AN IN VITRO PATHWAY FROM ES CELLS TO NEURONS AND GLIA

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ABSTRACT

Mouse embryonic stem (ES) cells can be induced to differentiate into neurons and glia in vitro. Induction protocols are straightforward and involve culture in the presence of retinoic acid. They result in an efficient conversion of undifferentiated ES cells to neural cells. Mature neurons produced have the key physiological, morphological and molecular properties of primary cultured neurons derived from the central nervous system. Most significantly, they form functional chemical synapses that utilize either glutamate, GABA or glycine as neurotransmitters. ES cell-derived glial cells also correspond well with their normal counterparts. During induction, ES cells undergo a series of developmental steps that resemble key stages in the early mouse embryo. This supports the hypothesis that the in vitro pathway is a valid model of the normal developmental pathway leading to neurons and glia. The in vitro system combines 3 experimental strengths. It is suitable for genetic manipulation, affords large numbers of cells and allows precise manipulation of the culture environment. It is thus suitable for a wide variety of mechanistic studies in the areas of neural development and cell biology.

Introduction Mouse embryonic stem (ES) cells strongly resemble cells of the inner cell mass and primitive ectoderm, which are transient structures found in the embryonic day 4-6 (E 4 –6 day) embryo. The primitive ectoderm, which is the source of all cells of the body, gives rise to the neural plate by E8. The neural plate is quickly transformed into the neural tube. At early stages, the neural tube consists predominantly of rapidly dividing stem cells. Stem cells later exit the cell cycle to give rise to the neurons and glia of the nervous system. ES cells have the inherent capacity to differentiate into all cell types of the nervous system as demonstrated by formation of chimeric mice from embryos in which ES cells are transplanted into the inner cell mass. At first glance, it would appear unlikely that the developmental lineage extending from ES cells to mature neurons and glia could be recapitulated in cell culture because the processes which give rise to the vertebrate nervous system, while not understood in detail, appear to be highly complex. Contrary to this expectation, it has proved possible to reconstitute, at least partially, this pathway in vitro starting with ES cells and ending with differentiated neurons and glia. The result is an experimental system combining 3 strengths. Genetics is available because the ES cell is the leading model for targeted modification of the genome. Since ES cells are a large-scale source of cells, biochemical investigation is feasible. Finally, tissue culture makes it

possible to alter the environment of cells in precise ways. Thus studies with the system have the potential for making important contributions to understanding neuronal and glial development and function. This review covers the literature in which ES cells have been induced to differentiate along a neural lineage in cell culture.

ES cells are maintained in vitro as totipotent stem cells by culture in the presence of the cytokine leukemia inhibitory factor (LIF). Most studies of in vitro differentiation of ES cells begin by culturing ES cells as small aggregates called embryoid bodies (EBs). EBs are formed by withdrawing LIF and culturing cells in dishes with a non-adhesive substratum. Standard EB cultures are composed of a wide variety of cell types, including a small proportion with a neuronal appearance (see for example Martin, 1981). Three papers published in 1995 demonstrated that more efficient in vitro differentiation of ES cells into neural cells is possible. All utilized retinoic acid (RA) but differ in the details of culture method. In our laboratories (Bain et.al, 1995), ES cells were cultured as EBs in the absence of LIF for 4 days. Culture was then continued for 4 additional days in the presence of RA. Next, aggregates were dissociated with trypsin and plated as monolayer cultures on an adhesive substrate. Within several days, large numbers of neuron-like cells appeared. The phenotype of these cells (reviewed below) justifies designating them as neurons. About 40% of the cells in the cultures were neurons. These grew on top of a layer of flat cells adhering to the substrate. Many of the flat cells were glia, but the exact percentage has not been determined. A study by Strubing et.al (1995) used a similar approach. EBs were formed by the 'hanging drop' culture method. These EBs were exposed to RA for the first 2 days of culture and then cultured in suspension for an additional 2 days in the absence of RA. Next, the EBs were cultured on an adhesive substrate; neurons appeared about 2 days after plating on the adhesive substrate. RA had a dramatic effect. All EBs exposed to RA gave rise to neurons whereas only 15% of control EBs not exposed to RA had neurons. In the study of Fraichard et.al (1995), EBs were formed in mass culture and exposed to RA for the first 2 days. They were then plated onto an adhesive substrate in the absence of RA. After 4-5 days, neuronal cells appeared. Thus 3 related protocols involving culture of EBs in the presence of RA result in extensive neuronal differentiation. Another approach to obtaining neurons from ES cells has been described (Okabe et.al, 1996). In this case, ES cells were cultured as EBs for 4 days in standard medium in the absence of added RA. Next the EBs were transferred to an adhesive substrate, to which they attach, and the standard medium replaced by a serum-free medium. Over the next several days, many cells die. Many of the surviving cells are tightly packed epithelial cells that are nestin positive. In the presence of bFGF these cells proliferate and go on to differentiate into neurons and glia.

