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Tyrosine Phosphorylation in Early Embryonic Growth Cones

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A large and growing body of evidence suggests that the regulation of tyrosine phosphorylation is important in the induction of axon growth. We have examined the subcellular distribution of enzymes regulating tyrosine phosphorylation in early embryonic brain, employing a preparation of isolated growth cone particles (GCPs). Because of the early developmental age and well-characterized nature of our tissue source, our GCP preparation offers some advantages over those described previously. As was found with other GCPs, our GCPs had relatively high levels of both the growth-associated protein GAP-43 and the intracellular tyrosine kinase pp60$^{c\text{-}src}$. In addition, we found that both total tyrosine kinase activity and total tyrosine phosphatase activity were concentrated two- to threefold in the GCPs relative to a neuronal membrane fraction. Two other nonreceptor tyrosine kinases, YES and FYN, were concentrated in the GCPs to a similar degree as that seen for SRC. In addition, we examined the developmental expression in brain of the three tyrosine kinases, using both a quantitative ELISA and Western blot analysis. Our results show that FYN, like SRC, reaches a peak of expression early in development, and declines thereafter. In contrast, expression of YES peaks later, and remains high in the adult brain. Immunofluorescence staining suggests that FYN is expressed both by neurons and by glia, and possibly by neuronal precursor cells. Our results implicate multiple tyrosine kinases as well as tyrosine phosphatases in growth cone function. In addition, the concentration of FYN in early embryonic growth cones combined with its early peak of expression suggests an important role for FYN in early neuronal development.

[Key words: neurite growth, growth cones, tyrosine phosphorylation,* src, fyn, *tyrosine kinases]

The regulation of axon growth and guidance is clearly complex, and involves both soluble factors, such as the neurotrophins, and substrate-acting neuronal growth factors, including extracellular matrix (ECM) proteins, immunoglobulin superfamily molecules, and cadherins (Bixby and Harris, 1991). In the case of the neurotrophins, progress in our understanding of their mechanism of action has resulted from the discovery that at least some high-affinity neurotrophin receptors are transmembrane protein tyrosine kinases (PTKs) of the *trk* family (Kaplan et al., 1991; Klein et al., 1991). A fair amount is known about the identity and structure of the neuronal receptors for the substrate-associated growth factors, which include β1 integrins as well as the cell adhesion molecules (CAMs) mentioned above. However, very little is known concerning their signal transduction mechanisms. A variety of experiments implicate intracellular Ca^{2+}, protein kinase C, and GTP-binding proteins in these transduction events (Bixby and Harris, 1991; Doherty et al., 1991; see also Hynes, 1992). In the case of β1 integrins, there is direct evidence for changes in tyrosine phosphorylation of a membrane protein caused by receptor activation (Kornberg et al., 1991). In addition, several lines of evidence suggest the involvement of tyrosine phosphorylation in substrate regulation of axon growth (see below).

Because the known receptors for CAMs and ECM proteins are not kinases, the PTKs postulated to be involved in axonal growth induced by these molecules are nonreceptor PTKs of the *src* or *abl* family (see Hanks and Quinn, 1991). The evidence for the involvement of these PTKs in axon growth is mainly indirect, and includes the observations that three *src*-family PTKs (SRC, YES, and FYN) are expressed at high levels in adult brain, that SRC expression peaks during the time of axon growth, and that there is a neuronal-specific form of SRC that is concentrated in growth cones (Cotton and Brugge, 1983; Brugge et al., 1985; Maness et al., 1988; Sudol et al., 1988; Cooke and Perlmutter, 1989; Ingraham et al., 1992). More recently, it has been shown that tubulin and vinculin are substrates for PTKs in growth cones, and that the tubulin phosphorylation can be inhibited by soluble fragments of neural CAMs (Igarashi et al., 1990; Matten et al., 1990; Atashi et al., 1992). Both of these substrates could be relevant to axonal growth regulation. Additionally, there is direct evidence that PTKs are involved in neuronal differentiation. First, genetic deletions indicate that the *Drosophila abl* gene product is necessary for correct axonal growth (Gertler et al., 1989; Elkins et al., 1990). Second, the v-*src* gene product can induce neuronal differentiation in PC12 cells, through a mechanism distinct from the NGF pathway (Alema et al., 1985; Cox and Maness, 1991). Finally, inhibition of PTKs in embryonic neurons can lead to a potentiation of neurite growth induced by a variety of substrates *in vitro* (Bixby and Jhabvala, 1992). Although the regulation of tyrosine phosphorylation is generally thought to take place through PTKs, it should be appreciated that much of the available evidence is also consistent with regulation by controlling the activity of protein tyrosine phosphatases (PTPs). Many PTPs have structures suggestive of transmembrane receptors (Saito and Streuli, 1991). In the best-studied example of signal transduction through

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nonreceptor PTKs, proper activation of T-cells (which requires the *src*-family member $p56^{lck}$), depends on the integrity of the PTP known as CD45 (Shaw et al., 1989; Garcia-Morales et al., 1990; Shaw and Thomas, 1991). In addition, it has recently been shown that several different PTPs are expressed on distinct subsets of axons during *Drosophila* development (Tian et al., 1991; Yang et al., 1991).

The expression of SRC, YES, and FYN in the nervous system has been studied to varying degrees. In the retina and cerebellum, SRC expression peaks during embryogenesis and declines with maturity, while YES expression reaches a peak in the adult (Sorge et al., 1984; Fults et al., 1985; Sudol et al., 1988). This has led to the idea that SRC is important during neuronal development, while YES has distinct functions, mainly in the adult (Sudol, 1988). Less is known about the developmental pattern of FYN expression. One recent immunohistochemical study demonstrated approximately equal levels of FYN protein in retinas from embryonic day 8–11 (E8–E11) and hatched chicks (other times were not examined), with the highest levels of staining in neuronal cell bodies (Ingraham et al., 1992).

Studies of growth cone transduction mechanisms are likely to be aided greatly by the use of preparations of isolated growth cones. In the 9 years since the pioneering studies of Pfenninger and colleagues (Pfenninger et al., 1983) and Gordon-Weeks and colleagues (Gordon-Weeks and Lockerbie, 1984), the value of such preparations has been amply demonstrated (e.g., Simkowitz et al., 1989; Meiri and Gordon-Weeks, 1990; Meiri and Burdick, 1991). Growth cone preparations have been made from E17–E18 rat brain (Pfenninger et al., 1983; Igarashi and Komiya, 1991), neonatal rat brain (Gordon-Weeks and Lockerbie, 1984), and E13–E14 chick brain (Cypher and Letourneau, 1991). In this article we report a growth cone preparation from early embryonic chick forebrain that we believe offers advantages over those described previously. We have used our growth cone preparation to examine the proteins regulating tyrosine phosphorylation in developing axons. Our results add significantly to the evidence supporting a role for tyrosine phosphorylation in growth cone function, and suggest that the situation may be more complex than previously believed.

Materials and Methods

Materials

Fertile White Leghorn chicken eggs were obtained from SPAFAS, Inc. (Norwich, CT). Antibodies were obtained from the following sources: anti-GAP-43 [monoclonal antibody (mAb) 10E8; Meiri et al., 1991] was a gift from Dr. K. Meiri (SUNY, Syracuse), anti-SRC (mAb 327) was purchased from Oncogene Sciences or was a gift from Dr. J. Brugge (University of Pennsylvania), anti-N-cadherin (NCD-2) was a gift from Dr. M. Takeichi (University of Kyoto), anti-YES (Sudol and Hanafusa, 1986) was a gift from Dr. M. Sudol (Rockefeller University), and anti-FYN and the FYN peptide were obtained from UBI (Lake Placid, NY). Alkaline phosphatase–coupled secondary antibodies were from Promega (Madison, WI). An assay kit for tyrosine kinases was obtained from GIBCO–Bethesda Research Labs (Gaithersburg, MD); subsequent phosphocellulose disks were from Whatman. Phosphotyrosine was obtained from Sigma (St. Louis, MO). All other chemicals were reagent grade.

