Heme Deficiency Interferes with the Ras-Mitogen-activated Protein Kinase Signaling Pathway and Expression of a Subset of Neuronal Genes

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Abstract
Defective heme synthesis in mammals has been suspected of causing neuropathy associated with porphyrias and lead poisoning. To determine the molecular action of heme in neuronal cells, we examined the effect of the inhibition of heme synthesis on nerve growth factor (NGF) signaling in PC12 cells. We found that the inhibition of heme synthesis by succinyl acetone interferes with NGF-induced neurite outgrowth in PC12 cells. Furthermore, we show that heme deficiency obliterates the activation of the signaling intermediates of the Ras-mitogen-activated protein kinase signaling pathway and its downstream target, the transcription activator cyclic AMP response element-binding protein. Strikingly, microarray expression analysis shows that the inhibition of heme synthesis selectively diminishes the induction of expression of a subset of neuron-specific genes by NGF, such as Ras and neurofilament proteins, whereas NGF induces the expression of several major classes of neuronal genes that encode regulatory and structural proteins at three days after induction. Our data provide insights into how heme deficiency interferes with NGF signaling and abrogates programs of neuronal gene expression, thus ultimately causing defective neuronal functions.

Introduction
Heme is central to oxygen sensing and utilization in virtually all living organisms. It is essential for the transport and storage of oxygen, for the generation of cellular energy by respiration, for the synthesis and degradation of steroids, lipids, and related compounds, for the detoxification of xenobiotics, and for controlling oxidative damage. Remarkably, heme directly regulates numerous processes involved in oxygen sensing and utilization (1–5). Defects in heme synthesis in humans are associated with numerous detrimental diseases, including anemia and porphyrias. One major class of porphyrias is acute porphyrias, which are associated with diverse neurological symptoms (6–9). These neurological manifestations include severe abdominal pain, vomiting, constipation, hypertension, tachycardia, and bladder dysfunction, which have been ascribed to autonomic neuropathy (6–9). Additional symptoms include peripheral neuropathy, such as motor weakness and sensory involvement, and mental symptoms without clear morphological findings in the cerebrum (7, 8). Interestingly, lead inhibits heme synthesis and causes neurological disturbances similar to those observed in individuals with acute porphyrias (10). Also, the inhibition of heme synthesis by succinyl acetone in dogs causes neurological disturbances (11). These different lines of evidence all point to a connection between defective heme synthesis and neuropathy.

How does defective heme synthesis cause such neurological disturbances? One possibility is that the accumulation of heme precursors, in particular, ALA4 in neuronal and perhaps hepatic cells, causes neurotoxicity (7, 8). However, recent results (8, 12) from animal models argue against this possibility. Mice with partially defective porphobilinogen deaminase develop porphyrinic neuropathy but do not accumulate a high level of ALA (12). Alternatively, neurotoxicity might be caused by a lack of heme in neuronal or perhaps hepatic cells. Because heme is required for the functions of numerous important hemoproteins in neuronal tissues, such as cytochrome P450, tryptophan dioxygenase, and nitric oxide synthase, it is conceivable that the lack of heme could cause neurological disturbances similar to those observed with acute porphyrias (10). Alternatively, heme deficiency may cause the malfunctioning of important signaling pathways and regulatory proteins, thereby leading to neuropathy.

To determine the role of heme in neuronal signaling and to probe the molecular events underlying neuropathy associated with defective heme synthesis, we examined the effects of the inhibition of heme synthesis by succinyl acetone on NGF signaling in the rat pheochromocytoma (PC12) clonal cells (13). NGF is a neurotrophic factor important for both the peripheral nervous system and the CNS (14, 15). It is critical for the survival, differentiation, and maintenance of neural crest-derived sensory and sympathetic neurons (15) and...
plays important roles in the hippocampus, cortex, and olfactory bulb of the CNS (14). Treatment of PC12 cells with NGF leads to slowing or cessation of cell division and differentiation into sympathetic-like neurons with extended neurites (13). We, therefore, used NGF-induced PC12 cells as a model to dissect the molecular events mediating heme action in neuronal signaling. We found that the inhibition of heme synthesis in PC12 cells specifically interferes with NGF-induced neuronal differentiation and with the activation of the Ras-MAPK signaling pathway and the expression of a subset of neuron-specific genes. These results strongly suggest that heme plays key roles in neuronal signaling and provides a basis for an in-depth molecular understanding of heme action in neuronogenesis and for elucidating the molecular mechanism underlying neuropathy associated with defective heme synthesis.

