SEQUENTIAL STEPS IN THE DETERMINATION OF CHROMAFFIN CELL FATE BY GLUCOCORTICOIDS

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ABSTRACT

The development of the sympathoadrenal (SA) lineage has been studied as a model system in which to investigate the mechanisms that control the timing of environmental influences on cell fate. Glucocorticoids (GC) play a key role in the fate of the SA progenitor, causing it to differentiate to an adrenal chromaffin cell, rather than to a sympathetic neuron. Previously, it has been shown that GC exert both positive and negative effects on developing chromaffin cells: they promote cell survival and the expression of an adrenergic phenotype, and inhibit the expression of neuronal properties. However, the time at which GC first influence cell fate, and the mechanism(s) which underlie its effect(s), have remained matters of controversy. In this thesis, it is shown that the positive and negative effects of GC on SA progenitors during development are temporally separated and pharmacologically distinct. Most SA progenitors are competent to respond to GC by inhibition of process outgrowth two days before they are competent to respond by induction of PNMT, a chromaffin-specific marker. Competence to express PNMT appears to be acquired according to a cell-autonomous "clock". The early inhibition of neuronal differentiation may be a prerequisite to subsequent PNMT expression, since sympathetic neuroblasts rapidly lose the capacity to express PNMT. The two effects of GC are both mediated via the type-II glucocorticoid receptor (GCR). However, lower concentrations of GC are required to inhibit neuronal differentiation than to promote the expression of PNMT, and the two effects show differential responsiveness towards the receptor-specific antagonist RU38486. That the two effects of GC in this system are pharmacologically separable suggests that they may be mediated via different interactions of the GCR with endogenous cellular transcription machinery. Such differential interactions may explain how the two effects of GC in this system are temporally separated. Taken together, the results presented here provide precedent for an inductive developmental event in which the timing of the effects of an instructive signal on a bipotential progenitor are controlled neither by the schedule of appearance of the signal, nor of its receptor, but rather by cell-intrinsic, developmental changes in the response properties of the cell.

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INTRODUCTION

INTRODUCTION

The development of an embryo proceeds as a carefully timed sequence of events that leads to the diversification of particular cell types and their organization into discrete macroscopic structures. In molecular terms, this sequence of events is understood to consist primarily of the ordered control of gene expression by both environmental and genetic cues. Moreover, the induction or repression of one set of genes is often a prerequisite to the subsequent induction or repression of a second set of genes, and so on. Thus, particular cell fates are essentially reflections of particular developmental histories that have progressed according to particular schedules. Despite the importance of these schedules of differentiation, however, the mechanisms that control their timing are not well understood.

One of the few systems in which the issue of timing has been carefully studied is the schedule of glial cell differentiation in the rat optic nerve (Raff, 1989; Lillien and Raff, 1990). In this system, O2A bipotential progenitor cells give rise first to oligodendrocytes at around the time of birth, and then subsequently also differentiate to type 2 astrocytes approximately two weeks later. The timing of these events appears to be controlled in two different ways by soluble factors that are produced by a third cell type, the type-1 astrocytes (Hughes and Raff, 1987; Richardson et al., 1988). One of these factors, PDGF, serves as a mitogen for O2A progenitors that is permissive for the running of a cell-autonomous clock that counts a certain number of cell

divisions (Noble et al., 1988; Raff et al., 1988). Once this number has been reached, cell division stops and the progenitors differentiate to oligodendrocytes. A second environmental signal, CNTF, acts differently: it appears to be instructive, rather than permissive, for the differentiation of the O2A progenitor into type-2 astrocytes (Hughes et al., 1988; Lillien et al., 1988). The time at which type-2 astrocytes differentiate appears to be directly determined by the time at which CNTF begins to be released by type-1 astrocytes (Lillien et al., 1988). Two different mechanisms for the temporal control of differentiation thus emerge from these studies: one involves the continued presence of an environmental signal that allows a cell-intrinsic program to run, while the other involves an instructive environmental signal whose delayed time of appearance controls the timing of an inductive event.

Studies of the O2A progenitor have yielded considerable insight into the temporal control of differentiation within glial cell lineages. Little is known, however, about the events that control the timing of developmental decisions within neuronal cell lineages. One of the few neuronal cell lineages whose development has been studied in considerable detail is the neural crest-derived sympathoadrenal (SA) lineage (Landis and Patterson, 1981; Patterson, 1990). While the mechanisms that underly the schedule of sympathoadrenal differentiation have not been previously well established, environmental factors which influence cell fate within this lineage have been identified and characterized.

The Sympathoadrenal Lineage

Transplantation experiments using the chick-quail chimera system have established that cells in the SA lineage arise from the trunk region of the spinal neural crest between the levels of somites 18 and 24 (LeDouarin, 1980). These cells, like other crest derivatives, appear to be initially multipotent (Baroffio et al., 1988; Bronner-Fraser and Fraser, 1989), and then subsequently become progressively restricted in their choice of cell fate, presumably by environmental factors that they encounter during the course of their migration. Ultimately, cells in the SA lineage give rise to the major catecholaminergic cell types: adrenal medullary chromaffin cells, sympathetic neurons, and small intensely fluorescent (SIF) cells (Landis and Patterson, 1981; Patterson, 1990). Chromaffin cells are relatively small, contain large amine-storing vesicles, display a characteristically round morphology, and synthesize norepinephrine and epinephrine. Sympathetic neurons, by contrast, have large cell bodies, contain much smaller vesicles, extend long processes, and synthesize norepinephrine, but not epinephrine (see e.g., Doupe et al., 1985a). The lack of epinephrine synthesis in sympathetic neurons is a consequence of their failure to express the enzyme phenylethanolamine N-methyltransferase (PNMT), which catalyzes the conversion of norepinephrine into epinephrine. SIF cells have properties that are intermediate between sympathetic neurons and chromaffin cells (Eranko, 1975), and can display either neuronal or chromaffin properties, depending on their hormonal milieu (Doupe et al., 1985b).

What are the environmental signals that influence the differentiation of cells in the SA lineage? Early studies both *in vivo* and *in vitro* clearly established nerve growth factor (NGF) as a survival factor for sympathetic neurons (Levi-Montalcini and Angeletti, 1963; Levi-Montalcini and Angeletti, 1968). Conversely, Wurtman and Axelrod showed that glucocorticoids (GC) produced by the adrenal cortex are required for the maintenance of epinephrine synthesis in chromaffin cells (Wurtman and Axelrod, 1966). That NGF and GC are critical environmental determinants of cell fate in the SA lineage was initially suggested by experiments *in vitro* that revealed the plasticity of chromaffin cells. Unsicker and coworkers demonstrated that NGF could induce mature chromaffin cells in dissociated cultures to transdifferentiate to cells resembling sympathetic neurons. Moreover, this effect of NGF on neuronal differentiation could be blocked by GC (Unsicker et al., 1978). Doupe, Landis and Patterson extended these studies in long-term postnatal chromaffin cell cultures, and clearly established GC as a survival factor for these cells. They showed, moreover, that long-term exposure to NGF converted chromaffin cells into cells that were indistinguishable from sympathetic neurons (Doupe et al., 1985a). More recent studies of postnatal chromaffin cells indicate that acidic and basic fibroblast growth factor (aFGF, bFGF), as well as NGF, can initiate this process of transdifferentiation (Claude et al., 1988; Stemple et al., 1988).

Studies on committed embryonic SA progenitor cells *in vitro* have strongly supported the suggestion from studies of postnatal cells, that NGF and GC respectively influence the initial development of sympathetic neurons and chromaffin cells. Using the monoclonal antibody B2 as a tag for

fluorescence-activated cell-sorting (FACS), Anderson and Axel were able to isolate a purified population of bipotential progenitor cells (SA progenitors) from embryonic day 14.5 (E14.5) rat adrenal glands that differentiated to sympathetic neurons in the presence of NGF, and to PNMT+ chromaffin cells in the presence of GC (Anderson and Axel, 1986). Carnahan and Patterson used the monoclonal antibody SA-1 to isolate bipotential progenitor cells by FACS from E13.5 rat superior cervical ganglia (SCG) that behaved in a similar manner in response to these environmental factors (Carnahan and Patterson, 1991), confirming earlier studies of neonatal SCG. Studies of the effects of bFGF, NGF and GC on an immortalized cell line derived from E14.5 SA progenitors have provided further evidence for a decisive role for these factors in the determination of cell fate within the SA lineage (Birren and Anderson, 1990).

The foregoing *in vitro* data indicate that neurotrophic factors and GC have opposite effects on SA progenitor cells. Moreover, they suggest that each of these factors have both positive and negative effects on these cells: neurotrophic factors promote the expression of neuronal properties and repress the expression of chromaffin properties; conversely, GC promote the expression of chromaffin properties and inhibit the expression of neuronal properties. These complementary influences are consistent with the pattern of antigenic marker expression and repression by SA progenitors *in vivo*. At E11.5, SA progenitors initially coexpress neuronal and chromaffin markers (Cochard and Paulin, 1984; Anderson and Axel, 1985; Anderson et al., 1991) (Appendix III, this volume). Progenitors that remain in the ganglia continue to express neuronal markers, but extinguish the expression of chromaffin

markers. Cells that migrate to the adrenal anlage display the converse behavior, extinguishing the expression of neuronal markers and continuing to express chromaffin markers (Anderson and Axel, 1985; Anderson et al., 1991) (Appendix III, this volume). Taken together, the *in vitro* and *in vivo* data suggest that both sympathetic neurons and chromaffin cells derive from a common embryonic progenitor cell whose choice of cell fate is controlled by the environmental factors that it encounters as it migrates. Those cells which arrest their migration in sympathetic ganglia encounter neurotrophic factors that promote neuronal differentiation. By contrast, cells that continue their migration to the adrenal gland encounter high levels of GC produced by the adrenal cortex, that act to promote chromaffin differentiation. It is interesting that although the adrenal cortex and medulla perform essentially independent physiological functions, GC produced by the cortex also appear to be necessary for both the maintenance of the medullary (chromaffin) phenotype (see above), and for the proper development of this phenotype.

While it is clear that particular environmental signals influence the fate of developing SA progenitor cells, the mechanisms that control the timing of their effects have not been well understood. The experiments presented in this thesis focus in particular on the temporal control by GC of chromaffin cell differentiation. Previous studies that have addressed this issue have assumed that the schedule of differentiation is determined either by the time of appearance of GC, or of its receptor. The results of the experiments in this thesis suggest that neither of these mechanisms is correct. This conclusion appears to fit well with recent studies of gene

regulation by steroid hormones which indicate that the presence of liganded receptor per se is not necessarily sufficient for activation or repression of transcription. Therefore, before proceeding further with a discussion of the issue of timing as it relates to the development of the adrenal gland, contemporary views on the control of gene expression by steroid hormones will be discussed. Several comprehensive reviews of this subject have been published in recent years (Evans, 1988; Evans and Arriza, 1989; Beato, 1989; Beato et al., 1989; Gustafsson et al., 1990; Pratt, 1990). As a prelude to this discussion, an overview of glucocorticoids and their receptors in the nervous system is presented.

Mechanisms of glucocorticoid hormone action

Glucocorticoid receptors in the nervous system

At least two types of glucocorticoid receptors (GCRs) have been identified and characterized in the nervous system, and these correspond in structure to the two primary types of GCRs described in other systems. The type-I GCR, the so-called "mineralocorticoid" receptor (MR), has an equally high affinity for both corticoisterone and the principal mineralocorticoid, aldosterone (Sheppard and Funder, 1987; Funder et al., 1988). By contrast, the type-II GCR (known as the "classical" GCR) has a much higher affinity than MR for synthetic glucocorticoids such as dexamethesone, but has a nearly 10-fold lower affinity for corticosterone, and an even lower affinity still for aldosterone (see, eg., Reul and De Kloet, 1985).

In the brain, expression of the type-II GCR (hereafter referred to simply as "GCR") is much more widespread than is the expression of MR. Expression of MR in the brain is restricted mostly to the hippocampus and a few other telencephalic structures, but is generally absent from the diencephelon. GR, by contrast, is widely expressed in both di- and telencephalic regions (Fuxe et al., 1985; Van Eekelen et al., 1988; Van Eekelen et al., 1991). In situ hybridization studies have revealed quantitative differences in the levels of GR mRNA present in various brain regions (Van Eekelen, et al., 1991). Thus, whereas the highest levels of GR message are detected in the hippocampus and in the parvocellular layer of the hypothalamic paraventricular nucleus, levels are more modest in regions such as the thalamus and basal ganglia, confirming earlier immunohistochemical studies (Fuxe, et al., 1985). The observation of such heterogeneity in levels of GR message expression suggests that perhaps different levels of functional receptor may be required for different cellular effects.

Despite the differences in localization of MR and GCR in the brain, a clear overlap in the expression of the two receptor types is evident in at least some sections of the hippocampus (notably the CA1 and CA2 regions) (Van Eekelen, et al., 1988). The studies of Evans and coworkers suggest that this overlapping expression may be functionally significant, insofar as it could, in principle, constitute a 'biphasic response system' (Evans and Arriza, 1989). Since plasma GC concentrations can vary from the subnanomolar range at certain points during the circadian rhythm cycle up to levels exceeding 100nM in response to stress, two receptor systems in the same cells may be

necessary to accomodate effective responses to such a wide range of steroid levels.

Effects of GC on neural development

In addition to their effects on sympathoadrenal development, GC also influence central nervous system ontogeny. Teratological studies have indicated that fetal exposure to high levels of GC profoundly affect brain development, causing the retardation of synpatogenesis, axonal outgrowth, cell division and myelinogenesis, among other abnormalities (see, eg., De Kloet et al., 1988). Nevertheless, low levels of GC appear to be essential for normal development of the CNS. That GC may be involved in establishing neuronal topography in the brain is not altogether surprising, considering the well-documented effects of androgens and estrogens on the development of neuronal connections in sexually dimorphic regions of the brain of passerine songbirds (for review of the effects of steroids in the bird song system, see, eg., Konishi, 1989). However, despite numerous reports on the phenomenology of GC effects on cortical neurons, little is known about the cellular and molecular mechanisms of these effects.

One aspect of GC regulation of brain development that has been studied in cellular and molecular detail is the control of myelination in the CNS. Type-II glucocorticoid receptors have been demonstrated to be localized at high levels within both oligodendrocytes and astrocytes (Vielkind et al., 1990). *In vivo* studies have shown that adrenalectomy causes a profound decrease in CNS myelination (Preston and McMorris,

1984), and several glial enzymes and lipids important for myelination appear to be directly GC-inducible (Dawson and Kernes, 1979; Kumar et al., 1986; Warringa et al., 1987; Kumar et al., 1989). GC have also been shown to stimulate development of oligodendrocytes in primary glial cultures, and to act as a survival factor for these cells (Warringa, et al., 1987). One of the most well-studied GC-inducible glial enzymes involved in myelination is glycerol-phosphate dehydrogenase (GPD). The continuous presence of GC appears to be required for both the induction and normal developmental increase in levels of this enzyme (Meyer, 1985). Recent studies using *in vitro* transcription assays indicate that the effect of GC on GPD expression occurs at the level of transcription (Kumar, et al., 1989). It is interesting that whereas GPD is clearly regulated by GC in the CNS, its expression in other tissues such as liver and skeletal muscle appears to be GC-independent (Doupe and Patterson, 1982).

Another well-studied case of GC effects on CNS development at the molecular level is the regulation by GC of glutamine synthetase (GS) expression in the retina. In the chick embryo, GS synthesis rises sharply around days E16-E17 of development, commensurate with a systemic rise in circulating GC levels (Moscona and Hubby, 1963; Moscona, 1972). Partial GS expression can be induced precociously by injections of GC as early as embryonic day 7-8, but not prior to this time (Paddington and Moscona, 1967; Soh and Sarkar, 1978; Moscona and Moscona, 1979; Patejunas and Young, 1987), even though functional GCR is clearly present in the developing retina as early as E6 (Lippman et al., 1974; Koehler and Moscona, 1975). It has therefore been suggested that other transcription factors may be required in

addition to the GCR in order to induce competence within the retina to express GS in response to GC (Patejunas and Young, 1987). Accordingly, the delay in GC-inducibility of GS expression may reflect a delay in the appearance of such an additional transcription factor. Interestingly, GS is GC-inducible in muscle as well as the retina (Feng et al., 1990), but its expression in liver and hippocampus appears to be independent of GC (Patejunas and Young, 1987; Tombaugh and Sapolsky, 1990). Thus, GC-inducibility of GS, like GPD (see above), is tissue-specific, further underscoring the idea that steroid inducibility may involve synergistic interactions between GCR and cell-type-specific regulatory factors. An understanding of the possible nature of such interactions is perhaps best appreciated in the context of the molecular details of steroid receptor structure and target-gene promoters.

Glucocorticoid receptor structure and function

The glucocorticoid and mineralocorticoid receptors are members of a family of nuclear receptor proteins that also includes the progesterone, estrogen, androgen, thyroid and retinoic acid receptors, as well as several others for which a ligand has yet to be identified (for review, see Evans, 1988). All of these molecules share a highly conserved cysteine-rich domain to which zinc atoms are liganded to form a structure for sequence-specific interactions with DNA (Klug and Rhodes, 1987; Evans and Hollenberg, 1988; Freedman et al., 1988; Severne et al., 1988). Similar to the structure first described for the Xenopus transcription factor TFIIIA (Miller et al., 1985), this "zinc finger" region, located between the N-terminal and C-terminal domains of these molecules (see below), defines the DNA-binding domain

of what is known as the steroid hormone receptor superfamily. The sequence of this domain also shows robust homology to the DNA-binding domain of the viral oncogene v-erb-A (Weinberger et al., 1985). It also shows considerable homology to the *knrl* gene, whose product is closely related to that of the drosophila gap segmentation gene *knirps*, thus emphasizing the potentially important role that steroid receptors may play in governing developmental decisions (Oro et al., 1989).

Steroid receptors as a class also have two other major structural domains. The C-terminal region contains the hormone binding site. Deletion of this region results in the generation of receptors that can activate transcription in the absence of hormone, suggesting that in the absence of hormone this region inhibits the DNA-binding and activation functions that reside elsewhere in the receptors (Godowski et al., 1987). The N-terminal region contains a potent transcriptional activation function, called τ1 or TAF1, within an acidic region (Hollenberg and Evans, 1988). It shows substantial variability in length and sequence among different receptor types as well as among different isoforms of receptor for the same hormone, and has been implicated in the synergistic interaction with other transcription factors. The unique characteristics of this region may significantly influence the efficiency with which a receptor can activate target gene expression (Evans and Arriza, 1989).

The DNA- and steroid-binding regions also contain activation functions, in addition to the one characterized in the N-terminal region (Hollenberg and Evans, 1988; Webster et al., 1988; Schena et al., 1989). In

addition, a nuclear localization signal lies in a region between the DNA- and steroid-binding regions (Picard et al., 1990). Glucocorticoid receptors appear to be unique in that the steroid-binding domain of these receptors contain a second nuclear localization signal (Picard and Yamamoto, 1987), in addition to the one contained in the DNA-binding domain. This second signal is close to a site at which unliganded GC receptor can interact with the heat shock protein hsp90. Such receptor-hsp90 complexes have been shown to be important in mediating the ligand-induced transformation of steroid receptors to the DNA-binding state (Pratt et al., 1988; Picard et al., 1990b). Presumably, the binding of GC to its receptor induces a dissociation of, or conformational change in this complex that unmasks the nuclear localization signal and leads to translocation of the receptor across nuclear pores. More recent evidence indicates that hsp90 serves not only as a steric hindrance to the passage of unliganded receptor to the nucleus, but also actively facilitates the subsequent response to hormonal signal (Picard et al., 1990b). That the presence of a second nuclear localization signal is unique to GC receptors fits well with the observation that whereas progesterone and estrogen receptors are predominantly located in the nucleus both in the absence and presence of hormone, unliganded GC receptors are primarily located in the cytoplasm (Welshons et al., 1984; Perrot-Applanat et al., 1985; Picard and Yamamoto, 1987).

DNA regulatory elements in glucocorticoid-responsive genes

Postively regulated genes. Many genes that are positively regulated by steroids have been characterized. The positive action of glucocorticoids are

conferred by the binding of liganded receptor to specific glucocorticoid responsive elements (GREs) that can be located anywhere from tens to thousands of nucleotides upstream of transcription initiation sites. The consensus sequence for GREs consists of a palindromic 15-mer (Beato, 1989) that binds receptor dimers (Chalepakis et al., 1990). Progesterone, androgens and mineralocorticoid receptors can all activate transcription through binding to these cognate elements (Strahle et al., 1987; Ham et al., 1988). Recent studies suggest that the specificity of transcriptional control by these hormones is likely to be mediated within cells by their differential expression of particular hormone receptors, rather than by a differential dependence on additional transcription factors (Strahle et al., 1989).

Negatively regulated genes. Far fewer genes that are negatively regulated by steroids have been studied in detail. Of those that have been studied, virtually all involve transcriptional repression by GC. In contrast to the positive GRE, a clear consensus sequence for negative regulation has been difficult to decipher. Several sequences to which GC receptor binds in promoters of negatively-regulated genes have been identified, and are referred to collectively as negative glucocorticoid responsive elements (nGREs). Some essential positions in positive GREs are also present in nGREs, but there appear to be particular differences as well. It is not yet clear whether in general, differences in nucleotide sequence in hormone responsive elements are sufficient to account for opposite effects on transcriptional control (Beato et al., 1989), but this idea was originally proposed based on studies of the bovine prolactin gene (Sakai et al., 1988). More recently, an nGRE has been characterized that overlaps a recognition element for the AP-1 class of transcription factors, and can confer either

positive or negative regulation, depending on which members of this class are also bound (see below) (Diamond et al., 1990).

Coordinate regulation of gene expression by glucocorticoid receptors and other transcription factors

Many studies have revealed that the regulation of transcription of steroid hormones can be modulated by other transcription factors, acting in concert with steroid receptors. These factors may act through binding to unique sites on DNA, or to steroid receptors themselves, or both. Such studies emphasize that the mere presence of liganded steroid receptor does not guarantee the efficient activation or repression of a steroid-regulatable gene; other factors may be necessary as well.

The induction of α_1-acidic glycoprotein (AGP) by GC, for example, can be inhibited by adding cycloheximide together with hormone (Klein et al., 1987). More recent studies of the AGP promoter reveal both functional GREs and sequences downstream of these receptor binding sites that are required for trans-activation of the AGP gene (Klein et al., 1988). Footprinting analysis has shown that these downstream sequences bind a cycloheximide-sensitive factor. Thus, the activation of AGP transcription appears to involve the direct action on the AGP promoter of both the GC receptor and a different labile transcription factor.

Studies of the MMTV promoter have revealed it to contain binding sites for at least three transcription factors: GC (or progesterone) receptor,

NF-1 and OTF-1 (oct-1). Mutations in the OTF-1 binding sites completely abolish the stimulatory effect of progesterone receptor on transcription. Moreover, binding of this steroid receptor to the GRE, facilitates the binding of OTF-1 to its cognate element. These results have suggested that the OTF-1 transcription factor binds to a unique site but interacts directly with the GC receptor, bound to its unique site, to coordinately regulate transcription (Bruggemeier et al., 1991). A similar case of protein-protein interactions is seen in the coordinate regulation of prolactin transcription in rat pituitary. Thus, estrogen receptor can mediate a large increase in prolactin expression by binding to an estrogen response element and synergistically interacting with the pituitary-specific pit-1 transcription factor bound to a different site (Simmons et al., 1990). Interestingly, OTF-1 and pit-1 are both members of the POU-domain family of transcriptional activators (Ingraham et al., 1990). Such synergistic protein-protein interactions between steroid receptors and POU domain factors may reflect a common mode of coordinate regulation of gene expression.

Recent studies have characterized a system in which different protein-protein interactions can result in either positive or negative transcriptional regulation by GC, at a single nGRE (Diamond, et al., 1990). Thus, an nGRE located within the rat proliferin promoter contains overlapping binding sites for both the GC receptor and members of the AP-1 class of transcription factors (see above). When jun-jun homodimers are bound to this nGRE, proliferin is only basally induced; liganded GC receptor confers a robust upregulation of transcription. By contrast, jun-fos heterodimers bind this nGRE in the absence of GC receptor to activate transcription, which can then

be repressed by the additional binding of liganded GC receptor. Regulation of transcription at this nGRE can thus involve either synergistic or antagonist protein-protein interactions between the two classes of transcription factors.

The examples offered here clearly indicate that the mere presence of hormone-bound receptor is not necessarily sufficient to regulate transcription of steroid-responsive genes; other factor(s) are likely to be required as well. Moreover, the regulation of different genes may require different classes of transcription factors. With this idea in mind, the concluding sections of this introduction present an overview of previous attempts to explain the timing of a maturation event in the development of adrenal chromaffin cells -- the onset of PNMT expression -- which occurs *in vivo* with striking temporal specificity. This discussion is introduced within the context of PNMT expression in general, and the structure of the PNMT gene in particular.

Expression of PNMT in the nervous system

In addition to its expression in the adrenal medulla, PNMT is also expressed in several regions of the central nervous system. For example, adrenergic neurons in the C1 regions of the brainstem send descending axons into the spinal cord that appear to synapse directly on adrenal preganglionic neurons (Bernstein-Goral and Bohn, 1988; Bernstein-Goral and Bohn, 1989). PNMT has also been detected in neurons that project to the paraventricular nucleus of the hypothalamus (Liposits et al., 1990). Both immunohistochemical and lesion studies indicate that PNMT is expressed

as well in at least some cell bodies of the hypothalamus itself (Ruggiero et al., 1985; Masana and Mefford, 1989). Additionally, PNMT has been detected in small amacrine neurons within the retina (see (Baetge et al., 1988) for references).

In contrast to the sympathetic nervous system, where PNMT is clearly GC-inducible, at least one study has suggested that expression of the adrenergic phenotype within the central nervous system is not under GC control (Bohn et al., 1987). In explants of embryonic rat brainstem isolated at E13, natural GC such as corticosterone had no effect on PNMT activity at any time in culture; the enzyme appeared even in the absence of steroid. Moreover, GC antagonists were unable to prevent the developmental increase in PNMT activity within this tissue. Dexamethesone had a slight inductive effect on enzyme levels after three weeks in culture, but this effect could not be blocked by GCR antagonists. Thus, the expression of PNMT, like the expression of GS and GPD (see above), may be differentially regulated in a tissue-specific manner.

The PNMT gene

In recent years, the PNMT gene has been cloned from rat, bovine and human genomic libraries (Batter et al., 1988; Sasaoka et al., 1989; Ross et al., 1990). All three genes are very similar to each other: their coding regions all span approximately two kilobases, consist of three exons, and contain several GREs as well as several putative binding sites for the transcription factor Sp1, to which the GREs are generally adjacent. Regulatory elements

for tissue-specific expression of PNMT appear to be present within a two-kilobase 5'-flanking region of the PNMT gene, since in transgenic mice, a chimeric transgene made up of this flanking sequence fused to the SV40 early region initiated tumors specifically within the adrenal medulla and retina (Baetge, et al., 1988). Transgenes were not expressed, however, in other known PNMT-immunoreactive brain regions such as the brainstem, consistent with the idea that normal PNMT expression in various tissues may be differentially regulated.

Only one recent study has investigated the transcriptional regulation of PNMT. Goodman and coworkers found that PNMT-β-galactosidase constructs containing a functional GRE are expressed when transfected into primary adult chromaffin cells, but not in PC12 cells (Ross, et al., 1990). Since both of these cell types express functional GCR, but only chromaffin cells express PNMT, these data support the idea that additional regulatory factors, differentially expressed in chromaffin cells and PC12 cells, may be necessary, in addition to functional GCR, in order to activate transcription of the PNMT gene.

Temporal control of chromaffin cell development

The developmental onset of epinephrine synthesis

In the rat, migrating SA progenitor cells invade the adrenal anlage between embryonic days 12 and 15 of development (Lempinen, 1964; Bohn

et al., 1981). There they encounter presumptive cortical cells, which are already present in the area by this time. After a delay of several days, the SA progenitors begin to express PNMT at around E17, and the levels of enzyme increase dramatically thereafter (Bohn et al., 1981).

Previous studies indicated that the timing of PNMT appearance (and the consequent synthesis of epinephrine) correlates with the time at which developing cortical cells are induced by ACTH to synthesize particularly high levels of GC (Cohen, 1976; Teitelman et al., 1979). Earlier experiments demonstrated, moreover, that hypophysectomy prevents the normal developmental increase in epinephrine synthesis by chromaffin cells (Margolis et al., 1966). Taken together, these data suggested that the production of GC by cortical cells induces chromaffin cell progenitors to express PNMT, and that the delay in the appearance of this enzyme reflects a delay in the time at which the inducing signal, GC, first appears. Such a view is analagous to the role of CNTF in controlling the time of type-2 astrocyte differentiation in the O2A lineage (see above).

Controversy over the role of GC as an inducing signal

Early experiments attempted to verify GC as the inducing signal for chromaffin cell differentiation *in vivo*. It was reasoned that if GC do indeed control the timing of PNMT appearance, then it ought to be possible to effect an earlier induction of PNMT by experimentally elevating levels of GC several days before the normal time of appearance of this enzyme. Such manipulations of GC levels *in vivo* produced negative results (Bohn et al.,

1981). Moreover, whole adrenal glands in organ culture were shown to induce PNMT even in the absence of exogenously added steroid (Teitelman et al., 1982). Although the concentrations of GC actually present in the vicinity of developing chromaffin cells could not be carefully controlled in either of these experiments, the authors of these studies concluded that GC are not required for the initial expression of epinephrine synthesis. This conclusion was bolstered by other studies indicating that low levels of PNMT are detectable in the developing adrenal 1 to 2 days before the surge in cortical steroidogenesis (Ehrlich et al., 1989). All of these studies were interpreted within the framework that GC are not required for the initial expression of PNMT, but do mediate a subsequent developmental increase in its expression. This interpretation, however, tended to confuse the two issues of whether GC control the timing of PNMT appearance, or simply are required for its expression.

In contrast to the results cited above, more recent experiments in dissociated cell culture have shown GC to be absolutely required for the initial appearance of PNMT (Seidl and Unsicker, 1989). Dose-response experiments further implied that the low levels of GC present in the fetal circulation prior to the surge in cortical steroidogenesis ought to be sufficient to induce PNMT prior to E17. These results suggested that although GC are necessary for the expression of PNMT, the appearance of these steroids is unlikely to control the time at which PNMT is initially expressed. However, this conclusion could not be critically tested because the chromaffin precursors were isolated at E16.3, only one day prior to the normal time of

PNMT appearance. Hence the ability of GC to induce precocious appearance of PNMT *in vitro* could not be addressed.

Radioligand binding studies to measure levels of GC receptor revealed a correlation between the time of PNMT appearance and the time at which levels of GC receptor first become detectable in embryonic chromaffin cells: receptors were measured at 5,000/cell on E17.3, but were not detectable on E16.3 (Seidl and Unsicker, 1989). Based on these results, the authors of these studies suggested that the delay in PNMT appearance can be attributed to a delay in the appearance of functional GC receptor within developing chromaffin cells. This would imply that chromaffin precursors are unresponsive to GC prior to E17. Such a conclusion, however, would preclude a role for GC in controlling the initial decision between the chromaffin and neuronal pathways which, based on the expression of markers *in vivo* (Anderson et al., 1991) (Appendix III, this volume), occurs several days earlier in development. On the other hand, if GC do inhibit neuronal differentiation at early times, then why is the expression of PNMT delayed?

