

**Inhibition of neurite outgrowth in differentiating mouse N2a neuroblastoma cells by phenyl saligenin phosphate: Effects on MAP kinase (ERK 1/2) activation, neurofilament heavy chain phosphorylation and neuropathy target esterase activity**

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*Abbreviations:* DFP, diisopropyl phosphorofluoridate; ERK, extracellular signal-regulated kinase; MAP kinase, mitogen-activated protein kinase; NFH, neurofilament heavy chain; NTE, neuropathy target esterase; OP, organophosphate; OPIDN; OP-induced delayed neuropathy; PSP, phenyl saligenin phosphate; TOCP, tri*ortho* cresyl phosphate; SCOTP, saligenin cyclic- O-tolyl phosphate; TPCP, tri*para* cresyl phosphate.

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## ABSTRACT

Sub-lethal concentrations of the organophosphate phenyl saligenin phosphate (PSP) inhibited the outgrowth of axon-like processes in differentiating mouse N2a neuroblastoma cells ( $IC_{50}$  2.5  $\mu$ M). A transient rise in the phosphorylation state of neurofilament heavy chain (NFH) was detected on Western blots of cell extracts treated with 2.5  $\mu$ M PSP for 4 h compared to untreated controls, as determined by a relative increase in reactivity with monoclonal antibody Ta51 (anti-phosphorylated NFH) compared to N52 (anti-total NFH). However, cross-reactivity of PSP-treated cell extracts was lower than that of untreated controls after 24 h exposure, as indicated by decreased reactivity with both antibodies. Indirect immunofluorescence analysis with these antibodies revealed the appearance of neurofilament aggregates in the cell bodies of treated cells and reduced axonal staining compared to controls. By contrast, there was no significant change in reactivity with anti- $\alpha$  tubulin antibody B512 at either time point. The activation state of the MAP kinase ERK 1/2 increased significantly after PSP treatment compared to controls, particularly at 4 h, as indicated by increased reactivity with monoclonal antibody E-4 (anti-phosphorylated MAP kinase) but not with polyclonal antibody K-23 (anti-total MAP kinase). The observed early changes were concomitant with almost complete inhibition of the activity of neuropathy target esterase (NTE), one of the proposed early molecular targets in organophosphate-induced delayed neuropathy (OPIDN).

*Keywords:* Phenyl saligenin phosphate; Mouse N2a neuroblastoma; Axon outgrowth; Neurofilament heavy chain; Neuropathy target esterase; MAP kinase (ERK1/2).

## 1. Introduction

Certain organophosphate compounds (OPs) are capable of inducing a delayed neurodegenerative condition in central and peripheral nervous system of man, birds and livestock [1-6]. This condition, which may arise up to 3 weeks after exposure to neuropathic OPs, is known as organophosphate-induced delayed neuropathy (OPIDN). Studies on animal models have shown that the onset of clinical signs of the disease is preceded by inhibition of neuropathy target esterase (NTE) and also by cytoskeletal disruption [4,5,7-10], suggesting that these lesions may represent key molecular events in the development of this delayed neurodegenerative condition. The ability to block the neurodegenerative effects of neuropathic OPs by  $\text{Ca}^{2+}$  channel blockers and calpain inhibitors also suggests that increased intracellular  $\text{Ca}^{2+}$  levels and calpain activation may be involved [11]. However, the precise molecular basis of OPIDN is poorly understood.

For many years NTE has been regarded as the primary target for neuropathic OPs, although its inhibition in non-neuronal cells and tissues that are not damaged has raised doubts about a direct role for its esterase activity in the pathogenesis of OPIDN. However, it has been shown that the ability to inhibit NTE alone is not sufficient to induce the chain of events leading to OPIDN. Indeed, a modification that involves an increase in negative charge at the active site is proposed to occur before the induction of OPIDN, a phenomenon known as ageing [5,12,13]. Furthermore, recent work suggests that NTE may have important non-esterase functions, the alteration of which may in some way trigger a series of molecular events leading to  $\text{Ca}^{2+}$  release and calpain activation [13].