Methods

Growth cone particle preparation. Growth cones were prepared essentially according to the procedure of Gordon-Weeks (1987), with minor modifications. Briefly, forebrains from E7 chick embryos were homogenized with a ground-glass homogenizer in 8 vol of BS buffer (0.32 M sucrose, 10 mM HEPES pH 7.4, with pepstatin, leupeptin, and phenylmethylsulfonyl fluoride) and spun at 1000 × *g* for 5 min (pellet = nuclear fraction), and the supernatant (S/N) was spun at 13,300 × *g* for 20 min. This pellet was rehomogenized in BS buffer and recentrifuged (13,300 × *g*, 20 min) to yield the P2 (pellet) and the S2 (S/N). The P2 was resuspended in 1 vol of BS buffer and layered onto a cushion of 7% Ficoll in BS buffer. The tube was spun at 58,000 × *g* for 20 min, and the layer on top of the Ficoll (growth cone particles, GCPs) and the bottom of the gradient (gradient pellet) were collected. Fractions were used immediately or quick frozen and stored at −80°C.

Electron microscopy. Freshly isolated GCPs (75 μl) were fixed by dropwise addition of 4% glutaraldehyde in cacodylate buffer, pH 7.2, until the volume was 1 ml, followed by incubation on ice for 25 min. The fixed membranes were spun down for 10 min in a microfuge at 10,000 rpm, and then resuspended in 100 μl of fixative and spun at 80,000 × *g* for 2 min in an airfuge. Membranes were postfixed in 1% OsO_4, dehydrated through a graded series of ethanols, and embedded in Epon. Sections were stained with lead citrate and uranyl acetate. For morphometric analysis, micrographs were overlaid with a grid pattern, and the intersections of the grid were scored according to the morphological types of profiles they contacted.

Western blotting. Fractions from the GCP preparation were solubilized in SDS sample buffer, run on 8% or 12.5% polyacrylamide gels, transferred to nitrocellulose, and probed with antibodies as described previously (Bixby and Zhang, 1990). Equal amounts of protein loading were confirmed by staining of blots with Ponceau S before blocking. Alkaline phosphatase reactions were stopped before apparent saturation, when blots were to be examined densitometrically. Densitometric analysis was performed with the JAVA video analysis system (Jandel Scientific), and was not strictly quantitative, owing to the probable nonlinearity of the enzyme reaction.

Tyrosine kinase assay. Assays were performed with the assay system of GIBCO–Bethesda Research Labs, following the manufacturer's instructions, which employ methods detailed by Casnellie (1991). The assay is based on the addition of ^{32}P-labeled PO_4 from ATP to the RR-SRC peptide, followed by precipitation of cellular proteins, binding of the peptide to phosphocellulose disks, and scintillation counting. Background radioactivity bound in the absence of RR-SRC peptide was subtracted from each sample.

Tyrosine phosphatase assay. PTP activity was measured essentially by the method of Maher (1991). The standard assay contained 20–50 μg of protein in a volume of 80 μl with 25 mM imidazole, pH 7.2, 0.1% β-mercaptoethanol, and 10 mM phosphotyrosine as substrate. The reaction was carried out for 10 min at 30°C and terminated by the addition of BSA to 2 mg/ml and trichloroacetic acid to 15%. Following precipitation, released PO_4 was measured by the method of Chen et al. (1956). Background levels of PO_4 in the presence of phosphotyrosine but the absence of exogenous protein were subtracted from each value. Background levels in the absence of phosphotyrosine were negligible. Although Maher (1991) reported linearity for up to 80 μg of protein for brain homogenates, it was necessary to stay below 40 μg in our assays, because of the high activities in the GCPs.

ELISA assays. Ninety-six well flat-bottom tissue culture plates were coated with nitrocellulose as described previously (Bixby and Zhang, 1990). Protein samples were solubilized in 0.5% 3-[(3-cholamidopropyl)-dimethylammono]-1-propane sulfonate (CHAPS), 150 mM NaCl, 20 mM HEPES (pH 7.4), spotted onto the nitrocellulose, and allowed to incubate for 20 min at room temperature (RT). Wells were rinsed twice with PBS, then twice with PBS, 0.5% nonfat dry milk, 0.2% Tween 20 before blocking with the latter buffer for 25 min. Plates were incubated with first antibody diluted in blocking buffer overnight at 4°C, washed three times with PBS, 0.5% Tween, and then incubated for 2 hr at RT in secondary antibody. Finally, plates were washed four times in PBS and then incubated with substrate solution (10 mM *p*-nitrophenylphosphate in 0.2 M Tris, pH 9, 5 mM $MgCl_2$) for varying times before optical densities were read at 410 nm. Concentrations of first antibody were determined using Western blots to determine the dilution at which antibodies were specific for the antigen of interest. Background readings for wells with no added membrane protein were subtracted from each point.

Immunofluorescence studies. Sections of the head from stage 31 embryos (fixed in 4% paraformaldehyde) were cut on a cryostat and stored at −80°C until use. Cultures of E7 forebrain neurons and E8 ciliary ganglion neurons were prepared on laminin-coated coverslips as described elsewhere (Bixby and Jhabvala, 1992) and fixed for 10 min in 3% paraformaldehyde in PBS. In both cases the cells were incubated overnight at 4°C with primary antibody diluted 1:150 in PBS, 1% goat serum, 0.02% saponin. Cells were washed four times in PBS and then

incubated for 1 hr at RT with goat anti-rabbit IgG coupled to rhodamine (Cappell), diluted 1:200 in PBS/goat serum/saponin. The cells were then washed four times in PBS and coverslipped for examination. For controls, a 10-fold excess (by weight) of the FYN peptide used to produce the antiserum (UBI) was added to the primary antibody and allowed to preincubate for 45 min before adding to the cells.

Results

A growth cone fraction from early embryonic chick brain

Previous growth cone preparations have been made from E17 or postnatal day 5 rat brains (Pfenninger et al., 1983; Gordon-Weeks and Lockerbie, 1984), and more recently, E14 chick brains (Cypher and Letourneau, 1991). Using the procedure of Gordon-Weeks (1987) with slight modifications, we have isolated a growth cone fraction from E7 chick forebrain. Of the two procedures established in the literature (Pfenninger et al., 1983; Gordon-Weeks and Lockerbie, 1984), this is the simplest and fastest, and appears to yield fractions of comparable purity to those isolated by the Pfenninger procedure. The source of tissue was chosen for several reasons. First, we chose tissue at an early stage of development, in the hopes of enriching for growth cones making outgrowth/pathway decisions rather than those at or near targets, which might be involved in early stages of synaptogenesis. Second, we chose not to use whole brain, since different regions of the brain have very different developmental schedules, which might complicate our results. Finally, it has been shown that the E7 chick forebrain, when dissociated, yields a population of cells that is more than 90% neuronal, and the outgrowth characteristics of these neurons have been partially analyzed (Kligman, 1982; Bixby and Jhabvala, 1992). It seemed advantageous to start with a tissue source that is both highly enriched in neurons and at least partially characterized. In our hands, the growth cone fraction constituted 2–3% of total homogenate protein (Table 1), roughly twofold higher than reported by Pfenninger et al. (1983) for E17 rat brain, but consistent with the relative paucity of differentiated non-neuronal cells in our starting material. We examined the morphology of the isolated growth cone fraction, for comparison with standard preparations, by fixing the growth cone particles in solution and spinning them down for staining and embedding. Electron micrographs of these fractions demonstrate that our preparation is comparable in homogeneity to those described earlier (Pfenninger et al., 1983; Gordon-Weeks and Lockerbie, 1984; Fig. 1*A*), and appears quite similar morphologically (Fig. 1*B*). "Growth cones" were defined as spherical particles 1–2 μm in diameter, which were filled with membranous cisternae and vesicles of various types, as well as filaments and occasional mitochondria. As seen previously, the majority of profiles are apparently either pinched-off growth cones, semi-intact growth cones, or growth cone "ghosts." Morphometric analysis revealed that more than 70% of profiles fell into this category (71%; n = 205). Of the remaining profiles, most were unidentifiable membranous structures, and 20% (or 5–6% of the total) were isolated mitochondria. These numbers are similar to those obtained from rat brain using a different protocol (Pfenninger et al., 1983). One difference between the GCPs prepared from E7 forebrain and those prepared from fetal rat brain using the same protocol is the apparent absence in our preparation of synaptic profiles (see Gordon-Weeks, 1987). These results indicate that a relatively simple procedure can be used to isolate a growth cone fraction from early embryonic chick brain. We will refer to this fraction as "growth cone particles" (GCPs), following established convention.

