cies (BC1-RNA) complementary to the ID sequence is present in brain, but absent in other organs such as liver and kidney. As shown in Fig. 1F, we confirm that the ~160 nucleotide RNA species is very evident in poly(A)$^+$ cytoplasmic RNA of the brain, but not in liver and kidney (*15*). In the latter organs, hybridization is observed as a faint smear throughout the blot. While no functional role of this brain cytoplasmic 160 nucleotide species has been shown experimentally, it might serve as a signal in selective transport via attachment to brain-specific mRNA-protein complexes, being freed from the complex once transport is complete. Other possibilities for this RNA might be in modulation of translation of mRNA or as a component of a signal recognition particle, perhaps analogous to 7 SL RNA (*16*).

The observation that the copy number of this sequence in mouse and rat varies by nearly an order of magnitude seems to be inconsistent with a regulatory role in the selective expression of genes (*17*). It seems that the copy number of an important transcriptional control element would be conserved relative to the number required for function, because the probability of deleterious location of such a sequence increases with copy number. Thus, while this 82 base pair repeat and respective small RNA's might have a control function, a role as a control element in governing brain specific gene expression at the transcriptional level seems improbable.

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10. The size determination of poly(A)$^+$ hnRNA hybridizing with ID sequences was based on the migration distances in these gels of rat 28*S* ribosomal RNA (~5000 nucleotides), 18*S* ribosomal RNA (~2000 nucleotides), and transfer RNA (~80 nucleotides). Since no RNA marker larger than 5 kilobases was used, the upper size limit of hybridizing transcripts was estimated by assuming the rate of migration of denatured RNA to be linearly proportional to $\log_{10}$ of the molecular weight. The distribution of molecules reacting with the probe does not necessarily represent the average steady-state size of poly(A)$^+$ hnRNA. Some of the smaller RNA molecules hybridizing with the probe might be fragments formed by degradation of larger molecules during purification and fractionation.
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24. Total nuclear RNA was extracted from isolated nuclei (*18*) in the presence of 10 m*M* vanadyl-ribonucleoside complex by the hot phenol method at *p*H 5.2 (*19*). In experiments with total nuclear RNA, samples were first treated with deoxyribonuclease I (200 μg/ml) for 75′ at 37°C and the RNA repurified by extraction with phenol-chloroform and precipitation with ethanol. This procedure eliminated all exogenous pBR322 DNA that was added to a control sample of nuclear RNA prior to deoxyribonuclease digestion. For dot blotting, samples quantified by absorbance at 260 nm were denatured in a phosphate-buffered solution containing 50 percent formamide and 6 percent formaldehyde (*20*) for 20 minutes at 67°C, cooled on ice, diluted with 5 volumes of 20× SSC, and then applied onto wet nitrocellulose papers. In Northern gel analysis, the RNA was denatured as described above, fractionated by electrophoresis in 1.5 percent agarose gels in the presence of 2*M* formaldehyde (*20*), and then blotted onto nitrocellulose (*21*). Blots were baked for 2 hours at 80°C and hybridized overnight at 42°C in 50 percent formamide: 0.6*M* NaCl, 40 m*M* Na:PO_4 (*p*H 7.4), 4 m*M* EDTA, 0.1 percent SDS, herring sperm DNA (100 μg/ml), and 5× Denhardt's solution (*22*). Hybridizations were carried out for 24 to 48 hours at 42°C in fresh solution containing heat-denatured ^{32}P-labeled probe. The hybridization solutions used in D and F also contained poly(A) (1 μg/ml). Blots were washed at 42°C in three changes (at 15-minute intervals) of 2× SSC and 0.2 percent sodium dodecyl sulfate (SDS), and finally in 0.2× SSC and 0.2 percent SDS for 30 minutes at 42°C. In some experiments, an additional 30-minute wash in 0.2× SSC and 0.2 percent SDS at 67°C was performed. The hybridization in A to C was done with nick-translated cloned plasmid p2A120 DNA [for sequence and restriction map, see (*1*)], specific activity 0.5×10^8 to 2×10^8 cpm/μg. In D and F, a 93 base pair restriction fragment comprising the last 65 nucleotides of the ID sequence plus short oligo(A) and oligo(C) tracts was used as probe. This fragment was obtained by digesting p2A120 DNA with the restriction endonucleases Dde I and Pst I and isolating the appropriate sized fragment from an 8 percent acrylamide gel (*23*). Prior to electrophoresis, the 3′ recessed ends generated by Dde I digestion were labeled by filling in with α ^{32}P-labeled deoxyribonucleotides using the Klenow fragment of *E. coli* DNA polymerase I (*23*).
25. We thank J. G. Sutcliffe for plasmid p2A120 DNA. Supported by NIH grant NS10813.