Physiological properties of ES cell-derived neurons The neurons derived from in vitro differentiation of ES cells express voltage- and ligand-gated ion channels. In mature cultures, nearly all of the cells with neuronal morphology are capable of generating action potentials. They have resting membrane potentials of -40 to -70 mV. Under voltage clamp, the cells display inward and outward currents when stimulated by a depolarizing voltage step (Bain et al., 1995; Fraichard et al., 1995; Strübing et al.,

1995). Inward Na+ currents inactivate rapidly and are blocked potently by the Na+ channel antagonist tetrodotoxin. Outward K+ currents include a delayed-rectifier type current, which can be blocked with Cs or tetraethylammonium, and in some cells, a transient outward current that is sensitive to 4-aminopyridine. In addition, the ES cell-derived neurons express voltage-gate calcium channels. As shown by Strübing et al. (1995), distinct components of whole-cell calcium current are sensitive to specific antagonists of P-, N- and L-type channels. The ES cell-derived neurons respond to a variety of different neurotransmitters and selective receptor agonists. The inhibitory transmitters _-aminobutyric acid and glycine activate Cl^--selective channels in most of the cells. Similarly, most of the neurons express one or more of the ionotropic glutamate receptors, which are named for the agonists AMPA (amino-3-hydroxy-5-methylisoxazole-4-propionic acid), NMDA (N-methyl-D-aspartate) and kainate. Moreover, distinct components of whole cell calcium current in ES cell-derived neurons can be regulated by G protein-coupled receptors for somatostatin, GABA and other transmitters (Strubing et.al, 1997). In all of these respects, the physiological properties of ES cell-derived neurons are similar to those of primary neurons.

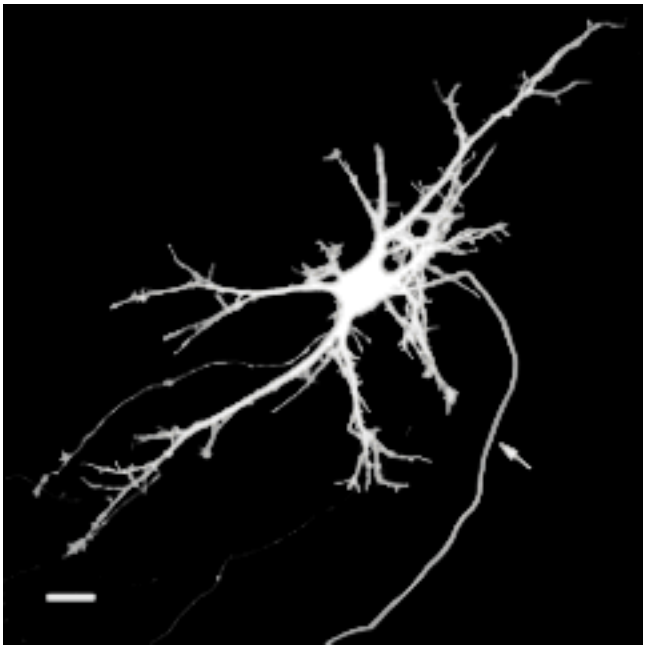

Fig. 1. Morphology of an ES cell-derived neuron revealed by DiI labeling 15 days after plating. The arrow points to an axon-like fiber. Scale bar=20 microns

