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Brain-derived neurotrophic factor induces NMDA receptor subunit one phosphorylation via ERK and PKC in the rat spinal cord

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Abstract

Brain-derived neurotrophic factor (BDNF) is involved in the modulation of synaptic transmission in the spinal cord, and several circumstantial lines of evidence suggest that it has the ability to modulate the activity of the NMDA receptor. Here we dissect the signalling mechanisms by which BDNF exerts its neuromodulatory role on the NMDA receptor subunit 1 (NR1). Using a preparation of adult isolated dorsal horn with dorsal roots attached, we found that electrical stimulation of roots induced a concomitant release of BDNF and an increased phosphorylation of NR1, which was partly prevented by the BDNF sequestering molecule, TrkB-IgG. Using a second approach *in vitro*, we confirmed that both exogenous glutamate and BDNF (but not other neurotrophins) were able to induce NR1 phosphorylation, in particular at residue Ser-897. NR1 phosphorylation induced by BDNF was blocked by a TrkB inhibitor, an ERK inhibitor and a PKC inhibitor but not a PKA inhibitor. Activation of PKC using exogenous PMA also led to NR1 phosphorylation. Together these data suggest that BDNF modulates the activity of the receptor by phosphorylation via the kinases ERK and PKC.

Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family that has received increasing interest as an important central neuromodulator of the first pain synapse in the dorsal horn of the spinal cord (Pezet *et al*., 2002c).

It is found in a subset of primary afferent fibres sensitive to nerve growth factor (NGF). During peripheral inflammation, NGF robustly increases BDNF expression in TrkA-expressing dorsal root ganglion cells (Cho *et al*., 1997a, b). BDNF is anterogradely transported in the axonal terminals of these cells in the spinal cord (Zhou & Rush, 1996; Michael *et al*., 1997) where it is released following activation of primary afferent fibres by noxious stimulation (Lever *et al*., 2001).

Different studies have revealed that BDNF may be pro- or anti-nociceptive. In mice, intrathecally delivered BDNF (ED_{50} 6 fmol) induced thermal hyperalgesia that was prevented by co-treating the mice with BDNF antisense oligonucleotide (Groth & Aanonsen, 2002). In addition, intrathecally delivered BDNF sequestering molecule TrkB-IgG reduced the pain behaviour in rats treated with formalin or carageenan (Kerr *et al*., 1999), supporting a pronociceptive role of BDNF. In contrast, in normal rats, intrathecally administered BDNF in the nanomolar range induced a short lasting thermal hypoalgesia mediated by GABAB receptors (Pezet *et al*., 2002a). The pharmacological effect of BDNF on acute pain threshold is complex and depends on the range of doses used.

Intrathecally delivered BDNF can phosphorylate its receptor TrkB and activate ERK (Pezet *et al*., 2002b), a key signal transduction kinase activated following nociceptive transmission in the spinal cord (Ji *et al*., 1999). The effects of endogenous BDNF are likely to be mediated by postsynaptic TrkB receptors whereas exogenous BDNF would recruit extrasynaptic TrkB receptors which are localized throughout the spinal cord (Zhou *et al*., 1993; Malcangio & Lessmann, 2003).

The role of BDNF is thought to be neuromodulatory (Pezet *et al*., 2002b), enhancing NMDA receptor-mediated responses (Kerr *et al*., 1999; Arvanian & Mendell, 2001; Heppenstall & Lewin, 2001; Groth & Aanonsen, 2002). However, a recent study has also involved the tetrotoxin-insensitive sodium channel Nav1.9 as a receptor coupled to TrkB, responsible for rapid BDNF-evoked depolarization (Blum *et al*., 2002). The NMDA receptor is a glutamate receptor found throughout the spinal cord, where it is known to have a key role in nociception as well as central sensitization. This channel comprises a combination of NR1 and NR2 subunits and contains several modulatory sites, enabling a large number of modulatory proteins to dock and alter its function (Raymond *et al*., 1994; Sheng *et al*., 1994; Cull-Candy *et al*., 2001).

It is still unclear how BDNF modulates the NMDA receptor (NMDAR). A recent study using electrophysiological recordings in neonatal rat spinal cord slices showed that BDNF facilitated synaptic efficacy in the spinal cord via the NMDA receptor in a PLC/PKC-dependent manner (Garraway *et al*., 2003). In a similar *in vitro* preparation of neonatal rat hemicord, we showed that BDNF modulates NMDAR phosphorylation (Slack & Thompson, 2002). We therefore hypothesize that BDNF exerts its role upon the NMDAR via this phosphorylation mechanism. Phosphorylation of the various NMDAR subunits has been reported in the spinal cord *in vivo* following chronic noxious stimulation (inflammation of the paw) (Guo *et al*., 2002) and the NR1 subunit was shown to be phosphorylated following acute noxious stimulation (capsaicin in the rat paw) (Zou *et al*., 2000).

Here we characterize the modulation of this phosphorylation by BDNF and study the signalling mechanisms involved, using *in vitro*

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adult hemisected spinal cord preparations to release BDNF in the dorsal horn or apply exogenously BDNF to the spinal cord.

Materials and methods

All experiments were performed in accordance with institutional and Home Office regulations.

Materials

BDNF, NGF, NT4, NT3 and TrkB-IgG were purchased from R&D Systems (Abingdon, Oxfordshire, UK). Glutamate, U0126, K252a, H89, phorbol-12-myristate-13-acetate (PMA), forskolin and chelerythrine were obtained from Calbiochem (Nottingham, Nottinghamshire, UK) and antibodies against total NR1 (CT), phospho-NR1 (Ser-897) and phospho-NR1 (Ser-896) were obtained from Upstate Biotech (Cambridge, Cambridgeshire, USA). All other materials were obtained from Sigma (Poole, Dorset, UK).

Electrical stimulation of adult sliced cord preparation

Lumbar spinal cords of adult male Wistar rats (250–300 g, B&K Universal Ltd, Grimston, UK) were dissected out rapidly from animals which were killed by decapitation and hemisected horizontally to give horizontal dorsal horn slices (400 μm thick) with dorsal roots attached (one slice per rat) (Malcangio & Bowery, 1994). The cords were mounted in the middle compartment of a three-compartment chamber, and continuously superfused (1 mL/min) with oxygenated Krebs' solution including 0.1% bovine serum albumin (BSA), 1 μg/mL aprotinin, 0.2 μg/mL bestatin, 0.1 mM benzethonium chloride, 1 mM benzamidine and 10 μg/mL leupeptin [to reduce loss of detectable BDNF-like immunoreactivity (LI) through surface adhesion and to prevent degradation]. The dorsal roots were positioned in the side compartments on bipolar platinum electrodes and immersed in mineral oil to avoid dehydration. Two different stimulation protocols for the dorsal roots were used: (i) continuous stimulation (CS) for 8 min at 1 Hz (480 pulses) and (ii) burst stimulation (BS), which was 75 trains of four pulses at 100 Hz separated by 0.2-s intervals (i.e. a total of 300 pulses in 15 s).