Thus, while there is general consensus that GC influence chromaffin cell fate, the time at which these steroids first act on SA progenitors has remained a matter of controversy. The cell culture experiments in Chapter 2 of the thesis were designed specifically to answer this question. Evidence is presented indicating that the delay in PNMT expression is due neither to a delay in the appearance of GC nor of its receptor. Rather, it is suggested that the timing of PNMT expression is controlled by cell-intrinsic changes in

progenitor cell responsiveness to GC. Progenitor cells appear competent to respond to GC at early times in culture by inhibition of process outgrowth, but *not* by the expression of PNMT. Competence to induce PNMT appears to develop with time in culture, according to a cell autonomous schedule. In the absence of steroid, most SA progenitors differentiate into neurons, which cannot acquire competence to express PNMT (see Appendix II). GC inhibit this neuronal differentiation, and in doing so, act as a permissive factor for the acquisition of competence. In this way, the early, negative effect of GC is a prerequisite for its later, positive effect. Pharmacological data indicate that the early and late effects of GC are both mediated via type-II GC receptors, but may involve different interactions of the GC receptor. Such differences may explain how the same signal (GC) can have two temporally-separated effects in this system.

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CHAPTER 2

GLUCOCORTICOIDS CONTROL TWO TEMPORALLY AND PHARMACOLOGICALLY DISTINCT EVENTS IN CHROMAFFIN CELL DIFFERENTIATION*

*A revised version of this manuscript, entitled "Changes in competence determine the timing of two sequential glucocorticoid effects on sympathoadrenal progenitors", by Arie M. Michelsohn and David J. Anderson, has been submitted for publication.

ABSTRACT

We have shown that *in vitro*, sympathoadrenal progenitor cells differentiate to adrenal medullary chromaffin cells in two distinct stages: an early inhibition of neuronal differentiation, followed by a subsequent induction of PNMT. Both of these events are controlled by glucocorticoids (GC). Cells are initially competent to respond to GC by inhibition of process outgrowth, but *not* by the expression of PNMT. Competence to express PNMT is acquired with time in culture according to a cell-autonomous, steroid-independent schedule. When cells are cultured in the absence of GC, most differentiate to sympathetic neurons, which lose the ability to acquire competence to express PNMT. Thus, by inhibiting neuronal differentiation at early times in culture, GC are permissive for the acquisition of competence to induce PNMT at later times. Both the early, negative and later, positive effects of GC are mediated via type-II GC receptors, but are pharmacologically distinct, suggesting that the two effects of GC may involve different interactions of the GC receptor with the cellular transcription machinery. Such differences may explain how the two effects of GC in this system can be temporally separated.

INTRODUCTION

The analysis of cell fate during vertebrate neural development has revealed that many progenitor cells are multipotent (Turner and Cepko, 1987; Baroffio et al., 1988; Holt et al., 1988; Wetts and Fraser, 1988; Bronner-Fraser and Fraser, 1989). This is consistent with the idea that the choice of cell fate may be controlled, in many instances, by environmental signals (e.g., see Patterson, 1978). If this is indeed the case, the fact that different cell types develop on relatively well-defined schedules in turn implies that the timing of environmental influences may be an important aspect of the control of cell fate. However, there are relatively few systems in which this problem is amenable to study. The timing of glial cell differentiation has been extensively analyzed in the O2A lineage (Raff, 1989). These studies have revealed that the schedule of oligodendrocyte production is determined by a cell-autonomous clock, whereas that of type-2 astrocyte development is determined by the time of appearance of one or more instructive signals (Lillien and Raff, 1990). One neurogenic progenitor cell that has been well-studied is the sympathoadrenal (SA) progenitor (Patterson, 1990). This neural crest-derived cell migrates either to the sympathetic ganglia, where it differentiates to sympathetic neurons, presumably under the influence of neurotrophic factors such as aFGF, bFGF and NGF (Anderson and Axel, 1986; Stemple et al., 1988; Claude et al., 1988; Birren and Anderson, 1990; Carnahan and Patterson, 1991), or to the adrenal gland, where it differentiates to chromaffin cells under the influence of adrenal corticosteroids (Unsicker et al., 1978; Doupe et al., 1985a,b; Anderson

and Axel, 1986; Carnahan and Patterson, 1991). While it is well-accepted that glucocorticoids (GC) are required for the maintenance of the chromaffin phenotype, the time at which these steroids first begin to exert their influence on SA progenitors has remained a matter of controversy (Anderson and Michelsohn, 1989; Ehrlich et al., 1989; Seidl and Unsicker, 1989).

Previous studies have used the expression of phenylethanolamine N-methyltransferase (PNMT, the chromaffin-specific enzyme that converts norepinephrine into epinephrine) as a marker for the earliest effects of GC on chromaffin cell development (Bohn et al., 1981; Teitelman et al., 1982; Jiang et al., 1989; Seidl and Unsicker, 1989). The major developmental onset of PNMT expression occurs at around embryonic day 17 (E17) in the rat (Teitelman et al., 1979; Bohn et al., 1981; Seidl and Unsicker, 1989). However, SA progenitors invade the developing adrenal gland as early as E13.5-E14.5 (Pankratz, 1931; Lempinen, 1964; Bohn et al., 1981). More recent studies indicate that such progenitors are already phenotypically distinct from their counterparts within sympathetic ganglia at this time (Anderson et al., 1991). For example, at E14.5, cells in the adrenal anlage have retained expression of the marker SA-1 and have extinguished or failed to express neuronal markers, whereas cells in the ganglia primordia have extinguished SA-1 and induced expression of the late neuronal marker, B2. Thus, the environment of the adrenal gland appears to influence the development of SA progenitors 2-3 days before the major onset of PNMT expression is detected.

These results have suggested that *in vivo*, chromaffin cell development proceeds through at least two stages: an early inhibition of neuronal differentiation, and a later induction of PNMT. This raises the question of whether GC mediate both events. Previous studies have shown that *in vitro*, GC are absolutely required for PNMT induction in embryonic SA progenitors (Anderson and Axel, 1986; Seidl and Unsicker, 1989). Studies on cultured postnatal chromaffin cells have shown, moreover, that GC block FGF- or NGF-induced neuronal differentiation (Unsicker et al., 1978; Doupe et al., 1985a; Stemple et al., 1988). These data suggest that *in vivo*, GC might also act to inhibit neuronal differentiation, prior to inducing PNMT. However, recent data have suggested that the delayed appearance of PNMT reflects the time at which SA progenitors first acquire functional GC receptor (Seidl and Unsicker, 1989). If true, this would preclude the possibility that GC control the early inhibition of neuronal differentiation. If, on the other hand, GC do exert an early, inhibitory effect on SA progenitors, it raises the question of how the induction of PNMT is delayed.

To address these issues, we have reconstituted the development of chromaffin cells from SA progenitors *in vitro*, and used this system to determine the time at which GC first influence cell fate. We have found that GC both inhibit neuronal differentiation and induce PNMT in SA progenitors, and that these two effects are temporally separated. Inhibition of process outgrowth is detected within 15 hours post-plating, whereas induction of PNMT is not detected in the majority of cells until 2-3 days *in vitro*, similar to its schedule of appearance *in vivo*. The delay in PNMT induction does not reflect the slow kinetics of accumulation of this protein,

but rather a delay in the ability of the cells to rapidly express this gene in response to GC. The delay does not appear to involve a qualitative change in GC receptor expression, since both early and late effects appear to be mediated by the type-II GC receptor. However, the two effects are pharmacologically distinct, suggesting that they may involve different interactions of the GC receptor. If SA progenitors are permitted to differentiate into sympathetic neurons, the capacity to induce PNMT is irreversibly lost (Anderson et al., 1991). Thus, the early inhibition of neuronal differentiation by GC is necessary to permit cells to subsequently acquire competence to express PNMT in response to steroid. This mechanism may serve to restrict the expression of epinephrine synthesis to those cells which have migrated to the adrenal gland. Taken together, these data reveal a two-step inductive process in chromaffin differentiation, in which the timing of the second event (PNMT expression) is controlled by neither the time of appearance of inducing signal, nor of its receptor, but rather by changes in the response properties of the cell. This mechanism fits well with recent insights into the molecular mechanisms of transcriptional regulation by the glucocorticoid receptor.

RESULTS

PNMT is expressed on schedule by differentiating SA progenitors *in vitro*

Migrating SA progenitors invade the developing adrenal gland on E13.5-E14.5 *in vivo*, but the expression of PNMT is not detected until several days later (Bohn et al., 1981; see also Appendices I and II). We first sought to determine whether PNMT could be expressed on schedule by isolated SA progenitors cultured in the presence of GC. SA progenitors were isolated from E14.5 adrenal glands by fluorescence-activated cell sorting using monoclonal antibody HNK-1 (Abo and Balch, 1981). Freshly isolated HNK-1+ cells express both TH (Fig. 1C) and SA-1 (Fig. 1A,B), an antigenic marker expressed by embryonic SA progenitors and by mature chromaffin cells (Carnahan and Patterson, 1991). By contrast, PNMT immunoreactivity is undetectable at the time of isolation (Fig. 1D).

The cells so isolated were plated on a collagen/poly-lysine/laminin substrate in steroid-stripped medium (see Materials and Methods), supplemented with 1μM corticosterone (CORT), or, in some experiments, the more potent agonist dexamethasone (DEX). Cultures were assayed for PNMT expression at either 1 (E14.5+1) or 3 (E14.5+3) days post-plating, by staining with a highly-specific anti-PNMT antibody (M. Bohn) and couterstaining with monoclonal anti-TH to identify the SA progenitor population. These assay points were chosen because they correspond to the equivalents of E15.5 and E17.5 *in vivo*, times when PNMT is expressed by few or most chromaffin cells, respectively (Appendix I).

In the presence of 1μM CORT, the *in vivo* schedule of PNMT induction appeared to be reproduced *in vitro*. At E14.5+1, very few of the TH+ SA progenitors expressed detectable PNMT (Fig. 2A,B; Fig. 3A, +1). By contrast, at E14.5+3, corresponding to E17.5 *in vivo*, over 70% of cells expressed the enzyme (Fig. 2C,D; Fig. 3A, +3). Similar results were obtained using the more potent GC agonist DEX (Fig. 2E,G), although a slightly higher level of PNMT expression was detected at E14.5+1 (Fig. 3B, +1). However, the overall staining intensity was much weaker at E14.5+1 than at E14.5+3, so that the quantitative data presented in Figure 3 overestimate PNMT expression at E14.5+1 relative to that measured at E14.5+3. The proportion of PNMT+ cells at E14.5+3 did not increase with longer incubations (not shown), and is similar to that measured *in vivo*. No PNMT expression was detected at any times in cultures incubated without GC (Fig. 4C), confirming the earlier observations of Seidl and Unsicker (1989). These data therefore indicate that in the presence of CORT, PNMT is expressed by isolated SA progenitors *in vitro* on a schedule that closely parallels the appearance of this enzyme *in vivo*. Moreover, the data indicate that GC are absolutely required for PNMT expression.

Early inhibition of neurite outgrowth by GC

Previous studies have suggested that the timing of PNMT appearance is controlled by the timing of the appearance of GC receptor at E17.3 (Seidl and Unsicker, 1989). This model predicts that SA progenitors should be

unresponsive to GC prior to E14.5+3 *in vitro*. We therefore sought an assay of GC-responsiveness in SA progenitors independent of the expression of PNMT. One such assay is the inhibition of neuronal differentiation. GC are known to inhibit process outgrowth from postnatal chromaffin cells (Unsicker et al., 1978), and to suppress the expression of neuron-specific genes in PC12 cells (Stein et al., 1988). In the case of E14.5 SA progenitors, 1μM CORT strongly inhibited process outgrowth from these cells when cultured for 3 days in the presence of the steroid (Fig. 4A vs. D, B vs. E). We next asked whether the inhibition of process outgrowth by GC could be detected earlier than the appearance of PNMT. When isolated E14.5 SA progenitors are cultured in the absence of steroid, the majority of the cells extend processes within the first 15 hours of culture (Fig. 5a). In the presence of either 1μM CORT or DEX, this short-term process outgrowth is clearly inhibited (Fig. 5b,c). These qualitative observations were quantified at 24 hours after plating (Figure 5d, NO ADD). Whereas over 60% of cell clusters bore processes in the absence of steroid, fewer than 30% of cell clusters did so in the presence of either 1μM CORT or DEX. (Note that this assay underestimates the inhibitory effect of steroid, since *all* cells in a cluster must lack processes in order for that cluster to be scored as round; see Materials and Methods.)

To address the possibility that the early effect of steroid is to permit the survival of a subpopulation of round cells rather than to inhibit neurite-outgrowth, we carried out serial observations of identified living progenitors. The fates of 56 cells grown in the absence of DEX, and 62 cells grown in its presence, were monitored in low-density cultures over a three

day period. Cells were initially identified on gridded dishes at 4 hours after plating. After a total of 24 and 48 hours in culture, each cell was scored as round, process-bearing or dead. 47% of initially round cells cultured in the absence of steroid extended processes within the first 24 hours (Fig. 6 upper, A; Fig. 6 lower, -DEX,DAY 1), whereas in the presence of 1μM DEX, 74% of such cells retained their round morphologies (Fig. 6 upper, B; Fig. 6 lower, +DEX, DAY 1). By day 2, 65% of initially round cells had extended processes in the absence of DEX (Fig. 6 lower, -DEX, DAY 2), while 76% remained round in the presence of steroid (Fig. 6 lower, +DEX, DAY 2). The fact that greater than 50% of the identified cells were round in the presence of DEX, and process-bearing in its absence, strongly implies that many individual cells are bipotential and that DEX therefore acts instructively to inhibit process outgrowth. In support of this interpretation, when cells that initially were process-bearing were followed, in many cases a retraction of such processes was observed within the first 24 hours of culture in DEX (Fig. 6 upper, C).

Thus, the analysis of both identified cells and cell populations supports the idea that GC exert an early inhibitory effect on neuronal differentiation in E14.5 SA progenitors. By contrast, little or no PNMT expression is detected in this cell population during the first 24 hours of culture (E14.5+1). Taken together, these results imply that SA progenitors at E14.5 possess functional glucocorticoid receptors, and that the delay in the appearance of PNMT is therefore unlikely to be due to a delay in the expression of such receptors.

The adrenal gland contains sufficient GC to inhibit neuronal differentiation at E14.5

The foregoing results indicate that *in vitro*, GC exert two temporally-separable effects on chromaffin cell differentiation. As early as E14.5, the steroids are able to inhibit neuronal differentiation. Two to three days later, expression of PNMT is detected, that is absolutely GC-dependent. If GC mediate an early inhibition of neuronal differentiation *in vivo*, then the E14.5 adrenal gland should contain sufficient concentrations of endogenous steroids to mediate this effect. Previous studies of GC content in the fetal rat adrenal gland measured a large surge in steroid levels between E16.5 and E17.5, by radioimmunoassay (Teitelman et al., 1982). However, these studies did not examine the content of GC two days earlier in development, when SA progenitors first invade the adrenal gland. To address this issue, the levels of various steroid hormones in fetal adrenal glands between E14.5 and E17.5 were analyzed by RIA (see Materials and Methods).

Micromolar or sub-micromolar levels of CORT, progesterone (PROG) and 11-deoxycorticosterone (DXC) are already present in developing adrenal glands at E14.5 (Table I). Consistent with previous studies (Roos, 1967; Teitelman et al., 1982), the levels of all three steroids increase substantially over the next several days. CORT is the predominant steroid present at all stages examined, although the relative concentration of PROG is higher at E14.5. DXC and CORT were undetectable in sympathetic ganglia, muscle or brain at E14.5 (Table I), indicating that these steroids are specifically concentrated in the adrenal gland. Although CORT levels at E17.5 (the time

of PNMT induction) are extremely high, the concentrations of the steroid present at E14.5 are easily sufficient to inhibit neuronal differentiation *in vitro* (see Fig. 7, below). Moreover, they are also sufficient to induce PNMT *in vitro* (see below), further supporting the idea that the schedule of PNMT appearance *in vivo* is not controlled by the time of appearance of GC within the adrenal gland.

Both effects of GC on SA progenitors are mediated by type-II GC receptors, but are pharmacologically separable

Our *in vitro* observations raise the question of how the same inducing signal, GC, can mediate two temporally distinct effects in the same responding cell type. One possible explanation is that the responses are mediated by different types of GC receptors; studies of the steroid receptor gene superfamily have indicated that there are at least two GC receptors, called type I and type II (see, eg., Evans and Arriza, 1989). To address this issue, we first examined the dose-response characteristics of the two different GC effects, after three days of exposure to the steroid. A significantly lower concentration of GC was required to inhibit process outgrowth than was required to induce PNMT (Fig. 7A). Half-maximal inhibition of process outgrowth was achieved at a CORT concentration of 40nM, whereas half-maximal PNMT expression required 200-250nM CORT. This difference was also apparent when DEX was used as the glucocorticoid (Figure 7B).

Despite this difference in dose-response profiles, the two effects of GC can be inhibited by the potent type-II GC receptor antagonist RU38486 (Teitelman, et al., 1982; Jung-Testas and Baulieu, 1983; Moguilewsky and Philibert, 1984; Nawata et al., 1988). The induction of PNMT by 100nM DEX was completely blocked by only a 5-fold excess of RU38486 (Fig. 7C, filled symbols). At this concentration of DEX, a 15-fold excess of RU38486 also blocked about 60% of the process-outgrowth inhibition (Fig. 7C, open symbols). However, 100nM DEX is an order of magnitude above the saturating dose for this inhibitory effect (Fig. 7B). At 10nM DEX, a near-saturating dose for process-outgrowth inhibition, a 50-fold excess of RU38486 was able to block 80% of the inhibitory effect of DEX (Fig. 7D, filled symbols). The inability of RU38486 to completely block the DEX-inhibition of process-outgrowth reflects the fact that, at high concentrations, this GC receptor antagonist on its own displays a partial agonist activity for this response; in other words, it inhibits process outgrowth (Fig. 7D, open symbols). Indeed, the residual 20% activity observed in 10nM DEX+1000nM RU38486 (Fig. 7D, filled symbols) can be accounted for by the agonist (i.e., process outgrowth-inhibitory) activity seen with RU38486 alone (Fig. 7D, open symbols at 1000nM RU38486). Such a partial agonist activity of RU38486 at high concentrations has been observed for steroid-inducible responses in other systems. Despite these quantitative differences in the efficacy of inhibition by a specific antagonist, the data support the idea that both the inhibition of process-outgrowth, and the induction of PNMT, are mediated via type-II GC receptors. Further support for this conclusion comes from the observation that neither process-outgrowth inhibition nor PNMT induction were blocked by a 50-fold excess of the type-I GC receptor antagonist RU28318

(Torelli et al., 1982; Ratka et al., 1989) (Table II). Moreover, a low concentration (50nM) of the specific type-II GC receptor agonist RU26988 (Teutsch et al., 1981) had comparable efficacy to DEX in both assays (Table II).

One consequence of the different dose-response profiles observed is that low concentrations of either CORT or DEX are able to inhibit process outgrowth, without inducing PNMT expression (Fig. 7A,B, open symbols). This in turn suggests that weak agonists of the GC receptor could have similar effects. We therefore examined the activity of PROG and DXC, two biosynthetic precursors to CORT which are known to be weak agonists of the GC receptor (Samuels and Tomkins, 1970), in the two bioassays. Inhibition of process outgrowth was observed using both DXC and PROG, whereas PNMT induction was not (Fig. 8 and Table II). PROG at 1μM was 61% as effective as DEX in inhibiting process outgrowth, but greater effects were not observed at higher concentrations (not shown). That this effect of PROG is not mediated via the progesterone receptor (PR) is suggested by the fact that a PR-specific agonist, R5020 (Philibert and Raynaud, 1973; Philibert et al., 1977; Haslam and Shyamala, 1979), showed little inhibition of process outgrowth compared to PROG, although it is more specific for the PR (Table II). The specificity of the PROG and DXC effects is further indicated by the fact that testosterone and β-estradiol had no significant effects in this system (Table II). These data therefore indicate that progesterone, a biosynthetic precursor of corticosterone present in the adrenal gland at E14.5 (Table I), is able to inhibit the neuronal differentiation of SA progenitors without inducing PNMT. The separation of these two effects is explained by the weak cross-

reactivity of progesterone towards the type-II GC receptor, and the lower threshhold for the inhibitory response.

The delay in PNMT appearance reflects the acquisition of competence to express the enzyme, and not simply the slow kinetics of protein accumulation

The foregoing data indicate that both process outgrowth inhibition and PNMT induction are likely to be mediated by the same type of GC receptor. The delay in PNMT appearance is therefore unlikely to be due to the appearance of a novel GC receptor. Another explanation is that the timing of PNMT appearance is controlled by the acquisition or loss of other molecules which interact with the GC receptor to control expression of PNMT. Such a mechanism would in effect constitute a cell-autonomous "clock". Alternatively, PNMT expression could be initiated from the start of the culture period, and the failure to detect it before E14.5+3 would simply reflect the slow kinetics of accumulation of this enzyme to immunocytochemically-detectable levels. If the latter were the case, then detectable PNMT immunoreactivity should appear in cultures with similar kinetics, irrespective of the time at which cells are initially exposed to GC.

To test this prediction, we compared the expression of PNMT in cells exposed to CORT for the first 24 hr of culture, to that in cells grown for two days in the absence of steroid and then exposed to CORT for the last 24 hr of culture. Thus, in both conditions, cells were assayed for PNMT expression

after a 24 hr exposure to CORT, but the time at which this exposure was initiated was different. As described earlier, when CORT was added at the time of plating and PNMT assayed at E14.5+1, very few of the cells were positive, and only weakly so (Fig. 9A,B; Fig. 10A, 24C). In striking contrast, when CORT was added at E14.5+2, PNMT was clearly detectable in 23% of cells 24 hr later (Fig. 9C,D; Fig. 10A, 48N/24C). Thus, the induction of PNMT occurs more rapidly in the third day of culture, although no steroid was present for the first two days. Moreover, this effect is underestimated by measuring the percentage of total cells that are PNMT-positive (Fig. 10A). This is because a high proportion of SA progenitors commit to neuronal differentiation in the first 48 hours of culture (Fig. 6; Fig. 9D), and such committed neuroblasts are unable to express PNMT in response to GC (Anderson et al., 1991). When the percentage of PNMT-positive cells is calculated for only those cells that retained a round morphology during the first 48 hours of culture (Fig. 10B, 48N/24C), over 65% of such cells are immunoreactive for PNMT upon a subsequent 24 hr exposure to CORT (Fig. 9C,D, arrows; Fig. 10C, 48N/24C). This value is within 25% of that measured for cells grown continuously in CORT for three days (Fig. 9G,H; Fig. 10C, 72C). By contrast, 5-fold fewer round cells express PNMT when steroid is added for the first 24 hours of culture (Fig. 10C, 24C).

These data suggest that when presentation of CORT is delayed for 2 days, subsequent PNMT expression only occurs in those cells which escape neuronal differentiation during the delay period. This implies that the total number of PNMT+ cells should be increased by a treatment that would inhibit neuronal differentiation without inducing PNMT during the delay

period. Such a requirement is filled by progesterone (see above). We therefore compared the percentage of PNMT+ cells that developed after a delayed exposure to CORT in cultures preincubated for two days with or without 1μM progesterone. As expected, pretreatment with PROG clearly increased the percentage of round cells compared to cultures grown in control medium (Fig. 9D,F; Fig. 10B, compare 48N/24C to 48P/24C), reflecting the ability of this steroid to inhibit neurite outgrowth (see above). Concomitantly, preincubation in PROG yielded an almost two-fold increase in the percentage of total cells expressing PNMT upon a subsequent 24 hr exposure to CORT (Fig. 9C,E; Fig. 10A, 48P/24C). However, the percentage of round cells expressing PNMT was similar whether or not PROG was included during the preincubation (Fig. 10C). These results are consistent with the idea that progesterone can "rescue" cells that would otherwise have differentiated into neurons, and maintain their program of chromaffin differentiation.

Taken together, these data indicate that most cells that have not yet committed to neuronal differentiation during the first 48 hr of culture are capable of rapidly expressing high levels of PNMT upon a subsequent exposure to CORT for 24 hr. By contrast, the majority of freshly-isolated E14.5 SA progenitors fail to display such a rapid PNMT induction. This difference suggests that the delay in PNMT appearance observed upon continuous exposure to CORT (Fig. 2A) does not reflect a slow rate of PNMT accumulation. Rather, SA progenitors acquire the capacity to rapidly express PNMT in response to CORT, as they develop in culture. We refer to this capacity as "competence". The fact that PROG increases the proportion of

cells that acquire competence, but cannot itself induce PNMT, further argues that competence reflects an underlying process distinct from the accumulation of immunoreactive enzyme. The acquisition of competence can occur in the absence of steroid, and follows a cell-autonomous schedule.

DISCUSSION

Migrating SA progenitor cells invade the adrenal gland primordium at E13.5-E14.5 and choose a chromaffin fate under the influence of the adrenal microenvironment. We have shown that chomaffin cell development proceeds through at least two distinct stages: an early inhibition of neuronal differentiation, and a subsequent induction of PNMT expression. We have reconstituted this sequence of events *in vitro*, using a purified population of E14.5 SA progenitor cells, and have shown that both stages of differentiation are dependent upon GC and are mediated by the type-II GC receptor. The two temporally-separated effects of GC are pharmacologically distinct, implying that they may involve qualitatively or quantitatively different interactions of the GC receptor. We have shown further that the delay in PNMT appearance does not reflect simply the slow kinetics of enzyme accumulation, but rather a timed, cell-intrinsic process that is necessary, in addition to GC, for PNMT expression. We propose, therefore, that the timing of PNMT appearance reflects a gain of competence for induction of this enzyme by GC. Although the molecular mechanisms underlying competence in this system are not yet known, it presents a clear case in which the timing of an inductive event (the expression of PNMT) is determined neither by the time of appearance of the inducing signal, nor of its receptor.

GC are required for the initial appearance of PNMT, but do not control its timing

The expression of detectable PNMT-immunoreactivity in SA progenitor cells is absolutely dependent upon the addition of exogenous GC. These results are in agreement with the recent studies of Seidl and Unsicker, who assayed PNMT expression in chromaffin cell precursors isolated at E16.3, using three independent methods of varying sensitivities (Seidl and Unsicker, 1989). The conclusion that GC are required for PNMT expression does not necessarily imply, however, that GC control the timing of this expression. Early studies of fetal steroidogenesis revealed a surge in GC synthesis whose timing seemed to correlate with the developmental induction of PNMT (see also Table I). This observation, taken together with the fact that PNMT expression is dependent upon GC in the adult (Wurtman and Axelrod, 1966; Ciaranello and Black, 1971) originally suggested that the surge in corticosterone production by the adrenal cortex controls the timing of PNMT appearance. However, our *in vivo* assays of adrenal steroid content indicate that micromolar or sub-micromolar concentrations of steroid are already present in the adrenal at E14.5, two days before the surge occurs, consistent with the previous studies of Roos (1967). These concentrations are more than sufficient to induce PNMT *in vitro*, in agreement with the studies of Seidl and Unsicker (1989). Nevertheless, induction of PNMT *in vivo* is delayed until E16.5-E17.5. A similar delay is observed *in vitro*, despite the presence of GC from the time of plating. Therefore, the timing of PNMT appearance is not controlled by the time of the appearance of GC.

Earlier studies led to the conclusion that GC are not required for the initial appearance of PNMT during development (Ehrlich et al., 1989). This

conclusion was based upon three observations: 1) the inability of intrauterine injections of GC prior to E17 to induce precocious PNMT expression *in vivo* (Bohn et al., 1981); 2) the expression of PNMT on schedule in organ cultures of adrenal glands in the absence of exogenously added steroid (Teitelman et al., 1982); and 3) the observation that PNMT is detectable *in vivo*, albeit at low levels and in a minority of cells, two days prior to the surge in adrenal corticosteroid synthesis (Ehrlich et al., 1989). Our data indicate that GC are required for the initial expression of PNMT, but do not control the timing of PNMT appearance. None of these earlier studies is inconsistent with this conclusion. For example, the inability to induce precocious PNMT expression *in vivo* by steroid injections is consistent with our demonstration that most SA progenitors are not competent to express PNMT at E14.5. In the case of the organ culture studies, the whole adrenal explants used contained an abundance of GC-producing cortical cells, which could provide a sufficiently concentrated source of endogenous GC to induce PNMT expression in the absence of exogenous steroid. Finally, since E14.5 adrenal glands contain concentrations of GC sufficient to induce PNMT (Table I) two days before the surge in cortical steroidogenesis, the early detection of PNMT in a small number of cells does not constitute evidence for the GC-independence of PNMT expression, as previously suggested.

Our data indicate that the majority of SA progenitor cells display a rapid biological response to GC -- neurite outgrowth inhibition -- 24-48 hours before they express PNMT. Our pharmacological data suggest that both the early and late effects of GC are mediated by type-II GC receptors.

Thus, although our results confirm the previous demonstration by Seidl and Unsicker that GC are required for PNMT expression, they do not support their conclusion that the timing of PNMT expression is controlled by the time of the appearance of the GC receptor. The failure of Seidl and Unsicker (1989) to detect receptors prior to E17.3 by radioligand binding could reflect a low sensitivity of their assay, or a difference in receptor affinity at E16.3 versus E17.3. Whatever the case, SA progenitors appear to possess functional GC receptors several days prior to the appearance of PNMT, suggesting that other mechanisms control the timing of the onset of epinephrine synthesis.

The timing of PNMT appearance is controlled by a cell autonomous "clock"

We have shown that most E14.5 SA progenitor cells are unable to respond to GC by rapid induction of PNMT within the first 24 hrs of culture. A higher proportion of cells acquires the ability to express PNMT, over time in culture. This observation suggests that the delay in PNMT appearance that is observed *in vitro* and *in vivo* is unlikely to simply reflect the slow kinetics of enzyme accumulation. Rather, SA progenitors appear to acquire competence to express PNMT during their development in culture.

Previous studies in other systems provide precedent for several mechanisms that could underlie the acquisition of competence. For example, in avian liver, five different yolk genes become inducible by b-estradiol at different ages (Evans et al., 1987). Full competence to induce

different genes is acquired gradually over time, as cells autonomously upregulate levels of estrogen receptor (ER) via an estrogen-independent mechanism. By analogy, perhaps process-outgrowth inhibition in SA progenitor cells requires a smaller number of GC receptors per cell than does PNMT induction. In that case, the acquisition of competence to express PNMT might be explained by an increase in the concentration of GC receptor to a critical threshhold. Consistent with this idea, an increase in the number of GC receptors per cell has been observed to occur concomitant with the expression of PNMT (Seidl and Unsicker, 1989).

An alternative mechanism for the acquisition of competence is changes in the expression of other regulatory factors that act in concert with the GC receptor to control PNMT gene transcription (see below). Studies of mice homozygous for deletions near the albino locus on chromosome 7 provide genetic evidence that factors other than GC receptor are required for the induction of some steroid-responsive genes (DeFranco et al., 1991). Hepatocytes in these mutant mice lack GC-inducibility of several liver-specific genes, yet possess wild type numbers of functional GCR, suggesting the absence of an accessory factor. Perhaps an analagous accessory factor controls competence to induce PNMT in chromaffin cells. In support of this idea, transfected PNMT-β-galactosidase constructs containing a GRE are not expressed in PC12 cells that contain functional GC receptors, but are expressed in adult chromaffin cells (Ross et al., 1990). This suggests that PNMT expression is dependent upon additional factor(s) differentially expressed in PC12 cells and chromaffin cells. The timing of PNMT

expression during development could then reflect the time at which such a regulator accumulates (or decays) to threshhold levels in SA progenitors.

