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Noradrenaline activation of neurotrophic pathways protects against neuronal amyloid toxicity

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Abstract

Degeneration of locus coeruleus (LC) noradrenergic forebrain projection neurons is an early feature of Alzheimer's disease (AD). The physiological consequences of this phenomenon are unclear, but observations correlating LC neuron loss with increased AD pathology in LC projection sites suggest that noradrenaline (NA) is neuroprotective. To investigate this hypothesis, we determined that NA protected both hNT human neuronal cultures and rat primary hippocampal neurons from amyloid- β ($A\beta_{1-42}$ and $A\beta_{25-35}$) toxicity. The noradrenergic co-transmitter galanin was also effective at preventing $A\beta$ -induced cell death. NA inhibited $A\beta_{25-35}$ -mediated increases in intracellular reactive oxygen species, mitochondrial membrane depolarization, and caspase activation in hNT neurons. NA exerted its neuroprotective effects in these cells by stimulating canonical β_1 and β_2 adrenergic receptor signaling pathways involving the activation of cAMP response element binding protein (CREB) and the induction of endogenous nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF). Treatment with functional blocking antibodies for either NGF or BDNF blocked NA's protective actions against $A\beta_{1-42}$ and $A\beta_{25-35}$ toxicity in primary hippocampal and hNT neurons, respectively. Taken together, these data suggest that the neuroprotective effects of noradrenergic LC afferents result from stimulating neurotrophic NGF and BDNF autocrine or paracrine loops via β adrenoceptor activation of the CREB pathway.

Introduction

A major feature of Alzheimer's disease (AD) is the selective degeneration of subcortical projection neurons mediating higher cognitive processes including noradrenergic locus coeruleus (LC) neurons (Adolfsson et al. 1979; Mann et al. 1983; Zarow et al. 2003; Grudzien et al. 2007). LC neurons provide the sole source of noradrenaline (NA) to the hippocampus and neocortex (Foote et al. 1983) and NA signaling plays an important role in various behaviors including selective attention, memory storage and retrieval, general arousal, vigilance and mood (Foote et al. 1983; Levine et al. 1990; Ressler and Nemeroff 1999; Berridge and Waterhouse 2003; Weinshenker 2008; Sara 2009). Degeneration of LC neurons and reductions in NA levels in LC target fields (Adolfsson et al. 1979; Mann et al. 1980; Palmer et al. 1987) are associated with the onset and duration of AD (Mann et al. 1984; Forstl et al. 1994; Zarow et al. 2003), suggesting a neuroprotective role for NA. In this regard, *in vivo* studies show that a loss of LC-derived NA impacts multiple aspects of AD-like neuropathology. For example, *N*-(2-chloroethyl)-*N*-ethyl-2 bromobenzylamine (DSP4)-induced LC damage increases amyloid- β ($A\beta$) deposition in the hippocampus and cortex and up-regulates markers of gliosis and inflammation in amyloid overexpressing mouse models of AD (Heneka et al. 2002; Heneka et al. 2006; Kalinin et al. 2007). Moreover, *in vitro*

experiments show that NA protects cultured neurons from amyloid induced toxicity (Madrigal et al. 2007), excitotoxicity (Madrigal et al. 2009), metabolic stress (Madrigal et al. 2009), and oxidative stress (Troade et al. 2001; Traver et al. 2005).

Despite the diverse repertoire for NA neuroprotection, the mechanisms underlying this action are not well understood. To address this problem, we first demonstrated that NA protects human hNT and rat primary hippocampal neurons against A β . We explored the neurotoxic sequela activated by A β exposure and tested whether they were sensitive to NA or specific noradrenergic receptor ligands. We also asked whether NA neuroprotection against A β involved the activation of neurotrophin-mediated pro-survival pathways. Our experiments suggest that NA can protect neurons from amyloid toxicity by inducing either nerve growth factor (NGF) or brain-derived neurotrophic factor (BDNF) expression through the activation of canonical β -adrenoceptor signaling cascades.

Materials and Methods

Neuronal cell culture

hNT neuronal cultures were derived from the human teratocarcinoma NT2 cell line (a gift from Virginia Lee, Univ. Penn) (Andrews et al. 1984; Lee and Andrews 1986). NT2 cells were maintained in OptiMem (Invitrogen, Carlsbad, CA) with 5% fetal bovine serum (FBS). For differentiation, cells were seeded at 25,000/cm² into T75 flasks in 1:1 DMEM/F-12 media (Invitrogen)/10% FBS, treated twice a week with 10 μ M all-*trans* retinoic acid (Sigma; St. Louis, MO) for 4 weeks and then seeded to new T75 flasks at 65,000/cm² and treated with the mitotic inhibitors cytosine arabinoside (1 μ M) and fluorodeoxyuridine (10 μ M, Sigma) for 2 weeks. This resulted in a layer of phase-bright, post-mitotic neuronal cells loosely attached atop a monolayer of non-neuronal cells. Neuronal enrichment was achieved by gently trypsinizing the top neuronal layer and replating at 125,000/cm² onto 2% Matrigel (BD Biosciences, San Jose, CA) and 10 μ M poly-D-lysine (Sigma)-coated black-walled 96 well plates (spectrophotometric assays), 24-well plates (PCR), 60 mm dishes (immunoblotting), or 18 mm² cover slips (fluorescence microscopy). hNT neurons were cultured for an additional 2 weeks in 1:1 DMEM/F-12 media/10% FBS. Rat E18 primary hippocampal neurons were purchased from Neuromics (Edina, MN) and cultured at ~35,000/cm² on poly-D-lysine using manufacturer protocols

A β neurotoxicity experiments

Differentiated hNT or primary hippocampal neurons were rinsed, pretreated with 10 μ M NA (Sigma, dissolved in water) for 5 minutes and then challenged with 10 μ M A β ₂₅₋₃₅, A β ₁₋₄₂, or reverse peptides (Sigma) in serum-free OptiMem. These concentrations were derived from comprehensive dose response testing during pilot studies (not shown). A β ₂₅₋₃₅ was dissolved in DMSO and applied without pre-aggregation, which results in the rapid formation of oligomeric and protofibril intermediates in aqueous solutions (Giuffrida et al. 2007; Millucci et al. 2009). A β ₁₋₄₂ was dissolved in DMSO and pre-aggregated for 16 hours at 37°C. Western blotting revealed an accumulation of SDS-soluble immunoreactive material migrating at ~40–48 kDa reminiscent of oligomeric amyloid (Walsh et al. 1999; Chromy et al. 2003) (not shown). In a parallel experiment, hNT cultures were pre-treated with 1 μ M galanin (dissolved in 0.1% trifluoroacetic acid) (Counts et al. 2002; Elliott-Hunt et al. 2007), a noradrenergic peptide co-transmitter, prior to A β ₁₋₄₂ exposure. Neuronal viability was determined by the Live/Dead cell viability assay (Invitrogen) (Lambert et al. 1998) following 4 days of A β challenge or by propidium iodide (PI) (Elliott-Hunt et al. 2007) following 48 hours challenge. For all experiments in this study, control (CTL) conditions included vehicle.

