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Amitriptyline is a TrkA and TrkB Receptor Agonist that Promotes TrkA/TrkB Heterodimerization and Has Potent Neurotrophic Activity

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SUMMARY

Neurotrophins, the cognate ligands for the Trk receptors, are homodimers and induce Trk dimerization through a symmetric bivalent mechanism. We report here that amitriptyline, an antidepressant drug, directly binds TrkA and TrkB and triggers their dimerization and activation. Amitriptyline, but not any other tricyclic or selective serotonin reuptake inhibitor antidepressants, promotes TrkA autophosphorylation in primary neurons and induces neurite outgrowth in PC12 cells. Amitriptyline binds the extracellular domain of both TrkA and TrkB and promotes TrkA-TrkB receptor heterodimerization. Truncation of amitriptyline binding motif on TrkA abrogates the receptor dimerization by amitriptyline. Administration of amitriptyline to mice activates both receptors and significantly reduces kainic acid-triggered neuronal cell death. Inhibition of TrkA, but not TrkB, abolishes amitriptyline's neuroprotective effect without impairing its antidepressant activity. Thus, amitriptyline acts as a TrkA and TrkB agonist and possesses marked neurotrophic activity.

INTRODUCTION

Neurotrophins, which include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), NT-3, and NT-4/5, are critical for the development and maintenance of the peripheral and the central nervous systems. Neurotrophins exert their physiological actions through two classes of receptors: Trk tyrosine kinase receptors and p75NTR. The Trk receptors are members of a transmembrane tyrosine kinases family (TrkA, TrkB, and TrkC). NGF primarily binds TrkA, BDNF and NT-4/5 mainly interact with TrkB, and NT-3 predominantly associates with TrkC, while having weaker interactions with TrkA and TrkB receptors. Docking of neurotrophic factors on Trk receptors initiates receptor homodimerization, autophosphorylation of cytoplasmic tyrosine residues on the receptors, and a cascade of cell signaling events including Ras/Raf/MAP kinase, PI3K/Akt, and PLC-γ1 (Kaplan and Stephens, 1994). These signals prevent apoptotic cell death, promote cellular differentiation and axon elongation, and upregulate choline acetyl transferase. Several neuronal cell types that are lost in certain pathologies express TrkA and respond to NGF, and NGF has been reported to reverse basal forebrain cholinergic atrophy, reduce cognitive decline, stimulate cholinergic fiber growth in humans with mild Alzheimer's disease (Mufson et al., 2008), and ameliorate peripheral diabetic neuropathies (Apfel, 2002). Other applications proposed for NGF include treatment of neuronal damage (Hughes et al., 1997) and targeting of neuroectoderm-derived tumors (Cortazzo et al., 1996; LeSauteur et al., 1995). In addition, neurotrophins such as BDNF act as key regulators of neurite outgrowth and synaptic plasticity, and it has been proposed that a deficit of these molecules may underlie the psychopathology of stress and depression, while antidepressants may act via neurotrophins to produce the molecular and behavioral changes associated with their efficacy (Altar et al., 1997; Duman et al., 1997). Thus, both NGF and BDNF might contribute to antidepressant-induced formation and stabilization of synaptic connectivity.

Nonetheless, the use of NGF for the treatment of these diseases has been limited by its poor pharmacological properties, such as the low blood-brain barrier permeability (Poduslo and Curran, 1996) and relevant side effects, like hyperalgesia (Apfel, 2002). To search for NGF and BDNF mimetics with better pharmacokinetic properties, tremendous efforts have been made to design small, proteolytically stable molecules with neurotrophic activity and specificity for TrkA or TrkB. For example, ligand mimicry and antibody mimicry strategies have been intensively explored to generate peptide analogues of two agonists directed to the extracellular domain (ECD) of TrkA or TrkB (Beglova et al., 2000; O'Leary and Hughes, 2003; Saragovi et al., 1991; Xie et al., 2000). Additionally, an NGF-mimetic peptide has recently been reported to partially mimic NGF and reduce neuropathic pain in rat (Colangelo et al., 2008). However, none of these antibodies and peptidomimetics can fully mimic NGF or BDNF function in animals.

In this paper, we show that tricyclic antidepressant amitriptyline, which is traditionally thought to exert its therapeutic effects via blockade of the serotonin and noradrenaline transporters, interacts directly with both TrkA and TrkB receptors on the

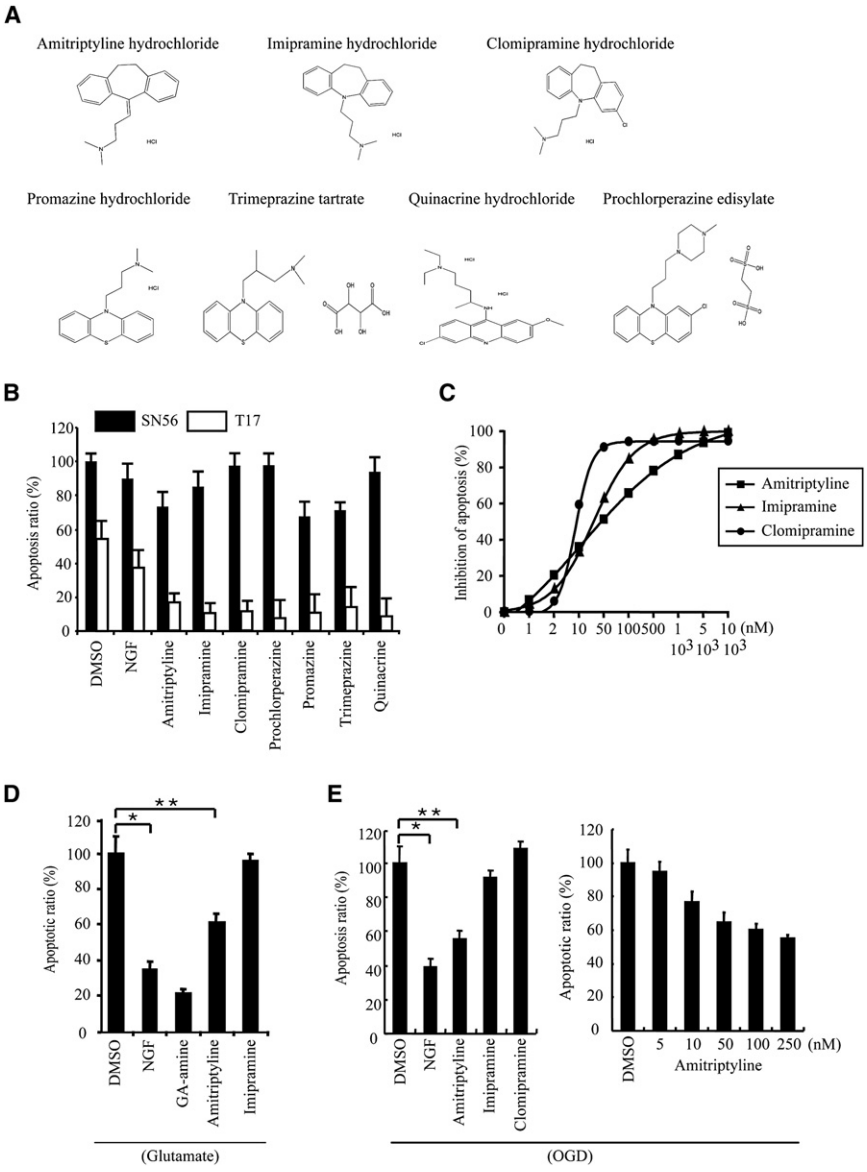


Figure 1. Amitriptyline Selectively Protects Hippocampal Neurons from Apoptosis

(A) Chemical Structures of tricyclic antidepressant drugs.

(B) Some of the tricyclic antidepressant drugs protect T17-TrkA cells but not parental SN56 cells from apoptosis.

(C) EC_{50} titration assays for promoting T17 cell survival. TrkA-overexpressing T17 cells were pretreated with various tricyclic antidepressant drugs for 30 min, followed by 1 μM staurosporine for 9 hr. Apoptosis was quantitatively analyzed. EC_{50} values are the drug concentrations, which prevent 50% of cells from apoptosis.

(D) Amitriptyline selectively prevents apoptosis in hippocampal neurons. Hippocampal neurons were pretreated with NGF (100 ng/ml), gambogic amide, and various tricyclic antidepressant drugs (0.5 μM) for 30 min, followed by 50 μM glutamate for 16 hr. Apoptosis was quantitatively analyzed.

(E) Amitriptyline prevents OGD-provoked neuronal apoptosis in hippocampal neurons. Hippocampal neurons were pretreated with various drugs (0.5 μM) for 30 min, followed by OGD for 3 hr. Apoptosis was quantitatively analyzed (left). Data represent the mean ± SEM of n = 4–5; one-way ANOVA, followed by Dunnett's test, *$p < 0.01$; **$p < 0.005$.

ECD. Further, our data reveal that amitriptyline induces TrkA and TrkB homo- and heterodimerization and activation in mouse brain, but that the heterodimerization is not required for Trk receptor activation. Truncation of amitriptyline binding motif on TrkA but not the corresponding region on TrkB abolishes the receptor homo- and heterodimerization. Moreover, amitriptyline suppresses neuronal apoptosis elicited by kainic acid (KA) in a TrkA-dependent manner. Hence, amitriptyline acts as a TrkA and TrkB receptor agonist and possesses marked neurotrophic activity.

