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TrkB Regulates N-Methyl-D-Aspartate Receptor Signaling by Uncoupling and Recruiting the Brain-Specific Guanine Nucleotide Exchange Factor, RasGrf1

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Abstract

Brain-derived neurotrophic factor (BDNF) facilitates multiple aspects of neuronal differentiation and cellular physiology by activating the high-affinity receptor tyrosine kinase, TrkB. While it is known that both BDNF and TrkB modulate cellular processes involved in learning and memory, exactly how TrkB cross-talks and modulates signaling downstream of excitatory ionotropic receptors, such as the NMDA receptor (NMDAR), are not well understood. A model that we have investigated involves the signaling molecule RasGrf1, a guanine nucleotide exchange factor for both Ras and Rac. We previously identified RasGrf1 as a novel Trk binding partner that facilitates neurite outgrowth in response to both nerve growth factor (NGF) (Robinson et al. in J Biol Chem 280:225–235, 2005) and BDNF (Talebian et al. in J Mol Neurosci 49:38–51, 2013); however, RasGrf1 can also bind the NR2B subunit of the NMDAR (Krapivinsky et al. in Neuron 40:775–784, 2003) and stimulate long-term depression (LTD) (Li et al. in J Neurosci 26:1721–1729, 2006). We have addressed a model that TrkB facilitates learning and memory via two processes. First, TrkB uncouples RasGrf1 from NR2B and facilitates a decrease in NMDA signaling associated with LTD (p38-MAPK). Second, the recruitment of RasGrf1 to TrkB enhances neurite outgrowth and pERK activation and signaling associated with learning and memory. We demonstrate that NMDA recruits RasGrf1 to NR2B; however, co-stimulation with BDNF uncouples this association and recruits RasGrf1 to TrkB. In addition, activation of TrkB stimulates the tyrosine phosphorylation of RasGrf1 which increases neurite outgrowth (Talebian et al. in J Mol Neurosci 49:38–51, 2013), and the tyrosine phosphorylation of NR2B (Tyr^{1472}) (Nakazawa et al. in J Biol Chem 276:693–699, 2001) which facilitates NMDAR cell surface retention (Zhang et al. in J Neurosci 28:415–24, 2008). Collectively, these data demonstrate that TrkB alters NMDA signaling by a dual mechanism that uncouples LTD and, in turn, stimulates neuronal growth and the signaling pathways associated with learning and memory.

Keywords N-methyl-D-aspartate (NMDA) receptor · Ras guanine-nucleotide exchange factor (RasGrf1) · Trophomyosin-related kinase B (TrkB) · Phosphotyrosine signaling · Neurobiology · Brain-derived neurotrophic factor (BDNF)

The TrkB receptor tyrosine kinase is widely expressed throughout the brain, in both pre- and post-synaptic neurons, and mediates intracellular signaling by BDNF in both the developing and mature nervous systems (Xu et al. 2000; Tanaka 2008). In the mature nervous system, BDNF and TrkB are essential to various aspects of synaptic plasticity (Carvalho 2008; Lu et al. 2008). Specifically, TrkB is essential for long-term potentiation (LTP) in the hippocampus as knockout mice of both BDNF and TrkB have impaired LTP and deficits in hippocampal-dependent spatial learning (Pozzo-Miller 1999; Minichiello et al. 1999). In addition, BDNF can modulate the activity of glutamate receptors; specifically, the NMDA and the α-amino-3-hydroxy-5-methyl-4-isoxazole-propionic acid (AMPA) receptors, both of which are important for excitatory synaptic transmission (Kakegawa 2004; Chapleau et al. 2008). BDNF and TrkB also mediate morphological changes essential for synaptic plasticity such as the formation and maintenance of dendritic spines (Gorski et al. 2003; Ji et al. 2005).

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LTP, a long-lasting enhancement of synaptic responses following neural stimulation, is a cellular correlate of learning and memory (Lu et al. 2008). The strengthening of these synapses requires activation of several different receptors including the NMDARs which mediate excitatory synaptic transmission (Lisman 2003; MacDonald 2006). NMDARs are heteromeric proteins and consist of two obligatory NR1 subunits and two NR2 (A-D) subunits (Kutsuwada 1992; Monyer 1992). NMDARs contribute to both LTP as well as LTD. In particular, NMDARs containing NR2A subunits promote LTP, while NR2B subunits promote LTD (Li et al. 2006; Kollen et al. 2008; Liu et al. 2004; Massey 2004).

In terms of understanding how BDNF facilitates synaptic plasticity and activity, it is well known that the facilitation of pre-synaptic neurotransmitter release is one major mechanism (Poo 2001; Schinder and Poo 2000) as well as the modulation of both post-synaptic ion channels (Schinder and Poo 2000; Manabe 2002; Rose et al. 2004) and NMDARs (Levine et al. 1998). Although the molecular mechanism(s) by which BDNF regulates NMDAR-mediated neural plasticity is poorly understood, it is known that activated TrkB stimulates the tyrosine phosphorylation of NR2B and increases the retention of NMDARs on the post-synaptic cell surface (Nakazawa et al. 2001; Prybylowski et al. 2005). NMDARs are channels for cations including calcium, with calcium influx being essential to LTP, learning, and memory (MacDonald 2006). Interestingly, activated TrkB also increases the open channel probability of NMDARs and is one mechanism by which it facilitates LTP (Levine and Kolb 2000).

Another protein that contributes to signaling in post-synaptic neurons in the mature brain is the guanine nucleotide exchange factor, RasGrf1 (Zippel et al. 1997). RasGrf2, an isoform of RasGrf1 with 80% sequence identity, is more widely expressed but is also present within neurons of the mature brain (Tian et al. 2004). Subcellular fractionation studies indicate that RasGrf1 is found in the post-synaptic density (PSD) and is primarily localized to the dendrites and soma of neuronal cells (Sturani et al. 1997; Zippel et al. 1997). RasGrfs have been suggested to be a missing link in NMDAR regulation of synaptic plasticity as they function as calcium sensors regulating the activation of GTPases (Ras and Rac) that regulate both LTP and LTD (Li et al. 2006). In addition, RasGrf1 has been shown to be tyrosine-phosphorylated by the Src (Kiyono et al. 2000b) and Ack1 (Kiyono et al. 2000a) kinases stimulating its Rac and Ras-GEF activities respectively. RasGrf1 also binds constitutively to the NR2B subunit of the NMDAR (Krapivinsky et al. 2003) and stimulates LTD (Li et al. 2006). In contrast, there is no interaction between RasGrf1 and either NR1 or NR2A (Krapivinsky et al. 2003).

We have previously shown that RasGrf1 is tyrosine phosphorylated by Trk receptors and facilitates neurite outgrowth in response to low doses of both NGF (Robinson et al. 2005) and BDNF (Talebian et al. 2013) in cell culture via the activation of both Ras and Rac. As RasGrf1 is a substrate of both NMDA and TrkB receptors, we have addressed a model that RasGrf1 is a key molecular link that facilitates receptor cross-talk between TrkB and NMDARs. Using brain slices from post-natal day 30 (P30) mice, we have examined interactions between NR2 subunits with both TrkB andRasGrf1, changes in NR2B and RasGrf1 tyrosine phosphorylation, as well as changes in the activation of the ERK and p38 MAP kinases in response to BDNF and/or NMDA.

Collectively, our results indicate that TrkB activation alters NMDAR signaling by a dual mechanism that both uncouples RasGrf1 from NR2B and decreases p38 Map kinase activity associated with LTD and, in turn, recruits RasGrf1 to TrkB to facilitate BDNF-dependent pErk activation, neurite outgrowth and intercellular communication.

Materials and Methods

Reagents

Antibodies to RasGrf1 (C-20; sc-224), RasGrf2 (sc-7591), Trk (C-14; sc-11), NMDA NR2B (NMDAε2; sc-20), and NR2A (NMDAε1; sc-9056) subunits, PSD-95 (sc-6926), Shc (C20; sc-288) as well as normal mouse (sc-2343), rabbit (sc-2027), and goat (sc-2028) IgG were from Santa Cruz. The mouse anti-TrkB antibody (1494) was from BioVision. A separate mouse anti-TrkB monoclonal (610102) and anti-ShcC (610643) antibodies were from Transduction Labs. Rabbit anti-Trk antibodies to the carboxyl-terminal 14 residues of TrkA have been previously described (Meakin et al. 1999). Anti-phosphotyrosine 100 (p-Tyr^{100}; 9411), pTyr^{516}-TrkB (4619), phospho-p38-MAPK (Thr^{180}/Tyr^{182}; 9211S), p38-MAPK (9212S), phospho-ERK (Thr^{202}/Tyr^{204}; 9106S), and ERK antibodies (9102S) were from cell signaling. Anti pTyr^{1472}-NR2B (M2442) was from Sigma. Anti-pTyr^{817}-TrkB (NB110-66653) and anti-pTyr ^{1336}-NR2B (NB300-295) were from Novus Biologicals. HRP-coupled goat anti-mouse (115-035-003), rabbit anti-goat (305-035-003) and goat anti-rabbit (111-035-003) antibodies were from Jackson Laboratories. Human recombinant BDNF was obtained from R & D systems and used at a final concentration of 100 ng/ml. H-89 dihydrochloride hydrate, used at a concentration of 10 μM, NMDA, used at a final concentration of 100 μM in conjunction with 10 mM KCl, as well as PP2 and PP3 (100 nM) were from Calbiochem. Tyrphostin AG879 and its negative control, AG 9 (Sigma; 26 mg/ml in DMSO), were used at a final concentration of 10 μM. The TrkB receptor agonist (7, 8-Dihydroxyflavone; 7,8-DHF) (Abcam Biochemicals) was used at a final concentration of 500 nM.