The early and late effects of GC may involve different interactions of the GC receptor

SA progenitors display an early inhibition of process outgrowth in response to GC that precedes the induction of PNMT. Our data suggest that these two effects of GC, though both mediated via type-II GC receptors, are pharmacologically distinct. Lower doses of GC are required for maximal inhibition of process-outgrowth than for PNMT induction. In addition, weak GCR agonists, such as progesterone, produce significant amounts of process-outgrowth inhibition without inducing PNMT. Finally, RU38486 displays full antagonist activity towards PNMT induction, but shows partial agonist activity towards process-outgrowth inhibition. Pharmacological differences in steroid-inducible responses within the same cell type have been seen in other systems. For example, tyrosine aminotransferase (TAT) induction in the FU5-5 rat hepatoma cell line occurs at an approximately 5-fold lower DEX concentration than is required for the induction of other steroid-inducible genes. Moreover, RU38486 displays partial agonist activity towards TAT induction, but behaves as a pure antagonist with respect to the induction of other genes such as glutamine synthetase or an MMTV-reporter gene construct (Mercier et al., 1983; Mercier et al., 1986; Simons et al., 1989). It has been suggested that such differential responsiveness of various genes to GC may reflect differences in the accessory factors that act in

concert with the GC receptor to regulate transcription at a given promoter (Meyer et al., 1990).

Recent studies have identified several factors that interact with the GC receptor to regulate transcription. For example, the GREs of at least some steroid-regulated genes overlap recognition elements for transcription factors in the AP-1 family. Steroid inducibility at such "composite GREs" has been shown to be dependent upon interactions between the GC receptor and c-fos and/or c-jun (Diamond et al., 1990). The affinity of a liganded GC receptor for such a composite GRE may therefore reflect not only its affinity for DNA, but also its affinity for other proteins bound at or near the site. Different steroid-inducible genes may therefore have different affinities for liganded GC receptor. According to this idea, the differing dose-response curves for the positive and negative effects of GC on SA progenitor cells could reflect the fact that these two effects require different concentrations of liganded receptor in the cell.

PNMT expression requires an earlier developmental decision

It has been well established that the fate of SA progenitor cells is controlled by environmental signals, and that GC promote chromaffin differentiation. Our observations reveal that GC control two distinct steps in chromaffin cell development: not becoming a neuron, and expressing PNMT. These steps are temporally and pharmacologically separable. Moreover, they are related to each other, since cells that differentiate into neurons are unable to express PNMT. Thus, GC appear to be necessary at

early times in chromaffin development to prevent cells from differentiating into neurons. We found that progesterone and 11-deoxycorticosterone, two biosynthetic precursors of corticosterone, are also able to inhibit the neuronal differentiation of SA progenitors. The presence of progesterone and 11-deoxycorticosterone in the adrenal gland at E14.5 (Table I) suggests that these steroids may also contribute to inhibiting the neuronal differentiation of migrating SA progenitors. In this way, an early action of adrenal corticosteroids on SA progenitors is a necessary prerequisite for a later inductive effect of these hormones (Fig. 11). Such a mechanism would explain why PNMT is expressed by cells in the adrenal gland and not by neurons in the sympathetic ganglia, even though the ganglia are exposed to a transient surge in circulating GC levels at E17.5 .

In vitro, a small proportion of cells escapes neuronal differentiation in the absence of GC and is able to express PNMT upon subsequent exposure to steroid. This observation would predict that a few cells in the sympathetic ganglia *in vivo* might transiently express PNMT upon exposure to a high level of corticosterone. In fact, a transient burst of PNMT expression is observed in the superior cervical sympathetic ganglia *in vivo* at E17.5, coincident with the surge in circulating GC (Bohn et al., 1982). Such ganglionic PNMT expression appears restricted to SIF cells, which are not yet committed to neuronal differentiation (Doupe et al., 1985b). These observations therefore support the idea that the acquisition of competence is not *dependent* upon the presence of GC, although *it is more likely to occur* in the presence of steroid.

Our results may also help to explain why some chromaffin cells do not express PNMT, even though they are located in the steroid-rich adrenal gland (Hillarp and Hokfelt, 1953; Eranko, 1955). The fact that the acquisition of competence is not a steroid-dependent event implies, conversely, that some cells may not acquire competence even in a high concentration of GC. Indeed, we observe that 20-30% of SA progenitors *in vitro* do not express PNMT, even in the presence of 1mM CORT. This percentage is similar to the proportion of PNMT-negative chromaffin cells *in vivo* (Doupe et al., 1985b). Thus, despite their location in the GC-rich environment of the adrenal gland, some SA progenitors may not acquire competence, and therefore would fail to express PNMT. The reason why some chromaffin cells would fail to acquire competence is not clear. However, as SA progenitors differentiate along the competing neuronal pathway, they reach a point at which they can no longer acquire competence (Fig. 11) (Anderson et al., 1991). It may be that a subset of SA progenitors in the adrenal gland advances along the neuronal pathway to this critical pont, without undergoing overt neuronal differentiation. Consistent with this idea, PNMT-negative chromaffin cells express some neuronal markers, such as L1 and the calcium-binding cerebellar Purkinje cell-specific protein (spot-35), not expressed in PNMT-positive chromaffin cells (Kondo et al., 1985; Langley, 1991). The mechanisms that control this heterogeneity in the chromaffin cell population are not clear.

The precise molecular mechanisms that underlie the temporal separation of GC effects on SA progenitor cells await future study. However, our results suggest that the developmental regulation of PNMT expression

is likely to occur through a more complex mechanism than previously considered. This mechanism involves cell-autonomous changes in the response properties of progenitor cells to GC. Our data thus underscore the possibility that the timing of inductive events can be controlled by mechanisms other than the appearance of an inducing signal or its receptor. Moreover, they emphasize that cell fate may involve progressive mechanisms consisting of temporally ordered, discreet steps. The SA lineage may provide a frutiful system for investigating such mechanisms in molecular detail.

MATERIALS AND METHODS

Isolation, Plating and Culture of Sympathoadrenal Progenitor Cells

Staged embryos were removed from timed-pregnant Simonson Albino rats (Simonson Labs). The morning following an overnight breeding was considered day 0.5 of gestation. Dissociated suspensions of adrenal tissue were prepared as previously described (Anderson and Axel, 1986). Sympathoadrenal progenitor cells were isolated by fluorescence-activated cell sorting using the monoclonal antibody HNK-1, as previously described (Anderson et. al., 1991). Isolated HNK-1+ cells were plated at a density of approximately 400 cells/mm^2 into 4 mm-diameter cloning rings placed on the surface of 24-well tissue culture plates that had been previously coated with a collagen/poly-D-lysine/laminin substratum and cultured in L15-CO_2 complete medium, as previously described (Anderson and Axel, 1986), except that FBS that had been previously stripped of endogenous steroids was substituted for rat serum. For single-cell tracking experiments, cells were plated at a density of approximately 25 cells/mm^2 in 35-mm dishes. Cells were identified at 4 hours post-plating and their positions marked by means of a gridded coordinate system embossed in the bottom of the dish using a BB press. Cells were photographed at this time and after a total of 24 and 48 hours in culture. When included, the steroids used in this study were diluted from stock solutions of 1mM in 95% ethanol. Dexamethesone, corticosterone, progesterone, 11-deoxycorticosterone, pregnenolone, testosterone and β-estradiol were purchased from Sigma. RU26988, RU28318 and RU38486 were the kind gifts of D. Philibert and J. Raynaud, Roussel-

Uclaf (Rommainville, France). R5020 (promegestone) was purchased from New England Nuclear.

Immunocytochemical and Morphological Assays

Cells were fixed in 4% formaldehyde in 25mM HEPES-buffered MEM + 5% sucrose for 10 minutes at room temperature. For quantitative analysis of PNMT and TH expression, cells were preblocked for 30 min. at room temperature in a solution containing 0.5M NaCl, 0.1M sodium phosphate buffer (pH 7.4), 10% horse serum, 3% NGS and 0.3% NP-40. Cells were then incubated with a monoclonal antibody directed against TH (Boeringer-Mannheim) used at a dilution of 1:100, and a rabbit polyclonal antiserum to PNMT (the kind gift of Dr. Martha Bohn), used at a dilution of 1:1000, overnight at 4^0C. Immunostaining was developed using FITC-Goat-anti-Mouse IgG and Rhodamine-Goat-anti-Rabbit antibodies (TAGO). Cells were counterstained with DAPI (Sigma) to verify the number of cells present in the tight clusters of cells which were often present in the cultures. PNMT expression was assayed as the percentage of TH+ cells that were also PNMT+ (TH is a specific marker of all cells in the sympathoadrenal lineage). >300 cells were counted for each determination. For double-labelling with HNK-1 and SA-1 antibodies, HNK-1+ cells isolated by FACS were fixed and subsequently preblocked in PBS+1%NGS+0.1% NP-40 for 30 minutes at room temperature, incubated with SA-1 monoclonal antibody ascites fluid (the kind gift of Dr. Josette Carnahan) at a dilution of 1:250 overnight at 4^0C, and developed using a rhodamine-Goat-anti-mouse IgG secondary antibody (Southern Biotech). To reduce non-specific binding, dilutions of this secondary antibody were precleared as described previously (Anderson et. al.,

1991). Controls lacking primary antibody exhibited no specific staining. For quantifying the extent of process-outgrowth, a cell-cluster was defined as "process-bearing" if any cytoplasmic extension could be detected originating from the cluster at 200x magnification. >200 clusters were counted for each determination. It was not possible to determine whether every cell in a cluster was process-bearing, because the tight clumping of cells precluded assignment of processes to individual cells. Nonetheless, for the purposes of this study, this measurment represents a highly stringent criterion, in that only clusters containing exclusively round cells were counted as non-process-bearing. Any systematic error introduced by this method would, if anything, underestimate the inhibition of neurite-outgrowth produced by GC, because only clusters in which all cells lacked processes would be scored as round.

For comparison of the relative effects of various steroids on process outgrowth inhibition or PNMT expression, values are presented as the percent of maximal activity, where maximal activity is the effect measured after culturing in 1 μM DEX for 3 days, the "standard" condition. The standard condition was performed in parallel with test conditions in each independent run of an experiment, as was a control incubation in the absence of steroid. This method served to normalize the data for variations in the extent of steroid effects observed from plating to plating, and permitted the pooling of data from different platings and different experiments. In the case of PNMT induction, the average maximal activity was 84% $PNMT^+$ cells (22 separate determinations from 13 different platings) (see Fig. 3). Since no PNMT was detected when cells were grown in the

absence of steroid, the % of maximal PNMT induction observed in a given test condition was calculated as (%PNMT$^+$ cells in test condition/%PNMT$^+$ cells in the standard condition) x 100%.

In the case of process outgrowth inhibition (poi), the inhibition produced by a given test steroid (%poi^{test}) was defined as:

$$\%poi^{test} = \frac{\%po^{NA} - \%po^{test}}{\%po^{NA}} \times 100\% \qquad (1)$$

where $\%po^{NA}$ = percent process-bearing clusters (po) in control medium (No Addition), and $\%po^{test}$ = percent process-bearing clusters in the presence of test steroid. This metric normalizes the data to the extent of process outgrowth obtained in control medium, which varies slightly from plating to plating (range = 70% - 93% for 13 independent platings). DEX (at $\geq$ 100nM) always produced the maximal inhibition of process outgrowth of any steroid tested, but the extent of inhibition also varied from plating to plating (range = 61% - 87% for 13 independent platings). Therefore, as in the case of PNMT induction the process outgrowth inhibition produced by a given dose of test steroid in any experiment was always normalized to the inhibition produced by 1μM DEX in the same experiment. The data are therefore presented as the relative efficacy of a given steroid in comparison to 1mM DEX:

$$\%\text{maximal inhibition}^{test} = \frac{\%poi^{test}}{\%poi^{DEX}} \times 100\% \qquad (2)$$

Substituting the expression for %poi from equation 1, this parameter can be calculated directly from the raw numbers obtained in an experiment as:

$$\%\text{maximal inhibition}^{test} = \frac{\%po^{NA} - \%po^{test}}{\%po^{NA} - \%po^{DEX}} \times 100\% \qquad (3)$$

For example, if in a particular experiment, $\%po^{NA} = 80\%$; $\%po^{DEX} = 30\%$; and $\%po^{corticosterone} = 40\%$, then $\%\text{maximal inhibition}^{corticosterone} = [(80\% - 40\%)/(80\% - 30\%)] \times 100\% = 80\%$; in other words, in this experiment, corticosterone was 80% as effective as (1μM) DEX at inhibiting process outgrowth.

Determination of Steroid Concentrations by Radioimmunoassay

Dissected tissue from sources indicated in the text were frozen in liquid nitrogen and shipped on dry ice to the Nichols Institute (San Juan Capistrano, CA) for analysis of progesterone, 11-deoxycorticosterone and corticosterones content by RIA. The procedures used by the analytic facility were in-house modifications of established protocols (Abraham et al., 1971; Nabors et al., 1974; Antonipillai et al., 1983), using highly specific antibodies. Sensitivity of the assays on tissue samples was estimated by the Nichols Institute to be in the 0.1μM range for the three steroid examined. As a test of the assays' specificity and accuracy, a series of solutions containing known concentrations of corticosterone, 11-deoxycorticosterone, progesterone and testosterone, in various combinations, were sent for analysis along with the experimental samples. Results of the assays on the known solutions were within 20% of the correct values for all samples examined, and no cross-reactivity between steroids was observed.

Table I. ***Steroid concentrations in developing adrenal glands as determined by radioimmunoassay.***

Adrenal glands from rat embryos between E14.5 and E17.5 were dissected, frozen in liquid nitrogen, and shipped on dry ice to the Nichols Institute for analysis of steroid content by RIA, as described in Materials and Methods. Sympathetic ganglia, brain and forelimb muscle were also dissected, as controls. Samples consisted of pooled tissue from 35,25,20,10,20,15 and 15 embryos from E14.5 adrenal, E15.5 adrenal, E16.5 adrenal, E17.5 adrenal, E14.5 sympathetic ganlia, E14.5 brain and E14.5 forelimb muscle, respectively. Values for concentrations of progesterone (PROG), 11-deoxycorticosterone (DXC) and corticosterone (CORT) were reported to us in units of ng steroid/g tissue. These values were then calculated as μM concentrations assuming a tissue density of 1 g/ml. We estimate that this value for tissue density is within 10% of the actual value, since the tissue samples sink in distilled water but float in a 30% sucrose solution in distilled water (density = 1.1 g/ml).

Table I.

Steroid Concentrations in Embryonic Adrenal Glands
as Determined by Radioimmunoassay

	CONCENTRATION (μM)[#]		
Sample	CORT	DXC	PROG
E14.5 Adrenal	3	0.4	0.7
E15.5 Adrenal	20	0.6	1.1
E16.5 Adrenal	200	7.2	2.4
E17.5 Adrenal	500	100	25
E14.5 Muscle	ND*	ND*	ND*
Brain	ND*	ND*	0.7
Symp. Ganglia	ND*	ND*	ND*

*ND=not detectable
[#]see legend for calculation

Table II. ***Effect of synthetic and endogenous steroids on process-outgrowth and PNMT induction in SA progenitors cells in culture.***

E14.5 HNK-1+ SA progenitor cells were cultured in the absence (No Addition) or the continued presence of various synthetic (A) or naturally occuring (B) steroids, at the indicated concentrations, for 3 days (until E14.5+3), at which time the cultures were assayed for process-outgrowth and PNMT expression as described in Materials and Methods. Values are expressed as the % of maximal activity, relative to 1μM dexamethesone (=100% process-outgrowth inhibition and 100% PNMT induction), as defined in Materials and Methods.

Table II.

Effects of synthetic and naturally occuring steroids on process-outgrowth and PNMT expression in SA progenitor cells in culture

Steroid	% inhibtion of process-outgrowth[a]	% induction of PNMT[a]	D(p)[b]
A. Synthetic agonists and antagonists			
Dexamethesone, 1μM	100	100	22(13)
Dex, 100 nM[c] + 5 μM RU28318 (type I GR antagonist)	101 ±3	100 ±2	4(2)
RU26988, 50nM (type-II GR agonist)	97 ± 2	88 ± 2	5(2)
R5020, 1μM (PR agonist)	12 ± 1	ND*	5(2)
B. Endogenous steroids (all 1μM)			
Corticosterone	89 ± 3	85 ± 4	10(6)
11-deoxycorticosterone	79 ± 10	16 ± 5	5(3)
Progesterone	61 ± 3	ND*	14(8)
β-estradiol	0 ± 3	ND*	4(2)
Testosterone	4 ± 4	ND*	4(2)
No Addition	0	ND*	24(14)

*ND = not detectable

[a]relative to 1μM DEX, as defined in Materials and Methods.
[b]D separate determinations from (p) independent platings.
[c]process outgrowth inhibition and PNMT induction in the presence of 100nM Dex (alone) was indistinguishable from that seen in 1μM Dex; see Materials and Methods; cf. Fig. 7B.

Figure 1. ***Antigen expression in freshly-isolated E14.5 SA progenitor cells.*** A. E14.5 HNK-1+ SA progenitor cells, isolated, plated and fixed as described in Materials and Methods. B. Same field as in (A), labelled with SA-1 monoclonal antibody as described in Materials and Methods. C. E14.5 HNK-1+ cells isolated, plated and fixed as in (A), stained for TH immunoreactivity as described in Materials and Methods. D. Same field as in (C), stained for PNMT immunoreactivity as described in Materials and Methods. A,B,C,D are matched exposures. Cells in A,B and C,D were plated, fixed and stained in parallel in the same experiment. Other experiments indicated that virtually all HNK-1+ cells were TH+, and that >70% of HNK-1+ cells were SA-1+ (Anderson et. al., 1991) (Appendix III).

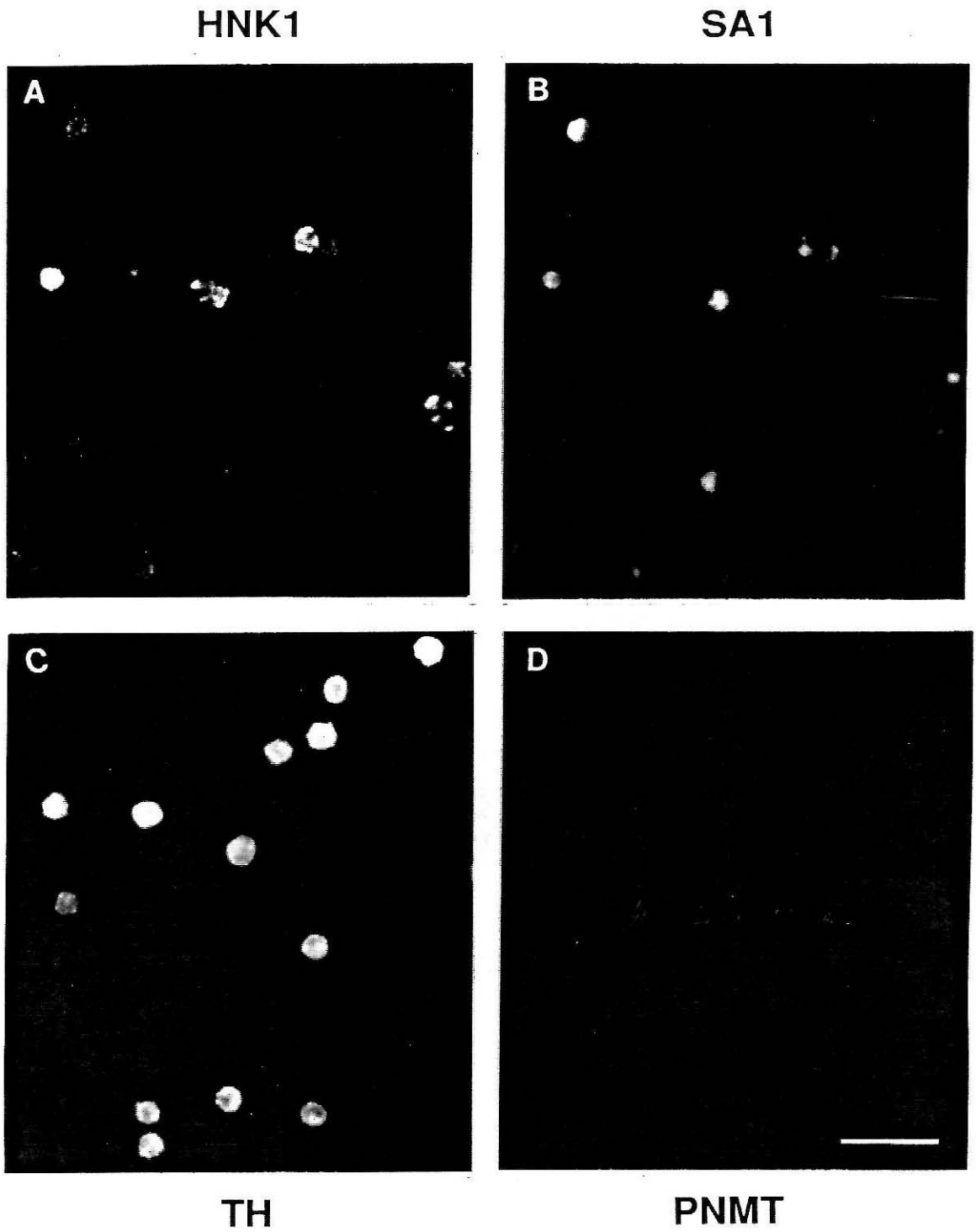

HNK1
SA1
A
B
C
D
TH
PNMT

Figure 2. ***PNMT expression*** **in vitro** ***(qualitative).***

E14.5 HNK-1+ cells were cultured in the continued presence of either 1μM CORT (A,B,C,D) or 1μM DEX (E,F,G,H) for either 1 day (A,B,E,F) or 3 days (C,D,G,H), and assayed for both PNMT expression (A,C,E,G; matched exposures) and TH expression (B,D,F,H; matched exposures) by double-label immunofluorescence, as described in Materials and Methods. (A,B);(C,D);(E,F);(G,H) are paired exposures of identical fields. All conditions shown were assayed in parallel in the same experiment. Scale bar = 50μ.

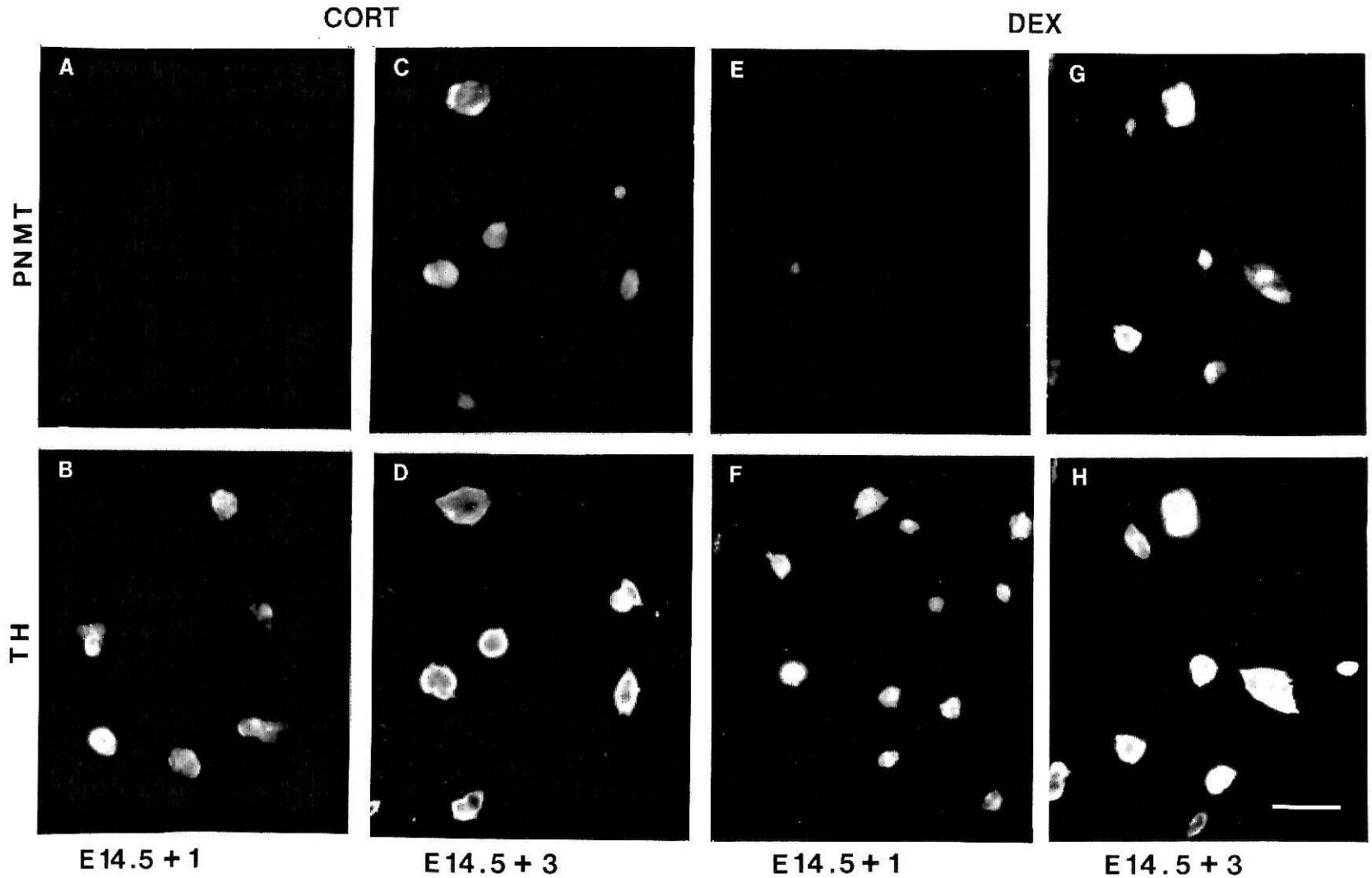

CORT
DEX
A
C
E
G
PNMT
B
D
F
H
TH
E14.5 + 1
E14.5 + 3
E14.5 + 1
E14.5 + 3

Figure 3. ***PNMT expression*** **in vitro** ***(quantitative).***

E14.5 HNK-1+ SA progenitor cells were cultured in the continued presence of either 1μM CORT (A) or 1μM DEX (B) for 1 (E14.5+1) or 3 (E14.5+3) days, at which times they were assayed for TH and PNMT expression by double-label immunofluorescence, as described in Materials and Methods. Values are expressed as the %TH+ cells that are PNMT+. Each bar represents the mean ± S.E.M. for D separate determinations from (*p*) independent platings: CORT +1: 10(5); +3: 10(6); DEX +1: 21(11); +3: 22(13).

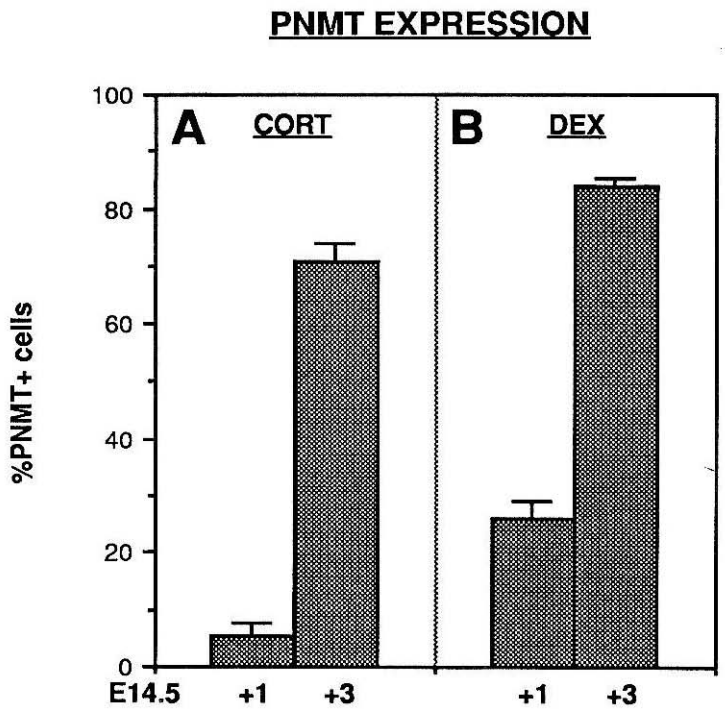

PNMT EXPRESSION
%PNMT+ cells
100
80
60
40
20
0
A
CORT
B
DEX
E14.5
+1
+3
+1
+3

Figure 4. ***E14.5 SA progenitor cells after 3 days in culture (14.5+3).***

E14.5 HNK-1+ SA progenitor cells were cultured in the absence (A,B,C) or presence (D,E,F) of 1μM dexamathesone (DEX) for 3 days (until E14.5+3), at which time they were fixed and stained for TH (B,E) and PNMT (C,F) immunoreactivity by double-label immunofluorescence, as described in Materials and Methods. A,B,C and D,E,F are sets of photographs of identical fields from conditions plated in the same experiment. A,D are phase-contrast micrographs; B,E and C,F are matched exposures. Scale bar = 50μ.

