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# Antagonistic effects of TrkB and p75$^{NTR}$ on NMDA receptor currents in post-synaptic densities transplanted into *Xenopus* oocytes

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**Abstract**

Brain-derived neurotrophic factor (BDNF) and its receptor TrkB are essential regulators of synaptic function in the adult CNS. A TrkB-mediated effect at excitatory synapses is enhancement of NMDA receptor (NMDA-R)-mediated currents. Recently, opposing effects of TrkB and the pan-neurotrophin receptor p75$^{NTR}$ on long-term synaptic depression and long-term potentiation have been reported in the hippocampus. To further study the regulation of NMDA-Rs by neurotrophin receptors in their native protein environment, we micro-transplanted rat forebrain post-synaptic densities (PSDs) into *Xenopus* oocytes. One-minute incubations of oocytes with BDNF led to dual effects on NMDA-R currents: either TrkB-dependent potentiation or TrkB-independent inhibition were observed. Pro-nerve growth factor, a ligand for p75$^{NTR}$ but not for TrkB, produced a reversible, dose-dependent, TrkB-independent and p75$^{NTR}$-dependent inhibition of NMDA-Rs. Fractionation experiments showed that p75$^{NTR}$ is highly enriched in the PSD protein fraction. Immunoprecipitation and pull-down experiments further revealed that p75$^{NTR}$ is a core component of the PSD, where it interacts with the PDZ3 domain of the scaffolding protein SAP90/PSD-95. Our data provide striking evidence for a rapid inhibitory effect of p75$^{NTR}$ on NMDA-R currents that antagonizes TrkB-mediated NMDA-R potentiation. These opposing mechanisms might be present in a large proportion of forebrain synapses and may contribute importantly to synaptic plasticity.

**Keywords**: brain-derived neurotrophic factor, neurotrophins, NMDA receptor, post-synaptic density, synapse.

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Neurotrophins, including nerve growth factor (NGF) and brain-derived neurotrophic factor (BDNF), bind to two structurally unrelated receptors, Trk receptor tyrosine kinases and the p75$^{NTR}$ receptor. NGF and BDNF act selectively on TrkA and TrkB, respectively, triggering well characterized intracellular cascades, but also acting directly on ionic channels on a rapid time scale (Huang and Reichardt 2003). In contrast, p75$^{NTR}$ is activated by all neurotrophins with

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*Abbreviations used*: BDNF, brain-derived neurotrophic factor; LTP, long-term potentiation; NGF, nerve growth factor; PSD, post-synaptic density; MBP, maltose-binding protein.

similar nanomolar affinity. By interacting with diverse intracellular adaptor proteins, $p75^{NTR}$ initiates various signaling cascades implicated in diverse, complex and sometimes opposing functions. $p75^{NTR}$ can promote survival, induce apoptosis, enhance neurite growth, facilitate growth cone collapse, mediate differentiation and enhance proliferation, however, no effect on ionic channels have yet been described (Chao 2003; Nykjaer *et al.* 2005). Recently, the precursors of neurotrophins, the pro-neurotrophins, have been identified as high-affinity ligands for $p75^{NTR}$ in complex with a co-receptor, sortilin. Therefore, pro-neurotrophins are now thought to be biologically active (Heymach and Shooter 1995; Mowla *et al.* 2001; Lu 2003).

In the adult forebrain, BDNF has emerged as an essential modulator of activity-dependent neuronal structure, function and synaptic plasticity (Poo 2001; Bramham and Messaoudi 2005; Dijkhuizen and Ghosh 2005). Its receptor TrkB is localized at both pre-synaptic and post-synaptic compartments of excitatory synapses within the forebrain (Yan *et al.* 1997; Drake *et al.* 1999; Aoki *et al.* 2000; Wyneken *et al.* 2001), where it is involved in the facilitation of long-term potentiation (LTP) as well as learning and memory processes (Yamada *et al.* 2002; Hariri *et al.* 2003; Kramar *et al.* 2004; Monteggia *et al.* 2004; Rattiner *et al.* 2004). Although the expression of $p75^{NTR}$ is high during early development, in the adult forebrain it was supposed to be largely restricted to the cholinergic basal forebrain (Yan and Johnson 1988; Lee *et al.* 1998; Dechant and Barde 2002). However, it has recently been shown that in the hippocampus, $p75^{NTR}$ may be associated to dendritic spines and post-synaptic densities (PSDs) (Woo *et al.* 2005). An emerging view is that both receptors have opposing effects on hippocampal synaptic plasticity: $p75^{NTR}$ facilitates long-term depression while TrkB facilitates LTP (Lu *et al.* 2005).

Post-synaptic densities are major sites of synaptic receptor clustering and signal integration in dendritic spines at excitatory synapses (Kennedy 2000; Kim and Sheng 2004). Here, the NMDA receptor (NMDA-R) is a core component of the PSD (Husi *et al.* 2000). Its NR2A and NR2B subunit C-terminal sequences (-XS/TXV) interact with the PDZ1 and PDZ2 domains of the scaffolding protein SAP90/PSD-95 (Sheng and Sala 2001). Interestingly, $p75^{NTR}$ also contains a potential PDZ-binding motif (-TSPV); an interaction with synaptic PDZ domain proteins has, however, not been shown. Rapid post-synaptic BDNF/TrkB-mediated effects include augmentation of NMDA-R channel open probability (Levine *et al.* 1998; Levine and Kolb 2000). Here, we studied whether $p75^{NTR}$ is contained in isolated forebrain PSDs and whether it is able to regulate NMDA-R currents. We show that NMDA-Rs are acutely down-regulated by $p75^{NTR}$ that is recruited to the NMDA-R complex via PSD-95. This adds a novel mechanism to the complex signaling pathways activated by $p75^{NTR}$ and supports the view that $p75^{NTR}$ and TrkB act antagonistically on synaptic NMDA-Rs on a rapid time scale.

## Materials and methods

### Materials

All chemical reagents were purchased from Sigma (St Louis, MO, USA), unless otherwise stated. Dynabeads were purchased from Dynal (Oslo, Norway). Tocris (Bristol, UK) provided us with NMDA and APV. Protein G Sepharose and glutathione Sepharose were from Amersham Biosciences (Freiburg, Germany). Recombinant *Escherischia coli*-derived BDNF was from Calbiochem (San Diego, CA, USA), pro-NGF from scil proteins (Halle, Germany) and K252a from Alomone (Jerusalem, Israel). The antibodies were provided as follows: Rex (loss-of-function anti-$p75^{NTR}$ antibody) was a generous gift from Louis F. Reichardt, anti-$p75^{NTR}$ for Western blot from Upstate (Lake Placid, NY, USA) and for immunoprecipitation from Alomone (Jerusalem, Israel), anti-maltose-binding protein (anti-MBP) was from New England BioLabs (Ipswich, MA, USA), anti-NR2A/B from Chemicon (Temecula, CA, USA), anti-Rab-5 and anti-TrkA from Santa Cruz (Santa Cruz, CA, USA). BD Biosciences (Franklin Lakes, NJ, USA) provided us with antibodies against nNOS (for Western blots), NR1, TrkB, PSD-95 and sortilin. nNOS antibody for immunoprecipitation was from Sigma. Horseradish peroxidase-conjugated secondary antibodies were provided by BioRad (Hercules, CA, USA).

### Subcellular fractionation

Sprague–Dawley rats (10 weeks old) were maintained and killed following the recommendations of the government agency Fondecyt, and the experimental protocols were approved by the Ethics Committee of Universidad de Los Andes.

For PSD isolation, the method of Carlin *et al.* (1980) was slightly modified. Cortices and hippocampi of 10 male Sprague–Dawley rats were homogenized in 5 mL/g wet weight of homogenization buffer [0.32 mol/L sucrose, 5 mmol/L HEPES, 0.5 mmol/L EGTA, pH 7.4, containing a protease inhibitor mixture (Boehringer Mannheim, Mannheim, Germany)] and subsequent fractionation followed the procedures described earlier (Wyneken *et al.* 2001), except that the discontinuous sucrose gradients were of 0.85/1/1.2 mol/L sucrose steps. This allowed, in addition to the collection of synaptosomes and synaptic membranes from the 1/1.2 mol/L interphase, the collection of light membranes from the 0.85/1 mol/L interphase.

