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DOWN SYNDROME

Restoration of Norepinephrine-Modulated Contextual Memory in a Mouse Model of Down Syndrome

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Down syndrome (trisomy 21) is the most common cause of mental retardation in children and leads to marked deficits in contextual learning and memory. In rodents, these tasks require the hippocampus and are mediated by several inputs, particularly those originating in the locus coeruleus. These afferents mainly use norepinephrine as a transmitter. To explore the basis for contextual learning defects in Down syndrome, we examined the Ts65Dn mouse model. These mice, which have three copies of a fragment of mouse chromosome 16, exhibited significant deficits in contextual learning together with dysfunction and degeneration of locus coeruleus neurons. However, the postsynaptic targets of innervation remained responsive to noradrenergic receptor agonists. Indeed, despite advanced locus coeruleus degeneration, we were able to reverse contextual learning failure by using a prodrug for norepinephrine called L-threo-3,4-dihydroxyphenylserine, or xamoterol, a β_1-adrenergic receptor partial agonist. Moreover, an increased gene dosage of *App*, in the context of Down syndrome, was necessary for locus coeruleus degeneration. Our findings raise the possibility that restoring norepinephrine-mediated neurotransmission could reverse cognitive dysfunction in Down syndrome.

INTRODUCTION

Down syndrome (DS) is a complex genetic disorder caused by the presence of a third copy of chromosome 21, resulting in triplication of ~300 genes. It is the most common source of congenital anomalies, with a prevalence of 1 per 733 live births in the United States; 5000 affected infants are born each year (*1*). Among several abnormalities in DS, intellectual deficiencies that affect the quality of life for both children and adults are of primary concern. Understanding the neurobiological basis of failed cognition in DS is thus a high priority because deciphering pathogenesis may lead to effective therapies.

Failed learning and memory is essentially universal in people with DS (*2*). We have pursued a strategy that emphasizes the initial documentation of phenotypes followed by discovery of underlying gene dose effects and molecular and cellular mechanisms (*3*–*5*). Among the many deficits present in children with DS, these individuals show severe defects in contextual tasks mediated by the hippocampus. This phenotype is both robust and significant, compromising the ability to carry out tasks of daily life. Cued recall, in which memory is elicited by certain sensory cues, is partially spared in DS (*6*, *7*); these tasks are modulated by the amygdala and, unlike the hippocampus, this region shows no change in structure in young people with DS (*8*). The hippocampus is markedly affected in DS (*6*–*8*). This brain region is essential for registering events with respect to time and space (*9*, *10*). By modulating contextual discrimination, in which spatial information is integrated with other salient features of the environment, the hippocampus is involved in generating appropriate responses to dynamic changes in milieu (*10*). Amnesic individuals with hippocampal damage fail in tests of contextual learning (*11*). Furthermore, hippocampal and entorhinal cortex damage has been shown to produce insensitivity to contextual changes in rodents (*10*, *12*), as has transient inactivation of the hippocampus using γ-aminobutyric acid type A ($GABA_A$) agonists (*13*). Contextual discrimination is made possible by accessing information from a number of afferent systems, both sensory and modulatory; sensory information is transmitted from the entorhinal cortex, whereas modulatory inputs originate in several populations including basal forebrain cholinergic neurons (BFCNs), norepinephrine (NE)–containing neurons of the locus coeruleus (LC), serotoninergic neurons of the raphe nuclei, and calretinin-positive neurons of the supramammillary area (*4*). Modulatory inputs extensively innervate the hippocampus. With respect to contextual discrimination, the LC, which is the sole source of NE-positive inputs, appears to play a defining role through the release of NE to act on β adrenoceptors. Indeed, studies in which the activity of LC afferents or β_1 receptors was selectively inhibited showed that NE neurotransmission is essential for this aspect of hippocampal function (*14*).

Whether the LC plays a role in contextual discrimination in humans is yet to be determined. Essential to demonstrating a link would be studies that dissect hippocampally driven contextual learning from cued learning, in which the amygdala plays a central role. Examining disorders in which the LC degenerates is one strategy for exploring LC function in humans. LC neurons are markedly affected in Alzheimer's disease (AD), DS, Parkinson's disease, Huntington's disease, dementia pugilistica, and Wernicke-Korsakov syndrome (*15*–*21*). In AD, LC neurons undergo more extensive degeneration than BFCNs (*18*) and the extensive neurofibrillary degeneration of the LC correlates well with the severity of cognitive decline (*20*). Furthermore, NE concentrations are significantly reduced in the temporal cortex of patients with AD (*21*). In DS, individuals show significant hippocampal dysfunction, including deficits in contextual discrimination (*22*). Although cued learning remains relatively intact, contextual learning is markedly impaired in both infants and adolescents with DS (*7*, *22*); it has been suggested that such deficits significantly impair learning (*6*).

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RESULTS

Cognitive deficits in Ts65Dn mice

To explore the pathogenesis of cognitive disabilities in DS, we carried out studies in the Ts65Dn mouse model of DS. This mouse is trisomic for a fragment of mouse chromosome 16 (MMU16) extending from *Mrpl39* to *Znf295* (Fig. 1A); it contains at least 104 mouse genes homologous to those present in three copies in DS (*23*, *24*). Ts65Dn mice recapitulate a variety of DS structural and functional changes (*24*). To investigate whether these mice show changes in contextual discrimination, we compared the performance of euploid (that is, 2N) mice with Ts65Dn mice in tests of contextual learning (Fig. 1, D and E). The contextual fear conditioning test, which registers fear-based responses as episodes of behavioral freezing, differentiates between contextual and cue-based learning. After training on day 1, Ts65Dn and 2N mice underwent a test of cued learning on day 2 and contextual learning on day 3. We found no abnormalities in cued learning in Ts65Dn mice (P = 0.1825) (Fig. 1D). In contrast, there was a marked deficit in Ts65Dn mice in contextual learning (P = 0.0324) (Fig. 1, D and E). Indeed, in this test, although 2N mice showed twice as much freezing relative to the training session, Ts65Dn mice showed no increase. Naïve Ts65Dn mice were also shown to have a significant disability in contextual learning (*25*, *26*).

