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Selective loss of noradrenaline exacerbates early cognitive dysfunction and synaptic deficits in APP/PS1 mice

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Abstract

Background—Degeneration of the locus ceruleus (LC), the major noradrenergic nucleus in the brain, occurs early and is ubiquitous in Alzheimer's disease. Experimental lesions to the LC exacerbate AD-like neuropathology and cognitive deficits in several transgenic mouse models of AD. Because the LC contains multiple neuromodulators known to affect A β toxicity and cognitive function, the specific role of noradrenaline (NA) in AD is not well understood.

Methods—To determine the consequences of selective NA deficiency in an AD mouse model, we crossed dopamine β -hydroxylase (DBH) knock-out mice with APP/PS1 mice, overexpressing mutant amyloid precursor protein and presenilin-1. DBH (–/–) mice are unable to synthesize NA but otherwise have normal LC neurons and co-transmitters. Spatial memory, hippocampal long-term potentiation (LTP), and synaptic protein levels were assessed.

Results—The modest impairments in spatial memory and hippocampal LTP displayed by young APP/PS1 or DBH(–/–) single mutant mice were augmented in DBH(–/–)/APP/PS1 double mutant mice. Deficits were associated with reduced levels of total Ca²⁺/calmodulin-dependent protein kinases II (CaMKII) and *N*-Methyl-D-aspartate receptor 2A (NR2A), increased *N*-Methyl-D-

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aspartate receptor 2B (NR2B) levels and were independent of A β accumulation. Spatial memory performance was partly improved by treatment with the NA precursor drug L-threo-DOPS.

Conclusions—These results indicate that early LC degeneration and subsequent NA deficiency in AD may contribute to cognitive deficits via altered levels of CaMKII and *N*-Methyl-D-aspartate receptors, and suggest that NA supplementation could be beneficial in early AD.

Keywords

Locus ceruleus; noradrenaline; norepinephrine; memory; degeneration; pathogenesis; Alzheimer's disease; CaMKII

Introduction

Alzheimer's disease (AD) is the most common cause of dementia. Despite great progress in the description of the classical pathologies, the early and initiating events that cause A β plaque deposition and neurofibrillary tangle formation remain elusive.

The brainstem LC projects widely and serves as the chief source of noradrenaline (NA) in the forebrain, where it modulates arousal, selective attention, learning and memory, stress reactivity, behavioral adaptation, sleep architecture and inflammation (1).

It has been known since the 1960's that LC degeneration is an early event of AD pathogenesis (2; 4; 6), and loss of LC neurons has even been observed in patients suffering from mild cognitive impairment (MCI) that are at high risk for developing AD over the next decade (8; 10). In fact, emerging evidence indicates that the LC is the site of the earliest detectable AD-like neuropathology in the brain (10; 13). In AD, NA concentrations in LC projection areas have been shown to be reduced (15; 17). LC neuron loss correlates with A β plaque and neurofibrillary tangle load as well as severity of dementia (11; 15; 19), and is more severe than and corresponds better to AD progression than degeneration of the cholinergic nucleus basalis of Meynert (21). Surviving LC neurons appear to undergo profound compensatory changes, including the upregulation of NA biosynthetic enzymes and dendritic and axonal sprouting (23). Interestingly, recent studies have reported that a putatively functional single nucleotide polymorphism in the NA biosynthetic enzyme, DBH, is associated with a reduction in plasma DBH activity and AD (2; 25; 27).

AD-like neuropathology and cognitive deficits in several transgenic mouse models is exacerbated by the chemical ablation of the LC using the neurotoxin *N*-[2-chloroethyl]-*N*-ethyl-2-bromobenzylamine (DSP-4) (3; 5; 7; 9; 12). However, LC neurons synthesize and release a vast array of neuromodulators in addition to NA, some of which are implicated in AD such as galanin and brain-derived neurotrophic factor (BDNF) (14; 16; 18). Thus, DSP-4 lesions cannot isolate the contribution of NA loss itself to AD-like neuropathology and to cognitive deficits. In addition, the brain's response to the acute neurotoxic insult itself may be different from the etiology of LC and NE loss in AD patients. Thus, despite the wealth of knowledge about the physiological relevance of the LC and the degree of its loss in neurodegenerative disorders, the consequences of specific NA loss in AD remain unclear (20). In this study we examined the behavioral consequence of NA loss in an APP over-

expression model of AD to look for relevant interactions of potential interest in understanding human AD. For that we crossbred APP/PS1 mice with DBH ($-/-$) mice that specifically lack NA but have normal LC neuron number, distribution of LC fibers, and LC co-transmitter levels (18; 22; 26; our unpublished data), and analyzed them for AD-like phenotypes, including A β levels, spatial memory performance. Additionally, we assessed synaptic plasticity and levels of learning-associated, postsynaptic proteins in synaptosomal preparations, including Ca²⁺/calmodulin-dependent protein kinases II (CaMKII) and *N*-Methyl-D-aspartate receptor 2A (NR2A), increased *N*-Methyl-D-aspartate receptor 2B (NR2B).

Methods

Animals

Hemizygous double transgenic mice APP_{swe}/PS1 E9 were maintained on a C57BL/6J background (28). DBH ($-/-$) mice were generated by targeted disruption of the DBH locus by replacing 3.4 kb of genomic sequence with a neomycin-resistance cassette and were on a 129/SvEv background. APP/PS1 males were bred to DBH ($+/-$) females to generate all the litters used in this study. DBH ($+/-$) mice were used as controls because they have normal catecholamine levels and are phenotypically indistinguishable from wild-type animals (24; 29-31). Similar DBH ($+/-$)/APP/PS1 mice were used as a control for the DBH ($-/-$)/APP/PS1 mice. Prevention of high fetal lethality in DBH ($-/-$) mice was done as previously described (29). Mice were housed in groups under standard conditions at a temperature of 22°C (\pm 1°C) and a 12 hour light/dark cycle with free access to food and tap water. Animal care and handling were performed according to the declaration of Helsinki and approved by local ethical committees. Mice were deeply anaesthetized with isoflurane and sacrificed.

Drugs and treatments

L-threo-dihydroxyphenylserine (DOPS) was generously provided by Dainippon-Sumitomo Pharmaceutical Company, Ltd. (Osaka, Japan). DBH ($-/-$) and DBH ($-/-$)/APP/PS1 mice were injected with DOPS solution (500 mg/kg, s.c.) supplemented with benserizide and ascorbic acid as previously described (29; 32). DBH ($+/+$) and DBH ($+/+$)/APP/PS1 mice were treated with vehicle solution. N-(2-Chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (dsp4; Sigma, #C8417) treatment was performed in a subgroup of DBH($+/-$)/APP/PS1 and DBH($-/-$)/APP/PS1 mice at days 1 and 7 at 50 mg/kg at 6 month of age. Thirty min before application of DSP4, animals received fluoxetine (10 mg/kg) in order to protect serotonergic fibers.