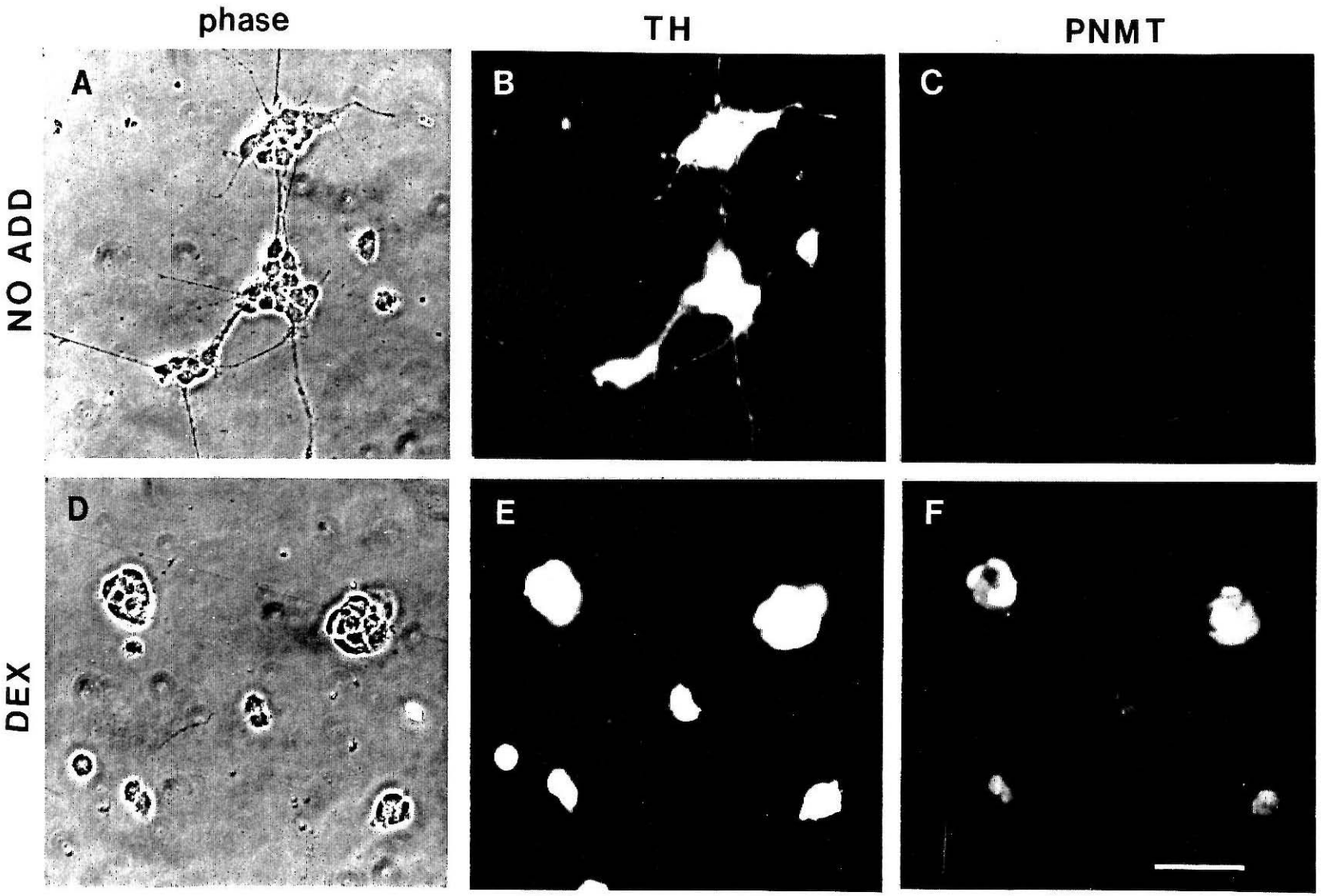
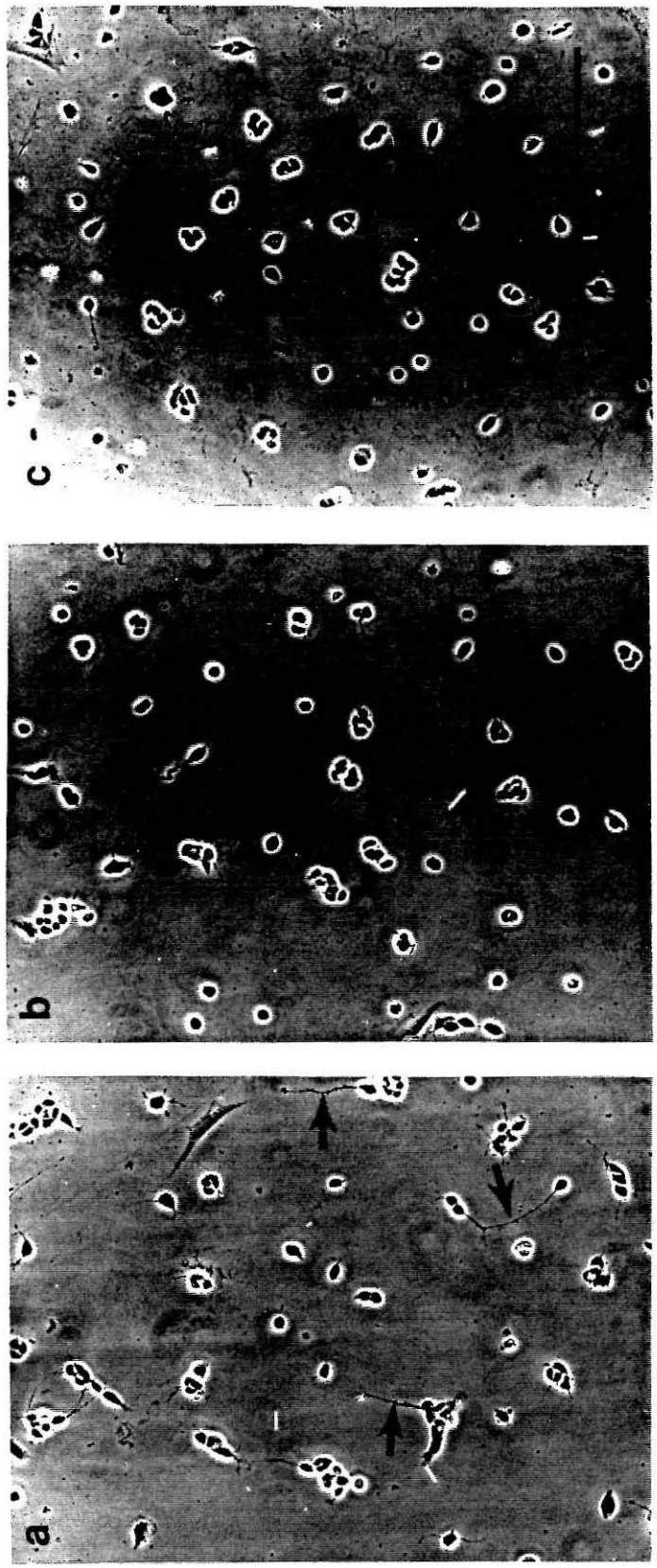

phase
TH
PNMT
NO ADD
DEX
A
B
C
D
E
F

Figure 5. ***Early inhibition of process-outgrowth by GC.***

E14.5 HNK-1+ SA progenitor cells were cultured in the absence (a,NO ADD) or presence of 1μM CORT (b,CORT) or 1μM DEX (c,DEX) for 15 hours, and fixed as described in Materials and Methods. When steroid was absent from the culture, many of the cell clusters extended processes (arrows), whereas in the presence of either CORT or DEX, the majority of cell clusters did not. Scale bar = 100μ. d. *Quantitation of process-outgrowth from E14.5 SA progenitors in the presence and absence of GC during the first 24 hours of culture.* Cells as in (a-c) were cultured for 24 hours in the absence of steroid or in the continued presence of either 1μM CORT or 1μM DEX for 24 hours (until E14.5+1). The percentage of cell clusters bearing processes was determined for each indicated condition according to the criteria described in Materials and Methods. Each bar represents the mean $\pm$ S.E.M. for 9-13 separate determinations from 5-8 independent platings.


a
NO ADD
b
CORT
c
DEX

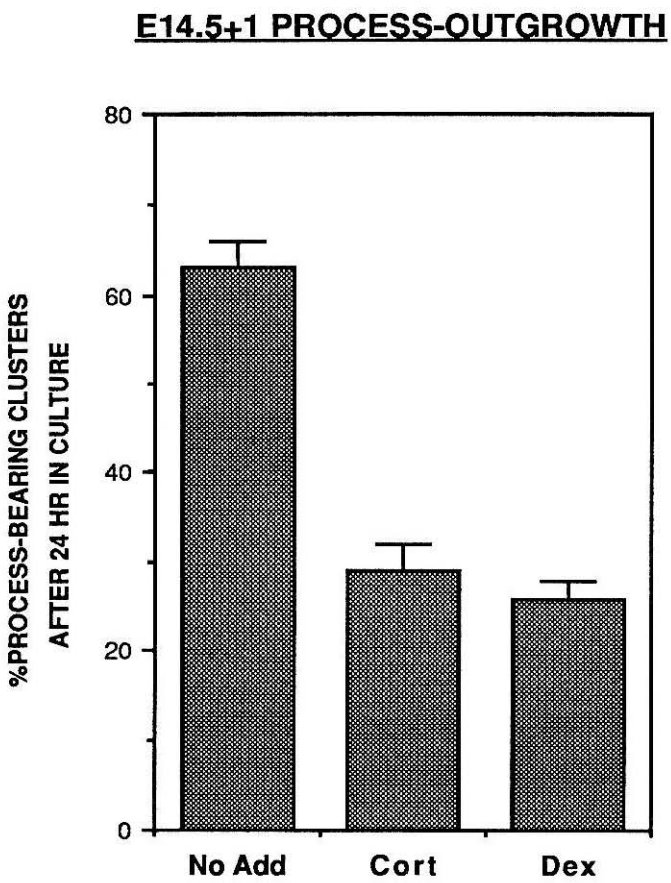
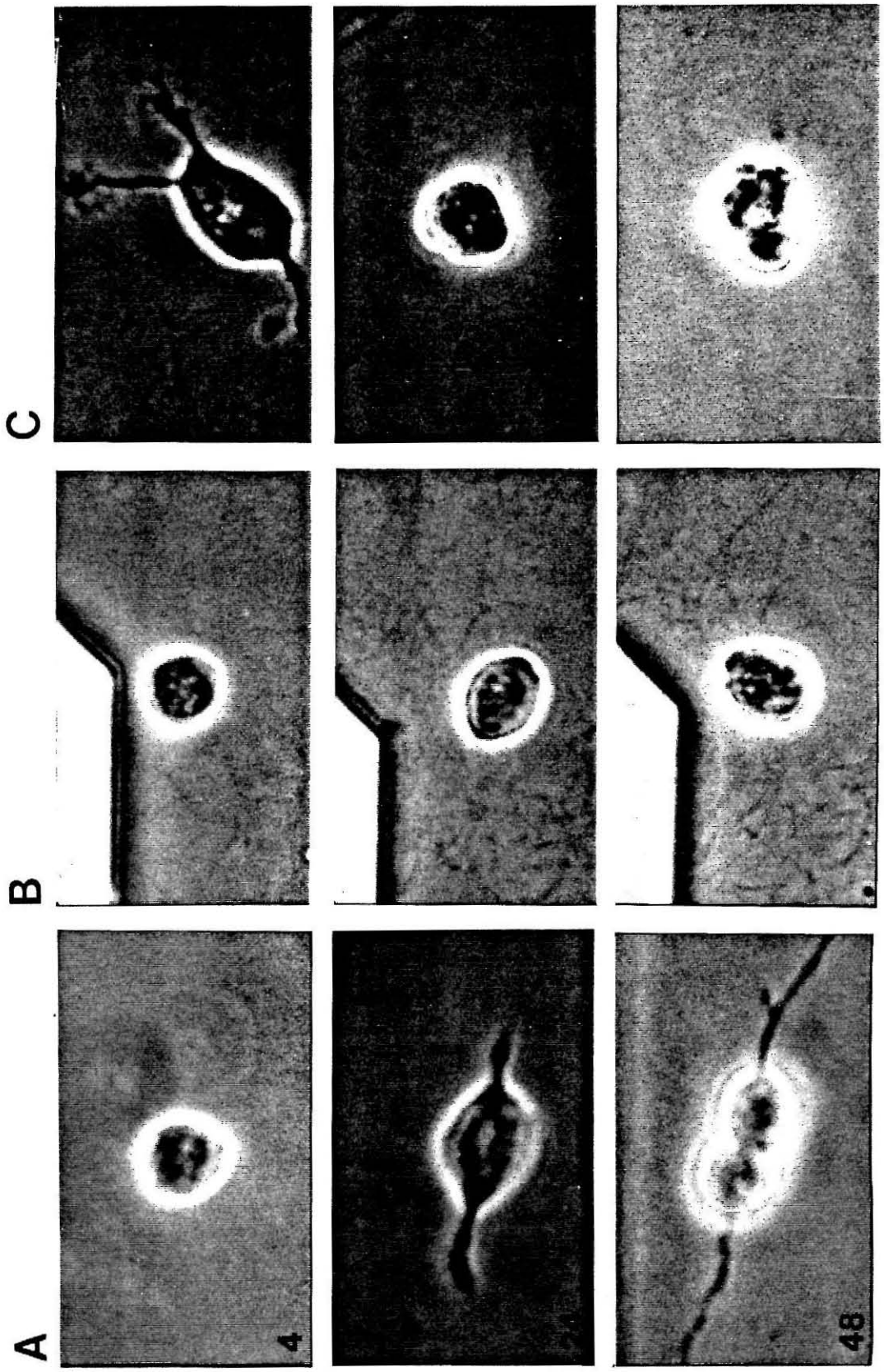
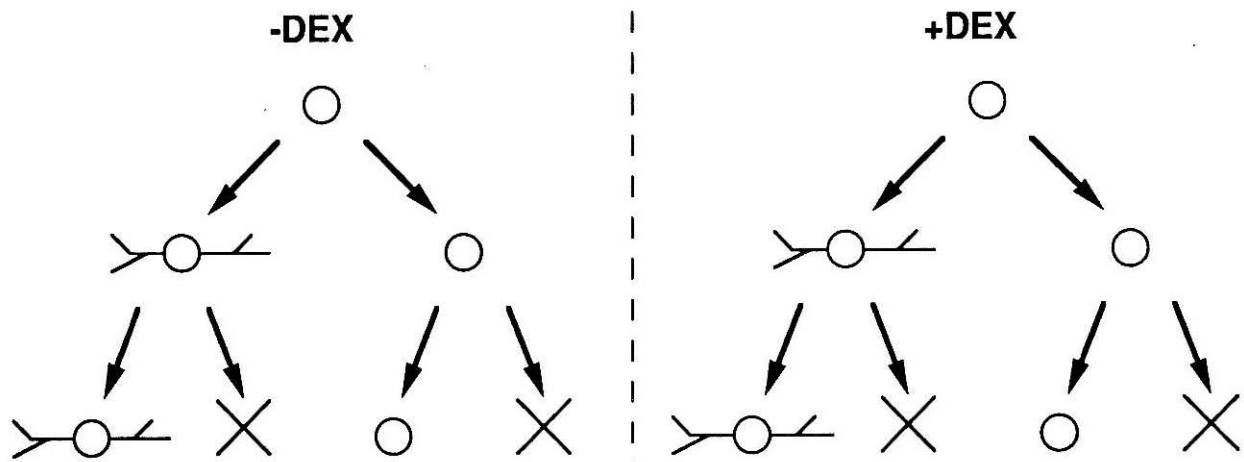

E14.5+1 PROCESS-OUTGROWTH
%PROCESS-BEARING CLUSTERS AFTER 24 HR IN CULTURE
80
60
40
20
0
No Add
Cort
Dex

Fig. 5d

Figure 6. ***Serial Observations of Identified Cells.***

Upper: E14.5 HNK-1+ SA progenitors were plated at low density on gridded dishes as described in Materials and Methods. Individual cells were identified and photographed at 4 hours post-plating, and subsequently thereafter following a total of 24 and 48 hours in culture. A: representative cell cultured in the absence of steroid throughout the culture period. B: representative cell identified at 4 hours post-plating and subsequently cultured in the continued presence of 1μM DEX. C: representative cell bearing processes at 4 hours post-plating and subsequently grown in the continued presence of 1μM DEX. Cells bearing processes comprised <10% of the HNK-1+ population observed at 4 hours post-plating. *Lower: Cell histories of identified cells.* E14.5 HNK-1+ SA progenitor cells were isolated, plated and identified as described in *Upper*. Only cells which displayed a round morphology at 4 hours post-plating were included in the quantitative analysis shown here. After a total of 24 (DAY 1) and 48 (DAY 2) hours in culture in the absence of steroid (left side) or in the continued presence of 1μM DEX (right side), each identified cell was scored as round, process-bearing or dead. A total of 56 cells were tracked in the absence of DEX; 62 cells were tracked in its presence. The values listed represent the percentage of initially identified cells with each of the following histories: extended processes and survived; extended processes and died; remained round and survived; remained round and died.


A
B
C
4
48



DAY 1	47	0	47	7	24	0	74	2
DAY 2	61	4	21	14	23	2	71	5

Fig. 6 lower

Figure 7. ***Dose-response curves for CORT- and DEX-induced process-outgrowth inhibition and PNMT induction in the absence or presence of RU38486.***

A. E14.5 HNK-1+ SA progenitors were cultured in the continued presence of different concentrations of CORT ranging from 1nM to 10μM, for 3 days. At E14.5+3, cultures were assayed for process-outgrowth (open symbols) and PNMT expression (filled symbols), as described in Materials and Methods. Values are presented as the percent of maximal activity, relative to 1μM DEX, as defined in Materials and Methods. Each point shown represents the mean ± S.E.M. from 4-7 separate determinations from 2-3 independent platings.

B. Cells as in (A) were cultured in the continued presence of different concentrations of DEX, ranging from 10pM to 1μM, for 3 days, and assayed for process-outgrowth inhibition (open symbols) and PNMT induction (filled symbols) at E14.5+3. Values are expressed as in (A). Each point represents the mean ± S.E.M. of 4-6 separate determinations from 2-4 independent platings.

C. Cells as in (A) were cultured in the continued presence of 100nM DEX, plus increasing concentrations of the type-II GC receptor antagonist RU38486 ranging from 10nM to 10μM, for 3 days. At E14.5+3, cultures were assayed for process-outgrowth inhibition (open symbols) and PNMT expression (filled symbols). Values are expressed as in (A). Each point represents the mean ± S.E.M. of 4 separate determinations from two independent platings.

D. Cells as in (A) were cultured in the continued presence of different concentrations of RU38486 ranging from 10nM to 10μM (open squares), or in the continued presence of 10nM DEX, plus increasing concentrations of RU38486 (filled diamonds), for 3 days. Cultures were assayed for process-outgrowth inhibition at E14.5+3. Values are expressed as in (A). PNMT expression was undetectable in cells cultured in the continued presence of all concentrations of RU38486 (alone) examined. Each point represents the mean ± S.E.M. of 4 separate determinations from two independent platings.

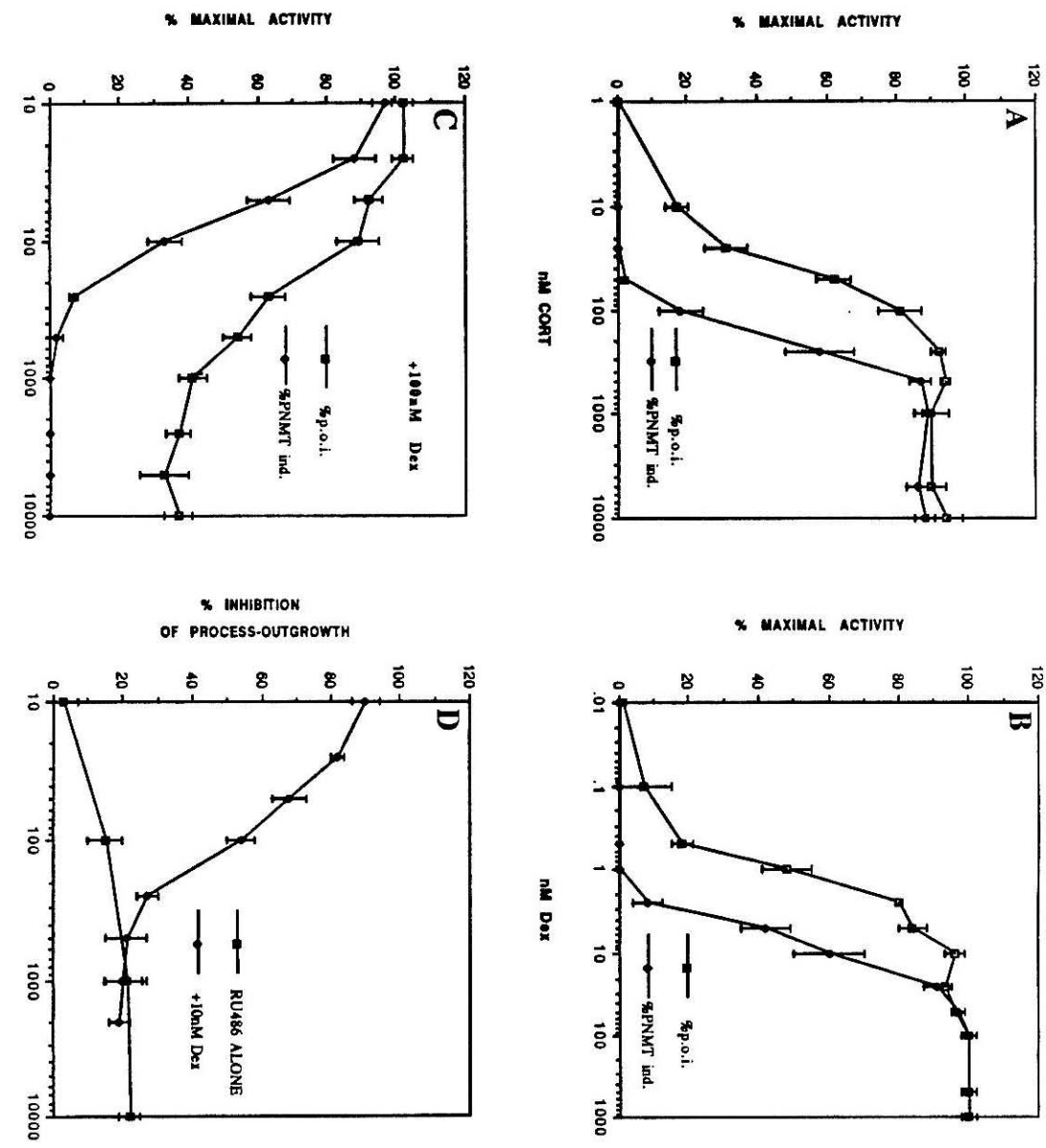

A
% MAXIMAL ACTIVITY
nM CORT
%p.o.i.
%PNMT ind.
B
% MAXIMAL ACTIVITY
nM Dex
%p.o.i.
%PNMT ind.
C
% MAXIMAL ACTIVITY
+100nM Dex
%p.o.i.
%PNMT ind.
nM RU486
D
% INHIBITION
OF PROCESS-OUTGROWTH
RU486 ALONE
+10nM Dex
nM RU486

Figure 8. ***Progesterone inhibits process-outgrowth from E14.5 SA progenitors, but does not induce PNMT expression.***

E14.5 HNK-1+ SA progenitors were cultured in the absence of steroid (A,B; same field) or in the continued presence of 1μM progesterone (C,D; same field) for 3 days. At E14.5+3, cells were fixed and stained for both TH (A,C; matched exposures) and PNMT (B,D; matched exposures) expression by double-label immunofluorescence, as described in Materials and Methods. The exposures of TH and PNMT immunoreactivity shown here are matched to those shown in Fig. 9. Scale bar = 50μ.

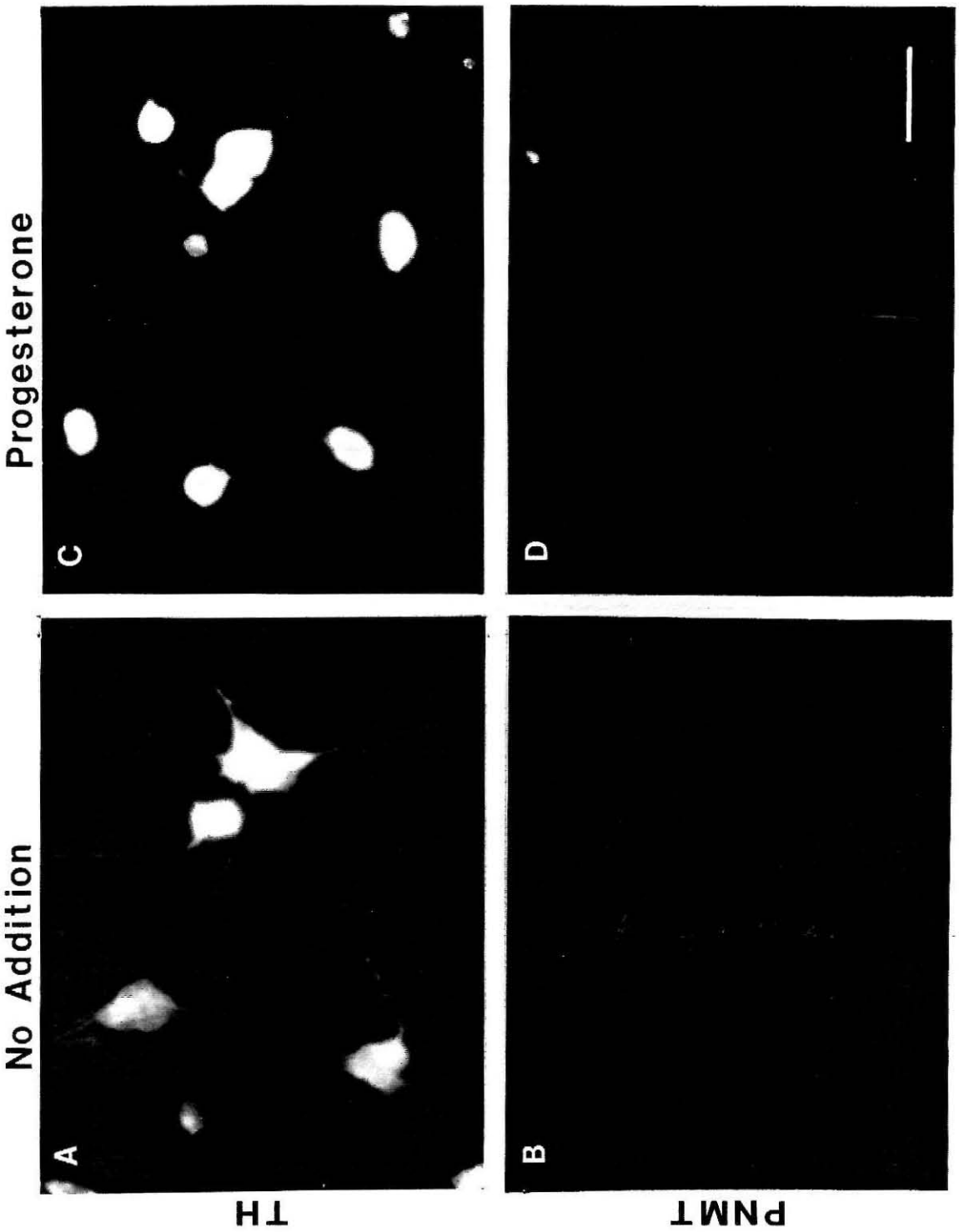

No Addition
Progesterone
A
B
C
D
TH
PNMT

Figure 9. ***PNMT expression after a 24-hour exposure to CORT given at two different times in culture.***

E14.5 HNK-1+ SA progenitor cells were cultured under the conditions listed below, and assayed for both PNMT (A,C,E,G; matched exposures) and TH (B,D,F,H; matched exposures), by double-label immunofluorescence, as described in Materials and Methods. A,B: in the continued presence of 1μM CORT for 24 hours, and photographed at E14.5+1. C,D: in the absence of steroid for 2 days (until E14.5+2, at which time PNMT expression was undetectable; data not shown), followed by a 24 hour exposure to 1μM CORT, photographed at E14.5+3. Note that the majority of cells within round clusters (arrows) are PNMT+. E,F: in the continued presence of progesterone for 2 days (until E14.5+2, at which time PNMT was undetectable; data not shown), followed by a 24 hour exposure to CORT in the absence of progesterone, photographed at E14.5+3. G,H: in the continued presence of 1μM CORT for 3 days, and photographed at E14.5+3. All conditions shown were plated, assayed and photographed in parallel in the same experiment. Scale bar = 50μ.

24C
48N/24C
48P/24C
72C
PNMT
TH
A
B
C
D
E
F
G
H

Figure 10. ***Quantitation of PNMT expression and process-outgrowth inhibition after a 24 hour exposure to CORT at two different times in culture.***

A. E14.5 HNK-1+ SA progenitor cells were cultured under the conditions described below, and assayed for PNMT expression as described in Materials and Methods. 24C: in the continued presence of 1μM CORT for 24 hours. 48N/24C: in the absence of steroid for 48 hours, followed by a 24 hour exposure to 1μM CORT. 48P/24C: in the continued presence of 1μM progesterone for 48 hours, followed by a 24 hour exposure to 1μM CORT, in the absence of progesterone. 72C: in the continued presence of 1μM CORT for 72 hours. The data shown are from one representative experiment in which all conditions were plated in triplicate and assayed in parallel. Each bar represents the mean ± S.E.M. of the triplicate determinations.

B. The identical conditions described in (A) were assayed for the percent of cell clusters displaying a round morphology. Each bar represents the mean ± S.E.M. of the triplicate determinations.

C. >100 cell clusters displaying a round morphology and containing a total of >300 cells were selected at random from among the triplicate wells plated in each of the conditions described in (A). The percent of cells within these round clusters that expressed PNMT was determined.

