From Embryonal Carcinoma Cells to Neurons: The P19 Pathway

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Summary

The differentiation of mammalian neurons during development is a highly complex process involving regulation and coordination of gene expression at multiple steps. The P19 mouse embryonal carcinoma cell line is a suitable model system with which to analyze regulation of neuronal differentiation. These multipotential cells can be maintained and propagated in tissue culture in an undifferentiated state. Exposure of aggregated P19 cells to retinoic acid results in the differentiation of cells with many fundamental phenotypes of mammalian neurons. Undifferentiated P19 cells are amenable to genetic manipulations such as transfection and establishment of stable clonal cell lines expressing introduced genes. Proteins that play a key role in the neuronal differentiation of P19 cells are beginning to be identified. These include retinoic acid receptors, the epidermal growth factor receptor and the transcription factors Oct-3 and Brn-2. The biological and technical advantages of this system should facilitate deeper analysis of the activities of proteins that play a role in neuronal differentiation.

Introduction

Mammalian neurons are highly specialized cells. At every level – anatomical, physiological and molecular – they express properties which make them exquisitely adapted to perform their function: the voluminous integration and analysis of information on a millisecond time scale. In development, neurons arise from much smaller and simpler progenitors. These cells are multipotential and are capable of giving rise to multiple types of neurons and also to glia, the support cells of the nervous system. At a stage of development which is narrowly defined for each type of neuron, progenitors withdraw from the cell cycle and rapidly change their form and function into those of neurons. A large array of processes, ranging from differential gene expression to growth and assembly of axons, dendrites and synapses, underlie the cellular transformation of progenitors into fully differentiated neurons. In the past few years a great deal has been learned about the cellular and molecular changes involved in each of these aspects of neuronal differentiation.

A great practical challenge facing this field is to establish model systems for analyzing the mechanisms by which developmental changes are regulated and coordinated. Over the past several years, an increasing number of laboratories have been studying the P19 teratocarcinoma cell line as a model of neuronal differentiation. These cells can easily be maintained in tissue culture in an undifferentiated state. They can also be induced to differentiate synchronously into neuron-like cells by a simple and reproducible procedure. In this review we will summarize studies demonstrating that P19 cells differentiate along a pathway leading to cells with many of the important features of neurons. We will then discuss experiments which are beginning to identify regulatory proteins that are essential for this differentiation process.

Basic characteristics of P19

The P19 cell line was isolated by Rogers and McBurney[1]. It was derived from a teratocarcinoma in C3H/He mice, produced by grafting an embryo at 7 days of gestation to the testes of an adult male mouse. The resulting tumour was excised, dissociated and plated for growth in tissue culture. A clonal cell line was selected which possessed the typical morphology of embryonal carcinoma (EC) cells and was designated P19[1].

P19 cells have a number of characteristics which make them a suitable model for studying early mammalian development. The cells possess a normal karyotype[1], sugggesting that they do not contain any gross genetic abnormalities. In addition, they are multipotential. Depending upon the treatment used, P19 EC cells can differentiate *in vitro* into derivatives of all three germ layers – endoderm, mesoderm and ectoderm. Similarly, when injected into mouse blastocysts, P19 EC cells differentiate into a broad range of cell types in the resulting chimeras[2]. These observations show that P19 cells have the capability to participate in many normal differentiation pathways.

P19 cells are eminently suitable for cell genetic approaches. They divide rapidly and maintain their ability to differentiate after many passages in culture. Stably transfected clonal sublines are readily obtained at high frequency. Such clones retain their ability to differentiate. Therefore both normal and mutated proteins can be overexpressed to determine possible effects on neuronal differentiation. Expression of endogenous proteins can be blocked by antisense RNA. Finally, the potential of creating specific mutations by homologous recombination exists.

Two key elements are involved in regulating P19 cellular differentiation *in vitro*. The first is the nature of the chemical inducer. The second is the formation of cellular aggregates during treatment with the inducer; this is necessary to stimulate the differentiation of some cell types. Exposure of aggregated P19 EC cells to dimethylsulfoxide leads to the formation of cells with many of the characteristics of cardiac and skeletal muscle[3]. Alternatively, treatment of aggregated cells with retinoic acid (RA) results in their differentiation into cells that resemble neurons, glia and fibroblast-like cells[4]. RA treatment of P19 EC cells growing in monolayers rather than aggregates stimulates the formation of endodermal and mesodermal derivatives[5].