Membrane depolarization, oxidative stress, and apoptosis assays

Plasma membrane depolarization—Differentiated hNT neurons were preloaded for 30 minutes with the voltage-sensitive fluorescent probe Bis - (1,3 - diethylthiobarbituric acid) trimethine oxonol (DiBAC₂; Molecular Probes/Invitrogen) prior to NA and A β treatments. DiBAC₂ enters depolarized cells where it binds to intracellular/membrane proteins or membrane and exhibits enhanced fluorescence (Loew 1982). Glutamate (Glu, 1 mM, dissolved in water) was used as a positive control. DiBAC₂ fluorescence was measured 5 minutes after A β or Glu application.

Oxidative stress—hNT neurons were pretreated with NA, challenged with A β _{25–35} for 1 hour and assayed with the reactive oxidation-sensitive fluorescent probe H₂DCFDA (2',7'-dichlorofluorescein diacetate, Molecular Probes/Invitrogen), which is nonfluorescent until the acetate groups are removed by intracellular esterases and the probe is oxidized (Babo and Charbonneau 1994). Hydrogen peroxide (100 μ M/10 μ M FeSO₄) was used as a positive control.

Mitochondrial membrane depolarization—hNT neurons were pretreated with NA and challenged with A β _{25–35} for 24 hours, incubated with JC-1 reagent (Stratagene, La Jolla, CA), and then analyzed by spectrophotometry and fluorescence microscopy. JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolcarbocyanine iodide) aggregates within the mitochondria with a red fluorescence at ~585 nm; mitochondrial membrane depolarization allows JC-1 into the cytosol, where it remains a monomer with a green fluorescence at ~515 nm (Cossarizza et al. 1993).

Apoptosis—hNT neurons were pretreated with NA, challenged with A β _{25–35} for 24 hours, and then assayed using the ApoFluor fluorescent pan-caspase-sensitive probe FAM-VAD-FMK (MP Biomedicals, Irvine, CA), which binds covalently to active caspases 1–9 (Smolewski et al. 2001). Staurosporine (1 μ M) was used as a positive control.

Adrenoceptor ligand experiments

The following adrenoceptor agonists were tested for their ability to mimic NA's protective effects against A β neurotoxicity: isoproterenol (ISO; β 1 and β 2, dissolved in water, 1–100 μ M dose range), salmeterol (SAL; β 2, DMSO, 100 nM - 10 μ M), cirazoline (CRZ; α 1, water, 100 nM - 10 μ M), and UK 14, 304 (UK; α 2, DMSO, 100 nM - 10 μ M). hNT cultures were pretreated with ligand for 5 minutes, challenged with A β _{25–35} for 48 hours and assessed for cell death by PI. The following adrenoceptor antagonists were tested for their ability to block NA's protective effects against A β neurotoxicity: CGP 20712 (CGP; β 1, dissolved in water, 100 nM - 10 μ M), ICI 118, 551 (ICI; β 2, water, 100 nM - 10 μ M), prazosin (PRZ; α 1, DMSO, 100 nM - 10 μ M), and yohimbine (YHB; α 2, water, 100 nM - 10 μ M). hNT cultures were pretreated with ligand for 10 minutes prior to NA or vehicle administration, challenged with A β _{25–35} for 48 hours, and then cell death was assessed by PI. All ligands were purchased from Tocris (Ellisville, MO). Dose ranges were based on ligand-specific dissociation constants and pilot testing.

cAMP pathway experiments

hNT neurons were pretreated with ISO (100 μ M), ISO + PKI [1 μ M, an inhibitor of protein kinase A (PKA), dissolved in water], or dibutyryl cAMP (1 μ M, a cell-permeable analog of cAMP, dissolved in water) for 5 minutes prior to challenge with A β _{25–35}. Cell death was assessed by PI after 48 hours. Reagents were purchased from Tocris.

Trk inhibitor experiments

hNT neurons were co-treated with NA and K252a (1 μ M, dissolved in DMSO), genistein (GEN, 10 μ M, DMSO), the inactive genistein analog, genistin [GEN(-), 10 μ M, DMSO], or vehicle for 5 minutes and then challenged with A β ₂₅₋₃₅ for 48 hours. Cell death was measured by PI. Inhibitors were purchased from Tocris.

Neurotrophin antibody experiments

hNT neurons were pretreated with NA for 5 minutes and then challenged with A β ₂₅₋₃₅ alone or A β ₂₅₋₃₅ in the presence of 1) polyclonal NGF antiserum with functional blocking capacity (AB1528, 1:100; Chemicon/Millipore, Temecula, CA), 2) a functionally inert monoclonal NGF antibody (MAB5260, 1:100, Chemicon), or polyclonal BDNF antiserum with functional blocking capacity (AB1779SP, 1:100, Chemicon). Fresh antibody was added to culture media after 24 hours. Cell death was assessed by PI 48 hours after the start of the experiment.

Quantitative real-time PCR (qPCR)

Differentiated hNT neurons were treated with NA for 30 min, 1 hr, or 2 hr and then processed for qPCR analysis using human-specific primer sets (SA Biosciences, Frederick, MD) for *ngf*, *bdnf*, and the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*). Samples were loaded in triplicate on 96 well plates and analyzed using SYBR green reporter dye on an Opticon 2 DNA Engine (Bio-Rad, Hercules, CA). Standard curves and cycle threshold (Ct) were measured using standards obtained from reference human brain total RNA (SA Biosciences). For graphical representation, Ct values were converted to signal intensity values using Easy Engine software (Bio-Rad).

Western blotting

Differentiated hNT cultures were treated with vehicle, NA (10 μ M), ISO (100 μ M), or dbcAMP (1 μ M) for a 5 minutes to 2 hours time range and then harvested in ice cold homogenization buffer (20 mM Tris, 1 mM EGTA, 1 mM EDTA, 10% sucrose, pH 7.4) containing protease inhibitors. Sample proteins (10 μ g/sample) were separated by SDS-PAGE (10% acrylamide) and transferred to polyvinylidene fluoride membranes (Immobilon P, Millipore) electrophoretically. Membranes were blocked in TBS/0.1% Tween-20/5% milk for 30 minutes at room temperature (RT) and incubated overnight at 4°C with rabbit antiserum to phosphorylated cAMP response element binding protein (pCREB; 1:1000, Upstate/Millipore). Blots were rinsed and incubated for 1 hour at RT with horseradish peroxidase-conjugated goat anti-rabbit 2° IgG (1:8,000; Pierce, Rockford, IL). pCREB was visualized by enhanced chemiluminescence (Pierce/Thermo, Rockford, IL) on a Kodak Image Station 440CF (Perkin Elmer, Wellesley, MA). Blots were then stripped and re-probed with rabbit CREB antiserum (Upstate/Millipore) and processed as described above. Immunoreactive bands were quantified by densitometry using Kodak 1D image analysis software. pCREB signals were normalized to CREB signals for quantitative analysis.

