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Prolyl hydroxylase inhibitors depend on extracellular glucose and HIF-2α to inhibit cell death caused by NGF deprivation: evidence that HIF-2α has a role in NGF-promoted survival of sympathetic neurons

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List of abbreviations: BH3, Bcl-2 homology 3; CM-H_2DCFDA, 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate; cpt-cAMP, 8-(4-chlorophenylthio) cyclic AMP; DHB, ethyl 3, 4-dihydroxybenzoic acid; DMOG, dimethyloxalylglycine; GFP, green fluorescent protein; 4-HT, 4-hydroxytamoxifen; HIF, hypoxia-inducible factor; JNK, c-Jun N-terminal kinase; NGF, nerve growth factor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; ROS, reactive oxygen species; SCG, superior cervical ganglion; TBS, tris-buffered saline

Abstract

Neurotrophins are critical for the survival of neurons during development and insufficient access to neurotrophins later in life may contribute to the loss of neurons in neurodegenerative disease, spinal cord injury, and stroke. Recently, the prolyl hydroxylase inhibitors ethyl 3, 4-dihydroxybenzoic acid (DHB) and dimethyloxalylglycine (DMOG) were shown to inhibit cell death in a model of neurotrophin deprivation that involves depriving sympathetic neurons of nerve growth factor (NGF). Here we show that treatment with DMOG or DHB reverses the decline in 2-deoxyglucose uptake caused by NGF withdrawal and suppresses the NGF deprivation-induced accumulation of reactive oxygen species (ROS). Neither DMOG nor DHB prevented death when NGF deprivation was carried out under conditions of glucose starvation, and both compounds proved toxic to NGF-maintained neurons deprived of glucose, suggesting that their survival-promoting effects are mediated through preservation of glucose metabolism. DHB and DMOG are well known activators of hypoxia-inducible factor (HIF), but whether activation of HIF underlies their survival-promoting effects is not known. Using gene disruption and RNA interference, we provide evidence that DMOG and to a lesser extent DHB require HIF-2α expression in order to inhibit NGF deprivation-induced death. Furthermore, suppressing basal HIF-2α expression, but not HIF-1α, in NGF-maintained neurons is sufficient to promote cell death. These results implicate HIF-2α in the neuroprotective mechanisms of prolyl hydroxylase inhibitors and in an endogenous cell survival pathway activated by NGF in developing neurons.

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Sympathetic neurons from the superior cervical ganglia (SCG) of newborn mice are widely used to study the regulation of cell survival by neurotrophins such as nerve growth factor (NGF). Soon after birth, the neurons in the SCG are reduced 30–40% during a period of programmed cell death that is regulated by the availability of NGF. Cell death coincides with the onset of target field innervation—neurons that bind sufficient NGF at the target live, those that fail to bind sufficient NGF die (Glebova and Ginty, 2005). Removing NGF from neonatal sympathetic neurons *in vitro* mimics the *in vivo* phenomenon, resulting in nearly complete cell death over 2–3 days (Deckwerth and Johnson, 1993).

Death induced by NGF withdrawal is apoptotic and characterized by a decline in glucose uptake, an increase in reactive oxygen species (ROS), activation of c-Jun N-terminal kinases (JNK) and its downstream target c-Jun, increased expression of select genes including the gene encoding the Bcl-2 homology 3 (BH3) domain protein Bim_{EL}, Bax-dependent release of cytochrome c from mitochondria, and caspase activation (reviewed by Freeman et al., 2004). These events are important for regulating the timing and extent of cell death. For example, mitochondria-derived ROS induced during NGF deprivation are implicated in triggering release of cytochrome c from mitochondria. Treatment with antioxidants blocks cytochrome c release and substantially delays death following NGF withdrawal (Kirkland and Franklin, 2001; Kirkland et al., 2002).

We recently reported that the prolyl hydroxylase inhibitors ethyl 3,4-dihydroxybenzoate (DHB) and dimethyloxalylglycine (DMOG) inhibit apoptosis in neurons deprived of NGF (Lomb et al., 2007). Treatment with either DHB or DMOG inhibited cytochrome c release and caspase activation. On the other hand, DHB but not DMOG inhibited phosphorylation of c-Jun and suppressed induction of Bim_{EL}. Thus, while DMOG and DHB may target a common

pathway upstream of cytochrome c release, DHB also impacts the JNK/c-Jun pathway. A likely common target of DMOG and DHB in NGF-deprived sympathetic neurons is the prolyl hydroxylase EGLN3.

EGLN3 mRNA and protein levels increase after NGF withdrawal (Lipscomb et al., 1999), and over-expressing EGLN3 promotes death in the presence of NGF through a mechanism that depends on its prolyl hydroxylase activity (Lipscomb et al., 2001; Lee et al., 2005). The three EGLN proteins are iron- and 2-oxoglutarate-dependent dioxygenases best known for their role in destabilizing hypoxia-inducible factor (HIF) (Schofield and Ratcliffe, 2004). Under normal oxygen tensions, EGLNs hydroxylate two proline residues in HIF alpha subunits (Epstein et al., 2001; Bruick and McKnight, 2001). Once hydroxylated, HIF-1α and HIF-2α bind the von Hippel-Lindau protein and its associated E3 ubiquitin ligase, resulting in their polyubiquitination and degradation by the proteasome (Ivan et al., 2001; Jaakkola et al., 2001). Under hypoxic conditions, or in the presence of iron chelators or 2-oxoglutarate analogs including DMOG and DHB, EGLN activity is inhibited (Hirsilä et al., 2003). Under these conditions, HIF-1α and HIF-2α accumulate in the nucleus where, in association with HIF-β, they regulate the transcription of a variety of genes that regulate the cell and tissue level response to oxygen deprivation (Kaelin, Jr. and Ratcliffe, 2008).

HIF activation has been associated with cell death and survival in neurons (Siddiq et al., 2005). In NGF-dependent sympathetic neurons, however, HIF may predominantly exert pro-survival effects. For example, expression of a stabilized HIF-1α that is resistant to proline hydroxylation inhibits death caused by NGF withdrawal (Xie et al., 2005). In addition, treatments that activate HIF such as hypoxia, the iron chelator desferrioxamine, and DHB and

DMOG, inhibit cell death in NGF-deprived neurons (Farinelli and Greene, 1996; Xie et al., 2005; Lomb et al., 2007).

The aim of this study was to investigate how DMOG and DHB inhibit cell death caused by neurotrophin deprivation. Results show that DMOG and DHB prevent the decline in glucose uptake and the accumulation of ROS that occur after NGF withdrawal. In neurons deprived of glucose, DMOG and DHB not only failed to inhibit death, but both compounds proved toxic even in the presence of NGF. Results from knocking out HIF-1α and knocking down HIF-2α in sympathetic neurons provide evidence that HIF-2α has a major role in the mechanisms by which DMOG and DHB inhibit NGF deprivation-induced death. Surprisingly, suppressing basal HIF-2α expression in NGF-maintained neurons was sufficient to promote cell death. These data implicate HIF-2α in the neuroprotective mechanisms activated by prolyl hydroxylase inhibitors and as an obligatory player in a survival pathway activated by NGF in developing neurons.

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Materials and Methods

Materials. NGF was purchased from Harlan Bioproducts for Science (Indianapolis, IN). Sheep anti-NGF antiserum was from Cedarlane Laboratories (Burlington, Ontario, Canada). DHB and 8-(4-chlorophenylthio) cyclic AMP (cpt-cAMP) were from Sigma-Aldrich (St. Louis, MO), DMOG was from Frontier Scientific (Logan, UT), and 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) was purchased from Invitrogen Corp. (Carlsbad, CA). Reagents for cell culture were from Invitrogen and other reagents were obtained from Sigma-Aldrich, unless otherwise indicated.

Cell culture. Primary cultures of sympathetic neurons were prepared from the SCG of newborn (postnatal day 0–1) C57BL/6J mice as described previously (Lipscomb et al., 1999) using procedures approved by the University Committee on Animal Resources at the University of Rochester. For obtaining neurons that are conditional for HIF-1α, mice homozygous for a HIF-1α allele in which exon 2 is flanked by loxP sites (HIF-1$\alpha^{f+/f+}$) (Ryan et al., 2000) were mated to HIF-1$\alpha^{f+/f+}$::ESRCre mice (Vangeison et al., 2008), which carry a ubiquitously expressed transgene that encodes a modified estrogen receptor ligand-binding domain/Cre recombinase fusion protein that can be activated by 4-hydroxytamoxifen (4-HT). The tamoxifen-inducible strain B6.Cg-Tg(cre/Esr1) 5Amc (The Jackson Laboratory, Bar Harbor, ME) was used to generate the original HIF-1$\alpha^{f+/f+}$::ESRCre mice. SCG from the progeny of these crosses were dissociated and plated separately. Genotyping was done as described by Ryan et al. (Ryan et al., 2000) and by the Jackson Laboratory at http://jaxmice.jax.org/pub-cgi/protocols/protocols.sh?objtype=protocol&protocol_id=288.

Cells were plated into medium consisting of 90% Minimum Essential Medium, 10% fetal bovine serum, 2 mM L-glutamine, 20 μM uridine, 20 μM fluorodeoxyuridine, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 ng/ml NGF. Cells were cultured for 5 days before being used for experiments. For experiments involving the presence and absence of added glucose, the culture medium consisted of 10% dialyzed fetal bovine serum (glucose reduced to 5 mg/dl; Atlanta Biologicals, Lawrenceville, GA) in either low glucose (5.5 mM) Dulbecco's Modified Eagle Medium (control media) or glucose-free Dulbecco's Modified Eagle Medium (glucose-depleted media). Accounting for the glucose in the serum, the glucose concentration in the glucose-depleted media is approximately 28 μM compared to 5.6 mM in the control media. To initiate NGF deprivation, neurons were washed twice with media lacking NGF prior to addition of the same media supplemented with NGF-neutralizing antiserum. Neurons were plated onto collagen-coated dishes except for confocal microscopy, where cells were plated on collagen-coated #1 cover glass, and for microinjections, where cells were plated on 35 mm glass-bottom dishes (MatTek Corporation, Ashland, MA) coated with polyornithine and laminin. All cultures were maintained at 37ºC in a humidified atmosphere of 5% CO_2 and 95% air.