RESULTS

Amitriptyline Selectively Protects Hippocampal Neurons from Apoptosis

Recently, we developed a cell-based screening approach for novel TrkA agonist discovery and reported that gambogic amide is a selective agonist for TrkA that possesses robust neurotrophic activity and prevents neuronal cell death (Jang et al., 2007). During the screening, we also surprisingly identified numerous tricyclic antidepressant compounds that selectively protected TrkA-expressing T17 cells but not parental SN56 cells lacking TrkA from apoptosis (Figures 1A and 1B). Titration assays with T17 cells for apoptosis inhibitory activity revealed that EC_{50} values were 8, 30, and 50 nM for clomipramine, imipramine, and amitriptyline, respectively (Figure 1C). TrkA and p75NTR are upregulated in hippocampal and cortical neurons under pathophysiological conditions (Kokaia et al., 1998; Lee et al., 1998). Moreover, neuroprotective effects of NGF in hippocampal and cortical neurons have been demonstrated in vitro and in vivo (Culmsee et al., 1999; Zhang et al., 1993). Therefore, to test whether the tricyclic compounds can also protect primary hippocampal neurons from apoptosis, we pretreated primary cultures with test compounds (0.5 μM each) for 30 min, followed by glutamate treatment. NGF, gambogic amide, or amitriptyline pretreatment significantly protected hippocampal neurons from apoptosis, while other tricyclic drugs tested had no effect (Figure 1D and data not shown).

NGF reduces cortical infarction and apoptosis in transgenic mice and protects PC12 cells from apoptosis in oxygen-glucose deprivation (OGD) (Beck et al., 1992; Guegan et al., 1998). To explore whether amitriptyline and/or other tricyclics could protect hippocampal neurons from OGD-provoked apoptosis, we pretreated primary cultures with various tricyclic drugs,

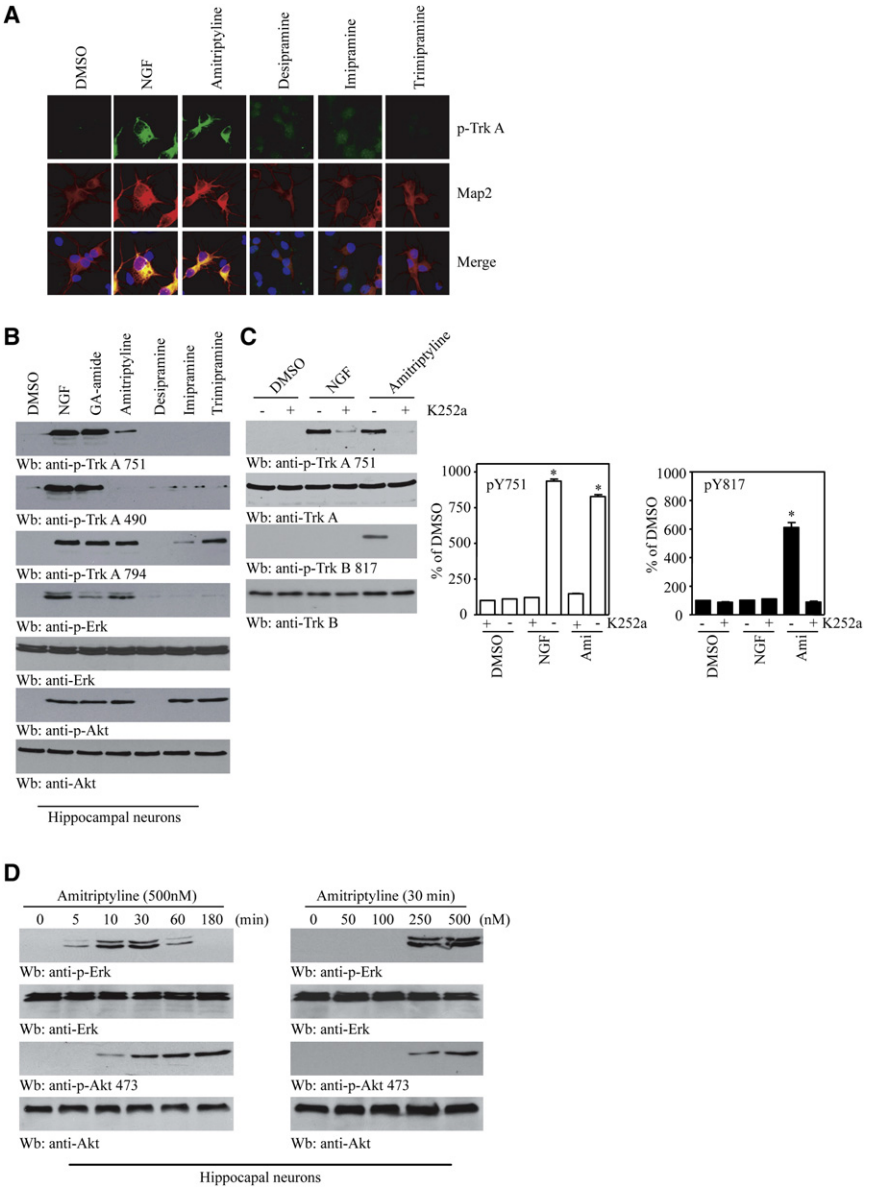


Figure 2. Amitriptyline Activates the TrkA Receptor and its Downstream Signaling Cascades

(A) Amitriptyline activates TrkA in hippocampal neurons. Hippocampal neurons were treated with NGF (100 ng/ml) or various tricyclic antidepressant drugs (500 nM) for 30 min. Immunofluorescent staining was conducted with anti-p-TrkA antibody.

(B) Amitriptyline activates TrkA downstream signaling cascades including Akt and MAP kinases. Hippocampal neurons were treated as described above. The cell lysates were analyzed by immunoblotting with various antibodies.

(C) Trk receptor inhibitor K252a (100 nM) blocks amitriptyline-provoked TrkA and TrkB activation. Data represent mean ± SEM (One-way ANOVA, Dunnett's test, *$p < 0.001$).

(D) Akt and MAP kinase activation kinetics and dosage assays by amitriptyline. Hippocampal neurons were treated with 500 nM amitriptyline for various time points. The cell lysates were analyzed by immunoblotting with various antibodies.

followed by OGD stimulation for 3 hr. Amitriptyline significantly suppressed apoptosis, whereas neither imipramine nor clomipramine exhibited any protective activity (Figure 1E, left). Titration assays showed that amitriptyline repressed neuronal apoptosis in a dose-dependent manner (Figure 1E, right). Thus, amitriptyline but not any other tricyclic antidepressant drugs selectively protects hippocampal neurons from apoptosis.

Amitriptyline Activates TrkA and its Downstream Signaling Cascades

NGF binds TrkA and elicits its autophosphorylation and downstream MAP kinase and PI3K/Akt pathway activation in primary hippocampal and cortical cultures that express demonstrable TrkA (Culmsee et al., 2002; Kume et al., 2000). To explore whether amitriptyline could stimulate TrkA, we treated hippocampal neurons with 0.5 μM amitriptyline or other tricyclic drugs for 30 min. Immunofluorescent staining showed that amitriptyline, like NGF, triggered TrkA tyrosine phosphorylation, whereas other tricyclic compounds did not (Figure 2A). Both Akt and Erk1/2 were markedly activated in NGF- or amitriptyline-treated hippocampal neurons. In contrast, none of the other tricyclic drugs was capable of simultaneously activating Akt and Erk1/2 (Figure 2B). It was worth noting that amitriptyline induced TrkA phosphorylation on both tyrosine Y751 and Y794. Surprisingly, Y490 was not phosphorylated at all. In contrast, NGF and gambogic amide activated all three tyrosine residues on TrkA receptor. Although trimipramine induced TrkA phosphorylation on Y794, it failed to induce phosphorylation on either Y490 or Y751 residues (Figure 2B). K252a is an inhibitor of the Trk receptors. K252a potently blocked amitriptyline-triggered TrkA tyrosine phosphorylation, indicating that the stimulatory effect by amitriptyline represents Trk receptor-dependent autophosphorylation. Strikingly, amitriptyline, but not NGF, also induced TrkB tyrosine phosphorylation, which was also blocked by K252a (Figure 2C). However, amitriptyline failed to provoke TrkC activation (see Figure S1 available online). Amitriptyline swiftly activated both MAP kinase and Akt signaling cascades in hippocampal neurons in a manner temporally similar to NGF (Figure 2D, left). Titration assays demonstrated that 250 nM amitriptyline stimulated both Erk1/2 and Akt signaling activation and the signal became stronger at 500 nM (Figure 2D, right). Pretreatment with anti-NGF or anti-BDNF failed to block the stimulatory effect of TrkA or TrkB by amitriptyline in cortical neurons, suggesting that amitriptyline provokes TrkA and TrkB activation independent of neurotrophins (Figure S2). Together, these results demonstrate