### Electrophysiology

We used the method developed by Miledi and colleagues to transplant heterologous cellular membranes into the *Xenopus laevis* oocyte plasma membrane (Marsal *et al.* 1995; Morales *et al.* 1995). This procedure allows the incorporation and physiological characterization of ion channels, which had been pre-assembled in their physiological cellular context, in frog oocytes. The method is especially suitable for the investigation of complex signaling mechanisms (Palma *et al.* 2005) and has been used to record detergent-treated membrane fractions (Palma *et al.* 2002). As much as 100–130 ng of PSDs suspended in 50 mmol/L HEPES, pH 7.4 (50–65 nL; 2–3 mg/mL) were injected into *Xenopus* oocytes. Following injection, *Xenopus laevis* oocytes were kept at 16°C in modified Barth's solution [MBS, in mmol/L: NaCl 88; KCl 1; $Ca(NO_3)_2$ 0.33; $MgSO_4$ 0.82; $NaHCO_3$ 2.4; HEPES 10; pH 7.4; supplemented with 100 IU/mL penicillin and 0.1 mg/mL streptomycin] until the electrophysiological recordings were performed. As

controls, oocytes from the same batch were injected with 65 nL of 50 mmol/L HEPES, pH 7.4.

About 12–16 h after membrane injection, membrane currents were recorded from voltage-clamped oocytes by using two microelectrodes filled with 3 mol/L KCl. The oocytes were placed in a recording chamber containing 0.5 mL oocyte Ringer's solution (115 mmol/L NaCl, 2 mmol/L KCl, 1.8 mmol/L $BaCl_2$, 5 mmol/L HEPES, adjusted to pH 7.4 with NaOH) at room temperature (20–22°C). The oocyte membrane potential was held at −60 mV and NMDA was applied continuously for 5 min. Between each agonist application, oocytes were superfused with oocyte Ringer's solution for 10 min (5 mL at 0.5 mL/min). About 90–95% of PSD-injected oocytes displayed a negative membrane potential (−30 to −60 mV), and about 60% of them expressed NMDA-R currents. In contrast, when non-delipidated synaptic membranes were injected, only 20% of them presented a negative (less than −30 mV) membrane potential, suggesting that synaptic membranes are more ootoxic than PSDs. The membrane currents generated in frog oocytes were clearly caused by activation of incorporated forebrain NMDA-Rs because control oocytes injected with 50 nL of 50 mmol/L HEPES never responded to NMDA. The following parameters of each NMDA response were calculated: current integral (total current) during agonist application, amplitude ($I_{peak}$) and $t_{0.5}$, the time $I_{peak}$ takes to decay by 50%.

Neurotrophins and drugs were added at the indicated final concentrations to the Ringer's solution 1 min before NMDA application. After neurotrophin application, oocytes were superfused for at least 20 min to return to the baseline NMDA response (10 mL at 0.5 mL/min). To estimate the apparent inhibition constants for pro-NGF, data were fitted, using least-square routines, to the following equation: $I = I_0 + (1 - I_0)*\{Ki/[(\text{pro-NGF}) + Ki]\}$, where $I$ is the normalized current and $1–I_0$ the maximal inhibition. Plastic material used to handle BDNF was blocked with 0.1 g/L bovine serum albumin (BSA) prior to use.

The WCP program, provided by John Dempster (University of Strathclyde, UK), was used for data acquisition and analysis. Unless otherwise specified, values given correspond to the average ± standard error of the mean (SEM). Data obtained in the same oocyte were compared using the paired Student's *t*-test.

### Magnetic bead immunoprecipitations and co-immunoprecipitations

Magnetic beads coated with an antibody against PSD-95 were used to further purify PSD-95-containing complexes from the Triton-extracted PSD fraction. During all incubation and washing steps described below, magnetic beads were continuously mixed with a rotary mixer and the beads were collected by placing the tubes in magnetic racks.

As much as 100 µL of magnetic beads were washed twice in 50 mmol/L HEPES, pH 7.4, and then incubated overnight at 4°C with 5 µg of PSD-95 antibody. The supernatant was discarded and the beads were washed twice with 50 mmol/L HEPES, pH 7.4 HEPES and twice with 50 mmol/L HEPES, pH 7.4, 0.5% Triton X-100. Before incubation with magnetic beads, the parent Triton X-100-derived PSD fraction (200 µg protein) was suspended in 50 mmol/L HEPES, 0.5% Triton, pH 7.4 and briefly sonicated. The PSD-95 antibody-coated beads were added and further incubated for 2 h at 4°C. Finally, the four times washed beads with attached PSDs were treated with sample buffer [2% sodium dodecyl sulphate (SDS), 12.5% glycerol, 2.5% -mercaptoethanol, 30 mmol/L Tris-HCl, pH 6.8] for performing Western blots.

For co-immunoprecipitations, 200 µg of PSD or light membrane proteins were solubilized during 2 h in solubilization buffer [50 mmol/L Tris-HCl pH 7.5, 150 mmol/L NaCl, 10 mmol/L EDTA, 2 mmol/L EGTA, 0.1% SDS, 1% Triton X-100, 1% CHAPS, 0.5% NP-40, 0.1% BSA and 50 mmol/L NaF plus proteases inhibitors], under constant agitation at 4°C. The remaining particulate material was discarded by centrifugation (5 min at 9500 *g*) and the corresponding primary antibody was added to each supernatant to interact overnight at 4°C (PSD-95, 5 µg; $p75^{NTR}$, 5 µg; nNOS, 2 µg). For controls, the corresponding normal IgG was used. Subsequently, 20 µL Protein G Sepharose (pre-washed with solubilization buffer and blocked with 0.2% BSA) were added and incubated for 1 h at 4°C under agitation. The samples were centrifuged for 5 min at 1000 *g* and the supernatants were discarded. The immunoprecipitates were washed three times with buffer containing 50 mmol/L Tris-HCl, pH 7.5; 150 mmol/L NaCl; 0.1% SDS; 1% Triton X-100, 1% CHAPS and 0.5% NP-40 plus protease inhibitors and were resuspended in 60 µL of electrophoresis loading buffer.

### Pull down assays

Glutathione-S-transferase (GST) fusion proteins of the PDZ domains of PSD-95 were constructed by subcloning PCR-amplified DNA fragments into the bacterial expression vectors pGHEB or pGEX5x1 (Amersham Pharmacia Biotech, Uppsala, Sweden). GST fusion proteins contained the following regions of PSD-95: PDZ1 (amino acid residues 62–161), PDZ2 (aa 148–249) and PDZ3 (aa 299–413).

Maltose-binding protein fusion protein of the C-terminal tail of $p75^{NTR}$ (aa 274–425) was constructed by subcloning the PCR-amplified fragment into the pMAL-c2 vector. This construct was used to test the direct interaction between PDZ3 (as a GST fusion protein) and the intracellular domain of $p75^{NTR}$ (as a MBP fusion protein); therefore, the presence of MBP was detected in the pull downs.

For pull downs, 100 µL of slurry glutathione Sepharose were washed three times with PBS and incubated in a final volume of 1 mL with 1 µmol/L of each of the GST fusion proteins. Unbound fusion protein was removed by three washes with PBS.

To test interaction with the native proteins, 200 µg of synaptosomal proteins were suspended in solubilization buffer (150 mmol/L NaCl, 50 mmol/L Tris-Cl pH 8.0, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate) and incubated overnight in the same buffer with GST-protein bound to Sepharose. After three washes, the bound proteins were eluted with SDS-polyacrylamide gel electrophoresis (PAGE) loading buffer.

