ORIGINAL ARTICLE

Amitriptyline protects against TNF-α-induced atrophy and reduction in synaptic markers via a Trk-dependent mechanism

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Abstract

Neuritic degeneration and synaptic loss are features of both neuroinflammation and neurodegenerative disease. The tricyclic antidepressant amitriptyline has neurotrophic and anti-inflammatory properties and acts as a novel agonist of the neurotrophin Trk receptors. Primary cortical neurons were treated with amitriptyline, nortriptyline and NGF and tested for neuronal complexity by Sholl analysis, protein expression by Western immunoblotting, and synapse number by colocalization of pre and postsynaptic makers. Amitriptyline (500 nmol/L) and its active metabolite nortriptyline (50 nmol/L) are found to induce neurite outgrowth in rat primary cortical neurons. Amitriptyline-induced neurite outgrowth is blocked by inhibition of Trk signaling using Trk antagonist K252a (200 nmol/L) but not by the neurotrophin inhibitor Y1036 (40 μmol/L), indicating that amitriptyline binds directly to the Trk receptor to initiate neurite outgrowth. MEK inhibitor PD98059 (10 μmol/L) also blocks amitriptyline-induced neurite outgrowth, implicating activation of the MAPK signaling pathway downstream of Trk receptor activation. Furthermore, pretreatment of primary cortical neurons with amitriptyline and nortriptyline prevents the effects of the proinflammatory cytokine TNF-α (10 ng/mL) on neurite outgrowth and colocalization of synaptic proteins. These findings suggest that amitriptyline and nortriptyline can exert neurotrophic effects in primary cortical neurons via activation of a Trk/MAPK signaling pathway. These compounds therefore have significant potential to be used in the treatment of neurodegenerative conditions where atrophy and loss of synaptic connections contribute to progression of disease.

Abbreviations

BDNF, brain-derived neurotrophic factor; CNS, central nervous system; ERK, extracellular signal-related kinase; IKK, IκB kinase; MAPK, mitogen-activated protein kinase; NFκB, nuclear factor κ B; NGF, nerve growth factor; NT-3/4, neurotrophin-3/4; p75NTR, p75 neurotrophin receptor; PI3K, phosphoinositide 3-kinase; TCA, tricyclic antidepressant; TNF-α, tumor necrosis factor α; Trk, tropomyosin-receptor-kinase.

Introduction

Amitriptyline is a tricyclic antidepressant that has been in clinical use since 1961 and is still in widespread use today (Leucht et al. 2012), both as an antidepressant and as a first-line treatment for neuropathic pain where its role in regulation of neuronal plasticity is thought to be involved in its mechanism of action (Verdu et al. 2008). As an antidepressant, amitriptyline inhibits reuptake of the monoamine neurotransmitters noradrenaline and

serotonin into the presynaptic neuron, thereby increasing their concentrations in the synaptic cleft and contributing to the antidepressant response. This action occurs via blockade of the noradrenaline and serotonin transporters with amitriptyline having a strong affinity for the serotonin transporter and a moderate affinity for the noradrenaline transporter (Tatsumi et al. 1997). Amitriptyline's mechanism of action in treatment of neuropathic pain is not likely to involve the noradrenaline and serotonin systems, as analgesia is often achieved at lower dosage than that required to achieve an antidepressant effect (Moore et al. 2015). Amitriptyline has an active metabolite, nortriptyline, which acts in a similar manner to amitriptyline, differing in its selectivity for inhibition of the noradrenaline transporter over the serotonin transporter (Tatsumi et al. 1997). Nortriptyline is currently prescribed as a second-generation tricyclic antidepressant for the treatment of major depressive disorder and childhood nocturnal enuresis and is also recommended for treatment of neuropathic pain (Derry et al. 2015). Amitriptyline and nortriptyline also act as antagonists of various other receptors in the central nervous system (CNS), including serotonergic, α_1-adrenergic, histamine and muscarinic cholinergic receptors (Gillman 2007). It is action on these receptors that leads to the associated adverse effects of these drugs.

More recently, amitriptyline has been shown to act as an agonist of tropomyosin-receptor-kinase (Trk) receptors (Jang et al. 2009). Trk receptors (TrkA, TrkB, and TrkC) are bound by the neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF) and neurotrophins-3 and -4 (NT-3, NT-4). Binding of neurotrophins to Trk receptors promotes neuronal survival and differentiation, with TrkA mediating the effects of NGF, TrkB being bound by BDNF, NT-3 and NT-4 and TrkC activated by NT-3. All four neurotrophins can also bind to the p75 neurotrophin receptor (p75NTR), which is thought to induce apoptosis and inhibition of neurite outgrowth, as well as mediating certain cell survival and inflammatory processes (Chao 2003). It has been demonstrated both in vitro and in vivo that amitriptyline directly binds both TrkA and TrkB receptors and induces their dimerization and activation (Jang et al. 2009). Amitriptyline induces neurite outgrowth in the PC12 cell line and injection of amitriptyline into mouse brain prevents excitotoxicity and neuronal apoptosis induced by kainic acid, with the TrkA receptor shown to have the greater role in mediating the neuroprotective effects of amitriptyline in vivo (Jang et al. 2009). Amitriptyline has been further demonstrated to induce phosphorylation of the TrkB receptor and to increase production of its cognate ligand, BDNF, in the hippocampus (Chadwick et al. 2011). In this study, amitriptyline also enhanced cognitive

function in a mouse model of Alzheimer's disease which occurred alongside increased neurogenesis in the dentate gyrus in vivo and increased neural progenitor cell development in vitro. Amitriptyline increases expression of *Ndrg4*, a gene that has been demonstrated to enhance neurite outgrowth and remodeling (Hongo et al. 2006). It has been hypothesized that these increases in markers of neuronal development may indicate a potential mechanism for the observed cognitive enhancement following amitriptyline treatment (Chadwick et al. 2011). Amitriptyline also has neurotrophic effects, increasing production of glial-derived neurotrophic factor through activation of fibroblast growth factor signaling (Hisaoka et al. 2011; Kajitani et al. 2012) and increasing BDNF expression in the brains of depressed patients (Hellweg et al. 2008). Additionally, nortriptyline has been demonstrated to increase TrkB phosphorylation in the hippocampus while also increasing BDNF mRNA expression in the dentate gyrus. These changes in neurotrophic signaling were found to correlate well with antidepressant and anxiolytic effects of nortriptyline in a rat model of major depression (Eriksson et al. 2012).

Inflammation in the CNS is known to be a major contributing factor to neurodegeneration and has been implicated in numerous CNS disorders including Alzheimer's disease, Parkinson's disease, multiple sclerosis, depression, and stroke (Allan and Rothwell 2003; Heneka and O'Banion 2007; Dantzer et al. 2008; Lassmann 2008; McGeer and McGeer 2008). Growth factors such as the neurotrophins represent potential targets for treatment of inflammatory damage in the CNS. However, the neurotrophins are limited as a therapeutic option due to their lack of blood–brain barrier permeability (Longo and Massa 2013) and associated adverse effects, such as NGF-induced hyperalgesia (McKelvey et al. 2013). For this reason, there is an evident need for brain-permeable drugs that will reduce neurodegeneration by targeting neuroinflammation. Amitriptyline has been shown to have anti-inflammatory and antinociceptive effects in animal models of inflammation (Abdel-Salam et al. 2003; Sadeghi et al. 2011). Amitriptyline treatment drives an anti-inflammatory phenotype by suppressing nitric oxide synthesis, pro-inflammatory cytokine release (Vismari et al. 2012), prostaglandin release (Yaron et al. 1999) neutrophil migration and mast cell secretion of proinflammatory mediators (Clemons et al. 2011; Gurgel et al. 2013). Amitriptyline and nortriptyline inhibit tumour necrosis factor α (TNF-α) and interleukin-1β release induced by lipopolysaccharide in rat mixed glial cell cultures (Obuchowicz et al. 2006).

