NCAM–dependent Neurite Outgrowth Is Inhibited in Neurons from *Fyn*-minus Mice

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Abstract. Src-related nonreceptor protein tyrosine kinases in nerve growth cones (p59^{fyn}, pp60^{c-src}, and pp62^{c-yes}) are potential intracellular signaling molecules for cell adhesion molecule–directed axonal growth. To determine whether src-related tyrosine kinases mediate NCAM-dependent neurite outgrowth, cultures of cerebellar and sensory neurons from *fyn*$^-$, *src*$^-$, and *yes*$^-$ minus mice were analyzed for neurite outgrowth on monolayers of NCAM140-transfected L fibroblasts. NCAM-dependent neurite outgrowth was selectively inhibited in cultures of cerebellar and dorsal root ganglion neurons from *fyn*$^-$, but not *src*$^-$ or *yes*$^-$ mice. Neurite outgrowth by *fyn*$^-$, *src*$^-$, or *yes*$^-$ neurons on untransfected fibroblast monolayers was unaffected, indicating that these kinases do not contribute significantly to axon growth on at least some integrins or other adhesive substrates present on fibroblasts. This study demonstrates that p59^{fyn} is an essential component of the NCAM signaling pathway leading to axonal growth.

PROMOTION or inhibition of axonal growth is mediated by interactions of cell surface receptors on neuronal growth cones with guidance cues in the local environment. The neural cell adhesion molecule (NCAM)[1] is a widely expressed member of the immunoglobulin superfamily of cell surface molecules found on nerve growth cones and neuronal processes. Of nearly 30 possible NCAM isoforms generated through alternative splicing of a single gene, there are three major isoforms: transmembrane forms of 180 (NCAM180) and 140 kD (NCAM140), which differ only in their cytoplasmic domains, and a 120-kD glycophosphatidyl inositol-linked form (NCAM120) (Reyes et al., 1991; Gennarini et al., 1986; Murray et al., 1986). Antibody blocking studies and transfection of NCAM cDNAs into fibroblast monolayers have demonstrated that NCAM is a potent substrate for neurite outgrowth in culture (Silver et al., 1984; Bixby et al., 1987; Fraser et al., 1988; Doherty et al., 1990a), and mice deficient in NCAM have defects in the olfactory bulb in vivo (Tomasiewicz et al., 1993; Kremer et al., 1994). NCAM-dependent neurite growth is mediated principally by a homophilic-binding mechanism whereby NCAM on the neuronal cell surface binds to NCAM on an opposing surface (Hoffman and Edelman, 1983; Doherty et al., 1990b), but NCAM may also participate in heterophilic binding to heparin sulfate proteoglycan or other heterophilic

ligands (Cole and Akeson, 1989; Murray and Jensen, 1992; Grumet et al., 1993).

The ability of the larger NCAM isoforms to promote neurite outgrowth is not due to adhesive force alone, but may result from intracellular signal transduction via the cytoplasmic domain. Binding of NCAM molecules or NCAM antibodies to PC12 cells activates second messenger cascades resulting in calcium influx through a pertussis toxin-sensitive calcium channel (Schuch et al., 1989; von Bolen et al., 1992; Saffell et al., 1992). Tyrosine-specific protein kinases appear to be proximal components in neural cell adhesion molecule signaling, as demonstrated by studies showing that triggering of NCAM, L1, or myelin-associated glycoprotein (MAG) inhibits tyrosine phosphorylation of tubulin and other substrates in membranes from a subcellular fraction enriched in nerve growth cones from fetal rat brain (Atashi et al., 1992). The specific tyrosine kinases mediating these effects have not been identified, but are likely to be nonreceptor PTKs of the *src* family (reviewed in Maness, 1992). Support for this hypothesis is provided by the findings that the *src*-related nonreceptor PTKs p59^{fyn}, pp60^{c-src}, and pp62^{c-yes} are concentrated in nerve growth cone membranes (Maness et al., 1988; Bare et al., 1993; Bixby and Jhabvala, 1993; Meyerson and Pahlman, 1993) and exhibit patterns of expression on developing axonal tracts similar to cell adhesion molecules (Sorge et al., 1984; Fults et al., 1985; Zhao et al., 1991; Bare et al., 1993). At least one of them, pp60^{c-src}, is upregulated during nerve regeneration (LeBeau, 1991; Ignelzi et al., 1992). These nonreceptor tyrosine kinases have highly homologous catalytic domains (~80% identity) and NH$_2$-terminal variable domains (5–30% identity) that are

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1. *Abbreviations used in this paper:* DRG, dorsal root ganglion; MAG, myelin-associated glycoprotein; NCAM, neural cell adhesion molecule.

likely to confer specificity to their molecular interactions at the membrane (Cooper, 1990). There are two forms of p59^{fyn} arising as a consequence of alternative mRNA splicing. One form is expressed in T-lymphocytes; the other is found in brain and other tissues (Cooke et al., 1989). PTKs can influence neurite growth, as seen by the ability of PTK inhibitors to increase the length of neuritic processes in primary neuronal cultures and in PC12 pheochromocytoma cells (Bixby and Jhabvala, 1992; Miller et al., 1993). Moreover, expression of pp60^{v-src}, an activated PTK expressed from the retroviral src gene, induces neurite extension in PC12 cells in the absence of nerve growth factor (Alema et al., 1985; Eveleth et al., 1989; Rausch et al., 1989; Cox et al., 1991).

