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HYPOXIA INDUCES NEURITE OUTGROWTH IN PC12 CELLS THAT IS MEDIATED THROUGH ADENOSINE A2A RECEPTORS

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Abstract—Development of the nervous system is a complex process, involving coordinated regulation of diverse cellular processes including proliferation, differentiation and synaptogenesis. Disturbances to brain development such as pre- and perinatal hypoxia have been linked to behavioural and late onset of neurological disorders. This study examines the effect of hypoxia on neurite outgrowth in PC12 cells. Hypoxia not only caused a rapid induction of neurite outgrowth, but also synergistically enhanced nerve growth factor (NGF)-induced neurite outgrowth up to 24 h. Transactivation of TrkA receptors was ruled out since the TrkA inhibitor K252a did not block hypoxia-induced neurite outgrowth. Adenosine deaminase prevented hypoxia-induced neurite outgrowth indicating that the effect is mediated by adenosine. Use of the specific adenosine A2A receptor agonist CGS21680 and antagonist 8–3(chlorostyryl)caffeine demonstrated that activation of this receptor is critical for hypoxia-induced neurite outgrowth. Hypoxia-induced neurite outgrowth was blocked by the adenylate cyclase inhibitor, MDL-12,330A, indicating a role for activation of this enzyme in the pathway. Hypoxia was further shown to cause a decrease in growth-associated protein (GAP)-43 levels and a lack of induction of βIII tubulin, in contrast to NGF treatment which resulted in increased cellular levels of both of these proteins. These findings suggest that hypoxia induces neurite outgrowth in PC12 cells via a pathway distinct from that activated by NGF. Thus, exposure to hypoxia at critical stages of development may contribute to aberrant neurite outgrowth and could be a factor in the pathogenesis of certain delayed developmental neurological disorders. © 2005 Published by Elsevier Ltd on behalf of IBRO.

Key words: adenosine, adenylate cyclase, βIII tubulin, GAP-43, nerve growth factor, neuronal differentiation.

Development of the nervous system is a highly complex process involving the temporal and spatial coordination of critical processes such as proliferation, migration, differentiation, synaptogenesis and programmed cell death. Vulnerable periods during development of the nervous system are sensitive to environmental insults or adverse conditions because they can interfere with these processes resulting in the development of late onset neurological disorders. For example, transient birth hypoxia has been shown to be a major risk factor in behavioural disorders (Berger and Garnier, 1999; El-Khodor and Boksa, 2000). Perinatal hypoxia has been linked with the development of schizophrenia in humans (Davies et al., 1998; Boksa and El-Khodor, 2003) although the biochemical mechanism underlying this is unknown. One current theory of the neuropathology of schizophrenia is that it is a developmental disorder of 'neural connectivity' (Benes, 2000; Penn, 2001) and as such it may be the result of interference with normal ontogeny of developmental processes such as neuronal differentiation in the nervous system. However, the mechanisms by which this occurs have not been elucidated and whether or not hypoxia plays a role in aberrant neuronal development is unknown.

Cellular exposure to hypoxic conditions results in alterations in gene transcription that promote expression of proteins involved in the glycolytic pathway and repress genes encoding components of the electron transport chain (Webster, 1987; Semenza et al., 1994). Activation by hypoxia of the basic helix–loop–helix transcription factor, hypoxia-inducible factor-1 (HIF-1), is largely responsible for these changes in gene transcription (Semenza and Wang, 1992; Wang et al., 1995). HIF-1 activation occurs through stabilisation of one of its two subunits, HIF-1α (Huang et al., 1996), which then dimerises with the constitutively expressed HIF-1 (Kallio et al., 1997).

Another important cellular response to low oxygen is altered adenosine metabolism with a consequent increase in the rate of adenosine release (Kobayashi et al., 2000). Adenosine has been shown to modulate neuronal function via adenosine receptors (Daval et al., 1991). To date, four adenosine receptors have been cloned and characterised (A1, A2A, A2B and A3) (Olah and Stiles, 1995) and have been suggested to influence neuronal differentiation, migration, synaptogenesis and survival (Marangos et al., 1982; Weaver, 1993, 1996; Paes-De-Carvalho, 2002; Stone, 2002; Aden et al., 2003; Schwarzschild et al., 2003). Rat pheochromocytoma (PC12) cells express adenosine A1, A2A and A2B receptors (Arslan et al., 1999). These cells have the ability to differentiate to a sympathetic neuron-like phenotype in response to the neurotrophic factor nerve growth factor (NGF; Greene and Tischler, 1976). Binding of NGF to TrkA receptors on PC12 cells induces receptor autophosphorylation and initiates the mitogen-activated protein kinase (MAPK) cascade that leads to neuronal differentiation of PC12 cells (Pang et al., 1995). Activation of adenosine A2A receptors, which are positively coupled to adenylate cyclase (Ribeiro et al.,

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Abbreviations: CGS21680, 2-[4-(2-carboxyethyl)phenethylamino]-5′-N-ethylcarboxamidoadenosine; CSC, 8-(3-chlorostyryl)caffeine; DMSO, dimethylsulphoxide; GAP-43, growth-associated protein of 43 kDa; HIF-1, hypoxia-inducible factor-1; MAPK, mitogen-activated protein kinase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NGF, nerve growth factor; PBS, phosphate-buffered saline; PMSF, phenylmethylsulfonyl fluoride.

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2002), has been shown to potentiate NGF-induced neurite outgrowth in PC12 cells (Cheng et al., 2002).

The aim of the present study was to examine the effect of hypoxia on neurite outgrowth and to further examine the effect of hypoxia on NGF-mediated neurite outgrowth. This investigation was carried out using PC12 cells which are a useful model to study neurite outgrowth.

EXPERIMENTAL PROCEDURES

Cell culture

PC12 cells (ECACC no. 88022401) were routinely maintained in RPMI 1640 medium supplemented with 5% foetal calf serum, 10% heat-inactivated horse serum, 50 U/ml penicillin, 50 mg/ml streptomycin and 2 mM L-glutamine (full medium). Cells were routinely subcultured every 4–5 days.

