Neprilysin Is Suppressed by Dual-Specificity Tyrosine-Phosphorylation Regulated Kinase 1A (DYRK1A) in Down-Syndrome-Derived Fibroblasts

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Amyloid-β peptide (Aβ) accumulation is a triggering event leading to the Alzheimer’s disease (AD) pathological cascade. Almost all familial AD-linked gene mutations increase Aβ production and accelerate the onset of AD. The Swedish mutation of amyloid precursor protein (APP) affects β-secretase activity and increases Aβ production up to ca. 6-fold in cultured cells; the onset age is around 50. Down syndrome (DS) patients with chromosome 21 trisomy present AD-like pathologies at earlier ages (40s) compared with sporadic AD patients, because APP gene expression is 1.5-fold higher than that in healthy people, thus causing a 1.5-fold increase in Aβ production. However, when comparing the causal relationship of Aβ accumulation with the onset age between the above two populations, early DS pathogenesis does not appear to be accounted for by the increased Aβ production alone. In this study, we found that neprilysin, a major Aβ-degrading enzyme, was downregulated in DS patient-derived fibroblasts, compared with healthy people-derived fibroblasts. Treatment with harmine, an inhibitor of dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), which is located in the DS critical region of chromosome 21, and gene knockdown of DYRK1A, upregulated neprilysin in fibroblasts. These results suggest that a decrease in the Aβ catabolic rate may be, at least in part, one of the causes for accelerated AD-like pathogenesis in DS patients if a similar event occurs in the brains, and that neprilysin activity may be regulated directly or indirectly by DYRK1A-mediated phosphorylation. DYRK1A inhibition may be a promising disease-modifying therapy for AD via neprilysin upregulation.

Key words Alzheimer’s disease; chromosome 21; down syndrome; dual-specificity tyrosine phosphorylation-regulated kinase 1A; neprilysin

Alzheimer’s disease (AD) brains are pathologically characterized by numerous senile plaques and neurofibrillary tangles, which consist of aggregation amyloid-β peptide (Aβ) and hyperphosphorylated tau, respectively, and prominent neuronal cell death. Aβ is generated from amyloid precursor protein (APP) by β- and γ-secretase-mediated sequential cleavages, followed by Aβ degradation by neprilysin (NEP). Aβ in healthy brains is maintained at a constant level by an equilibrium between the production and degradation rates. An imbalance arising by a subtle change in either rate leads to Aβ accumulation.

The Swedish double mutation (K670N/M671L) in APP, one of the most well-known mutations in familial AD, leads to a dramatic increase in total Aβ production. a,b The average onset age of Swedish familial AD is around 55 years. a On the other hand, Down syndrome (DS) is one of the most frequently occurring chromosomal abnormalities. a,b Approximately 95% of DS is caused by standard trisomy 21, which is an extra copy of chromosome 21. The remaining causes, chromosomal translocation and mosaicism, account for 1–5 and 1–2% of DS cases, respectively. DS patients have unique physical characteristics and suffer from various complications, such as congenital heart disease, leukemia and AD. a,b,c Because of the location of the APP gene on chromosome 21, it is generally accepted that Aβ is theoretically produced 1.5-fold more than normal via overexpression of APP in DS, compared with normal 21 disomy people, which is probably why DS patients present AD-like pathologies and symptoms at earlier ages (40s) compared with sporadic AD patients. a,b,c

Given that the average age presenting AD-like pathologies in DS patients is over 40, a,b there is a poor association between the level of Aβ production and the onset age of AD (prominent appearance of the pathologies), when comparing between familial AD patients with the Swedish mutation and DS patients. Moreover, the DS mouse model, Ts1Cje, with partial trisomy of murine chromosome 16 (corresponding to human chromosome 21) including the DS critical region, but not the APP gene, shows distinct learning and behavioral abnormalities. a,b,c,d Thus, DS-like and AD-like symptoms could also be caused by the partial chromosomal translocation. These findings suggest that there are other factors exacerbating AD development in the DS critical region on chromosome 21.