The recent development of mice carrying null mutations in the fyn (Appleby et al., 1992; Stein et al 1992), src (Soriano et al., 1991), and yes genes (Soriano, P., unpublished results) has allowed a direct test of the hypothesis that non-receptor PTKs are components of adhesion molecule signaling pathways. Despite the widespread localization of pp60^{c-src} in developing axonal tracts, src- mice exhibit no obvious neurological defects, but have osteopetrosis, a bone remodeling deficiency associated with osteoclast malfunction (Soriano et al., 1991; Horne et al., 1992). However, cerebellar granule cells from src- mice are strikingly impaired in their ability to extend neurites on the cell adhesion molecule L1 in culture, indicating that pp60^{c-src} is on the L1 signaling pathway for neurite outgrowth (Ignelzi et al., 1994). These results can be reconciled if the L1-pp60^{c-src} pathway is functionally compensated by another adhesion system in vivo. For example, NCAM in association with a different nonreceptor PTK might perform this compensatory function in src- neurons on axonal tracts bearing both cell adhesion molecules. p59^{fyn} is a candidate PTK for mediating NCAM-dependent neurite growth, because it is colocalized with NCAM on many axonal tracts in the developing central and peripheral nervous system and in the adult olfactory system (Gennarini et al., 1986; Bare et al., 1993; Yagi et al., 1993). Fyn- mice display some neurological defects, including loosely organized dendrites of CA1 pyramidal cells of the hippocampus, increased neuronal number, and blunted long-term potentiation (Grant et al., 1992). Mice carrying another mutation in the fyn gene show partially impaired myelination (Umemori et al., 1994).

To determine whether p59^{fyn}, pp60^{c-src}, or pp62^{c-yes} function in the NCAM signaling pathway, cultures of cerebellar granule cells and sensory neurons from fyn, src, and yes null mutant mice were analyzed for neurite outgrowth on fibroblasts transfected with a full length NCAM140 cDNA. Here it is reported that NCAM-dependent neurite outgrowth was selectively inhibited in fyn-, but not src- or yes- cerebellar granule cells and sensory neurons in culture. These results demonstrate that p59^{fyn} is an essential component of the NCAM signaling pathway in neurons leading to neurite outgrowth, and suggest that src-related kinases have nonredundant but compensatory functions in adhesion molecule-directed axonal growth.

Materials and Methods

Mouse Mutants and Genotyping

Homozygous fyn- (Stein et al., 1992), yes- (Soriano, P., unpublished results) and heterozygous src- (Soriano et al., 1991) mutant mice were bred to produce homozyous pups. Wild type C57Bl or C57Bl/129Sv mice were bred to produce control mice born on the same day as the mutant mice. The genotypes of the src heterozygote intercrosses were determined by a multiplex PCR. For the PCR reaction, two oligonucleotide primers were generated from c-src genomic sequences (Soriano et al., 1991), and another primer was generated from the neo gene of the targeting vector. The genomic c-src sense primer 2069 (5'-AGCAACAAGAGCAAGCCCAAG-GAC-3'), the genomic c-src antisense primer 2455 (5'-GTGACGGTGTCC-GAGGAGTTGAAG-3'), and the neo-ATG antisense primer (5'-AATC-CATCTTGTTCAATGGCCGATC-3') were used simultaneously in the multiplex PCR reaction with genomic DNA isolated from the tails of 4-d-old pups. The sizes of the PCR reaction products unambiguously defined the genotypes of the mice. Template DNA was isolated by incubating a 1-cm length of tail overnight at 55°C in 700 μl of digestion buffer (0.5 mg/ml proteinase K, 1% SDS, 100 mM NaCl, 100 mM EDTA, 50 mM Tris HCl, pH 8.0). After phenol/chloroform extraction and ethanol precipitation, the DNA (1 μg) was amplified in Taq polymerase buffer containing 1.5 mM MgCl$_2$, Taq polymerase (4 U), 0.2 mM each dNTP, 1 μM primer 2069, 0.5 μM primer 2455, 0.5 μM primer neo, and 4% formamide in a final volume of 100 μl. Amplifications were carried out using a "hot start" at 94°C for 6 min, followed by 30 cycles of the following conditions: 1 min denaturation at 94°C, 1 min annealing at 65°C, and 3 min extension at 72°C. Multiplex PCR products were visualized by ethidium bromide staining of DNA fragments after electrophoretic separation on a 4% agarose gel. The wild type genotype produced a single 200-bp DNA fragment; the src- homozygous genotype produced a single 400-bp DNA fragment; and the heterozygous genotype produced DNA fragments of 200 and 400 bp.

Cell Culture Procedures

Mouse L-fibroblasts transfected with a full-length cDNA clone encoding the 140-kD isoform of NCAM (gift from Dr. Richard Akeson's laboratory, Children's Hospital Medical Center, Cincinnati, OH) and untransfected L fibroblasts were maintained on plastic flasks in DME with high glucose containing 10% fetal calf serum (GIBCO BRL, Gaithersburg, MD). For co-culturing neurons on fibroblasts monolayers, untransfected and NCAM140-transfected L cells were seeded at 8 × 10^4 cells per chamber of a multiwell dish (Lab-Tek, Naperville, IL), which had been coated with 0.025% poly-L-lysine (Sigma Chemical Co., St. Louis, MO) and 0.45 mg/ml rat tail collagen in 30% ethanol. Cells were grown to confluency. Cultures were maintained at 37°C in a 5% CO$_2$ atmosphere.

Cerebellar neuronal cultures, consisting primarily of granule cells, were prepared from the cerebellum of postnatal day 4 mice as described (Schnitzer and Schachner, 1981). The cerebellum was incubated for 10 min in 1% trypsin/0.05% DNase at room temperature, washed twice with Ca^{2+}/Mg^{2+}-free Hank's buffered salt solution, and resuspended in a 0.05% DNase solution. Cells were dissociated by mechanical trituration and plated sparsely onto the confluent L cell monolayers (5 × 10^3 cells per well). Dorsal root ganglion (DRG) neurons isolated from postnatal day 4 mice were incubated in 0.5% trypsin for 20 min at 37°C, and washed twice with F14 media (GIBCO BRL). Cells were dissociated by mechanical trituration and plated sparsely onto confluent L cell monolayers in multiwell dishes (1.5 DRG per chamber). Cerebellar co-cultures were maintained in Earle's basal media supplemented with 2.5 mg/ml glucose and 10% horse serum (GIBCO BRL). DRG co-cultures were maintained in F14 medium supplemented with N1 additives (GIBCO BRL), 10% horse serum, 5% fetal calf serum, and 2.5S β-NGF (50 ng/ml; Boehringer Mannheim, Indianapolis, IN).