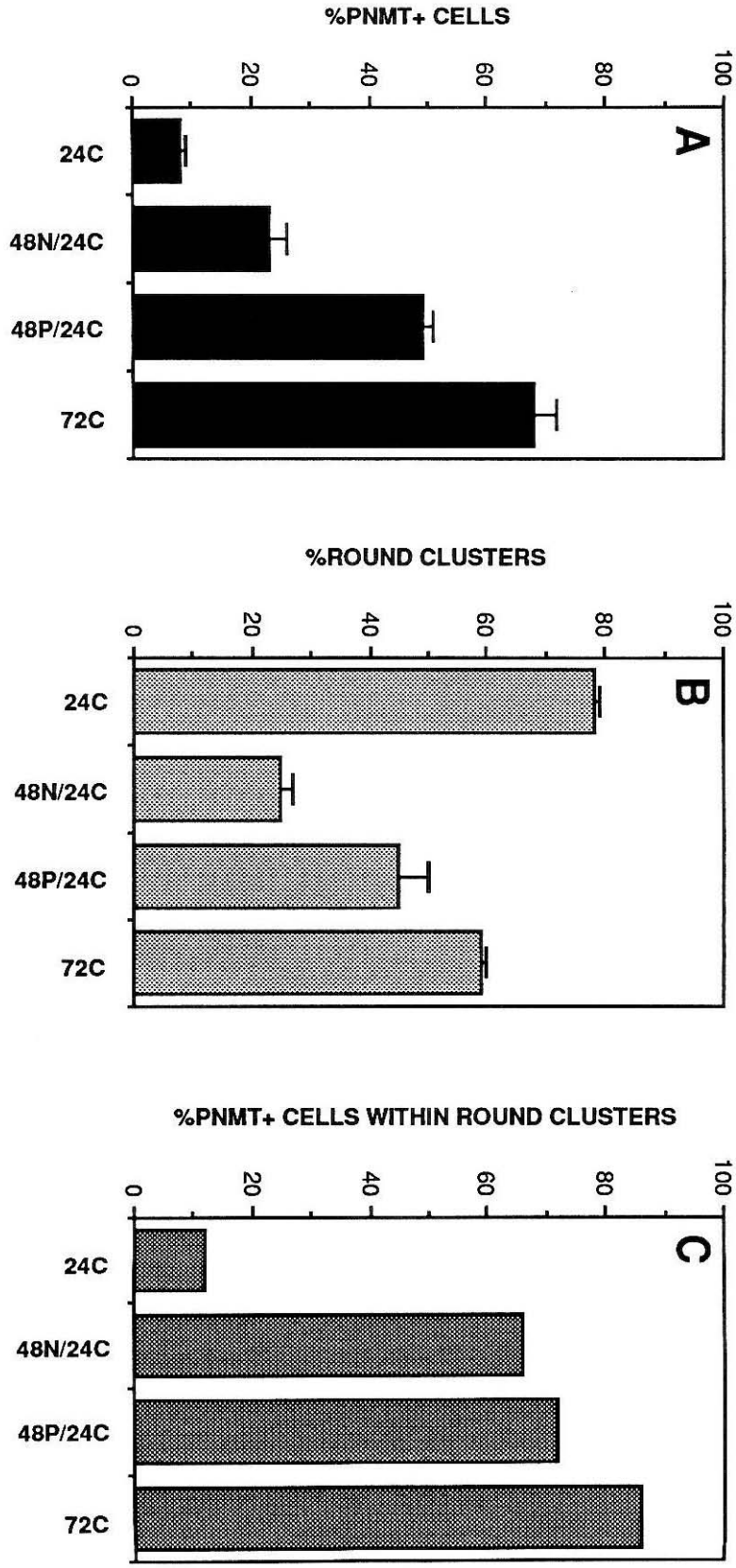
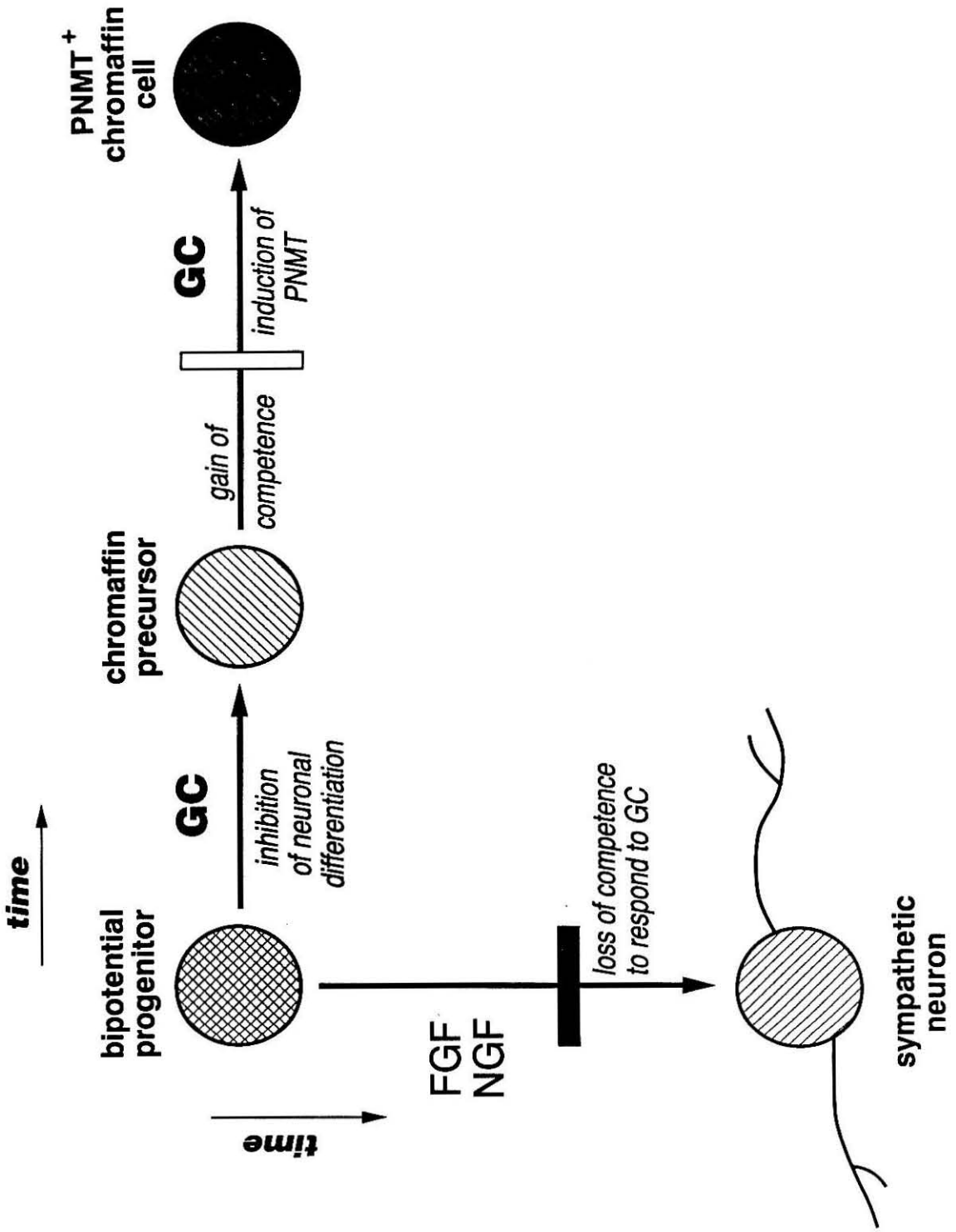

%PNMT+ CELLS
0
20
40
60
80
100
24C
48N/24C
48P/24C
72C
A
%ROUND CLUSTERS
0
20
40
60
80
100
24C
48N/24C
48P/24C
72C
B
%PNMT+ CELLS WITHIN ROUND CLUSTERS
0
20
40
60
80
100
24C
48N/24C
48P/24C
72C
C

Figure 11. ***MODEL: Glucocorticoids control two sequential steps in chromaffin cell differentiation.***

Bipotential SA progenitor cells (expressing both neuron- and chromaffin-specific genes) that migrate to the adrenal anlage are exposed to GC, which inhibit these cells from differentiating into neurons. In doing so, GC are permissive for the acquisition of competence to express PNMT upon a continued exposure to steroid. Thus, early exposure to GC is a prerequisite for the subsequent induction of epinephrine synthesis. By contrast, progenitor cells that migrate to the sympathetic ganglia primordia undergo a program of neuronal differentiation, which blocks the acquistion of competence, thus precluding the expression of PNMT.


time
bipotential progenitor
GC
inhibition of neuronal differentiation
chromaffin precursor
gain of competence
GC
induction of PNMT
PNMT+ chromaffin cell
time
FGF
NGF
loss of competence to respond to GC
sympathetic neuron

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APPENDIX I

TIME COURSE OF PNMT APPEARANCE *IN VIVO*

RESULTS

The initial objective of the experiments described in Chapter 2 was to investigate the timing of PNMT expression *in vitro*, using a quantitative single-cell assay. As a standard for comparison, we first determined the schedule of PNMT appearance *in vivo* using a similar single-cell assay. Dissociated cell suspensions of purified chromaffin cells or their precursors from rat embryos of different ages were isolated as described in Materials and Methods (below), and the percentage of TH+ cells that expressed PNMT at each age was determined by double-label immunofluorescence. As shown in Figure 1, the onset of PNMT expression *in vivo* is well-defined. At E14.5 and E15.5, detectable PNMT is present in fewer than 1% of isolated cells. By E16.5, approximately 30% of the TH+ adrenal cells are PNMT-positive. One day later, at E17.5, PNMT appears in the majority of cells (70%). By E18.5, the percentage of PNMT+ cells (81%) approaches the maximum values previously reported for mature populations of postnatal chromaffin cells (Doupe et al., 1985a). Our results confirm at the single-cell level previous data documenting the developmental appearance of PNMT immunoreactivity in histological sections (Bohn et al., 1981) and mRNA (Anderson and Michelsohn, 1989) (Appendix II). [n.b. for references, see bibliography for Chapter 2.]

MATERIALS AND METHODS

SA progenitor cells were isolated from rat embryos from between 14.5 and 18.5 (E14.5-E18.5) days of age by fluorescence-activated cell sorting using monoclonal antibody HNK-1, as described in Chapter 2. Sorted, dissociated suspensions of cells were plated at a density of approximately 400 cells/mm^2 within cloning rings onto 24-well tissue culture plates that were previously coated with 0.5mg/ml ploy-D-lysine. Cells were allowed to settle onto the substrate for approximately 20 minutes, after which time they were fixed,, double-stained with anti-TH and PNMT antibodies, and assayed for the percentage of TH+ cells that were PNMT+ (as described in Chapter 2).

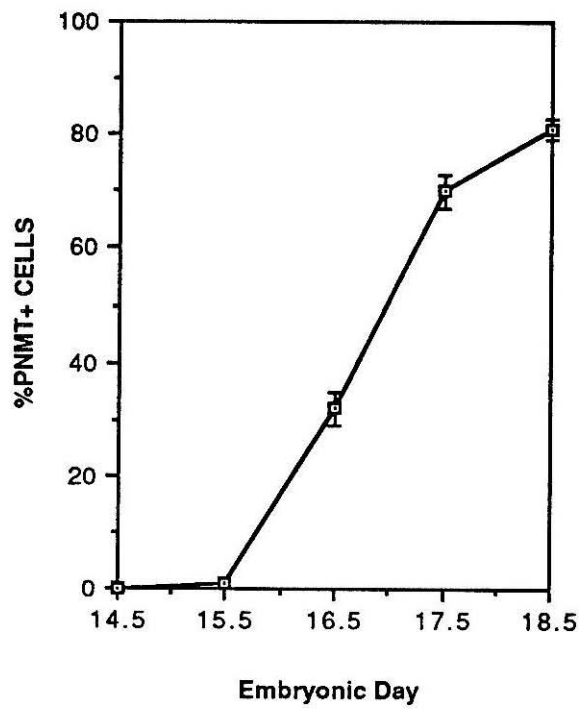

Figure 1. ***PNMT expression*** **in vivo.**

Purified HNK-1+ cells were isolated, allowed to adhere briefly to tissue-culture wells, fixed, and then stained for TH and PNMT immunoreactivity, as described in Materials and Methods. Values are expressed as %TH+ cells that are PNMT+. Each point represents the mean $\pm$ S.E.M. for D separate determinations from (*p*) independent platings: E14.5: 13(6); E15.5: 15(5); E16.5: 8(3); E17.5: 8(3); E18.5: 5(2).

APPENDIX II

ROLE OF GLUCOCORTICOIDS IN THE CHROMAFFIN-NEURON DEVELOPMENTAL DECISION

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ABSTRACT

Chromaffin cells and sympathetic neurons develop from a common, neural crest-derived progenitor cell. The developmental fate of this cell differs depending upon whether it migrates to the sympathetic ganglion or to the adrenal gland primordium, suggesting that local environmental signals control its differentiation. Glucocorticoid (GC) is a good candidate for an important adrenal environmental signal. These steroids are known to regulate PNMT, an adrenal-specific enzyme. However, *in vivo* observations suggest that the adrenal microenvironment influences the phenotype of sympathoadrenal progenitor cells as early as E14.5, two days before PNMT is first expressed by developing chromaffin cells. Using cDNA probes, we find that GC receptor mRNA can be detected in the embryonic adrenal at least one full day before the initial appearance of PNMT mRNA. This observation is compatible with the idea that the apparent early influence of the adrenal microenvironment reflects the action of GC on progenitors which have migrated into this environment. In support of this, we show that similar influences can be exerted by GC on PC12 cells, which contain GCR mRNA but do not express or induce PNMT mRNA. Taken together, these data suggest that other factors in addition to the presence of the GCR may be necessary for the developmental appearance of PNMT expression.

INTRODUCTION

Neural crest cells give rise to a wide array of differentiated derivatives following their migration from the top of the neural tube. The development of this heterogeneous cell population raises the question of the relative contributions of cell lineage and environmental determinants to its differentiation (LeDouarin, 1986). Among the derivatives of the neural crest are the endocrine chromaffin cells of the adrenal medulla, and the principal sympathetic neurons of the paravertebral autonomic ganglia. We have focused on the differentiation of these cells from the so-called *sympathoadrenal lineage* (Landis and Patterson, 1981) as a model system for addressing general questions of neural crest development and plasticity.

It has clearly been shown that chromaffin cells and sympathetic neurons develop from a common determined progenitor cell *in vitro* (Doupe et al, 1985b; Anderson and Axel, 1986; Carnahan and Patterson, 1988.) *In vivo*, this progenitor can first be detected on E11.5 in the sympathetic ganglion primordia of the rat, by expression of tyrosine hydroxylase (Cochard et al, 1979) and other, more recently-developed markers (Anderson, Carnahan and Patterson, unpublished.) Subsequently, some of these progenitors continue their migration ventrally to invade the adrenal *anlagen*, where they develop into chromaffin cells (Bohn et al, 1981; Teitelman et al, 1982) (Figure 1.) Others remain in the ganglion primordia, where they develop into sympathetic neurons. This behavior suggests that local environmental signals in the ganglion and the adrenal gland may control the choice of differentiation pathways by this bipotential progenitor cell.

One environmental factor likely to be of importance in controlling chromaffin development is glucocorticoid (GC). Unsicker et al (1978) first showed that dexamethasone (dex), a synthetic glucocorticoid, could impair the NGF-induction of neurite outgrowth from neonatal chromaffin cells *in vitro*. It was suggested from this result and others (Unsicker et al, 1985) that the presumably high local concentration of glucocorticoids in the adrenal gland (produced by the cortex) were important for maintaining the chromaffin phenotype. Subsequently, it was shown that glucocorticoids also function as a *survival factor* for neonatal chromaffin cells (Doupe et al, 1985a). A role for glucocorticoid in the *embryonic* development of chromaffin cells was suggested by *in vitro* experiments showing

that sympathoadrenal progenitor cells from E14.5 fetal rat adrenal glands would invariably develop into sympathetic neurons unless supplied with exogenous GC (Anderson and Axel, 1986). In these experiments, the effect of GC on E14.5 progenitor cells was not measured until 4 days following their initial exposure to the steroid; thus, it was formally possible that responsiveness to steroid was actually initially acquired during the 4-day culture period. Nevertheless, this finding was interpreted as being consistent with the idea that the migration of progenitor cells into the adrenal primordium at E13.5-E14.5 would expose them to a high concentration of steroid, which would then inhibit the cell from differentiating along the neuronal pathway and promote its differentiation along the endocrine pathway (Figure 2).

Glucocorticoid also acts to increase the expression of phenylethanolamine-N-methyltransferase (PNMT), the rate-limiting enzyme in epinephrine biosynthesis which is expressed by chromaffin cells but not by sympathetic neurons (Wurtman and Axelrod, 1966, Bohn et al, 1982; Grothe et al, 1985). The expression of PNMT during embryonic adrenal development first occurs between E16.5 and E17.5, several days after progenitor cells have first invaded the adrenal primordium (Bohn et al, 1981; Teitelman et al, 1982.) This phenomenon raises the question of what factors are responsible for the delay in the expression of this gene. As the appearance of PNMT correlates temporally with a sharp rise in corticosterone synthesis by the adrenal cortex, and as PNMT is a GC-inducible enzyme, a logical explanation initially was that the time of appearance of PNMT was controlled by the time of appearance of its inducer. Such an explanation, however, would be at odds with the suggestion that GC exerts an important influence several days earlier in development (E14.5), when it would act to inhibit the neuronal differentiation of progenitor cells. Indeed, measurements by Roos (1967) showed that steroidogenesis by the embryonic adrenal could be detected as early as E12.5 in the rat.

One resolution to this apparent paradox was suggested by experiments which indicated that the initial appearance of PNMT occurred on schedule whether or not corticosteroids were removed, or added prematurely, implying that induction of this gene is *independent* of the presence of steroids (Bohn et al, 1981; Teitelman et al, 1982.) However, these experiments were performed *in vivo* and in organ culture, under conditions where it was impossible to prove the unequivocal elimination of all endogenous sources of

steroid. Nevertheless, more recent experiments have indicated that PNMT appears on schedule in cultures of purified E14.5 adrenal progenitors, despite the presence of micromolar dex from the time of plating (Michelsohn, A. and Anderson, D.J., unpublished), in agreement with earlier *in vivo* studies (Bohn et al, 1981). Therefore, the time of PNMT appearance is clearly not controlled by the time of appearance of its inducer (GC). However, more recent experiments (see Seidl et. al., 1988 and this volume) have suggested that the appearance of detectable PNMT *in vitro* nonetheless *requires* the presence of GC.

The apparent dependence of correctly-timed PNMT expression upon the presence of inducer suggests another explanation for the delay in PNMT appearance: a delay in the appearance of the *receptor* for the inducer, i.e., the GC receptor (GCR). Indeed, Seidl et. al. (this volume) present convincing data that functional GCR cannot be detected in purified chromaffin precursors until E17.5, the time at which PNMT first appears. If this explanation were valid, it would imply that the progenitor cells which migrate into the adrenal gland at E14.5 are initially *unresponsive* to steroid. In this paper, however, we present evidence that E14.5 progenitors in the adrenal gland primordium already are phenotypically distinct from their ganglionic counterparts at this early age. Specifically, they appear to be *inhibited* from undergoing neuronal differentiation, implying an early effect of the adrenal microenvironment. Moreover, such an inhibition can be mimicked, at least in part, by GC on a sympathoadrenal lineage-derived cell line (PC12) (Greene and Tischler, 1976) which neither expresses nor induces PNMT mRNA in response to the steroid. Taken together, these data are consistent with the idea that progenitors could be responsive to GC *prior* to their expression of PNMT. In support of this, GC receptor (GCR) mRNA appears in the embryonic adrenal at least one full day before the appearance of PNMT mRNA. These data imply that GC exert an early influence on chromaffin cell differentiation, and therefore that factors other than the appearance of the GCR must be responsible for the delay in the initial activation of the PNMT gene.

MATERIALS AND METHODS

A full-length bovine PNMT cDNA probe (Batter et al, 1988) was used to screen an adult rat adrenal medulla λgt10 cDNA library

(Anderson and Axel, 1985). Approximately 50,000 clones were screened at high stringency. Positives were picked, the phage grown up and insert DNA isolated by restriction with EcoRI. These isolated inserts in turn were radiolabeled and used to probe Northern blots of adrenal medullary RNA to confirm the identity of the clone (see Figure 5). RNA isolation, Northern blotting and hybridization with single-stranded cRNA probes prepared by SP6 in vitro transcription were all performed as described previously (Anderson and Axel, 1985). For the developmental Northern blot series, embryos were designated as 0.5 days of gestation the morning a plug was detected following a midnight breeding. Fluorescence-activated cell sorting and immunohistochemical techniques were carried out as detailed elsewhere (Anderson and Axel, 1986; Stein et al, 1988b).

RESULTS

Sympathoadrenal progenitors in the E14.5 adrenal gland are antigenically-distinct from their ganglionic counterparts.

In an earlier study, we identified a monoclonal antibody, called B2, which transiently labels developing neurons in the sympathetic ganglion (Figure 3C, arrowhead.). Although this cell-surface marker is expressed by a subset of cells within the adrenal gland (Anderson and Axel, 1986), it is absent from the majority of TH-positive cells in the gland in the same histological section (Figure 3A, small arrow; compare with C). That $B2^+$ cells are advanced in neuronal differentiation is indicated by the appearance of these cells immediately following their isolating by sorting: these cells exhibit a strikingly more neuronal morphology (Figure 4C,D) than their $B2^-$ adrenal counterparts (Figure 4A,B.) Similarly, progenitor cells which migrate into the adrenal gland at E14.5 extinguish expression of SCG10 (Figure 3B), a neural-abundant vesicle protein (Stein et al, 1988b) which is first expressed at E11.5; in contrast cells which remain in the ganglion sustain and up-regulate SCG10 expression (Figure 3B, arrowhead.) These data imply that progenitors which migrate into the adrenal gland are *inhibited* from undergoing neuronal differentiation. More recent data indicate that in addition to this apparent inhibitory influence of the adrenal microenvironment, this environment *maintains* the expression of some newly-defined chromaffin-specific antigens, which initially appear in progenitors at E11.5 but which are lost by ganglionic cells beginning on E13.5 - E14.5 (Anderson, Carnahan and Patterson, unpublished observations.) *In vitro*, the persistence of these

antigens depends upon the presence of dex (Carnahan and Patterson, personal communication.)

Taken together, these more recent data suggest that developing chromaffin cells become phenotypically distinct from developing sympathetic neurons at least two to three days before the initial appearance of PNMT. The ability of synthetic GC to promote these phenotypic distinctions *in vitro* in progenitor cells which do not yet express PNMT (Anderson and Axel, 1986; Carnahan and Patterson, personal communication) suggests that GC are the adrenal environmental determinant responsible for these early differences. To begin to examine this issue at the molecular level, we have cloned a rat PNMT cDNA and used it to study the relationship between expression of PNMT mRNA and that of the GC receptor mRNA.

Isolation of a rat PNMT cDNA

We cloned a rat PNMT cDNA by screening an adult adrenal medulla cDNA library (Anderson and Axel, 1985) with a bovine PNMT cDNA probe (Batter et. al., 1987). Hybridization of this rat cDNA probe to total adult adrenal medulla RNA revealed an intense band of approximately 1100 nucleotides (Figure 5B). An identically-sized band was revealed using the bovine probe (Figure 5A), confirming the identity of the rat clone. However, the intensity of the hybridization signal obtained using the rat probe appeared more than ten-fold greater than that seen with the bovine probe, reflecting species divergence in the nucleotide sequence of the rat and bovine genes (B. Kaplan, personal communication). The size of the rat PNMT cDNA is about 800 bp, indicating that it is not full-length. Nevertheless, it is useful as a hybridization probe to detect specific expression of this gene.

PC12 cells respond to GC, but neither express nor induce PNMT mRNA

In order to determine whether PC12 cells would be a useful model system in which to study the regulation of PNMT gene expression, we performed Northern blot analysis of RNA obtained from this cell line in the presence and absence of 5 μM dex. This experiment revealed that PC12 cells do not detectably express PNMT mRNA (Figure 5, lanes PC12$^-$). We estimate that the sensitivity of our Northern hybridization procedure, using high-specific activity single stranded cRNA probes, is sufficient to permit the detection of PNMT mRNA at an abundance of 1 copy per cell. Moreover, PC12 cells

did not induce PNMT mRNA upon exposure to GC (Figure 5, PC12+) for several days. However, PC12 cells contain GCRs and respond in other ways to GC: they increase expression of TH mRNA (Figure 6B) (Lewis et al, 1983; Leonard et al, 1987), and show a decreased magnitude of SCG10 mRNA induction by NGF (Figure 6A). Leonard et. al. (1987) also identified several other genes which are induced by GC in PC12 cells, as well as several genes whose NGF-induction in these cells is inhibited by GC. Thus, PC12 cells display both positive and negative responses to GC, but neither express nor induce PNMT mRNA.

Expression of GCR mRNA precedes that of PNMT mRNA during adrenal development

We next examined the expression of PNMT mRNA at various stages of development. Total RNA isolated from whole embryonic adrenal glands was analyzed by Northern blotting using a PNMT cRNA probe. In agreement with previously published immunohistochemical data (Bohn et al, 1981; Teitelman et al, 1982), we found that PNMT mRNA was undetectable at E15.5, but was clearly expressed one day later, at E16.5 (Figure 7, PNMT). However, TH mRNA was present at E15.5 and its amount did not change between E15.5 and E16.5 (Figure 7, TH), indicating that the absence of PNMT mRNA at E15.5 was not due simply to the absence of sympathoadrenal lineage cells in the tissue used to prepare RNA. Unfortunately, the small quantities of material available at E14.5 precluded a measurement of PNMT mRNA at this early age. Note that the ratio of PNMT to TH mRNA appears to increase between E15.5 and birth, implying a selective induction of this gene rather than simply proliferation of the PNMT+ cell population.

The availability of a cloned probe for the GCR (Miesfeld et al, 1984) next permitted us to examine the appearance of the receptor mRNA in relationship to that of the PNMT mRNA. The same Northern blot was stripped and rehybridized with a GCR cRNA probe. This experiment revealed the characteristic 7 kb GCR mRNA at all stages of development examined (Figure 7, GCR). However, this band is less readily apparent in the E15.5 sample because the slight RNA degradation in this sample (Figure 7, ACTIN) affects a large mRNA to a greater extent than a smaller one; nevertheless, the presence of RNA hybridizing to the GCR probe in this lane is clear. Therefore, at least some GCR mRNA is present in the embryonic adrenal gland fully one day prior to the appearance of PNMT mRNA.

The apparent degradation of the E15.5 mRNA sample precludes a quantitative comparison of GCR mRNA levels in the adrenal between E15.5 and E16.5; it is possible that some up-regulation of the transcript occurs over this developmental interval when PNMT mRNA is being induced. However, in support of the idea that PNMT expression does not necessarily reflect increased GCR mRNA expression, we observed that there was no large difference in the amount of GCR mRNA between the adrenal medulla and neonatal superior cervical sympathetic ganglion (Figure 7C, SCG), although PNMT mRNA did not appear to be expressed in the ganglion (Figure 7A), consistent with previous immunohistochemical studies (Bohn et al, 1982.) Because NGF inhibits the ability of GC to induce TH mRNA in PC12 cells (Figure 6B; see also Leonard et al, 1987), it was of interest to determine whether this inhibition was due to a depression of GCR mRNA levels. Northern hybridization revealed, however, no significant difference in the levels of GCR mRNA between untreated and NGF-treated PC12 cells (Figure 7, lanes $PC12^-$ and $PC12^+$.)

DISCUSSION

In an effort to determine whether the timing of PNMT expression is likely to be controlled by the time of appearance of the GCR in developing chromaffin cells, we have examined the relative time of appearance of the mRNAs for these two proteins using cloned probes. Our data indicate that GCR mRNA is qualitatively detectable in the embryonic adrenal gland at E15.5, one full day prior to the appearance of PNMT mRNA. Moreover, our results show clearly that progenitor-like sympathoadrenal lineage cells (represented by the PC12 line) can express GCR mRNA and respond in several ways to GC, without either expressing or inducing PNMT mRNA. A similar result was obtained in the sympathetic ganglia. Taken together, these and other data (cf. Figure 3) appear consistent with the hypothesis that embryonic sympathoadrenal progenitors contain GCR and respond to GC several days prior to the appearance of PNMT, and therefore imply that other factors in addition to the presence of GCR may be necessary for the developmental appearance of PNMT expression. For example, expression of the gene could require the appearance of a temporally-regulated *trans*-activator protein, or a change in chromatin structure. Such mechanisms have been suggested to underly the selective regulation of tissue-specific

genes by steroid hormones in other systems (Burch and Weintraub, 1983.)

The conclusions drawn above must, however, be tempered by several important caveats at the present time. First, the mere expression of GCR mRNA does not mean that a functionally active GCR is necessarily present. Second, as the RNA samples analyzed were from whole adrenal glands and not from purified progenitor cells, it is possible that the GCR mRNA detected was contributed by other cells in the gland (eg, cortical cells). (However, the specific activity of the GCR mRNA is virtually identical in adult medulla and embryonic whole adrenal glands (as well as in SCG and PC12 cells), suggesting that most if not all cells in the samples analyzed express GCR mRNA.) Third, the demonstration that the presence of functional GCRs in PC12 is not *sufficient* for expression of PNMT logically does not exclude the possibility that such receptors may nevertheless be *necessary* for PNMT expression. These caveats could account for the difference between the results reported here and the findings of Seidl et al (this volume), who measured no GCR in purified E16.5 chromaffin precursors using a radioligand binding assay, and who showed a dramatic induction of receptors over the next few days.

If, in fact, there are no functional GCRs in chromaffin cells prior to E17.5, other models must be advanced to account for the apparent effects of the adrenal microenvironment on sympathoadrenal progenitor phenotype at E14.5 (Figure 3). One possibility is that prior to E17.5, the main effect of the adrenal microenvironment is to *exclude* inducers of *neuronal* differentiation which may be located in the sympathetic ganglion primordia (eg., see Stemple et al, 1988.) The suppression of neuronal differentiation observed within the adrenal at this early stage would in that case result from the absence of a neural inducer, rather than the presence of an inhibitor (GC). This model would be compatible with an initial unresponsiveness of E14.5 progenitors to GC. A second possibility is that adrenal corticosteroids *do* suppress the neuronal differentiation of progenitors at E14.5, but that the effect of GC is mediated *indirectly,* through some other cell type present in the adrenal. This model would be consistent with our finding of GCR mRNA in the adrenal prior to E17.5. Neither of these two models, then, require that GCRs appear in developing chromaffin cells until E17.5, as suggested by the data of Seidl et al.

Another model that could reconcile our data with those of Seidl et al is one in which PNMT induction does not occur until a *threshhold* concentration of GCR is reached, but that levels of GCR below this threshhold are still sufficient to mediate the earlier apparent effects of steroids on progenitor cells. Studies of other steroid-inducible genes are consistent with the idea that different genes within the same cell may require different levels of steroid receptor to be induced, perhaps due to intrinsic differences in the affinities of these genes for the steroid-receptor complex (Barton and Shapiro, 1988.) In this respect, it is of interest that the bovine PNMT gene contains five consensus Glucocorticoid-Responsive Elements (GREs) (Batter, et. al., 1988); perhaps all of these sites must be occupied by the hormone-receptor complex in order for expression of the gene to occur. The observation of Seidl et. al. that the rise in functional GCR occurs during the same period in which corticosterone synthesis is rising is, furthermore, consistent with the idea that the steroid may induce its own receptor. The delay in PNMT appearance could, then, reflect the time required for GCR levels to be induced above a threshhold level necessary for activation of PNMT transcription. Our present data do not rule out a rise in GCR mRNA levels during adrenal development. It is also possible that the rise in steroid-binding activity observed by Seidl et al occurs by a post-transcriptional activation of the receptor. Precedent for an autoinduction of steroid receptors exists in other systems (Barton and Shapiro, 1988.). A self-reinforcing action of GC on chromaffin differentiation would account for other features of the chromaffin-neuron developmental decision, as discussed elsewhere (Anderson, 1989).

Thus, the data presented here unfortunately do not settle the issue, but (especially when taken together with the results of Seidl et al) make it evident that the regulation of PNMT expression and chromaffin differentiation by GC are likely to be more complex than previously appreciated. However, our data clearly show that the presence of functional GCRs in a sympathoadrenal lineage-derived cell (the PC12 cell) is insufficient to achieve expression of the PNMT gene, and that nevertheless GC is able to exert effects on these cells similar to those observed on early progenitors (ie., suppression of neural-specific gene expression.) In fact, the early inhibition of neuronal differentiation in the developing chromaffin population could actually be *required* for subsequent expression of PNMT. This is because cells differentiating along the neuronal pathway apparently rapidly lose the ability to express PNMT in

response to GC (Bohn et. al., 1982, Anderson and Axel, 1986; Anderson, 1988). Thus, the ability to express PNMT at E16.5 may be the consequence of a cell lineage decision made several days earlier in development. Our data are consistent with the idea that GC is an important environmental determinant of this earlier decision.

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FIGURE LEGENDS

Figure 1.

Schematic indicating the migratory route taken by cells in the sympathoadrenal lineage. The diagram illustrates a section through an E14.5 embryo, with earlier developmental events superimposed. (After Anderson, 1988.)

Figure 2.

A model for sympathoadrenal development. The diagram illustrates the changes in cellular morphologies and factor-responsiveness thought to occur at different stages in the lineage. The cells labeled "primitive neuron" correspond to the B2+ cells (see Figure 4.) These cells are still mitotic. (From Anderson and Axel, 1986).

Figure 3.

Differential expression of neural-specific markers in the E14.5 sympathetic ganglion and adrenal gland. (A) The overall distribution of sympathoadrenal progenitors in the adrenal gland and sympathetic ganglion (large arrowhead) is shown by TH staining. *Medial* is to the right. (B,C) nearby sections stained with either anti-SCG10 (B) or B2 (C). Only the region containing the sympathetic ganglion (large arrowhead) and medial portion of the adrenal gland is shown, and the magnification is twice as high as in (A). Note that while the ganglion (arrowhead) is strongly positive for both SCG10 (B) and B2 (C), most of the cells within the adrenal gland are negative (compare to the region bounded on the left by the small arrow in (A)). However, a few scattered SCG10+ cells can be seen, and there is a characteristic cluster of B2+,SCG10+ cells at the extreme medial margin of the adrenal (small arrows in (B) and (C).) This region will later become the extra-adrenal ganglionic complex.

Figure 4.

Sympathoadrenal precursors at two different developmental stages can be isolated by fluorescence-activated cell-sorting with different antibodies. *HNK-1* recognizes a carbohydrate epitope present on the surfaces of all TH+ cell types in the embryonic adrenal gland, but absent from cortical and mesenchymal cells. *B2* recognizes a surface carbohydrate epitope which at E14.5 is

expressed in the embryo exclusively by neuroblasts in the sympathetic ganglia, and by a subset of cells within the adrenal gland. Cells isolated using monoclonal antibody B2 (C,D) have extended long processes by 12-24 hrs after plating. Those isolated with HNK-1 (from the $B2^-$ fraction) are more fully bipotential (A,B), although with time many of these cells begin to extend processes as well (arrow, A). The $B2^+$ cells contain a subpopulation which is less mature and still responsive to dex (arrow, C). (After Anderson, 1988.)

Figure 5.

Identification of a rat PNMT cDNA clone and analysis of PC12 cells. Two identical Northern blots containing the RNA samples indicated (10 µg/lane) were hybridized and washed to high stringency with a bovine PNMT cDNA probe (A) or a newly-isolated rat PNMT cDNA probe (B). Note that the rat probe detects an 1100 bp mRNA identical in size to that seen using the bovine probe. No detectable PNMT mRNA is seen in PC12 cells with or without 5 µM dexamethasone using either probe.

Figure 6.

