A Small Molecule p75\textsuperscript{NTR} Ligand, LM11A-31, Reverses Cholinergic Neurite Dystrophy in Alzheimer’s Disease Mouse Models with Mid- to Late-Stage Disease Progression

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Abstract

Degeneration of basal forebrain cholinergic neurons contributes significantly to the cognitive deficits associated with Alzheimer’s disease (AD) and has been attributed to aberrant signaling through the neurotrophin receptor p75 (p75\textsuperscript{NTR}). Thus, modulating p75\textsuperscript{NTR} signaling is considered a promising therapeutic strategy for AD. Accordingly, our laboratory has developed small molecule p75\textsuperscript{NTR} ligands that increase survival signaling and inhibit amyloid-\(\beta\)-induced degenerative signaling in \textit{in vitro} studies. Previous work found that a lead p75\textsuperscript{NTR} ligand, LM11A-31, prevents degeneration of cholinergic neurites when given to an AD mouse model in the early stages of disease pathology. To extend its potential clinical applications, we sought to determine whether LM11A-31 could reverse cholinergic neurite atrophy when treatment begins in AD mouse models having mid- to late stages of pathology. Reversing pathology may have particular clinical relevance as most AD studies involve patients that are at an advanced pathological stage. In this study, LM11A-31 (50 or 75 mg/kg) was administered orally to two AD mouse models, Thy-1 hAPP\textsuperscript{Lond/Swe} (APPL/S) and Tg2576, at age ranges during which marked AD-like pathology manifests. In mid-stage male APPL/S mice, LM11A-31 administered for 3 months starting at 6–8 months of age prevented and/or reversed atrophy of basal forebrain cholinergic neurites and cortical dystrophic neurites. Importantly, a 1 month LM11A-31 treatment given to male APPL/S mice (12–13 months old) with late-stage pathology reversed the degeneration of cholinergic neurites in basal forebrain, ameliorated cortical dystrophic neurites, and normalized increased basal forebrain levels of p75\textsuperscript{NTR}. Similar results were seen in female Tg2576 mice. These findings suggest that LM11A-31 can reduce and/or reverse fundamental AD pathologies in late-stage AD mice. Thus, targeting p75\textsuperscript{NTR} is a promising approach to reducing AD-related degenerative processes that have progressed beyond early stages.

Introduction

Degeneration of basal forebrain cholinergic neurons (BFCN) and their neurites is a major contributing factor to the cognitive dysfunction associated with Alzheimer’s disease (AD). It precedes neuron loss and is associated with mild cognitive impairment, a predecessor of AD [1]. BFCN atrophy involves aberrant signaling through the neurotrophin receptor p75 (p75\textsuperscript{NTR}); p75\textsuperscript{NTR} is abundantly expressed by these neurons in adulthood [2] and its signaling can positively or negatively affect the condition of BFCNs depending on the presence of several factors including coreceptors and ligands [3,4]. For example, nerve growth factor (NGF) signaling through p75\textsuperscript{NTR} may promote cell survival or death depending on the presence of its high-affinity receptor TrkA [3,4]. Neuronal degeneration also occurs when amyloid-\(\beta\) (\(\text{A}\beta\))\textsuperscript{1–40} binds to p75\textsuperscript{NTR} [5,6]. Moreover, BFCNs were found to degenerate when \(\text{A}\beta\) oligomers were delivered to the brains of wild-type (WT) but not p75\textsuperscript{NTR} deficient mice [7], and this degeneration was prevented by functionally removing the neurotrophin-binding domain of the receptor in an AD mouse model [8]. Together, these observations indicate that p75\textsuperscript{NTR} signaling plays a necessary role in enabling \(\text{A}\beta\)-induced degeneration and implicates it as an AD therapeutic target [9,10,11].
Accordingly, our laboratories have developed small molecule, non-peptide ligands of p75NTR that promote survival-related signaling, inhibit Aβ-induced degenerative signaling, and reduce degeneration in Aβ-exposed neuronal cultures [10,12,13]. One such ligand, LM11A-31, is a water soluble amino acid derivative that reaches the CNS [14,15] and has structural and chemical features similar to the NGF loop 1 domain, which is known to interact with p75NTR [12]. LM11A-31 prevents BFCN atrophy and cognitive deficits in an AD mouse model when administration starts at early pathological stages of the disease, shortly after Aβ plaques appear [14,16]. A frequent occurrence in AD drug development is that compounds eliciting promising results in preclinical models fail to produce positive outcomes in clinical trials. One potential reason for this failing is that, in most preclinical studies, therapy is initiated in animals exhibiting little or no pathology or behavioral symptoms, while clinical studies of AD therapies involve symptomatic patients at more advanced pathological stages [17,18]. Until it is possible to reliably identify early stages of AD pathology in the clinical setting, a key strategy for preclinical testing of potential therapeutics will be to apply them to symptomatic animals with moderate to severe neuropathology which better corresponds to the clinical population. Therefore, this study examined whether LM11A-31 could arrest or reverse the degeneration of cholinergic neurites in two well characterized AD models, Thy-1 hAPP/+/Swe (APPL/S) and Tg2576 mice, with treatment beginning in mid- to late stages of disease progression characterized by the presence of abundant amyloid deposits, well established BFCN degeneration, and memory deficits apparent in multiple testing paradigms [14,16,19,20,21].