Statistical analysis

All experiments were repeated 3 times independently. Data was analyzed by one-way ANOVA with Tukey's *post hoc* testing ($\alpha = 0.05$) except for western blot experiments, which were analyzed with Dunnett's *post hoc* testing ($\alpha = 0.05$). Student's *t* test was used for qPCR comparisons ($\alpha = 0.05$).

Results

Noradrenaline provides neuroprotection against amyloid

Differentiated human hNT neurons exhibit morphological and neurochemical properties similar to primary hippocampal and cortical neurons (Fig. 1A) (Lee and Andrews 1986; Pleasure et al. 1992; Tamagno et al. 2000). To test whether these cells were vulnerable to A β toxicity, cultures were treated with 10 μ M A β _{25–35} (see Methods). Cellular degeneration resembling apoptosis (e.g., pyknotic soma) was seen within 24 hours under light microscopy and peaked at ~ 96 hours (Fig. 1B). An approximately 50% increase in cell death was measured by the LIVE/DEAD assay after 96 hours of exposure to either A β _{25–35} or A β _{1–42} (Fig. 1D). To determine whether NA protects neuronal cells against this A β toxicity cultures were pretreated with 10 μ M NA prior to A β administration. Qualitative (Fig. 1C) and quantitative (Fig. 1D) analysis revealed that NA provided neuroprotection against both A β _{25–35} and A β _{1–42}. Propidium iodide (PI) staining was used after 48 hours to corroborate the protective effects of NA against A β _{25–35} and A β _{1–42} toxicity (Fig. 1E). NA neuroprotection against the toxic effect of amyloid treatment was also shown in experiments using rat E18 primary hippocampal cultures (Fig. 1F). Despite the structural differences in A β _{25–35} and A β _{1–42}, the similar toxicity profile of these two amyloid species in our model is reminiscent of findings from biochemical and electrophysiological comparative amyloid studies (Chen et al. 2000; Frozza et al. 2009) and may reflect a similar aggregation state, as previously described (Pike et al. 1993; Pike et al. 1995). Finally, we demonstrated that galanin, a neuropeptide which colocalizes with noradrenergic LC neurons and has neuroprotective properties (Elliott-Hunt et al. 2004; Hokfelt 2005; Counts et al. 2009), also provided neuroprotection against amyloid in the hNT cultures (Fig. 1G) similar to a report using septal cholinergic neurons (Ding et al. 2006).

Noradrenaline does not prevent A β -induced membrane depolarization

Following the demonstration that NA was neuroprotective against A β in our culture systems, we explored the mechanism(s) by which NA prevented A β neurotoxicity. Depolarization of the plasma membrane is a well-established physiological property of A β (Good et al. 1996; Blanchard et al. 1997; Hartley et al. 1999) and may be an initial contributor to a neurotoxic cascade which includes oxidative stress, mitochondrial membrane depolarization, and apoptosis (Mattson 2006). We found that 5 minutes of A β _{25–35} treatment depolarized hNT cultures as measured by the voltage sensitive probe DiBAC₂ and that pretreatment of the cultures with 10 μ M NA did not prevent A β -induced depolarization (Fig. 2).

Noradrenaline inhibits A β -induced oxidative stress, mitochondrial depolarization, and caspase activation

Since NA did not prevent A β -mediated plasma membrane depolarization, we investigated its effects on more downstream events underlying A β neurotoxicity. To this end, 10 μ M A β _{25–35} treatment of hNT neurons induced a significant 40% increase in reactive oxygen species (ROS) concentration after one-hour treatment (Fig. 3A). Pretreatment with 10 μ M NA abolished the generation of ROS induced by A β (Fig. 3A). The effects of amyloid on mitochondrial membrane potential in hNT cultures were evaluated using the JC-1 mitochondrial reagent assay. Treatment of the cultures with 10 μ M A β _{25–35} for 24 hours caused a significant 55% decrease in the ratio of intramitochondrial JC-1 to intracellular JC-1 (Fig. 3B), reflecting the depolarization of the mitochondrial membrane following amyloid exposure. Pretreatment with 10 μ M NA restored the mitochondrial membrane potential to control levels (Fig. 3B). This protective effect of NA in mitochondria was corroborated by fluorescence microscopy. As shown in Fig. 3D, A β reduced whereas NA pretreatment maintained levels of intramitochondrial JC-1 aggregates, a proxy for intact mitochondria. Finally, we measured caspase activation in hNT cultures following 24 hours

of 10 μM $\text{A}\beta_{25-35}$ with or without NA. The 40% increase in caspase activation in the presence of $\text{A}\beta$ alone was inhibited by NA pretreatment (Fig. 3C).

Noradrenaline neuroprotection is mediated by β -adrenergic receptors

To test whether NA neuroprotection was mediated by adrenoceptor activation, hNT cultures were pretreated with α_1 , α_2 , β_1 , or β_2 -adrenoceptor specific agonists at a range of doses prior to the administration of $\text{A}\beta$ (see Materials and Methods). As shown in Figure 4A, both the β -adrenoceptor agonist isoproterenol (ISO, 100 μM) and the β_2 -specific agonist salmeterol (SAL, 10 μM) mimicked the neuroprotective effects of 10 μM NA against 10 μM $\text{A}\beta_{25-35}$, whereas the α_1 agonist cirazoline (CRZ, 100 nM - 10 μM) and α_2 agonist UK 14, 304 (UK; 100 nM - 10 μM) were not protective. Likewise, pretreatment with the β_1 antagonist CGP 20712 (CGP, 10 μM) or β_2 -antagonist ICI 118, 551 (ICI, 10 μM) completely prevented NA from reducing amyloid-mediated cell death (Fig. 4B). Neither the α_1 antagonist prazosin (PRZ, 100 nM - 10 μM) nor the α_2 antagonist yohimbine (YHB, 100 nM - 10 μM) blocked NA neuroprotection (Fig. 4B).

To explore the role of β -adrenoceptor activation in preventing downstream events related to $\text{A}\beta$ neurotoxicity, we pretreated hNT cultures with 10 μM CGP or ICI prior to NA and evaluated their effects on $\text{A}\beta$ -induced oxidative stress, mitochondrial depolarization, and caspase activation, as described above. In the presence of either antagonist, 10 μM NA failed to prevent 10 μM $\text{A}\beta_{25-35}$ -induced mitochondrial depolarization (Fig. 5B) or caspase activation (Fig. 5C); however, the antagonists did not prevent NA from reducing $\text{A}\beta$ -mediated oxidative stress (Fig. 5A).