The same Sepharose-bound PDZ domains were incubated with 250 nmol/L of the MBP-$p75^{NTR}_{CT}$ fusion protein to test direct interaction between PDZ domains and the C-terminal segment of $p75^{NTR}$. Incubations and washes were done with PBS, 0.1% Triton X-100. To test that the interaction with the PDZ domain was specifically mediated by the C-terminus of $p75^{NTR}$, competition experiments with wild-type and mutant peptides corresponding to the 10 C-terminal amino acids of $p75^{NTR}$ were performed. The active (CSESTATSPV) and inactive peptides (CSESTATSPT) were added at a final concentration of 250 nmol/L to the pull-down mixture. For

 

pull downs, the potential ligands were used at a concentration of 250 nmol/L, based on the reported binding affinities between PDZ domains and their ligands (Songyang *et al.* 1997).

## Results

### Opposing effects of BDNF on NMDA-R currents

Injection of PSDs into oocytes leads to the incorporation of NMDA-Rs, which upon activation by 100 μmol/L NMDA plus 5 μmol/L glycine display inward currents. For each NMDA current, the following parameters were calculated: total current ($I$, current integral), current amplitude ($I_{peak}$) and $t_{0.5}$, the time $I_{peak}$ takes to decay by 50% (Fig. 1e). $t_{0.5}$ is a measure of current inactivation. Application of agonists elicited currents of variable $I$, $I_{peak}$ and $t_{0.5}$. In this study, NMDA-R $I_{peak}$ control currents ranged from −10 to −100 nA with a mean ± SD of −22 ± 25 nA (102 cells from ten frogs; six different PSD preparations). Inactivation kinetics ranged

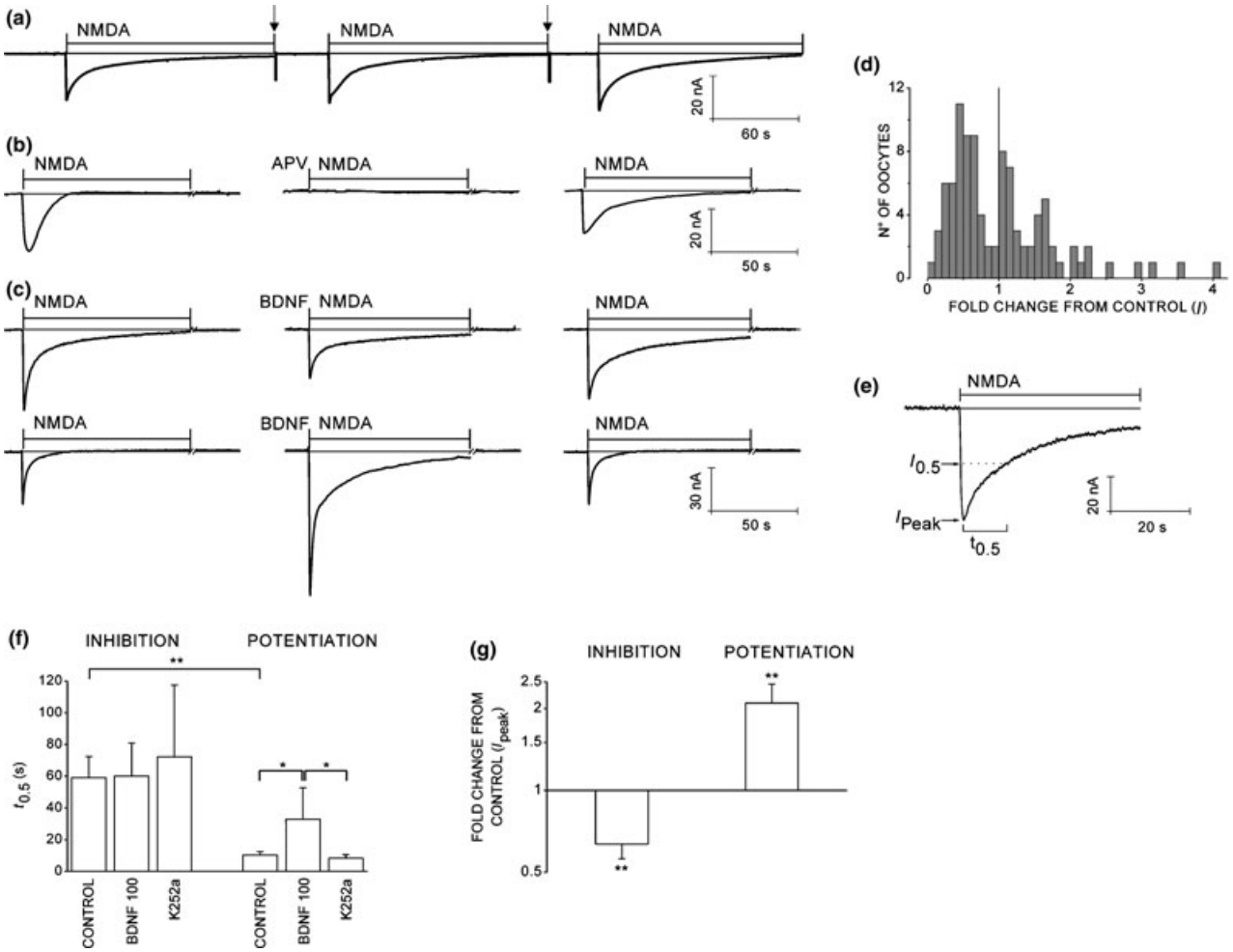


**Fig. 1** As much as 100 ng/mL brain-derived neurotrophic factor (BDNF) has a dual effect on NMDA receptor (NMDA-R) currents. (a) Consecutive responses of post-synaptic density (PSD)-injected oocytes to 100 μmol/L NMDA + 5 μmol/L glycine. The membrane potential was clamped at −60 mV. The agonists were washed out for 5 min (arrow) between each NMDA application. (b) 100 μmol/L APV reversibly inhibited 100 μmol/L NMDA-evoked currents. In these and subsequent recordings, 100 s following agonist application are shown. The breaks indicate that the recording continued for 200 additional seconds, then, agonists and/or antagonists were washed out for 5 min. (c) The NMDA-induced current is shown before and after 1-min pre-incubation of the oocyte with 100 ng/mL BDNF, and following washout of BDNF for 20 min. In different oocytes, BDNF did either inhibit (upper panel) or potentiate (lower panel) NMDA-R currents. (d) Frequency histogram of fold total current change over control ($I_{BDNF}/I_{control}$) after BDNF in 97 oocytes. (e) In addition to the current integral ($I$), the amplitude of the response ($I_{peak}$), as well as $t_{0.5}$, the time the current takes to decay by 50%, was calculated for each response to NMDA. (f) NMDA-Rs clearly inhibited by BNDF (displaying a $I_{BDNF}/I_{control}$ fraction < 0.7) were analyzed separately from those potentiated by BDNF (a $I_{BDNF}/I_{control}$ fraction > 1.5). NMDA-R currents potentiated by BDNF displayed a mean $t_{0.5}$ of 10 ± 2 s, that was significantly shorter that the mean $t_{0.5}$ of NMDA-Rs inhibited by BDNF (58 ± 8 s). Potentiation induced increases in $t_{0.5}$ that were inhibited by the TrkB antagonist K252a. (g) $I_{peak}$ changed similarly to $I$.