A number of other studies have shown that there is a major rearrangement of the axonal cytoskeleton and substantial changes in the phosphorylation states of key cytoskeletal proteins in

microtubule and neurofilament networks within 24 hours of exposure to neuropathic OPs [1, 7-10]. However, the ability of OPs to disrupt specific cell signalling pathways has not yet been thoroughly investigated. Moreover, there is disagreement as to the exact nature of the changes [8,9] and the relationship between NTE inhibition and cytoskeletal alterations remains to be established.

Detailed *in vivo* studies of the molecular basis of OPIDN are hampered by the costly and time-consuming nature of experiments on large numbers of laboratory animals. This has led to the development of *in vitro* alternatives to animal studies and, in particular, the use of cultured nerve cells [14]. One approach has been to measure the effects of neuropathic and non-neuropathic OPs on NTE activity in neuroblastoma cell lines [15-18], which is potentially very useful in the screening of OPs for their potential to cause OPIDN. A second approach has been to study the effects of neuropathic and non-neuropathic OPs on the outgrowth of neurites by neuronal and glial cells induced to differentiate *in vitro* [19-23]. Interestingly, the neuropathic properties of protoxicants can be enhanced in cultured cells either by bromination [15,16] or by co-incubation with NADPH-activated rat liver microsomes [15,16,20], suggesting that *in vitro* cellular models are able to simulate at least some of the neurodegenerative effects of OPs *in vivo*. Furthermore, in a number of the above mentioned studies the authors have reported selective inhibition of neurite outgrowth by neuropathic OPs, although the involvement of NTE in the inhibition of neurite outgrowth remains to be established.

Our work has focused on the sub-lethal effects of OPs on the outgrowth of axon-like processes by differentiating N2a cells. Our previous findings show that the neuropathic *ortho* isomer of tricresyl phosphate (TOCP) inhibits axon outgrowth to a greater extent than the non-neuropathic isomer tri*paracresyl* phosphate [20]. This effect, which is most evident following shorter

exposure times, is enhanced by incubation in the presence of NADPH-activated microsomes. Since TOCP is thought to be metabolised into active (in terms of ability to induce OPIDN) congeners such as saligenin cyclic-*O*- tolyl phosphate (SCOTP) [24-27], our findings suggest that differentiating N2a neuroblastoma cells represent a useful model for mechanistic studies of the neurodegenerative effects of OPIDN-inducing OPs. As the exact nature of the microsomal activation products *in vitro* is not known, it is important to show that known *in vivo* metabolites are able to induce neuropathic changes in this cell model. The work presented here focuses on the effects of phenyl saligenin phosphate (PSP; a neuropathic homologue of SCOTP) on neurite outgrowth, cytoskeletal proteins, NTE activity and MAP kinase activation *in vitro*.

## **2. Materials and methods**

### *2.1. Cell lines and reagents*

Mouse N2a neuroblastoma cells were purchased from ICN (Thane, UK). Cell culture plastics were supplied by SLS Laboratory Supplies (Nottingham, UK). Cell culture reagents and monoclonal antibodies against  $\alpha$ -tubulin (clone B512) and NFH (clone N52) were obtained from Sigma-Aldrich Co. Ltd. (Poole, UK). Mouse anti-phospho ERK 1/2 (E-4) and rabbit anti-total ERK 1/2 (K-23) were purchased from Santa Cruz Biotechnology (Santa Cruz, USA). Fluorescein isothiocyanate (FITC)-conjugated and alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin and goat anti-rabbit immunoglobulin secondary antibodies were obtained from DakoCytomation (Ely, UK). Monoclonal antibody Ta51 hybridoma culture supernatant [28] was a gift from Dr M. Carden (Biological Laboratory, University of Kent).

Phenyl saligenin phosphate (PSP) synthesis was based on the method of SCOTP synthesis by Nomeir and Abou-Donia [29] using phenyl phosphorodichloridate in place of *o*-cresyl phosphorodichloridate. Chemical purity was confirmed by NMR and mass spectrometry.

### *2.2. Maintenance of cultured cells*

The cells were grown in T-25 flasks at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub> - 95% air, in Dulbecco's Modified Eagle's Medium (DMEM) containing 10% fetal bovine serum, 1 mM glutamine, penicillin G (100 units/ml) and streptomycin (100 µg/ml). They were maintained in the logarithmic phase of growth and passaged at 4-5 day intervals.

