# Stimulation of neuropeptide Y gene expression by brain-derived neurotrophic factor requires both the phospholipase C$\gamma$ and Shc binding sites on its receptor, TrkB

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In PC12 cells, it has been previously reported that nerve growth factor stimulates neuropeptide Y (NPY) gene expression. In the current study we examined the signalling pathways involved in this effect by transiently expressing in PC12 cells the receptor (TrkB) for the related neurotrophin, brain-derived neurotrophic factor (BDNF). BDNF caused a 3-fold induction of luciferase expression from a transiently co-transfected plasmid possessing the firefly luciferase gene under the control of the NPY promoter. This effect of BDNF was completely blocked by either a Y484F mutation in TrkB (which blocks high-affinity Shc binding to TrkB) or by a Y785F substitution [which blocks the binding, phosphorylation and activation of phospholipase C$\gamma$ (PLC$\gamma$)]. Activation of the NPY promoter by neurotrophin-3 in PC12 cells overexpressing TrkC was also completely blocked by a naturally occurring kinase insert which prevents the high-affinity binding of Shc and PLC$\gamma$. NPY promoter activation by BDNF was blocked by PD98059, suggesting a role for mitogen-activated protein kinase (MAP kinase). Stimulation of NPY gene expression by PMA, but not by BDNF, was blocked by Ro-31-8220, a protein kinase C inhibitor, excluding a role for this serine/threonine protein kinase in the effect of BDNF. In addition, BDNF did not cause an elevation in cytosolic $Ca^{2+}$ concentration. Taken together, our results suggest that stimulation of the NPY promoter by BDNF requires the simultaneous activation of two distinct pathways; one involves Shc and MAP kinase, and the other appears to be PLC$\gamma$-independent but requires an intact tyrosine-785 on TrkB and so may involve an effector of TrkB signalling that remains to be identified.

## INTRODUCTION

Neuropeptide Y (NPY) is a 36-amino-acid peptide neurotransmitter expressed by a subset of sympathetic neurons [1]. A major role of this polypeptide is as a satiety factor; indeed, it has recently been shown to play a crucial role in the regulation of food intake by leptin [2]. Other factors may regulate NPY gene expression, including nerve growth factor (NGF) [3]; however, the molecular mechanism by which extracellular stimuli stimulate NPY gene expression is not known.

The PC12 cell line has proven a useful model cell system for studying the regulation of the NPY promoter [4]. These cells express TrkA, the receptor for NGF, which promotes the differentiation of PC12 cells into sympathetic-like neurons [5]. At the same time, NGF activates a programme of changes in gene transcription, including the induction of a number of early response genes such as c-*fos* [6], as well as late response genes such as those encoding NPY [4], the neurosecretory protein termed VGF [7] and transin [8].

Analysis of the NPY promoter reveals a number of potential enhancer elements, including a putative AP-1 site and two AP-2 sites. Both of these sites may contribute to the activation of the NPY promoter by NGF [9]. NPY gene expression can also be stimulated by cell-permeant cAMP analogues, such as chlorophenylthio-cAMP, which activate cAMP-dependent protein kinase, and by phorbol esters via activation of protein kinase C [10]. There is some evidence for a role for protein kinase C in the activation of the NPY promoter by NGF, based on the use of calphostin C, a protein kinase C inhibitor [11]. However, the same study argued against a role for cAMP-dependent protein kinase in mediating the effect of NGF [11]. Indeed, whether cAMP-dependent protein kinase plays a role in NGF action remains controversial [11–14].

In recent years, substantial advances have been made in our understanding of the early signalling events initiated upon binding of NGF to its receptor in PC12 cells. For example, a number of tyrosine residues in Trk receptors are autophosphorylated following ligand stimulation [15–17]. When phosphorylated, tyrosine-785 in TrkB forms a binding site for phospholipase C$\gamma$ (PLC$\gamma$) through its Src-homology 2 (SH2) domain [18]. The adapter protein Shc binds via its PTB (phosphotyrosine binding) domain to phosphorylated tyrosine-484 [19]. Once phosphorylated, Shc binds to the Grb2/Sos complex via the SH2 domain of Grb2. This in turn leads to the activation of Ras, due to GDP : GTP exchange by Sos. A hallmark of NGF action in PC12 cells is the resulting sustained activation of mitogen-activated protein kinase (MAP kinase) [20–22]. As a consequence, mutagenesis of the Shc binding site on TrkA has been shown to block the sustained activation of MAP kinase [23] and neurite outgrowth [24] in response to NGF.