Table 1. Growth cone fractionation procedure

Fraction	Total protein	% Total protein
Homogenate	12.4 mg	(100)
P1 (nuclei)	2 mg	16
S1	9.3 mg	75
S2 (cytosol)	7.1 mg	57
P2 (membranes)	1.6 mg	13
GCP	0.35 mg	2.8
Gradient pellet	0.25 mg	2

The total amounts of protein in the indicated fractions and the percentage of the total protein in each fraction are provided for a typical growth cone isolation. There appears to be some loss of protein during the procedure, as the calculated yield is 91% for P1 + S1 compared with the homogenate. (S2 + P2 are expected to comprise less protein than the S1, because protein is lost in the wash step.) It is likely that some of the membranes are distributed in the Ficoll, since the pellet from this gradient makes up only a small fraction of the protein (15% of the membranes).

Although no proteins have been discovered to be specific to growth cones or GCPs, it has been shown that several different proteins are concentrated in these fractions, including an 80 kDa phosphoprotein, a membrane protein known as p34, the cellular *src* gene product SRC, and the growth-associated protein GAP-43 (Ellis et al., 1985; Meiri et al., 1986; Maness et al., 1988; Nelson et al., 1989; Simkowitz et al., 1989). To assess the degree to which our GCPs are comparable to others in this regard, we performed a Western blot analysis of various fractions from our procedure, using mAbs against GAP-43 and SRC. In accord with previous results (Meiri et al., 1986), GAP-43 was readily detectable in the membrane but not the soluble fraction, and was highly enriched in the GCPs (Fig. 2*A*). Densitometric analysis of the blots suggested that the GAP-43 concentration in the GCP was more than sevenfold higher than that in the membrane fraction. Similarly, the c-*src* gene product was enriched in membranes (not shown), and most highly concentrated in the GCPs (2.5–3-fold over crude membranes; Fig. 2*B* and data not shown). Our GCPs therefore share at least some biochemical properties with well-characterized previous preparations. The degree to which SRC (and other PTKs; see below) was concentrated in our GCPs appeared to be somewhat less than that seen previously, though a direct comparison cannot be made (Maness et al., 1988). This difference may be due, at least partially, to differences in the tissue source from which the GCPs were prepared (see Discussion).

Tyrosine kinase and phosphatase activities are concentrated in embryonic growth cones

Our hypothesis is that tyrosine phosphorylation is an important regulatory element in growth cone activity. In accord with this view, previous results using GCPs from E17 rat brain suggested that tyrosine kinase activity is concentrated in growth cones (Igarashi et al., 1991). In this case, however, the comparison was made not to membranes, but to the nuclear fraction and to the "C" fraction (which contains mitochondria and endoplasmic reticulum), which would not be expected to concentrate PTK activity. Additionally, the results were not quantitative. To assess whether PTK activity is concentrated in early embryonic growth cones relative to other neuronal membranes, we performed tyrosine kinase assays on various fractions from our GCP preparation. Our assay employed a modified peptide from SRC (RRLIEDAEYAARG) as phosphate acceptor. The PTK

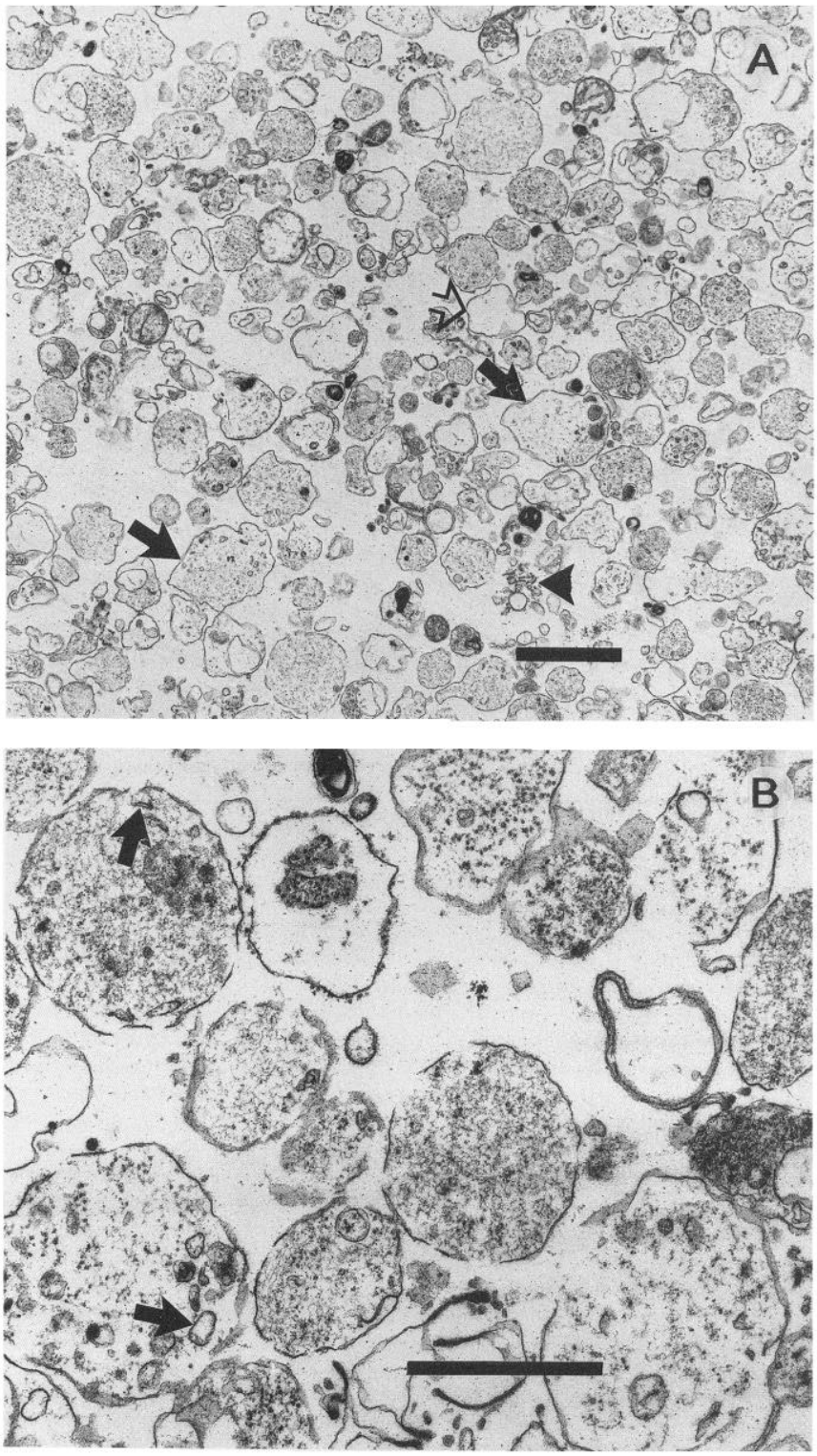

Figure 1. Electron microscopic examination of GCPs from E7 chick forebrain. *A*, Low-power view of the preparation, which shows a section through a typical region of the growth cone pellet. Most of the particles are either sealed growth cones (*solid arrows*), lysed growth cones, or growth cone "ghosts" (*open arrow*). Contamination includes some unidentified membrane fragments (*arrowhead*) and a few free mitochondria. Note that some of the fragments may be connected to GCPs not visible in this section (e.g., Gordon-Weeks and Lockerbie, 1984). We examined sections at several levels through two different pellets, to assess homogeneity of the pellet (not shown). *B*, Higher-power view of some of the GCPs, showing the typical morphology. GCPs are characterized by microfilaments and membrane cisternae of various kinds (*arrows*). Scale bars: *A*, 2 μm; *B*, 1 μm.

