The dopaminergic system promotes neprilysin-mediated degradation of amyloid-β in the brain

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Editor's summary

The accumulation of amyloid-β in the brain is associated with progressive cognitive impairment in Alzheimer's disease. Watamura *et al*. found that a treatment commonly used to treat a symptom of Parkinson's disease also induced the breakdown of amyloid-β. In mice, dopamine release in the brain increased the abundance and activity of the enzyme neprilysin, which degraded amyloid-β specifically in the working memory center of the brain. Levodopa induced these effects and improved cognitive function in

aged and Alzheimer's disease model mice, suggesting that the approach might be explored for patients with Alzheimer's disease. —Leslie K. Ferrarelli

Abstract

Deposition of amyloid-β (Aβ) in the brain can impair neuronal function and contribute to cognitive decline in Alzheimer's disease (AD). Here, we found that dopamine and the dopamine precursor levodopa (also called L-DOPA) induced Aβ degradation in the brain. Chemogenetic approaches in mice revealed that the activation of dopamine release from ventral tegmental area (VTA) neurons increased the abundance and activity of the Aβ-degrading enzyme neprilysin and reduced the amount of Aβ deposits in the prefrontal cortex in a neprilysin-dependent manner. Aged mice had less dopamine and neprilysin in the anterior cortex, a decrease that was accentuated in AD model mice. Treating AD model mice with levodopa reduced Aβ deposition and improved cognitive function. These observations demonstrate that dopamine promotes brain region–specific, neprilysin-dependent degradation of Aβ, suggesting that dopamine-associated strategies have the potential to treat this aspect of AD pathology.

INTRODUCTION

Pathogenic accumulation of amyloid-β (Aβ) peptide, caused by an imbalance of anabolic and catabolic activity in the brain, is believed to trigger Alzheimer's disease (AD). Identified familial mutations on *APP*, *PSEN1*, and *PSEN2* genes induce excessive production of toxic Aβ species, such as $A\beta_{42}$ and $A\beta_{43}$, or alter the $A\beta_{42/40}$ ratio, leading to Aβ deposition in the brain (*1*–*5*). Chronic attenuation of Aβ catabolism may accelerate this Aβ deposition (*6*).
Neprilysin (NEP) is a potent Aβ-degrading enzyme in the brain (*7*, *8*). The expression and activity of NEP decrease with aging, particularly in patients with AD (*9*–*12*), and mice lacking the NEP (*MME*) gene exhibit approximately twofold Aβ levels in the brain (*8*). Furthermore, impaired NEP activity in *APP* transgenic mice is associated with high levels of toxic Aβ oligomer at synapses, which cause abnormal plasticity and learning and memory dysfunction before the deposition of Aβ plaques (*13*). In contrast, neuronal overexpression of NEP after the administration of an adeno-associated virus (AAV) vector carrying the *MME* gene in AD model mice reduces toxic Aβ oligomer levels and deposition of Aβ plaques, resulting in the rescue of aberrant learning and memory function without serious adverse effects (*14*). Exploring targetable elements for promoting NEP-mediated degradation of Aβ may thus be beneficial. A genome-wide association study demonstrated that specific *MME* variants have been identified as risk factors for AD, suggesting that NEP has an etiological significance in AD (*15*).

Our group has previously found that the neuropeptide somatostatin (SST) enhances NEP activity (*16*) and, of the five SST receptor (SSTR1–5) subtypes, SSTR1 and SSTR4 (SSTR1/4) redundantly regulate NEP (*17*). We have also reported that α-endosulfine (ENSA), an endogenous ligand for the adenosine 5′-triphosphate–sensitive potassium channel, modulated NEP activity downstream of SST signaling (*18*). In this study, we aimed to explore alternative candidates that up-regulate NEP activity independently of the SST-ENSA pathway.

RESULTS

Identification of DA as a NEP regulator in vitro

We carried out in vitro screening of compounds potentially capable of up-regulating NEP activity using a mouse cortical/hippocampal and basal ganglia–derived coculture system (*16*–*19*). Our group previously reported that SST increased NEP activity (*16*). Because SST is a hormone released from the hypothalamus (*20*), we analyzed the effect of other hypothalamic hormones. Only dopamine (DA) increased NEP activity in cocultured neurons (Fig. 1A); this effect was not seen in cortical/hippocampal or basal ganglia neurons (fig. S1).

Fig. 1. DA regulates Aβ levels in conditioned medium via NEP up-regulation.