In contrast with the Shc → Ras → MAP kinase pathway, the role of PLC$\gamma$ is less well understood. PLC$\gamma$ stimulation, by

Abbreviations used: BDNF, brain-derived neurotrophic factor; CAT, chloramphenicol acetyltransferase; GFP, green fluorescent protein; MAP kinase, mitogen-activated protein kinase; NGF, nerve growth factor; NPY, neuropeptide Y; NT-3, neurotrophin-3; PLC$\gamma$, phospholipase C$\gamma$; SH2, Src-homology 2.

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tyrosine phosphorylation, results in generation of the second messengers diacylglycerol, which activates protein kinase C, and Ins(1,4,5)$P_3$, which increases the intracellular $Ca^{2+}$ ion concentration [25–27]. The activation of protein kinase C by NGF in PC12 cells has been reported [11,25]. However, there is controversy as to whether cytosolic $Ca^{2+}$ ion concentrations are elevated; for example, there is some evidence for an NGF-induced increase in cytosolic $Ca^{2+}$ [28–30], but other reports fail to confirm this [31,32]. Despite this, mutagenesis of the PLC$\gamma$ binding site would be expected to block any subsequent signalling events dependent on PLC$\gamma$ activation, although there are opposing reports as to whether or not mutagenesis of tyrosine-785 blocks NGF-dependent neurite outgrowth in PC12 cells [24,33].

Several naturally occurring splice variants of TrkC, the receptor for the related neurotrophic factor neurotrophin-3 (NT-3), exist. These include those with 14- and 39-amino-acid inserts within the tyrosine kinase homology region [34]. While PC12 cells do not express TrkB or TrkC, when they are heterologously expressed these receptors can mediate brain-derived neurotrophic factor (BDNF)- and NT-3-dependent neurite outgrowth. The kinase inserts, however, block NT-3-mediated neurite outgrowth via TrkC [34,35]. This is probably the result of the inability of the kinase insert variants to form high-affinity complexes with Shc and PLC$\gamma$ [16]; as a consequence, the sustained activation of MAP kinase is attenuated [36].

We have examined the mechanism by which TrkB, when heterologously expressed in PC12 cells, stimulates the NPY promoter by taking advantage of a panel of TrkB tyrosine phosphorylation site mutants. These include TrkB[Y484F] and TrkB[Y785F], which are unable to form high-affinity complexes with Shc and PLC$\gamma$ respectively. In addition, we have studied the activation of this promoter by NT-3 in cells expressing TrkC and its naturally occurring kinase insert variant TrkC.ki14. Our data point to a concerted role for the Shc and PLC$\gamma$ binding sites in the activation of this promoter by the Trk family of receptors.

## MATERIALS AND METHODS

### Materials and cell culture

Tfx-50 transfection reagent and the Dual-Luciferase Reporter Assay kit were from Promega Corp. (Madison, U.S.A.). PD98059, a MAP kinase kinase inhibitor, was from Calbiochem (Nottingham, U.K.). Ro-31-8220, a protein kinase C inhibitor, was from Calbiochem. BDNF and NT-3 were generously donated by Dr. G. Yancopoulos (Regeneron Pharmaceuticals, New York, NY, U.S.A.). PC12 cells were routinely cultured on collagen-coated plates in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) foetal calf serum, 5% (v/v) horse serum, 10 mM Hepes (pH 7.4), 2 mM glutamine, 50 units/ml penicillin and 50 $\mu$g/ml streptomycin. Unless otherwise stated, all biochemicals were from Sigma, and general-purpose laboratory reagents were of analytical grade and were from BDH (Poole, Dorset, U.K.).