### *2.3. Induction and assessment of N2a cell differentiation*

Cells were taken at 60 - 80 % confluence, seeded in 24-well culture dishes at a density of 50,000 cells/ml in 0.5 ml growth medium, and induced to differentiate by serum withdrawal and the addition of dibutyryl cAMP, as described previously [19,20,30]. They were then incubated for up to 24 h in the presence and absence of PSP to yield various final concentrations, as indicated in Results. Cell growth and viability were assessed under all experimental conditions employed by the reduction of methyl blue tetrazolium using the method of Denizot and Lang [31].

For morphological assessment, at the end of the incubation period the cells were fixed and stained with Coomassie brilliant blue and the outgrowth of axon-like processes was determined using an inverted light microscope, as described previously [19,20,30]. This involved the quantification of axon-like processes, defined according to Keilbaugh et al. [32] as neurites greater than 2 cell body diameters in length. Small extensions were defined as outgrowths between 0.5 and 2 cell body diameters in length and the numbers of cells with round or flattened morphology were also determined.

### *2.4. Western blot analysis*

For immunoblot analysis, N2a cells were induced to differentiate in the presence and absence of PSP as described above, except that they were seeded in to 10 ml of growth medium in T-25 flasks. Cell monolayers were then solubilised by boiling in electrophoresis sample buffer comprising 62.5 mM Tris, 2% w/v sodium dodecyl sulphate (SDS), 10% w/v glycerol, 5% w/v  $\beta$ -mercaptoethanol and 0.002% w/v bromophenol blue or in 0.5% w/v SDS. Protein was estimated on acetone-precipitated cell extracts by the method of Lowry *et al.* [33] with minor modifications, using bovine serum albumin (BSA) as the standard. Equal amounts of cell protein

were subjected to gel electrophoresis in the presence of SDS (SDS-PAGE) using a 7.5% polyacrylamide resolving gel overlaid with a 4% stacking gel [34].

Separated proteins were then electrophoretically transferred onto nitrocellulose membrane filters [35]. The resultant Western blots were blocked with 3% w/v BSA in Tris-buffered saline (BSA/TBS) and then probed overnight at 4°C with appropriate dilutions of primary antibodies in BSA/TBS [20]. These included mouse monoclonal anti- $\alpha$ -tubulin (B512), mouse monoclonal anti-total neurofilament heavy chain (N52) or rat monoclonal anti-phosphorylated neurofilament heavy chain (Ta51). Alternatively, they were probed with mouse monoclonal anti-phosphorylated ERK1/2 MAP kinase (clone E-4) or rabbit polyclonal anti-total ERK 1/2 (K-23). After washing extensively with TBS/Tween-20, blots were probed with appropriate dilutions of secondary antibodies and enzyme substrates, as described previously [20]. Antibody reactivity on developed blots was quantified densitometrically using Quantiscan software (BioSoft) and values expressed as a percentage of their corresponding controls. The phosphorylation status of ERK 1/2 was also determined as the ratio between the levels of reactivity with anti-phosphorylated ERK and anti-total ERK compared to controls.

### *2.5. Indirect immunofluorescence staining*

N2a cells were induced to differentiate for 24 h in the presence and absence of 2.5  $\mu$ M PSP. They were then fixed with methanol and stained with antibodies that recognise total neurofilament heavy chain (NFH) independent of its phosphorylation state (clone N52) or NFH phosphorylated in its KSP domain (clone Ta51) followed by FITC-labelled secondary antibodies, as described previously [19].



## *2.6. Measurement of neuropathy target esterase activity*

For assays of cellular NTE activity, cells were induced to differentiate as above, except that they were seeded into 40 ml of growth medium in T-75 cell culture flasks. At the end of the incubation period, cells were harvested by centrifugation at 1200 g for 10 min, and then washed by resuspension and recentrifugation in PBS. Pellets were stored at - 20°C until required. NTE activity was determined in homogenates of control (untreated) and PSP-treated cells by the absorbance difference for phenyl valerate hydrolysis between samples exposed to paraoxon only and those exposed to both paraoxon and mipafox, as previously described [36].