(**A** to **D**) NEP activity assay after treatment of cocultured mouse cortical/hippocampal and basal ganglia neurons with hypothalamic hormones (A), pituitary hormones (B), neurotransmitters (C), or catecholamines (D) for 24 hours; *n* = 3 to 8 independent biological replicates per treatment. (**E** and **F**) ELISAs for $A\beta_{40}$ (E) and $A\beta_{42}$ (F) in conditioned medium after treatment of cocultured cells from WT mice with DA for 24 hours; *n* = 6 independent biological replicates per treatment. (**G** and **H**) As described in (E) and (F), from *Mme* KO mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ by one-way ANOVA with Dunnett's multiple comparison test [(A) and (D)], Student's *t* test (E), or Mann-Whitney *U* test (F). In (A) to (H), data are shown as means ± SEM of the specified number of independent experiments (*n*), each the mean of three technical replicates. For (A) to (F), details of test compounds and concentrations are provided in table S1. CRF, corticotropin-releasing factor; GHRF, growth hormone releasing hormone; MCH, melanin-concentrating

hormone; OX-A, orexin-A; OX-B, orexin-B; TRH, thyrotropin-releasing hormone; ACTH, adrenocorticotropic hormone; BE, β-endorphin; FSH, follicle stimulating hormone; GH, growth hormone; OXT, oxytocin; PRL, prolactin; Ach, acetylcholine; Ade, adenosine; GABA, γ-aminobutyric acid; L-Glu, L-glutamic acid; Gly, glycine; 5-HT, serotonin; Tyr, L-tyrosine; NA, noradrenaline; AD (in this figure only), adrenaline; Quin, quinpirole.

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The hypothalamic neurotransmitters SST and DA are known to inhibit the release of growth hormone from the pituitary gland (*21*, *22*). When NEP activity was measured in cocultured neurons after treatment with pituitary hormones (Fig. 1B) and other neurotransmitters (Fig. 1C), no statistically significant effects were observed.
Screening other catecholamines (noradrenaline and adrenaline synthesized on the DA biosynthesis pathway), DA precursors [L-tyrosine and L-3,4-dihydroxyphenylalanine (L-DOPA)], and D1-like and D2-like receptor agonists revealed that only D2-like receptor agonism significantly enhanced NEP activity (Fig. 1D). We measured the levels of $A\beta_{40}$ and $A\beta_{42}$ in conditioned media from primary cocultured neurons derived from wild-type (WT) and NEP knockout (*Mme* KO) mice after treatment with DA. DA significantly reduced both $A\beta_{40}$ and $A\beta_{42}$ levels in conditioned media from WT mice (Fig. 1, E and F), but not from *Mme* KO mice (Fig. 1, G and H), suggesting that DA decreased Aβ levels in a NEP-mediated fashion.

Activation of DAergic neurons in the VTA regulates NEP expression in the PFC

To analyze the physiological role of DA in NEP regulation in the brain, we used designer receptors exclusively activated by designer drugs (DREADD) system to DAT-Cre mice, which express Cre recombinase under the transcriptional control of the endogenous DA transporter promoter (*23*). First, targeted injections of Cre-dependent double-floxed inverted open reading frame (DIO) vectors, carrying mCherry-tagged stimulatory (hM3Dq) or inhibitory (hM4Di) designer receptors, were conducted to the ventral tegmental area (VTA) selectively, before clozapine *N*-oxide (CNO; 1 mg/kg) was administered for 4 weeks (Fig. 2A). The expression of the DREADD-fused mCherry tag in DAT-Cre mice was highly specific for the VTA, with approximately 40 to 50% colabeling of mCherry and tyrosine hydroxylase (TH), a marker of DA-releasing (DAergic) neurons (Fig. 2B). CNO administration induced high expression of c-Fos in TH-positive VTA neurons in DAT-Cre mice expressing hM3Dq, but not hM4Di, which was indistinguishable from that of controls (Fig. 2C).

Fig. 2. Chronic activation of DA neurons in the VTA modulates NEP activity in the PFC.

(**A**) Schematic of the DREADD system applied to DAT-Cre mice. (**B** and **C**) Colabeling of TH with mCherry (B) and c-Fos (C) in the VTAs of DAT-Cre mice infected with the viral vectors AAV8-hSyn-DIO-mCherry, AAV8-hSyn-DIO-hM3D(Gq)-mCherry, and AAV8-hSyn-DIO-hM4D(Gi)-mCherry at the age of 5 months; *n* = 5 to 7 mice in each group, each with three technical replicates. Scale bars, 100 μm. (**D**) Immunoblotting of APP, CTFs, NEP, IDE, and ECE-1 and β-actin in anterior cortices from mice in (B) and (C). Band intensities were normalized to that of β-actin; *n* = 6 or 7 mice for each group, each with three technical replicates. (**E**) NEP activity in anterior cortices of mice in (B) and (C); *n* = 6 or 7 mice for each group, each with three technical replicates. (**F** and **G**) ELISAs for $A\beta_{40}$ (F) and $A\beta_{42}$ (G) in the tris-soluble fraction of anterior cortices from mice in (B) and (C); *n* = 5 mice for each group, each with three technical replicates. (**H** and **I**) Immunostaining of Aβ (green), detected by N1D antibody, from frontal cortices of 12-month-old App^{NL-F}; DAT-Cre mice infected with or without the viral vectors AAV8-hSyn-DIO-mCherry and AAV8-hSyn-DIO-hM3D(Gq)-mCherry, with statistical analysis of immunoreactivity of Aβ; *n* = 4 mice for each group, each with three technical replicates. Scale bar, 500 μm. $*P < 0.05$ and $**P < 0.01$ by (C) Kruskal-Wallis test with Dunn's multiple comparison test, [(F) and (G)] Student's *t* test, or [(D), (E), and (I)] one-way ANOVA with Tukey's multiple comparison test. ns, not significant.