Dual-specificity tyrosine phosphorylation-regulated kinase 1A (DYRK1A), an intranuclear and cytoplasmic protein encoded in the DS critical region on chromosome 21, plays an important role in developmental processes and tissue homeostasis, and its dysregulation has been associated with many pathologies observed in humans. DYRK1A phosphorylates serine or threonine residues of the substrate protein, and is a multifunctional protein kinase with a variety of substrates including transcription factors, splicing regulators and synapse-related proteins. a,b,c It has been reported that DYRK1A phosphorylated the microtubule-associated protein tau a,b,c and APP a,b,c leading to development of AD pathology.

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In this study, we focused on the earlier formation of AD-like pathologies in DS patients and analyzed fibroblasts established from DS patients and from healthy control individuals. We found that the NEP activity in the DS patient-derived fibroblasts was significantly lower than that in healthy controls. NEP mRNA and protein expression levels were also decreased. NEP activity was rescued by pharmacological inhibition and RNA interference (RNAi)-mediated knockdown of DYRK1A in the DS fibroblasts. Our findings suggest that dysfunction of the Aβ overexpression may be one of the causes for AD-like pathologies at earlier ages in DS, and that DYRK1A is a potential drug target for AD treatment.

MATERIALS AND METHODS

Cell Culture Human fibroblasts (TIG-119 and TIG-120 derived from 6-month-old male and female Japanese healthy controls, respectively; Detroit 539 and Detroit 532 derived from female Caucasian and 2-month-old male Caucasian children with DS, respectively) were obtained from JCRB Cell Bank (Osaka, Japan). TIG-119 and TIG-120 were cultured in Eagle’s minimum essential medium (EMEM; Wako Pure Chemical Industries, Ltd., Osaka, Japan) containing 10% fetal bovine serum (FBS; SAFC Biosciences, Inc., Lenexa, KS, U.S.A.) and 100 U/mL penicillin/100 µg/mL streptomycin (Nacalai Tesque, Inc., Kyoto, Japan). Detroit 532 and Detroit 539 were cultured in EMEM containing 10% FBS, 100 U/mL penicillin/100 µg/mL streptomycin, nonessential amino acids (Nacalai Tesque, Inc.), 1 mM sodium pyruvate (Nacalai Tesque, Inc.) and 0.1% lactalbumin hydrolysate (Sigma-Aldrich Co., LLC, St. Louis, MO, U.S.A.). All cells were cultured at 37°C in a humidified atmosphere containing 5% CO2. We observed that the growing speed of DS fibroblasts was extremely slow, as reported in the case of DS-derived induced pluripotent stem (iPS) cells.15 This appears to be one of the characteristics of DS-derived cells. Therefore, we added 1 mM sodium pyruvate and 0.1% lactalbumin hydrolysate to the medium, to optimize and increase the cell growth speed according to the protocol by JCRB Cell Bank. We confirmed that the DYRK1A and membrane metallo-endopeptidase (MME) genes expression at protein level and NEP activity were not affected by the presence or absence of these additives (data not shown).

Harmine Treatment of Fibroblasts A DYRK1A inhibitor, harmine (also known as banisterine), was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan) and dissolved in dimethyl sulfoxide (DMSO).13,16 Human fibroblasts from DS patients were plated at 8×10^4 cells/well in 6-well plates. The fibroblasts were treated with harmine at the concentrations indicated in Fig. 3 for 72 h, and then harvested. The final concentration of DMSO was 0.1% in all groups.

Small Interfering RNA (siRNA) Transfection into Fibroblasts The DYRK1A sequence was obtained from Gene Bank accession number NM_0013963.3. The DYRK1A siRNA duplex was designed and synthesized by Sigma-Aldrich Co., LLC. The duplex sequences were: GAGCUAUGGACGUUA AUUUUdTdT and AAAUUAACGUCCAUGCUCdTdT. The fibroblasts from DS patients were plated at 8×10^4 cells/well in 6-well plates. After the medium was changed to medium without antibiotics, siRNA reverse transfection was performed with Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific Inc., Waltham, MA, U.S.A.) according to the manufacturer’s instructions. The fibroblasts were harvested 72 h after the transfection.