activity in our homogenates was similar to that seen by Maher (1991) in homogenates of whole E7 brain, using poly-GluTyr as substrate (16 pmol/min/mg protein vs 27 pmol/min/mg; Fig. 3). Most of this activity was found in the membrane fraction (P2); activity in this fraction was three times as high as in the soluble fraction. This is also in agreement with the results of Maher (1991), who found that 61% of PTK activity in E7 brain partitioned into the detergent phase of a two-phase system. Most importantly, we found that PTK activity was highest in the GCPs, almost 6-fold higher than the homogenate and 2.5-fold higher than the membrane fraction. The GCP fraction contained half of the activity found in the membrane fraction, even though it constituted only 15–20% of the protein in this fraction. This result implies that PTK activity is concentrated in early em-

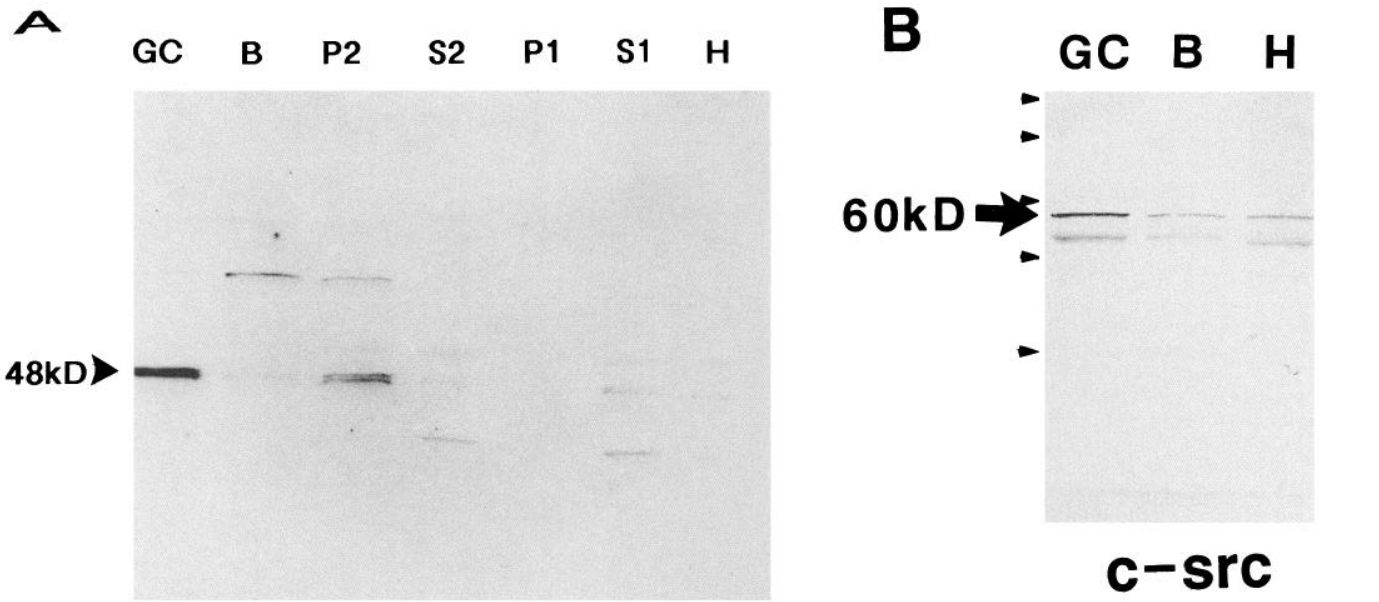

Figure 2. Western blots of fractions from GCP preparation (12.5% polyacrylamide gels), probed with an mAb to GAP-43 (*A*) or an mAb to SRC (*B*). Fractions include *H*, homogenate; *S1*, low-speed S/N; *P1*, nuclear fraction; *S2*, soluble fraction; *P2*, membrane fraction; *B*, bottom of the Ficoll gradient; and *GC*, growth cone fraction. *A*, *Arrow* at 48 kDa indicates the position of GAP-43 in these gels. Faint reactivity is visible in the homogenate, which is concentrated in the membrane fraction, and most highly concentrated in the GCPs. *B*, *Arrow* at 60 kDa indicates position of pp60$^{c\text{-}src}$. Reactivity is concentrated in the GCPs. Concentration of SRC in GCPs relative to neuronal membranes was determined in three other gels (not shown). The lower (minor) band of SRC reactivity may be the "non-neuronal" form of SRC. Molecular weight markers (116 kDa, 97 kDa, 66 kDa, 45 kDa, 29 kDa) are indicated by *arrowheads*.

bryonic growth cones, even compared to other neuronal membranes. These results extend previous observations showing that SRC activity is concentrated in growth cones (Maness et al., 1988). In agreement with the qualitative results of Igarashi and Komiya (1991), the heavier fractions of the crude membranes (Fig. 3, column B) and the nuclear fraction (not shown) both had very low levels of PTK activity compared to the GCPs.

In principle, regulation of tyrosine phosphorylation can be at the level of either PTK activity, PTP activity, or some combination of the two. We therefore examined whether PTP activity, like PTK activity, might be concentrated in embryonic growth cones, by using the PTP assay of Maher (1991). As expected from previous results, we found PTP activity to be roughly 1000-fold higher per mg protein than PTK activity (e.g., Tonks et al., 1991). The PTP activity in the homogenate was very similar to that described by Maher (1991) in whole E7 brain (15.7 vs 19.2 nmol/min/mg protein; Fig. 4). Unlike the PTK activity, PTP activity was found to be roughly the same in the homogenate, soluble, and membrane fractions (Fig. 4). This probably reflects the existence in brain of soluble as well as membrane-associated or transmembrane PTPs (Lombroso et al., 1991; Saito and Streuli, 1991). Despite this broad sub-

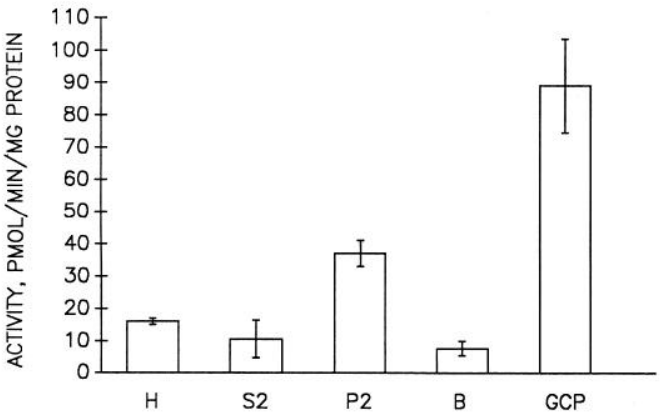

Figure 3. Tyrosine kinase activities in fractions from the growth cone preparation, measured using the RR-SRC peptide as substrate. Total PTK activity (minus background) is given for the homogenate (*H*), soluble fraction (*S2*), membrane fraction (*P2*), bottom of the Ficoll gradient (*B*), and GCPs. Activity is concentrated in the membranes, but is two- to threefold higher in the GCPs. Points are the mean ± SEM for three experiments run in duplicate.

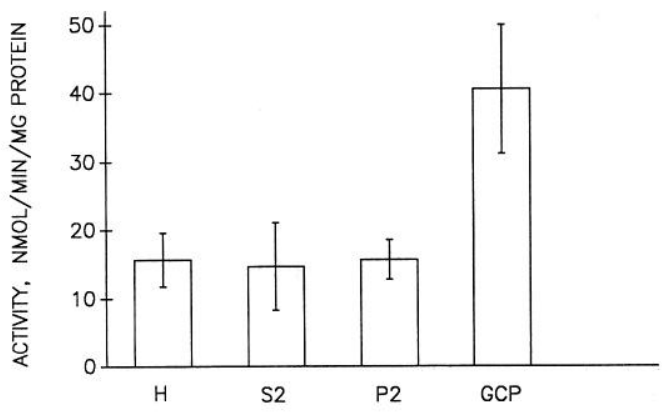

Figure 4. Tyrosine phosphatase activities in fractions from the growth cone preparation, measured using phosphotyrosine as substrate. Total PTP activities are approximately the same in the homogenate (*H*), soluble (*S2*), and membrane (*P2*) fractions, but were low in the nuclear fraction (not shown). PTP activity is concentrated two- to threefold in the GCPs relative to any other fraction. Points are means ± SEM for three experiments run in duplicate or triplicate.