### Plasmid constructs

The plasmid pRL.SV40, which places the *Renilla retinformis* luciferase gene under the control of the non-regulated constitutive simian virus 40 promoter, was from Promega. Wild-type and point-mutated TrkB cDNAs in the mammalian expression vector pcDNAneoI have been described previously [15,16]. Full-length TrkC and the kinase insert variant TrkC.ki14 were cloned into pcDNAneoI, and have also been described previously [16]. The plasmid pNPY.CAT contains a $-750$ to $-50$ fragment of the NPY gene placed upstream of the chloramphenicol acetyltransferase (CAT) gene, and was a gift from Professor J. Allen (University of Glasgow, Glasgow, U.K.) [11]. This promoter fragment was amplified by PCR and subcloned into the *Kpn*I and *Bgl*II sites of the pGL3-Basic luciferase vector (Promega) to give the plasmid pNPY.Luc. The GAL4–Elk-1 fusion plasmid, pSG424.Elk-1-(83–428), was used in conjunction with the luciferase reporter plasmid pGAL4.Luc, which possesses five GAL4 binding sites upstream of the firefly luciferase gene. The plasmid pCMX.GFP contains the green fluorescent protein (GFP) from *Aqueoria victoria* under the control of the cytomegalovirus promoter, and was a gift from Dr. J. Pines (Wellcome/CRC, Cambridge, U.K.). All plasmids were purified by two successive CsCl gradients.

### Transient transfection of PC12 cells

PC12 cells were seeded at a density of $3.5 \times 10^5$ cells per well of a 12-well plate (Costar). The cells were transfected 24 h later with 50 ng of receptor expression plasmid, 0.5 $\mu$g of pNPY.Luc and 0.5 $\mu$g of pRL.SV40 per well in complete medium using 2.2 $\mu$l of Tfx-50 reagent per $\mu$g of DNA for 2 h, according to the manufacturer's instructions. The next day, the cells were serum starved for 2 h and treated with or without BDNF (100 ng/ml) or NT-3 (100 ng/ml) for 20 h. Where inhibitors were used, cells were preincubated for 30 min with 100 nM Ro-31-8220 or 50 $\mu$M PD98059 prior to the addition of BDNF.

### Reporter gene assays

Cells were washed once in ice-cold PBS, extracted in ice-cold lysis buffer and assayed sequentially for the firefly and *Renilla* luciferase activities using a Berthold Lumat LB9501 luminometer, according to the manufacturer's instructions. The ratio of firefly luciferase activity to *Renilla* luciferase activity provided a specific measure of NPY promoter activity corrected for variations in transfection efficiency. All experiments were performed in triplicate and on at least three separate occasions. All results are presented as means $\pm$ S.E.M.

### Cell microinjection and calcium imaging

PC12 cells were seeded on 22 mm glass coverslips coated with poly-L-lysine (50 $\mu$g/ml) and laminin (10 $\mu$g/ml). These cells were microinjected 24 h later with 0.1 mg/ml pCMX.GFP and 0.05 mg/ml TrkB expression plasmid (pcDNAneo.TrkB) using an Eppendorf semi-automatic microinjector. Cells cultured on a round coverslip were washed once with HBM [115 mM NaCl, 5 mM KCl, 1 mM $KH_2PO_4$, 0.5 mM $MgSO_4$, 1.25 mM $CaCl_2$, 25 mM Hepes (pH 7.4) and 15 mM glucose] and incubated at room temperature in the same medium supplemented with indo-1-AM (5 mM) and BSA (1 mg/ml). After this initial loading, the cells were washed and incubated for a further 30 min in HBM, to allow dye hydrolysis. Cells thus loaded were placed in a perfusion chamber at room temperature on the stage of a Nikon Diaphot inverted microscope with 20 $\times$ objective. Imaging was performed using a Bio-Rad MRC-1024 two-photon confocal system running LaserSharp software (version 2.1T) incorporating the Time-Course module (version 1.0; Hemel Hempstead, U.K.). For visualization of GFP, cells were excited with the 488 nm line of a 100 mW argon-ion laser and fluorescence was collected at 515 nm. For indo-1 imaging, the cells were excited with light at 720 nm (pulse width 80–130 fs; repetition rate 82 MHz) from a titanium:sapphire laser (Spectra-Physics, Hemel Hempstead, U.K.). Emission was monitored in two channels containing a 405/35 nm band-pass filter and a 460 nm long-pass filter respectively. Media, with or without test compounds, were perfused

over the slide chamber (volume 1 ml) via a gravity-fed perfusion system (2–3 ml/min.).