PC12 cells respond both negatively and positively to glucocorticoid. (A), dex inhibits the ability of NGF to induce the neural-specific SCG10 mRNA. (B) dex induces TH mRNA. (C) control using actin probe to show that equivalent amounts of mRNA were loaded in each lane. (D,E) quantitation of the results shown in (A) and (B). (F), dex inhibits the basal levels of SCG10 expression, independent of its ability to interfere with NGF induction. After Stein et al, 1988a.

Figure 7.

Expression of GCR mRNA precedes that of PNMT mRNA during embryonic adrenal gland development. 10 µg of total RNA from the indicated tissues at various developmental stages were analyzed by Northern blotting. The same blot was hybridized sequentially with the probes indicated to the right. E15.5 -E20.5 and P3 (postnatal day 3) represent RNA isolated from whole adrenal glands; Ad. indicates RNA from adult adrenal medulla. $PC12^-$ and $PC12^+$ indicate RNA from PC12 cells treated without or with 50 ng/ml NGF for 24 hrs, respectively. Note that while no PNMT mRNA is detectable at E15.5, TH mRNA is present at this stage. The ratio of TH mRNA to actin

mRNA is comparable between E15.5 and E17.5, during a period when PNMT mRNA is increasing. (C) Arrowhead indicates the 7 kb GCR mRNA; *upper* and *lower* arrows indicate the 28*S* and 18*S* rRNAs, respectively. Some degradation of the E15.5 RNA sample has occurred (see also D), accounting for the smear of hybridization signal obtained with the GCR probe. Nevertheless the 7 kb band can be detected in this lane. Note the presence of GCR and TH mRNAs in the P3 Superior Cervical Ganglion (P3), but the absence of PNMT mRNA.

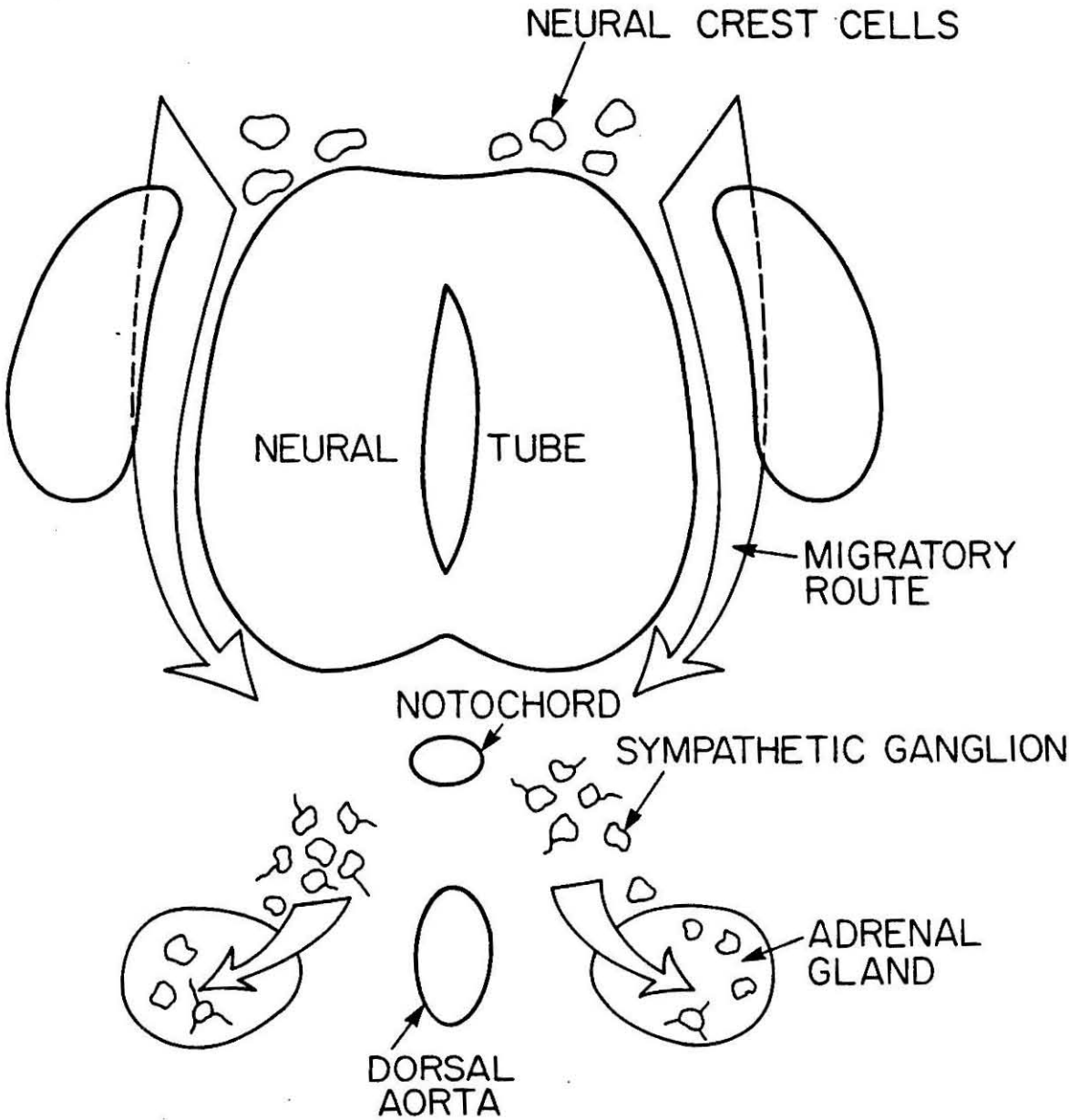
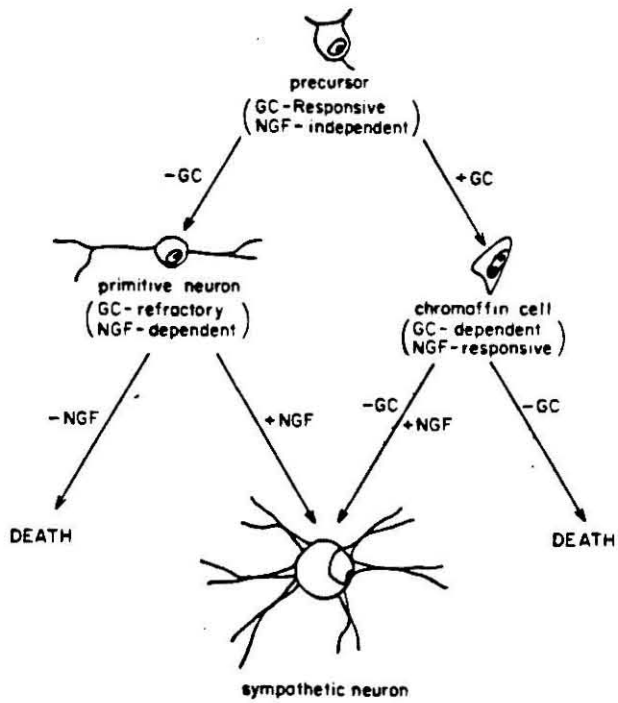

NEURAL CREST CELLS
NEURAL TUBE
MIGRATORY ROUTE
NOTOCHORD
SYMPATHETIC GANGLION
ADRENAL GLAND
DORSAL AORTA

Fig. 1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NGF-independent)
-GC
+GC
primitive neuron
(GC-refractory
NGF-dependent)
chromaffin cell
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NGF-responsive)
-NGF
+NGF
-GC
+NGF
-GC
DEATH
DEATH
sympathetic neuron

Fig. 2

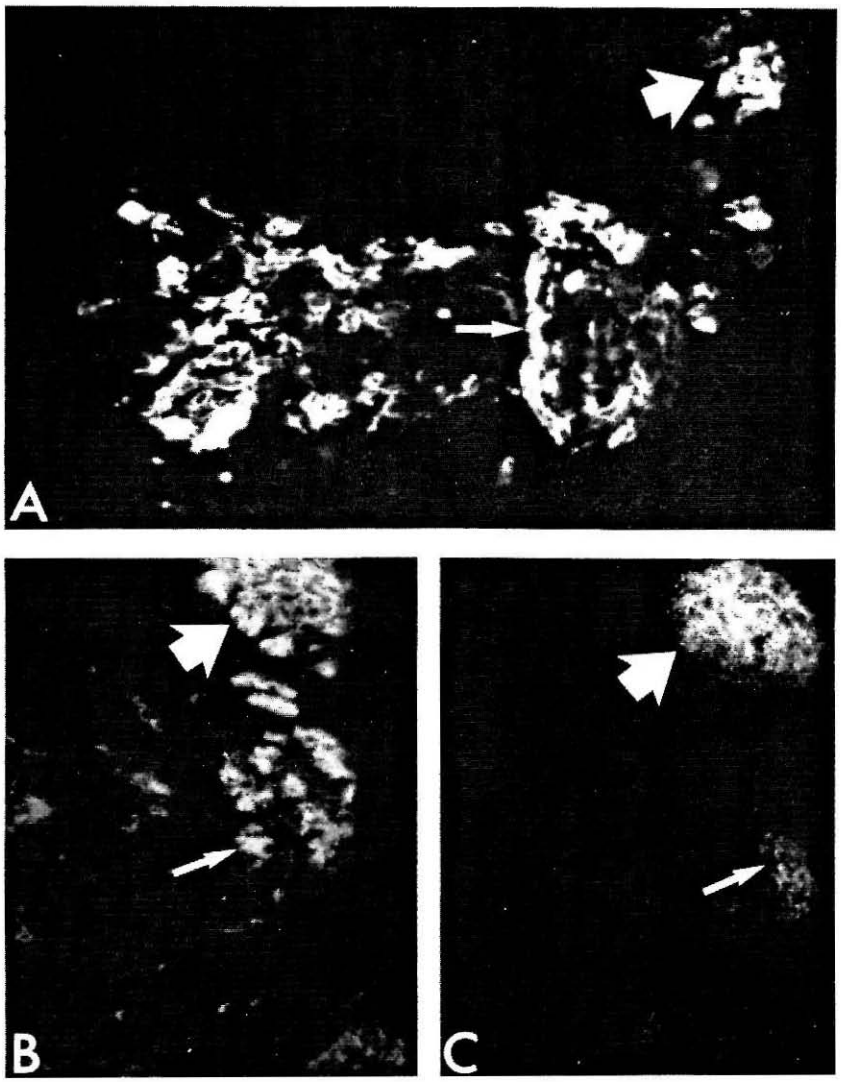


Fig. 3

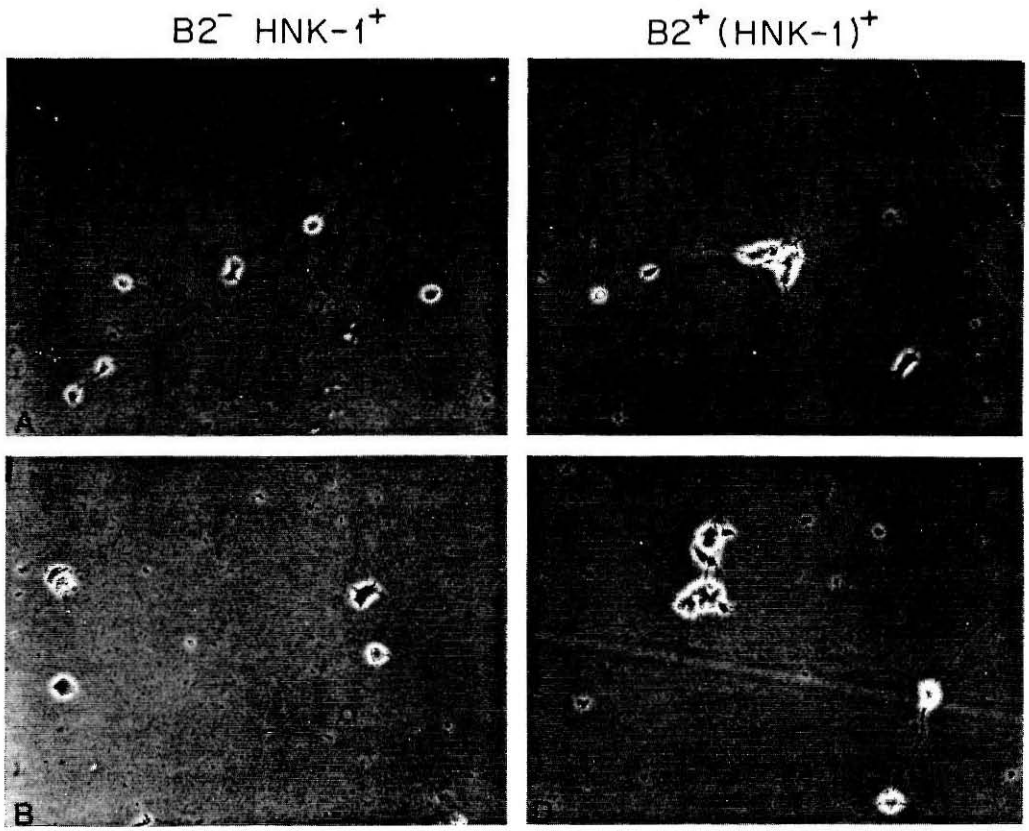


Fig. 4

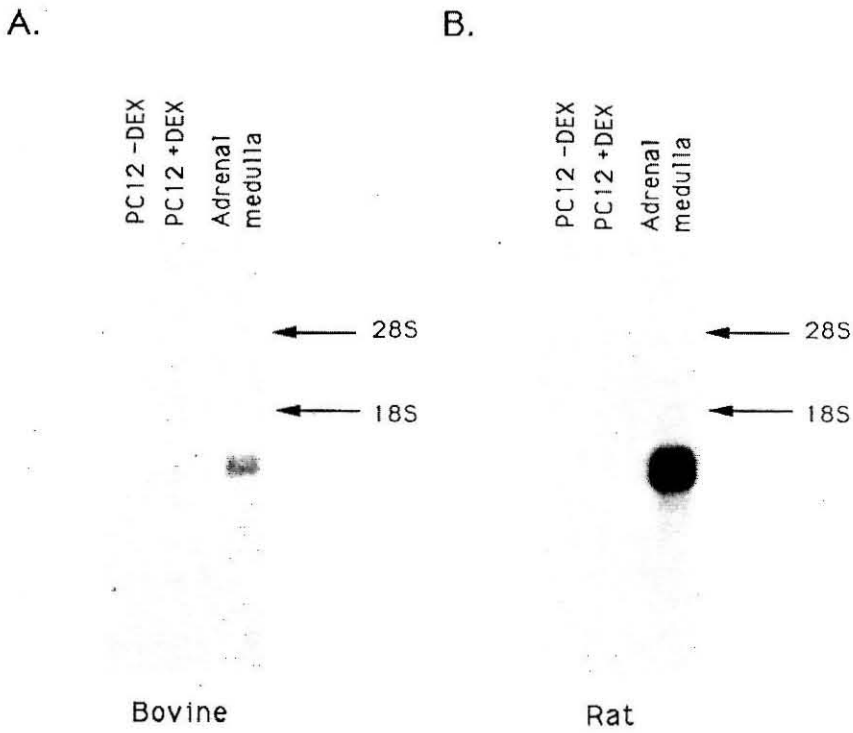
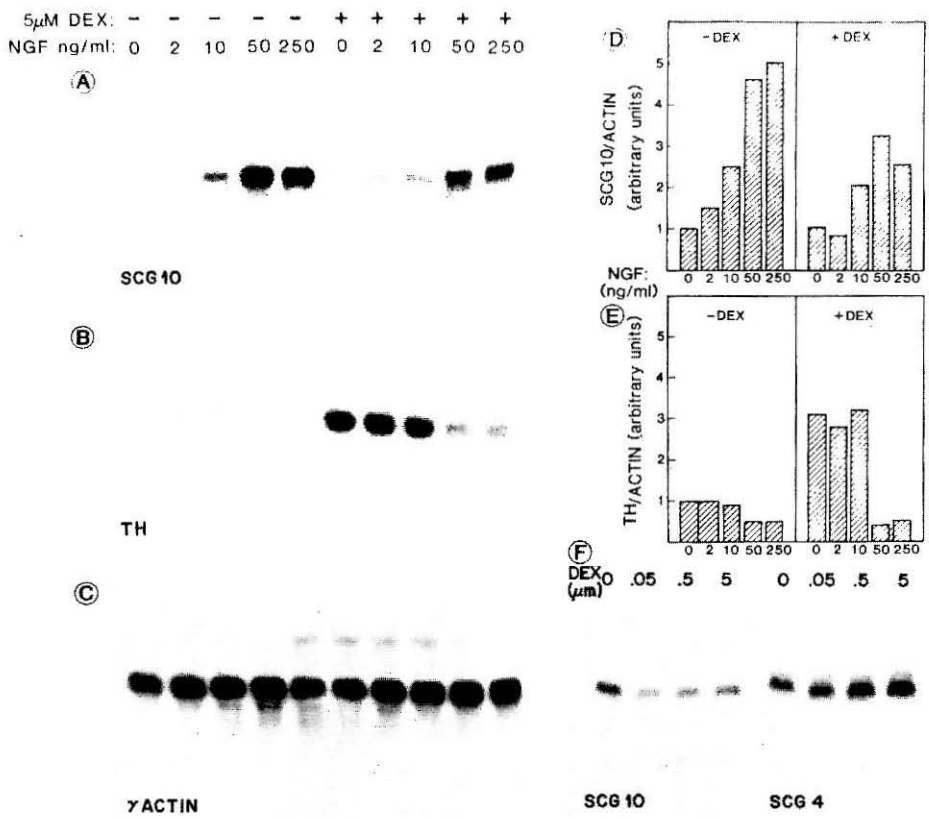

A.
B.
PC12 -DEX
PC12 +DEX
Adrenal medulla
28S
18S
Bovine
PC12 -DEX
PC12 +DEX
Adrenal medulla
28S
18S
Rat

Fig. 5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NGF ng/ml: 0 2 10 50 250 0 2 10 50 250
A
SCG10
B
TH
C
γACTIN
D
-DEX
+DEX
SCG10/ACTIN (arbitrary units)
NGF: (ng/ml)
0 2 10 50 250 0 2 10 50 250
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SCG10
SCG4

Fig. 6

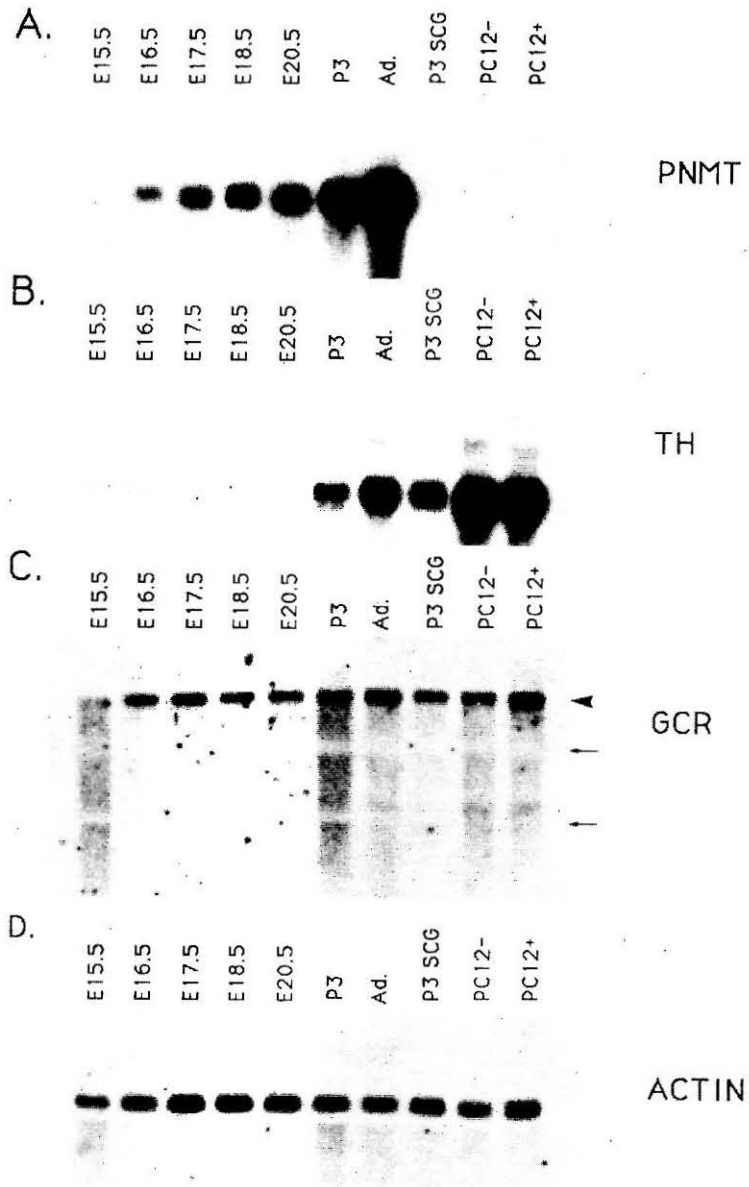
A.
E15.5
E16.5
E17.5
E18.5
E20.5
P3
Ad.
P3 SCG
PC12-
PC12+
PNMT
B.
TH
C.
GCR
D.
ACTIN

Fig. 7

APPENDIX III

Antibody Markers Identify a Common Progenitor to Sympathetic Neurons and Chromaffin Cells *in vivo*, and Reveal the Timing of Commitment to Neuronal Differentiation in the Sympathoadrenal Lineage

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ABSTRACT

Using specific antibody markers and double-label immunofluorescence microscopy, we have followed the fate of progenitor cells in the sympathoadrenal sublineage of the neural crest, in developing rat embryos. Such progenitors are first recognizable in the primordial sympathetic ganglia at embryonic day (E) 11.5, when they express tyrosine hydroxylase (TH). At this stage, the progenitors also co-express neuronal markers such as SCG10 and neurofilament, together with a series of chromaffin cell markers called SA1-5 (Carnahan and Patterson, 1991a.) The observation of such doubly-labeled cells is consistent with the hypothesis that these cells represent a common progenitor to sympathetic neurons and adrenal chromaffin cells. Subsequent to E11.5, expression of the chromaffin markers is extinguished in the sympathetic ganglia, but retained by cells within the adrenal gland. Concomitant with the loss of the SA1-5 immunoreactivity, a later sympathetic neuron-specific marker, B2, appears in sympathetic ganglia. In dissociated cell suspensions, some B2$^+$ cells that co-express SA1 are seen. This implies a switch in the antigenic phenotype of developing sympathetic neurons, rather than a replacement of one cell population by another. The SA1➔B2 transition does not occur for the majority of cells within the adrenal primordium. *In vitro*, most B2$^+$ cells fail to differentiate into chromaffin cells in response to glucocorticoid. Instead, they continue to extend neurites, and then die. Taken together, these data imply that the SA1➔B2 transition correlates with a loss of competence to respond to an inducer of chromaffin differentiation. Thus, the development of sympathoadrenal

derivatives is controlled both by environmental signals, and by changes in the ability of differentiating cells to respond to such signals.

An important problem in developmental neurobiology concerns the mechanism of diversification of the cell types that derive from the neural crest. The dissection of this process has been aided by the use of monoclonal antibodies, which have proven useful tools for the study of neural development (Mirsky, 1982, Reichardt, 1984). Such antibodies can provide molecular markers of differentiation, and can reveal important differences between cells that are otherwise indistinguishable. The preceding paper (Carnahan and Patterson, 1991a) describes the generation and characterization of a novel set of monoclonal antibodies (SA1-5) that, in the postnatal rat, specifically label adrenal chromaffin cells, the major endocrine derivative of the neural crest. These antibodies provide a set of reagents that can be used to identify chromaffin cells and their precursors in developing embryos.

Studies of postnatal (Doupe et al., 1985a; Doupe et al., 1985b; Unsicker et al., 1978) and of embryonic chromaffin cells (Anderson and Axel, 1986, Seidl and Unsicker, 1989a, Seidl and Unsicker, 1989b) have suggested that these adrenal medullary cells derive from a progenitor whose alternate fate is to become a sympathetic neuron. However it has been difficult to establish whether this lineage relationship holds *in vivo*. To address this issue, we have examined in sections of developing rat embryos the pattern of expression of the chromaffin-specific SA antigens in relationship to that of neuron-specific markers. Neuronal markers in this lineage include intracellular proteins such as neurofilament 68 kD (Cochard and Paulin, 1984) and SCG10 (Anderson and Axel, 1985; Stein et al., 1988b), as well as cell surface antigens such as B2 (Anderson, 1988, Anderson and Axel, 1986). Because several of these markers are sequentially expressed in an overlapping manner, the developmental segregation of sympathetic neurons and chromaffin cells from their precursor(s) can be traced *in situ*.

We find that SA1$^+$ cells within the sympathetic ganglion primordium initially co-express SCG10 and TH, suggesting that they have the potential to become either sympathetic neurons or chromaffin cells. Subsequently, SA1 immunoreactivity is lost and B2 immunoreactivity appears in these sympathetic neuroblasts. By contrast, this antigenic switch does not occur for most of the developing adrenal medullary population. Experiments *in vitro* suggest that the SA1➡B2 switch correlates with a loss of competence to respond to glucocorticoids, and commitment to the neuronal pathway of differentiation.

MATERIALS AND METHODS

Immunocytochemistry

Staged embryos were fixed in 4% paraformaldehyde, embedded and sectioned in a cryostat as described (Carnahan and Patterson, 1991a). Double-labeling was performed using an SA1 ascites fluid diluted 1:2000, together with: undiluted B2 hybridoma supernatant ·(a gift from J. Dodd, Columbia University); polyclonal rabbit anti-TH diluted 1:250 (Eugene Tech Inc., NJ); or polyclonal rabbit anti-SCG10 diluted 1:250 (Stein et al., 1988a.) Sections were incubated overnight at 4°C with primary antibody, in the presence of 1% normal goat serum (NGS) and 0.1% Nonidet P 40 (NP40). After thorough washing to remove unbound primary antibody, sections incubated with SA1 plus anti-TH or SA1 plus anti-SCG10 were developed using Rhodamine Goat anti-rabbit IgG at 1:500 plus FITC Goat anti-Mouse IgG at 1:200 (TAGO, Inc., Burlingame, CA). Sections incubated with SA1 plus B2 were developed using Rhodamine Goat anti-Mouse IgM (TAGO) and FITC Goat anti-Mouse IgG (Southern Biotech, Inc., Atlanta, GA), both at 1:100. To reduce nonspecific binding, dilutions of secondary antibody were pre-cleared by incubating for 1 hr at 4°C in 10% normal rat serum, 5% NGS, and then centrifuged for 10 min. at 14,000 xg. Controls lacking primary antibody exhibited no

specific staining. Sections were mounted in para-phenylenediamine/glycerol and examined in an Olympus OMT-2 inverted microscope. All micrographs in this paper illustrating SA1 vs. B2 staining were photographed and printed using identical exposure times, so that staining intensity is directly comparable between the figures. For the detection of PNMT immunoreactivity, a rabbit anti-PNMT antiserum (the generous gift of Dr. Martha Bohn) was used at a dilution of 1:1000, and visualized using an FITC-conjugated Goat anti-rabbit IgG secondary antibody (TAGO) at 1:200.

Isolation and culture of $B2^+$ and $B2^-$ cells by FACS

$B2^+$ cells were isolated from dissociated suspensions of E14.5 adrenal glands by surface labeling and FACS as previously described (Anderson and Axel, 1986). $B2^-$ cells were isolated using monoclonal antibody HNK-1 (Abo and Balch, 1981), which labels all TH^+ cells within the E14.5 adrenal gland (Anderson and Axel, 1986; Birren and Anderson, 1990). We used HNK-1 rather than the SA(1,2&4) cocktail (Carnahan and Patterson, 1991b) to isolate $B2^-$ progenitors, because the SA antigens are poorly expressed on the cell surface in E14.5 adrenal suspensions (J. Carnahan, unpublished observations). As all $B2^+$ cells are also HNK-1^+, however, in order to isolate the $B2^-$ fraction, the $B2^+$ cells first had to be eliminated by complement lysis using monoclonal antibody B2. The remaining $B2^-$ cells were then labeled with HNK-1 ascites diluted 1:500 and FITC Goat anti-Mouse IgM (TAGO). Virtually all HNK-1^+, $B2^-$ cells are TH^+ and over 80% are $SA1^+$, indicating that HNK-1 (in combination with B2 elimination) is an sympathoadrenal lineage surface marker comparable to the SA antibodies, for the E14.5 adrenal gland. $B2^+$ and HNK-1^+ ($B2^-$) cells were separated from unlabeled cells by FACS using an Ortho instrument (Ortho Diagnostics, Inc.) Approximately 1-2% of the input cells were $B2^+$ while 6-7% were $B2^-$, HNK-1^+. Cells were cultured on a collagen/poly-D-lysine/laminin substratum in steroid-stripped L15-CO_2 complete medium as previously described (Anderson and Axel, 1986), except that fetal *calf serum* was

substituted for rat serum. When included, dexamethasone was diluted from a stock solution of 10^{-3} M in 95% ethanol, to a final concentration of 1 μM. For quantifying the inhibition of process outgrowth, a cell cluster was defined as "process-bearing" if any cytoplasmic extension could be detected originating from the cluster. It was not possible to determine whether every cell in a cluster was process-bearing, because the tight clumping of the cells precluded assignment of processes to individual cells. Nonetheless, for the purposes of this study this measurement represents a highly stringent criterion, in that only clusters containing exclusively rounded cells were counted as non-process-bearing. Any systematic error introduced by this method would, if anything, underestimate the difference in process outgrowth between $B2^-$ and $B2^+$ cells. For single-cell tracking experiments, cells were identified 24 hrs after plating and their positions marked by means of a gridded coordinate system embossed in the bottom of the dish using a BB press. Cells were photographed at that time and every 24 hrs thereafter for the next 2 days.

RESULTS

Transient co-expression of neuronal- and chromaffin-specific markers by cells in embryonic sympathetic ganglia

In the postnatal rat, the SA1-5 series of monoclonal antibodies specifically labels adrenal chromaffin cells but not sympathetic neurons (Carnahan and Patterson, 1991a). In developing embryos, however, expression of the SA1 antigen is detectable in cells of the primordial sympathetic ganglia, beginning at E11.5 (Figure 1A; see also Carnahan and Patterson, 1991a). At this time, neural crest cells have just aggregated in clusters adjacent to the dorsal aorta, and can be recognized by their expression of tyrosine hydroxylase (TH), a lineage marker for adrenergic neural crest derivatives (Cochard et al., 1979). Double-labeling indicates that all SA1$^+$ cells appear to co-express TH, and vice-versa (Fig. 1B). No expression of SA1 can be detected in premigratory or in migrating neural crest cells one day earlier in development (data not shown).

The initial expression of SA1 coincides not only with that of TH, but also with that of neural-specific markers such as SCG10 (Anderson and Axel, 1985; Stein et al., 1988b) and neurofilament 68 Kd subunit (NF68) (Cochard and Paulin, 1984). To determine whether such neuronal markers are co-expressed in the same cells as express SA1, double-label immunohistochemistry was performed using a rabbit anti-SCG10 antibody (Stein et al., 1988b), mouse monoclonal SA1 antibody and species-specific secondary antibodies. Within the ganglionic cluster, SCG10 (Figure 1D) is expressed in an overall pattern that is co-extensive with that of SA1 (Figure 1C). At higher magnification (Figure 1, E,F), individual cells labeled by both anti-SCG10 and SA1 are visible; many of these cells are process-bearing (1E,F; arrows). It is difficult to determine whether all SA1$^+$ cells are also SCG10$^+$, however, because the SCG10 antigen is membrane bound (Stein et al., 1988b) and concentrated in the perinuclear region (Figure 1D, arrow) whereas the SA1 antigen exhibits a punctate, cytoplasmic *distribution* (Fig.