Results

**Succinyl Acetone Inhibits Heme Synthesis in PC12 Cells and Interferes with NGF-induced Neurite Outgrowth.** Cells grown in regular medium synthesize heme continuously and provide sufficient heme for the proper functioning of various molecular and cellular processes. Thus, to reveal the potential roles of heme in neuronal cells, we created intracellular heme-deficient conditions by using succinyl acetone, which has been shown to be a potent and specific inhibitor of the second enzyme involved in heme synthesis, ALA dehydratase, in mammalian cells (16). Indeed, measurement of intracellular heme synthesis (17) showed that heme synthesis in PC12 cells was reduced by more than 10-fold by 1 mM succinyl acetone (Fig. 1). However, another potential inhibitor of heme synthesis, N-methyl protoporphyrin (an inhibitor for ferrochelatase), was not effective in inhibiting heme synthesis in PC12 cells (Fig. 1).

Remarkably, in succinyl acetone-treated cells, NGF-induced neurite outgrowth was greatly diminished (Fig. 2): The number of neurites was greatly reduced in succinyl acetone-treated cells; in addition, the length of neurites was significantly decreased. The effect was most prominent at 10 days after NGF induction when neurites had fully extended (Fig. 2). Measurement of the neurites shows that the majority (>80%) of the neurites in NGF-induced cells were longer than 220 μm (the average length was 280 ± 130 μm) whereas the majority of the neurites in succinyl acetone-treated, NGF-induced cells were shorter than 60 μm (the average length was 45 ± 27 μm). The addition of exogenous heme to succinyl acetone-treated cells largely restored neurite outgrowth (Fig. 2), supporting the idea that the arrest of neurite outgrowth is caused by a lack of heme synthesis. Together, these data show that succinyl acetone inhibits heme synthesis and causes the arrest of NGF-induced neuronal differentiation, which suggests that heme is critical for NGF signaling.

**Succinyl Acetone Obliterates the Activation of ERK and MEK by NGF, but not TrkA.** The Ras-MAPK signaling pathway mediates both NGF-dependent neuronal differentiation and survival (18–20). Binding of NGF to the receptor TrkA causes activation of TrkA, a tyrosine kinase receptor, and autophosphorylation on tyrosine residues (20, 21). Phosphorylated tyrosine 490 provides a docking site for Shc, which is tyrosine-phosphorylated after receptor activation and is then able to bind the adaptor Grb2. Binding of Grb2 causes accumulation of Grb2-SOS complexes near the cell membrane in which SOS (a guanine nucleotide exchange factor) activates Ras. Activated (GTP-bound) Ras binds and activates Raf, which in turn activates MEKs. Activated MEK1/2 in turn activates the p44/p42 MAPK (the ERK1/2). Therefore, to determine whether heme deficiency interferes with the NGF-induced activation of the Ras-MAPK signaling pathway, we initially examined the effect of succinyl acetone on the activation of ERK1/2, which is mediated by the phosphorylation of its Thr-202 and Tyr-204 residues (19, 22).

As shown previously (19, 22), NGF greatly induced ERK1/2 (p44/p42) phosphorylation (Fig. 3A), detected by using an antibody specific for phosphorylated (Thr-202/Tyr-204) ERK1/2. In contrast, in heme-deficient cells treated with succinyl acetone, ERK1/2 phosphorylation was greatly reduced, particularly at early times (Fig. 3C). The total amount of ERK1/2 was unaffected by addition of both NGF and succinyl acetone (Fig. 3, B and D). The addition of heme to succinyl acetone-treated cells largely reversed the inhibition on ERK1/2 activation (Fig. 3E), as was the case for neurite outgrowth (Fig. 2). These results suggest that the effect of succinyl acetone on neuronal signaling is largely, if not entirely, caused by heme deficiency.