Exogenous stimulation of P28 rat hemicords

Experiments were performed on 28-day-old male Wistar rats (referred to as P28 rats and considered young adults) (B&K Universal Ltd). Spinal cords were rapidly obtained by hydraulic extrusion and hemisected along the central axis. The hemisections were then transferred into a Perspex chamber, which was superfused with oxygenated Krebs and were left to recover for 1 h and then transferred to 10-mL wells for stimulation. Each well was individually oxygenated and contained 10 mL Krebs with BDNF and/or other compounds.

For the investigation of the role of extracellular calcium in the induction of NR1 phosphorylation following extracellular BDNF stimulation, calcium was not included in the Krebs' solution and 10 mM EDTA was added to chelate any remaining calcium ions present.

Western immunoblot analysis

After stimulation hemicords were immediately frozen at −80 °C and samples processed as described previously (Slack & Thompson, 2002). Briefly, cords were homogenized in lysis buffer with protease inhibitors. The extracts were centrifuged and the supernatant retained. After protein titration the equivalent of 20 μg of total protein was added to loading buffer made of 2% sodium dodecyl sulphate (SDS), 100 mM DTT, 10% glycerol and 0.25% bromophenol blue, and boiled for 5 min. Proteins were separated on a 10% acrylamide resolving gel and transferred to a PVDF membrane using a semi-dry transfer unit (Bio-Rad, Hercules, USA). After 30 min transfer in 20% methanol transfer buffer the membranes were blocked in 5% BSA in Tris-buffered saline (TBS) containing 0.1%Tween-20 (TBST) and were incubated overnight at room temperature in rabbit anti-phospho-NR1 (Ser-897) antibody (diluted 1 : 3000) or rabbit anti-phospho-NR1 (Ser-896) antibody (diluted 1 : 500) (lot no. 19960, Upstate, Cambridge, Cambridgshire, UK). Blots were washed in TBST and incubated in peroxidase-conjugated donkey anti-rabbit IgG (1 : 5000, Amersham Pharmacia, Chalfont St Giles, UK) for 1 h at room temperature. All antibodies were diluted in TBST. The linearity of film density against the total phosphorylated protein was verified by a control experiment in which different amounts of extracts loaded to SDS-PAGE were proportional to the integrated pixel density of the film.

Protein bands were visualized using an enhanced chemi-luminescence detection kit (ECL Plus) (Amersham Pharmacia) followed by autoradiography using Hyperfilm MP (Amersham Pharmacia). The blots were then washed in TBST and stripped twice for 7 min in acidic stripping buffer (1.5% glycine, 0.1% SDS, 1% Tween 20, pH 2.2) then twice in 0.01 M phosphate-buffered saline (PBS), before blocking for 1 h in 5% BSA in TBST and reprobed for total NR1 by overnight incubation at room temperature in primary polyclonal antibody mouse anti-NR1 CT specific for all NR1 isoforms (1 : 250) (Upstate). Blots were then washed, incubated for 1 h with secondary antibody (preoxidase-conjugated donkey anti-mouse IgG, 1 : 5000, Amersham Pharmacia) and visualized as above. Gels were scanned and captured using Adobe Photoshop. The densitometric analysis was made using Scion Image software by measuring the surface area of the band (pixel squares) as well as the average pixel intensity (using a grey scale of 256 levels) for each band. Results were expressed as the level of phosphorylation for each sample, i.e. as the ratio (in arbitrary units) of the phosphorylated form (phospho-NR1) over total form of NR1 (total NR1).

Release of BDNF from dorsal horn slices: processing of collected samples

Throughout the experiment, 3-mL fractions of the superfusates were collected in ice-cooled siliconized tubes to maximize BDNF recovery. In the TrkB-IgG experiments, TrkB-IgG was superfused (500 ng/mL for 30 min) prior and during the electrical stimulation. To quantify BDNF, samples were desalted, concentrated using an Ultrafree-15 centrifugal device 10K (Waters Associates, Watford, UK), freeze-dried and stored at −80 °C. All samples from one experiment were then processed on 1 day for BDNF content. Samples were reconstituted in 300 μL of block and sample buffer (Promega, Southampton, UK) and duplicates of 100 μL assayed for BDNF-LI content by ELISA using Nunc MaxiSorp 96-well plates (Roskilde, Denmark). BDNF standards (100 μL of a range of concentrations of 7.8–500 pg/mL solutions) were run in duplicate for each plate following a protocol previously described [modified from the manufacturer's instructions (Emax ImmunoAssay kit; Promega (Lever *et al.*, 2001)].

Statistical analysis

Statistics were calculated using an ANOVA on Rank's method. *P*-values < 0.05 were considered significant.

Results

The effects of glutamate and d-serine upon NR1 phosphorylation in an in vitro *model*

The endogenous NMDA receptor agonist glutamate was used as a positive control to assess NR1 phosphorylation in adult spinal cords. In addition, we used co-application with a coagonist (D-serine – which acts at the D-glycine modulatory site) to enhance the effect of glutamate as both agonists can modulate NMDA receptor function.

P28 rat hemicords were incubated with glutamate (10 μM) or with glutamate plus D-serine (100 μM) for 10 min. Western blotting of samples showed that cords stimulated with glutamate had increased levels of NR1 phosphorylation compared with control untreated cords (Fig. 1). Co-application of glutamate with D-serine induced a modest and non-significant increase in phosphorylation levels above those of glutamate-only treated cords (Fig. 1).

Endogenous BDNF induces NR1 phosphorylation in adult rat dorsal horn of the spinal cord in vitro

The adult rat 'dorsal horn – with attached dorsal roots' is a valuable preparation for the investigation of endogenous mediators released following nociceptive stimulation. Recent work in our laboratory using this preparation showed that burst (but not continuous) stimulation of L4–L5 dorsal roots of this preparation results in the release of glutamate, substance P (SP) and BDNF from the dorsal horn (Lever *et al.*, 2001).

Here we confirm that continuous stimulation of dorsal roots for 8 min at 1 Hz (i.e. 480 pulses) did not induce BDNF release (Fig. 2B). Analysis of NR1 phosphorylation levels in the same preparations showed a concomitant lack of change following stimulation (Fig. 2A, lane 2 vs. lanes 1 and 3). However, following a regime of burst stimulation of dorsal roots (BS – 75 trains of four pulses at 100Hz, i.e. 300 pulses in total) BDNF was released (Fig. 2B) and a marked increase in phospho-NR1 levels was induced (lane 4 vs. lanes 1 and 3), showing that NR1 phosphorylation is produced when BDNF is released along with glutamate and SP.

We further analysed the nature of the neuromodulators released from central terminals of sensory neurons using a BDNF sequestering fusion protein, TrkB-IgG, in the superfusate prior to and during burst stimulation. There was a significant decrease in BDNF detected in ELISA assays of the superfusate from these experiments (Fig. 2). In spinal cords subject to burst stimulation and treated with TrkB-IgG, the ratio of phospho-NR1/total NR1 was significantly decreased (Fig. 2A, lane 5 vs. lane 4). This suggests that the chimera prevented BDNF binding to endogenous TrkB and prevented the modulation of NR1 phosphorylation.