Cell Lines, Transfections, Immuno-Precipitations, and Western Blots

HEK 293 T cells (Graham et al. 1977) were cultured under standard conditions in DMEM with 5% supplemented calf serum, 5% FBS (Hyclone), and 50 μg/ml gentamycin sulfate (Sigma). Cells (1.5×10^6 per 100 mm dish) were co-transfected using the calcium phosphate approach with 0.5 to 5 μg of pCMX-rat TrkB and pEFP-RasGrf1 (Anborgh et al. 1999; Meakin and MacDonald 1998). Following 48-h expression, cells were lysed in NP40 lysis buffer (1% Nonidet P-40, 137 mM NaCl, 20 mM Tris [pH 8.0], 0.5 mM EDTA) containing 1 mM PMSF, 1 mM sodium orthovanadate (Na_3VO_4), 10 μg/ml aprotinin, and 10 μg/ml leupeptin. Lysates were immunoprecipitated (IPed) with the indicated antibodies (1.0 μg) in addition to 5 μl of washed Pansorbin (Calbiochem) or 2 μl γ-bind sepharose (Amersham Pharmacia Biotech) at 4 °C overnight. After washing three times with lysis buffer, bound proteins were re-suspended in 25 μl Laemmli sample buffer containing 100 mM DTT and heated at 65 °C for 10 min. Samples were resolved on 6–12% SDS-PAGE gels and transferred to PVDF. Blocking prior to primary antibody incubation was performed for 1 h at RT in 10% milk powder and PBS with 0.1% tween-20 (TBS-T) or 2% BSA (for phospho-antibodies). Westerns were blotted in 10 ml 10% milk powder or 2% BSA with primary antibody at 4 °C for 16 h followed by washing in TBS-T for 30 min. Westerns were then incubated in 10 ml 10% milk powder or 2% BSA and secondary antibody for 1 h at room temperature. Blots were washed for 30 min in TBS-T and exposed to enhanced chemiluminescence reagents (BioRad) and visualized on a BioRad ChemidocTM MP system.

Neural Slice Analyses

Animal procedures were performed in accordance with guidelines of the Canadian Council on Animal Care (CCAC) and the Animal Care and Veterinary Services (ACVS) at Western University. P30–35 male CD1 mice were sacrificed with CO_2 and their cortices dissected into ice-cold fresh 95% O2/5% CO2 saturated Kreb's Ringer Solution. Cortices were embedded in 2% agarose and 300 μm coronal slices from the middle portion of each hippocampus were cut with a vibrotome (frequency 8 Hz, speed 4 Hz). Four slices of cortex and 10 slices of hippocampus were placed into each well of a 6-well plate and incubated in 2 ml Kreb's Ringer Solution for 1 h at 37 °C with 95% O_2/5% CO_2. Slices were then stimulated for 10 min at 37 °C with 100 ng/ml BDNF or 100 μM NMDA with 10 mM KCl. Slices were removed and immediately lysed in lysis buffer A (10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, and 1 mM $NaVO_3$). Samples were homogenized with a Polytron (2×30 s) followed by centrifugation at 1400 rpm for 10 min at 4 °C. The supernatant was removed, transferred to a microcentrifuge tube, and centrifuged again for 20 min at 4 °C, 14,000 rpm. Supernatants were removed, kept on ice, and protein concentrations determined (Bio-Rad DCTm Protein Assay). Lysates (750 μg to 1.5 mg) were IPed with anti-NR2B, anti- Trk, anti-NR2A, anti-RasGrf1, anti-phosphotyrosine antibodies, or normal IgG (1 μg) in Lysis buffer A. Samples were resolved on 6–12% SDS-PAGE gels and analyzed by Western blot as described above. For analysis of ERK and p38-MAPK phosphorylation, Laemmli sample buffer with 100 mM DTT was added to whole cell lysates from treated P30 slices, 10–60 min post-stimulation, and samples incubated at 65 °C for 10 min. Samples were separated by 12% SDS-PAGE and Western blotted. Relative densities were evaluated compared to control lanes in each figure. The means of 3–6 separate slice preparations were evaluated and relative densities statistically analyzed by paired student *t* tests for significant differences (P value < 0.05) between two different stimulation conditions. To determine the long term changes in pERK activation, by BDNF and NMDA (up to 1-h post-stimulation), the mean of 10 separate slice preparations were evaluated and the relative density of pERK to total ERK determined.

Primary antibody dilutions for Western blotting are as follows: RasGrf1 (1:10,000), RasGrf2 (1:5000), Trk (1:20,000), TrkB 1494 (1:5000), TrkB, NR2B, NR2A, PSD-95, and ShcC (1:2000), pTyr100 (1:10,000), $pTyr^{516}$-TrkB and $pTyr^{817}$-TrkB (1:5000), phospho-p38-MAPK and p38-MAPK (1:2000), phospho-ERK, and ERK (1:5000), NR2B $pTyr^{1472}$, or $pTyr^{1366}$ (1:5000). HRP-coupled goat anti-mouse and goat anti-rabbit antibodies were used at 1:10,000 dilution while HRP-coupled rabbit anti-goat was used at 1:5000 dilution.

Statistical Analysis

All of the experiments were conducted at least three times. One way analysis of variance (ANOVA) with Tukey multiple comparison tests were used to analyze the difference of means among each groups. P value < 0.05 is considered statistically significant.

Results

BDNF Increases Tyrosine Phosphorylation of NR2B and Decreases NR2B-RasGrf1 Binding Previous studies have shown that RasGrf1 and RasGrf2 bind the NR2B and NR2A subunits of NMDARs (Krapivinsky et al. 2003) and that they regulate NMDA activation of ERK and p38-MAPK respectively (Li et al. 2006). In addition, we have shown a direct interaction between RasGrf1 and TrkA in vitro (MacDonald et al. 1999) as well as neurotrophin-dependent co-

immunoprecipitation with Trk receptors in PC12 cells (Robinson et al. 2005). Thus, we addressed the nature of these interactions in mouse P30 cortical/hippocampal brain slices and how they were affected by the addition of BDNF and NMDA. Moreover, as the NR2B subunit of the NMDAR is tyrosine phosphorylated by Fyn kinase at Tyr^{1472} in the YEKL motif, which uncouples the receptor from clathrin-mediated endocytosis and increases receptor activity (Nakazawa et al. 2001; Prybylowski et al. 2005), we also addressed changes in the phosphorylation status of NR2B and its ability to bind RasGrf1. As shown in Fig. 1a, we found basal levels of NR2B Tyr^{1472} phosphorylation in unstimulated lysates (left panel, lane 1); however, phosphorylation levels increased more than twofold following BDNF stimulation (lane 2). In comparison, stimulation with NMDA did not stimulate Tyr^{1472} phosphorylation (Fig. 1a, lane 3) and co-stimulation of NMDA with BDNF decreased levels of BDNF-induced phosphorylation (lane 4). Levels of NR2B immunoprecipitated were verified by blotting with the anti-NR2B antibody (Fig. 1a, lower panel). Quantification of $pTyr^{1472}$ levels are shown in the right panel. In addition to phosphorylation at Tyr^{1472}, NR2B is also phosphorylated at Tyr^{1336}, by Src family kinases, which negatively regulates NMDAR activity. Specifically, $pTyr^{1336}$ traffics NR2B containing NMDRs to the extra-synaptic membrane (Goebel-Goody et al. 2009) as well as stimulates calpain-dependent cleavage of the NR2B C-terminal tail which eliminates intracellular signaling (Wu et al. 2007). Thus, we also addressed if BDNF and/or NMDA altered the phosphorylation status of NR2B at Tyr^{1336}. As shown in Fig. 1b, we found basal levels of NR2B Tyr^{1336} phosphorylation in unstimulated lysates (left panel, lane 1) and the levels increased approximately 1.5-fold following BDNF stimulation (lane 2). In comparison, NMDA stimulated a 2.5-fold increase in Tyr^{1366} phosphorylation (Fig. 1b, lane 3) and co-stimulation of NMDA with BDNF reduced phosphorylation to levels comparable to BDNF treatment alone (Fig. 1b, lane 4). Levels of NR2B immunoprecipitated were verified by blotting with the anti-NR2B antibody (lower panel) and quantification of $pTyr^{1366}$ levels are shown in the right panel. Together, these results suggest that BDNF stimulates NMDAR activity.