Materials and Methods

Ethics statement

All animal procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Studies were performed at Stanford University with protocols approved by its Institutional Animal Care and Use Committee and at the Palo Alto Veteran’s Administration Hospital with approval of its Committee on Animal Research. Protocols included efforts to minimize animal suffering and numbers of mice used.

AD mouse models

Two mouse lines were used in these studies: 1) Thy-1 hAPP transgenic line 41 C57BL/6 which over-express human amyloid precursor protein (APP) 751 [hAPP751] containing the London (V717I) and Swedish (K670M/N671L) mutations under the murine Thy1 promoter [19], which will herein be referred to as APP<sup>V717I</sup> and 2) Tg2576, which over-express the 695 amino acid human isofrom of APP with the “Swedish” mutation [22].

p75<sup>NTR</sup> ligand

LM11A-31 [2-amino-3-methyl-pentanoic acid (2-morpholin-4-yl-ethyl)-amide] is a water soluble isoleucine derivative (MW 243.3) for structure and detailed pharmacological characterization see Massa et al. (2006) and Knowles et al. (2013). LM11A-31 was custom manufactured by Ricerca Biosciences (Painesville, OH) at >97% purity, which was assessed by liquid chromatography/mass spectroscopy (LC-MS/MS) analysis.

LM11A-31 treatment

Both lines of transgenic mice and their gender- and age-matched non-transgenic (WT) littermates were randomly assigned to either vehicle or LM11A-31 treatment groups. For male APP<sup>V717I</sup> mice, treatment started at two different ages: 1) 6–8 month old mice were treated for 3 months prior to euthanizing at 9–11 months old ("mid-stage pathology" cohort), and 2) 12–13 month old mice were treated for 1 month and euthanized at 13–14 months of age ("late-stage pathology" cohort). For both age groups, LM11A-31 was dissolved in sterile water and administered daily by oral gavage at 50 mg/kg/day. This dose was chosen based on positive effects of the ligand on histological and behavioral endpoints in previous studies from our laboratory [14,16]. Furthermore, previous pharmacokinetic studies indicated that LM11A-31 (50 mg/kg) delivered daily by oral gavage for 2 weeks resulted in brain concentrations of 1904 nM [14], which greatly exceeds the in vitro effective dose [12]. Food was withheld 4 hours prior to dosing to aid in compound absorption. Vehicle-treated mice received an equivalent volume per weight of sterile water by the same route. Since LM11A-31 did not significantly affect any measure analyzed in the 6–8 month old WT mice, this group was not included in the study using 12–13 month old mice. Starting at the first week of treatment, APP<sup>V717I</sup> mice weighed significantly less than WTs at both of the age groups studied, as neuropathology is present at the ages that treatment was started [14,16,19] (Figure 1).