Characteristics of the BDNF modulation of NR1 phosphorylation

In the present study, we wished to investigate the mechanisms of NR1 phosphorylation by BDNF and chose a P28 (young adult) *in vitro* model, which permits considerable pharmacological manipulation. Following incubation of P28 spinal cords with BDNF for 20 min (20 ng/mL), a significant increase in the state of phosphorylation of NR1 was detected (Fig. 3A, first column), confirming and extending our findings in the neonatal hemicords stimulated with BDNF (Slack & Thompson, 2002). Application of BDNF onto horizontally sliced spinal cords (where only the dorsal horn was present) showed similar increasing effects on NR1 phosphorylation compared with the P28 hemicord sliced vertically (containing both a dorsal and a ventral horn) (Supplementary material, Fig. S1). Co-administration of glutamate (10 μM) and BDNF (20 ng/mL) for 20 min did not further increase the phosphorylation levels of NR1 observed with BDNF alone or glutamate alone, suggesting there is no potentiating effect of BDNF upon glutamate-induced NR1 phosphorylation (Supplementary material, Fig. S2).

The effect of BDNF upon NR1 phosphorylation following 20 min stimulation (20 ng/mL) was blocked by incubation with K252a (100 nM) before and during BDNF treatment (Fig. 3A). In the presence of K252a, NR1 phosphorylation levels were statistically indistinguishable from levels in the absence of BDNF (Fig. 3A).

In order to evaluate the possibility that BDNF induced phosphorylation of NR1 following release of glutamate, we examined the effect of BDNF on NR1 phosphorylation in the absence of calcium ions (which are indispensable for release to occur). Dissection of the P28 spinal cords and incubation with BDNF in calcium-free Krebs complemented with the calcium chelator EDTA

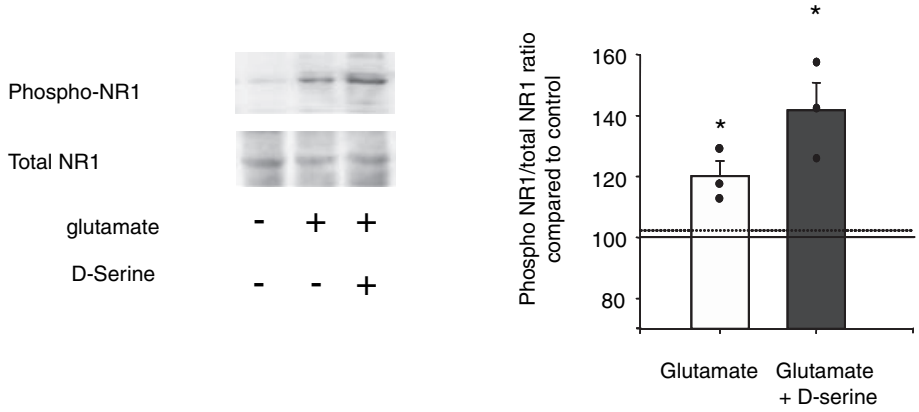

FIG. 1. Glutamate induces NR1 Ser-897 phosphorylation. Glutamate (10 μM, 10 min) and glutamate with the NMDA co-agonist D-Serine (100 μM, 10 min) induce NR1 phosphorylation in P28 rat hemicords stimulated *in vitro*: $n = 5$, $^*P < 0.05$. Filled circles show individual data. The graph represents Western blot analysis of the bands. A ratio of phospho-NR1 over total NR1 was calculated for each sample and results were expressed as a percentage of control levels (100%). The full line represents 100% (control levels) and dotted line represents SEM of the control group NR1 levels.