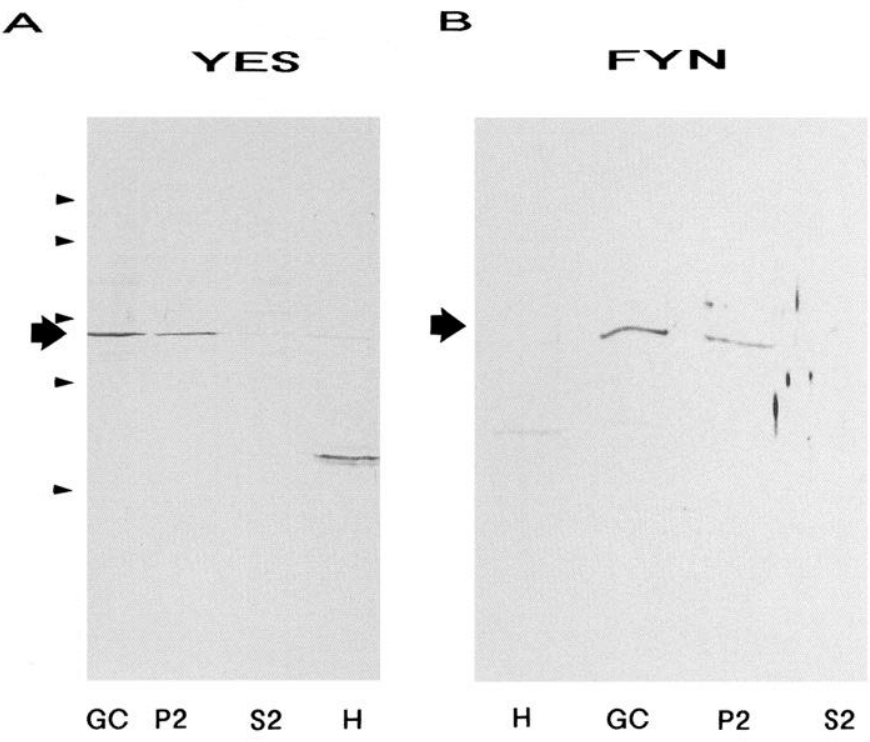

Figure 5. Western blots of fractions from growth cone preparation (12.5% gels), probed with polyclonal antibodies to YES (*A*) or to FYN (*B*). The positions of FYN and YES at 60 kDa are shown with *arrows*. Both YES and FYN reactivities are concentrated in the growth cone fraction, to a similar degree. A low-molecular-weight band is recognized by the anti-YES antibody in the homogenate; this band was concentrated in the nuclear fraction (not shown), essentially absent from the membrane fraction, and is clearly not YES. These results were confirmed in three other gels (not shown), representing two different GCP preparations. Molecular weight markers and abbreviations are as in Figure 2.

cellular distribution, PTP activity was concentrated 2.6-fold in the GCPs relative to the homogenate, soluble, or membrane fraction. Our data indicate that each of the two classes of enzymatic activity responsible for the control of tyrosine phosphorylation is concentrated in early embryonic growth cones.

Two other members of the src *family are also concentrated in growth cones*

Besides SRC, two other nonreceptor PTKs, YES and FYN, are known to be expressed at relatively high levels in adult brain (Cooke and Perlmutter, 1989; Sudol, 1989; Zhao et al., 1991). Although these have not been studied as intensively as SRC in the nervous system, it is possible that they may also play a role in the embryonic growth cone. We used Western blot analysis to examine the presence of YES and FYN in subcellular fractions of E7 chick forebrain. As expected from previous results, both YES and FYN segregated with the membrane fraction, and were essentially absent in the soluble fraction (Fig. 5). More importantly, both of these SRC family members were concentrated in the GCPs relative to crude neuronal membranes (Fig. 5). The relative concentration of YES and FYN in the GCPs (1.8–3-fold higher than the P2; $n = 3$ for each) was similar to that seen for SRC (not shown). In control experiments using the NCD-2 mAb and an antiserum to NCAM (Bixby and Reichardt, 1987), the amount of N-cadherin and NCAM found in the GCPs was similar to that found in the membrane fraction (0.8–1.2× relative to the P2 concentration), which suggests that our results with PTKs are not simply the result of purification of plasma membranes during the GCP preparation. Because the Western blot assay is not strictly quantitative, the relative concentrations are not known exactly. However, the results can be taken as clear evidence that YES and FYN are concentrated in GCPs. These results suggest that all three of the *src*-family PTKs known to be highly expressed in the CNS are concentrated in early embryonic growth cones.

FYN expression peaks during early embryogenesis

The assumption that SRC is involved in the differentiation of neurons is based largely on its pattern of expression. One argument commonly invoked is that SRC expression peaks during embryonic development, and tapers off after hatching, which suggests a role in neuronal differentiation (Sorge et al., 1984; Fults et al., 1985). In contrast, YES expression rises somewhat later during development and remains high in the adult retina and cerebellum (Sudol et al., 1988). Little is known concerning the developmental pattern of FYN expression in the brain, but preliminary results from this laboratory suggested that *fyn* RNA is highly expressed in the chick forebrain at E5–E10, even compared to *src* (K. Bodden and J. L. Bixby, unpublished observations). We therefore examined the expression of FYN protein during embryonic development of the brain, using a quantitative ELISA assay. Control experiments using various amounts of E17 membrane protein showed that the assay was linear up to ≈25 μg of membrane protein (not shown). We then used the assay to measure the amount of SRC protein in various fractions of the GCP preparation, to compare our ELISA assay with the semiquantitative Western blots. The amount of SRC protein in the GCP fraction was 2.2× the amount in the membrane fraction, using the ELISA assay, which compares reasonably well with our previous Western blot results. Our preliminary results therefore suggest that the ELISA assay can be used to assess quantities of *src* family PTKs in developing brain.

We examined PTK protein levels in brain membranes taken from E5 to posthatch chicks. As expected from earlier results in the cerebellum and retina (Sorge et al., 1984; Fults et al., 1985; Sudol et al., 1988), SRC expression in the brain peaked

around E11, a time when neural differentiation is proceeding, and expression continued to decline after hatching (Fig. 6*A*). Interestingly, we found that FYN expression also peaked in the early embryo, and also declined at later stages of development (Fig. 6*A*). In fact, the peak of FYN expression in our experiments was even earlier than that of SRC. This result is consistent with a role for FYN in early neuronal development. By comparison, the YES protein would be expected to reach its peak of expression later and to remain at high levels in mature animals (Sudol et al., 1988). The results of our ELISA assay on YES are in agreement with this prediction. YES levels started to increase around E13, but reached a peak at E20, and remained high in adult chicken brain (Fig. 6*B*). The apparent biphasic expression of YES during brain development was also seen in cerebellum and retina (Sudol et al., 1988). To ensure that our ELISA results accurately reflected recognition of the FYN protein, rather than a contaminating activity, we also performed Western blots, using membrane proteins from brains of various embryonic ages (Fig. 7). Using this assay, we found that SRC expression peaked around E11–E14, and that FYN expression peaked at E8–E10, reaching a value roughly 1.5× that at E19 (average of three blots). For comparison, the SRC peak measured in this way was also about 1.5× the E19 value ($n = 3$). Although these data are not as reliable quantitatively as the ELISA data, they are certainly consistent with them. In summary, the results of these assays suggest complementary or similar roles for SRC and FYN in early neuronal development, and a somewhat different (but perhaps overlapping) role for YES. Because FYN is concentrated in neuronal growth cones and reaches a peak early in embryonic development, it will be important to examine its potential role in neuronal differentiation.