As both the anti-inflammatory role and action of amitriptyline as a Trk receptor agonist represent potential therapeutic targets for stimulating neuronal growth and

synaptogenesis, we hypothesized that amitriptyline and nortriptyline bind directly to Trk receptors on primary neurons to induce neurite outgrowth and increase colocalization of synaptic markers. We used different antagonists of neurotrophic signaling pathways to determine the mechanism by which neurite outgrowth occurs. We also used an inflammatory challenge, namely TNF-α-induced neuronal atrophy and reduced synaptic colocalization, to determine whether the neurotrophic effects of amitriptyline and nortriptyline are protective in neuroinflammatory conditions. In summary, amitriptyline and nortriptyline were found to increase neurite outgrowth and colocalization of synaptic markers and to protect against the atrophic effects of TNF-α.

Materials and Methods

Materials

Amitriptyline and nortriptyline were obtained from Sigma-Aldrich, Arklow, Ireland. TNF-α was obtained from R&D Systems Abington, Ireland. K252a was obtained from Sigma-Aldrich, Ireland. Y1036 was obtained from Millipore, Cork, Ireland. PD98059 was obtained from Tocris Bioscience, Bristol, UK. Anti-βIII-tubulin antibody was obtained from Promega Southampton, UK, antisynaptophysin antibody was obtained from Pierce, Waltham USA, and anti-PSD-95 antibody was obtained from Fisher Scientific, Dublin Ireland. Secondary antibodies for fluorescent staining were obtained from Invitrogen, Dun Laoghaire USA, and for Western immunoblotting from GE Life Sciences, Buckinghamshire, UK. Primary antibodies for Western immunoblotting were obtained from Cell Signaling, Danvers USA. Cell culture reagents were obtained from Sarstedt, Wexford, (Ireland) and all other reagents were obtained from Sigma-Aldrich (Ireland) unless otherwise stated. All animal procedures conformed to the European Council Directive 2010/63/EU and were approved by the Comparative Medicine Ethics Committee of the University of Dublin, Trinity College.

Preparation of primary cortical neurons

Primary cortical neurons were isolated and prepared from 1-day-old Wistar rats (Comparative Medicine Unit, Trinity College Dublin). Briefly, the rats were decapitated, meninges removed and the cortices isolated. Cortical tissue was cross-chopped in prewarmed complete Neurobasal media (cNBM, 1% (v/v) penicillin/streptomycin, 1% (v/v) Glutamax, 0.1% (v/v) Fungizone and 1% (v/v) B27 supplement) and dissociated in trypsin-EDTA for 2 min at 37°C. Prewarmed complete Dulbecco's minimal essential media (cDMEM:F12, 10% (v/v) foetal bovine serum, 1% (v/v) penicillin/streptomycin, 0.1% (v/v) Fungizone) was added and the tissue was triturated, followed by centrifugation at 2000g for 3 min at 20°C. The resultant supernatant was discarded and the pellet resuspended in cDMEM. This cell suspension was triturated to a homogeneous suspension, passed through a sterile mesh filter (40 μm) and centrifuged at 2000× rpm for 3 min at 20°C. The supernatant was discarded and the pellet was resuspended in cNBM. The cell suspension was diluted to 3×10^5 cells/mL for Sholl analysis and synaptic marker experiments and 1×10^6 cells/mL for Western blot experiments and plated onto poly-d-lysine-coated coverslips in 24-well plates. Cells were cultured in an incubator containing 5% CO_2, 95% air at 37°C. Neurons were treated after 3 days in vitro (DIV) for Sholl analysis and Western blot experiments and after 18–21 DIV for synaptic marker experiments. All drugs were dissolved in cNBM, where control wells received cNBM alone (+dimethyl sulfoxide (DMSO) vehicle where appropriate).

Immunocytochemistry and imaging for Sholl analysis

Neuronal cultures were fixed in ice-cold methanol for 20 min. Non-specific interactions were blocked using 4% normal goat serum (NGS) in phosphate-buffered saline (PBS) for 2 h at room temperature. Coverslips were incubated with primary antibody against mouse βIII-tubulin (1:1000 in PBS), a specific marker for neurons, at 4°C overnight before incubation with fluorescent secondary antibody goat anti-mouse Alexa Fluor 488 (1:2000 in PBS) at room temperature for 90 min. Coverslips were mounted on microscope slides using Vectashield mounting medium with DAPI (Vector Labs, Peterborough, UK.). Images were taken using an epifluorescent microscope and AxioVision Rel. 4.8 software (Carl Zeiss MicroImaging, Cambridge, UK.). Neurons were viewed at 200× magnification. Neuronal morphology was measured using Sholl analysis, a widely used method for quantification and graphic representation of the characteristics of neurites (Sholl 1953). The procedure was adapted from Gutierrez and Davies (2007). Care was taken to capture representative images of neurons with clearly distinguishable neurites. Five neurons were analyzed per coverslip and averaged so that 1 coverslip = 1 *n*. The number of primary neurites, the number of neuritic branches, relative neurite length and the Sholl profile of neurons were measured. Primary neurites were defined as neurites extending from the cell body while a neuritic branch was defined where a neurite branched in two for at least 5 μm. Relative neurite length refers to the total number of estimated intersections per neuron and this value is linearly proportional to the metric length of the neuritic arbor (Gutierrez and Davies 2007). The Sholl profile of a

neuron represents the number of neuritic intersections versus the radial distance from the cell soma. Data was collected with the use of a simple computer program (Matlab R2012b, MathWorks, Cambridge, UK.) script.

Immunocytochemistry and imaging for analysis of synaptic markers

Neuronal cultures were fixed in ice-cold methanol for 20 min. Nonspecific interactions were blocked and cells permeabilized using 50% NGS in PBS with the addition of 0.2% Triton-X for 30 min at room temperature. Coverslips were incubated in a cocktail of primary antibodies against rabbit synaptophysin (1:500 in PBS) and mouse PSD-95 (1:500 in PBS) at 4°C overnight before incubation with fluorescent secondary antibodies goat anti-mouse Alexa Fluor 488 (1:1000 in PBS) and goat anti-rabbit Alexa Flour 546 (1:1000 in PBS) at room temperature for 90 min. Coverslips were mounted on microscope slides using Vectashield mounting medium with DAPI. Images were taken using an epifluorescent microscope and Axio-Vision Rel. 4.8 software. Neurons were viewed at 400× magnification. Images were taken of cells that were at least two cell diameters away from their nearest neighbors. Synaptic colocalization was measured by examining the expression of the pre-synaptic marker, synaptophysin, and postsynaptic marker, PSD-95. This procedure was adapted from Ippolito and Eroglu, using the ImageJ analysis programme (National Institutes of Health) with the Puncta Analyzer plug-in (Ippolito and Eroglu 2010).

Western immunoblotting

Neuronal cells were lysed in NP-40 lysis buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 1% NP-40) and protein concentration was determined by the Bradford protein assay method (Bradford 1976). Samples were equalized and diluted 1:4 in 4× Laemmli sample buffer (1M Tris-HCl, 25% sodium dodecyl sulphate, 50% glycerol, 10% β-mercaptoethanol, 2% bromophenol blue) and boiled for 5 min at 65° prior to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblotting being performed. 10–15 μg total protein was loaded per well. Proteins were separated on 10% gels, transferred onto polyvinylidene fluoride (PVDF) membrane and blocked for 1 h at room temperature with 5% bovine serum albumin (BSA) in Tris-buffered saline with 0.05% Tween (TBS-T) prior to incubation at 4°C overnight with primary antibodies against rabbit phosphorylated extracellular signal-related kinase (ERK), total ERK and β-actin (1:1000 in TBS-T). Blots were incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit or goat anti-mouse secondary antibody (1:2000 in TBS-T)

at room temperature for 1 h. Blots were exposed to Immobilon Western HRP substrate (Millipore) for 5 min and developed using a Fujifilm Luminescent Image Analyzer (LAS-3000). Protein bands were quantified using ImageJ software. Blots were stripped and reprobed with additional antibodies (three antibodies/blot). The β-actin levels were used as loading controls.