Cell survival. For cell survival assays, equal numbers of neurons were plated onto collagen-coated 2-well chambered cover glasses (Thermo Fisher Scientific, Waltham, MA). At the end of the treatment period, cells were rinsed with phosphate-buffered saline (PBS), fixed in 4% paraformaldehyde in PBS, and then stained with Hoechst 33,342 (Invitrogen). Cell morphology was examined using phase-contrast and epifluorescence microscopy at a magnification of 400×. For each treatment group, the number of healthy neurons in 6 randomly selected fields in each of two duplicate wells was counted. The resulting data were normalized to the number of healthy

neurons in 6 randomly selected fields in identically plated NGF-maintained cultures. Healthy neurons were distinguished by a round, smooth, and refractile soma with a clearly discernible nuclear membrane, one or two visible nucleoli, and chromatin that was stained uniformly with Hoechst dye and that filled the nucleus. Dying or dead cells exhibited condensed, fragmented, or clumped Hoechst-stained chromatin, atrophied and rough soma, and fragmented neurites. In some cases, an extra well from each treatment group was not subjected to fixation, but instead was stained with the vital dye trypan blue. Trypan blue positive cells were visualized under bright-field microscopy. All cell counts were performed in a blinded manner and all survival data are reported as means ± S.E.M. from at least 3 independent experiments. Images were captured using a Dage-MTI CCD camera and Scion Image software (Scion Corp., Frederick, MD).

Measurement of ROS. Procedures for measuring ROS using fluorescence confocal microscopy are described in detail elsewhere (Kirkland et al., 2007). Cultures were incubated in the indicated experimental medium containing the redox-sensitive dye CM-H_2DCFDA (10 μM) for the final 20 min of each treatment period. After 20 min at 35°C, the cells were washed twice with Leibovitz's L-15 medium containing the experimental treatments and left in the last wash for confocal microscopy using a Nikon (Melville, NY) C1 laser-scanning head mounted on a Nikon Eclipse TE 300 inverted microscope. Neurons were chosen at random and scanned by the confocal microscope keeping laser power, confocal pinhole size, and photomultiplier gain at constant levels during an experiment. CM-H_2DCFDA was excited with the 488 nm line of the confocal laser and the green photomultiplier channel was used for image acquisition. The intensity of each neuron was normalized to that of NGF-maintained neurons receiving the same

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concentration of dye for the same time as the experimental cells. Normalized data are shown as fold change from the intensity of the dye measured in sibling cultures of neurons maintained since the time of plating in NGF-containing medium. Data were collected from experiments done with neurons from at least two separate platings.

2-deoxyglucose uptake assay. Glucose uptake assays were performed as described previously (Deckwerth and Johnson, 1993). Briefly, sympathetic neurons (~30,000 cells/well) were plated on collagen-coated wells of a 24-well dish in complete NGF-containing medium, except for 3 wells, which were plated in the same medium lacking NGF in order to generate cultures consisting only of non-neuronal cells (see below). All treatments were initiated 5 d after plating and were carried out in triplicate wells. At the end of a treatment period, cells were labeled in Leibovitz's L-15 media containing 2.5 μC/ml 2-deoxy-D-[2,6-^{3}H]glucose (53 Ci/mmol; GE Healthcare Bio-Sciences, Piscataway, NJ) for 10 min at 37°C. Cells were rinsed three times and then incubated in 500 μl of lysis buffer consisting of 1% SDS, 1 mM EDTA, and 10 mM Tris (pH 7.5). Tritium in the lysates was measured using a liquid scintillation counter. For each experiment, glucose uptake was also measured in the 3 wells of cells that were plated and maintained in the absence of NGF. The average radioactivity taken up by these non-neuronal cells was subtracted from that measured in wells containing neurons. Each experiment was repeated three times using neurons from different dissections.

Plasmids and microinjections. Short hairpin RNAs (shRNAs) targeting three different sequences in mouse HIF-2α and a control shRNA targeting firefly luciferase were designed and constructed using strategies described by others (Paddison et al., 2004). Oligonucleotides

corresponding to these sequences were obtained from Sigma-Genosys (St. Louis, MO) and after annealing, the resulting double-stranded DNAs were cloned into pSHAG-1 (Paddison et al., 2002) downstream of the U6 RNA polymerase III promoter. The resulting plasmids were verified by automated DNA sequencing. pBOS-H2BGFP, which expresses histone H2B fused to enhanced green fluorescent protein (GFP), was purchased from BD Biosciences (San Jose, CA). Mouse HIF-1α and HIF-2α expression plasmids were obtained from M. Celeste Simon (University of Pennsylvania).

Microinjections were carried out as described previously (Barone et al., 2008). The injection buffer consisted of 100 mM potassium chloride and 10 mM potassium phosphate (pH 7.4) and contained 4 mg/ml rhodamine-conjugated dextran, 50 μg/ml pBOS-H2BGFP, and 75 μg/ml of shRNA expression plasmid. After injection, neurons were returned to the incubator overnight to allow time for any neurons damaged by the injection process to die and for expression of the GFP-histone fusion protein. After ~12 h, the number of healthy neurons expressing GFP-histone was determined. Cell viability was assessed at the end of an experiment by examining the morphology of the nuclei of injected neurons. Nuclei with a rounded shapes and a diffuse pattern of GFP-histone fluorescence were scored as healthy. When viewed under phase-contrast microscopy, healthy neurons exhibited a distinct nuclear membrane, one or more prominent nucleoli, and an intact and refractile cell body. Cells with condensed, indented, or otherwise irregularly-shaped chromatin and nuclei were considered unhealthy. Percent survival was calculated from the ratio of the number of healthy GFP-histone expressing cells counted at the end of the experiment to the initial number of GFP-histone expressing cells determined 12 h after injection. All cell counts were performed in a blinded manner.

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Reverse-transcription PCR. RNA purification, reverse transcription and PCR reactions were performed as described previously (Lipscomb et al., 1999). Oligonucleotide primers corresponding to sequences in exons 1 and 3 of HIF-1α amplified a 481 basepair product in cDNA from wild type, HIF-1α$^{f+/f+}$, and HIF-1α$^{f+/f+}$::ESRCre neurons before treatment with 4-HT, and a 290 basepair product from HIF-1α$^{f+/f+}$::ESRCre neurons after 4-HT treatment. PCR products were separated by electrophoresis on agarose gels and viewed after staining with ethidium bromide.

Immunoblotting. COS-7 cells were transfected with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Whole cell lysates were prepared in Laemmli sample buffer, separated by SDS-PAGE, and then transferred to nitrocellulose membranes. Membranes were blocked in 5% fat-free milk and 0.1% Tween-20 in Tris-buffered saline (TBS) for 30 min and then incubated overnight at 4˚C with the following antibodies diluted 1:1000 in 1% fat-free milk and 0.1% Tween-20 in TBS: anti-HIF-2α (cat. #NB 100-122, Novus Biologicals); anti-HIF-1α (cat. #NB 100-449, Novus Biologicals); anti-α-tubulin (cat. #T5168, Sigma-Aldrich). Membranes were rinsed 3–5 times and then incubated with a 1:10,000 dilution of horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse antibody (Bio-Rad Laboratories, Hercules, CA) for 1 hr. Protein-antibody complexes were detected with SuperSignal West Pico chemiluminescence reagent (Thermo Fisher Scientific).

Immunofluorescence. Cells were rinsed once with PBS, fixed with 3% paraformaldehyde in PBS for 15 min, and then incubated in 5% goat serum (Invitrogen) and 0.3% Triton X-100 in TBS for 30 min. The cells were then incubated overnight at 4°C with anti-HIF-2α antibody (cat.

#NB 100–122, Novus Biologicals, Littleton, CO) diluted 1:500 in TBS containing 1% goat serum and 0.3% Triton X-100. After 3 washes with TBS, cells were incubated for 2 hr at 4˚C with Alexa Fluor-conjugated goat anti-rabbit secondary antibody (Invitrogen) diluted 1:500 in 1% goat serum and 0.3% Triton X-100 in TBS. Cells were rinsed twice with TBS before being viewed under phase-contrast and epifluorescence microscopy at a magnification of 400×.

Results

DMOG or DHB treatment after NGF withdrawal rescues neurons from cell death. When sympathetic neurons from newborn mice are deprived of NGF, the vast majority of cells undergo apoptosis within 48 h. Treatment with DMOG (1 mM) or DHB (800 μM) at the time of NGF withdrawal inhibits most of this cell death (Lomb et al., 2007). Nevertheless, a variety of agents including the protein synthesis inhibitor cycloheximide, the pan-caspase inhibitor BAF, and NGF itself inhibit cell death even when added many hours after initiating NGF deprivation (Edwards et al., 1991; Deckwerth and Johnson, 1993; Deshmukh et al., 1996). Therefore, we asked whether neurons already deprived of NGF could be 'rescued' by subsequent treatment with prolyl hydroxylase inhibitors. To parallel cultures we added cycloheximide or re-added NGF to neurons that had already been deprived of NGF for various amounts of time. In each case, survival was assessed at 48 h after initiating NGF deprivation. Past experiments done with rat sympathetic neurons showed that adding cycloheximide 16 h after NGF withdrawal or re-adding NGF 22 h after NGF withdrawal prevented the death of ~50% of the neurons when measured 2 d later (Deckwerth and Johnson, 1993). Our results with mouse sympathetic neurons were similar, with just under half-maximal survival when cycloheximide (1μg/ml) was added at 12 h or NGF (50 ng/ml) was added at 24 h after initiating NGF deprivation (Fig. 1A and B). In the same experiments, DMOG (1 mM) and DHB (800 μM) prevented death of the vast majority of neurons when added coincident with NGF removal or up to 12 h after NGF withdrawal. DMOG and DHB still inhibited a significant fraction of the cell death (30–40%) even when added 18 h after NGF withdrawal, a time when cycloheximide addition failed to provide any survival advantage. These results suggest that prolyl hydroxylase inhibitors can inhibit death pathways

activated during NGF deprivation at points downstream of those blocked by a protein synthesis inhibitor but upstream of those affected by NGF itself.

DMOG and DHB suppress the accumulation of ROS in NGF-deprived neurons. An increase in mitochondria-derived ROS occurs in mouse sympathetic neurons between 6 and 18 h after NGF withdrawal. ROS induction in these cells helps promote subsequent cell death events including the release of cytochrome c from mitochondria into the cytoplasm (Kirkland et al., 2002). Since prolyl hydroxylase inhibitors prevent cytochrome c release after NGF withdrawal (Lomb et al., 2007), we sought to determine if DMOG and DHB treatment might also suppress the NGF deprivation-induced increase in ROS. To measure ROS, we employed a procedure used previously with NGF-deprived sympathetic neurons (Kirkland and Franklin, 2001; Kirkland et al., 2002; Kirkland et al., 2007). Neurons were deprived of NGF in the presence or absence of DMOG or DHB for 24 h and then loaded with the redox-sensitive dye CM-H_2DCFDA, which fluoresces when oxidized by H_2O_2 and other ROS generated downstream of superoxide dismutation. Consistent with the previous studies, we detected a 4–5 fold increase in ROS 24 h after NGF withdrawal (Fig. 2A). Treatment with either DHB or DMOG during NGF deprivation suppressed the accumulation of ROS to levels that were only slightly above the basal level measured in NGF-maintained neurons. Lower concentrations, which failed to provide significant protection from cell death (Lomb et al., 2007), either did not decrease or only modestly decreased ROS.