Next, changes in the co-immunoprecipitation of RasGrf1 with NR2B were assayed in lysates using both control antibodies and antibodies to NR2B. As shown in Fig. 2a (lane 3), basal levels of interaction between NR2B and RasGrf1 were detected in the absence of any stimulation, relative to negative controls (lanes 1, 2), and this was significantly increased, almost twofold, in the presence of NMDA (Fig. 2a, lane 5). However, interaction between RasGrf1 and NR2B was not observed following BDNF stimulation (lane 4) and, in fact, the NMDA-induced increase in interaction was lost in the presence of BDNF (lane 6). These results suggest that BDNF-mediated TrkB activation alters NMDAR signaling by stimulating the dissociation of RasGrf1 from NR2B. The levels of NR2B immunoprecipitated were verified using the anti-NR2B antibody (Fig. 2a, lower panel) and the quantification levels of NR2B-RasGrf1 interaction, normalized to the level of NR2B, are shown in Fig. 2b. To address whether the BDNF-induced effects were specific for NR2B, or also affected the association of RasGrf2 with the NR2A subunit of the NMDAR (Li et al. 2006), BDNF-dependent changes in the co-immunoprecipitation between NR2A and RasGrf2 were investigated. As shown in Fig. 2c (lanes 3 and 4), there was no basal or BDNF-induced co-immunoprecipitation between

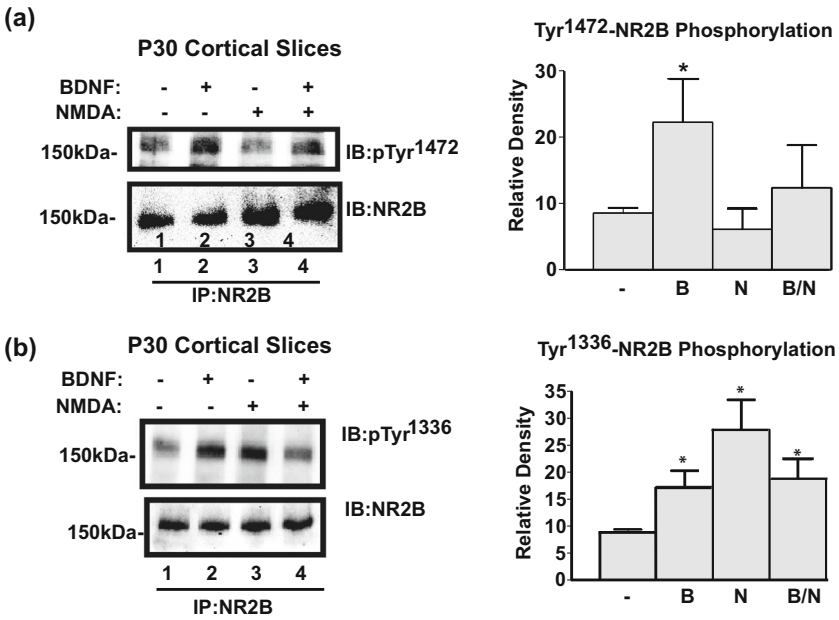

Fig. 1 Phosphorylation of NR2B in P30 cortical slices. Cortical slices were lysed after BDNF and/or NMDA stimulation, and lysates (3 mg) IPed with NR2B antibodies. **a** Tyrosine phosphorylation assessment of NR2B subunit in response to BDNF and/or NMDA ($n = 3$). **b** Tyrosine phosphorylation assessment of NR2B subunit in response to BDNF and/or NMDA ($n = 3$)

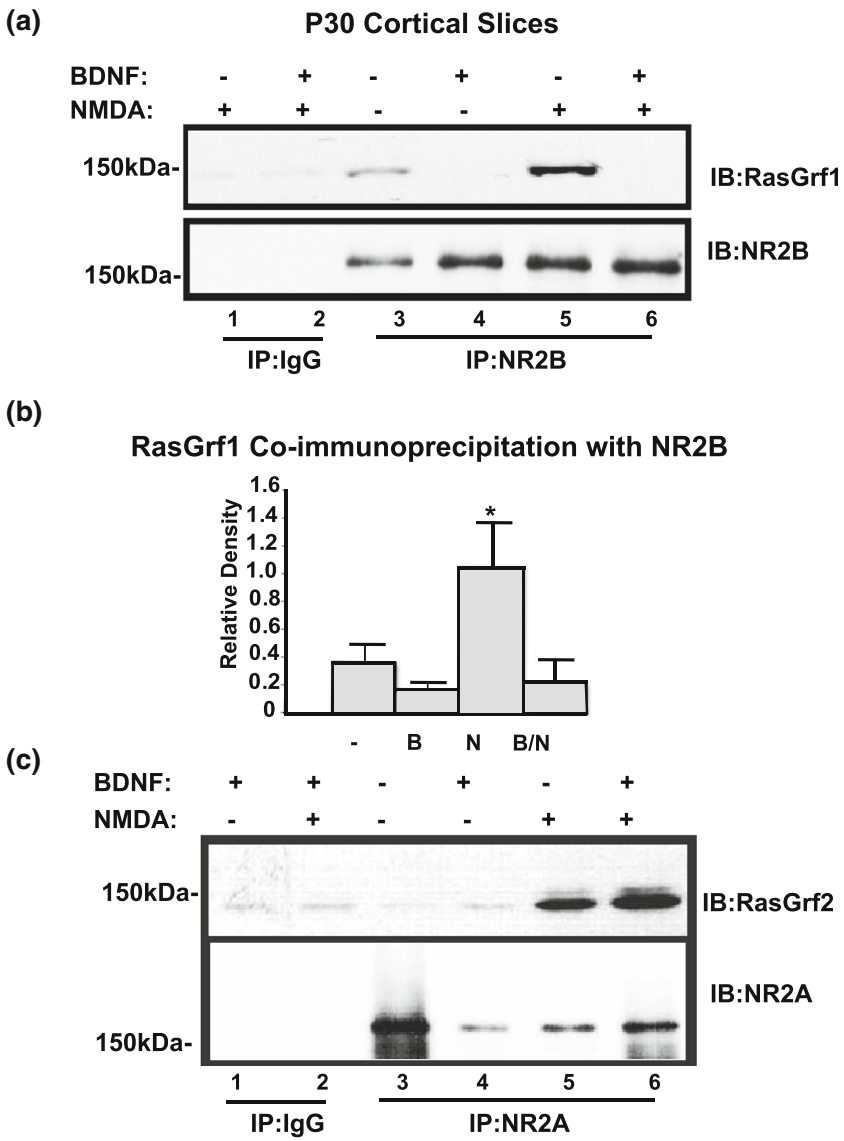

Fig. 2 Co-immunoprecipitations of NR2B, NR2A, RasGrf1, and RasGrf2 in P30 cortical slices. Cortical slices were lysed after BDNF and/or NMDA stimulation, and lysates (3 mg) IPed with anti-NR2B or NR2A antibodies. IPs were analyzed by SDS-PAGE, and Western blots performed with the indicated antibodies. **a** Interaction between NR2B and RasGrf1 upon BDNF and/or NMDA stimulation in P30 slices ($n = 3$). Goat IgG was used as a negative control. **b** Quantification of the levels of interaction. **c** Interaction between NR2A and RasGrf2 upon BDNF and/or NMDA stimulation in P30 slices ($n = 3$). Goat IgG was used as a negative control

RasGrf2 and NR2A. In contrast, RasGrf2 was co-immunoprecipitated with NR2A in response to NMDA stimulation (Fig. 2c, lane 5) relative to unstimulated cells (lane 3) and negative controls (lanes 1 and 2). However, in contrast to RasGrf1 interaction with NR2B (Fig. 2a), BDNF did not stimulate a loss of NMDA-induced interaction between NR2A and RasGrf2 in the presence of BDNF (lane 6). The levels of TrkB tyrosine phosphorylation were next analyzed in P30 mouse cortical slices. As shown in Fig. 3a, TrkB shows basal levels of tyrosine phosphorylation which is not significantly affected by co-stimulation with NMDA (lanes 1 and 3); however, levels of TrkB tyrosine phosphorylation are significantly increased in the presence of BDNF (lane 2). Co-stimulation with NMDA; however, resulted in a small but insignificant reduction in the levels of BDNF-induced TrkB tyrosine phosphorylation (lane 4). Quantification of the TrkB tyrosine phosphorylation levels are shown in the right panel. To determine which TrkB tyrosine residues are primarily phosphorylated following BDNF stimulation, we used phosphospecific antibodies to the juxtamembrane residue ($pTyr^{516}$), a known docking site for the Shc family of adapters (ShcA, ShcB, ShcC, ShcD), the fibroblast growth factor (FRS) family of adapters (FRS2 and FRS3), as well as the conformational changes required to expose the HIKE domain on TrkB to enable RasGrf1 binding (Obermeier et al. 1994; Meakin et al. 1999; Liu and Meakin 2002; Atwal et al. 2000; Dixon et al. 2006). We also used an antibody directed to the C-terminal residue on TrkB ($pTyr^{817}$) which provides a docking site for the recruitment and activation of PLCγ-1 (Loeb et al. 1994; Stephens et al. 1994; Obermeier et al. 1994). As shown in Fig. 3b, we find that BDNF stimulation does not increase the basal levels of TrkB phosphorylation at residue Tyr^{817}. In contrast, we find that BDNF significantly increases TrkB phosphorylation at the Tyr^{516} residue (Fig. 3c) and while both