The expression levels for both voltage- and ligand-gated currents, measured as current density per unit capacitance, increase with time as the cells mature (Bain et al., 1995; Strübing et al., 1995). Voltage-gated Na+ and K+ currents can be detected within a day or two after EBs are plated on an adhesive substrate. Over the first 6 to 10 days their density increases to a plateau of 150 to 200 pA / pF. These expression levels are comparable to the density of functional ion channels observed in primary cultures of dissociated neural tissue (e.g. MacDermott and Westbrook, 1986; Nerbonne and Gurney, 1989; Wu and Barrish, 1994). Transmitter-gated currents are first detected on the 2nd or 3rd day after plating and increase in density to reach steady levels by day 12 to 16. Over this same time period the cells produce abundant outgrowth of neurites, establish distinct axonal and dendritic compartments and begin to form functional synaptic connections. Immunostaining indicates that many different neuron-specific antigens are broadly expressed in ES cells with neuronal morphology. These include including, NCAM, neurofilaments, class III _-tubulin, MAPs and synaptophysin (Bain et.al, 1995, Fraichard et.al, 1995; Strubing et.al, 1995; Finley et.al, 1996; Okabe et.al, 1996).

The establishment of axons and dendrites becomes apparent when individual cells are labeled with lipophilic dyes, such as DiI (Fig. 1). As is the case for primary neurons in culture and in vivo, antibodies to MAP-2 stain the dendrites, and to a lesser extent the soma, of ES cell-derived neurons, whereas antibodies to GAP-43 label axons (Finley et al., 1996). In relatively mature cultures, 12 to 14 days after plating, punctate localization of synaptic vesicle antigens, including SV2, synapsin and synaptophysin, is observed at sites of presumptive intercellular transmission (Strübing et al., 1995; Finley et al., 1996; Okabe et al., 1996). Related studies of ES cell differentiation into striated muscle have demonstrated the co-accumulation of nicotinic acetylcholine receptors with other synaptic proteins, including agrin and synaptophysin (Rohwedel et.al, 1998). Thus, the segregation of proteins into distinct biochemical compartments is recapitulated during the in vitro differentiation ES cells.

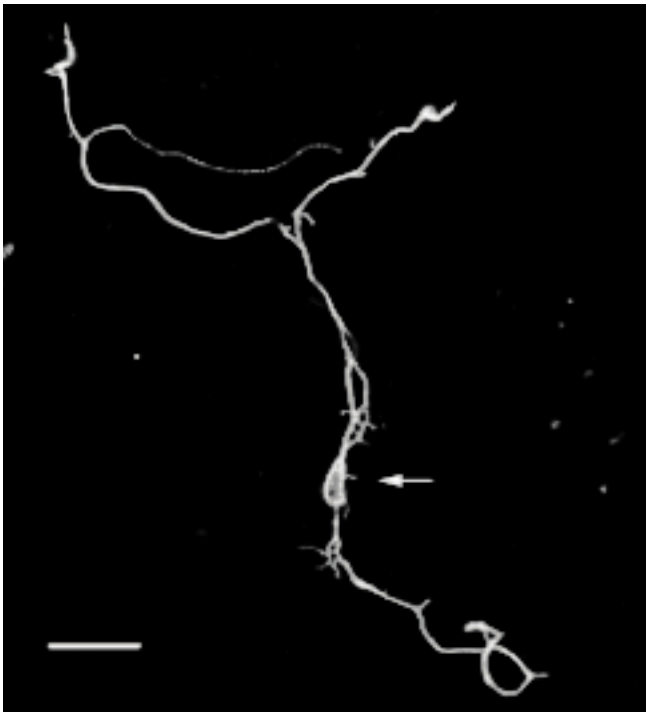

Fig. 2. GABA-like immunoreactivity in a culture of ES cell-derived neurons 5 days after plating. The arrow points to the cell soma. Scale bar=30 microns