NA quantification

Brains were dissected and washed in ice-cold PBS. Specific brain regions were dissected and frozen on dry ice. Samples were homogenized in sample buffer (0.1 M perchloric acid, 0.01% EDTA and 0.08 ng/ μ l 3,4-dihydroxybenzylamine – used as an internal standard) and subjected to two rounds of centrifugation (14,000 \times g, 20 min each, 4 °C). The supernatant was injected onto a C18 reverse-phase column (Spherisorb ODS-I 5 μ m, 250 \times 4.6 mm; Techlab). The isocratic mobile phase consisted of 0.15 M chloroacetic acid, 0.2 mM EDTA,

0.86 mM sodium octyl sulfate, 6% acetonitrile and 2.5% tetrahydrofuran, pH 3. The flow rate was 1.2 ml/min. NA was detected by an electrochemical detector (Coulochem II, Chelmsford, MA).

In-situ hybridization for tyrosine hydroxylase

In-situ hybridization (ISH) was performed as previously described by Yaylaoglu *et al.* (7). The following TH-specific (NM_009377) PCR primers were used: forward 5'-GATTGCAGAGATTGCCTTCC- 3' and reverse 5'-CCTGTGGGTGGTACCCTATG-3'. The Agilent's 2100 Bioanalyzer was used for both quality control and quantification of RNA probes.

Long-term potentiation in vitro

Electrophysiological recordings were performed on brain slices from 4 month-old mice (Figure 1A). Brains were sliced sagittally in 400 μ m sections using a vibratome and maintained at 29°C in an oxygen-enriched atmosphere in artificial cerebrospinal fluid (aCSF) (containing in mM: 124 NaCl, 4 KCl, 1.24 NaH₂PO₄, 1.3 MgSO₄, 26 NaHCO₃, 10 D-glucose, and 1 CaCl₂) for 30 min, followed by incubation in aCSF, 2 mM CaCl₂ for 30 min. Slices were moved to an interface-type recording chamber. Extracellular field excitatory postsynaptic potentials (fEPSPs) were recorded in the pyramidal cell layer of the CA1 region using glass microelectrodes (resistance 0.7 – 2 M Ω) filled with aCSF. The stimulating electrode was placed in the CA2 region within the Schaffer collaterals. Stimuli were applied at constant current, at duration of 0.1 ms. The initial slope of evoked fEPSPs was measured as an index of synaptic strength. Basal synaptic transmission was assessed by plotting the stimulus intensity (mA) against the peak slope of the evoked fEPSP to generate input-output relations. For long term potentiation (LTP) experiments, a 20 min baseline was recorded with an interpulse interval of 1 min at a stimulus intensity that evoked a response of approximately 30% of maximum fEPSP. The LTP was induced by a theta burst consisting of 4 trains of 10 pulses at 100 Hz separated by 200 ms. Two-stimulus protocols with stimulus intervals of 30, 50, 75 and 100 ms at 30% maximal stimulation intensity were used to assess paired pulse facilitation (PPF).

Behavioral studies

Mice were single housed in the testing room under a reversed light/dark cycle for at two weeks prior to the start of behavioral experiments. Tests were performed during the dark phase. Equal numbers of males and females at the age of 4 months were used (Figure 1A). As no differences between genders were observed, data were combined. Behavior was scored by a blinded observer.

Open field exploration (OF)

Mice were placed in the center of a lit (20-30 lux) chamber of the open field apparatus (61x61x61cm). Animals were tracked by an automatic monitoring system (Noldus Ethovision, The Netherlands) for 5 min. The area was virtually divided into a center (40 cm edge lengths), a corridor (7.5 cm along the walls) and four corner squares (10 cm edge lengths), which partly overlapped with the corridor area. The time spent in each area and

frequency of urination and defecation were monitored. The experiment was repeated on three consecutive days.

Morris water maze test (MWM)

The test was conducted in a pool consisting of a circular tank (1 m diameter) filled with opacified water at 24 °C. The water basin was dimly lit (20-30 lux) and surrounded by a white curtain. The maze was virtually divided into four segments (quadrants). In one of the quadrants, a hidden platform (15x15 cm) was present 1.5 cm below the water surface. Mice were trained to find the platform, using three external cues placed asymmetrically as spatial references. They were let into the water in a quasi-random fashion to prevent strategy learning. Mice were allowed to search for the platform for 40 s; if the mice did not reach the platform in the allotted time, they were placed manually on it. The mice were allowed to stay on the platform for 15 s before the next trial was started. Mice received 4 trials per day for 8 consecutive days. Movements of the mice were recorded by a computerized tracking system (Noldus, The Netherlands). On day 9 the mice were tested for latency to find a visible platform.

Brain lysate extraction

Brains were extracted from 4 and 12 month-old mice (Figure 1A) as previously described (9).

Synaptosomal preparation

After brain dissection, cerebellum and brainstem were removed and the forebrain was immediately homogenized in 9 vol. of forebrain weight in 0.32 M sucrose buffer (50 mM Tris acetate, 1 mM EDTA, 5 mM pyrophosphate, 5 mM NaF, 2 mM Na₂VO₄, 1 mM PMSF, 1:500 diluted proteinase inhibitor cocktail, pH 7.4). The homogenization was performed by 15 slow up and down strokes with a teflon-glass homogenizer (0.15 mm clearance) at 700 rpm. Cellular debris and nuclei were pelleted by centrifugation at 800 g for 5 min. The supernatant was layered on top of a discontinuous sucrose density gradient consisting of 1.4 M and 1.0 M sucrose buffer. The gradient was centrifuged at 54,000 × g for 90 min and the synaptosomal fraction was removed from the interface of the 1.4 M and 1.0 M sucrose layers. The resultant fraction was diluted with H₂O and sedimented at 17,000 × g for 15 min. The supernatant was removed and the synaptosomal pellet was stored at -80°C.

Western blot analysis

Protein concentration in the RIPA soluble and synaptosomal fraction was determined using the BCA Protein Assay kit (Pierce). Protein samples (10 – 50 µg) were separated by 4-12% NuPAGE (Invitrogen, Karlsruhe, Germany) using MES or MOPS buffer and transferred to nitrocellulose membranes. For detection of Aβ, blots were boiled for 5 min in water. Aβ was detected using antibody 6E10 (Covance, Münster, Germany), APP and CTF using antibody C-terminal 140 (gift from J. Walter, Bonn), and PS1 using antibody PS1-NT (Calbiochem). Tubulin (antibody E7; DSHB) was used as a loading control. In synaptosomal fractions protein levels of CaMKII (Santa Cruz), CaMKII phosphorylation (R&D Systems), ERK2 (Santa Cruz), GluR1 (Millipore), GluR1 phosphorylation (Millipore), NR1 (BD

Pharmingen), NR2A (Millipore), NR2B (Millipore) and phosphorylation of NR2B (Calbiochem) were assessed. RabGDP dissociation inhibitor (GDI) (Synaptic Systems) was used as an internal control. Primary antibodies were detected with horseradish peroxidase-conjugated secondary antibodies. Immunoreactivity was detected by enhanced chemiluminescence reaction (Millipore, Schwalbach) and luminescence intensities were analyzed using a Chemidoc XRS (Biorad, München, Germany).

