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Differential effects of NgCAM and N-cadherin on the development of axons and dendrites by cultured hippocampal neurons

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Summary

A fundamental step in neuronal development is the acquisition of a polarized form, with distinct axons and dendrites. Although the ability to develop a polarized form appears to be largely an intrinsic property of neurons, it can be influenced by environmental cues. For example, in cell cultures substrate and diffusible factors can enhance and orient axonal development. In this study we examine the effects of growth on each of two cell adhesion molecules (CAMs), NgCAM and N-cadherin, on the development of polarity by cultured hippocampal neurons. We find that although the same pattern of development occurs on control substrates and the CAMs, the CAMs greatly accelerate the rate and extent of development of axons—axons form sooner and grow longer on the CAMs than on the control substrate. In contrast, the CAMs have opposite effects on dendritic development—N-cadherin enhances, but NgCAM reduces dendritic growth compared to control. These results provide further evidence that the development of polarity is largely determined by a cell-autonomous program, but that environmental cues can independently regulate axonal and dendritic growth.

Introduction

A crucial early step in neuronal differentiation is the development of a polarized form, with structurally and functionally distinct axons and dendrites. Dissociated cell cultures from the embryonic rat hippocampus have been widely used as a model to study the development of neuronal polarity. When hippocampal neurons are plated onto poly-L-lysine (PLL)-treated substrates, the substrate most commonly used for such cultures, they develop axons and dendrites similar to those produced *in vivo*. The sequence of this development is well-defined and predictable (Dotti *et al.*, 1988). During the dissociation process, any neurites that may have formed *in vivo* are lost, so that when the cells are plated they exhibit a round morphology. Shortly after attachment, the neurons produce a lamellipodial veil that surrounds the soma (stage 1). Within a few hours, the lamellipodium condenses into several short neurites, which are approximately 10–20 μm long. These neurites are not distinguishable as axons or dendrites and therefore are simply termed minor processes. The minor processes undergo alternating periods of growth and partial retraction, exhibiting a slow net growth over the next several hours (stage 2). After 12–36 hours in culture, one of the minor processes enters a prolonged period of growth and acquires axonal characteristics, thus defining the cell's polarity (stage 3). Over the next few days, the remaining minor processes acquire dendritic characteristics (stage 4).

These morphological changes are paralleled by molecular changes. The membrane-associated proteins GAP-43 and L1, which are initially expressed in all neurites, become segregated to the axon as soon as it forms (Goslin *et al.*, 1990; van den Pol & Kim, 1993). The microtubule-associated protein tau, which is present in all neurites, becomes dephosphorylated at the tau-1 epitope only in the nascent axon in early stage 3 cells (Mandell & Banker, 1996). Similarly, other molecules such as the microtubule-associated protein MAP2, the transferrin receptor, and the low density lipoprotein receptor-related protein (LRP) become segregated to

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dendrites as they develop in late stage 3 and stage 4 (Caceres *et al.*, 1986; Brown *et al.*, 1997; T. Esch, M. Jareb, & G. Banker, unpublished observations).

Many classes of cell adhesion molecules influence neurite elongation by a variety of cell types, including hippocampal neurons, but their effects on the development of polarity have not been studied in detail (Doherty *et al.*, 1992; Lochter & Schachner, 1993; Esch *et al.*, 1999; see Baldwin *et al.*, 1996; Burden-Gulley & Lemmon, 1997 for reviews). In the present study, we examined the development of hippocampal neurons on substrates of two different types of cell adhesion molecules, NgCAM, the chick homolog of L1 (both members of the Immunoglobulin superfamily), and N-cadherin (N-cad). Both L1 and N-cad mediate homophilic adhesion and both are expressed by hippocampal neurons (Lemmon *et al.*, 1989; van den Pol & Kim, 1993; Benson & Tanaka, 1998), but their subcellular localization differs. N-cadherin is expressed on the surface of both axons and dendrites of hippocampal neurons (Benson & Tanaka, 1998) whereas L1 is polarized to the axon (van den Pol & Kim, 1993; Jareb & Banker, 1998). We found that neurons follow the same developmental sequence on N-cad & NgCAM as they do on PLL, but both CAMs accelerate axonal development. NgCAM and N-cad differ in their effects on dendritic development. NgCAM inhibits, whereas N-cad enhances dendritic growth.