Formation of functional connections between ES cell-derived neurons can be detected as spontaneous or evoked synaptic currents (Strübing et al., 1995; Finley et al., 1996; Okabe et al., 1996). Synaptic events are first detected between 8 and 10 days after plating. Both excitatory and inhibitory connections are observed. Studies of evoked transmission indicate that the majority of ES cell-derived neurons are excitatory (approximately 80%) while the remaining 20% are inhibitory neurons (Finley et al., 1996). These percentages are remarkably similar to those observed in primary cultures of cerebral cortex and hippocampus (Huettner and Baughman, 1988), suggesting that the mechanisms which determine the proportion of excitatory and inhibitory cells may be similar for neurons derived from ES cells and for neurons in the brain.

Inhibitory synaptic currents in ES cell-derived neurons are blocked either by bicuculline or by strychnine, suggesting that they are mediated by GABA or glycine, respectively. Immunostaining with antibodies to GAD or to GABA (Fig.2) confirm that a subpopulation of ES cell-derived neurons are GABAergic. Excitatory synaptic currents in ES cell-derived neurons are mediated by glutamate receptors. Both AMPA and NMDA receptors contribute to the postsynaptic responses (Strübing et al., 1995; Finley

et al., 1996), as is true for CNS neurons in vivo. Thus, electrophysiological recordings from ES-derived neurons, as well as the subcellular localization of neural antigens, provide compelling evidence that the cells acquire relatively mature neuronal properties.

Glial cells All cultures of ES cells induced to differentiate in a neural direction contain populations of non-neuronal cells in addition to neurons. Although these have not been investigated as thoroughly as neurons, there is abundant evidence that many of them are glia. Fraichard et.al, (1995)observed many examples of cells staining for glial fibrillary acidic protein (GFAP) or O4 antigen, markers for astrocytes and oligodendrocytes respectively. Bain et.al (1995) showed that GFAP mRNA is expressed in cultures of differentiated cells but not in undifferentiated cultures. Angelov et.al, (1998) observed the presence of cells with astroglial, oligodendroglial and microglial marker expression. Interestingly, these appeared in a time-dependent manner that parallels their appearance during brain development. These data suggest that the in vitro system is suitable for analyzing the development and function of glial cells. The presence of glial cells also has a significant bearing on the question of how the in vitro system is related to the normal nervous system. In normal development stem cells of the early CNS give rise to both neurons and glia. Presence of glial cells in the in vitro system strongly suggests that a similar multipotential precursor is generated in the course of ES cell neural differentiation.

Developmental pathway from ES cells to neurons The protocols reviewed above are successful at inducing ES cells to differentiate into neurons and glia. They were discovered, not by reference to normal early embryonic development, but by trial and error. This raises a very basic issue: what is the relationship of the in vitro pathway and the normal pathway of development in the embryo? There are three broad possibilities as to how the in vitro pathway could be related to normal development. The first is that there is little relation. It is imaginable that culture conditions in general, and RA in particular, turns on sets of genes resulting in neuronal differentiation in a way that bears little or no resemblance to the natural process. Normal neural development proceeds through a series of cellular progenitors that give rise to mature neurons. It is conceivable that RA directly turns on large numbers of the genes responsible for the neuronal phenotype (channels, receptors, vesicle components etc.). This would be possible if each of these genes had a retinoic acid response element. In this model, early cellular progenitors are 'skipped' and cells go directly from ES cells to terminally differentiated neurons and glia. A second possibility is that the pathway in vitro bears substantial resemblance to the in vivo pathway but that there are also significant differences. Such differences would be imposed by culture conditions and the reduced morphological order of EBs relative to the normal embryo. Finally, it is conceivable that the pathways in vitro and in vivo are exactly the same. A priori, this is highly unlikely because it would suggest that the cellular arrangements found in the developing brain had no influence on the course of neuronal development.