Statistical analysis of data

All values are expressed as mean ± standard error of the mean (SEM). Data were analyzed using a one- or two-way analysis of variance (ANOVA) followed, where appropriate, by a Newman–Keuls, Bonferroni (Sholl analysis) or Fisher's Least Significant Difference (LSD) (Western immunoblotting) post hoc test (GB-Stat). A $P < 0.05$ was considered statistically significant.

Results

Amitriptyline and nortriptyline induce neurite outgrowth via a direct action on neurons

Amitriptyline has been shown to act as an agonist of Trk receptors and to induce neurite outgrowth in PC12 cells through Trk receptor activation (Jang et al. 2009). Therefore, we hypothesized that amitriptyline may act directly on neuronal Trk receptors to induce neurite outgrowth in primary cortical neurons. Primary cortical neurons were treated with amitriptyline (50, 500 nmol/L) or nortriptyline (50, 500 nmol/L) for 24 h. Neuronal complexity was assessed using Sholl analysis. Amitriptyline significantly increased the number of primary neurites ($P < 0.001$; Fig. 1a), neuritic length ($P < 0.001$; Fig. 1b), number of neuritic branches ($P < 0.001$; Fig. 1c) and enhanced the Sholl profile ($P < 0.001$; Fig. 1d). Treatment with nortriptyline significantly increased the number of primary neurites ($P < 0.001$; Fig. 1a), relative neurite length ($P < 0.001$; Fig. 1c) and enhanced the Sholl profile ($P < 0.001$; Fig. 1d).

Inhibition of Trk receptor signaling blocks amitriptyline-induced neurite outgrowth

To confirm the mechanism by which amitriptyline induces neurite outgrowth, the effect of inhibition of Trk receptor signaling using the antagonist K252a was investigated. K252a is widely used as an inhibitor of Trk signaling (Tapley et al. 1992). Primary cortical neurons were pretreated with K252a (200 nmol/L) for 30 min followed by stimulation with amitriptyline (500 nmol/L) or NGF (10 ng/mL) for 24 h. Sholl analysis was performed as
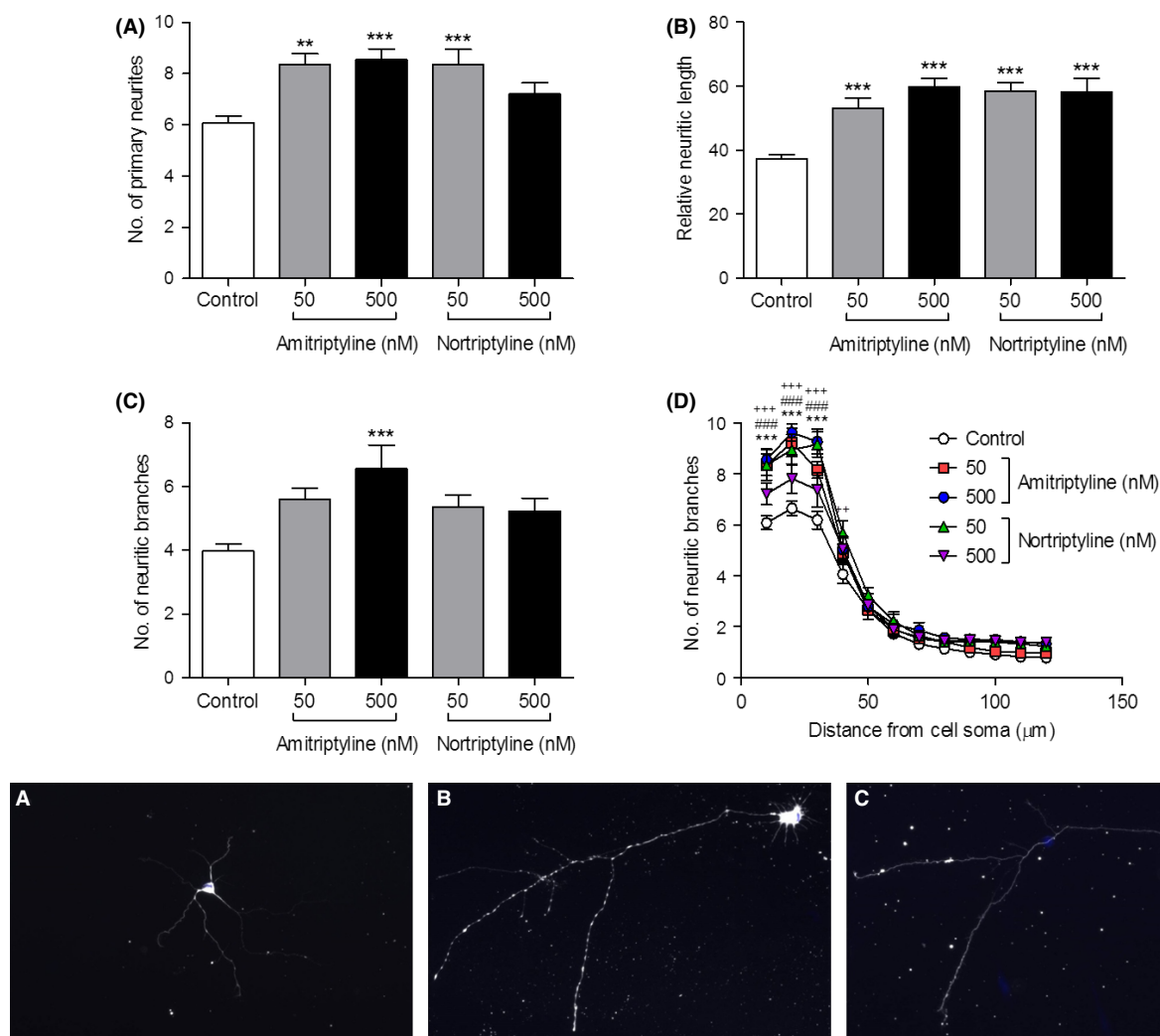
Figure 1. Amitriptyline and nortriptyline increase neurite outgrowth in primary cortical neurons. Effect of amitriptyline and nortriptyline on (A) number of primary neurites, (B) relative neuritic length and (C) number of neuritic branches. **$P < 0.01$, ***$P < 0.001$ versus control (one-way ANOVA followed by post hoc Newman–Keuls). (D) Effect of amitriptyline and nortriptyline on the Sholl profile. ***$P < 0.001$ amitriptyline (50 nmol/L), ###$P < 0.001$ amitriptyline (500 nmol/L), ++$P < 0.01$, +++$P < 0.001$ nortriptyline (50 nmol/L) versus control (two-way repeated measures ANOVA followed by post hoc Bonferroni). Data expressed as mean ± SEM (n = 11–14 coverslips from two independent experiments). Images show representative neurons treated with (A) control and (B) amitriptyline (500 nmol/L) and (C) nortriptyline (50 nmol/L). Neurons are stained with anti-βIII-tubulin antibody (white) and DAPI (blue). Scale bar = 50 μm.

before. Amitriptyline increased the number of primary neurites, neuritic branches, and relative neurite length as before (Fig. 2). K252a had no effect on neuronal complexity in its own right (Fig. 2). K252a blocked the increase by amitriptyline on the number of primary neurites ($P < 0.01$; Fig. 2A), relative neurite length ($P < 0.01$; Fig. 2B) and the number of neuritic branches ($P < 0.01$; Fig. 2C). NGF significantly increased the number of primary neurites ($P < 0.01$; Fig. 2A), neurite length ($P < 0.01$; Fig. 2B) and neuritic branches ($P < 0.01$; Fig. 2C). K252a blocked the increase by NGF on the number of primary neurites ($P < 0.01$; Fig. 2A), relative

neurite length ($P < 0.01$; Fig. 2B) and the number of neuritic branches ($P < 0.01$; Fig. 2C).