We next investigated whether chronic activation of VTA DAergic neurons could affect Aβ production and expression of Aβ-degrading enzymes. Western blot analysis showed that DAT-Cre mice expressing hM3Dq significantly increased NEP expression only, in anterior cortices (Fig. 2D). No significant differences were observed in expression levels of full-length amyloid precursor protein (APP), the ratio of APP C-terminal fragments generated by α-secretase (CTF-α) and β-secretase (CTF-β), or other Aβ-degrading enzymes such as insulin-degrading enzyme (IDE) and endothelin converting enzyme 1 (ECE-1). Chronic treatment with CNO facilitated NEP activity in the anterior cortices of DAT-Cre mice expressing hM3Dq (Fig. 2E), with

significant reductions in both $A\beta_{40}$ and $A\beta_{42}$ levels in the tris-HCl–buffered saline (Ts) fractions (Fig. 2, F and G). These effects were not seen in *Mme* KO mice (fig. S2, A to D).
We subsequently used high-performance liquid chromatography (HPLC) to measure monoamine neurotransmitter levels in anterior cortices 2 hours after the final CNO injection and to investigate whether the DREADD system properly activated VTA DAergic neurons. We found that treatment with CNO elevated DA-related metabolites such as 3,4-dihydroxyphenylacetic acid and 3-methoxy-4-hydroxyphenyl acetic acid in the anterior cortices of both *Mme* KO mice and DAT-Cre mice expressing hM3Dq (figs. S3 and S4).
To investigate whether the activation of VTA DAergic neurons could affect Aβ deposition, we used *App* knock-in (App^{NL-F}) mice, which develop Aβ plaques without overexpression of *APP* gene containing pathogenic mutations (*24*), and administered CNO to App^{NL-F}; DAT-Cre mice for 8 weeks. Aβ deposition in the prefrontal cortices (PFCs) of App^{NL-F}; DAT-Cre mice expressing hM3Dq was significantly lower than in control mice (Fig. 2, H and I, and fig. S5, A to C), suggesting that the DAergic system controls Aβ levels through modulation of NEP expression in the PFC.

Effect of L-DOPA on the regulation of NEP expression

To further analyze the physiological effect of DA on NEP regulation in the brain, we treated App^{NL-F} mice with L-DOPA, which is converted to DA after penetrating the blood-brain barrier (*25*). Benserazide, a peripherally acting amino acid decarboxylase inhibitor, was administered simultaneously to allow L-DOPA to efficiently reach the brain (*26*). Coadministration of L-DOPA (12 mg/kg) with benserazide (3 mg/kg), five times over 3 days, increased phosphorylation levels at the Thr^{34} residue of DA- and cAMP-regulated neuronal phosphoprotein 32 (DARPP-32). DARPP-32 mediates DA signaling; it is distributed primarily not only in the striatum but also in the cerebral cortex and hippocampus (*27*, *28*). Increased levels of phosphorylated DARPP-32 in App^{NL-F} mice were accompanied by enhanced NEP expression in anterior cortices, but not in either posterior cortices or hippocampi (Fig. 3, A and B). We thus measured the Ts-fractioned and guanidine-HCl (GuHCl)–fractioned $A\beta_{40}$ and $A\beta_{42}$ levels and found a significant decrease in $A\beta_{42}$ levels in the anterior cortices of L-DOPA treated App^{NL-F} mice (Fig. 3, C and D).

Fig. 3. Short-term L-DOPA treatment increases NEP expression.

(**A** and **B**) Immunoblotting of phosphorylated DARPP-32 (p-DARPP-32), DARPP-32, NEP, and β-actin in anterior and posterior cortices and hippocampi of 3-month-old App^{NL-F} mice after treatment with or without L-DOPA (12 mg/kg) five times for 3 days with benserazide (3 mg/kg) by intraperitoneal injection. Band intensities for p-DARPP-32 and NEP were normalized to those of total DARPP-32 and β-actin, respectively; n = 5 mice for each group, each with three technical replicates. (**C** and **D**) ELISAs for $A\beta_{40}$ and $A\beta_{42}$ in the tris-soluble

(Ts) fraction (C) and guanidine-soluble (GuHCl) fraction (D) of anterior cortices of 3-month-old *App*$^{NL-F}$ mice after treatment with or without L-DOPA (12 mg/kg) five times for 3 days with benserazide (3 mg/kg) by intraperitoneal injection; *n* = 6 mice for each group, each with three technical replicates. In (A) to (D), data are shown as means ± SEM; ***P < 0.001 and ****P < 0.0001 by two-way ANOVA with Tukey's multiple comparison test (B) or Student's *t* test [(C) and (D)].

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Effect of chronic L-DOPA administration on amyloid pathology in the *App*$^{NL-F}$ mouse model of AD

To assess the potential for a therapeutic effect of L-DOPA in AD, we analyzed Aβ levels in *App*$^{NL-F}$ mice implanted with drug pellets allowing continuous release of L-DOPA (10 mg/kg per day) for 3 months (*29*). Long-term L-DOPA administration significantly increased the phosphorylation level of DARPP32 and increased NEP expression (Fig. 4, A and B); Aβ levels were decreased in both anterior and posterior cortices (Fig. 4, C and D), but not in the hippocampus (Fig. 4, A to D). Immunohistochemical analysis with the 4G8 antibody indicated that L-DOPA treatment decreased amyloid density in the anterior and posterior cortices and the hippocampal subicula of *App*$^{NL-F}$ mice compared with those of the placebo group (Fig. 4, E and F).