Treatment of cells

PC12 cells were plated onto glass coverslips coated with poly-L-lysine (0.001%) at a density of 7.5×10^4 cells/33 mm dish in 2 ml of medium. Prior to experimental treatment the cells were grown overnight in full medium, after this time the medium was changed and replaced with RPMI 1640 media supplemented with 1% horse serum, 50 U/ml penicillin, 50 mg/ml streptomycin and 2 mM L-glutamine (low serum medium). NGF treatment (2.5s mouse NGF from Alomone Laboratories, Jerusalem, Israel) was carried out by including 50 ng/ml NGF in low serum medium for the required time. The medium containing NGF was changed daily. Hypoxia treatment was achieved using the $InVivo_2$ hypoxia workstation (Ruskinn Technology, Dublin, Ireland) set to maintain conditions of 0.5% O_2. The culture medium was exchanged daily with fresh medium that had been equilibrated with 0.5% O_2. For chemical treatments stocks of adenosine (Sigma-Aldrich Ireland Ltd., Dublin, Ireland), 8-(3-chlorostyryl)caffeine (CSC; Sigma) and MDL-12,330A (Calbiochem, Merck Biosciences Ltd., Nottingham, UK) were made up in water whilst 2-[4-(2-carboxyethyl)phenethylamino]-5′-*N*-ethylcarboxamidoadenosine (CGS21680) and SB202190 (Calbiochem) were dissolved in dimethylsulphoxide (DMSO). Concentrations used were as indicated in the Results section and the final concentration of dimethylsulphoxide did not exceed 4%.

Neurite outgrowth assay

Following treatment the cells were washed in ice-cold phosphate-buffered saline (PBS) and fixed with 10% paraformaldehyde. The cells were stained with haematoxylin and eosin to improve visualisation. The coverslips were mounted with Mowiol (Calbiochem) and allowed to dry before viewing under phase contrast conditions with a Zeiss Axiovert S100 microscope. From each slide at least 100 cells from five randomly selected fields were counted. Cells were classed as differentiated if they exhibited an outgrowth extending from the cell which was at least 1.5 times the diameter of the cell. Measurements were carried out using NIH Scion image software.

MTT assay for neuronal survival

Integrity of cellular function was measured by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Sigma-Aldrich) tetrazolium salt assay (Mosmann, 1983). This colorimetric assay is based on the capacity of the mitochondrial enzyme succinate dehydrogenase to transform the yellow MTT tetrazolium salt into blue MTT formazan crystals in viable cells. The level of conversion provides an indication of mitochondrial metabolic function. PC12 cells were plated into 33 mm dishes at a density of 2.5×10^5 cells/dish in 2 ml medium. Cells were grown overnight in full medium and then switched to low serum medium. The cells were allowed to grow untreated for another 24 h. MTT tetrazolium salt was dissolved in Hanks' balanced salt solution to a final concentration of 5 mg/ml and filtered to remove particulate matter. Following exposure to hypoxia the cells were incubated with 100 μl of the MTT solution for 30 min at 37 °C in the hypoxia chamber and then centrifuged at $3000\times g$ for 4 min. The medium was then removed and 100 μl of DMSO was added to dissolve the formazan crystals. This solution was vortexed and transferred to a 96 well plate. The absorbence of each well was obtained using a Dynatech MR5000 plate counter at test and reference wavelengths of 550 nm and 650 nm respectively.

Western blot analysis

PC12 cells were plated into 25 cm^2 tissue culture flasks at a density of 5×10^5 cells/flask in 5 ml full medium. Cells were grown overnight in full medium and the medium was then changed to low serum medium. The cells were then allowed to grow untreated for another 24 h. Following exposure to treatment the cells were harvested by gentle scraping and were then washed in ice cold PBS. The cell pellet was then resuspended with 100 μl of whole cell lysis buffer (20 mM HEPES, pH 7.5 containing 350 mM NaCl, 1 mM $MgCl_2$, 0.5 mM EDTA, 0.1 mM EGTA, 1% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride [PMSF], 2 μg/ml aprotinin and 0.5 μg/ml pepstatin). Pellets were triturated until completely resuspended and the lysates were then centrifuged at $21{,}000\times g$ for 2 min to remove any insoluble particles. Supernatants were retained for analysis. Protein concentration was determined by Bradford assay using bovine serum albumin as standard. Cell lysates were boiled for 4 min in sample buffer containing 0.31 M Tris–HCl, pH 6.8, 1% SDS, 5% glycerol, 3.6 M β-mercaptoethanol, 5 mM PMSF, and 0.05% Bromophenol Blue.

Samples containing 20–25 μg protein were separated on 12% SDS–polyacrylamide gels at 100 V. The proteins were transferred to nitrocellulose membranes (Schleicher and Schuell UK Ltd., London, UK) at 110 V for 100 min. Nitrocellulose membranes were blocked with 5% non-fat dry milk in PBS containing 0.1% Tween-20 for 2 h at room temperature. Membranes were then incubated with primary antibodies: overnight at 4 °C with anti-growth-associated protein-43 (GAP-43; clone 91E-12 obtained from Sigma) or for 1 h at room temperature with anti-βIII tubulin (clone TU-20; obtained from AbCam Ltd., Cambridge, UK), both diluted 1:1000 in PBS containing 0.2% Tween-20 and 1% BSA or with anti-p38 MAPK (obtained from Biosource UK, Nivelles, Belgium) diluted 1:1000 in PBS containing 0.2% Tween-20 and 3% BSA. Incubation with horseradish peroxidase-labelled goat anti-mouse (diluted 1:10,000 in PBS, 1% BSA and 0.2% Tween-20) was carried out for 3 h at room temperature. Detection of bands was carried out with Supersignal West Pico chemiluminescent kit (Pierce; Medical Supply Company Ltd., Dublin, Ireland).