For treatment of Tg2576 mice, LM11A-31 was delivered via drinking water. To determine dosing, LM11A-31 was administered to C57BL/6 mice ad libitum in drinking water for 3 months at targeted doses of 10, 50, 75 and 100 mg/kg/day (n = 3–4 mice/dose). Brain concentrations of the ligand were determined by LC-MS/MS at Absorption Systems (Exton, PA; for methods see [14]). A targeted dose of 75 mg/kg/day produced a brain concentration of 256±66 nM. This concentration was higher than that resulting from 10 and 50 mg/kg/day doses and the concentration (100 nM) that provided neuroprotection in in vitro studies [13], but very similar to that seen with 100 mg/kg/day (Figure 2). Therefore, 75 mg/kg/day was selected as the target dose for the Tg2576 study. Female Tg2576 mice and their non-transgenic (nTg) littermates were allowed ad libitum access to drinking water containing LM11A-31 (0.6 mg/ml; targeted dose 75 mg/kg) for 3 months starting at 14 months of age. Water bottles were refilled every 3 days and were weighed before and after filling to estimate doses received. Starting from treatment week 6, Tg2576 mice weighed significantly less than nTg mice; LM11A-31 did not significantly affect the body weights of either genotype (Figure 3A). Tg2576 mice consumed significantly more water than nTg mice (Figure 3B) and because of their lower body weights those in the treatment group received higher mean daily doses of LM11A-31. Actual mean dose per treatment group in mg/kg/day was: 79 for nTg and 119 for Tg2576 mice.

Immunohistochemistry

After treatment, all mice were deeply anesthetized with avertin (2,2,2-tribromoethanol; 425 mg/kg) and perfused with heparinized saline. Brains were removed, post-fixed in 4% paraformaldehyde for 24 hours at 4°C, and then cryoprotected in 30% sucrose. Brains were sectioned (40 μm, coronal) using a freezing microtome. Free-floating sections (every 8th section in a 1 to 16th series) were processed for immunohistochemical localization of choline acetyltransferase (ChAT; 1:600; Millipore, Billerica, MA), as a marker for BFCNs, or p75NTR using an antibody that recognizes the extracellular domain of the receptor (1:800; Neuromics, Edina, MN). Sections were incubated in the Vectastain Elite ABC kit (Vector Laboratories, Burlingame, CA) solution and visualized with a diamino-benzidine substrate kit.
Amyloid plaques were labeled with 1% Thioflavin-S (ThioS) stain.

Microscopy and quantitative analysis

All immunostaining was visualized with a Leica DM 5000B or a Zeiss AxioImager M2 light microscope. Digitized images were acquired with the latter microscope using AxioVision software v4.7. Images for figures were acquired with a Zeiss AxioImager M2 light microscope using a 10, 20 or 63× oil objective and AxioVision software v4.7. All quantitative analyses were performed by experimenters that were blind to the treatment and genotype conditions.

In the basal forebrain, ChAT immunolabeling was viewed with a 40× objective and, using methods adapted from previous work [8,14,16], was evaluated in the vertical limb of the diagonal band of Broca (VDB), which was defined as the area below the anterior commissure (anterior part) at the rostro-caudal level of the islands of Calleja and before the emergence of the horizontal diagonal band (−Bregma 0.98 mm [23]). After this anatomical criteria was applied only one section qualified for analysis when every 8th section was processed. Brain sections were analyzed with NeuroLucida v8 image analysis software (MBF Bioscience) using Figure 1. Body weight is not affected by treatment with LM11A-31 in male APP/L5 mice. Body weights of (A) 9–11 month old male APP/L5 mice treated with LM11A-31 (50 mg/kg) for 3 months (WT Veh, n = 9 mice; WT-31, n = 10; APP Veh, n = 10; APP-31, n = 9) and (B) 13–14 month old male APP/L5 mice treated with LM11A-31 (50 mg/kg) for 1 month (WT Veh, n = 5 mice; APP Veh, n = 4; APP-31, n = 5). APP mice weighed significantly less than WT mice in both of the age groups examined. LM11A-31 did not affect body weight at either age or genotype. Statistical significance was determined using repeated measures ANOVA with Dunnett’s post-hoc test. doi:10.1371/journal.pone.0102136.g001