Inhibition of MAPK/ERK signaling blocks amitriptyline-induced neurite outgrowth

Activation of Trk receptors leads to activation of several signaling pathways. Of these, the mitogen activated protein kinase (MAPK) pathway, is widely associated with neurite outgrowth.

To investigate the involvement of the MAPK signaling pathway in amitriptyline-induced neurite outgrowth, the
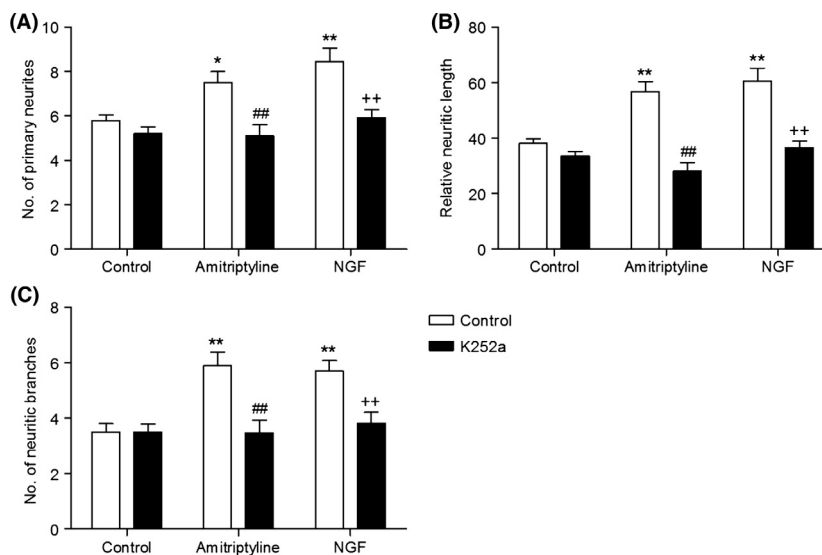
Figure 2. Amitriptyline-induced neurite outgrowth is blocked by inhibition of Trk signaling. Effect of amitriptyline and NGF on levels of (A) number of primary neurites, (B) relative neuritic length and (C) number of neuritic branches following pre-treatment with Trk antagonist K252a. *$P < 0.05$, **$P < 0.01$ versus control, ##$P < 0.01$ versus amitriptyline control, ++$P < 0.01$ versus NGF control (two-way ANOVA followed by post hoc Newman–Keuls). Data expressed as mean $\pm$ SEM ($n = 11$–14 coverslips from three independent experiments).

ability of the MAPK kinase (MEK) inhibitor PD98059 to attenuate amitriptyline-induced increase in neuronal complexity was examined. Primary cortical neurons were pretreated with PD98059 (10 μmol/L) for 30 min followed by stimulation with amitriptyline (500 nmol/L) or NGF (10 ng/mL) for 24 h. Sholl analysis was performed as before. Amitriptyline and NGF increased the number of primary neurites, the number of neuritic branches and relative neurite length as before (Fig. 3). PD98059 had no effect on neuronal complexity in its own right (Fig. 3). PD98059 blocked the effect of amitriptyline on the number of primary neurites ($P < 0.01$; Fig. 3A), neurite length ($P < 0.01$; Fig. 3B) and the number of neuritic branches ($P < 0.01$; Fig. 3C). PD98059 also blocked the effect of NGF on the number of primary neurites ($P < 0.05$; Fig. 3A), neurite length ($P < 0.01$; Fig. 3B) and the number of neuritic branches ($P < 0.05$; Fig. 3C).

Inhibition of Trk receptor signaling reduces nortriptyline-induced ERK phosphorylation

Activation of the MAPK pathway leads to increased phosphorylation of ERK1/2. Therefore, the effect of Trk inhibition on amitriptyline-, nortriptyline- and NGF-induced ERK1/2 phosphorylation was examined. Primary cortical neurons were pretreated with K252a (200 nmol/L) for 15 min following by stimulation with amitriptyline (500 nmol/L), nortriptyline (50 nmol/L) or NGF (10 ng/mL) for 15 min before neurons were harvested and protein levels of phosphorylated ERK1/2 and total ERK1/2

were detected by Western immunoblotting. Nortriptyline significantly increased phosphorylation of ERK1/2 ($P < 0.05$, Fig. 3D) while amitriptyline and NGF trended towards an increase. K252a significantly reduced ERK phosphorylation levels following amitriptyline ($P < 0.05$; Fig. 3D), nortriptyline ($P < 0.01$; Fig. 3D) and NGF ($P < 0.01$; Fig. 3D) treatment.

Amitriptyline increases neurite outgrowth in the presence of inhibited neurotrophin signaling

NGF and BDNF are the cognate ligands for the TrkA and TrkB receptors, respectively. Y1036 is a small molecule inhibitor that binds to NGF and BDNF and prevents their binding to neurotrophin receptors (Eibl et al. 2010). The effect of inhibition of NGF and BDNF signaling on amitriptyline-induced neurite outgrowth was examined. Primary cortical neurons were pretreated with Y1036 (40 μmol/L) for 30 min followed by stimulation with amitriptyline (500 nmol/L) or a combination of NGF (10 ng/mL) and BDNF (10 ng/mL). Sholl analysis was performed as before. Amitriptyline increased the number of primary neurites, neuritic branches and relative neurite length as before (Fig. 4). NGF and BDNF increased the number of primary neurites, the number of neuritic branches and relative neurite length (Fig. 4). Y1036 had no effect on neurite outgrowth in its own right (Fig. 4). Y1036 prevented NGF- and BDNF-induced increase in number of primary neurites ($P < 0.01$; Fig. 4A), neuritic
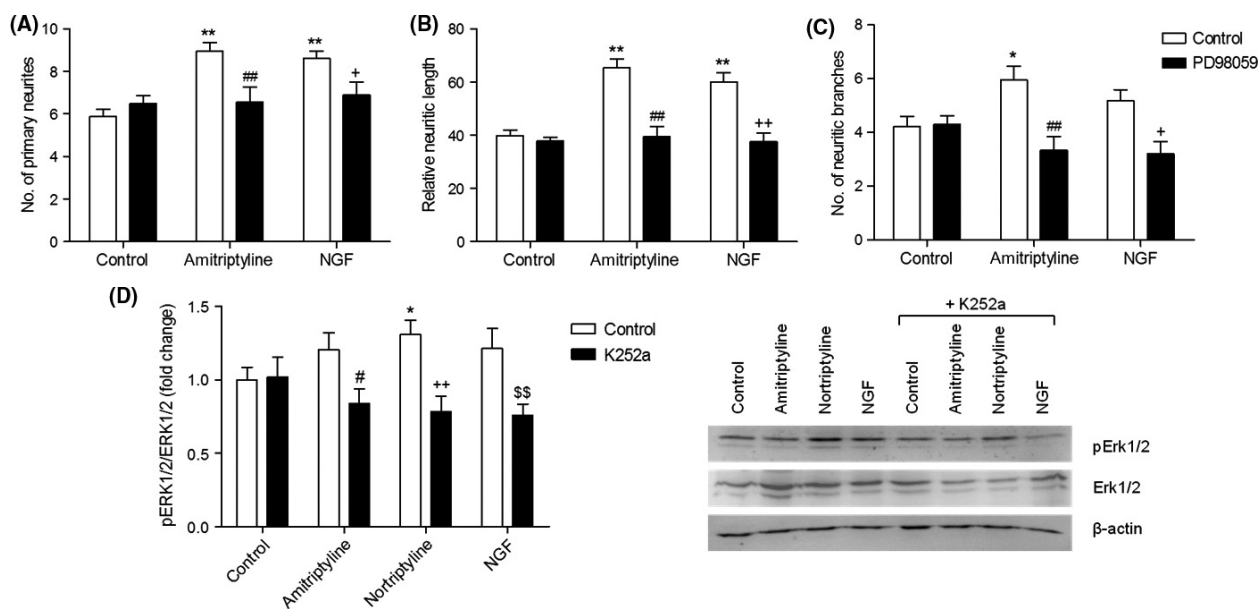
Figure 3. Amitriptyline- and nortriptyline-induced increase in neurite outgrowth involves MAPK/ERK signaling downstream of Trk activation. Effect of amitriptyline and NGF on levels of (A) number of primary neurites, (B) relative neuritic length and (C) number of neuritic branches following pretreatment with MAPK inhibitor PD98059. *$P < 0.05$, **$P < 0.01$ versus control, #$P < 0.05$, ##$P < 0.01$ versus amitriptyline control, +$P < 0.05$, ++$P < 0.01$ versus NGF control (two-way ANOVA followed by post hoc Newman–Keuls). Data expressed as mean ± SEM ($n = 12–15$ coverslips from three independent experiments). (D) Effect of amitriptyline, nortriptyline and NGF on ERK1/2 phosphorylation following pretreatment with Trk antagonist K252a. *$P < 0.05$ versus control, #$P < 0.05$ versus amitriptyline control, ++$P < 0.01$ versus nortriptyline control, $$$P < 0.01$ versus NGF control (two-way ANOVA followed by post hoc Fisher's LSD). Data normalized to β-actin levels and expressed as mean ± SEM ($n = 10–12$ from three independent experiments).