## *2.7. Statistical analysis*

Unless indicated otherwise, axon outgrowth, NTE activity and densitometric peak areas of bands revealed on probed Western blots of cells/cell extracts following exposure to OPs were expressed as a percentage of control values  $\pm$  S.E.M. When appropriate, the statistical significance of differences from corresponding controls was established using the Mann-Whitney U-test for non-parametric distributions or by ANOVA, with 95% confidence limits.

## 2. Results

Since our previous work suggested that metabolic activation of TOCP could enhance its ability to inhibit the outgrowth of axon-like processes [20], it was of interest to determine the direct effects of active congeners of TOCP on neurite outgrowth in the present work. Experiments were therefore carried out with PSP, a structural analogue of SCOTP that has the ability to cause OPIDN *in vivo* without the need for metabolic activation [37]. Preliminary work (not shown) indicated that PSP inhibited the outgrowth of axon-like processes at various exposure times between 4 and 48 h, with IC<sub>50</sub> values all within the range of 2.2 to 3.0 μM. Accordingly, a concentration of 2.5 μM was adopted for subsequent experiments. As indicated in Fig. 1, there was an apparent decrease in the outgrowth of neurites compared to controls in cells induced to differentiate for 4 and 24 h in the presence of 2.5 μM PSP. Similar effects were observed at 8 h and 48 h time points (not shown). Quantitative assessment of cell morphology following treatment with this concentration of PSP confirmed that there was a statistically significant reduction in the outgrowth of axon-like processes compared to controls (Table 1). By contrast, no significant effect was observed on the number of smaller neurites (extensions) or on the proportion of round and flat cell (Table 1).

In order to study the molecular basis of these effects in more detail, Western blots of extracts from cells induced to differentiate in the presence and absence of 2.5 μM PSP were probed with antibodies that recognise key proteins involved in the process of neurite outgrowth. These included antibodies against NFH (clones N52 and Ta51, which recognise total NFH and phosphorylated NFH, respectively), α-tubulin (clone B512) and MAP kinase (ERK1/2; monoclonal antibody E-4 and polyclonal antibody K-23, which recognise activated and total ERK 1/2, respectively). As shown in Fig. 2 and Table 2, cross-reactivity of an approximately 200

kDa polypeptide with N52 (which recognises NFH independently of its phosphorylation state) shows a slight but significant decrease compared to controls at 4 h and after 24 h exposure. By contrast, reactivity with Ta51 (which recognises a phosphorylated epitope in the KSP domain of NFH) was significantly higher than control values at 4 h, followed by a decline compared to control values at 24 h exposure. By contrast, reactivity of cell extracts with monoclonal anti-tubulin antibody B512 showed no significant difference from controls at either time point.

In order to determine whether the observed relative increase in reactivity with Ta51 compared to N52 in PSP-treated cell extracts reflected altered activation of MAP kinase (ERK 1/2), of which NFH is a known substrate during neuronal cell differentiation [38,39], blots were also probed with antibodies against activated and total ERK. As shown in Fig. 2 and Table 2, total ERK levels were slightly lower than controls at 4 h exposure but showed no significant change from controls at 24 h. By contrast, reactivity of cell extracts with anti-phospho ERK antibody was 240% of control values at 4 h and 157% of control values at 24 h, corresponding to phosphorylation ratios of 3.6 and 1.6, respectively.

The possibility that the observed change in NFH phosphorylation in PSP-treated cells might cause neurofilament disruption was further investigated by indirect immunofluorescence staining. As shown in Fig. 3, differentiated cells exhibited relatively strong axonal staining (vertical arrows) with both monoclonal antibodies N52 and Ta51. By contrast, PSP-treated cells showed relatively strong aggregate staining (horizontal arrows) in the cell body with both antibodies and staining of axon-like processes was relatively weak.

Since NTE inhibition is thought to be an early lesion occurring within hours of exposure to PSP both *in vivo* and *in vitro*, but its relationship to cytoskeletal changes or cell signalling is not yet

known, it was of interest to measure NTE activity at early time points in differentiating N2a cells. Indeed, the activity of NTE in extracts from differentiating N2a cells exposed for 4 h or 8 h to 2.5  $\mu$ M PSP was greatly reduced to  $2.6 \pm 1.4$  % and  $6.3 \pm 4.5$  % of the corresponding control values, respectively (4 independent experiments).