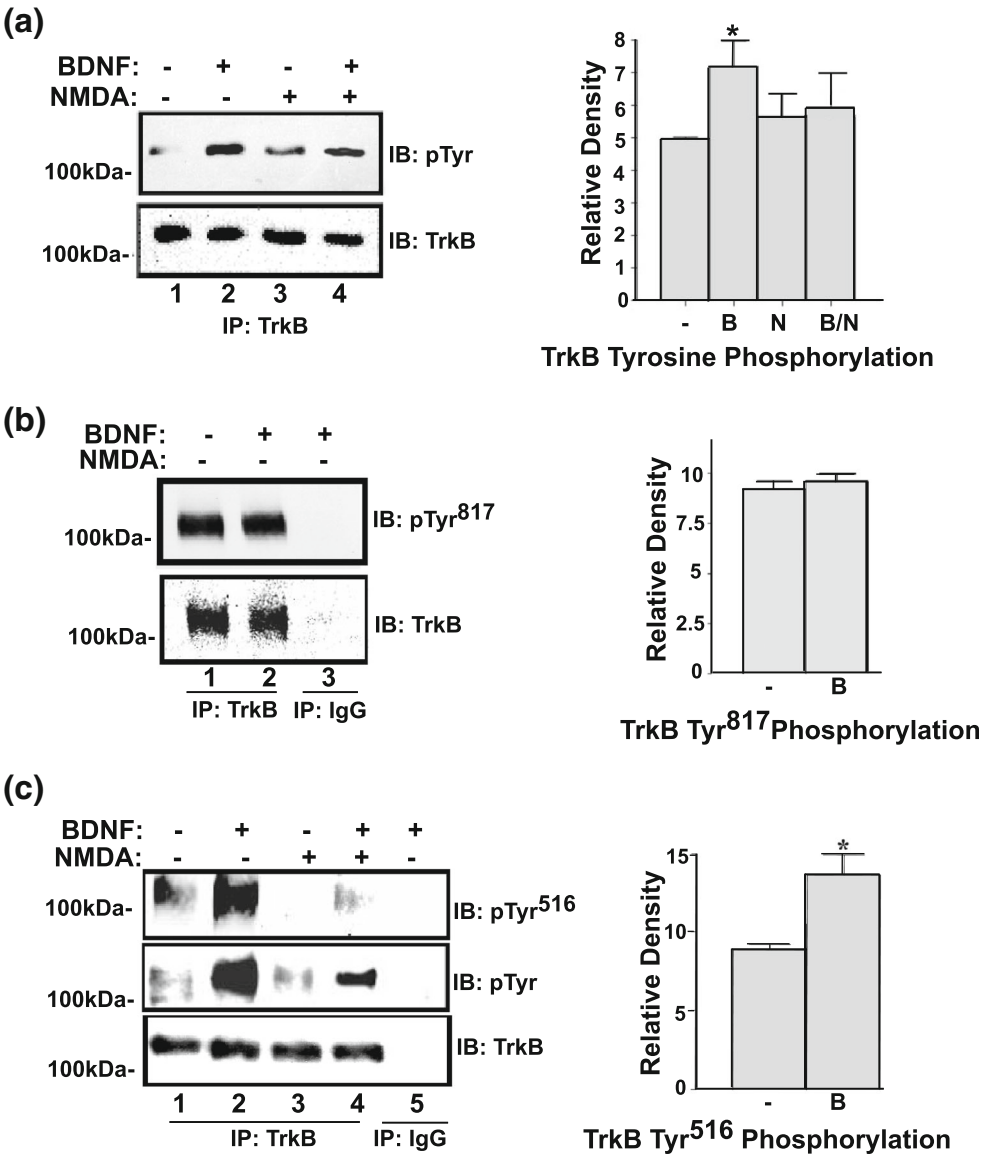

Fig. 3 TrkB phosphorylation in P30 brain slices. Cortical slices were lysed after BDNF and/or NMDA stimulation, and lysates (2 mg) IPed with Trk (C-14) or control IgG. IPs were analyzed by SDS-PAGE, and Western blots performed with the indicated antibodies. **a** BDNF stimulates an increase in the tyrosine phosphorylation of TrkB. **b** BDNF does not stimulate an increase in Tyrosine^{817} on TrkB. **c** BDNF stimulates an increases in phosphorylation of Tyrosine^{516} on TrkB ($n = 3$)

basal levels and BDNF-induced pTyr^{516} levels are reduced in the presence of NMDA (lanes 3 and 4), low levels of pTyr^{516} as well as total pTrkB phosphorylation are still detectable. These observations suggest that the basal level of TrkB tyrosine phosphorylation, and those observed in the presence of NMDA (Fig. 3a), are primarily at pTyr^{817} which does not change in the presence of BDNF stimulation (Fig. 3b) and the BDNF-induced increase in phosphorylation of TrkB is at pTyr^{516} (Fig. 3c). The immunoprecipitation levels of TrkB were verified in all treatments using an anti-TrkB antibody.

Inhibition of both TrkB and PKA, but Not Src Kinase, Decreases BDNF Modulation of NR2B-RasGrf1 Interaction To address the TrkB-dependent specificity and intracellular kinases involved in regulating the ability of BDNF and NMDA to modulate NR2B-RasGrf1 interaction, we examined the effects of three separate inhibitors, namely, Tyrphostin (Trk), H89 (PKA), and PP2 (Src family kinases) on NR2B/RasGrf1 interactions. As expected, in the presence of the negative control, increased interactions between NR2B and RasGrf1 were observed after NMDA stimulation (Fig. 4a, lane 6), relative to unstimulated cells (lane 4), and this interaction was lost in the presence of BDNF (Fig. 4a, lane 7). However, when slices were pre-treated with the TrkB inhibitor Tyrphostin, the interaction between NR2B and RasGrf1 increased in the presence of NMDA, and the level of inhibition was reduced, but still strongly detected, in the presence of both BDNF/NMDA (Fig. 4a, compare lanes 10, 11 versus lanes 6, 7) confirming that TrkB activation regulates RasGrf1/NR2B interaction. The levels of RasGrf1 and NR2B were verified by loading the whole cell lysate as a positive control (Fig. 4a, lane 1). Quantification of the Tyrphostin effects on NR2B-RasGrf1