1C,arrowhead). Nevertheless a substantial number of double-positive cells can be observed. For technical reasons, double-labeling with monoclonal SA1 and NF68 antibodies was not possible. However, the timing of TH and NF68 appearance are coincident (Cochard et al., 1979; Cochard and Paulin, 1984), and TH and NF68 are co-expressed in sympathoadrenal precursors (Anderson and Axel, 1986). It is therefore likely that SA1$^+$ ganglionic cells co-express NF68 as well as SCG10.

The co-expression of SA1, TH and SCG10 in individual ganglionic cells persists through E12.5 (Figure 2A,B,C,D). After this time, the expression of SA1 in the sympathetic ganglia begins to decline (see also Carnahan and Patterson, 1991a). However expression of SCG10, TH and NF is maintained, as previously described (Anderson and Axel, 1986, Cochard et al., 1979; Cochard and Paulin, 1984).

Cells in sympathetic ganglia begin to express B2 coincident with the loss of SA1

Previously, monoclonal antibody B2 (J. Dodd, unpublished) was shown to specifically label a population of neuronal precursors in E14.5 sympathetic ganglia, as well as a subset of cells within the adrenal gland (Anderson and Axel, 1986). As B2, like the SA antibodies, appears to be highly specific for cells of the sympathoadrenal lineage, it was of interest to determine the relative timing of expression of these two markers. In contrast to SA1, no staining with B2 can be detected in sympathetic ganglia, or elsewhere in the embryo, at E11.5 (not shown). At E12.5, faint patches of punctate B2 staining can occasionally be seen (Fig. 2F). These patches overlap some of the SA1$^+$ cells (Figure 2E). Over the next few days of development (E13.5 - E15.5), the intensity of B2 expression in the sympathetic ganglia increases, while that of SA1 decreases (compare exposure-matched Figures 2F, 3B and 4B), until SA1 expression appears completely replaced by that of B2.

The increase in B2 expression appears to occur in a rostrocaudal gradient, with more anteriorly-located ganglia (Figure 3B) expressing higher levels than those located more posteriorly (Figure 3D). SA1 staining does not appear to fade in a complementary rostro-caudal manner, however (Figure 3A,C). Within "transitional" sympathetic ganglia at E14.5, a complementary pattern of SA1 and B2 expression is seen: regions of a given ganglion that are high in SA1 (Figure 4A, arrow) are low in B2 (Figure 4B, arrow); conversely regions that are high in B2 are low in SA1 (Figure 4A,B). However the overall domains of SA1 and B2 expression in such ganglia appear roughly co-extensive. By E15.5-16.5, however, virtually all detectable SA1 staining has disappeared, and the ganglia are uniformly strongly $B2^+$. Taken together, these data indicate that expression of B2 occurs subsequent to that of SA1 during sympathoadrenal development, but that there is a period of transient overlap between these two markers.

The SA1➡ B2 transition does not occur in the adrenal medulla

Chromaffin cells derive from sympathoadrenal progenitors that continue migrating from the vicinity of the sympathetic ganglia to invade the adrenal gland primordium. This invasion begins between E13.5 - E14.5 (Bohn et al., 1981; Teitelman et al., 1982). These invading progenitors express SA1 (not shown). In contrast to their counterparts in the sympathetic ganglia, however, they appear for the most part to maintain expression of this antigen after settling within the adrenal (Figure 4C). For example, at E14.5, many cells expressing high levels of SA1 can be observed deep within the adrenal gland (Figure 4C,E; arrowhead), whereas in the adjacent sympathetic ganglion virtually no SA1 staining is detectable (Fig. 4C, arrow). Conversely, the sympathetic ganglia exhibit a high level of B2 labeling (Fig. 4D, arrow), whereas only faint B2 staining is detected within the adrenal gland. In some sections, areas of intense B2 staining could be observed within the adrenal primordium (Fig. 4F, arrowhead.) However, these regions correspond to the future extra-adrenal ganglionic complex (EAGC) (Lempinen, 1964), a structure which

segregates from the adrenal gland proper, and eventually degenerates towards the end of gestation (Aloe and Levi-Montalcini, 1979).

Cells classified as "chromaffin cells" by histochemical stains have been identified in the embryo in locations outside of the adrenal gland (Lempinen, 1964). Such "extra-adrenal chromaffin cells" also appear to undergo the SA1 ➡ B2 transition, like the cells in the sympathetic ganglia but unlike those within the adrenal gland (Figure 5). For example, clusters of cells expressing SA1 can be observed in the para-aortic region (Fig. 5A, arrows) at E14.5. Patches of $B2^+$ cells are present in some, but not all, of these $SA1^+$ para-aortic clusters (Fig. 5B, arrows). At higher magnification, the domains of B2 and SA1 staining appear to be partially overlapping (Fig. 5C,D; arrowhead), similar to the patterns in the transitional sympathetic ganglia (see above).

The appearance of B2 correlates with a loss of competence for chromaffin differentiation

The foregoing data indicated that neuroblasts in the sympathetic ganglia undergo a developmental change beginning on E13.5, in which they gradually extinguish expression of SA1 and acquire expression of B2. By contrast, this change does not appear to occur for the majority of cells that migrate to the adrenal gland. In order to determine whether this change in antigenic phenotype reflects changes in the developmental properties of sympathoadrenal progenitors, we studied in culture the behavior of $B2^+$ and $B2^-$ cell populations isolated from E14.5 adrenal glands. The adrenal gland was chosen for this experiment, because previous studies had indicated that, in addition to the majority $B2^-$ chromaffin precursor population, a subset of $B2^+$ cells is present (Anderson and Axel, 1986; see also Fig. 4F); thus, both populations can be obtained from the same tissue. After isolating the $B2^+$ cells, the $B2^-$ TH^+ cells were separated from non-sympathoadrenal $B2^-$ cells using monoclonal antibody HNK-1 (see Materials and Methods). This antibody

identifies sympathoadrenal lineage cells in the adrenal gland, since all HNK-1$^+$ cells are also TH$^+$ (Birren and Anderson, 1990). The majority (over 80%) of the B2$^-$, HNK-1$^+$ cells are also strongly SA1$^+$, as determined by staining of fixed, permeabilized cells (n=300). By contrast, 81% of freshly-isolated B2$^+$ cells are SA1$^-$. This immunochemical fractionation thus separated the sympathoadrenal progenitor population from the E14.5 adrenal gland into predominantly B2$^+$, SA1$^-$ and B2$^-$, SA1$^+$ cells.

B2$^+$ cells appeared more advanced in their state of neuronal differentiation, and less able to develop into chromaffin cells, than the B2$^-$ SA1$^+$ cells. For example, after 24 hrs in culture, 81% of B2$^+$ cell clusters were process-bearing, whereas only 47% of the B2$^-$ clusters bore neurites (Fig. 6A, No Add). In addition, the processes on B2$^+$ cells tended to be longer, on average, than those on B2$^-$ cells (Anderson, 1988). By day 3, however, 76% of cell clusters in the B2$^-$ population had extended processes (data not shown), indicating that most if not all of these cells are neuronal precursors but that their differentiation lags behind that of the B2$^+$ cells.

The two cell populations also differed significantly in their ability to differentiate into chromaffin cells, as assessed by morphology and antigenic phenotype, in response to the synthetic glucocorticoid dexamethasone (dex). For example, after 24 hrs of culture in 1 μM dex, the majority of B2$^-$ cell clusters exhibited a round, chromaffin morphology: only 13% were process-bearing (Fig. 6A, DEX, hatched bars). By contrast, under these same conditions, over 60% of the B2$^+$ cells bore neurites (Fig. 6A, DEX, solid bars). Thus, dex inhibited process outgrowth 72% in the B2$^-$ population, but only 24% in the B2$^+$ population. Chromaffin differentiation was also assessed by staining cells with antibodies to the chromaffin-specific marker enzyme phenylethanolamine-N-methyltransferase (PNMT). After 3 days of culture in dex, over 80% of the B2$^-$ cells exhibited PNMT immunoreactivity, whereas less than 50% of the B2$^+$ cells expressed PNMT (Fig. 6B). Taken together, these data indicate that the B2$^-$ population is less

advanced in neuronal differentiation, and has a greater capacity for chromaffin differentiation, than the B2$^+$ population.

Although the B2$^+$ population on average is more advanced in neuronal differentiation than the B2$^-$ population, at the single cell level it is heterogeneous. Previous studies indicated that many B2$^+$ cells continue to extend neurites and then die in glucocorticoid, but that a subset of cells can undergo chromaffin differentiation (Anderson and Axel, 1986). Serial observations of identified cells now indicate that these two subpopulations can be identified prospectively, by their different morphologies. We followed the fates of 72 individual B2$^+$ cells in low-density cultures with dex, over a 3 day period. 43 of these cells were initially process-bearing; of those, 86% died over the ensuing culture period (Fig. 7, arrow). The process-bearing cells that survived did not express detectable PNMT (not shown). On the other hand, of the 19 cells that survived and displayed a chromaffin phenotype, 84% derived from precursors initially lacking processes (Fig. 7, arrowhead). (The remaining 16% derived from cells initially bearing short processes.) These data indicate a strong correlation between the initial morphology of B2$^+$ cells and their response to glucocorticoid: cells with short processes or a round morphology are likely to survive and differentiate into chromaffin cells, whereas process-bearing cells are likely to continue to extend neurites, and then die. The response of sympathoadrenal precursors to glucocorticoid can therefore be predicted based upon a combination of cell morphology and antigenic phenotype.

Expression of SA1 and B2 in late gestational embryos

The preceding observations revealed a good correlation between the position of sympathoadrenal lineage cells in the embryo and their antigenic phenotype, around E14.5 - E15.5. Cells that migrate to the adrenal gland maintain expression of SA1 and do not induce B2, whereas cells that migrate to the sympathetic ganglia extinguish expression of SA1 and induce B2. At later stages of gestation, however (ca. E18.5), some violations of

this "rule" were observed. For example, scattered patches of B2$^+$ cells can be observed deep within the adrenal medulla (Fig.8B). Conversely, patches of SA1$^+$ cells can be observed *outside* of the adrenal microenvironment, in the EAGC (Fig. 8C). The EAGC also contains patches of brightly-stained B2$^+$ cells (Fig. 8D, arrow). However, these patches exhibit a complementary, non-overlapping distribution relative to the patches of SA1$^+$ cells (Fig. 8D, arrowheads; compare to 8C). Thus, the mutual exclusivity of high B2 and high SA1 labeling persists, despite the fact that some cells of each phenotype appear in the "inappropriate" location.

The SA1$^+$ and B2$^+$ patches can also be distinguished by their relative levels of TH expression: SA1$^+$ patches are intensely stained with anti-TH antibody (Fig. 8E,F), whereas B2$^+$ patches are more weakly stained (not shown). B2$^+$ patches conversely stain more intensely than SA1$^+$ patches with neuronal markers such as SCG10 and NF68 (not shown.) This pattern of marker expression supports the idea that the B2$^+$ patches contain cells with a more neuronal phenotype than the SA1$^+$ patches.

DISCUSSION

Evidence for a bipotential sympathoadrenal progenitor in vivo

Neural crest cells in the ventro-lateral migration pathway make a late developmental decision between the expression of an endocrine or a neuronal phenotype. *In vitro* studies (Anderson and Axel, 1986; Carnahan and Patterson, 1991b; Doupe et al., 1985b; Seidl and Unsicker, 1989b) have provided evidence that this decision is made by a progenitor cell whose choice of cell fate is determined, at least in part, by environmental factors. However, it has been difficult to verify this lineage relationship *in vivo*, because of the difficulties involved in tracing the fates of migratory cells in the mammalian embryo. In this study, we report the observation that at least some cells in embryonic sympathetic ganglia transiently co-express neural-specific and chromaffin-specific

markers. Cells that remain in the ganglion primordia lose expression of the chromaffin markers (SA1-5) and retain expression of the neuronal markers. Conversely, cells that migrate to the adrenal primordium lose expression of the neuronal markers (Anderson and Axel, 1986; Vogel and Weston, 1990) and retain expression of SA1-5. These data are consistent with the idea that the transiently dual-phenotype cells are bipotential progenitors of chromaffin cells and sympathetic neurons, which selectively repress expression of the neuronal or chromaffin markers according to their final choice of cell fate.

An alternative explanation is that the disappearance of SA1 immunoreactivity from the ganglion primordium at E14.5 is due to the death or emigration of $SA1^+$ cells. This seems unlikely, because initially the $SA1^+$ cells are also TH^+ and $SCG10^+$, and cells with a TH^+, $SCG10^+$ phenotype remain in the ganglia even as SA1 disappears. Moreover, we observe the concomitant appearance of a second marker, B2, in cells which are still weakly $SA1^+$ (see below). Thus, the cells which are initially $SA1^+$ appear to remain in the ganglion primordium and to change their antigenic phenotype, rather than to disappear. Furthermore, the isolation of $SA1^+$ cells from E13.5 sympathetic ganglia has revealed that 90% of these cells initially express neurofilament 140 kD, and that these cells have the capacity to develop either into chromaffin cells or sympathetic neurons, depending upon culture conditions (Carnahan and Patterson, 1991b). Taken together, these data indicate that the $SA1^+$, $SCG10^+$ cells observed in embryonic sympathetic ganglia are likely to be precursors of sympathetic neurons *in vivo*.

The phenomenon of transient marker co-expression followed by mutually-exclusive segregation is reminiscent of several other cell lineages, wherein bi- or multi-potential progenitors have been shown to give rise to closely-related but distinct cell types. In the immune system, for example, $CD4^+$ and $CD8^+$ peripheral T lymphocytes have been shown to derive from a progenitor that is initially double-positive for both of these cell-surface markers (Carbone et al., 1988; Fowlkes et al., 1988). In the pituitary

gland, somatotrophs expressing growth hormone and lactotrophs expressing prolactin are thought to develop from precursors which transiently co-express both hormones (Hoeffler et al., 1985). Finally, in the endocrine pancreas, embryonic islet progenitor cells have been shown to initially co-express peptides, such as insulin and glucagon, which later segregate to different subpopulations of mature endocrine cells (Alpert et al., 1988).

This phenomenon could reflect the fact that the co-expressed genes are regulated, in part, by transcription factors which are common to all cells of a given lineage (Bodner et al., 1988; Ingraham et al., 1988). The later restriction of these markers to different subtypes of cells within the lineage could reflect the superposition of additional regulatory mechanisms, which fine-tune the pattern of gene expression. In the sympathoadrenal lineage, for example, the expression of neuronal markers such as SCG10 can be up-regulated by FGF and NGF (factors which promote neuronal differentiation), and down-regulated by glucocorticoid (which promotes chromaffin differentiation) (Stein et al., 1988a). Conversely, chromaffin-abundant markers such as TH (Fig. 8E,F) are up-regulated by glucocorticoid and down-regulated by NGF (Leonard et al., 1987; Stein et al., 1988a). In this way, a repertoire of lineage-specific gene expression established in progenitor cells by a common transcriptional program may be fine-tuned by differences in local environment, generating further phenotypic diversification.

Developing sympathetic neuroblasts undergo a switch in antigenic phenotype

Our antibody markers revealed a relatively late developmental event in the differentiation of sympathetic ganglionic neuroblasts: as expression of the SA1 antigen declines, the cells induce the expression of a surface antigen recognized by monoclonal antibody B2. At intermediate times, both SA1 and B2 are expressed in the same ganglia, and the pattern of expression of these two markers appears co-extensive. Eventually,

SA1 expression becomes undetectable and the cells in the ganglia uniformly express high levels of B2. This pattern strongly suggests that the SA1➡B2 transition reflects a change in marker expression within a single population of cells, rather than the replacement of one population by another. This interpretation is reinforced by the complementary patterns of SA1 and B2 staining seen within individual transitional ganglia: regions that are intensely $SA1^+$ are weakly $B2^+$, and vice-versa. Such inhomogeneous staining further suggests that the SA1➡B2 switch occurs asynchronously for cells within a given ganglion.

The SA1➡B2 transition provides an indication that the cells of the developing sympathetic ganglia are changing, in a way that is not otherwise detectable *in vivo*. In what way are the cells changing? The fact that expression of SA1 precedes that of B2 suggests that cells expressing the former antigen are at an earlier stage of neuronal differentiation than cells expressing the latter. Consistent with this idea, cells isolated by sorting with a cocktail of SA antibodies have a rounded morphology (Carnahan and Patterson, 1991b), whereas cells isolated by sorting with B2 tend to extend processes shortly after plating (Anderson, 1988). Moreover, most $B2^-$, $SA1^+$ cells can be induced to differentiate into chromaffin cells at high frequency by glucocorticoid (see also Carnahan and Patterson, 1991b). By contrast, process-bearing $B2^+$ cells are refractory to such an induction. As demonstrated previously (Anderson and Axel, 1986), a minority of $B2^+$ cells remain responsive to glucocorticoid, but most of these cells initially exhibit a rounded morphology. The developmental heterogeneity within the adrenal $B2^+$ population is consistent with the fact that 19% of freshly-isolated $B2^+$ cells express SA1. This could reflect the gradual nature of the SA1➡B2 transition, as suggested by the overlapping expression of SA1 and B2 in sections of E14.5 sympathetic ganglia. Nevertheless, cells with a $B2^+$, $SA1^-$ antigenic phenotype and a process-bearing morphology have lost competence to respond to glucocorticoid and appear committed to

neuronal differentiation (Figure 9). The loss of responsiveness to glucocorticoid may serve to "insulate" immature sympathetic neurons from the steep rise in circulating fetal corticosterone levels, which begins on E16.5 (Teitelman et al., 1982). Loss-of-competence is thought to be a feature of inductive processes in other embryonic systems (Grainger and Gurdon, 1989; Gurdon, 1987).

Control of the SA1➡B2 transition

Cells that arrest migration in the sympathetic ganglia extinguish SA1 and induce B2, whereas those that migrate into the adrenal primordium mostly fail to make this switch. Instead, chromaffin cells maintain expression of SA1 into adulthood. This observation suggests that the SA1➡B2 switch may be controlled by factors in the micro-environments encountered by sympathoadrenal progenitors. SA antigen-positive progenitors from sympathetic ganglia reduce levels of SA1 expression in the absence of dexamethasone, but maintain expression in the presence of dex (Carnahan and Patterson, 1991b). Moreover, chromaffin cells from neonatal rats lose SA staining in the presence of NGF and absence of glucocorticoid (Carnahan and Patterson, 1991a). These *in vitro* results suggest that *in vivo*, progenitors within the adrenal primordium may be prevented from undergoing the SA1➡B2 transition by the high local concentration of adrenal corticosteroids. The SA1➡B2 transition may also be promoted by factors which stimulate neuronal differentiation, such as FGF and NGF (Birren and Anderson, 1990; Carnahan and Patterson, 1991b; Stemple et al., 1988).

The maintenance of SA1 and failure to induce B2 are first apparent in the adrenal gland as early as E14.5 (Figure 4), suggesting that glucocorticoids and their receptor are functional at this early stage. mRNA encoding the glucocorticoid receptor has been detected in the adrenal gland as early as E15.5 (Anderson and Michelsohn, 1989). However, using a radioligand binding assay, Seidl and Unsicker (Seidl and Unsicker, 1989b) have reported that glucocorticoid receptor is undetectable in adrenal medullary

precursors prior to E16.5. The reason for this apparent discrepancy is not clear. Chromaffin precursors may contain a low level of receptor that is sufficient to mediate the inhibition of neuronal differentiation; alternatively the SA1→B2 transition may be suppressed in the early adrenal gland by factors other than steroid hormones. However, recent radioimmunoassay data indicate that the E14.5 adrenal gland contains micromolar quantitites of glucocorticoid (A. Michelsohn and D.J. Anderson, in preparation.) Whatever the case, the SA1 and B2 markers provide evidence that progenitors in the adrenal gland are phenotypically distinct from their ganglionic counterparts two days before they express PNMT, the only other marker previously able to distinguish chromaffin cells from sympathetic neurons (Bohn et al., 1981; Teitelman et al., 1982).

The correlation between the antigenic phenotype of sympathoadrenal progenitors and their location in the embryo is not perfect: occasional clusters of $B2^+$ cells can be found within the adrenal gland, and clusters of $SA1^+$ cells are found outside of it, late in gestation. The $B2^+$ clusters probably correspond to clusters of NPY^+ cells observed by Henion and Landis (Henion and Landis, 1990). Similar clusters of cells with a neuronal phenotype have been found within the human fetal adrenal gland as well (Cooper et al., 1990). Our *in vitro* results provide a possible explanation for this phenomenon: these $B2^+$ neuronal cells derive from precursors that have lost competence to respond to glucocorticoid, either prior to or shortly after migration to the adrenal primordium. The majority of these cells disappear by the first postnatal week, probably being eliminated by cell death (Henion and Landis, 1989).

By the same token, at E18.5 some $SA1^+$ cells can be found *outside* of the adrenal environment. Since the SA1→B2 switch appears to occur slowly and asynchronously, these cells may have failed to induce B2 by E16.5, and then have been prevented from doing so subsequently by the surge in fetal GC levels that occurs on E16.5-17.5 (Teitelman et al., 1982). The decision to retain the SA1 antigen may, moreover, become

stabilized with further chromaffin development. Interestingly, the SA1$^+$ cells in the para-aortic and extra-adrenal region are found in clusters, as are the B2$^+$ cells. This suggests that once cells decide whether or not to make the SA1➡B2 switch, they may reinforce their immediate neighbors to do likewise. Such a "community effect" has been demonstrated for the process of mesodermal induction in amphibia (Gurdon, 1988). Alternatively, such clusters could reflect an aggregation (Henion and Landis, 1990) or clonal expansion of phenotypically similar cells.

Using a series of antibody markers, we have defined a sequence of events during the development of the sympathoadrenal lineage in the rat embryo (Fig. 9). Bipotential sympathoadrenal progenitors initially co-express both chromaffin-specific and neuron-specific markers, and then differentiate into neurons or endocrine cells depending upon the environment to which they migrate. Subsequently, additional changes in marker expression are detected: cells in the sympathetic ganglia switch from an SA1$^+$ to a B2$^+$ phenotype, whereas this switch fails to occur for the majority of cells within the adrenal gland. Studies of isolated sympathoadrenal progenitors *in vitro* indicate that this switch correlates with a loss of competence to respond to glucocorticoids, an inducing signal for chromaffin differentiation. Taken together, these data indicate that the development of sympathetic neurons and adrenal chromaffin cells is controlled not only by environmental signals, but also by changes in the ability of cells to respond to such signals. Such changes illustrate one way in which the developmental capacities of initially multipotent cells can become restricted during differentiation.

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FIGURE LEGENDS

Figure 1. Initial expression of SA1 occurs in developing sympathetic ganglia.

Sections are through sympathetic ganglion primordia, at E11.5 (A,B) the same section was doubly-labeled with SA1 (FITC) and TH (Rhodamine). (C,D) the same section was doubly-labeled with SA1 and polyclonal anti-SCG10. Arrows indicate individual doubly-labeled cells. (E,F) higher magnification view illustrating two process-bearing cell bodies (arrows) doubly-labeled by SA1 (F) and SCG10 (E). Arrowheads indicate the process from one cell, which is also doubly-labeled. Asterix in C and D indicates autofluorescent blood cells in the dorsal aorta. The specific staining patterns illustrated were not observed in controls lacking primary antibody. Scale bar = 33 μM.

Figure 2. Co-expression of SA1 with TH and SCG10 in E12.5 sympathetic ganglia.

A,B; C,D; and E,F represent doubly-labeled sections, respectively, stained with the antibodies indicated below the panels. In all cases the fluorochrome used for SA1 was FITC. In (F), a faint patch of punctate B2 staining can be detected in a region containing $SA1^+$ cells (arrow, E and F). However many ganglia showed no detectable staining with B2 at this stage of development. Asterisks indicate autofluorescent blood cells. Scale bar, 33 μM.

Figure 3. Initial appearance of B2 in sympathetic ganglia.

A,B and C,D represent doubly-labeled sections through two different axial levels in the trunk region of an E13.5 embryo. The (A,B) pair lies more anterior (rostral) than the (C,D) pair. Panels A and C were photographed and printed using identical exposure times, as were B and D. Note that the B2 staining in (B) overlaps the region of the

sympathetic ganglion containing SA1$^+$ cells in (A). The B2 staining shows a characteristic punctate appearance. In the more caudally-located ganglion (D) the intensity of B2 staining is much weaker (compare with panel B). Compare the intensity of B2 staining in (B) to that in Fig. 2F, one day earlier in development. Arrowheads (C,D) indicate SA1$^+$ cells which exhibit faint B2 labeling. da, dorsal aorta.

Figure 4. The SA1➡B2 switch occurs in sympathetic ganglia, but not in the adrenal gland.

(A,B) doubly-labeled pair from a section through an E14.5 sympathetic ganglion. Note that the levels of B2 staining by this stage are quite high (compare with exposure-matched panels 2F and 4B,D). Note also that faint SA1 staining in (A) can still be detected in the region of the ganglion which is strongly B2$^+$. Arrow indicates a region which is still strongly SA1$^+$ (A), but weak for B2 (B). (C,D) and (E,F) represent doubly-labeled pairs from two nearby sections through the adrenal gland at E14.5. Arrowhead (C,E) indicates strongly SA1$^+$ chromaffin cells deep within the adrenal medullary region. Note that these same regions are negative or only faintly positive for B2 (D,F). Arrow (C,D) indicates the nearby sympathetic ganglion; note the intense B2 staining (D) and low SA1 staining (C). In (F), arrowhead indicates a patch of B2$^+$ in the region that will become the extra-adrenal ganglionic complex (EAGC); note that this region also contains some brightly SA1$^+$ cells. Photographic exposure times are identical with those in Figs. 4 and 2 (E,F) for SA1 and B2, respectively. Scale bar 63 μM.

Figure 5. Expression of SA1 and B2 by extra-adrenal chromaffin cells in the para-aortic region.

(A,B) and (C,D) represent doubly-labeled pairs from two nearby sections through the lumbar region of an E14.5 embryo, posterior to the region containing the adrenal gland. Arrowheads in (A) indicate the sympathetic ganglia. da, dorsal aorta. Arrows in

(B) indicate patches of B2$^+$ cells in the para-aortic region; some of these patches are also SA1$^+$ in (A). Note, however, a large SA1$^+$ patch in (A) (arrow, left) that is not labeled by B2 in (B). Similar patches in other sections are also labeled by anti-TH and anti-SCG10 (not shown). (C,D) is a higher magnification view of para-aortic cells. Note the large patch of SA1$^+$ cells that is not labeled by B2 (D, arrow), and the intense B2 staining that overlaps the SA1$^+$ region. Scale bar (A,B) 134 μM. (C,D) are four times the magnification of (A,B).

Figure 6. B2$^+$cells show a reduced capacity for chromaffin differentiation compared to B2$^-$ cells.

B2$^+$ and B2$^-$ (HNK-1$^+$) cells were isolated from E14.5 adrenal glands (see Materials and Methods) and cultured with or without 1 μM dexamethasone ("DEX"). (A) After 24 hrs in culture, the population was scored for the percentage of cell clusters bearing processes (see Materials and Methods). Only the TH$^+$ cell population was counted; contaminating flat cells were not included. Note that in DEX, only 13% of B2$^-$ clusters had processes, whereas 61% of B2$^+$ clusters had processes. The method of analysis probably underestimates the difference between B2$^+$ and B2$^-$ cells (see Materials and Methods). (B) After 3 days in culture, the population was fixed and stained with an anti-PNMT antibody, and the percentage of surviving cells expressing PNMT determined.. Time course experiments indicate that most cells that can express PNMT have done so by 3 days of culture in DEX (A. Michelsohn and D. Anderson, unpublished.) Data are the mean ±SEM of 4-6 determinations from two independent experiments.

Figure 7. B2$^+$ cells bearing processes fail to respond to glucocorticoid

Individual B2$^+$ cells were identified 24 hrs.after plating in 1 μM DEX and photographed every 24 hrs for the next two days. Numbers indicate hours of culture.

Arrow indicates an example of a pair of process-bearing cells that failed to respond to DEX. Note at 48 hrs the phase-bright pycnotic figures near the remaining two phase-gray living cells, suggestive of cell division followed by cell death. At 72 hrs only a single pycnotic figure is visible (arrow). Arrowhead indicates an example of a non-process-bearing cell (or cell cluster) that survived and displayed a rounded chromaffin morphology. Antibody staining indicated that cells of this phenotype were $PNMT^+$ (not shown). Note that process-bearing cells were also observed to die in cultures lacking dex (not shown), indicating that the death of these cells is not caused by glucocorticoid. The extent of cell death is greater in the low-density cultures used in these single cell-tracking experiments, than in the mass cultures used in population experiments; therefore the results of the two types of experiments are not quantitatively comparable.

Figure 8. Patches of $B2^+$ and $SA1^+$ cells that violate the positional "rule."

Shown are sections from an E18.5 embryo. (A,B) doubly-labeled pair through the adrenal gland. Note the large patch of $B2^+$ cells (B), which is $SA1^-$ (A). In (A), the SA1 staining is obscured by a high level of autofluorescence, but is clearly visible as punctate labeling at the margin of the $B2^+$ zone. (C,D) doubly-labeled pair through the extra-adrenal ganglionic region. Note the intensely-stained patch of $B2^+$ cells (arrow, D) which is $SA1^-$ (C). Arrowheads in (D) indicate two less intensely-stained $B2^+$ patches, which flank a $SA1^+$ patch (C). (E,F) doubly-labeled pair through an EAGC, illustrating adjacent patches of $SA1^+$, TH^{hi} cells, and $SA1^-$, TH^{lo} cells. The $SA1^-$ patches would be $B2^+$ (as, for example, in C and D, arrowheads.) Scale bar, 62 μM.

Figure 9. Schematic diagram illustrating sequence of antigenic changes in the sympathoadrenal lineage.

The earliest bipotential progenitors are SA1$^+$ and B2$^-$. They co-express neuron-specific markers such as SCG10 and chromaffin-specific markers such as SA1-5, as illustrated by the double cross-hatching. In this study, progenitor cells were isolated from E14.5 adrenal glands using monoclonal antibody HNK-1 in combination with B2-mediated complement lysis. Most but not all of these cells are also SA1$^+$; those cells lacking SA1 may have initiated neuronal differentiation. In the absence of glucocorticoids, these progenitors differentiate along the neuronal pathway, a process promoted by FGF and perhaps other, as yet unidentified factors. Cells that are B2$^+$ and SA1$^-$ and which bear processes have lost competence to respond to glucocorticoid ("committed neuroblast"). For clarity, intermediate stages of development between the bipotential progenitor and committed neuroblast are omitted.