DMOG and DHB might suppress ROS by blocking pathways that lead to their generation, by promoting pathways that lead to their removal, or through direct antioxidant effects. If DMOG or DHB were to react directly with ROS or to suppress ROS by rapidly

stimulating antioxidant mechanisms, then their presence during the final 20 min CM-H_2DCFDA loading period might be sufficient to suppress any ROS that accumulate during NGF deprivation. This was the result obtained when NGF was added back to NGF-deprived neurons during the 20 min dye-loading period (Kirkland et al., 2007). In contrast to NGF re-addition, treatment with DMOG or DHB during the dye-loading period failed to diminish the ROS that accumulate after 24 h of NGF deprivation (Fig. 2B). We next asked whether addition of DMOG or DHB at earlier times after initiating NGF withdrawal would reduce the ROS burden measured at 24 h. Results from these experiments reveal that both DMOG and DHB can suppress the ROS induction if added within 6 h of NGF withdrawal (Fig. 2C), but only DHB can prevent the accumulation of ROS when added 12 h after NGF withdrawal. The observation that initiating DMOG treatment 12 h after NGF withdrawal inhibits the majority of cell deaths (see Fig. 1C) without blocking the accumulation of ROS, indicates that these processes—promoting cell survival and suppressing ROS—can be dissociated.

DMOG and DHB prevent a drop in glucose uptake during NGF deprivation. Within 12 h of NGF withdrawal, the capacity of sympathetic neurons to take up glucose from the culture medium is greatly diminished (Deckwerth and Johnson, 1993). Recent studies have implicated glucose uptake and utilization as important anti-apoptotic targets of signaling pathways activated by growth factors (Hammerman et al., 2004). In addition, DHB has been shown to stimulate glucose uptake in cultured neonatal myocytes (Wright et al., 2003). To determine if prolyl hydroxylase inhibitors can influence the ability of NGF-deprived neurons to take up glucose, cells were deprived of NGF in the presence or absence of DMOG and glucose uptake was estimated by determining the intracellular accumulation of the non-hydrolysable glucose analog

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2-deoxyglucose. In control neurons, NGF withdrawal resulted in a steady decline in glucose uptake over the first 12 h, similar to previous results obtained with rat sympathetic neurons (Deckwerth and Johnson, 1993). Addition of DMOG at the time of NGF withdrawal largely prevented the drop in glucose uptake, at least during the first 12 h of NGF deprivation (Fig. 3A). Even after 24 h, glucose uptake in DMOG-treated, NGF-deprived neurons declined only about 30%, compared to about 70% in control NGF-deprived neurons. DHB also prevented the drop in glucose uptake after NGF withdrawal. However, in contrast to DMOG, DHB treatment actually stimulated glucose uptake to levels 2–3 times that of NGF-maintained neurons (Fig. 3B). Thus, prolyl hydroxylase inhibitors can inhibit or prevent a decline in glucose uptake after NGF withdrawal.

Inhibition of cell death by DMOG and DHB depends on extracellular glucose. The observations described above led us to ask whether the anti-apoptotic effects of DMOG and DHB depended on glucose in the culture medium. To address this question, neurons were cultured in the presence of NGF and 5.5 mM glucose for 5 days and then subsequently deprived of NGF under glucose-starved or glucose-replete conditions in either the presence or absence of DMOG and DHB. In control experiments, we found that NGF-maintained neurons could be cultured for at least 48 h in either glucose-replete or glucose-starved media without any overt detrimental effects (Fig. 4A and B, +NGF). In the presence of added glucose, neurons deprived of NGF but treated with DMOG or DHB remained viable and appeared similar to NGF-maintained neurons (Fig. 4A and C). In contrast, the vast majority of neurons in glucose-starved cultures died in response to NGF deprivation, regardless of whether DMOG or DHB was present (Fig. 4B and C). To determine if the combination of glucose deprivation and NGF deprivation

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was simply incompatible with sympathetic neuron survival, we treated cells with a different survival-promoting agent, the membrane permeable cAMP analog cpt-cAMP (Rydel and Greene, 1988). In the presence or absence of extracellular glucose, cpt-cAMP treatment almost completely inhibited cell death caused by NGF deprivation (Fig. 4A and B, bottom rows). Thus, extracellular glucose is critical for DMOG and DHB to inhibit death of NGF-deprived neurons, while it is expendable for survival mediated by cAMP.

DMOG and DHB are toxic to NGF-maintained neurons cultured in the absence of added glucose. While carrying out the experiments described above, we also treated neurons maintained in the presence of NGF with DMOG and DHB. As expected, DMOG and DHB had little effect on the morphology of neurons cultured in glucose-replete media (Fig. 5A). However, the vast majority of NGF-maintained neurons treated with DMOG or DHB and starved of glucose died (Fig. 5B and C). Glucose-deprived, DMOG- and DHB-treated neurons looked similar to neurons that had been deprived of NGF, exhibiting an atrophied soma and fragmented neurites, condensed and fragmented chromatin, and the ability to take up the vital dye trypan blue. Thus, DMOG or DHB treatment carried out in the absence of added glucose overrides the survival-promoting effects of NGF and results in neuronal death.

Effects of disrupting HIF-1α on survival promoted by DMOG and DHB. To determine the extent to which the survival promoting effects of prolyl hydroxylase inhibitors depend on HIF-1 induction, we utilized HIF-1$\alpha^{f+/f+}$::ESRCre mice that allow for tamoxifin-inducible disruption of the HIF-1α gene (Vangeison et al., 2008). We first verified that we could disrupt HIF-1α expression in sympathetic neurons from these mice by treating cultures with varying

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concentrations of 4-HT (data not shown). From these experiments, we found that exposing neurons to 1 μM 4-HT for 24 h nearly completely eliminated the exon-2-containing wild type HIF-1α transcript (Fig. 6A).

Neurons isolated from HIF-1α$^{f+/f+}$::ESRCre mice were treated with or without 4-HT and the next day either deprived of NGF or re-fed with fresh NGF-containing media. The survival of control cells not exposed to 4-HT, both before and after NGF deprivation, was indistinguishable to that of neurons from wild type mice (data not shown). Likewise, in the absence of 4-HT DMOG and DHB inhibited death to nearly the same extent as in wild type neurons (compare Fig. 6B with Fig. 1C and D). Addition of 4-HT and subsequent loss of HIF-1α did not significantly affect cell survival in the presence of NGF, nor did it alter the extent of cell death that occurred after NGF withdrawal. However, loss of HIF-1α partially reduced survival promoted by DMOG while having no effect on survival promoted by DHB (Fig. 6B). Because treatment with 4-HT failed to reduce survival of DMOG-treated neurons from HIF-1α$^{f+/f+}$ littermates lacking ESRCre (data not shown), it is likely that the reduction in DMOG-promoted survival of HIF-1α$^{f+/f+}$::ESRCre neurons was in fact due to loss of HIF-1α expression. Thus, while HIF-1 partially contributes to the neuroprotective effects of DMOG, HIF-1-independent mechanisms are also involved. Moreover, HIF-1 is not required for the ability of DHB to inhibit cell death caused by NGF deprivation.

Effects of inhibiting HIF-2α expression on survival promoted by DMOG and DHB. To assess the importance of HIF-2 for protection from cell death mediated by DMOG and DHB, we microinjected neurons with plasmids expressing shRNAs targeting HIF-2α (shHIF2α). To validate this approach, we first tested three different shHIF2α sequences for their ability to

inhibit expression of a co-transfected mouse HIF-2α cDNA. Two of three shHIF2α sequences (#1 and #3) specifically reduced HIF-2α protein levels without affecting HIF-1α expression (Fig. 7A, lanes 4 and 6). To further test the efficacy of shHIF2α(1) and shHIF2α(3), sympathetic neurons were microinjected with plasmids expressing mouse HIF-2α together with the shHIF2α vectors or or a negative control vector (shLuc). HIF-2α expression was readily detectable in neurons injected with the control shLuc plasmid, while little or no HIF-2α was detected in neurons co-injected with shHIF2α(1) or shHIF2α(3) (Fig. 7B). Although we have been unable to detect endogenous HIF-2α protein in sympathetic neurons by immunofluorescence, the ability of shHIF2α to reduce expression of ectopic HIF-2α in neurons makes it likely that the endogenous HIF-2α is also reduced in the same cells.

To determine if knockdown of HIF-2α affects the ability of DMOG and DHB to promote survival, sympathetic neurons were microinjected with shHIF2α or shLuc plasmids and the next day deprived of NGF in the presence or absence of DMOG and DHB. In preliminary experiments, we noticed that NGF-deprived neurons injected with shHIF2α died more quickly than uninjected neurons or neurons injected with shLuc. For this reason, cell survival was determined at 24 h after NGF withdrawal in these experiments. As expected, both DMOG and DHB inhibited cell death in NGF-deprived neurons injected with control shLuc plasmid (Fig. 7C and D). In contrast, DMOG failed to provide protection from cell death after knocking down HIF-2α expression (Fig. 7C). DHB, on the other hand, retained at least some ability to protect shHIF2α-expressing neurons from NGF deprivation-induced death, although the level of protection was less than that seen in control shLuc-injected neurons (Fig. 7D). Together these results suggest that HIF-2 is critical for survival promoted by DMOG, whereas DHB appears to act through HIF-2-dependent and HIF-2-independent mechanisms.

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HIF-2α is required for NGF-promoted survival of sympathetic neurons. Because knockdown of HIF-2α prior to NGF deprivation resulted in greater cell death than was seen after NGF withdrawal alone, we wondered whether HIF-2α might function as part of an NGF-induced cell survival pathway. NGF-maintained sympathetic neurons were microinjected with plasmids expressing shHIF2α or shLuc and monitored over several days. As early as 36 hr after injection, we detected morphological changes in many of the shHIF2α-injected neurons (but not in those injected with shLuc) that were consistent with cell death (Fig. 8B). When cell survival was assessed at 60 h after microinjection, only about 20% of the neurons injected with either shHIF2α(1) or shHIF2α(3) were still healthy compared to control shLuc-injected neurons (Fig. 8A). These effects were specific in that injection of shHIF2α(2), which failed to inhibit HIF-2α expression (see Fig. 7A), did not decrease cell survival. Neither DMOG nor DHB significantly inhibited the cell death associated with knockdown of HIF-2α in NGF-maintained neurons (Fig. 8C). These results provide initial evidence for a role for HIF-2α in NGF-promoted survival of developing sympathetic neurons.