Sandwich ELISA for A β ₁₋₄₀ and A β ₁₋₄₂

Quantitative determination of A β ₁₋₄₀ and A β ₁₋₄₂ from brain extracts was performed using the human Amyloid A β ₁₋₄₀ and A β ₁₋₄₂ ELISA kit (The Genetics Company, Switzerland) according to the manufacturer's instructions.

Statistical analysis

Behavioral data of the MWM test were analyzed by two-way ANOVA with repeated measurements (independent between subjects variables: APP/PS1 transgene, DBH (-/-) and DOPS treatment, dependent between subjects variables: latency or distance, independent within subject variable: day). All other experimental data were analyzed by two-way ANOVA unless stated otherwise. Student's t-test for intergroup comparison were performed subsequently. Statistical evaluation was done with SPSS 17 and GraphPad Prism 5.02 (San Diego, CA).

Results

Loss of noradrenergic innervation by DBH

DBH gene deficiency resulted in NA levels below our limit of detection in all adult brain regions tested (Figure 1B and data not shown). Lack of DBH had no effect on LC topology or LC size in the presence and in the absence of the APP/PS1 transgenes (Figure 1C). Our findings are in agreement with previous reports (22; 24; 26; 30; 34-36).

NA depletion impairs long term but not short term synaptic plasticity in CA1

Because hippocampal synaptic plasticity is crucial for spatial memory formation, we recorded long term potentiation (LTP) of CA1 neurons *in vitro* in the single and double mutant mice. Schaffer collaterals were stimulated and fEPSP were recorded in the pyramidal cell layer. Peak slopes of fEPSP recordings, averaged over a time course 5 min before (baseline), and after LTP induction were recorded (Figure 2 A,B). Early and late stages of LTP were assessed through responses at 10 min (T1) and 60 min (T2), respectively. APP/PS1 and DBH (-/-) single mutant mice showed a 50% reduction in the early (T1) and 58% reduction in the late (T2) LTP responses, as compared to DBH (+/-) mice. The ability to form LTP was nearly absent in the DBH (-/-)/APP/PS1 double mutant mice (reduction T1=80%, T2=79%) (Figure 2B). Factors were additive without interaction as shown by two-way ANOVA. Next, short term synaptic plasticity was assessed through paired pulse facilitation (PPF). PPF recorded in CA1 with interstimulus intervals of 30, 50, 75 and 100 ms was similar in all genotypes tested (Figure 2C). Furthermore, PPF was the same before and after LTP induction, suggesting a postsynaptic mechanism for LTP modulation.

NA deficiency modifies plasticity related synaptosomal proteins

To further analyze synaptic alterations of APP/PS1 mice modulated by NA deficiency, synaptic fractions were analyzed for potential changes in learning-associated proteins (Figure 2D). Interestingly, we found that the 60 kDa (β) subunit of CaMKII was downregulated in DBH ($-/-$)/APP/PS1 mice. In parallel, NMDAR subunit composition was altered by DBH deficiency. The NR2A subunit of the NMDA receptor was decreased in DBH ($-/-$), DBH ($+/-$)/APP/PS1, and DBH ($-/-$)/APP/PS1, while the NR2B subunit was upregulated in DBH ($-/-$)/APP/PS1 mice. Other proteins tested, including the 50 kDa (α) subunit of CamKII, phosphorylated forms of CamKII, extracellular-signal-related kinase (ERK) 2, glutamate receptor 1 (GluR1)- or NR1 subunit as well as phosphorylation levels of GluR1 at Ser 845 or of NR2B at Tyr 1472, were unaffected (data not shown).

Genetic NA depletion augments cognitive deficits in APP/PS1 mice

In order to correlate the above changes of hippocampal synaptic plasticity to spatial memory performance, the MWM test was performed (Figure 3 A-D, Table 1). All mice learned to find a hidden platform by spatial navigation. However, both APP/PS1 and DBH($-/-$) mice had increased escape latencies and longer distances to platform as compared to controls, and the combination of NA deficiency and the presence of APP/PS1 produced the greatest spatial memory impairment. In the OF test, DBH ($-/-$) caused a strong reduction in horizontal but not vertical activity, as described previously (38-40), while presence of the APP/PS1 transgene induced a slight increase in locomotor activity (Figure 3E). No differences in time spent at the center were observed between genotypes, suggesting that NA deficiency does not influence anxiety-like behavior in this paradigm (Figure 3F), as shown previously (Marino et al., 2005; Schank et al., 2008).

DOPS partially rescues spatial memory impairment induced by NA depletion

To investigate whether acute NA restoration rescues spatial memory impairment in APP/PS1 mice with DBH deficiency, the NA precursor DOPS was administered with the peripheral aromatic acid decarboxylase (AADC) inhibitor benserazide to restrict NA production to the brain. Controls received vehicle injections. Daily MWM training was performed 5 hours after DOPS treatment, a time when cerebral NA levels peak in DBH ($-/-$) mice (Thomas et al., 1998). DOPS treatment reduced the latency of DBH ($-/-$)/APP/PS1 mice compared to untreated double mutants, but animals still performed worse than DBH ($+/-$)/APP/PS1 mice. DOPS also tended to reduce distance travelled to platform, but the effects were not significant.

APP processing is not influenced by DBH ($-/-$)

A β peptides have been reported to impair synaptic function, LTP and memory performance (Selkoe, 2008), and we have previously shown that LC ablation by the neurotoxin DSP-4 increases A β levels (3). We analyzed A β levels at 4 months (prior to A β deposition in APP/PS1 mice) and at 12 months of age (an age at which A β deposition is already high in APP/PS1 mice). In all genotypes lacking the APP/PS1 transgenes, endogenous APP were detected (Figure 4). DBH ($-/-$) did not alter levels of APP, CTFs, presenilin-1 or SDS-soluble A β in APP/PS1 mice (Figure 4). These results suggest that the increase in A β levels

observed previously in DSP-4 treated mice is not caused by a specific loss of NA per se. To determine whether neurotoxic lesion of LC neurons could still exacerbate AD-like neuropathology, even in the absence of NA, we treated DBH (+/-)/APP/PS1 and DBH (-/-)/APP/PS1 mice with DSP-4 at 6 month of age. Analysis at 12 month of age revealed, as previously reported, that DSP-4 tended to increase A β in single mutant APP/PS1 mice. Surprisingly, DSP-4 also increased total A β levels in double mutant mice (Figure 5A and B). This was verified for soluble and insoluble A β ₁₋₄₀ and A β ₁₋₄₂ levels by ELISA (Figure 5C and D). These results suggest that LC lesions may exacerbate AD-like neuropathology even in the absence of NA.