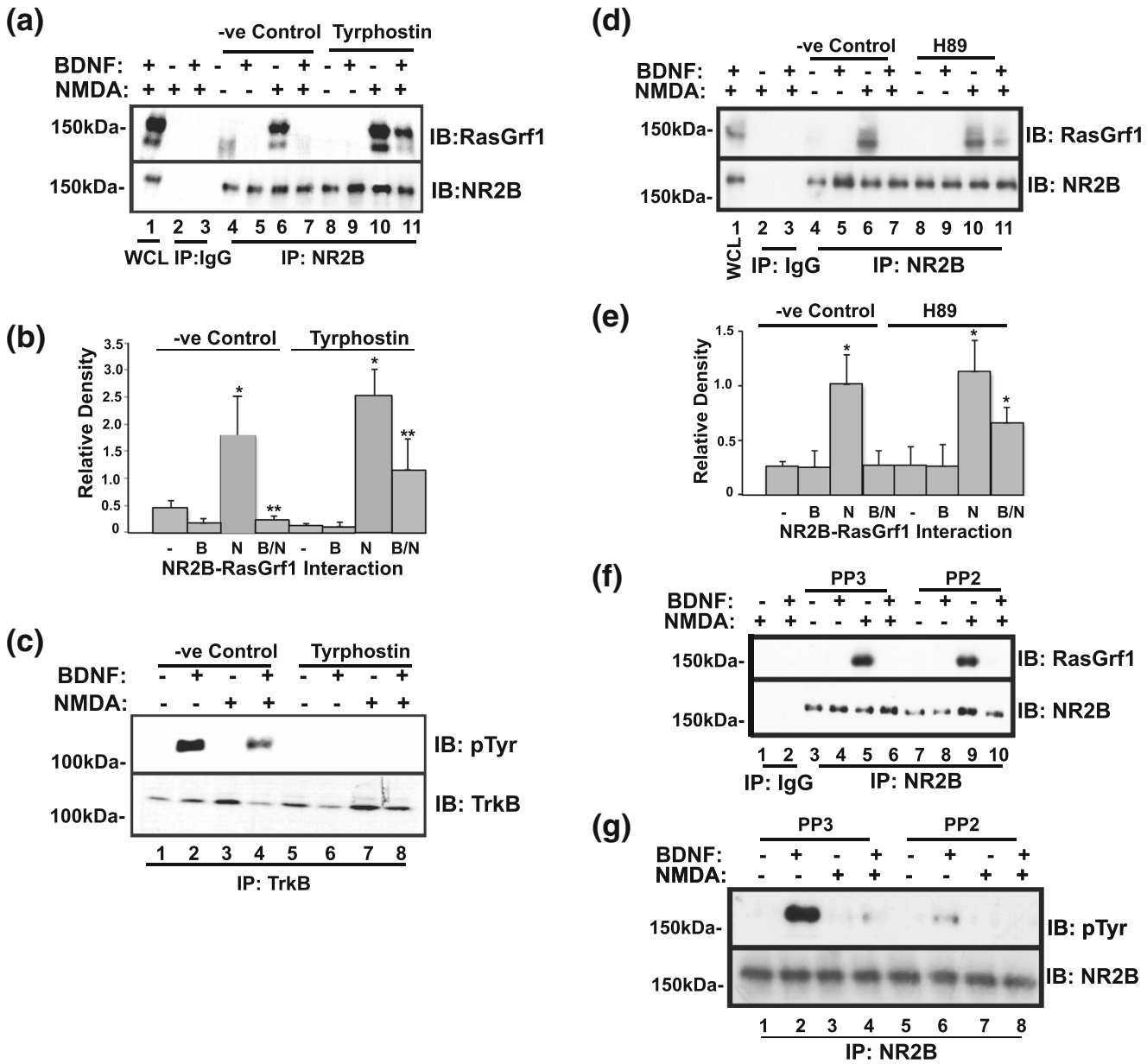

Fig. 4 Interaction between NR2B and RasGrf1 in presence of Tyrphostin, H89, and PP2. Cortical slices were pre-treated with Tyrphostin, AG 9, H89, PP3, PP2, stimulated with BDNF and/or NMDA, and lysates were IPed with the indicated antibodies. The pellet or whole cell lysate (WCL) was separated by SDS-PAGE, and Western blots performed with the indicated antibodies. **a** Interaction between RasGrf1 and NR2B in control and Tyrphostin-treated slices. **b** Quantification of changes in RasGrf1-NR2B interaction. **c** Tyrphostin effectively reduced BDNF-induced tyrosine phosphorylation of TrkB. **d** H89 pre-treatment of slices reduced the BDNF-induced decrease in NMDA stimulated NR2B and RasGrf1 interaction. **e** Quantification of H89-induced changes in RasGrf1-NR2B interaction. **f** Interaction between NR2B-RasGrf1 was unaffected in presence of Src inhibitor, PP2, relative to cells treated with the negative control, PP3. **g** PP2 significantly decreases BDNF-induced tyrosine phosphorylation of NR2B. ($n = 3$)

interactions are shown in Fig. 4b and verification that Tyrphostin blocks TrkB kinase activity are shown in Fig. 4c.

Protein kinase A (PKA) is an important regulator of multiple protein and receptor interactions within the PSD including the interaction between the PSD adapter protein, PSD-95, and the NMDAR (Dell'Acqua 2006). As well, PKA has been shown to regulate the subcellular enrichment of TrkB into active dendritic spines in hippocampal neurons in a PSD-95 and activity-dependent manner (Ji et al. 2005). PKA also regulates BDNF and TrkB-mediated synapse formation (Taniguchi 2006) and is an upstream activator of RasGrf1 within neurons (Baouz et al. 2001). Therefore, we asked whether the BDNF-induced loss of interaction between RasGrf1 and NR2B might depend on PKA. As expected, in the absence of an inhibitor, NMDA-induced interaction between NR2B and RasGrf1 and this was blocked by BDNF

(Fig. 4d, lanes 6, 7). However, in the presence of the PKA inhibitor H89, the NMDA-dependent binding of RasGrf1 and NR2B was still detected albeit significantly reduced in the presence of BDNF (lanes 10 and 11). Quantification of the H89 dependent changes in BDNF/NMDA-induced binding of NR2B and RasGrf1 are shown in Fig. 4e.

Lastly, it is well known that Src kinases play important roles in both NMDAR and TrkB signaling pathways and they are also upstream activators of RasGrf1 (Yamada 2003; Kiyono et al. 2000b). In this respect, the Src kinase family member, Fyn, has been shown to be an important regulator of NR2B subcellular location, activity and the open channel probability of NMDARs (Yamada 2003, 2004; Wu et al. 2007). To assay the potential involvement of Src family kinases as regulators of NR2B/RasGrf1 interaction, we assayed if the Src family kinase inhibitor, PP2, relative to its negative control PP3, could block the BDNF-dependent loss of binding (Fig. 4f). As expected, NMDA resulted in an increase in the interaction between NR2B and RasGrf1 in PP3-treated slices (lane 5), and this interaction was lost in the presence of BDNF (lane 6). However, the Src family inhibitor PP2, unlike Tyrphostin, had no effect on the interaction between NR2B and RasGrf1 in the presence of both NMDA and BDNF (lanes 9 and 10). Since NR2B is a target of Src kinases downstream of activated TrkB, we evaluated the effectiveness of PP2 in inhibiting Src activity in the slices. As shown in Fig. 4g, BDNF enhances tyrosine phosphorylation of NR2B (lane 2), but in the presence of PP2 there was a significant decrease in phosphorylation levels (lane 6) confirming activity of the inhibitor.

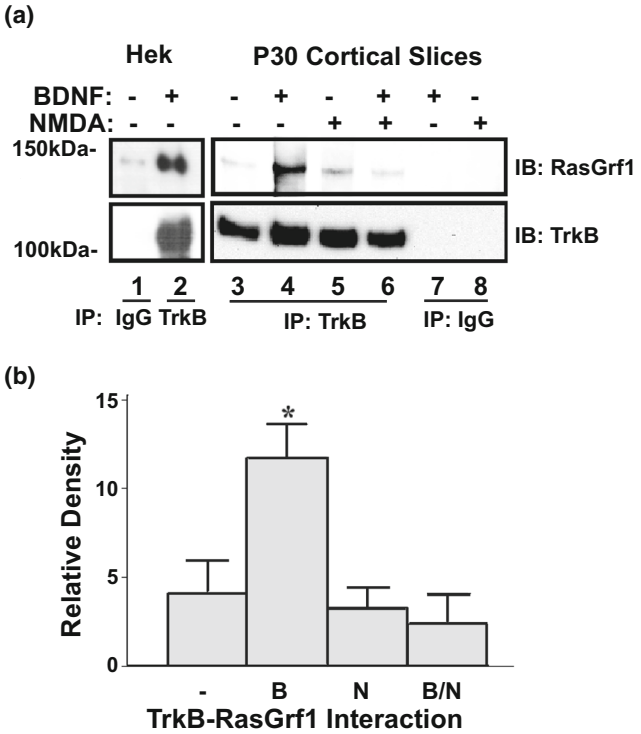

Fig. 5 Interaction between Trk and RasGrf1 in P30 brain slices. Transfected HEK cells and cortical slices were lysed after BDNF and/or NMDA stimulation, and lysates (2 mg) IPed with the anti-Trk (C-14) antibody or normal rabbit IgG as a negative control. IPs were analyzed by SDS-PAGE and Western blots performed with the indicated antibodies ($n = 3$). **a** An enhanced interaction (almost threefold) between TrkB and Ras-Grf1 occurs in response to BDNF relative to the unstimulated control. Co-stimulation with NMDA decreases this interaction. **b** Quantification of the interaction between Trk and RasGrf1, normalized to the level of Trk in each lane

BDNF Increases TrkB-Dependent Binding and Tyrosine Phosphorylation of RasGrf1 Our previous studies demonstrated a direct interaction between TrkA and RasGrf1 in yeast two-hybrid assays (MacDonald et al. 1999) mediated by the binding of RasGrf1s N-terminal PH domain to the HIKE domain on TrkA (Robinson et al. 2005). Thus, the interaction between TrkB and RasGrf was assessed by co-immunoprecipitation of lysates prepared from P30 cortical slices to determine if BDNF, in addition to uncoupling RasGrf1 from NR2B, can stimulate the recruitment of RasGrf1 to TrkB. As a control for TrkB/RasGrf1 co-immunoprecipitation, TrkB and RasGrf1 co-transfected HEK cells were also assayed. As shown in Fig. 5a, we observe basal co-immunoprecipitation of RasGrf1 with control rabbit IgG antibodies in co-transfected (TrkB/RasGrf1) BDNF-treated HEK293 cells but there is a significant increase when anti-Trk antibodies are used (compare lanes 1 and 2). In lysates prepared from P30 cortical slices, we also observe a basal interaction between RasGrf1 and TrkB in unstimulated slices (Fig. 5a, lane 3); however, in the presence of BDNF, this interaction was significantly increased, almost 2.5-fold (lane 4), indicating that BDNF stimulation significantly increases TrkB/RasGrf1 interaction in brain tissue. While the basal levels of TrkB and RasGrf1 are not affected by treatment with NMDA (lane 5), slices co-stimulated with BDNF and NMDA significantly reduced TrkB/RasGrf1 co-immunoprecipitation to the levels comparable to baseline. As an additional control, RasGrf1 is not non-specifically precipitated by normal rabbit IgG under either condition (lanes 7 and 8). The levels of immunoprecipitated TrkB were verified using an antibody against TrkB (Fig. 5a, lower panel) and quantification of the interaction levels are presented in Fig. 5b. Collectively, these data indicate that BDNF uncouples RasGrf1 from NR2B (Fig. 1) and recruits it directly to TrkB.