#### 4. Discussion

The work presented here shows that PSP is able to inhibit the outgrowth of axon-like processes by differentiating N2a cells with no significant effect on the outgrowth of smaller processes or cell shape. Since PSP is an analogue of one of the main neuropathic metabolites of TOCP [37], the observed inhibition of neurite outgrowth is in good agreement with our previous work, in which the neuropathic effect of TOCP *in vitro* was enhanced by metabolic activation with NADPH activated rat liver microsomes [20]. It is also consistent with the findings from *in vivo* studies of the pathogenesis of OPIDN, in which PSP and other metabolites of TOCP are more effective than TOCP in the induction of OPIDN [1,2,20,21,23,32]. The fact that the longer (i.e. "axon-like") processes are more sensitive to PSP treatment *in vitro* agrees with previous studies using TCP (mixed isomers) and TOCP, in which the outgrowth of smaller processes was also unaffected [19,20]. These observations are also consistent with the finding that axons of longer neurons tend to be more vulnerable to neuropathic OPs *in vivo* [1].

Interestingly, the observation of decreased reactivity of PSP-treated cell extracts with N52 is in agreement with our previous findings for TOCP [20]. The fact that it is also in agreement with a more recent *in vivo* study using sciatic nerves of hens treated with TOCP [40] indicates that this cellular model is capable of predicting the pattern of *in vivo* delayed toxicity of OPs. Since the epitope recognised by N52 is independent of its phosphorylation state, this suggests that there may be a decrease in the levels of NFH at this time point. This could be attributed to increased degradation of NFH by proteases such as cathepsin D or calpain [41,42]. Indeed, activation of calpain has been shown to occur in response to OP treatment *in vivo* [11]. Alternatively, the reduced reactivity could be caused by decreased synthesis of NFH. In this respect, it has been shown that the OP pesticide chlorpyrifos is able to reduce the levels of DNA and protein

synthesis *in vivo* [43]. However, the OP compound diisopropyl phosphorofluoridate (DFP) causes an increase in levels of neurofilament mRNA [44], suggesting that some OPs are able to induce expression of NFH, although the levels of protein were not measured in that work. It may therefore be that structurally different groups of OPs are able to inhibit neurite outgrowth through a variety of mechanisms.

Quantitative analysis of Western blots probed with Ta51, indicated that PSP induced a significant increase in reactivity concomitant with reduced levels of N52 reactivity (total NFH) at the 4-hour time point. Since Ta51 recognises NFH in a phosphorylation- dependent manner and the binding of N52 is independent of phosphorylation state, this result indicates an increase in the phosphorylation state of NFH at this time point. The subsequent decrease in Ta51 reactivity following 24 h exposure suggests that the increase in NFH phosphorylation at 4 h is transient. Furthermore, the levels of reactivity at 24 h exposure are similar to those found in TOCP-treated cells [20] indicating that sustained inhibition of neurite outgrowth is associated with reduced levels of NFH.

The fact that PSP but not TOCP, TCP or TPCP causes a transient increase in NFH phosphorylation suggests that PSP may have some distinct molecular targets compared to the other compounds and that increased NFH phosphorylation may require metabolic activation of TOCP. As mentioned in our previous work, smaller increases in the phosphorylation state of NFH may be masked by proteolytic degradation. Interestingly, Suwita *et al.* [10] demonstrated an increased level of *in vitro* Ca<sup>2+</sup>/calmodulin activated protein kinase-dependent phosphorylation of NFH in spinal cord neurofilaments isolated from TOCP-treated hens, suggesting that altered phosphorylation of NFH and other cytoskeletal proteins was an early event in the pathogenesis of OPIDN. This suggests that increased phosphorylation of

cytoskeletal proteins is a key early event following exposure to TOCP and, presumably, its activated metabolites *in vivo*. However, work by Jortner et al. [9] suggested that reactivity of the phosphorylated NFH specific antibody SMI31 with PSP-treated hen spinal cord neurofilaments was lower than controls between 4 days exposure and the onset of clinical OPIDN. This suggests that, if it occurs *in vivo*, the OP-induced increase in phosphorylation of NFH observed by Suwita et al. [10] is not sustained for more than a few days.