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an unbiased automated scanning procedure (Meander Scan) that allows fields within a user-defined contour to be seen without overlap. In every third field, all neurons, and processes originating from them, were manually traced while focusing in the z-plane throughout the section to accurately assess volume and branching and to clearly discern the origin and end of neurites emanating from a particular cell. The software program moves the field of view to track the neurite throughout the VDB and realigns the tracing ensuring that the neurite is traced in its entirety in the x- and y-planes. Neurolucida Branched Structure Analysis was used to compute the volume, length, surface area, and branching order of the neurites.

Using methods previously established in our laboratory [14], ChAT immunostaining and ThioS staining in the motor and primary somatosensory cortices (between ~Bregma 1.18–0.74 [23]) was visualized with a 10× objective and one digitized image was taken from each side of the brain in 3 sections per animal for a total of 6 fields/animal (sample field = 900 × 670 μm²). The same sample fields were analyzed for both stains. Clusters of ChAT-stained dystrophic neurites were manually traced by outlining their perimeter using Neurolucida v8 and the Branched Structure Analysis function was used to compute the area. ThioS staining was quantified from the digital images using histogram thresholding in Image Pro Plus v6.3 software (Media Cybernetics). The threshold was set manually to identify dense immunolabeling that was distinct from the background. The immunostained area was expressed as a percentage of the total area analyzed.

Figure 4. LM11A-31 normalizes increased p75NTR levels in the basal forebrain of late-stage APPL/S mice. Representative photomicrographs show p75NTR immunostaining in the vertical limb of the diagonal band of the basal forebrain in (A) WT Vehicle (Veh), (B) APP Veh, and (C) APP LM11A-31 (-31) mice at 13–14 months old. Scale bar = 40 μm. Quantitative analysis showed that, at this age, the area occupied by (**p < 0.01) and (D) the density (p < 0.05) of p75NTR immunostaining is increased in APP Veh compared to WT Veh mice, while the increase in the number of p75NTR-stained cells was not statistically significant. LM11A-31 normalized p75NTR levels (*p < 0.05 vs. APP Veh for both area and density). Statistical significance was determined using a one-way ANOVA with Dunnett’s post-hoc test (WT Veh, n = 5 mice; APP Veh, n = 4; APP-31, n = 5).

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p75NTR-immunolabeling was visualized with a 20× objective in 2 sections per mouse in the basal forebrain (both medial septum and VDB), defined as the area above and below the anterior commissure (anterior part) at the rostro-caudal level of the islands of Calleja and before and just after the emergence of the horizontal diagonal band (~Bregma 0.98–0.86 mm [23]). Three to four non-overlapping images were taken per section each using a 450×335 μm² sample field. p75NTR-immunostained soma and neurites were automatically thresholded after background subtraction using ImageJ. The immunostaining area and density of both soma and neurites was measured and the number of soma was manually counted.