We have previously shown that RasGrf1 becomes tyrosine phosphorylated in response to NGF and BDNF in transfected cells, resulting in the activation of both Ras and Rac, and the facilitation of neurite outgrowth (Robinson et al. 2005; Talebian et al. 2013). Thus, we addressed the ability of BDNF to stimulate RasGrf1 tyrosine phosphorylation in P30 slice cultures. We initially found weak levels of BDNF-induced tyrosine phosphorylation of RasGrf1 when lysates were immunoprecipitated with anti-RasGrf1 antibodies and Western blots probed with anti-pTyr antibodies (data not

shown), but when we reversed the approach and immunoprecipitated lysates with anti-pTyr antibodies and probed Western blots with antibodies to RasGrf1 and TrkB, to monitor levels of activated TrkB, we found that BDNF stimulated an approximate 1.5-fold increase in the tyrosine phosphorylation of RasGrf1, in cortical slice cultures (Fig. 6, lanes 1 and 2), that NMDA alone did not affect basal levels of RasGrf1 tyrosine phosphorylation (lane 3) but the co-stimulation of BDNF with NMDA significantly reduced levels of tyrosine phosphorylation to basal levels (lane 4) consistent with the fact that NMDA reduced BDNF activation of TrkB (Fig. 3).

Since BDNF is a peptide and may only stimulate cells on the surface of the slices, not those within the slice, we also utilized the small molecule 7,8–dihydroxyflavone (7,8-DHF), which can cross the blood brain barrier and binds with high affinity to TrkB (Jang et al. 2010), to see if the levels of RasGrf1 tyrosine phosphorylation increased. As shown Fig. 6a (lane 5), DHF effectively stimulated the tyrosine phosphorylation of both TrkB and RasGrf1. In fact, the levels of RasGrf1 tyrosine phosphorylation were stronger in the presence of DHF relative to BDNF (compare lanes 2 and 5). Lastly, co-stimulation of slices with DHF and NMDA reduced levels of RasGrf1 tyrosine phosphorylation comparable to our observations with BDNF. Quantification levels of RasGrf1 tyrosine phosphorylation, relative to controls, are shown in Fig. 6b.

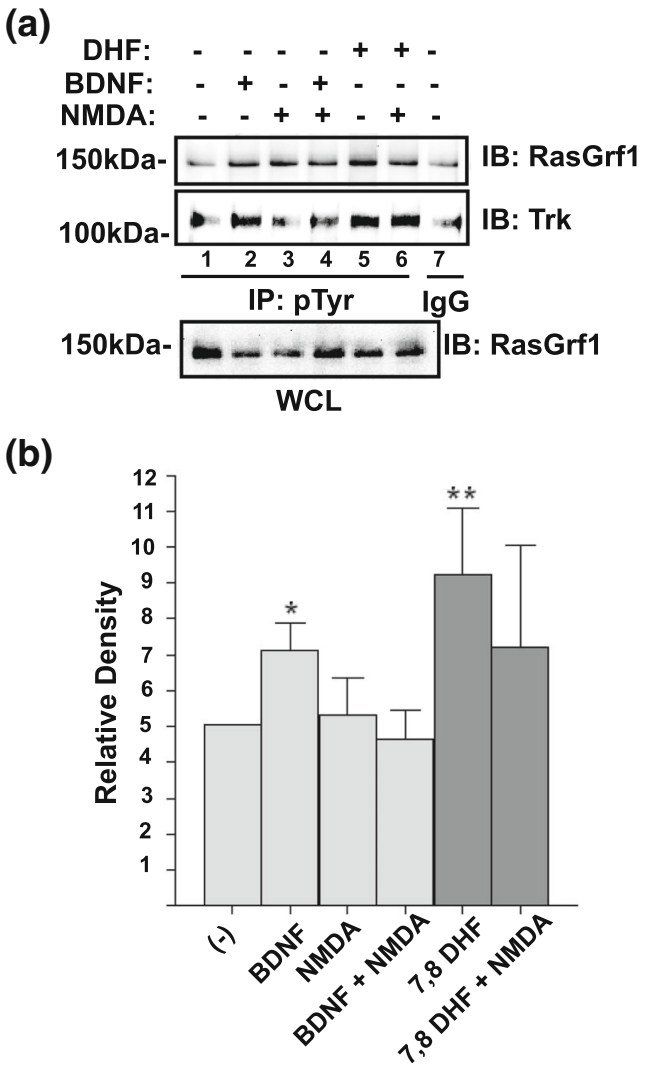

Fig. 6 TrkB activation stimulates RasGrf1 tyrosine phosphorylation in P30 brain slices. Cortical slices were lysed after BDNF, 7,8-DHF and/or NMDA stimulation, and 2 mg of lysates were IP with anti-pTyr and control IgG antibodies. IPs or a WCL (100 μg) were analyzed by SDS-PAGE, and Western blots performed with the indicated antibodies. Both BDNF and 7,8-DHF stimulate an increase in the tyrosine phosphorylation of RasGrf1 ($n = 4$)

BDNF Recruits the ShcC Adapter to TrkB and Uncouples the Binding of PSD-95 to NR2B We then investigated the interaction between TrkB and the ShcC adapter, a known Trk receptor adapter which binds pTyr^{516} and is highly expressed in the post-natal brain, and how this was affected by BDNF and NMDA. As expected, ShcC is tyrosine phosphorylated in response to BDNF activation of TrkB (Fig. 7a) and co-immunoprecipitates with TrkB (Fig. 7b, lane 2). However, unlike the interaction between TrkB and RasGrf1, co-stimulation of P30 brain slices with BDNF and NMDA did not reduce the binding of ShcC to TrkB (Fig. 7b, lane 4). This observation, in comparison to the effect of NMDA on RasGrf1 binding to TrkB (Fig. 5), suggests a differential strength in the binding affinities of ShcC versus RasGrf1 to TrkB. In this respect, it is known that the phosphotyrosine binding (PTB) domain (present on Shc) binds with high affinity (nM) to NPXpY motifs (Pawson and Nash 2000), at Tyr^{516} on TrkB, and while some PH domains (present on RasGrf1) have been shown to bind with high affinity (nM) to phosphoinositols in the membrane, they bind with lower affinity (μM) to protein targets within the cell (Lemmon et al. 1996; Agamasu et al. 2015; Lemmon 2007). The observation that co-stimulation of slice cultures with BDNF and NMDA significantly decreased the binding of the RasGrf1 PH domain to the HIKE domain on TrkB, which is conformationally exposed following phosphorylation of Tyr^{516}, but did not affect the binding of ShcC to TrkB, indicates that RasGrf1 binding to TrkB is much weaker and sensitive to conformational changes.

Next, since RasGrf1 is highly expressed in the PSD (Zippel et al. 1997) and PSD-95 is a major regulator of NMDAR signaling that facilitates the phosphorylation of NR2B by Src family kinases (van Zundert et al. 2004), it is likely that NMDA would facilitate interaction between NR2B and PSD-95. Thus, the interaction between NR2B and PSD-95 was determined in P30 cortical slices. As shown in Fig. 7c, a basal level of interaction was observed between NR2B and PSD-95 in the absence of any treatment (lane 1), but this interaction was significantly increased in the presence of NMDA (lane 3)