length ($P < 0.01$; Fig. 4B) and neuritic branches ($P < 0.01$; Fig. 4C). Y1036 prevented amitriptyline-induced increase in number of neuritic branches ($P < 0.01$; Fig. 4C) but not amitriptyline-induced increase in primary neurites (Fig. 4A) or neuritic length (Fig. 4B).

TNF-α reduces neurite outgrowth in primary cortical neurons

The proinflammatory cytokine TNF-α has been widely demonstrated to contribute to neurodegeneration under inflammatory conditions in the CNS. Primary cortical neurons were treated with TNF-α (1, 10, 100 ng/mL) for 48 h before Sholl analysis was performed as before. TNF-α significantly reduced the number of primary neurites ($P < 0.01$; Fig. 5A), neurite length ($P < 0.05$; Fig. 5B), neuritic branches ($P < 0.05$; Fig. 5C) and the Sholl profile ($P < 0.001$; Fig. 5D).

Amitriptyline, nortriptyline and NGF prevent TNF-α-induced reduction in neurite outgrowth

As amitriptyline and nortriptyline have been shown to increase neurite outgrowth and enhance neuronal com-

plexity, it was investigated whether amitriptyline and nortriptyline could protect neurons from the reduction in neurite outgrowth induced by TNF-α. Primary cortical neurons were pretreated with amitriptyline (500 nmol/L), nortriptyline (50 nmol/L) or NGF (10 ng/mL) for 2 h followed by addition of TNF-α (10 ng/mL) for 48 h. These doses were selected as they were previously found to be sufficient to increase/decrease neurite outgrowth in this study and previous studies (Gutierrez et al. 2008; Jang et al. 2009). Sholl analysis was performed as before. Amitriptyline, nortriptyline and NGF increased the number of neuritic branches and relative neurite length as before (Fig. 6A, B) but had no effect on primary neurites. TNF-α reduced number of neuritic branches and neurite length as before (Fig. 6A, B) but had no effect on primary neurites. Amitriptyline prevented the reduction in neurite length ($P < 0.01$; Fig. 6B) and the number of neuritic branches ($P < 0.01$; Fig. 6C) induced by TNF-α. Nortriptyline prevented the reduction in neurite length ($P < 0.01$; Fig. 6B) and the number of neuritic branches ($P < 0.01$; Fig. 6C) induced by TNF-α. NGF prevented the reduction in neurite length ($P < 0.01$; Fig. 6B) and the number of neuritic branches ($P < 0.01$; Fig. 6C).
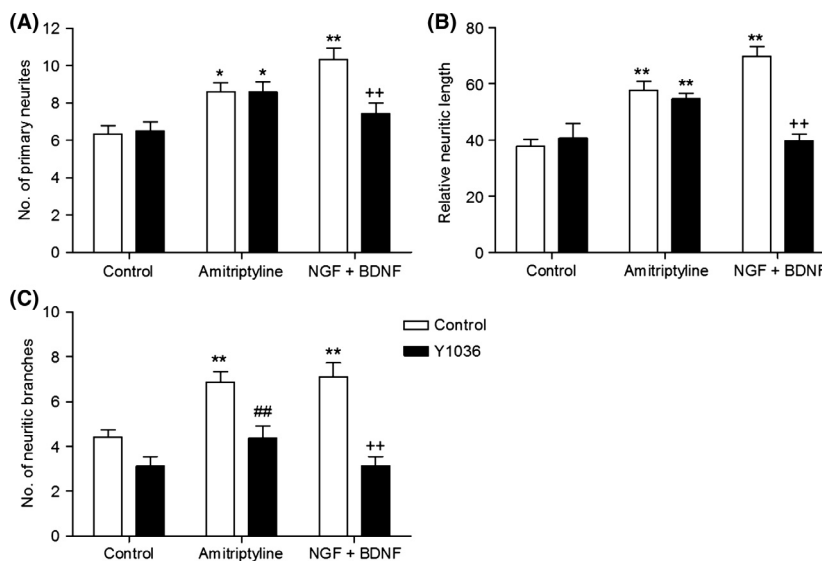
Figure 4. Amitriptyline increases neurite outgrowth in the presence of inhibition of neurotrophin signaling. Effect of amitriptyline and NGF + BDNF on (A) number of primary neurites, (B) relative neuritic length and (C) number of neuritic branches following pretreatment with neurotrophin antagonist Y1036. *$P < 0.05$, **$P < 0.01$ versus control, ##$P < 0.01$ versus amitriptyline control, ++$P < 0.01$ versus NGF + BDNF control (two-way ANOVA followed by post hoc Newman Keuls). Data expressed as mean ± SEM ($n = 11$–20 from three independent experiments).