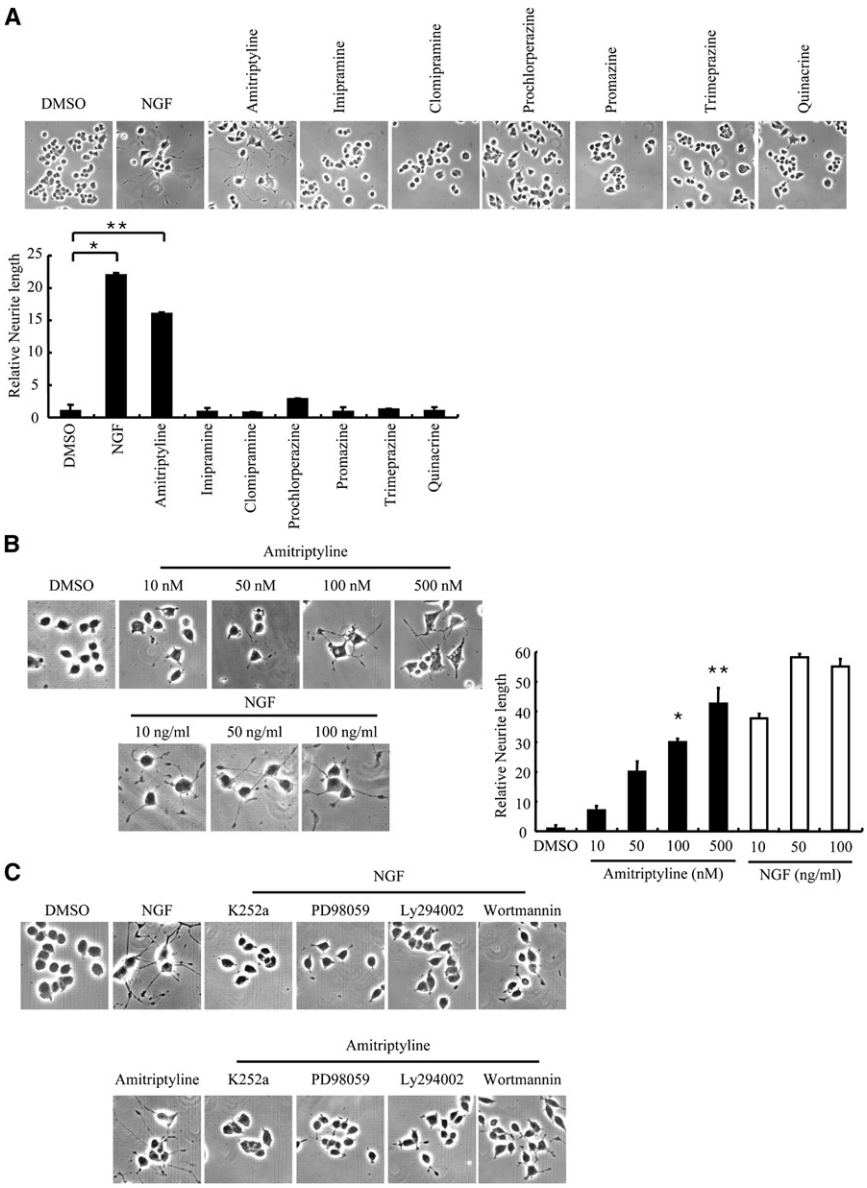


Figure 3. Amitriptyline Provokes Neurite Outgrowth in PC12 Cells

(A) Amitriptyline but not other tricyclic compounds provokes neurite outgrowth in PC12 cells. PC12 cells were treated with amitriptyline and other compounds (500 nM) for 5 days in 2% FBS and 1% HS medium. The drug-containing medium was replenished every other day. Amitriptyline induced neurite outgrowth as potently as NGF (top). The relative neurite length was quantified (bottom).

(B) Dose-dependent effect of neurite outgrowth. Amitriptyline (100 nM) was able to provoke neurite outgrowth in PC12 cells (One-way ANOVA, Bonferroni post hoc test, *$p < 0.01$; **$p < 0.005$).

(C) K252a, PI3K, and MAP kinase inhibitors abolish amitriptyline-provoked neurite outgrowth in PC12 cells.

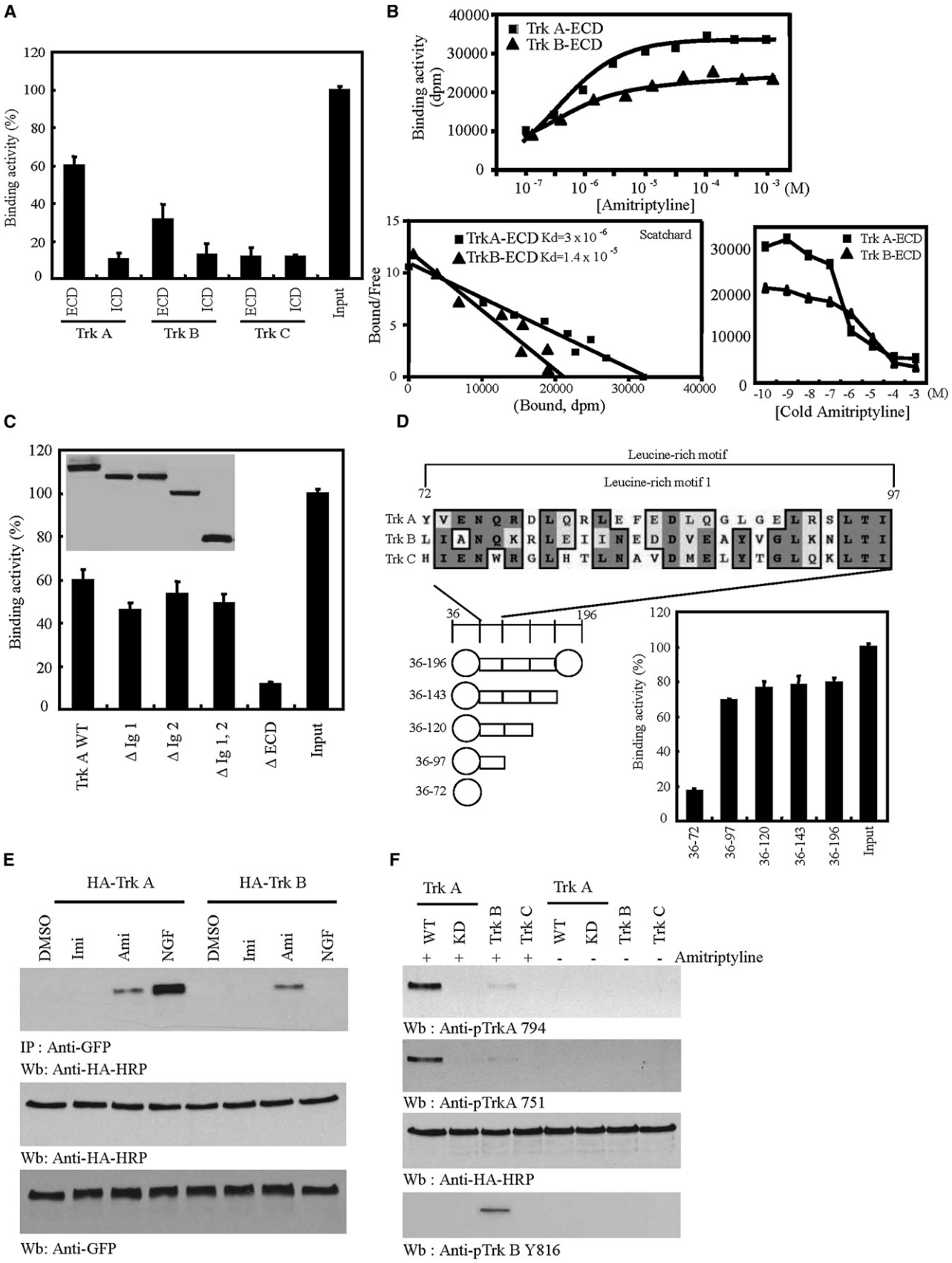
that amitriptyline strongly induces TrkA and TrkB receptor phosphorylation and activation in a dose-dependent manner.

Amitriptyline Induces Neurite Outgrowth in PC12 Cells

One of the most prominent neurotrophic effects of NGF is to trigger neurite outgrowth in neuronal cells and incur differentiation. To assess whether amitriptyline and/or other antidepressant drugs possess this activity, we incubated PC12 cells with NGF or various antidepressant drugs (0.5 μM) for 5 days. As expected, NGF induced pronounced neurite sprouting in PC12 cells after 5 days of treatment. Among all tested tricyclic antidepressant drugs, only amitriptyline markedly triggered demonstrable neurite outgrowth in PC12 cells, and the neurite network generated was comparable to that initiated by NGF (Figure 3A). Titration assays revealed that 100 nM amitriptyline was sufficient to provoke substantial neurite sprouting in PC12 cells (Figure 3B). Because PI3K and MAP kinase signaling pathways are required for NGF's neurite outgrowth effect, we performed experiments using pharmacological agents, including K252a, PI3K inhibitors, and PD98058 (MEK1 inhibitor), which all substantially blocked the neurite outgrowth effect by NGF (Figure 3C). These inhibitors also inhibited amitriptyline's neurotrophic activity, indicating that amitriptyline's neurite outgrowth effect is Trk receptor dependent. Thus, amitriptyline possesses notable neurotrophic activity and robustly induces neurite outgrowth.