At present we are in the very early stages of addressing this fundamental issue. The data we do have however, suggests that the second alternative best describes the situation. Our reasoning is given with reference to the protocol utilized in our laboratories (Bain et.al, 1995; Finley et.al, 1996). We term this the “4-/4+ protocol”; it entails culture of EBs for 4 days in the absence of RA followed by culture for 4 days in the presence of RA. There are strong parallels between events that take place in the E4-6 embryo and EBs during the first 4 days of culture. Oct3/4 is a gene encoding a transcription factor that is expressed in early, pluripotent embryonic cells (Okamoto et.al, 1990; Rosner et.al, 1990). Its expression is extinguished between E5 and E8 as committed lineages emerge. Oct3/4 is strongly expressed in ES cells and its expression goes down in cultured EBs as they mature (Shen et.al, 1992). Thus expression in the embryo and EBs follow a similar pattern. Development of a visceral endoderm-like layer in EBs provides a second major parallel between EBs and the early embryo. The visceral endoderm (VE) is one of the earliest cell layers to emerge in the post-implantation embryo, where it grows to surround the embryo by E5. The outer layer of EBs consists of distinctive cells strongly resembling VE cells based on marker expression (Bielinska et.al, 1996; Duncan et.al., 1997). In this regard the morphological appearances of the E5 embryo and EBs cultured for 4 days are strikingly similar. A third parallel relates to cavity formation. A major event in the E4-5 period of development is the formation of the pro-amniotic cavity via a process of cavitation of the inner cell mass. Cavitation also occurs in EBs. The parallels between cavitation in the embryo and that in EBs have been described in an elegant study (Coucovanis and Martin, 1995). Taken together, these results clearly demonstrate strong morphological and molecular parallels between normal embryonic development and events in EBs during the first 4 days of culture. At the time RA is added, considerable development beyond the undifferentiated ES cell has taken place.

There is also evidence based on gene expression studies that the next stages of differentiation in the EBs after the addition of RA at 4 days resembles that of the embryo (Bain et.al, 1996; Li et.al, 1998). Based on these results, the conclusion that RA induces neurons by skipping the precursor stage and directly turning on late genes characteristic of terminal differentiation is virtually ruled out. Wnt-1 and MASH1 are regulatory genes expressed in early neural development within about a day of formation of the neural plate. They are not expressed in undifferentiated ES cells or EBs cultured for 4 days in the absence of RA. Addition of RA at to EBs already cultured for 4 days in its absence induces the expression of wnt-1 within 2 days and MASH1 within 4 days. Markers for more advanced neural differentiation including intermediate neurofilaments and the genes for glutamic acid decarboxylase (GAD 65 and GAD67) are turned on after wnt-1 and MASH1. These results reveal a good correspondence between the order of gene expression in the in vitro model and the embryo. Other support for this conclusion comes from a study that uses gene targeting (Li et.al, 1998). A line of ES cells was created in which the SOX2 gene was targeted. The SOX2 gene codes for a DNA binding protein selectively expressed in the early neural plate. The targeting event placed a promotorless neomycin cassette in the SOX2 gene so that only cells expressing SOX2 are neomycin resistant. ES cells were differentiated in

vitro by the 4-/4+ protocol. After neural differentiation, cultures were selected with neomycin resulting in pure cultures of neural precursor cells. Gene expression in selected cultures was analyzed. A number of regulatory genes expressed in neural precursor cells were expressed in the selected cultures, including SOX-1, pax6, pax3, mash1, and delta 1. Thus, cells undergoing neural differentiation in vitro express a number of the regulatory genes expressed in very early brain development. This supports the idea that the in vitro pathway towards mature neurons proceeds through a precursor state resembling that in vivo.