Neurite Extension Assay

Cultures of neurons on fibroblast monolayers were maintained for 40 (cerebellar cells) or 20 h (DRG neurons), and then fixed for 1 h in 4% formalin, added directly to the medium. Cultures were washed in ice-cold PBS, and incubated in 2% horse serum (GIBCO BRL), 0.5% BSA (Sigma Chemical Co.), 0.05% (vol/vol) Tween-20, 0.05% (vol/vol) Triton X-100 for 30 min to block nonspecific binding sites and permeabilize the cells. Fixed cells were incubated overnight with a mouse monoclonal antibody (1:3,000) directed against GAP-43 (clone 91E12; gift of Drs. David Schreyer, Queen's University, Kingston, Canada, and Pate Skene, Duke University, Durham, NC; Schreyer and Skene, 1991), followed by biotinylated goat anti-mouse IgG (1:200) and complexes of avidin-biotinylated peroxidase (Vector Laboratories, Burlingame, CA). Reaction product was developed with diaminobenzidine (0.5 mg/ml) as substrate for 10 min, followed by dehydration and mounting with Permount.

GAP-43 immunostained images of cells were recorded under Nikon

phase contrast optics onto a super VHS video recorder, and the length of the longest neurite per cell was measured on a Hammamatsu C2400 image processor using cursor overlay. For measurement of neurites from cerebellar neurons, only neurites longer than ~8 μm and not in contact with other cells were measured. For DRG neurons, neurites of any length not in contact with other cells were measured. Cerebellar and DRG neuron attachment to L fibroblast monolayers was assayed by counting at least 300 cells from a total of at least 100 randomly selected fields.

ELISA Determination of NCAM in Neuronal Cultures

The relative level of NCAM expression on wild type and *fyn⁻* cerebellar neurons was determined using ELISA. Five replicate cultures of 80,000 cerebellar neurons from each genotype were plated in individual wells of a 96 well microtiter plate that had been coated with 0.025% poly-L-lysine. After 40 h, cultures were fixed for 1 h with 4% formalin, which was added directly to the culture medium. Cultures were washed with ice-cold PBS and incubated for 1 h with 10% BSA to block nonspecific sites. Cultures were then incubated for 1.5 h at 37°C with a 1:500 dilution of an affinity-purified NCAM polyclonal antibody recognizing all three NCAM isoforms (gift of M. Schachner, Swiss Federal Institute of Technology, Zurich, Switzerland; Simon et al., 1991), a 1:250 dilution of the NCAM-180-specific polyclonal antibody 4299 (gift of John Hemperly, Becton Dickinson Research Center, Triangle Park, NC), or a 1:1,000 dilution of tubulin polyclonal antibody (Sigma Chemical Co.) to normalize the amount of protein present in replicate wells. Pilot experiments established these dilutions to provide conditions of antibody excess. A linear relationship was established between antibody binding and the number of wildtype cerebellar neurons plated (5,000–160,000 cells per well). In controls, the NCAM polyclonal antibody did not bind to nontransfected L fibroblasts, but did bind effectively to NCAM purified from adult mouse brain (Kadmon et al., 1990) after adsorption to microtiter wells (1 μg/well). After two washes in PBS/0.05% Tween-20, cultures were incubated for 1 h with a 1:2,500 dilution of horseradish peroxidase-conjugated anti-rabbit IgG (Pierce Chemical Co., Rockford, IL) and washed twice in PBS/0.05% Tween-20 and once in 0.1 M acetate buffer. Finally, cultures were incubated with 1 mg/ml (2.2-azino-*bis*-3-ethylbenzthiazoline-6-sulfonic acid) (Sigma Chemical Co.), and 0.01% (vol/vol) H_2O_2 in acetate buffer. After 5–10 min, the reaction was stopped by addition of 0.6% SDS, and the absorbance of the reaction product measured spectrophotometrically at 405 nm using a WS050 Wellscan automatic microplate reader. Results were averaged and the standard error of the mean determined.

Results

Neurite Outgrowth in Response to NCAM Is Selectively Abolished in Fyn⁻ Cerebellar Neurons

Cerebellar granule cells provide an important model for neurite outgrowth, due to their well-characterized migratory pathway (Rakic, 1971; Burgoyne and Cambray-Deakin, 1988). The molecular mechanism for granule cell migration involves several cell adhesion molecules, including NCAM and L1, which are present on developing axonal pathways in the cerebellum in vivo (Chuong, 1990; Persohn and Schachner, 1987). In addition, p59^fyn (Bare et al., 1993; Yagi et al., 1993), pp60^c-src (Fults, et al., 1985), and pp62^c-yes (Zhao et al., 1991) are known to be expressed in developing cerebellar granule cells. To determine whether p59^fyn was essential for NCAM-directed neurite outgrowth, cultures of cerebellar neurons from wild type and homozygous *fyn⁻* mice (postnatal day 4) were analyzed for neurite outgrowth on confluent monolayers of mouse L cells stably transfected with a full length cDNA encoding NCAM140 (Liu et al., 1993). The NCAM140 isoform used for transfection lacks the VASE insert in the fourth immunoglobulin domain, which downregulates the neurite outgrowth-promoting activity of NCAM (Doherty et al., 1992). Cerebellar cultures consist of ~90% granule cell neurons, 5–7% stellate and basket neurons, and the remainder oligodendroglia and astrocytes (Schnitzer and Schachner, 1981). At 40 h after plating, cultures were fixed and neurons visualized on the fibroblast monolayers by staining for GAP43 immunoreactivity (Fig. 1). GAP43 is a neuronal-specific marker that strongly stains the cell body and processes of cerebellar and DRG neurons, although it may be excluded from some dendrites of cultured neurons (Goslin et al., 1988). As described previously, neurite lengths were measured by computer-assisted digitized image analysis.