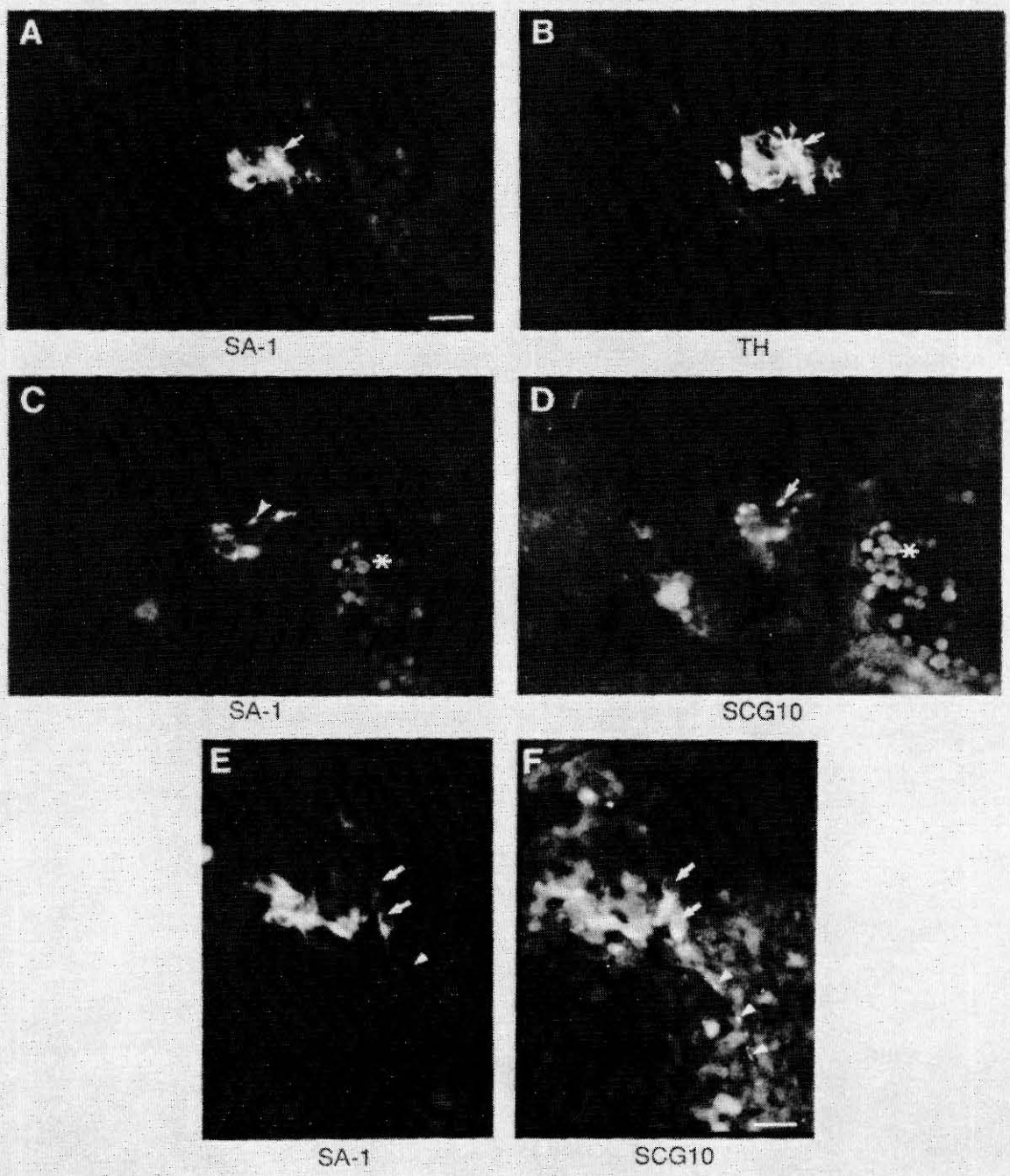


Fig. 1

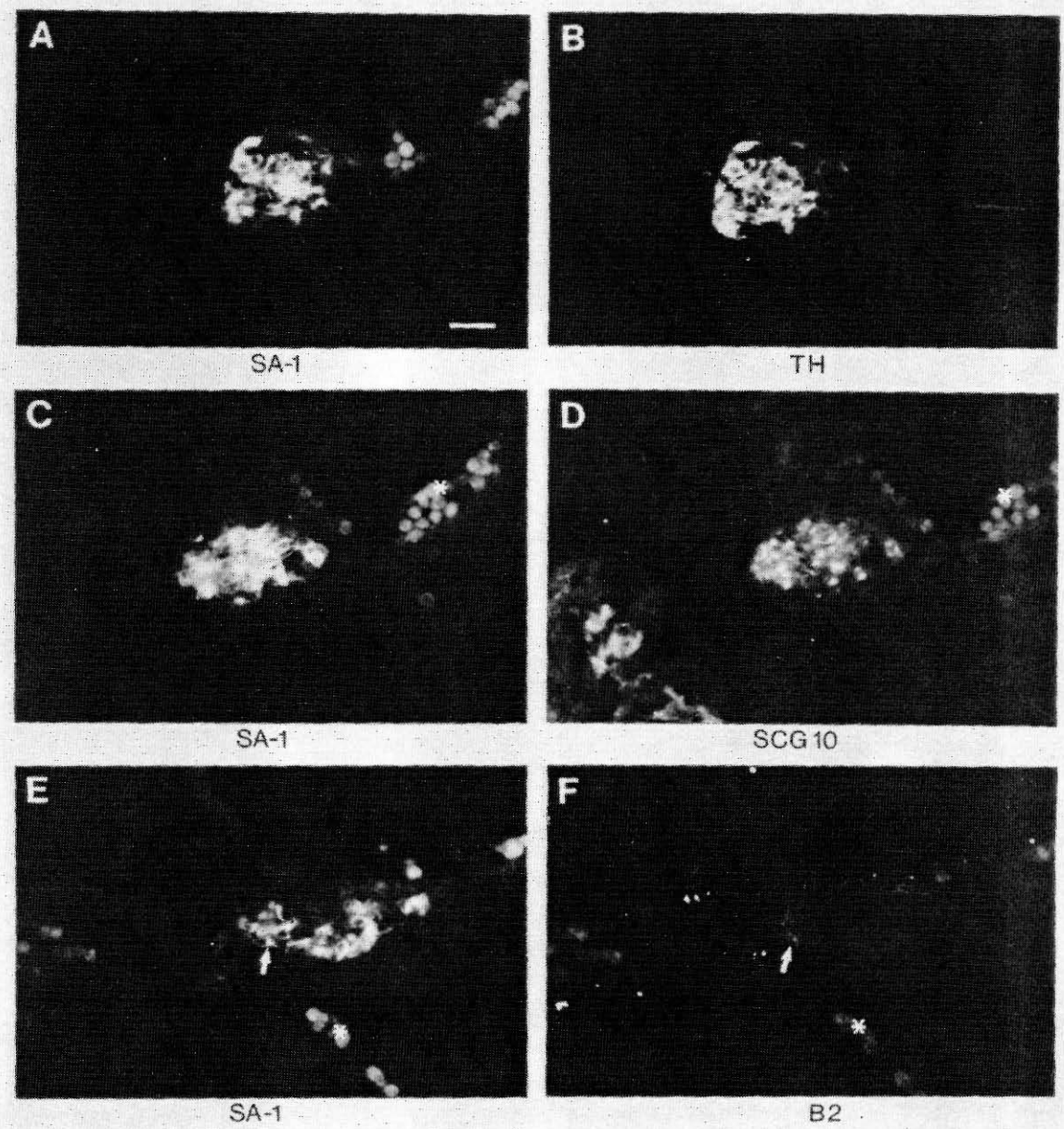


Fig. 2

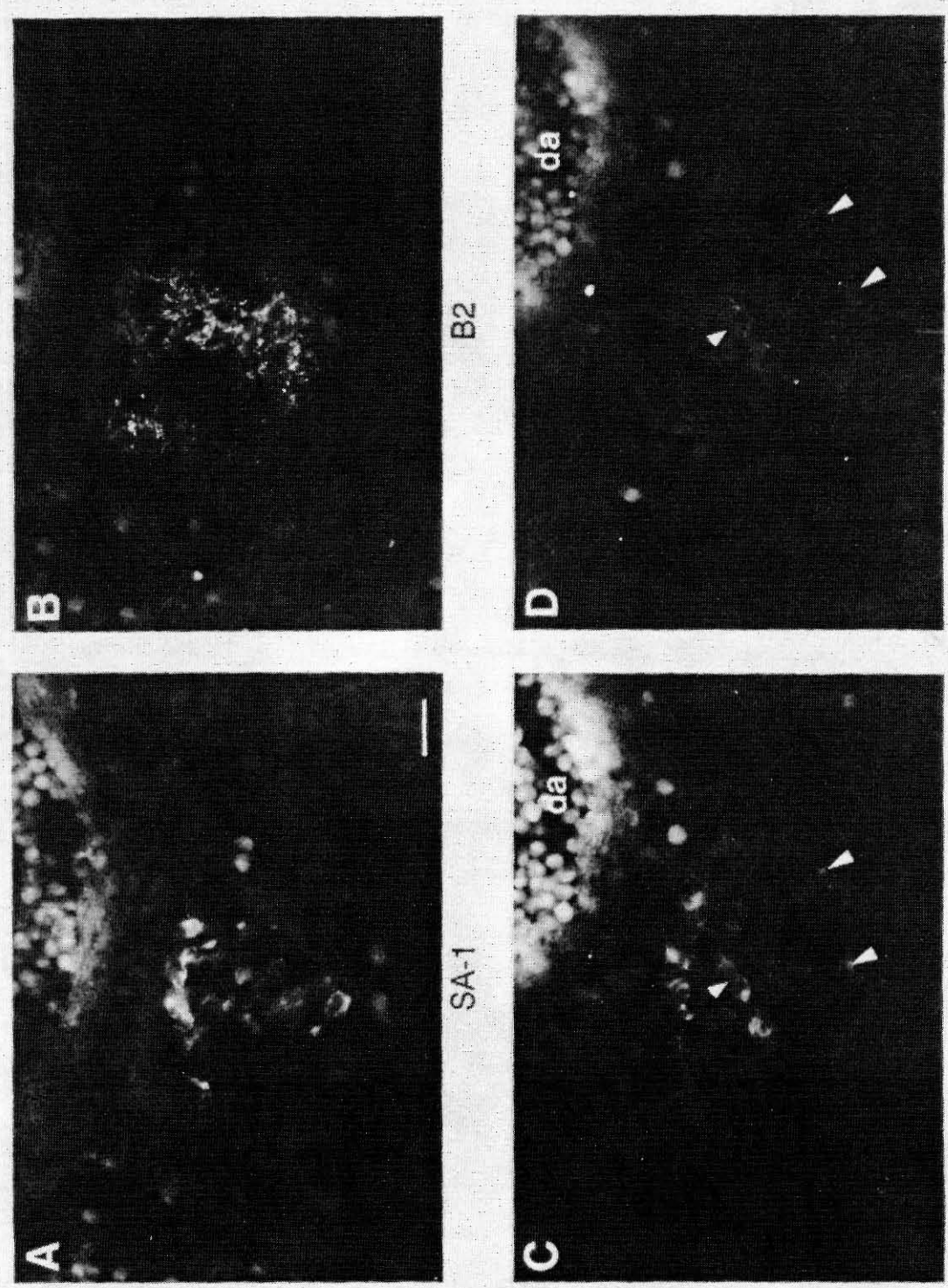


Fig. 3

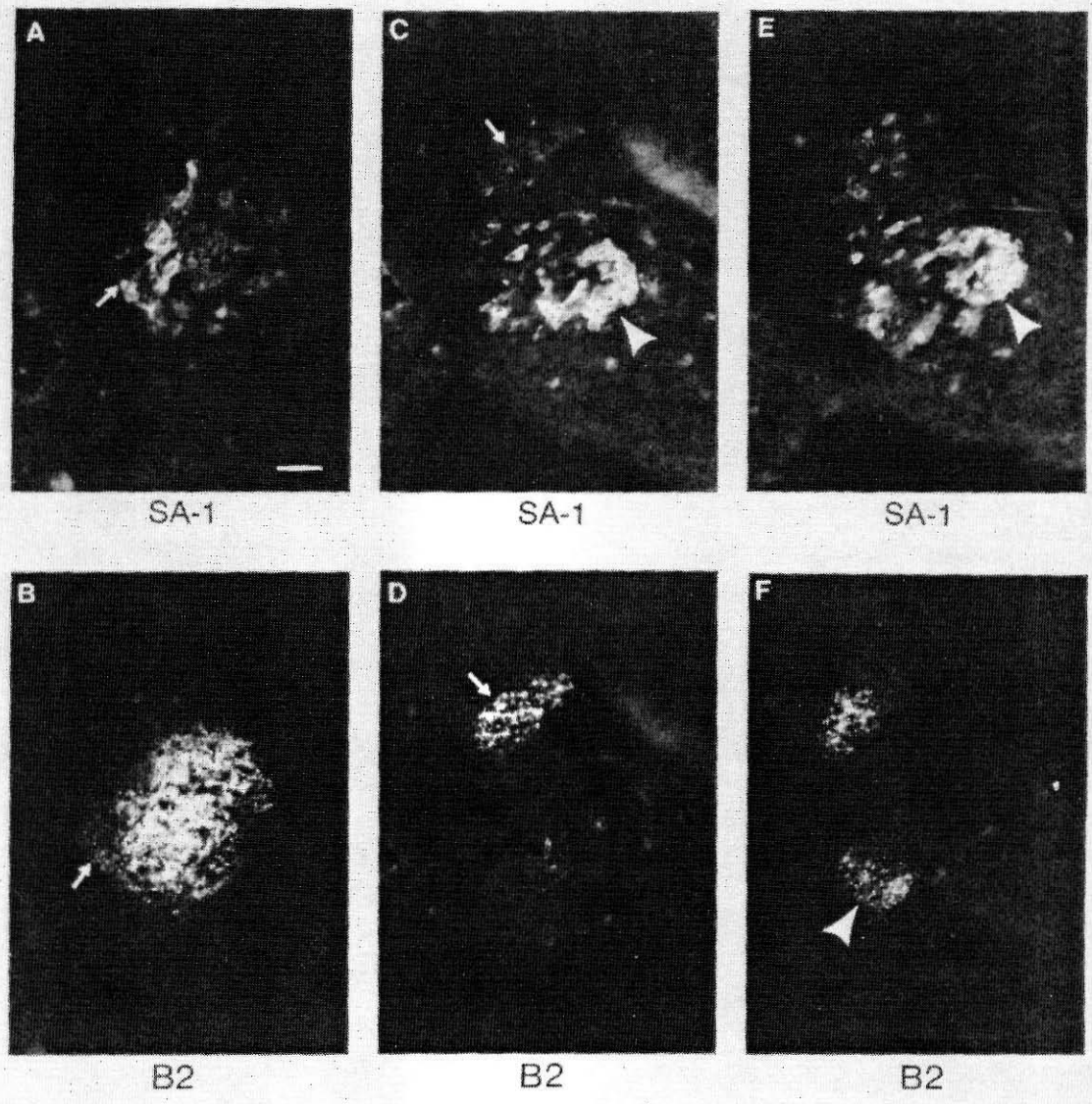


Fig. 4

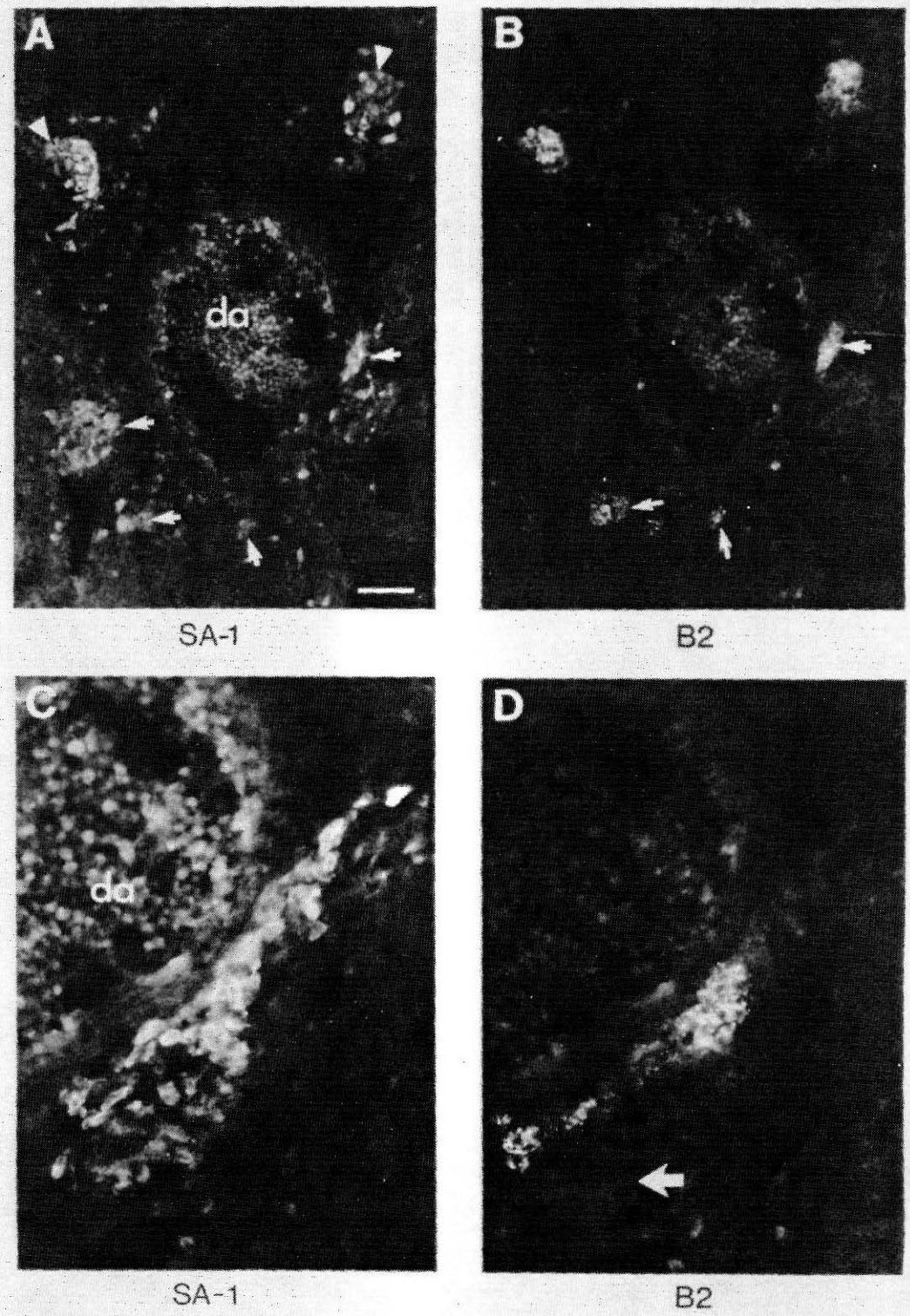
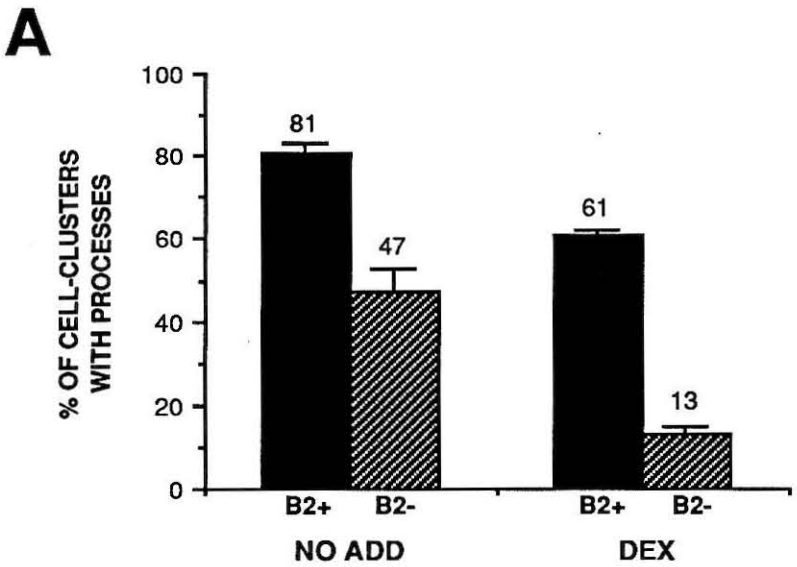


Fig. 5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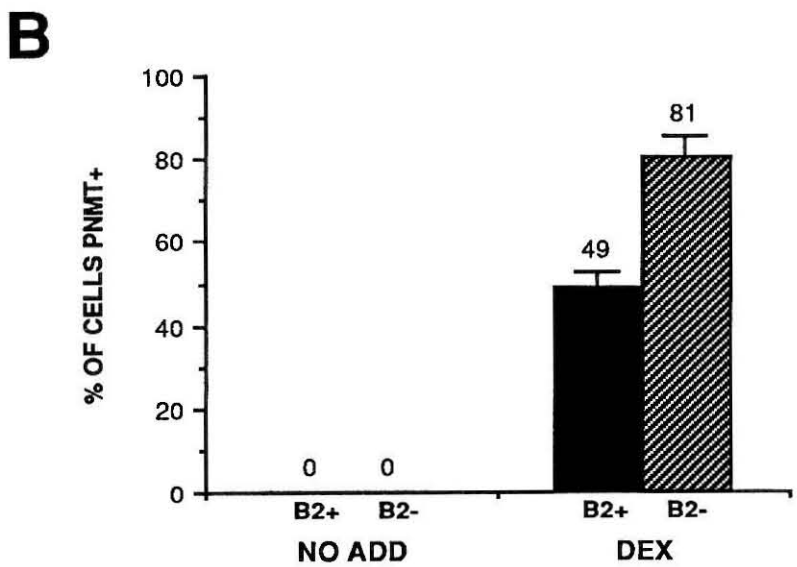
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Fig. 6

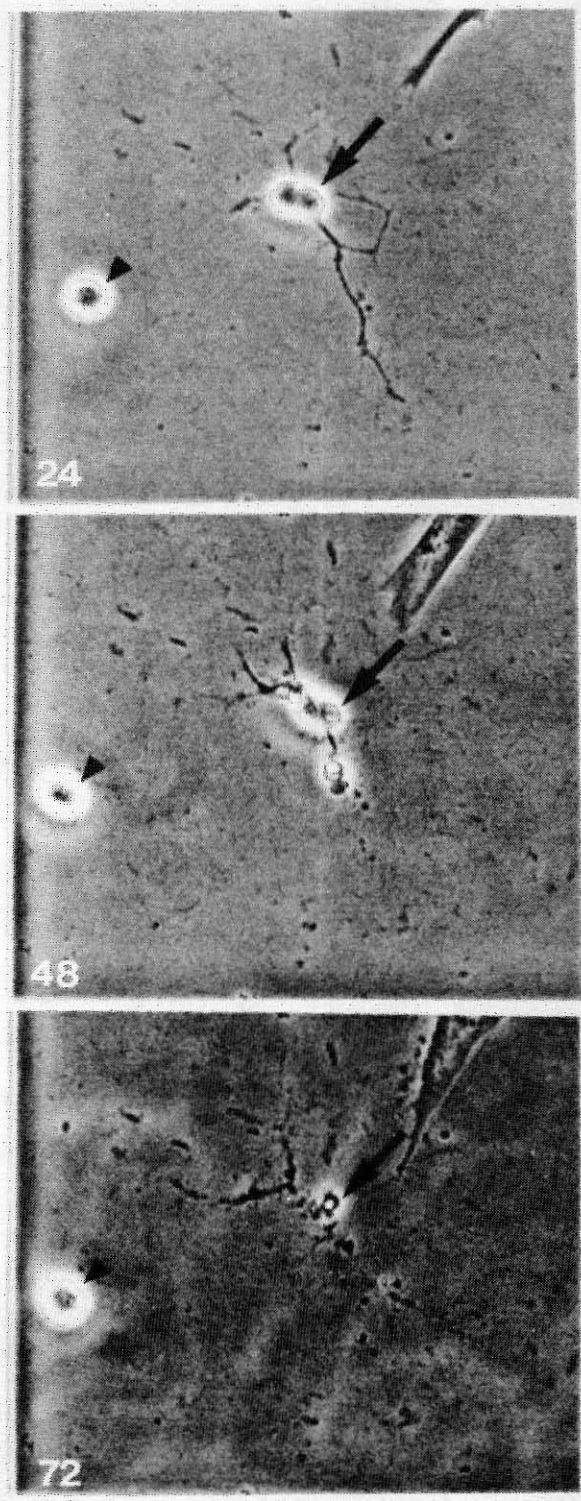

Fig. 7

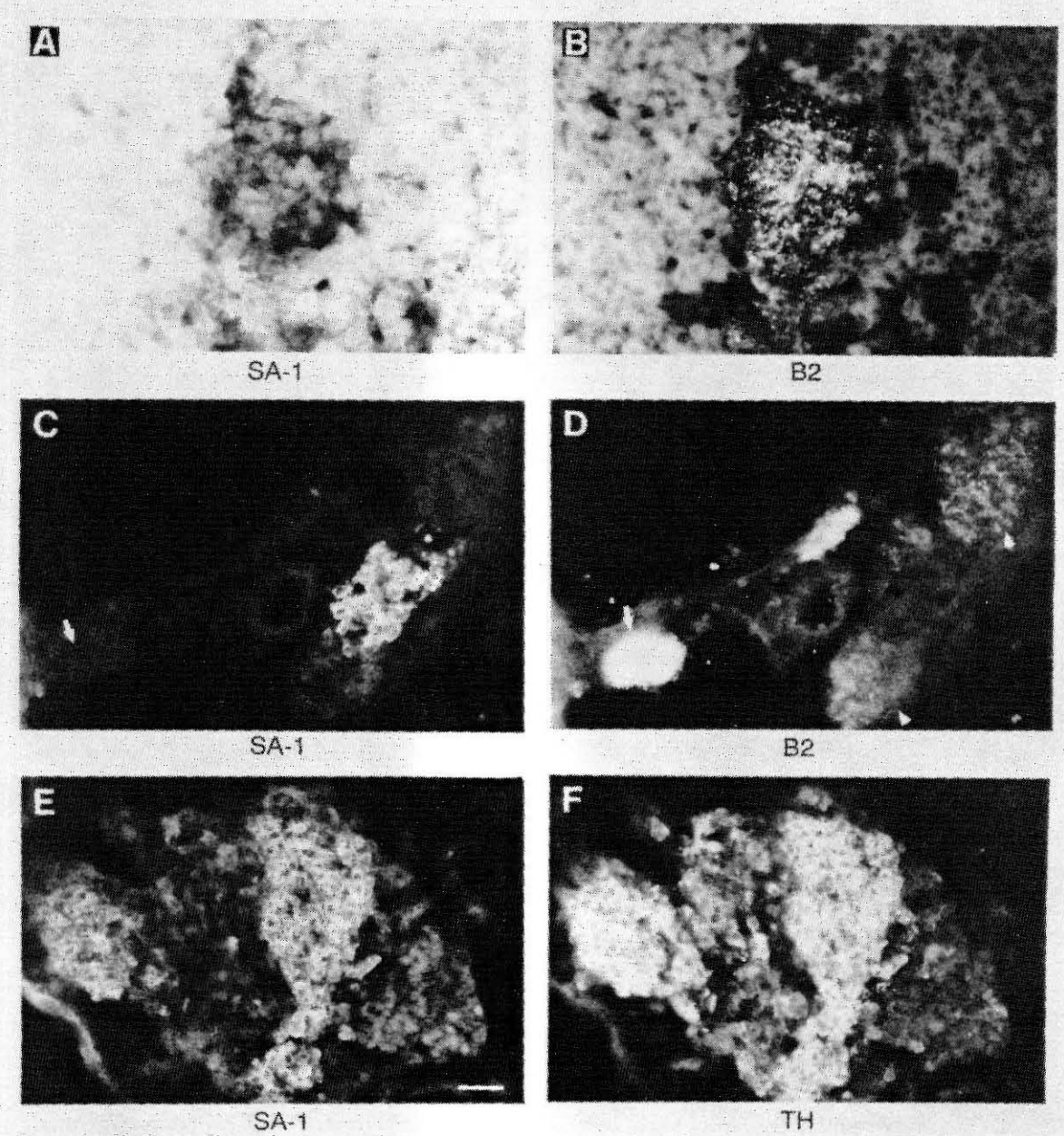

Fig. 8

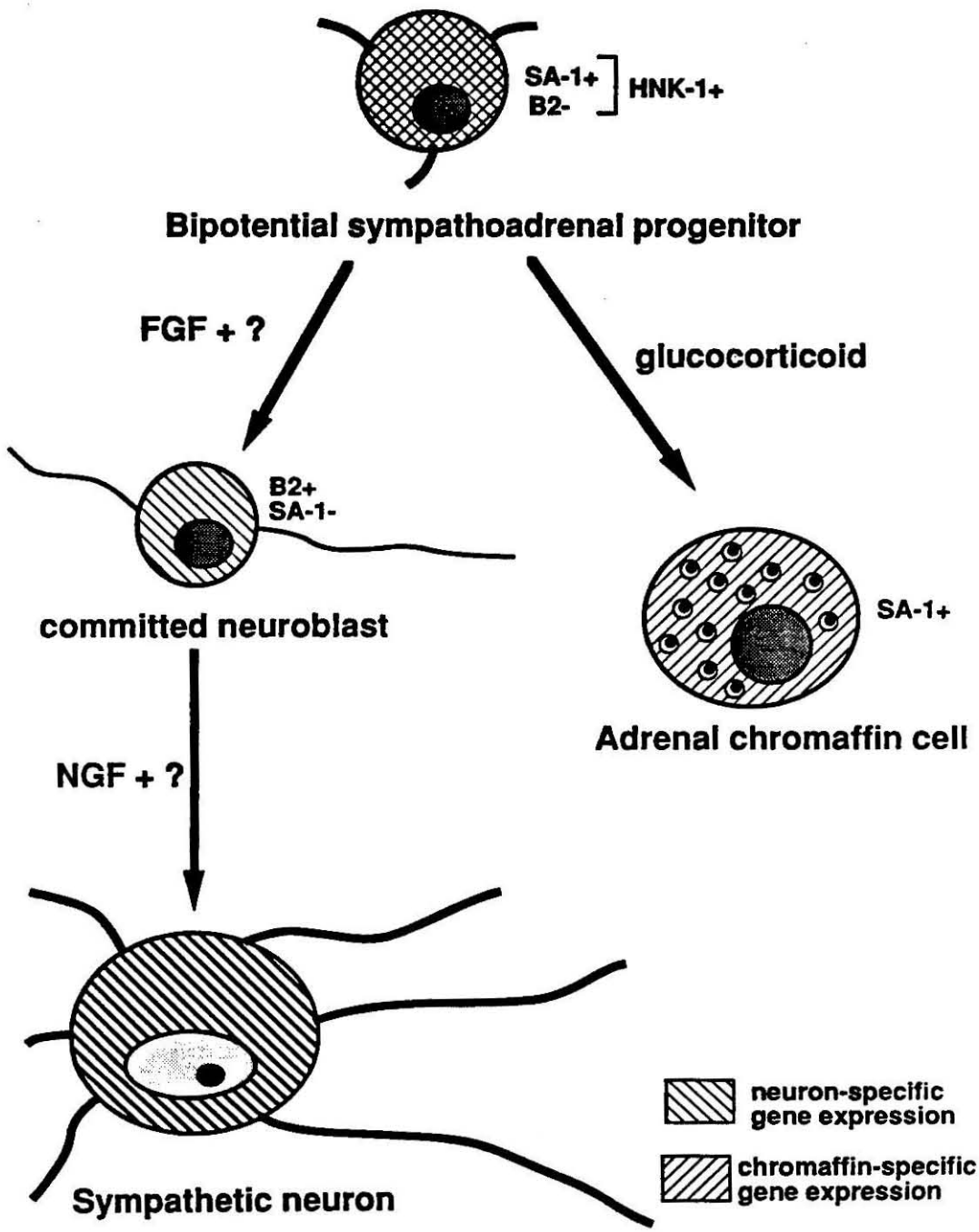
SA-1+
B2-
HNK-1+
Bipotential sympathoadrenal progenitor
FGF + ?
glucocorticoid
B2+
SA-1-
committed neuroblast
SA-1+
Adrenal chromaffin cell
NGF + ?
Sympathetic neuron
neuron-specific gene expression
chromaffin-specific gene expression

Fig. 9