## RESULTS AND DISCUSSION

### Transient transfection of PC12 cells with wild-type TrkB and mutant receptor constructs

In order to examine the signalling pathways involved in BDNF-induced NPY gene expression, we transiently overexpressed TrkB in PC12 cells. In parallel, we transfected TrkB mutants in which the phosphotyrosine binding sites for the downstream signalling molecules Shc and PLC$\gamma$ had been substituted with phenylalanine residues.

BDNF caused an approx. 2.5-fold induction of luciferase expression in cells expressing wild-type TrkB (Figure 1). A K540R mutation in the active site of TrkB, which results in a kinase-dead receptor incapable of binding ATP, completely obliterated this induction, but had no effect on basal promoter activity (Figure 1). Both a Y484F mutation, which blocks binding of Shc, and a Y785F mutation, which blocks binding of PLC$\gamma$, prevented activation of the NPY promoter by BDNF to a similar extent to that caused by the K540R mutation. A double mutant Y484F/Y785F, which blocks binding of both Shc and PLC$\gamma$, also failed to signal (results not shown).

To confirm that both the Shc and PLC$\gamma$ sites are necessary for BDNF to activate the promoter, the TrkB[Y484F] and TrkB[Y785F] single mutants were co-expressed. As shown in Figure 1, there was a partial restoration of the response, such that the level of induction was about 50% of that observed with wild-type TrkB. This partial restoration of signalling is likely to be the result of tyrosine phosphorylation in *trans* between the two mutant receptors, and thus complementation between the signalling pathways subsequently activated [37].

The data suggest, therefore, that both the Shc and PLC$\gamma$ binding sites on TrkB are required for the ability of this receptor to mediate the activation of the NPY promoter by BDNF.

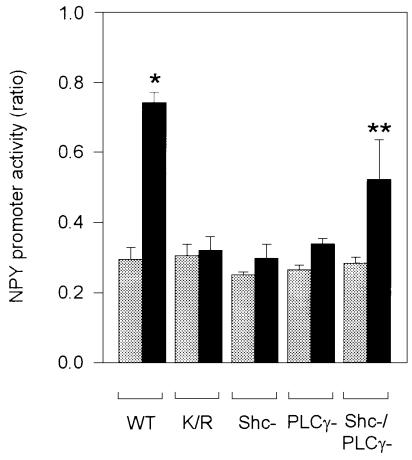


**Figure 1 Y484F and Y785F mutations in Trk block activation of the NPY promoter by BDNF**

PC12 cells were transiently transfected with pNPY.Luc, pRL.SV40 and pcDNAneo containing wild-type TrkB (WT), TrkB[K540R] (K/R), Y484F (Shc–), Y785F (PLC$\gamma$–) or Y484F and Y785F single mutants together (Shc–/PLC$\gamma$–). The cells were then incubated for 20 h in the absence (grey bars) or presence (filled bars) of 100 ng/ml BDNF. Cell lysates were prepared and assayed for expression levels of firefly luciferase (Luc) and *Renilla* luciferase (Ren). The Luc/Ren ratio provides a measure of the specific activity of the NPY promoter corrected for variations in transfection efficiency, as the simian virus 40 promoter is not regulated by BDNF. Significance of differences compared with controls: $^{*}P < 0.01$; $^{**}P < 0.05$.

**Table 1 The TrkC kinase insert blocks activation of the NPY promoter by NT-3**

PC12 cells were transiently transfected with pNPY.Luc, pRL.SV40 and pcDNAneo containing wild-type TrkC or TrkC.ki14, as indicated. The cells were then incubated for 20 h in the absence or presence of 100 ng/ml NT-3. Cell lysates were prepared and assayed for the levels of expression of firefly luciferase (Luc) and *Renilla* luciferase (Ren), as described in the legend to Figure 1. Results are means ± S.E.M.; $^{*}P < 0.01$ compared with control.

| Conditions | Ratio (Luc/Ren) |
|---|---|
| TrkC–NT-3 | 0.12 ± 0.003 |
| TrkC + NT-3 | 0.22 ± 0.02* |
| TrkC.ki14–NT-3 | 0.13 ± 0.003 |
| TrkC.ki14 + NT-3 | 0.10 ± 0.01 |