Methods

PROTEINS AND ANTIBODIES

Poly-L-lysine was obtained from Sigma. Nitrocellulose (0.45 μm pore size, grade BA85) was obtained from Schleicher and Schuell. NgCAM was purified from chick brains using an affinity column conjugated with 8D9 antibodies (Lemmon & McLoon, 1986). N-cadherin was prepared using antibody NCD-2 (Hatta & Takeichi, 1986). Rabbit anti-L1 was prepared as in Brittis *et al.* (1995). Rabbit anti-laminin was obtained from Telios and rabbit anti-tubulin from Sigma; monoclonal antibodies TuJ1 (against neuronal tubulin), AP14 (against MAP2), and tau-1 (against a dephosphorylated epitope of tau) were generously provided by Tony Frankfurter (University of Virginia).

SUBSTRATE PREPARATION

Acid-cleaned glass coverslips were incubated in 1 mg/ml PLL in borate buffer overnight at room temperature, and rinsed with distilled water. For PLL controls, these coverslips were placed in Minimal Essential Medium (MEM) containing 10% horse serum for plating. For NgCAM and N-cad coverslips, the PLL-coated coverslips were air dried before a thin layer of nitrocellulose solution (1 by 2.5 cm strip dissolved in 18 ml of methanol; Lagenaur & Lemmon, 1987) was applied. The coverslips were once again air dried. NgCAM (50 μg/ml, determined by dilution series to give maximal effects on neurite length) or N-cadherin (20 μg/ml, determined by dilution series) was applied to the coverslips and incubated for 15–20 min. at 37°C. Coverslips were rinsed two times in water and placed into culture medium for plating.

CELL CULTURE

Hippocampal cultures were prepared as described previously (Goslin *et al.*, 1998). Hippocampi were dissected from 18 day fetal rats and treated with trypsin (0.25% for 15 min. at 37°C) and dissociated by trituration with a Pasteur pipette. Cells were plated at a density of about 200 cells/cm^2 on substrate-coated glass coverslips in MEM with N2 supplements (Bottenstein & Sato, 1979; Bottenstein, 1985), 0.1% ovalbumin, and 0.1 mM pyruvate. Control PLL coverslips were put into MEM containing 10% horse serum. After allowing 2 hours for cell attachment, coverslips were transferred to dishes containing a confluent monolayer of astroglia in culture medium.

ANALYSIS OF NEURONAL MORPHOLOGY

Early development

At various times during the first 24 hours in culture, cells were fixed for 20–30 minutes in 4% paraformaldehyde in phosphate-buffered saline (PBS) containing 4% sucrose, permeabilized in 0.25% Triton X-100 for 5 min., and rinsed several times in PBS. Coverslips were blocked with 10% bovine serum albumin (BSA) in PBS for 1 hour at 37°C, and then incubated with anti-tubulin antibodies (1 : 200 in 3% BSA/PBS) for 1 hour at 37°C or overnight at 4°C. Coverslips were rinsed 3 times (15 min. each) with PBS, and incubated with biotinylated secondary antibodies (1 : 600 in 3% BSA/PBS) for 1 hour at 37°C. Coverslips were again rinsed 3 times (15 min. each) with PBS and incubated with an Avidin DH:biotinylated horseradish peroxidase H complex (Vectastain Elite ABC, Vector). Cells were then visualized by reaction with 0.02% H_2O_2 in 50 mM Tris-HCl containing 0.5 mg/ml diaminobenzidine (DAB). Cells were dehydrated through a series of ethanol dilutions (25%–100%) and xylene, and mounted on glass microscope slides with Gurr Fluoromount.

Dendritic development

After 7 days in culture, cells were fixed for 30–40 minutes in 4% paraformaldehyde in PBS containing 4% sucrose, permeabilized with methanol at −20°C, and rinsed briefly in PBS. Coverslips were blocked, incubated with antibodies against MAP2 (1 : 200 in 3% BSA/PBS) and appropriate secondary antibodies, and stained as described above. The changes in fixation protocol were necessary to minimize cell detachment.

Data analysis

Images of randomly selected cells were recorded with a Neuvicon video camera and recorded on an Optical Memory Disk Recorder (OMDR). The number of processes was counted and their length was measured from the base of the process to the end of tubulin or MAP2 labeling, using Image-1 software (Universal Imaging, Chester, PA). A criterion of 65 μm was used as a morphological definition for axons in cells fixed during the first 24 hours in culture. This number was based

on our analysis of the distribution of all process lengths on PLL, and on previous time-lapse studies of cells growing on PLL which suggested that axons rarely retract after attaining this length. Immunostaining for tau-1 in other cells confirmed that almost all processes of this length are axons.