Discussion

The aim of this study was to provide insight into the mechanisms by which DMOG and DHB protect neurons from NGF deprivation-induced death. In addition, we sought to determine whether the protective effects of DMOG and DHB are mediated through activation of HIF-1 and HIF-2. The results suggest that DMOG and DHB utilize overlapping mechanisms to inhibit cell death. For example, DMOG and DHB prevented cell death to a similar extent when added back to neurons at various times after NGF withdrawal. Moreover, both compounds inhibited the decline in glucose uptake in NGF-deprived neurons (DHB > DMOG), and they both failed to inhibit cell death in glucose-starved cells. Both compounds also inhibited the accumulation of ROS during NGF deprivation, although DHB suppressed ROS when added at later times after NGF withdrawal compared to DMOG. Finally, HIF expression is critical for neuroprotection by DMOG and DHB, with HIF-2 having the largest role.

We recently showed that treatment with DMOG or DHB blocks the release of cytochrome c from mitochondria in NGF-deprived neurons. We also reported, however, that DHB but not DMOG inhibits the induction of c-Jun, JNK-phosphorylated c-Jun, and BIM_{EL} during NGF-deprivation (Lomb et al., 2007). Like the present study, our previous results suggested the existence of overlapping survival pathways activated by DMOG and DHB, yet they also identified a distinct pathway acted on only by DHB—namely the JNK/c-Jun/Bim_{EL} pathway. The possibility that DHB targets an additional cell death pathway, one not targeted by DMOG, may help explain the finding that inhibiting HIF-2α only partially reduced the neuroprotection by DHB, while completely eliminating survival promoted by DMOG.

Withdrawal of NGF results in a gradual accumulation of ROS that remain at peak levels for at least 24 h (Greenlund et al., 1995). This accumulation of ROS after 24 h of NGF-

deprivation was almost completely blocked by DMOG and DHB. A likely source of the ROS that accumulate following NGF-withdrawal is superoxide generated at mitochondria (Kirkland and Franklin, 2001). DMOG and DHB treatment could suppress ROS by stimulating HIF-2α activity and increasing the expression of genes encoding antioxidant enzymes such as *superoxide dismutase-2* (Scortegagna et al., 2003). In addition, DMOG- and DHB-mediated stimulation of glucose uptake followed by an increased flux of glucose through the pentose phosphate pathway could also cause a decrease in ROS. In this scenario, an increase in NADPH generated by the pentose phosphate pathway could lead to higher levels of reduced glutathione, potentially resulting in more efficient detoxification of superoxide-derived H_2O_2 (Maher, 2006). The finding that DHB increases glucose uptake to a greater extent than DMOG could explain why DHB suppresses ROS at later times after NGF withdrawal compared to DMOG.

Both DMOG and DHB became toxic to neurons starved of glucose, suggesting that their ability to promote glucose uptake is a critical part of the mechanism by which they inhibit cell death. This prediction is in line with other studies that have implicated maintenance of glucose uptake and utilization as a mechanism by which growth factors promote cell survival (Hammerman et al., 2004). Given that HIF is a major regulator of genes involved in glucose uptake and utilization (Denko, 2008), it is likely that the ability of DMOG and DHB to promote glucose uptake in NGF-deprived neurons involves HIF activation. One potential HIF target gene that might contribute to the increase in glucose uptake encodes the glucose transporter Glut1, and unlike the genes for several glycolytic enzymes, *Glut1* gene expression can be regulated by both HIF-1 and HIF-2 (Hu et al., 2003). But why do DMOG and DHB become toxic to NGF-maintained neurons when cultured in the absence of added glucose? Recent studies have revealed that HIF induction not only promotes glycolysis, but it can also suppress aerobic

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respiration (Papandreou et al., 2006; Kim et al., 2006). Thus, if neurons exposed to prolyl hydroxylase inhibitors were to become largely dependent on glycolysis to produce ATP as a result of HIF activation, then in the absence of adequate amounts of glucose they may fail to generate sufficient ATP and ultimately succumb to bioenergetic collapse.

NGF deprivation is accompanied by upregulation of the HIF prolyl hydroxylase EGLN3 (Lipscomb et al., 1999). Recently, the loss of EGLN3 in mouse neurons and in neuronal PC12 cells was associated with a reduction in cell death caused by NGF deprivation (Lee et al., 2005; Bishop et al., 2008; Schlisio et al., 2008). When examined after the period of programmed cell death, SCG from *EGLN3*-null mice had a greater number of neurons compared to the ganglia from wild type mice (Bishop et al., 2008). Thus, in the absence of EGLN3, sympathetic neurons appear to be less sensitive to NGF deprivation-induced death. Crossing *EGLN3*-null mice to mice heterozygous for HIF-2α prevented the increase in SCG neuron number, suggesting that HIF-2 activation (presumably resulting from the absence of EGLN3) could prevent sympathetic neurons from undergoing programmed cell death *in vivo* (Bishop et al., 2008). Based on these results and our finding that the survival promoting effects of prolyl hydroxylase inhibitors also depend on HIF-2α, it is likely that EGLN3 is a principal target of DMOG and DHB in sympathetic neurons. Thus, our results provide pharmacological support for the hypothesis that EGLN3 promotes death in NGF-deprived neurons in part by suppressing HIF-2α. In addition, our results obtained using shHIF2α provide the first evidence that basal HIF-2α expression in neurons maintained under normoxic conditions is critical for NGF to promote survival.

In summary, the results of this study build on our understanding of the mechanisms by which prolyl hydroxylase inhibitors protect neurons from cell death while providing evidence that HIF-2α plays a critical role in neurotrophin-mediated cell survival. If extended to other

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types of neurons, these results could have ramifications for the advancement of prolyl hydroxylase inhibitors as a therapeutic strategy for stabilizing HIF and for treating disorders brought on by ischemia including stroke (Freeman and Barone, 2005).

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Footnotes

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Figure legends

Fig. 1. DMOG and DHB rescue neurons from cell death caused by NGF withdrawal. Sympathetic neurons from neonatal mouse SCG were maintained in culture in the presence of NGF for 5 days prior to initiating NGF deprivation. Additional control cultures were maintained continually in the presence of NGF. Treatments with the indicated agents were initiated at 0, 6, 12, 18, and 24 h after the withdrawal of NGF. **A**, NGF (50 ng/ml); **B**, cycloheximide (CHX, 1 μg/ml); **C**, DMOG (1 mM); **D**, DHB (800 μM). Forty-eight hours after initiating NGF deprivation, cells were fixed and stained with Hoechst 33,342 to permit visualization of their nuclear morphology. Percent survival represents the fraction of neurons exhibiting a rounded and diffusely stained nucleus, intact nuclear membrane, one or more prominent nucleoli, and a round and refractile cell body when viewed under epifluorescence and phase-contrast microscopy. Cells with condensed, fragmented, or otherwise irregularly-shaped chromatin and nuclei were scored as unhealthy. Graphs depict the means ± standard errors of the mean (S.E.M.) of the data from 3 independent experiments. Means significantly different from the NGF-deprived control cultures are indicated with asterisks ($p<0.05$, ANOVA followed by Dunnett's *post hoc* test).

Fig. 2. DMOG and DHB prevent the accumulation of ROS following NGF withdrawal. A, Sympathetic neurons were maintained in the presence of NGF or deprived of NGF for 24 h. Where indicated, DMOG or DHB was added to the media at the onset of NGF deprivation. At the end of the 24 h treatment period, cells were loaded with the redox-sensitive dye CM-H_2DCFDA for 20 min, and relative fold changes in ROS compared to neurons maintained

continuously in the presence of NGF were determined as described in Materials and Methods (*n* = 110–245 neurons). **B**, Neurons were maintained in the presence of NGF or deprived of NGF for 24 h. At the end of the 24 h period, DMOG (1 mM) or DHB (800 μM) was added where indicated for a total of 20 min, coinciding with the CM-H_2DCFDA loading period. Relative changes in ROS were determined in 39–82 neurons. **C**, NGF-deprived neurons were treated with either 1 mM DMOG (■) or 800 μM DHB (▲) at 6, 12, and 18 h after removal of NGF from the culture medium. The data for addition of DMOG and DHB at 0 hr and 24 h after NGF withdrawal are from the graphs shown in A and B, respectively. The average change in intensity measured after 24 h of NGF deprivation in the absence of either drug (●, -NGF) is shown for comparison. All cultures were loaded with CM-H_2DCFDA for 20 min at the end of the 24 h of NGF withdrawal; ROS were measured in 70–319 neurons. For each graph in A–C, fold change in CM-H_2DCFDA intensity represents the change from the intensity of the dye measured in sibling cultures of neurons maintained continuously in the presence of NGF (*n* = 39–71 neurons). Error bars indicate S.E.M. Asterisks indicate significant differences from the NGF-deprived control (**A)** or the NGF-maintained control (**B**) ($p<0.01$, ANOVA followed by Dunnett's *post hoc* test).

Fig. 3. DMOG and DHB stimulate glucose uptake in NGF-deprived neurons. A, Sympathetic neurons were maintained in the presence of NGF or deprived of NGF for 6, 12, or 24 h. NGF deprivation was carried out in the presence (▲) or absence (■) of DMOG (1 mM). For the final 10 min of each treatment period, the cells were incubated in serum-free medium containing [^{3}H]-2-deoxy-D-glucose (2DOG) at 37°C. After several rinses, cells were lysed and the amount of [^{3}H]-2DOG present in each lysate was determined by scintillation counting. Data

were corrected for the 2DOG uptake measured in parallel cultures devoid of neurons and then normalized to the values from cultures maintained in the presence of NGF at each time point. **B**, Sympathetic neurons were maintained in the presence of NGF, or deprived of NGF for 24 h in the presence or absence of DHB (800 μM). At the end of the treatment period, cultures were incubated in serum-free medium containing [^{3}H]-2DOG for 10 min at 37°C, rinsed several times, and then lysed. 2DOG uptake was determined as described above. Results in **A** and **B** represent means ± S.E.M. from 3 independent experiments. Asterisks indicate a significant difference in the mean ($p<0.05$) from corresponding NGF-deprived controls.