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Superinduction of c-*fos* by Nerve Growth Factor in the Presence of Peripherally Active Benzodiazepines

Abstract. *Alterations in proto-oncogene expression after stimulation of rat pheochromocytoma (PC12) cells by nerve growth factor (NGF) have been investigated. A specific stimulation of* c-fos *messenger RNA and protein was detected 30 minutes after treatment. This induction was enhanced more than 100-fold in the presence of peripherally active benzodiazepines. The effect was specific as very little change was observed in the levels of* c-ras^{Ha}, c-ras^{Ki}, *c-*myc, *and N-*myc *messenger RNA's. Under the conditions used here, NGF treatment ultimately results in neurite outgrowth, with a reduction or cessation of cell division. Thus, stimulation of the* c-fos *gene in this system appeared to be associated with differentiation and not with cellular proliferation. The effect of benzodiazepines was stereospecific and represents a novel action of these compounds at the level of gene expression.*

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Neoplastic transformation is a consequence of the subversion of normal growth regulatory mechanisms. Furthermore, several retroviral oncogenes and their cellular progenitors are intimately associated with the normal processes of proliferation and differentiation. Many oncogene products share sequence homology with growth factors and their receptors (*1*), while expression of the c-*myc* and c-*fos* proto-oncogenes is stimu-

lated by a variety of polypeptide growth factors and other mitogenic agents (*2*). On the basis of these observations, investigators have suggested that the c-*myc* and c-*fos* oncogenes are directly involved in the stimulation of mitogenesis. However, the induction of c-*fos* and c-*myc* has been observed in the absence of mitogenesis (*3*), and c-*fos* expression occurs in various differentiated cells that have reduced proliferative capacities (*4*). To distinguish between the potential roles of c-*fos* in proliferation and differentiation, we chose to study a system in which treatment with growth factor results in growth and differentiation with a concomitant reduction or cessation of cell division (*5*).

Nerve growth factor (NGF) is a multimeric protein that modulates the differentiation of several neuronal cell types in vivo and in vitro (*6*). In the rat pheochromocytoma cell line PC12 (*7*), NGF acts via a specific receptor (*8*) to induce a series of alterations that include neurite growth, modification of the cytoskeleton, and changes in neurotransmitter synthesis, as well as induction of the enzyme ornithine decarboxylase (ODC) (*9*). Certain types of peripheral benzodiazepines (BZD's) can interact with NGF to modify its action both on neurite outgrowth and on the induction of ODC (*10*). The mechanism of action of NGF and BZD's at the level of gene expression is unclear (*11*).