Statistical analysis

Results are expressed as means±S.E.M. Statistical analysis was made using Student unpaired *t*-test or repeated measures ANOVA followed by appropriate post hoc tests.

RESULTS

Hypoxia induces neurite outgrowth in PC12 cells

Exposure of PC12 cells to hypoxia (0.5% O_2) for 3 days was found to induce neurite outgrowth (Fig. 1A). The cells underwent morphological changes that were comparable to those induced by NGF (Fig. 1A) which is a well-known inducer of neuronal differentiation in these cells (Greene

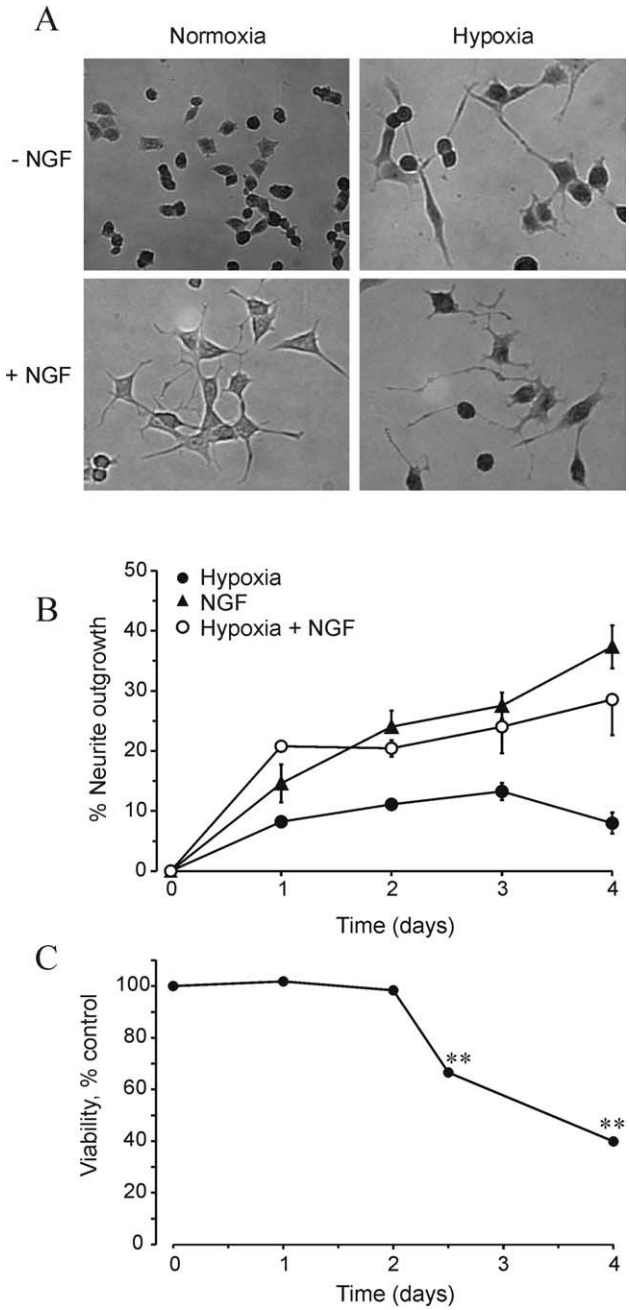

Fig. 1. Hypoxia induces neurite outgrowth in PC12 cells. (A) PC12 cells were cultured in medium containing low serum for 24 h prior to exposure to hypoxia, NGF or combined hypoxia/NGF for 3 days. Cells were stained with eosin and haematoxylin to aid visualisation of neurites. (B) Neurite outgrowth was quantified by counting the number of cells exhibiting neurites which were longer than 1.5 times the cell diameter. The proportion of cells with neurites was expressed as a percentage of the total number of cells. Values are the mean±S.E.M. of three independent experiments. The data were analysed using ANOVA with Dunnett's post hoc test. All values were significantly different from untreated cells ($P<0.01$). (C) PC12 cells were exposed to hypoxia and cell viability was measured by an MTT assay. Values are the mean±S.E.M. of three independent experiments. The data were analysed using ANOVA with Dunnett's post hoc test. ** $P<0.01$ compared with values for untreated cells.

and Tischler, 1976; Yankner et al., 1990). Cells exposed to hypoxia became flattened and extended long processes (Fig. 1A). Naive PC12 cells did not show any basal neurite outgrowth. A combination of hypoxia and NGF treatment also stimulated neurite outgrowth (Fig. 1A). The induction of neurite outgrowth by hypoxia was quantified over 0–4 days and compared with that induced by NGF treatment. There was a time-dependent increase in neurite outgrowth as a result of hypoxia exposure (Fig. 1B) reaching a maximum of 13.3±1.4% after 3 days in hypoxia. This was significantly lower than the level of outgrowth due to NGF treatment, which reached 27.5±2.2% after 3 days (Fig. 1B). Prolonged exposure of the cells to hypoxia beyond 3 days caused a decrease in the level of neurite-bearing cells which is in contrast to the continued increase observed with NGF treatment (Fig. 1B). This was due to a decrease in cell survival (measured using an MTT assay) in cultures exposed to hypoxia for periods longer than 2 days (Fig. 1C).

Lack of involvement of HIF-1 in hypoxia-induced neurite outgrowth

Many intracellular responses to hypoxia involve changes in gene expression that are coordinately regulated by HIF-1 (Semenza and Wang, 1992; Wang et al., 1995). Treatment of the cells with $CoCl_2$ under normoxic conditions is known to mimic many elements of the hypoxic response (Huang et al., 1996) through its ability to stabilise HIFα (Kallio et al., 1997). In order to examine whether HIF-1 activation played a role in neurite outgrowth, PC12 cells grown in normoxic conditions were exposed to 0–100 μM $CoCl_2$ for 3 days and the level of neurite outgrowth determined. None of the concentrations tested induced neurite outgrowth (data not shown) and in fact, higher concentrations of $CoCl_2$ ($\geq$50 μM) caused cell degeneration (data not shown).