Neurite outgrowth by wild type neurons on the NCAM-expressing monolayers was stimulated relative to outgrowth on control L cell fibroblasts as seen in the distribution of neurite lengths (compare Fig. 2, *A* and *B*), and as described previously (Doherty et al., 1990*a*). The NCAM-dependent component of neurite extension was evident in an increased mean neurite length of the cultures on NCAM (Table I). Remarkably, the NCAM-dependent component of neurite extension was completely eliminated in the *fyn⁻* cerebellar neuron cultures, as seen in the shift in distribution of neurite lengths in the *fyn⁻* neurons growing on NCAM-transfected monolayers to lower values compared to wild type neurons on NCAM-transfected monolayers (Fig. 2 *A*). The mean neurite length of *fyn⁻* neurons on NCAM-transfected monolayers was the same as that of *fyn⁻* neurons on control monolayers (Table I). Blocking experiments with antibodies that bind to β_1 integrins have shown that neurite outgrowth on nontransfected 3T3 fibroblast monolayers depends in part (~30%) on neuronal integrins (Doherty et al., 1991). The neurite length distribution of *fyn⁻* cerebellar neurons on nontransfected L cell fibroblasts was the same as that of wild type neurons (Fig. 2 *B*). Furthermore, *fyn⁻* neurons are indistinguishable from wild type neurons when grown on laminin absorbed to nitrocellulose (Ignelzi et al., 1994). These results indicated that a functional *fyn* gene was required for NCAM-dependent neurite outgrowth by cerebellar neurons but not for neurite outgrowth supported by adhesion molecules present on the surface of nontransfected L fibroblasts including at least some integrins, which transduce signals from laminin.

Cerebellar cultures from homozygous null mutant mice lacking functional *src* (Soriano et al., 1992) or *yes* (Soriano, P., unpublished data) genes were also examined for neurite extension on NCAM-transfected and -nontransfected L cells. In contrast to *fyn⁻* cerebellar neurons, the *src⁻* and *yes⁻* cerebellar neurons showed no impairment in neurite outgrowth on NCAM-transfected monolayers relative to wild type neurons (Fig. 2, *C* and *D*; Table I). Neither the distribution nor the mean neurite length in the *src⁻* or *yes⁻* cultures was significantly changed relative to the wild type neuronal cultures on NCAM-transfected monolayers. These results demonstrated that neither the c-*src* nor *yes* gene was needed for NCAM-dependent neurite outgrowth by cerebellar neurons. The *src⁻* and *yes⁻* minus neurons also showed no difference in neurite length distribution or mean neurite length on nontransfected L cell monolayers (Fig. 2 *B*, Table I). The apparent lack of requirement of functional c-*src*, c-*yes*, or *fyn* genes for neurite growth supported by integrins that transduce laminin signals is in agreement with other studies in which neurite growth by *src⁻*, *yes⁻*, and *fyn⁻* cerebellar neurons was unaltered on laminin-coated nitrocellulose (Ignelzi et al., 1994).
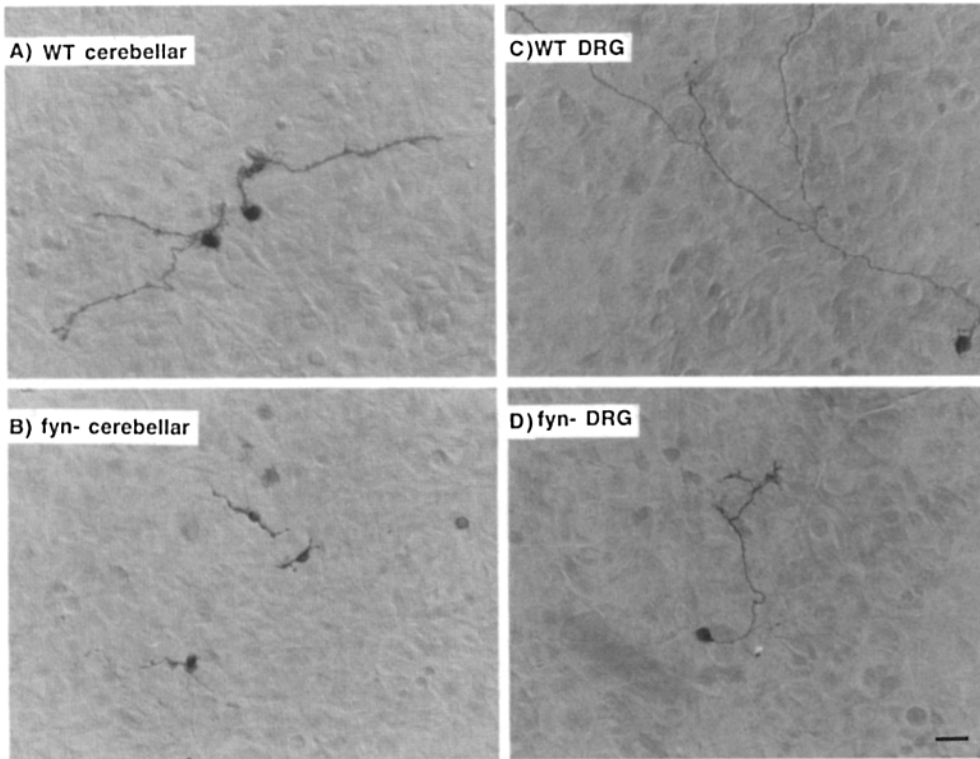
Figure 1. Cultures of cerebellar and DRG neurons on NCAM140-transfected and -nontransfected L cell monolayers. Cerebellar and sensory neurons from fyn⁻ and wild type mice were plated onto confluent monolayers of NCAM140-transfected mouse L cell fibroblasts, and neurites were allowed to extend for 40 and 20 h, respectively. Neurons were visualized by immunoperoxidase staining with GAP-43 antibodies. (A) Wild type cerebellar neuron co-cultures. (B) Fyn⁻ cerebellar neuron co-cultures. (C) Wild type DRG neuron co-cultures. (D) Fyn⁻ DRG neuron co-cultures. A–D are the same magnification. Bar, 25 μm.