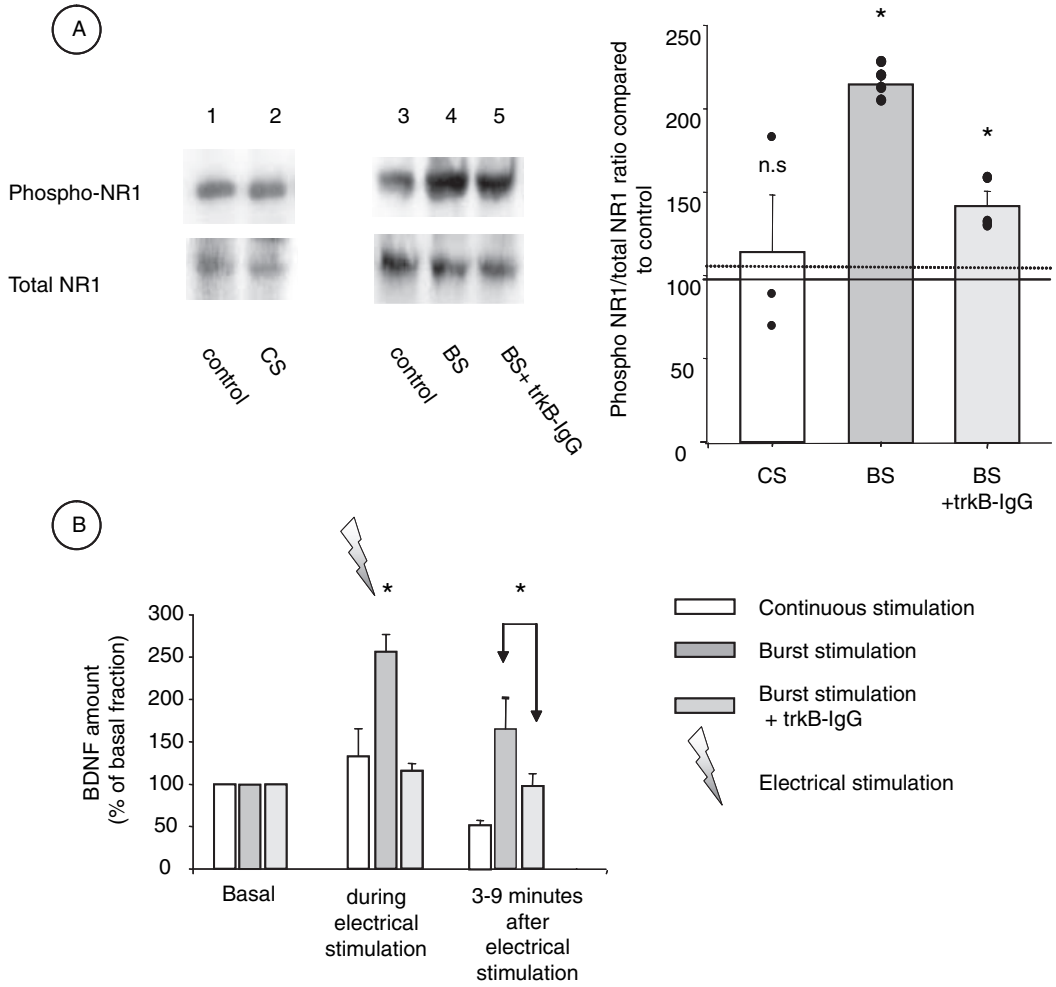

FIG. 2. Continuous electrical stimulation of the dorsal roots of the adult rat spinal cord does not induce NR1 Ser-897 phosphorylation. However, burst electrical stimulation does, and this is partially blocked by TrkB-IgG, which prevents BDNF detection in the superfusate. (A) Dorsal roots of a hemisected cord preparation were stimulated with continuous stimulation (CS) for 8 min at 1 Hz (480 pulses) or with burst stimulation (BS), which was 75 trains of four pulses at 100 Hz separated by 0.2-s intervals (i.e. a total of 300 pulses). Western blotting of cords stimulated with CS showed no significant increase in phospho-NR1 levels compared with control unstimulated cords. Cords stimulated with BS show a marked increase in phospho-NR1 levels vs. control cords. Incubation of the cords 20 min prior and during BS with the BDNF sequestering molecule TrkB-IgG results in a partial but significant decrease in phospho-NR1 levels (lane 5 vs. lane 4) compared with BS-only cords (lane 2), but levels were still significantly higher than control levels (lane 3), suggesting other neurotransmitters co-released with BDNF also phosphorylated NR1. $n = 5$, $^*P < 0.05$. Quantification of blots and representation of control on the graph is as per Fig. 1. (B) BDNF is released during BS but not CS and incubation of cords with trkB-IgG prevents BDNF detection in the superfusates of cords stimulated with BS. Superfusates were collected prior, during and 9 min after electrical stimulation of the dorsal roots and assessed for the presence of BDNF by ELISA. The graph represents levels of BDNF as a percentage of basal levels (100%). $n = 4$–5, $^*P < 0.001$.

(10 mM) for 1 h did not modify the increase in NR1 phosphorylation induced by BDNF (20 ng/mL, 20 min) (Fig. 3A, second column), suggesting that extracellular calcium-mediated release of transmitters is not critical in the mechanisms of BDNF-induced increase in NR1 phosphorylation.

The NMDA receptor 1 has several sites of phosphorylation and it has been reported that the phosphorylation state of the site Ser-896 can be modulated by noxious stimulation *in vivo* (Brenner *et al.*, 2002). We therefore investigated whether BDNF could activate NR1 phosphorylation at this site (in the P28 rat hemicord *in vitro* preparation). Two bands were identified at the correct molecular weight on the Western blot membranes probed with the phospho-Ser-896-specific antibody but only one could be labelled for total NR1 as well, and therefore was considered as the NR1 band (Fig. 3B). Analysis was restricted to this band. There was no statistical difference between levels of Ser-896 phosphorylation between control and treated cords ($n = 5$, Fig. 3B), suggesting that BDNF regulates the phosphorylation site of residue 897 and not 896.

In order to determine whether other Trk receptor activation would activate NR1, we stimulated P28 rat hemicords for 20 min with either BDNF (20 ng/mL) (as standard), NGF (100 ng/mL), neurotrophin3 (NT3, 20 ng/mL) or neurotrophin 4 (NT4, 200 ng/mL). Neither NGF, NT3 nor NT4 induced a significant increase in phospho-NR1 compared with control levels (Fig. 4).

Signal transduction mechanisms involved in BDNF-induced NR1 phosphorylation

Involvement of the MEK/ERK pathway

Previous studies in the spinal cord suggest that the neurotrophic factors, and BDNF in particular, lead to stimulation of the MEK/ERK