Amitriptyline, nortriptyline and NGF increase colocalization of synaptic markers and prevent TNF-α-induced reduction in colocalization

Loss of synaptic connections is a feature of many neurodegenerative disorders. As amitriptyline and nortriptyline have been shown to increase neurite outgrowth, we examined whether this increase coincided with an increase in colocalization of synaptic markers. We also determined whether amitriptyline and nortriptyline could protect against TNF-α-induced reduction in colocalization. Primary cortical neurons were treated with amitriptyline (500 nmol/L), nortriptyline (50 nmol/L) or NGF (10 ng/mL) for 24 h before synaptic markers were examined by measurement of colocalization of the presynaptic marker synaptophysin and postsynaptic marker PSD-95. Amitriptyline ($P < 0.001$; Fig. 7A), nortriptyline ($P < 0.05$; Fig. 7A) and NGF ($P < 0.01$; Fig. 7A) significantly increased the number of colocalized synaptic puncta. Neurons were treated with TNF-α (10 ng/mL) for 48 h before analysis. TNF-α significantly decreased colocalization ($P < 0.001$; Fig. 7B). To investigate whether the Trk agonists could protect against reduction in colocalization induced by TNF-α, neurons were pretreated with amitriptyline (500 nmol/L), nortriptyline (50 nmol/L) or NGF (10 ng/mL) for 2 h followed by addition of TNF-α (10 ng/mL) for 48 h before colocalization of synaptic

markers was examined. Amitriptyline increased colocalization and TNF-α decreased colocalization as before (Fig. 7C). Nortriptyline and NGF alone had no effect on colocalization at this timepoint. Amitriptyline, nortriptyline, and NGF significantly prevented reduction in colocalization ($P < 0.01$; Fig. 7C).

Discussion

The results of this study demonstrate, for the first time, that amitriptyline and its active metabolite nortriptyline increase neurite outgrowth and colocalization of synaptic markers in rat primary cortical neurons and have the ability to protect neurons against an atrophic stimulus. The induction of neurite outgrowth by amitriptyline and nortriptyline is comparable to that induced by the endogenous TrkA agonist NGF, as previously reported by our laboratory (Day et al. 2014). The therapeutic plasma concentration of amitriptyline ranges from 360 to 900 nmol/L (O'Donnell and Shelton 2011) and brain concentrations are likely to be higher due to the lipophilic nature of the drug. Therefore, the 500 nmol/L concentration of amitriptyline used in this study falls within its therapeutic range. Amitriptyline is metabolized in the liver by N-demethylation whereby it is converted to nortriptyline, a secondary amine (Breyer-Pfaff 2004). The desmethyl metabolite nortriptyline retains biological activity and therefore may also account for part of the
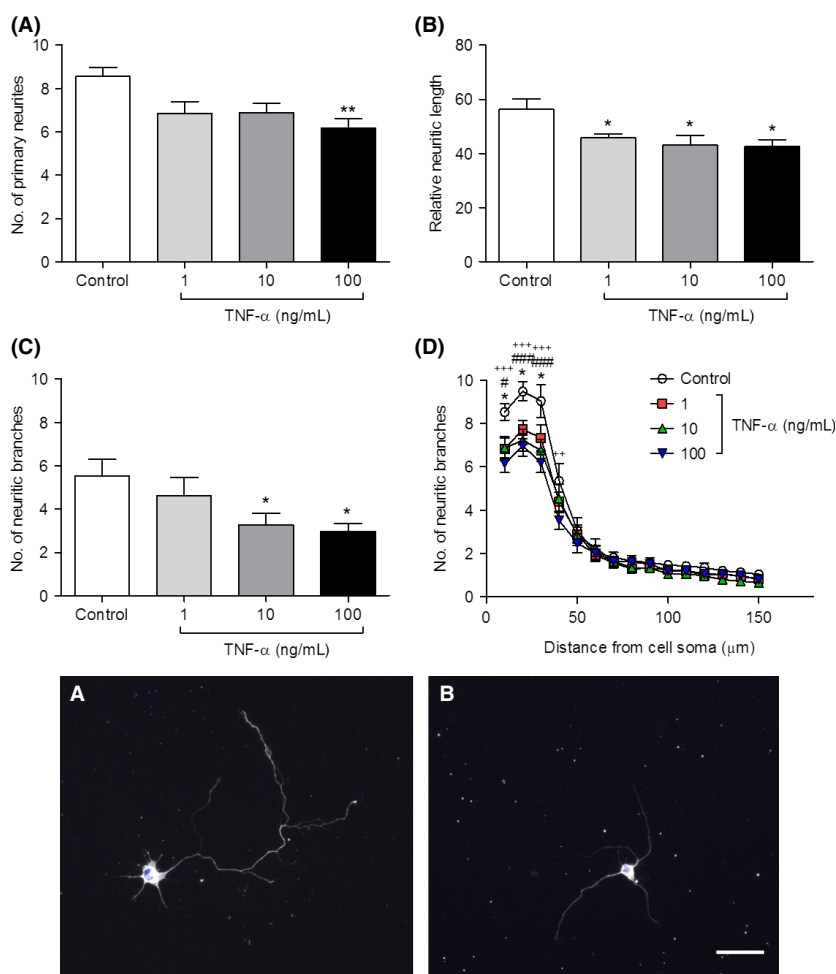
Figure 5. TNF-α decreases neurite outgrowth of primary cortical neurons. Effect of TNF-α on (A) number of primary neurites, (B) relative neuritic length and (C) number of neuritic branches. *$P < 0.05$, **$P < 0.01$ versus control (one-way ANOVA followed by post hoc Newman–Keuls). (D) Effect of TNF-α on the Sholl profile. *$P < 0.05$ TNF-α (1 ng/mL), #$P < 0.05$, ###$P < 0.001$ TNF-α (10 ng/mL), ++$P < 0.01$; +++$P < 0.001$ TNF-α (100 ng/mL) versus control (two-way repeated measures ANOVA followed by post hoc Bonferroni). Data expressed as mean ± SEM ($n = 8$–12 from two independent experiments). Images show representative neurons treated with (A) control and (B) TNF-α (10 ng/mL). Neurons are stained with anti-βIII-tubulin antibody (white) and DAPI (blue). Scale bar = 50 μm.

activity of amitriptyline. It was found in these studies that amitriptyline and nortriptyline increased neurite outgrowth while having no effect on neuronal viability (data not shown), indicating that these drugs do not simply improve viability but increase the complexity of neuronal structure. It has previously been shown that alternative tricyclic compounds have no effect on neurite outgrowth (Jang et al. 2009). In our laboratory, we have also found clomipramine and selective serotonin reuptake inhibitor fluoxetine to have no effect on neurite outgrowth at identical endpoints to those examined with amitriptyline and nortriptyline (data not shown). Amitriptyline- and nortriptyline-induced increase in co-localization of synaptic markers is a novel finding. While no effect on synaptic growth has previously been

reported, amitriptyline has been demonstrated to have effects on synaptic plasticity. Amitriptyline stimulates transcription of the early growth response-1 (Egr-1) in glial cells (Chung et al. 2007). Egr-1, also known as NGF-induced protein A, is a plasticity-related gene that has functions including enhancement of synaptic protein expression (James et al. 2004). Amitriptyline itself has been found to increase expression of plasticity-related genes synaptophysin, synaptotagmin, ampiphysin, neo-genin, and cAMP response element-binding protein (CREB) (Drigues et al. 2003). This increase in synaptic gene expression potentially contributes to the increase in synapse markers observed in this study.