Hypoxia-induced neurite outgrowth is not mediated through TrkA

In order to examine whether hypoxia-induced neurite outgrowth was mediated via activation of the TrkA receptor, the effect of K252a (a potent inhibitor of the tyrosine kinase activity of TrkA) was assessed. K252a (50 nM and 100 nM) was found to inhibit NGF-induced neurite outgrowth (Table 1). In contrast, K252a did not affect hypoxia-induced neurite outgrowth (Table 1). This excludes an involvement of TrkA activation in hypoxia-induced neurite outgrowth.

Effect of hypoxia on GAP-43 and βIII tubulin expression

GAP-43 is a neuronal specific axonal marker that is upregulated following NGF-mediated neurite outgrowth (Das et al., 2004) and class III β tubulin is a neuronal specific isoform of β tubulin (Lee et al., 1990). Since it appears that hypoxia and NGF cause neurite outgrowth via different mechanisms, it was decided to examine intracellular markers of neuronal differentiation. Western blot analysis showed a time-dependent increase in GAP-43 expression

Table 1. Effect of K252a on induction of induction of neurite outgrowth by hypoxia and NGF[a]

Treatment	Neurite outgrowth % cells
NGF alone	33.0±5.3
NGF plus 50 nM K252a	16.9±2.9*
NGF plus 100 nM K252a	4.3±1.5**
Hypoxia alone	9.3±2.9
Hypoxia plus 50 nM K252a	12.1±4.3
Hypoxia plus 100 nM K252a	12.1±3.4

[a] PC12 cells were pretreated with K252a (50 nM or 100 nM) for 30 min prior to exposure to hypoxia or NGF for 3 days. Neurite outgrowth was quantified. The data are the mean±SEM of three independent experiments. The data were analysed using ANOVA with Tukey-Kramer posthoc test.
* $P<0.05$, ** $P<0.01$ versus no K252a.

during 0–24 h NGF treatment (Fig. 2A). In contrast, hypoxia caused a time-dependent decrease in GAP-43 expression (Fig. 2A). Quantification of this data by densitometry showed that there was a 4.7-fold increase in GAP-43 expression at 24 h of NGF exposure and this was significantly different from control levels ($P<0.05$, $n=4$, data not shown) whilst there was a 4.5-fold decrease in GAP-43 expression at 24 h of hypoxia exposure ($P<0.01$, $n=4$, data not shown). Similarly, Western blot analysis showed a time-dependent increase in βIII tubulin expression following 2–4 day of NGF treatment (Fig. 2B) and in contrast, hypoxia did not induce βIII tubulin expression during 0–4 day exposure (Fig. 2B).

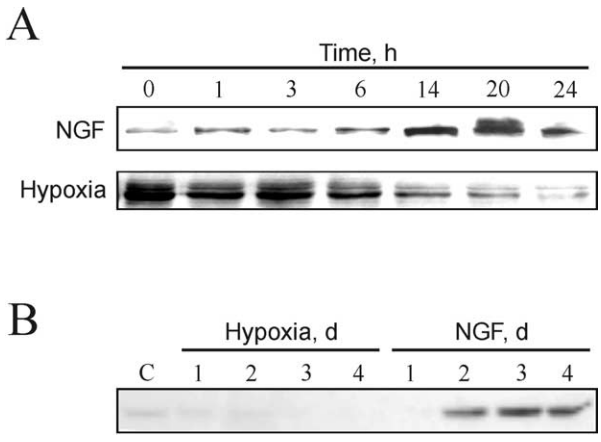

Fig. 2. Effect of hypoxia on GAP-43 and βIII tubulin expression. (A) PC12 cells were exposed to NGF or hypoxia for 0–24 h. Total protein extracts (25 μg/lane) were subjected to SDS-PAGE, followed by Western blot analysis, using a monoclonal antibody to GAP-43. Representative blots for treatment with hypoxia and NGF (four separate experiments) are shown. (B) PC12 cells were exposed to NGF or hypoxia for 0–4 days. Total protein extracts (25 μg/lane) were subjected to SDS-PAGE, followed by Western blot analysis, using a monoclonal antibody to βIII tubulin. The blot shown is representative of three separate experiments.

Table 2. Effect of adenosine deaminase on induction of induction of neurite outgrowth by hypoxia[a]

Treatment	Neurite outgrowth % cells
Hypoxia alone	15.35±0.71
Hypoxia plus ADA	0.91±0.06***

[a] PC12 cells were pretreated with 1 unit/ml adenosine deaminase (ADA) for 30 min prior to exposure to hypoxia for 2 days. Neurite outgrowth was quantified. The data are the mean±SEM of four independent experiments. The data were analysed using Student's unpaired *t* test.
*** $P<0.001$ versus no adenosine deaminase.

Hypoxia-induced neurite outgrowth is mediated through A2A receptor activation

Adenosine has previously been shown to be released from cells upon exposure to hypoxia (Kobayashi et al., 2000). A role for exogenous adenosine in hypoxia-induced neurite outgrowth was examined by incubating PC12 cells with adenosine deaminase during 2 day exposure to hypoxia. Adenosine deaminase was found to completely block neurite outgrowth by hypoxia (Table 2; $P<0.001$).