The changes in NFH phosphorylation suggested that disruption of cell signalling pathways might occur following exposure to PSP. It was of particular interest to study the effects of this OP on the activation of the MAP kinase ERK 1/2, since this protein kinase is a convergence point of cell signalling events involved in neurite outgrowth and it is known to phosphorylate NFH [38,39]. The increase in reactivity of anti-activated (phosphorylated) ERK but not anti-total ERK antibody with cell extracts following 4 h exposure to PSP suggests that PSP treatment leads to increased ERK activation, which may account for the increased level of NFH phosphorylation at this time point. Reactivity with probed blots of extracts exposed to PSP for 24 h indicates a smaller increase in activation over control levels at this time point than at 4 h, possibly due to phosphatase activity or negative feedback phosphorylation of MAP kinase kinases such as MEK [45]. Thus, it may be that the decreased levels of NFH phosphorylation at 24 h occurs as a combination of both down regulation of ERK activation and the proteolytic degradation suggested earlier.

A recognised early target for OPIDN-inducing OPs is NTE, although a link between NTE inhibition and cytoskeletal disruption had not been previously established in the differentiating N2a model. The almost complete loss of NTE activity observed at the 4 h time point in PSP-treated differentiating N2a cells (Table 3) is similar to that observed by Ehrich *et al.* [46] who

used mitotic human neuroblastoma cells treated with 10  $\mu$ M PSP. This result confirms that NTE inhibition is another key early event following exposure of differentiating N2a cells to PSP. The precise way in which NTE inhibition and ageing are related to the observed changes in MAP kinase activation and NFH levels and phosphorylation is not yet known. It has been suggested that endoplasmic reticulum-associated NTE may have the ability to hydrolyse lipids or act as an ion channel [47,48]. It may therefore be that the disruption of these properties is involved in the response to OP exposure in this cellular system.

In conclusion, the work presented in this paper shows that exposure of differentiating N2a cells to PSP causes selective inhibition of the outgrowth of axon-like processes. This is the first demonstration that inhibition of neurite outgrowth in a differentiating neuronal cell system is associated with reduced NTE activity (i.e. a proposed early molecular target of OPs *in vivo*). In addition, it is the first report of transiently enhanced phosphorylation of KSP domains on NFH and increased MAP kinase activation *in situ*. Further work on differentiating N2a cells will help to unravel the critical early molecular events following intoxication with neuropathic OP compounds.

