は統合失調症不一致例が存在するため，統合失調症は遺伝因子と出生の季節や場所，社会経済状況，母体感染などの環境因子が相互に組み合わさって発症に至る複雑精神疾患であると考えられている[1]．

本総説では，統合失調症病態仮説と発症に関与していることが示唆されている遺伝子（発症脆弱性遺伝子）について概説する（表1）．さらに，臨床試験が行われている新規抗精神病薬の候補化合物についても述べる．

1．統合失調症病態仮説

1）ドパミン仮説

統合失調症では中脳―辺縁系ドパミン作動性神経系の機能の亢進が陽性症状を，中脳―皮質系ドパミン作動性神経系の機能低下が陰性症状や認知障害の発現に関与していると考えられている．臨床において使用されている定型および非定型抗精神病薬のほとんどがドパミンD2受容体に作用することから，ドパミンD2受容体と統合失調症との関連性について検討されている．遺伝学的研究において，Taq A2およびCys311Serの多型が統合失

NAGAI Taku, NABESHIMA Toshitaka, YAMADA Kiyofumi/＊名古屋大学大学院医学系研究科医療薬学・医学部附属病院薬剤部，§名城大学大学院薬学研究科薬品作用学

表 1. 統合失調症の発症脆弱性遺伝子

遺伝子名	染色体領域	脳内発現変化	機能
COMT	22q11	増加もしくは減少	カテコールアミン分解
DARPP-32	17q12	減少	ドパミン作動性神経系の細胞内情報伝達
PPP3CC	8p21	減少	カルシウム依存性セリン/スレオニンフォスファターゼ
BDNF	11p13	減少	神経栄養因子
NRG1	8p12-21	上昇	NMDA 受容体機能調節，神経発達，神経伝達，シナプス可塑性
DTNBP1	6p22	減少	ドパミンおよびグルタミン酸作動性神経伝達
G72	13q34	増加	DAAO 活性化
PRODH	22q11	変化なし	プロリン分解
RGS4	1p21-22	減少	G 蛋白質共役型受容体シグナル抑制
DISC1	1p42	変化なし	神経突起の伸長，神経細胞の移動
CHRNA7	15q13-14	減少	コリン作動性神経伝達
AKT1	14q22-32	減少	PI3K シグナル伝達

（著者作成）

調症と有意に関連することが示されている[2]．また，健常者において線条体ドパミン D2 受容体結合に影響を与える C957T の機能的多型について，統合失調症患者との関連性が見出されている[3]．ドパミン D2 受容体以外についても関連研究がなされ，D3 受容体では Ser9Gly の多型は統合失調と有意な関連を示すことが報告されている[4]．一方，D1 および D4 受容体では有意な関連は認められない[5]．

2）グルタミン酸仮説

グルタミン酸受容体には 3 種類のイオンチャネル型受容体（NMDA 受容体，AMPA 受容体とカイニン酸受容体）と代謝型受容体が存在する．phencyclidine（PCP）あるいはケタミンなどの NMDA 受容体拮抗薬は統合失調症の陽性症状と陰性症状に類似した症状を惹起することから，統合失調症にグルタミン酸作動性神経系の機能低下が関与しているという仮説である．実際，統合失調患者の線条体ではグルタミン酸作動性神経のマーカーである神経型グルタミン酸トランスポーターの excitatory amino acid transporter（EAAT）-3 および vesicular glutamate transporter-1 の発現が健常者にくらべて減少していることが示されている[6]．遺伝学的研究では，*EAAT-2* 遺伝子（*SLC1A2*）と *EAAT-4* 遺伝子（*SLC1A6*）に統合失調症との関連が認められている[7][8]．また，NMDA 受容体に関する遺伝学的研究では，NMDA 受容体 NR1 サブユニット遺伝子（*GRIN1*）および *NR2B* サブユニット遺伝子（*GRIN2B*）の多型は統合失調症と有意な関連を示すことが報告されている[9][10]．