In particular, stimulation of the adenosine A2A receptor has been shown to induce differentiation in PC12 cells (Charles et al., 2003). Therefore, it was decided to examine whether the adenosine A2A receptor was involved in hypoxia-induced neurite outgrowth. PC12 cells were pretreated with 0–20 nM CSC (a specific antagonist of the adenosine A2A receptor) for 0.5 h prior to exposure to hypoxia for 3 days. There was a dose-dependent decrease in neurite outgrowth (Fig. 3A). The proportion of cells bearing neurites in cultures exposed to hypoxia alone decreased significantly in the presence of CSC (Fig. 3A; $P<0.01$, $n=3$). To verify the involvement of the adenosine A2A receptor in neurite outgrowth by hypoxia, PC12 cells were exposed to a range of concentrations of adenosine or the specific adenosine A2A receptor agonist (CGS21680) under normoxic conditions and the extent of neurite outgrowth was determined. Both of these adenosine receptor agonists caused induction of neurite outgrowth (Fig. 3B, C). The maximum level of neurite-bearing cells was 8.5% for 50 μM adenosine ($P<0.01$, $n=3$) and 5.1% for 50 nM CGS21680 ($P<0.001$, $n=3$).

Adenosine A2A receptor activation is known to stimulate adenylate cyclase (Schulte and Fredholm, 2003). In order to examine whether hypoxia-induced neurite outgrowth involved activation of this enzyme the effect of the adenylate cyclase inhibitor, MDL-12,330A, on hypoxia-induced neurite outgrowth was determined. MDL-12,330A was found to significantly inhibit the effect of hypoxia (Fig. 4).

Hypoxia synergistically enhances NGF-induced neurite outgrowth

Previous reports have shown that purines, e.g. guanosine (Gysbers and Rathbone, 1996a,b) and adenosine (Huffaker et al., 1984; Muroi et al., 2004), can synergistically enhance NGF-induced neurite outgrowth in PC12 cells. Although there

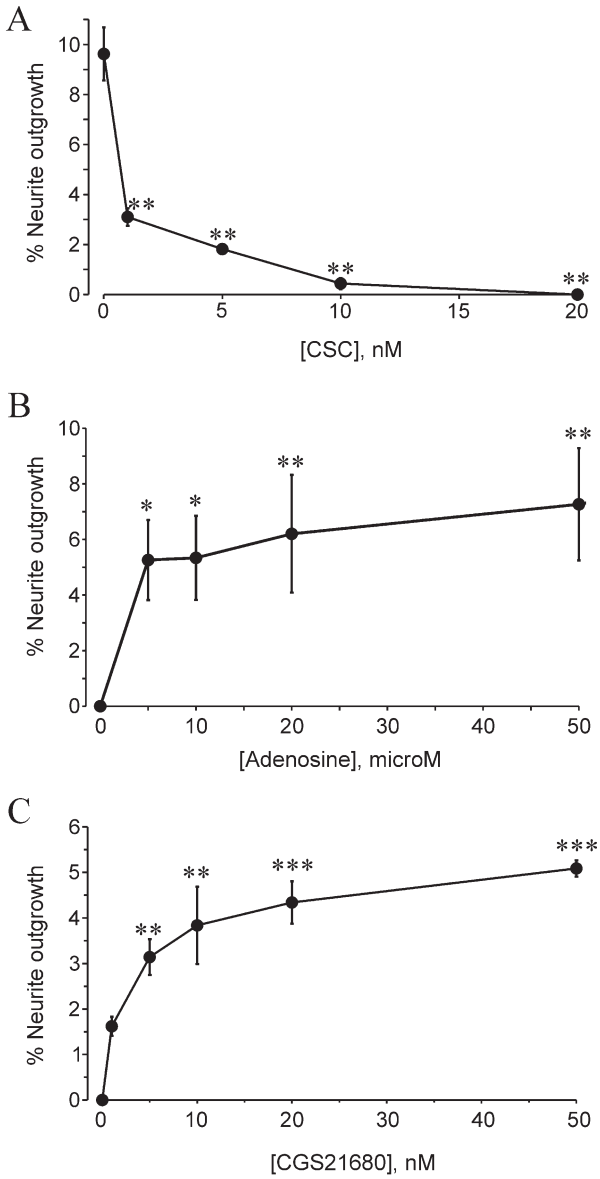

Fig. 3. Hypoxia-induced neurite outgrowth is mediated through A2A receptor activation. (A) PC12 cells were exposed to 0–20 μM CSC for 30 min prior to exposure to hypoxia for 3 days and the level of neurite outgrowth quantified. Values are the mean S.E.M. of three separate determinations. The data were analysed using ANOVA with Dunnett's post hoc test. ** $P<0.01$, versus absence of CSC. PC12 cells were exposed to 0–50 μM adenosine (B) or 0–50 nM CGS21680 (C) for 3 days and the level of neurite outgrowth quantified. Values are the mean±S.E.M. of three separate determinations. The data were analysed using ANOVA with Dunnett's post hoc test. * $P<0.05$, ** $P<0.01$, *** $P<0.001$ versus untreated cells.

did not appear to be any enhancement of the effect of hypoxia on NGF-induced neurite outgrowth at 2, 3 or 4 days (Fig. 1B), at the earliest time tested (1 day) neurite outgrowth due to the combination of the two stimuli was slightly higher than that due to NGF alone. This suggested that hypoxia might enhance the initiation of neurite outgrowth due to NGF. Therefore, it was decided to examine the effect of hypoxia on NGF-induced neurite outgrowth at earlier times (0–24 h). It

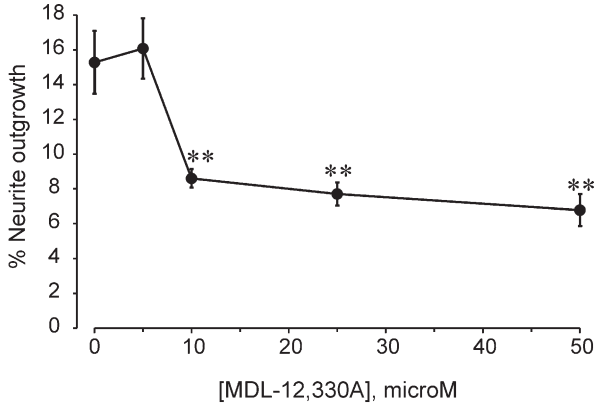

Fig. 4. Hypoxia-induced neurite outgrowth involves adenylate cyclase. PC12 cells were pretreated with 0–50 μM MDL-12,330A and then exposed to hypoxia for 2 days (MDL-12,330A was included in the medium during this time) and the level of neurite outgrowth quantified. Values are the mean±S.E.M. of three separate determinations. The data were analysed using ANOVA with Dunnett's post hoc test. ** $P<0.01$ versus untreated cells.

was found that at the earlier times hypoxia synergistically enhanced the level of neurite outgrowth due to NGF (Fig. 5A). The combination of the two stimuli caused a rapid increase in the proportion of cells with neurites. The synergistic effect of hypoxia was completely antagonised by CSC (Fig. 5B), demonstrating that this effect is mediated through the adenosine A2A receptor.