**Table 2 PD98059 blocks the BDNF-dependent activation of the MAP kinase-responsive transcription factor Elk-1**

PC12 cells were transiently transfected with pSG424.Elk-1-(83–428), pGL3.G5E4$\Delta$38, pRL.SV40 and pcDNAneo containing wild-type TrkB. The cells were then preincubated for 30 min in the absence or presence of 50 $\mu$M PD98059, and then for 20 h with or without BDNF (100 ng/ml) as indicated. Subsequent cell lysates were assayed for the expression levels of firefly luciferase (Luc) and *Renilla* luciferase (Ren), as described in the legend to Figure 1. Results are means ± S.E.M.; $^{*}P < 0.01$ compared with control.

| Conditions | Ratio (Luc/Ren) |
|---|---|
| Control | 2.07 ± 0.66 |
| BDNF | 31.77 ± 5.62* |
| BDNF + PD98059 | 7.08 ± 1.32 |

### Activation of the NPY promoter by NT-3 in cells expressing TrkC

PC12 cells transiently overexpressing TrkC, the receptor for NT-3, exhibited a 2-fold induction of the NPY promoter (Table 1). We have shown previously that a naturally occurring 14-amino-acid tyrosine kinase insert in TrkC blocks the ability of this receptor to form a high-affinity complex with both Shc and PLC$\gamma$ [16]. If the high-affinity binding of both Shc and PLC$\gamma$ is required to mediate the activation of the NPY promoter by NT-3, then we would predict that the TrkC.ki14 variant would be ineffective at stimulating the NPY promoter. The results shown in Table 1 demonstrate that this was indeed the case.

### MAP kinase, but not protein kinase C, is necessary for activation of the NPY promoter by BDNF

The tyrosine phosphorylation of Shc ultimately results in the activation of Ras and the MAP kinase cascade. Therefore we investigated the effect of inhibition of MAP kinase activation on activation of the NPY promoter by BDNF. To do this, we took advantage of PD98059, a membrane-permeant inhibitor of MAP kinase kinase activation by Raf-1 [38]. As the transfection efficiency of PC12 cells is very low (< 5%; A. G. Williams, unpublished work), we could not demonstrate directly that this inhibitor blocked MAP kinase activation by BDNF under the conditions used. We thus investigated the effect of PD90859 on activation of the Elk-1 transcription factor by BDNF. It is well established that trans-activation of a GAL4–Elk-1 fusion protein by growth factors is dependent on MAP kinase phosphorylation of serine-383 of Elk-1 [39]. As clearly demonstrated in Table 2,

**Table 3 MAP kinase, but not protein kinase C, is involved in the activation of the NPY promoter by BDNF**

PC12 cells were transiently transfected with pNPY.Luc, pRL.SV40 and pcDNAneo containing wild-type TrkB. The cells were preincubated for 30 min with 50 $\mu$M PD98059 or 100 nM Ro-31-8220 as indicated, and then for 20 h with or without BDNF (100 ng/ml). The relative levels of firefly luciferase (Luc) and *Renilla* luciferase (Ren) were measured in cell lysates. Results are means $\pm$ S.E.M.; $^*P < 0.05$ compared with control. †Indicates not significantly different from BDNF-stimulated condition.

| Conditions | Ratio (Luc/Ren) |
|---|---|
| Control | $0.15 \pm 0.04$ |
| BDNF | $0.43 \pm 0.08^*$ |
| BDNF + PD98059 | $0.03 \pm 0.003$ |
| BDNF + Ro-31-8220 | $0.51 \pm 0.06$† |

**Table 4 PMA-stimulated NPY gene expression is blocked by Ro-31-8220**

PC12 cells were transiently transfected with pNPY.Luc, preincubated for 30 min with 100 nM Ro-31-8220 as indicated, and then treated for 20 h with or without 1.6 $\mu$M PMA. The subsequent cell lysates were assayed for the level of firefly luciferase expression. Results are means $\pm$ S.E.M.; $^*P < 0.005$ compared with control. Activity is expressed as relative light units (RLU).

| Conditions | Luciferase activity (RLU) |
|---|---|
| Control | $10.40 \pm 2.19$ |
| PMA | $28.12 \pm 0.38^*$ |
| PMA + Ro-31-8220 | $12.72 \pm 3.83$ |

this inhibitor almost completely blocked the effect of BDNF on the activation of a GAL4-Elk-1 fusion protein.