RNA Isolation and Real-Time PCR The mRNA expression levels of APP, DYRK1A, RCAN1, and MME were measured by real-time PCR. RNA isolation, cDNA synthesis and real-time PCR were performed as previously reported.37 All data were normalized to the endogenous reference gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) expression. The following primers were used for quantitative RT-PCR amplifications: APP: forward: 5’-GGGGTTCAAAACAAGTGCAATC-3’, reverse: 5’-TGCTGATCTTGTGACAGTG-3’; DYRK1A: forward: 5’TGAATTGACCAACACAGTGCACG-3’, reverse: 5’-AGGACGCTGACACACACCGA-3’; RCAN1: forward: 5’-AGCCTTGTGCTGACGGAAC-3’, reverse: 5’-AGTACACGTTTCACCCAT-3’, reverse: 5’-GGGACGCTGAACTCAATG-3’, reverse: 5’TCTCCTGTGACAGCCTCTAC-3’, GAPDH: forward: 5’-GACACCGTACAGGTCTGAAGAC-3’, reverse: 5’TGGTGAGAGAGCGGACATT-3’, reverse: 5’TCTCCTGTGACAGCCTCTAC-3’; MME: forward: 5’-GGGACGACTGAACTCAATG-3’, reverse: 5’TGGTGAGAGAGCGGACATT-3’, reverse: 5’TCTCCTGTGACAGCCTCTAC-3’.

Western Blot Analysis The harvested cells were lysed in lysis buffer (% Triton X-100, 50 mM Tris–HCl, pH 7.4) supplemented with a protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany) and a phosphatase inhibitor cocktail (Nacalai Tesque, Inc.). The cell lysates were incubated for 1 h on ice, and centrifuged at 21900×g for 30 min at 4°C. The resultant supernatants were used for analyses and their protein concentration was determined using a bicinchoninic acid protein assay kit (TaKaRa Bio Inc.). Equal protein amounts of each sample were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and separated proteins in the gels were transferred to polyvinylidene difluoride membranes (Merck, Darmstadt, Germany) under semidy or wet conditions. Subsequently, after blocking with 0.5% casein, the membranes were probed with the appropriate primary antibody at 4°C overnight, and then reacted with the appropriate secondary antibody, horseradish peroxidase conjugated anti-mouse, anti-rabbit or anti-goat immunoglobulin G (GE Healthcare UK Ltd., Buckinghamshire, England). The protein bands were visualized with an enhanced chemiluminescence detection method, and the image showing immunoreactive protein bands was captured by a LAS-4000 densitometer (GE Healthcare UK Ltd.). Science Laboratory 2001 Image Gauge software (GE Healthcare UK Ltd.) was used to analyze signal intensity. Goat anti-NP polyclonal antibody (Bio-Technne Corporation, Minneapolis, MN, U.S.A.), rabbit anti-DYRK1A polyclonal antibody and mouse anti-β-actin antibody (Sigma-Aldrich Co., LLC) were diluted to 0.1, 1 µg/mL and 1:5000, respectively, with blocking solution.

NEP Activity Assay Cellular NEP activity was determined as described previously.18 NEP-dependent neutral endopeptidase activity in the cell lysate was fluorometrically measured using an indirect coupled enzyme assay based on the decrease in the rate of digestion produced by the NEP inhibitor, thiorphan. In brief, the cell lysate was incubated in 100 mM 2-(N-morpholino)ethanesulfonic acid (MES) (pH 6.5) with 0.1 mM succinyl-Ala-Ala-Phe-4-methylcoumaryl-7-amide (MCA; Bachem AG, Bubendorf, Switzerland) as a substrate at 37°C for 1 h, and then leucine aminopeptidase (approximate 5 µg/mL, Sigma-Aldrich Co., LLC) and 10 µM phosphorami-
don (Peptide Institute, Inc., Osaka, Japan) were added for 30 min to remove the phenylalanine residue from the Phen-MCA generated by the neutral endopeptidase. The intensity of the free 7-aminomethylcoumarin was measured with excitation at 390 nm and emission at 460 nm using the microplate spectrometer, Infinite M-1000 (Tecan Group Ltd., Männedorf, Switzerland).

