Antidepressant Drugs Transactivate TrkB Neurotrophin Receptors in the Adult Rodent Brain Independently of BDNF and Monoamine Transporter Blockade

Tomi Rantamäki¹*, Liisa Vesa¹✉, Hanna Antila¹, Antonio Di Lieto¹, Päivi Tammela², Angelika Schmitt³, Klaus-Peter Lesch³, Maribel Rios⁴, Eero CASTRÉN¹

¹ Sigrid Juselius Laboratory, Neuroscience Center, University of Helsinki, Helsinki, Finland, ² Centre for Drug Research, Faculty of Pharmacy, University of Helsinki, Helsinki, Finland, ³ Molecular Psychiatry, Laboratory of Translational Neuroscience, Department of Psychiatry, Psychosomatics and Psychotherapy, University of Würzburg, Würzburg, Germany, ⁴ Department of Neuroscience, Tufts University School of Medicine, Boston, Massachusetts, United States of America

Abstract

Background: Antidepressant drugs (ADs) have been shown to activate BDNF (brain-derived neurotrophic factor) receptor TrkB in the rodent brain but the mechanism underlying this phenomenon remains unclear. ADs act as monoamine reuptake inhibitors and after prolonged treatments regulate brain bdnf mRNA levels indicating that monoamine-BDNF signaling regulate AD-induced TrkB activation in vivo. However, recent findings demonstrate that TrkB receptors can be transactivated independently of their neurotrophin ligands.

Methodology: In this study we examined the role of BDNF, TrkB kinase activity and monoamine reuptake in the AD-induced TrkB activation in vivo and in vitro by employing several transgenic mouse models, cultured neurons and TrkB-expressing cell lines.

Principal Findings: Using a chemical-genetic TrkB⁶¹⁶A mutant and TrkB overexpressing mice, we demonstrate that ADs specifically activate both the maturely and immaturely glycosylated forms of TrkB receptors in the brain in a TrkB kinase dependent manner. However, the tricyclic AD imipramine readily induced the phosphorylation of TrkB receptors in conditional bdnf⁻/⁻ knock-out mice (132.4±8.5% of control; P = 0.01), indicating that BDNF is not required for the TrkB activation. Moreover, using serotonin transporter (SERT) deficient mice and chemical lesions of monoaminergic neurons we show that neither a functional SERT nor monoamines are required for the TrkB phosphorylation response induced by the serotonin selective reuptake inhibitors fluoxetine or citalopram, or norepinephrine selective reuptake inhibitor reboxetine. However, neither ADs nor monoamine transmitters activated TrkB in cultured neurons or cell lines expressing TrkB receptors, arguing that ADs do not directly bind to TrkB.

Conclusions: The present findings suggest that ADs transactivate brain TrkB receptors independently of BDNF and monoamine reuptake blockade and emphasize the need of an intact tissue context for the ability of ADs to induce TrkB activity in brain.

Introduction

TrkB (tropomyosin-related kinase B) neurotrophin receptor transduces intracellular signaling events that are critical for neuronal differentiation, survival and plasticity throughout life [1–5]. Brain-derived neurotrophic factor (BDNF) is the main endogenous ligand for TrkB [3], but recent evidence demonstrates that TrkB can also be transactivated independently of BDNF or other neurotrophins through neuromodulator receptors [6,7] and small molecules [8,9].

Abnormal TrkB receptor signaling has been linked to a number of central nervous system (CNS) diseases such as mood and memory disorders and addiction [10–12]. Accumulating evidence suggests that antidepressant drugs (AD) that regulate the brain levels of monoamine neurotransmitters serotonin and norepinephrine, act at least partially by activating TrkB receptor signaling in brain [13,14]. ADs have been shown to rapidly induce the phosphorylation and activation of TrkB receptors in the rodent cortex and hippocampus [13,15]. When administered chronically, ADs also increase BDNF mRNA and protein levels and TrkB phosphorylation in brain [13,15,16]. Furthermore, animal studies suggest that many of the behavioral and functional actions of ADs are attenuated in mice with reduced BDNF signaling in brain.
Levels of BDNF are reduced in the brain and serum of depressed patients and the levels are returned back to normal range upon a successful treatment with ADs [10,19].