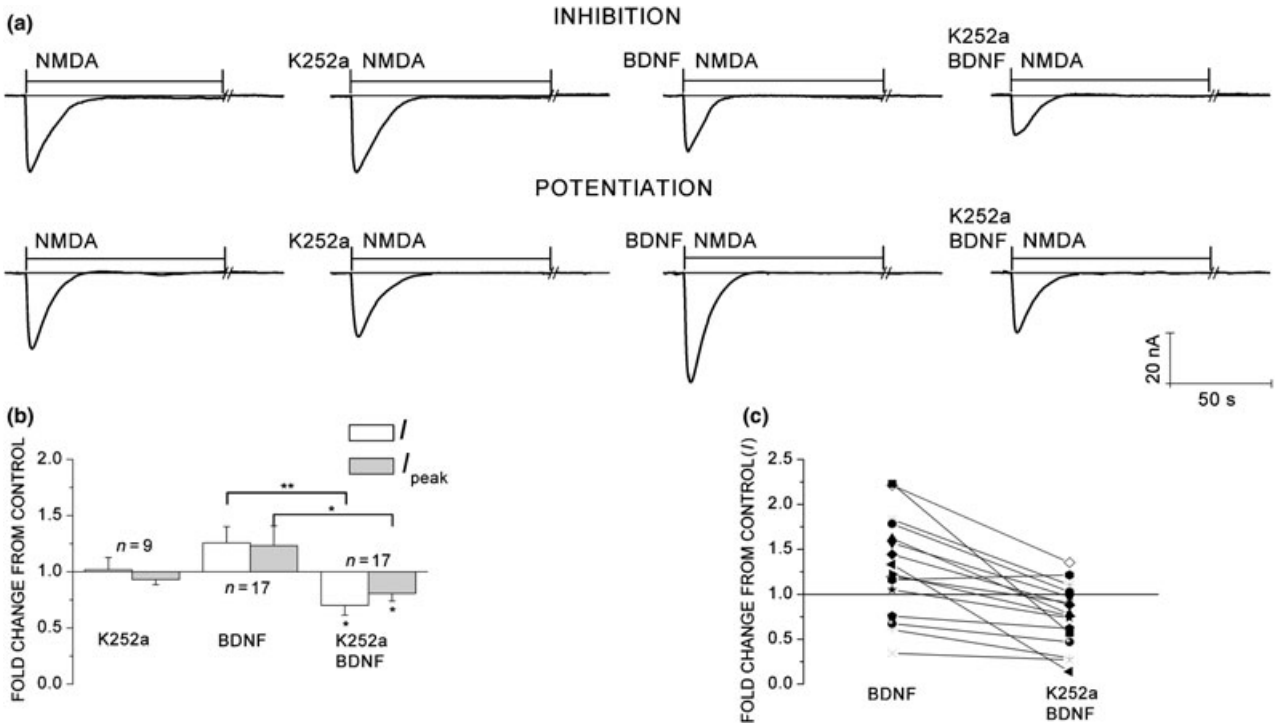


**Fig. 2** Brain-derived neurotrophic factor (BDNF) mediates down-regulation of NMDA receptor (NMDA-R) currents in the presence of the TrkB inhibitor K252a. (a) Representative recordings of an oocyte where BDNF induced an inhibition of NMDA-R currents that was insensitive to K252a (upper panel). In a different oocyte (lower panel), the potentiation by BDNF was reversed by K252a. (b) $I$ and $I_{peak}$ changed similarly. (c) In paired experiments, it is shown that K252a mainly reverses potentiation ($n = 17$; $p < 0.05$).

from 1 to 300 s, indicating that in some oocytes, current returned to basal levels only after washout of the agonists (e.g. Fig. 1a and c). However, each oocyte elicited reproducible inward currents when the agonists were washed out for 5 min between each application (Fig. 1a). NMDA currents were blocked by APV in a dose-dependent manner (not shown), and a complete and reversible blockade by 100 μmol/L APV was obtained (Fig. 1b).

After repetitive control recordings, oocytes were pre-incubated for 1 min with recombinant BDNF (100 ng/mL). Then, the agonists NMDA plus glycine were applied again in the continuous presence of BDNF. In different oocytes, either inhibition (Fig. 1c, upper panel) or potentiation (Fig. 1c, lower panel) by BDNF could be observed. In contrast, when α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) was used as agonist, no effect was observed following 1-min BDNF application (not shown). NMDA-R currents returned to control values after washing out BDNF for 20 min. In 97 experiments of this type, the total current ($I$) of the response was calculated and the frequency distribution of changes relative to control values was plotted (Fig. 1d). A whole range of responses, from strong inhibition to fourfold potentiation, could be observed. To examine whether $t_{0.5}$ could predict whether an oocyte would be inhibited or potentiated by BDNF, we calculated the mean $t_{0.5}$ for oocytes that were either clearly inhibited ($I_{control}/I_{BDNF} < 0.7$) or potentiated ($I_{control}/I_{BDNF} > 1.5$) (Figs 1f and 3e). We found that BDNF inhibited both rapidly ($t_{0.5} < 50$s) as well as slowly (50s $< t_{0.5} <$ 300s) inactivating NMDA-R currents, but potentiated only rapidly inactivating NMDA-Rs. In other words, when an NMDA-elicited current inactivated slowly, it was always inhibited by BDNF. This behaviour may be due to differential expression of NR2A (rapidly desensitizing) and NR2B (slowly desensitizing) NMDA subunits, but other factors, like association with differential PSD proteins in different oocytes, may also play a role. When BDNF inhibited NMDA-Rs, $t_{0.5}$ did not change compared with controls, but $t_{0.5}$ increased significantly when BDNF potentiated NMDA-Rs, revealing differential effects on current kinetics.

We next examined whether these effects depended on TrkB by applying the Trk antagonist K252a. 100 ng/mL K252a by itself does not alter NMDA-R currents (Fig. 2a and b), but, in the presence of BDNF, a net inhibition of both $I$ and $I_{peak}$ is observed (Fig. 2b). The effect of K252a on $t_{0.5}$ is shown in Fig. 1f, and revealed that it blocked the increase of $t_{0.5}$ of NMDA-R currents that were potentiated by BDNF. The $I$ values of 17 paired experiments are shown in Fig. 2c. These data suggest that 100 ng/mL BDNF has a diverse effect on NMDA-R currents: a TrkB-dependent potentiation

 

that can be blocked by K252a and a TrkB-independent inhibition. In addition, when TrkB is blocked, BDNF causes inhibition of NMDA-Rs. Previous studies have revealed a bidirectional mode of neurotrophin action, mediated either by TrkB or $p75^{NTR}$ (Lu *et al.* 2005; Rosch *et al.* 2005). Reportedly, $p75^{NTR}$ as well as Trk receptors can be activated by mature neurotrophins with nanomolar affinity (Esposito *et al.* 2001), whereas pro-neurotrophins activate $p75^{NTR}$ with subnanomolar affinity, at least in the presence of the co-receptor sortilin (Nykjaer *et al.* 2004).

When we applied 10-fold lower BDNF concentrations to oocytes that displayed inhibitory responses at 100 ng/mL, we obtained a potentiation of NMDA-R currents (not shown, Figs S1a and S1b). This was confirmed in oocytes that where inhibited by 100 ng/mL BDNF, in which sequentially, doses ranging from 0.5 to 300 ng/mL were applied. Typically, each oocyte could be tested with three different BDNF concentrations. The dose–response curve revealed a dual effect: potentiation at doses of 10 ng/mL (0.35 nmol/L for the active dimer) and inhibition at higher concentrations, where the maximal inhibition was about 50%. Next, we explored the possibility that BDNF was acting in opposite directions through its two receptors, $p75^{NTR}$ and TrkB, with slightly different affinities. To test whether BDNF indeed could activate $p75^{NTR}$, we performed blocking experiments with the Rex antibody that blocks $p75^{NTR}$. To this end, PSDs were incubated for 1 h with 0.05 μg/μL Rex and then injected into oocytes. In this case, BDNF did not inhibit NMDA-Rs at any of the concentrations tested, whereas PSDs incubated with a control IgG led to both inhibition as well as potentiation of NMDA-Rs by 100 ng/mL BDNF (not shown). These results suggest that BDNF, acting through $p75^{NTR}$, is able to acutely inhibit NMDA-R currents. To further test the role of $p75^{NTR}$, and to completely eliminate the participation of TrkB on NMDA-Rs, we used pro-NGF instead of pro-BDNF to stimulate $p75^{NTR}$, because pro-BDNF might interact with TrkB (Fayard *et al.* 2005). As pro-NGF could potentially act on TrkA, we performed Western blots using anti-TrkA antibody. TrkA could not be detected in PSDs, but was present in PC12 cell homogenates that were used as positive controls (not shown). Therefore, we concluded that pro-NGF should primarily activate $p75^{NTR}$ in our experiments.