Fig. 4. Long-term L-DOPA treatment improves Aβ pathology and memory function in *App*$^{NL-F}$ mice through increased NEP expression.

(**A** and **B**) Immunoblotting of phosphorylated DARPP-32 (p-DARPP-32), DARPP-32, NEP, and β-actin in anterior and posterior cortices and hippocampi of 18-month-old *App*$^{NL-F}$ mice after treatment with or without L-DOPA (10 mg/kg per day) for 3 months by an implanted pellet. Band intensities for p-DARPP-32 and NEP normalized to those of DARPP-32 and β-actin, respectively; *n* = 5 mice for each group, each with three technical replicates.

(**C** and **D**) ELISAs for $Aβ_{40}$ and $Aβ_{42}$ in tris-soluble [Ts; (C)] and guanidine-soluble [GuHCl;

(D)] fractions of anterior and posterior cortices and hippocampi of 18-month-old *App*$^{NL-F}$ mice after treatment with or without L-DOPA (10 mg/kg per day) for 3 months by an implanted pellet; *n* = 4 mice for each group, each with three technical replicates. (**E** and **F**) Immunostaining of Aβ (green), detected by 4G8 antibody, from 18-month-old *App*$^{NL-F}$ mice after treatment with or without L-DOPA (10 mg/kg per day) for 3 months by an implanted pellet; *n* = 4 mice for each group, each with three technical replicates. Scale bar, 500 μm. (**G**) Freezing ratio of 18-month-old *App*$^{NL-F}$ mice after treatment with L-DOPA (10 mg/kg per day) for 3 months by an implanted pellet followed by 14 days of depletion, and control mice (placebo group); *n* = 8 or 9 mice for each group. All quantitative data are shown as means ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$ by Student's *t* test (C) or two-way ANOVA with (B) Tukey's multiple comparison test or [(F) and (G)] Bonferroni's multiple comparison test.

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After the above findings, we investigated functional memory in *App*$^{NL-F}$ mice using a contextual fear-conditioning test. Because DA is known to modify cognitive and motor functions (*30*–*33*), experimental conditions were first defined using three WT groups: placebo, L-DOPA, and L-DOPA plus 2 weeks of depletion. L-DOPA administration significantly increased DA abundance and signaling and NEP expression in the anterior and posterior cortices (fig. S6, A to D), resulting in significant prolongation of freezing time in 18-month-old WT mice (fig. S6E). In the group with 2 weeks of depletion after 3 months of L-DOPA treatment, DA level and freezing ratio recovered somewhat (fig. S6, A and E), although DA signaling and NEP expression were still elevated relative to the placebo group (fig. S6, B to D). We, therefore, assessed memory function in *App*$^{NL-F}$ mice implanted with L-DOPA pellets for 3 months with a subsequent 2 weeks of depletion.

We observed an aberrant memory parameter on *App*$^{NL-F}$ mice at 18 months compared with age-matched WT mice (fig. S6F). Eighteen-month-old *App*$^{NL-F}$ mice treated with L-DOPA for 3 months with a subsequent 2 weeks of depletion exhibited a longer prolongation of freezing time compared with controls (placebo group) (Fig. 4G). These results suggest that chronic L-DOPA administration restores abnormal memory function in *App*$^{NL-F}$ mice by reducing the Aβ levels through NEP up-regulation.

Expression of NEP is associated with aging, Aβ pathology, and DA signaling in the anterior cortex

Although we demonstrated that the DA system dynamically controls Aβ levels through NEP-mediated actions, it is unclear whether aging, one of the major risk factors for AD, and Aβ pathology could affect the relationship between endogenous DA signaling and NEP expression. To explore this, we compared endogenous DA levels in the anterior cortices, posterior cortices, and hippocampi of 5- and 15-month-old WT mice and 15-month-old App^{NL-F} mice.
We found that 15-month-old WT mice showed a significant reduction of endogenous DA levels compared with 5-month-old WT mice. This effect could be seen more strongly in 15-month-old App^{NL-F} mice (Fig. 5A). These age- and genotype-dependent effects on DA signaling and NEP expression were confirmed in the anterior cortex (Fig. 5, B to E), but not in the posterior cortex or the hippocampus (Fig. 5, A to E), indicating that DA could link closely to AD pathogenesis exclusively in the anterior cortex.

Fig. 5. The molecular link between the DAergic system and NEP with or without Aβ pathology.

(**A**) ELISA for DA in the anterior and posterior cortices and hippocampi from 5- and 15-month-old WT mice and 15-month-old App^{NL-F} mice; $n = 6$ mice for each group, each with three technical replicates. (**B** to **E**) Immunoblotting of phosphorylated DARPP-32 (p-DARPP-32), DARPP-32, NEP, and β-actin in anterior and posterior cortices and hippocampi of 5- and 15-month-old WT mice and 15-month-old App^{NL-F} mice. Band intensities for p-DARPP-32 and NEP normalized to those of DARPP-32 and β-actin, respectively; $n = 4$ mice for each group, each with three replicates. In (A) and (C) to (E), data are shown as means ± SEM; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, and $^{****}P < 0.0001$ by two-way ANOVA with Tukey's multiple comparison test.