In order to confirm that synergy between hypoxia and NGF is through the adenosine A2A receptor the induction of neurite outgrowth by a combination of either NGF plus adenosine or NGF plus CGS21680 was compared with the expected outgrowth if the effects were not synergistic (i.e. additive). The combination of NGF plus adenosine induced neurite outgrowth that was greater than that expected when the values for separate NGF and adenosine treatment were added (Fig. 5C); however, this was not statistically significant. Combined treatment with NGF plus CGS21680 led to neurite outgrowth that was significantly different from that expected when the values for separate NGF and CGS21680 treatment were added (Fig. 5C, $P<0.05$). This indicates that there is synergy between NGF and adenosine A2A receptor activation in the induction of neurite outgrowth.

Involvement of p38 MAPK in hypoxia-induced neurite outgrowth

There is evidence that diverse signalling pathways including activation of p38 MAPK can lead to induction of neurite outgrowth (Morooka and Nishida, 1998; Iwasaki et al., 1999; Hansen et al., 2000; Choi et al., 2001; Ishii et al., 2001; Takeda and Ichijo, 2002). In the present study, both NGF and hypoxia were found to stimulate a rapid increase in the level of phosphorylated p38 MAPK (Fig. 6A). Hypoxia-induced phosphorylation occurred slightly earlier than that due to NGF treatment (3 h compared with 6 h). In order to examine the role of p38 MAPK in hypoxia-induced neurite outgrowth, a specific inhibitor, SB202190, was included during exposure of the cells to hypoxia. However,

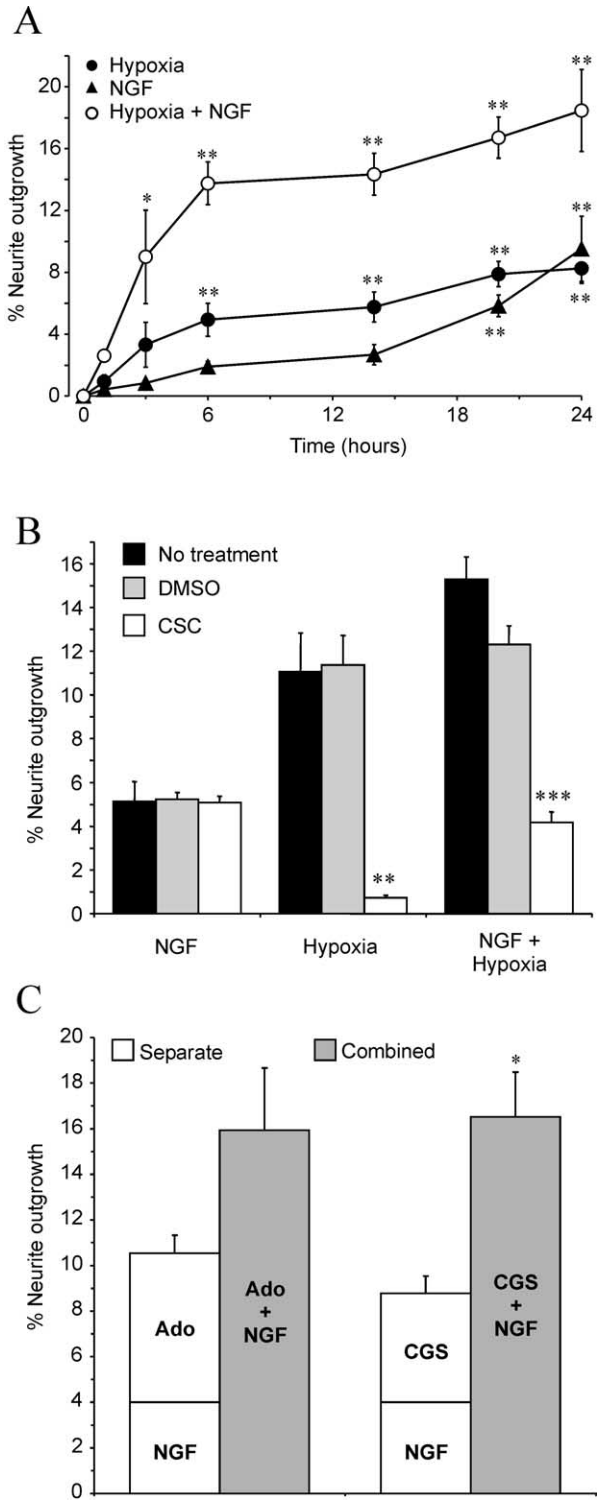

Fig. 5. Co-treatment with hypoxia enhances NGF-induced neurite outgrowth. (A) PC12 cells were treated with hypoxia, NGF or combined hypoxia/NGF for up to 24 h and the levels of neurite outgrowth were examined. Values are the means±S.E.M. of three independent determinations. The data were analysed using ANOVA with Dunnett's post hoc test. * $P<0.05$, ** $P<0.01$, versus untreated cells. (B) PC12 cells were treated with CSC (5 nM) for 30 min prior to exposure to NGF, hypoxia or combined hypoxia/NGF for 9 h. The levels of neurite outgrowth were determined. Values are the mean±S.E.M. of three independent experiments. The data were analysed by Student's t-test. ** $P<0.01$, *** $P<0.001$, versus untreated cells. (C) PC12 cells were treated separately with NGF, adenosine (Ado; 50 μM) or CGS21680 (CGS; 10 nM) or with a combination of NGF plus Ado or NGF plus CGS. The level of neurite outgrowth after 9 h was quantified. Values are the mean±S.E.M. of four independent experiments. The sum of the values obtained from separate NGF and Ado or NGF and CGS treatments (separate) were compared with the actual values that were obtained from combined NGF plus Ado or NGF plus CGS treatments (combined) using Student's t-test. * $P<0.05$ versus the sum of separate treatments.