Neuronal expression of FYN

Only one study to date has analyzed the expression pattern of FYN in the embryonic CNS, using the E11 retina (Ingraham et al., 1992). This study concluded that FYN is a product of differentiated retinal neurons and possibly Müller glia. To examine the expression pattern of FYN in the early embryonic CNS, we stained sections of stage 31 (E7) CNS with the FYN antiserum. In the brain, there was punctate immunoreactivity, often concentrated over cell bodies (Fig. 8*A,B*). FYN staining was not uniform in the brain, with highly immunoreactive regions near those with relatively low staining (Fig. 8*B*). In the developing neural retina, cells were outlined with FYN staining; this was most prominent at the ventricular surface next to the pigment epithelium (Fig. 8*C,D*), but extended throughout most of the thickness of the retina (Fig. 8*C*). At this time, there are still numerous mitotic figures in this region (not shown), suggesting the possibility that FYN is expressed by precursor cells. It is possible, however, that the stained cells are newly formed, migrating neurons, especially the ganglion cells (Ingraham et al., 1992).

To see whether FYN is expressed by differentiated neurons and/or glia, we stained cultures of ciliary ganglion neurons with the FYN antibody. In these cultures, FYN immunoreactivity was found in both neurons and glia (Fig. 9*A*). In the neurons, immunoreactivity was sometimes concentrated in the distal neurite and proximal growth cone (Fig. 9*B*, open arrows), but other growth cones were stained to the same degree as their neuritic shafts (Fig. 9*B*, solid arrow). In cultured sensory neurons, for comparison, SRC often appears at similar levels in the neurite and growth cone (Maness, 1986). Immunoreactivity could also be seen in thin projections from the neurite (Fig. 9*A*). The distal regions of growth cones were stained only very lightly, consistent with background staining (Fig. 9*C*), suggesting that FYN is excluded from the tips of growth cones. Similar observations were made in cultures of E7 forebrain neurons (not shown).

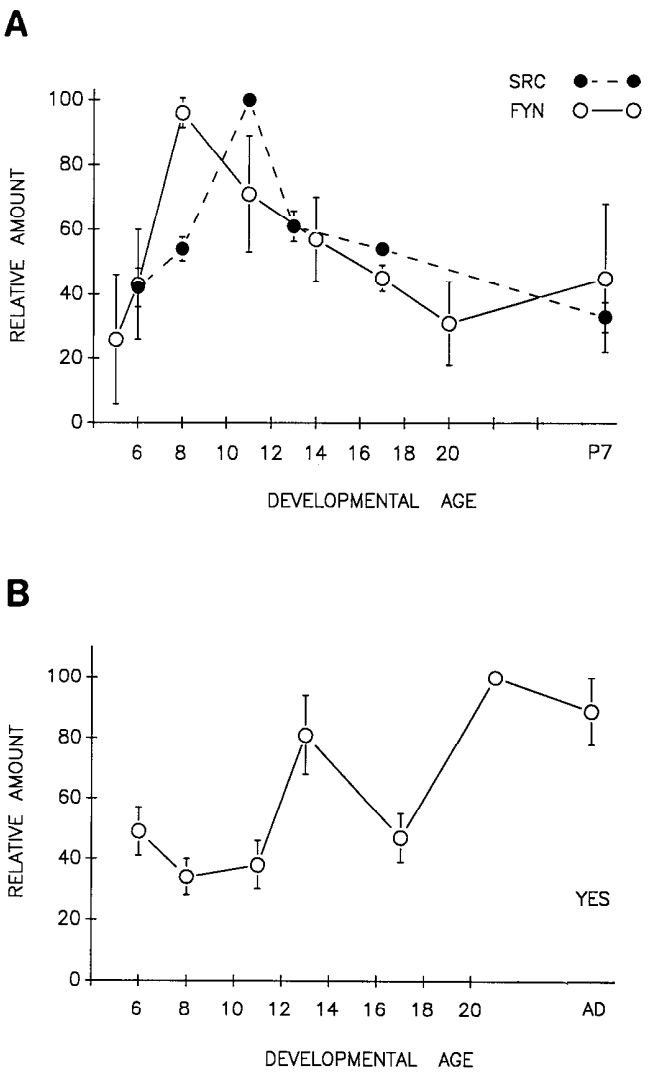

Figure 6. Developmental profile of expression of SRC, FYN, and YES proteins, measured using the ELISA assay. The relative amounts (mean optical density as a percentage of maximum) are plotted versus developmental age (*6–20* = E6–E20; *P7* = 7 d posthatch; *AD* = adult). *A*, Profile of SRC expression (*solid circles, broken line*) is plotted with the profile of FYN expression (*open circles, solid line*). Both proteins reach a peak during early embryogenesis, and decline steadily thereafter. The pattern of SRC expression is consistent with earlier observations in the cerebellum and retina. Points are mean ± SEM for three experiments run in duplicate or triplicate. The apparently earlier rise in FYN expression is real; the assays were run in the same plate with the same protein samples. *B*, Pattern of expression of YES contrasts with that of SRC and FYN. Levels are low at early times, with a possibly biphasic rise starting about E13. Significantly, YES expression remains high in the adult brain, as shown previously in the cerebellum.

Discussion

We have described a preparation of "growth cone particles" that we believe has a number of advantages for the study of axonal

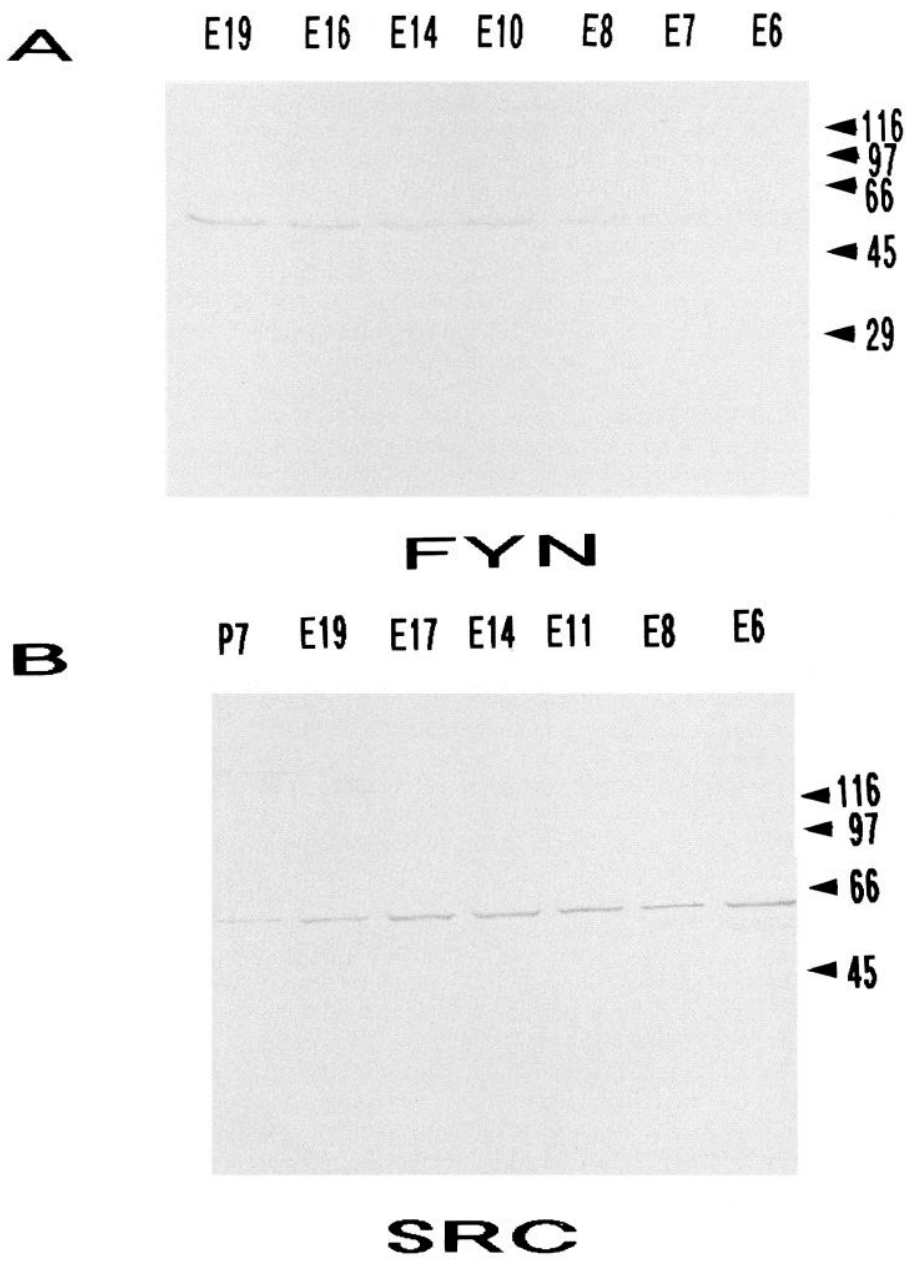

Figure 7. Western blots of membrane fractions from developing brain, probed with antibodies to FYN (*A*) and SRC (*B*). Both antibodies recognize predominately a 60 kDa band at all ages tested. In the case of FYN, a second band at 180 kDa also appeared in some blots (not shown), which disappeared when fresh, well-reduced samples were used and probably is oligomers of FYN. The FYN protein peaks at E8–E10, while the SRC band reaches a peak at E11–E14 ($n = 3$ experiments each for FYN and SRC). Molecular weight markers are given in kDa (*arrowheads*).