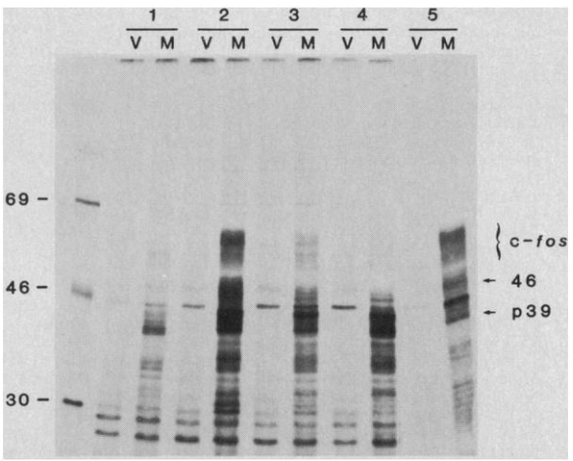

Fig. 1. Time course of c-*fos* protein induction by NGF. Cultures of PC12 cells in 35-mm dishes were treated with NGF (300 ng/ml) for (lanes 1) 0 minutes, (lanes 2) 30 minutes, (lanes 3) 60 minutes, and (lanes 4) 120 minutes. At each time point, [^{35}S]methionine (200 μCi/ml; Amersham) was added for an additional 15 minutes. Cell extracts were prepared, treated with control antiserum (V) and c-*fos* peptide antiserum (M), and analyzed by SDS–polyacrylamide gel electrophoresis as described (*14*). (Lanes 5) As a control, NIH 3T3 cells stimulated with 20 percent fetal bovine serum (*12*) are also shown. The numbers on the left indicate the molecular sizes of ^{14}C-methylated marker proteins in kilodaltons (Amersham). Each lane represents the immunoprecipitate from equivalent amounts of cell lysate in terms of radioactivity in trichloroacetic acid (TCA)-insoluble protein. The positions of the c-*fos*, p39, and the 46-kD proteins are indicated.

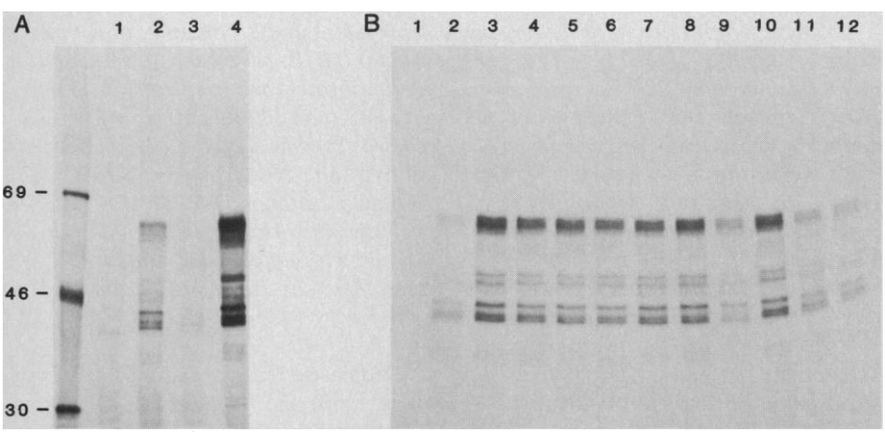

Fig. 2. Superinduction of c-*fos* protein by BZD's. (A) Cultures of PC12 cells were (lane 1) untreated, or treated with (lane 2) NGF (100 ng/ml), (lane 3) 100 μ*M* 7-3351, or (lane 4) NGF plus 100 μ*M* 7-3351 for 30 minutes. Cells were labeled for 15 minutes with [^{35}S]methionine, and lysates were prepared and immunoprecipitated with *fos*-specific peptide antiserum (M) (*14*). The numbers on the left indicate molecular size markers (kD). (B) PC12 cells were (lane 1) untreated, (lane 2) treated with NGF (100 ng/ml), or (lanes 3-12) treated with NGF (100 ng/ml) in the presence of 100 μ*M* of the following BZD's: (lane 3) 5-4864, (lane 4) 5-3464, (lane 5) 7-3351, (lane 6) 5-6531, (lane 7) 5-6993, (lane 8) 5-6669, (lane 9) 11-6893, (lane 10) 11-6896, (lane 11) 5-4023, (lane 12) 5-2180. Lysates were prepared and treated with M-*fos* peptide antiserum as described above.

The level of expression of c-*fos* in PC12 cells was measured by immunoprecipitation and Northern blot analyses as described (*12*). The c-*fos* protein migrates on sodium dodecyl sulfate (SDS)–gels with an apparent size of 55 to 62 kilodaltons (kD), the heterogeneity being a result of post-translational modification (*13*) (Fig. 1). The peak of synthesis of the c-*fos* product was at 30 minutes after NGF treatment. In addition to the c-*fos* protein, p39 [the cellular protein to which c-*fos* is complexed (*14*)] and a series of proteins in the 46-kD size range were also detected. A small amount of p39 was detected in unstimulated PC12 cells while maximum levels occurred at 60 to 120 minutes after stimulation (Fig. 1). As p39 is detected only by its ability to bind to c-*fos*, the detection of p39 in the apparent absence of c-*fos* protein represents the binding of newly synthesized, and therefore radioactive, p39 to previously synthesized, nonradioactive c-*fos* protein. The 46-kD products (Fig. 1) were also observed after stimulation of fibroblasts with serum (*12*) and are thought to represent an antigenically related protein that is also induced by growth factors. A candidate gene for this role is r-*fos* (*15*), which has homology to c-*fos* in the exact region of *fos* used to predict the peptide to which antisera were raised (*14*).