Amitriptyline has previously been found to inhibit neurite outgrowth in cerebral rat and chick embryos
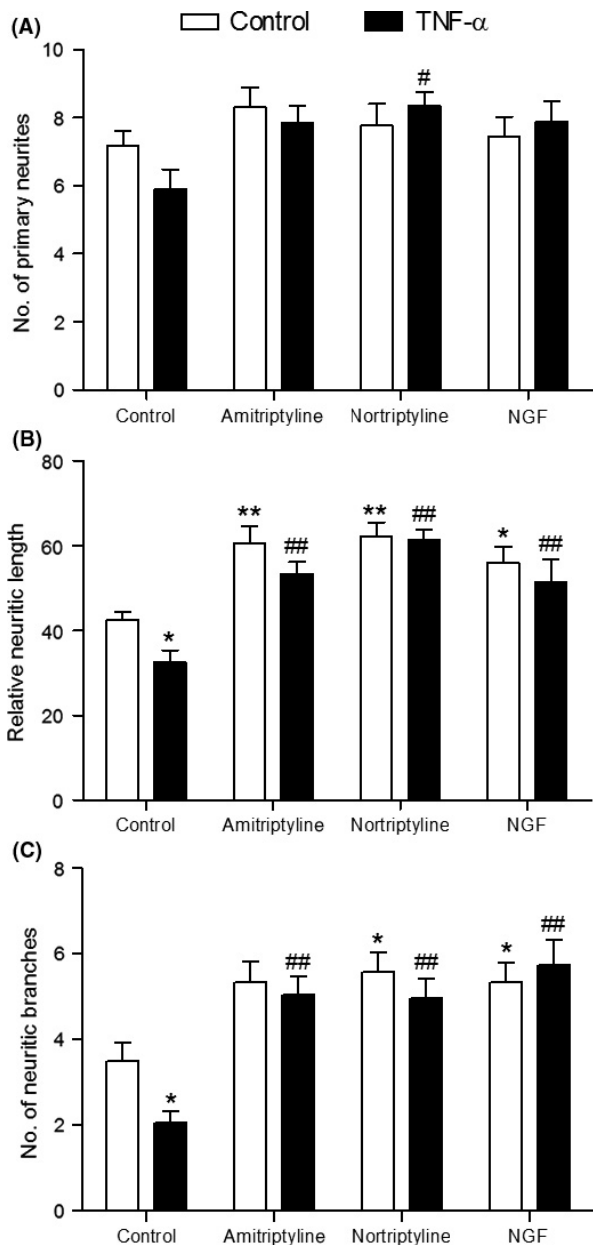
Figure 6. Amitriptyline and nortriptyline prevent TNF-α-induced neuronal atrophy. Effect of TNF-α on (A) number of primary neurites, (B) relative neuritic length and (C) number of neuritic branches following pretreatment with amitriptyline, nortriptyline and NGF. *$P < 0.05$, **$P < 0.01$ versus control, #$P < 0.05$, ##$P < 0.01$ versus TNF-α control (two-way ANOVA followed by post hoc Newman–Keuls). Data expressed as mean ± SEM ($n = 12$–16 from three independent experiments).

(Farbman et al. 1988). This study involved use of explants which contain immune cells that possibly contribute to the observed reduction in outgrowth. In line with this result, it was found in the present study that conditioned media from amitriptyline-treated primary cortical mixed glial cells has no effect on neurite outgrowth (data not shown). It is therefore possible that amitriptyline-induced activation of immune cells, such as microglia which were present in the mixed glial cultures, may have an inhibitory or no effect on neurite outgrowth. This indicates that direct targeting of neurons is necessary to promote outgrowth with amitriptyline and led to the hypothesis that amitriptyline acts on neuronal Trk receptors to induce growth. Inhibition of Trk signaling using antagonist K252a blocked amitriptyline-induced neurite outgrowth, confirming a Trk receptor role in this mechanism. NGF was utilized as a positive control in this experiment and NGF-induced increases in neuronal complexity were also blocked by K252a. However, it should be noted that K252a has more recently been demonstrated to inhibit the activity of several other tyrosine and serine/threonine kinases in vitro (Martin et al. 2011). Therefore, use of more specific Trk inhibitors would be of interest for further studies.

Trk activation results in autophosphorylation of tyrosine residues, recruitment of adaptor proteins and activation of the MAPK and phosphoinositide 3-kinase (PI3K) pathways (Huang and Reichardt 2003). The MAPK pathway, in particular the ERK1/2 branch, has been found to be favored following NGF-induced Trk activation (Segal 2003) and is also widely implicated in neurite outgrowth (Thomas and Huganir 2004). Therefore, increased phosphorylation of ERK1/2 was investigated as a marker of increased Trk activation and K252a-induced reversal of ERK phosphorylation used to confirm the Trk involvement. Nortriptyline significantly increased ERK phosphorylation while amitriptyline and NGF trended towards an increase. K252a significantly reduced amitriptyline-, nortriptyline- and NGF-induced levels of phosphorylated ERK, demonstrating that Trk receptor activation is involved in the ERK signaling induced by all three compounds and therefore likely involved in their induction of neurite outgrowth. Inhibition of this pathway via the MEK inhibitor PD98059 blocked amitriptyline- and NGF-induced neurotrophic activity, further implicating the Trk/MAPK pathway in induction of neurite outgrowth. Inhibition of neurotrophin signaling using the neurotrophin antagonist Y1036 prevented amitriptyline-induced increases in neuritic branching but did not prevent amitriptyline-induced increase in number of primary neurites and neuritic length. The reason for the effect on neuritic branching is unclear, however, the mechanisms underlying neurite extension and branching are not identical (Gallo 2011) and it is possible that amitriptyline requires some convergence with neurotrophins to induce neuritic branching. Neurotrophin-induced activation of PI3K signaling leads to formation of axonal branches
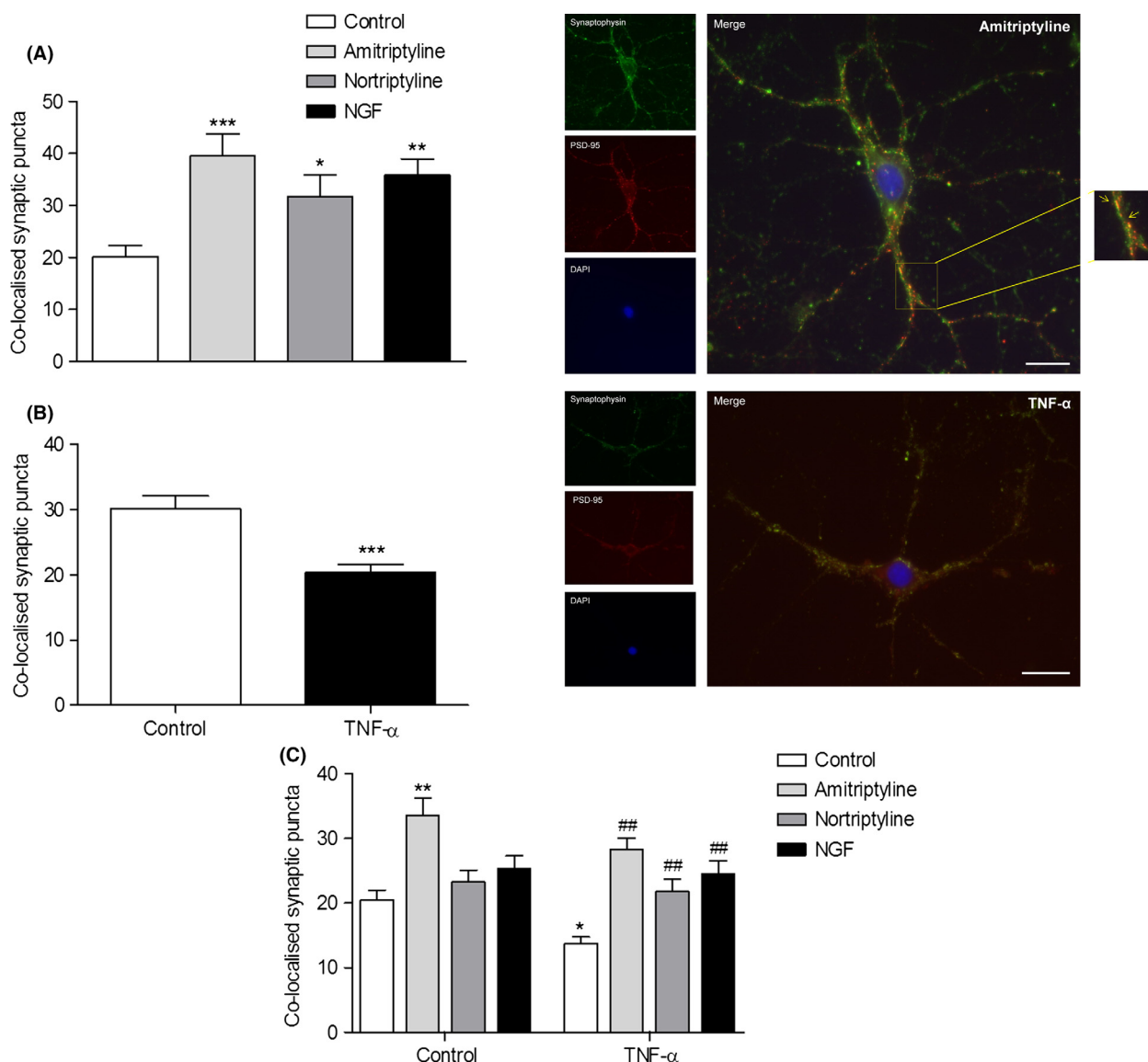
Figure 7. Amitriptyline and nortriptyline prevent TNF-α-induced reduction in colocalization of synaptic markers. (A) Effect of amitriptyline, nortriptyline and NGF on colocalization of synaptic puncta. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ versus control (one-way ANOVA followed by post hoc Newman– Keuls). (B) Effect of TNF-α on colocalization of synaptic puncta. *$P < 0.001$ versus control (unpaired t-test). (C) Effect of TNF-α on colocalization following pretreatment with amitriptyline, nortriptyline and NGF. *$P < 0.05$, **$P < 0.01$ versus control, ##$P < 0.01$ versus TNF-α control (two-way ANOVA followed by post hoc Newman–Keuls). Data expressed as mean $\pm$ SEM ($n = 70$–120 neurons from three independent experiments). Images show representative neuron treated with amitriptyline (500 nmol/L) and TNF-α (10 ng/mL). Neurons are stained with antisynaptophysin (green), anti-PSD-95 (red) and DAPI (blue). Yellow arrows indicate examples colocalized synaptic puncta. Scale bar = 20 μm.