In this study we have examined several potential molecular mechanisms of AD-induced TrkB activation in vivo and in vitro. We show that both mature and immature forms of TrkB can be specifically tyrosine phosphorylated by ADs, but neither the endogenous ligand BDNF, nor the serotonin transporter (SERT), the principal target of many ADs, is required for this effect. However, the observation that ADs or serotonin (5-HT) or norepinephrine (NE) do not activate TrkB phosphorylation in vitro argues that ADs do not directly bind to TrkB receptors.

Results

Antidepressant drugs specifically activate TrkB receptors in mouse brain

Previous studies suggest that BDNF-TrkB signaling is critical for the behavioral effects of ADs [13,17] and that ADs activate Trk receptors in vivo. We have previously shown that several independent antibodies raised against phosphorylated tyrosines Y705/706 or Y816 within the intracellular domain of TrkB all show increased phospho-TrkB levels after acute and chronic AD treatment [13,15], while no increase is detected with antibodies against the shc binding site at pY515. Furthermore, immunoprecipitation with Trk specific antibodies and probing with pTyr-antibodies also reveals increased TrkB phosphorylation [13,15,20]. However, since phospho-Trk antibodies are not completely specific for TrkB, we investigated whether the protein phosphorylated by the acute AD treatment is indeed TrkB. We pretreated TrkB<sup>F616A</sup> knock-in mice [21] with NaPP1, a chemical that specifically inhibits TrkB kinase activity in these mutant mice, and then injected the mice acutely with imipramine. Whereas imipramine readily induced rapid activation of brain TrkB in vehicle-treated TrkB<sup>F616A</sup> knock-in mice, NaPP1 treatment abolished this effect (Figure 1A–B). Furthermore, when we treated transgenic mice over-expressing flag-tagged TrkB receptors in adult neurons (TrkB<sup>TK+</sup>) [22,23] with imipramine, we observed enhanced phosphorylation of the TrkB specific band when compared to wild-type mice (data not shown). Importantly, when TrkB receptors were immunoprecipitated with a Flag antibody from TrkB<sup>TK+</sup> mouse brain homogenates, phospho-TrkB signal was more intense in samples of imipramine treated animals (Figure 1C). Collectively, these data demonstrate that imipramine specifically induce phosphorylation of TrkB receptors, if present in mouse brain.

The immaturely glycosylated form of TrkB is phosphorylated by antidepressants

As we have previously shown [13], an additional low-molecular weight (LMW) phospho-Trk –immunoreactive protein (about 105 kDa) is robustly phosphorylated in the rodent brain after single or repeated AD treatment (Figure 2A). This phosphorylated protein is detected by the same antibodies that demonstrate the phosphorylation of TrkB after AD treatment (Figure S1A–B) and has been detected following TrkB immunoprecipitation and hybridization to pTyr antibodies [13,20]. AD-induced phosphorylation of both the full-length TrkB and the 105 kDa protein is also readily detected in different brain regions including striatum, midbrain and cerebellum (data not shown), but, similar to full-length TrkB, its phosphorylation is diluted in whole brain homogenate (Figure S1C). However, this band cannot be reliably detected by antibodies against the non-phosphorylated intracellular domain of Trk receptors (Figure 2A).

This lower molecular weight protein might represent an immaturely glycosylated form of catalytic TrkB [24], as TrkB transactivation has been shown to coincide with accumulation of intracellular immaturely glycosylated TrkB species [6,7,25]. We therefore further examined the glycosylation structure of this protein using endoglycosidase-H (Endo-H) that cleaves immature high-mannose rich N-glycans out of proteins. Endo-H digestion produced a slight reduction in the molecular weight of the mature full-length TrkB, suggesting that the immature TrkB still contains immature-type glycan residues (Figure 2B) as also observed before for TrkA [26]. Importantly, Endo-H treatment strongly reduced the molecular weight of 105 kDa protein (Figure 2B), suggesting that essentially all the glycan residues in this protein represent immature high-mannose rich N-glycans. These data are consistent with the interpretation that the 105 kDa protein represents an immaturely glycosylated and intracellularly located species of TrkB. This interpretation is further supported by the observations that the basal phosphorylation levels of this phosphoprotein are increased in the brains of TrkB over-expressing mice (Figure 2C) and that the activation of this band is lost after 1NaPP1 treatment in the TrkB<sup>F616A</sup> mice (Figure 1B).