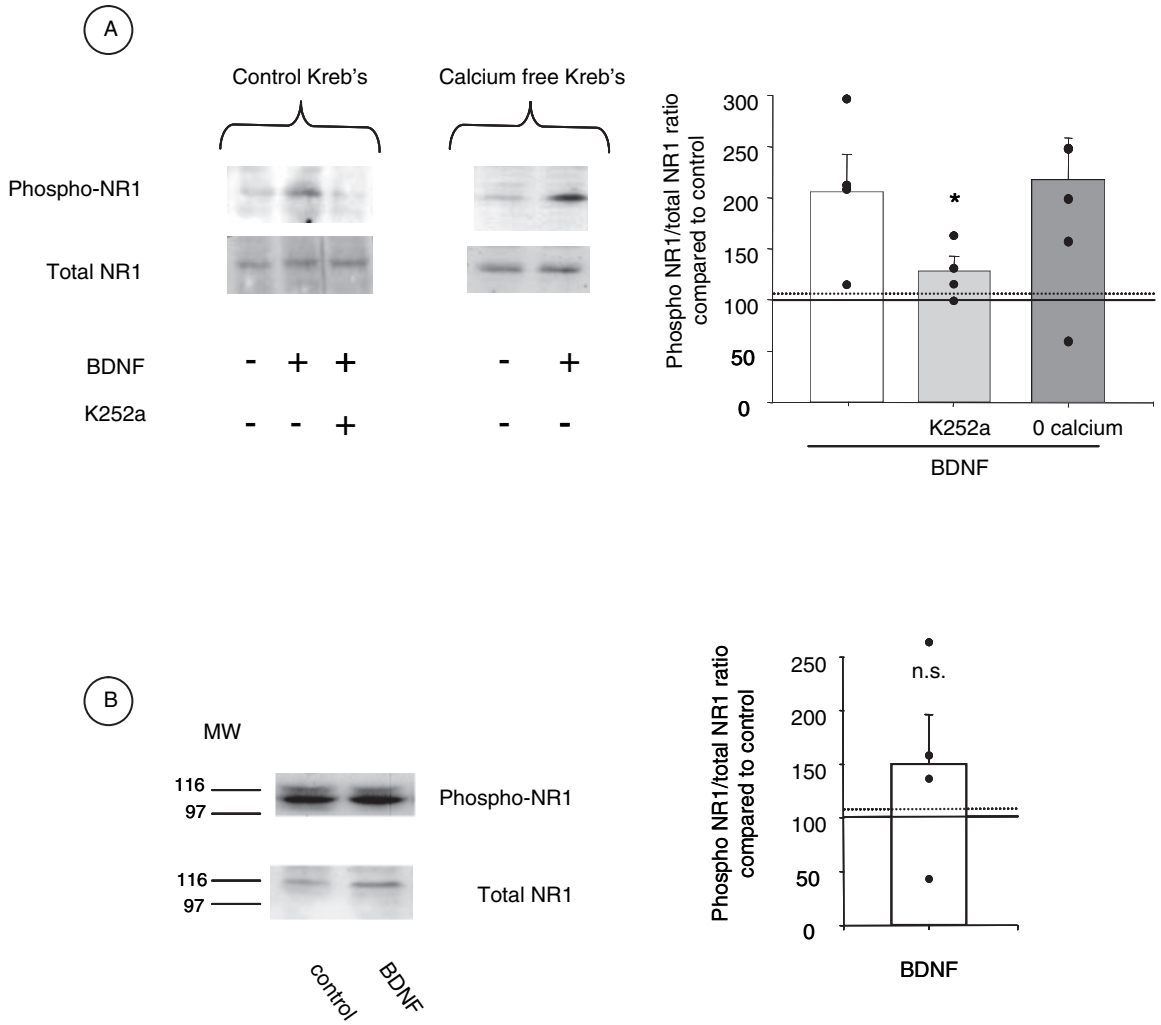

FIG. 3. Characteristics of BDNF modulation of NR1 Ser-897 phosphorylation. (A) Left column: the TrkB receptor kinase inhibitor K252a inhibits BDNF-induced NR1 phosphorylation. P28 rat cords were incubated prior and during BDNF stimulation (20 ng/mL, 20 min) with K252a (100 nM). Cords treated with the inhibitor showed a marked decrease in NR1 phosphorylation compared with both BDNF-only treated and control cords. $n = 4$, $P < 0.05$. Right column: NR1 Ser-897 phosphorylation induced by BDNF is independent of extracellular calcium. P28 rat hemicords were left to recover and stimulated in calcium-free Krebs modified with EDTA, a calcium-chelating agent (10 mM). Cords stimulated with BDNF (20 ng/mL, 20 min) showed a marked increase in NR1 phosphorylation compared with control untreated cords. $n = 5$, $^*P < 0.05$. (B) The amino acid Ser-896 of the NR1 subunit is not phosphorylated following BDNF stimulation of cords *in vitro*. Two bands are recognized by the antibody raised against phospho-Ser-896. A molecular-weight marker run in parallel shows the size of the proteins recognized as approximately 116 kDa and 97 kDa. The molecular weight of NR1 is 117 kDa. Total NR1 reprobing of the membranes confirmed that only the top band represented the NR1 protein. Quantification of blots and representation of control on the graph is as in Fig. 1.

pathway (Pezet *et al*., 2002b). Much less is known about the potential involvement between the MEK/ERK pathway and functional activation of the NMDA receptor.

We used the MEK inhibitor U0126 (which inhibits both active and inactive forms of MEK), and found that P28 rat cords incubated with this compound (10 μM), 10 min prior and throughout BDNF stimulation (20 ng/mL, 20min) showed reduced levels of NR1 phosphorylation back to control levels (Fig. 5A), indicating that the MEK pathway was necessary for BDNF-induced NR1 phosphorylation.

PKC is required for BDNF induced NR1 phosphorylation

PKC has been involved in the signalling mechanisms of nociceptive transmission in the dorsal horn, and has been shown to modulate the NMDA receptor, via a modulation of NR1 (Xiong *et al*., 1998; Lan *et al*., 2001). We hypothesized that the phosphorylation of NR1 could be mediated by PKC.

Incubation of P28 rat hemicords with the PKC inhibitor chelerythrine (3 μM) for 5 min prior and throughout the period of BDNF addition resulted in a significant reduction in the phosphorylation of NR1 induced by BDNF (Fig. 5B).

Endogenous stimulation of PKC using the phorbol ester PMA (1 μM, 10 min) showed a significant increase in phospho-NR1 levels (Fig. 5C, lanes 2 and 5 vs. lanes 1 and 4).

Furthermore, when chelerythrine chloride (3 μM) was added to PMA-stimulated P28 rat spinal cords, there was a marked decrease in phospho-NR1 levels (Fig. 5C, lane 3 vs. lane 2), confirming that PMA induced pNR1 via PKC. Together, these data support the notion that PKC is involved in intracellular mechanisms of BDNF-induced NR1 phosphorylation. In order to determine whether these pathways were activated independently of each other or were part of a single phosphorylation cascade leading to NR1 phosphorylation, the effect of U0126 upon PMA-induced NR1 phosphorylation was investigated.

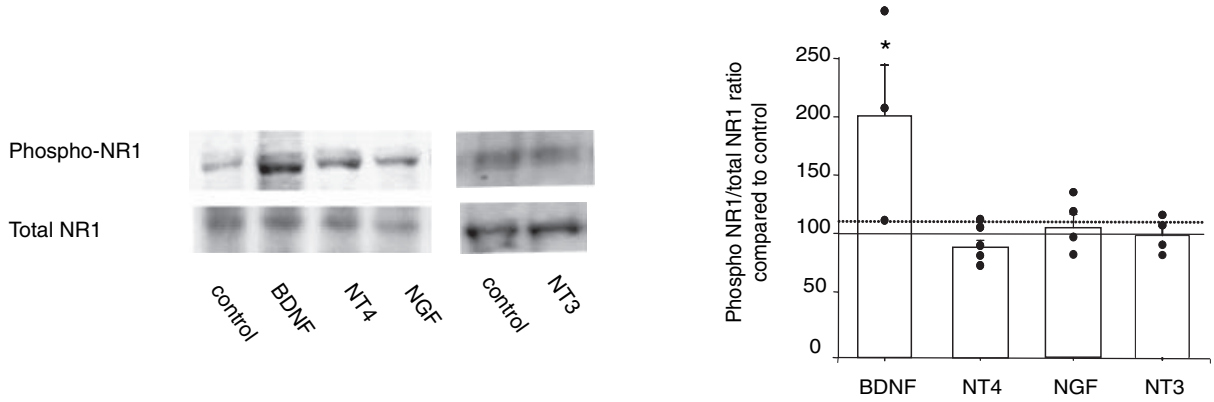

FIG. 4. Although BDNF induced significant NNRI Ser-897 phosphorylation (*$P < 0.05$), other members of the neurotrophin family do not induce NR1 Ser-897 phosphorylation. P28 rat hemicords were incubated for 20 min with BDNF as control (20 ng/mL), NGF (100 ng/mL), NT3 (20 ng/mL) or NT4 (200 ng/mL). Neither NGF, NT3 nor NT4 induced a significant increase in phospho-NR1 levels compared with control levels. $n = 4$. Quantification of blots and representation of control on the graph is as in Fig. 1.

In the presence of PMA (PKC activator) plus U0126 (ERK inhibitor) NR1 phosphorylation levels were not significantly different to levels in PMA-only treated spinal cords (Fig. 5C, lane 6 vs. lane 5). Therefore, inhibition of ERK did not prevent PKC-induced NR1 phosphorylation, indicating that MEK is activated independently of PKC.

PKA is not required for BDNF-induced NR1 phosphorylation

Previous studies of NR1 have suggested that PKA is responsible for phosphorylation at the Ser-897 site (Tingley *et al*., 1997; Zou *et al*., 2000). Using inhibitors and activators of the PKA pathway, we investigated whether PKA was involved in BDNF-mediated NR1 phosphorylation. Incubation of P28 rat hemicords for 5 min prior and during BDNF stimulation (20 ng/mL, 20 min) with the PKA inhibitor H89 (50 nM) did not prevent BDNF-induced NR1 (Fig. 6A). At higher concentrations, H89 (10 μM) will inhibit BDNF-induced NR1 phosphorylation (data not shown); however, the effects of H89 become non-specific at this concentration. In support of these data, we further show that forskolin (5 μM, 10 min) failed to induce NR1 phosphorylation above control levels (Fig. 6B). Forskolin will increase cyclic AMP levels and indirectly activate PKA. Thus we conclude that PKA is not involved in the BDNF-induced NR1 phosphorylation.