Nesting behavior is another test that measures hippocampus-based cognition. Previous studies have shown that nesting behavior can be used to define the integrity of hippocampal function (*27*, *28*). It correlates with failed contextual discrimination and spatial learning in rodents (*29*). In tests of nesting, mice placed in a novel cage were provided with nesting material in the form of "nestlets" of known weight (see Materials and Methods; fig. S1). Unlike 2N mice, Ts65Dn mice used a relatively small portion of their nestlets (P = 0.0012) (Fig. 1G and fig. S1) and their nests were poorly formed. These findings are evidence that hippocampal function, and context discrimination in particular, is markedly affected in Ts65Dn mice.

Morphological alteration in the LC

Contextual learning requires the participation of the LC. The LC is the sole source of the norepinephrinergic inputs that engage β-adrenergic receptors in the hippocampus. Indeed, studies in which the activity of LC afferents or β_1 receptors was selectively inhibited showed that NE neurotransmission is essential for this aspect of hippocampal function (*14*). Whether the LC plays a role in contextual discrimination in humans is yet to be defined, but this neuronal population is markedly affected in a number of neurodegenerative disorders (*15*–*21*).

Given similar deficits in contextual learning in DS and the mouse model, we asked whether LC degeneration was also present. Using an antibody against tyrosine hydroxylase (TH), NE-containing neurons were examined throughout the rostral-caudal axis of the LC. Unbiased stereology (see Materials and Methods) was used to estimate the total number and cell profile area of TH-immunoreactive neurons in the LC (Figs. 1, B and C, 2, B and C, and 3, A to E). We first examined mice at 6 months of age because this age was used for behavioral testing. The TH-immunoreactive cell number was significantly lower in Ts65Dn mice at this age. A similar pattern was observed in the size of TH-immunoreactive cell profile areas (Fig. 2, B and C). Studying mice at ages before and after 6 months showed that the degeneration of LC neurons persisted at 18 months, whereas in 3-month-old mice there were no significant differences in either parameter. The temporal pattern of changes was reminiscent of those documented for BFCNs in Ts65Dn mice (*3*, *5*) in that the differences were due both to a decrease in the number of neurons and failure to show an increase in number with aging. The latter almost certainly reflects a failure to increase TH content in NE-expressing neurons in Ts65Dn mice. Our data on the increased number and size of TH-immunoreactive neurons from 3- to 6-month-old 2N mice are in accordance with recent studies showing a significant increase in TH gene expression from 3 to 6 months, which is

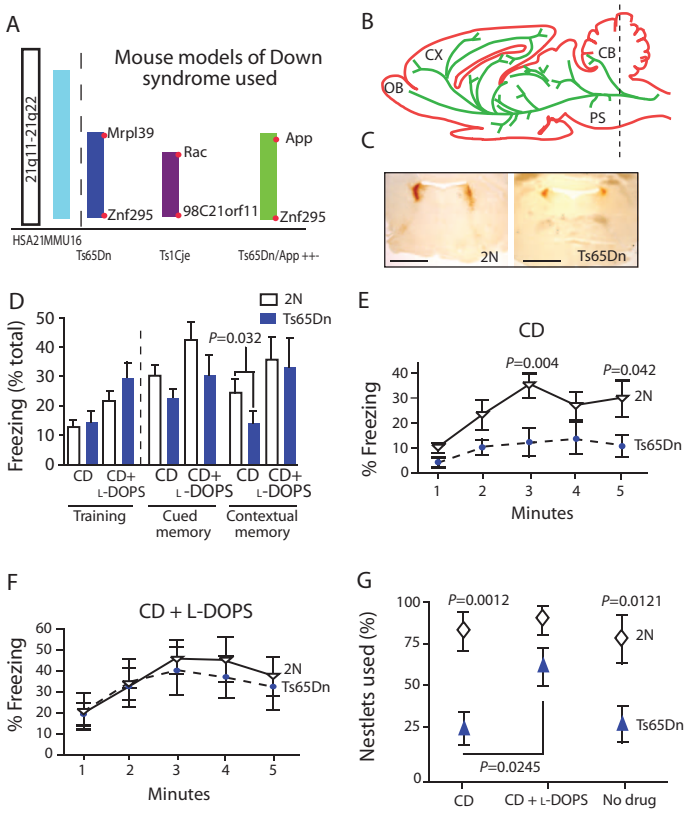

Fig. 1. (**A**) Schematic representation of trisomic regions of chromosome 16 in mouse models of DS used for this study. HSA21, human chromosome 21. (**B**) The anatomical localization of NE neurons in the rodent brain. CB, cerebellum; OB, olfactory bulb; PS, pons; CX, cortex. (**C**) Micrographs depicting immunocytochemical visualization in coronal sections of LC in 2N and Ts65Dn mice at the age of 6 months using TH antibody. Scale bar, 1 mm. (**D**) Comparison of freezing behavior between 2N and Ts65Dn mice treated with carbidopa in cued and contextual learning tests. A significantly shorter freezing time was seen in Ts65Dn mice in the contextual learning relative to 2N mice (P = 0.0324, n = 13 for 2N mice and n = 10 for Ts65Dn mice). (**E**) Quantification of the percent of freezing during the first 5 min of the contextual learning test. Unlike Ts65Dn mice, the 2N group improved with each minute (P = 0.0002). (**F**) Treatment of 2N and Ts65Dn mice with L-DOPS led to a significant improvement in contextual memory in Ts65Dn mice. As a result, there was no significant difference between the two groups (P = 0.5247). (**G**) Ts65Dn mice used significantly lower amounts of their nestlets relative to 2N mice at 6 months of age (P = 0.0012). Treating the mice with L-DOPS led to a significant improvement in the nesting behavior in Ts65Dn mice. The beneficial effects of L-DOPS vanished 2 weeks later (P = 0.0121, n = 10 for each group). "No drug" refers to a group of mice that were not receiving any treatments at the time.

suggested to be due to phenotypic robustness of TH expression in preexisting neurons during early aspects of aging (*30*).

The LC innervates most brain regions; its projections are organized topographically. The anterior pole of the LC innervates the hypothalamus, whereas the posterior supplies the hippocampus. The neurons between the subregions innervate the hippocampus, cerebellum, cortex, and spinal cord (Fig. 3D). We examined the possibility that changes in LC cells would differentiate the different regions of this area. The severity of degeneration in Ts65Dn mice was greatest in the caudal LC (Fig. 3E), a subregion with extensive projections to the hippocampus (*31*).