MOLECULAR MARKERS OF POLARITY

Cells were fixed at various times after plating and permeabilized with Triton X-100 as described above. Blocking and incubation with primary antibodies (1 : 100 tau-1 or 1 : 200 anti-L1 in 3% BSA/PBS) and biotinylated secondary antibodies were done as described above. Following incubation with secondary antibodies, coverslips were rinsed with PBS and incubated with fluorescein-conjugated streptavidin for 1 hour at 37°C. Coverslips were again rinsed 3 times (15 min. each) with PBS, rinsed once briefly in water, and then mounted on glass slides in a polyvinyl alcohol-based mounting medium containing 2% diazobicyclo-octane. Fluorescence images were acquired using a Zeiss axiophot microscope, a Photometrics AT-200-cooled charge coupled device (CCD) camera, and Metamorph image analysis software (Universal Imaging).

Results

To determine the effects of NgCAM and N-cad on the development of polarity by hippocampal neurons, cells were cultured on coverslips coated with these substrates and fixed at different times after plating. The length and number of processes were compared to those of cells from the same culture preparation grown on PLL-coated coverslips. In most cases N-cad and NgCAM were tested in separate experiments, and therefore are not directly compared to each other.

Examination of neurons grown on NgCAM or N-cad for 8 or 24 hours in culture revealed that their basic morphology was similar to that of cells grown on PLL. On all three substrates the cells developed a polarized morphology, with a single axon (or occasionally two axons) and minor processes, which were distinctly shorter (Fig. 1). There was, however, a striking enhancement of axonal growth on both the CAMs relative to PLL. This effect was greatest on NgCAM, on which axons were on average approximately three times longer than axons on PLL at 24 hours (Table 1). In some cells grown on NgCAM the axon was over a millimeter long at this time. The effect of N-cad was not as great, but was still significant. In addition to increasing the length of axons, both substrates increased the percentage of cells with multiple axons (Table 1).

The growth of minor processes was also influenced by different substrata. Perhaps the most striking effect was a reduction in the number of minor processes that formed when neurons were grown on NgCAM (Table 1). On this substrate, cells with only a single minor process were common (Fig. 1); such cells were never observed on PLL. The number of minor processes that formed when cells were cultured on N-cad was also slightly, but significantly, fewer than on PLL. On the other hand, N-cad increased the length of minor processes to a greater degree than did NgCAM (Table 1). Because cells formed multiple axons more commonly on NgCAM and N-cad, some of the apparent increase in the average length of minor processes on these substrates may also be attributed to the inclusion of neurites that were becoming axons, but had not yet exceeded the 65 μm cutoff we used to categorize neurites as axons.

In addition to elongating more rapidly, axons formed sooner on NgCAM and N-cad than on PLL (Figs. 1 and 2). About 50% of neurons growing on NgCAM or N-cad had formed axons by 12 hours after plating. This number increased to greater than 90% by 24 hours after plating. In contrast, less than 5% of neurons grown on PLL had axons after 12 hours, and only 70% had axons after 24 hours in culture.

To determine whether the acceleration of axon formation could be accounted for by an acceleration in the formation of minor processes, we monitored the initial outgrowth of processes at earlier times after plating (Fig. 3). After 3 hours in culture, the earliest time point examined, slightly more neurons grown on N-cad had processes than neurons on PLL. Although fewer processes formed on NgCAM, processes formed at similar rates on NgCAM and PLL. Therefore, at most only a small portion of the acceleration of axon formation by N-cad or NgCAM can be attributed to an overall effect on process formation.

To determine if neurons cultured on N-cad or NgCAM became molecularly polarized, we immunostained cultures with several antibodies that selectively stain axons or dendrites. Axonal markers were enriched in the nascent axons of neurons grown on these substrates as early as 5 hours after plating. The dephosphorylated isoform (tau-1 epitope) of the microtubule-associated protein tau was polarized to the same extent in cells grown on NgCAM (Fig. 4C and D) or N-cad (Fig. 4E and F) for 5 hours as it was in cells of an equivalent morphological stage grown for 24 hours on PLL (Fig. 4A and B). The cell adhesion molecule L1 was similarly polarized at these times (data not shown). In cells with two axons, both expressed tau-1 and L1 immunostaining (data not shown).

