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pp60$^{c\text{-}src}$ expression in the developing rat brain

(cellular oncogene/protein-tyrosine kinase/neuron)

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ABSTRACT **We have studied pp60$^{c\text{-}src}$ expression in the striatum, hippocampus, and cerebellum of the developing rat brain. In the striatum, pp60$^{c\text{-}src}$ protein kinase activity peaks during embryonic development and then declines in the adult. The peak activity occurs in the striatum on embryonic day 20 (E20) when it is 18- to 20-fold higher than the activity in fibroblasts and 4- to 5-fold higher than the activity in the striatum at E15 or in the adult striatum. In the hippocampal region, pp60$^{c\text{-}src}$ activity reaches a maximum shortly after birth but remains high throughout life. On postnatal day 2 (P2) the activity in the hippocampus is 9- to 13-fold higher than the activity in fibroblasts and twice as high as the activity in the hippocampus at E18. In the cerebellum, the kinase activity remains constant from E20 onward and is 6- to 10-fold higher than that observed in fibroblasts. The increase in pp60$^{c\text{-}src}$ kinase activity observed during the development of the striatum and hippocampus is due to an increase in the amount of pp60$^{c\text{-}src}$ protein and to an increase in the specific activity of the kinase. The increase in specific activity in these regions coincides with the peak periods of neurogenesis and neuronal growth. In the striatum, we have found that the increase in pp60$^{c\text{-}src}$ activity also parallels the increase observed in culture as embryonic striatal neurons differentiate. Taken together, our results are consonant with the idea that pp60$^{c\text{-}src}$ is the product of a developmentally regulated gene that is important for the differentiation and/or the continuing function of neurons.**

The cellular gene c-*src*, which is homologous to the transforming gene of the Rous sarcoma virus, is highly conserved throughout evolution and encodes a 60-kDa membrane-associated phosphoprotein, pp60$^{c\text{-}src}$, which is a protein-tyrosine kinase (1–4).

High levels of pp60$^{c\text{-}src}$ have been found in the developing vertebrate nervous system and also in the nervous system of *Drosophila* (5–13). In the chicken neural retina (7) and cerebellum (9) pp60$^{c\text{-}src}$ appears to be expressed in neurons at the onset of differentiation and persists in mature, fully differentiated cells. Similarly, differentiated neurons from embryonic rat brain express high levels of two forms of pp60$^{c\text{-}src}$ that have elevated specific activity (8, 12, 13). When embryonal carcinoma cells (14) or primary neurons from developing rat striatum (13) are induced to differentiate in culture, there is an increase in the amount of pp60$^{c\text{-}src}$, and, in the case of striatal neurons, there is also an increase in the specific activity of the pp60$^{c\text{-}src}$ kinase. Together, these studies suggest that pp60$^{c\text{-}src}$ is the product of a developmentally regulated gene that may be important for the initial differentiation and continuing function of neurons.

Determining where and when pp60$^{c\text{-}src}$ is expressed in the developing nervous system may provide useful clues as to its functional role in neurons. To investigate this, we have analyzed pp60$^{c\text{-}src}$ expression in three well-defined and carefully studied regions of the developing rat brain: the striatum, hippocampus, and cerebellum (15–20). The striatum (or caudate-putamen) develops over a relatively short period during the latter third of gestation, with the peak period of neurogenesis in rats occurring around embryonic day 16 (E16) or E17 (20–22). Neurogenesis in the hippocampal region (including the hippocampus and the associated dentate gyrus) and the cerebellum occurs over a much longer period, beginning in late embryonic life and continuing for some time after birth. In the rat hippocampal region, neurogenesis begins at E15 and continues—mainly in the dentate gyrus—until well after postnatal day 18 (P18) (23–26). In the cerebellum, granule cells are formed from E17 to P15 in mice (27) and primarily during the second postnatal week in rats (28–30).

MATERIALS AND METHODS

Cell Culture and Tissue Lysates. Rat fibroblast 208F cells and striatal neurons from day 15 Sprague–Dawley rat embryos were cultured as described (13). Tissues were dissected, pooled (0.5–4 tissues per pool), and frozen on dry ice prior to homogenization in a modified RIPA buffer (13).

Immune Complex Protein Kinase Assays. Lysate protein concentrations and immune complex protein kinase assays using monoclonal antibody (mAb) 327 (31) were performed as described (13, 32–36). The pp60$^{c\text{-}src}$ kinase activity was linearly related to the concentration of cell protein.