To determine whether changes in LC neuron cell bodies are linked to changes in the hippocampus innervation, we examined monoaminergic terminals in the hippocampus by examining and quantifying staining for vesicular monoamine transporter (VMAT2) (*32*, *33*). A majority of monoaminergic projections to the hippocampus, particularly in the dentate gyrus (DG), contain NE (*33*). Comparing Ts65Dn and 2N mice at age 3 months, the DG of Ts65Dn mice showed a significant increase in VMAT2 staining; in particular, there was a marked increase in the number of bright puncta. In contrast, by 6 months, the optical density of VMAT2 staining in Ts65Dn mice showed an overall decrease of ~20% relative to 2N mice (Fig. 2D and fig. S2). These observations are evidence for changes in LC terminals that precede those detected in neuronal somas.

Biochemical changes in the hippocampus of Ts65Dn mice

To determine whether the morphological change in axon terminals in the hippocampus was correlated with changes in NE, we examined the concentrations of this neurotransmitter. There was a significant age-related reduction in NE concentrations. Comparing the hippocampus in Ts65Dn and 2N mice at 4.5 months of age, there was a 16% reduction in Ts65Dn that was not statistically significant. At 18 months, however, the decrease in Ts65Dn was significant (P = 0.0007), averaging 31%. Examining absolute values for NE, there was a decrease with age in both 2N and Ts65Dn mice, but only the change in the Ts65Dn hippocampus was significant (P = 0.019) (Fig. 2F). These findings gave additional evidence for dysfunction in the terminals of the LC neurons in Ts65Dn mice.

Norepinephrine-mediated neurotransmission

We examined the postsynaptic targets of LC axons in the hippocampus. Noradrenergic neurotransmission is affected in part through β_1-adrenergic receptors. The β_1-adrenergic receptor is present on the postsynaptic targets of LC axons in the hippocampus and has been shown to play an important role in cognition, including contextual learning (*14*). Regarding hippocampal hilar neurons expressing the β_1-adrenergic receptor gene, immunostaining for these receptors is readily detected on their cell bodies. In Ts65Dn mice at 3 months of age, there was a marked increase in the size of β_1-adrenergic receptor–immunoreactive cells ($P < 0.005$) (Fig. 3F). The increase in size was also seen at age 6 months. At 6 months, we also documented a more than 90% increase in the number of β_1-adrenergic receptor–immunoreactive neurons in the Ts65Dn hippocampus (P = 0.0173) (Fig. 3F). At the same time, by examining β_1 immunoreactive in the entire hippocampus, we noted an overall increase that was significant ($P < 0.001$). These findings are evidence for changes in the postsynaptic targets of LC neurons. Our data in Ts65Dn mice showing degenerative changes in LC terminals and reduced NE concentrations suggest that there is a compensatory increase in postsynaptic receptors in response to decreasing norepinephrinergic transmission.

The continued presence of NE receptors on postsynaptic targets raised the possibility that postsynaptic mechanisms activated by NE remained functional even with the presence of LC dysfunction and degeneration. To test whether NE responses are still present in the DG of Ts65Dn mice, we studied the effect of isoproterenol, an agonist at both β_1- and β_2-adrenergic receptors and a widely used agent for studying the functional integrity of adrenergic receptors. For these studies, we

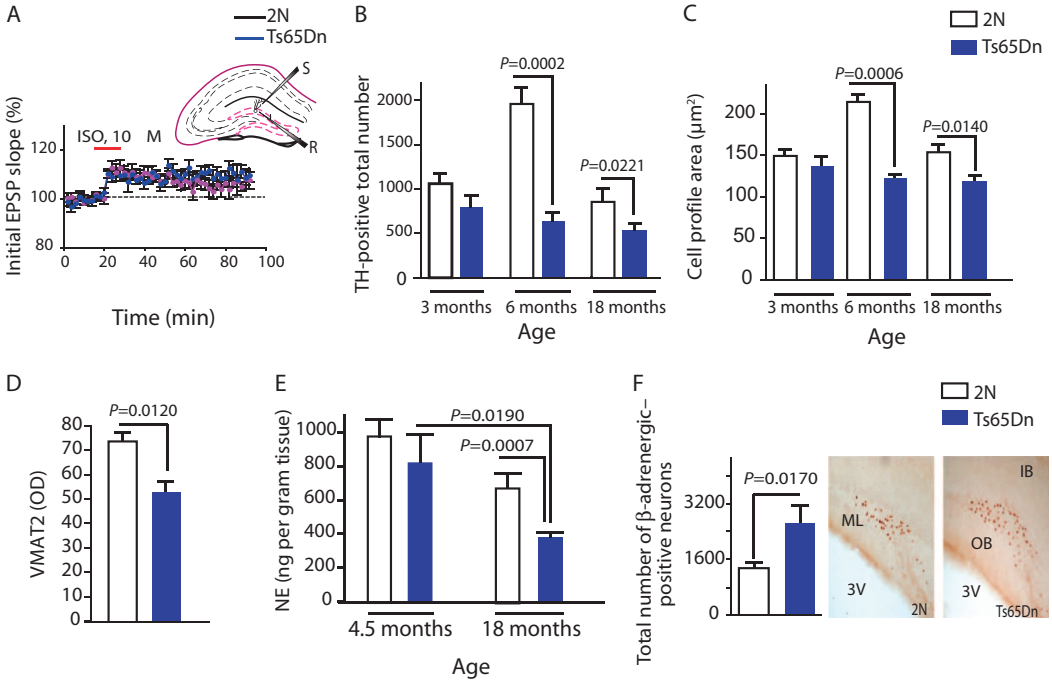

Fig. 2. (**A**) Representative field EPSP responses recorded from the granule cell layer after stimulation of the middle molecular layer before and after application of a β-adrenergic agonist, isoproterenol (ISO) (n = 3 for each group). (**B** and **C**) TH-positive neuron number (B) and cell area (C) in the LC at 3 to 18 months of age. (**D**) VMAT2 staining in the DG of 6-month-old Ts65Dn mice (P = 0.0120, n = 3 for each group). (**E**) Average concentrations of NE in 4.5- and 18-month-old Ts65Dn mice compared with 2N. (**F**) Total number of β_1-adrenergic–positive neurons in the polymorphic layer (hilus) of the DG in Ts65Dn mice at age 6 months (P = 0.0173, n = 6 for 2N mice and n = 5 for Ts65Dn mice). 3V, third ventricle; ML, molecular layer; OB, outer blade; IB, inner blade of the DG.

applied stimulating current to the middle molecular layer of the DG in acute hippocampal slices, in the absence and presence of isoproterenol, while recording in the DG granule cell layer. In slices taken from mice at 3 months of age, there were increases in both the slope and the amplitude of excitatory postsynaptic potentials (EPSPs) in both 2N and Ts65Dn mice. The same was true at age 6 months. At 6 months of age, the responses to isoproterenol in Ts65Dn slices were consistently more robust (Fig. 2A and figs. S3 and S4). Thus, responsiveness to NE was retained despite degenerative changes in LC neurons and their terminals in the hippocampus.