DISCUSSION

Using a combination of chemogenetic and pharmacological approaches, we demonstrated that DA regulates NEP expression, particularly in the anterior cortex, where DAergic innervation is

received from the midbrain. Modulation of Aβ dynamics and improvement of abnormal memory function were observed in *App*$^{NL-F}$ mice treated with L-DOPA.

Increased NEP activity was seen after DA treatment of cocultured cortical/hippocampal and basal ganglia neurons but not in single cultured neurons. This suggests that the DA-stimulating factor(s) that regulate NEP activity in this cocultured system are secreted from either cortical/hippocampal or basal ganglia neurons. To explore the mechanisms underlying NEP regulation downstream of DA, it will be necessary to identify the specific DA-mediated molecular interactions between cortical/hippocampal and basal ganglia neurons. We conducted further experiments to reveal which subtypes of DA receptor could be responsible for the regulation of NEP and found that the D2-like receptor agonist quinpirole, but not the D1-like selective agonist SKF-38383, predominantly controlled NEP activity in the cocultured system. Considering these results, performing proteomics using conditioned media from cortical/hippocampal and/or basal ganglia neurons after treatment with DA or quinpirole may help to identify one or more mediators that are capable of modulating NEP activity and expression.

Degeneration of TH-positive cells is closely linked to the pathophysiology of AD (*34*–*38*). In Tg2576 mice [an AD mouse model overexpressing the *APP* gene containing the Swedish mutation (*39*)], the loss of DAergic neurons in the VTA, but not in the substantia nigra pars compacta, precedes the deposition of Aβ plaques (*40*–*42*), suggesting specific vulnerability of VTA DAergic neurons in AD. In addition, there is a pathological association between VTA disruption and memory impairment in patients with early AD (*43*–*45*). Our study aimed to clarify the involvement of VTA DAergic neurons in the pathogenesis of AD. We found that the activity of VTA DAergic neurons plays a key role in the control of Aβ dynamics through modulation of NEP expression in the PFC, where accumulated Aβ fibril is observed at the earliest preclinical AD stage (*46*). Therefore, the VTA-PFC DAergic neural circuit could be a potential therapeutic target to attenuate the development of Aβ pathology, especially in the preclinical stages of AD.

Hippocampal NEP regulation is likely to be controlled independently of a DA pathway, given that L-DOPA treatment effects on Aβ dynamics were mostly observed in the anterior cortex rather than in the hippocampus. This notion is supported by the tendency of *App*$^{NL-F}$ mice to exhibit decreased NEP expression, despite having endogenous DA levels and signaling intensity similar to those of control animals. These findings imply the presence of alternative mechanism(s) regulating NEP activity in the hippocampus exclusively. We previously demonstrated that an SST-ENSA pathway predominantly regulated hippocampal NEP activity (*16*–*18*).

Regional and cell type–specific differences in the regulation of NEP activity and/or expression might be accounted for by variations in the distribution of relevant neurotransmitters and receptors. Previous studies have identified distinct ligands, such as opioids and substance P, that are capable of up-regulating NEP for monocytes and bone marrow cells, respectively (*47*, *48*). It will be important to investigate whether combined pharmacological approaches targeting both the anterior cortex and the hippocampus could simultaneously enhance NEP-mediated Aβ degradation. Identifying the biological cross-talk between SST and DA signaling will support this idea. A previous study reported that midbrain DA neurons regulate the activity of both choline acetyltransferase-expressing neurons and D2 receptor–expressing medium spiny neurons in the striatum via SST-expressing interneurons, which may suggest the overlap of signaling

from neurotransmitters related to NEP actions (*49*). The therapeutic strategy that can help optimize such neurotransmitters would be a potential approach as well.
In the present study, aged *App*$^{NL-F}$ mice exhibited lower DA levels/signaling in the anterior cortex than age-matched WT mice, possibly because of a negative feedback loop between DAergic activity and Aβ levels. To break this cycle, long-term L-DOPA treatment might serve as a beneficial approach for lowering Aβ in the brain. However, given that the long-term use of L-DOPA encompasses intrinsic issues with complicated disabling fluctuations and dyskinesias (*50*), any clinical intervention with L-DOPA in patients with AD should be designed carefully to optimize therapeutic benefits and minimize adverse effects.
Although the question of what type of Aβ species is toxic remains open, a previous study showed that the catecholaldehyde DOPAL (3,4-dihydroxyphenylacetaldehyde), one of the metabolites of DA, modulates the Aβ aggregation process. DOPAL stabilizes Aβ oligomer species including dimers and trimers, which potentially exert toxic effects on the human neuroblastoma cells, and inhibits Aβ fibril formation in vitro (*51*). We found that activation of DAergic neurons in the VTA significantly increased $Aβ_{40}$ levels in the anterior cortices of *Mme* KO mice, with a similar tendency toward $Aβ_{42}$ levels in the Ts fractions that are known to contain Aβ monomer and oligomer species (*52*, *53*). Despite some limitations to our study, DA likely regulates Aβ dynamics through its metabolite independently of NEP-mediated degradation in vivo. Further analysis will be required to address whether the NEP-sensitive or DOPAL-sensitive Aβ populations could overlap in the brain.
In conclusion, we provide evidence that DA is a NEP up-regulator in the PFC and enhances Aβ degradation in vivo. Long-term treatment with L-DOPA could serve as a beneficial approach to target Aβ pathology. Our findings give previously unidentified insights into the molecular link between DA and NEP and suggest potential alternative therapeutic strategies for lowering Aβ in AD.