Immunoblot Analysis. The amount of pp60$^{c\text{-}src}$ in tissue lysates was determined as described (37) and was shown to be linear over a 5-fold range of cell protein.

Phosphopeptide Mapping. One-dimensional phosphopeptide mapping was done in 12% NaDodSO$_4$/polyacrylamide gels (acrylamide/bisacrylamide, 39:1) with 100 ng of *Staphylococcus aureus* V8 protease (Miles) as described (38).

RESULTS

pp60$^{c\text{-}src}$ Protein Kinase Activity in the Striatum Peaks During Embryonic Development. To study pp60$^{c\text{-}src}$ protein kinase activity during development the striatum was dissected from groups of embryonic and postnatal rats. E15 was the earliest stage at which we could dissect sufficient striatal tissue for analysis. The tissue was homogenized in RIPA buffer, and lysates were standardized so that each contained 80 μg of protein. Lysates of a rat fibroblast cell line, 208F, were prepared in parallel. Proteins were immunoprecipitated with excess mAb 327, a mAb specific for pp60$^{c\text{-}src}$, incubated with [γ-^{32}P]ATP and the exogenous substrate enolase, and

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Abbreviations: mAb, monoclonal antibody; E, embryonic day; P, postnatal day.

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analyzed on $NaDodSO_4$/polyacrylamide gels. The results of a representative experiment are shown in Fig. 1*A*. A 60-kDa phosphoprotein was detected, and *S. aureus* V8 protease mapping (not shown) confirmed that it was $pp60^{c\text{-}src}$. The $pp60^{c\text{-}src}$ protein kinase activity was similar whether measured by autophosphorylation (p60) or phosphorylation of enolase and varied systematically during development. Activity in the striatum peaked toward the end of gestation, at E20, and then declined in the adult. ^{32}P incorporation into $pp60^{c\text{-}src}$ or enolase was quantified by scanning densitometry of autoradiograms, and the combined results (based on 2–4 experiments at each stage) are shown in Fig. 4 *Top*. At E20 $pp60^{c\text{-}src}$ protein kinase activity in the striatum was 18- to 20-fold higher than in 208F fibroblasts and 4- to 5-fold higher than the activity in the striatum at E15 or in the adult brain.

To determine whether the differences observed in $pp60^{c\text{-}src}$ kinase activity were due to different levels of $pp60^{c\text{-}src}$ and/or to changes in its specific activity, immunoprecipitates made with excess mAb 327 (from equivalent amounts of the same tissue lysates used for the assays in Fig. 1*A*) were separated on $NaDodSO_4$ gels prior to transfer to nitrocellulose. The filters were incubated with excess mAb 327 and ^{125}I-labeled sheep anti-mouse immunoglobulin. Two ^{125}I-labeled proteins were detected in each lane (Fig. 1*B*). Additional experiments with a truncated form of $pp60^{c\text{-}src}$ or mAb 327 alone (not shown) confirmed that the upper protein was $pp60^{c\text{-}src}$ and that the lower was mouse IgG heavy chain. $pp60^{c\text{-}src}$ was quantified by scanning densitometry of autoradiograms and the combined results of two experiments, each done in duplicate, are shown in Fig. 4 *Top*. A small increase in the level of $pp60^{c\text{-}src}$ protein was observed during development: the amount of protein in the striatum at E20 was 2.6-fold higher than that found in fibroblasts and 1.6-fold higher than that in the striatum at E15. In addition, the specific activity of $pp60^{c\text{-}src}$ in tissue extracts of the striatum (calculated by dividing the total protein kinase activity by the amount of $pp60^{c\text{-}src}$ protein) increased ≈3-fold between E15 and E20 (Table 1) and then declined somewhat in the adult. The peak in the specific activity of the $pp60^{c\text{-}src}$ kinase during embryonic development coincides with the peak period of neurogenesis in the striatum, which in rats occurs at E16 or E17 (20–22).