The selective inhibition of NCAM-dependent neurite outgrowth in fyn⁻ neurons was not accompanied by an inability of the cells to extend neurites. NCAM-expressing monolayers were a highly effective substrate, with >89% of neurons extending neurites in all cultures (Table I). On NCAM-transfected monolayers, fyn⁻ and wild type cultures exhibited no significant differences in the density of cells attached to the monolayer (cells/mm²), the density of cells that extended neurites (cells with neurites/mm²), or the percent of cells with neurites (Table I). On nontransfected
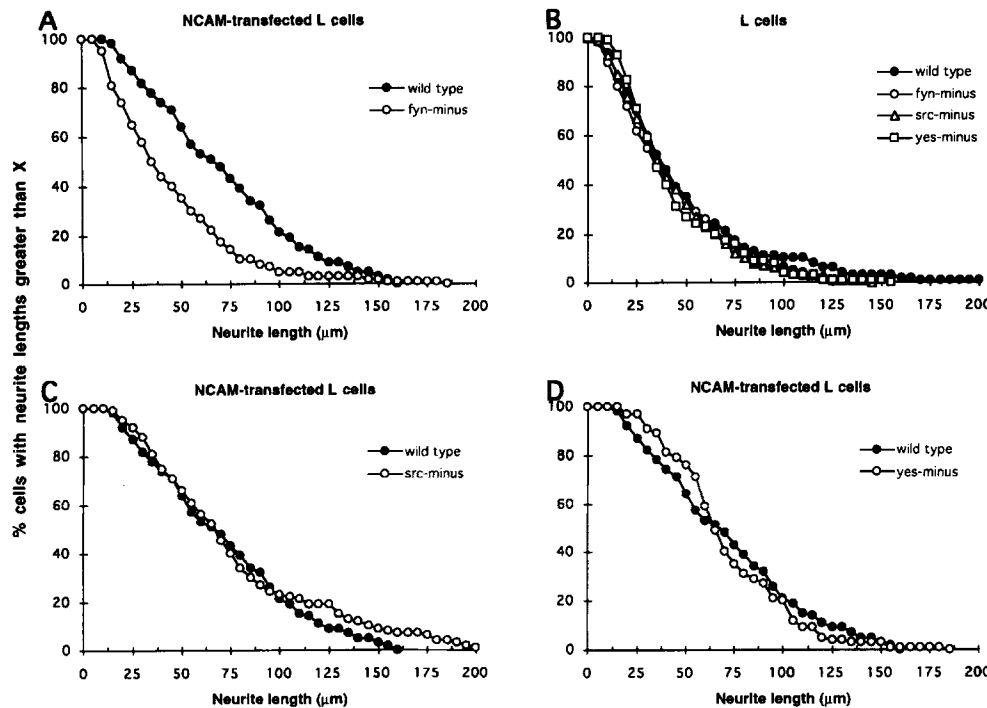
Figure 2. Distribution of neurite lengths in fyn⁻, src⁻, and yes⁻ cerebellar cultures on NCAM140-transfected and -nontransfected fibroblast monolayers. Cerebellar granule cells from mutant and wild type mice were plated onto confluent monolayers of NCAM140-transfected and -nontransfected mouse L cell fibroblasts. Neurites were allowed to extend for 40 h. Cells were fixed, stained for GAP-43 immunoreactivity, and neurite lengths were measured by video-enhanced phase contrast microscopy. Graphs show neurite length distributions of (A) fyn⁻ and wild type cerebellar neurons on NCAM-transfected fibroblasts, (B) fyn⁻, src⁻, and yes⁻ and wild type neurons on untransfected fibroblasts, (C) src⁻ and wild type neurons on NCAM140-transfected fibroblasts, and (D) yes⁻ and

wild type neurons on NCAM-140 transfected fibroblasts. The experiments with fyn⁻ neurons have been repeated five times, and the experiments with src⁻ and yes⁻ neurons three times, with similar results.

Table I. Neurite Extension of Fyn-, Src-, and Yes-minus Cerebellar Neurons on NCAM

	Genotype	Neurite length	n	Cells/ mm²	Cells with neurites/ mm²	Percent cells with neurites
		(mean ± SEM)				
NCAM	WT	70.6 ± 3.1*	151	33	30	91
	fyn⁻	44.9 ± 2.5*	174	33	30	91
	src⁻	76.6 ± 3.6	166	33	31	94
	yes⁻	71.1 ± 3.7	100	29	27	93
L cells	WT	48.2 ± 3.0	158	25	23	92
	fyn⁻	43.6 ± 2.4	166	27	24	89
	src⁻	48.2 ± 2.3	152	29	26	90
	yes⁻	43.8 ± 3.3	100	23	21	91

Neurite lengths of cerebellar neurons on L fibroblasts and L fibroblasts transfected with NCAM 140 kD were measured 40 h after plating. *n* is the number of cells measured for mean neurite length. Cell density was determined by counting at least 300 total cells from 100 random fields.
* Denotes a statistically significant difference of $P < 0.005$.

monolayers, cell attachment, the density of cells with neurites, and percent of cells with neurites in the *fyn⁻* cultures was not reduced compared to wild type cultures. No differences in these parameters were noted in the *src⁻* or *yes⁻* cultures on NCAM-transfected or control monolayers. The average number of neurites per cell in both *fyn⁻* and wild type cerebellar neuron cultures was the same (~1.6 neurites/cell), indicating that the reduced neurite extension by *fyn⁻* neurons was not a consequence of elaboration of additional neuronal processes. There was little, if any neurite fasiculation in these sparse cultures. No obvious differences were observed in the general morphology of cultured neurons or in gross cerebellar architecture of the *fyn⁻* mice at the light microscope level on postnatal day 4 or in the adult, although subtle changes might have been undetectable.

