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Norepinephrine protects against A β toxicity via TrkB

Xia Liu^a, Keqiang Ye^{a,*}, and David Weinshenker^{b,*}

^aDepartment of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA

^bDepartment of Human Genetics, Emory University School of Medicine, Atlanta, GA 30322, USA

Abstract

The locus coeruleus (LC), the brainstem noradrenergic nucleus that is the sole source of norepinephrine (NE) in the forebrain, is one of the first structures affected in Alzheimer's disease. Experimental ablation of the LC exacerbates, while increasing NE abates, AD-like neuropathology and cognitive deficits in animal models of the disease. Some neuroprotective effects of NE appear to be mediated by tropomyosin-related kinase B (TrkB), the canonical receptor for brain-derived neurotrophic factor (BDNF). Here, we report that NE dosedependently protected primary cortical and LC neurons from amyloid- β (A β) toxicity. The neuroprotective effects of NE were fully prevented by the Trk receptor antagonist K252a but only partially attenuated by adrenergic receptor antagonists and not mimicked by adrenergic agonists. Activation of TrkB by NE in cortical and LC neurons was confirmed by immunoblot and immunocytochemistry for phospho-TrkB. These results indicate that NE can activate TrkB and protect against A β toxicity, at least in part, via adrenergic receptor-independent mechanisms, and have implications for the consequences of LC degeneration in AD and potential therapies for the disease.

Keywords

norepinephrine; brain-derived neurotrophic factor; Alzheimer's disease; amyloid- β ; tropomyosin-related kinase B; neuroprotection; locus coeruleus

Introduction

Alzheimer's disease (AD) is the most common cause of dementia and is characterized pathologically by β -amyloid plaques, tau neurofibrillary tangles, and neuronal death in the brain. While loss of neurons in the entorhinal cortex, hippocampus, and basal forebrain is considered central to AD pathogenesis, LC degeneration and loss of forebrain norepinephrine (NE) is ubiquitous in AD, occurs early, and correlates better with plaques, tangles, and cognitive impairment than neuronal death in other brain regions [1–3]. In fact, a series of recent papers indicates that aberrant tau in the LC is the first detectable sign of AD-

*Address correspondence to David Weinshenker, Department of Human Genetics, Emory University School of Medicine, Whitehead 301, 615 Michael St., Atlanta, GA 30322, Phone (404) 727-3106, Fax (404) 727-3949, dweinsh@emory.edu or Keqiang Ye, Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Whitehead 145, 615 Michael St., Atlanta, GA 30322, Phone (404) 712-2814, Fax (404) 712-2979, kye@emory.edu.

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like neuropathology in the human brain [4–8]. Experimental LC lesions in amyloid β -protein precursor (*A β PP*) transgenic mice also lead to more severe AD-like forebrain neuropathology and memory deficits, while increasing NE transmission partially ameliorates neuroinflammation, A β load, and cognitive impairment [9–13].

While most evidence points to a neuroprotective role of NE in AD, the underlying mechanisms are unclear. In some cases, adrenergic receptors, particularly the β -subtype, have been implicated. For example, β -adrenergic agonists have potent anti-inflammatory properties, can protect cultured neurons from A β toxicity, and enhance A β phagocytosis by microglia, while β -adrenergic antagonists have the opposite effects [12, 14–16]. However, in some cases, the ability of NE to protect cultured neurons from AD-related insults such as oxidative stress and A β cannot be fully mimicked by adrenergic agonists or blocked by adrenergic antagonists, suggesting the existence of non-canonical signaling mechanisms [16–19]. Moreover, the neuroprotective effects of NE have been assessed in hippocampal neurons, cortical neurons, and immortalized cell lines, but not LC neurons. Although amyloid burden in the brainstem is relatively low, it is very high in LC projection areas like the hippocampus and cortex. One theory to explain the death of LC neurons in AD is that amyloid damages LC axons and terminals in the forebrain, which over time leads to cell body death in the brainstem; thus, assessing amyloid toxicity in cultured LC neurons, which retain processes, could provide important insight into LC degeneration in AD.

Tropomyosin-related kinase B (TrkB), the cognate receptor for brain-derived neurotrophic factor (BDNF), has emerged as a candidate for mediating the neuroprotective effects of NE in the context of AD. Activation of TrkB by BDNF or other agonists can protect neurons from A β toxicity [20–24], while blockade of TrkB attenuates the neuroprotective effects of NE against A β toxicity in culture [16]. The purpose of this study was to examine both cortical and LC neurons to further investigate the relative contributions of TrkB and adrenergic receptor activation to the neuroprotective effect of NE against A β toxicity.