The dendritic marker MAP2 was segregated in neurons grown for 7 days on NgCAM or N-cad (Fig. 5). In all cases, the MAP2-stained processes exhibited the characteristic taper of dendrites. As on PLL (Fig. 5A and B), MAP2-labeling in neurons grown on N-cad (Fig. 5E and F) extended to the ends of processes and these ends were clearly identifiable with phase optics. In cells grown on NgCAM (Fig. 5C and D), however, a long, thin, axon-like process almost always extended

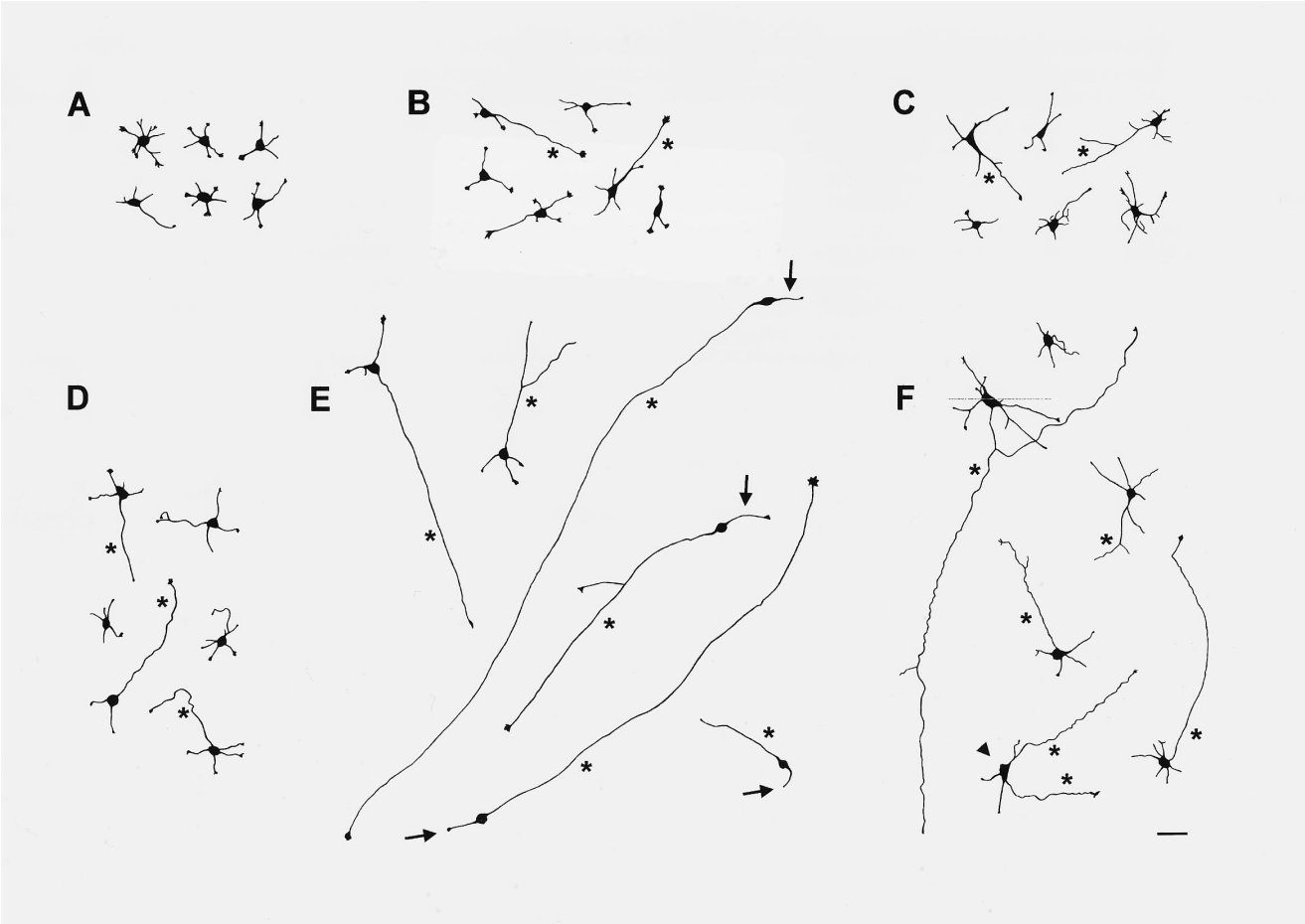

Fig. 1. The morphology of neurons grown on PLL, NgCAM and N-cadherin. After 8 h. in culture (A–C) neurons grown on PLL (A) had several minor processes but only about 1% had axons. Neurons grown on NgCAM (B) or N-cad (C) also had several minor processes, and more neurons had developed axons (asterisks). After 24 hours in culture (D–F), some neurons grown on PLL (D) and almost all neurons grown on NgCAM (E) or N-cad (F) had developed axons. The axons of neurons grown on NgCAM and N-cad were much longer than those on PLL, and some cells grown on these substrates had more than one axon (F, arrowhead). Many neurons grown on NgCAM had only one minor process besides the axon (E, arrows). The cells illustrated were selected from more than 200 cells at each time point in order to illustrate the range of morphologies observed; they are arranged as composites to conserve space. Scale bar, 25 μm.

beyond the end of the MAP2 labeling. With our techniques it was impossible to determine whether these processes were axons of other cells running along the labeled dendrites, or whether the MAP2 staining was present in the proximal portion of an otherwise axonal process. Attempts to label single cells were unsuccessful due to cell detachment (see below).