In the Hippocampal Region $pp60^{c\text{-}src}$ Protein Kinase Activity Reaches Its Maximum During Postnatal Life. The earliest stage at which we could easily obtain sufficient hippocampal tissue was E18. Tissue lysates were standardized so that each contained 75 μg of protein, and $pp60^{c\text{-}src}$ was precipitated and phosphorylated as described above. As measured by autophosphorylation or enolase phosphorylation, $pp60^{c\text{-}src}$ protein kinase activity reached its maximum in postnatal rats and remained high throughout adult life (Fig. 2*A*). In this experiment, protein kinase activity was measured by counting excised gel pieces (not shown) as well as by densitometric scanning of autoradiograms (Fig. 4 *Middle*). Both methods gave similar results. $pp60^{c\text{-}src}$ protein kinase activity was highest in 4- and 18-month-old (M4 or M18) rats when measured by autophosphorylation and at P2 when measured by enolase phosphorylation. In principle, the phosphoryl-

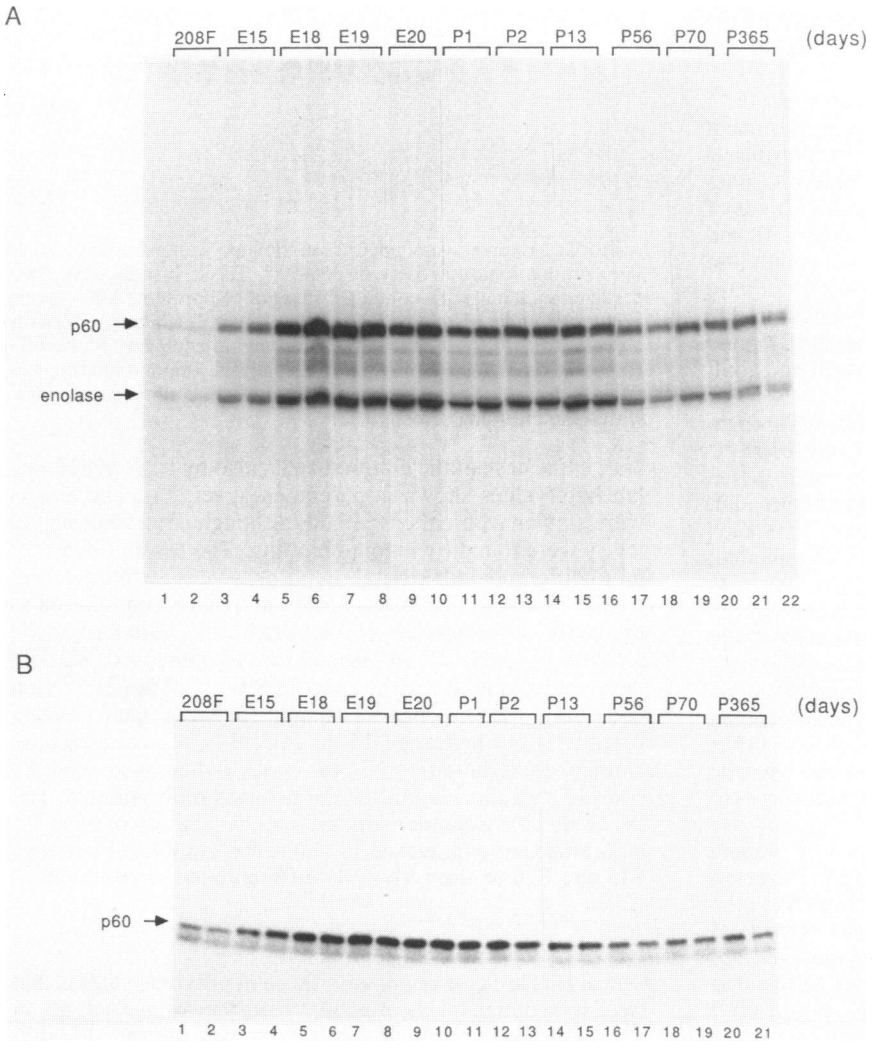

FIG. 1. Expression of $pp60^{c\text{-}src}$ in striatum. Striata were dissected from embryonic (E) or postnatal (P) rats, homogenized in RIPA buffer, and lysates were standardized such that each contained 80 μg of cell protein. Lysates of a rat fibroblast cell line (208F) were prepared in parallel. (*A*) *In vitro* protein kinase activity of $pp60^{c\text{-}src}$. Proteins were immunoprecipitated with excess mAb 327, incubated with [γ-^{32}P]ATP and enolase, and resolved on 7% $NaDodSO_4$/polyacrylamide gels. Each lane represents an independent pool of one to four striata from rats at each age. Exposure time, 4 hr. (*B*) Immunoblot analysis. Proteins were immunoprecipitated with excess mAb 327 (from equivalent amounts of the same tissue lysates used for the *in vitro* kinase assays above) and resolved on 7% $NaDodSO_4$/polyacrylamide gels prior to transfer to nitrocellulose. The filters were incubated with excess mAb 327 and ^{125}I-labeled sheep anti-mouse immunoglobulin, and ^{125}I-labeled proteins were detected by autoradiography. Exposure time, at −70°C with an intensifying screen, 4 hr.