3）神経発達障害仮説

統合失調症の神経発達障害仮説は，胎児期および新生児期における何らかのイベントが初期の脳発達に異常をもたらし，生じた神経回路の形成不全や神経機能の変化が統合失調症の発症を引き起こすというものである．MRI（magnetic resonance imaging）研究から，統合失調症における脳の構造異常が実証されており[11]，初回エピソードの統合失調症患者において認められる著しい所見は脳室の拡大である．全脳容積は脳室拡張に伴い縮小するが，その縮小率はわずかである．その他に海馬の萎縮が統合失調症患者では観察されている．実際，妊婦の母体感染，周産期イベントあるいは分娩時の合併症は出生時の統合失調症の発症リスクを増大させる[12]．また，小児の中枢神経系の感染または出生時の低酸素状態は精神発達障害をきたすリスクを 5 倍に増加させることが報告されている[12]．

2．発症脆弱性遺伝子と統合失調症の病態

1）Catechol-O-methyl transferase

口蓋心顔面症候群（vero-cardio-facial syndrome：VCFS）は染色体 22q11 領域の欠損により顔面や心臓の奇形が認められる遺伝疾患である．VCFS 患者の約

30%は統合失調症を発症することから，22q11領域に発症脆弱性遺伝子が存在する可能性が示唆されている．catechol-O-methyl transferase（*COMT*）遺伝子は22q11領域に存在する．COMTはドパミンなどのカテコールアミンを分解する酵素であり，前頭前野の細胞外ドパミン遊離量や認知機能の調節に関与している分子である．COMTには溶解型および膜貫通型が存在し，それぞれに酵素活性に影響する機能的多型が存在することが確認されている．溶解型についてはコドン108が，膜貫通型についてはコドン158がバリンまたはメチオニンとなる多型が存在する．メチオニン型はバリン型の25%程度の活性を有する．シナプス間隙のドパミン量が少ないと推定されるバリン型COMTを有する統合失調症患者では，作業記憶能力および前頭葉皮質の活動低下が認められる[13]．

2）Dopamine and cAMP-regulated phosphoprotein of 32kDa

dopamine and cAMP-regulated phosphoprotein of 32kDa（DARPP-32）はドパミン作動性神経系の細胞内情報伝達に重要な役割を果たしている分子である．DARPP-32は前頭前皮質や帯状回に高発現しており，ヒトにおいてDARPP-32の発現異常は前頭前皮質の体積減少や活動低下，作業記憶および実行機能の障害につながることが報告されている[14]．統合失調症患者の死後脳解析において背外側前頭前皮質のDARPP-32の発現が減少していること[15]，DARPP-32遺伝子の高頻度ハプロタイプが統合失調症と有意に関連していることが報告されている[14]．興味深いことに，DARPP-32遺伝子の位置する染色体17q12領域は統合失調症の発症脆弱性遺伝子座位であることが示されている[16]．

3）Calcineurin

calcineurinはカルシウム依存性セリン/スレオニンフォスファターゼであり，DARPP-32の脱リン酸化に関与する分子である．calcineurinのγサブユニットをコードする*PPP3CC*遺伝子の多型解析において統合失調症と相関が認められている[17]．また，*calcineurin* KOマウスでは多動，社会性行動の低下，感覚情報処理能力の障害およびNMDA受容体アンタゴニストに対する反応性が低下していることが報告されている[18]．

4）Brain-derived neurotrophic factor

brain-derived neurotrophic factor（BDNF）は神経の発達，再生および生存を促進する作用を有している神経栄養因子である．BDNFは中脳辺縁系ドパミン作動性神経系の発達およびドパミンD3受容体の発現調節に重要な役割を果たしており，統合失調症の神経病態にBDNFが関与していると考えられている．メタ解析において，BDNF遺伝子C270Tの多型は統合失調症に関連していることが示されている[19]．一方，*BDNF*遺伝子のG196AおよびVal66Metの多型については，統合失調症との関連性が認められない[19][20]．