Amitriptyline Binds TrkA and TrkB Receptors and Triggers their Dimerization and Autophosphorylation

To determine whether Trk receptors directly bind amitriptyline, we conducted an in vitro binding assay with purified Trk ECD and intracellular domain (ICD) proteins and [^{3}H]amitriptyline. Remarkably, the ECD but not ICD from TrkA and TrkB receptors bound to amitriptyline, with TrkA exhibiting stronger binding (3 μM TrkA binding constant and 14 μM TrkB) (Figure 4A). Gradual increase of cold amitriptyline concentration progressively diminished [^{3}H]amitriptyline binding to the ECD of TrkA and TrkB receptors, indicating specific binding (Figure 4B). In contrast, amitriptyline was unable to compete with NGF or BDNF for binding to TrkA or TrkB ECD, respectively (data not shown). This might be due to its low affinity to the receptors. To map which region is involved in TrkA binding to amitriptyline, we systematically deleted Ig1, Ig2, and Ig1+2 domains in TrkA receptor and found that none of the immunoglobulin-like domains were required for the interaction with amitriptyline, while removal of the entire ECD domain significantly diminished the binding by amitriptyline (Figure 4C). Truncation assays with a variety of TrkA ECD proteins showed that the first leucine-rich motif (LRM) (aa 72–97) was essential for full binding of amitriptyline to TrkA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Binding activity (dpm)
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TrkB-ECD Kd=1.4 x 10 -5
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Bound/Free
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C
Binding activity (%)
Trk A WT
Δ Ig 1
Δ Ig 2
Δ Ig 1, 2
Δ ECD
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D
Leucine-rich motif
Leucine-rich motif 1
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Trk C
36-196
36-143
36-120
36-97
36-72
Binding activity (%)
E
HA-Trk A
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NGF
IP : Anti-GFP
Wb: Anti-HA-HRP
Wb: Anti-GFP
F
Trk A
WT
KD
Trk B
Trk C
Amitriptyline
Wb : Anti-pTrkA 794
Wb : Anti-pTrkA 751
Wb : Anti-HA-HRP
Wb : Anti-pTrk B Y816

(Figure 4D, bottom). Sequence alignment for the first LRM (aa 72–97) of Trk receptors is shown (Figure 4D, top).

We next sought to determine whether the binding by amitriptyline to Trk receptors incurs their dimerization. Coimmunoprecipitation demonstrated that amitriptyline, but not imipramine, elicited TrkA homodimerization as NGF. Strikingly, amitriptyline uniquely caused TrkA to heterodimerize with TrkB (Figure 4E, top). Amitriptyline elicited marked tyrosine phosphorylation in TrkA and TrkB, but not in TrkC receptor when HEK293 cells were individually transfected by TrkA, TrkB, or TrkC (Figure 4F). In contrast, TrkA-KD (kinase-dead) was not tyrosine phosphorylated, indicating that tyrosine phosphorylation of Trk receptors provoked by amitriptyline is exerted by the receptors themselves but not by any other cytoplasmic tyrosine kinases. Hence, amitriptyline mimics NGF and induces TrkA dimerization and autophosphorylation and also possesses novel TrkA-TrkB heterodimerization activity.

Amitriptyline Activates TrkA and TrkB Receptors and Prevents KA-Triggered Neuronal Apoptosis in Mouse Brain

To assess whether amitriptyline can induce Trk receptor activation in mouse brain, we injected amitriptyline (15 mg/kg, i.p.) into mice. Amitriptyline induced both TrkA (Y794) and TrkB (Y816) activation in mouse brain after 4 hr and the effect persisted at 8 hr (Figure 5A, left top and third panels). In contrast, imipramine failed to activate any of the receptors (Figure 5A, right). Consequently, we also observed robust Akt and ERK1/2 activation with the same time course, supporting that amitriptyline can provoke both TrkA and TrkB receptor activation in mice. Although imipramine failed to induce phosphorylation of the Trk receptors, it did promote phosphorylation of Akt and ERK1/2 (Figure 5A, right). The onset of Trk receptor activation fits with the peak plasma concentration of amitriptyline that is reached within 6 hr. RT-PCR analysis showed that neither TrkA nor TrkB mRNA levels were altered after treatment by amitriptyline or imipramine (Figure 5B), indicating that acute treatment with amitriptyline or imipramine might not significantly regulate Trk receptor transcription.

To explore whether amitriptyline can block the excitotoxicity initiated by KA, we injected the mice with saline or amitriptyline (15 mg/kg, i.p.), followed by saline or KA (25 mg/kg, i.p.). Five days after treatment, TUNEL staining revealed significant apoptosis in the hippocampus of KA-treated mice compared to vehicle control or amitriptyline alone. KA-provoked apoptosis was substantially diminished by pretreatment with amitriptyline, while administration of amitriptyline after KA was not as effective (Figure 5C). Quantitative analysis revealed that amitriptyline suppressed KA-induced cell death by 70% when injected prior to KA, compared to 45% when injected after (Figure 5C, right). Immunohistochemical analysis showed that TrkA and TrkB were significantly activated in the hippocampus by amitriptyline. In addition, amitriptyline evidently augmented the expression of TrkA but not TrkB (Figure 5D). Hence, amitriptyline strongly provokes TrkA and TrkB activation in mouse brain and protects hippocampal neurons from KA-triggered cell death.

Amitriptyline Promotes TrkA and TrkB Heterodimerization in Mouse Brain

To test whether amitriptyline regulates Trk receptor expression, we fed the mice with various antidepressant drugs subchronically and chronically. After 5 or 28 days, we monitored TrkA and TrkB protein expression levels and activation by immunoblotting. In 5 days, amitriptyline prominently provoked TrkA tyrosine phosphorylation, whereas imipramine, fluoxetine, and control vehicle had no effect (Figure 6A, left top). TrkA expression levels in amitriptyline-treated mice were higher than other drugs or saline-treated mice (left second panel). Remarkably, TrkB was activated by all three drugs compared to saline, and total TrkB protein levels were higher in imipramine- and fluoxetine-treated mice than in amitriptyline- and saline-treated mice (Figure 6A, third and fourth panels). Compared to vehicle control and other antidepressant drugs, RT-PCR analysis revealed that TrkA transcription was substantially augmented after amitriptyline. Nevertheless, TrkB mRNA levels remained similar in all samples (Figure 6A, right), indicating that subchronic treatment with antidepressant drugs does not significantly affect TrkB receptor transcription. We made the similar observation in 28 day treated samples (data not shown). The comparable effects were recapitulated in 5-HT1a null mice, suggesting that serotonin receptor 5-HT1a is not implicated in these events (Figure S3). Thus, these findings support the idea that amitriptyline activates TrkA receptor and upregulates its expression in both subchronic and chronic treatment conditions. However, imipramine and fluoxetine are more potent than amitriptyline in provoking TrkB activation and elevating TrkB protein levels.

Coimmunoprecipitation revealed that amitriptyline strongly provoked endogenous TrkA to bind endogenous TrkB receptor in the brain after subchronic treatment. In contrast, imipramine or fluoxetine failed (Figure 6B). Cotransfection and pull-down assays demonstrated that amitriptyline induced TrkA and TrkB homodimerization like NGF and BDNF, but also triggered

Figure 4. Amitriptyline Binds TrkA and TrkB Receptors and Triggers their Dimerization and Autophosphorylation

(A) In vitro binding assay with Trk recombinant proteins. [^{3}H]Amitriptyline (0.1 μCi) was incubated with purified ECD and ICD recombinant proteins of Trk receptors (5 μg each) at 30°C for 1 hr. The mixture was subjected to filter/vacuum assay. After extensive washing, the filter paper was analyzed in liquid scintillation counter.

(B) Quantitative analysis of the binding between amitriptyline and TrkA and TrkB ECD. TrkA and TrkB ECD binding curve by [^{3}H]amitriptyline (top). Scatchard plot for K_d analysis (bottom left). Cold aminitryptyline competed with [^{3}H]amitriptyline for binding to TrkA or TrkB ECD (bottom right).

(C) ECD domain in TrkA is essential for amitriptyline to bind TrkA receptor.

(D) Amitriptyline associates with the first LRM in the N terminus of ECD of TrkA. Protein sequence alignment between the LRM motif from TrkA and the counterparts from TrkB and TrkC (top). In vitro binding assay was conducted with a variety of fragments of TrkA ECD recombinant proteins (bottom). Data represent mean ± SEM.

(E) Amitriptyline provokes TrkA dimerization. GFP-TrkA and HA-TrkA or HA-TrkB were respectively cotransfected into HEK293 cells and treated with 0.5 μM amitriptyline or imipramine for 30 min. GFP-TrkA was immunoprecipitated with anti-GFP antibody, and the coprecipitated proteins were analyzed with anti-HA antibody.

(F) Amitriptyline-triggered TrkA or TrkB tyrosine phosphorylation in HEK293 cells individually transfected by TrkA or TrkB, respectively. Kinase-dead TrkA displayed negligible tyrosine phosphorylation.