Thus, treatment of PC12 cells with NGF results in a transient induction of c-*fos* similar in degree and time course to that observed after treatment of fibroblasts with mitogenic growth factors (*2*). Immunofluorescence analysis has indicated that c-*fos* induction was not restricted to a subpopulation of NGF-stimulated PC12 cells. In these studies, PC12 cells were maintained in growth medium prior to induction and thus represented an asynchronous population of proliferating cells. Thus, there was no absolute requirement for a progression from the G_0 to the G_1 phase of the cell cycle for c-*fos* induction. Analysis of the dose-response relation indicated that an NGF concentration of 50 to 100 ng/ml was the smallest concentration that gave the maximum induction of c-*fos*. For the PC12 cells used in this study, this was also the minimum concentration of NGF required to induce and support vigorous neurite outgrowth.

The induction of ODC by NGF is stimulated by a variety of BZD's (*10*). Treatment of PC12 cells with the BZD 7-3351 alone at a concentration of 100 μ*M* had little effect on the rate of c-*fos* protein synthesis (Fig. 2A). However, a significant overstimulation of c-*fos* synthesis was detected in the presence of NGF; the levels of both p39 and the 46-

kD protein were also increased. Dose response analysis indicated that 25 to 100 μM was the minimal concentration range of BZD's that gave the maximum superinduction of c-*fos* at several NGF concentrations. Superinduction also caused an increase in the degree of post-translational modification of c-*fos*, and perhaps also of p39 (Fig. 2). The major change in cells treated with NGF plus 7-3351 was an increase in the highly modified 62-kD form of c-*fos*. Indeed, in some experiments, an even larger form of approximately 80-kD was also observed (Fig. 2). It is possible that either the enzymes or the substrates involved in the modification of c-*fos* could be induced, or the increased level of c-*fos* protein may allow more efficient modification. The expression of p39 appeared to increase concomitantly with c-*fos*. However, as p39 is detected only by its ability to bind to the c-*fos* product, its expression may not be modulated. In this event, there must be sufficient p39 available in nonstimulated cells to accommodate the increased amount of c-*fos* after stimulation.

The derivative 7-3351 is not the only BZD that can superinduce c-*fos* (Fig. 2B). BZD's that bind only to the central receptor of brain and not to PC12 cells (5-2180 and 5-4023) (*13*) were essentially inactive (Fig. 2B). A pharmacologically distinct BZD binding site, usually referred to as the peripheral BZD site (*16*), is found on a wide variety of cell types including PC12 (*10*). Although many BZD's that bind to PC12 cells at the peripheral BZD site (peripheral-type BZD's) were active in superinducing c-*fos* (Fig. 2B), individual affinity constants did not predict biological activity. The c-*fos* response to BZD's was stereospecific; 11-6896 was active, but its enantiomer (11-6893) was inactive (Fig. 2B). However, both ligands bound equally well to PC12 cells (*10*). This stereoselective structure-activity relation has been shown for the BZD modulation of NGF-induced neurite outgrowth (*10*) and for the direct BZD induction of differentiation of Friend erythroleukemia cells (FEL) (*17*).

We next determined the effects of NGF and 7-3351 on the steady-state level of c-*fos* messenger RNA (mRNA). The maximum level of c-*fos* mRNA was detected 30 minutes after treatment with NGF (Fig. 3A). Simultaneous addition of 7-3351 increased the absolute level of c-*fos* mRNA, but did not change the rate of induction. After 60 minutes, the amount of c-*fos* mRNA was reduced by a factor of five in both situations, and dropped to the basal level after 120 minutes (Fig. 3A). This profile of mRNA induction is similar to that obtained after stimulation of fibroblasts with mitogenic growth factors (*12*). A small amount of stimulation of c-*fos* mRNA was obtained with 7-3351 alone (Fig. 2A). Densitometric analysis indicated that NGF treatment resulted in an approximately 20-fold increase in the level of c-*fos* mRNA. In the presence of 7-3351, this was increased to more than 100-fold. The level of c-*fos* mRNA in superinduced PC12 cells is much higher than that in fibroblasts transformed by the FBJ murine sarcoma virus (Fig. 3A). In previous studies, induction was shown to affect the rate of transcription of c-*fos* rather than the half-life of the mRNA (*18*). Although this has not yet been demonstrated directly for NGF, the identical induction profile makes it seem likely. The superinduction of c-*fos* by BZD's also appears to be at the level of transcription because the decrease in c-*fos* mRNA at 60 minutes and 120 minutes after induction occurs at the same rate as that after stimulation by NGF alone.