Material and Methods

Primary neuron culture

All experiments were approved by the Emory Institutional Animal Care and Use Committee. Primary rat cortical neurons and LC neurons were cultured as previously described [25]. Briefly, E17 rat pups were decapitated, and cortex was extirpated, cross-chopped, and suspended by pipetting for gentle separation in 5% fetal calf serum (FCS), 5% horse serum (HS) DMEM. The cell suspension was then centrifuged at 250 \times g for 5 min. This operation was repeated again. Cells were seeded into 10 polyethyleneimine-coated dishes and 12-well plates including coated coverslips and incubated at 37°C in 5% CO₂/95% air. After 3 h, culture medium was changed to Neurobasal containing B-27 supplement (Invitrogen, Grand Island, NY) and incubated for 4 d. For maintenance, half the medium was changed to fresh Neurobasal/B27 every 4 d. After a week, the dished cultured neurons were used in experiments. Approximately 15–25% of the neurons in the LC cultures were noradrenergic, as assessed by TH immunostaining (data not shown). Primary cortical neuron cultures were also made from TrkB F616A knock-in mice, as described [19].

To assess the effect of NE on A β -induced toxicity, NE (50–500 nM) was added to the medium 20 min before A β treatment. Neurons were then exposed to 10–40 μ M A β _{25–35} or A β _{1–42} and incubated for 18 h in serum-free OptiMem, as described [16, 24]. A β _{25–35} was dissolved in DMSO and applied without pre-aggregation, which results in the rapid formation of oligomeric and protofibril intermediates [26, 27], while A β _{1–42} was dissolved in N2 medium and preaggregated for 4 days at 37°C, as described [16, 24]. Caspase activity was detected by Caspase-Glo 3/7 assay (Promega, Madison, WI). Neuronal apoptosis was detected with the *in situ* cell death detection kit (Roche Diagnostics, Indianapolis, IN). The apoptotic index was expressed as the percentage of TUNEL-positive neurons out of the total number of MAP2-positive neurons. To assess the effect of TrkB and adrenergic receptor antagonists, neurons were pretreated with the Trk antagonist K252a (100 nM) or a cocktail of the α 2-adrenergic antagonist yohimbine (10 μ M) + the α 1-adrenergic antagonist prazosin (50 nM) + the β -adrenergic antagonist propranolol (10 μ M) 30 min prior to NE or isoproterenol (β -adrenergic agonist, 100 μ M) exposure. Because some of these experiments were part of a larger panel of studies testing various agents in A β toxicity assays, the “control” and “A β ” bars in Fig. 2B are the same as in Fig. 1C of our previously published paper [24].

Immunofluorescence staining

For immunofluorescence and TUNEL staining in the A β experiments, cells were incubated overnight at 4°C with anti-MAP2 or anti-TH antibody. After being washed with TBS, the cells were incubated with Alexa Fluor 488-coupled secondary antibodies. The cells were then incubated with TUNEL reagent for 1 h at room temperature (RT). After a PBS wash, images were acquired through an AxioCam camera on an Axiovert 200M microscope (Zeiss). For the TrkB experiments, primary cortical and LC neurons were seeded on poly-L-lysine-coated coverslips in 12-well dishes. After 7 DIV, the neurons were treated with 100 ng/ml BDNF (Peptron; Daejeon, Republic of Korea) or 100 nM NE for 30 min, and then washed with PBS. Cells were fixed with 3% formaldehyde in PBS at RT for 15 min, then permeabilized and blocked with 0.4% Triton X-100 and 2% FBS in PBS at RT for 15 min, washed with PBS three times, and treated with anti-MAP2 (1: 200) or anti-TH (1:250) and anti-pTrkB 706 antibody (1:100) overnight. After staining with FITC-or rhodamine-conjugated secondary antibody, the coverslips were mounted on slides. Fluorescent images were taken by Olympus IX71 fluorescence microscope.