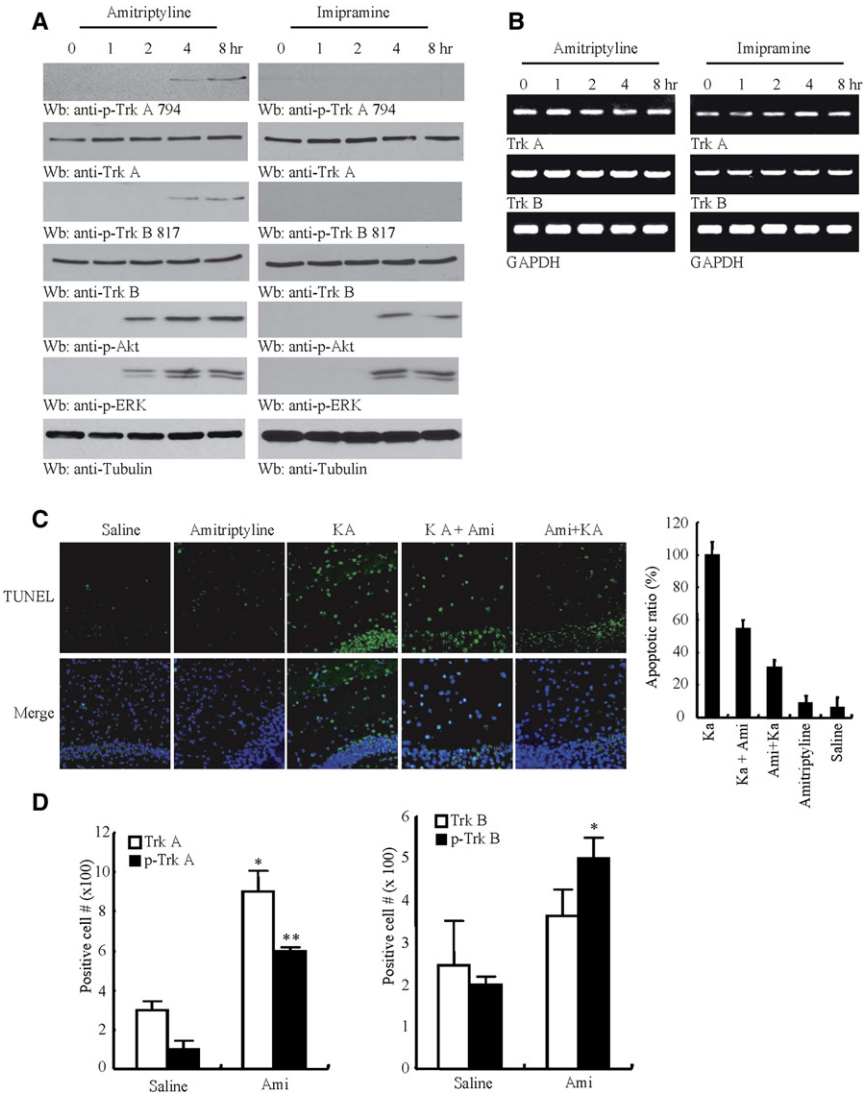


Figure 5. Amitriptyline Activates TrkA and TrkB Receptors and Prevents KA-Triggered Neuronal Apoptosis in Mouse Brain

(A) Amitriptyline but not imipramine activates TrkA and TrkB receptors in mouse brain. Two- to three-month-old C57BL/6 mice were intraperitoneally injected with 15 mg/kg amitriptyline and 20 mg/kg imipramine for various time points. Immunoblotting was conducted with various indicated antibodies.

(B) Amitriptyline and imipramine do not alter TrkA or TrkB transcription. RT-PCR analysis of TrkA and TrkB in mouse brain.

(C) Amitriptyline diminishes KA-triggered hippocampal neuronal cell death. Two- to three-month-old C57BL/6 mice were intraperitoneally injected with 15 mg/kg amitriptyline either before or after KA (25 mg/kg) administration. In 5 days, the brain slides were analyzed with TUNEL assay. Green shows apoptotic nuclei, which were also stained with DAPI.

(D) Amitriptyline increases TrkA expression and provokes TrkA and TrkB activation in hippocampus. Data represent mean ± SEM (n = 3/group, One-way ANOVA, Bonferroni post hoc test, *p < 0.01; **p < 0.01).

heterodimerization between TrkA and TrkB receptors in HEK293 cells. In contrast, imipramine and fluoxetine did not induce either of these effects (Figure 6C). To explore the role of amitriptyline binding motifs in mediating TrkA and TrkB receptor heterodimerization, we deleted the binding motif and transfected the truncated receptors into HEK293 cells. Deletion of amitriptyline binding motif on TrkA completely abolished the homo- and heterodimerization activity (Figure 6D, left). In contrast, truncation of the similar region on TrkB failed to abolish the stimulatory activity by amitriptyline (Figure 6D, right), indicating that the deleted fragment on TrkB might not be the binding spot for amitriptyline. Notably, the cotransfected wild-type TrkA and TrkB were potently phosphorylated by amitriptyline. Interestingly, truncated TrkB but not deleted TrkA was still capable of being activated by amitriptyline. In contrast, truncated TrkA and TrkB were robustly activated by NGF or BDNF, respectively (Figure 6D), suggesting that amitriptyline binding motif is not required for neurotrophins to trigger Trk receptor activation.

Previous studies show that TrkA immunoglobulin-like ligand binding domains inhibit spontaneous dimerization and activation of the receptor (Arevalo et al., 2000). To explore whether amitriptyline elicits receptor dimerization via blocking the autoinhibitory effect by the Trk receptors, we transfected the truncated receptors with different tags into HEK293 cells. Cotransfection of truncated TrkA failed to form a homodimer regardless of amitriptyline (Figure S4A), supporting that amitriptyline induces TrkA dimerization not through blocking its autoinhibitory effect by the binding motif on TrkA. In contrast, amitriptyline elicited potent homodimer by the truncated TrkB receptors as BDNF, though TrkB activation by amitriptyline was evidently reduced as compared to BDNF. Ligand binding assays demonstrated that deletion of 72–97 residues abolished the binding activity by amitriptyline on TrkA but not on TrkB (Figures S4B and S4C). This finding might explain why the truncated TrkB is still able to form a homodimer by amitriptyline. Hence, Trk receptor binding by amitriptyline is essential for the receptor homo- and heterodimerization.

Investigation of Amitriptyline Role in Prevention of Neuronal Apoptosis Reveals Distinct Effect on TrkA and TrkB

To assess whether amitriptyline's neurotrophic activity is solely mediated through TrkA receptor, we explored its ability to promote neuronal survival in wild-type neurons and those that lack TrkA. Heterozygous (TrkA +/−) males and females were crossed and hippocampal neurons were harvested from newborn (P0) pups. Amitriptyline triggered TrkA tyrosine phosphorylation in wild-type but not TrkA knockout neurons

(Figure 7A, left second panel). Fluoxetine also elicited weak tyrosine phosphorylation in both TrkA and TrkB receptor in a TrkA-dependent manner (Figure 7A, right second and fourth panels). Interestingly, these experiments additionally revealed a different effect of amitriptyline on TrkB in the absence of TrkA. In contrast to TrkA, amitriptyline activated TrkB in both wild-type and TrkA −/− neurons, indicating that TrkA is not required for amitriptyline-induced phosphorylation of TrkB and amitriptyline activates TrkB in a TrkA-independent way.

We next assessed the apoptotic pathway by examining caspase-3 activation at baseline and following glutamate exposure. In the absence of glutamate treatment, a low amount of active caspase-3 was demonstrable in TrkA null neurons but absent in wild-type neurons, consistent with the substantial neuronal cell death that has been observed in TrkA null mice (Smeyne et al., 1994). Amitriptyline completely inhibited the basal caspase-3 activation in TrkA −/− neurons (Figure 7A, left fifth panel). Caspase-3 activation by glutamate was moderately enhanced in TrkA −/− neurons, suggesting that TrkA normally inhibits the apoptotic response to excitotoxicity. Amitriptyline pretreatment partially blocked glutamate-induced caspase-3 activation in both wild-type and TrkA −/− neurons, suggesting that activation of TrkB by amitriptyline in TrkA −/− neurons contributes to this protective action. Neither fluoxetine nor imipramine inhibited caspase-3 activation in wild-type or TrkA −/− neurons (Figure 7A, right fifth panel). To determine whether activation of TrkA by amitriptyline requires TrkB, we extended our analysis to TrkB −/− neurons. Amitriptyline, but not imipramine, selectively activated TrkA in both wild-type and TrkB −/− neurons (Figure 7B, third panel). Depletion of TrkB incurred spontaneous caspase-3 activation, which was completely blocked by amitriptyline but not imipramine. Moreover, amitriptyline also markedly suppressed glutamate-provoked caspase-3 activation in both wild-type and TrkB −/− neurons, whereas imipramine had no protective effect (Figure 7B, bottom). These results indicate that the activation of TrkA by amitriptyline is independent of TrkB and TrkA activation in TrkB −/− neurons might account for the protective effect of amitriptyline pretreatment.