N-cad and NgCAM had opposite effects on the length and branching of MAP2-labeled neurites (Fig. 6). The total length of the dendritic arbor of neurons grown on N-cad for 7 days was greater (Fig. 6A), and there were more branches (Fig. 6B) on N-cad than on PLL. In contrast, the MAP2-positive processes on NgCAM were relatively short and unbranched after 7 days. The reduced dendritic development of neurons grown on NgCAM may have been due in part to the fact that neurons were not well-attached to the substrate at this age. In fact, many somata detached completely and remained tethered to the substrate only by the axon bundles that ran over them (data not shown).

Discussion

Our results demonstrate that the same basic pattern of neuronal development—the initial formation of unspecified minor processes followed by axonal and then dendritic development—occurs on all of the substrates examined, but the timing of these events, the rate of neurite growth, and even the number of neurites that develop, differ greatly. When compared with PLL, the biological substrate molecules we and others (Lein *et al.*, 1992) have examined (including NgCAM, N-cad and laminin) dramatically accelerate development, particularly axon specification. On the other hand, their

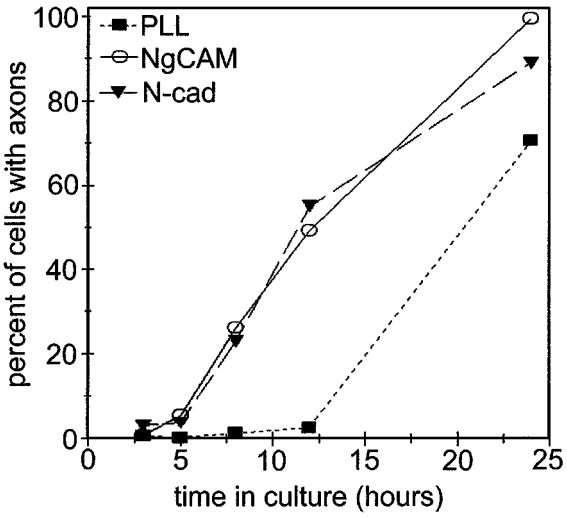

Fig. 2. The time course of axonogenesis on different substrates. Neurons grown on different substrates were fixed at varying times after plating and the percent of cells with axons was determined (see Methods). Axons formed much sooner on NgCAM and N-cad than on PLL. Each point represents combined data from at least 2 different experiments and at least 200 cells.

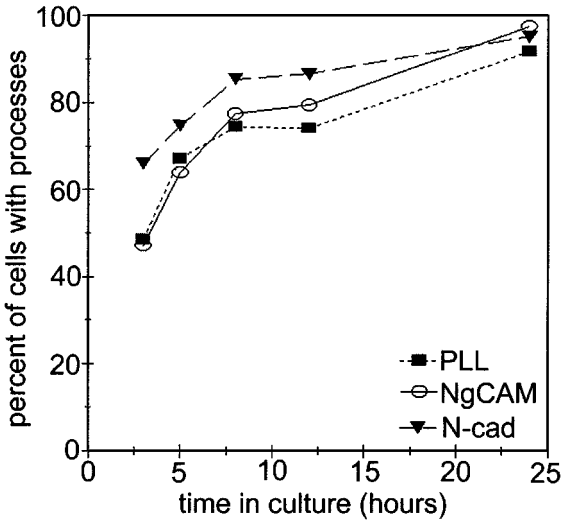

Fig. 3. The time course of process formation on different substrates. Neurons grown on different substrates were fixed at varying times after plating, and the percent of cells with at least one process was determined. Processes formed slightly sooner on N-cad than on PLL or NgCAM. Each point represents data from a total of at least 200 cells combined from at least 2 different experiments.

effects on dendritic development are quite different. When compared with PLL, N-cad stimulates dendritic development, NgCAM inhibits dendritic development, and laminin has little or no effect. These results suggest that the basic events that lead to the development of polarity by hippocampal neurons in culture are largely governed by a cell-autonomous program,