Antidepressant-induced TrkB activation does not require BDNF

Previous studies have shown that acute AD treatment does not influence BDNF mRNA or protein levels [13,16]. Since it has recently been suggested that pro and mature forms of BDNF might have different capacities to activate TrkB [27], we investigated the effects of acute antidepressant treatment on proBDNF cleavage in brain. However, we were not able to detect any proBDNF signal in mouse brain, even if the antibody readily detected the recombinant proBDNF control protein (Figure S2A). Nevertheless, acute fluoxetine treatment, with a dose and time point (30 mg/kg; 1 hour) which induced TrkB phosphorylation in mouse hippocampus [13,15], failed to produce any significant changes in the levels of the mature BDNF (mBDNF) in mouse brain as detected with western blotting (Figure S2A). Furthermore, fluoxetine did not influence the activity of tissue plasminogen activator (tPA), the major regulator of pro-BDNF cleavage into mBDNF (Figure S2B). These data suggest that ADs do acutely not influence BDNF levels or processing in brain.

Although BDNF is the main ligand of TrkB, recent evidence suggests that TrkB can also be activated independently of BDNF in neurons [6,8,9]. We therefore used conditional BDNF mutant mice (BDNF<sup>2L/2L<sup>Ck-cre</sup></sup>) lacking BDNF in forebrain regions [28] to investigate whether BDNF is required for the AD-induced TrkB activation in brain in vivo. Imipramine readily induced tyrosine phosphorylation of both the mature and the immature glycosylated forms of TrkB in the hippocampus of conditional BDNF<sup>2L/2L<sup>Ck-cre</sup></sup> mice (Figure 3). Similarly, imipramine produced an increase in brain TrkB phosphorylation in heterozygous <sup>bdnf</sup><sup><sup>+/−</sup></sup> null mice and wild-type mice (data not shown). These data demonstrate that ADs activate TrkB receptors in the mouse brain in a manner independent of BDNF.

Adenosine has been shown to transactivate TrkB receptors via adenosine<sup>2A</sup> signaling in the absence of BDNF in vitro and in vivo [6,29] and to enhance TrkB signaling [30]. Furthermore, some ADs have been shown to acutely increase the extracellular levels of adenosine by reducing adenosine reuptake [31]. We therefore tested whether prior pharmacological inhibition of
adenosine A2A receptors with ZM241385 might block the acute effects of ADs on TrkB phosphorylation. We found that, imipramine increased the phosphorylation of TrkB receptors similarly in mice pretreated with saline or active dose [32,33] of ZM241385 (Figure S3), suggesting that A2A receptors were not involved.

Amitriptyline, but not imipramine, was recently shown to directly bind and transactivate TrkB receptors in vitro [9]. We therefore tested whether amitriptyline, imipramine or other selected drugs could directly phosphorylate TrkB receptors in two different cell models: fibroblast expressing catalytic TrkB receptors and E18 rat primary hippocampal and cortical neuronal cultures. In both of these cells, BDNF produces a robust phosphorylation of TrkB. However, exposure to tested ADs, including amitriptyline, or other tested drugs did not regulate TrkB phosphorylation status in these cultures (Figures 4A, S4).

We further tested whether ADs might potentiate the pTrkB response induced by a small dose of BDNF or whether depolarization of neurons might render them sensitive to ADs in vitro. Imipramine did not facilitate BDNF-induced TrkB phosphorylation in flag-precipitated pool of protein from the brains of mice over-expressing flag-tagged catalytic TrkB receptors. Data is presented as percentage of control/saline ± standard error of mean (SEM). *<0.05, **<0.01; two-way ANOVA with Newmann-Keuls post hoc test.

doi:10.1371/journal.pone.0020567.g001

TrkB activation by antidepressant drugs is not mediated by the serotonin transporter or monoamine transmitters

Essentially all clinically used antidepressant drugs acutely increase the extracellular levels of NE and/or 5-HT in brain and we therefore investigated the role of these monoamines in the AD-induced TrkB transactivation in vivo and in vitro. First we examined the effect of fluoxetine, a prototypic SERT selective reuptake inhibitor (SSRI), on TrkB receptor phosphorylation in SERT knockout mice (sert−/−). A 6–10 fold up-regulation of extracellular 5-HT levels in sert−/− mice [34] did not regulate basal TrkB phosphorylation levels in hippocampus when compared to the wild-type controls (89.43%±6.43% of wild-type, P = 0.19, Student t-test). Importantly, fluoxetine readily induced the phosphorylation of both the mature and immature forms of TrkB in the brains of sert−/− mice in a manner indistinguishable of the wild-type mice (Figure 5A), indicating that SERT is dispensable to the fluoxetine-induced TrkB autophosphorylation.