Statistical analyses

GraphPad Prism v5 (GraphPad Software, San Diego California) was used to determine significance using a one-way Analysis of Variance (ANOVA) with Dunnett’s multiple comparison post-hoc test and, when appropriate, a two-tailed Student’s t-test. For planned comparisons in which a direction of difference was predicted, a one-tailed Student’s t-test was used. A 2×2 contingency table with a Fisher’s Exact test was used to determine the statistical significance of analyses with categorical variables (e.g., presence or absence of dendrite branching). For body weights, a repeated measures ANOVA with Dunnett’s post-hoc test was used. Results are expressed as group mean ± SEM and statistical significance was set at p < 0.05.
Figure 5. LM11A-31 prevents and/or reverses basal forebrain cholinergic neurite atrophy in mid-stage APP<sup>LS</sup> mice. Representative photomicrographs show ChAT-immunostained neurons in VDB of the basal forebrain of (A) WT Veh, (B) WT LM11A-31 (-31), (C) APP<sup>LS</sup> (APP) Veh, and (D) APP-31 mice at 9–11 months of age. Arrowheads indicate the distal part of neurites. Below each photomicrograph are reconstructed drawings from NeuroLucida tracings of two ChAT-stained neurons per treatment group. The left drawing is the neurite and corresponding soma indicated by the arrowhead in the photomicrograph (orientations were altered). The right drawing is of a cell outside the field displayed in the photomicrograph but within the field analyzed. Scale bar in A = 20 μm and also applies to the line drawings. Quantification indicates that treating APP<sup>LS</sup> mice with LM11A-31 for 3 months increases the (E) length, (F) area occupied by, and (G) branching of BFCN neurites compared to those given vehicle. Statistical significance was determined using an ANOVA with Dunnett’s post-hoc test and, for branching, a 2×2 contingency table with Fisher’s exact test (WT Veh, n = 9 mice; WT-31, n = 10; APP Veh, n = 10; APP-31, n = 9). **p<0.01 and ***p<0.001 vs. WT Veh; +p<0.05 and ++p<0.01 vs. APP Veh.

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Results

Effects of LM11A-31 on p75NTR levels in basal forebrain of AppL/S mice

Since levels of p75NTR have been shown to increase in AD patients [24,25] and mouse models [26,27] with late-stage pathology and p75NTR is the target of LM11A-31, we determined whether p75NTR immunostaining was increased in the basal forebrain of APPL/S mice at the ages involved in this study, and if levels were affected by the ligand, which could suggest target engagement. P75NTR immunostaining did not differ between 9–11 month old APPL/S and WT mice given vehicle and LM11A-31 treatment had no effect on this measure (data not shown). In contrast, in late-stage APPL/S mice (13–14 months of age), the area occupied by p75NTR staining associated with neurons and neurites was significantly increased by 37±68.5% and density was elevated by 31±65.2%, compared to WT mice (Fig. 4A–E). The number of p75NTR-stained cells increased slightly but this difference was not statistically significant (Fig. 4F). The increased area of p75NTR immunostaining is most likely due to increased p75NTR levels in neurons/neurites since the staining density significantly increased but the number of p75NTR-stained cells did not. Furthermore, previous reports showed that BFCN number, as assessed with ChAT immunostaining, was unaltered in APPL/S mice compared to WTs [8,14]. LM11A-31 treatment inhibited the increases in p75NTR immunostaining in the basal forebrain of APPL/S mice. This finding is consistent with our prior in vitro studies demonstrating that LM11A-31, like NGF, induces p75NTR endocytosis and proteolytic processing and that the latter also occurs after administration to APPL/S mice [16]. Thus, p75NTR is present in abundance on the cells of interest in this study, showing feasibility of the ligand acting through its intended target, and LM11A-31 treatment decreases p75NTR levels, which is consistent with target engagement.

LM11A-31 prevents progression of and/or reverses cholinergic neuritic dystrophy in mid-stage AppL/S mice

Since we previously found that treating 3–5 month old male and female APPL/S mice with LM11A-31 for 3 months slowed the progression of cholinergic neurite dystrophy [14,16], we sought to determine if LM11A-31 could prevent the progression of, and/or reverse, cholinergic neurite dystrophy with treatment beginning at a mid-pathological stage of the disease (6–8 months old). APPL/S mice develop dense amyloid deposits in the frontal cortex and hippocampus at 3–4 and 5–7 months of age, respectively [19], and amyloid deposition progressively increases thereafter. BFCN loss does not occur in APPL/S mice at 6–8 months, but atrophy of cholinergic neurites is evident [1,8,14,16]. For example, the length of BFCN neurites in APPL/S mice is ~30% shorter than those in WT mice at that age [14,16]. Moreover, hippocampal projection fibers of BFCNs are modulated by p75NTR in WT mice [28] as are ChAT-containing neurites in APPL/S mice [8]. Finally, mild
cognitive deficits are first seen in APP<sup>L/S</sup> mice at 3 months [21], are significantly worse at 6–8 months, and then progressively increase [14,16,20,21].