**Statistical Analysis** All data are expressed as the mean±standard deviation (S.D.). The data were analyzed by a one-way or two-way ANOVA followed by post-hoc test, in which Student–Newman–Keuls test was applied, except for a dose–response study of harmine if it passed the Shapiro–Wilks normality test (SigmaPlot software, ver.13; Systat Software Inc., San Jose, CA, U.S.A.). When the normality test was not passed at ANOVA, Dunn’s test was performed. Analysis of a dose–response study of harmine was performed by Dunnett’s test as post-hoc test. P values below 0.05 were considered to be significant.

**RESULTS**

**NEP mRNA Level Is Significantly Decreased in Fibroblasts from DS Patients** First, to determine whether the expression of three genes, including APP, located on chromosome 21 is increased in two cell lines of fibroblasts form DS patients (DS fibroblasts), we performed quantitative real-time PCR analysis (Fig. 1A). The genes encoding DYRK1A and regulator of calcineurin 1 [RCAN1; also known as calcipressin-1 or DS critical region protein 1 (DSCR1)] are strongly associated with DS phenotypes \(^{19}\) and have been suggested to have a relationship with the AD pathogenesis. \(^{20,21}\) The mRNA levels of APP, DYRK1A and RCAN1 were significantly higher in the DS fibroblasts than in fibroblasts from healthy controls (control fibroblasts), except that in DS fibroblast Detroit 539 the expression of DYRK1A was significantly lower than that in control fibroblast TIG-119. Interestingly, the DS fibroblasts showed lower levels of MME, which encodes NEP, compared with the control fibroblasts. Next, we analyzed protein levels of DYRK1A in the fibroblasts using quantitative western blot analysis (Fig. 1B). DYRK1A protein level in DS fibroblast Detroit 539 was significantly higher than that in both control fibroblasts, but that in DS fibroblast Detroit 532 showed a significant higher value, only when compared with that in control fibroblast TIG-119. Thus, DYRK1A in DS fibroblast Detroit 532 appears to be upregulated at translational level, but not at transcriptional level.

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**Fig. 1. Expression Levels of APP, DYRK1A, RCAN1 and MME (NEP) in Fibroblasts from Healthy Controls and DS Patients**

(A) The mRNA expression levels of APP, DYRK1A, RCAN1 and MME in fibroblasts from two healthy controls (TIG-119 and TIG-120) and two DS patients (Detroit 532 and Detroit 539) were determined by quantitative real-time PCR analysis. All values were normalized to GAPDH expression. Data represent the mean±S.D. \((n=4)\), and were analyzed by a one-way ANOVA, followed by post-hoc Student–Newman–Keuls test. Asterisks show significant differences between the indicated groups \((p<0.05)\).

(B) Left panel. Representative result of western blot showing gene expression of DYRK1A at protein level in fibroblasts from two healthy controls (TIG-119 and TIG-120) and two DS patients (Detroit 532 and Detroit 539). Right panel. The protein levels of DYRK1A in fibroblasts from two healthy controls (TIG-119 and TIG-120) and two DS patients (Detroit 532 and Detroit 539) were determined by quantitative western blot analysis. All values were normalized to β-actin expression. Data represent the mean±S.D. \((n=4)\), and were analyzed by a one-way ANOVA, followed by post-hoc Student–Newman–Keuls test. Asterisks show significant differences between the indicated groups \((p<0.05)\).
NEP Activity Is Significantly Decreased in Fibroblasts from DS Patients  
Next, we analyzed the protein levels of NEP in cell lysates of control and DS fibroblasts using the quantitative western blot method (Fig. 2A). Consistently with the MME mRNA expression levels in fibroblasts, the protein levels of NEP were remarkably decreased in the DS fibroblasts, compared with the control fibroblasts. Furthermore, a reduction in NEP activity in the DS fibroblasts was observed; the activity was decreased to at least half that in the control fibroblasts (Fig. 2B). It has been suggested that a decline in NEP activity in DS patients leads to Aβ accumulation in addition to enhancement of Aβ production through increased APP gene expression.