Discussion

Early AD is associated with LC degeneration, but the causes and consequences have remained enigmatic. Transgenic mouse models of AD lack the rampant loss of neurons in the brain, suggesting that A β accumulation alone is insufficient to cause neurodegeneration *in vivo* (41). While some studies have reported a modest, late-onset decrease of LC neurons in APP transgenic models (42; 43), others failed to detect the LC or NA loss characteristic of human AD (3; 44). Modeling LC degeneration has been limited to the neurotoxin DSP-4, which has several disadvantages since it ablates NA and all LC co-transmitters, some of which (e.g. galanin, BDNF) have been implicated in AD and secondly, the physical damage to noradrenergic neurons induced by the DSP-4 could activate inflammatory brain mechanisms that modulate A β deposition. Finally, questions have arisen concerning the specificity and efficacy of DSP-4 for LC neurons and the compensatory responses of surviving LC neurons following the lesion (45-47). To isolate the effects of specific NA loss, we bred APP_{swe}/PS1 E9 mice with DBH (-/-) animals. Because DBH deficiency results in a complete lack of NA but leaves LC neurons and co-transmitters intact, any phenotype observed in these mice can be attributed to NA depletion alone (18; 22; 26; our unpublished data).

Spatial memory

While memory dysfunction defines early AD, most APP transgenic mice are only mildly impaired. Since NA facilitates many stages of memory (1), we hypothesized that specific NA dysfunction would exacerbate cognitive deficits in APP/PS1 mice. At an age (4 months) prior to plaque deposition, mice carrying the APP/PS1 transgene or depleted of NA were modestly deficient in the MWM test, while the combination had an additive effect and robustly impaired spatial memory. The pre-plaque spatial memory loss caused by APP overexpression is likely caused by soluble A β oligomers (48-52). Although peripheral catecholamines have been implicated in learning and memory (53), the effects of DBH (-/-) on memory were centrally mediated, since hippocampal LTP was similarly impaired and selective restoration of NA to the brain partially rescued the water maze deficit in the double mutants.

Synaptic plasticity

DBH (-/-) or the presence of APP/PS1 each individually partially impaired LTP, but LTP suppression was strongest by combining APP/PS1 with NA depletion. These results mirror

our findings in the MWM and provide the electrophysiological underpinnings for the cognitive deficits. While high-frequency stimulation (HFS)-induced LTP in the dentate gyrus is generally enhanced by NA or β -adrenergic receptor (β AR) agonists (54), the effects of NA on CA1 LTP are more complicated and sometimes contradictory. Some studies have shown that NA increases LTP via a β AR-dependent mechanism (54-56), while other found no effect of β AR blockade or NE depletion (32; 57). These discrepancies may be attributed to different protocols used, specific brain region surveyed (e.g. stratum radiatum vs. pyramidal cell layer), and readout analyzed (e.g. EPSP slope vs. population spike amplitude). Our results indicate a critical role for NA in synaptic plasticity, particularly in the presence of AD-like neuropathology.

A β accumulation

We and others have shown that acute reductions of forebrain NA levels using DSP-4 increased A β deposition. Surprisingly, we did not observe changes of A β in DBH(-/-)/APP/PS1 mice. By contrast, administration of DSP-4 to DBH(-/-)/APP/PS1 mice markedly increased soluble and insoluble A β 40 and 42. There are several ways to explain how DSP-4 can affect mice that already lack NA. First, the increase in A β deposition following DSP-4 lesions could be due to the loss of LC co-transmitters. However, we do not favor this explanation because APP/PS1 mice carrying a mutation in *Ear2* that prevents the development of ~70% of the LC neurons also do not have increased A β (Figure S1). Second, it is possible that compensatory mechanisms that prevent the increase in A β are engaged during postnatal development in DBH (-/-)s. Third, although DSP-4 may possess neurotoxic properties completely independent from LC neurons, this is unlikely given the extensive literature on its specificity and our protection of serotonergic neurons with a SERT inhibitor. The explanation we favor is that the process of LC neuronal cell death itself may trigger brain responses, such as inflammation, that potentiate A β deposition and/or impair clearance. An important remaining question is whether LC neuron degeneration in clinical AD resembles DSP4-induced damage and triggers and/or exacerbates neuropathology in LC projection regions, or whether the consequences of LC loss in AD are restricted to the deterioration of cognition and are unrelated to A β levels.

Synapse composition

Because the detrimental effects of NA depletion on spatial memory and synaptic plasticity appeared to be independent of A β levels or changes in presynaptic function, we evaluated the levels of learning-associated, postsynaptic proteins in synaptosomal preparations. DBH (-/-) decreased CaMKII subunit levels in APP/PS1 mice, with a greater effect on the β subunit, while CaMKII phosphorylation levels remained unaffected. These differences may have direct functional consequences, as CaMKII is a critical component of long-term plasticity and spatial memory (58).

NMDARs are tetramers composed of two obligatory NR1 subunits and 2 other subunits, mostly hetero- or homomeric NR2A and NR2B (59), and are regulated by CaMKII. DBH (-/-) decreased NR2A levels and increased NR2B levels, while NR1 levels remained unchanged. These data imply that while NA depletion alters the composition of NMDA receptors, which is critical for gating and conductance. NR1/NR2B channels have greater

conductance (59) and a higher affinity for CaMKII than NR2A containing receptors (58). Interestingly, impaired LTP in CaMKII mutant mice was accompanied by a selective reduction of NR2A at the synapse without effecting NR2B localization (60). Furthermore, A β oligomers inhibit LTP by increasing activation of NR2B-containing receptors (61). We hypothesize that the LTP and memory deficits associated with NA depletion in APP/PS1 mice are caused, at least in part, by a reduction in CaMKII activity and a shift from NR2A-containing NMDARs to NR2B-containing NMDARs. A reduction of NR1 and phosphorylated CaMKII levels were found in the frontal cortex and HC of AD brains (62), suggesting that LC degeneration and NA depletion may contribute to CaMKII signaling and NMDA receptor function alterations in AD.