growth-related events. As mentioned, our preparation relies on procedures developed by Gordon-Weeks and colleagues. Several advantageous features of this preparation, including the speed with which it can be obtained and the relative homogeneity and apparent physiological integrity of the GCPs, have been described (Gordon-Weeks, 1987; Meiri and Burdick, 1991). However, the tissue source we have chosen offers unique advantages for some kinds of investigation. The early stage of development we chose is likely to be before many target recognition– or synaptic formation–related events have occurred. This should provide a population of GCPs in a state appropriate for the examination of axonal growth responses. The absence of synaptic profiles in our preparation is consistent with this idea. Second, the cells in the E7 forebrain have been demonstrated to be almost entirely neuronal following a culture period of 18 hr, making it possible to exclude significant glial contamination. It should be noted, however, that the E7 forebrain contains neuronal precursor cells, and some of these may differentiate during 18 hr *in vitro*, making it difficult to estimate the magnitude of this "contamination." Finally, with respect to our own interests in substrate-induced axon growth and tyrosine phosphorylation, we have shown that the neurons contained in the E7 forebrain (1) respond to L1, N-cadherin, and laminin by producing neurites, and (2) respond to inhibition of tyrosine kinases with a potentiation of this growth (Bixby and Jhabvala, 1992). The GCPs prepared from this tissue therefore have, in principle, the physiological characteristics necessary for the biochemical elucidation of these events.

The degree to which SRC is concentrated in our GCPs, relative to the membrane fraction, is apparently less than that seen previously using GCPs from E18 rat brain (Maness et al., 1988). Judging from the electron microscopy, this does not appear to

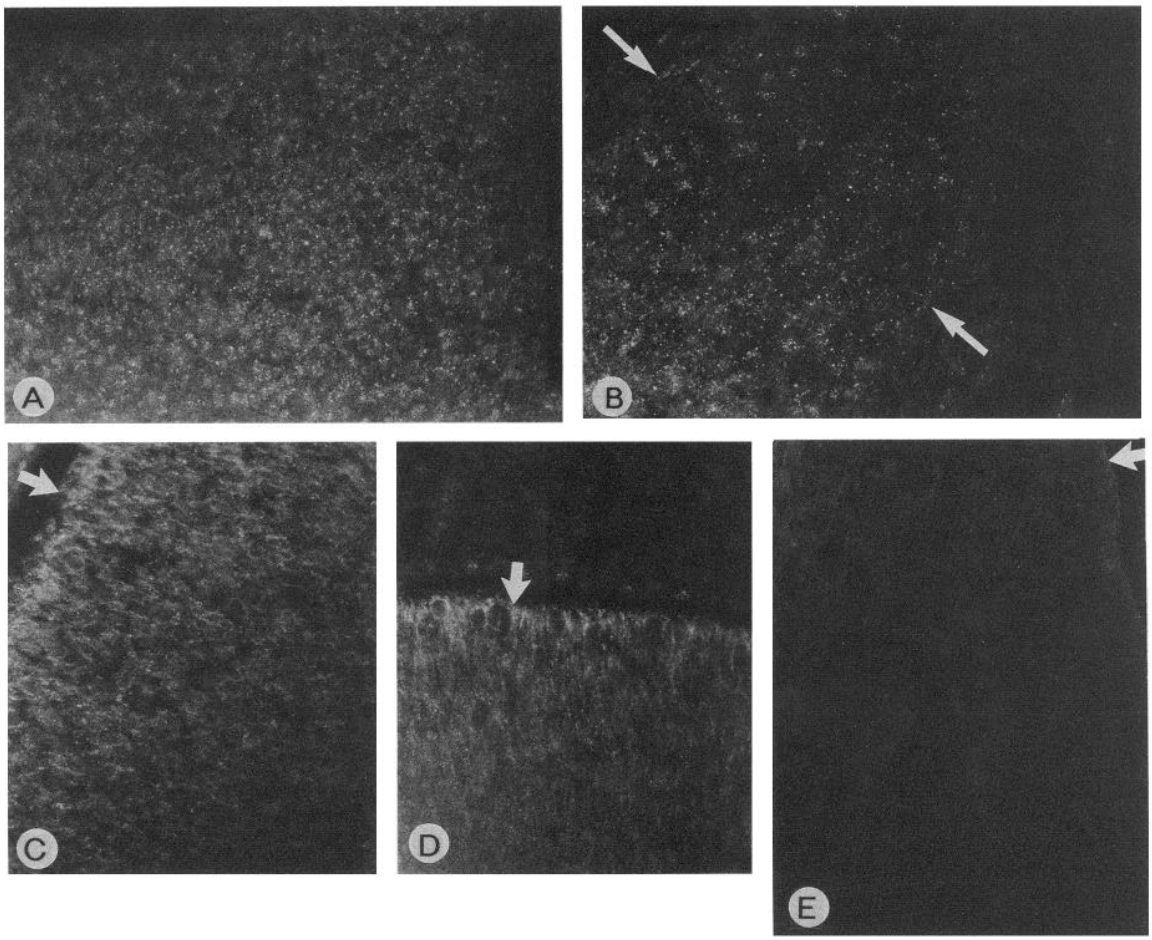

Figure 8. Immunofluorescence localization of FYN in E7 CNS. *A, B,* and *E*, brain; *C* and *D,* retina. The section shown in *E* is a control, where the FYN antibody was incubated with the peptide used to prepare the antibody. Cells in the brain are stained in a punctate manner, and areas of staining are interspersed with areas of low immunoreactivity (*B*). *Arrows* in *B* show the borders of a brain nucleus that was visible in the phase contrast image. In the stage 31 retina, staining outlines cells through much of the thickness of the neural retina (*C*), but is concentrated at the ventricular border near the pigment epithelium (*arrows* in *C* and *D*). Staining is confined to the neural portion of the retina (*D*). Staining is abolished by incubation with the FYN peptide (*E*). *Arrow* in *E* represents ventricular border.

be due to greater contamination of our preparation with irrelevant material. Rather, we believe that at least some of the discrepancy is due to differences in the tissue of origin. Brains from E18 rats would be expected to have considerable numbers of glial cells (Jacobson, 1978). Therefore, comparison of a growth cone fraction with any other would be a comparison of *neuronal* growth cones with *neuronal and glial* fractions. In contrast, we are comparing different neuronal fractions largely with each other (and with precursor cell membranes; see above). If more of the SRC is in neurons than in glia (Brugge et al., 1985), the relative purification seen in GCPs from E18 rats would be expected to be greater than in our GCPs. In any case, our results allow us to suggest that SRC (as well as YES and FYN) is concentrated in growth cones relative to other parts of the neuron, by about threefold.

Much of the evidence linking SRC to growth cone function is based on patterns of gene expression and subcellular localization of the protein. There are many other PTKs expressed in the nervous system, but their distributions are comparatively poorly understood, making comparison with SRC problematic. We have now shown that total PTK activity in the E7 forebrain is concentrated in growth cones and, in particular, that YES and FYN are also concentrated in these fractions. These findings suggest two conclusions. First, they add to a growing body of evidence linking the regulation of tyrosine phosphorylation to growth cone function. Second, they suggest that it may be useful to examine the role of other PTKs besides SRC in these functions. Because YES is expressed later in development than SRC, and remains elevated in adult brain, it has been suggested that YES performs different functions than SRC in the development of the nervous system (Sudol et al., 1988). However, the concentration of YES in early embryonic growth cones emphasizes the possibility that at least some of the biological roles of this PTK are similar to those of SRC.