TrkA F592A knockin mice can be selectively blocked by the inhibitor 1NMPP1, which results in Trk null phenotypes (Chen et al., 2005). Consistent with previous reports, NGF-provoked TrkA, Akt, and Erk1/2 phosphorylation was selectively blocked by 1NMPP1 but not K252a, a pattern that was mimicked by amitriptyline treatment (Figure 7C). Imipramine and fluoxetine failed to activate TrkA but did stimulate Akt and Erk1/2, although these activities were not blocked by 1NMPP1 or K252a, indicating that these antidepressants were acting via a TrkA-independent pathway (Figure 7C, third and fifth panels).

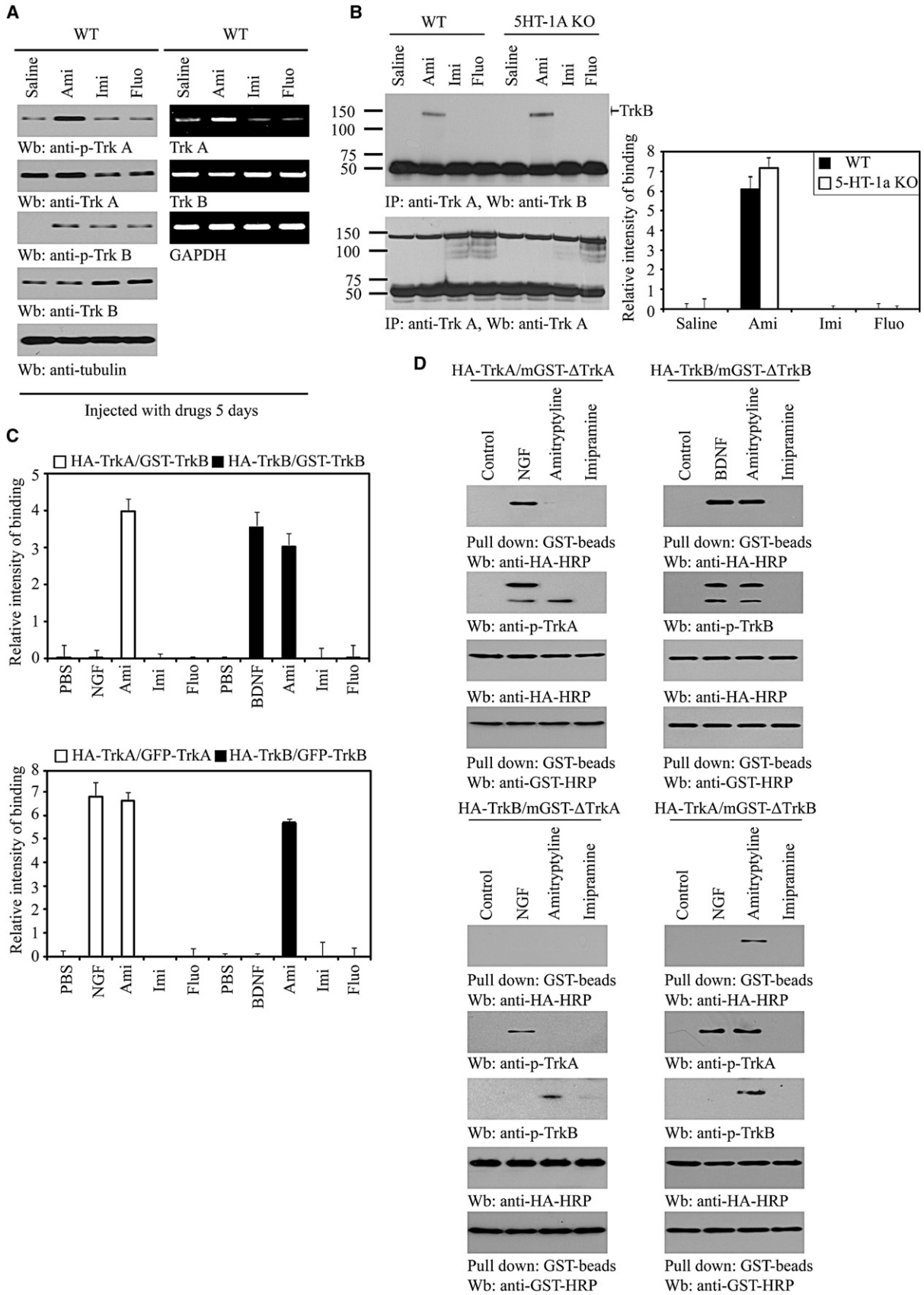
We next tested whether our in vitro results could be recapitulated in vivo by assessing KA-induced caspase-3 activation in TrkA F592A mice. As expected, 1NMPP1, amitriptyline alone, or 1NMPP1 + amitriptyline combined treatment had no effect on caspase-3 in TrkA F592A mice. KA provoked significant caspase-3 activation that was suppressed by amitriptyline and this protective effect was abolished by 1NMPP1 pretreatment (Figure 7D, top). 1NMPP1 also blocked TrkA F592A phosphorylation by amitriptyline and imipramine did not activate TrkA under any condition (Figure 7D, middle). KA-provoked caspase-3 activation in 1NMPP1-sensitive TrkB F616A knockin mice was also inhibited by amitriptyline. However, in contrast to the TrkA F592A mice, this protective effect persisted in the presence of 1NMPP1 (Figure 7E). Although imipramine weakly activated TrkB F616A, it failed to suppress caspase-3 activation by KA (Figure 7E). These results demonstrate that amitriptyline selectively activates both TrkA and TrkB receptors in mice, but TrkA is more important than TrkB in mediating the neuronal survival functions by amitriptyline.

DISCUSSION

In this study, we show that amitriptyline binds to a motif in the first LRM in the TrkA receptor ECD with a K_d of ~3 μM. Interestingly, amitriptyline also interacts with the ECD from TrkB but not TrkC with a decreased binding affinity (K_d ~14 μM). A previous study suggests that brain [amitriptyline] in treating depression is around 5–7 μm (Glotzbach and Preskorn, 1982). To treat pain associated peripheral neuropathy, [amitriptyline] is 2-fold lower (Esser and Sawynok, 1999). Hence, amitriptyline affinity to TrkA might be sufficient to exert its biological actions. The known K_d for NGF to TrkA and BDNF to TrkB is ~1 nM in the absence of p75NTR. These differences in binding affinities are consistent with the quantitative dose-response studies that amitriptyline is some 100-fold less potent than the neurotrophin ligands (Figure 3).

NGF is a homodimer and it binds to both the Ig2 and LRM domain on TrkA and brings two TrkA receptors together, which subsequently autophosphorylate each other. NGF selectively binds the 24 amino acids (aa 97–120) of the second LRM with a K_d of ~1.3 nM (O'Connell et al., 2000). Here we show that amitriptyline can only bind the first LRM domain (aa 72–97) and promotes TrkA receptor dimerization. In addition, we also show that amitriptyline binds both TrkA and TrkB receptors and provokes TrkA to interact with TrkB, leading to a heterodimer. However, NGF fails to do so (Figure 4). Previous studies show that BDNF, NT-3, and NT-4 bind to the LRM3 cassette of TrkB, whereas NGF does not. These binding characteristics clearly reflect in vivo specificities. A more precise mapping of the regions responsible for binding BDNF, NT-3, and NT-4 identified the second LRM of TrkB as a functional unit capable of binding all three neurotrophins (Windisch et al., 1995). Why does amitriptyline but not NGF or BDNF provoke the heterodimer formation by TrkA and TrkB? NGF and BDNF bind p75NTR, while amitriptyline does not (data not shown), suggesting that these two categories of molecules bind neurotrophin receptors in fundamentally different ways.

Antidepressant drugs and electroconvulsive stimuli significantly influence brain neurotrophin concentrations. It has been proposed that BDNF and NGF may play a role in depression (Angelucci et al., 2000). Subchronic treatment with lithium increases NGF content in brain of adult rat, supporting that NGF may be implicated in the mechanism of antibipolar treatments (Hellweg et al., 2002). To test whether amitriptyline exerts its antidepressant action through the TrkA receptor, we conducted forced swim tests with TrkA F592A mice. Blocking TrkA signaling with 1NMPP1 did not significantly alter the immobility. Subchronic treatment of mice with amitriptyline or imipramine substantially decreased the immobility no matter whether TrkA receptor was blocked or not. Moreover, both drugs exhibited demonstrable


A
WT
WT
Saline
Ami
Imi
Fluo
Wb: anti-p-Trk A
Trk A
Wb: anti-Trk A
Trk B
Wb: anti-p-Trk B
GAPDH
Wb: anti-Trk B
Wb: anti-tubulin
Injected with drugs 5 days
B
WT
5HT-1A KO
150
100
75
50
TrkB
IP: anti-Trk A, Wb: anti-Trk B
IP: anti-Trk A, Wb: anti-Trk A
Relative intensity of binding
WT
5-HT-1a KO
C
HA-TrkA/GST-TrkB
HA-TrkB/GST-TrkB
Relative intensity of binding
PBS
NGF
Ami
Imi
Fluo
BDNF
HA-TrkA/GFP-TrkA
HA-TrkB/GFP-TrkB
D
HA-TrkA/mGST-ΔTrkA
HA-TrkB/mGST-ΔTrkB
Control
NGF
BDNF
Amitryptyline
Imipramine
Pull down: GST-beads
Wb: anti-HA-HRP
Wb: anti-p-TrkA
Wb: anti-p-TrkB
Wb: anti-GST-HRP

antidepressant effect in NGF +/+ and +/− mice (Figure S5). Thus, TrkA signaling might be dispensable for at least some of the therapeutic actions of amitriptyline. This finding fits with previous reports that amitriptyline increased BDNF but not NGF concentration in serum of depressed patients (Hellweg et al., 2008). Moreover, normal TrkB signaling is required for the behavioral effects typically produced by antidepressants (Saarelainen et al., 2003). Conceivably, amitriptyline exerts its antidepressant action through TrkB but not TrkA receptor.