DYRK1A Inhibition Rescues NEP Activity in Fibroblasts from DS Patients  
Even a subtle reduction in NEP activity causes an imbalance between Aβ production and degradation rates, triggering the long-term AD pathological activity causes an imbalance between Aβ production and destruction.22) As NEP was suppressed in the DS fibroblasts compared with the control fibroblasts (Figs. 1, 2), we speculated that rescuing the suppressed NEP activity or expression would prevent or decelerate the development of AD pathology and of AD-like pathology observed in DS patients.

We treated the two DS fibroblast cell lines from different DS patients with the DYRK1A inhibitor, harmine, to inhibit the catalytic activity of DYRK1A (Fig. 3A). The pharmacological inhibition of DYRK1A at a concentration of at least 5 μM enhanced NEP activity by 1.5–2.0-fold in both DS fibroblasts. The increased degree of NEP activity was close to the difference observed between the control and DS fibroblasts (Figs. 2A, B).

It has been reported that harmine is capable of inhibiting recombinant monoamine oxidase A (MAO A) and DYRK1B, a DYRK1A homologue. In a test-tube, the IC50 values for MAO A and DYRK1B are 2 and 166 nM, respectively,22,23) whereas for DYRK1A it is 33 nM.20) To exclude the involvement of these molecules in the modulation of NEP activity, we evaluated the knockdown effects of DYRK1A in the two DS fibroblast lines using a specific siRNA (Figs. 3B, C). We confirmed using western blot analysis that DYRK1A expression in the DS fibroblasts was suppressed by the knockdown. The knockdown of DYRK1A significantly increased NEP activity and protein amount by 1.5–2.0-fold in both DS fibroblasts. The level of increase was almost the same as that observed by the harmine treatment, of which the effective dosage was much higher than the IC50 value for DYRK1A in the test-tube.

DISCUSSION

In the present study, we demonstrated that NEP activity is suppressed in DS fibroblasts, compared with the control fibroblasts, via downregulation of gene expression at the mRNA and protein levels, and that inhibition of DYRK1A can rescue the NEP activity. This study suggested that it may be possible to pharmacologically upregulate decreased NEP activity in the brains of AD and DS patients.

Here, we first analyzed the expression levels of several genes located on the DS critical region of chromosome 21, to characterize two fibroblast cell lines established from DS patients, because it has been reported that the gene expression of APP is elevated in the adult brain, but is not significantly changed in the fetal brain of DS patients.24) and that DYRK1A and RCAN1 are upregulated in the brain of not only DS patients, but also of AD model mice and late-onset AD patients.25) The expression levels of APP, DYRK1A and RCAN1 were elevated almost consistently at the transcriptional or translational level (Figs. 1, 2). Therefore, we focused on these genes in the present study. We found that NEP activity was downregulated via functional roles of DYRK1A in DS fibroblasts.