### Inhibition of NMDA-R currents by pro-NGF involves $p75^{NTR}$

One-minute incubations of oocytes with 150 ng/mL pro-NGF led to a reversible inhibition of NMDA-R currents (Fig. 3a). The inhibition by pro-NGF was dose-dependent (Figs 3b and c) and the fit of the relationship between the remaining NMDA-R current and the pro-NGF dose yielded an apparent inhibition constant of 11.4 ng/mL (i.e. $K_i$ ~0.36 nmol/L for the monomer). Pro-NGF also reduced $t_{0.5}$ (Fig. 3d), however, the distribution of $t_{0.5}$ values shows that responses that inactivated both rapidly as well as slowly are inhibited by pro-NGF. Indeed, NMDA-Rs in 35 oocytes were inhibited by 100 ng/mL of pro-NGF irrespective of their $t_{0.5}$ (Figs 3e and 4d,e). Similarly to the total current, $I_{peak}$ and $t_{0.5}$ were reduced in a dose-dependent manner by pro-NGF by maximally 49.5% and 82%, and yielded Ki values of 0.39 and 0.4 nmol/L, respectively. The pro-NGF induced inhibition was not affected when Trk receptors were blocked with K252a (Fig. 4a). In five independent experiments, the inhibition of $I$ obtained with 100 ng/mL pro-NGF alone was of 67.8 ± 9.5%, and in the presence of both pro-NGF and K252a it was of 69.9 ± 7.9%. Figure 4b shows that the inhibition of NMDA-R currents by pro-NGF could be prevented when PSDs were incubated with 0.05 μg/μL Rex antibody before injection into oocytes ($n$ = 12). PSDs incubated with a control IgG (0.5 μg/μL) did not reverse the pro-NGF-induced inhibition ($n$ = 5, Figs. 4c, d and e). These experiments show that pro-NGF is able to inhibit acutely and reversibly the responses evoked by NMDA and that this effect is mediated via $p75^{NTR}$.

### $p75^{NTR}$ is anchored to the NMDA-R complex in isolated post-synaptic densities

To assess the presence of $p75^{NTR}$ in the PSD protein fraction used for oocyte injection we performed an immunoblot analysis with the subcellular fractions obtained during the PSD preparation (Wyneken *et al.* 2001). As much as 20 μg of homogenate, crude membrane fraction (P2), synaptosomes and synaptic membranes, but 20 or 5 μg (asterisks in Fig. 5a, for PSD-95 and $p75^{NTR}$) of the PSD fraction were subjected to SDS-PAGE, blotted onto nitrocellulose membranes and analysed for the presence of $p75^{NTR}$ and the PSD marker protein PSD-95 (Fig. 5a). Analysis of five independent PSD preparations revealed a 19.2 ±2.4-fold enrichment of $p75^{NTR}$ over the homogenate ($p < 0.01$). This was very similar to the enrichment of PSD-95 (18.8 ± 2.9, $n$ = 5, $p < 0.01$). In contrast to $p75^{NTR}$, neither TrkB nor neuronal nitric oxide synthase (nNOS) are enriched in PSDs and the truncated form of TrkB is almost not detectable. Interestingly, sortilin, the co-receptor for $p75^{NTR}$ that contributes to the high-affinity binding site for pro-neurotrophins, could not be detected in PSDs, nor Rab-5, a marker for the early endosomal compartment. In magnetic bead-assisted immunoprecipitation assays of PSDs using an anti PSD-95 antibody, a further enrichment of $p75^{NTR}$ was found, whereas the relative content of TrkB and nNOS decreased even more (Fig. 5b). This suggests a direct or indirect physical link between $p75^{NTR}$ and PSD-95. To test for a possible interaction of $p75^{NTR}$ and PSD-95 in PSDs, we performed co-immunoprecipitation assays from purified PSD fractions as well as from pooled light membrane fractions, which are thought to contain no PSDs, but instead are enriched in various intracellular membranes including trafficking organelles (Cohen *et al.* 1977; Carlin *et al.* 1980; Lah and Levey 2000; Wyneken *et al.* 2006). Both $p75^{NTR}$ and PSD-95 are

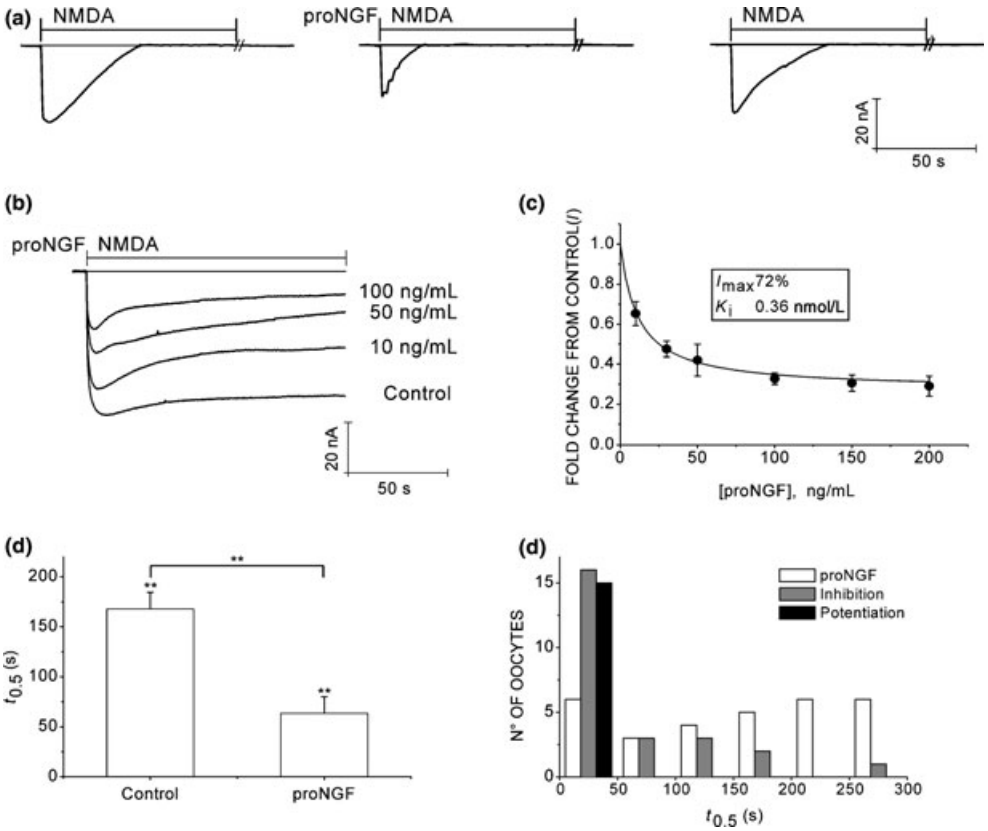


**Fig. 3** Pro-nerve growth factor (pro-NGF) produces a dose-dependent and reversible inhibition of NMDA receptor (NMDA-R) currents that is mediated by $p75^{NTR}$. (a) Representative recording of an oocyte where 150 ng/mL pro-NGF (3.1 nmol/L for the monomer) induced a reversible inhibition of NMDA-R currents. (b) Representative recordings of an oocyte, after application of indicated doses of pro-NGF. (c) The mean fractional NMDA-R current in the presence of increasing concentrations of pro-NGF was fitted with the following equation: $I = I_0 + (1 - I_0) \times \{Ki/[(\text{pro-NGF}) + Ki]\}$, where $I$ is the normalized current and $1 - I_0$ the maximal inhibition. (d) $t_{0.5}$ decreases in the presence of 100 ng/mL pro-NGF. (e) $t_{0.5}$ distribution of control NMDA currents that were subsequently inhibited by pro-NGF, and inhibited or potentiated by brain-derived neurotrophic factor.

present in the light membrane fractions, where they may serve as reservoir for dendritic recycling, or may be present in endosomes (Lee *et al.* 2001; Chetkovich *et al.* 2002). Especially, $p75^{NTR}$ is thought to be present in intracellular signaling endosomes (Bronfman *et al.* 2003). In anti-$p75^{NTR}$ immunoprecipitates from PSDs, PSD-95, nNOS and the NMDA-R subunits NR2A/B could be detected (Fig. 6a). This was found in five independent experiments, in which the nitrocellulose membranes were re-probed with the different antibodies. In contrast, anti-$p75^{NTR}$ co-immunoprecipitated only nNOS from light membrane fractions, but not the other proteins, which are known components of the NMDA-R complex. Similarly, in co-immunoprecipitation experiments using an antibody against PSD-95, $p75^{NTR}$ could be detected when PSDs were used as starting material, but not when light membranes were used. nNOS and NR2A/B subunits were found in both precipitates (Fig. 6b). These results suggest that the close association of $p75^{NTR}$ with PSD-95 is presumably restricted to the PSD. In nNOS precipitates, $p75^{NTR}$ was not detected, whereas PSD-95 and NR2A/B were present in precipitates from the PSD fraction (Fig. 6c).