Having confirmed that PD90859 blocks Elk-1 activation, and thus the stimulation of MAP kinase by BDNF, we next investigated its effect on the activation of the NPY promoter by BDNF. Again, as shown in Table 3, this inhibitor completely blocked activation of the NPY promoter by BDNF. Indeed, the activity of the NPY promoter was reduced to levels considerably lower than were observed under basal conditions (Table 3). These data demonstrate that MAP kinase is necessary for activation of the NPY promoter by BDNF, consistent with the effect of the Y484F mutation, which blocked Shc binding (Figure 1). The data also suggest that there is a tonic level of MAP kinase activation in the basal state, which is blocked by PD98059 and which is responsible for the elevated basal NPY promoter activity that we observe.

The role of protein kinase C as a potential downstream effector of PLC$\gamma$ signalling (i.e. diacylglycerol production) was also investigated, as it has been reported previously that calphostin C blocked activation of the NPY promoter by NGF in PC12 cells [11]. The phorbol ester PMA, which activates protein kinase C, caused an approx. 3-fold stimulation of the NPY promoter (Table 4). This effect was completely prevented by the protein kinase C inhibitor Ro-31-8220 at 100 nM (Table 4). We have previously reported that this concentration of Ro-31-8220 blocks PMA- but not neurotrophin-stimulated MAP kinase activation [36]. Under the conditions used, however, this inhibitor had no effect on the ability of BDNF to activate the NPY promoter (Table 3), ruling out a role for protein kinase C in mediating this effect.

It is not clear why we found that TrkB-mediated NPY promoter activation does not require protein kinase C, whereas

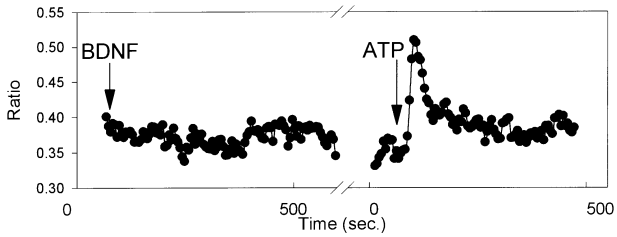


**Figure 2 BDNF does not promote a rise in the intracellular $Ca^{2+}$ concentration**

PC12 cells were microinjected with pCMX.GFP and pcDNAneo.TrkB, and then imaged 24 h later by confocal microscopy as described in the Materials and methods section. Cells expressing TrkB were identified by the expression of GFP in single-photon mode (excitation 488 nm; emission 515 nm) and then the intracellular $Ca^{2+}$ concentration was measured using indo-1 in two-photon mode (excitation 720 nm; emission 485 nm). The data for a representative GFP/TrkB-expressing cell are shown. BDNF (100 ng/ml) was added and the cell imaged over a 500 s period. Then the BDNF was removed and the cell was allowed to recover, before addition of ATP (100 $\mu$M) and imaging for another 500 s period. The data are shown as the ratio of indo-1 fluorescence (405/485 nm).

TrkA-dependent activation by NGF was apparently blocked by calphostin C [11]. However, calphostin C, which has an $IC_{50}$ for protein kinase C of 50 nM [40], was used in the previous study at a high concentration (20 $\mu$M [11]) and thus may have had a non-specific effect on another protein kinase. Alternatively, TrkA- but not TrkB-mediated activation of the NPY promoter requires protein kinase C. While we cannot exclude the possibility that BDNF activates an Ro-31-8220-insensitive isoform of protein kinase C, we have been unable to demonstrate any change in protein kinase C activity in response to NGF in PC12 cells, or any effect of calphostin C (5–500 nM) on BDNF- or NGF-stimulated NPY gene expression (A. G. Williams, unpublished work).