Because the selectivity of the ADs against different transporters is only relative, we performed chemical lesion experiments to reduce brain 5-HT (by pCPA treatment) and NE levels (by DSP4) and used selective 5-HT and NE transporter blockers citalopram and reboxetine, respectively. As noted before [15], citalopram and reboxetine produced a non-significant trend of increase in TrkB autophosphorylation levels in pCPA and DSP4 treated mice,
respectively (Figure 5B). However, citalopram and reboxetine induced a strong and highly significant increase in the phosphorylation of the immuno-glycosylated form of TrkB in pCPA and DSP4 treated mice, respectively (Figure 5B). These observations suggest that even when brain 5-HT and NE levels are very low, ADs can activate at least the immuno-glycosylated form of TrkB.

Finally, we tested whether NE or 5-HT would directly regulate TrkB phosphorylation in cultured primary neurons. Under conditions where BDNF robustly induced TrkB phosphorylation, incubation with different concentrations of NE or 5-HT did not regulate TrkB phosphorylation levels in primary neuronal cultures (Figure 5C). Collectively, these data suggest that TrkB receptor is activated in mouse brain by ADs independent of monoamine reuptake inhibition.

Discussion

Emerging evidence suggests a key role of the BDNF-TrkB signaling in the regulation of many of the molecular and behavioral actions of ADs. ADs acutely and chronically increase TrkB signaling [13,15]. Moreover, chronic, but not acute, AD treatment increases BDNF synthesis in the rodent brain [16,35]. BDNF injection and TrkB activation produce AD-like responses in rodents [36–38], while mice deficient of BDNF or with inhibited TrkB signaling do not respond to ADs in the forced swim test [13,17], the classical paradigm for AD effectiveness. These data suggest that, at least in rodents, activation of TrkB receptors induced by BDNF is essential for the antidepressant effect. However, we show here that rapid activation of TrkB in response to AD administration in vivo does not require BDNF release. This finding does not rule out the role of BDNF in regulating TrkB activation following chronic AD treatment. Since acute AD treatment increases phosphorylation of CREB, a critical upstream regulator of BDNF synthesis in a TrkB dependent manner [13], it is tempting to speculate that this ligand-independent TrkB activation is contributing to AD-induced BDNF synthesis in brain [39] which further leads to BDNF-dependent TrkB phosphorylation after prolonged AD administration.

Fluoxetine and SSRIs act primarily by blocking 5-HT reuptake in brain and BDNF, through TrkB, is a crucial regulator of serotonergic innervation [40,41]. However, neither the SERT nor the monoamines 5-HT or NE appear to be required for the activation or TrkB by the ADs. We have previously shown that representatives of all the different chemical classes of ADs similarly increase TrkB phosphorylation in mouse brain, suggesting that the monoamine independent TrkB activation may be a common feature for all the ADs. Accumulating evidence has shown that ADs, including fluoxetine and tricyclic ADs, have several additional targets in cells such as neurotransmitter receptors [42,43], ion channels [44], Sigma-1 receptors [45] and adenosine

Figure 2. Antidepressant drugs activate the immuno-glycosylated form of TrkB. A) Acute imipramine treatment induces the phosphorylation (Y816) of full-length and low-molecular weight (LMW; ~105 kDa) TrkB receptors in mouse brain. n=6/group. B) Antidepressant-induced ~105 kDa protein is sensitive to Endo-H digestion. A representative blot of triplicate data. C) Total TrkB, phosphorylated TrkB (Y816) and phosphorylated ~105 kDa protein levels are increased in the brains of mice over-expressing catalytic TrkB receptors. n=5/group. Data is presented as percentage of control ± standard error of mean (SEM). *p<0.05, ***p<0.005; unpaired two-tailed t-test. doi:10.1371/journal.pone.0020567.g002