Restoration of brain norepinephrine concentrations

Continued in vitro responsiveness of the targets of NE-positive innervation suggested that the same might be true in vivo. To test the idea, we asked whether restoring NE concentrations would rescue contextual fear conditioning in 6-month-old mice. We used an NE prodrug that readily crosses the blood-brain barrier (BBB). L-Threo-3,4-dihydroxyphenylserine (L-DOPS) or droxidopa is a synthetic amino acid (*34*). L-DOPS is metabolized by L-aromatic amino acid decarboxylase within NE-containing neurons to yield NE. To evoke NE increases in only the central nervous system (CNS), we administered L-DOPS (1 mg/g) together with carbidopa (CD) (0.125 mg/g), a peripheral L-aromatic amino acid decarboxylase inhibitor that does not cross the BBB (*14*, *35*). CD administration alone served as the control. L-DOPS concentrations were measured 6 hours after administration in both 2N and Ts65Dn mice. L-DOPS was present in several tissues, particularly the kidneys. In the hippocampus, no significant differences were found in either L-DOPS ($P = 0.5628$; fig. S5) or NE ($P = 0.4726$) concentrations between 2N and Ts65Dn mice. In both 2N and Ts65Dn mice, L-DOPS injections markedly increased hippocampal NE concentrations (fig. S5). Because brain NE reaches its maximum concentrations within 5 hours of subcutaneous administration (*14*, *34*), mice were tested at this time point. Treatment of 6-month-old Ts65Dn with L-DOPS (1 mg/g; 20 mg/ml) led to a significant improvement in contextual memory (Fig. 1, D and E). In the contextual test, L-DOPS restored fully the difference in freezing between 2N and Ts65Dn mice (Fig. 1, E and F) [$P = 0.5247$, one-way analysis of variance (ANOVA)]. In the control group, in contrast, a significant difference remained ($P = 0.0002$, one-way ANOVA). When performance was measured on a minute-by-minute basis, L-DOPS treatment was associated with increased freezing after the first minute, a pattern shared with 2N mice treated as controls or with L-DOPS. A similar beneficial effect of L-DOPS was found in nesting behavior. Treating young adult Ts65Dn mice and their controls with L-DOPS significantly ($P = 0.0245$) improved nesting in Ts65Dn mice (Fig. 1G). With cessation of treatment, Ts65Dn mice again showed poor nesting behavior ($P = 0.0121$) (Fig. 1G). These findings show that contextual discrimination can be rescued in a mouse model of DS by increasing tissue concentrations of NE. They provide compelling support for the view that LC dysfunction markedly contributes to failure in contextual learning in this model.

The increased β_1-adrenergic receptor expression that we observed raised the possibility that these receptors were involved in transducing the beneficial effects of L-DOPS in Ts65Dn mice. To test this idea, we treated Ts65Dn mice with xamoterol, a β_1-adrenergic receptor partial