## BDNF does not increase the intracellular $Ca^{2+}$ concentration in PC12 cells expressing TrkB

As the other product of PtdIns$P_2$ hydrolysis by PLC$\gamma$ is Ins(3,4,5)$P_3$, we investigated whether an increase in intracellular $Ca^{2+}$ could mediate the effect of BDNF on the NPY promoter. To do this, we co-microinjected PC12 cells with two plasmids, one to allow overexpression of TrkB and the other carrying GFP. Cells expressing TrkB were identified by their bright green fluorescence during excitation with a 488 nm argon-ion laser. These cells were loaded with the $Ca^{2+}$-sensitive dye indo-1, and the intracellular levels of $Ca^{2+}$ were measured over time using two-photon confocal microscopy. Despite the fact that cells co-expressing TrkB and GFP exhibit BDNF-induced neurite outgrowth (A. G. Williams, unpublished work), the addition of BDNF failed to increase intracellular $Ca^{2+}$ concentrations under conditions where ATP elicited a rapid rise via a $P_{2\mu}$-purinergic-dependent mechanism (Figure 2). In addition, NGF addition had no detectable effect on $Ca^{2+}$ levels in either GFP-expressing cells or non-transfected cells (A. G. Williams, unpublished work). These data rule out the possibility that changes in intracellular $Ca^{2+}$ are involved in activation of the NPY promoter by BDNF.

## Concluding remarks

The data described in the present study demonstrate that at least two signalling pathways must be activated simultaneously by TrkB to elicit BDNF-stimulated NPY gene expression, as

measured via the activation of an NPY promoter/reporter construct. We propose that one is a Shc-dependent pathway, which is initiated by the binding of Shc to phosphotyrosine-484 on TrkB; this results in the activation of MAP kinase, which is also required in order for BDNF to stimulate the NPY promoter. The other is initiated by tyrosine-785, which has been identified previously as a PLC$\gamma$ binding site. However, as we can find no role for either $Ca^{2+}$ or an Ro-31-8220-inhibitable protein kinase C in BDNF-mediated activation of the NPY promoter, the effect may be mediated by another, hitherto unidentified, protein that interacts at this site; such a protein may possess an SH2 domain. The issue of whether neurotrophins regulate protein kinase C activity or intracellular $Ca^{2+}$ concentration in PC12 cells thus remains controversial (see Introduction section). The controversy could be explained by the use of different clonal populations of the cells by the various groups.

Quite how, and where, these two signalling pathways ultimately converge to stimulate the NPY promoter is not known. There are two potential regulatory elements within the $-750$ to $-50$ NPY promoter fragment used in this study, i.e. the AP-1 and AP-2 sites. It is possible that the two pathways independently activate the transcriptional apparatus that binds to each of these elements. Alternatively, they could converge at a more proximal step, such as the activation of MAP kinase.