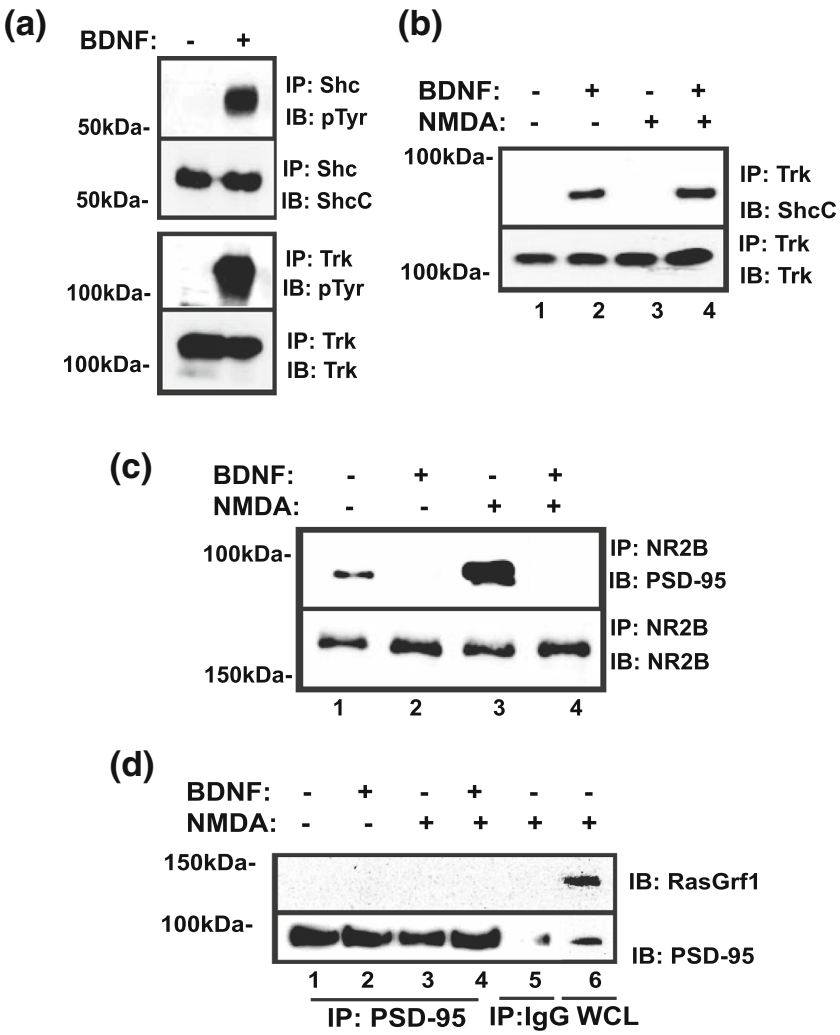

Fig. 7 Analysis of signaling molecules activated downstream of TrkB and NR2B in P30 mouse brain slices. Cortical slices were lysed after BDNF and/or NMDA stimulation, and immunoprecipitated (IP) with the indicated antibodies. IPs or WCL were analyzed by SDS-PAGE and Western blots performed with the indicated antibodies ($n = 3$). **a** Levels of TrkB and ShcC tyrosine phosphorylation in response to BDNF. **b** Interaction between ShcC and TrkB after BDNF and/or NMDA stimulation. **c** Interaction between PSD-95 and NR2B after BDNF and/or NMDA stimulation. NMDA stimulation increases the NR2B-PSD-95 association while co-treatment with BDNF uncouples the interaction. **d** No interaction occurs between PSD-95 and RasGrf1 in the absence or presence of BDNF and/or NMDA

similar to what was observed between RasGrf1 and NR2B (Fig. 2a). However, co-stimulation of NMDA with BDNF dissociated the interaction between NR2B and PSD-95 (lane 4) similar to the dissociation of RasGrf1 from NR2B (Fig. 2) and RasGrf1 from TrkB (Fig. 5). Collectively, these results raised the possibility that RasGrf1 might also affect NMDA signaling via interaction with PSD-95. Thus, interaction between PSD-95 with RasGrf1 was also tested; however, we found no co-immunoprecipitation between RasGrf1 and PSD-95 under any conditions (Fig. 7d).

BDNF Stimulates Extracellular Signal-Regulated Kinase and Alters NMDA-Mediated p38-MAPK Activation Previous studies have indicated that NR2B/RasGrf1 interaction increases the activation of p38-MAP kinase, while both TrkB and NR2A/RasGrf2 enhance the activation of ERK 1 and 2 (Li et al. 2006). Thus, the phosphorylation/activation levels of p38-MAPK and ERK were evaluated in response to BDNF and/or NMDA stimulation. As shown in Fig. 8a, basal levels of ERK1/2 phosphorylation were found in untreated conditions (lane 1) and BDNF, but not NMDA, stimulated a small (25%), but statistically significant, increase in ERK phosphorylation (lanes 1 and 2). However, when slices were co-stimulated with BDNF and NMDA, there was a significant reduction in the levels of pERK comparable to unstimulated (lanes 1 and 4). Quantification levels of phosphorylated ERK, relative to levels of ERK expression, are shown in the right panel.

With respect to changes in the levels of p38-MAPK phosphorylation, p38-MAPK was inactive in cortical slices in the absence of any treatment (Fig. 8b, lane 1). Low levels of phosphorylation were stimulated by BDNF (lane 2) but a substantially greater increase in p38-MAPK phosphorylation was stimulated by NMDA (Fig. 8b, lane 3), which is opposite to what was observed for Erk (Fig. 8a). When slices were co-stimulated with BDNF/NMDA, there was a decrease in the level of p38-MAPK phosphorylation similar to the levels seen with BDNF treatment alone (Fig. 8b, lane 4). Collectively, these observations indicate that TrkB and NMDAR signaling cross-talk and they can decrease the mechanism by which they each activate ERK and p38-MAPK. Quantification levels of

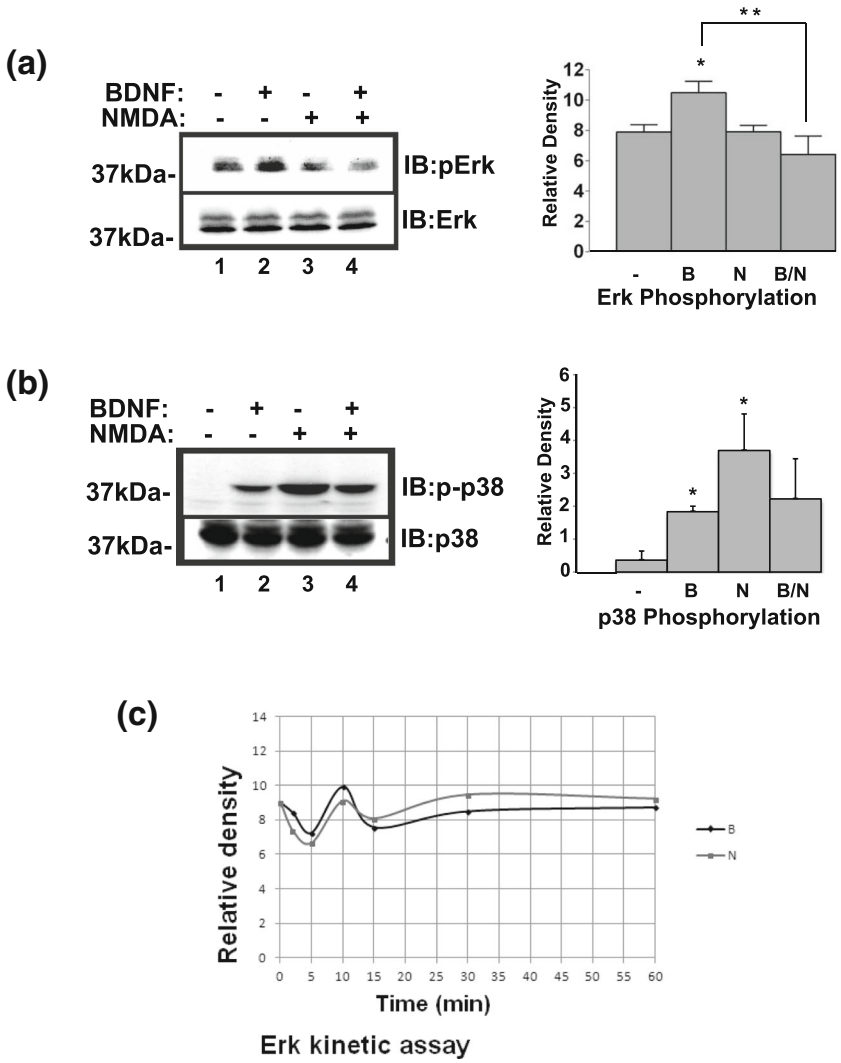

Fig. 8 Activation of ERK/p38-MAPK in response to BDNF and/or NMDA. Cortical slices were lysed after BDNF and/or NMDA stimulation and WCL (100 μg) analyzed by SDS-PAGE and Western blots performed with the indicated antibodies ($n = 3$). **a** Levels of pERK in P30 slices after BDNF and/or NMDA stimulation. Quantification of changes in ERK phosphorylation are shown in right panel ($n = 3$). **b** Levels of p38-MAPK phosphorylation in P30 slices after BDNF and/or NMDA stimulation. Quantification of the changes in p38 phosphorylation are shown in right panel ($n = 3$). **c** Kinetics of BDNF and NMDA induced changes in pErk levels in unstimulated cells relative to stimulated cells at 2 min, 5 min, 10 min, 15 min, 30 min, and 60 min time points ($n = 10$)

phosphorylated p38-MAPK, relative to p38-MAPK expression, are shown in Fig. 8b (right panel).

Lastly, we investigated the kinetics of BDNF and NMDA-induced changes in the activation levels of ERK at 2, 5, 10, 15, 30, and 60 min post-treatment ($n = 10$). As shown in Fig. 8c, changes in the levels of pERK rapidly decreased post-stimulation at 5 min and the BDNF, but not the NMDA, treated slices showed a transient increase of approximately 25% at 10 min post-stimulation, but these levels rapidly decreased at 15 min and slowly returned to baseline levels by 30 to 60 min post-treatment.