To further ascertain the effect of heme deficiency on the activation of the Ras-MAPK signaling pathway, we examined the effect of succinyl acetone on the activation/phosphorylation of TrkA, Raf, and MEK1/2, which are upstream of ERK1/2 in the Ras-MAPK signaling pathway (20). Using antibodies specific to phospho(Tyr-490)-TrkA, phospho(Ser259)-Raf, and phospho(Ser-217/221)-MEK1/2, we found that MEK1/2 and Raf activation, like ERK activation, at early times of NGF induction, was greatly reduced by succinyl acetone (Fig. 4, A and B), whereas the activation of TrkA was unaffected by succinyl acetone (Fig. 4A). In sum, ERK activation was reduced at all times (Fig. 3), whereas MEK activation was delayed and reduced (Fig. 4C). Raf activation was delayed (Fig. 4B) and was
reduced more than 10-fold by succinyl acetone at 5 and 10 min after NGF induction; at 30 min postinduction, Raf activation was unaffected. Evidently, the impact of succinyl acetone was magnified as the signal was transmitted downstream of TrkA.

Succinyl Acetone Diminishes NGF-induced CREB Activation. The main downstream target of the Ras-ERK1/2 signaling pathway is the transcriptional activator CREB (23, 24). CREB is activated by the M, 90,000 ribosomal S6 kinases (p90RSKs), which are activated by phosphorylated ERK1/2 (25). Once activated by the phosphorylation of Ser-133, CREB activates the transcription of many downstream target genes (24). We reasoned that the lack of ERK1/2 activation in heme-deficient cells should also diminish the activation of CREB. Thus, we examined the effect of heme deficiency on NGF-induced CREB activation by using an antibody specific for phospho(Ser-133)-CREB. As expected, NGF greatly induced CREB activation (Fig. 5A), but CREB activation at early times (from 5 to 60 min) was completely suppressed by the inhibition of heme synthesis. For a control, we show that the levels of total cellular CREB were unaffected by NGF or succinyl acetone (Fig. 5B). These data suggest that the inhibition of heme synthesis by succinyl acetone interferes with the activation of the Ras-MAPK signaling pathway by NGF and, thus, ultimately diminishes the activity of the transcriptional activator CREB.

Inhibition of Heme Synthesis by Succinyl Acetone Selectively Affects the Expression of a Subset of NGF-affected Neuron-specific Genes. The above experiments show that heme deficiency obliterates the activation of the Ras-MAPK signaling pathway and its downstream transcription factor, which suggests that heme deficiency should ultimately cause changes in neuronal gene expression. In addition to the genes affected by the Ras-MAPK signaling pathway and CREB, heme deficiency may affect the expression of numerous other neuron-specific genes. Therefore, to

![Image of Fig. 2](image_url)

**Fig. 2.** The effect of heme deficiency on NGF-induced PC12 cell neuronal differentiation. PC12 cells were treated with no reagent (None), or succinyl acetone alone (SA), or NGF alone (NGF), or NGF and succinyl acetone (NGF+SA), or NGF and heme and succinyl acetone (NGF+SA+heme) for 10 days. Scale bar, 100 μm.

![Image of Fig. 3](image_url)

**Fig. 3.** The effect of heme deficiency on p44/42 MAPK (ERK1/2) phosphorylation during NGF-induced PC12 cell differentiation. A, a time course of ERK1/2 activation by NGF, PC12 cells were treated with no reagent (N, Lane 1), or succinyl acetone alone (SA, Lane 2), or NGF alone (NGF; Lanes 3–7). Proteins were extracted from PC12 cells induced with NGF for 5 (Lane 3), 10 (Lane 4), 30 (Lane 5), 60 (Lane 6), or 180 min (Lane 7). The same amount of total cellular proteins was loaded in each lane. Activated ERK1/2 proteins were detected by Western blotting with an antibody specific for phospho(Thr-202/Tyr-204)-p44/42 (ERK1/2). The positions of phospho-p44/42 (p-p44/42) and p44/42 are marked. B, the total ERK1/2 protein level in NGF-induced cells. Total cellular ERK1/2 proteins were detected by using an antibody specific for both phosphorylated and nonphosphorylated p44/42 (ERK1/2). The same blot in A was stripped and reprobed here. C, a time course of ERK1/2 activation by NGF in heme-deficient cells. PC12 cells were treated with no reagent (N, Lane 1), or succinyl acetone alone (SA, Lane 2), or NGF and succinyl acetone (NGF+SA, Lanes 3–7). Proteins were extracted from PC12 cells induced with NGF for 5 (Lane 3), 10 (Lane 4), 30 (Lane 5), 60 (Lane 6), or 180 min (Lane 7). The same amount of total cellular proteins was loaded in each lane. Activated ERK1/2 proteins were detected by Western blotting with an antibody specific for phospho(Thr-202/Tyr-204)-p44/42 (ERK1/2). D, the total ERK1/2 protein levels in NGF-induced heme-deficient cells. The same blot in C was stripped and reprobed with an antibody specific for both phosphorylated and nonphosphorylated p44/42 (ERK1/2). E, the effect of heme add-back on ERK1/2 phosphorylation. PC12 cells were treated with no reagent (Lane 1), NGF alone (Lanes 2–4), NGF and heme and succinyl acetone (Lanes 5–7), or NGF and succinyl acetone (Lanes 8–10). Proteins were extracted from PC12 cells induced with NGF for 10 (Lanes 4, 7, and 10), 30 (Lanes 3, 6, and 9), or 60 min (Lanes 2, 5, and 8). Activated ERK1/2 proteins were detected by Western blotting with an antibody specific for phospho(Thr-202/Tyr-204)-p44/42 (ERK1/2). F, the total ERK1/2 protein levels in NGF-induced heme-sufficient and -deficient cells. The same blot in E was stripped and reprobed with an antibody specific for both phosphorylated and nonphosphorylated p44/42 (ERK1/2) here.
gain a broad idea of the genes the expression of which is affected by heme deficiency, we used the powerful microarray technology to systematically identify NGF-affected and heme-regulated neuronal genes. Because of their well-designed controls, we used the Affymetrix Rat Neurobiology U34 array. This array includes more than 1200 mRNA sequences for neuron-specific genes. These genes include sequences for membrane channels, receptors, synthases, transcription factors, oncogenes, and CNS-disease specific genes.