Characteristics of P19 neurons

Treatment of aggregated P19 stem cells with retinoic acid for four days results in cells that are capable of differentiating into neuron-, glia- and fibroblast-like cells when the induced aggregates are dispersed and plated under suitable conditions. In the remainder of this review we will refer to the neuron-like cells as 'P19 neurons' for convenience, with the understanding that they may differ from normal neurons in some respects. A number of observations suggest that the P19 neurons closely resemble neurons present in the mammalian nervous system. First, their morphology is similar to cultured brain cells. P19 neurons in tissue culture have small cell bodies with long, elaborate processes similar to axons and dendrites in appearance. Second, P19 neurons are stably post-mitotic, as are normal neurons. Third, electron microscopic observation has revealed structures with the pre- and post-synaptic features of chemical synapses[6]. In agreement with this, functional synapses can be detected between P19 neurons using electrophysiological techniques[7]. Fourth, a variety of neurotransmitters and associated gene transcripts and enzymes are expressed in P19 neurons. Choline acetyltransferase (ChAT)[8], enzymes of catecholamine (CA) synthesis including tyrosine hydroxylase (TH), dopamine β-hydroxylase (DBH) and phenylethanolamine N-methyl transferase (PNMT)[9], transcripts of the glutamic acid decarboxylase (GAD) 1 and 2 genes[10], and GAD enzyme[11] have all been detected in various studies. Fifth, functional ionotropic glutamate receptors of both the NMDA and AMPA/kainate types are present on P19 neurons[12,13]. These receptors are hallmarks of neurons located in the central nervous system and are not expressed in most of the commonly used neuronal cell lines. In fact, the only other cell line known to express these receptors is Ntera2, a human retinoic acid-inducible EC line[14]. Sixth, P19 neurons express a number of cell surface carbohydrate antigens characteristic of neurons[15]. Finally, a variety of neuron-specific genes and proteins not directly involved in neurotransmission are expressed in P19 neurons. These include neurofilaments (NF)[4], nerve-specific enolase (NSE)[9], various microtubule-associated proteins (MAPs)[16], a neuron-specific form of β-tubulin called Mβ-6[17], and the amyloid precursor protein (APP)[17]. Of particular relevance to differentiation, the mammalian homologs of several genes important in neural differentiation in *Drosophila*, such as Motch[18], MASH[19] and Wnt-1[20], are expressed in neuronally differentiating P19 cultures.

Taken together, these data show that P19 neurons bear a strong resemblance to normal mammalian neurons. However,it should be emphasized that many questions about P19 neurons remain to be answered. For example, neurons in the nervous system usually express a single non-peptide neurotransmitter; it is not yet known whether P19 neurons do the same. In addition, while functional synapses have been detected on P19 neurons, little data as to the details of their physiology are available. The present data supports the idea that P19 neurons express what may be thought of as 'core' neuronal phenotypes. Whether they model any part of the diversification of neurons that occurs in the intact nervous system is an open question that requires much additional investigation.

Patterns of gene expression during P19 differentiation

During neuronal differentiation *in vivo* the expression of key regulatory genes occurs in a defined temporal sequence. It is important to know whether a similar sequence is followed during the differentiation of P19 neurons. While this issue has not been examined in great detail, there are a few examples to suggest that a temporal pattern of gene expression also occurs in P19 differentiation. MASH-1 and Motch, the mammalian homologs of *Drosophila* neurogenic regulatory genes, are expressed during the early stages of P19 neuronal differentiation, as is the Wnt-1 cellular oncogene; transcript levels of these genes rise dramatically about two days after RA treatment of aggregated P19 cells is begun[18-20]. In contrast, the expression pattern for GAD1 and GAD2 transcripts, which encode a neurotransmitter-synthesizing enzyme, is quite different[10]. Here the dramatic rise in transcript levels occurs 8-10 days following the start of RA treatment. Levels of other neuronally expressed genes rise at intermediate time points. Additional data are needed to determine whether a regulatory cascade of gene expression occurs during the neuronal differentiation of P19 cells.