NA activates the cAMP 2nd messenger pathway

Since hNT neurons express β_1 and β_2 adrenoceptors (Fennell et al. 1998) and NA stimulation of these receptors mediated most aspects of neuroprotection in our assays, we tested whether the canonical β -adrenoceptor cAMP 2nd messenger pathway was activated in our paradigm. First, we demonstrated that 100 μM ISO-mediated protection of hNT cultures against amyloid was reversed by ~70% in the presence of the cAMP-dependent protein kinase A inhibitor PKI (1 μM , Fig. 6A). Furthermore, treatment of the hNT cultures with the membrane soluble cAMP mimetic dibutyryl cAMP (dbcAMP, 1 μM) mimicked ISO by preventing amyloid-induced cell death by ~85% (Fig. 6A).

We also immunoblotted detergent lysates of hNT cultures treated with 10 μM NA, 100 μM ISO, or 1 μM dbcAMP to measure levels of phosphorylated cAMP response element binding protein (pCREB), a major downstream target of cAMP involved in neuroprotective signaling (Mantamadiotis et al. 2002; Jancic et al. 2009). pCREB levels were elevated ~6–8 fold after 30 minutes of NA, ISO, or dbcAMP exposure (Fig. 6B, C), suggesting that activation of this pathway mediates NA neuroprotection.

NA confers neuroprotection against amyloid via neurotrophic pathways

When considering the possible CRE-containing gene targets of pCREB which might mediate NA neuroprotection in our model, we noted that 1) genes encoding the neurotrophins nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF) contain CREs (Tabuchi et al. 2002; McCauslin et al. 2006) and are up-regulated in response to CREB activation (Tabuchi et al. 2002; McCauslin et al. 2006; Chen and Russo-Neustadt 2009) and 2) hNT cultures express the Trk high affinity neurotrophin receptors for NGF (TrkA) and BDNF (TrkB) (Piontek et al. 1999). Therefore, we tested the hypothesis that NA induces NGF and/or BDNF expression to stimulate Trk-mediated neurotrophic signaling. To this end, quantitative real-time PCR (qPCR) was performed to measure *ngf* and *bdnf* mRNA levels in hNT cultures following one hour of 10 μM NA treatment. *Bdnf* mRNA levels were

~40% higher at this endpoint in NA-treated compared to untreated cultures (Fig. 7A), whereas *ngf* mRNA levels were increased ~25% following NA exposure (Fig. 7B). To test the involvement of Trk pathway stimulation in NA neuroprotection of hNT neurons, the Trk inhibitors K252a (1 μ M) or genistein (10 μ M) were co-incubated with 10 μ M NA prior to the administration of 10 μ M A β _{25–35}-induced cell death. These inhibitors effectively blocked NA protection against amyloid (Fig. 8A). Moreover, functional blocking antibodies to NGF or BDNF prevented NA neuroprotection against amyloid, providing more direct evidence for the involvement of these neurotrophic pathways (Fig. 8B). Significantly, NGF and BDNF functional antibodies also reduced NA neuroprotection by ~50% when A β _{1–42} was applied to rat E18 primary hippocampal cultures (Fig. 8C)

Discussion

In the present study, we show that NA protects target neurons from amyloid toxicity by preventing A β -induced increases in oxidative stress, mitochondrial dysfunction and the activation of apoptotic pathways. Moreover, we found that NA exerts its neuroprotective properties primarily through β -adrenergic stimulation of cAMP production and pCREB signaling, which in turn activates both NGF and BDNF-mediated pro-survival pathways. Taken together, these findings support the hypothesis that NA confers neuroprotection against amyloid toxicity in target fields through the activation of a neurotrophic autocrine or paracrine loop. Hence, a potential consequence of LC neurodegeneration may be the reduction in NGF and/or BDNF signaling in LC projection sites in AD.

A recent investigation has shown that NA protects rat primary cortical neurons against amyloid and that one potential mechanism involved antioxidant protection through the stimulation of glutathione levels and activation of peroxisome proliferator activated receptor delta, which boosts antioxidant systems through the detoxification of superoxide radicals (Madrigal et al. 2007). Whether these effects were receptor-mediated is unclear. We found evidence that NA prevented amyloid-induced ROS in a receptor-independent manner in a well-established cell culture model for human hippocampal neurons (Lee and Andrews 1986; Pleasure et al. 1992; Tamagno et al. 2000). These observations complement a previous study demonstrating a receptor-independent role for NA in preventing neuronal cell death via low-level oxidative stress, perhaps via the catechol ring structure of NA since its diphenolic moiety pyrocatechol was also protective (Traver et al. 2005). On the other hand, our adrenoceptor pharmacological and protein signaling analysis indicated that NA's role in stabilizing mitochondrial membrane permeability, preventing caspase activation, and promoting neuronal survival in A β -treated cultures was dependent on β -adrenoceptor activation of neurotrophins. This suggests a dual function for NA in neuroprotection via receptor-dependent and independent pathways.

Direct application of recombinant neurotrophins like BDNF blocks amyloid toxicity in neuronal cultures (Arancibia et al. 2008). However, the present investigation revealed that NA stimulates either an adrenoceptor-mediated autocrine or paracrine loop to induce endogenous neurotrophin production resulting in neuroprotection. Previous work demonstrated that NA up-regulates BDNF and phosphorylated pan-Trk levels in hippocampal cultures and that these responses can be reduced by Trk pathway inhibitors, suggesting a neuronal feed-forward BDNF production loop which involve adrenoceptor-Trk transactivation through G proteins (Chen et al. 2007). While we cannot rule out this latter signaling event in the present study, the prevention of NA's neuroprotective effects by BDNF and NGF functional blocking antibodies places the induction and release of these neurotrophins upstream of Trk-mediated pro-survival functions. Intriguingly, noradrenergic innervation of glia and glial expression of β -adrenoceptors (Schubert et al. 1976; Hosli et al. 1982), combined with the role of astro- and microglia in neurotrophin production (Miklic et

al. 2004; Riley et al. 2004), raises the possibility that NA activates a paracrine loop wherein β -adrenoceptor-expressing glial cells are stimulated to produce NGF and/or BDNF (Juric et al. 2008), which in turn are secreted and activate neuronal TrkA and TrkB receptors to provide protection against amyloid. This is in line with a recent finding that astroglial release of the chemokine MCP-1 can mediate NA neuroprotective properties (Madrigal et al. 2009). However, further studies are needed to test whether a paracrine neurotrophic loop is stimulated by NA.

A critical question arising from the present findings and previous reports of NA anti-inflammatory action (Feinstein et al. 2002; Heneka et al. 2002; Heneka et al. 2003) is whether NA both suppresses A β production and protects against A β toxicity. For instance, NA signaling reduces nitric oxide (NO) synthase expression in astrocytes (Feinstein 1998) in a cAMP-dependent manner (Galea and Feinstein 1999). This suggests the involvement of β -adrenoceptor and CREB in NA-mediated reductions of NO levels. Since NO activates A β production (Blasko and Grubeck-Loebenstein 2003), which in turn can stimulate a neurotoxic inflammatory cascade through the further induction of NO (Puzzo et al. 2006), NA may act to prevent a vicious cycle of NO and A β production. Furthermore, the observation that NA signaling induces BDNF production via cAMP and CREB to prevent A β toxicity suggests that activation of β -adrenoceptors by this neurotransmitter regulates the balance between amyloid and neurotrophin production to maintain neuronal survival. This would provide a unifying theory for NA's putative role in regulating amyloidosis and neurotrophism and shed even greater insight on the mechanistic consequences for LC degeneration during the pathogenesis of AD.