Fig. 4. DMOG and DHB fail to inhibit cell death in glucose-starved neurons. **A**, Sympathetic neurons were maintained in standard culture medium containing 5 mM glucose and 50 ng/ml NGF for their first 5 d *in vitro*. On day 5, the culture medium was replaced with fresh NGF-containing medium or with medium lacking NGF and supplemented with neutralizing anti-NGF antiserum. Where indicated, DMOG (1 mM), DHB (800 μM), and cpt-cAMP (400 μM) were added at the time of NGF withdrawal. After 48 h, one well from each treatment group was stained with Hoechst 33,342 and images were captured under phase-contrast and fluorescence microscopy. A second well was stained with trypan blue (TB) and imaged under bright-field microscopy. **B**, Sympathetic neurons were treated as described above except that on day 5, glucose-depleted culture medium containing NGF, or lacking NGF and supplemented with neutralizing anti-NGF antiserum, was added to each well. DMOG, DHB, and cpt-cAMP were also included at the time of NGF withdrawal where indicated. All images were captured at a magnification of 100×. Scale bar, 60 μM. The images shown are representative of results obtained in 3–4 independent experiments. **C**, Neurons were treated as described above and cell

survival was quantified 48 h later by staining the cultures with trypan blue and determining the fraction of cells that excluded the dye. Results represent the means ± S.E.M. from 3 independent experiments; asterisks indicate significant differences compared to the NGF-maintained controls ($p < 0.01$, ANOVA followed by Dunnett's *post hoc* test).

Fig. 5. DMOG and DHB promote death in the presence of NGF in neurons starved of glucose. Sympathetic neurons were maintained in standard culture medium containing 5 mM glucose and 50 ng/ml NGF for their first 5 d *in vitro*. On day 5, the culture medium was replaced with fresh medium containing glucose and NGF, with or without DMOG (1 mM) or DHB (800 μM). After 48 h, one well from each treatment group was stained with Hoechst 33,342 and images were captured under phase-contrast and fluorescence microscopy. A second well was stained with trypan blue (TB) and imaged under bright-field microscopy. **B**, Sympathetic neurons were treated as described above except that on day 5, glucose-depleted culture medium containing NGF with or without DMOG or DHB was added to the cells. Magnification equals 100× for all images. Scale bar, 60 μM. The images shown are representative of results obtained in 3–4 independent experiments. **C**, Neurons were treated as described above and cell survival was quantified 48 h later by staining the cultures with trypan blue and determining the fraction of cells that excluded the dye. Results represent the means ± S.E.M. from 3 independent experiments; asterisks indicate significant differences compared to the corresponding NGF-maintained control ($p < 0.01$, ANOVA followed by Dunnett's *post hoc* test).

Fig. 6. Effects of disrupting HIF-1α expression on survival promoted by DMOG and DHB. **A**, On the fourth day *in vitro*, sympathetic neurons from HIF-1$\alpha^{f+/f+}$::ESRCre mice were treated

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with or without 4-HT (1 μM) for 24 h. RNA was then isolated from the cultures, reverse transcribed to cDNA, and analyzed by PCR with primers that span exon 2 in HIF-1α: lane 1, no cDNA control reaction; lane 2, without 4-HT; lane 3, with 4-HT; M, molecular weight markers. In the absence of 4-HT, the majority of the HIF-1α amplification product corresponded to a 481 basepair cDNA that includes exon 2. After 4-HT treatment, the only detectable HIF-1α amplification product was the 290 basepair cDNA lacking exon 2; this transcript does not result in HIF-1α protein expression (Ryan et al., 2000; Vangeison et al., 2008). Note that a small amount of the 290 basepair amplification product was sometimes seen in the absence of 4-HT, presumably due to leaky Cre recombinase activity in these cells. **B**, Neurons from HIF-$1\alpha^{f+/f+}$::ESRCre mice were treated with or without 4-HT for 24 h and then maintained in the presence of NGF or deprived of NGF, either with or without DMOG (1 mM) or DHB (800 μM). After 48 h of NGF deprivation, cells were fixed and stained with Hoechst dyes. Survival was assessed as described in Fig. 1. Results represent the means ± S.E.M. from 3 independent experiments. Asterisks indicate significant differences compared to the corresponding NGF-deprived controls ($p < 0.05$, ANOVA followed by Dunnett's *post hoc* test).

Fig. 7. Suppressing HIF-2α expression blocks DMOG-mediated protection and partially reduces DHB-mediated protection from NGF deprivation-induced death. **A**, COS-7 cells were transfected with expression plasmids for either mouse HIF-2α (top panels, lanes 2–6) or HIF-1α (bottom panels, lanes 2–6). Some cells were co-transfected with an shRNA vector targeting luciferase (shLuc, lane 3), or with one of 3 different shRNAs targeting HIF-2α: shHIF2α(1), lane 4; shHIF2α(2), lane 5; shHIF2α(3), lane 6. The next day, cell lysates were prepared and analyzed by immunoblotting with antibodies against HIF-2α or HIF-1α. Lane 1

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represents untransfected control lysates. Equivalent amounts of each lysate were also immunoblotted for α-tubulin as a control for protein loading. **B**, Sympathetic neurons were microinjected with an expression plasmid for mouse HIF-2α together with plasmids expressing shHIF2α(1), shHIF2α(3), or shLuc. Each injection solution also contained an additional plasmid expressing a GFP-histone fusion protein to aid in the visualization of injected cells and in the analysis of nuclear morphology. After 24 h, cells were fixed and processed for immunofluorescence with an antibody against HIF-2α. Representative phase-contrast and epifluoresence images show readily detectable HIF-2α immunofluorescence in cells co-injected with shLuc, but not in cells co-injected with shHIF2α(1) or shHIF2α(3). Scale bar, 20 μM. **C** and **D**, Sympathetic neurons were microinjected with shLuc, shHIF2α(1), or shHIF2α(3) vector along with a plasmid expressing GFP-histone. The baseline number of healthy injected cells was determined 12 h later. At that time, NGF deprivation was initiated in the presence or absence of 1 mM DMOG (**C**) or 800 μM DHB (**D**). The number of injected cells that remained healthy after 24 h of NGF deprivation was expressed as a percentage of the total number of cells injected. Results are expressed as means ± S.E.M. from 4–11 dishes that were injected for each condition with 100–150 neurons injected per dish. The survival of neurons injected with either of the shHIF2α vectors was significantly less than that of neurons injected with shLuc (*, $p<0.01$). DMOG treatment resulted in an increase in the survival of shLuc-injected neurons (#, $p<0.001$), but it had no significant effect on neurons injected with shHIF2α(1) or shHIF2α(3) ($p>0.05$). For every shRNA injected, DHB treatment resulted in significantly greater survival compared to the corresponding untreated control (#, $p<0.001$), although the level of protection by DHB in shHIF2α(1) and shHIF2α(3) expressing neurons was less than that seen in control

shLuc-injected neurons ($p<0.01$). Data were analyzed using ANOVA and Bonferroni *post hoc* tests.

Fig. 8. Suppressing HIF-2α expression induces death in NGF-maintained neurons. A, Sympathetic neurons were microinjected with a plasmids expressing shHIF2α or shLuc, along with a plasmid expressing GFP-histone. After 12 h, the baseline number of healthy injected cells was determined, after which the medium was replaced with fresh NGF-containing medium. After an additional 48 h, the percentage of injected neurons that remained healthy was determined. **B**, Neurons were microinjected as outlined above and 12 h later the medium was replaced with fresh NGF-containing medium, either with or without DMOG (1 mM) or DHB (800 μM). Phase-contrast and corresponding fluorescence images of injected cells were captured 60 h after microinjection. Results with the shHIF2α(3) vector are shown. Magnification, 400×; scale bar, 20 μM. **C**, Neurons were microinjected with shLuc or shHIF2α(3) as described above and survival was assessed 60 h later. Results represent means ± S.E.M. from a total of 6–8 dishes for each condition with 100–150 neurons injected per dish. Asterisks indicate a significant difference from control cells injected with shLuc ($p<0.01$, ANOVA followed by Dunnett's *post hoc* test). Differences between the untreated shHIF2α-injected neurons and those treated with DMOG or DHB were not significant ($p>0.05$, ANOVA followed by Bonferroni *post hoc* tests).

Figure 1

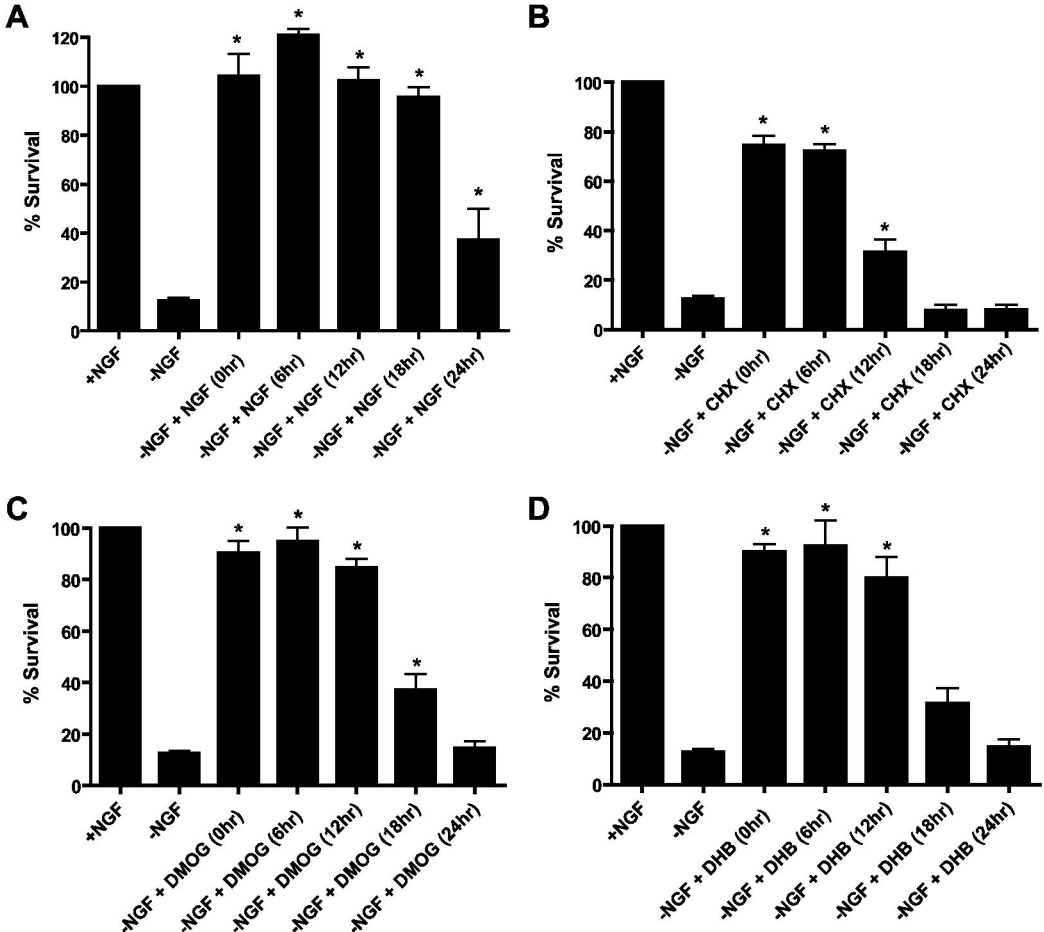

A
% Survival
120
100
80
60
40
20
0
+NGF
-NGF
-NGF + NGF (0hr)
-NGF + NGF (6hr)
-NGF + NGF (12hr)
-NGF + NGF (18hr)
-NGF + NGF (24hr)
B
% Survival
100
80
60
40
20
0
+NGF
-NGF
-NGF + CHX (0hr)
-NGF + CHX (6hr)
-NGF + CHX (12hr)
-NGF + CHX (18hr)
-NGF + CHX (24hr)
C
% Survival
100
80
60
40
20
0
+NGF
-NGF
-NGF + DMOG (0hr)
-NGF + DMOG (6hr)
-NGF + DMOG (12hr)
-NGF + DMOG (18hr)
-NGF + DMOG (24hr)
D
% Survival
100
80
60
40
20
0
+NGF
-NGF
-NGF + DHB (0hr)
-NGF + DHB (6hr)
-NGF + DHB (12hr)
-NGF + DHB (18hr)
-NGF + DHB (24hr)