Factors involved in neuronal differentiation of P19 cells

For reasons mentioned briefly above, the P19 system is highly suitable for analyzing the basic mechanisms that underlie the differentiation of neurons. Recent studies have begun to exploit the advantages of this system and have already given valuable insights into mechanisms of neuronal differentiation. A number of regulatory proteins which appear to be critical to the neuronal differentiation of P19 cells have been identified. First, the well-characterized family of retinoic acid receptors is probably involved in mediating the differentiation-inducing activity of RA. Second, the epidermal growth factor receptor, a member of the receptor tyrosine kinase family, also appears to be required. Third, the transcription factor Oct-3 seems to be involved. Finally, Brn-2, another transcription factor, appears to be required for neuronal differentiation in this system.

Differentiation of P19 cells into neurons requires exposure of the aggregated cells to RA. The effects of this molecule in a variety of biological systems appear to be mediated through the action of an ensemble of receptors and binding proteins. There are two major families of receptors – the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). Each family consists of three types of receptors, designated α, β and γ[21]. All these proteins are products of separate genes[21], and several isoforms of each type can be generated by alternative splicing[21]. Both the RARs and RXRs act as ligand-inducible transactivating factors, which regulate the transcription of target promoters[21]. In addition to these receptors, mammalian cells also possess a number of cytoplasmic retinoid and retinoic acid-binding proteins, which it

has been proposed control the concentration of free RA within the cell[21].

The expression patterns of the RARs during P19 neuronal differentiation have been analyzed [22]. Undifferentiated cells express low levels of both RARα and RARγ mRNA, while RARβ mRNA is undetectable. Upon RA treatment of aggregated cells, the amount of RARα mRNA increases rapidly, while that encoding RARγ is strongly repressed. There is also a swift accumulation of high levels of RARβ mRNA.

Direct evidence for a functional role of the RARs in P19 differentiation has come from studies of a mutant cell line derived from P19. This line, known as RAC65, does not differentiate when treated with RA; instead, the cells maintain the undifferentiated EC phenotype[8]. Recently, a number of studies have determined that RAC65 cells contain a mutation in the RARα gene[23,24] which leads to the loss of 70 amino acids from the C terminus of the RARα protein. The truncated RARα acts as a dominant repressor of transcription from RA-responsive gene promoters[23,24]. One possible conclusion from these results is that RARα function is essential for neuronal differentiation in P19 cells. This hypothesis is supported by the observation that RA responsiveness in RAC65 cells can be at least partially restored by the expression of high levels of the normal untruncated RARα[24]. However,it is important to note that similar truncations in the human RARα molecule have been found to be potent inhibitors of all three classes of RAR receptors[25], so in RAC65 cells the actions of the β and γ, as well as the α, receptors are presumably blocked by the truncated RARα molecule. Therefore, while the RAC65 data strongly suggest that the RAR family plays a key role in the neuronal differentiation of P19 cells, they do not definitively implicate any particular RAR in this process. Furthermore, expression of the truncated RARα in normal P19 cells does not completely block RA responsiveness[23]. This raises the possibility that RAC65 cells may possess other mutations important for their nonresponsive phenotype. In this regard, it is worth noting that the expression of the gene encoding the cytoplasmic retinoic acid-binding protein is abnormal in RAC65 cells[26].

All three RARs act as inducible *trans*-acting factors which regulate gene expression[21]. Therefore, it is likely that the primary function of these molecules in the neuronal differentiation of P19 cells is to activate and/or repress the expression of a set of genes required for differentiation. The identification of such regulated genes, and the determination of their functions, remain critical goals in the study of P19 differentiation. While beyond the scope of this review, it should be noted that evidence is beginning to accumulate to suggest that RA and its receptors are also involved in neural differentiation *in vivo*[27]. Expression of other proteins involved in RA recognition, such as the RXRs, during the neuronal differentiation of P19 EC cells has not yet been examined in detail.

Another regulatory protein that appears to be critical for P19 differentiation is the epidermal growth factor receptor (EGFR). The EGFR is a cell surface glycoprotein, which possesses a tyrosine kinase in its cytoplasmic domain that is activated upon binding EGF. Undifferentiated P19 cells do not contain any EGFR located on the cell surface, although a small amount is present intracellularly[28]. However, levels of both the mRNA and protein are dramatically upregulated by RA treatment of aggregated P19 cells, beginning after about 48 h of exposure to the inducer[28]. In addition, biochemically active EGFR molecules begin to appear on the cell surface[28].