As expected, there are also important differences between in vitro ES models and normal embryonic development. We have assayed for the expression of the cholineacetyltransferase (ChaT) gene with negative results (Bain G. and Gottlieb, D, unpublished results). This shows that either the pathway leading to motoneurons is not initiated or that it fails to reach the stage at which ChAT is expressed. The gene for tyrosine hydroxylase is expressed only transiently (Bain et.al, 1995). This suggests that the pathway leading to catecholaminergic neurons is initiated but not maintained. There is also a deviation in the pattern of wnt-1 expression (Bain et.al, 1995). In normal development, wnt-1 expression is transient. In the in vitro system expression persists for at least 5 days post-plating. Future investigations will surely reveal other differences between the CNS and the in vitro model as it stands now. These findings will provide valuable insights into the degree to which neuronal phenotypes depend on the context of an intact nervous system or are, alternatively, independent of such a context.

Neural differentiation of ES cells with targeted gene mutations One of the greatest potentials of this system is to analyze the effects of mutations on neurons without the need to make mice. This phase of research is just beginning. Rohwedel et.al, 1998 showed that loss of beta-integrin accelerates neuronal differentiation. The gene for GD3 synthase has been disrupted and the effects on neuronal differentiation assayed (Kawai et.al, 1998). Mutant cells lacked b series gangliosides due to the disruption but differentiated as well as wild-type cells. While negative, the result is significant because it contradicts the established idea that this family of gangliosides is necessary for neuronal differentiation. The use of a line with a targeted mutation in the SOX2 gene (Li et.al, 1998) has been described above. We anticipate a large number of studies with mutations in neural genes will be forthcoming soon.

Gene trap screening Gene trap experiments with ES cells is an active area of research. In vitro differentiation of ES cells affords a way of screening for trapping of tissue-specific genes. The neuronal in vitro pathway has proven useful for identifying gene trap ES cell lines with trapping events in neural expressed genes (Baker et.al, 1997; Salminen et.al, 1998; Xiang et.al, 1998) All 3 papers identified lines mutated in neural genes. This approach should lead to the identification of other novel neural genes

Transplantation studies Transplantation of neurons and neural precursors into the CNS is currently an active area of research (see Brustle and McKay 1996 and

Gage, 1998 for recent overviews). These efforts are motivated by the possibility of affording treatment in cases where the brain has been damaged by traumatic injury, stroke or neuro-degenerative diseases. The ES cell system has the potential of making significant contributions to transplantation research for several reasons. For example, it offers a large-scale source of cells. Cells for transplantation can be taken from any point along the entire developmental sequence from the earliest precursors to mature neurons and glia. Also, a large variety of relevant mutations obtained by gene targeting will be available, some of which may give more favorable results than normal cells. Thus far only a few transplantation utilizing ES cell-derived neurons and glia have been done. Deacon et.al (1998) transplanted ES cells into the injured adult rat striatum. The transplanted ES cells engrafted well and integrated into the host striatum. Some of the transplanted cells differentiated into dopaminergic neurons while others differentiated into serotinergic neurons. Brustle et.al (1997) transplanted ES cell-derived neural precursors into the fetal rat brain. These precursors were prepared by the method of bFGF driven expansion (Okabe et.al., 1996) and transplanted into the cerebral ventricles of E16-18 rat fetuses. Results were examined within several weeks of birth. The transplanted cells differentiated into neurons, astrocytes and oligodendrocytes. Transplanted cells were integrated widely into the host CNS. We anticipate that transplantation of ES cell-derived neurons and glia will be carried out using a number of CNS injury and disease models. The availability of human ES cells (Shamblott et.al, 1998; Thompson et.al, 1998) adds special interest to this research area.