(Ketschek and Gallo 2010; Kalil and Dent 2014) and it is possible that amitriptyline does not induce as potent a stimulation of PI3K signaling. Pretreatment with anti-NGF and –BDNF antibodies has been shown to fail to block activation of TrkA and TrkB by amitriptyline in primary cortical neurons (Jang et al. 2009). This suggests that amitriptyline activates Trk receptors independent of neurotrophin action.

Neuroinflammation and the increased expression of proinflammatory cytokines in the CNS have widely been shown to contribute to various neurodegenerative diseases. One of the hallmark characteristics of neuroinflammation is an increased expression of proinflammatory cytokines in the brain, of these TNF-α is among the most prominent (Block and Hong 2005). Proinflammatory cytokines such as TNF-α contribute to neurodegeneration

by increasing immune activation, excitotoxicity and apoptosis (Smith et al. 2012) as well as by altering glial function (DiProspero et al. 1997), inhibiting neurotrophic activity (Golz et al. 2006), reducing neuritic outgrowth (Neumann et al. 2002) and promoting synaptic degeneration (Centonze et al. 2009). In this study, TNF-α was found to significantly reduce neurite outgrowth and synaptic colocalization of primary cortical neurons. The reduction in outgrowth was not accompanied by reduction in neuronal viability, indicating that treatment of neurons at 3 DIV with TNF-α specifically affects neurite outgrowth. However, reduction in synaptic marker expression in neurons at 18–21 DIV was accompanied by a reduction in viability (data not shown), indicating that these more mature neurons are more susceptible to TNF-α-induced damage. TNF-α has previously been shown to inhibit outgrowth via activation of the inhibitory Rho GTPase RhoA, leading to cytoskeletal changes (Mathew et al. 2009), and also by activation of nuclear factor κ B (NFκB) (Gutierrez et al. 2008). Release of microglial TNF-α coincides with synaptic loss and dendritic spine loss in a model of multiple sclerosis (Centonze et al. 2009). Reduced neuronal complexity and synaptic connectivity as the result of increased cytokine signaling has the potential to contribute to several dysfunctional conditions in the CNS. These conditions may be improved with the use of drugs with anti-inflammatory and neurotrophic properties.

Both amitriptyline and nortriptyline protect primary cortical neurons against TNF-α-induced atrophy and synaptic marker loss. NGF also produced a similar protective effect, indicating that the protection provided by amitriptyline and nortriptyline acts via the Trk pathway. It is possible that amitriptyline and nortriptyline simply compensate for TNF-α-induced suppression by promoting compensatory outgrowth and synaptic growth. Another mechanism by which this effect may occur is via convergence of the Trk and TNF-α signaling pathways on the NFκB transcription factor system. TNF-α has previously been found to inhibit neurite outgrowth by an NFκB-dependent mechanism (Gutierrez et al. 2008; Nolan et al. 2014). A recent study has found that activation of TrkA by NGF has anti-inflammatory effects including reduced phosphorylation of NFκB inhibitor IκB and reduced NFκB translocation (Prencipe et al. 2014). It has also been recently shown that amitriptyline decreases NFκB translocation in an astrocyte cell line (Valera et al. 2014). It is therefore possible that, through Trk activation, amitriptyline and nortriptyline interfere with NFκB signaling downstream of TNF-α, preventing its detrimental effects on neurons. It is also important to note that the protective effect of amitriptyline and nortriptyline on reduction in synaptic markers occurs in more mature neurons. This highlights the ability of these drugs to protect against neurodegenerative conditions in further developed neurons and may indicate an efficacy in adult neurodegeneration.

Neuronal atrophy and synaptopathy are features of several neurodegenerative disorders that are also associated with a neuroinflammatory component, such as Alzheimer's disease (Nimmrich and Ebert 2009; Teipel et al. 2013), Parkinson's disease (Overk and Masliah 2014) and multiple sclerosis (Wegner et al. 2006). The loss of synaptic connections and failure of neuronal and neuritic regeneration represent critical obstacles in the treatment of such diseases (Conforti et al. 2007). Atrophy and synaptopathy are also observed in psychiatric and mood disorders, such as schizophrenia, autism and depression (Duman and Monteggia 2006; Duman and Aghajanian 2012). The delayed therapeutic effect of antidepressant drugs has led to research into alternative mechanisms by which antidepressants may act in the brain. Changes involving increased plasticity, neurotrophic signaling and synaptogenesis are thought to occur (Seo et al. 2014). It is possible that antidepressant drugs such as amitriptyline facilitate neurite outgrowth as a means of enhancing synaptogenesis in the brain. This action may also increase synapse formation when neuronal complexity is challenged in the brain during neuroinflammation. An in vitro system such as that used here is invaluable for the examination of subtle changes in neuronal morphology and an increase in neurite outgrowth in culture can indicate an increased capacity for regeneration in vivo (Fournier et al. 2003). Therefore, examination of neuritic regeneration and synaptic formation in vivo, while beyond the scope of this work, would be of interest for further studies.

Collectively, the results of this study demonstrate that amitriptyline and its metabolite nortriptyline have the ability to induce neurite outgrowth and an increase in synaptic marker colocalization in primary cortical neurons and can also mitigate the atrophic effects of the proinflammatory cytokine TNF-α. Both compounds therefore demonstrate potential therapeutic benefit as neurotrophic drugs. Use of neurotrophins for treatment of CNS disorders is impeded by their low blood–brain barrier permeability and associated adverse effects. Alternative Trk agonists, such as amitriptyline and nortriptyline, therefore have promising potential to be used in the treatment of inflammatory neurodegenerative conditions.

Acknowledgements

Disclosures

None declared.

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