While tricyclics are generally regarded as comparable antidepressants, why is amitriptyline uniquely effective versus the other tricyclics? Imipramine differs chemically from amitriptyline only in the presence of an exocyclic double bond in amitriptyline, which is absent in imipramine (Figure 1A). The exocyclic double bond inhibits the free rotation of the side chain of amitriptyline, rendering it slightly more "rigid." Thus, this chemical feature may account for the selective and potent Trk agonistic activity of amitriptyline. Consistently, it has been shown before that amitriptyline but not imipramine possesses robust anticholinergic activity (Snyder and Yamamura, 1977). Tricyclic antidepressants like amitriptyline inhibit the monoamine reuptake inactivation of norepinephrine and serotonin neurotransmitters. They are potent blockers of muscarinic cholinergic (Snyder and Yamamura, 1977), alpha adrenergic, and 5-HT receptors (Peroutka and Snyder, 1980). To explore whether serotonin is implicated in amitriptyline's action, we depleted serotonin with pCPA and found that amitriptyline's stimulatory effect on TrkA was not significantly affected. Moreover, serotonin itself was unable to provoke TrkA or TrkA activation in primary neurons (data not shown), suggesting that serotonin might not be implicated in amitriptyline's agonistic effect. Amitriptyline has anticholinoceptor actions on both the CNS and peripheral organs. The best correlation to the biological activity of amitriptyline is the anticholinergic actions. For instance, inhibition of nAChRs by mecamylamine had antidepressant-like effects and potentiated the antidepressant activity of amitriptyline when the two drugs were used in combination. Mice lacking high-affinity nAChRs showed no behavioral response to amitriptyline. Hence, the antagonism of nAChRs might be an essential component of the therapeutic action of antidepressants like amitriptyline (Caldarone et al., 2004). In alignment with this finding, we show that mecamylamine also strongly elicited neurite sprouting processes in PC12 cells (Figure S6). Our data demonstrate that amitriptyline exhibits potent neurotrophic activities including neurite outgrowth in PC12 cells, neuronal survival in primary neurons, and neuroprotection in mice, which mimic the primary neurotrophic effects of NGF. It is also noteworthy that amitriptyline effectively treats chronic neuropathic pain (Ho et al., 2008; Watson, 2000). Unlike NGF, which causes intense pain, conceivably, amitriptyline achieves its pain-reducing effects and might be entirely distinct from NGF/TrkA mechanisms involving peripheral nerves.

SIGNIFICANCE

Neurotrophins exert the physiological functions through provoking neurotrophin receptor (TrkA, TrkB, and TrkC) homodimerization. However, it remains elusive whether the ligand has to be a homodimer in order to trigger the receptor dimerization. Moreover, it is unknown whether the Trk receptors can form a heterodimer or not. In the current study, we show that the small tricyclic antidepressant drug amitriptyline provokes both TrkA and TrkB homo- and heterodimerization and activation in transfected HEK293 cells and primary neurons. Amitriptyline elicits potent TrkA and TrkB activation in mice with a similar temporal pattern. Thus, we demonstrate that amitriptyline acts as a novel agonist for both TrkA and TrkB. Strikingly, this small molecule but not any other antidepressant drugs selectively stimulates endogenous TrkA/TrkB heterodimer formation in mouse brain. This finding provides a molecular mechanism in dimerization and activation of transmembrane receptor tyrosine kinase. Endogenous cognate ligands for Trk receptors are homodimers, which can only selectively bind to one of the Trk receptors, whereas amitriptyline binds to both TrkA and TrkB receptors, leading to a heterodimer formation. Hence, this discovery establishes a proof-of-concept model for identifying small molecular agonists and antagonists for receptor tyrosine kinase. Conceivably, using the established screening assay, numerous small compounds can be identified for mimicking growth factors including EGF, insulin, netrins, etc.

EXPERIMENTAL PROCEDURES

Cells, Reagents, and Mice

Mouse septal neuron × neuroblastoma hybrid SN56 cells were created by fusing N18TG2 neuroblastoma cells with murine (strain C57BL/6) neurons from postnatal day 21 septa. SN56 cells were maintained at 37°C with 5% CO_2 atmosphere in DMEM containing 1 mM pyruvate and 10% FBS. T17 cells, stably transfected with rat TrkA, were cultured in the same medium containing 300 μg/ml G418. NGF and BDNF were purchased from Roche. The specificity of anti-p-TrkA 794 has been previously described (Jeanneteau et al., 2008; Rajagopal et al., 2004). The specificity of anti-p-TrkB 816 has been described before (Arevalo et al., 2006). Anti-TrkB antibody was obtained from Biovision. Anti-TrkA was obtained from Cell Signaling. The chemical library containing 2000 biologically active compounds was from the Spectrum Collection (MicroSource Discovery System, Inc.). $TrkA^{F592A}$ and $TrkB^{F616A}$ mice have been

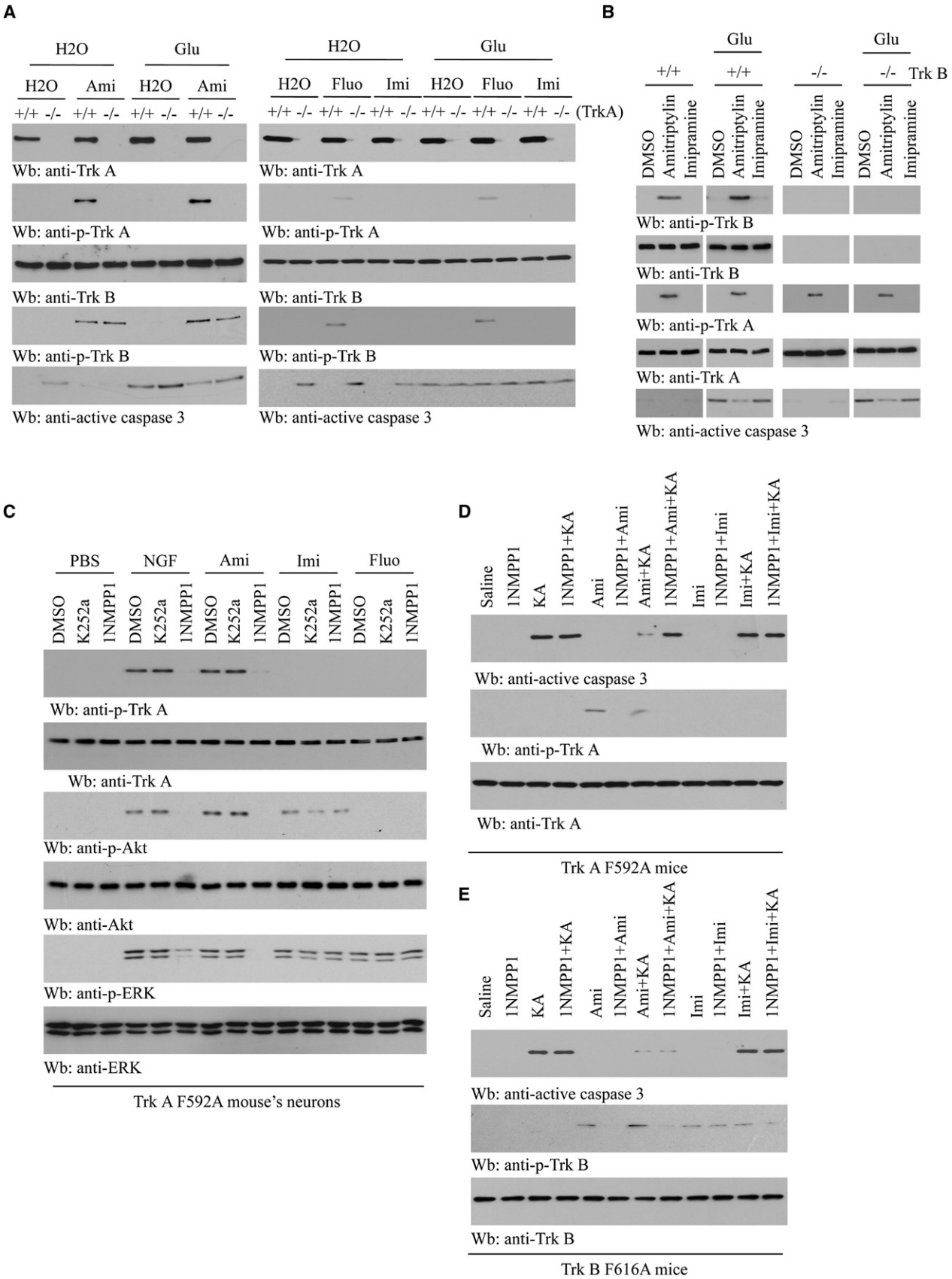
Figure 6. Amitriptyline Promotes TrkA and TrkB Heterodimerization in Mouse Brain

(A) Amitriptyline provokes TrkA phosphorylation and upregulates its expression mice. Two- to three-month-old mice were intraperitoneally injected with various antidepressant drugs for 5 days. The doses were 15 mg/kg, 20 mg/kg, and 25 mg/kg for amitriptyline, imipramine, and fluoxetine, respectively.