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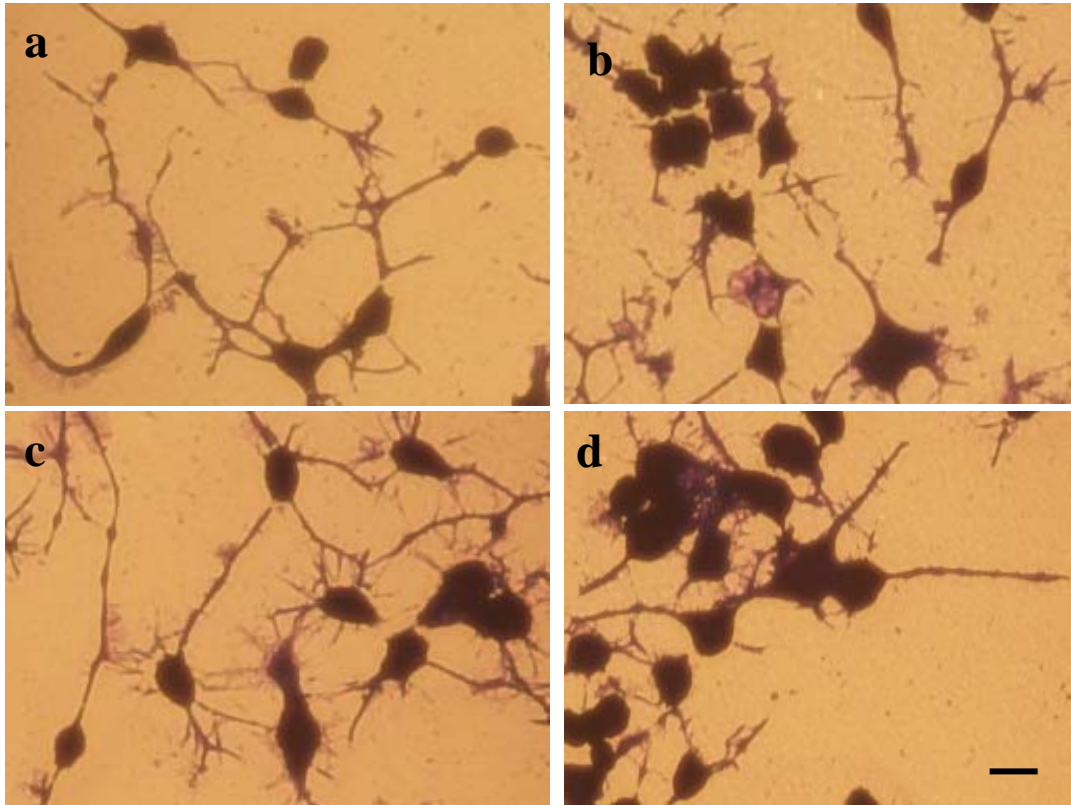
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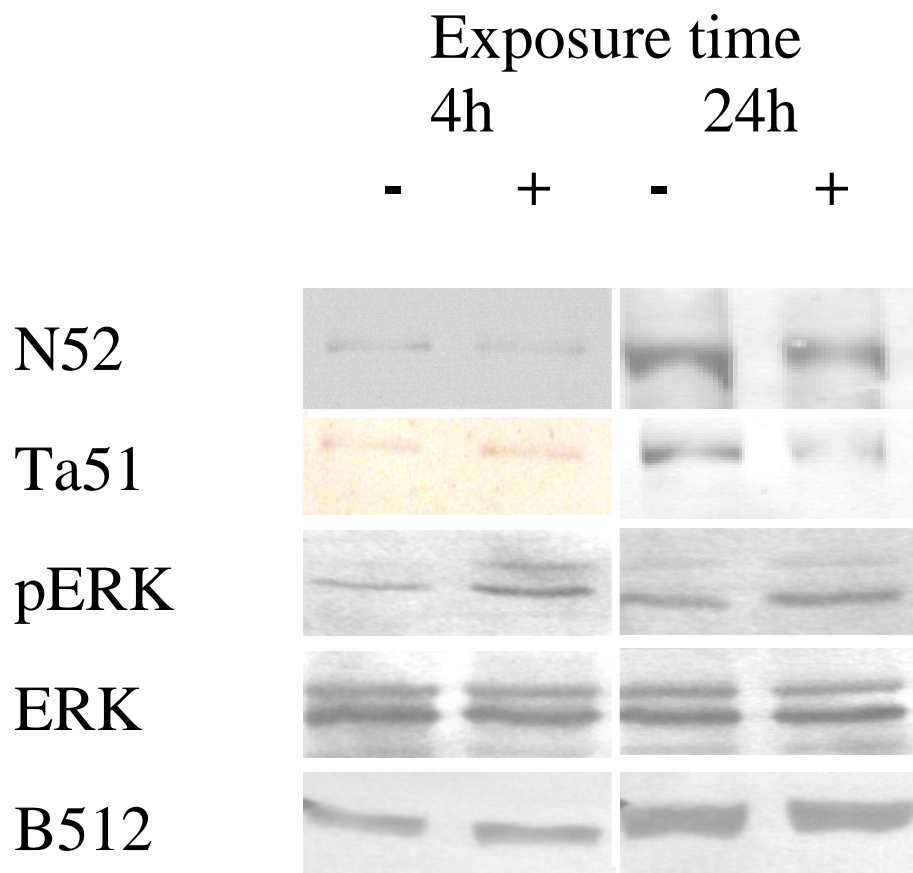
**Fig. 1 Effects of PSP on the morphology of differentiating N2a cells**

N2a cells were induced to differentiate for 4 h (a, b) or 24 h (c, d) in the presence (b, d) and absence (a, c) of 2.5  $\mu$ M PSP, as described in Materials and Methods. Shown are images of typical fields of cells viewed by phase contrast microscopy after 4 h differentiation (a, b) or using an inverted light microscope following methanol fixation and staining with Coomassie blue after 24 h differentiation (c, d). Bar represents 15  $\mu$ m.