The inhibition of neurite outgrowth by *fyn⁻* neurons was not due to reduced levels of NCAM expression. An ELISA was performed using an NCAM polyclonal antibody that recognizes all three major isoforms of NCAM, or an NCAM polyclonal antibody that recognizes only the 180-kD isoform to quantitate the relative levels of NCAM180 and other isoforms in wild type and *fyn⁻* cerebellar neuron cultures (see Materials and Methods). The relative amount of total NCAM (all three isoforms) was nearly identical in wild type

(2.1 absorbance units ± 0.1) and *fyn⁻* cerebellar neuronal cultures (2.0 absorbance units ± 0.1). The relative amount of the NCAM180 isoform in wild type (1.2 absorbance units ± 0.1) and *fyn⁻* neurons (1.2 absorbance units ± 0.1) was also the same. Since neurons prepared from postnatal day 4 mouse cerebellum express the NCAM180 and NCAM140 isoforms with only small amounts of NCAM120 (He et al., 1987; Gegelashvili et al., 1993), the relative level of NCAM140 and NCAM120 isoforms did not appear to be appreciably altered in *fyn⁻* neurons. Cerebellar neurons isolated from postnatal day 4 mice do not express the VASE exon, which downregulates the ability of NCAM140 to promote neurite outgrowth (Doherty et al., 1992; Liu et al., 1993). It should be noted that this assay does not distinguish neuritic NCAM from somal NCAM, and may include surface and nonsurface NCAM since fixation permeabilizes cells to some extent.

Neurite Outgrowth in Response to NCAM is Inhibited in Fyn⁻ Dorsal Root Ganglion Neurons

The cerebellar granule cell differs from many other neurons in its mechanism of elaborating neuronal processes, because the cell soma migrates along Bergmann glia as it descends into the internal granule layer, while extending a short axon that forms the parallel fibers (Ramon y Cajal, 1911). In contrast, projection neurons such as sensory neurons of the DRG, extend long axonal processes from the cell body after migration. DRG neurons cultured from postnatal mice express all three isoforms of NCAM (Seilheimer and Schachner, 1988) without the VASE exon (Small and Akeson, 1990). To examine whether the absence of a functional *fyn* gene altered the ability of DRG neurons to extend neurites on NCAM140-transfected L cells, cultures of DRG neurons from wild type and homozygous *fyn⁻* mice (postnatal day 4) were analyzed for neurite growth on confluent monolayers of NCAM140-transfected L cells and nontransfected L cells at 20 h after plating. Neurite outgrowth was inhibited in *fyn⁻* DRG neuronal cultures on the NCAM-expressing monolayers as shown by the shift in distribution of neurite lengths to lower values compared to wild type DRG neurons (Fig. 3 A). Neurite outgrowth on nontransfected L cell monolayers was nearly identical for *fyn⁻* and wild type DRG neurons (Fig. 3 B). Examination of mean neurite lengths showed that the NCAM-mediated component of neu-
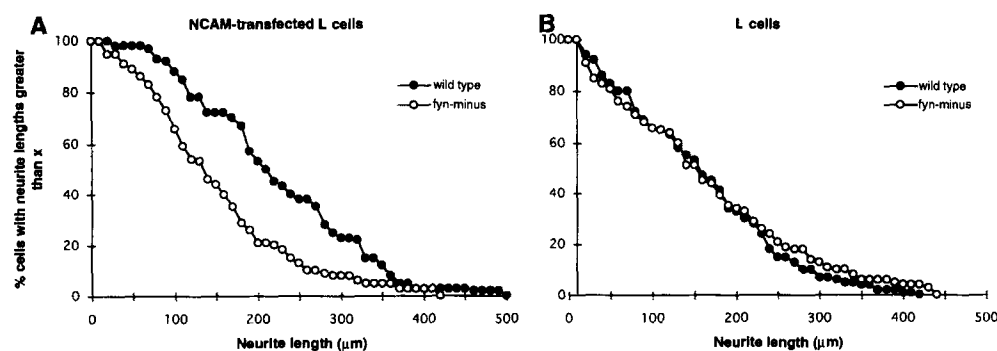
Figure 3. Distribution of neurite lengths in *fyn⁻* DRG neurons on NCAM140-transfected and -nontransfected fibroblast monolayers. DRG neurons from *fyn⁻* and wild type mice were plated onto confluent monolayers of NCAM140-transfected and -nontransfected mouse L cell fibroblasts. Neurites were allowed to extend for 20 h. Cells were fixed, stained for GAP-43 immunoreactivity, and neurite lengths were measured by video-enhanced phase contrast microscopy. Graphs show neurite length distributions of (A) *fyn⁻* and wild type DRG neurons on NCAM-transfected fibroblasts, and (B) *fyn⁻* and wild type DRG neurons on nontransfected fibroblasts. These experiments have been repeated twice with similar results.