Figure 3. Role of BDNF in antidepressant-induced rapid TrkB activation in brain. Imipramine (30 mg/kg, 30 min, i.p.) readily increases the phosphorylation of TrkB receptors (Y816) in forebrain specific BDNF−/− knock-out mice (BDNF<sup>−/−</sup> Drake) n=4/group. Data is presented as percentage of control ± standard error of mean (SEM). *p<0.05; unpaired two-tailed t-test. doi:10.1371/journal.pone.0020567.g003
reuptake proteins [31] that could potentially be involved in regulating TrkB signaling. Since adenosine-A2A receptor signaling has been linked to TrkB signaling [30], we tested the role of this receptor in AD-induced TrkB response by pharmacologically blocking this receptor before imipramine challenge. However, no change was observed compared to control treatment indicating that adenosine is not a critical regulator of TrkB activation in response to AD treatments.

While all the different ADs readily induce TrkB autophosphorylation in rodent brain [13,15], neither these compounds nor monoamines do, in our hands, induce TrkB phosphorylation in vitro in cultured cortical or hippocampal neurons, or in cell lines stably expressing TrkB receptors. A recent study reported that amitriptyline, but not imipramine, binds to TrkB receptors, induces their dimerization and autophosphorylation in cultured hippocampal neurons [9]. The reasons that underlie the discrepancy between that study and our results with amitriptyline are currently unclear, however, they may be related to the culture conditions used. The lack of activation of TrkB by ADs in cultured neurons is in line with our recent unpublished observations showing that the ability of ADs to activate TrkB in vivo is developmentally regulated: ADs do not activate TrkB in

Figure 4. Antidepressant drugs amitriptyline and imipramine do not regulate TrkB phosphorylation in primary neurons. A) Whereas BDNF (20 ng/ml; 15 min) robustly increases the phosphorylation of TrkB (Y816) in E18 rat cortical and hippocampal neurons (14 DIV), amitriptyline (left & middle; 0.5 μM, 5 μM; 15 min) and imipramine (0.5 μM, 5 μM; 50 μM; 15 min) produces no change on TrkB phosphorylation. Representative blot of triplicate data. B) Imipramine pre-treatment (4, 12, 40 μM; 15 min) did not facilitate BDNF-induced (5 ng/ml; 15 min) TrkB phosphorylation in E18 rat cortical neurons as measured with phospho-TrkB ELISA. n = 4/group. C) Imipramine pre-treatment (4, 12, 40 μM; 15 min) did not regulate TrkB phosphorylation in its own or in combination with depolarization stimuli (50 mM KCl; 15 min) as measured with phospho-TrkB ELISA. n = 4/group. Data is presented as percentage of control ± standard error of mean (SEM). *p<0.05; one-way ANOVA with Newmann-Keuls post hoc test.
doi:10.1371/journal.pone.0020567.g004
embryonic or early postnatal mice, but the ability of these compounds to induce pTrkB response appears only around postnatal day 15 (P15) (ADL, TR and EC, submitted). Taken together, these data suggest us that ADs do not directly bind to TrkB, but, instead, emphasize the importance of developmental processes and intact tissue context in the ability of small molecule weight drugs such as ADs to activate TrkB autophosphorylation in vivo.

Materials and Methods

Animal experiments - Wild-type (C57/BL6), TrkB.TK+ mutant [22,23], tkrB22/22A knock-in [21], BDNF2L/2LCk-cre [28] and sert–/– knock-out mice [46,47] were used in animal experiments. Mice were group-housed in standard laboratory conditions and food and water were freely available. All the experiments were carried out according to the guidelines of the Society for Neuroscience and were specifically approved by the University of Helsinki Committee on Animal Experiments (permit: HY 137-05) or the County Administrative Board of Southern Finland (permit: ESLH-2007-09085/15-23). Unless otherwise stated all the tested chemicals used in these studies are purchased from Sigma-Aldrich.