Summary

Our results suggest that LC degeneration and NA depletion, as are observed in MCI and early AD, contribute to cognitive impairment independent of A β plaque formation by impairing synaptic integrity and plasticity. These consequences may be mediated by alterations in synapse composition and function, such as changes in CaMKII and NMDAR subunits. No existing AD therapies take LC degeneration and NA loss into account. We found that DOPS, a compound used to treat congenital DBH deficiency and neurogenic orthostatic hypotension (63), partly reversed cognitive impairment induced by NA depletion in a mouse model of AD. Thus, NA supplementation may also help alleviate cognitive and behavioral dysfunction in early MCI and AD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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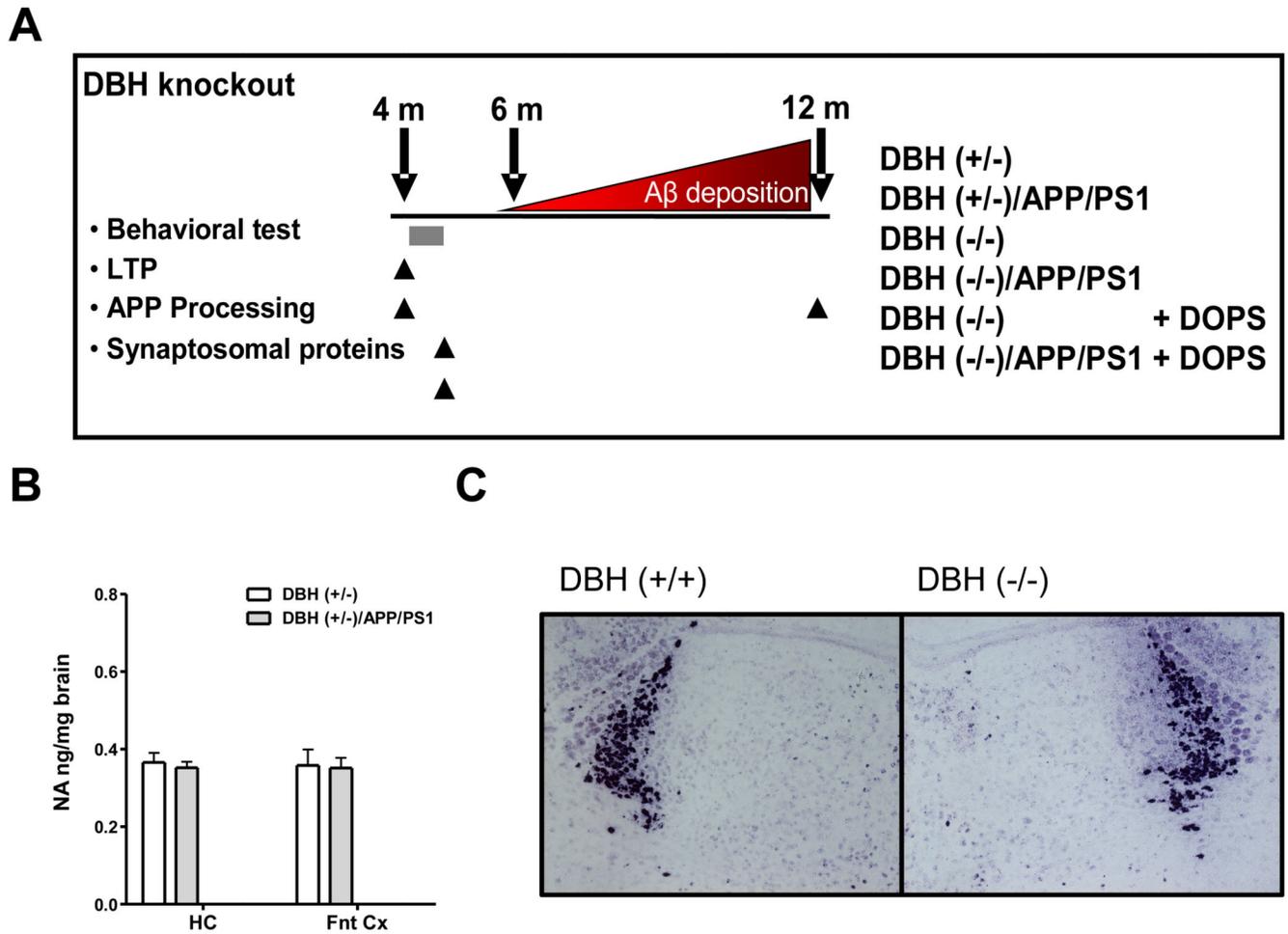


Figure 1. DBH (-/-) depletes NA but leaves LC neurons intact

A) Experimental time course. DBH(+/-) or (-/-) mice, with or without APP/PS1, were tested for spatial memory performance by the MWM test at the age of 4 months (pre-plaque deposition stage). Brains were subsequently removed for analysis. LTP was assessed in another subset of mice of identical age. APP processing was assessed at 4 and 12 months of age. Learning associated proteins in the forebrain were determined subsequent to behavioral testing. Mice were treated with DOPS to investigate if NA supplementation could rescue the spatial memory impairment. **B)** HPLC assessment of NA level. In DBH(-/-) and DBH(-/-)/APP/PS1 mice, the level of NA is below the limit of detection in the hippocampus (HC) and frontal cortex (Fnt Cx). **C)** Tyrosine hydroxylase (TH) in-situ hybridization in the LC of DBH(+/-), DBH(-/-), DBH (+/-)/ APP/PS1, and DBH (-/-)/ APP/PS1 mice mice.