MATERIALS AND METHODS

Chemicals

Screening compounds (hypothalamic hormones, pituitary hormones, neurotransmitters, catecholamines, and DAergic agonists) and supporting information are listed in table S1. All were assayed in vitro at the concentrations stated.

Cell culture

Primary cortical/hippocampal neurons, basal ganglia, and mixed-cell cultures were prepared from male and female embryonic C57BL/6Ncrslc and *Mme* KO mice at day E18 (*18*). Brain regions were excised by scalpel and treated with 5 ml of 0.25% trypsin solution (Nacalai Tesque, 32777-44) at 37°C for 15 min. Two hundred fifty microliters of 1% deoxyribonuclease I (DNase I) was added by pipette and mixed. Subsequently, centrifugation was performed at 1500 rpm for 5 min, and 5 ml of Hank's buffered salt solution containing 250 μl of 1% DNase I was added to the pellet and incubated in a water bath at 37°C for 5 min. An additional 10 ml of Hank's buffered salt solution was added to the mixture and centrifuged at 1500 rpm for a further 5 min. The resulting pellet was added to a neurobasal medium with B27 Plus Supplement (Thermo Fisher Scientific, 17504044) and 25 μM glutamine (Thermo Fisher Scientific, 05030-149). The

cells were filtered using a cell strainer with 100-μm nylon mesh (Falcon 2360) and seeded on poly-L-lysine–coated 6- or 96-well plates (Falcon, 353046 or Corning, 356640). Cortical/hippocampal and basal ganglia neurons were mixed in a 9:1 ratio as cocultured neurons. Cells were seeded at 1×10^4 cells per well on 96-well plates or at 1×10^6 cells per well on 6-well plates. All in vitro assays were performed on cultures after 14 days.

NEP activity assay

Primary cultures, composed of cortical/hippocampal and/or basal ganglia neurons (1×10^4 cells per well) or membrane fractions (0.1 μg/μl) from brain tissues, were prepared in 96-well plates. Test compounds (table S1) in addition to the assay medium were added to cultures after 14 days in vitro, and, after a 24-hour treatment period, cell surface NEP activity assay was performed as previously described (*19*). Cultured neurons were incubated with substrate mixture [50 μM suc-Ala-Ala-Phe-MCA (Sigma-Aldrich, S8758) and 10 nM Z-LLLal (Peptide Institute, 3175-V) in 50 mM MES (pH 6.5)] with or without 10 μM thiorphan (Sigma-Aldrich, T6031) at 37°C for 60 min. With this substrate, NEP releases suc-Ala-Ala and phe-MCA as products (suc-Ala-Ala-Phe-MCA → suc-Ala-Ala + phe-MCA). After the addition of leucine aminopeptidase (0.1 mg/ml; Sigma-Aldrich) and 0.1 mM phosphoramidon, the reaction mixture was incubated at 37°C for a further 30 min, which liberated 7-amino-4-methylcoumarin (AMC) (phe-MCA → phe + AMC). AMC fluorescence was measured by the plate reader (TECAN) at excitation and emission wavelengths of 380 and 460 nm, respectively. A standard curve was constructed with free AMC.

Animals

Pregnant C57BL/6Ncrslc mice were purchased from Japan SLC for the generation of primary cultures. Male *App*$^{NL-F}$ mice were generated in our laboratory (*24*, *54*). *Mme* KO mice were obtained from C. Gerard (*55*). DAT-Cre mice were purchased from the Jackson Laboratory (*23*), and the heterozygous line was used for all experiments in this study. All animals were maintained under standard housing conditions, with lights on at 08:00 and lights off at 20:00. All animal experiments were conducted according to the guidelines of the RIKEN Center for Brain Science.

Viral vectors and injection

DAT-Cre mice at 3 months of age were anesthetized with a triple mixed anesthetic [Domitor (0.3 mg/kg), Dormicum (4 mg/kg), Bettlefar (5 mg/kg)] administered by intraperitoneal injection. AAV-hSyn-DIO-mCherry (AAV8) (Addgene, #50459), AAV-hSyn-DIO-hM3D(Gq)-mCherry (AAV8) (Addgene, #44361), and AAV-hSyn-DIO-hM4D(Gi)-mCherry (AAV8) (Addgene, #44362) were injected into the bilateral VTA (anteroposterior, −3.15 mm; mediolateral, ±0.5 mm; dorsoventral, −4.0 mm) in a total volume of 1 μl (2.2×10^{13} vg/ml) using a Hamilton syringe (Altair Corporation), at a constant flow rate of 0.1 μl/min using a Legato 130 syringe pump (KD Scientific, Holliston, MA). After injection, mice were administered Antisedan (3 mg/kg) and maintained in cages with free access to food and water.