Figure 2

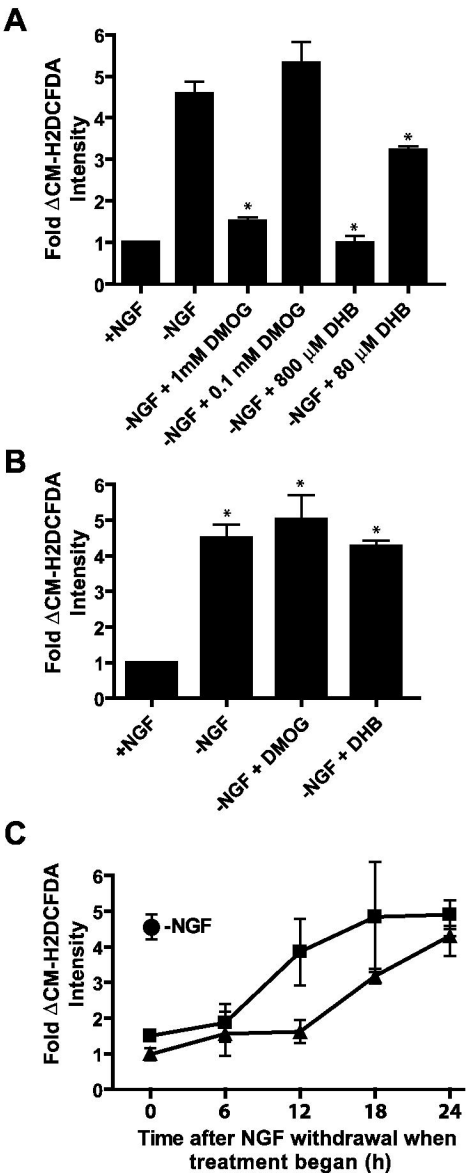
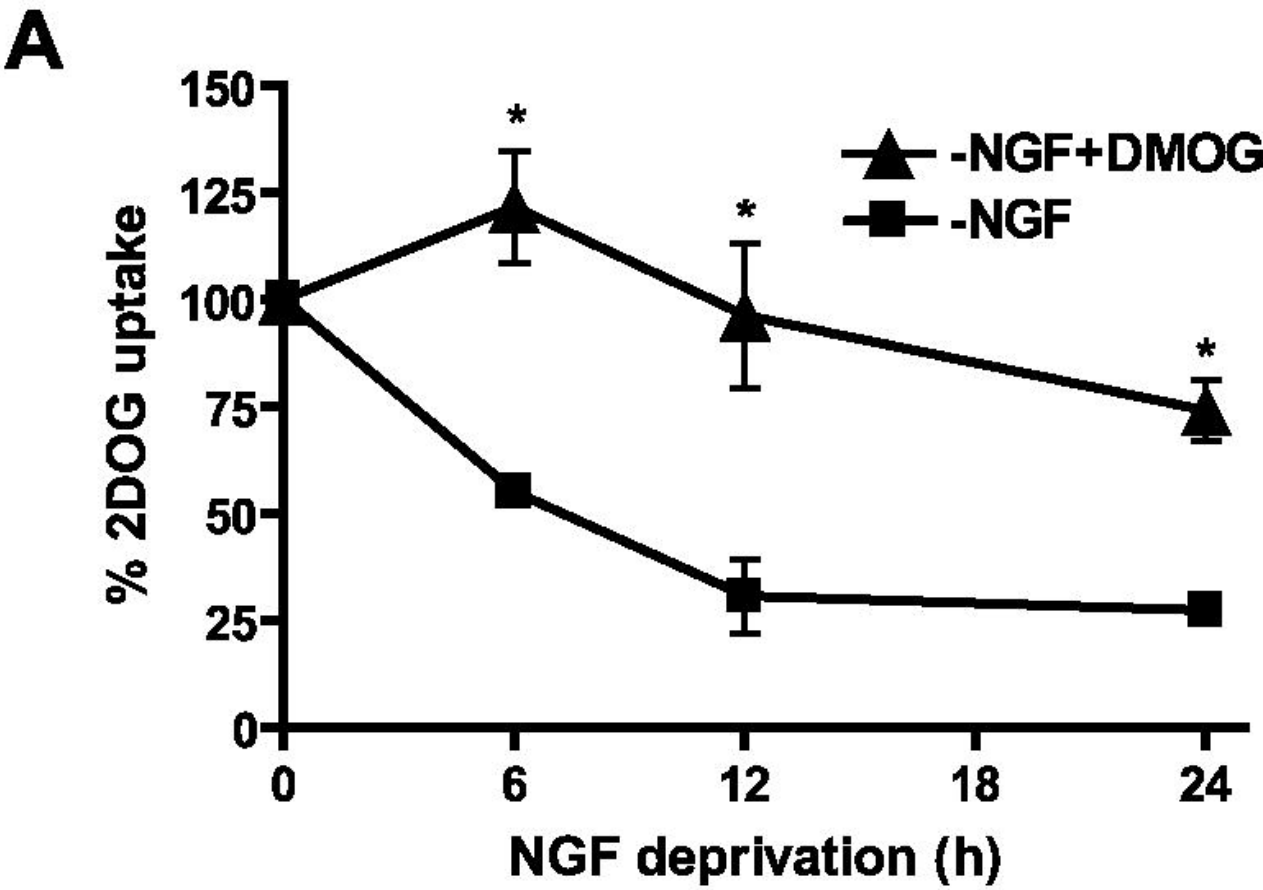

A
Fold ΔCM-H2DCFDA Intensity
0
1
2
3
4
5
6
*
*
*
+NGF
-NGF
-NGF + 1mM DMOG
-NGF + 0.1 mM DMOG
-NGF + 800 μM DHB
-NGF + 80 μM DHB
B
Fold ΔCM-H2DCFDA Intensity
0
1
2
3
4
5
6
*
*
*
+NGF
-NGF
-NGF + DMOG
-NGF + DHB
C
Fold ΔCM-H2DCFDA Intensity
0
1
2
3
4
5
6
-NGF
0
6
12
18
24
Time after NGF withdrawal when treatment began (h)

Figure 3


A
150
125
100
75
50
25
0
% 2DOG uptake
*
*
*
-NGF+DMOG
-NGF
0
6
12
18
24
NGF deprivation (h)