Direct experimental evidence for a functional role of the EGFR in P19 neuronal differentiation has been obtained from both gain-of-function and loss-of-function experiments. P19 cells modified to express large amounts of the human EGFR in the undifferentiated stem cells no longer require RA to induce differentiation. Instead, treatment of the aggregated cells with EGF triggers extensive neuronal differentiation[29]. Conversely, inhibition of EGFR function in P19 cells, either by antisense RNA or by the expression of a dominant negative form of the receptor which blocks the activity of the endogenous receptor, severely curtails the differentiation of neurons in RA-treated cultures[30]. Taken together, these experiments indicate that the EGFR plays a critical role in the pathway which leads to the neuronal differentiation of P19 cells.

The mechanism by which the EGFR works in these cells is unknown. Presumably, the tyrosine kinase activity of the receptor is involved. This activity must be activated by the binding of a ligand to the receptor. Both epidermal growth factor (EGF) and transforming growth factor α (TGFα) activate the EGFR. P19 cells produce TGFα[31], so this molecule may be the activator which stimulates the EGFR during P19 neuronal differentiation. Further studies of TGFα expression and activity in these cells are required to test this hypothesis. There is no report of EGF being produced by P19 cells.

Another regulatory protein that may play an important role in P19 differentiation is Oct-3. Like the RARs, Oct-3 is a *trans*-activating factor which modulates transcription from target gene promoters[32]. Studies in mammalian embryos have shown that Oct-3 is expressed only in pluripotent early embryonic cells and germ cells. It is downregulated as these cells differentiate[32]. These observations suggest that Oct-3 may be required in pluripotent cells and that its downregulation may be a prerequisite for differentiation.

As predicted from its expression pattern in embryos, Oct-3 is present in undifferentiated P19 cells, but is rapidly downregulated when the cells are treated with RA and begin to differentiate. Oct-3 mRNA is dramatically reduced within 12 hours of the beginning of RA treatment[33], while Oct-3 protein disappears from the cells within 48 h[34]. Consequently, the expression of genes dependent on Oct-3 will be inhibited, while those which are repressed by Oct-3 may become activated in the differentiating cells.

A recent study has demonstrated a strong correlation between Oct-3 downregulation and the onset of neural differentiation[34]. The expression of Oct-3 was manipulated by the phenomenon of hybrid extinction. In general, when two different cell types are fused, the expression of many cell type-specific genes is repressed in the resulting hybrids[35]. Undifferentiated P19 stem cells were fused to fibroblasts and several hybrid lines were isolated. In all these lines, both Oct-3 mRNA and protein were absent. Their morphology was

distinctly different from either parental line, featuring large cell bodies and long processes. They also expressed two antigenic markers of early neural tube differentiation: nestin, an intermediate filament protein expressed in neuroepithelial stem cells[36], and Brn-2, a transcription factor present in the developing and adult brain[37]. Neither marker is expressed in either of the parental lines. When Oct-3 activity was restored in the hybrid cells through the transfection of an Oct-3 expression vector, the cells adopted a morphology similar to undifferentiated P19 cells and extinguished nestin and Brn-2 expression. Remarkably, cells isolated from these lines, which had lost the ability to express the exogenous Oct-3 (presumably due to the instability of the expression construct), reverted back to the differentiated morphology of the original hybrid cell lines and also regained nestin and Brn-2 expression. These results demonstrate a very strong correlation between the downregulation of Oct-3 expression and the induction of neural differentiation.

As mentioned briefly above, Brn-2 is a transcription factor that is expressed in the developing and adult brain[37]. Recently, this transcription factor has been shown to be expressed at high levels during the RA-induced neuronal differentiation of P19 cells[38]. Elimination of Brn-2 expression in differentiating P19 cells by anti-sense RNA blocks neural differentiation; neither neurons nor glia are generated[38]. However, a heterogeneous population of cells, which includes muscle-like cells, results from the induction that normally gives rise to the neural lineage, indicating that the general competence to differentiate does not depend on Brn-2 expression.

The RARs, EGFR, Oct-3 and Brn-2 all appear to play key regulatory roles early in the pathway which leads to neuronal differentiation in P19 EC cells. However, at least one protein which may regulate neuronal differentiation at a later stage in this pathway has been identified. The microtubule-associated protein MAP2 is expressed in P19 neurons but not in EC cells[39]. When MAP2 expression was blocked in differentiating P19 cells by antisense RNA, the expression of a number of neuronal-specific markers was not repressed. However, these cells did not extend neurites or withdraw from the cell cycle[39], events which normally occur during terminal differentiation of P19 neurons. These results indicate that MAP2 plays an important role in the terminal stages of the pathway which causes neuronal differentiation in P19 EC cells. It is not yet clear whether MAP2 plays a similar role in neuronal differentiation *in vivo*.