this combination of treatment caused the cells to die (data not shown). Since the adenosine A2A receptor has been shown to mediate the effect of hypoxia (Fig. 3), the effect of SB202190 on CGS21680-induced neurite outgrowth was examined. It was found that SB202190 inhibited CGS21680-induced neurite outgrowth in a dose dependent manner, with essentially complete inhibition occurring at 2.5 μM SB202190 (Fig. 6B; $P<0.01$).

DISCUSSION

The data presented in this study demonstrate that exposure to hypoxia stimulates a rapid induction of neurite outgrowth in PC12 cells. An involvement of HIF-1 was ruled out since $CoCl_2$, which chemically stabilises HIF-1α (Kallio et al., 1997), did not induce neurite outgrowth. In contrast, hypoxia-induced neurite outgrowth in PC12 cells was blocked by the adenosine A2A receptor antagonist, CSC, and could be mimicked by adenosine and the CGS21680 (a specific adenosine A2A receptor agonist), indicating that the effect is mediated through adenosine A2A receptors. Moreover, activation of adenosine A2A receptors is known to activate adenylate cyclase (Ribeiro et al., 2002) and the present study shows that inhibition of adenylate cyclase with MDL-12,330A blocks hypoxia-induced neurite outgrowth. Transactivation of Trk neurotrophin receptors (in the absence of NGF) by activation of adenosine A2A receptors has previously been reported (Lee and Chao, 2001; Piiper et al., 2002). However, despite the demonstrated involvement of the adenosine A2A receptor, hypoxia-induced neurite outgrowth was not suppressed by a selective inhibitor of TrkA, indicating that transactivation of TrkA by adenosine A2A receptors could not provide a mechanism to explain how hypoxia caused neurite outgrowth in the present study.

Purines such as adenosine have increasingly been recognised as important intercellular trophic signalling molecules in the nervous system, influencing cell growth, differentiation and death. Adenosine (Huffaker et al., 1984; Braumann et al., 1986; Muroi et al., 2004), inosine (Braumann et al., 1986) and guanosine (Gysbers and Rathbone, 1992, 1996a,b) have all been reported to induce neurite outgrowth or to enhance NGF-induced neurite outgrowth. Induction of neurite outgrowth in PC12 cells by the bacterial nucleoside N^6-methyldeoxyadenosine has been demonstrated to be mediated by adenosine A2A receptors (Charles et al., 2003). Moreover, chronic hypoxia has been reported to enhance adenosine release in PC12 cells by

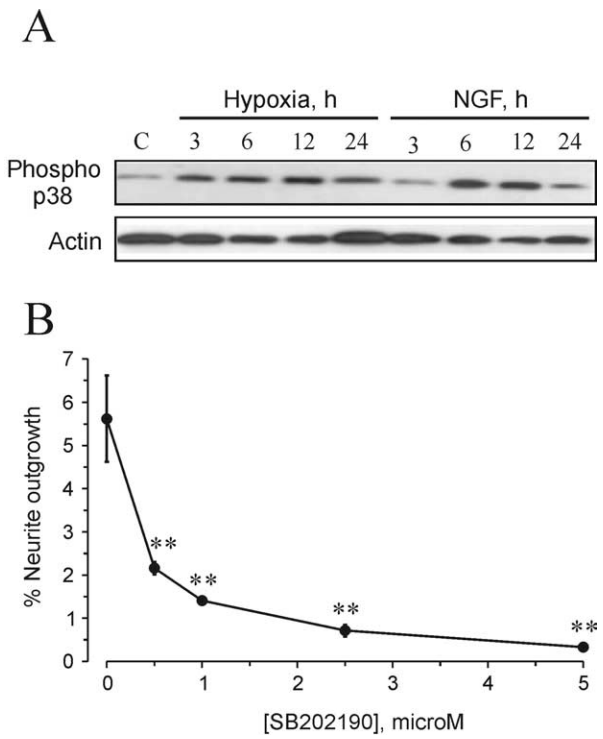

Fig. 6. Involvement of p38 MAP kinase in hypoxia-induced neurite outgrowth. (A) PC12 cells were exposed to hypoxia or NGF for 0–24 h. Total protein extracts (25 μg/lane) were subjected to SDS-PAGE, followed by Western blot analysis, using a monoclonal antibody against phosphorylated p38 MAP kinase. The blot shown is representative of four separate experiments. (B) PC12 cells were exposed to 0–5 μM SB202190 for 30 min prior to exposure to CGS21680 (10 nM) for 2 days and the level of neurite outgrowth quantified. Values are the mean±S.E.M. of three separate determinations. The data were analysed using ANOVA with Dunnett's post hoc test. ** $P<0.01$, versus absence of SB202190.

altering adenosine metabolism and membrane transport (Kobayashi et al., 2000). By including adenosine deaminase in the culture medium during exposure to hypoxia, neurite outgrowth was completely abrogated. Thus, the present findings suggest that an increase in extracellular adenosine, acting at A2A receptors, mediates neurite outgrowth by chronic hypoxia.