Table II. Neurite Extension of Fyn-minus DRG Neurons on NCAM

Substrate	genotype	Neurite length	n	Cells/mm²	Cells with neurites/mm²	Percent cells with neurites
		(mean ± SEM)				
NCAM	WT	228 ± 8*	146	8	8	92
	fyn⁻	161 ± 9*	106	10	9	90
L cells	WT	156 ± 8	147	10	8	83
	fyn⁻	154 ± 9	124	6	5	79

Neurite lengths of DRG neurons on L cells and NCAM 140-transfected L cells were measured 20 h after plating. n is the number of cells measured for mean neurite length. Cell density was determined by counting at least 300 total cells from 100 random fields.
* Denotes a statistically significant difference of $P < 0.005$.

Table III. Neurite Extension of Small and Large Diameter DRG Neurons from Wild Type and Fyn-minus Mice on NCAM

Substrate	Genotype	Small (≤20 μm)	n	Large (>20 μm)	n
		(mean ± SEM)		(mean ± SEM)	
NCAM	WT	229 ± 8	132	242 ± 37	14
	fyn⁻	163 ± 10*	88	152 ± 26*	18
L cells	WT	153 ± 8	128	177 ± 27	19
	fyn⁻	152 ± 11	93	160 ± 19	31

Neurite lengths (mean ± SEM) of DRG neurons on NCAM 140-transfected L cells and on control L cells from the same experiment shown in Table II were measured 20 h after plating and grouped according to cell body diameter into small (≤20 μm) and large (>20 μm) DRG neurons. n is the number of cells measured for mean neurite length.
* Denotes a statistically significant difference of $P < 0.005$.

rite growth was completely blocked in the fyn⁻ cultures (Table II). Sensory neurons extended neurites at a greater rate than cerebellar granule cells, as seen by the larger values for mean neurite length in the DRG cultures 20 h after plating compared to the cerebellar neuron cultures 40 h after plating (Table I).

The complete loss of NCAM-dependent neurite growth by fyn⁻ DRG neurons was not accompanied by a reduction in the ability of the cells to attach to the substrate and extend neurites. On NCAM-transfected monolayers, fyn⁻ and wild type cultures showed no significant differences in the density of cells attached to the monolayers (cells/mm²), density of cells that extended neurites (cells with neurites/mm²), or the percent of cells with neurites (Table I). Similarly on control monolayers, there was no change in these parameters in the fyn⁻ DRG cultures. The lower density of adherent cells in the DRG cultures compared to the cerebellar cultures was due to a lower initial plating density, which was required to minimize fasciculation among the rapidly extending neurites of the sensory neurons. Because the average number of neurites per cell was 1.9 in both fyn⁻ and wild type cultures, inhibition of NCAM-dependent neurite extension in fyn⁻ DRG cultures was not due to elaboration of superfluous numbers of neurites. No differences in neurite branching or general morphology of the fyn⁻ DRG neurons were noted.

Myelinated and nonmyelinated neurons are enriched respectively in populations of large and small diameter DRG neurons in culture, and have different functional modalities including differences in neurite elongation in their response to cell adhesion molecules (Seilheimer and Schachner, 1988; Johnson et al., 1989). It was of interest to ask if fyn⁻ DRG neurons with cell body diameters of <20 μm (small) and >20 μm (large) were differentially affected in neurite outgrowth ability on NCAM140-transfected and -nontransfected L cells. Neurite growth of both small and large DRG neurons from fyn⁻ mice was completely inhibited on NCAM-transfected monolayers (Table III). Neurite growth on control monolayers was unimpaired. These results indicated that both subpopulations of DRG neurons were equally compromised for NCAM-directed neurite growth and that the inhibition of neurite growth due to NCAM appeared to be complete.

In conclusion, these results indicate that p59ᶠʸⁿ, but not pp60ᶜ⁻ˢʳᶜ or pp62ᶜ⁻ʸᵉˢ, was required for NCAM-directed neu-

rite extension by cerebellar granule cells and by sensory neurons in culture.

Discussion

Here we report that NCAM-dependent neurite extension by fyn⁻ neurons growing on monolayers of NCAM140-transfected L cells was selectively abolished, demonstrating an essential function for p59ᶠʸⁿ in the intracellular signaling pathway for NCAM-mediated axonal growth. The finding that NCAM-dependent neurite growth was completely lost in fyn⁻ cerebellar neurons and in large and small dorsal root ganglion neurons, suggests that p59ᶠʸⁿ has a general function in NCAM-dependent axonal growth in the central and peripheral nervous system.

NCAM-mediated neurite extension was specific for p59ᶠʸⁿ, since neurite growth by src⁻ or yes⁻ neurons was unaffected relative to wild type neurons. Moreover, p59ᶠʸⁿ was not associated with other cell adhesion molecule-activated pathways as fyn⁻ neurons showed no impairment in neurite growth on L1 or laminin (Ignelzi et al., 1994), or on nontransfected fibroblasts. Taken together with the previous study (Ignelzi et al., 1994), the present findings demonstrate that at least two src-related PTKs are components of distinct signaling pathways for neurite outgrowth directed by cell adhesion molecules of the immunoglobulin superfamily. pp60ᶜ⁻ˢʳᶜ mediates L1 signaling (Ignelzi et al., 1994), while p59ᶠʸⁿ is an essential component of the NCAM-signaling pathway. A difference noted in the action of p59ᶠʸⁿ and pp60ᶜ⁻ˢʳᶜ in adhesion molecule signaling was that fyn⁻ neurons were completely inhibited in their neurite extension on NCAM-transfected monolayers, whereas src⁻ neurons were inhibited by only 50% on L1. Thus, NCAM signaling in fyn⁻ neurons, unlike L1 signaling in src⁻ neurons, did not appear to be compensated, even partially, by other cellular components. However, in view of the demonstrated threshold effect of NCAM in promoting neurite growth (Doherty et al., 1990a), it is possible that at higher concentrations of NCAM a component of neurite outgrowth independent of fyn action might be triggered. Since cultured cerebellar neurons express primarily NCAM140 and 180, and cultured DRG neurons express all three isoforms, it was not possible to deduce if p59ᶠʸⁿ-mediated neurite outgrowth occurred in response to homophilic binding of NCAM140 on the fibroblast monolayers to a specific NCAM isoform on the neuronal cell surface or to a heterophilic NCAM receptor.