Table 1. Relative specific activity of pp60$^{c\text{-}src}$ protein kinase in striatal neurons

	pp60$^{c\text{-}src}$ level	Specific activity	
		Autophosphorylation	Enolase phosphorylation
208F	1.3 (1.3)	1.1 (1.1)	1.1 (1.1)
Culture			
2 day	1.0	1.0	1.0
11 day	1.6 (1.2)	10.8 (14.4)	5.1 (6.8)
Tissue			
E15	1.5	2.8	2.8
E20	2.5	8.0	6.7

Proteins were immunoprecipitated with excess mAb 327 from lysates of cultured striatal neurons or tissue extracts and analyzed for *in vitro* kinase activity. Relative protein levels were determined by immunoblotting and, in cultured cells, also by labeling with [^{35}S]methionine (values in parentheses; ref. 13). Radiolabeled proteins were detected by autoradiography and quantitated by scanning densitometry. Values are expressed relative to those for undifferentiated neurons and represent the mean of duplicate plates of cells or between two and four independent pools of tissue. Each pool contained tissue from two to four embryos at each age. Actual values varied <20% from the mean within experiments and <40% from the mean between experiments. Two-day cultures, when most neurons are undifferentiated; 11-day cultures, when most neurons are clearly differentiated.

ation of an exogenous substrate is a more accurate measure of protein kinase activity than autophosphorylation. Therefore, the results with enolase phosphorylation may more closely reflect the true activity of pp60$^{c\text{-}src}$ in the hippocampal region. The activity at P2 was 9- to 13-fold higher than the activity in rat fibroblasts and 2-fold higher than in the hippocampus at E18.

Immunoblot analysis showed that the amount of pp60$^{c\text{-}src}$ in the hippocampal region varied little during development and was ≈2-fold higher than the level found in fibroblasts (Figs. 2*B* and 4 *Middle*). As measured by enolase phosphorylation the specific activity of pp60$^{c\text{-}src}$ in tissue extracts of the hippocampal region increased ≈2-fold between E18 and P2 and remained more or less constant throughout adult life. The increase in specific activity of the pp60$^{c\text{-}src}$ kinase coincides with the major period of neurogenesis in the hippocampal region, which begins in the rat at E15 and continues—mainly in the dentate gyrus—until well into adult life (23–26).

pp60$^{c\text{-}src}$ Protein Kinase Activity Remains Constant Throughout the Development of the Cerebellum. To study pp60$^{c\text{-}src}$ protein kinase activity during cerebellar development, tissue was dissected at several stages from E20 until well into adult life. Again tissue lysates were standardized so that each contained 75 μg of protein, and pp60$^{c\text{-}src}$ was precipitated, phosphorylated, and quantified as before. pp60$^{c\text{-}src}$ protein kinase activity, as measured by autophosphorylation or enolase phosphorylation, varied somewhat but remained fairly high (approximately 6- to 10-fold that in fibroblasts) throughout development (Figs. 3*A* and 4 *Bottom*). Immunoblot analyses confirmed that the amount of pp60$^{c\text{-}src}$ in the cerebellum varied little during development and was 1- to 2-fold higher than in fibroblasts (Figs. 3*B* and 4 *Bottom*). The specific activity of pp60$^{c\text{-}src}$ in the cerebellar tissue extracts was 4- to 5-fold that of pp60$^{c\text{-}src}$ in fibroblasts.