Discussion

The neurotransmitter glutamate is thought to be a key factor in the induction of synaptic plasticity in the dorsal horn. Other neuromodulators co-released are thought to act in the dorsal horn by modulating its release and postsynaptic effects. It has already been shown that BDNF modulates the NMDA receptor by phosphorylating the NR2A and NR2B subunits in postsynaptic densities isolated from rat spinal cord (Di Luca *et al*., 2001) or the NR1 subunit in the neonatal spinal cord (Slack & Thompson, 2002) and in hippocampal synaptoneurosomes (pre- and postsynaptic densities) (Suen *et al*., 1997). In the present study we have first confirmed that BDNF modulates NR1 phosphorylation in rat spinal cords, secondly investigated the characteristics of this effect and thirdly determined the intracellular pathways responsible for the modulation of the NMDAR in the spinal cord by BDNF.

Endogenous glutamate and BDNF induce NR1 phosphorylation

Recent work in our laboratory using an *ex vivo* preparation of hemisected slice preparation of adult rat spinal cord (dorsal horn only) showed that electrical stimulation of dorsal roots induced release of neurotransmitters and neuromodulators, including BDNF, SP and glutamate, and suggested that BDNF was mainly of sensory neuron origin (Lever *et al*., 2001, 2003), although it has been reported in motorneurons and other intrinsic neurons (Scarisbrick *et al*., 1999). Depending on the stimulation protocol, one or more of these substances were selectively released. Here we used this preparation for a biochemical study of the effects of endogenous BDNF upon the NMDA receptor. Burst stimulation induced BDNF release and NR1 phosphorylation, which was partially prevented by incubation with TrkB-IgG. It is likely that the remaining increase in NR1 phosphorylation observed under these conditions is due to the co-release of glutamate, as exogenous glutamate also induced NR1 phosphorylation in the *in vitro* preparation. Exogenously applied BDNF also induced NR1 phosphorylation. This key investigation of a physiological role of BDNF at the synapse in the spinal cord suggests that endogenous BDNF is partially responsible for NR1 phosphorylation as a means to modulate the NMDA receptor.

In order to investigate the biochemistry involved in BDNF-induced postsynaptic modulation of the NR1 subunit phosphorylation we have used an isolated hemisected spinal cord preparation. We could not use the *ex vivo* hemisected slice preparation used above as electrical stimulation of roots induces BDNF release as well as glutamate and therefore biochemical studies of the candidate pathways would not decipher whether BDNF or glutamate were responsible for the NR1 phosphorylation effects, nor could we use pharmacological blocks to tease out the contribution of different transmitters.

We have previously shown that exogenous BDNF effects on NR1 phosphorylation were blocked by incubation of cords with the sequestering molecule TrkB-IgG (Slack & Thompson, 2002). Here we have further shown that the tyrosine kinase inhibitor K252a prevented BDNF-induced NR1 phosphorylation. K252a is, however, a broad-spectrum tyrosine kinase inhibitor. This confirms our hypothesis that BDNF acts via the tyrosine kinase receptor TrkB. Further investigations of the selectivity of Trk receptor requirement for NR1 phosphorylation showed that NGF, which can bind its high-affinity receptor TrkA or the pan-neurotrophin (low-affinity) receptor p75, was unable to induce NR1 phosphorylation at Ser-897. This suggests that the TrkA and p75 receptors are not involved in this specific phosphorylation. As BDNF is also able to bind p75, this suggests that BDNF effects must be solely via TrkB. We expected an effect of NT4 upon NR1 phosphorylation, as it is a ligand of TrkB, but did not see any increase in phosphorylation after treatment. This may be explained by a difference in binding