Growth cone–enriched membranes from fetal rat brain contain high levels of $p59^{fyn}$ (Bare et al., 1993) and are very active in the endogenous phosphorylation of tubulin and other substrates on tyrosine residues (Aubry and Maness, 1988; Matten et al., 1990). It is paradoxical that addition of purified extracellular fragments of NCAM or NCAM antibodies to the growth cone–enriched membranes causes inhibition of protein tyrosine phosphorylation (Atashi et al., 1992), while the results presented here show that $p59^{fyn}$ acts as a neurite growth–promoting factor in neuronal cells on NCAM-expressing monolayers. This disparity can be explained if the growth cone membranes, which have only membrane bound components, lack cytosolic $p59^{fyn}$ substrates or effector molecules necessary for appropriate regulation of kinase activity and neurite outgrowth. These cytosolic components may be recruited to the complex only upon NCAM binding. Alternatively, extracellular NCAM fragments and antibodies may behave differently from substrate-bound NCAM in modulating tyrosine kinase activity, perhaps by binding to different determinants on the NCAM molecule, or in the case of the NCAM fragments, by binding to a heterophilic receptor. In this regard, it is interesting that affinity-purified NCAM polyclonal antibodies have been found to trigger protein tyrosine phosphatase activity in growth cone membranes (Klinz, S. G., M. Schachner, and P. F. Maness, manuscript submitted for publication).

$p59^{fyn}$ regulates multiple pathways of signal transduction through direct or indirect association with diverse receptors. $p59^{fyn}$ has been shown to associate with MAG in a subcellular myelin fraction (Umemori et al., 1994) and with the nicotinic acetylcholine receptor in electric organ (Swope and Huganir, 1993). In a pro-B cell line, $p59^{fyn}$ binds and regulates the interleukin-2 receptor, while in platelets $p59^{fyn}$ associates with CD36 (Huang et al., 1991; Kobayashi et al., 1993). The thymic form of $p59^{fyn}$ binds directly to the CD3 and Thy-1 components of the T cell receptor (Samelson et al., 1990) in a molecular association involving the $p59^{fyn}$ NH_2-terminal variable domain (Cooke et al., 1989). A distinct region of the $p59^{fyn}$ molecule, the SH2 (src-homology 2) domain binds to a tyrosine autophosphorylation site in the β-platelet–derived growth factor receptor (Kypta et al., 1990). A direct association of NCAM with $p59^{fyn}$ through its SH2 domain is unlikely, because transmembrane isoforms of NCAM are not known to be phosphorylated on tyrosine residues within their cytoplasmic domains. Conversely, NCAM does not have an SH2 domain, which could potentially bind to autophosphorylated $p59^{fyn}$.

NCAM-induced neurite outgrowth is the final event in a signaling pathway that comprises a number of intermediate steps. As shown in PC12 cells and other neurons in culture, homophilic NCAM binding activates second messenger systems that result in pertussis toxin–sensitive calcium influx (Schuch et al., 1989; Doherty et al., 1991; von Bohlen und Halbach et al., 1992). Although we do not know whether $p59^{fyn}$ influences calcium channel opening in neurons, calcium flux induced by T cell receptor activation is compromised in fyn^- T lymphocytes (Soriano et al., 1992; Appleby et al., 1992), indicating that $p59^{fyn}$ lies upstream of calcium channel opening in these cells. Other agents, such as the fibroblast growth factor receptor acting through its tyrosine kinase receptor induce neurite outgrowth in cerebellar neuron cultures by activating a cascade that involves

calcium channel opening (Williams et al., 1994). It is not clear whether nonreceptor tyrosine kinases such as $p59^{fyn}$ and $pp60^{c\text{-}src}$ are common elements of cell adhesion molecule and fibroblast growth factor pathways leading to neurite outgrowth, or whether they are components of only the NCAM and L1 pathways, respectively.

In view of the complete inhibition of NCAM-dependent neurite growth in fyn^- neurons in culture and the expression of $p59^{fyn}$ on many developing axonal tracts (Bare et al., 1993), it is surprising that fyn^- mice do not display a more severe neurological phenotype. However, the loosely organized dendrites of CA1 pyramidal cells observed in the hippocampus of fyn^- mice (Grant et al., 1992) could be a consequence of deficient NCAM-dependent neurite growth, and might contribute to the impairment in long term potentiation and spatial learning observed in the mutant mice. Other studies have shown that fyn^- mice have a reduced myelin content (Umemori et al., 1994), but other regions of the nervous system of fyn^- mice are normal, suggesting that there are compensatory systems at most sites in vivo.

A possibility consistent with the present work and related findings (Ignelzi et al., 1994) is that there is a redundancy in adhesion systems that regulate axon outgrowth in vivo, each involving a different cell adhesion molecule and nonreceptor PTK. NCAM is functionally associated with $p59^{fyn}$ and L1 is functionally associated with $pp60^{c\text{-}src}$. Consistent with this idea, redundant axon outgrowth systems have been demonstrated in Drosophila mutants involving the cell adhesion molecule fasciclin I and the abl nonreceptor tyrosine kinase (Elkins et al., 1990). The double null mutants exhibit aberrant growth cone navigation, whereas single mutants in either gene show unimpaired axonal growth. In vertebrates, the existence of multiple families of cell adhesion molecules that participate in axonal growth over cellular surfaces, such as NCAM, L1, cadherins, and integrins (reviewed in Bixby and Harris, 1991; Reichardt and Tomaselli, 1991; and Doherty and Walsh, 1992), coupled with distinct nonreceptor tyrosine kinases, might serve to minimize pathfinding errors during development of the nervous system.

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