5）Neuregulin 1

アイスランドの統合失調症患者における全ゲノム連鎖解析からみいだされた分子としてneuregulin 1（NRG1）およびNRG1受容体ErbB4がある．*NRG1*および*ErbB4*ヘテロKOマウスでは，感覚情報処理機構の障害および新規環境における多動を示し，これらの障害は非定型抗精神病薬のclozapineにより改善する[21]．特に，*NRG1*プロモーター領域の遺伝的多様性が統合失調症患者に認められる前頭葉および側頭葉の活動低下，精神症状の進行と関連していることが示唆されている．前頭葉皮質の錐体神経細胞において*NRG1*プロモーターの活性化は細胞表面に存在するNMDA受容体の細胞内移行を促進し，その機序としてクラスリンが関与していることが見出されている[22]．また，統合失調症患者の前頭葉皮質においてNRG1刺激によるErbB4の活性化はNMDA受容体NR2Aサブユニットのチロシンリン酸化を減少させる[23]．その他にもNRG1は，神経発達，神経伝達およびシナプス可塑性に関与していることが知られている．

6）Dystrobrevin binding protein 1

dystrobrevin binding protein 1（dysbindin）は認知機能や記憶に関与している分子であり，統合失調症患者の脳内におけるdysbindinの発現が健常者にくらべて減

少している[24]．遺伝学的研究においても，*dysbindin* 遺伝子（DTNBP1）のSNPsと統合失調症との有意な相関が示されている[25]．dysbindinはシナプス小胞，postsynaptic density（PSD）および微小管に局在し，ドパミンおよびグルタミン酸作動性神経系の機能制御に関与している．自然変異によって *DTNBP1* 遺伝子が欠損したマウス（sandyマウス）では，社会性行動および記憶の障害が観察される[26]．また，sandyマウスではシナプス小胞の膜融合に必要なSNAP25と結合するsnapinの発現が減少し，シナプス小胞の形態学的異常と小胞の開口放出に障害が認められる[25]．したがって，dysbindinの減少によるsnapinの不安定化が統合失調症で認められる神経伝達および行動の異常に関与している可能性が示唆されている．

7）G72

Chumakovら[27]は統合失調症との連鎖が示唆されている13q34染色体の5Mb領域について191個のSNPsをマーカーとして連鎖解析を行った．その結果，統合失調症と関連する65-kbの領域を発見し，この遺伝子領域にコードされるG72を同定した．その後の機能解析により，G72はD-amino acid oxidase（DAAO）を活性化する作用を有していることが示された．DAAOはD-セリンなどのD-アミノ酸を酸化する酵素であり，*DAAO* KOマウスでは脳内のD-セリン濃度が上昇している[28]．一方，統合失調症患者の前頭前野背外側部では健常者にくらべてG72の発現が有意に増加していることが報告されている[29]．D-セリンはNMDA受容体の活性化作用を有していることから，G72の発現増加に伴うD-セリンの減少が統合失調症におけるNMDA受容体の機能低下に関与している可能性がある．

8）Proline dehydrogenase

proline dehydrogenase（PRODH）はプロリンを分解する酵素であり，*PRODH* 遺伝子は染色体22q11領域にコードされている．22q11領域の多型解析では，*PRODH* 遺伝子SNPのハプロタイプが有意に統合失調症と相関した．特に，このハプロタイプは統合失調症の早期発症例で多く認められた[30]．また，*PRODH* 遺伝子変異を伴う先天性高プロリン血症において，統合失調症との関連性が示唆されている[31]．*PRODH* 遺伝子KOマウスは感覚情報処理の障害を示し，脳内のグルタミン酸およびGABAの含量が野生型マウスにくらべて減少している[32]．

9）Regulator of G-protein signaling 4

regulator of G-protein signaling 4（RGS4）はG蛋白質共役型受容体の下流シグナルを抑制する分子であり，group 1/5代謝型グルタミン酸受容体のシグナル伝達に関与している．連鎖解析によりRGS4の多型と統合失調症との関連が示唆されている．統合失調症患者の前頭前野背外側部において，RGS4の発現が減少していることが報告されている[33]．また，実験動物では，ストレス負荷やグルココルチコイド投与によってRGS4の発現が変化することから，環境因子との関連性が示唆されている[34]．