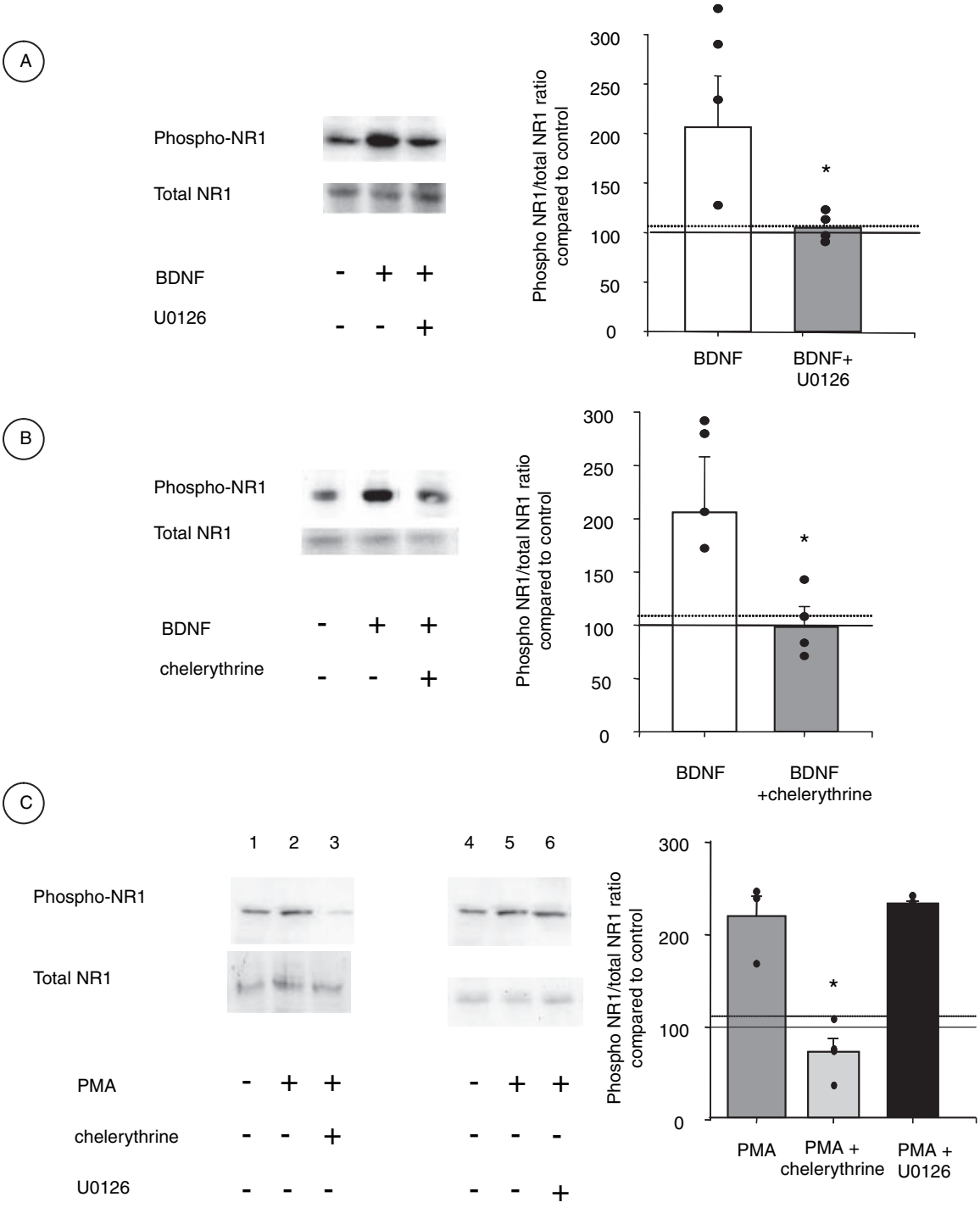

FIG. 5. The MEK and PKC pathways are involved in BDNF-induced NR1 Ser-897 phosphorylation. (A) The MEK inhibitor U0126 prevents BDNF-induced NR1 phosphorylation. P28 rat hemicords were incubated prior and during BDNF stimulation (20 ng/mL, 20 min) with U0126 (10 μM). Cords treated with the inhibitor showed a marked decrease in NR1 phosphorylation compared with both BDNF-only treated and control cords. $n = 5$, $^*P < 0.05$. (B) The PKC inhibitor chelerythrine inhibits BDNF-induced NR1 phosphorylation and the PKC activator PMA stimulates NR1 phosphorylation. P28 rat hemicords were incubated prior and during BDNF stimulation (20 ng/mL, 20 min) with chelerythrine chloride (3 μM). Cords treated with the inhibitor showed a marked decrease in NR1 phosphorylation compared with both BDNF-only treated and control cords. $n = 5$, $^*P < 0.05$. (C) Modulation of NR1 phosphorylation is via PKC independently of MEK. P28 rat hemicords incubated with PMA (1 μM, 10 min) showed a marked increase in NR1 phosphorylation levels compared with untreated control cords (lanes 2 and 5 vs. lanes 1 and 4). When chelerythrine (3 μM) was added to cords stimulated with PMA (1 μM, 10 min) there was a marked decrease in phospho-NR1 levels compared with PMA-only treated cords (lane 3 vs. lane 2). Blocking the MEK pathway with U0126 (10 μM) while stimulating PKC with PMA (1 μM) for 10 min did not prevent PMA-induced NR1 phosphorylation. $n = 4$, $^*P < 0.05$. Quantification of blots and representation of control on the graph is as in Fig. 1.

affinity for TrkB compared with BDNF (Knusel *et al*., 1994) or that concentrations or times of incubation were not appropriate for detection of changes in phosphorylation levels. We did not expect an effect of NT3 as it acts throught the TrkC receptor. These data suggest that TrkB is a major receptor involved in neurotrophin-induced NR1 phosphorylation.

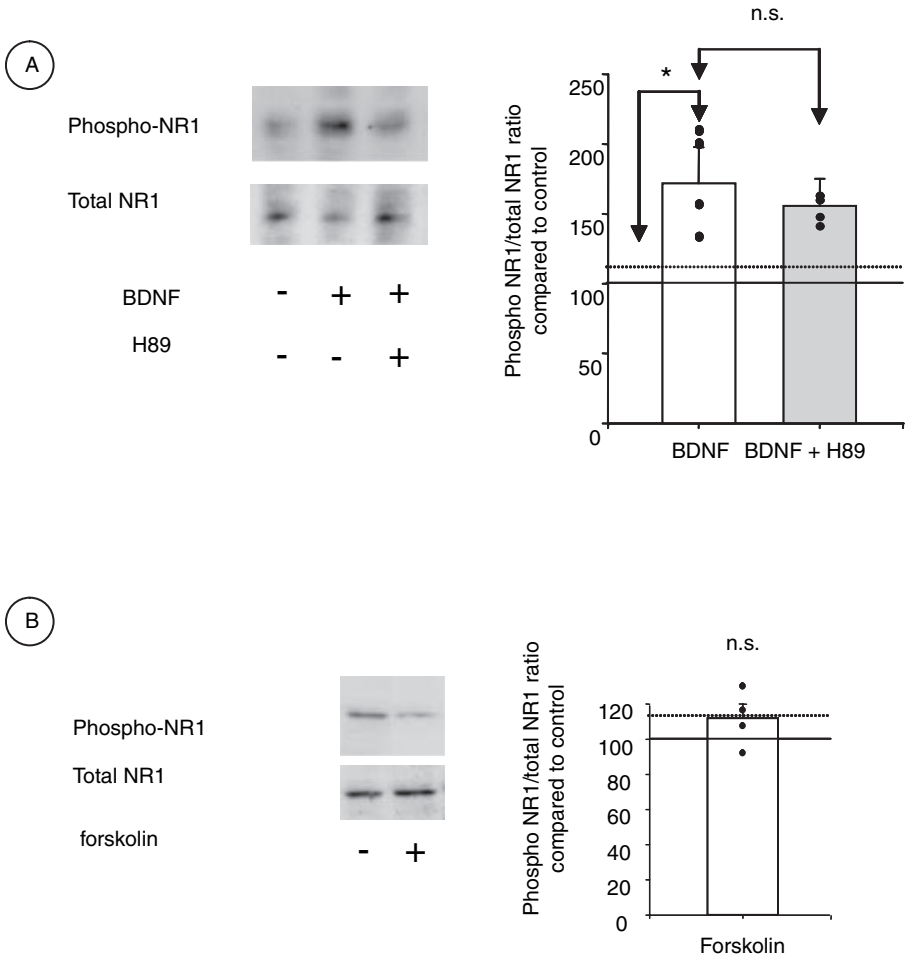

FIG. 6. PKA does not appear to be involved in the pathway leading to NR1 Ser-897 phosphorylation. (A) The PKA inhibitor H89 does not inhibit BDNF-induced NR1 phosphorylation. P28 rat cords incubated prior and during BDNF stimulation (20 ng/mL, 20 min) with H89 (50 nM) showed similar levels of NR1 phosphorylation compared with both BDNF-only treated cords. $n = 4$, $^*P < 0.05$. (B) The PKA activator forskolin does not activate NR1 phosphorylation. P28 rat cords incubated with forskolin (1 μM, 10min) showed no significant (n.s.) increase in NR1 phosphorylation levels compared with untreated control cords. Quantification of blots and representation of control on the graph is as in Fig. 1.

In the brain BDNF can increase glutamate release from cerebrocortical nerve terminals, astrocytes (Pascual *et al*., 2001) or hippocampal neurons (Li *et al*., 1998). As TrkB receptors are located pre- and postsynaptically in the spinal cord, BDNF could have induced the release of glutamate in our system, which would have mediated NR1 phosphorylation. To consider this possibility, we incubated hemicords with BDNF in the absence of extracellular calcium to block release and found that BDNF still induced NR1 phosphorylation. This suggests that the effect of BDNF upon NR1 was not due to the release of glutamate in the dorsal horn. This idea is supported by a recent study in the spinal cord, in which BDNF did not induce the release of glutamate (Pezet *et al*., 2002a).

A recent study has focused on the phosphorylation state of the Ser-896 residue of the NR1 subunit and its possible role in the cord (Brenner *et al*., 2002). Following noxious stimulation of rat hindpaw, a marked increase in phospho-Ser-896 was observed, which was reduced by co-administration of a NMDA-receptor antagonist. In our study we did not see any effects of BDNF upon phosphorylation of Ser-896. This suggests that the nociceptive effects of BDNF may be specific to Ser-897 and that the Ser-896 residue modulation in nociception may be affected by other neurotransmitters co-released with BDNF during nociceptive transmission in the cord (e.g. SP or glutamate).