In vivo drug treatments - Mice received a single i.p. injection of tested chemical or saline and after indicated time the mice were killed with CO2 and brain area of interest dissected on a cooled plastic dish. Next the samples were lyzed in NP++ buffer (157 mM NaCl, 20 mM Tris, 1% NP-40, 10% glycerol, 48 mM NaF, H2O, 2× Complete inhibitor mix (Roche) and 2 mM Na3VO4), incubated on ice (>30 min) and centrifuged (+4°C for 15 min, 16100 g). The supernatant was processed further as described below. The following chemicals were used: fluoxetine-HCl (Orion Pharma), citalopram-HBr (GlxsoSmithKline; GSK), imipramine-HCl, moclobemide (kind gift from F Hoffmann-La Roche Ltd), clomipramine-HCl, amitriptyline-HCl, reboxetine (GSK). Reboxetine and moclobemide were first stock-dissolved in DMSO, citofalopram in ethanol, others directly in saline. In order to inhibit TrkB kinase activity prior drug administration in TrkB+/-/A mutant mice, mice were pre-treated with 25 μM of 1NaPP1 (kindly provided by Prof. Jari Yli-Kauhaluoma, Univ. Helsinki, Finland) for 7 days (in drinking solution) and further co-injected i.p. (83 ng/g) with imipramine or vehicle. ZM241358 was injected i.p. to block adenosine A2A receptors 30 min prior imipramine treatment [33]. Brain NE and 5-HT levels were depleted using DSP-4 (brain NE levels 920% of control; P<0.01, t-test) and pCPA (brain 5-HT levels >15% of control; P<0.005, t-test) injections as described previously [15].

Tissue plasminogen activator (tPA) SDS-PAGE zymography - For tPA activity assay, freshly dissected brain samples were homogenized into buffer consisting of 0.1 M Tris-HCl (pH 8.0), 2.5% Triton-X-100, 10 mM leupeptin, 10 μg/ml aprotinin, 1 mM phenylmethanesulfonyl fluoride (PMSF). Samples and controls (human recombinant tPA) were loaded under non-reducing conditions in SDS-PAGE containing 6 human plasminogen (Sigma-Aldrich) and pre-heated non-fat dry milk at low current density (~15–20 mA) over night (O/N) at cold bath. Next the gels were rinsed thoroughly with 2.5% Triton X-100 to remove SDS and allow proteins to renature. Next the gels were rinsed thoroughly with 10 mM CaCl2 50 mM Tris-HCl (pH 7.6) to remove Triton X-100 and the caseinolyis was allowed to occur by incubating the gels at +37°C for 16–24 h in the same solution. Caseinolyis areas were shown as translucent areas when the gels were stained with Coomassie Brilliant Blue.

In vitro experiments - For the primary neuronal cultures, hippocampi or cortex was dissected from E18 rat embryos and the tissue dissociated in a papain solution (in mg: 10 DL-Cystein-HCl, 10 bovine serum albumin (BSA), 250 glucose, ad 50 ml PBS;
10 min, 37°C). Next the cells were triturated and suspended in a medium containing 9.8 ml of Ca^2+/Mg^2+ free HBBS, 1 mM sodium pyruvate, 10 mM HEPES and 10 μl DNase I. The cells were plated onto poly-L-lysine coated 12-24 well culture plates at a cell density of 0.5×10^6 ml^(-1) (hippocampal) or 1×10^5 ml^(-1) (cortical). Cells were maintained in neurobasal medium (+2% B27 supplement, 1% penicillin/streptomycin, 1% glutamine and 25 μM glutamic acid; 5% CO_2, 37°C) for 14 to 15 days in vitro (DIV) before treatments. Parental MG87 and MG87-trkB fibroblasts [48] were cultured in 12-48 well culture plates in Dulbecco’s Modified Eagle’s Medium (DMEM) (+10% fetal calf serum, 1% PEST, 1% L-Glutamine, 400 μg/ml G418; 5% CO_2, 37°C) and were stimulated under confluent conditions. The following chemicals were used for the experiments: BDNF (Peprotech), imipramine, amitriptyline, desipramine, chlorpromazine, phentolamine, clozapine, lithium (chloride salt). After the treatments, the medium was discarded and the cells lysed in NP+ buffer and processed for western blot analysis or for phospho-Trk ELISA described below.