We examined the effects of LM11A-31 on the length and surface area of ChAT-immunolabeled neurites in the VDB because it provides a larger cholinergic projection to the cortex and hippocampus than the medial septum [29,30]. The mean number of neurons analyzed per mouse was 71 ± 3. The length of ChAT-containing neurites of BFCNs appeared shorter in vehicle-treated mid-stage APP<sup>L/S</sup> mice (9–11 months old) than any of the other groups (Fig. 5A–D). Quantitative analyses showed that ChAT-stained neurites were 39 ± 5% shorter in vehicle-treated APP<sup>L/S</sup> mice relative to their WT littermates (Fig. 5E) and their surface area was decreased by 41 ± 6% (Fig. 5F). All mice of both genotypes had ChAT-positive neurites with 2<sup>nd</sup> order branching, however, significantly fewer APP<sup>L/S</sup> mice had neurites with 3<sup>rd</sup> order branching compared to WT mice (Fig. 5G).

Treating mid-stage APP<sup>L/S</sup> mice with LM11A-31 eliminated neurite degeneration as the length and surface area of their ChAT-stained neurites was equal to that of vehicle-treated WT mice (Fig. 5B, C). Furthermore, the reduction in branching complexity seen in vehicle-treated APP<sup>L/S</sup> mice was markedly reduced with LM11A-31, as 6 of 9 treated APP<sup>L/S</sup> mice had ChAT neurites with 3<sup>rd</sup> order branching compared to 1 of 10 in the vehicle group (Fig. 2G). LM11A-31 did not affect the length, surface area or branching of BFCN neurites in WT mice (Fig. 5D, E–F).

Since basal forebrain neurons are the main source of cholinergic innervation to the cortex [30] and cholinergic fibers become increasingly dystrophic with age in the vicinity of cortical amyloid plaques [14,31,32,33], we examined the effects of LM11A-31 treatment on cholinergic dystrophic neurites in this region. Large clusters of dystrophic ChAT-stained neurites were prominent in the cortex of vehicle-treated APP<sup>L/S</sup> mice (Fig. 6A), but were absent in WT mice (not shown). LM11A-31 decreased the total area occupied by ChAT-immunostained clusters in APP<sup>L/S</sup> mice (Fig. 6B,C) by reducing the number of ChAT clusters (Fig. 6D) not the area per cluster (Fig. 6E).

LM11A-31 did not affect the percentage of the area occupied by ThioS staining in the cortex of APP<sup>L/S</sup> mice (0.38 ± 0.07% for vehicle group vs. 0.43 ± 0.08% for LM11A-31). These results corroborate our previous studies indicating that fibrillar forms of Aβ were not affected by the ligand in APP<sup>L/S</sup> mice at earlier stages.
disease stages [14,16]. In addition, we previously reported that soluble Aβ(1-42) levels were not affected by the same LM11A-31 treatment [14]. Therefore, decreases in Aβ levels are unlikely to contribute to LM11A-31’s prevention and/or reversal of ChAT neurite dystrophy. 

LM11A-31 reverses cholinergic neuritic dystrophy in late-stage APPL/S mice

Given the prominent cholinergic neurite degeneration already present in APP<sup>L/S</sup> mice at 6–8 months of age [14,16] when treatment in the present study was initiated, the above findings that LM11A-31 treatment largely normalized cholinergic neurites suggested that the ligand, to a significant extent, reversed rather than simply slowed cholinergic neurite dystrophy. In order to further evaluate the existence of a reversal effect, we determined whether similar results could be obtained with a shorter (1 month) LM11A-31 treatment beginning in late-stage APPL/S mice (12–13 months old) with more advanced pathology.