Neuronal differentiation of P19 EC cells is dependent upon cellular aggregation during the induction period[4]. Conditions that reduce adhesion in the aggregates also inhibit the formation of neurons[40]. While aggregation is clearly essential, little is known about the mechanism by which it promotes differentiation. However, some intriguing observations have been made. First, the expression of the RARs in response to RA is affected by aggregation[22]. Cells in monolayer culture exposed to RA show a rapid increase in the expression of both RARα and RARβ mRNAs; RARγ mRNA remains at a constant level. In contrast, when the cells are aggregated during RA treatment, RARβ mRNA is induced more rapidly and RARγ mRNA is quickly repressed. The maximal levels of the RARα and RARβ mRNAs are also reduced in comparison to the monolayer cultures. Second, the amounts of several tyrosine-phosphorylated proteins have been observed to decrease during RA treatment of aggregated P19 EC cells; some of this downregulation can be induced by aggregation alone[41]. It is not clear at present whether these particular aggregation-related effects play a role in the neuronal differentiation of P19 EC cells.

Evidence that neuronal differentiation in P19 cells may be activated independently of exogenous RA treatment has recently been obtained[42]. P19 EC cell lines containing a β-galactosidase reporter gene integrated into random sites in the genome were isolated. One such integration was found to occur in the Pax-3 gene, which encodes a DNA binding protein expressed during early neurogenesis[43]. The activity of the reporter gene was found to follow the expression pattern of the disrupted gene. Undifferentiated cells showed no activity, but neuronally differentiating cells expressed it. Uninduced cells carrying the integrated reporter were mixed with RA-induced parental cells and cultured together as aggregates. Upon replating in monolayer culture, at least some of the marked cells expressed the reporter gene. In addition, some cells with neuronal morphology present in these cultures were found to express the reporter. These results indicate that neuronally differentiating P19 cells express an inducing activity which can trigger neuronal differentiation in neighboring cells independently of exogenous RA treatment. The basis of this intriguing activity is unknown.

Conclusion

The ability of P19 cells to differentiate into cells with the appearance of neurons was discovered 12 years ago. Since then a substantial body of data has been gathered which, taken together, strongly supports the conclusion that P19 neurons express many of the most characteristic and functionally relevant genes and phenotypes of neurons (see Fig. 1). The mechanisms that orchestrate this expression are beginning to be explored; there are exciting leads as to how they work, but much remains to be discovered.

The fact that P19 cells differentiate from multipotential cells, with characteristics of early embryonic cells, into neurons, in an *in vitro* environment, at first seems to conflict with the picture of neuronal development derived from the study of the normal embryo. P19 neuronal differentiation occurs in the absence of the steps of early embryogenesis associated with the normal formation of the nervous system and which are thought to play crucial regulatory roles: formation of the primitive ectoderm, gastrulation, formation of ectoderm and mesoderm and inductive interactions between ectoderm and mesoderm to form neuroectoderm. How can we reconcile the normal or near normal end of P19 neuronal development with the very unconventional path used to get there? Three broad possibilities suggest themselves. The simplest is to assume that most neural genes have RA response elements and that RA directly induces their expression in a coordinate way. This seems unlikely. Although detailed analysis of neural gene promoters is in its infancy, there is no indication