Phospho-Trk ELISA - An enzyme-linked immunosorbent assay (ELISA) method was developed to easily measure the level of phosphorylated Trk receptors from cultivated cells (Figure S4A). Whereas in Trk expressing cells the assay readily detects BDNF- or NGF-induced Trk phosphorylation, such induction is not detected in cells not expressing Trk receptors (data not shown). Moreover, BDNF-induced trkB phosphorylation is lost if the cells are pretreated with Trk kinase inhibitor k252a (data not shown). Representative blots showing imipramine-induced phosphorylation of ~205 kDa protein in mouse hippocampus. n = 6/group. Data is presented as percentage of control ± standard error of mean (SEM).

**Supporting Information**

**Figure S1** Diverse antidepressant drugs induce ~105 kDa protein phosphorylation in the mouse brain. A) Representative blot showing the time-response (30 min, 60 min, 120 min) of fluoxetine-induced (20/30 mg/kg, i.p.) phosphorylation of TrkB and ~105 kDa protein (Y1816 in left; Y705/6 in right) in mouse hippocampus. B) Representative blots showing antidepressant-induced phosphorylation of ~105 kDa in mouse hippocampus. C) Representative blots showing imipramine-induced phosphorylation of ~105 kDa in mouse striatum, midbrain and whole brain homogenate. Abbreviations: FLX = fluoxetine, SAL = saline; CIT = citalopram; AMI = amitriptyline; CLO = clomipramine; MOC = moclobemide; REB = reboxetine; MW = molecular weight; TrkB.extr = antibody directed against the extracellular portion of TrkB receptors.

**Figure S2** Acute fluoxetine treatment did not regulate BDNF protein levels or tPA (tissue plasminogen activator) activity. A) Representative blot showing mature-BDNF specific band in western blot from brain homogenates and pro- and mature-BDNF specific bands from respective control lanes as detected by polyclonal BDNF antibody (N-20/sc-546; Santa Cruz). Acute fluoxetine did not regulate mature-BDNF levels in mouse hippocampus. n = 6/group. B) Representative zymography showing caspase inhibition at the level of recombinant tPA. Acute fluoxetine did not regulate tPA activity levels in mouse hippocampus. n = 6/group. Data is presented as percentage of control ± standard error of mean (SEM).

**Figure S3** Blockade of adenosine-A2A receptor signaling does not prevent antidepressant-induced TrkB activation. Acute imipramine treatment (30 mg/kg, i.p.; 30 min; n = 6/group) induces essentially similar changes on TrkB phosphorylation in vehicle and adenosine-A2A receptor antagonist (ZM241385; 1 mg/kg, i.p.; 30 min) pre-treated mice. A representative blot in left showing imipramine-induced phosphorylation of TrkB and ~105 kDa protein in mouse brain. Data is presented as percentage of control/saline ± standard error of mean (SEM). *P<0.05; two-way ANOVA with Newman-Keuls post hoc test.

**Figure S4** Phospho-TrkB enzyme-linked immunosorbent assay (ELISA). A) Dose-response of BDNF (2, 8, 20 ng/ml, 15 min) on TrkB phosphorylation in TrkB expressing fibroblasts cultivated in 48-well plates. n = 4/group. B) Whereas BDNF produces robust TrkB phosphorylation in TrkB expressing fibroblasts cultivated in 24-well plates, all the tested drugs at selected doses did not have any effect on TrkB phosphorylation (compounds incubated for 15 min). n = 3/group. Data is presented as percentage of control ± standard error of mean (SEM). Abbreviations: IMI = imipramine; PHE = phentolamine; FLX = fluoxetine.
fluoxetine; CLOZ = clozapine; Li = lithium chloride; CHLOR = chlorpromazine.

(TIF)

Acknowledgments

The authors would like to thank the laboratory members of Professor Eero Castren, especially Outi Nikkilä, Henri Autio, Ettore Tiraboschi and Juha Knuutila for excellent technical and academic assistance. Professor Jari Vi-Kauhaluoma (University of Helsinki, Finland) is thanked for providing the NaPP1 inhibitor, and Professor Moses Chao (Skirball Institute, NY, USA) is thanked for providing phospho-TrkB Y816 antibody.

Author Contributions

Conceived and designed the experiments: TR ADL PT K-PL MR EC. Performed the experiments: TR IV HA ADL PT AS. Analyzed the data: TR ADL. Wrote the paper: TR EC.