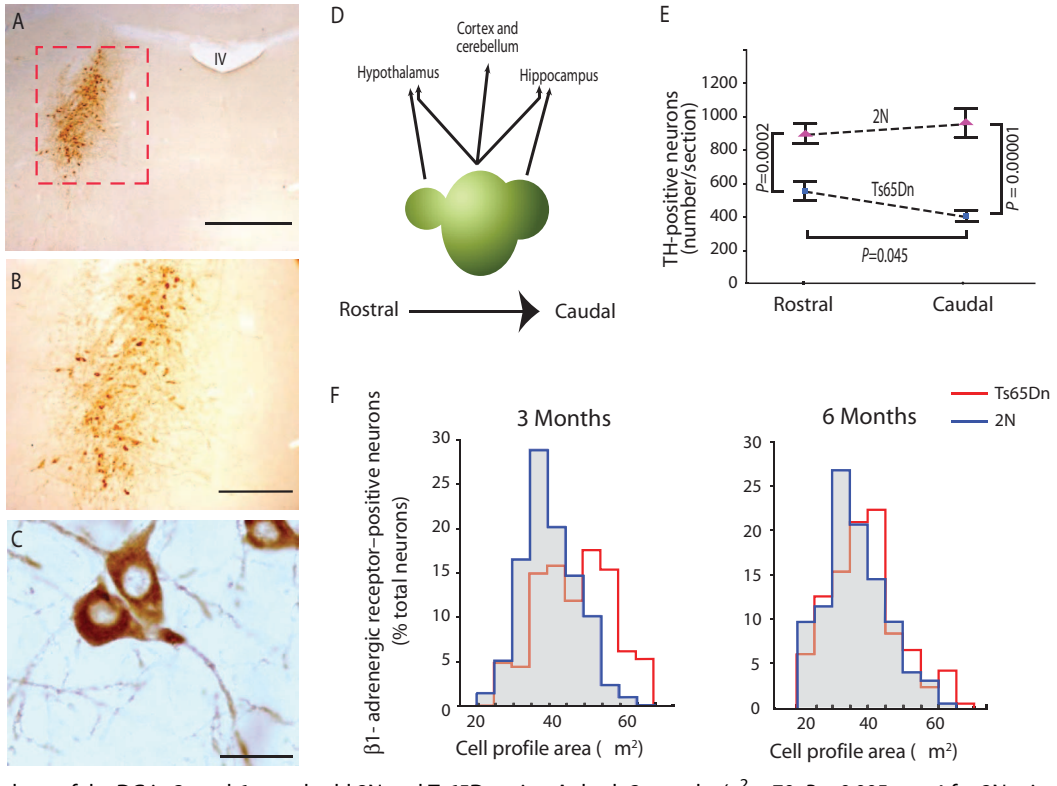

Fig. 3. (**A** to **C**) Immunocytochemical visualization of TH-positive neurons in the LC region. Scale bars, 500 μm (A), 200 μm (B), and 20 μm (C). IV, fourth ventricle. (**D**) Schematic representation of LC projections to cortical and subcortical targets. The rostral part of the LC in rodents projects to the hypothalamus. The core of the LC projects to the hypothalamus, cortex, and spinal cord, and the caudal part of the LC projects exclusively to the hippocampus. (**E**) Density of TH-immunoreactive neurons across the rostral-caudal axis of the LC in 6-month-old 2N and Ts65Dn mice. Although there was a reduction in neuronal density across the rostral-caudal axis of the LC, degeneration of TH neurons was more severe in the caudal part of the LC. There was a significant reduction in the density of TH-positive neurons in Ts65Dn mice relative to the rostral region of the LC ($P = 0.045$). (**F**) Frequency distribution of cell area of β_1-positive neurons in the polymorphic layer of the DG in 3- and 6-month-old 2N and Ts65Dn mice. At both 3 months ($\chi^2 = 70$, $P < 0.005$, $n = 4$ for 2N mice and $n = 5$ for Ts65Dn mice) and 6 months ($\chi^2 = 24$, $P < 0.01$, $n = 4$ for 2N mice and $n = 5$ for Ts65Dn mice), there was a significant shift to higher values in the frequency distribution of cell profile area in Ts65Dn mice.

agonist. Unlike cued learning, which was not affected in Ts65Dn mice, treatment with xamoterol restored failed contextual learning in Ts65Dn mice (Fig. 4, A and B) ($P = 0.032$). These findings are evidence that β_1-adrenergic receptors play a vital role in mediating the effects of increasing norepinephrinergic transmission in the Ts65Dn hippocampus. Moreover, they show that pharmacologically targeting functionally intact postsynaptic neurons can restore contextual learning.

Exploring the genetic basis of LC degeneration

To decipher the underlying genetic basis for LC degeneration in Ts65Dn mice, we compared mouse models of DS harboring different triplicated fragments of MMU16 (Fig. 1A). This approach has been used to identify a role for *App* in the pathogenesis of BFCN degeneration in Ts65Dn mice (*3*). Even in old age, Ts1Cje mice showed no significant changes in size or number of LC neurons (Fig. 4C). The lack of apparent LC degeneration in Ts1Cje mice suggested that the responsible gene(s) is located on MMU16 fragment between *Gabpa* and *Sod1* (Fig. 1A); this region contains ~32 genes, including *App* (*23*, *24*). To test whether increased dose for *App* contributes to LC degeneration, we examined these neurons in Ts65Dn mice bearing either two or three copies of *App*. Deleting the third copy of *App* in Ts65Dn mice eliminated the decrease in the number of LC neurons, suggesting that *App* overexpression is necessary for LC degeneration (Fig. 4D). A question raised was whether deleting the extra copy of *App* and normalizing the apparent morphology of LC neurons in Ts65Dn mice would necessarily restore cognition in these mice. To address this point, we compared nesting behavior among Ts65Dn mice with two versus three copies of *App*. As expected, we found a significant deficit in nesting behavior in Ts65Dn mice (Fig. 4E) ($P = 0.0013$). However, deleting an extra copy of *App* in Ts65Dn mice did not restore nesting in these mice. As a result, no significant difference was detected between Ts65Dn mice with two versus three copies of *App* ($P = 0.6658$) (Fig. 4E). To determine whether increased concentrations of APP were sufficient to cause LC degeneration, we examined mice that overexpress a mutant APP_{Swe} transgene. There was a significant decrease in LC neurons in these mice ($P = 0.0317$) (Fig. 4F). This latter finding is consistent with a recent study showing degeneration of LC neurons in *APP/PS1* transgenic mice (*36*). Together, the findings are evidence that *APP/App* gene dose plays a conspicuous role in the degeneration of LC neurons in these mouse models.