In contrast to the large fluctuations of c-*fos* mRNA synthesis, very little change was observed in the synthesis of mRNA from a variety of other oncogenes (Fig. 3B). As also noted in stimulated fibroblasts (*18*) the levels of c-*ras*^{Ha} and c-*ras*^{Ki} were altered no more than two- to threefold in PC12 cells by the various treatments. Surprisingly, little change was detected in the amount of c-*myc* mRNA, a gene induced along with c-*fos* in many other situations (*2, 4*). At 120 minutes after stimulation there was a threefold increase in c-*myc*; but in contrast to c-*fos*, there was no superinducing effect of 7-3351 on c-*myc* expression (Fig. 3B).

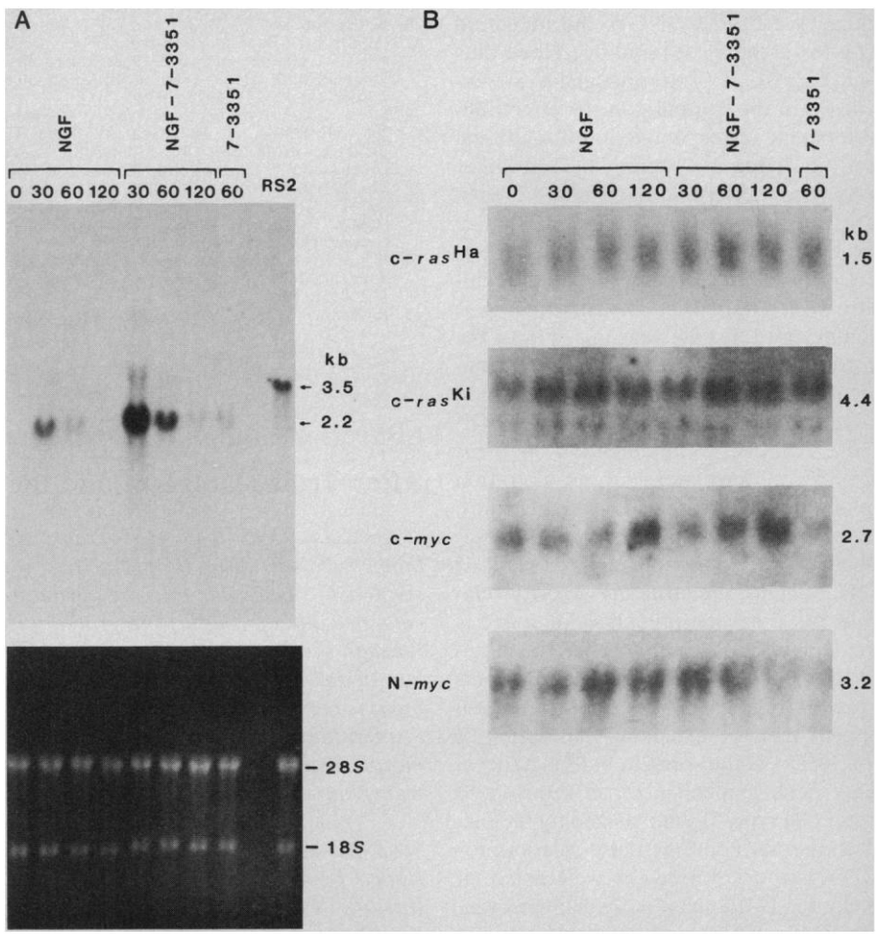

Fig. 3. Analysis of oncogene mRNA levels after treatment with NGF and 7-3351. (A) PC12 cells were treated with NGF (300 ng/ml), 100 μM 7-3351, or NGF (300 ng/ml) plus 100 μM 7-3351 for 0, 30, 60, or 120 minutes as indicated. RNA was isolated (*25*) and 10-μg fractions were analyzed by electrophoresis on agarose-formaldehyde gels and transferred to nitrocellulose (*26*). As a control, 10 μg of RNA from the FBJ murine sarcoma virus–transformed nonproducer cell line (RS2) was also analyzed. The filter was hybridized with a ^{32}P-labeled, nick-translated *fos* DNA fragment (corresponding to the entire c-*fos* coding region) (*27*). Before transfer, the gel was stained with ethidium bromide (10 μg/ml) for 30 minutes, destained for 40 minutes, and photographed under ultraviolet light. The positions of 28*S* and 18*S* ribosomal RNA's are indicated. (B) Identical aliquots of RNA were separated on agarose-formaldehyde gels and transferred to nitrocellulose as described above. Filters were hybridized with c-*ras*^{Ha} (*28*), c-*ras*^{Ki} (*29*), c-*myc* (*30*), and N-*myc* (*19*) probes as indicated (*31*).

Virtually no modulation was observed in the level of N-*myc* mRNA during the time course analyzed. N-*myc* is frequently amplified and expressed at high levels in neuroblastoma cells (*19*). Thus, treatment of PC12 cells by NGF and 7-3351 results in a very dramatic and specific stimulation of the c-*fos* oncogene.

The actions of NGF on PC12 cells may be divided into two categories. The first category consists of effects that occur relatively soon after addition of NGF and that do not require transcription, such as the altered phosphorylation of endogenous substrates (*20*). The second category contains the more long-term actions that are transcription-dependent. Alterations requiring transcription include the elevation of ODC activity (*21*), the increase in microtubule-associated proteins (*22*), and the generation of neurites (*23*). Of the transcription-dependent changes caused by NGF, the induction of c-*fos* is the most rapid described thus far (Fig. 3). If proto-oncogenes are involved in the normal control of cell development, c-*fos* could fulfill a critical early function in coupling NGF to other long-term transcription-dependent events. This is supported by the finding that c-*fos* is superinduced when certain