AD research has shown that almost all AD-linked mutations of the genes, presenilins and APP, increase the anabolic activity of Aβ, especially longer Aβ (Aβ42), to accelerate the
onset of the disease. In general, the steady state level of biological molecules is determined by a constant balance between anabolic and catabolic rates. Impairment of this metabolic balance leads to $\mathrm{A}\beta$ accumulation in the brain. It has been reported repeatedly that the gene expression of the major $\mathrm{A}\beta$-degrading enzyme, NEP, in sporadic AD brains is decreased from the early stages of the disease development, and there is an inverse relationship between $\mathrm{MME}$ (NEP) gene expression and $\mathrm{A}\beta$ accumulation, indicating that downregulation of NEP is at least one of the causes of sporadic AD. The present study suggests that reduction in the $\mathrm{A}\beta$ catabolic rate may be, at least in part, one of the causes for accelerated AD-like pathogenesis in DS patients.

Shi et al. have characterized neuronal cells differentiated from DS patient-derived iPS cells and observed that the amount of $\mathrm{A}\beta$ in the culture medium was increased up to 8-fold compared with that from the control neurons differentiated from healthy people-derived iPS cells. Interestingly, the amount of $\mathrm{A}\beta$ in the culture medium of DS neurons was not completely decreased by (3,5-difluorophenylacetyl)-l-alanyl-l-2-phenylglycine $t$-butyl ester (DAPT), an inhibitor of $\gamma$-secretase that is responsible for $\mathrm{A}\beta$ production, and remained at 4-fold increased levels compared with that of the control neurons. Although the gene expression level of NEP in the DS neurons was not analyzed in this study, based on our data we think that the higher levels of $\mathrm{A}\beta$ maintained in the culture medium of DS neurons after DAPT treatment may be attributed to downregulation of NEP.

The mechanisms of transcriptional and translational regulation of the $\mathrm{MME}$ gene as well as the post-translational regulation of NEP in the brain are still unclear. In the present study, the knockdown effect of DYRK1A on the upregulation of NEP was unambiguous, whereas the upregulation of NEP by the DYRK1A inhibitor, harmine, appeared to be saturated at concentrations of more than 5 $\mu\text{M}$ in the two DS-derived fibroblast cell lines, of which the base line NEP activity was slightly, but not significantly, different (Fig. 3). As a two-way ANOVA revealed no interaction between the harmine treatment and the individual fibroblasts, the sensitivity to the drug was almost the same. These results suggest that the effect of
harmine is restricted to DYRK1A and that NEP activity may be directly regulated by DYRK1A-mediated phosphorylation or any indirect actions via DYRK1A signaling pathway-mediated gene expression.

RCAN1 is known as a regulator of calcineurin 1, which is a protein phosphatase. In DS patients, the calcineurin-nuclear factor of activated T cells (NFAT) signaling pathway, which is implicated in cancer metastasis, is suppressed by overproduction of DYRK1A and RCAN1. However, long-term inhibition of the calcineurin-NFAT signaling pathway by the calcineurin inhibitor, cyclosporine A, showed no effects on the expression level of MME mRNA in human neuroblastoma SH-SY5Y cells. Therefore, the transcription factor, NFAT, may not be directly responsible for the regulation of MME gene expression. Apart from NFAT, genes encoding transcription factors and regulators (e.g., BACH1, GABPA, ERG, ETS2, RUNX1 and SIM2) are located on chromosome 21, and some of them have been reported to be upregulated in DS patients. The expression level of the transcription factor, GATA1, which is located on chromosome X, has been reported to be altered in DS patients. Further studies are required to determine the detailed mechanism of the decreased NEP expression in DS patients.

Growing evidence that NEP expression level is reduced in the hippocampus and cerebral cortex of AD patients from the early stages of disease development and also with aging in humans indicates a strong relationship between the expression level of NEP and AD pathology. Given that in general elderly women have a higher prevalence of AD than elderly men, gender difference is one of key factors in development of AD. Because a representative female sex hormone, 17β-estradiol, induces the expression of MME gene, gender-specific regulatory mechanism of MME gene expression in the brains may be implicated in higher prevalence of AD in elderly women. In fact, it is reported that in AD-model mice NEP expression levels located on chromosome X, has been reported to be altered in DS patients. Further studies are required to determine the regulation of MME gene expression in DS patients. The authors declare no conflict of interest.

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