CNO treatment

One month after AAV injection, CNO (1 mg/kg; Nacalai Tesque, 16166-44) was given systemically by intraperitoneal injection two times a day with at least 6-hour intervals, 5 days/week, for 4 weeks to DAT-Cre mice and 8 weeks to *App*$^{NL-F}$; DAT-Cre mice. The mice were euthanized 2 hours after the final CNO injection.

Immunohistochemistry

Brains were dissected and fixed by 4% PFA overnight. Fixed brains were then processed and embedded in paraffin. The sagittal and coronal sections were prepared with 30 consecutive 4-μm sections. The slides were pretreated with an autoclave in citrate buffer or formic acid, before being placed in a blocking solution for 1 hour, then incubated overnight with the following primary antibodies: anti-Aβ (4G8, mouse, 1:400 dilution; Covance), anti-Aβ [N1D, rabbit, 1:200 dilution (*56*)], anti-TH (AB152, rabbit, 1:2,000 dilution; Sigma-Aldrich Merck), anti-DsRed (sc-390909, 1:500 dilution; Santa Cruz Biotechnology), and anti–c-Fos (sc-166940, 1:100 dilution; Santa Cruz Biotechnology). Slides were then incubated in a secondary antibody for 1 hour and visualized with enhanced immunofluorescence (TSA Biotin Systems, PerkinElmer) according to the manufacturer's instructions. Fluorescence was retained in the mounting medium with 4′,6-diamidino-2-phenylindole. Whole-slide images were obtained using NanoZoomer (Hamamatsu Photonics) and analyzed for positive signals using Definiens Tissue Studio 3 (Definiens AG). To analyze the efficiency of the DREADD system, we manually counted the number of c-Fos–positive cells normalized to total TH-positive cells in the VTA as previously described, with small modifications (*57*). The PFC was defined by selecting the same bregma (according to the Pexinos mouse brain atlas, bregma 1.33 to 3.53 mm) as previously described, with minor modifications (*58*). The regional demarcation between anterior and posterior cortices was defined using the point of bregma 0.0 mm (anterior cortex: 0.00 to 3.53 mm, posterior cortex: −4.03 to 0.00 mm).

Western blotting

Brain tissues were homogenized in lysis buffer [50 mM tris (pH 7.5), 150 mM NaCl, 2 mM KCl, 1% Triton X-100, protease inhibitor, and phosphatase inhibitor cocktail; Roche Diagnostics, 11697498001] using a multi-beads shocker MB (Yasui-Kikai). Lysates were centrifuged at 15,000 rpm at 4°C for 30 min. We added supernatant to the sample buffer and loaded it onto a 10% SDS-containing polyacrylamide gel. Proteins transferred onto polyvinylidene difluoride membrane were incubated in 1% blocking solution for 1 hour, then the following primary antibodies: anti-NEP (AF1126, goat 1:500 dilution; R&D Systems), antibodies recognizing the N-terminal region of APP (22C11, mouse, 1:1000 dilution; Chemicon), antibodies recognizing the C-terminal region of APP (A8717, rabbit, 1:1000 dilution; Sigma-Aldrich), anti-IDE (ab32216, rabbit, 1:5000 dilution; Abcam), anti–ECE-1 (RB20006, rabbit, 1:1000 dilution; Abgent), anti–phospho-DARPP-32 (Thr^{34}, rabbit, 1:1000 dilution; R&D Systems), anti–DARPP-32 (EP720Y, rabbit, 1:1000 dilution; Abcam), or anti–β-actin (AC-15, mouse, 1:5000 dilution; Sigma-Aldrich) overnight and lastly in a 1:5000 dilution of secondary antibody for 1 hour. Enhanced chemiluminescence was performed with ECL Select according to the manufacturer's instructions (GE Healthcare). Quantitative densitometry was carried out using the ImageJ software.

Preparation of membrane fractions from brain tissue

Brain tissues were homogenized in tris buffer [50 mM tris (pH 8), 0.25 M sucrose, and EDTA-free complete protease inhibitor cocktail (Roche Diagnostics, 05056489001)] and centrifuged at 3600 rpm and 4°C for 30 min. Collected supernatants were centrifuged at 70,000 rpm at 4°C for 20 min. Resultant pellets were solubilized in tris buffer containing 1% Triton X-100 and incubated on ice for 1 hour before centrifugation at 70,000 rpm at 4°C for 20 min. Protein concentrations of membrane fractions in collected supernatant samples were measured by BCA Protein Assay Kit (Thermo Fisher Scientific, 23225) for measuring the NEP activity.