The Increase in Specific Activity of the pp60$^{c\text{-}src}$ Kinase During Striatal Development Parallels the Increase Observed in Culture as Striatal Neurons Differentiate. Table 1 shows the levels and specific activities of pp60$^{c\text{-}src}$ in tissue extracts of the striatum between E15 and E20 (Figs. 1 and 4 *Top*) compared to those of pp60$^{c\text{-}src}$ in striatal neurons dissociated at E15 that were allowed to differentiate in culture (13). Relative protein levels were determined by immunoblotting

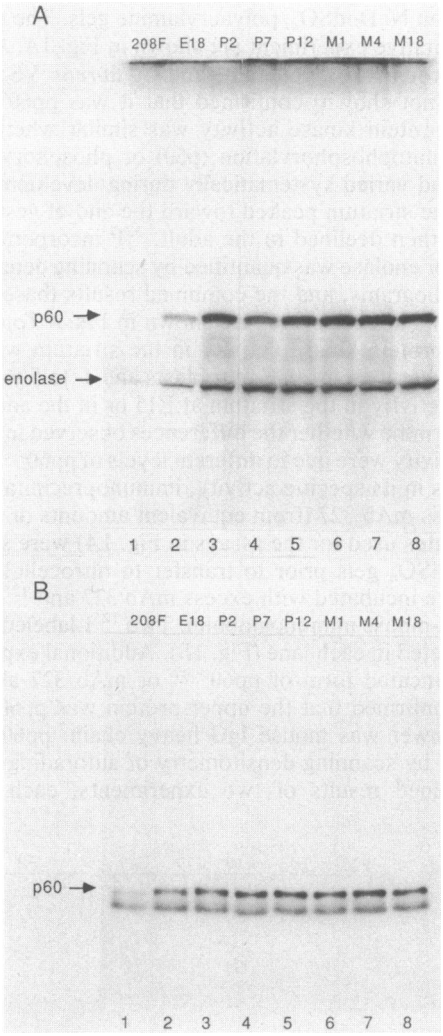

FIG. 2. Expression of pp60$^{c\text{-}src}$ in the hippocampal region. (*A*) *In vitro* protein kinase activity of pp60$^{c\text{-}src}$. Tissue lysates were standardized such that each contained 75 μg of cell protein. Assays were performed as described in the legend to Fig. 1. Each lane represents a pool of one to four hippocampi from rats at each age. M, month. Exposure time, 8 hr. (*B*) Immunoblot analysis. Immunoblotting was done as described in the legend to Fig. 1. Exposure time, at −70°C with an intensifying screen, 9 hr.

and, in the case of the cultured cells, also by [^{35}S]methionine labeling (values shown in parentheses; ref. 13). The results were similar with either method, although somewhat higher levels were found by immunoblotting. The level of pp60$^{c\text{-}src}$ in undifferentiated striatal neurons (2-day cultures) was similar to that in fibroblasts (208F) and increased <2-fold as the cells differentiated (11-day cultures). Similarly, the amount of pp60$^{c\text{-}src}$ in striatal tissue increased <2-fold between E15 and E20. The specific activity of pp60$^{c\text{-}src}$ from undifferentiated neurons was similar to that of pp60$^{c\text{-}src}$ from fibroblasts but increased 11- to 14-fold (when measured by autophosphorylation) or 5- to 7-fold (when measured by enolase phosphorylation) as the neurons differentiated (ref. 13; Table 1). By comparison, the specific activity of pp60$^{c\text{-}src}$ in striatal tissue increased to about the same level between E15 and E20 as the activity in differentiated striatal cells.

DISCUSSION

Our results are consonant with those in previous studies that have shown high levels of pp60$^{c\text{-}src}$ protein kinase activity in the developing vertebrate central nervous system. In addi-