Immunoprecipitation and Western blot

Rat primary cultured cortical neurons were treated with vehicle, BDNF (100 ng/ml) or NE (500 nM) for 20 min and then harvested in ice-cold homogenization buffer [10 mM sodium- β -glycerophosphate, 50 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EDTA (pH 8.0), 1% Triton X-100, 1.5 mM Na₃VO₄, 50 mM NaF, 10 mM sodium-pyrophosphate, pH 7.4] containing protease inhibitors. Total protein from rat neurons was mixed in 800 μ l lysis buffer and added to a tube with 25 μ l bed volume of Protein A/G beads (Santa Cruz Biotechnology, Dallas, TX) and 1 mg anti-TrkB antibody (1:1000). The mixture was rotate overnight at 4°C, then beads were washed 3–4 times with 1 ml homogenization buffer. Beads were incubated with 30 μ l of 1x loading buffer for 5 min at 95°C. Eluted sample proteins were separated by SDS-PAGE (9% acrylamide) and transferred to nitrocellulose

membranes (BioRad, Hercules, CA) electrophoretically. Membranes were blocked in TBS/0.1% Tween-20(TBST)/5% milk for 30 min at RT and incubated overnight at 4°C with anti-PY99 antibody (1:1000) in 3% BSA/TBST incubation buffer. Blots were washed 3 times and incubated for 1 h at RT with horseradish peroxidase-conjugated goat anti-mouse secondary IgG (1:2000, Fisher Scientific). The pTrkB band was visualized by enhanced chemiluminescence (Thermo Scientific/Pierce, Rockford, IL) on x-ray film.

Reagents

Adrenergic compounds, K252a, and A β _{25–35} were obtained from Sigma-Aldrich (St. Louis, MO), BDNF was obtained from Peptron (Daejeon, Republic of Korea), and 1NMPP1 was obtained from Toronto Research Chemicals (Toronto, Ontario). Antibodies used were antityrosine hydroxylase (EMD Millipore, Billerica, MA), anti-MAP2 (Sigma-Aldrich, St. Louis, MO) anti-TrkB (BioVision, Milpitas, CA and Cell Signaling, Danvers, MA), anti-pTrkB 706 (Santa Cruz Biotechnology, Inc., Dallas, TX), anti-pTrkB 817 (Epitomics, Burlingame, CA), and anti-PY99 (Santa Cruz Biotechnology).

Statistical Analysis

All data are presented as mean \pm SEM. Statistical analysis were performed using either Student's t-tests were used when comparing 2 groups, and ANOVA followed by Sidak's or Tukey's posthoc tests was used when comparing more than 2 groups using Prism 6.0 for Macintosh (Graphpad, La Jolla, CA). Data were normally distributed. The level of significance was set at $p < 0.05$.

Results

Norepinephrine protects cortical neurons from A β toxicity

It has been reported that NE can protect cultured hippocampal neurons from A β toxicity in a Trk receptor-dependent manner. To confirm and extend these results, we exposed cultured cortical neurons to the A β _{25–35} fragment and assessed toxicity in the presence of NE with or without the Trk receptor antagonist K252a. The increase in caspase-3 activity following A β _{25–35} treatment (20 μ M) was dose-dependently suppressed by NE (50–500 nM) in cortical neurons (one-way ANOVA: $F_{4,10} = 520$, $p < 0.0001$) (Fig. 1A), and the protective effect of NE (100 nM) was abolished by K252a (100 nM; one-way ANOVA: $F_{3,8} = 1293$, $p < 0.0001$) (Fig. 1B).

Although A β _{25–35} is commonly used in primary culture studies, it is a synthetic fragment not found endogenously in AD brains. To confirm our results with full length A β , we assessed the effects of NE on A β _{1–42} toxicity in cortical neurons. NE significantly reduced cell death elicited by 10 μ M A β _{1–42} (one-way ANOVA: $F_{2,9} = 932.80$, $p < 0.0001$) (Fig. 2), although the rescue was not complete. Cell death was still higher than control in cultures treated with A β _{1–42} + NE, and some of the surviving neurons looked abnormal (hypertrophic cell bodies and thickened projections) (Fig. 2). Similar results were obtained using a higher dose of A β _{1–42} (20 μ M) (data not shown).

Protection of locus coeruleus neurons from A β toxicity by norepinephrine requires Trk receptor activation but not canonical adrenergic receptors

Because LC degeneration is a hallmark of AD, occurs early in the disease, and exacerbates AD-like neuropathology and cognitive deficits in A β PP transgenic mice, identifying ways of protecting these neurons is of high clinical significance. We have shown before that the small molecule TrkB agonist 7,8-dihydroxyflavone (7,8-DHF) can protect LC neurons from A β toxicity, but the effects of NE have never been tested. Similar to what we observed in cortical neurons, NE (500 nM) protected against A β ₂₅₋₃₅ (40 μ M)-induced cell death in LC neurons in a TrkB-dependent manner (one-way ANOVA: $F_{2,3} = 41.97$, $p < 0.01$) (Fig. 3A).