Discussion

LTP and LTD, two molecular processes that regulate learning and memory, are differentially regulated by glutamate activated NMDARs in the brain. In particular, the NR2B subunit regulates LTD, via RasGrf1, Rac, and p38-MAPK activation, with no effect on LTP. In contrast, the NR2A subunit regulates LTP, via RasGrf2, Ras, and ERK activation, but does not affect LTD (Fig. 9a) (Lu et al. 2008; Liu et al. 2004). Interestingly, BDNF has been shown to increase NMDA signaling by stimulating glutamate release from both hippocampal and cortical pre-synaptic membranes, via a TrkB, Src, and PLCγ-1 dependent pathway, as well as increasing the post-synaptic open channel probability of NR2B containing NMDARs (Levine and Kolb 2000; Zhang et al. 2013). While the later data demonstrates that TrkB stimulates intracellular cross-talk with NMDARs, the signaling mechanism(s) that mediate the receptor cross-talk have not been fully identified. Here, we demonstrate that RasGrf1 is a key signaling molecule involved in regulating a TrkB-dependent cross-talk mechanism with NR2B containing NMDARs.

We utilized acute brain slices, containing both cortex and hippocampus from P30 mice, since these areas co-express BDNF and TrkB associated with neuronal plasticity, LTP, and LTD (Li et al. 2006). We found that BDNF stimulated

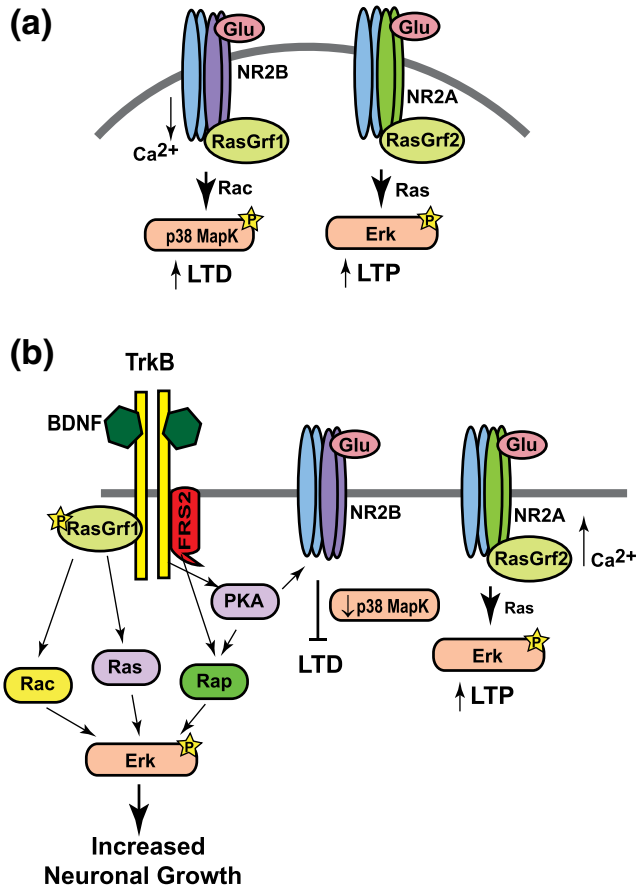

Fig. 9 Schematic diagram depicting the model of how RasGrf1 mediates cross-talk between TrkB and NMDA receptors. **a** RasGrf1 binds to the NR2B subunit of the NMDA receptor. This leads to p38-MAP kinase activation, low Ca^{2+} influx and LTD. **b** In the presence of BDNF, TrkB is activated, leading to activation of the FRS2 adapter and PKA, both of which increase activation of the GTPase, Rap1. TrkB activation also induces a conformation change which exposes the HIKE domain that facilitates the recruitment of RasGrf1 away from NR2B resulting in its tyrosine phosphorylation and the subsequent activation of both the Ras and Rac GTPases. All three GTPases (Ras, Rac, and Rap) result in increased levels of Erk activation and neurite outgrowth which will increase the potential for increased neuronal contact. TrkB activation also leads to Fyn-mediated phosphorylation of NR2B at Tyr^{1472} and an increase in intracellular Ca^{2+} levels. The loss of RasGrf1 binding to NR2B decreases the levels of p38-MAPK activation associated with LTD but does not affect RasGrf2 interaction with NR2A and the potential for increased activation of LTP

tyrosine phosphorylation of the NR2B subunit of the NMDAR at Tyr^{1472} (Fig. 1), consistent with previous reports (Lin et al. 1998; Levine et al. 1998; Levine and Kolb 2000) which is essential to increase its open-channel probability and retain it at the cell membrane (Nakazawa et al. 2001; Prybylowski et al. 2005). However, co-stimulation with NMDA reduced phosphorylation levels to background. Moreover, we observed a significant increase in the interaction between the NR2B subunit of the NMDAR and RasGrf1 in the presence of NMDA (Figs. 2 and 9a); however, BDNF uncoupled RasGrf1 binding to NR2B and recruited it directly to TrkB (Figs. 2, 5, and 9). A direct role for TrkB kinase activity being essential to a decrease in NMDA stimulated NR2B-RasGrf1 binding was confirmed with the use of the Trk kinase inhibitor, Tyrphostin (Fig. 4). A role for PKA, but not Src family kinases, also being involved in NMDA-dependent RasGrf1 binding to NR2B, was demonstrated with the use of the PKA inhibitor, H89, and the Src family kinase inhibitor PP2 (Figs. 4d–g, 9b). PKA performs a variety of important functions that regulate receptor trafficking and signaling including TrkB localization and activation in dendritic spines (Skeberdis et al. 2006), phosphorylation and activation of the Ras GEF function of RasGrf1 (Baouz et al. 2001), and facilitating Ca^{2+} influx through NR2B containing NMDARs in dendritic spines by a mechanism that involves the phosphorylation of NR2B at Ser^{1166} (Murphy et al. 2014).

In addition to BDNF, we found that TrkB and RasGrf1 also interacted in response to the TrkB receptor agonist, 7,8 DHF (Figs. 6 and 9). While DHF binds TrkB with a slightly lower affinity than BDNF (Jang et al. 2010), it is still in the nM range (*K*d of 15 nM versus 1.7 nM). BDNF also stimulated a significant increase in TrkB phosphorylation of TrkB at Tyr^{516}, equivalent to Tyr^{499} on TrkA, which are the docking sites for the Shc and FRS2 adaptor proteins (Meakin et al. 1999; Atwal et al. 2000; MacDonald et al. 1999). Phosphorylation at Tyr^{516} is also required to stimulate the conformational change that exposes the HIKE domain on TrkB to enable binding to the PH domain on RasGrf1 (Robinson et al. 2005). Baseline phosphorylation levels of TrkB at Tyr^{817}, the docking site that recruits and activates PLCγ-1, were not increased in the presence of BDNF (Fig. 3). Importantly, TrkB activation does not affect RasGrf2 binding to the NR2A subunits (Fig. 2) and cannot interfere with NMDA mediated activation of Erk and LTP (Figs. 8 and 9).

While BDNF recruits and couples both ShcC and RasGrf1 to TrkB, co-stimulation with NMDA uncouples RasGrf1 from TrkB but does not affect TrkB binding to ShcC. This reflects a higher binding affinity of the PTB domain on ShcC to the NPXpY motif (Pawson and Nash 2000), at Tyr^{516} (nM range), as compared to the lower binding affinity of the PH domain on RasGrf1 to the conformationally exposed HIKE domain on TrkB (Lemmon et al. 1996; Lemmon 2007; Agamasu et al. 2015). Conversely, we observed an increased interaction between NR2B and PSD-95 in the presence of NMDA; however, a loss of this interaction occurred by co-stimulation with BDNF (Fig. 7), similar to what was observed between NR2B and RasGrf1. This suggested that RasGrf1 might be part of a complex with PSD-95; however, no interaction between PSD-95 and RasGrf1 was observed.

We found that BDNF, but not NMDA, stimulated a small, but significant, increase in ERK phosphorylation; however, co-stimulation with NMDA significantly reduced phosphorylation levels comparable to background. In contrast, NMDA stimulation significantly increased phosphorylation of p38-

MAPK, while co-stimulation with BDNF reduced the levels comparable to those observed with BDNF alone (Fig. 8). These results are in agreement with work of Li et al. (2006) in which interaction between RasGrf1 and NR2B activated p38-MAPK, but not ERK, associated with increases in LTD, while the association of RasGrf2 with NR2A facilitates ERK activation and LTP (Li et al. 2006).

Collectively, we have identified a novel signaling pathway within the mature cortex/hippocampus in which BDNF-mediated TrkB activation modifies NMDAR signaling via RasGrf1. Specifically, while NMDA stimulates the binding of RasGrf1 to the NR2B subunit of NMDARs, and facilitates both p38-MAPK activation and LTD (Li et al. 2006) (Fig. 9a), co-stimulation with BDNF and activation of TrkB stimulates the tyrosine phosphorylation of RasGrf1, the uncoupling of RasGrf1 from NR2B and it's direct recruitment and binding to TrkB (Fig. 9b). This decreases p38-MAPK activation that would, in turn, enable LTP. Moreover, since we have shown that RasGrf1 increases neuronal growth in response to BDNF (Talebian et al. 2013), these studies provide a molecular understanding of how BDNF/TrkB signaling facilitates learning and memory as well as provides a link between BDNF stimulated neuronal differentiation and synaptic plasticity.

Author Contribution SOM conceived the study. KRB and AT conducted the experiments and all three authors contributed to writing the paper.

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Compliance with Ethical Standards

Conflict of Interest The authors declare that they have no conflict of interest.

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