Finally, GST pull-down assays using the recombinant PDZ domains of PSD-95 were performed. GST-Sepharose beads were loaded with equal amounts of fusion proteins. After incubation with solubilized synaptic membranes, beads were extensively washed and the bound material was eluted with 50 μL of electrophoresis loading buffer. Ten microlitres were subjected to SDS-PAGE for Coomassie staining as control for adequate presence of fusion proteins (Fig. 7a, lower panel), while the remaining sample was used for SDS-PAGE and subsequent Western blotting. $p75^{NTR}$ was pulled down with PDZ3 of PSD-95, while, as expected, in the same experiment, PDZ1 and 2 pulled down the NMDA-R subunits NR2A and/or B (Fig. 7a, upper panels). To test for a direct interaction between $p75^{NTR}$ and PSD-95, pull-down assays with the recombinant purified intracellular domain of $p75^{NTR}$, expressed as a maltose-binding fusion protein

 

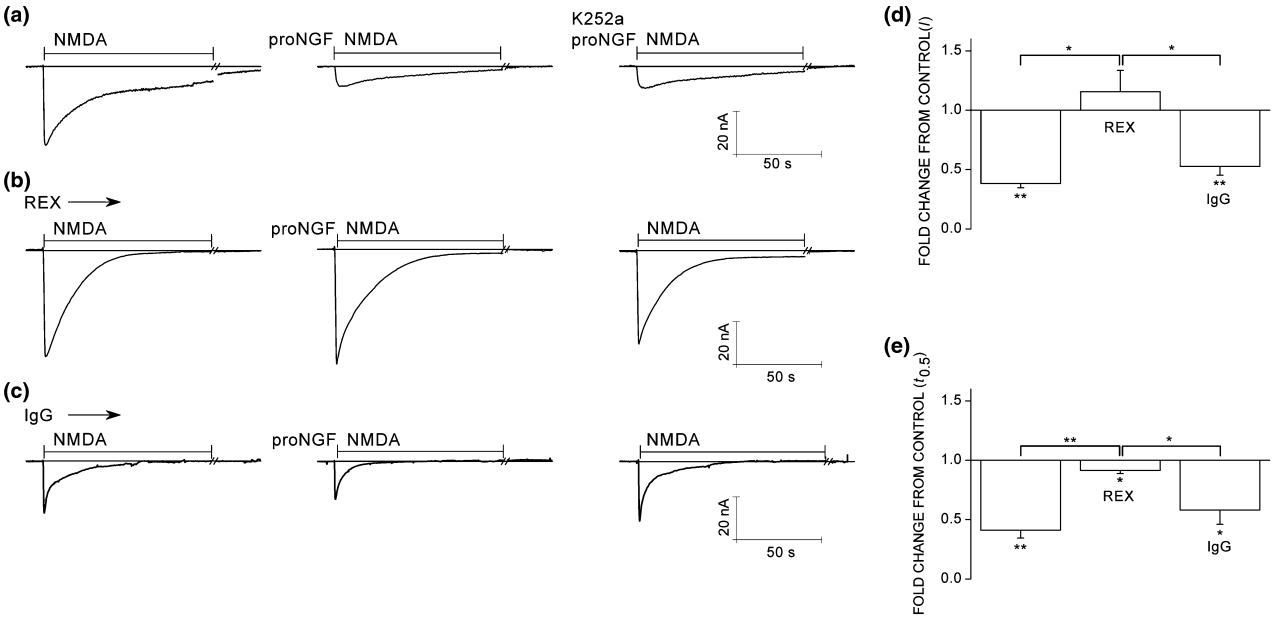


**Fig. 4** The inhibition of NMDA receptors (NMDA-Rs) by pro-nerve growth factor (pro-NGF) depends on p75$^{NTR}$ but not on TrkB. (a) Representative recordings of an oocyte showing that 100 ng/mL pro-NGF inhibited the NMDA-R currents both in the presence or absence of the Trk receptor inhibitor, K252a. (b) Representative recordings of an oocyte showing that the inhibition of NMDA-R currents was reversed by pre-incubation of post-synaptic densities (PSDs) with 0.05 μg/μL Rex, a p75$^{NTR}$ function-blocking antibody. (c) Pre-incubation of post-synaptic densities with a control IgG (0.5 μg/μL) did not affect inhibition by pro-NGF. (d, e) Rex reversed the effect on *I* as well as on $t_{0.5}$ ($n$ = 5), whereas the control IgG did not ($n$ = 4).

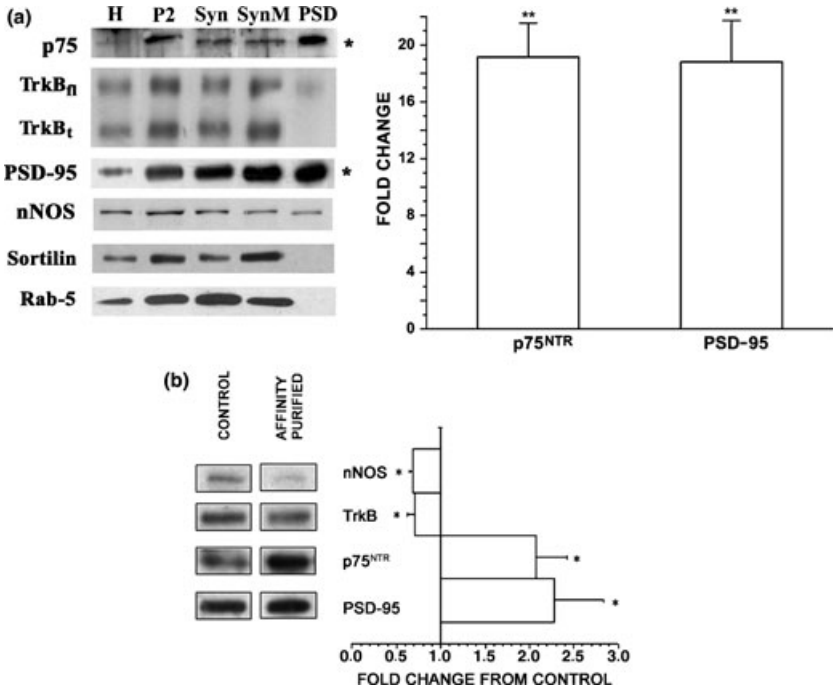


**Fig. 5** The p75$^{NTR}$ is enriched in isolated post-synaptic densities (PSDs). (a) Left panel: Immunoblot analysis of subcellular fractions using antibodies against p75$^{NTR}$, TrkB, PSD-95, nNOS, Sortilin and Rab-5 (H, homogenate; P2, crude membrane fraction; Syn, synaptosomes; SynM, synaptic membranes; PSD, post-synaptic density fraction). Lanes were loaded with 20 μg of protein except for the PSD lanes labeled with *, where only 5 μg were loaded . Right panel: quantification of enrichment of p75$^{NTR}$ and PSD-95 in PSDs over homogenate in five independent PSD preparations. (b) Purification of PSDs with magnetic beads using a PSD-95 antibody. Representative blots and mean enrichment over parent PSD preparations is shown.