It has been reported that the ability of NE to protect cultured hippocampal neurons from A β toxicity may not always involve canonical β , $\alpha 1$, or $\alpha 2$ G protein-coupled adrenergic receptors, and our results support this idea. The neuroprotective properties of NE (100 nM) in LC neurons were only partially attenuated by a cocktail of $\alpha 1$ (prazosin, 50 nM) + $\alpha 2$ (yohimbine, 10 μ M) + β (propranolol, 10 μ M) adrenergic receptor antagonists and were not recapitulated by the β -agonist isoproterenol (10 μ M) (Fig. 3B). By contrast, the Trk receptor antagonist K252a (100 nM) completely abolished the pro-survival effects of NE (Fig. 3B). A two-way ANOVA showed a main effect of antagonist pretreatment ($F_{2,9} = 19.33$, $p < 0.001$), agonist treatment ($F_{2,9} = 173.6$, $p < 0.0001$), and a pretreatment x treatment interaction ($F_{4,9} = 19.72$, $p < 0.001$). Posthoc tests revealed a significant protective effect of NE ($q = 18.02$, $p < 0.0001$) and NE + adrenergic receptor antagonist cocktail ($q = 10.20$, $p < 0.001$), but not NE + K252a ($q = 3.13$, $p > 0.05$), compared to vehicle, and cell death was significantly higher in the NE + K252a group compared to the NE + adrenergic antagonist cocktail group ($q = 7.08$, $p < 0.05$). Isoproterenol had no effect on A β toxicity under any condition.

Norepinephrine induces TrkB phosphorylation in primary cortical and locus coeruleus neurons

Upon agonist stimulation, TrkB undergoes dimerization and autophosphorylation. To confirm that NE treatment caused bona fide TrkB activation, we conducted immunoprecipitation assays with cortical neuron lysates. BDNF (100 ng/ml) and NE (500 nM) elicited evident TrkB tyrosine phosphorylation (Fig 4A, top panel). Immunoblotting analysis with neuronal lysates using an antibody against p-TrkB 817 also showed that both BDNF and NE escalated TrkB activation compared to control (Fig 4A, middle panel). Total TrkB levels were unaffected by treatment (Fig. 4A, bottom panel). To further explore whether NE can selectively provoke TrkB activation, we employed TrkB F616A knock-in mice. Whereas wild-type TrkB neurons can be inhibited by K252a, TrkB F616A can be selectively blocked by the specific inhibitor 1NMPP1, resulting in a virtual TrkB-null phenotype [28, 29]. We found that NE-provoked TrkB phosphorylation was selectively reduced by 1NMPP1, but not K252a, in TrkB F616A neurons (Fig 4B). Immunofluorescence staining confirmed that both BDNF and NE provoked robust TrkB phosphorylation in primary cortical (Fig. 4C) and LC (Fig. 4D) neurons.

Discussion

Some neuroprotective properties of NE depend on the activation of canonical adrenergic receptors, while others appear to be mediated by independent mechanisms. Here we show that NE triggers TrkB phosphorylation and that TrkB, but not adrenergic receptors, is essential for the ability of NE to protect cultured cortical and LC neurons from A β toxicity.

Neuroprotective effects of NE in AD

As discussed in the Introduction, LC degeneration and loss of forebrain NE is ubiquitous in postmortem AD brains. LC pathology is evident as early as mild cognitive impairment, a prodromal stage of AD, and perhaps even decades before symptom onset. Experimental lesions of the LC exacerbate, while increasing NE levels ameliorate, AD-like neuropathology in rodent A β PP models of AD.

Some neuroprotective effects of NE against A β toxicity involve adrenergic receptors, most notably the β -adrenergic receptor. For instance, the β -adrenergic receptor agonist isoproterenol ameliorates neuroinflammation produced by A β in vivo [14]. In cultured cells, activation of β -adrenergic receptors facilitates the phagocytosis of A β and attenuates A β -induced cell death [12, 16], while β -adrenergic antagonists abolish the ability of NE to block A β -induced mitochondrial membrane depolarization and caspase activation [16]. α -adrenergic drugs were ineffective in these assays. On the other hand, the increase in survival of cholinergic neurons exposed to oxidative stress following NE treatment could not be mimicked by adrenergic receptor agonists nor blocked by adrenergic receptor antagonists [17], and the antagonists also failed to alter the antioxidant effects of NE in A β -exposed cells [16]. A recent study showed that the ability of NE to protect against A β -mediated oxidative stress and toxicity in SK-N-SH cells could not be blocked by a cocktail of α - and β -antagonists [19]. Thus, at least some neuroprotective properties of NE appear to be mediated by canonical adrenergic receptor-independent mechanisms.