The present findings are also informative in light of NA's long-recognized role in higher cognitive processes such as memory and attention (Berridge and Waterhouse 2003; Weinschenker 2008; Sara 2009). Relevant to the memory deficits which are central to the clinical diagnosis of AD, NA is critical for certain types of hippocampal learning and memory tasks that are impaired in this disease. Electrophysiological studies show that NA stimulation of β -adrenoceptors profoundly influences long term potentiation - a model for memory consolidation - and is required for hippocampal dendritic protein expression (Gelinias and Nguyen 2005) and memory formation (Katsuki et al. 1997; Gelinias et al. 2008; Sara 2009), whereas rodents lacking NA signaling have specific defects in memory retrieval (Thomas and Palmiter 1997; Schimanski et al. 2007). Given the established role for CREB-BDNF (Korte et al. 1996; Patterson et al. 1996) and NGF (Woolf et al. 2001; Conner et al. 2009) in hippocampal synaptic plasticity, NA regulation of these neurotrophins through β -adrenoceptors may contribute to hippocampal learning and memory function in addition to essential neuronal survival signaling.

We also found that the LC noradrenergic co-transmitter GAL protected hNT cultures from A β toxicity. The mechanism for this is unclear, but GAL has been shown to protect hippocampal organotypic cultures from excitotoxicity (Elliott-Hunt et al. 2004; Elliott-Hunt et al. 2007) and primary septal neurons from amyloid (Ding et al. 2006) via the GAL receptor GALR2. GALR2 couples to G_{q/11} proteins to activate phospholipase C and protein kinase C (Wang et al. 1998; Wittau et al. 2000), suggesting a different G protein-coupled pathway for cell survival relative to NA-stimulated G_s proteins. While the neuroprotective effects of NA and GAL co-administration were not additive in our model, these findings also support the hypothesis that LC deafferentation of projection zones in AD may impact several neurodegenerative sequela underlying the disease process.

In summary, the present findings provide data to a growing list of potential mechanisms by which noradrenergic signaling might exert neuroprotective properties (Troade et al. 2001; Heneka et al. 2002; Traver et al. 2005; Chen et al. 2007; Madrigal et al. 2007; Madrigal et

al. 2009), particularly against diffusible amyloid in brain areas associated with cognitive dysfunction during the progression of AD. With respect to AD therapy, current β -adrenoceptor agonists improve cognition but have a side effect profile that includes tachycardia and other arrhythmias (Friedman et al. 1999), limiting their use as cognitive enhancing agents despite their integral involvement in memory formation and hippocampal cholinergic activity (Watabe et al. 2000) as well as regulating amyloid and neurotrophin production. However, our findings offer alternative targets which activate the same neuroprotective pathways elucidated here but circumscribe supraphysiological β -adrenoceptor activation, including strategies such as targeted BDNF delivery (Nagahara et al. 2009) or NA replacement (Weinshenker 2008). Considering the up-regulation of β -adrenoceptors observed in end-stage AD (Kalaria et al. 1989), this latter alternative might maintain endogenous receptor levels and activity early in the disease process without deleterious physiological side effects.

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Abbreviations

Aβ	amyloid- β peptide
BDNF	brain-derived neurotrophic factor
CREB	cAMP response element binding protein
GAL	galanin
NA	noradrenaline
NGF	nerve growth factor

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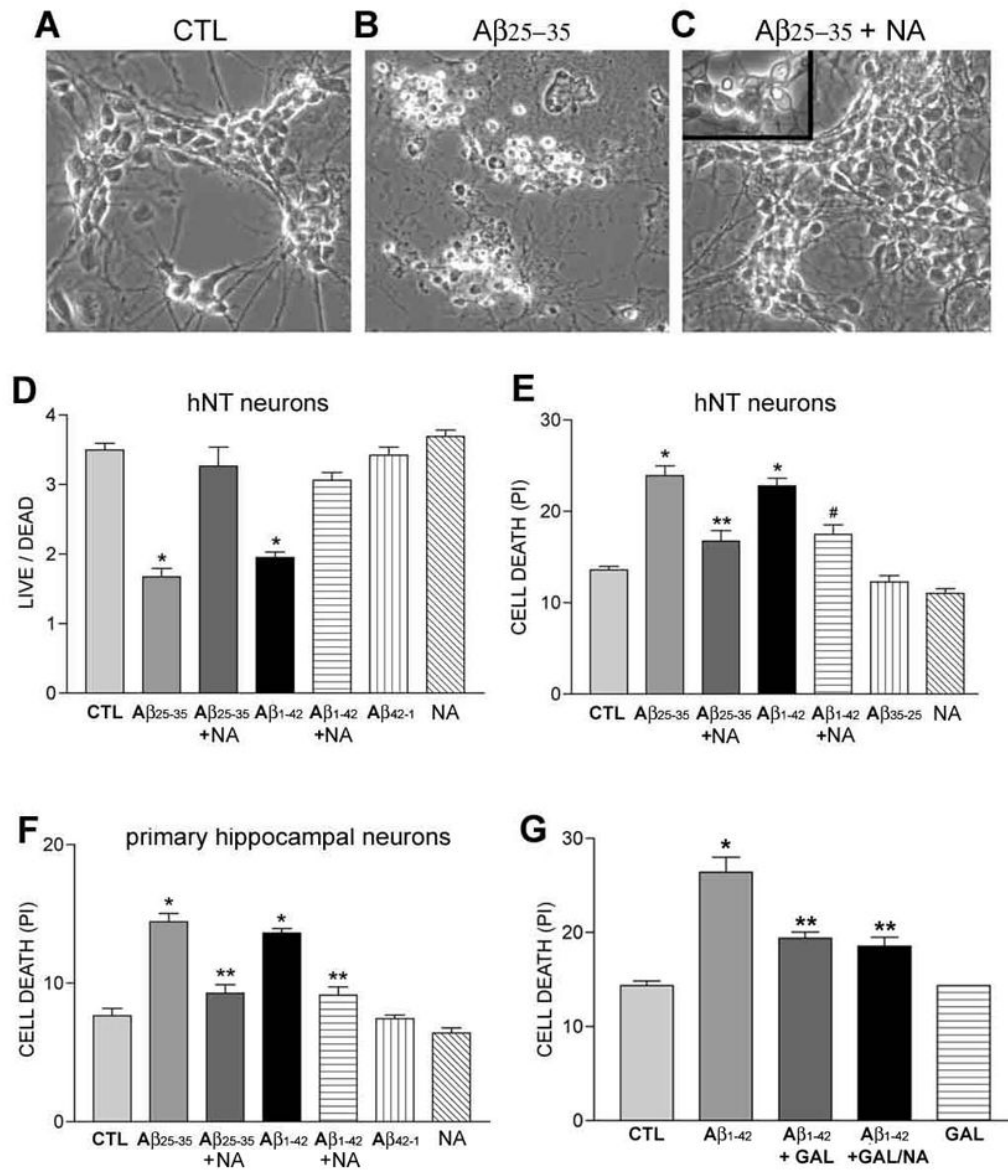
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**Figure 1.**

NA protects hNT and primary hippocampal neurons from A β toxicity.