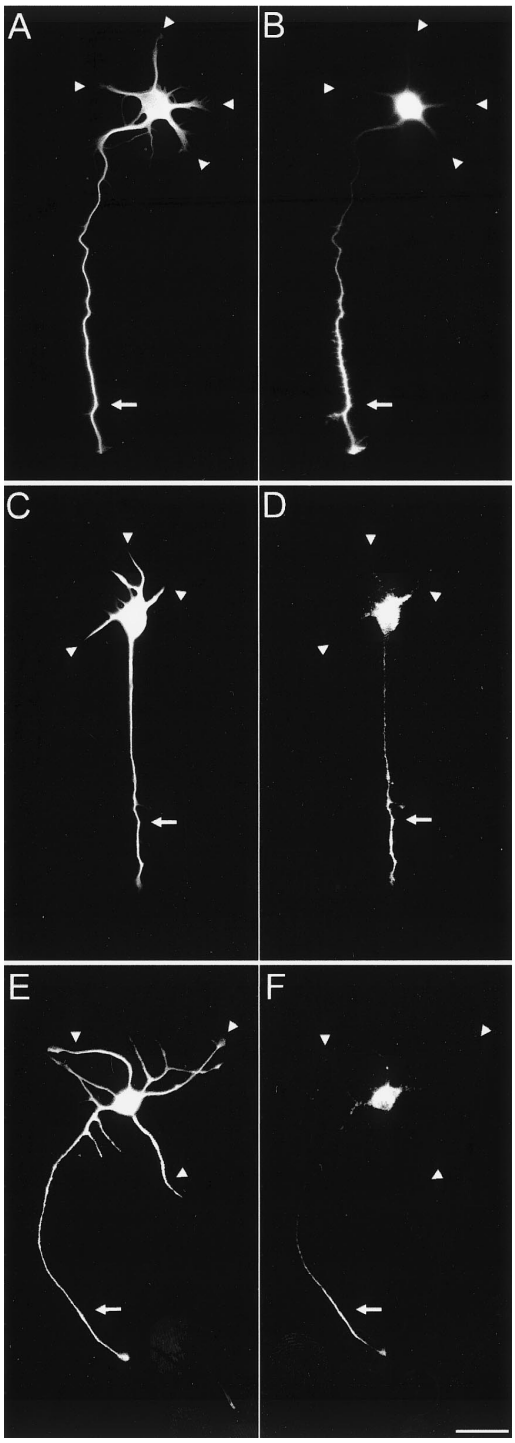

Fig. 4. Polarization of dephosphorylated tau (tau-1) in young axons on different substrates. Neurons were double-immunolabeled with antibodies against tubulin (A, C, E) and tau-1 (B, D, F). Neurons grown on PLL (A, B) were fixed after 24 hours in culture. At this stage minor processes (arrowheads) were devoid of tau-1 staining, which instead was restricted to the distal axon (arrows). Neurons grown on NgCAM (C, D) or N-cad (E, F) were fixed after 5 hours in culture. At these earlier times, neurons grown on NgCAM and N-cad expressed dephosphorylated tau in a polarized distribution comparable to that seen on PLL several hours later. Scale bar, 25 μm.