peripheral-type BZD's are present in the culture with NGF (Fig. 2). This finding is significant for two reasons. First, the concentrations of BZD's used are sufficient to modify both the neurite and ODC responses of PC12 cells to NGF (*10*). That is, a modification in c-*fos* expression correlates with an alteration in biological response. Furthermore, both of these responses occur subsequent to the induction of c-*fos* (Fig. 1) (*24*). Second, the structure-activity relation for the superinduction of c-*fos* is indistinguishable from that for the modification of NGF-induced neurite outgrowth (Fig. 2B) (*10*). Thus, it would appear that the mechanism of action of the BZD's is the same in both instances. That such a mechanism is relevant to other cell types is emphasized by the fact that the structure-activity relation for BZD's reported here (as well as previously for PC12 cells) is the same as that for the direct BZD induction of hemoglobin synthesis in FEL cells (*17*). The same mechanism appears to modify differentiation in cells of two quite distinct phenotypes. It is possible that c-*fos* fulfills a similar function in the other cell types in which it is expressed. However, the differentiated or physiological state of the stimulated cell may determine the nature of the long-term events to which c-*fos* action is coupled.

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Migration and Differentiation of Cerebral Cortical Neurons After Transplantation into the Brains of Ferrets

Abstract. *Cells from the cerebral proliferative zones of newborn ferrets were labeled with tritiated thymidine and a fluorescent dye and were transplanted as a single-cell suspension into the occipital region of newborn ferrets. The transplanted cells became thoroughly integrated into the host environment: many cells migrated through the intermediate zone and into the cortical plate, where they developed as pyramidal neurons. Other transplanted cells came to resemble glial cells. After 1 to 2 months most transplanted neurons had taken up residence in layer 2 + 3, the normal destination of neurons generated on postnatal days 1 and 2. Thus the sequence of morphological differentiation and the eventual laminar position of the isochronically transplanted neurons closely paralleled that of their normal host counterparts.*

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The laminar arrangement of neurons in the mammalian cerebral cortex is one of the most striking features of cortical organization. Not only do most neurons in a given layer tend to share certain morphological features, but they also have similar patterns of connectivity and physiological properties (*1*). Tritiated thymidine "birth-dating" studies have revealed that neurons in a layer undergo their terminal mitotic divisions at about the same time and that the layers are generated in an inside-out manner, with cells of the deepest layers being born first and those of the most superficial layers last (*2, 3*). That cortical neurons in a lamina share common birth dates and