Our results with FYN strongly suggest a role for this PTK in neuronal differentiation, including growth cone function. Previous studies showed that FYN, like SRC, has an alternatively spliced form found mainly in the nervous system (Cooke and Perlmutter, 1989). In this article we also demonstrate that FYN is concentrated in embryonic growth cones, and that FYN expression reaches a peak in the early embryonic brain. Immunofluorescence studies show that FYN is expressed in both neurons and glia, when cultured neurons are examined, and that neuronal staining includes both axons and growth cones, with

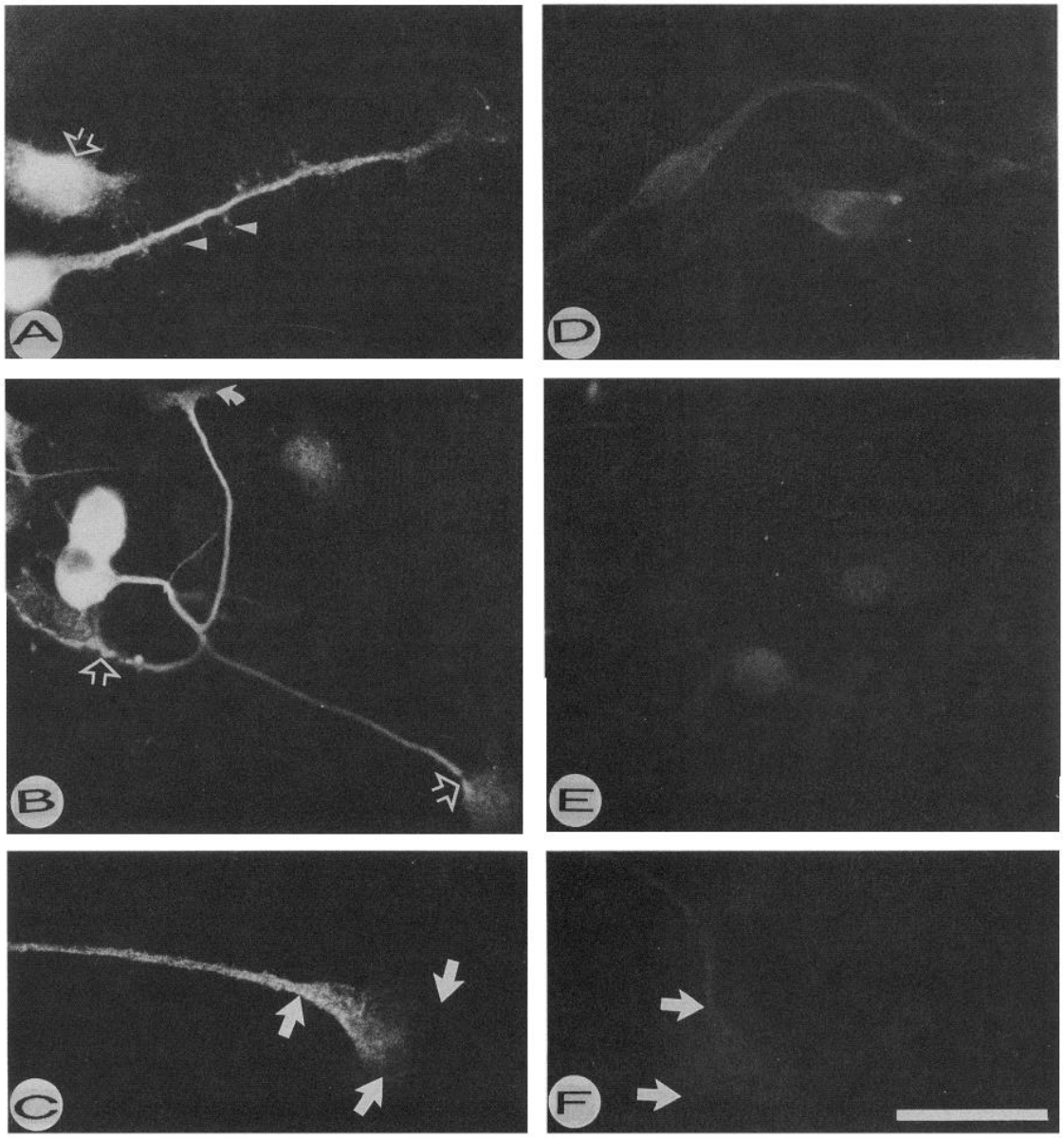

Figure 9. Cells from the E8 ciliary ganglion were cultured overnight and then stained with the FYN antibody. *A–C* are examples of FYN immunoreactivity, and *D–F* are corresponding controls, incubated in the presence of the FYN peptide. In *A*, the *arrowheads* point to filamentous FYN-positive processes emanating from the neurite, and the *open arrow* points to a ganglionic non-neuronal cell that is FYN positive. In *B*, the *open arrows* point to neurites in which staining is increased in the distal neurite and proximal growth cone, and the *solid arrow* points to a neurite/growth cone that is more uniformly stained. The *arrows* in *C* and *F* demarcate the borders of growth cones, showing that the distal growth cone is poorly stained or negative. Scale bar: 20 μm for *A, B, D,* and *E*; 10 μm for *C* and *F*.

some concentration in the growth cones. In summary, the concentration of FYN in growth cones, its early peak of expression, and the presence of a neural form of the protein argue that FYN is just as likely as SRC to play a key role in neuronal differentiation.

FYN is also highly expressed by cells at the ventricular surface in the early embryonic retina. This may represent expression by newly formed neurons, rather than mitotic precursors (see Ingraham et al., 1992). Nevertheless, in view of the potential role of FYN in neuronal proliferation (Grant et al., 1992), it will be important to examine this issue.

Our results with YES, FYN, and SRC can be used to support the idea of "functional redundancy" among different nonreceptor PTKs in the embryonic nervous system. Indeed, the lack of gross neural defects in transgenic mice lacking SRC expression has been explained by invoking this concept (Soriano et al., 1991). However, it seems very likely that each of the SRC family PTKs has unique functions during normal development. Each PTK has its own developmental pattern of expression (Fig. 6), its own unique distribution *within* the CNS (Sudol et al., 1988; Ingraham et al., 1992; present results), and each is likely to have unique functional domains outside of the kinase domain (e.g., Espino et al., 1992). It is clear that further studies will be required to elucidate the relative contributions of SRC, YES, and

FYN to axonal growth as well as other aspects of neuronal differentiation. However, the present results underscore that this examination should not be limited to SRC.

A great deal of study has been devoted to PTKs, with much less attention being paid to PTPs, both in general and in the nervous system in particular. This is likely due to the prominence of PTKs as retroviral transforming proteins and growth factor receptors, the apparent lack of PTP substrate specificity *in vitro*, and the fact that PTP activities are so high as to suggest "constitutive" and unregulated activation. Recently, however, it has become clear that PTPs are a large and diverse family of enzymes with potential for regulation and substrate specificity (Saito and Streuli, 1991; Trowbridge, 1991). The potential involvement of PTPs in axonal growth has been emphasized by the finding that different "receptor-type" PTPs are expressed on different subsets of axons in the *Drosophila* embryo (Tian et al., 1991; Yang et al., 1991). We have now shown that PTP activity is concentrated in early embryonic growth cones compared to any other subcellular fraction. Because of the predominatcly neuronal starting material, this concentration is not explained by purification of neuronal membranes in general. We suggest, therefore, that PTPs are likely to have important roles in the growth cone, presumably including axon elongation. Although only one PTP has been identified that is specifically expressed in the nervous system (Lombroso et al., 1991), we have recently identified several novel PTPs that are expressed in embryonic brain, and that may prove to be selectively expressed (C. Bedolla and J. L. Bixby, unpublished observations). It will likely be necessary to continue to expand our knowledge of PTPs in the nervous system, if we are to elucidate the role of tyrosine phosphorylation in neuronal differentiation.

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