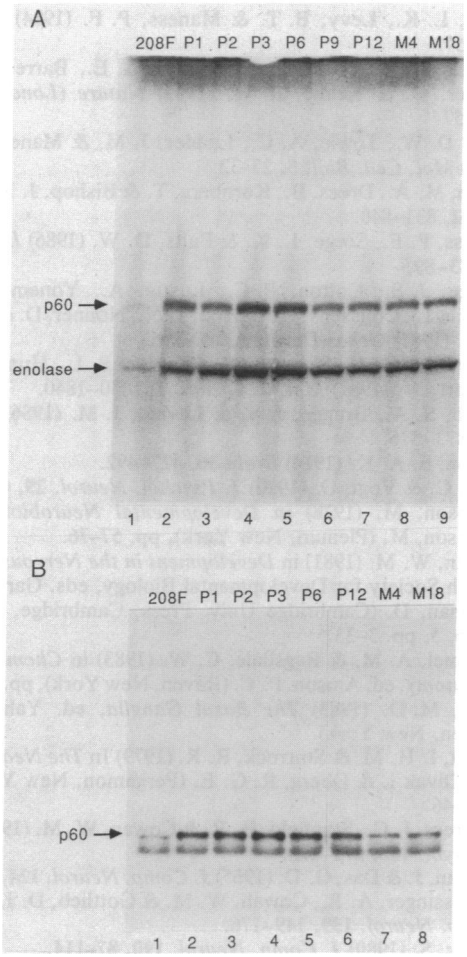

FIG. 3. Expression of pp60$^{c\text{-}src}$ in cerebellum. (*A*) *In vitro* protein kinase activity of pp60$^{c\text{-}src}$. Tissue lysates were standardized such that each contained 75 μg of protein. Assays were carried out as described in the legend to Fig. 1. Each lane represents a pool of one or two cerebella from rats at each age. M, month. Exposure time, 8 hr. (*B*) Immunoblot analysis. Immunoblotting was done as described in the legend to Fig. 1. Exposure time, at −70°C with an intensifying screen, 9 hr.

tion, we have found that pp60$^{c\text{-}src}$ kinase activity varies between different regions of the rat brain, and in any given region, at different times during development. The observed increase in kinase activity is due to higher levels of pp60$^{c\text{-}src}$ and to an increase in the specific activity of the enzyme. Although pp60$^{c\text{-}src}$ activity in the striatum and hippocampal region is highest during neurogenesis it is not clear whether it is involved in cell proliferation or in the differentiation and maturation of postmitotic neurons. In either event, it is evident that pp60$^{c\text{-}src}$ is important during neuronal development.

Although we cannot exclude the possibility that pp60$^{c\text{-}src}$ is enzymatically activated in the nonneuronal (glial) cells, on the whole we think this is unlikely for several reasons. First, in most regions of the central nervous system neuronal differentiation precedes the major phase of glial cell formation, and in the regions we examined the peak in pp60$^{c\text{-}src}$ kinase activity generally occurred at the time when either neurogenesis or neuronal differentiation was at or near its peak. Second, when relatively pure populations of neurons or astrocytes are cultured from embryonic rat brains, pp60$^{c\text{-}src}$ specific activity is found to be between 6- and 12-fold times higher in neurons than in astrocytes or fibroblasts (refs. 8 and 13; Table 1). It seems probable, therefore, that the activated pp60$^{c\text{-}src}$ is principally in neurons rather than in the surrounding glial cells. It remains to be determined (from immunocytochemical studies) in just which classes of neurons the activated pp60$^{c\text{-}src}$ is maximally expressed and to define more precisely their state of differentiation or maturation during the period of maximal pp60$^{c\text{-}src}$ expression.