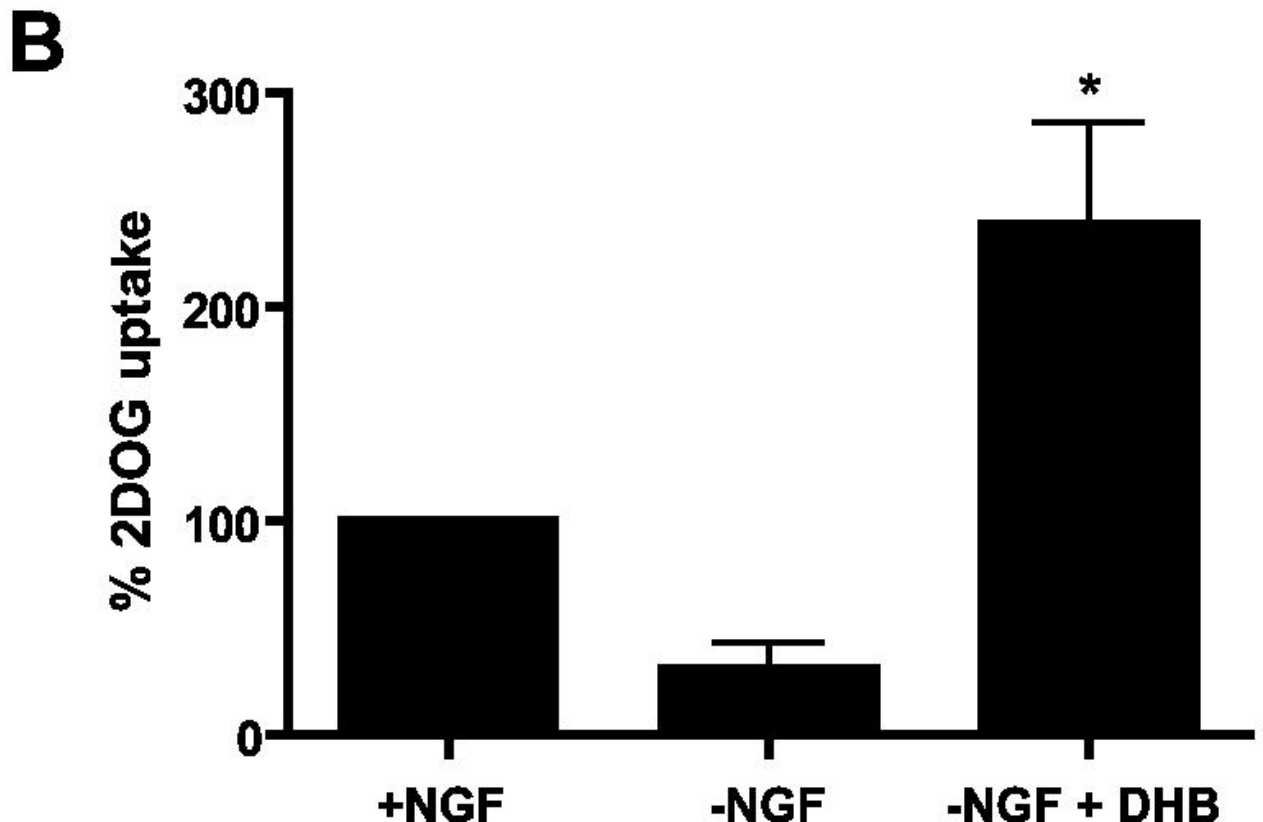
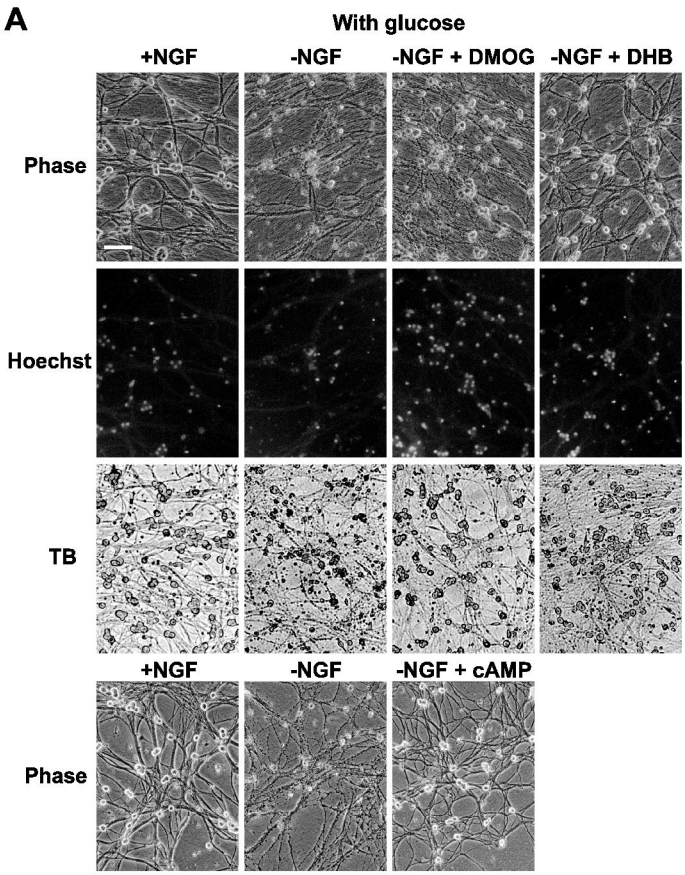
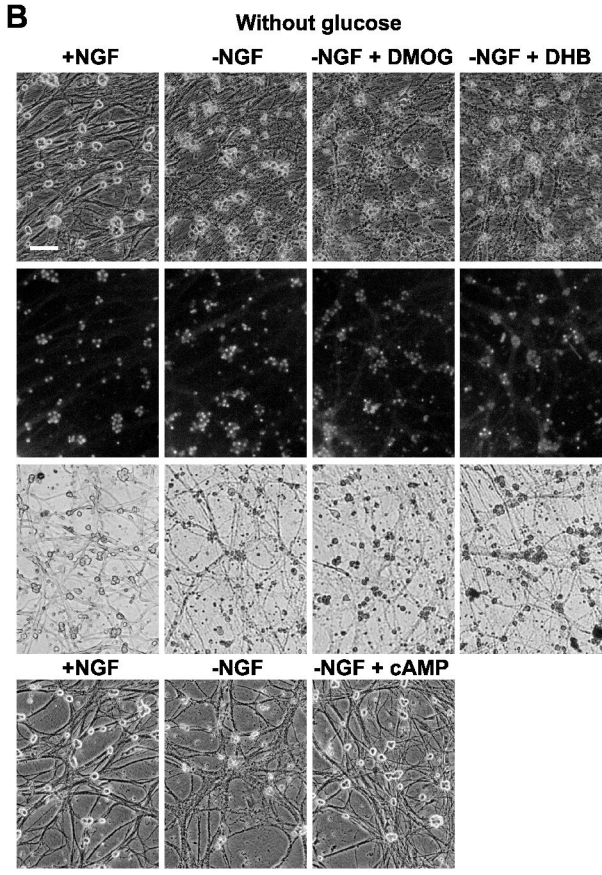

B
300
200
100
0
% 2DOG uptake
*
+NGF
-NGF
-NGF + DHB

Figure 4


A
With glucose
+NGF
-NGF
-NGF + DMOG
-NGF + DHB
Phase
Hoechst
TB
+NGF
-NGF
-NGF + cAMP
Phase


B
Without glucose
+NGF
-NGF
-NGF + DMOG
-NGF + DHB
+NGF
-NGF
-NGF + cAMP

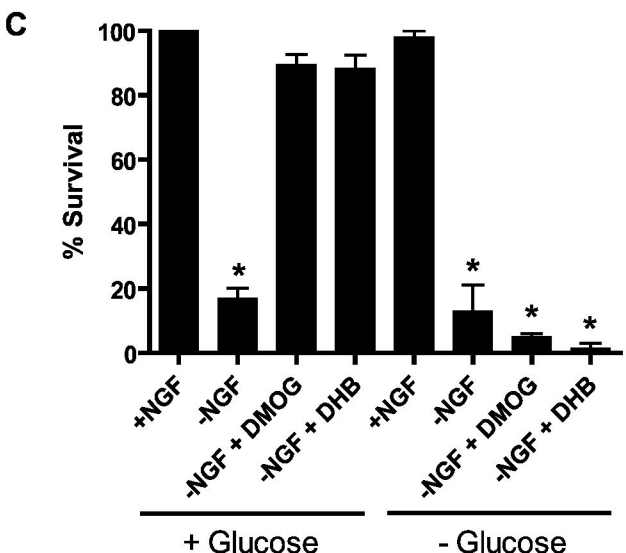

C
% Survival
100
80
60
40
20
0
*
*
*
*
+NGF
-NGF
-NGF + DMOG
-NGF + DHB
+NGF
-NGF
-NGF + DMOG
-NGF + DHB
+ Glucose
- Glucose

Figure 5

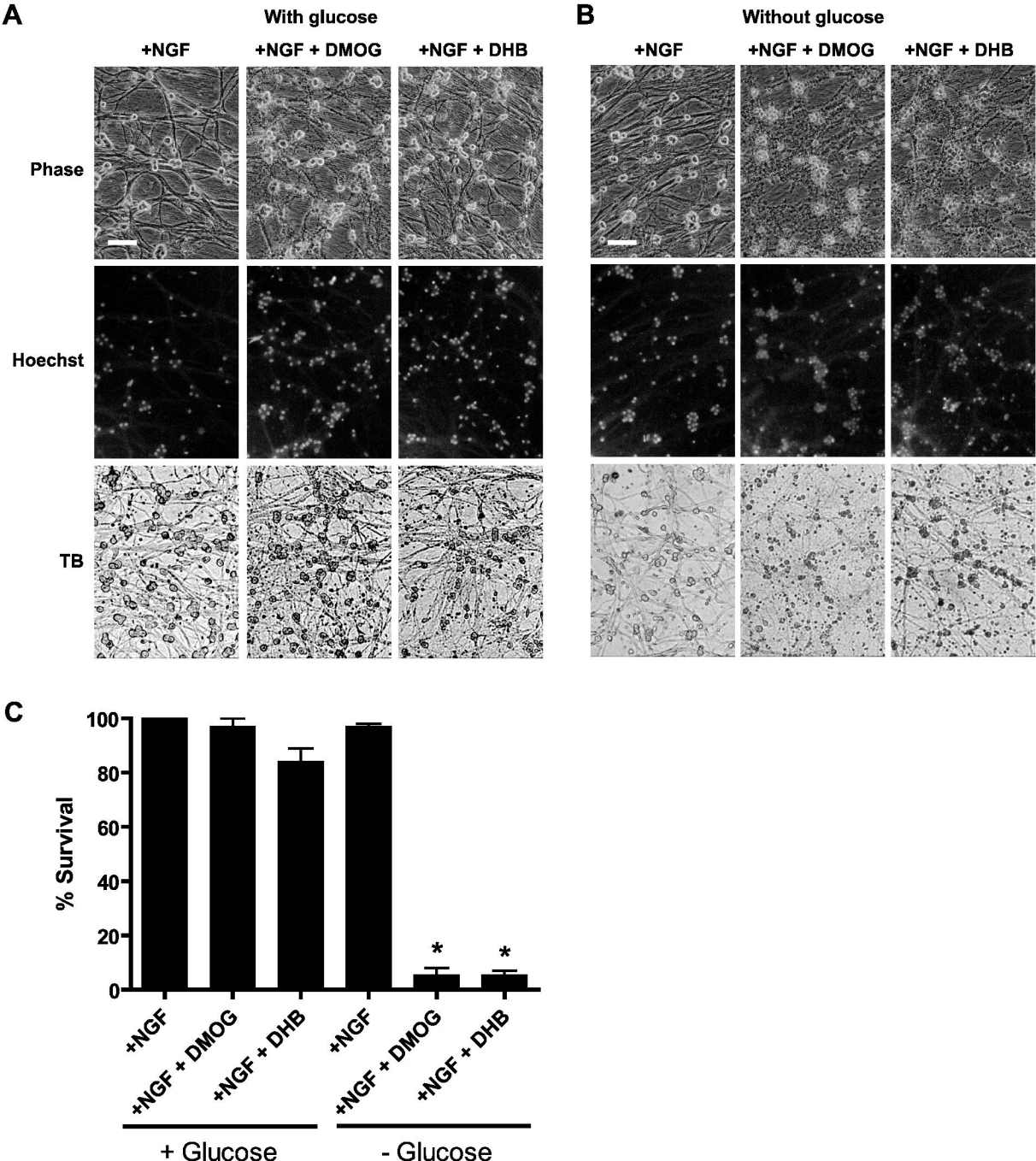

A
With glucose
+NGF
+NGF + DMOG
+NGF + DHB
Phase
Hoechst
TB
B
Without glucose
+NGF
+NGF + DMOG
+NGF + DHB
C
% Survival
100
80
60
40
20
0
*
*
+NGF
+NGF + DMOG
+NGF + DHB
+NGF
+NGF + DMOG
+NGF + DHB
+ Glucose
- Glucose