The neuroprotective effects of NE require TrkB activation

A loss of neurotrophic support is also associated with AD. For example, both BDNF and TrkB levels are decreased in postmortem AD brains [30], while we and others have shown that activation of TrkB signaling is neuroprotective in animal and cellular models of AD [22–24, 31]. Importantly, TrkB is required for the neuroprotective effects of NE in cultured hippocampal and hNT neurons following A β exposure [16]. To extend these findings, we assessed the contribution of adrenergic receptors and TrkB to NE-mediated protection against A β toxicity in cortical and LC neurons. We found that the ability of NE to prevent A β -induced cell death was fully prevented by the Trk antagonist K252a but only partially attenuated by adrenergic antagonists and not recapitulated by isoproterenol. These results suggest that canonical G protein-coupled adrenergic receptors and TrkB may act in concert to transduce NE-mediated neuroprotection. While many similarities between our data and those reported by Counts and Mufson [16] exist, the adrenergic receptor-independent effects of NE on A β -induced neuronal death were more pronounced in our experiments. Some potential explanations are differences in cell type examined (hippocampal neurons and a

neuronal cell line vs. LC neurons) and cell death assay used (Live/Dead cell viability assay or propidium iodide vs. TUNEL staining).

Activation of TrkB by NE

To further investigate the relationship between NE and TrkB, we assessed TrkB activation in cultured cortical and LC neurons and found that NE provoked robust TrkB phosphorylation as measured by western blot and immunocytochemistry, although the exact nature of the interaction remains to be elucidated. There are several ways that NE could activate TrkB. First, NE may function via increases in BDNF, as reported previously for protection against A β toxicity [16]. Alternatively, NE may transactivate TrkB via Src family kinase activity, as shown for adenosine, pituitary adenylate cyclase-activating peptide, and zinc [32–35]. Finally, it has not escaped our notice that NE and the small molecule TrkB agonist 7,8-dihydroxyphenylserine [36] share structural similarities (i.e. a catechol ring), and thus a direct physical interaction cannot be ruled out. Interestingly, the neuroprotective effect of NE against oxidative stress in cultured cholinergic neurons and SK-N-SH cells also appears to be mediated by the catechol ring [17, 19].

The nature and pattern of NE-TrkB interactions that we observed is reminiscent of those that occur during noradrenergic neuron development. The specification of NE neurons requires NE and TrkB activation but not canonical adrenergic receptors [37–40]. Thus, NE-mediated activation of TrkB may be important for multiple aspects of neuronal development and function.

Implications for Alzheimer's disease

There is an overall loss of trophic support in AD; BDNF and TrkB levels are reduced, while cell death is rampant in postmortem AD brains. LC pathology appears very early in AD, and loss of LC neurons and forebrain NE are pathological and neurochemical hallmarks of the disease. Our data suggest that LC degeneration could exacerbate AD neuropathology because NE activation of TrkB is depressed. BDNF is co-expressed in LC neurons [41, 42], and BDNF released from noradrenergic terminals has neurotrophic properties [43, 44]. Thus, LC degeneration might deprive the brain of two TrkB activators. BDNF itself promotes noradrenergic neuron survival in aged animals [45, 46], suggesting that progressive loss of autocrine support could hasten the degeneration of this nucleus during AD progression. We and others have shown that TrkB agonists ameliorate AD-like neuropathology in *A β PP* transgenic mice [23, 24]. Drugs such as L-3,4-dihydroxyphenylserine that elevate NE levels are also neuroprotective in animal models of AD [10, 13], perhaps in part because they increase TrkB activation, and are potential therapies for disease treatment in humans.