References

1. McAllister AK, Katz LG, Lo DC (1999) Neurotrophins and synaptic plasticity. Annu Rev Neurosci 22: 295–318.
2. McAllister AK, Lo DC, Katz LG (1995) Neurotrophins regulate dendritic growth in developing visual cortex. Neuron 15: 791–803.
3. Huang Ej, Reidl EF (2001) Neurotrophins: Roles in neuronal development and function. Annu Rev Neurosci 24: 677–736.
4. Poo MM (2001) Neurotrophins as synaptic modifiers. Nat Rev Neurosci 2: 24–32.
5. Thoenen H (1995) Neurotrophins and neuronal plasticity. Science 270: 593–598.
6. Lee FS, Chao MV (2001) Activation of trk neurotrophin receptors in the absence of neurotrophins. Proc Natl Acad Sci U S A 98: 3533–3536.
7. Rajagopal R, Chen ZY, Lee FS, Chao MV (2004) Transactivation of trk neurotrophin receptors by G protein-coupled receptor ligands occurs on intracellular membranes. J Neurosci 24: 6630–6638.
8. Huang YZ, McNamara JO (2010) Mutual regulation of src family kinases and neurotrophin receptor trkB. J Biol Chem 285: 8207–8217.
9. Jang SW, Liu X, Chan CB, Weishenker D, Hall RA, et al. (2009) Amipristine is a TrkB and TrkA receptor agonist that promotes TrkA/TrkB heterodimerization and has potent neurotrophic activity. Chem Biol 16: 644–656.
10. Pezet S, Malcangi M (2004) Brain-derived neurotrophic factor as a drug target for CNS disorders. Expert Opin Ther Targets 8: 391–399.
11. Schindowksi K, Belardi B, Bies E (2008) Neurotrophic factors in Alzheimer’s disease: Role of axonal transport. Genes Brain Behav 7 (Suppl 1): 43–56.
12. Rantamäki T, Castrén E (2008) Targeting TrkB neurotrophin receptor to treat depression. Expert Opin Ther Targets 12: 703–15.
13. Saarelainen T, Hendolin P, Lucas G, Koponen E, Sairanen M, et al. (2003) Administration of the trkB neurotrophin receptor is induced by antidepressant drugs and is required for antidepressant-induced behavioral effects. J Neurosci 23: 349–357.
14. Sairanen M, Lucas G, Enfors P, Castrén M, Castrén E (2003) Brain-derived neurotrophic factor and antidepressant drugs have different but coordinated effects on neuronal turnover, proliferation, and survival in the adult dentate gyrus. J Neurosci 23: 1099–1094.
15. Rantamäki T, Hendolin P, Kankaanpää A, Mijatovic J, Pippinen P, et al. (2007) Pharmacologically diverse antidepressants rapidly activate brain-derived neurotrophic factor receptor trkB and induce phosphodiesterase-gamma signaling pathways in mouse brain. Neuropsychopharmacology 32: 2152–2162.
16. Niemö N, Mironoš S, Duman RS (1995) Regulation of BDNF and trkB mRNA in rat brain by chronic electroconvulsive seizure and antidepressant drug treatments. J Neurosci 15: 7539–7547.
17. Monteggia LM, Luikart B, Barrot M, Thoebold D, Malliokova I, et al. (2007) Brain-derived neurotrophic factor conditional knockouts show gender difference in both short-term and long-term behavioral treatments. J Neurosci 15: 7539–7547.
18. Chen B, Dowlatshahi D, MacQueen GM, Wang JF, Young LT (2001) Increased hippocampal BDNF immunoreactivity in subjects treated with antidepressant medication. Biol Psychiatry 50: 260–265.
19. Sen S, Duman R, Sanacora G (2008) Serum brain-derived neurotrophic factor, depression, and antidepressant medications: Meta-analyses and implications. Biol Psychiatry 64: 327–332.
20. Woyteken U, Sandoval M, Sandowal S, Jorquera F, Gonzalez I, et al. (2006) The effects of acute and long-term lithium treatments on trkB neurotrophin receptor activation in the mouse hippocampus and anterior cingulate cortex. Neuropsychopharmacology 30: 593–598.
21. Wang LJ, Zhang C, Zhang L, Zhang J, Wang J, et al. (2008) In vivo and in vitro functional analysis of Neurotrophic Tyrosine Receptor Kinase B (Neuropathy Target Estrogen Related Receptor) in the limbic system. PLoS ONE 3: e20567.