Exposure of PC12 cells to hypoxia induced a higher level of neurite outgrowth than adenosine alone suggesting that hypoxia may have additional effects. In fact, hypoxia has been reported to stimulate the expression of the A2A receptor in PC12 cells (Kobayashi and Millhorn, 1999) and in contrast, adenosine A2A receptor agonists (such as adenosine) are reported to cause down-regulation of A2A receptor expression during both normoxia and hypoxia (Kobayashi and Millhorn, 1999). Thus, a dual effect of hypoxia in causing adenosine release and an upregulation of the receptor could explain why neurite outgrowth by hypoxia is greater than with adenosine A2A receptor agonists alone. Feedback-regulated expression of the adenosine A2A receptor might also contribute to the drop in neurite outgrowth seen after 4 days of hypoxia exposure. However, it is also likely that the cells are dying and can no longer withstand the severe depletion of energy.

Exposure to hypoxia in the presence of NGF was found to accelerate the initial phase of induction of neurite outgrowth. At early times of exposure to hypoxia and NGF there is a synergistic effect by the two treatments but this effect is only transient and is ameliorated by 2 days. It is likely that this initial synergy is mediated by cAMP since the adenosine A2A receptor is positively coupled to adenylate cyclase leading to an increase in intracellular cAMP (Schulte and Fredholm, 2003). Synergistic induction of neurite outgrowth was also observed at early times of combined CGS21680 and NGF treatment. A similar result was obtained at early times of treatment with adenosine and NGF; however, it was not statistically significant. These findings are in agreement with other groups who have reported synergistic enhancement of NGF-induced neurite outgrowth by adenosine (Muroi et al., 2004), the adenosine analog 5′-*N*-ethylcarboxamideadenosine (Guroff et al., 1981) and intracellular cAMP (Heidemann et al., 1985; Charles et al., 2003).

Differential regulation of GAP-43 and βIII tubulin expression by NGF and hypoxia was observed in the present study. NGF induced an upregulation in the expression of both GAP-43 and βIII tubulin. On the other hand, hypoxia caused a downregulation in GAP-43 and did not induce βIII tubulin expression. GAP-43 has a role in pathfinding during neurite outgrowth and is preferentially localised in the growth cone and elongating axon of developing neurons. Neurons growing *in vivo* or *in vitro* express high levels of GAP-43 at the beginning of neurite outgrowth (Perrone-Bizzozero et al., 1986; Meiri et al., 1988; Costello et al., 1990; Dani et al., 1991). Beta III tubulin is recognised as a marker of neuronal cells and its upregulation is associated with neuronal differentiation (Menezes and Luskin, 1994). NGF is well known to increase GAP-43 expression in PC12 cells during differentiation (Costello et al., 1990). This NGF-induced increase in GAP-43 expression is due to protein kinase C-dependent stabilisation of the GAP-43 mRNA (Perrone-Bizzozero et al., 1993). The divergent regulation of GAP-43 expression and neurite outgrowth has been demonstrated in various systems. For example, neurite outgrowth in the absence or repression of GAP-43 expression has been reported in cultured adult rat dorsal root ganglion neurons (Andersen et al., 2000a), the serotonergic CNS cell line RN46A (Andersen et al., 2000b) and certain clones of PC12 cells (Baetge and Hammang, 1991; Burry and Perrone-Bizzozero, 1993). Neurite outgrowth without increased GAP-43 is associated with cAMP signalling (Burry and Perrone-Bizzozero, 1993; Andersen et al., 2000a,b). These reports support the present finding that GAP-43 expression and neurite outgrowth can be uncoupled and that hypoxia-induced neurite outgrowth is mediated by activation of adenosine A2A receptors coupled to adenylate cyclase. However, the fact that cells fail to express βIII tubulin when treated with hypoxia suggests that the outgrowths are qualitatively different from those induced by NGF. To our knowledge this is the first demonstration of neurite outgrowth in PC12 cells in the absence of an induction of βIII tubulin.

Activation of ERK and p38 MAP kinase pathways are required for NGF-induced neuronal differentiation in PC12 cells (Cowley et al., 1994; Morooka and Nishida, 1998). Cyclic AMP has been reported to induce neurite outgrowth and differentiation through p38 MAPK activation (Hansen et al., 2000) or through a pathway dependent on protein kinase A and phosphatidylinositol-3 kinase activity (Sanchez et al., 2004). The role of p38 MAPK in hypoxia-induced neurite outgrowth was investigated, since activation of the cAMP pathway has previously been reported to induce a rapid, dose-dependent phosphorylation and activation of this enzyme (Hansen et al., 2000). The present study demonstrates that both hypoxia and NGF induce phosphorylation of p38 MAPK suggesting that the pathways are not completely divergent. Hypoxia-induced neurite outgrowth was inhibited by SB202190 indicating that activation of p38 MAPK is essential in this process. Taken together, these findings are in agreement with those of Hansen et al. (2000) who show that induction of neurite outgrowth by cAMP requires p38 MAPK activation.

Correct pathfinding and connection formation in the brain is critical for the normal development and functioning of the brain. Exposure of the developing foetus to hypoxia during the pre- or perinatal period is linked to long term neurological deficits and the development of behavioural abnormalities (Davies et al., 1998; Berger and Garnier, 1999; El-Khodor and Boksa, 2000; Boksa and El-Khodor, 2003). Based on the present findings these effects of hypoxia could be mediated by adenosine A2A receptor-dependent induction of inappropriate neurite outgrowth or enhancement of neurotrophin-induced neurite outgrowth, leading to aberrant pathfinding and connection formation. Neurites that are induced by hypoxia are biochemically distinct from those induced by NGF in that hypoxia fails to induce GAP-43 or βIII tubulin expression. This suggests that these neurites may display defective pathfinding capacity since this protein is preferentially localised in the growth cone and elongating axon of developing neurons. Thus, the present findings may provide a mechanism of how exposure to hypoxia during critical periods of development of the nervous system can lead to long term neurological abnormalities. Further work is necessary to investigate the effects of hypoxia on differentiation in primary neuronal cell cultures.

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