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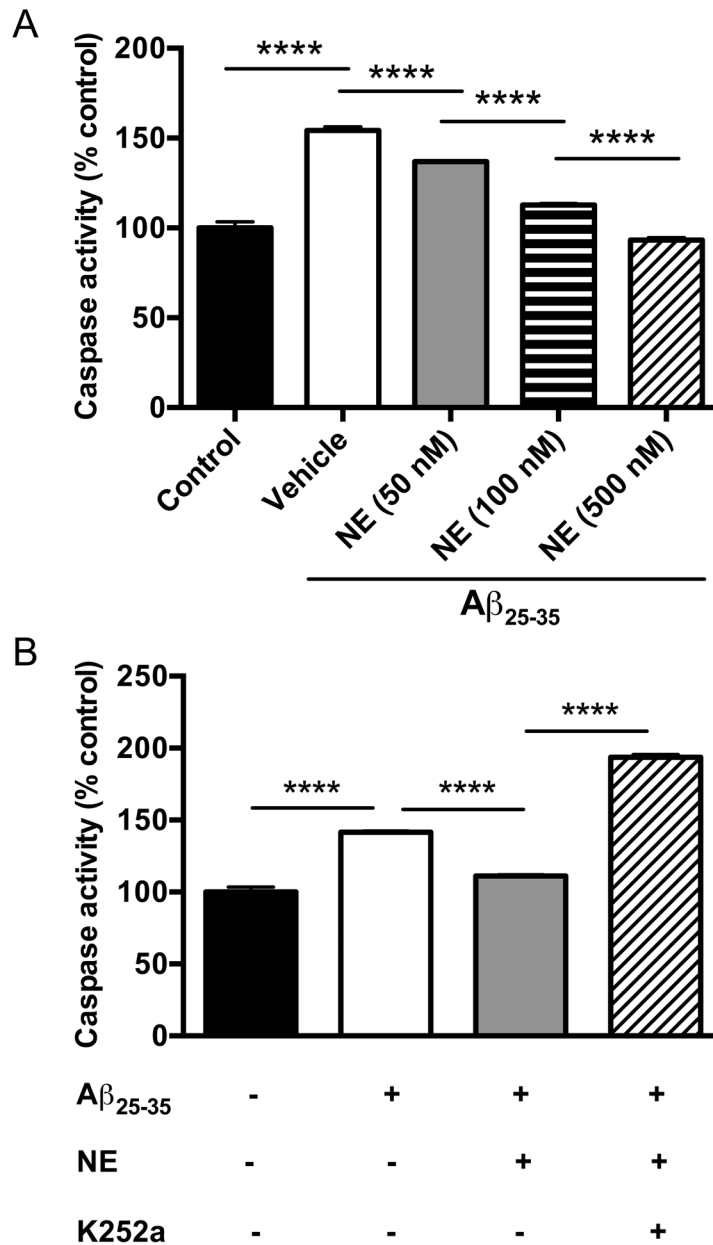


Fig. 1. Norepinephrine attenuates A β_{25-35} -induced neurotoxicity in cultured cortical neurons
 (A) Cultured cortical neurons were exposed to A β_{25-35} (20 μ M) for 18 h in the absence or presence of NE (50–500 nM). Shown is the mean \pm SEM caspase activity (n=3). (B) Cultured cortical neurons were exposed to A β_{25-35} (20 μ M) for 18 h in the presence or absence of NE (100 nM) and the Trk inhibitor K252a (100 nM). Shown is the mean \pm SEM caspase activity (n=3). ****p<0.0001.