10）Disrupted-in-schizophrenia-1

disrupted-in-schizophrenia-1（*DISC1*）はスコットランドにおいて統合失調症や気分障害など精神障害発生率の高い家系より発見された遺伝子である[35]．この家系において精神障害を発症したヒトでは，*DISC1* の1番染色体長腕と11番染色体長腕の相互転座が認められる．染色体の転座によりC末端アミノ酸の一部が欠質した変異型DISC1が産生される．ヒトの脳内におけるDISC1の発現は大脳皮質および海馬に認められる．DISC1はkinesin-1，NUDEL，LIS1，14-3-3εなどの蛋白と結合することが示され，神経突起の伸長および神経細胞の移動を制御していることが見出されている[36]．変異型DISC1を導入したトランスジェニックマウスにおいて，多動，感覚情報処理障害および意欲低下が観察されている[37]．タモキシフェンによる遺伝子発現誘導システムを用いて生後7日目に変異型DISC1を誘導させたトランスジェニックマウスでは作業記憶障害および意欲低下，神経突起および神経伝達に障害が認められる[38]．一方，3ヵ月齢に変異型DISC1を誘導させたトランスジェニックマウスではこれらの異常は認められないことから，発達期における変異型DISC1の発現が統合失調症の発症に関与していると考えられる．

11）α7 nicotinic acetylcholine receptor

統合失調症患者ではコリン作動性神経系の障害が認められる．アセチルコリンの作用する受容体にはムスカリン受容体およびニコチン受容体があり，認知機能に重要な役割を果たしている．遺伝学的研究において，*α7 nicotinic acetylcholine receptor* 遺伝子（*CHRNA7*）のプロモーター領域の変異が統合失調症患者に多いことが報告されている[39]．*CHRNA7* 遺伝子の変異を有する統合失調症患者では事象関連電位 P50 抑制に代表される聴覚情報処理の障害が認められることから，*CHRNA7* 遺伝子変異は受容体の機能に関与していると考えられる．一方，統合失調症患者の死後脳では，ニコチン受容体の発現が減少していることが示されている[40]．単一光子放射断層撮影（single photon emission computed tomography：SPECT）を用いた研究において，皮質，視床および大脳基底核のムスカリン受容体の発現が減少していることが報告されている[41]．

12）AKT1

phosphatidylinositide 3-kinase（PI3K）経路は，細胞の増殖，分化，移動，生存などを担う重要な細胞内情報伝達系の1つである．Emamian ら[42]は，統合失調症患者の末梢リンパ球と脳において，AKT1 蛋白質とリン酸化 GSK3β のレベルが健常者にくらべて低下していること，*AKT1* 遺伝子のハプロタイプは統合失調症と有意な相関を示すことを報告している．また，*AKT1* KO マウスでは，アンフェタミンによる感覚情報処理機構の障害効果が増大することから，AKT1 遺伝子が統合失調症に関与している可能性が示唆されている．

3．新規抗精神病薬の開発

統合失調症の治療薬として，ドパミン D2 受容体拮抗作用を有する定型抗精神病薬の他，ドパミン D2 受容体拮抗作用に加えてセロトニン受容体やその他の神経伝達物質受容体に作用する非定型抗精神病薬が臨床で用いられている．定型抗精神病薬および非定型抗精神病薬は陽性・陰性症状を改善するが，これら既存の薬物で適切な治療を行っても認知障害は改善されにくいことが問題となっている．現在，神経伝達物質および受容体を標的として，認知障害に対する治療薬の開発が盛んに行われている．