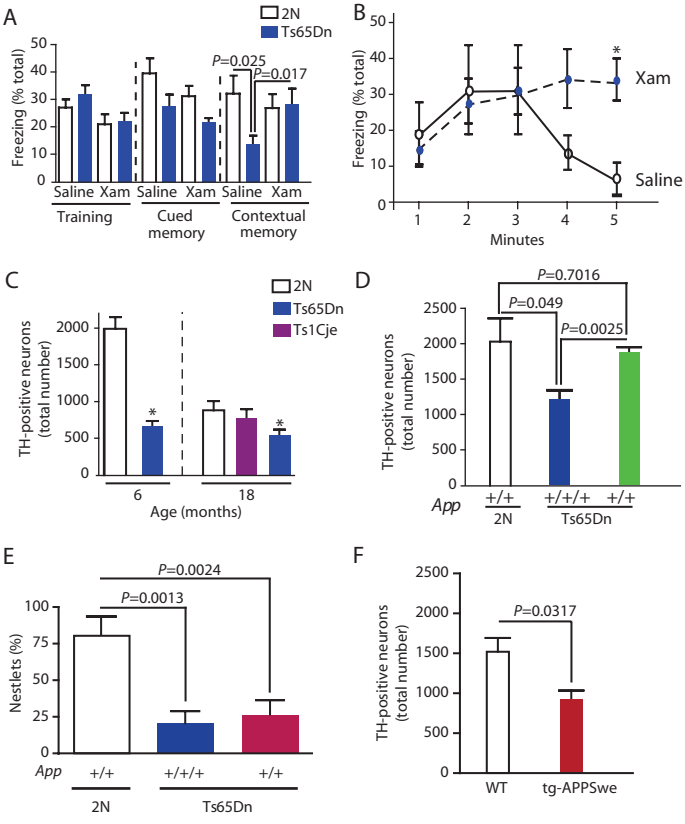

Fig. 4. (**A**) Contextual learning in Ts65Dn mice treated with saline or xamoterol (Xam). There was a significant improvement in contextual learning in Ts65Dn mice treated with xamoterol relative to saline-treated Ts65Dn mice ($P = 0.0175$, $n = 7$ for both groups). (**B**) A similar pattern was found in the contextual day ($P = 0.032$, $n = 7$ for saline-treated Ts65Dn mice and $n = 8$ for xamoterol-treated Ts65Dn mice). (**C**) The LC neuron degeneration was significant only in Ts65Dn mice (*$P < 0.05$, $n = 6$ for Ts1Cje mice). This suggests that the triplication of the region between *Mrpl39* and *Rac* must be responsible for degeneration of LC neurons. (**D**) There was a significant difference ($P = 0.0025$) between Ts65Dn with either two or three copies of *App* ($n = 4$ for Ts65Dn/*App*+/+/– mice and $n = 5$ for Ts65Dn/*App*+/+/+ mice). (**E**) Nesting behavior in Ts65Dn mice with two or three copies of *App*. There was a significant deficit in nesting behavior in Ts65Dn mice with three copies of *App* ($P = 0.0013$, $n = 9$ for 2N mice and $n = 8$ for Ts65Dn mice). However, deleting the extra copy of *App* in these mice did not lead to a significant improvement in nesting behavior ($P = 0.6658$, $n = 11$ for Ts65Dn/*App*+/+/– mice). (**F**) Number of TH-positive neurons in LC of 18-month-old APP_{Swe} transgenic (tg-APPSwe) and wild-type (WT) mice ($n = 5$, $P = 0.0317$).

DISCUSSION

In this study, we linked marked defects in hippocampally mediated contextual learning in a model of DS to LC dysfunction and demonstrated that these deficits can be restored by treatments targeted at correcting deficient NE neurotransmission. The most important implication of our work is that postsynaptic targets of degenerating neurons may remain responsive and functional well after the presence of advanced disease in their presynaptic inputs. If so, treatments that target still-functional elements of neuronal circuits may restore circuit function. In particular, treatments targeted to restore the loss of NE inputs to the hippocampus may prove effective in enhancing cognition in people in whom these neurons are affected.

The age of 6 months was appropriate for testing because of the presence of both degenerative and apparent compensatory processes. Although we found both decreased number and size of LC neurons (Fig. 2, B to D) and reduced VMAT2 expression in presynaptic neurons (Fig. 2D), we detected increased numbers of β_1-positive postsynaptic neurons in 6-month-old Ts65Dn mice (Fig. 2F). Furthermore, it has already been shown that the hippocampal β-adrenergic receptor transduction is significantly reduced in these mice at a similar age (*37*).

Our findings reinforce the importance of NE neurotransmission to contextual learning. It appears that LC modulates the impact of this information on hippocampal function to enhance contextual discrimination. The entorhinal cortex (*38*) gives rise to the medial perforant path carrying navigational information and the lateral perforant path

conveying sensory information. NE, released by the LC, is believed to differentially affect perforant path inputs. For example, stimulating NE-positive inputs potentiates the population spike amplitude of the medial perforant path while depressing synaptic potentials in the lateral perforant path (*38*–*42*). The net effect is thought to be an enhanced perception of spatial context. NE-containing inputs also appear to modulate directly or indirectly other neuronal systems with efferents to the hippocampus, including cholinergic neurons of the basal forebrain and serotoninergic neurons of the raphe nuclei (*43*). Indeed, NE release from LC axons may play a defining role in cholinergic and serotoninergic neurotransmission. Given the degeneration of these other neuronal systems in the Ts65Dn mouse as well as in DS and AD, it might be argued that dysfunction of the LC represents only one of several deficiencies and that rescuing NE concentrations would have no effect on cognition. Our data argue that this is not the case. Instead, they indicate that restoring NE neurotransmission is effective even when these other neurons are affected.

The GABAergic system also plays a significant role in cognition. For instance, we and others recently reported that treatment with $GABA_A$ receptor antagonists is able to improve both synaptic plasticity (*44*) and cognition (*45*) in Ts65Dn mice. There is a considerable overlap in the distribution of GABAergic and catecholaminergic innervation in the brain (*46*). NE is able to activate or conversely inhibit (*47*) GABAergic neurons, and GABA can augment the release of NE in brain slices (*48*). Therefore, it is possible that there is an overlap between mechanisms by which $GABA_A$ antagonists and L-DOPS improve learning in Ts65Dn mice.

Unlike Ts65Dn mice, the 18-month-old Swedish *APP* mutant transgenic mice used in this study show high Aβ accumulation in their brain. In this regard, the relation between *App* and LC degeneration was investigated in the context of both high concentrations of APP and significant Aβ accumulation. On the basis of these results, we can conclude that although increased gene dose for *App* is necessary for degeneration of LC neurons in the context of DS, it is not yet possible to state that it is also sufficient.

In investigating mechanism(s) by which *App* gene dose affects pathogenesis, it is important to note that the LC appears to be affected more severely by pathogenic factors than targets of innervation. Indeed, changes in LC axon terminals preceded the observed changes in soma size and number, a finding reported also for mouse models of AD (*34*). Conceivably, other measures may define an even earlier onset of pathogenesis. For example, Ts65Dn mice at age 2 months fail in tests of contextual discrimination, and our own findings are consistent with changes in nesting behavior as early as 3 months. In view of the findings for LC in people with DS and those with AD, and the corresponding changes detected in the mouse models of these disorders, it is possible that LC dysfunction contributes to cognitive changes in both children and adults.

We found that deleting an extra copy of *App* in Ts65Dn mice did not completely restore hippocampus-mediated nesting behavior (Fig. 4E). This finding suggests that although increased *App* gene dose alone can account for the degenerative changes in LC cell body size and number, this is not the case for the defects in contextual learning. The most plausible conclusion is that other genes combine with *App* to affect the degeneration of LC neurons. This perspective is quite exciting because it provides an intellectual paradigm for exploring the neurobiology of DS. In the past, it has been envisioned that one and the same gene would affect both the cell bodies and the terminals of degenerating neurons. This is not the case, at least as judged by these new data. Thus, one must now entertain the possibility that different genes affect various aspects of the neurodegenerative phenotype. Moreover, our data argue that it may be necessary for treatments to address separately the mechanisms engaged by different genes. In this regard, the L-DOPS data suggest that one would be able to therapeutically affect changes in neurotransmission that are partially independent of treatments aimed at preventing the degeneration of the neuron cell bodies that provide the corresponding efferents.