Although the Ras-MAPK and other signaling pathways are activated at early times of NGF induction, the expression of most neuron-specific genes are highly induced 3 days after NGF treatment (26–28). Thus, 3 days after NGF induction should be the optimal time point for identifying many NGF-induced neuron-specific genes and genes the expression of which is affected by succinyl acetone. Thus, to gain a relatively comprehensive description of neuron-specific genes and how succinyl acetone may affect their expression, we examined gene expression patterns in NGF-induced PC12 cells at 3 days after NGF induction. We prepared mRNAs from uninduced cells, from NGF-induced cells, and from cells induced with NGF in the presence of succinyl acetone. RNA probes were generated and then hybridized to the U34 arrays. Analysis of control genes showed that the probes worked properly. First, signal variations from 3/H11032,5/H11032, and the middle of control genes, including /H9252-actin and /H9252-GAPDH, were less than 30%. Second, differences in mRNA levels of both /H9252-actin and /H9252-GAPDH in untreated, NGF-induced, and succinyl acetone-treated, NGF-induced cells were less than 30%. Third, signals were not detected from probe sets containing sequences complementary to sense mRNAs (the probes contain sequences of the antisense mRNAs).

The expression analysis systematically revealed the major neuronal genes the expression of which is altered by NGF (Table 1). Only those genes the expression of which was indisputably changed (judged by the most strict standards of Affymetrix) in response to NGF or succinyl acetone by more
than 3-fold are shown (Table 1). Genes whose expression may be changed by NGF or succinyl acetone but whose mRNA levels were too low to satisfy all of the criteria for a clear call of “decrease” or “increase” are not shown. Thus, certain genes that are not shown in Table 1 may also be affected by NGF. These genes include TGF (transforming growth factor)-β and neuronal NOS (nitric oxide synthase), whose expression was mathematically detected to be decreased but was not called as changed by the program, presumably because of their low mRNA levels. Quantitative RT-PCR analysis (data not shown) showed that the expression of nNOS and TGF-β was induced by NGF but suppressed by succinyl acetone.

Several lines of new information emerge from this analysis. Most important, several major classes of neuronal structural genes were induced by NGF, whereas a few genes were suppressed (Table 1). First, induced genes include mRNAs of five (groups of) neurotransmitter receptors, mRNAs of six (groups of) proteins important for synaptic vesicle release, and mRNAs of six (groups of) neuronal cytoskeletal and extracellular proteins (Table 1). Second, important signaling intermediates were induced by NGF. These include β-arrestins, which mediate signaling by G-protein-coupled receptor tyrosine kinases (29), Rab1B, which participates in the regulation of vesicular transport (30), JAK1, which has recently been shown to mediate signaling by the ciliary neurotrophic factor (31, 32), Ca2+/calmodulin-dependent protein kinase I (33), a splicing product of the p85α regulatory subunit of PI3-kinase (34), p38 MAPK (35), and p21 c-Ki-Ras (36). In contrast, Gβ1, which is essential for establishing cocaine sensitization (37), was suppressed by NGF. Many of these signaling intermediates have been shown to be expressed in neuronal cells (29, 31, 32). Our data here confirm their roles in NGF signaling and neuronal functions.