Within the 13–14 month old group, vehicle-treated APP<sup>L/S</sup> mice had ChAT-stained neurites in the basal forebrain that were 58±5% shorter and occupied 60±4% less area than those of WT mice (Fig. 7A–E). Furthermore, all of the vehicle-treated WT mice at this age had neurites with ≥3<sup>rd</sup> order branching, while only 1 of 4 APP<sup>L/S</sup> mice had this characteristic (Fig. 7F). LM11A-31 prevented deficits in both neurite length and area in 13–14 month old APP<sup>L/S</sup> mice compared to those given vehicle (Fig. 7D,E). It also increased the number of APP<sup>L/S</sup> mice that had neurites with ≥3<sup>rd</sup> order branching (3 out of 5 mice), however this increase did not reach statistical significance (Fig. 7F). In the cortex, LM11A-31 did not affect the total area occupied by or the number of ChAT neurite clusters but did significantly decrease the area per cluster (Fig. 8A–E). As in 9–11 month old APP<sup>L/S</sup> mice, LM11A-31 did not affect the area of ThioS staining in the cortex of the late-stage APP<sup>L/S</sup> mice (0.60±0.14% for vehicle group vs. 0.51±0.08% for LM11A-31). These results suggest that LM11A-31 may slow cluster expansion and coalescence and/or promote their disintegration.
Cholinergic neuritic dystrophy is ameliorated with LM11A-31 treatment in mid- to late-stage Tg2576 mice

To further assess the robustness of the effect of LM11A-31 in slowing progression of degeneration, we determined whether the ligand would be effective in reducing neurite degeneration in another well-characterized AD mouse model, Tg2576 mice. LM11A-31 was administered to female Tg2576 mice and their nTg littermates for 3 months starting at 14 months of age. In these mice, cognitive deficits are seen at 3 months of age and are progressive [34,35,36]. Insoluble Aβ levels incrementally increase beginning at 6 months of age [37] and Aβ deposition is evident at 11 months [38]. Cholinergic dystrophic neurites are not seen at 8 months of age but occur in the cortex by 16 months [39].

In comparison to nTg mice, vehicle-treated Tg2576 mice at 17 months old had shorter ChAT-stained neurites in the basal forebrain that occupied less area (Fig. 10A,B). These defects were prevented in Tg2576 mice given LM11A-31, which had cholinergic neurites resembling those of nTg mice. Dendrite branching complexity was also decreased in vehicle-treated Tg2576 mice but not in those given LM11A-31, compared to nTg mice (Fig. 10C). Finally, LM11A-31 did not affect the number of cholinergic dystrophic neurite clusters in the cortex, but significantly reduced the total area they occupied as well as the mean area per cluster (Fig. 10D–F). LM11A-31 did not affect any measure in nTg mice. These findings confirmed the ability of LM11A-31 treatment to prevent neurite degeneration in a second mouse model. The extent to which treatment might reverse degeneration in this model will require studies at later disease stages.

Discussion

This study showed that administering the p75NTR ligand LM11A-31 to APPL/S mice with mid- to late-stage pathology slowed the progression of cholinergic neurite degeneration in the basal forebrain and cortex and this effect was confirmed in the Tg2576 model. Importantly, LM11A-31 reversed degeneration when it was administered for a brief duration (1 month) and initiated at an age (12–13 months) that was about four times older than the age of detectable pathology onset (3–4 months old). To our knowledge, this report is the first to demonstrate that a CNS penetrating small molecule can function at a specific target to reduce and/or reverse fundamental AD pathologies in late-stage AD mice.