(B) Amitriptyline triggers TrkA and TrkB receptor heterodimerization in mouse brain. Wild-type and 5-HT1a null mice were treated with various antidepressant drugs for 5 days. TrkA was immunoprecipitated, and its coprecipitated proteins were analyzed by immunoblotting.

(C) Amitriptyline elicits TrkA and TrkB receptor homo- and heterodimerization. Differentially tagged TrkA and TrkB receptors were cotransfected into HEK293 cells and treated with various antidepressant drugs for 30 min. Data represent mean ± SEM of n = 3.

(D) Amitriptyline binding motif on TrkA is essential for Trk receptor homo- and heterodimerization. HA-tagged TrkA or TrkB was cotransfected with GST-ΔTrkA or GST-ΔTrkB into HEK293 cells, followed by NGF, BDNF, amitriptyline, or imipramine treatment. Truncated TrkA or TrkB was pulled down by glutathione beads and monitored by anti-HA antibody.


A
H2O
Glu
H2O
Ami
H2O
Ami
+/+ -/- +/+ -/- +/+ -/- +/+ -/-
Wb: anti-Trk A
Wb: anti-p-Trk A
Wb: anti-Trk B
Wb: anti-p-Trk B
Wb: anti-active caspase 3
H2O
Glu
H2O
Fluo
Imi
H2O
Fluo
Imi
+/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/- +/+ -/- (TrkA)
Wb: anti-Trk A
Wb: anti-p-Trk A
Wb: anti-Trk B
Wb: anti-p-Trk B
Wb: anti-active caspase 3
B
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Glu
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-/-
-/-
Trk B
DMSO
Amitriptylin
Imipramine
Wb: anti-p-Trk B
Wb: anti-Trk B
Wb: anti-p-Trk A
Wb: anti-Trk A
Wb: anti-active caspase 3
C
PBS
NGF
Ami
Imi
Fluo
DMSO
K252a
1NMPP1
Wb: anti-p-Trk A
Wb: anti-Trk A
Wb: anti-p-Akt
Wb: anti-Akt
Wb: anti-p-ERK
Wb: anti-ERK
Trk A F592A mouse's neurons
D
Saline
1NMPP1
KA
1NMPP1+KA
Ami
1NMPP1+Ami
Ami+KA
1NMPP1+Ami+KA
Imi
1NMPP1+Imi
Imi+KA
1NMPP1+Imi+KA
Wb: anti-active caspase 3
Wb: anti-p-Trk A
Wb: anti-Trk A
Trk A F592A mice
E
Saline
1NMPP1
KA
1NMPP1+KA
Ami
1NMPP1+Ami
Ami+KA
1NMPP1+Ami+KA
Imi
1NMPP1+Imi
Imi+KA
1NMPP1+Imi+KA
Wb: anti-active caspase 3
Wb: anti-p-Trk B
Wb: anti-Trk B
Trk B F616A mice

described previously (Chen et al., 2005). TrkA^{F592A} and TrkB ^{F616A} mice and TrkA +/−, TrkB +/−, and NGF +/− C57BL/6 mice were bred in a pathogen-free environment in accordance with Emory Medical School guidelines. All chemicals not included above were purchased from Sigma.

Cell-Based Screen

T17 cells were seeded in a 96 well plate at 10,000 cells/well in 100 μl of complete medium. Cells were incubated overnight, followed by 30 min pretreatment with 10 μM compounds in DMSO (10 mM stock concentration was obtained from the Spectrum Collection library). The cells were then treated with 1 μM staurosporine for 9 hr. One hour before the termination of the experiment, 10 μM MR(DEVD)2, a cell permeable caspase-3-activated fluorescent dye, was introduced. Cells were fixed with 4% paraformaldehyde for 15 min. Cells were washed with PBS and incubated with 1 μg/ml Hoechst 33342 for 10 min. Cover slides were washed with PBS, mounted, and examined using a fluorescence microscope.

Binding Constant Determination

Purified TrkA ECD or ICD proteins were incubated with different [^{3}H-amitriptyline] at 4°C for 10 min in 1 ml of binding buffer (50 mM Na-K phosphate [pH 7.1], 200 mM NaCl, and 2 nM ^{3}H-amitriptyline [68,000 cpm]). After the incubation, the reaction mixture was loaded on filter paper. The mixture was washed with 3 × 5 ml of washing buffer (100 mM Tris [pH 7.1]). The dried filter paper was put into a small vial and subjected to liquid scintillation counter analysis. The value of the dissociate constant and the number of sites were obtained from Scatchard plots by using the equation $r/[L]free = n/K_d - r/K_d$, where r is the ratio of the concentration of bound ligand to the total protein concentration and n is the number of binding sites.

KA/Amitriptyline Drug Administration

Male C57BL/6 mice aged 60 days were injected intraperitoneally with a single dose of either 30% ethanol in saline or KA (25 mg/kg) (Sigma) or 7,8-dihydroxyflavone (5 mg/kg) followed by KA. Animals were continually monitored for 2 hr for the onset of seizure activity. At 5 days after treatment, animals were anesthetized and perfused with 4% paraformaldehyde in 0.1 M phosphate-buffered saline. Brains were removed, post-fixed overnight, and processed for paraffin embedding. Serial sections were cut at 5 μm and mounted on slides (Superfrost-plus; Fisher). The slides were processed for TUNEL staining to assess the degree of DNA fragmentation.

Immunohistochemistry Staining

Brain tissues were fixed in 4% paraformaldehyde overnight followed by paraffin embedding. Sections of 6 μm were cut. For immunohistochemical staining, brain sections were deparaffinized in xylene and rehydrated in graded alcohols. Endogenous peroxidase activity was blocked by 3% hydrogen peroxide for 5 min and all slides were boiled in 10 mM sodium citrate buffer (pH 6.0) for 10 min. Phosphorylated TrkA, TrkA, phosphorylated TrkB, and TrkB were detected using specific antibodies and Zymed Histo-SP AEC kit (Invitrogen). Slides were then counterstained with hematoxylin.

TrkA F592A and TrkB F616A Mice Treatment with KA

Two- to four-month-old TrkAF592A and TrkBF616A mice were pretreated with 1NMPP1 in drinking water (25 μM) 1 day before amitriptyline (15 mg/kg) treatment. After 4 hr, KA (25 mg/kg) was intraperitoneally injected into the mice. The mice were housed for an additional 4 days with 1NMPP1 in the drinking water. At day 5, animals were anesthetized and perfused and treated as described above. The brain slides were processed for TUNEL staining to assess the degree of DNA fragmentation.

SUPPLEMENTAL DATA

Supplemental Data include six figures and can be found with this article online at http://www.cell.com/chemistry-biology/supplemental/S1074-5521(09)00177-X.

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Figure 7. Amitriptyline Prevents Neurons from Apoptosis in a TrkA-Dependent Manner

(A) Amitriptyline activates TrkB independent of TrkA receptor. Cortical neurons (TrkA −/−) were pretreated with a variety of compounds for 30 min and the cell lysates were analyzed by immunoblotting with anti-p-TrkB and anti-p-TrkA.

(B) Amitriptyline activates TrkA in TrkB null neurons. Cortical neurons from TrkB +/− × TrkB +/− mice were treated with amitriptyline or imipramine for 30 min. Amitriptyline but not imipramine activated TrkA receptor in both wild-type and TrkB knockout neurons (top and third panels).

(C) Amitriptyline selectively activates TrkA F592A, which can be blocked by 1NMPP1. The primary cultures were pretreated for 30 min with either K252a (100 nM) or 1NMPP1 (100 nM), followed by 0.5 μM amitriptyline, imipramine, or fluoxetine for 30 min. Amitriptyline-provoked TrkA activation was selectively inhibited by 1NMPP1 but not by K252a (top).

(D) Amitriptyline suppresses KA-induced neuronal cell death in TrkA F592A mutant mice, which can be blocked by 1NMPP1. TrkA F592A knockin mice were treated with the following reagents: saline, 1NMPP1, KA, 1NMPP1 + KA, amitriptyline, 1NMPP1 + amitriptyline, and 1NMPP1 + amitriptyline + KA, as described in the Experimental Procedures. Immunoblotting was conducted with the indicated antibodies.

(E) TrkB activation is dispensable for the neuroprotective effect of amitriptyline.

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