Signalling mechanisms involved in BDNF-induced NR1 phosphorylation are via the ERK and PKC pathways

We found that inhibition of MEK during BDNF stimulation prevented NR1 phosphorylation, establishing the MEK/ERK pathway as a signalling cascade, leading TrkB activation to NR1 phosphorylation. BDNF induces phosphorylation of ERK in the spinal cord (Pezet *et al*., 2002b), and in other parts of the CNS (Heumann, 1994; Gooney & Lynch, 2001; Huang & Reichardt, 2001). ERK has a role in hyperalgesia (Ji *et al*., 1999), and in early and plate phases of long-term potentiation (LTP) in the hippocampus (Giovannini *et al*., 2001).

ERK1/2 phosphorylation is known to be rapid *in vivo* and *in vitro* in the CNS (1–5 min in the rat dorsal horn) (Ji *et al*., 1999; Pezet *et al*., 2002b) or in the hippocampus following LTP (Giovannini *et al*., 2001). Its role in signalling mechanisms and long-term processes (e.g. gene regulation) is well established (Giovannini *et al*., 2001).

We prevented BDNF stimulation of NR1 phosphorylation by inhibiting PKC, directly induced NR1 phosphorylation by biochemically activating PKC, and prevented the latter with a PKC inhibitor. These data suggest that PKC is involved in a pathway leading to NR1 phosphorylation. Our data from the use of PMA and U0126 suggests

that PKC is not upstream of ERK or that the pathways are independently involved in NR1 phosphorylation.

The PKC pathway has been shown to be responsible for NR1 phosphorylation in other models (Tingley *et al*., 1993) and several studies have shown a pivotal role of PKC in the modulation of the NMDA receptor function in the CNS (Logan *et al*., 1999; Liao *et al*., 2001). The role of PKC in NMDA receptor-mediated LTP is well established: through both receptor trafficking (exocytosis) and channel gating actions (increasing channel open probability) (Lan *et al*., 2001; Chen & Huang, 1992). Although several subtypes of PKC exist (Dekker & Parker, 1994) there has been considerable evidence for a role of PKCγ (located in lamina II of the spinal cord) in pain-related signalling mechanisms, which themselves involve NMDA receptor activity (MacDonald *et al*., 2001; Martin *et al*., 2001).

Together, these studies suggest that PKC-mediated NR1 phosphorylation is likely to modify the NMDA receptor channel properties. This may suggest a role of BDNF as a modulator of synaptic plasticity in the spinal cord via PKC modulation of the NMDA channel. Interestingly, BDNF activated ERK phosphorylation in the dorsal horn in neurons that were immunonegative for the PKCγ isoform (Pezet *et al*., 2002a). This may suggest that NR1 phosphorylation was modulated by ERK in some neurons, and by PKC in others, or that other PKC isoforms were co-responsible with ERK for NR1 phosphorylation.

Several lines of evidence have suggested that PKA may be involved in NR1 phosphorylation following exogenous activation (Tingley *et al*., 1997) or nociceptive activation (Zou *et al*., 2002). In the present study we were unable to modulate BDNF-induced NR1 phosphorylation with a PKA inhibitor. Nor did direct stimulation of adenylate cyclase (with forskolin) induce NR1 phosphorylation. These two experiments seem to rule out a role for PKA. We believe that the effects seen in the study by Zou *et al*. probably involve other signalling mechanisms and receptor activation.

Functional relevance of the modulation of NR1 by phosphorylation mechanisms

Little is known about the role of phosphorylation of the NR1 subunit. The subunit was thought to be key for structure and stability of the receptor complex and until recently a modulatory role was assigned only to the NR2 subunit (Cull-Candy *et al*., 2001). The NR1 subunit has been viewed as the location site for glycine modulation, whereas the glutamate high-affinity binding site is thought to be on NR2 subunits (Kuryatov *et al*., 1994; Anson *et al*., 1998, 2000). However, recent studies suggest that NR1 phosphorylation may enhance the opening probability of the channel following glutamate binding. The role of PKC in phosphorylating the NMDAR has been already reported in NR2-subunit transfected oocytes: PKC modulated NR2 phosphorylation to alter receptor gating and trafficking; a PKC activator increased the channel opening probability and induced a rapid delivery of functional NMDARs to the cell surface (Lan *et al*., 2001). Addition of the constitutively active fragment of PKC to dissociated hippocampal neurons enhanced the NMDA current and the probability of the NMDA receptor channel opening (Xiong *et al*., 1998). We show modulation by BDNF of residue Ser-897 but not Ser-896 and suggest that other residues may also be modulated by BDNF, such as Ser-889 and Ser-890, which may also be modulated by PKC as observed in transfected oocytes (Zheng *et al*., 1999) (there are, however, currently no commercially available antibodies for studies similar to that presented here).

Our data are supported by the findings of Garraway *et al*. (2003), who reported that blockade of the PLC/PKC pathway prevented the BDNF facilitation of evoked synaptic currents in a spinal cord slice preparation. In order to examine further the functional relevance of the regulation by BDNF of the NMDAR and particularly focus on the NR1 subunit, it would be interesting for future *in vitro* studies to examine with electrophysiological recordings: (i) the response and properties of the NMDA receptor in dorsal horn neurons in a cord slice preparation, while altering biochemical pathways and stimulating with glutamate and BDNF; or (ii) the effects of BDNF upon NMDA channel properties in transfected cells containing NR1 with mutations of the phosphorylation site.

Our study suggests the following mechanism: following nociceptive neuronal activation, BDNF stored at the presynaptic terminal is released and binds to its high-affinity receptor TrkB located postsynaptically, activating MEK and PKC. This leads to NR1 phosphorylation and modulation of the function of the channel. BDNF modulation of dorsal horn NMDA receptor is likely to be relevant in conditions of peripheral inflammation when sensory-neuron-derived BDNF is up-regulated and NMDA receptors increase dorsal horn neuron excitability.

Supplementary material

The following supplementary material may be found on: http://www.blackwellpublishing.com/products/journals/suppmat/EJN/EJN3656/EJN3656sm.htm

FIG. S1. BDNF induces NR1 phosphorylation in both the horizontal sliced spinal cord preparation and the hemicord preparation.

FIG. S2. Lack of potentiating effect of BDNF upon glutamatergic induction of NR1 phosphorylation.

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Abbreviations

BDNF, brain-derived neurotrophic factor; BS, burst stimulation; BSA, bovine serum albumin; CS, continuous stimulation; LTP, long-term potentiation; NGF, nerve growth factor; NMDA, *N*-methyl-D-asparte; NMDAR, NMDA receptor; NR1, NMDA receptor subunit 1; NT3, neurotrophin 3; NT4, neurotrophin 4; PMA, phorbol-12-myristate-13-acetate; SDS, sodium dodecyl sulphate; SP, substance P.

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