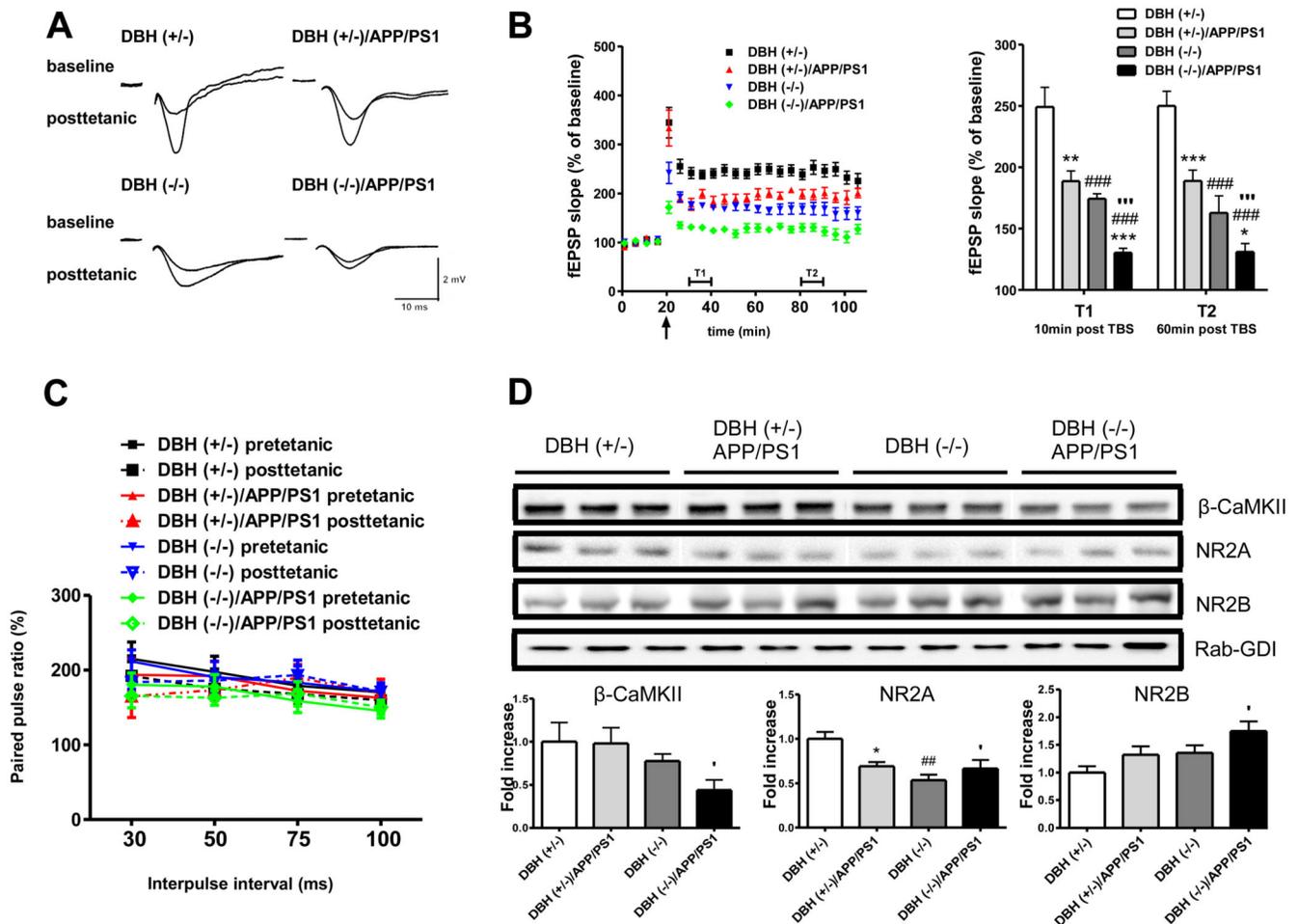


Figure 2. NA depletion impairs CA1 hippocampal long term potentiation and synapse composition in APP/PS1 transgenic mice

A) and **B)** Long term potentiation. Representative fEPSP response obtained before and after LTP-inducing stimulus. Lower plot depict fEPSP slope as percentage of baseline as the mean of 5 min. APP/PS1 and DBH(-/-) single mutant mice are impaired in the early and late phase of LTP. The most aggravated impairment is found in DBH(-/-)/APP/PS1 mice. **(B, right panel)** Quantification of the magnitude of LTP. The fEPSP slope as percentage of the baseline is depicted at time point 1, directly after decay of posttetanic potentiation, and time point 2, 60 min after LTP induction ($n=5$ animals per group, Student's t-test: DBH(+/-) versus DBH(+/-)/APP/PS1 ** p 0.01 for T1, *** p 0.001 for T2; DBH(+/-) versus DBH(-/-) ### p 0.001 for T1 and T2; DBH(-/-) versus DBH(-/-)/APP/PS1 *** p 0.001 for T1, * p 0.05 for T2; DBH(+/-)/APP/PS1 versus DBH(-/-)/APP/PS1 ### p 0.001 for T1 and T2; DBH(+/-) versus DBH(-/-)/APP/PS1). **C)** Paired pulse facilitation. The ratio of paired fEPSP at inter-pulse intervals of 30, 50, 75 and 100 ms is not changed between the groups. LTP had no impact on the facilitation. **D)** Immunoblot of learning associated proteins in synaptosomal preparations of DBH(-/-) and DBH (+/-) mice on a wild-type or APP/PS1 background and densitometric analysis. Fold increase was calculated with respect to the expression of the specific target in the DBH(+/-) control group. The β -CaMKII is decreased by DBH (-/-) and strongly reduced in DBH (-/-)/APP/PS1 mice. NR2A is decreased by

both parameters APP/PS1 and DBH(-/-) while NR2B is increased by both (n = 4 per group of animals, Student's t-test for β -CaMKII: DBH(+/-)/APP/PS1 versus DBH(-)/APP/PS1 #p < 0.05; DBH(+/-) versus DBH(-)/APP/PS1 'p < 0.05; for NR2A and NR2B: DBH(+/-) versus DBH(-)/APP/PS1 'p < 0.05).

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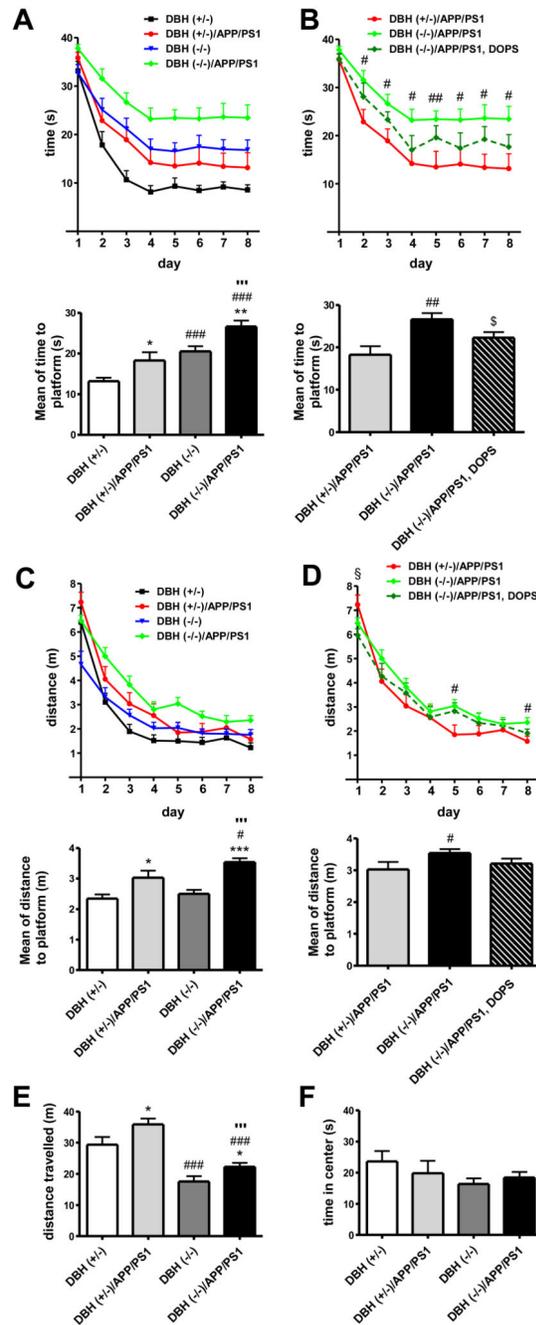