The mechanism responsible for APP-mediated pathogenesis is yet to be defined. As one possibility, there may exist a trophic deficiency whose manifestations arise in the target, a suggestion for which earlier studies provide support (*3*, *39*). It is possible that local changes in the synthesis or release of a trophic factor or its ability to signal in a retrograde fashion are impaired (*3*, *49*, *50*). Of particular interest is the possibility that brain-derived neurotrophic factor may play a role, especially in view of the fact that this protein serves as a trophic factor for LC neurons (*39*).

In this study, two agents acted to restore contextual learning. In the case of L-DOPS, the drug is metabolized by LC terminals to produce NE. In the case of xamoterol, the drug directly accesses NE receptors. The fact that both agents were effective suggests that receptor activation is functioning, thus reflecting the need for neuronal LC axonal terminals. If this circumstance also applies to humans, restoring NE concentrations in the hippocampus may act to enhance contextual learning even in patients in which LC degeneration is advanced. Future clinical trials attempting to increase NE neurotransmission in people with DS, and possibly AD, may show cognitive benefits. To avoid peripheral nervous system activation, a regimen with drugs that can be targeted specifically to the CNS, as was the case herein for L-DOPS (plus carbidopa), would be preferred. The use of β-adrenergic antagonists with access to the CNS might impair contextual learning (*14*, *51*). Our findings raise concern for the use of such agents in patients with cognitive difficulties involving the LC and the hippocampus.

Clinical implications

There has been limited success to date in treating cognitive symptoms in DS. Most efforts have focused on augmenting cholinergic function, particularly with donepezil, with some limited benefits reported in both young (*52*) and older (*53*–*55*) individuals. Furthermore, memantine, an antagonist of *N*-methyl-D-aspartate receptors, is now being tested in a large group of DS patients, the result of which is yet to be released (*56*).

Our findings suggest that enhancing NE neurotransmission may be useful in treating cognitive disability in DS. An important question is which age group to target. The murine studies reported herein suggest that young adults with DS, in whom pathology is present but not advanced, may be appropriate. If the status of LC neurons and their targets in mice mirrors those in humans, at this stage of the disorder postsynaptic adrenergic receptors will be present and responsive to pharmacologically induced increases in brain NE concentrations. We envision a plan to test the efficacy of droxidopa in young adults with DS. The NE prodrug droxidopa is now in a phase III clinical trial for orthostatic hypotension; in another clinical trial (phase II), it is being tested, both with and without the peripheral aromatic amino acid decarboxylase inhibitor carbidopa, for the ability to ameliorate CNS-mediated effects in fibromyalgia. Analogous experimental therapeutic regimens could be applied in DS to test clinically the

hypothesis that raising NE concentrations in the CNS ameliorates cognitive impairment in these disorders. Depending on the status of preclinical toxicology and human studies, it may be possible to advance directly into efficacy studies in DS. Evidence that cerebrospinal fluid (CSF) NE concentrations are responsive to therapeutic intervention and perhaps correlate with cognitive function encourages this approach (*57*, *58*). The initial step would be dose-ranging phase II pharmacodynamic, safety, and efficacy studies with droxidopa plus carbidopa. On the basis of experience with cholinergic augmentation therapies in AD (*59*), we expect that a 12-week exposure would be sufficient for assessment of clinical effects. Therefore, it would be appropriate to begin with a 12-week exposure to a range of droxidopa doses in combination with an optimal dose of carbidopa to evaluate safety and impact on CSF NE concentrations and to explore efficacy in terms of cognitive performance, global clinical status, function, and behavioral measures. An ongoing multicenter trial in aging individuals with and without a dementia diagnosis (*60*) supports the utility of a number of assessment tools, including a test of praxis to assess cognition, a clinician's global assessment of change utilizing caregiver input, and behavior and function (by informant questionnaire); these measures are robust across a wide range of intellectual functioning (*61*). Optimal doses may then be explored in combination, possibly with and without a standard cholinergic intervention, to assess additive or synergistic benefit. Optimal monotherapy or combined therapy regimens would then be assessed in pivotal 6-month placebo-controlled phase III trials.

It appears that the use of L-DOPS in humans has not been associated with life-threatening side effects. The results of multiple clinical trials in adults have shown that the side effects of L-DOPS include nausea, headache, increased blood pressure, hallucination, anorexia, lightheadedness, palpitations, dry mouth, irritability, upset stomach, vomiting, abdominal pain, chest pain, akinesia, laryngeal dyspnea, syncope, urinary tract infection, elevated lactate dehydrogenase, and electrocardiogram abnormalities (*34*, *62*). In children, an oral dose of 100 mg has been associated with near-fainting, gradual decline in blood pressure, dizziness, lightheadedness, and fatigue (*63*).

Studies in other DS age groups can also be contemplated. In children with DS, one could use a similar strategy and, possibly, take advantage of other agents used to increase the brain NE concentrations that have already been used in children. Prime examples include NE reuptake inhibitors such as atomoxetine, a nonstimulant drug approved and widely used for the treatment of attention-deficit hyperactivity disorder, although there is a concern that atomoxetine may increase the chance of suicide. Combination therapy regimens may be particularly appropriate for later life, when accumulating synaptic pathology and the burden of amyloid plaques and neurofibrillary tangles and the other manifestations of AD may affect efficacy. Adding memantine to these protocols may also prove effective.

Whether patients with late- or early-onset AD would respond to increasing NE concentrations in the CNS is uncertain. Protocols described in the present study could easily be applied to transgenic mouse models of AD to assess the effects of raising NE concentrations in the CNS on both pathological and behavioral AD-related features. Past attempts to enhance cognition by increasing NE neurotransmission met with mixed results. For example, combining cholinesterase inhibitor treatment with yohimbine or clonidine, both targeting primarily α_2-adrenergic receptors, did not result in overall benefit, although some individuals apparently improved (*60*, *61*). The limited response may have been due to binding to α_2-adrenergic receptors instead of β_1-adrenergic receptors and the extent of synaptic dysfunction in established dementia. In any event, previous studies have been very small and exploratory; positive results from a phase II trial of droxidopa and carbidopa in mildly demented DS patients would likely warrant further investigation in AD.

MATERIALS AND METHODS

For extensive description of methods used, see the Supplementary Material.