We confirmed the data (Table 1) by RT-PCR, by Northern blotting, and, if antibodies were available, by Western blotting. Fig. 6 shows results from Northern blotting analysis of mRNA levels of several key neuron-specific proteins, including the small neurofilament protein NF-L, the rVAT, the Ras-related Rab1B protein, and the p21 c-Ki-Ras protein. The data shown in Fig. 6 are largely consistent with the results shown in Table 1. The variations in β-actin mRNA levels were within 2-fold. The mRNA levels of NF-L, rVAT, Rab1B, and p21 Ras were enhanced at least 3-fold by NGF and were reduced to levels similar to those in uninduced cells by succinyl acetone (see Fig. 6 and Table 1). Furthermore, our data are consistent with previous studies of NGF-induced expression of several well-characterized genes. For example, the synthesis of the microtubule-associated protein MAP1B, p53, and nicotinic acetylcholine receptor have previously been shown to be induced by NGF (38). In particular, the induction of p53 plays an important role in NGF-induced PC12 differentiation (38). The agreement between our data and previous studies supports the validity of this expression analysis. Notably, the hypoxia-inducible factor HIF-1, presenilin-2 (39), an Alzheimer-associated protein, neuropilin (40), the receptor for vascular endothelial growth factor, monamine oxidase A (41), and several other neuronal proteins and enzymes were also induced by NGF. Leptin receptor (42),
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Rab1B, p21 c-Ki-Ras, and Northern blotting analysis with radiolabeled cDNA probes for NF-L, rVAT, ured by using a PhosphorImager (Molecular Dynamics).

None
PC12 cells were treated with no reagent (None, Lane 1), or succinyl acetone alone (SA, Lane 2), NGF alone (Lane 3) or NGF and succinyl acetone (NGF+SA, Lane 4). mRNAs were extracted and subjected to Northern blotting analysis with radiolabeled cDNA probes for NF-L, rVAT, Rab1B, p21 c-Ki-Ras, and β-actin. Radioactivity was revealed and measured by using a PhosphorImager (Molecular Dynamics).

Second, we showed that the inhibition of heme synthesis by succinyl acetone abolished the activation of the Ras-MAPK signaling pathway by NGF. The activation of ERK, MEK, Raf, and the transcription activator CREB, but not TrkA, at the early time of NGF induction, was obliterated by the succinyl acetone-mediated inhibition of heme synthesis (Figs. 3–5). Third, we showed, by using the Affymetrix GeneChip expression analysis, that NGF induced the expression of several major classes of neuronal structural proteins and signaling components (Table 1; Fig. 6). Remarkably, the inhibition of heme synthesis affected only a few of these NGF-affected genes (Table 1), which suggests that heme plays a specific and selective role in NGF signaling and neuronal gene expression. These studies in PC12 cells provide compelling evidence for the role of heme in neuronal signaling at the molecular level and shed light on how defective heme synthesis causes neuropathy in porphyrias and lead poisoning.

These results are consistent with previous studies showing that heme can counteract the toxic effects of lead in mouse dorsal root ganglia (47). Because succinyl acetone may cause the accumulation of ALA, it raises the possibility that accumulation of ALA, not heme deficiency, accounts for the changes in the activation of the Ras-MAPK pathway and in neuronal gene expression. Although our data cannot entirely exclude this possibility, several lines of evidence argue against it. First, heme reversed the effects of succinyl acetone on the activation of the Ras–ERK1/2 pathway in PC12 cells, even when added together with NGF or only 1 h prior to NGF addition. In such a short time, the presumably accumulated ALA level is unlikely to be significantly reduced. Thus, the effect of succinyl acetone on the Ras–ERK1/2 signaling pathway is unlikely to be solely caused by the accumulation of ALA. Second, mice with partially defective porphobilinogen deaminase develop porphyric neuropathy but do not accumulate a high level of ALA (12). Thus, heme deficiency is likely responsible, at least in part, for the changes in signaling pathways and in gene expression caused by succinyl acetone, and for the neuropathy associated with acute porphyrias.