Photomicrographs show human hNT neurons after (A) 4 days in control conditions (CTL), (B) 4 days exposure to 10 μ M A β , or (C) 4 days exposure to 10 μ M A β in the presence of 10 μ M NA. Note the pyknotic cells (C, inset) suggesting A β toxicity to some neurons in the NA co-treated cultures. D) Bar graph shows NA protection of hNT neurons against both A β_{25-35} and A β_{1-42} . Cultures were assayed by LIVE/DEAD. *, $p < 0.001$ vs. CTL E) Bar graph shows NA protection of hNT neurons after 2 days of A β_{25-35} or A β_{1-42} treatment via corroborative propidium iodide (PI) assays. *, $p < 0.001$ vs. CTL; **, $p < 0.001$ vs. A β_{25-35} ; #, $p < 0.01$ vs. A β_{1-42} . F) Bar graph shows NA protection of rat primary hippocampal neurons after 2 days of A β_{25-35} or A β_{1-42} treatment via PI assay. *, $p < 0.001$ vs. CTL; **, $p < 0.001$ vs. A β ; $n = 8$ /treatment group in 3 independent experiments (i.e., $n = 24$ total/group) for each assay. Note that no effects were seen with reverse A β peptides in any of the assays. G) The NA peptide co-transmitter galanin (GAL) also conferred neuroprotection against 2 days of A β_{1-42} treatment. Co-treatment with GAL and NA did not significantly

improve survival over GAL alone. Similar effects were observed with A β ₂₅₋₃₅ (not shown)
*, $p < 0.001$ vs. CTL; **, $p < 0.05$ vs. A β Values in the Y-axis for LIVE/DEAD and PI
experiments are arbitrary spectrophotometric units.

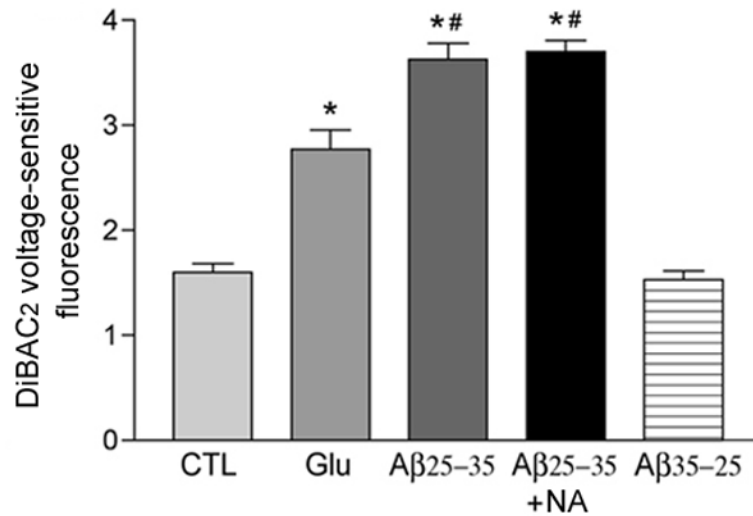
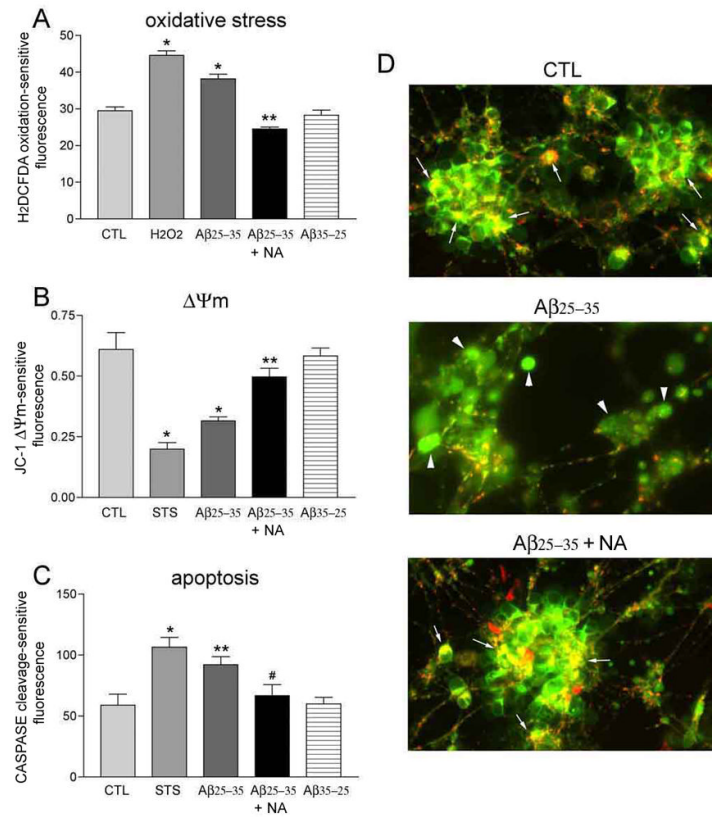


Figure 2.

NA does not prevent A β -induced neuronal plasma membrane depolarization. A) Bar graph shows that 10 μ M NA pre-treatment failed to prevent hNT plasma membrane depolarization induced after 1 hour of 10 μ M A β ₂₅₋₃₅ exposure, as assayed using MTT. Glutamate (Glu) was used as a positive control. *, $p < 0.001$ vs. CTL; #, $p < 0.01$ vs. Glu. B) Bar graph shows that NA did not prevent A β -induced depolarization as measured by DiBAC2 after 5 minutes of A β treatment. No effect was observed with reverse A β ₃₅₋₂₅ peptide. *, $p < 0.001$ vs. CTL; #, $p < 0.01$ vs. Glu. Values in the Y-axis are arbitrary spectrophotometric units.