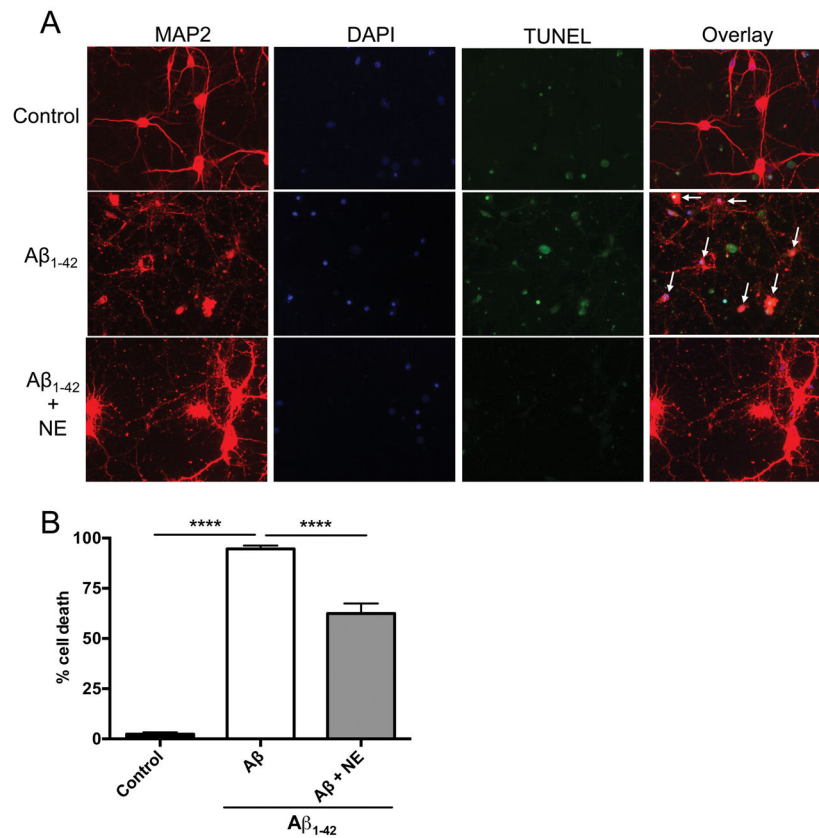


Fig. 2. Norepinephrine attenuates A β ₁₋₄₂-induced neurotoxicity in cultured cortical neurons
 Cultured cortical neurons were exposed to pre-aggregated A β ₁₋₄₂ (10 μ M) for 18 h in the presence or absence of NE (500 nM). Neurons were immunostained with the neuronal marker MAP2 (red) and the nuclear marker DAPI (blue), and neuronal apoptosis was detected by TUNEL staining (green). Shown are (A) representative immunofluorescent images, and (B) the mean \pm SEM percentage of apoptotic cells (n=4 experiments, 150–200 total cells examined per condition). White arrows indicate apoptotic neurons. ****p<0.0001.

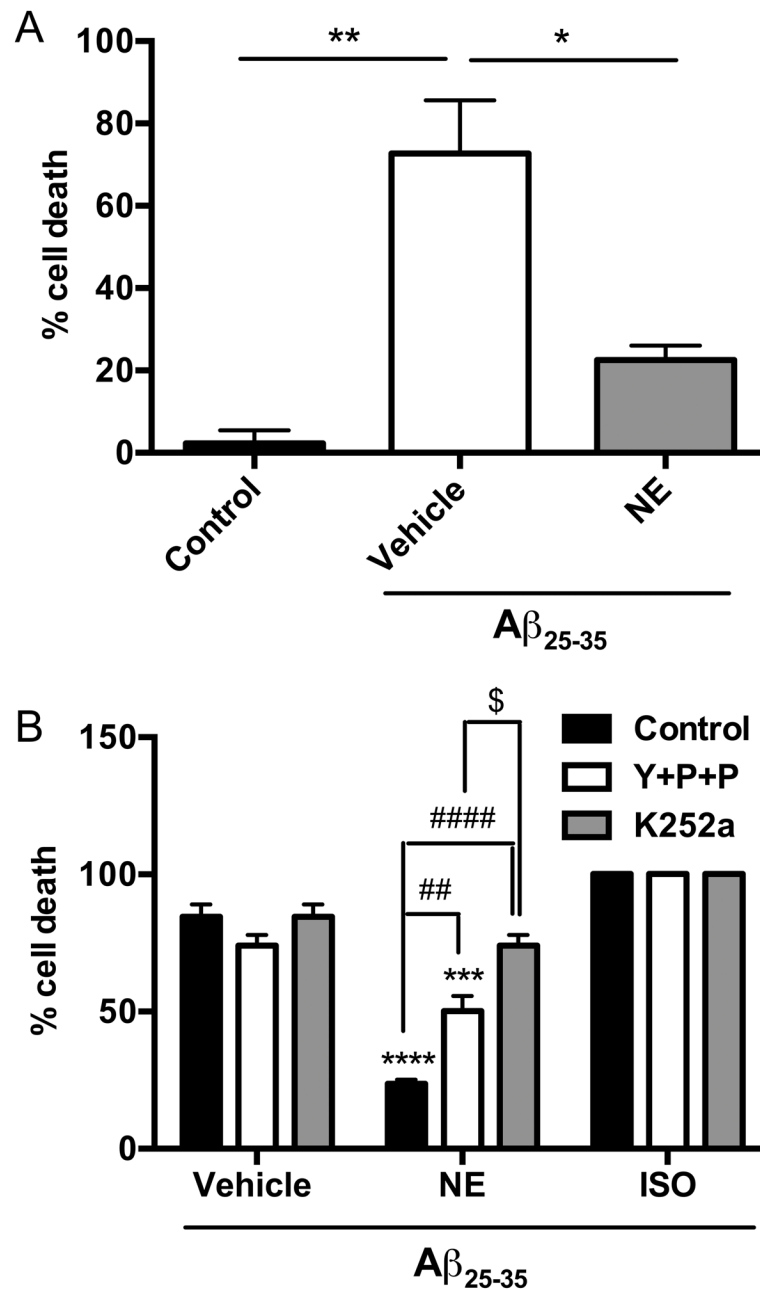


Fig. 3. Attenuation of $A\beta_{25-35}$ -induced neurotoxicity in cultured locus coeruleus neurons requires TrkB but is partially independent of adrenergic receptors