It is conceivable that heme deficiency may cause neuronal dysfunction because many important neuronal proteins, such as nitric oxide synthase and heme oxygenase, require heme for proper functioning. Even yeast cells require a low level of heme for maintaining vital cellular functions (3). In yeast, the heme activator protein Hap1 is the key mediator of heme action, although it is increasingly clear that other regulatory proteins, such as the Hap2/3/4/5 complex, also mediate heme regulation (3). In mammalian cells, heme regulation is even more complex; heme acts on not only transcription factors, such as NF-Y, but also kinases, such as the heme-regulated inhibitor kinase HRI (2, 48, 49). Although a class of heme-regulated proteins has been shown to contain a conserved heme-responsive motif (50), many heme-regulated proteins, such as NF-Y, do not contain typical heme-responsive motifs. In PC12 cells, our data suggest that heme acts on a component of the Ras-MAPK signaling pathway downstream of TrkA but upstream of Raf, or Raf itself. In addition, heme may act directly on certain transcription factors and cause changes in neuronal gene expression. Our

Discussion
Here we have presented morphological, biochemical, and gene expression data suggesting that heme plays a specific and selective role in NGF signaling and neuronal gene expression. First, we showed that the inhibition of heme synthesis in PC12 cells interfered with neurite outgrowth, but the addition of exogenous heme relieved this effect (Fig. 2).
data here provide a basis for further molecular analyses of heme action in neuronal cells.

The Ras-MAPK signaling pathway is critical not only for NGF-induced neuronal differentiation but also for neuronal survival (20, 21, 51). Likewise, CREB activation is important for both neuronal differentiation and neuronal survival (24, 25). Our data show that the effect of heme deficiency on the Ras-MAPK signaling pathway is 2-fold. First, at early times, immediately after NGF induction, the rapid activation of the pathway by NGF is obliterated by the inhibition of heme synthesis (Figs. 3–5). Second, at late times of NGF induction, the increase in p21 Ras expression and in the expression of signaling components of other important neuronal signaling pathways, such as Rab1B and JAK1 (Table 1), is diminished by the inhibition of heme synthesis. Thus, the effect of heme deficiency on the Ras-MAPK signaling pathway not only may interfere with the initiation of neuronal differentiation but may also impact on neuronal maintenance and survival. This reasoning would provide a molecular explanation for the previously observed role of heme in preventing neuronal degeneration (47) and for neurological disturbances associated with porphyrias (8).

The expression analysis (Table 1) shows that the inhibition of heme synthesis by succinyl acetone in PC12 cells does not cause a systemic failure of cellular functions. The inhibition of heme synthesis by succinyl acetone may lower the intracellular heme concentration to a level that can still support critical housekeeping cellular functions but not neuronal functions. The number of genes the expression of which is affected by heme deficiency is small compared with the number of genes the expression of which is affected by NGF (Table 1). This suggests that heme deficiency, and thus heme, has a specific and selective effect on neuronal gene expression. Heme deficiency reduced NGF induction of the expression of three signaling components, Rab1B, JAK1, and p21 Ras, and three neuronal structural proteins, NF-L, MAP1B, and rVAT (Table 1). This reduction of the expression of these major neuronal structural proteins provides an explanation for the lack of neurite outgrowth and neuronal differentiation in succinyl acetone-treated cells. Taken together, these data provide a sketch of the molecular events mediating heme action in NGF signaling. Evidently, inhibition of heme synthesis would initially diminish the activation of NGF signaling pathways, such as the Ras-MAPK signaling pathway. Then, this lack of activation would interfere with the activation of transcription factors, such as CREB, and with the expression of signaling components that ultimately permit the synthesis of neuronal structural proteins for the formation of neuronal cells.

Finally, the expression analysis also provides several new insights into NGF-induced gene expression in PC12 cells. First, the data show that several major classes of neuronal structural proteins, including neurotransmitter receptors, synaptic vesicle proteins, and neuronal cytoskeletal proteins, are systematically induced by NGF (Table 1). This result is expected but, nonetheless, new. Second, somewhat unexpectedly, the expression of quite a few signaling components, including Ras, Rab1B, and JAK1 (Table 1), is induced at late times of NGF induction. Previously, it was thought that signaling pathways, such as the Ras-MAPK pathway, led to the specific programs of immediate early gene and DRG expression (52). Whereas immediate early genes often encode transcription factors, DRGs are believed to be those genes that encode primary structural proteins and enzymes that contribute to the neuronal phenotype of NGF-treated PC12 cells. Our data (Table 1) show that DRGs include genes encoding not only structural proteins and enzymes but also signaling components and transcription factors such as p53 and HIF-1. These results suggest that neuronal differentiation and maintenance require a complex network of expression programs of both regulatory and structural genes.