Aβ ELISA

To determine levels of murine or humanized $A\beta_{40}$ or $A\beta_{42}$ in a culture medium or the brain, samples were quantified using a commercially available Aβ ELISA (enzyme-linked immunosorbent assay) kit (Wako) as described previously (*59*). Briefly, the conditioned media on coculture primary neurons plated in six-well plates (1×10^6 cells per well) were collected after 24-hour incubation with 1 μM DA. The conditioned medium was extracted with 5 M GuHCl and measured after dilution in the manufacturer-provided dilution buffer. Graphs represent the average of three well replicates, and the repeatability of the results was confirmed in at least three independent experiments. For quantifying soluble and insoluble Aβ within the brain, brain homogenates were extracted with Ts followed by centrifugation at 70,000*g* for 20 min. The supernatants were then diluted with 5 M GuHCl as the Ts soluble–fractioned samples. For the assay of guanidine-solubilized Aβ, the 70,000*g* pellet suspensions were solubilized with 5 M GuHCl as the insoluble-fractioned samples. The analysis was carried out using the plate reader at 450 nm.

Monoamine analysis

Monoamines in tissue extracts were measured by HPLC coupled to an electrochemical detection system (EICOM) as previously described (*60*). For measurement of monoamines and metabolites, samples were separated with a 3.0-by-150 mm EICOMPAK SC-5ODS column using a mobile phase [43.7 mM citrate, 39.3 mM sodium acetate, 17% methanol, sodium 1-octanesulfonate (190 mg/liter), and EDTA (5 mg/liter) (pH 3.5)], pumped at 0.5 ml/min. The graphite working electrode (WE-3G) potential was set at +0.75 V, and the column temperature was maintained at 25°C. The samples were derivatized with *o*-phthalaldehyde for 30 s before gradient separation on a reverse-phase 3 × 150 mm HPLC column (Hypersil, 3 μm, C18, Keystone Scientific) using sodium acetate [35 mM (pH 5.9) adjusted with glacial acetic acid] and 1% (v/v) tetrahydrofuran as the aqueous solvent and 70% acetonitrile, 15% methanol, and 15% sodium acetate [35 mM final concentration (pH 7.65)] (adjusted with glacial acetic acid) as the organic solvent for measurement of amino acids. The flow rate was set at 0.6 ml/min. The column was maintained at 30°C. Analytes were detected by fluorometry. HPLC data were automatically collected and analyzed using EzChrom Elite (Scientific Software Inc., Tokyo, Japan) using regularly generated standards from external standards and normalization to internal standards.

L-DOPA administration

Male *App*$^{NL-F}$ mice at the age of 3 months received five intraperitoneal injections across a 3-day period (minimum injection interval, 8 hours); L-DOPA (12 mg/kg) dissolved in 1 N HCl was administered with benserazide (3 mg/kg; Sigma-Aldrich, B7283).

Drug pellet for L-DOPA

For long-term L-DOPA treatment, pellets including L-DOPA or placebo for sustained release (10 mg/kg per day for 90 days; Innovative Research of America) were implanted subcutaneously in each animal's neck under light anesthesia as previously described (*29*). To examine the effect of Aβ pathology on behavior, fear conditioning experiments were conducted in *App*$^{NL-F}$ mice implanted with L-DOPA pellets for 3 months with subsequent 2 weeks of depletion.

DA ELISA

DA levels were measured in tissue homogenates dissected from the fixed brain, using a commercially available DA ELISA kit (LDN BA E-6300), as described previously (*61*). Briefly, dissected brain tissue was homogenized in phosphate-buffered saline containing 0.1 N HCl. The mixture was then incubated for 20 min at room temperature before being centrifuged at 10,000*g* for 30 min. Supernatants were collected and diluted properly for measuring. The analysis was carried out using a plate reader set to 450 nm and the reference wavelength at 650 nm.

Fear conditioning, context acquisition

A contextual fear conditioning protocol was performed using a method similar to that in a previous report (*62*), with slight modifications. Briefly, all chambers had the same standard design, light, and background noise. Mice were placed into the conditioning chamber; there was a 4-min preshock period (baseline period), followed by exposure to a sequence of foot shocks (0.75 mA, 2 s) for the conditioning. One minute after the last shock, the animals were returned to their home cage. This was repeated for 3 days. Freezing behavior was automatically recorded by video-tracking software (O'HARA & Co) during the 4-min period before the shock on each day to form an acquisition curve. The experiment was performed with randomization and blinding to the conditions.

Statistical analysis

For comparisons of the means between the two groups, Student's *t* tests were used for statistical analysis. If the normality test was not passed, then a Mann-Whitney *U* test was performed. For comparisons of the means among three or more groups, one- or two-way analysis of variance (ANOVA) was performed followed by a post hoc test; the Tukey, Dunnett, or Bonferroni test was applied if the data passed the Shapiro-Wilk normality and the Brown-Forsythe equal variance tests. If the normality test or the equal variance test was not passed at ANOVA, then a Kruskal-Wallis one-way ANOVA on the ranks was performed. Each statistical test in this study is described in an xls file in data file S1. Sample size determinations were conducted by the power calculation informed by previous studies where possible (*18*, *63*).

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Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper or the Supplementary Materials and are available from the corresponding authors upon request.

Supplementary Materials

This PDF file includes:

Figs. S1 to S6
Table S1

- **DOWNLOAD**
- **1.89 MB**

Other Supplementary Material for this manuscript includes the following:

Data file S1

- **DOWNLOAD**
- **27.95 KB**

MDAR Reproducibility Checklist

- **DOWNLOAD**
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