(MBP-p75$^{NTR}_{CT}$), were performed. Therefore, MBP was detected in the Western blots. Again, an interaction with PDZ3 was observed (Fig. 7b, upper panel). The specificity of this interaction was tested performing competition experiments with a synthetic peptide comprising the 10 C-terminal amino-acids of p75$^{NTR}$. As shown in Fig. 7c, pull down in

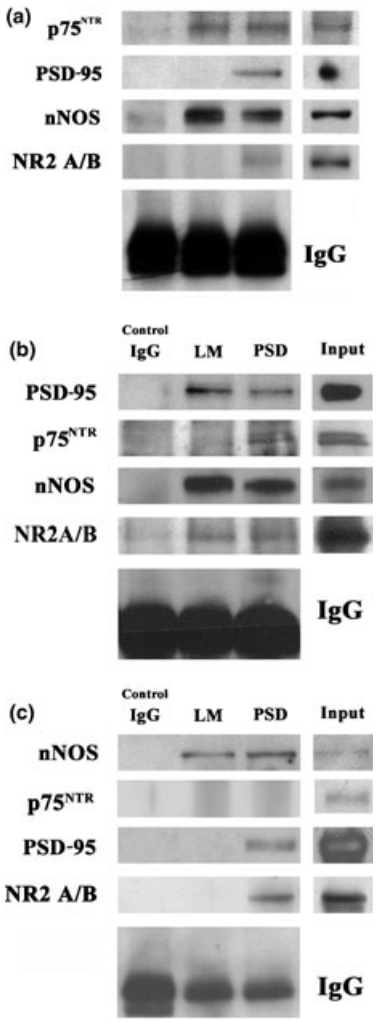


**Fig. 6** The p75$^{NTR}$ interacts with PSD-95 in post-synaptic densities (PSDs). (a) p75$^{NTR}$ was immunoprecipitated (first row) from the PSD and the light membrane (LM) fractions with anti-p75 antibody. Constituents of the NMDA-R complex and nNOS co-immunoprecipitated from PSDs, whereas in the light membrane precipitate, only nNOS was present. (b) PSD-95 (first row) was immunoprecipitated and the blots were re-probed with the same antibodies as in (a). In PSD immunoprecipitates, p75$^{NTR}$ was detected, whereas nNOS and NR2A/B were present in both precipitates. (c) nNOS (first row) was immunoprecipitated and re-probed with the same antibodies as in (a). p75$^{NTR}$ could not be detected in these precipitates, whereas PSD-95 and NR2A/B co-immunoprecipitated from PSDs.

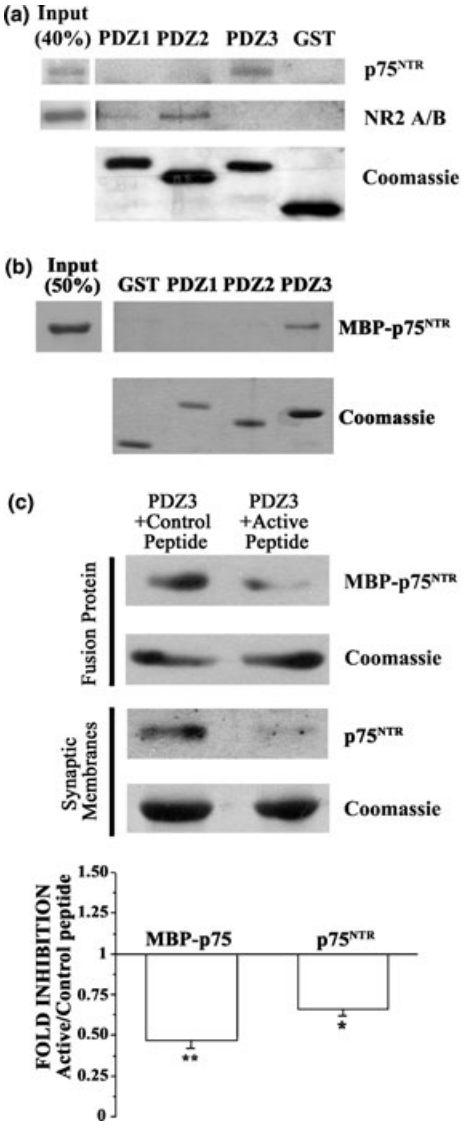


**Fig. 7** The p75$^{NTR}$ interacts with the PDZ3 domain of post-synaptic density-95 (PSD-95). Pull-down assays were performed with GST-fusion proteins bound to glutathione Sepharose. GST fusion proteins contained single PDZ domains of PSD-95. (a) Incubations were performed with solubilized synaptosomes, bound proteins were resolved by SDS-PAGE, transferred to nitrocellulose membranes and probed for p75$^{NTR}$ and NR2A/B. Lower panel: Coomassie staining revealed similar elution of each of the recombinant proteins. (b) Incubations were performed with a maltose binding protein (MBP)-fusion protein containing the C-terminus of p75$^{NTR}$ and probed for MBP. (c) The pull downs were done in the presence of a decapeptide that contained the C-terminal sequence of p75$^{NTR}$ to compete for binding. This peptide was either wild type (active peptide), or the last amino acid was changed (V to T, inactive or control peptide). The mean inhibition in three independent experiments is plotted.

the presence of the authentic peptide interferes with the interaction with the full-length protein (from synaptic membranes) as well as with the p75$^{NTR}$ intracellular domain (contained in a MBP fusion protein), whereas a mutant peptide (Val to Thr replacement of the terminal amino acid) had no effect.

Taken together, our results indicate that p75$^{NTR}$ is associated with the PSD through a direct interaction with the PDZ3 domain of PSD-95. In contrast, TrkB is not associated with the central core of the PSD and an interaction with p75$^{NTR}$ was not detectable.

 

## Discussion

### Recording of NMDA-R currents in PSDs

The NMDA-R multi-protein complex plays an essential role in signaling pathways that mediate synaptic plasticity. In this study, we have used PSDs derived from adult rat forebrain to investigate post-synaptic mechanisms assuming that native protein constituents and interactions are conserved in this preparation (Wyneken *et al.* 2004). Our studies were done with PSDs derived from the forebrain from adult animals. It was quite surprising to find a $p75^{NTR}$-mediated effect with this preparation, because $p75^{NTR}$ expression in the adult was thought to be restricted mainly to the basal cholinergic forebrain system (Lee *et al.* 1998; Dechant and Barde 2002). The extracellular medium in our recording system was precisely controlled (especially regarding neurotrophin levels, that are endogenously released in other experimental systems such as cell culture), and the post-synaptic element is clearly separated from the pre-synaptic release machinery. This is particularly relevant as neurotrophin-activated mechanisms can act both pre- and post-synaptically. On the other hand, the system should be rather complete with respect to the involved components, in contrast to injection of distinct transcripts (e.g. for $p75^{NTR}$, NMDA-R subunits, TrkB etc.) into oocytes – a system that has been used previously to study signaling through $p75^{NTR}$ (Mischel *et al.* 2001).

### Opposite and rapid effects of TrkB and $p75^{NTR}$ on NMDA-R currents

The essential finding of this study is that $p75^{NTR}$ acutely and reversibly can inhibit NMDA-Rs present in adult forebrain synapses. Neurotrophins exert long-term effects, lasting from hours to days, but TrkB, in addition, can mediate rapid effects that occur within seconds of BDNF application (Kovalchuk *et al.* 2004; Rose *et al.* 2004). Here, we report an acute effect mediated by the $p75^{NTR}$ that has not been observed before, and that opposes TrkB-mediated potentiation of NMDA-Rs. Both high nanomolar concentrations of BDNF and subnanomolar concentrations pro-NGF can acutely and reversibly inhibit NMDA-Rs. In contrast, Woo *et al.* (2005) observed an up-regulation of NR2B subunit-containing NMDA-Rs after long-term $p75^{NTR}$ activation that probably depends on regulation of NMDA-R surface expression. Differences in preparations and time scales may contribute to this apparent contradiction. An NMDA-R subunit-specific effect is suggested by the fact that slowly inactivating NMDA-R currents show inhibition by 100 ng/mL BDNF, while fast inactivating currents show potentiation. However, pro-NGF acts on all oocytes tested, and in all of them, $t_{0.5}$ was significantly reduced. Overall, TrkB increased $t_{0.5}$ while $p75^{NTR}$, when activated by pro-NGF, reduced it, confirming opposing effects of both receptors at this level.