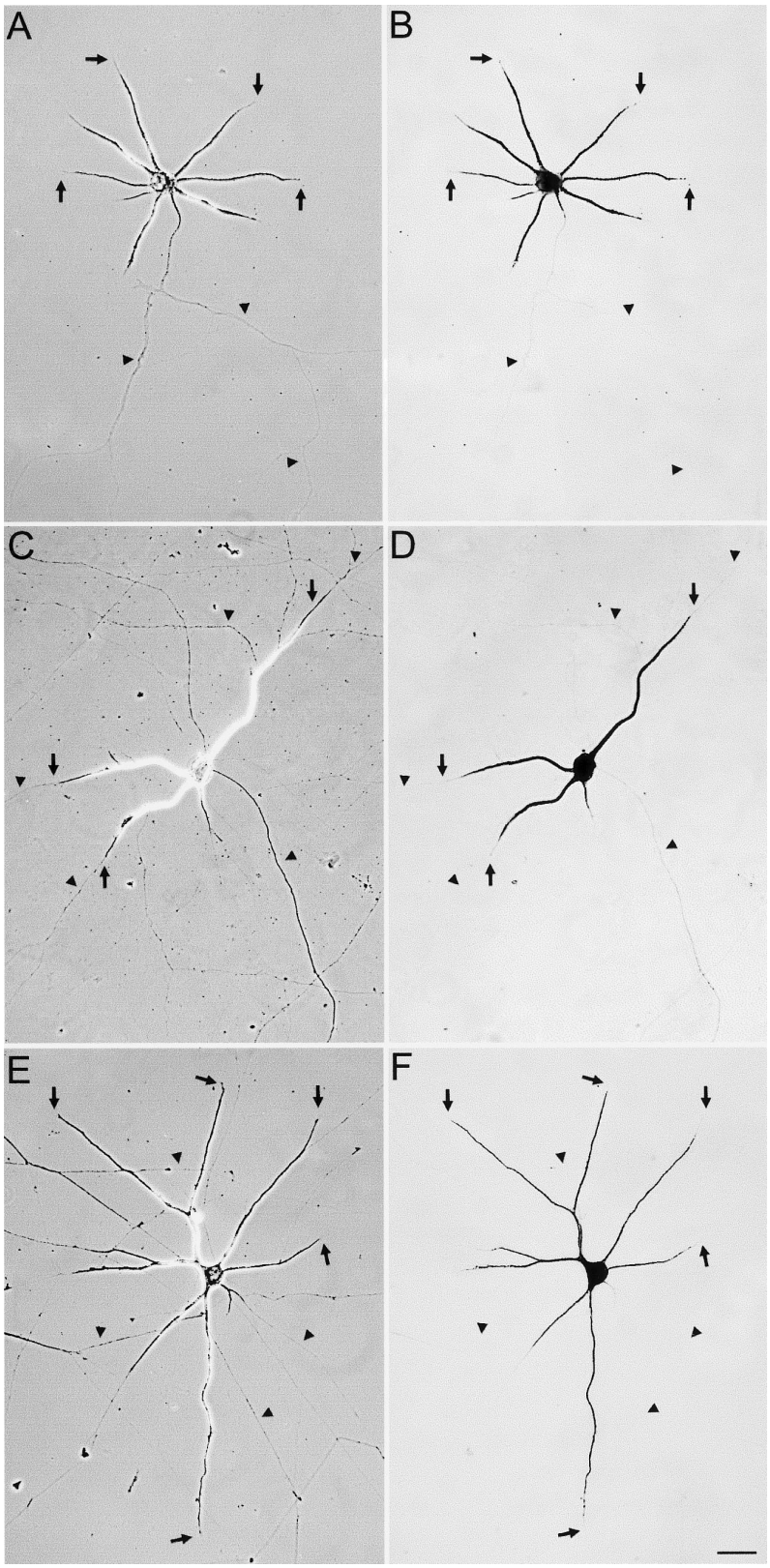

Fig. 5. Polarization of MAP2 on different substrates. Phase contrast (A, C, E) and bright field (B, D, F) images of neurons fixed after 7 days in culture and immunolabeled with antibodies against MAP2. MAP2 was polarized on all three substrates. On PLL (A, B) and N-cad (E, F) MAP2 was restricted to processes that had a dendritic morphology and distinct endings (arrows). Axons (arrowheads) had only background levels of staining. On NgCAM (C, D), MAP2 was mostly restricted to portions of processes that had a dendritic morphology, but beyond the end of the MAP2 labeling there was nearly always an axon-like process. Some axons appeared to have above-background MAP2 labeling. Scale bar, 25 μm.