The length and branching complexity of ChAT-stained dendrites in the basal forebrain was reduced in mid- and late-stage APPL/S mice as well as Tg2576 mice. LM11A-31 reversed these changes. Dendritic abnormalities and decreases in higher order branching have also been observed in the hippocampus, basal forebrain, and/or cortex of Tg2576 mice [40] and in other AD mouse models and patients [32,41,42,43,44]. Shorter and less branched dendrites are correlated with dementia [41,45], most likely due to decreased neuronal connectivity, and may therefore contribute to the memory deficits described in APPL/S and Tg2576 mice. LM11A-31 may have exerted its effects on dendritic length and complexity via activation of the PI3K/AKT pathway as the ligand increases AKT signaling [12] and this pathway has been shown to increase dendrite branching and alter dendritic arbor shape [46].

Dystrophy of cholinergic neurites in the basal forebrain and cortex is exacerbated in an age-dependent manner in APPL/S mice. These results are consistent with those seen in other mouse models.
models of AD [31,32,33]. Notably, LM11A-31 reversed the effect of aging on cholinergic neurite dystrophy and eliminated the age-related increase in p75NTR levels in the basal forebrain of APPL/S mice. This latter effect could be due to LM11A-31-induced receptor internalization and/or processing or it could be secondary to decreasing pathology, as numerous mechanisms of cellular injury, including those related to AD, up-regulate p75NTR [3,25,47,48]. The LM11A-31-induced decrease in p75NTR levels may account, in part, for the reversal of cholinergic deficits by decreasing the abnormally high p75NTR/Trk ratio in the basal forebrain found in human AD and mouse models [25,26,47,48]. The reversal effects in aged APPL/S mice occur even when treatment is shortened to 1 month and initiated after appreciable pathology has developed. This reversal holds particular relevance to translating a small molecule p75NTR ligand to the clinic, wherein AD patients, even at early clinical stages, would more likely be in pathologically advanced stages of disease prior to treatment.

LM11A-31-treated APPL/S mice with mid-stage pathology had fewer dystrophic neurite clusters in the cortex that occupied a smaller area but did not decrease in size. Although a reversal effect did not occur in the cortex of late-stage APPL/S mice, just 1 month of LM11A-31 treatment reduced cluster size without affecting their number or overall area. Perhaps a longer treatment time would have reversed the total area and size of the clusters to the levels of the 9–11 month old vehicle-treated APPL/S mice. Nevertheless, reducing either the size or the number of dystrophic neurite clusters could be beneficial and an indicator of slowing degenerative processes.

The positive results achieved here with LM11A-31 further validate p75NTR as a therapeutic target for AD. The ability to reverse neurite degeneration in the context of ongoing, late-stage Aβ accumulation is particularly notable in terms of the array of fundamental AD-related signaling mechanisms modulated by LM11A-31. p75NTR and TrkA signaling stimulated by NGF binding are essential for BFCN function and survival, and this signaling is disrupted in AD brains [49,50,51]. Furthermore, degenerative signaling is augmented in AD due, in part, to increased p75NTR/Trk ratios [52,53,54], elevated proNGF levels [15,55,56,57], and Aβ interactions either directly and/or indirectly with p75NTR [58]. LM11A-31 competes with NGF and proNGF binding to p75NTR but not TrkA [12,15] and has a signaling profile that is distinct from NGF [12]. Our previous in vitro studies showed that LM11A-31 inhibited Aβ-induced degenerative signaling (including excess activation of GSK3β, cdk5 and JNK), activated survival signaling that is compromised in AD (AKT and NFκB), and prevented excess tau phosphorylation in a p75NTR-dependent manner [13]. These actions and the present in vivo results showing prevention and reversal of BFCN degeneration are consistent with the idea that LM11A-31 can bind to p75NTR to simultaneously increase survival signaling while decreasing degenerative signaling to ameliorate mid- to late-stage AD-related pathology.
In all, these results suggest that targeting of p75NTR is a promising approach to modulating AD-related degenerative mechanisms in a way that is sufficiently robust to affect pathological processes that have progressed beyond early stages. Future studies will include behavioral analyses to assess whether reversing these neuropathologies improves functional outcomes.

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Author Contributions
Conceived and designed the experiments: DAS JKK FML. Performed the experiments: DAS JKK NPB GB CF. Analyzed the data: DAS. Wrote the paper: DAS FML.

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