Species other than mouse ES cells have been described in fish (Sun et.al, 1995; Hong et.al, 1996), chicken (Pain et.al, 1996), rhesus monkey, (Thompson et.al, 1995), marmoset (Thompson et.al, 1996) and humans (Shamblott et.al, 1998; Thomson et.al, 1998). RA appears able to induce chicken ES cells to differentiate into neurons although no details have yet been published (Pain et.al, 1996). Neural structures figure prominently in teratomas derived from rhesus ES cells(Thompson et.al, 1995; 1998). An interesting feature is the tendency of these cells to form organized neuroepithelia. Human ES cells have a similar propensity, forming organized neuroepithelia in teratomas created in mice (Thompson et.al, 1998) or in EBs in culture (Shamblott et.al, 1998).

Unanswered questions and future prospects The investigation of ES cell-derived neural cells is in its infancy, having started only 4 years ago and many basic questions about the nature of the neurons and glia derived in vitro remain unanswered. Of these many questions, 2 are perhaps the most basic. The first concerns the exact mechanism by which RA induces the neural pathway. Here new information at both the cellular and molecular levels is needed. At the cellular level we need to know which cells in EBs respond directly to RA. One hypothesis is that most cells in early EBs are bipotential, with the ability to form either ectodermal or mesodermal derivatives, and that RA induces the ectodermal pathway. At this stage we can't rule out more complex models such as one in which RA acts on a small percentage of cells and these cells then provide a second type of signal to the cells which actually become neural. At the molecular level we have little mechanistic

information about how RA exerts its neural-inducing action. The most firmly established mode of RA action is through the RAR and RXR families of receptors/transcription factors. These families consist of multiple genes. The number of distinct signaling proteins is much greater than the number of genes because these proteins form both homo and heterodimers. Where in this large set of potential gene targets and regulatory activities is the actual site of RA's neural inducing ability? Our understanding of the in vitro system will be greatly advanced when we have an answer.

Another important unanswered question concerns the phenotype of neurons generated in vitro. We already know they are polarized (that is they have axons and dendrites), make synapses and utilize glutamate, GABA or glycine as transmitters (see above). In all their properties, the ES cell-derived neurons and glia more closely resemble cells in the CNS than the peripheral nervous system. But where do they fit in the classification schemes for neurons and glia in the brain and spinal cord? For example, GABAergic neurons in the CNS are a diverse set including many distinct morphological and biochemical types. Do the GABAergic neurons in ES cell-derived cultures correspond closely to one or more of these types? Alternatively, might the ES cell-derived neurons represent a 'blend' of phenotypes of various types of GABAergic neurons? Such questions are only now beginning to be investigated. One obvious starting point will be to investigate which region specific genes are expressed in ES cell-derived neurons. For instance, HOX gene expression is confined to the hindbrain and spinal cord. Expression of HOX genes in the model would suggest that ES cell-derived neurons had a positional identity corresponding to the caudal portion of the nervous system

What we have learned thus far suggests that the ES cell-derived neural system accurately models a number of major aspects of development spanning the period of E4-E16 in development. Thus even in its present state, its suitable for many types of experiments. Its great strength is that genetic, biochemical and cell biological investigations are all feasible. With future technical developments the system will become even more powerful. For instance, the approach towards generating drug resistant cell lines via knock-ins described for the SOX2 gene (Li et.al, 1998) should be general. It should be possible to make lines in which subsets of neurons can easily be selected thus generating pure cultures of say GABAergic neurons or glutaminergic neurons. This would open the door to many experiments now precluded by cellular heterogeneity. An even more ambitious goal is to direct the differentiation of ES cells along particular sub-pathways of neuronal differentiation. At present we know very little about the regulatory pathways responsible for this sort of choice. It is very likely that many of these will be discovered. In fact the ES cell system should be extremely useful in that effort. As we learn more, its conceivable that we may eventually be able to start with ES cells and efficiently direct them through a series of steps so that they all end up as a single neuronal type. This goal should inspire many future efforts.

Acknowledgments- Work in the authors labs was supported by RR12309 and the Alan A. and Edith L. Wolff Charitable Trust (DIG) and NS 30888 (JH) We thank Dr. Michael Finley for providing Figures 1 and 2. We also thank Dr. Raphael Kopan and Dr. Jeanne Nerbonne for helpful comments on the manuscript.

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