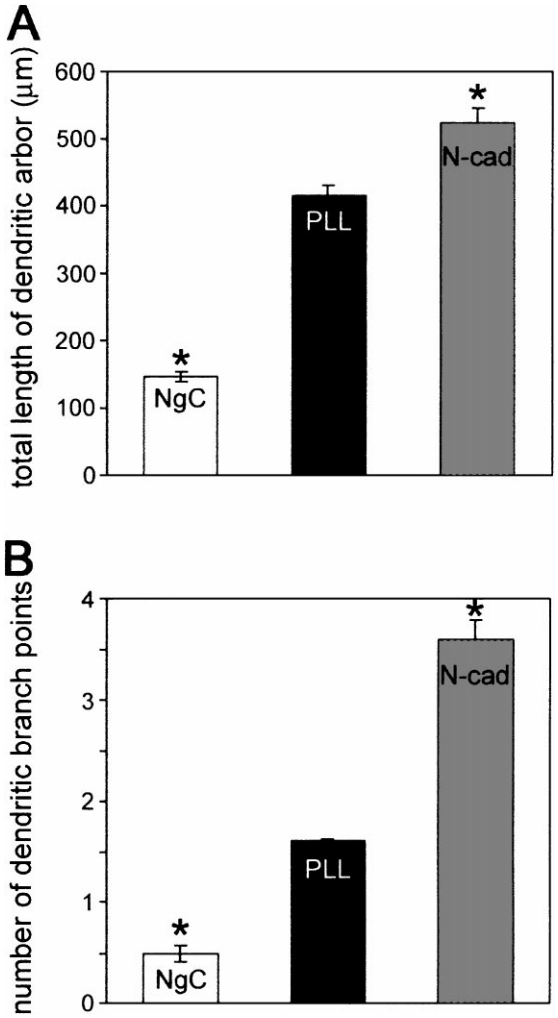

Fig. 6. Effects of substrates on dendritic growth. Neurons were fixed after 7 days in culture and immunolabeled for MAP2. MAP2 labeling was used to measure the total length of the dendritic arbor (A) and the number of branch points per cell (B). Dendritic growth was decreased in cells grown on NgCAM and increased in cells grown on N-cadherin relative to PLL. Data were combined from 2 experiments each for NgCAM and N-cadherin. Error bars = SEM. $N = 167$ for NgCAM, 336 for PLL, and 181 for N-cad. Asterisks indicate significant difference compared to PLL ($p < .001$).

but that substrate-attached cues have an important role in regulating some aspects of this development.

Our results differ somewhat from a previous analysis of hippocampal neurons cultured on L1, the mammalian homolog of NgCAM (Lochter & Schachner, 1993). In that study, growth on L1 increased the length of the cell's longest process, but the magnitude of this effect was much smaller than in the present study. This difference may be explained by technical differences in how the substrate was prepared. NgCAM/L1 does not bind as efficiently to polyornithine, the base substrate used by Lochter and Schachner, as it does to nitrocellulose, which was used in this study. Furthermore, the orientation of the bound protein varies across base substrates and can affect the extent to which a molecule promotes neurite growth (Schaefer & Lemmon, 1998). In the present study neurons were co-cultured with astroglia, but in the Lochter and Schachner study they were not. This may also be a factor.

After 5–7 days in culture, neuronal somata began to detach from NgCAM, but not from the other substrates. This is not surprising, because L1, the endogenous ligand for NgCAM, is excluded from the somatodendritic domain as cells mature (van den Pol & Kim, 1993; Winckler *et al.*, 1999). This effect may have been augmented by phosphacan, which is secreted by glia and binds to NgCAM with a higher affinity than does L1 (Milev *et al.*, 1994; Sakurai *et al.*, 1996)

Growth on either NgCAM or N-cad enhanced neurite elongation compared with growth on PLL, but in different ways. On NgCAM, the axons grew much more rapidly than on PLL, but there were fewer minor processes and dendritic growth was reduced. On N-cad, both dendrites and axons grew more rapidly than on PLL. The different effects of growth on NgCAM and N-cad probably reflect the different distribution of the endogenous binding partners for these adhesion molecules. L1, the endogenous ligand for NgCAM, is present primarily in growth cones in stage 2 cells and becomes polarized to the axon soon after it arises (van den Pol & Kim, 1993; T. Esch, M. Jareb G. Banker, unpublished observations), whereas N-cad is expressed in all processes at all developmental stages (Benson & Tanaka, 1998).

In addition to growing faster, axons formed sooner on NgCAM and N-cad than on PLL. Laminin, which also enhances the growth rate of hippocampal axons, similarly accelerates axon formation (Lein *et al.*, 1992). What could explain these observations? It has been hypothesized that during stage 2 of development "competitive" interactions among the minor processes determine which one of them ultimately becomes the axon (Dotti *et al.*, 1988; Goslin & Banker, 1989). During this stage of development individual minor neurites undergo alternating episodes of growth and retraction until one neurite, apparently by chance, undergoes an extended period of elongation and acquires an axonal identity. The stochastic nature of axon specification can be eliminated by local presentation of substrate molecules: if a single minor process of an undifferentiated cell contacts laminin or NgCAM, that process immediately develops as the cell's axon (Esch *et al.*, 1999). During stage 2, periods of growth and retraction of minor processes are mirrored by increases and decreases in the concentration of proteins, including CAMs, within their growth cones (Goslin & Banker, 1990; Bradke & Dotti, 1997; M. Jareb & G. Banker, unpublished data; D. Benson, personal communication). In a uniform environment, these variations in protein concentrations might substitute for environmental

variations in substrate molecules in specifying axons. Although the axon-promoting substrates we have examined do not cause consistent changes in the length of minor processes, as measured in fixed cultures, they may alter their dynamic characteristics, perhaps increasing the rate of elongation during periods of growth or enhancing the molecular and biochemical changes in their growth cones. If so, this could reduce the time needed for one neurite to become specified as the axon. Direct observations of living neurons will be required to evaluate this possibility. Such observations might also offer an explanation for why cells are more likely to form two axons when cultured on NgCAM or N-cad.

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