Mice used

Ts65Dn mice. Ts65Dn mice have three copies of a fragment of the MMU16 extending from *Mrpl39* to *Znf295* (Fig. 1A). The Ts65Dn mouse colony was maintained by crossing Ts65Dn females (stock number, 001924; Jackson Laboratory) to C57BL/6JEi × C3H/HeSnJ (B6EiC3Sn) F1 males (Jackson Laboratory). Genomic DNA isolated from tail was genotyped with multiplex real-time polymerase chain reaction with *App* and *ApoB* primers to identify 2N and Ts65Dn mice. 2N and Ts65Dn mice (3, 4.5, 6, 9, and 18 months old) were used for these studies. All the studies were approved by the Stanford University Committee on Animal Research.

Ts1Cje mice. Ts1Cje mice have triplication of a fragment of MMU16 extending from *98C21orf11* to *Rac*.

To generate Ts1Cje mice on a similar genetic background with Ts65Dn mice, we crossed Ts1Cje mice on the C57BL/6JEi background with C3H/HeSnJ mice and crossed the resulting Ts1Cje mice to B6EiC3Sn F1 mice. Five 18-month-old Ts1Cje and five age-matched 2N mice were used.

Ts65Dn:*App*+/+/– mice. To generate Ts65Dn:*App*+/+/– mice, we mated Ts65Dn female mice with male mice hemizygous for *App*, in which *App* was inactivated by deleting the *App* promoter (*64*). The latter group was kept on the C57BL/6JEi × C3H/HeSnJ (B6EiC3Sn) F1 background. As a result, Ts65Dn mice with the three copies of *App* (Ts65Dn:*App*+/+/+) and two copies of *App* (Ts65Dn:*App*+/+/–) were generated. For studying the effects of deleing an extra copy of *App*, we used five 2N, five Ts65Dn:*App*+/+/+, and four Ts65Dn:*App*+/+/– mice.

***APP*$_{Swe}$ and their nontransgenic littermates.** *APP*$_{Swe}$ and their nontransgenic littermates were maintained on a mixed (C3H/HeJ and C57BL/6J) background. The *APP*$_{Swe}$ mouse expresses a chimeric mouse or human *APP695* containing the human Aβ domain and mutations (K595N, M596L) linked to familial AD. Five 18-month-old *APP*$_{Swe}$ and five age-matched wild-type mice were used.

Immunocytochemistry

Histological analyses were performed blind to genotype. LC neurons were identified by immunocytochemical staining for TH (Fig. 3, A to C) (*36*).

Behavioral studies

Fear conditioning. Contextual and tone-cued fear conditioning tests were performed with the Fear Conditioning Video Tracking System (Med-Associates). Each mouse was handled for 5 days to reduce stress. Then, the mice underwent 1 day of training, a tone-cued in novel context testing day, and a contextual testing day. In the first day, mice went through the training session. The shock was delivered after the end of the tone. Therefore, an empty trace interval interposed between

the tone and the shock in each conditional stimulus–unconditional stimulus pairing. On the second day (tone-cued testing day), mice were placed in the novel context (new olfactory and visual cues) for 3 min and subsequently presented three tone presentations (same as the training day) without any shocks. On the last day of the experiment, mice were placed in the context similar to the training day for 5 min without any tones or shocks. Two groups of male Ts65Dn and 2N mice were injected either with carbidopa (2.5 mg/ml) or with a combination of L-DOPS (20 mg/ml) and carbidopa (2.5 mg/ml) (50 μl/g subcutaneously) 5 hours before the start in all 3 days of the experiment. Six-month-old 2N (CD, $n = 13$; CD + L-DOPS, $n = 11$) and Ts65Dn (CD, $n = 10$; CD + L-DOPS, $n = 9$) mice were used for this study. Because in all behavioral testing(s) we were able to reproducibly detect deficits in contextual learning Ts65Dn mice, the lack of difference in cued learning between 2N and Ts65Dn mice could not be due to insufficient statistical power.

Nesting behavior analysis. The nesting experiment was performed with 10 pairs of 6-month-old male Ts65Dn (CD, $n = 10$; CD + L-DOPS, $n = 10$) and 2N (CD, $n = 10$; CD + L-DOPS, $n = 10$) mice. The nesting behavior was analyzed in three different periods (sham, drug, and no treatment) (see Supplementary Material for details).

Statistical methods for behavioral studies. The data were tested with one-way ANOVA between the genotypes. The significance of genotype effects was confirmed by Student's *t* test and a nonparametric Mann-Whitney test; χ^2 distribution was used comparing frequency distributions. All data in the study are presented as means ± SEM. We performed statistical analyses with Statistica software (version 6.0).

Norepinephrine and L-DOPS determination

Tissue preparation. Mice were deeply anesthetized with sodium pentobarbital (200 mg/kg intraperitoneally) and brains were extracted immediately. Mice aged 4.5 months ($n = 4$ pairs of 2N and Ts65Dn) and 18 months (2N, $n = 8$; Ts65Dn, $n = 6$) were used for this study.

High-performance liquid chromatography method. An ultraviolet high-performance liquid chromatography machine (Varian) equipped with an autosampler and two pumps was used for this study (*65*, *66*). See the Supplementary Material for details.

Electrophysiological recordings

Three- and 6-month-old 2N and Ts65Dn mice were anesthetized and decapitated. The brain was quickly removed and immersed for 2 to 3 min in ice-cold artificial CSF (ACSF). The hippocampus was extracted and cut in ice-cold ACSF with a vibratome into 350-μm-thick transverse slices, which were allowed to recover in oxygenated ACSF before experimental recordings.

SUPPLEMENTARY MATERIAL

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Materials and Methods
References
Fig. S1. Photographs showing nesting in 2N and Ts65Dn mice.
Fig. S2. VMAT2 staining in the inner molecular layer of the dentate gyrus in 3- and 6-month-old mice.
Fig. S3. Recorded responses from the granule cell layer after stimulation of the middle molecular layer.
Fig. S4. The effects of releasing (10 μM) isoproterenol (ISO) in the middle molecular layer of dentate gyrus on changes in population spike amplitude of field EPSPs.
Fig. S5. NE concentrations in 6-month-old L-DOPS–treated mice.

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Competing interests: A.S., W.C.M., and J.V. have submitted a U.S. patent application related to this work and entitled "Method of improving cognitive functions in individuals with Down's syndrome and Alzheimer's disease."

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