Figure 6

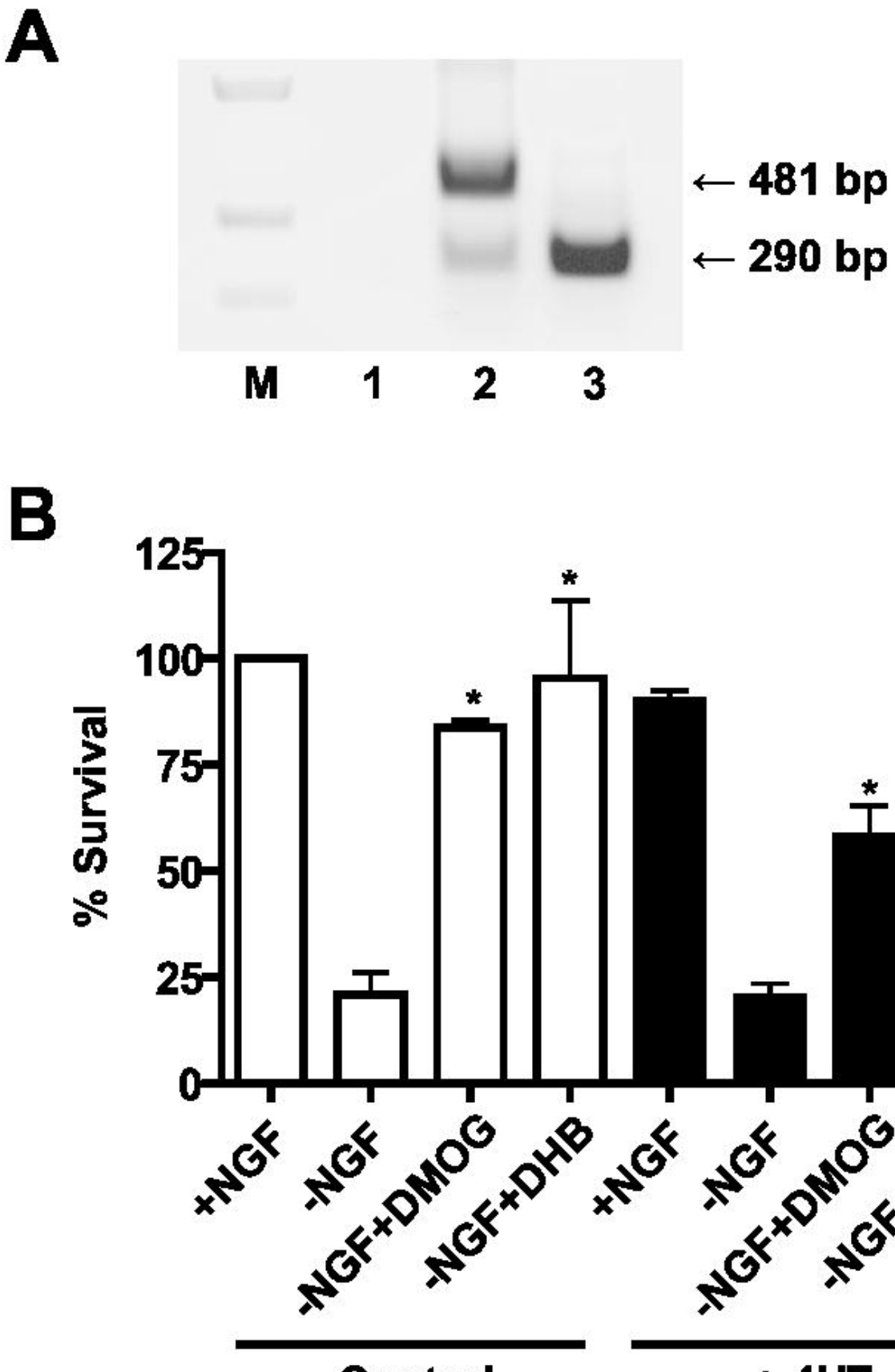

A
← 481 bp
← 290 bp
M
1
2
3
B
% Survival
125
100
75
50
25
0
*
*
*
*
+NGF
-NGF
-NGF+DMOG
-NGF+DHB
+NGF
-NGF
-NGF+DMOG
-NGF+DHB
Control
+ 4HT

Figure 7

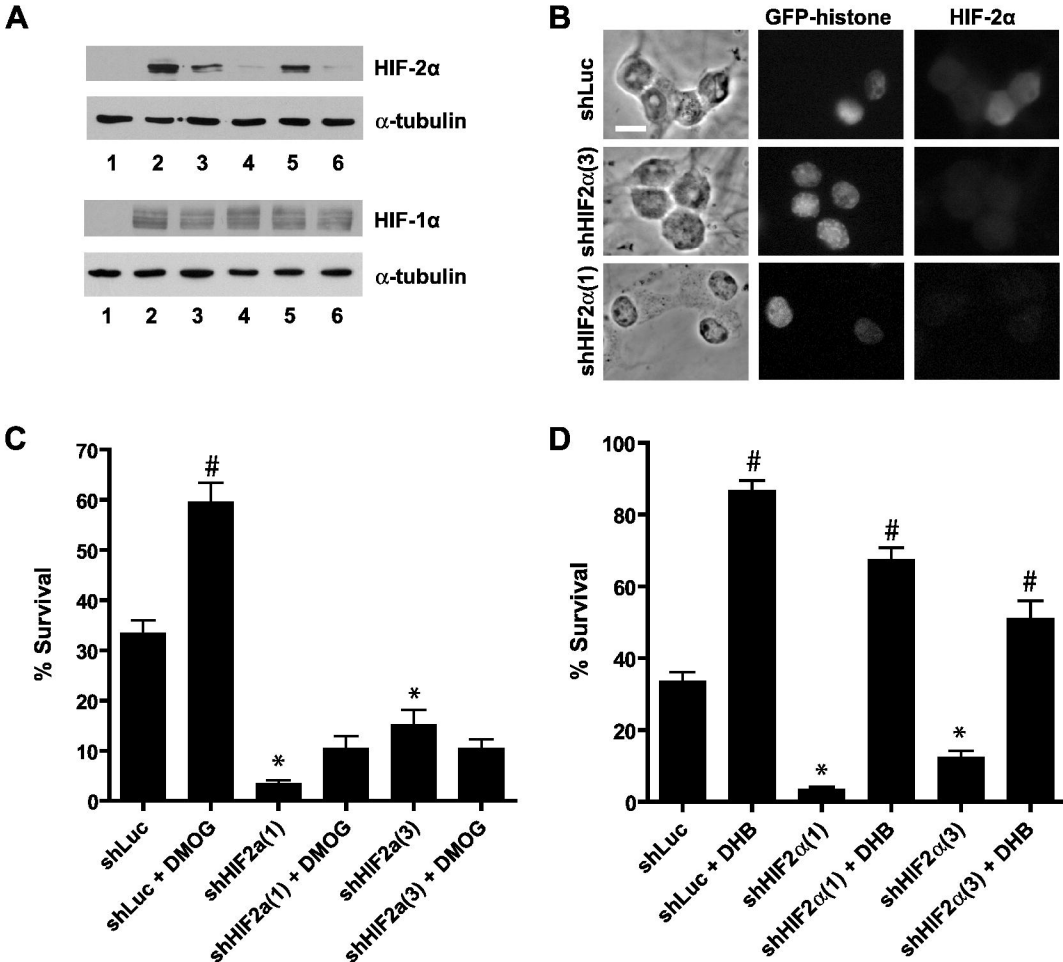
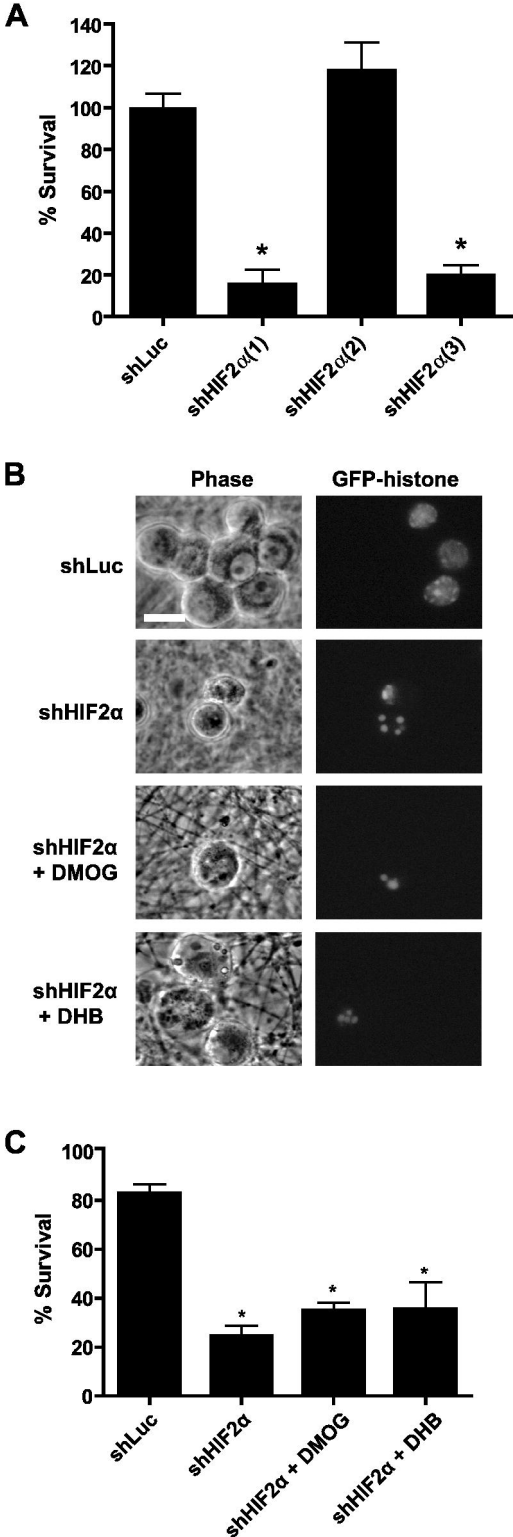

A
HIF-2α
α-tubulin
1 2 3 4 5 6
HIF-1α
α-tubulin
1 2 3 4 5 6
B
GFP-histone
HIF-2α
shLuc
shHIF2α(3)
shHIF2α(1)
C
% Survival
70
60
50
40
30
20
10
0
#
*
shLuc
shLuc + DMOG
shHIF2a(1)
shHIF2a(1) + DMOG
shHIF2a(3)
shHIF2a(3) + DMOG
D
% Survival
100
80
60
40
20
0
#
*
shLuc
shLuc + DHB
shHIF2α(1)
shHIF2α(1) + DHB
shHIF2α(3)
shHIF2α(3) + DHB

Figure 8


A
% Survival
140
120
100
80
60
40
20
0
*
*
shLuc
shHIF2α(1)
shHIF2α(2)
shHIF2α(3)
B
Phase
GFP-histone
shLuc
shHIF2α
shHIF2α
+ DMOG
shHIF2α
+ DHB
C
% Survival
100
80
60
40
20
0
*
*
*
shLuc
shHIF2α
shHIF2α + DMOG
shHIF2α + DHB