(A) LC neurons were exposed to $A\beta_{25-35}$ (40 μ M) for 18 h in the presence or absence of NE (500 nM). Shown is the mean \pm SEM percentage of apoptotic cells determined by TUNEL staining (n=2). *p<0.05, **p<0.01. (B) LC neurons were exposed to $A\beta_{25-35}$ (40 μ M) for 18 h in the presence or absence of NE (100 nM) or the β -adrenergic receptor agonist isoproterenol (ISO, 100 μ M), with or without K252a (100 nM) or a cocktail (Y+P+P) of the α 2-adrenergic antagonist yohimbine (10 μ M) + the α 1-antagonist prazosin (50 nM) + the β -antagonist propranolol (10 μ M). Shown is the mean \pm SEM percentage of apoptotic cells determined by TUNEL staining (n=2). ***p<0.001, ****p<0.0001 compared to

vehicle; ## $p < 0.01$, #### $p < 0.0001$ compared to NE alone; \$ $p < 0.05$ compared to NE with Y+P +P.

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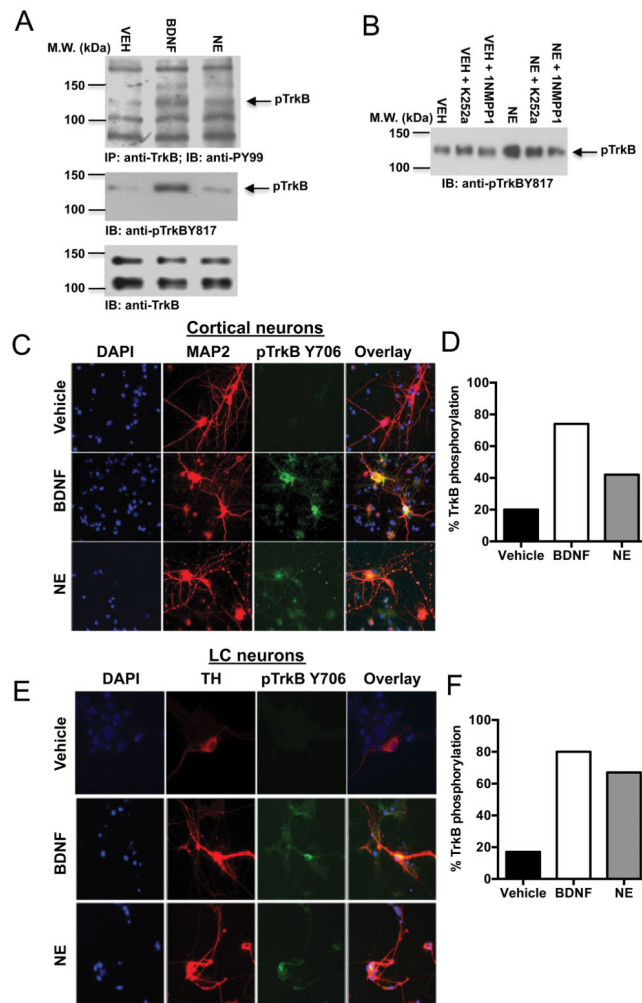


Fig. 4. NE triggers TrkB phosphorylation in cultured cortical and locus coeruleus neurons (A) Primary cortical neurons were treated with vehicle, BDNF (100 ng/ml), or NE (500 nM) for 15 min. Total TrkB was immunoprecipitated with anti-TrkB antibody, followed by immunoblotting analysis with anti-PY99 antibody (top panel). Total lysates were also analyzed with anti-pTrkB 817 antibody (middle panel) and anti-TrkB antibody (bottom panel). (B) Primary cortical neurons from TrkB F616A knock-in mice were incubated with vehicle, K252a (100 nM), or 1NMPPI (100 nM) for 2 h and then treated with vehicle or NE (500 nM) for 15 min, followed by immunoblotting analysis with anti-pTrkB 817 antibody. Primary cortical (C, D) and LC (E, F) neurons were pretreated with vehicle, BDNF (100 ng/ml) or NE (500 nM) for 15 min. Neurons were fixed and costained with the nuclear marker DAPI (blue), the neuronal marker MAP2, or the catecholaminergic marker TH (red), and anti-pTrkB 706 (green). Shown are representative immunofluorescent images (C, E) and % of the 150–200 total neurons counted for each condition that were also pTrkB-positive (D, F).