**Figure 3.**

NA inhibits Aβ-induced oxidative stress, mitochondrial depolarization, and caspase activation. A) Bar graph shows that 1 hour of 10 μM Aβ₂₅₋₃₅ results in increased reactive oxygen species (ROS) in hNT neurons. 10 μM NA restored ROS to CTL levels. Hydrogen peroxide (H₂O₂) was used as a positive control. No effect was seen with reverse Aβ. *, $p < 0.001$ vs. CTL; **, $p < 0.001$ vs. Aβ. B) NA prevented depolarization of the hNT mitochondrial membrane potential ($\Delta\psi_m$) after 24 hours of treatment with Aβ. $\Delta\psi_m$ was measured using a JC-1-dependent ratio of intact (red/green) to depolarized (green) mitochondria. Staurosporine (STS) was the positive control. *, $p < 0.001$ vs. CTL; **, $p < 0.05$ vs. Aβ. C) 24 hours of Aβ exposure increases caspase activation, which was inhibited by NA. *, $p < 0.001$ vs. CTL; **, $p < 0.05$ vs. CTL; #, $p < 0.05$ vs. Aβ; $n = 8$ /treatment group in 3 independent experiments for each assay. D) Fluorescent photomicrographs show overlays of intramitochondrial JC-1 aggregates (red) and intracellular JC-1 (green) in hNT cultures treated 24 hours in CTL (*top*), Aβ (*middle*), or Aβ with NA (*bottom*) conditions. Arrows in *top* and *bottom* panels show distinct red + green JC-1 colocalization indicating healthy mitochondria. Arrowheads in *middle* panel show diffuse green JC-1 staining of depolarized mitochondria. Values in the Y-axis for (A–C) are arbitrary spectrophotometric units.

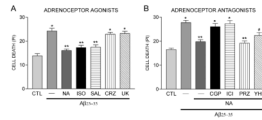
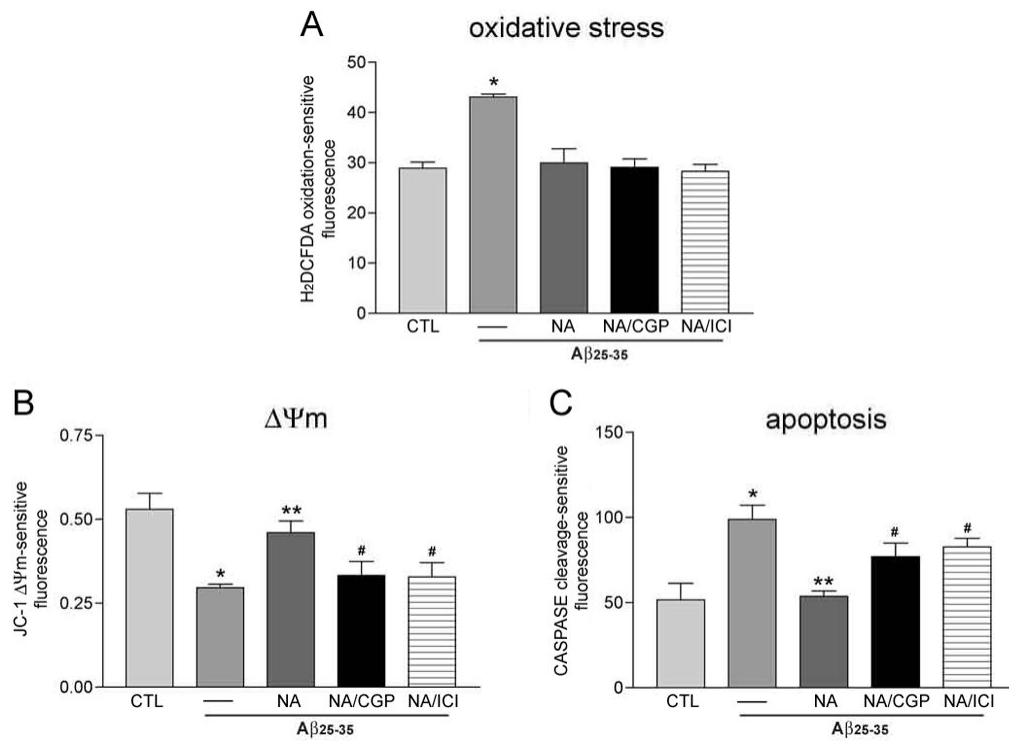


Figure 4.

NA neuroprotection is mediated by B-adrenergic receptors. A) Bar graph shows cell death after 48 hours of 10 μ M $A\beta_{25-35}$ exposure following pretreatment of hNT neurons with 10 μ M NA or adrenoceptor agonists isoproterenol (ISO, β), salmeterol (SAL, β_2), cirazoline (CRZ, α_1), or UK 14,304 (UK, α_2) [all doses 10 μ M except ISO (100 μ M)]. *, $p < 0.001$ vs. CTL; **, $p < 0.001$ vs. $A\beta$. B) Bar graph shows cell death after 48 hours of $A\beta_{25-35}$ alone, pretreated with NA, or co-treated with NA following pretreatment with adrenoceptor antagonists CGP 20712 (CGP, β_1), ICI 118, 551 (ICI, β_2), prazosin (PRZ, α_1), or yohimbine (YHB, α_2) (all doses 10 μ M). *, $p < 0.001$ vs. CTL; **, $p < 0.001$ vs. $A\beta$; #, $p < 0.05$ vs., $A\beta$; $n = 8$ /treatment group in 3 independent experiments for each assay.

**Figure 5.**

NA uses both receptor-dependent and independent signaling to inhibit cell death pathways. A. β -adrenoceptor antagonists CGP and ICI (10 μ M each) failed to block 10 μ M NA protection of hNT neurons against ROS induced by 1 hour of 10 μ M A β_{25-35} exposure. *, $p < 0.01$ vs. CTL. On the other hand, CGP and ICI pretreatment did inhibit NA's ability to block A β -mediated mitochondrial membrane depolarization (B) and caspase activation (C) after 24 hours. *, $p < 0.01$ vs. CTL; **, $p < 0.01$ vs. A β ; #, $p < 0.05$ vs. NA; $n = 4$ /treatment group in 3 independent experiments.

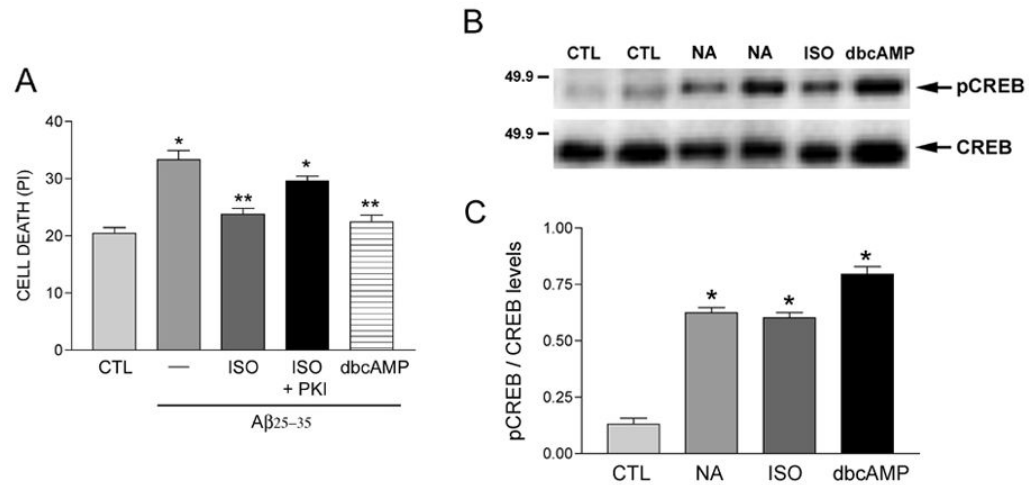


Figure 6.