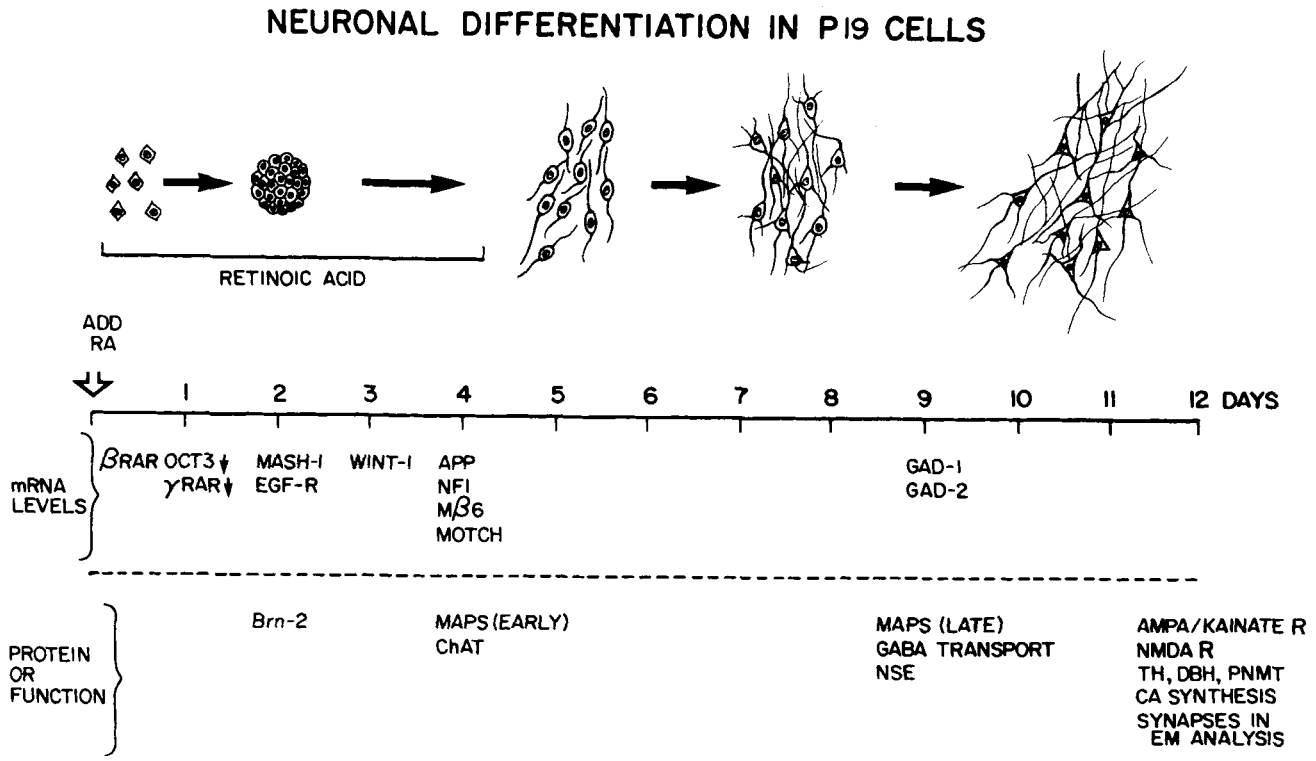

Fig. 1. Features of differentiation of P19 cells into neuron-like cells. Top: a schematic view of cellular morphology during an interval beginning with the start of induction and ending 12 days later. When aggregates are removed from retinoic acid they are trypsinized and the cell suspensions are plated on an adhesive substrate to promote outgrowth of neuronal processes. Flat glial-like cells also appear in cultures but these are omitted for purposes of illustration. Middle: mRNA levels which change dramatically; the place on the time line indicates approximate time after RA addition of maximal change in mRNA levels. All are upregulated, except for Oct-3 and RARγ which are downregulated, as indicated by arrows. Proteins or functions which are upregulated are indicated below. References documenting all these changes are cited in the text.

that the typical neurally expressed gene contains retinoic acid response elements. A second possibility is that induced aggregates of P19 cells contain multiple cell types, including those with the phenotype of early mesodermal cells. In this model many of the signals supplied by normal early mesoderm are supplied by these cells. This hypothesis assumes that it is not important that mesodermal cells be in an organized tissue for induction to take place. A third way of thinking about P19 induction is that the normal genetic apparatus underlying differentiation is highly organized in a functional sense. According to this view a small number of factors, presumably some RA responsive genes and one or more genes induced by aggregation, initiate a regulatory cascade which now becomes relatively independent of other extracellular signals. The downstream genes are those of normal differentiation and are the ones actually responsible for regulating the large numbers of genes that give the neuronal phenotype. Discovering which of these models approximates the truth will be an exciting research area, which may clarify mechanisms of normal development as well. Some of the answers will undoubtedly come from further research on regulatory genes already implicated in the pathway, such as those coding for RARs, EGFR, Oct-3 and Brn-2. Other key genes are likely to be discovered.

Much of this review has concentrated on the use of P19 as a developmental model. It should also be emphasized that the system has the requirements for making important contributions to other areas of neurobiology. P19 stem cells can be transfected with appropriate expression constructs and the role of the expressed genes tested in the context of P19 neurons. In a similar vein, the system offers the opportunity to study the effect of genes implicated in pathogenic processes in the environment of an appropriate model neuron.

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