<b>Incubation time</b>	<b>Treatment</b>	<b>Axons</b>	<b>Extensions</b>	<b>Round cells</b>	<b>Flat cells</b>
4h	CONTROL	32.35 ± 2.2	29 ± 1.1	16 ± 0.5	84 ± 0.5
	2.5 µM PSP	13.2 ± 1.4*	29 ± 1.1	15 ± 0.6	85 ± 0.6
24h	CONTROL	35.9 ± 6.5	24 ± 0.8	19 ± 0.8	81 ± 0.8
	2.5 µM PSP	18.5 ± 1.1*	28 ± 1.5	25 ± 2.0	75 ± 2.0

**Table 1 Effects of PSP on neurite outgrowth in differentiating N2a cells**

N2a cells were induced to differentiate for 4 or 24 h in the presence and absence of 2.5 µM PSP, stained with Coomassie blue and assessed for axon outgrowth, outgrowth of small extensions and round/flat cell morphologies, as described in Materials and methods. Data are expressed as number per 100 cells ± SEM from 4 independent experiments. Significance of differences from controls was determined by ANOVA (\*  $p < 0.05$ ).



**Fig. 2 Western blotting analysis of extracts from N2a cells induced to differentiate in the presence and absence of PSP.** N2a cells were induced to differentiate for 4 h or 24 h in the presence (+) and absence (-) of 2.5  $\mu$ M PSP, as indicated, after which extracts were solubilised, separated by SDS-PAGE and transferred onto nitrocellulose membrane filters as described in Materials and Methods. Shown are Western blots of equal amounts of control and PSP-treated cell protein probed with antibodies that recognise total NFH (N52), phosphorylated NFH (Ta51), activated ERK (pERK), total ERK (ERK) and total  $\alpha$ -tubulin (B512), followed by an appropriate alkaline phosphatase-conjugated secondary antibody.



	Exposure time	
	4 h	24 h
Antibody	Densitometric peak area (% control)	
N52	75.9 ± 10.4 *	74.2 ± 15.9
Ta51	168.2 ± 24.1*	66.5 ± 24.3
pERK	301.0 ± 27.4*	168.6 ± 7.6*
ERK	82.9 ± 6.8*	102.8 ± 6.9
B512	83.8 ± 11.2	104.7 ± 11.3

**Table 2 Effects of PSP on the levels of cytoskeletal proteins and ERK in N2a cells induced to differentiate in the presence and absence of PSP.** N2a cells were induced to differentiate in the presence (+) and absence (-) of 2.5 µM PSP for 4h or 24 h (as indicated), solubilised, separated by SDS-PAGE and subjected to Western blotting as described in Materials and Methods. They were then probed with antibodies against total NFH (N52), phosphorylated NFH (Ta51), activated ERK (pERK), total ERK (ERK) and total  $\alpha$ -tubulin (B512), as described in Materials and Methods. Densitometric analysis was performed using Quantiscan software. All values were normalised to the levels of  $\alpha$ -tubulin reactivity and are expressed as mean % control values  $\pm$  SEM for a minimum of 3 independent experiments. Asterisk indicates a significance of  $p < 0.05$  compared to the corresponding control.

**Fig. 3 Effects of PSP on neurofilament heavy chain distribution in differentiating N2a cells.**

N2a cells were induced to differentiate for 24 h in the presence (b, d) and absence (a, c) of 3  $\mu$ M PSP; they were then stained by indirect immunofluorescence using monoclonal antibodies N52 and Ta51, which recognise total NFH (a, b) and NFH phosphorylated in its KSP domain (c, d), respectively. Downward arrows indicate the position of axon-like processes, horizontal arrows indicate cell body accumulation of NFH staining and the bar represents 10  $\mu$ m.