NA activates the cAMP 2nd messenger pathway. A) Bar graph shows that ISO (100 μ M)-mediated neuroprotection against 10 μ M A β in hNT neurons is blocked by PKI (1 μ M), an inhibitor of PKA. Application of dbcAMP (1 μ M), a permeable cAMP analog, mimicked ISO treatment. *, $p < 0.001$ vs. CTL; **, $p < 0.001$ vs. A β ; $n = 8$ /treatment group in 3 independent experiments. B) Western blot showing increased phosphorylated CREB (pCREB) immunoreactivity after 30 minutes in NA-treated hNT cultures. This effect was mimicked by ISO and dbcAMP. C) Quantitative analysis revealed an approximately 6–8 fold increase in pCREB immunoreactivity following NA, ISO, or dbcAMP treatments ($n = 6$). *, $p < 0.001$ compared to CTL.

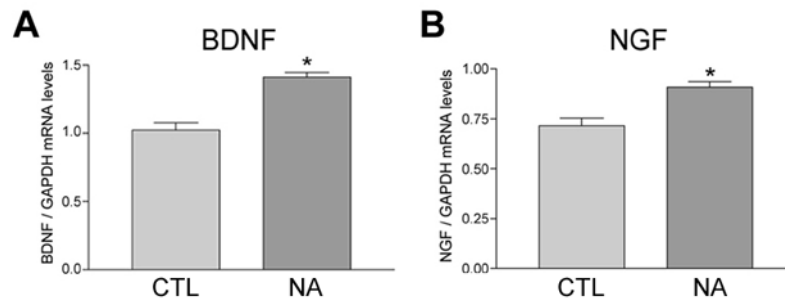
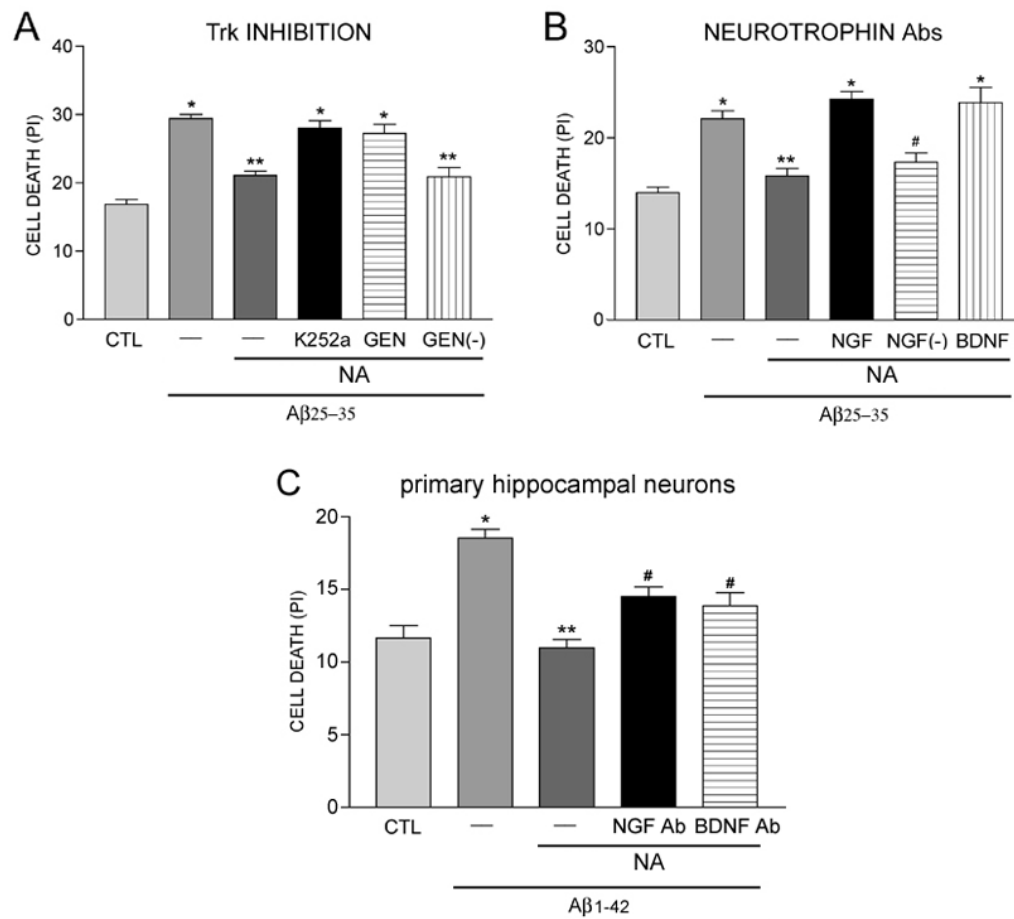


Figure 7.

NA treatment increases BDNF and NGF mRNA levels in hNT neurons. A) Bar graph shows increased *bdnf* mRNA levels in hNT cultures following 1 hour of 10 μM NA, as measured by quantitative real-time PCR (qPCR) using human *bdnf*-specific primers. *, $p < 0.05$ vs. CTL. B) One hour of NA treatment also resulted in increased *ngf* mRNA levels in the hNT cultures. *, $p < 0.05$ vs. CTL; $n = 3$ /treatment group in 3 independent experiments.

**Figure 8.**

NA neuroprotection is dependent on BDNF and NGF. A) Trk high-affinity neurotrophin receptor pathways are involved in NA neuroprotection. Bar graph shows that 10 μ M NA protection against 10 μ M $A\beta_{25-35}$ neurotoxicity was blocked by the Trk pathway inhibitors K252A (1 μ M) and genistein (10 μ M). Genistein [GEN(-), 10 μ M], an inactive genistein analog, served as a negative control. *, $p < 0.001$ vs. CTL; **, $p < 0.001$ vs. $A\beta$. B) Bar graph shows that functional blocking antibodies for NGF (1:100) and BDNF (1:100) reverse NA neuroprotection against $A\beta$ in hNT neurons. Another NGF antibody without functional blocking capability [NGF(-), 1:100] served as a negative control. *, $p < 0.001$ vs. CTL; **, $p < 0.01$ vs. $A\beta$, #, $p < 0.05$ vs. $A\beta$; $n = 8$ /treatment group in 3 independent experiments. C) Bar graph shows that functional NGF and BDNF blocking antibodies reverse 10 μ M NA neuroprotection against 10 μ M $A\beta_{1-42}$ in rat E18 primary hippocampal cultures. *, $p < 0.001$ vs. CTL; **, $p < 0.01$ vs. $A\beta$, #, $p < 0.05$ vs. NA. $n = 4$ /treatment group in 3 independent experiments.