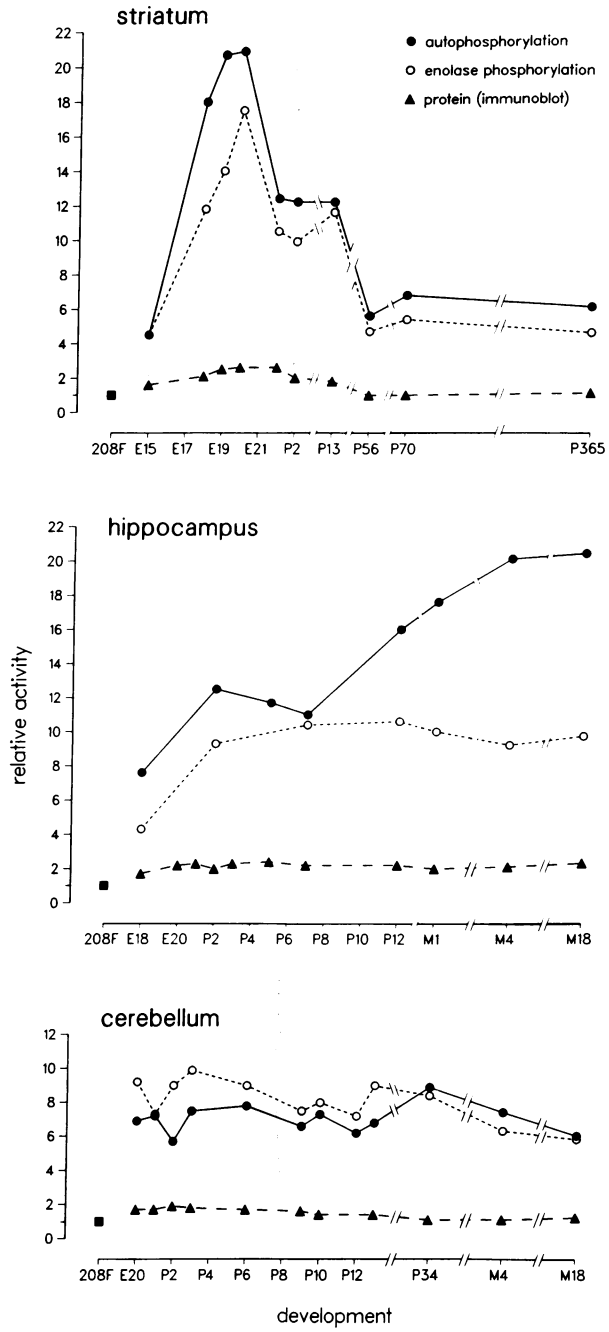

FIG. 4. Comparison of pp60$^{c\text{-}src}$ expression in different regions of the developing rat brain. Tissue lysates of striatum, hippocampus, or cerebellum were analyzed for pp60$^{c\text{-}src}$ levels and *in vitro* protein kinase activity. Between two and four independent sets of data were averaged for each tissue. Actual values for independent pools of tissue varied <20% from the mean within experiments and <40% from the mean between experiments. The data are expressed relative to those obtained for fibroblasts (■). M, month.

Similar increases in specific activity of pp60$^{c\text{-}src}$ were observed in tissue extracts of the striatum between E15 and E20 and in striatal neurons dissociated at E15 and allowed to differentiate for several days in culture. However, shortly after plating the specific activity of pp60$^{c\text{-}src}$ in striatal cells was lower than that *in vivo* at E15. One possible explanation

for this may be that when cells are dissociated and placed in culture at E15, only the less differentiated neurons containing low specific activity $pp60^{c\text{-}src}$ survive the initial plating.

The level of $pp60^{c\text{-}src}$ protein declines somewhat in the striatum of older rats. It is not clear why this is so, but one possibility is that it reflects the progressive increase in the number of nonneuronal cells. However, we should point out that we have not observed a similar decrease in $pp60^{c\text{-}src}$ levels in the hippocampal region where late gliogenesis also occurs.

$pp60^{c\text{-}src}$ is known to be expressed at high levels in the granule and Purkinje cells of the molecular and internal granular layers of the developing chicken cerebellum (9). Moreover, mutant mice in which granule and Purkinje cells degenerate show decreased levels of $pp60^{c\text{-}src}$ in the cerebellum (39). However, we observe that $pp60^{c\text{-}src}$ protein kinase activity remains constant during development of the cerebellum—at least from E20 onward. Since considerable numbers of granule cells are generated postnatally, further studies are needed to determine how $pp60^{c\text{-}src}$ activity is regulated in these cells during development.

Wiestler and Walter (40) have recently found that $pp60^{c\text{-}src}$ protein kinase activity in the mouse forebrain and midbrain peaks at E18 and then declines somewhat postnatally, whereas activity in the cerebellum remains constant throughout development. Since the brain of an E18 mouse is at roughly the same stage of development as that of a rat at E20 (22), it would appear that the pattern of $pp60^{c\text{-}src}$ expression in the developing mouse brain is similar to that in the rat.

Cultured neurons have been found to contain a unique form of $pp60^{c\text{-}src}$ (8, 12, 13) that has a 6-amino acid insertion at residue 117 (41, 42) and migrates more slowly on $NaDodSO_4$ gels than fibroblast $pp60^{c\text{-}src}$. We have observed this unique form of $pp60^{c\text{-}src}$ in cultured striatal cells labeled metabolically with [^{35}S]methionine (not shown) and we have also detected it in tissue extracts, when proteins were phosphorylated *in vitro* (e.g., Fig. 2*A*, lane 4); however, it is poorly separated from the fibroblast form in our gels.

The critical question that remains is whether the observed increase in $pp60^{v\text{-}src}$ protein kinase activity in neurons reflects some specific aspect of their differentiation. The observation that $pp60^{v\text{-}src}$ can cause PC12 pheochromocytoma cells to differentiate in culture (43) is consonant with the view that activated $pp60^{c\text{-}src}$ is capable of inducing differentiation. A role for $pp60^{c\text{-}src}$ in differentiation is also suggested by the finding that there are significant increases in $pp60^{c\text{-}src}$ protein kinase activity when primary neurons (13), embryonal carcinoma cells (14), and myeloid cells (44, 45) differentiate in culture.

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