前述したように，統合失調症の陰性症状・認知障害には中脳―皮質系ドパミン作動性神経系の機能低下が関与していると考えられており，現在臨床ではドパミン D2 受容体部分作動薬であるアリピプラゾールの使用が試みられている．一方，前頭前皮質ではドパミン D1 受容体がドパミン D2 受容体にくらべて高発現していること，ドパミン D1 受容体は作業記憶などの認知機能に関与していることから[43]，ドパミン D1 受容体作動薬の開発が進められている．dihydrexidine（DAR-0100）はドパミン D1 受容体に対して高親和性を示し（IC_{50} 10nM），ドパミン D1 受容体を介したアデニール酸シクラーゼ活性化作用はドパミンにくらべて 70 倍の強さを有する[44]．臨床研究において，DAR-01000 の単回皮下注射は認知機能に有意な改善効果は認められなかったが，投与直後から脳血流が著しく増加することが示されている[45][46]．

コリン作動性神経系は注意，記憶，情報処理速度および感覚情報処理などの能力に関係しており，統合失調症患者ではこれらの機能に障害が認められる．azabicyclic aryl amide を基本骨格とした新規 α7 nicotinic acetylcholine receptor（α7 nAChR）作動薬として PHA-709829 が開発されている．PHA-709829 は α7nAChR に対して高い親和性（Ki 3.4nM）と中枢移行性（脳/血漿濃度比 1.5）を示す．PHA-709829 はアンフェタミンを投与したラットに認められる聴覚情報処理の障害を改善する[47]．一方，Olincy ら[48]は天然アルカロイド anabaseine の誘導体である 3-(2,4-dimethoxybenzylidene) anabaseine（DMXB-A）を開発した．DMXB-A は α7 nAChR 部分作動薬であり，統合失調症患者を対象とした臨床試験では認知機能および事象関連電位 P50 抑制の障害に対して有意な改善効果が認められている．最近報告された第二相臨床試験において，DMXB-A はプラセボと比較して陰性症状を有意に改善するが，認知機能に対しては DMXB-A とプラセボとのあいだに有意な差は認められなかった[49]．しかし，DMXB-A は服薬前のベースラインと比較して注意・覚醒，作業記憶および言語学習を改善する．

NMDA 受容体は神経伝達物質としてのグルタミン酸

結合部位の他に，グリシン結合部位が存在する．グリシン結合部位を刺激しても，グリシン単独では神経伝達を生じさせる効果はないが，グルタミン酸結合部位にアゴニストが結合するのに必須であり，チャンネル開口頻度を上昇させて神経伝達を促進する．実験動物において，グリシン結合部位のアゴニストであるD-serineはPCPによる異常行動を抑制する．また，D-serineおよびD-cycloserineは統合失調症患者の陰性症状および認知障害を改善することが報告されている[50)51)]．一方，グリシントランスポーターを阻害することにより，間接的に細胞外のグリシン濃度を増加させる試みもなされている．統合失調症患者において，グリシントランスポーター阻害薬であるsarcosineは陽性症状，陰性症状および認知障害を改善することが報告されている[51)]．

実験動物においてGroup 2/3代謝型グルタミン酸受容体アゴニスト（LY404039）がPCPによって惹起される異常行動を抑制する．LY404039はバイオアベイラビリティーが低いことから，経口投与可能なLY404039のプロドラックであるLY2140023が開発されている[51)]．臨床試験において，LY2140023は非定型抗精神病薬のオランザピンとほぼ同程度の改善効果を示し，オランザピンに認められるプロラクチンの上昇，錐体外路系の障害や体重増加などの副作用は認められない[52)]．したがって，LY2140023は有効性および安全性を備えた新規抗精神病薬となる可能性が高い．

おわりに

これまでに，統合失調症の発症脆弱性に関与することが示唆されている遺伝子が数多く報告されている．本稿では主要な遺伝子について概説し，その内容を**表1**にまとめた．統合失調症の病態は複雑であり，いまだ病因の解明には至っていないのが現状である．しかし，統合失調症の発症に関与している脆弱性遺伝子が同定され，その機能が徐々に明らかになりつつある．現在のところ，神経伝達物質受容体を中心に新しい抗精神病薬の開発が進められ，有力な化合物も見出されている．今後，統合失調症の発症脆弱性遺伝子もしくは遺伝子産物を創薬ターゲットとした分子標的治療薬の開発が期待される．

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