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## REFERENCES

1 Tatemoto, K., Carlquist, M. and Mutt, V. (1982) Nature (London) **269**, 659–660
2 Schwartz, M. W., Seeley, R. J., Campfield, L. A., Burn, P. and Baskin, D. G. (1996) J. Clin. Invest. **98**, 1101–1106
3 Minth-Worby, C. A. (1994) J. Biol. Chem. **269**, 15460–15468
4 Allen, J. M., Martin, J. B. and Heinrich, G. (1987) Mol. Brain Res. **3**, 39–43
5 Greene, L. A. and Tischler, A. S. (1976) Proc. Natl. Acad. Sci. U.S.A. **73**, 2424–2428
6 Milbrandt, J. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 4789–4793
7 Hawley, R. J., Scheibe, R. J. and Wagner, J. A. (1992) J. Neurosci. **12**, 2573–2581
8 Machida, C. M., Scott, J. D. and Ciment, G. (1991) J. Cell Biol. **114**, 1037–1048
9 Higuchi, H., Nakano, K. and Miki, N. (1992) Biochem. Biophys. Res. Commun. **189**, 1553–1560
10 Sabol, S. and Higuchi, H. (1990) Mol. Endocrinol. **4**, 384–392
11 Balbi, D. and Allen, J. M. (1994) Mol. Brain Res. **23**, 310–316
12 Kalman, D., Wong, B., Horvai, A. E., Cline, M. J. and OLague, P. H. (1990) Neuron **2**, 355–366
13 Ginty, D. D., Fanger, G. R., Wagner, J. A. and Maue, R. A. (1992) J. Cell Biol. **116**, 1465–1473
14 Damon, D. H., D'Amore, P. A. and Wagner, J. A. (1990) J. Cell Biol. **110**, 1333–1339
15 Guiton, M., Gunn-Moore, F. J., Stitt, T. N., Yancopoulos, G. D. and Tavaré, J. M. (1994) J. Biol. Chem. **269**, 30370–30377
16 Guiton, M., Gunn-Moore, F. J., Glass, D. J., Geis, D. R., Yancopoulos, G. D. and Tavaré, J. M. (1995) J. Biol. Chem. **270**, 20384–20390
17 Middlemas, D. S., Meisenhelder, J. and Hunter, T. (1994) J. Biol. Chem. **269**, 5458–5466
18 Obermeier, A., Halfter, H., Wiesmuller, K. H., Jung, G., Schlessinger, J. and Ullrich, A. (1993) EMBO J. **12**, 933–941
19 Obermeier, A., Lammers, R., Wiesmuller, K. H., Jung, G., Schlessinger, J. and Ullrich, A. (1993) J. Biol. Chem. **268**, 22963–22966
20 Traverse, S., Gomez, N., Paterson, H., Marshall, C. and Cohen, P. (1992) Biochem. J. **288**, 351–355
21 Young, S. W., Dickens, M. and Tavaré, J. M. (1994) FEBS Lett. **338**, 212–216
22 Marshall, C. J. (1995) Cell **80**, 179–185
23 Baxter, R. M., Cohen, P., Obermeier, A., Ullrich, A., Downes, C. P. and Doza, Y. N. (1995) Eur. J. Biochem. **234**, 84–91
24 Stephens, R. M., Loeb, D. M., Copeland, T. D., Pawson, T., Greene, L. A. and Kaplan, D. R. (1994) Neuron **12**, 691–705
25 Heasley, L. E. and Johnson, G. L. (1989) J. Biol. Chem. **264**, 8646–8652
26 Debernardi, M. A., Rabin, S. J., Colangelo, A. M., Brooker, G. and Mocchetti, I. (1996) J. Biol. Chem. **271**, 6092–6098.
27 Zirrgiebel, U., Ohga, Y., Carter, B., Berninger, B., Inagaki, N., Thoenen, H. and Lindholm, D. (1995) J. Neurochem. **65**, 2241–2250
28 Pandiella-Alonso, A., Malgaroli, A., Vicentini, L. M. and Meldolesi, J. (1986) FEBS Lett. **208**, 48–52
29 Nikodijevic, B. and Guroff, G. (1991) J. Neurosci. Res. **28**, 192–199
30 Dickens, G., Lavarreda, M., Zheng, W. H. and Guroff, G. (1997) J. Neurosci. Res. **47**, 271–276
31 Bush, A. B., Borden, L. A., Greene, L. A. and Maxfield, F. R. (1991) J. Neurochem. **57**, 562–574
32 Ohmichi, M., Zhu, G. and Saltiel, A. R. (1993) Biochem. J. **295**, 767–772
33 Obermeier, A., Bradshaw, R. A., Seedorf, K., Choidas, A., Schlessinger, J. and Ullrich, A. (1994) EMBO J. **13**, 1585–1590
34 Lamballe, F., Tapley, P. and Barbacid, M. (1993) EMBO J. **12**, 3083–3094
35 Valenzuela, D. M., Maisonpierre, P. C., Glass, D. J., Rojas, E., Nunez, L., Kong, Y., Gies, D. R., Stitt, T. N., Ip, N. Y. and Yancopoulos, G. D. (1993) Neuron **10**, 963–974
36 Gunn-Moore, F. J., Willams, A. G. and Tavaré, J. M. (1997) Biochem. J. **322**, 193–198
37 Canossa, M., Rovelli, G. and Shooter, E. M. (1996) J. Biol. Chem. **271**, 5812–5818.
38 Alessi, D. R., Cuenda, A., Cohen, P., Dudley, D. T. and Saltiel, A. R. (1995) J. Biol. Chem. **270**, 27489–27494
39 Treisman, R. (1994) Curr. Opin. Gen. Dev. **4**, 96–101
40 Kobayashi, E., Nakano, H., Morimoto, M. and Tamaoki, T. (1989) Biochem. Biophys. Res. Commun. **159**, 548–553

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