It remains to be established whether the TrkB-dependent potentiation on NMDA-R currents in intact dendritic spines is more pronounced than $p75^{NTR}$-dependent inhibition, as observed in our experiments with isolated PSDs. The relative magnitude of potentiation versus inhibition, e.g. at 100 ng/mL BDNF, may be biased towards inhibition in our experiments because $p75^{NTR}$ is directly anchored to the NMDA-R complex, whereas TrkB seems to be loosely attached to the PSD core complex. At least, TrkB is not enriched in the PSD fraction and does not co-immunoprecipitate with PSD-95 (not shown). Although TrkB has been shown to be associated to the PSD by immunostaining and following fractionation, no quantitative studies are available, nor are the molecular links to the PSD known (Aoki *et al.* 2000; Wu *et al.* 2004). These links may change in response to extensive synaptic stimulation, when TrkB is recruited massively to the PSD (Wyneken *et al.* 2001; Ji *et al.* 2005). The temporal and spatial release pattern of endogenous ligands for each receptor type will also define the direction of the effect on NMDA-Rs (Kalb 2005).

### What might be the endogenous ligands for $p75^{NTR}$?

High levels of BDNF at excitatory synapses, e.g. associated with pathological processes, could lead to an overall NMDA-R inhibition rather than potentiation. Similarly, the levels of neurotrophins determine the balance between cell survival (mediated by Trks) and apoptosis (mediated by $p75^{NTR}$) during development (Chao 2003). Our results are consistent with previous findings (Rodriguez-Tebar *et al.* 1990; Fayard *et al.* 2005) showing that mature BDNF binds to $p75^{NTR}$. However, in a more physiological situation, the endogenous ligand for the $p75^{NTR}$ might be pro-BDNF, as proposed by Woo *et al.* (2005).

Our experiments show that pro-NGF can also activate the $p75^{NTR}$ in PSDs. NGF is synthesized within the basal forebrain cholinergic neuron target regions, such as the hippocampus and cerebral cortex, where it maintains the survival of the cholinergic system after injury and regulates cholinergic neurotransmitter levels (McAllister *et al.* 1999; Rossi *et al.* 2002). Little is known about a relationship between NGF and glutamatergic neurotransmission: NGF regulates the expression of proteins of the NMDA-R complex in the rat visual cortex (Cotrufo *et al.* 2003) and induces the release of glutamate from cortical synaptosomes (Rossi *et al.* 2002). NGF has a lower cortical expression level than BDNF (Phillips *et al.* 1990), but pro-NGF is the predominant form of NGF in the brain, it is bioactive and is up-regulated in Alzheimer's disease (Fahnestock *et al.* 2001, 2004), although it is still unknown whether pro-NGF is effectively secreted. Another potential source for pro-NGF at excitatory synapses are astrocytes (Toyomoto *et al.* 2005).

The inhibition of NMDA-R total currents ($I$) by pro-NGF occurred with an apparent affinity of approximately 0.36 nmol/L for the monomer (or 0.13 nmol/L for the dimer). The maximal inhibition obtained with pro-NGF on $I$ was about 70%, but $t_{0.5}$ was inhibited by 82%. Higher

BDNF doses (approximately 3.5 nmol/L for the dimer) also led to an incomplete inhibition of NMDA-Rs (about 50% when TrkB was not blocked by K252a, Fig. S1). Pro-NGF binds with high affinity ($10^{-11}$ mol/L) to $p75^{NTR}$ when this receptor interacts with sortilin (Nykjaer *et al.* 2004). This does not occur, however, in PSDs, because sortilin is excluded from the PSD fraction used in our study. The low $K_i$ obtained may reflect the participation of a yet unknown co-receptor.

Finally, the amyloid-beta peptide that is released at excitatory synapses is able to bind to $p75^{NTR}$ (Teng and Hempstead 2004). Non-toxic amounts of secreted amyloid-beta reduces LTP and glutamatergic transmission (Kamenetz *et al.* 2003). It is possible that this effect is mediated by $p75^{NTR}$, since in addition it is known that non-toxic concentrations of aggregated amyloid-beta induce the Ras-Erk pathway and stimulate the outgrowth of neurites via binding to $p75^{NTR}$ (Susen and Blochl 2005). Together with the increased pro-NGF levels in Alzheimer disease, this may contribute to synaptic plasticity dysregulation and early cognitive impairment. Therefore, the identity of the physiological ligands for $p75^{NTR}$ at excitatory synapses could depend on the intensity and temporal characteristics of the stimulus, on the brain region, and on health or disease state of the brain.

### Interaction of $p75^{NTR}$ with the NMDA-R complex in PSDs

Several subcellular markers have been used to show that the isolated PSD fraction is homogeneous (Smalla *et al.* 2000). In this study, we used in addition Rab-5 as a marker for early endosomes. The strong enrichment of $p75^{NTR}$ in PSDs, which parallels essentially that of PSD-95, implies that most of the adult forebrain PSDs contains this receptor. This extends previous findings, where $p75^{NTR}$ immunoreactivity was found in PSDs of the hippocampal CA1 region and dentate gyrus (Dougherty and Milner 1999; Woo *et al.* 2005). $p75^{NTR}$ is expressed in very low levels in the adult central nervous system, but it is strongly up-regulated after brain insults (Dechant and Barde 2002; Nykjaer *et al.* 2005). Previous proteomic studies, which did not detect this receptor in the PSD proteome, seem to confirm this notion (e.g. Husi *et al.* 2000; Li *et al.* 2004; Peng *et al.* 2004; Yoshimura *et al.* 2004). Nonetheless, we could clearly show, using specific antibodies, that $p75^{NTR}$ is present in the PSD in the adult brain. Thus, although the absolute levels of this protein in PSDs seem to be low, it might play a significant role in synaptic plasticity mechanisms, especially because it is strategically linked to the NMDA-R complex.

The co-immunoprecipitation studies suggest that the interaction of $p75^{NTR}$ with PSD-95 is specific and restricted to the PSD. Although the light membrane fraction contains both proteins, we could not co-immunoprecipitate them from this heterogeneous subcellular fraction, which contains early and recycling endosomes, endoplasmic reticulum, synaptic vesicles as well as Golgi and plasma membranes (Seidenbecher *et al.* 2002). Colocalization of $p75^{NTR}$ and PSD-95 in cell culture have been shown by Woo *et al.* (2005).

The pull-down experiments indicate that both the native $p75^{NTR}$ protein, as well as its recombinant intracellular domain, interact only with the third PDZ domain of PSD-95. In contrast, the NR2B C-terminus was able to bind to the PDZ 1 and 2 domains. Muller *et al.* (1996) have found that the NR2B tail binds with different affinities to the three PDZ domains of the closely related scaffolding protein SAP102 when measured by ELISA, but only to PDZ2 in overlay experiments. In accordance with this, in our assays the NR2A/B subunits could be pulled down more efficiently with the PDZ2 domain than with PDZ1. In the pull down experiments with synaptosomes, other adaptor proteins may modulate binding or act as a bridge between proteins. The pull downs between the two recombinant fragments of $p75^{NTR}$ and PDZ3 show that a direct physical interaction is possible.

Multiple, partly antagonistic functions have been assigned to TrkB and $p75^{NTR}$ in synaptic plasticity (Lu *et al.* 2005; Rosch *et al.* 2005), cell survival (Nykjaer *et al.* 2005) and structural plasticity (Zagrebelsky *et al.* 2005). A functional antagonism of both neurotrophin receptor systems has now become overt also at a rapid time scale, supporting the view of multiple and complex actions of neurotrophins at synapses. The present observations are of special relevance given the role of neurotrophins in learning and memory, and under pathological conditions including depression, epilepsy and neurodegenerative disorders.

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## Supplementary material

The following supplementary material is available for this article online:

**Fig. S1.** The brain-derived neurotrophic factor (BDNF)-induced inhibition is dose-dependent.

This material is available as part of the online article from http://www.blackwell-synergy.com

 

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