Figure 3. DOPS partially rescues spatial memory impairment induced by NA depletion
A) and **C)** MWM test. Spatial memory performance was assessed by the MWM test. Shown is the latency and distance to escape to a hidden platform on 8 consecutive days. Bar graphs represent the mean of time and distance to the platform for each genotype. APP/PS1 and DBH(-/-) single mutant mice show a mild memory dysfunction compared to the corresponding control group. The combination of NA deficiency and the APP/PS1 transgene causes the most profound cognitive impairment. (DBH: n 313 animals per group for Student' t-test results see table 1. **B)** and **D)** Morris water maze test after DOPS treatment. DOPS

treated DBH(-/-)/APP/PS1 mice improved their performance compared to the untreated groups (DBH: n³12 animals per group, Student's t-test for time over all days: DBH(+/-)/APP/PS1 versus DBH(-/-)/APP/PS1 ^{##}p 0.01; DBH(-/-)/APP/PS1 versus DBH(-/-)/APP/PS1 + DOPS ^{\$}p 0.05, distance over all days: DBH(+/-)/APP/PS1 versus DBH(-/-)/APP/PS1 [#]p 0.05. E) and F) Open Field exploration. The factor DBH(-/-) strongly decreased the horizontal activity (distance travelled) while the APP/PS1 transgene increased it. The time spent in the center of the apparatus was comparable in all genotypes. (DBH: n³13 animals per group: Student's t-test: DBH(+/-) versus DBH/APP/PS1 (+/-) *p 0.05; DBH(+/-) versus DBH(-/-) ^{###}p 0.001; DBH(-/-) versus DBH(-/-)/APP/PS1 *p 0.05; DBH(+/-)/APP/PS1 versus DBH(-/-)/APP/PS1 ^{###}p 0.001; DBH(+/-) versus DBH(-/-)/APP/PS1.

For all figures unless otherwise indicated: data points and bars represent mean + SEM. If an effect of the independent variables was found in the two-way ANOVA, Student's test for intergroup comparison were performed subsequently and results are depicted: * indicates an influence of factor APP/PS1 (control versus APP/PS1; KO versus KO/APP/PS1), # of factor KO (control versus KO; APP/PS1 versus KO/APP/PS1), ' of the combination of factors APP/PS1 and KO (control versus KO/APP/PS1). *p£0.05; **p£0.01 and *** p£0.001.

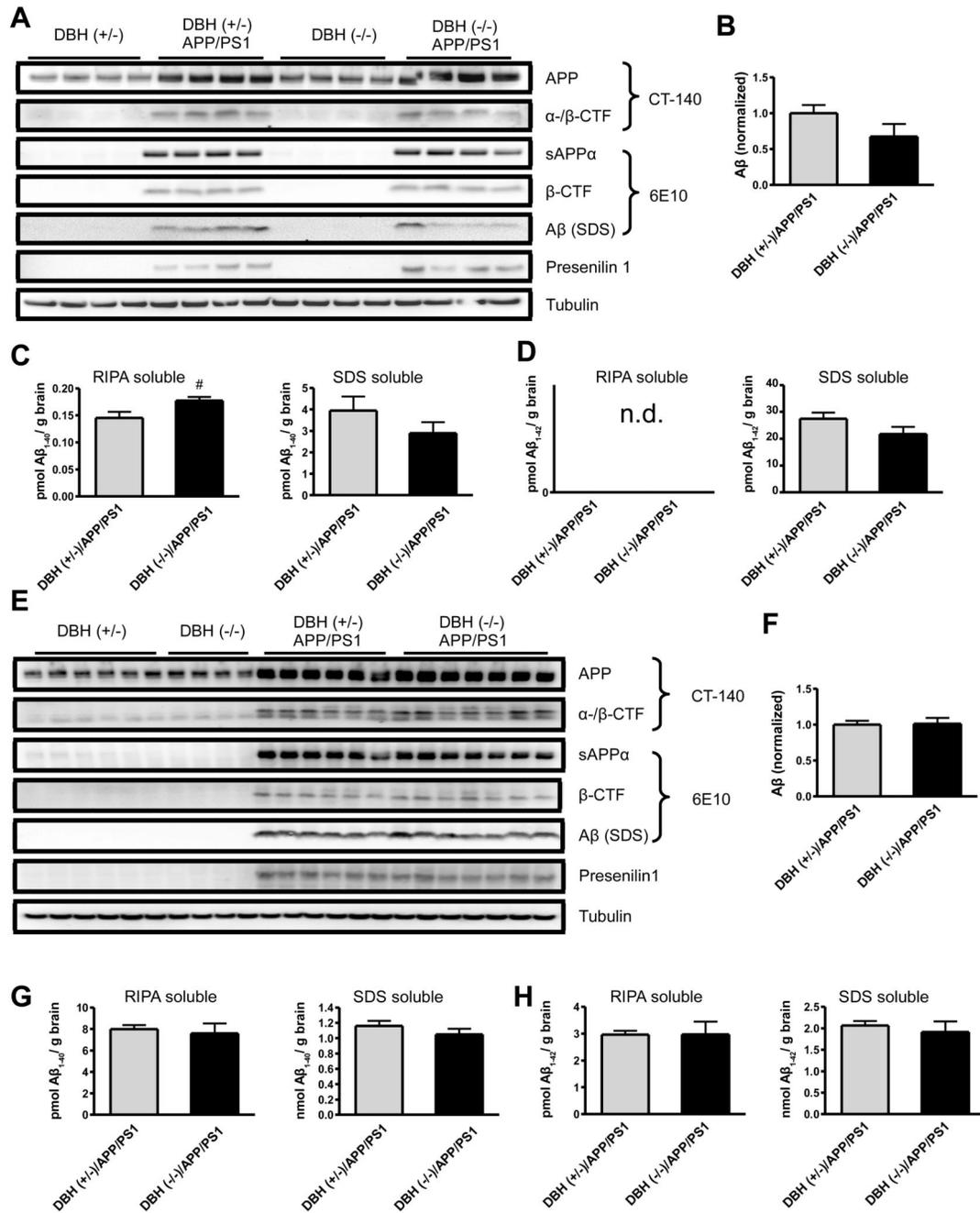


Figure 4. APP processing and A β burden are not exacerbated by NA deficiency

A) Immunoblot of APP processing products. Forebrain extracts from 4-month old DBH(+/-), DBH(+/-)/APP/PS1, DBH(-/-) and DBH(-/-)/APP/PS1 were analyzed for APP processing by immunoblot analysis. Total APP, CTF, α -CTF, β -CTF, sAPP α , PS1 and A β levels are not changed by NA deficiency indicating that loss of DBH does not affect APP processing. **B)** Densitometric evaluation of A β immunoblots. Quantification of RIPA-soluble and RIPA-insoluble A β 1-40 (**C**) and RIPA-insoluble A β 1-42 (**D**) by sandwich ELISA. Forebrain extracts of DBH(-/-)/APP/PS1 mice show a slight increase of A β 1-40 in

the RIPA soluble fraction compared to DBH(+/-)/APP/PS1 mice. The absence of DBH has no effect on the RIPA insoluble A β level. (n=4 per group of animals, Student's t-test: DBH (+/-)/APP/PS1 versus DBH(-/-)/APP/PS1 #p = 0.05). **E**) Immunoblot of APP processing products from 12 month-old mice. Total APP, CTF, α -CTF, β -CTF, sAPP α , PS1 and A β levels are not changed by NA deficiency. **F**) Densitometric evaluation of A β on immunoblots. Quantification of RIPA-soluble and RIPA-insoluble A β 1-40 (**G**) and A β 1-42 (**H**) by sandwich ELISA. The absence of DBH does not alter A β 1-40 and A β 1-42 levels (n = 4 per group of animals).