**Materials and Methods**

**Cell Culture and Inhibition of Heme Synthesis.** PC12 cells (American Type Culture Collection) were maintained in HAM’s F-12 medium (Cellgro) with 10% horse serum and 5% fetal bovine serum. To create intracellular heme deficiency, cells were cultured in medium containing heme-depleted serum and 1 mM succinyl acetone. Heme-depleted serum was generated by treating serum with ascorbic acid for 2 h, followed by dialysis against PBS and filter sterilization. The depletion of heme is verified by measuring the serum optical absorbance at 405 nm. Typically, serum absorbance is reduced at least 50% after treatment with ascorbic acid. For neuronal differentiation, PC12 cells were cultured on polylysine-coated dishes and treated with 20 ng/ml NGF (grade II; Boehringer Mannheim). For the inhibition of heme synthesis, PC12 cells were pretreated with succinyl acetone 12–24 h prior to the addition of NGF, and the cells were continuously treated with succinyl acetone for the duration of the experiments. For heme add-back experiments, 10 μM heme was added to the cells 1 h prior to the NGF addition.

For the measurement of heme synthesis, PC12 cells were treated with 0, 0.5, or 1 mM succinyl acetone, or 2 μM N-methyl protoporphyrin IX for 12–24 h, and then were incubated with 0.4 μCi of radiolabeled ALA for 24 h. Heme was extracted from these cells by using acetone–hydrochloric acid and diethyl ether, and the amount of radiolabeled heme was measured as described previously (17, 53). The incorporation of radioactivity into the extracted heme allows the measurement of heme biosynthesis. The amount of radiolabeled heme was measured by scintillation counting.

**Western Blotting Analysis.** PC12 cells were grown on polylysine-coated culture plates and treated, and proteins were extracted, as described previously (22, 54). Briefly, PC12 cells were washed twice in PBS, and whole-cell extracts were prepared by adding 10 packed cell volumes of 1× sample buffer [2% SDS, 100 mM DTT, 60 mM Tris (pH 6.8), and 10% glycerol] and boiled for 5 min. Protein concentrations were determined by using Bradford Protein assays (Bio-Rad). Twenty μg of proteins was analyzed on 12% SDS-PAGE and transferred onto the Immuno-Blot polyvinylidene difluoride membrane (Bio-Rad). The membranes were probed with polyclonal antibodies, followed by detection

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5 D. Bishop, personal communication.
with a chemiluminescence Western blotting kit (Boehringer Mannheim). Polyclonal anti-phospho(Thr-202/Tyr-204)-MAPK (ERK1/2), anti-ERK1/2, anti-phospho(Tyr-421/422)-TrkA, anti-TrkA, anti-phospho(Ser-259)-Raf, anti-phospho(Ser-217/221)-MEK1/2, anti-MEK1/2, anti-CREB, and anti-phospho-CREB (Ser-133) antibodies were purchased from New England Biolabs, Inc.

RNA Extraction and Northern Blotting Analysis. Treated or untreated PC12 cells were collected, and total RNA was extracted by using TRIzol reagent (Life Technologies, Inc.). Northern blotting analysis was carried out as described previously (55). Radiolabeled cDNA probes for NF-L, rVAT, Rab1B, p21 c-Ki-Ras, and β-actin mRNA were synthesized by using cDNA obtained from RT-PCR amplification. Radioactivity was revealed and measured by using a PhosphorImager (Molecular Dynamics).

Affymetrix GeneChip Expression Analysis. The rat neurobiology U34 arrays were purchased from Affymetrix, Inc. For preparation of probes, total RNAs were extracted from PC12 cells treated without reagent, with 30 ng/ml NGF, or with 30 ng/ml NGF and 1 mM succinyl acetone. The synthesis of cDNAs and biotin-labeled cRNAs were carried out exactly as described in the Affymetrix GeneChip Expression Analysis Technical Manual (2000). Probe hybridization and data collection were carried out by the Albert Einstein College of Medicine GeneChip processing center. Data analysis was performed by using the Affymetrix Microarray suite version 4.0.

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