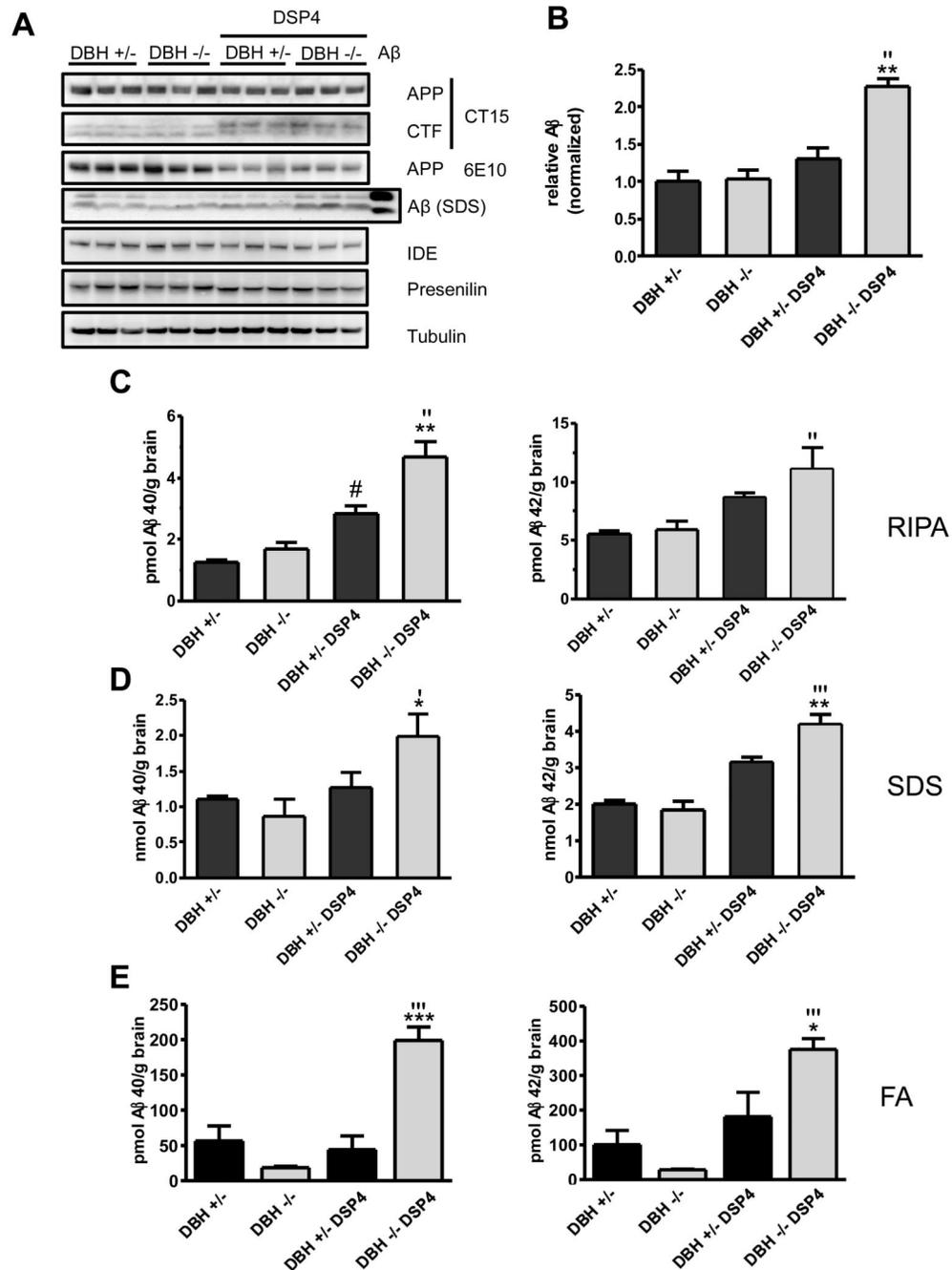


Figure 5. DSP-4 treatment increases A β burden in the absence of NA

DBH (+/-) or (-/-) mice carrying the APP/PS1 transgene were treated with the LC neurotoxin N-(2-Chloroethyl)-N-ethyl-2-bromobenzylamine hydrochloride (DSP4) or vehicle. (A) Immunoblot analysis and densitometric quantification (B) revealed that DBH(-/-)/APP/PS1 mice did not show any increase in A β levels unless they received an additional treatment with DSP4. Quantification of RIPA-soluble and RIPA-insoluble A β 1-40 (C) and A β 1-42 (D) by sandwich ELISA. (n = 4 per group of animals), *p 0.05, **p 0.05 DBH(-/-)

-)+DSP4 versus DBH(+/-)+DSP4; 'p 0.05, "p 0.01, ""p 0.001 DBH(-/-)+DSP4 versus DBH(-/-); #p 0.05 DBH(+/-)+DSP4 versus DBH(-/-).

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Table 1**Intersubject effects of two-way ANOVA with repeated measurements:**

Factor	F-value (time)	p-value (time)	F-value (distance)	p-value (distance)
DBH (-/-)	$F_{(1,53)} = 26.83$	###p 0.001	$F_{(1,53)} = 4.37$	#p 0.05
APP/PS1 (Sv129/C57BL6)	$F_{(1,53)} = 13.71$	***p 0.001	$F_{(1,53)} = 29.18$	***p 0.001

Intersubject comparison with Student's t-test:

Comparison	p-value (time)	p-value (distance)
DBH (+/-) versus DBH (+/-) / APP/PS1	*p 0.05	*p 0.05
DBH (-/-) versus DBH (-/-) / APP/PS1	**p 0.01	***p 0.001
DBH (+/-) versus DBH (-/-)	###p 0.001	p 0.05
DBH (+/-)/APP/PS1 versus DBH (-/-) / APP/PS1	###p 0.001	#p 0.05
DBH (+/-) versus DBH (-/-)/APP/PS1	''p 0.001	''p 0.001